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Immunoprevention of Mammary Carcinoma in HER-2/neu Transgenic Mice Is IFN-γ and B Cell Dependent

Patrizia Nanni,* Lorena Landuzzi,‡ Giordano Nicoletti,*,† Carla De Giovanni,*, Ilaria Rossi,*, Stefania Croci,*, Annalisa Astolfi,* Manuela Iezzi,‡ Emma Di Carlo,‡ Piero Musiani,‡ Guido Forni,§ and Pier-Luigi Lollini2*§

A vaccine combining IL-12 and allogeneic mammary carcinoma cells expressing p185 neu completely prevents tumor onset in HER-2/neu transgenic BALB/c mice (NeuT mice). The immune protection elicited was independent from CTL activity. We now formally prove that tumor prevention is mainly based on the production of anti-p185 neu Abs. In the present studies, NeuT mice were crossed with knockout mice lacking IFN-γ production (INO-γ−/−) or with B cell-deficient mice (μMT). Vaccination did not protect NeuT-IFN-γ−/− mice, thus confirming a central role of IFN-γ. The block of Ab production in NeuT-μMT mice was incomplete. About one third of NeuT-μMT mice failed to produce Abs and displayed a rapid tumor onset. By contrast, those NeuT-μMT mice that responded to the vaccine with a robust production of anti-p185 neu Ab displayed a markedly delayed tumor onset. In these NeuT-μMT mice, the vaccine induced a lower level of IgG2a and IgG3 and a higher level of IgG2b than in NeuT mice. Moreover, NeuT-μMT mice failed to produce anti-MHC class I Abs in response to allogeneic H-2d molecules present in the cell vaccine. These findings show that inhibition of HER-2/neu carcinogenesis depends on cytokines and specific Abs, and that a highly effective vaccine can rescue Ab production even in B cell-deficient mice. The Journal of Immunology, 2004, 173: 2288–2296.

The ability of spontaneous immune responses to control carcinogenic processes (1) is leading to the concept that a selective stimulation of the immune system could further decrease, possibly abolish, cancer incidence (2). Proof of principle was obtained in various models of induced and spontaneous carcinogenesis using cytokines (3, 4) and vaccines based on DNA, peptides, proteins, or cells expressing tumor Ags (5–11). We have recently shown that a combination of IL-12 and allogeneic tumor cells expressing the membrane protein product of the HER-2/neu (p185 neu) administered to healthy HER-2/neu transgenic mice reduced by 90–100% the risk of mammary carcinoma and more than doubled life expectancy (12). Cytokine release and Ab production appeared to be the immune mechanisms responsible for this efficient and long-term protection, whereas CTLs appeared to play no role (12). This was a provocative finding, because overwhelming experimental evidence shows that CTLs are of pivotal importance in the resistance to transplantable tumor challenges and in the cure of existing cancer lesions (13). In this study, we formally show that prevention of mammary carcinoma with IL-12 and allogeneic cell vaccines expressing p185 neu requires intact IFN-γ and B cell responses.

Materials and Methods

Mice

Transgenic BALB/c (H-2d) female mice, designated here as NeuT mice, overexpressing the transforming activated rat HER-2/neu oncogene under control of the mouse mammary tumor virus promoter (12) were bred under specific pathogen-free conditions by Charles River (Calco, Italy). The establishment of IFN-γ gene knockout HER-2/neu transgenic mice (NeuT-IFN-γ−/− mice) has been described previously (12). To obtain B cell-deficient HER-2/neu transgenic mice, one female μMT mouse (knockout for the Ig μ-chain gene) on BALB/c genetic background, a kind gift from Dr. T. Blankenstein (Max-Dellbruck Center for Molecular Medicine, Berlin, Germany), was crossed with one NeuT male mouse. Heterozygous knockout/transgenic F1 mice were backcrossed with female μMT to obtain mice homozygous for the μ-chain knockout allele and heterozygous for the HER-2/neu transgene, designated here as NeuT-μMT. The level of B cells was routinely monitored by flow cytometry using mAb RA3-6B2 (BD Pharmingen, San Diego, CA). Individually tagged virgin females used in the experiments were treated according to protocols approved by Institutional Review Boards. Mammary pads were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters. Progressively growing masses of ≥3 mm in mean diameter were regarded as tumors. Growth was monitored until all 10 mammary glands displayed a tumor or until a tumor exceeded a mean diameter of 1.5 cm, at which time mice were sacrificed for humane reasons.

Cells

TT12 and N202.1E cell clones were derived from mammary carcinomas of FVB-neuN #202 mice (H-2b), transgenic for the rat HER-2/neu protooncogene (14). TT12 cells (referred to as NeuH-2b) expressed high levels of p185 neu; N202.1E cells (referred to as NeuH-2b) lacked p185 neu. Cells were cultured in Dulbecco’s modified minimal essential medium supplemented with 20% FBS (Invitrogen, Milan, Italy) at 37°C in a humidified 5% CO2 atmosphere. In vaccine experiments, cells were treated with 40 μg/ml mitomycin C (Sigma-Aldrich, Milan, Italy) to block cell proliferation. Surface expression of p185 neu and class I H-2d molecules was assessed by flow cytometry using mAbs 7.16.4 (p185 neu, Oncogene Research Products, Cambridge, MA), KHI14 (H-2k, BD Pharmingen), 34-7-23S (H-2d, Cedarlane, Hornby, Ontario, Canada), and 28-14-8S (H-2dL, BD Pharmingen).

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Starting at the sixth week of age, mice received successive 3-wk courses of four twice-weekly i.p. vaccinations with $2 \times 10^6$ allogeneic Neu/H-2$^q$ mammary carcinoma cells in 0.4 ml of PBS, followed by five daily i.p. administrations of recombinant mouse IL-12 (kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA) in the third week. In the first course, the five injections were of 50 ng of IL-12 in 0.2 ml of PBS supplemented with 0.01% mouse serum albumin (MSA$^3$; Sigma-Aldrich); the subsequent injections were of 100 ng of IL-12. After 1 wk of rest, the course was repeated until mice were sacrificed or were 1 year old. Hereafter, mice receiving this combined treatment are referred to as vaccinated mice. Control mice received either mock vaccination or MSA administration. Their tumor progression mirrored that of untreated mice.

**Morphologic and immunohistochemical analysis**

Tissue samples were processed as described previously for histologic evaluation or for immunohistochemistry (15). The following Abs were used: anti-endothelial cells (anti-CD31, clone mEC-13.324; provided by A. Vecchi, Istituto M. Negri, Milan, Italy); anti-p185$^{neu}$ (C-18; Santa Cruz Biotechnology, Santa Cruz, CA), anti-proliferating cell nuclear Ag (PCNA; Ylem, Milan, Italy), and anti-CD45/B220 (BD Pharmingen).

**Whole-mount preparation of mammary glands**

Whole-mount preparations were performed as reported by Medina (16). Briefly, the skin of euthanized 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-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histolemon, and stored in methylsalicylate (Sigma-Aldrich). Digital pictures were taken with a Nikon Coolpix 995 (Nital, Turin, Italy) mounted on a stereoscopic microscope (MZ6; Leica Microsystems, Milan, Italy).

**IFN-$\gamma$ production by spleen cells**

Spleens were collected from vaccinated and control mice, and single-cell suspensions were prepared, washed in PBS, and resuspended in RPMI 1640 supplemented with 10% FBS. Spleen cells ($5 \times 10^7$ cells/ml) were then restimulated with mitomycin C-treated target cells ($5 \times 10^4$ cells/ml) for 6 days at 37°C in RPMI 1640 with 10% FBS containing 10 U/ml recombinant mouse IL-2 (PeproTech, Rocky Hill, NJ). Supernatants were assayed for IFN-$\gamma$ production by ELISA purchased from Endogen (Woburn, MA).

**Ab response**

Mice were routinely bled from a lateral tail vein, and sera were stored frozen at $-80^\circ$C. Analysis of Ab content of sera diluted 1/65 was performed by indirect immunofluorescence followed by flow cytometry. Neu/
H-2<sup>a</sup> and Neu<sup>wt</sup>/H-2<sup>a</sup> cells were used as targets for Ab binding. Total Ig binding was evaluated using a FITC-conjugated goat anti-mouse IgG (H + L) chains secondary Ab (Euroclone, Milan, Italy). For Ig subclass analysis, the following secondary FITC-conjugated mAbs were purchased from BD Pharmingen: anti-mouse IgG1 clone A85-1, anti-mouse IgG2a clone R19-15, anti-mouse IgG2b clone R12-3, anti-mouse IgG3 clone R40-82, anti-mouse IgM clone R6-60.2, anti-mouse IgA clone C10-3, and anti-mouse IgE clone R35-72.

**Complement-mediated and Ab-dependent cellular cytotoxicity (ADCC)**

Complement-mediated cytotoxicity test was performed as described previously (12). The ADCC test was performed according to Sung et al. (17). Neu-positive H-2<sup>a</sup> mammary tumor cells (12) were plated at 10<sup>5</sup> cells/well in 96-multiwell plates and allowed to attach overnight. Cultures were incubated for 2 hr on ice with sera from experimental mice at different dilutions. After washing, spleen cells from normal BALB/c mice were added at 50:1 E:T ratio and plates were incubated at 37°C overnight. Nonadherent cells were carefully washed out, 20 μl of WST-1 solution (Roche Diagnostics, Milan, Italy) was added to each well, and absorbance at 450 nm was read after an additional 4-h incubation.

**Immunoprecipitation and Western blot analysis**

Neu/H-2<sup>a</sup> and Neu<sup>wt</sup>/H-2<sup>a</sup> cells were lysed for 30 min on ice with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 150 mM NaCl plus phosphatase and protease inhibitors, 1 mM PMSF, 1 μg/ml aprotinin, 50 mM NaF, and 10 mM sodium pyrophosphate (all reagents were purchased from Sigma-Aldrich). Cell lysates were normalized at a protein concentration of 4 mg/ml with lysis buffer and diluted 1/3 with TBS (50 mM Tris-HCl (pH 7.5) plus 150 mM NaCl) to decrease detergent concentration. Cell lysates (500 μg of total protein each sample) were immunoprecipitated with 3 μg/ml anti-rat neu mAb 7.16.4 (Ab-4; Oncogene Research Products) or with 20 μl of sera from untreated or vaccinated mice for 2 h at 4°C. Immunoprecipitated proteins were eluted following overnight incubation with protein A-agarose (Santa Cruz Biotechnology); washed twice in TBS, and denatured at 100°C for 10 min in 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) containing SDS and 5% 2-ME (Sigma-Aldrich). Proteins were then separated on 7.5% SDS-PAGE gels (Ready Gel; Bio-Rad) and transferred onto polyvinylidene fluoride membranes (Bio-Rad). After blocking with PBS containing 0.1% Tween 20 and 5% nonfat dry milk, membranes were incubated overnight at 4°C with anti-rat Neu polyclonal Ab diluted 1/200 in blocking buffer (C18; Santa Cruz Biotechnology). Finally, the presence of immunoprecipitated Neu-2</p>
by the combined treatment in transgenic mice were an abundant production of IFN-γ by T cells, in particular by CD8+ lymphocytes, and a strong Ab response directed against p185neu. To analyze their importance in the protection from mammary carcinoma afforded by vaccination, NeuT transgenic mice were crossed with knockout mice deficient in IFN-γ release (NeuT-IFN-γ−/−) or in B cell immunity (NeuT-μMT).

Although the combined treatment protected almost all NeuT mice, none of the NeuT-IFN-γ−/− or NeuT-μMT were protected (Fig. 1). Differences between NeuT-μMT and NeuT-IFN-γ−/− were apparent in tumor latency and in the number of carcinomas per mouse (tumor multiplicity), which were significantly (p < 0.01 at least) reduced by vaccinations in B cell-deficient mice, but not in mice lacking IFN-γ.

p185neu expression and neoangiogenesis in the mammary gland of vaccinated IFN-γ- or B cell-deficient mice

Pathological studies revealed that by the third week of age the mammary glands of NeuT mice developed foci of atypical hyperplasia, which subsequently extended to all mammary glands. Foci of carcinoma in situ, first apparent around the 15th week of age, evolved to invasive carcinomas by the 20th week of age and were present in all the glands 10–12 wk later.

In NeuT mice vaccinated with Neu/H-2q cells plus IL-12, the mammary glands showed the development of a limited number of atypical hyperplastic foci often surrounded by reactive cells. These hyperplastic foci decreased with time so that, at 45–50 wk of age, the mammary glands were free of hyperplastic foci and tumors. In NeuT-IFN-γ−/− and NeuT-μMT mice treated with IL-12 or Neu/H-2q cells plus IL-12, the invasive lobular carcinomas that eventually developed in mammary glands were histologically very similar to those of untreated NeuT mice.

Importantly, in NeuT mice, the combined treatment resulted in a scarce expression of p185neu, the HER-2/neu gene product that, if present, was mainly confined to the cytoplasm of mammary duct epithelial cells, which rarely stained for PCNA, a marker of tumor cell proliferation (Fig. 2, B and F). By contrast, a marked cytoplasmic and membrane p185neu expression, similar to that found in
hyperplastic and neoplastic lesions of untreated mice (Fig. 2A), was displayed by neoplastic cells of carcinomas developed in both NeuT-μMT and NeuT-IFN-γ−/− mice (C and D). Expression of p185neu was associated with a marked expression of PCNA (Fig. 2). The microvessel count by anti-CD31 immunostaining (Fig. 2, I–L) revealed a significant (p < 0.005) decrease in the number of microvessels supplying hyperplastic or neoplastic lesions in vaccinated NeuT and NeuT-μMT mice when compared with that of untreated NeuT mice (vessel count in vaccinated NeuT mice, 6.1 ± 1.7; in vaccinated NeuT-μMT, 9.8 ± 1.2; in vaccinated NeuT-IFN-γ−/−, 11.7 ± 1.9; in untreated NeuT mice, 14.4 ± 2.2). A similar reduction in microvessel density was observed in NeuT-μMT mice treated with IL-12 only (9.0 ± 1.2; in vaccinated NeuT-μMT mice, 1.8 and 9.2 ± 1.1, respectively).

Vaccination elicits Ab production in B cell-deficient NeuT-μMT mice

It has been reported that B cells from μMT mice, in particular on a BALB/c genetic background, can bypass the IgM defect and undergo class switch leading to Ig secretion (18–21). We found that only one third of NeuT-μMT mice receiving the vaccine was truly unresponsive, whereas the remaining two thirds of mice produced abundant high-titer Abs against the vaccine (Fig. 3A). We then compared the serum Ab levels of Ab-proficient NeuT-μMT mice with those of the two parental mouse lines, NeuT and μMT (Fig. 3B). The frequency of Ab production in response to the vaccine was 100% both in NeuT and μMT mice, and the levels of serum Abs were also similar. It could be concluded that an effective vaccine elicited very high levels of Abs even in B cell-deficient μMT mice. Only when the two genetic lesions were combined in the same mouse (i.e., in NeuT-μMT mice), we observed a lack of anti-vaccine Abs production in about one third of mice and reduced Ab levels in the remaining two thirds. This may be attributed to the fact that HER-2/neu expression makes NeuT-μMT mice tolerant to HER-2/neu gene product p185neu (9).

Ab dependence of tumor prevention in NeuT-μMT mice

The bimodal distribution of Ab responses among vaccinated NeuT-μMT mice prompted us to re-evaluate the survival curves shown in Fig. 1. A clear difference in survival became evident when vaccinated NeuT-μMT mice were stratified according to their Ab response (Fig. 5). Mice that did not produce Igs in response to vaccination were prone to early onset of mammary carcinoma with a slight delay in comparison to untreated mice. On the contrary, tumor onset was significantly delayed in vaccinated NeuT-μMT mice able to mount a sizeable Ab response (Fig. 5). The analysis of whole-mount preparation of mammary glands before the onset of macroscopic carcinomas (16-wk-old mice) showed the presence of diffuse atypical hyperplasia and occasional in situ carcinomas in both NeuT and Ab-deficient NeuT-μMT mice, whereas in the mammary glands of Ab-proficient NeuT-μMT mice, hyperplastic nodules were rare and in situ carcinomas were absent (Fig. 6).

IFN-γ production by NeuT-μMT lymphocytes

The survival curves and tumor multiplicities of Ab-deficient NeuT-μMT clearly showed a residual protection from mammary carcinoma that was completely absent in NeuT-IFN-γ−/− mice (compare Figs. 1 and 5). IFN-γ, besides its well-known activities on B and T cells, can directly inhibit the growth of preneoplastic and neoplastic mammary cells (12); moreover, it can hamper tumor growth through the induction of the antiangiogenic chemokines monokine induced by IFN-γ and IFN-γ-inducible protein 10.
We found that IFN-γ release by spleen cells of vaccinated NeuT-μMT mice was clearly detectable in vitro, although at a level lower than that of vaccinated NeuT mice (Fig. 7). After in vitro restimulation with the vaccine, splenocytes from both NeuT and NeuT-μMT mice released comparable amounts of IFN-γ. The residual protection from mammary carcinoma produced by vaccination in Ab-negative NeuT-μMT mice provides an estimate of the relative importance of non-B cell-mediated effects of IL-12 and IFN-γ in this system.

**Specificity, isotype, and activity of Abs elicited by vaccination in NeuT-μMT mice**

Mammary carcinogenesis was significantly delayed by vaccination in Ab-proficient NeuT-μMT mice; however, all mice eventually succumbed to progressive tumors, unlike NeuT mice that remained tumor free for >1 year. This suggests that the Ab response of B cell-deficient NeuT-μMT mice was lacking in comparison with that of NeuT mice. We have already shown that anti-vaccine Ab level of NeuT-μMT mice was quantitatively inferior to that of NeuT mice (Fig. 3). Western blot analysis of Ab specificity showed that sera from Ab-proficient NeuT-μMT mice specifically recognized and immunoprecipitated p185neu molecules from HER-2/neu-positive transgenic cell lysates; however, a strong quantitative difference was evident in comparison to sera from NeuT mice (Fig. 8A). Sera from Ab-proficient NeuT-μMT mice showed a marginal activity against denatured p185 neu, and sera from Ab-deficient mice were again negative (Fig. 8B), thus indicating the absence of Abs against cryptic determinants. We never observed a significant difference between sera of untreated and vaccinated mice when tested on cell lysates of p185 neu-negative cells.

We then studied the spectrum of Ig isotypes produced by NeuT, NeuT-IFN-γ-/-, and NeuT-μMT mice in response to vaccination (Fig. 9). We reported previously (12) that the absence of tumor prevention in NeuT-IFN-γ-/- mice was associated with the lack of IgG2a, IgG2b, and IgG3 subclasses, whereas IgG1 production was similar to that of NeuT mice. The isotype spectrum of NeuT-μMT mice was clearly distinct from that of NeuT-IFN-γ-/-; however, it showed some subtle differences in comparison with NeuT
mice. In particular, both IgG2a and IgG2b were produced by NeuT-μMT mice, but the IgG2a:IgG2b ratio was reversed in comparison with NeuT mice. IgG3 production was low in vaccinated Neu mice, with the lowest levels in NeuT-IFN-κ µMT mice.

Abs against p185nu may affect tumor growth in several ways, including direct inhibition of p185nu complement-mediated cytotoxicity, and ADCC (22-24). In vitro exposure of Neu/H-2q cells to sera from vaccinated mice did not affect p185nu phosphorylation status (data not shown), but determined a decrease up to 50% of total p185nu level. This is in agreement with the increased ubiquitin-mediated degradation induced by neu-specific Abs (22). The study of complement-mediated cytotoxicity mediated by sera of vaccinated mice, showed a decreased activity in sera from immunodeficient mice, with the lowest levels in NeuT-IFN-γ (-/+) (Fig. 10A). Only sera from immunocompetent NeuT mice determined significant levels of ADCC (Fig. 10B).

Finally, a conspicuous hole was found in the repertoire of Abs induced by vaccination in NeuT-μMT mice. The vaccine contains MHC-allogeneic mammary carcinoma cells; therefore, it is expected to induce, in addition to p185-specific Abs, other Abs recognizing H-2q class I glycoproteins and other Ags (e.g., mammary tissue-specific Ags). NeuT-μMT mice, at variance with Neu mice, failed to produce Abs recognizing a p185-negative, H-2q class I-positive mammary carcinoma clone (Fig. 11A) deriving from a HER-2/nu transgenic mammary carcinoma (14). In complement-mediated cytotoxicity tests, sera of Ab-proficient NeuT-μMT mice did not lyse allogeneic lymphocytes of the H-2q haplotype (Fig. 11B). This indicates that the allogeneic Ab response of NeuT-μMT mice is defective, and suggests that the response against subdominant Ags present in the vaccine could be also impaired.

**Discussion**

HER-2/nu transgenic mice lacking either IFN-γ or B cells were not protected from mammary carcinoma by a highly effective vaccination combining IL-12 and MHC allogeneic cells expressing the p185nu Ag.

IFN-γ release was the most conspicuous T cell response present in vaccinated HER-2/nu transgenic mice, in the absence of detectable CTL (12). The complete disappearance of protection from mammary carcinoma in transgenic mice lacking IFN-γ confirmed the pivotal role of this cytokine. Morphological data indicated that IFN-γ production by reactive cells was fundamental to elicit an efficient antitumor response (12). Its absence, in fact, resulted in a scarce intratumoral recruitment of reactive cells, which were also unable to produce proinflammatory cytokines and chemokines (data not shown). Inhibition of tumor neangiogenesis, mainly induced by IL-12-elicited IFN-γ, contributed to the effectiveness of the combined treatment, because the vascular network supplying...
mammary hyperplasia in NeuT or carcinomas in NeuT-μMT mice was reduced in comparison with untreated NeuT and treated NeuT-IFN-γ−/− mice.

However, from a heuristic point of view, the pleiotropic action of IFN-γ, which is simultaneously a regulatory molecule in most antitumor immune responses and a direct effector acting on tumor cells themselves, did not allow a clear dissection of further immune mechanisms.

We found that different immune treatments were able to delay mammary carcinogenesis (25), but the jump from delay to effective prevention of carcinogenesis was invariably accompanied by the appearance of anti-p185neu Abs (6, 12), thus indicating a fundamental role for the B cell response in combination with T cell-derived cytokines (8, 22, 26). Abs directed against membrane oncoproteins like p185neu may play multiple antitumor roles that include block of mitogenic signal transduction through inhibition of receptor dimerization and induction of internalization and recycling along with immune-mediated functions like complement-mediated cytotoxicity and ADCC (8, 12, 22–24, 27, 28).

To formally demonstrate the role of Abs in cancer prevention, we vaccinated B cell-deficient, HER-2/neu transgenic NeuT-μMT mice. To our surprise, two thirds of NeuT-μMT mice responded to vaccination with a copious production of specific Abs of various Ig subclasses. This result is in general agreement with recent findings that μMT mice of BALB/c background are capable of mounting “natural” and Ag-induced Ab responses (20, 21). In μMT mice, Abs are produced by plasma cells deriving from B cells that completed development either via the expression of IgD or possibly through premature switching to IgG1 or other isotypes. It has been reported that immunization with either T cell-dependent or -independent Ags induces Ab responses only in about one third of μMT mice (20), whereas our vaccine was able to induce specific Abs in 100% of μMT mice. This finding points to the great potency of this combination of IL-12, allogeneic MHC, and p185neu.

At variance with μMT mice, NeuT-μMT mice were not uniformly able to mount Ab responses to our vaccine. HER-2/neu transgenic mice are tolerant to the p185neu protein (9); thus, the difference in the proportion of vaccine responses between μMT and NeuT-μMT mice may result from the immunological tolerance of the latter. This dichotomous behavior of NeuT-μMT mice in response to our vaccine was exploited to investigate long-term prevention of mammary carcinoma in the absence of Ab response. Without protective Abs, the preventive effect of the vaccine vanished, whereas a high Ab response went along with a delayed onset of mammary carcinomas.

The mean latency of tumors in vaccinated, Ab-deficient NeuT-μMT mice was about 1 month longer than in untreated mice. A similar delay (3 wk) was induced by the administration of IL-12 alone, without allogeneic cells. These data can be compared with the complete inefficiency of vaccination in NeuT-IFN-γ−/− mice, allowing us to dissect immune-mediated from direct antitumor effects of IFN-γ. It could be concluded that the major contribution of IFN-γ in the prevention of mammary carcinoma was its activity of immune mediator, in particular for what concerns the induction of a switch to Th1-type Igs. On the contrary, direct antitumor activities, such as the induction of antiangiogenic chemokines (monokine induced by IFN-γ and IFN-γ-inducible protein 10), or the inhibition of preneoplastic cell proliferation, in the absence of Abs, produced only a short delay in tumor appearance.

Vaccination induced a significant delay of mammary carcinogenesis in Ab-proficient NeuT-μMT mice, but all mice eventually developed tumors. By contrast, NeuT mice were completely protected by vaccination. This suggests that the B cell deficit extant in Ab-proficient NeuT-μMT mice precluded a complete prevention of mammary carcinoma, a fact that could shed further light on the nature of protective Abs. As mentioned above, quantitative differences in Ab titers may play a role in lowering the protection. Even Ab-proficient NeuT-μMT mice had significantly lower levels of anti-p185neu Abs than NeuT mice. Major qualitative differences among NeuT, NeuT-IFN-γ−/−, and NeuT-μMT were evident in the IgG subclasses elicited by vaccination. An abundant IgG1 response in NeuT-IFN-γ−/− mice, which were not protected by vaccination, indicates that the γ1 chain was not involved in prevention. NeuT-IFN-γ−/− mice lacked anti-vaccine IgG2a and IgG2b, two Ab subclasses that were produced both by NeuT and by Ab-proficient NeuT-μMT mice. In NeuT mice, the IgG2a/IgG2b ratio was skewed in favor of IgG2a, whereas in NeuT-μMT, the reverse was true. It has been shown that, in the absence of μ-chains, the γ2b chain can support B cell development (29, 30); thus, the imbalance found in NeuT-μMT mice could be the consequence of the alternative pathways leading to B cell survival and differentiation in this knockout mouse (20). From the point of view of cancer prevention, the imbalance found in NeuT-μMT suggests that a response skewed to IgG2a might provide a better protection from mammary carcinoma. IgG3 were induced by vaccination in NeuT mice, but not in NeuT-IFN-γ−/− or in NeuT-μMT mice. Because μMT mice are specifically deficient in IgG3 production (20, 21), the NeuT-μMT model could not be used to assess the relative importance of this isotype in cancer prevention.

An intriguing alteration in the specificity of nonprotective, “by-stander” Abs was evident. We found that vaccination induced protective Abs directed against p185neu both in NeuT and in NeuT-μMT mice; thus, we believe that quantitative and isotypic differences discussed above explain the lower degree of cancer prevention obtained in Ab-proficient NeuT-μMT mice. However, the combined vaccine is expected to induce a wide range of Abs recognizing determinants other than p185neu. In particular, the expression of allogeneic MHC molecules by cells in the vaccine
should elicit anti H-2^a class I Abs. Such Abs are a byproduct of preventive efficacy, because mice receiving IL-12 plus allogeneic cells lacking p185^{new} are not protected from mammary carcinoma (12). The very low level of anti-class I Abs found in vaccinated, Ab-proficient NeuT-\(\mu\)MT mice indicates a profound alteration in the ability to respond to multiple Ags. A possible explanation is that the selection caused by the p185^{new} Ag irreversibly shapes the development of B cells in deficient NeuT-\(\mu\)MT mice (31, 32).

In conclusion, we showed that B cell responses regulated by IFN-\(\gamma\) were a key mechanism mediating prevention of mammary carcinoma in mice vaccinated with allogeneic tumor cells and IL-12. This conclusion is important both to direct further investigation of the B cell-mediated mechanisms at work in the HER-2/new system (e.g., Ag presentation, cytokine release, complement-mediated cytotoxicity, ADCC, direct down-modulation of p185^{new}) and to design similar attempts at preventing cancer in humans.

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