Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas

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Since the mechanisms by which specific immunity destroys *Her-2/neu* carcinoma cells are highly undetermined, these were assessed in BALB/c mice vaccinated with plasmids encoding extracellular and transmembrane domains of the protein product (p185^{neu}) of the rat *Her-2/neu* oncogene shot into the skin by gene gun. Vaccinated mice rejected a lethal challenge of TUBO carcinoma cells expressing p185^{neu}. Depletion of CD4 T cells during immunization abolished the protection, while depletion of CD8 cells during the effector phase halved it, and depletion of polymorphonuclear granulocytes abolished all protection. By contrast, Ig μ-chain gene KO mice, as well as Fcγ receptor I/III, β-2 microglobulin, CD1, monocyte chemoattractant protein 1 (MCP1), IFN-γ, and perforin gene KO mice were protected. Only mice with both IFN-γ and perforin gene KOs were not protected. Although immunization also cured all BALB/c mice bearing established TUBO carcinomas, it did not cure any of the perforin KO or perforin and IFN-γ KO mice. Few mice were cured that had knockouts of the gene for Ig μ-chain, Fcγ receptor I/III, IFN-γ, or β-2 microglobulin. Moreover, vaccination cured half of the CD1 and the majority of the MCP1 KO mice. The eradication of established p185^{neu} carcinomas involves distinct mechanisms, each endowed with a different curative potential.

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Introduction

Many experimental and human tumors express tumor-associated antigens (TAAs) that are recognized by T cells (1) and antibodies (2). The role of T lymphocytes in reacting against TAAs in the eradication of established tumors (3) is widely documented. In most cases, tumor inhibition rests on the direct lytic activity of cytotoxic CD8 T lymphocytes (1–4), whereas CD4 T cells activated through the indirect presentation of TAAs by antigen-presenting cells (APCs) are required to support a CD8 T cell response (5, 6). Tumor eradication also depends on noncytotoxic CD4 (7) and CD8 (6) lymphocytes releasing high amounts of proinflammatory cytokines that recruit distinct effector leukocytes at the tumor site. This series of events gives rise to a complex delayed-type hypersensitivity reaction in the tumor site that involves several distinct cell types (8).

Most experimental evidence supports the notion that tumor eradication rests predominantly, if not exclusively, on T lymphocyte reactivity (9). However, the importance of antibody in the inhibition of solid tumors is not clearly defined and remains controversial. Some experimental data have shown that antibodies might enhance tumor growth by hindering the cell-mediated mechanisms on which tumor inhibition may depend (10, 11). Spontaneous anti-TAA antibodies are frequently detected in the serum of cancer patients (2, 12, 13). Although their titer may increase with the progression of the tumor (14), this rise actually correlates with a poor prognosis (15). Moreover, the presence of B cells and a nonprotective antitumor humoral immune response may inhibit the induction of protective T cell–dependent antitumor immunity (16).

On the other hand, the enhanced TAA cross-presentation that takes place in the presence of antibodies (17), as well as the binding of anti-TAA antibodies to the Fcγ receptors (FcγRs) on APC surfaces (18), may result in the eradication of established tumors in synergy with CD8 T cells (19). In addition, antibodies can sensitize tumors for complement and antibody-dependent cytotoxicity, or they may activate antibody-dependent cellular cytotoxicity (ADCC) through their binding to FcγR on leukocyte membrane (20, 21). Furthermore, the results of several vaccination trials have shown that improved survival correlates with the induction of antibodies to TAA (22, 23), and noticeable inhibitions of tumor progression can be obtained through the passive administration of mAb (24, 25).

Besides the activation of immunological functions, antibodies directed against oncogenic tyrosine kinase receptors directly impair the proliferative activity of the target cells. Among these receptors, the protein product of the *Her-2/neu* oncogene (p185[neu]) has been studied extensively. Antibodies may induce a p185[neu] functional block (26), downregulate its expression on the cell membrane, and impede its ability to form the homo or heterodimers that spontaneously transduce proliferative signals to the cells (26–28). Antibodies hamper the three-dimensional growth of *Her-2/neu* mouse mammary carcinoma cells (29) and play an important role in delaying the onset of mammary carcinomas in *Her-2/neu* transgenic mice (27, 30–32). However, the importance of reaction mechanisms dependent on anti-p185[neu] antibodies in the resistance to a p185[neu] tumor is controversial. Although in a few systems antibodies appear to play an important role in the rejection of p185[neu] tumors (33, 34), in others T cell reactivity appears to be sufficient for tumor rejection (35–38).

In this study, the relative contribution of antibody-mediated reactivity and cell-mediated reactivity was assessed in the rejection of a lethal challenge and the eradication of an established carcinoma formed by a transplantable cell line (TUBO cells) expressing rat p185[neu] (r-p185[neu]) (30). In this experimental system, r-p185[neu] is both a xenogeneic surrogate TAA that differs from mouse p185[neu] in less than 6% of the amino acid residues (39) and a membrane receptor that directly regulates the oncogenic behavior of TUBO cells in vitro and in vivo (29, 30). WT and various immunodeficient BALB/c mice were immunized with DNA plasmids coding for the extracellular and transmembrane domains of r-p185[neu] (p185 plasmids) (30) shot into the skin by a "gene gun" (40). Gene gun DNA immunization is an efficient way to elicit tumor immunity through antigen cross-presentation (40–43). After gene gun vaccination, distinct cellular and humoral immune mechanisms independently led to the rejection of the lethal TUBO cell challenge. By contrast, only the combination of several cellular and humoral mechanisms led to the eradication of established tumors.

Methods
Mice. In these experiments, 7-week-old virgin female WT and gene KO BALB/c mice (H-2[d]) were used. WT

BALB/cnAnCr (BALB/c) mice were from Charles River Laboratories (Calco, Italy); mice with the Ig μ heavy-chain gene KO (16) (BALB-μIgKO) were kindly provided by Thomas Blankenstein (Freie University, Berlin, Germany); mice with the FcγRI/III gene KO (44) (BALB-FcγRI/IIIKO) were from Taconic Laboratories (Germantown, New York, USA); mice with the β2-microglobulin gene KO (BALB-β2mKO) (45) and the IFN-γ gene KO (BALB-IFNγKO) (46) were from Jackson Laboratories (Bar Harbor, Maine, USA); mice with the CD1 gene KO (47) (BALB-CD1KO) were kindly provided by Luc Van Kaer (Vanderbilt University, Nashville, Tennessee, USA); mice with the MCP-1 gene KO (48) were kindly provided by Barrett Rollins (Dana Farber Institute, Boston, Massachusetts, USA); mice with the perforin (pfp) gene KO (BALB-pfpKO) and the IFN-γ gene double KO (49) (BALB-IFNγ-pfpKO) were from the Peter MacCallum Cancer Institute (East Melbourne, Australia). Mice were treated properly and humanely in accordance with European Community guidelines. When required, 22, 18, 14, 8, and 5 days before tumor challenge (day 0) or 2, 5, 8, 11, and 13 days after, a few mice received intraperitoneal injections of 0.2 ml of HBSS containing a 1:20 dilution of antiasialo GM1 rabbit anti-serum (Wako Chemicals GmbH, Dusseldorf, Germany) or 200 μg of anti-CD4 (GK1.5 hybridoma, L3T4, American Type Culture Collection, Rockville, Maryland, USA), anti-CD8 (TIB-105 hybridoma, Lyt 2 ATCC), or anti-polymorphonuclear cell (PMN) mAb (RB6-8C5 hybridoma kindly provided by R.L. Coffman, DNAX Inc., Palo Alto, California, USA). Flow cytometry of residual blood and spleen cells collected 3 days after the last injection showed that target leukocytes were selectively decreased to below 1 per 5000 cells.

Cell lines. TUBO cells (30) are a cloned line derived from a BALB/c mice transgenic for the transforming *r-Her-2/neu* oncogene (BALB-NeuT). N202.1A and N202.1E (32) are cloned cell lines derived from a FVB mice (H-2[q]) transgenic for the *r-Her-2/neu* proto-oncogene (FVB-NeuN) (32). F1F is a newborn WT BALB/c mouse–derived skin fibroblast line spontaneously transformed after the 15th in vitro passage (6). F1F were transfected with the full-length r-Her-2/neu gene (F1F-neu) cloned into pCDNA3 (Invitrogen, San Giuliano Milanese, Italy) or transduced with the β-galactosidase gene (F1-βgal) by means of the LBSN retroviral vector as described previously (50). r-p185[neu] is highly expressed on the cell membrane of TUBO (30), N202.1A (32), and F1F-neu cells (data not shown), but it is absent on N202.1E (32), TSA mammary carcinoma cell line (30), F1F (30), and F1F-βgal (data not shown) cell surfaces. H-2 D[d] and H-2 D[q] glycoproteins are expressed by all BALB/c and FVB tumors, respectively (30, 32), but no cell lines expressed H-2 class II glycoproteins (data not shown). N202.1A, N202.1E, TSA, F1F, F1F-βgal, and F1F-neu cells were cultured in RPMI 1640 medium supplemented with 10% FBS, and TUBO cells were cultured in DMEM with 20% FBS. Neomycin (800 μg/ml) was added to the culture medium of F1F-βgal and F1F-neu cells. Media were from BioWhittaker Europe (Verviers, Belgium), FBS was from Life

Technologies (San Giuliano Milanese, Italy), and neomycin was from Life Technologies (Milan, Italy).

Tumor challenge. Mice were challenged subcutaneously in the right flank with 0.2 ml of a single suspension containing the minimal lethal dose of TUBO (10^5) (30), F1F (10^4) (6), F1F-βgal (10^6), and F1F-neu (5×10^3) cells. The cages were coded, and the incidence and the growth of tumors were evaluated twice weekly in a blind fashion. Neoplastic masses were measured with calipers in the two perpendicular diameters, and the mean diameter was recorded for 200 days. At the end of this period, tumor-free mice were classed as survivors. Mice were killed for humane reasons when the tumor exceeded 10 mm in mean diameter.

DNA expression vectors and vaccination. The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, California, USA) by deleting the SV40 promoter, the neomycin resistance gene, and SV40 poly(A). The sequence for the extracellular and transmembrane domains of mutated r-p185^{neu} were generated from the PCR product using primers 3′-CGCAAGCTTCATCATG-GAGCTGGC-5′ and 3′-CGGAATTCGGGCTGGCTCTCT-GCTC-5′ and primers 3′-CGCAAGCTTCATGGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-5′, respectively, as previously described in detail (30). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hind*III and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the plasmid used in this work (p185 plasmid). *Escherichia coli* strain DH5α was transformed with p185 plasmid and then grown in Luria-Bertani medium (Sigma-Aldrich, St. Louis, Missouri, USA). Large-scale preparation of the plasmid was conducted by alkaline lysis using Endofree Quiagen Plasmid-Giga kits (Quiagen, Chatsworth, California, USA). DNA was then precipitated, suspended in sterile saline at a concentration of 1 mg/ml, and stored in aliquots at –20°C for subsequent use in immunization protocols.

Gene gun plasmid administration. Mice were vaccinated with empty plasmids or p185 plasmids shot intradermally by a hand-held Helios Gene Gun system (Biorad, Hercules, California, USA) (40). Plasmids were precipitated onto 1.6-μm gold particles. Coated particles were then washed in absolute ethanol and inserted into a Tefzel tube (Biorad) that was cut into 0.5-inch cartridges. In this way, 0.25 mg of gold coated with 0.465 μg of plasmid DNA was administered by each shot cartridge. Each vaccination consisted of three shots (2.79 μg of plasmid DNA per shot) administered with a 400-psi helium gas pulse in three distinct areas of shaved abdominal skin. In prevention experiments, mice were vaccinated on days 21 and 7 before tumor challenge (day 0). In other experiments, mice were first vaccinated when they presented a palpable tumor mass of 2, 4, or 6 mm in mean diameter and were boosted 7 days later.

Cellular cytotoxicity. CTLs were generated by culturing 10^7 responder lymphocytes and 5×10^5 stimulator TUBO cells pretreated with 40 mg/ml of mitomycin-C for 40 minutes for 6 days (6). Cytotoxicity was assayed in a 48-hour [^{3}H]thymidine release assay (30) by mixing various concentrations of effector cells with 5×10^3 labeled target cells at 50:1, 25:1, 12:1, and 6:1 effector/target ratios in round-bottom 96-well microtiter plates in triplicate as described previously. The values were expressed as the percentage of specific lysis (6).

Cytofluorometric evaluation of anti–r-p185^{neu} antibodies. Sera of six WT BALB/c mice shot with empty vector or p185 plasmid were collected 2 weeks after the first vaccination and pooled. The ability of sera to bind r-p185^{neu} was evaluated by flow cytometry. N202.1A or N202.1E cells (2×10^5) from in vitro cultures were washed twice with cold PBS supplemented with 2% BSA and 0.05% sodium azide and stained in a standard indirect immunofluorescence procedure with 50 μl of a 1:10 dilution of control or immune sera in PBS-azide-BSA. A fluorescein-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) was used as a second step Ab. The cells were resuspended in PBS-azide-BSA containing 1 mg/ml of propidium iodide to gate out dead cells and evaluated with a FACScan (Becton Dickinson and Co., Mountain View, California, USA). The specific N202.1A binding potential of the sera was calculated according to the following equation: ([% positive cells with test serum][fluorescence mean]) × serum dilution. A total of 5×10^3 viable cells were analyzed in each evaluation.

Morphological analysis. Groups of three WT BALB/c and KO mice were sacrificed at the following times: WT BALB/c mice vaccinated before tumor cell challenge were sacrificed 3 and 7 days after the last shot, and groups of WT BALB/c and KO mice shot when the tumor reach 2 mm in mean diameter were sacrificed each week beginning the second week after the first shot until the end of the experiment. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa and the trichrome method. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 minutes with antidendritic cells (NLDC 145; Cederlane, Hornby, Ontario, Canada), anti-CD4, anti-CD8a (both from Sera-Lab, Crawley Down, Sussex, United Kingdom), anti-Mac-1 (anti-CD11b/CD18), anti–Mac-3, anti-Ia (all from Boehringer Mannheim, Milan, Italy), anti-PMN leukocytes (RB6-8C5, provided by R.L. Coffman), antiasialo GM1 (Wako Chemicals GmbH), antiendothelial cells (mEC-13.324), anti–ELAM-1 (E selectin; both provided by A. Vecchi, Istituto M. Negri, Milan, Italy), anti-ICAM-1 (CD54), anti–VCAM-1, anti–IL-4, anti–IL-6 (PharMingen, San Diego, California, USA), anti-IL-1β (Genzyme, Cambridge, Massachusetts, USA), anti–TNF-α (Immuno Kontact, Frankfurt, Germany), anti-IFN-γ (provided by S. Landolfo, University of Turin), and TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, California, USA) Ab's. To evaluate the expression of r-p185^{neu}, paraffin-embedded sections were tested with anti-p185^{neu} antibodies (C-18-G, Santa Cruz Biotechnology). After washing, sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Laboratories, Burlingame, California,

Table 1
Specificity of the reaction elicited by *Her-2/neu* plasmids shot into WT BALB/c mice by gene gun

Immunization with BALB/c mice:	Tumor takes per mouse after a challenge with the indicated cell line:			
	TUBO (p185[neu] positive)	F1F (p185[neu] negative)	F1F-βgal (p185[neu] negative)	F1F-neu (p185[neu] positive)
Empty vector	10/10 (0%)	ND	ND	5/5 (0%)
p185 plasmid	0/16 (100%)	6/6 (0%)	5/5 (0%)	0/5 (100%)

WT BALB/c mice were immunized twice with p185 plasmid or empty vector 21 and 7 days before tumor challenge (day 0) with tumor cells expressing (TUBO and F1F-neu) or not expressing (F1F and F1F-βgal) p185neu. Tumor takes and percentage of protected mice were evaluated for 100 days after challenge. A significant protection was observed against TUBO and F1F-neu cells in mice vaccinated with p185 plasmids ($P < 0.001$, Yates's corrected χ^2 test). ND, not done.

USA) for 30 minutes. Unbound antibody was removed by washing, and the slides were incubated with ABC complex/AP (Dako). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blinded fashion. From mice with multiple tumors, one sample per tumor growth area and 10 randomly chosen fields in each sample were evaluated for each point determination. Positive cells were counted under a ×400-field microscope (×40 objective and ×10 ocular lens, 0.180 mm^2 per field). The expression of adhesion molecules, cytokines, and mediators was defined as absent (–) or scarcely (±), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding Ab (30, 32).

Statistical analysis. Differences in tumor takes were evaluated by Yates' corrected χ^2 test, and differences in the number of tumor-infiltrating cells were evaluated by the two-tailed Student's *t* test.

Results

WT BALB/c mice do not react against r-p185[neu] expressed by tumor cells. In mice, r-p185[neu] is a xenogeneic surrogate TAA, since it differs from mouse p185[neu] in less than 6% of the amino residues (39). Despite this difference, the TUBO and F1F-neu cell challenges grew progressively in all WT BALB/c mice (Table 1), and their growth kinetics were not different from those observed in transgenic BALB-NeuT mice, from which TUBO cells were derived (30). During TUBO and F1F-neu tumor growth, no anti–r-p185[neu] antibodies were detectable in the sera of WT BALB/c mice, nor were CTL activity or cytokine release found when splenocytes from TUBO- and F1F-neu–bearing WT BALB/c mice were restimulated in vitro with TUBO cells (30) (data not shown). In addition, the reactive cell infiltrate associated with the growth of TUBO and F1F-neu tumors in both WT BALB/c and BALB-NeuT mice was equally marginal and not significantly different from that associated with the fully syngeneic F1F and TSA tumors (data not shown).

Gene gun DNA vaccination protects WT BALB/c mice against an r-p185[neu] tumor challenge. Despite the ostensible immunological invisibility of r-p185[neu] expressed by proliferating tumor cells, both TUBO and F1F-neu tumor cell challenges were specifically rejected by WT BALB/c mice preimmunized with p185 plasmids administered 21 and 7 days before challenge (Table 1).

TUBO- and F1F-neu tumor cells first formed small aggregates that were quickly and massively infiltrated by reactive leukocytes consisting of dendritic cells, macrophages, PMNs, CD8 and CD4$^+$ lymphocytes, and NK cells. The prevalence of CD8 lymphocytes and PMNs was about fivefold that of TUBO tumors growing in untreated mice. In addition, a relevant expression of endothelial adhesion molecules (ICAM-1, ELAM-1, and VCAM-1) and cytokines (IL-1β, TNF-α, IFN-γ, and IL-4) was observed. Expression of r-p185[neu] in tumor cells was mainly confined to the cell cytoplasm (data not shown).
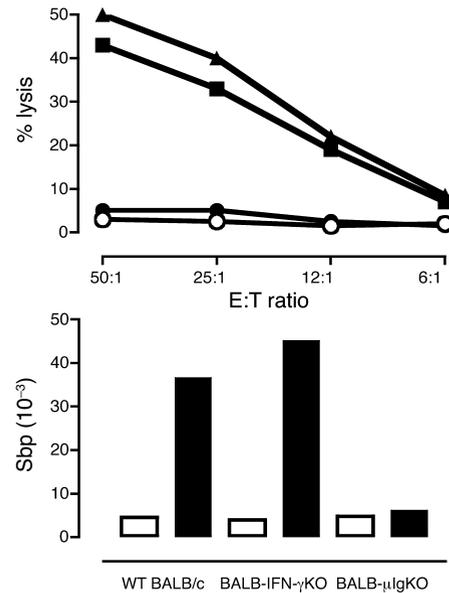
Figure 1
Cytotoxic and antibody response to p185[neu] of WT BALB/c mice shot at 2 week intervals with p185 plasmids. Spleens and sera were collected 2 weeks after the last immunization. The upper panel shows cytotoxicity of splenocytes evaluated in a 48-hour [^{3}H]thymidine release assay against TUBO (squares, H-2^d, p185[neu] positive), F1F-neu (triangles, H-2^d, p185[neu] positive), N202.1A (filled circles, H-2^q, p185[neu] positive); and F1F (open circles, H-2^d, p185[neu] negative) targets. No significant cytotoxicity was found in mice shot with empty plasmids (data not shown). The lower panel shows the specific binding potential titer of anti-p185[neu] Ab in a pool of sera from WT BALB/c, BALB-IFNγKO, and BALB-μIgKO mice shot with empty (white bars) or p185 (black bars) plasmids.

Vaccination with p185 plasmids elicited both significant cytolytic activity restricted to r-p185^{neu} H-2 D^d target cells (Figure 1, upper panel) and high titers of anti–r-p185^{neu} antibodies (Figure 1, lower panel). The memory elicited was still able to protect all mice when the TUBO cell challenge was performed 140 days after the second plasmid administration by gene gun (data not shown).

Immune mechanisms leading to the rejection of an r-p185^{neu} tumor challenge. To assess the relative weight of immune mechanisms elicited by gene gun vaccination, both WT BALB/c mice with depletions in selected leukocyte populations and BALB/c mice with null mutations in genes regulating immunological functions were challenged with TUBO cells (Table 2). The depletion of CD4 cells during the induction of the response, but not during the effector phase, abolished any capacity to reject the tumor challenge. Similarly, the removal of CD8 lymphocytes and PMNs during the effector phase (though not during the immunization) strongly impaired the capacity of immunized mice to reject TUBO cells. These selected leukocyte depletions did not influence TUBO cell growth in nonimmunized mice (data not shown).

Irrespective of the missing gene, TUBO cells grew out in all nonimmunized BALB/c KO mice but were rejected by all mice that had been immunized. Only in mice lacking both functioning pfp and IFN-γ genes and in mice with depletion of PMNs was there a complete failure to elicit protective immunity. In immunized mice, PMN antitumor activity can be triggered and guided by cytokines released by T cells and antibody (20, 51). Even when immunized mice were challenged with larger amounts, the disappearance of TUBO cells was so quick as to preclude insight into the pathological cellular events involved.

Gene gun DNA vaccination eradicates established r-p185^{neu} carcinomas. To assess the curative potential of DNA gene gun vaccination, WT BALB/c mice bearing established 2-, 4-, and 6-mm mean diameter TUBO carcinomas received the two plasmids shots 7 days apart (Figure 2). The 2-mm mean diameter solid carcinomas were measurable between 8 and 12 days after the TUBO cell challenge. In all mice untreated (Figure 2, d–f) or shot with the empty vector (Figure 3a), masses grew progressively, giving rise to large, p185^{neu}-positive carcinomas with a moderate leukocyte infiltrate associated with a slight production of proinflammatory cytokines (Table 3 and Figure 3, a, c, e, g, and i). By contrast, in mice shot with p185 plasmid tumor, masses grew for the first 20 days and then started to shrink, and no tumor was palpable 50 days after the first shot (Figures 2a and 3b). A marked infiltration by several leukocyte populations (Table 3 and Figure 3h) expressing high levels of IL-1, IL-4, IL-6, and TGF-β1 (Table 3 and

Table 2
Ability of p185 plasmid–vaccinated WT BALB/c mice with immunosuppression by mAb administration and immune-related gene KOs to reject a lethal TUBO challenge

	mAb immunosuppression				
Mice	mAb specificity	Time of administration	p185 plasmid vaccination	Tumor takes per challenged mouse	
WT BALB/c	none	—	–	15/15	0%
WT BALB/c	none	—	+	0/15	100%
WT BALB/c	anti-CD4 mAb during immunization		+	10/10	0%
WT BALB/c	anti-CD4 mAb	after challenge	+	0/10	100%
WT BALB/c	anti-CD8 mAb during immunization		+	0/10	100%
WT BALB/c	anti-CD8 mAb	after challenge	+	5/10	50%
WT BALB/c	anti-PMN mAb during immunization		+	0/8	100%
WT BALB/c	anti-PMN mAb	after challenge	+	8/8	0%
BALB-μIgKO	—	—	–	5/5	0%
BALB-μIgKO	—	—	+	0/5	100%
BALB-FcγRI/IIKO	—	—	–	8/8	0%
BALB-FcγRI/IIKO	—	—	+	0/6	100%
BALB-β2mKO	—	—	–	5/5	0%
BALB-β2mKO	—	—	+	0/6	100%
BALB-CD1KO	—	—	–	5/5	0%
BALB-CD1KO	—	—	+	0/5	100%
BALB-MCP1KO	—	—	–	5/5	0%
BALB-MCP1KO	—	—	+	0/5	100%
BALB-IFNγKO	—	—	–	5/5	0%
BALB-IFNγKO	—	—	+	0/5	100%
BALB-pfpKO	—	—	–	5/5	0%
BALB-pfpKO	—	—	+	0/5	100%
BALB-IFNγ-pfpKO	—	—	–	5/5	0%
BALB-IFNγ-pfpKO	—	—	+	5/5	0%

Untreated WT BALB/c and WT BALB/c mice that received Ab 22, 18, 14, 8, and 5 days before challenge or 2, 5, 8, 11, and 13 after challenge or KO BALB/c mice were immunized with p185 plasmid or empty vector 21 and 7 days before tumor challenge (day 0). Tumor takes and percentage of protected mice were evaluated for 100 days after challenge.
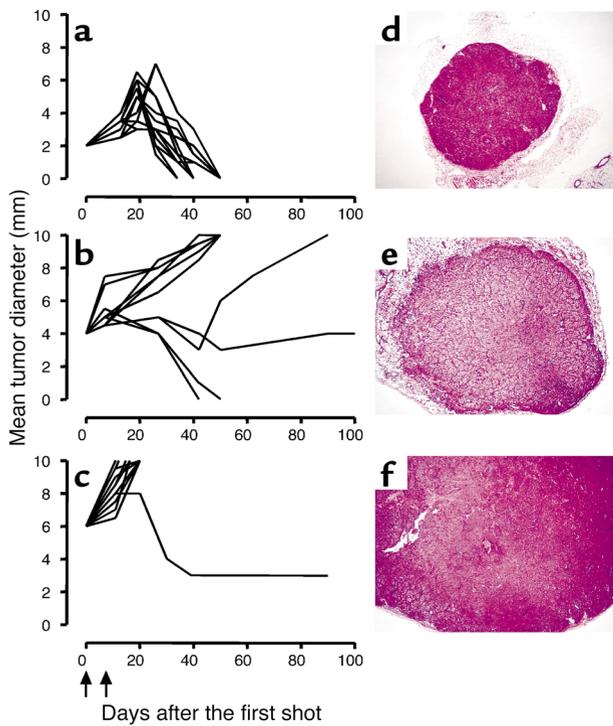
Figure 2

Ability of gene gun vaccination to cure established TUBO carcinomas. The left panels show growth and regression of 2-mm (**a**), 4-mm (**b**) or 6-mm (**c**) mean diameter carcinomas after two p185 plasmid shots (arrows). No modulation of tumor growth was observed after shots with empty plasmids (data not shown). The right panels show histological stages of the tumors. When the first shot was performed, the 2-mm carcinomas (**d**) consisted of well-defined neoplastic lobules surrounded by a delicate stroma. The 4-mm carcinomas (**e**) consisted of an established mass invading the subcutaneous fibroadipose tissue and almost completely occupying the microscope field. The 6-mm carcinomas (**f**) could no longer be fully contained within the microscope field. These carcinomas show an expansive and invasive growth pattern. Magnification, ×25.

pathologically examined 100 days after the first shot. In one mouse the tumor grew, shrank, and regrew, whereas the p185[neu]-expressing tumors of the other seven mice grew progressively (Figure 2b).

No evidence of a reduced growth rate was found for 11 of the 12 mice that received the first shot of p185 plasmid when the tumor was 6 mm in mean diameter, 25 days after challenge. Unfortunately, these mice had to be sacrificed for humane reasons within 16–25 days after the first shot, possibly before the immune reaction was fully established. In one mouse, the tumor

Figure 3j) was associated with ICAM-1, ELAM-1, and VCAM-1 expression by the activated endothelial cells of tumor microvessels. Residual neoplastic cells displayed low and cytoplasm-confined expression of r-p185[neu] (Figure 3f). As the rejection progressed, an extremely marked fibrotic reaction led to complete disintegration and replacement of the tumor mass (Figure 3d).

Four-millimeter mean diameter TUBO tumors were evident between 16 and 19 days after challenge (Figure 2e). In 4 of the 12 mice immunized with p185 plasmids, tumors grew slowly for the first 20 days after vaccination, then tumors shrank rapidly in 3 mice and no tumor was palpable within 60 days after the first shot. In the fourth mouse, the tumor formed a stable mass about 4 mm in mean diameter that was diagnosed as a fibrotic scar deprived of neoplastic cells when it was

Figure 3

Morphological events associated with the eradication of the 2-mm mean diameter TUBO carcinomas in immunized WT BALB/c mice. Twenty days after the first shot with empty vector, a large, solid carcinoma (**a**) with a delicate stroma and without appreciable collagen deposition (**c**) formed by round, monomorphous, r-p185[neu]-expressing neoplastic cells (**e**) was evident. Immunohistochemistry showed that the tumor mass was moderately infiltrated by PMNs (**g**) and characterized by a very slight production of the fibrogenic cytokine TGF-β1 (**i**). At the same time, mice shot with p185 plasmids displayed small neoplastic cell aggregates (**b**), with an evident down-modulation of p185[neu] expression on the cell membranes (**f**), surrounded by a massive collagen deposition and devastating fibrotic reaction evidenced as blue-green staining by the trichrome method (**d**). A marked infiltration by PMN (**h**) and expression of TGF-β1 (**j**) were evident among and inside the neoplastic cell aggregates. Magnification, ×630.

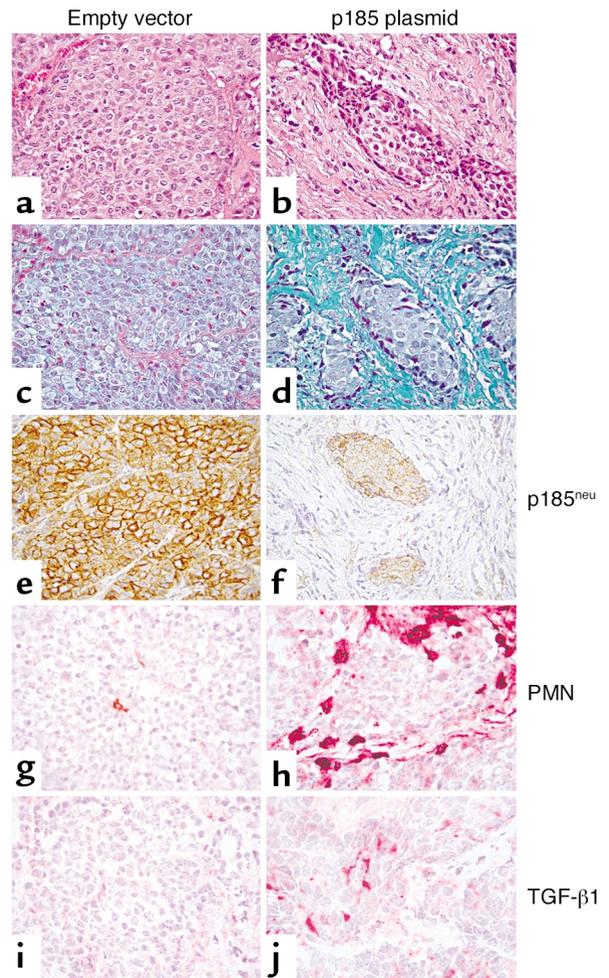Empty vector — p185 plasmid

p185[neu]

PMN

TGF-β1

Table 3
Immunohistochemical analysis of TUBO tumor growth and rejection area in untreated or immunized WT BALB/c mice 20 days after the tumor reached 2 mm in mean diameter

	Untreated mice	Mice vaccinated with p185 plasmid
Reactive cells		
Dendritic cells	ND	4 ± 1
Macrophages	18 ± 4	24 ± 6
PMNs	7 ± 2	20 ± 4
CD8 lymphocytes	6 ± 2	40 ± 8
CD4+ lymphocytes	ND	30 ± 6
NK cells	9 ± 3	20 ± 4
Endothelial adhesion molecules		
ICAM-1	+	++
ELAM-1	–	+
VCAM-1	–	+
Cytokines		
IL-1β	±	++
TNF-α	±	+
IFN-γ	–	++
IL-4	–	++
IL-6	±	++
TGF-β	–	++

The vaccine was administered when tumors reached 2 mm in mean diameter and 7 days later. Twenty days after the first shot, the reactive cells present in the tumor were evaluated at ×400 magnification in a 0.180-mm^2 field. At least three samples (one sample per tumor growth area) and 10 randomly chosen fields per sample were evaluated. Results are expressed as means ± SD of positive cells per field evaluated on cryostat sections by immunohistochemistry. The expression of endothelial adhesion molecules, cytokines, and chemokines was defined as absent (–) or scarcely (±), moderately (+), or strongly (++) present on cryostat sections stained with the Ab. WT BALB/c mice challenged with p185 plasmid show significant protection as compared with untreated mice ($P < 0.005$, two-tailed Student's t test). ND, not detected.

progressively regressed (Figure 2c). The rejection of 4- to 6-mm TUBO carcinomas was associated with a reaction similar to that described for 2-mm carcinomas.

Eradication of r-p185^neu carcinomas in immunodeficient mice. The p185 plasmid immunization by gene gun elicits an immunity sufficient to cure 2-mm mean diameter tumors. The relative weight of immune mechanisms elicited was therefore reassessed in this more demanding situation. The first striking result was that the great majority of BALB-μIgKO mice failed to be cured, even if immunization induced temporary tumor regression and delayed tumor growth (Figure 4a). The crucial role of antibody in r-p185^neu tumor cure is supported by the inability of vaccination to cure BALB-FcγRI/IIIKO mice (Figure 4b). Poor effectiveness was also observed in BALB-β2mKO mice (Figure 4g). Although the pattern of tumor shrinkage in BALB-CD1KO (Figure 4h) and BALB-MCP1KO (Figure 4d) mice was not substantially different from that in WT BALB/c mice, tumors evaded the vaccine-induced immunity in a few mice from each of these groups, a feature never observed in WT BALB/c mice. Also, the great majority of BALB-IFNγKO mice (Figure 4e) were not cured, even if the vaccination markedly delayed tumor growth. Neither cure nor

delayed tumor growth was evident for BALB-pfpKO (Figure 4f) or BALB-IFNγ-pfpKO (Figure 4c) mice.

Tumors progressively growing in the various groups of immunized KO mice consistently displayed high cell-membrane expression of p185^neu and a reactive infiltrate lower than that of untreated WT BALB/c mice. Regressing tumors showed reactive infiltration and cytokine expression similar to those associated with tumors regressing in immunized WT BALB/c mice, even if these were sometimes weaker (data not shown).

Discussion

The surrogate model TAA addressed in these experiments (p185^neu) is the protein product of the *Her-2/neu* (*Erb-B2* in humans) oncogene. This is a well-characterized signaling tyrosine kinase transmembrane receptor (52) whose overexpression in human carcinomas correlates with poor prognosis (53). Patients with Her-2/neu-expressing tumors may spontaneously develop anti-p185^neu antibody and T cell responses (12), while the passive transfer of anti-p185^neu monoclonal antibodies inhibits tumor growth and extends survival in a limited population of Her-2/neu patients (25). Because of the causal association of p185^neu expression with tumor progression (52), p185^neu is an attractive TAA for various forms of immunotherapy. In mice, r-p185^neu is an interesting surrogate TAA, since it is a well-defined heterologous protein that differs from mouse p185neu in less than 6% of the amino acid residues (39). Despite this difference in the amino acid sequence between mouse and rat, the expression of r-p185^neu on the membrane of tumors transplanted in WT BALB/c mice does not elicit a detectable antibody and cellular response, nor does it impair their growth, moreover the reactive cell infiltrate is marginal and not higher than that commonly associated with syngeneic tumors (30 and present findings).

This apparent immunological invisibility of r-p185^neu may rest on the inability of tumor cells expressing r-p185^neu to prime WT BALB/c T and B cells that recognize the xenogeneic r-p185^neu. Presumably, rat epitopes of r-p185^neu expressed on tumor cell membranes are insufficient to generate immunity in the absence of an effective cross-priming such as that which takes place after particle bombardment by gene gun. In effect, we observed that DNA vaccination of WT BALB/c mice with p185 plasmids administered by a gene gun not only elicited completely protective immunity against a subsequent challenge of r-p185^neu-expressing TUBO cells, but the reaction triggered was sufficiently strong to eradicate palpable TUBO carcinomas.

Teasing out the mechanisms responsible for this protective immunity, we observed that the rejection of a tumor challenge by preimmunized mice had different requirements than the eradication of an established carcinoma. The induction of the anti–r-p185^neu immune response rests on CD4 lymphocytes, whose removal during the immunization period abolished the induction of protective immunity. The cross-presentation by dendritic cells of antigens encoded by plasmids shot into the
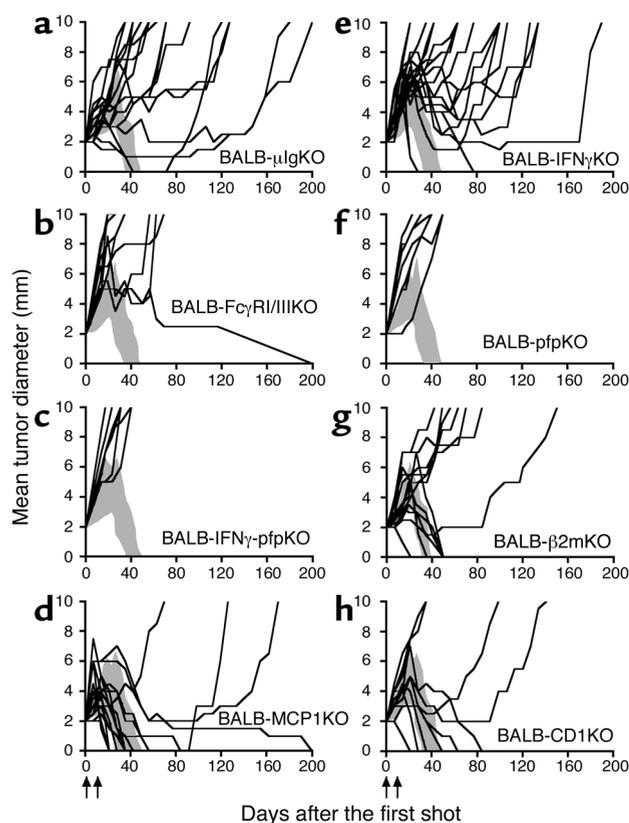
Figure 4

Mechanism of r-p185 carcinoma eradication. When the tumor was 2 mm in diameter, various KO mice were shot with p185 plasmid. Although all WT BALB/c mice were cured (20 of 20 cured at day 200) (Figure 2a), vaccination did not cure any BALB-pfpKO mice (0 of 7 cured, $P < 0.0001$ versus WT BALB/c) (**f**) or BALB-IFNγ-pfpKO mice (0 of 7 cured, $P < 0.0001$) (**c**). Vaccination did not cure 89% of BALB-μIgKO mice (2 of 18 cured, $P < 0.0001$) (**a**), 88% of BALB-FcγRI/IIIKO mice (1 of 8 cured, $P < 0.001$) (**b**), 86% of BALB-IFNγKO mice (3 of 21 cured, $P < 0.0001$) (**e**), and 78% of BALB-β2mKO mice (2 of 9 cured, $P < 0.001$) (**g**). Vaccination cured 54% of BALB-CD1KO mice (6 of 11 cured, $P < 0.006$) (**h**) and 80% of BALB-MCP1KO mice (12 of 15 cured, $P < 0.07$) (**d**). Arrows show the days of the shots, and the gray area shows the growth and the rejection of the tumor in WT BALB/c mice bearing 2-mm tumors.

dermis accounts for this central role of CD4 T cells in the induction of the protective response (40–43). Although the marked decrease of CD8 cells in BALB-β2mKO mice did not reduce protection, protection was noticeably reduced by the depletion of CD8 T cells during the rejection of TUBO cells. BALB-β2mKO mice lack NK T cells and have desensitized NK cells, no FcγRs, and some small populations of CD8 T cells (45). NK T cells can suppress Th1 responses, so in their absence the response of the small T cell population might be more effective (54). By contrast, BALB/c mice treated with antibodies to CD8 are devoid of CD8 T cells and probably also of CD8 DCs. In these immunized mice, the lack of resistance may directly result from the almost complete absence of CD8 T cells. Alternatively, CD8 DCs may be required for an effective cross-presentation of r-p185[neu] after gene gun

vaccination. Surprisingly, protection afforded by vaccination was also abolished by depletion of PMNs. Indeed, pathological analysis of the TUBO cell rejection area supports a key role for both of these leukocyte populations, since they were very well represented and closely intermingled with damaged tumor cells. A similar critical role of PMNs was repeatedly observed in the cytokine-activated rejection of transplantable tumors (51, 55). Although the antitumor activity of the resting PMNs is marginal, it can be elicited and guided by cytokines released by T cells (55), and the powerful destructive activity of PMNs can be guided by antibodies bound to FcγRI/III (20). However, the rejection of p185[neu]-expressing TUBO cells by immunized mice took place even in the absence of antibodies (BALB-μIgKO mice) (16), FcR-mediated ADCC (BALB-FcγRI/IIIKO mice) (44), NK cells that actively mediate ADCC (56), and NK T cells (47) associated with impaired macrophage function (57) (BALB-CD1KO mice). The rejection of the TUBO cell challenge even took place in the absence of pfp-mediated cytotoxicity (BALB-pfpKO mice) (49) and was not impaired by the absence of IFN-γ (BALB-IFNγKO mice) (46) or when macrophage recruitment was defective (BALB-MCP1KO mice) (48). Although numerous combinations of antibody depletion and gene KO may provide a more definite identification of the mechanisms involved, present data show that no single immune deficiency, besides PMNs, was crucial for the rejection of an r-p185[neu] cell challenge. This finding suggests that the reaction elicited by p185 plasmids administered by gene gun was based on the induction of multiple mechanisms. In this way, a specific immune deficiency can be compensated by the numerous residual mechanisms. The complete abrogation of protection observed when two gene deficiencies (IFN-γ and pfp) that do not individually impair the challenge rejection are combined (BALB-IFNγ-pfpKO mice) endorses this compensatory interpretation.

When the role of the distinct immune mechanisms was assessed in the eradication of an established and actively growing carcinoma, the results were diametrically different, since all the mechanisms assayed played a more or less essential role. Although immunization cured all WT BALB/c mice bearing a 2-mm carcinoma, almost no cure was observed in the absence of antibody, and none at all was observed in FcγRI/IIIKO mice. Therefore, antibody and presumably also ADCC are prerequisites for tumor cure. The absence of pfp-dependent lytic mechanisms, an impairment in CD8 effector lymphocytes (BALB-β2mKO mice), and the decrease or absence of NK T cells (BALB-β2mKO mice and CD1KO mice) either abolished or strongly reduced the ability of immunized mice to eradicate the carcinoma. Carcinoma cure was also dramatically impaired in the absence of IFN-γ (BALB-IFNγKO mice). Even if the defective macrophage recruitment in BALB-MCP1KO mice poorly affected the ability to cure, carcinoma grew in 20% of these mice. The ability to generate an effective antitumor attack that overcomes the kinetics of tumor proliferation and tumor escape mechanisms appears to result from the

combination of multiple immune mechanisms, each of which is necessary for efficient protection.

Anti-p185[neu] antibodies appear to have a differential role in the protection against p185[neu] tumors depending on the context in which their role is evaluated. In the absence of anti-p185[neu] antibodies, other pre-established reaction mechanisms protect against a lethal tumor challenge (35–38), whereas in a situation in which a rising immunity had to deal with the eradication of a preexisting tumor, their role became critical (34). These antibodies can directly impair the proliferation kinetics of p185[neu]-expressing tumors by interfering with the growth-signaling function of the p185[neu] receptor expressed on the tumor cell membrane (25–29). The marked membrane downmodulation of r-p185[neu] and diminished nuclear positivity of proliferating cell nuclear antigen (PCNA) characterizing the cure of TUBO carcinomas points to a direct inhibitory activity, since reduced r-p185[neu] expression was sufficient for the impairment of tumor growth ability in vivo (27, 28, 30, 32). However, observations of pathology highlighted a massive PMN and NK infiltration during carcinoma eradication. In the presence of the high titer of anti–r-p185[neu] antibodies elicited by p185 plasmid immunization, PMNs and NK cells may be activated to mediate ADCC (20, 58). The central role played by cells expressing FcγRI/III and by PMNs strongly support this concept. The number of PMNs and NK cells — and, less markedly, that of macrophages — infiltrating the tumor was significantly lower in carcinomas that continue to grow than in those that did regress. The mechanisms that regulate the tumor recruitment and local activation of these nonspecific effectors (51, 55) may play a significant role in deciding the growth or rejection of established carcinomas in immunized mice.

The massive cytokine expression in the tumor rejection area may explain not only the intratumoral recruitment of nonspecific and specific reactive cells but also the devastating fibrotic reaction that accompanied tumor regression. In fact, in addition to a high expression of TGF-β1, a well known collagen-inducing mediator, other cytokines such as IL-1β and IL-4, which promote fibroblast proliferation and collagen deposition, are widely produced during the rejection process (58).

In conclusion, present data suggest that a lethal challenge by r-p185[neu]-positive carcinoma cells can be completely rejected by preimmunized mice, not only in the absence of antibodies and FcγRI/III-positive cells but also in the absence of pfp-dependent cytotoxicity. It can be significantly inhibited even in the absence of CD4 and CD8 T effector cells. This rejection pattern suggests that several immune mechanisms can independently lead to tumor challenge rejection. However, when the induction of the immune response has to cope with the proliferative kinetics of a palpable tumor, efficient tumor eradication only results from the cooperation of these various mechanisms. Not only do antibodies, FcγRI/III-positive cells, IFN-γ, and pfp play a critical role, but complete protection also requires NK T cells and efficient macrophage recruitment. Although it is morphologically well documented that a multicell-mediated reaction is associated with tumor rejection (51, 59), we have been able to show here the importance of each component in the process. The additional effect resulting from this cooperation implies that an antitumor vaccine able to trigger a multifaceted reaction, and not only a CTL or antibody response, could be particularly effective. In this case, the choice of the target TAA is critically important. p185[neu] is one of many tyrosine kinase receptors directly involved in cell growth and carcinogenesis that are expressed on the cell surface (52). Those TAAs that cannot be lost or downmodulated without impairing the cell oncogenic potential appear to be suitable targets for both T cell– and antibody-orchestrated immune reactivity (60).

With the use of a xenogeneic surrogate TAA, a few of the multiple and sometimes redundant mechanisms required for the immune eradication of tumors expressing membrane oncogenic tyrosine kinase receptors have been teased apart. However, the use of r-p185[neu] as an antigenic target in a mouse system makes a direct assessment of the relevance of these data to an active immunization against self-p185[neu] problematic. After gene gun immunization against the xenogeneic p185[neu], the amino acid sequences that differ between rat and mouse can provide various types of T cell help and thus allow the elicitation of an effective immune response characterized by high-intensity and high-affinity effector mechanisms. However, gene gun vaccination may not be equally suitable for breaking down tolerance and inducing an effective immune response in the case of human p185[neu], since it is a self-tolerated antigen widely expressed at low levels in multiple tissues.

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