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PRECLINICAL DEVELOPMENT OF CANCER VACCINES

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Esame finale anno 2009

To Davide, Bianca and Chiara.
Their love made the
writing of this thesis possible.

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ABSTRACT

Triplex cell vaccine is a cancer immunopreventive cell vaccine that can prevent almost completely mammary tumor onset in HER-2/*neu* transgenic mice.

A future translation of cancer immunoprevention from preclinical to clinical studies should take into account several aspects. The work reported in this thesis deals with the study of three of these aspects: vaccine schedule, activity in a therapeutic set-up and second-generation DNA vaccines.

An important element in determining human acceptance and compliance of a treatment protocol is the number of vaccinations. In order to improve the vaccination schedule a minimal protocol was searched, i.e. a schedule consisting of a lower number of administrations than standard protocol but with a similar efficacy. A candidate optimal protocol was identified by the use of an *in silico* model, SimTriplex simulator. The *in vivo* test of this schedule in HER-2/*neu* transgenic mice only partially confirmed *in silico* predictions. This result shows that *in silico* models have the potential ability to aid in searching of optimal treatment protocols, provided that they will be further tuned on experimental data. As a further result this preclinical study highlighted that kinetic of antibody response plays a major role in determining cancer prevention, leading to the hypothesis of a threshold that must be reached rapidly and maintained lifetime.

Early clinical trials would be performed in a therapeutic, rather than preventive, setting. Thus, the activity of Triplex vaccine was investigated against experimental lung metastases in HER-2/*neu* transgenic mice in order to evaluate if the immunopreventive Triplex vaccine could be effective also against a pre-existing tumor mass. This preclinical model of aggressive metastatic development showed that the vaccine was an efficient treatment also for the cure of micrometastases. However the immune mechanisms activated

against tumor mass were not antibody dependent, i.e. different from those preventing the onset of primary mammary carcinoma.

DNA vaccines could be more easily used than cellular ones. A second generation of Triplex vaccine based on DNA plasmids was evaluated in an aggressive preclinical model (BALBp53neu female mice) and compared with the preventive ability of cellular Triplex vaccine. It was observed that Triplex DNA vaccine was as effective as Triplex cell vaccine, exploiting a more restricted immune stimulation.

CANCER IMMUNOPREVENTION

The immune surveillance theory, that was proved both in animal models and human studies, states that the immune system is physiologically active in the prevention of spontaneous tumors. On the contrary, several decades of attempts to further stimulate antitumor immunity for therapeutic approaches have given poor results. The better efficiency of immune system in preventing rather than cure tumors has led, in recent years, tumor immunologists to suggest that the immune system, if adequately stimulated before tumor onset, could be able to protect the organism from specific cancers (*Lollini et al., 2006a*).

An obvious approach consists in prevention of tumors related to infectious agents, such as human hepatitis B virus (HBV) and human papilloma virus (HPV), closely related to liver and cervical carcinoma respectively. In fact, vaccination against HBV has reduced the incidence of hepatocellular carcinoma, whereas vaccines against HPV are expected to greatly reduce the incidence of cervical carcinoma. A less obvious approach deals with the immunoprevention of tumors caused by endogenous factors. Vaccines targeting these factors could be a new promising approach in order to control endogenous molecules and protect from tumor development. Another approach to control tumor onset could be chemoprevention, consisting of chronic administrations of drugs targeting components of metabolic or signaling pathways, such as tamoxifen to reduce the risk of breast cancer. The limits of chemoprevention are the partial efficacy and the serious side effects. Cancer immunopreventive approaches, such as vaccines, could reduce the amount of side effects thanks to the selective action and probably could require less invasive procedures (*Lollini et al., 2006a*).

Cancer immunoprevention has insofar produced good results in preclinical studies, although it is still far from the clinic and several questions need to be answered before progressing into clinical trials (*Lollini et al., 2006a*).

The choice of a right target is an important issue to develop an effective vaccine. Up to now many tumor antigens have been described, but only a few molecules proved to be good target antigens for cancer immunoprevention. Tumor associated molecules that are essential for tumor growth and progression could be suitable cancer vaccine targets, since they cannot be easily downmodulated or negatively selected in precancerous lesions under the pressure of a specific immune attack. Lollini and colleagues have defined these molecules as oncoantigens (*Lollini et al., 2006a*). Oncoantigens should be poorly expressed by normal adult tissues, but overexpressed by tumor (*Cavallo et al., 2007*). Moreover the presence of target antigens on the cell membrane seems to be an optimal condition to obtain both a cell-mediated and an antibody-mediated immune response. Molecules eligible to be defined as oncoantigens include tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR), the insulin-like growth factor-1 receptor (IGF1R) and the vascular endothelial growth factor receptor (VEGFR). Other potential oncoantigens could be receptors of B- and T- cells. Adhesion and surface molecules involved in survival, invasion and malignancy could be part of oncoantigen class as well (*Lollini et al., 2005b*).

The development of cancer vaccines should progress through different phases and one of the first steps is the validation of the vaccine in appropriate animal models (*Wei et al., 2008*). Preclinical studies on antitumor vaccines are usually based on the evaluation of vaccine efficacy leading to the acute rejection of a subsequent tumor challenge. A more realistic alternative consists of models which develop autochthonous tumors induced by carcinogens or by genome manipulation. Genetically engineered mice (GEMs), harboring activated oncogenes and/or inactivated tumor-suppressor genes, are prone to develop cancer by molecular and genetic alterations that could resemble the stepwise progression of human cancer (*Quaglino et al., 2008*). GEMs are interesting models for cancer prevention thanks to the slow tumor progression, generally comprising invasion and metastasis, and to the presence of a long-

lasting interaction between the evolving tumor and the host immune system. Two main relevant issues in the choice of a model are the type of promoter, that will drive the pattern of transgene expression, and the existence (and eventually breakage) of immune tolerance to transgene-encoded proteins, in analogy to what happens for most tumor antigens in humans (*Lollini et al., 2006a*).

Vaccines consisting of live, irradiated or genetically modified tumor cells, dendritic cells, proteins, peptides or naked DNA have been tested in GEMs. The degree of protection varies among models and within the same model, ranging from delayed tumor onset to complete protection from tumor onset for one year or more. The most successful vaccines in preclinical studies consisted of combinations of the target tumor antigen with other immunological signals acting as adjuvants, such as microbial CpG sequences, cytokines, allogeneic glycoproteins of the major histocompatibility complex (MHC) and/or co-stimulatory molecules. On the whole the efficacy of a tumor preventive vaccine depends both on a high level of the antigen and on the adjuvants. Tumor antigens, both in humans and GEMs, are mostly self-antigens overexpressed by tumor. Adjuvant molecules, inducing an aspecific immune response, contribute to break the immune tolerance to tumor antigens. Therefore the efficacy of a vaccine is based on its ability to reverse a tolerant state and activate low-avidity immune reaction mechanisms that escape the central tolerance (*Lollini et al., 2006a*).

Concerning the immune mechanisms induced by immunopreventive vaccines, a coordinate induction of T-helper cells, cytotoxic T lymphocytes and, above all, antibodies seems to determine vaccine protective ability in preclinical models. In order to ensure a long-term immune response, vaccines should be able to elicit immune memory mechanisms that are believed to persist for a very long period. This could only be possible by repeated boost vaccinations (*Lollini et al., 2006a*).

Besides immunopreventive approaches such as vaccines, other cancer immunopreventive strategies include passive administration of antibodies

against surface oncoproteins, such as trastuzumab, and active stimulation of immune system by non-specific immunomodulators, such as interleukin (IL-) 12 or bacterial products (*Lollini et al., 2005a*).

Among target tumor antigens for cancer immunopreventive strategies the human epidermal growth factor receptor 2 (referred to as HER-2 or ErbB2) is one of the widely studied. This receptor can lead to neoplastic transformation, it is overexpressed in a fraction of human breast cancers and is a surface molecule. Such features permit to include HER-2 in the class of oncoantigens (*Lollini et al., 2005b*).

CANCER IMMUNOPREVENTION IN HER-2/*neu* TRANSGENIC MICE

HER-2

HER-2/ErbB2 is a member of the human epidermal growth factor receptor family of tyrosine kinases which includes EGFR/ErbB1, HER-3/ErbB3 and HER-4/ErbB4. The general structure of these molecules consists of an extracellular ligand-binding domain, a single-span transmembrane domain and an intracellular domain that contains the conserved catalytic-kinase domain and the carboxy-terminal tail (Figure I) *(Sergina and Moasser, 2007)*. The rodent homologue of HER-2 is the oncogene *neu.* The product of HER-2/neu genes is a protein of 185 KDa (p185).

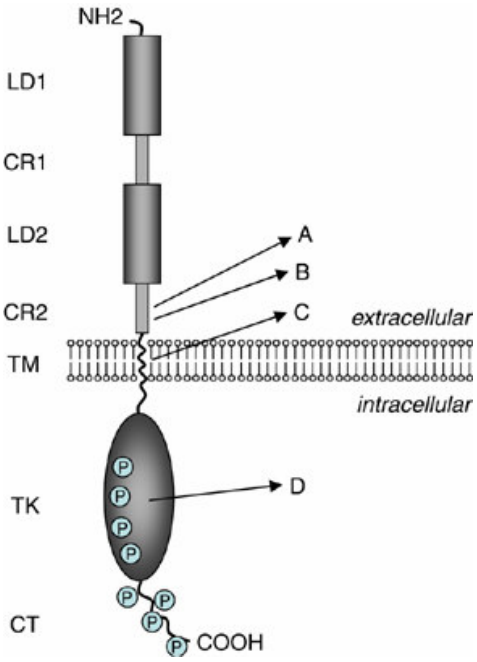


Figure I. Structure of the HER-2 and *neu* proteins. The domain structure is shown on the left and consists of two ligand-binding regions (LD1 and LD2), two cysteine-rich regions (CR1 and CR2), a short transmembrane domain (TM), a catalytic tyrosine kinase domain (TK) and a carboxy terminal tail (CT). Sites of tyrosine phosphorylation within TK and CT domains are indicated by circled P. The letters on the right point to specific areas that are altered or mutated in certain naturally occurring or experimentally induced cancers *(Moasser, 2007b)*.

The signaling cascade is initiated by the ligand-mediated interaction of two HER family members. The extracellular domain of HER proteins has two conformations: closed when inhibited and open when activated. Ligands

promote a change of the extracellular domain structure and then dimerization of two HER family members. Finally the transphosphorylation of their intracellular domains causes the activation of several downstream pathways. Pairing between HER proteins seems to be closely connected to several and specific signaling activities. Heterodimers are favorite over homodimers and HER-2 has the strongest catalytic kinase activity, therefore HER-2-containing heterodimers have the most powerful signaling functions. In detail, HER-2 and HER-3 form the most active complex of the family, although both of them are functionally incomplete receptor molecules. In fact HER-2 extracellular domain constitutively presents only the activated conformation since the receptor lacks ligand-binding activity and its signaling function is engaged by its ligand-bound heterodimeric partners. HER-3 catalytic domain is constitutively inactive thus HER-3 exploits the kinase domain of its partners *(Moasser, 2007b)*.

The HER family controls a comprehensive network of survival, growth, metabolism and motility responses by activation of several signaling pathways as phosphoinositide 3-kinase (PI3K)/Akt, Raf/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK), Janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT-3), Src, phospholipase C-γ (PLC γ) *(Sergina and Moasser, 2007)*.

HER-2 is involved in the development of human cancers: 25-30% of breast cancers show HER-2 gene amplification and overexpression of its product. HER-2 overexpression is a predictor of mammary tumors with a worse biologic behavior and prognosis (*Slamon et al., 1989*). Moreover HER-2 protein overproduction is found in cancers of esophagus, stomach, ovaries and endometrium. Evidences suggest that HER-2 amplification is an early event in human breast tumorigenesis. HER-2 amplified breast cancers are more responsive to some cytotoxic chemotherapeutic agents, show resistance to some hormonal agents and have an increased propensity to metastasize to the brain. Somatic mutations within HER-2 catalytic domain have been described in a small subset of lung cancers (see Figure I, D); this genetic alteration promotes

an increased kinase activity and transforming efficacy. Siegel and colleagues (*Siegel et al., 1999*), and Castiglioni and colleagues (*Castiglioni et al., 2006*) reported data on an alternative HER-2 transcript. The lack of a 48 bp coding exon and consequently a 16 aminoacids deletion from juxtamembrane region (see Figure I, B), produces an alternative HER-2 protein (ΔHER-2) with a higher ligand-independent signaling activity and transforming potency (*Moasser, 2007b*).

The role of the different HER-2 related pathways in tumor development is not completely defined. Surely HER-2 overexpression leads to an increase of HER-2 homodimers and heterodimers. Such increased number of complexes promotes the loss of polarity and a deregulation of cell adhesion. Moreover downstream signals such as PIK3, β4 integrin, src, and others, contribute to an invasive phenotype. A few number of studies reported an upregulation of cyclin D1, E and cdk6 and degradation of p27 in HER-2 overexpressing tumors. Thus, HER-2 overexpression produces a change in proliferative, survival, invasive and metabolic functions. Some authors also proposed a role in carcinogenesis for genes amplified with HER-2 as neighbors, for molecules considered transcriptional targets of HER-2, for the membrane protein MUC4 and for other pathways (*Moasser, 2007b*).

Since HER-2 is directly involved in tumor pathogenesis, it is a prototypic oncoantigen and HER-2 inhibitors should be highly effective treatments for HER-2-driven cancers. The most successfully developed drug is a humanized anti-HER-2 monoclonal antibody named trastuzumab. Several clinical studies showed that this drug, developed and commercialized by Genetech Inc. as Herceptin (South San Francisco, CA, USA), induces improvements in the clinical management of patients with HER-2-amplified breast cancer. The most beneficial clinical use of trastuzumab has been in combination with various cytotoxic chemotherapies. The biggest impact of this drug has been in the treatment of patients with potentially curable early-stage breast cancer since

this drug prolongs disease survival, after surgical resection, and reduces the chances of disease-free recurrence (*Moasser, 2007a*). Trastuzumab kills tumor cells through multiple mechanisms whose relative importance is not yet definitively established. The interaction of this drug with immune system by antibody-dependent cellular cytotoxicity (ADCC) is proved (*Weinberg, 2007*). Concerning other mechanisms, the study of trastuzumab effect on tumor cell HER-2 expression has produced conflicting results (*Moasser, 2007a*). It is known that trastuzumab inhibits the cleavage of a part of HER-2 extracellular domain by extracellular proteases reducing the formation of truncated HER-2 that has an increased transforming efficiency. Finally trastuzumab can show an anti-angiogenic activity (*Weinberg, 2007*). Other drugs have been developed and they are being tested in clinics such as anti-HER-2 monoclonal antibody pertuzumab and HER kinase inhibitors (*Moasser, 2007a*).

HER-2/*neu* TRANSGENIC MICE

HER-2/neu transgenic mice are likely the most extensively studied models for the evaluation of immunopreventive approaches against mammary cancer. These models, developed in the last 20 years, comprise various transgenic mouse lines differing in the genetic background (FVB, BALB/c, CD-1), the type of promoter, the type of oncogene and the species of origin (*Ursini-Siegel et al., 2007*).

The most popular promoters were the long terminal repeats (LTR) of the mouse mammary tumor virus (MMTV) and the promoter of the whey acidic protein (WAP). These two promoters induce a high level expression of the oncogene specifically in the mammary epithelium. Both MMTV-LTR and WAP promoters are hormonally sensitive promoters. The MMTV-LTR is active throughout mammary development and its transcriptional activity increases during pregnancy. On the contrary, the WAP promoter is only active in the mid-pregnant mammary gland. Consequently it is evident that the

phenotypes exhibited by WAP and MMTV transgenic mice may depend upon the developmental stage of the individual mouse examined. Few models express the transgene under the control of the endogenous promoter *(Hutchinson and Muller, 2000*).

For what concerns the type and the origin of HER-2/neu oncogene, several mouse models were developed. The rat *neu* oncogene can transform mammary epithelial cells *in vitro* and transforming activity is caused by a point mutation within the transmembrane domain (see Figure I, C) producing the V664E-mutated protein named neuT. This mutation promotes receptor dimerization and enhances the tyrosine kinase activity. The activated *neu* oncogene (neuT) expressed in mouse mammary tissue induces adenocarcinomas. The *c-neu* protooncogene, when overexpressed in murine mammary tissue, also induces tumor formation, but this mainly occurs through acquirement of deletion mutations within the extracellular juxtamembrane region that promote dimerization and enhanced kinase activity (see Figure I, A). Other mouse models were developed using human wild-type HER-2 or mutated HER-2 V659, although this genetic alteration was never found in human cancers (*Moasser, 2007b*).

HER-2 transgenic mice have been used for studies of cancer immunopreventive approaches in the Laboratory of "Immunology and Biology of Metastasis" (Cancer Research Section, Experimental Pathology Department) directed by Prof. Pier-Luigi Lollini. Hereafter the main characteristics of these HER-2 transgenic mice will be summarized.

BALBneuT

BALBneuT female mice have the genetic background of BALB/c mice and carry the rat *neu* activated oncogene under the control of the MMTV promoter (*Boggio et al., 1998*).

BALBneuT female mice develop multifocal mammary carcinomas with a short latency, about 20 weeks of age. Within 33 weeks of age lobular carcinomas are palpable in all 10 mammary glands. This rapid onset and the total gland involvement suggest that the expression of activated HER-2/*neu* in these mice requires few, if any, additional genetic events to transform the mammary epithelial cells (*Boggio et al., 1998*).

Mammary carcinogenesis in BALBneuT mice resembles the mains aspects of human breast carcinomas. Starting at 3 weeks of age, an atypical hyperplasia of small lobular ducts and lobules is evident and epithelial cells show expression of $p185^{neu}$. These proliferating areas progress to carcinoma *in situ* at about 11 weeks of age and then the neoplastic regions evolve in lobular carcinomas, macroscopically evident around 20 weeks of age. Tumor formation proceeds independently and multifocally in all ten mammary glands and, later, gives rise to metastases detectable in the lungs. Single metastatic cells are already present in the lung and bone marrow at time of non-invasive atypical hyperplasia (*Di Carlo et al., 1999*; *Husemann et al., 2008*).

BALBp53neu

BALBp53neu mice are bigenic mice obtained by crossing BALBneuT mice with BALB/c mice with heterozygos p53 knockout. Such combination of genetic defects induces a fast growth of multiple tumor types. BALBp53neu female mice develop salivary gland carcinomas at about 13-15 weeks of age and mammary tumors at about 19-20 weeks. Therefore the growth of salivary tumors precedes the complete development of mammary carcinogenesis. At sacrifice, about 30% of female mice are affected by both types of tumors. BALBp53neu male mice are prone to develop salivary gland carcinomas and pelvic rhabdomyosarcoma at about 13-15 weeks of age. Since these two different tumors show latency time and growth rate similar, lethality can result from either type of neoplasms (*Croci et al., 2004*).

TRIPLEX VACCINE

Cancer immunopreventive studies on rat HER-2/*neu* transgenic mice have produced a good number of approaches able to delay and/or reduce tumor onset up to a complete protection from tumor carcinogenesis driven by HER-2/*neu* oncoantigen (*Lollini et al., 2006a*).

One of the most effective vaccines was set up in the BALBneuT model in the Laboratory of "Immunology and Biology of Metastasis" and was based on allogeneic *neu*-expressing mammary cancer cells combined with IL-12. Vaccine cells derived from a mammary carcinoma of a FVBneuN #202 ($H\text{-}2^q$) mouse, transgenic for the rat protooncogene *c-neu*. Such vaccine, referred to as Triplex vaccine, expresses the $p185^{neu}$ oncoantigen and MHC molecules of the $H\text{-}2^q$ haplotype (allogeneic for $H\text{-}2^d$ BALBneuT mice). The third component of Triplex vaccine is IL-12. At first recombinant IL-12 was used and then vaccine cells were engineered with the genes coding for murine IL-12 (*Nanni et al., 2001*; *De Giovanni et al., 2004*).

The vaccination with IL-12 engineered allogeneic neu^+ mammary carcinoma cells started at 6 weeks of age and was repeated lifelong according to a schedule named Chronic. This protocol consisted of 4-week cycles: in the first two weeks mice received two intraperitoneal (i.p.) vaccine injections per week, then mice had two weeks of rest. Vaccine cells were pretreated with mitomycin C to block the proliferation and then $2x10^6$ cells in Phosphate Buffer Solution (PBS) were injected into each mouse (*De Giovanni et al., 2004*). The Triplex vaccine produced an almost complete protection from *neu*-driven mammary carcinogenesis (*Nanni et al., 2001*; *De Giovanni et al., 2004*).

The main actors of the immune response elicited by the Triplex vaccine were interferon gamma (IFN-γ) and anti-$p185^{neu}$ antibodies. IFN-γ, produced by T helper 1 (Th1) lymphocytes and cytotoxic T lymphocytes (CTL), through IL-12 induction, promotes the isotypic switch to Th1-type immunoglobulins G (IgG). Moreover IFN-γ causes block of cell proliferation and apoptotic cell death. The

killing of p185^{neu+} mammary carcinoma cells is mediated by classical immune mechanisms as well as by downmodulating the p185^{neu} receptor. Moreover IFN-γ can induce production of antiangiogenic factors. Anti-p185^{neu} antibodies (also referred to as anti-*neu* antibodies), specifically IgG2a and IgG2b murine subclasses, showed antitumor activities mediated both by immune mechanisms, such as ADCC, and by induction of p185^{neu} recycling and inhibition of receptor dimerization (*Nanni et al., 2001*; *De Giovanni et al., 2004*). The importance of IFN-γ and anti-*neu* antibodies was confirmed by studies in BALBneuT mice lacking antibodies or IFN-γ production. In these mice Triplex vaccine loss the ability to prevent mammary carcinoma onset (*Nanni et al., 2004*).

The Triplex vaccine was also studied in BALBp53neu mice, which are a more aggressive model, towards different tumor types (salivary and mammary in females, salivary and rhabdomyosarcoma in males). At one year of age the life-long administration of Triplex vaccine can prevent tumors in more than 60% of females and 40% of males mice (*Croci et al., 2004*).

TRIPLEX VACCINE:
FROM PRECLINIC TO CLINIC

Previous studies clearly demonstrated the potency of Triplex cell vaccine as cancer immunopreventive approach. A future human application of cancer immunoprevention, and specifically of Triplex vaccine, will require specific studies aimed at the definition, in preclinical models, of the optimal conditions for a successful translation. The development of some of these preclinical aspects was the overall aim of the work presented in this thesis and was focused on three main directions:

1) The success of Triplex cell vaccine in BALBneuT female mice was obtained through a cell vaccine administered lifetime with a 4-week schedule. The attempt to obtain equally effective protocols but with shorter vaccination periods or with less vaccine administrations should be pursued. This issue is relevant in a translational perspective, because the number of vaccinations is an important element in determining human acceptance and compliance. Then a new protocol was predicted by a mathematical simulator of immune system and evaluated in BALBneuT mice.

2) Early human trials will necessarily be performed in advanced cancer patients in a therapeutic, rather than preventive, set-up. Therefore the therapeutic activity of the Triplex vaccine was investigated against experimental lung metastases in BALBneuT mice.

3) DNA vaccines could be of easier clinical use in comparison with cellular vaccines. A second generation of Triplex vaccine based on DNA plasmids was evaluated in BALBp53neu female mice and compared with the preventive ability of cellular Triplex vaccine.

SECTION 1

OPTIMIZATION OF THE TRIPLEX VACCINE SCHEDULE

INTRODUCTION

Triplex vaccine schedule

The Triplex cell vaccine was able to prevent mammary carcinogenesis in HER-2/neu transgenic mice. BALBneuT mice were treated according to a very intensive 4-week cycle schedule. The treatment started at 6 weeks of age. Each vaccination cycle consisted of two injections per week for the first two weeks, followed by two weeks of rest (*Nanni et al., 2001*; *De Giovanni et al., 2004*).

This schedule gave excellent results, but it consisted of a huge number of injections over the entire lifetime, feature that should be hardly translatable to humans. Looking for simpler vaccination protocols, shorter schedules have already been tested in BALBneuT model (Figure II). These protocols were based on three vaccination cycles starting at different stages of mammary carcinogenesis: at 6 weeks of age, corresponding to mice with atypical hyperplasia ("Early"), 10 weeks of age corresponding to *in situ* carcinoma ("Late") and 16 weeks of age corresponding to incipient invasive carcinoma ("Very Late"). The results of the previous vaccinations were compared with lifetime vaccination (referred to as "Chronic").

BALBneuT female mice in the Early group showed the highest delay of tumor onset (median latency of tumors: 55 weeks of age) although all mice were positive for mammary tumors at 63 weeks of age. The Late schedule had a shorter median latency of tumors (about 40 weeks) even if the tumor-free survival curve was still significantly different from untreated mice one (median latency of tumors corresponding to about 20 weeks). Finally the tumor-free survival curve of the Very Late group was not significantly different from that

of untreated mice. Thus, a progressive loss of vaccine efficacy directly related to the advancement of tumor progression has been shown by these i*n vivo* studies. The administration of the Triplex vaccine had necessarily to start when only atypical hyperplasia was present in order to give a complete prevention of mammary carcinogenesis (*Nanni et al., 2007*).

Early protocol was simple and effective, but it only produced a delay of tumor onset: three vaccination cycles were not sufficient to keep mice tumor-free at one year of age (*De Giovanni et al., 2004*; *Nanni et al., 2007*).

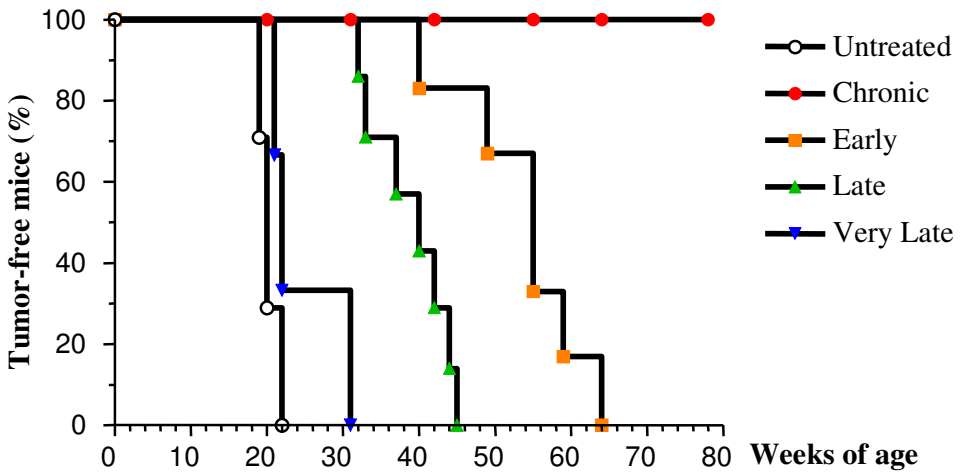


Figure II. Tumor-free survival curves of BALBneuT female mice (6-7 mice for each group) subjected to various vaccination protocols with Triplex vaccine (*Nanni et al., 2007*).

Two main indications were inferred to design new schedules for the Triplex vaccine: the treatment should not start after the 6th week of mouse age and only 12 administrations, i.e. three vaccination cycles, concentrated in the first three months of treatment are not sufficient to maintain mice tumor-free up to 1 year of age. The definition of these conditions could contribute to the improvement of the vaccination protocol for the Triplex vaccine and *in silico* tools could help to design a new schedule in order to optimize Triplex vaccine treatment.

Tumor immunology modeling

Biological sciences rely on modeling systems since models are fundamental to conceptualize, understand, test and predict phenomena. The modeling systems include both *in vivo* and *in vitro* models, such as genetically engineered mice or tumor cell lines respectively. Another class of models is defined by *in silico* approaches. In this context the term *in silico* means simulations of chemico-biological processes performed by computational and mathematical strategies. *In silico* modeling is a useful tool for several disciplines since *in silico* experiments can be performed in a fast and relatively cost-efficient way, compared to most wet-lab studies, thanks to the possibility to tune multiple parameters reproducibly and over a wide range (*Deisboeck et al., 2009*).

The study of immune mechanisms has been producing new knowledge for the design or the improvement of vaccines (*Kaufmann, 2007*). Immunomics is a new discipline arising from the cooperation of different fields, including informatics, genomics, proteomics, clinical medicine and obviously immunology. Immunomics could offer a great contribution to vaccinology by the development of algorithms for the design and discovery of new vaccines or the improvement of pre-existing vaccines. Among immunomics research products, *in silico* techniques have been developed to identify suitable vaccine candidates (T cell and B cell epitope prediction, reverse vaccinology), to discover allergen and adjuvant molecules and to enhance the efficiency of protein expression (codon optimization) (*Davies and Flower, 2007*).

The evolution of mathematical immunology led to the design of models able to explain general immunologic phenomena using basic molecular features and some of these models tried to describe the full immune system. One of the first models of this class was the IMMSIM model developed by Seiden and Celada (*Louzoun, 2007*; *Forrest and Beauchemin, 2007*). IMMSIM belongs to the category of agent based modeling, an approach that describes the interactions of autonomous entities (cells, agents). An agent can represent a cluster of cells, a

single cell, a protein or a gene. Agents interact with their environment and the other agents according to a set of literature-based rules. Each agent can assume different states as consequence of its interactions. The environment is represented by the simulation space and contains non cellular parameters relevant for the experimental question being addressed, for example concentrations of diffusible factors, such as chemokines or cytokines, or nondiffusible factors, such as the extracellular matrix. The simulation space in which the agents move can be given closed, open or periodic boundaries (*Thorne et al., 2007*).

SimTriplex model

The modeling of the immune system can be combined with the simulation of cancer growth, so that the ability of cancer vaccines to enhance an immune response against tumors can be tested in *in silico* models. These models are defined as immune system-tumor competition models (*Lollini et al., 2006c*).

SimTriplex, described by Pappalardo and colleagues (*Pappalardo et al., 2005*) is an immune system-vaccine tumor competition model developed on Celada-Seiden IMMSIM model. It reproduces at the cellular level the behavior of immune cells of Triplex vaccinated as well as untreated BALBneuT mice.

This model belongs to the agent based modeling family, thus including a variety of cellular and molecular entities. Cell entities comprise B lymphocytes, T helper and cytotoxic T lymphocytes. Moreover other agents are represented, such as macrophages, dendritic cells and plasma cells. Each cell entity of SimTriplex model has four attributes: *position*, *specificity*, *state* and *age*.

Position refers to the *lattice-cell* in which entities are located. This parameter is relevant since the interactions are possible only between entities co-located in the same *lattice cell*.

Specificity refers to the set of receptor molecules which characterize each entity defining its ability to bind target molecules (co-receptors, peptides,

epitopes). A clonotypic set of cell agents is characterized by the receptor which is represented by a bit-string of length *l*; hence the potential repertoire scales as *2l*. The binding is described as a matching between binary strings with fixed directional reading frame. The Hamming distance measures the match between bit-strings of length *l*: two bits match if they are complementary. Thus, 0 matches 1 and *vice versa*. If the number of matches is above the binding threshold, which is a parameter of the simulation, then the agents interact. The binding is maximal when all corresponding bits are complementary and so the Hamming distance is equal to the bit string length (*Forrest and Beauchemin, 2007*).

The *state* defines the internal state of each cell entity and the number of states is different among several entity types. Each cell can be in a different internal state and all cells are tracked individually throughout the course of an experimental run.

Finally the *age* is related to the entity age structure. To keep track of the age of cellular entities, the number of time-steps, since the cell birth, is counted. To simulate memory cells, the half-life of T helper, cytotoxic T and B cells is increased after successful interaction with target antigens. The death probability reaches 1 when the age gets to twice the half-life.

Molecular entities included in the simulator are antigens, antibodies, cytokines (IL-2, IFN-γ), MHC I/II and danger signals. Molecular entities do not have internal states and thus do not need to be modelled individually. They are defined for their position and lifetime. Diffusion on the lattice is described by appropriate change of their concentrations.

All these entities are governed by a set of rules. These rules, about 15, resume the main activities of the immune system: phagocytosis, immune activation, opsonization, infection and cytotoxicity.

The model reproduces a portion of mammary tissue (1 mm^3 of the mouse) that is represented as two-dimensional triangular lattice (six neighbour sites), with periodic boundary conditions in both directions. Cells and molecules are

free to move across the lattice sites. At each time step, representing 8 hours of real time, cells and molecules residing on the same lattice are able to interact with each other.

The tumor growth and the Triplex vaccine activity are reproduced by the cancer cells agents, which encode their tumor-associated antigens, and the vaccine cells agents, which include tumor-associated antigens, IL-12 and allogeneic MHC I, respectively. These two cell entities interact with immune entities and between them. The growth of cancer cells is reproduced including three new cancer cells in the lattice at every time step. Once inserted in the system, cancer cells duplicate, and very rarely die by apoptosis. The cancer cell duplication is included as a probabilistic event at each cancer cell once per cycle. The cancer cell population grows following an exponential law with parameters chosen to fit qualitatively the tumor growth observed in the real mice. The model assumes that a solid tumor is formed when the number of cancer cells in the lattice is over 10^5. Vaccine cells are modified tumor cells, which can not duplicate and have a half life of 1 day (*Pappalardo et al., 2005*).

The model has been tuned and validated against existing *in vivo* experiments as described by Motta and colleagues (*Motta et al., 2005*).

AIM

This part of the study had two goals: 1) to identify a candidate optimal vaccination schedule for the Triplex cell vaccine through the SimTriplex simulator and a genetic algorithm; 2) to test its efficacy *in vivo* in BALBneuT mice in comparison with Chronic and other schedules.

RESULTS

In silico research of optimizing vaccination protocols for Triplex

SimTriplex simulator can simulate in about 30 seconds an *in vivo* experiment lasting one year. Taking into account that the actual duration of one *in vivo* experiment is 400 days and that vaccinations are usually administered twice weekly, the number of days available for vaccine administration is about 100. So the possible different schedules are 10^{30}. Although an *in silico* experiment is faster than a real one, the number of possible schedules is too high (about 10^{30}) and the time required to test them exceeds any possible present or future computational capacity (*Nicoletti et al., 2009*).

Thus, looking for an optimized vaccination schedule for Triplex cell vaccine, the SimTriplex simulator was used in combination with a specific genetic algorithm, in which a vaccine schedule is represented as a binary vector in which each position is a time step of the simulator. If a vaccine was administered at that time step, the vector element is set 1, otherwise is 0. The thus constructed binary vector represents a vaccine schedule. The genetic algorithm tried to find the best vector which minimizes the objective functions. In order to set up the objective functions three fundamental and competing requirements were considered: 1) any schedule had to be an effective one, i.e. the mouse survival time must reach 400 days; 2) the best schedule had to provide mice survival with the minimal number of vaccine injections; 3) the number of cancer cells had to be unable both to induce an anergic state of T lymphocytes and to form carcinoma *in situ*. Moreover the schedule had to be optimal not only for a mouse but for a set of mice. Finally, other practical constraints applied to the genetic algorithm were that the vaccinations had to be administered only in working days, with a maximum of two injections per week, each followed by two/three days of rest (*Lollini et al., 2006b*; *Lollini et al., 2008*).

Through *in silico* tools a candidate optimal protocol named Genetic was identified. It was characterized by 32 injections, i.e. almost 50% less than the Chronic protocol, and a predicted tumor-free survival at 63 weeks of age of 81% (Figure 1). The Genetic protocol was then tested *in vivo* in order to evaluate the efficacy of the schedule in BALBneuT mice and validate the simulator.

In vivo experimental design

The preventive ability of the Triplex vaccine consisting of allogeneic IL-12 engineered *neu*$^{+}$ mammary carcinoma cells (Neu/H-2^{q}/IL-12) administered according to the Genetic schedule was evaluated in BALBneuT female mice. The experimental design consisted of five experimental groups of mice: Untreated, Chronic-63, Early, Genetic and Heuristic (Figure 1).

Untreated mice represented the "negative control" group. Previous experiments indicated that all BALBneuT female mice, if untreated, develop mammary tumors around the 20th week of age. Thus this group showed the baseline level of mammary carcinogenesis.

The "positive control group" included mice treated with Triplex cell vaccine according to the Chronic protocol. Previous experiments showed that the tumor-free survival was about 85-100% at more than one year of age. The treatment started at 6 weeks of age and was interrupted at the 63rd week of age. Mice received 60 injections of Triplex vaccine. This schedule was therefore referred to as Chronic-63.

A third group of mice was treated according to the Early protocol. As reported above, this treatment induced a strong delay in tumor onset but shortly after a year of age all mice were positive for tumor. The previously obtained tumor-free survival curve was intermediate between the Untreated and Chronic schedule curves and significantly different from both of them. The Early

protocol started at the 6th week of age. The last administrations were performed at 15 weeks of age and the schedule consisted of a total of 12 injections.

The fourth group was treated according to the Genetic protocol. The vaccination started at the 6th week and ended at the 55th week. This schedule consisted of 32 administrations irregularly distributed over the time with a higher density at earlier time points. So this schedule was different from Chronic-63 in two aspects, *i.e.* number of vaccinations and intervals. The predicted tumor-free survival was 81% at 63 weeks of age.

In the last group mice were treated according to a schedule named Heuristic. This treatment consisted of almost the same number of vaccinations, 30 injections, as the Genetic schedule, but regularly spaced (from the 6th to the 63rd week of age). SimTriplex simulator predicted a 65% tumor-free survival for this protocol at 63 weeks of age.

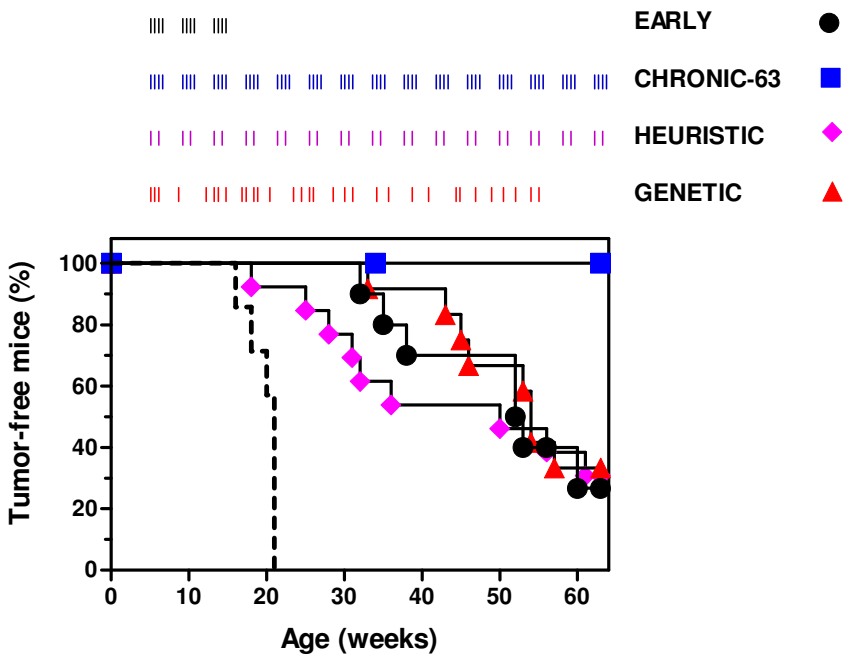


Figure 1. Tumor-free survival curves of BALBneuT female mice vaccinated according to various schedules: Chronic-63 (n=11), Early (n=10), Heuristic (n=13), Genetic (n=12). Untreated mice (dotted line, n=7). The vaccination schedules are shown at the top of the figure. Each bar represents an injection. Significance of differences with Mantel-Haenszel test: Genetic *vs* Chronic-63, $p<0.01$; Early/Heuristic *vs* Chronic-63, $p<0.001$. Survival curves of Early, Genetic and Heuristic groups were not statistically different from one another.

In vivo experiments: tumor-free survival

All treatments started at the 6th week of age. Mice were thereafter examined weekly for the presence of palpable mammary carcinomas.

The tumor-free survival curve of the Untreated BALBneuT group showed that all mice developed the first tumor within the 21st week of age (Figure 1). Thus the baseline level of mammary carcinogenesis was overlapping historical data. The follow-up of the experiment at more than one year of age showed that the kinetics of tumor onset were significantly different for all four treated mice groups compared to the Untreated group. Therefore a protective ability of Triplex cell vaccine was obtained with all the vaccination schedules. However, only the Chronic-63 protocol vaccination gave a complete protection from mammary carcinogenesis (Figure 1).

The tumor-free survival curve of mice treated with Genetic, Early and Heuristic protocols were significantly different compared to the Chronic-63 one but they did not significantly differ among them (Figure 1).

In the Early protocol a progressive fall in tumor-free survival curve was observed, as expected (Figure 1).

At 63 weeks of age Genetic group had 33% tumor-free mice, a value lower than that expected by the simulator prediction (81%). Tumor-free mice in the Heuristic protocol was 31%, lower than that predicted by the simulator (65%) (Figure 2).

Therefore, the simulator correctly ranked the preventive efficacy (Genetic better than Heuristic), but further improvements should be made to obtain a more precise prevision in terms of tumor-free survival curves.

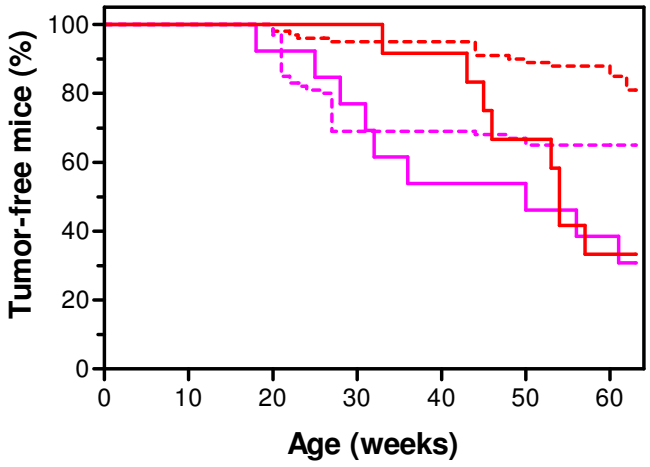


Figure 2. Comparison between tumor-free survival curves obtained by *in vivo* (solid lines) and *in silico* (dotted lines) experiments. Genetic schedule (red), Heuristic schedule (pink).

Triplex efficacy and aging

As reported above, the Chronic-63 schedule was interrupted at 63 weeks of age, i.e. after 60 administrations. At that time all mice were free from tumors. Prolonging the follow-up after the 63rd week of age (Figure 3), it was observed a fast fall in the percentage of tumor-free mice with 100% of mice positive for tumors within about 100 weeks. This result proved that prevention requires periodic boosts even after a year of repeated vaccinations. With life-long Chronic vaccination, tumor onset was only observed after 100 weeks of age (data not shown).

We compared the fall of tumor-survival curve of Early and Chronic-63 schedules. In both groups mice were all free from tumors at the end of vaccination, but kinetics of tumor onset thereafter were different. The gap between the last vaccination and the median latency of mammary tumor was 37 weeks for Early group and only 15 weeks for the Chronic-63 group. The Early protocol induced a more lasting anti-tumor response in young mice than the Chronic-63 protocol in old mice (Figure 3).

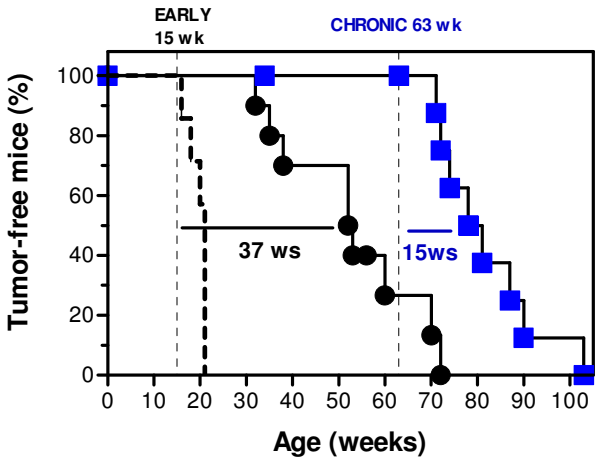


Figure 3. Tumor-free survival curves of BALBneuT mice vaccinated according to Early (black circle) or Chronic-63 (blue square) schedules; Untreated mice (dotted line). The grey thin lines indicates the last vaccination cycle for Early (left) and Chronic-63 schedules (right). The gap between the last vaccination and the median latency of mammary tumors was 37 weeks for Early group and only 15 weeks for the Chronic-63 group.

In vivo experiments: tumor progression

Even though tumor-free survival curves of Genetic, Heuristic and Early protocols were not significantly different among them, some differences in tumor progression was observed when tumor multiplicity (i.e. the mean number of tumors per mouse) was examined. Genetic protocol showed a lower tumor multiplicity than Heuristic, with a significant difference between 35 and 49 weeks of age. The tumor progression of the Early group was intermediate between Heuristic and Genetic ones, although this curve was not significantly different from the curves of the two other protocols (Figure 4).

Genetic and Chronic groups showed similar kinetics of tumor progression up to 50 weeks of age, whereas after this time the Genetic group showed a rise in tumor multiplicity (Figure 4).

Genetic protocol was more effective in controlling the development of multiple tumors compared to Early and Heuristic schedules. On the whole the total number of tumors observed in Genetic group was lower than the number

of tumors of the two other protocols at 63 weeks of age. Thus, considering the cumulative number of tumors at this age, the Genetic schedule prevented 71% of tumors while Early and Heuristic schedules 41% and 48% respectively, in comparison to untreated controls.

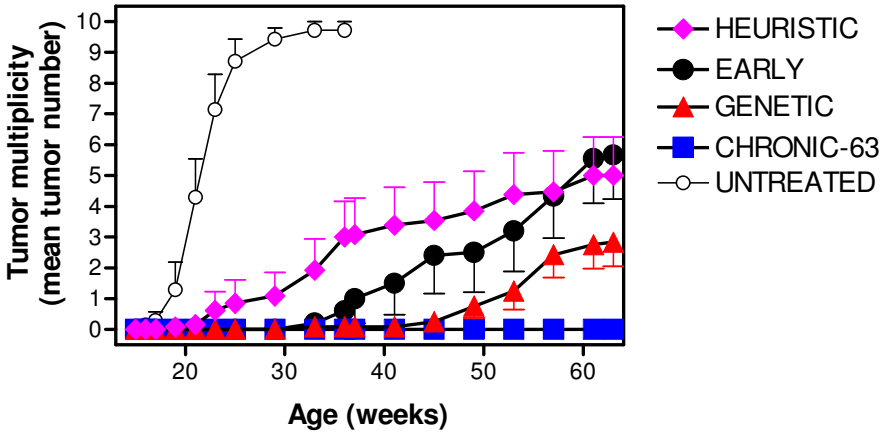


Figure 4. Tumor multiplicity per mouse (mean; *bars,* SE). Chronic-63 (n=11), Early (n=10), Heuristic (n=13), Genetic (n=12); Untreated mice (n=7). Statistical significance: Genetic *vs* Heuristic $p<0.05$ between 35 and 49 weeks of age; Genetic *vs* Early not significant; Heuristic *vs* Early not significant; Genetic *vs* Chronic-63 $p<0.05$ at least between 54 and 63 weeks (Student *t* test).

In vivo experiments: anti-vaccine antibodies

Previously published data proved that anti-vaccine antibodies are essential for cancer immunoprevention induced by Triplex cell vaccine. To study such effector mechanism in relation to the efficacy of different vaccination schedules, mice were bled every four weeks, starting from 9 weeks of age, to quantify the serum levels of anti-vaccine antibodies (Figure 5). The mean value of anti-vaccine antibodies for each group was compared at fixed time points, i.e. after 4, 8, 12,…weeks of vaccination, although each protocol consisted of a different number of injections during the same period. As an example in the

first eight weeks of vaccination, Chronic-63 and Early consisted of 8 administrations, Heuristic 4, Genetic 5.

All treated mice produced anti-vaccine antibodies but differed in antibody kinetics and levels. Chronic and Early protocols (which share the same schedule and only differ after the stop of vaccinations for Early group at 15 weeks of age) showed a very fast increase in anti-vaccine antibody levels, which reached the top at about 17 weeks of age with median fluorescence intensities approaching 1000 arbitrary units. After the stop of vaccination, however the antibody curve of the Early group began to fall. Genetic schedules caused a slightly slower rise in antibody level, reaching a maximal value similar to those of Chronic and Early groups but only after 21 weeks of age. Heuristic group showed a very slow rise in antibody level.

A comparison between these data and tumor-free survival curves suggested that vaccine-induced antibodies must reach quickly a safety threshold (identified above 700 arbitrary units of median fluorescence intensity) in order to prevent mammary carcinoma. Then, this level should be kept above the threshold for the entire lifespan of the mouse. A slow increase of antibody titer or a decrease of this level correlate with tumor onset at 1 year of age, as proved by Heuristic and Early protocols that were less protective than other schedules.

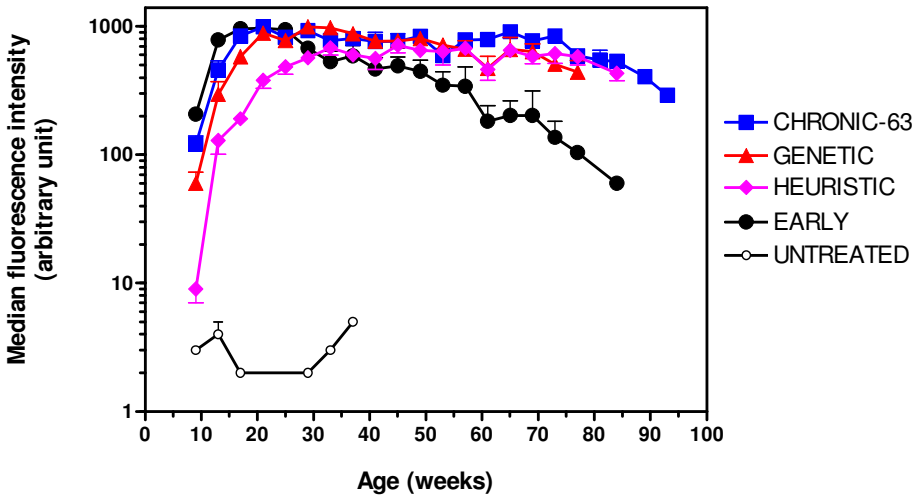


Figure 5. Kinetics of anti-vaccine antibodies (mean with n= 2-13; *bars,* SE). Sera of mice (diluted 1:65) were investigated for the presence of antibodies through binding to Neu/H-2^q cells and cytofuorometric analysis.

The results reported above will be included in a manuscript in preparation.

DISCUSSION

Triplex cell vaccine could prevent almost completely mammary tumor onset in BALBneuT female mice if administered according to a life-long protocol referred to as Chronic. However, no previous studies investigated if all the administrations of the Chronic schedule are necessary. The present research work aimed at looking for an optimal schedule for the Triplex cell vaccine, i.e. a schedule with a reduced number of treatments but maintained efficacy.

The *in silico* model SimTriplex driven by a genetic algorithm, led to the design of a vaccination protocol, referred to as Genetic, that was tested *in vivo* in BALBneuT female mice. It consisted of only 32 administrations, against 60 injections of the Chronic-63 protocol (i.e. a Chronic schedule stopped at 63 weeks of age). *In silico* runs predicted a high protective ability of the Genetic protocol with 81% of tumor-free mice at the end of experiment. The *in vivo* testing included five experimental groups of mice: Untreated, Chronic-63, Early, Genetic and Heuristic. The latter schedule consisted of almost the same number of vaccinations, 30 injections, as the Genetic schedule, but regularly distributed. SimTriplex simulator predicted for this protocol 65% tumor-free mice at 63 weeks of age.

The tumor-free survival curves of the Genetic and Heuristic groups produced by *in vivo* experiments only partially confirmed the prediction of the simulator, giving evidence of a protective ability lower than expected.

Though the first version of SimTriplex did not include the concept of multiple mammary areas, taking into account the overall rate of mammary tumors developed in each mouse within the 63rd week of age, the Genetic schedule was able to prevent the 71% of tumors and the Heuristic protocol 48% in comparison to the untreated mice. These data were closer to the simulator prediction than tumor-free survival curves.

As reported (*Nanni et al., 2004*), anti-vaccine antibodies are a predictive marker of the protection level induced by the Triplex vaccine. The comparison

of antibody kinetics among groups showed a slower increase of antibodies for the Heuristic group. The slow rise of antibody level was probably caused by the small number of injections (only 2) in the first month of treatment compared with 4 administrations of the Chronic-63, Early and Genetic protocols.

As a consequence the first month of treatment, and in particular the first two weeks, seem to be relevant to ensure a fast increase of anti-vaccine antibodies. In this way an antibody level above the 700 arbitrary units of median fluorescence, identified as safety threshold, might be reached within the third month of the treatment.

The anti-vaccine antibody curve of the Early treatment suggested another issue; the absence of boosts after the first three months of vaccination was followed by a progressive decrease of antibodies that paralleled the fall in the protective ability. Consequently an effective immunopreventive schedule for the Triplex vaccine should maintain a level of anti-vaccine antibodies above the threshold for the entire life-span of the mouse in order to ensure a high protection.

The anti-vaccine antibody curve of the Genetic group showed a more rapid increase than the Heuristic one during the first weeks of treatment. On the other hand, the anti-vaccine antibody level of the Genetic group reached after a month of treatment, was slightly lower than the antibody levels of Chronic-63 and Early groups, even if in the first month all the three schedules consisted of 4 injections. A reason could be that in Genetic schedule the 4^{th} administration was performed too late (on 24^{th} day of treatment) to give a contribution to the increase of antibody level in the first month (first point of the curve). Then the mean antibody level of the group remained over the threshold until about 50 weeks of age, much longer than the Early schedule. Subsequently it started to decrease and consequently the tumor-free survival, too. These two peculiarities of the Genetic curve may justify its lower efficacy compared with the Chronic-63 protocol.

The tumor-free survival curve produced by mice treated with the Chronic-63 protocol showed that the absence of boosts after 63 weeks of age caused a fast tumor onset since after only 15 weeks 50% of treated mice had already developed mammary carcinoma. Therefore multiple vaccination cycles, i.e. 60 administrations, were not sufficient to ensure a prolonged immune protection against tumor after the end of vaccination: further boosts are required. Dominguez and colleagues (*Dominguez and Lustgarten, 2008*) showed that tolerant mice cannot develop memory responses against self tumor antigens. This consideration could justify the rapid increase of tumor onset in BALBneuT mice after stopping the Chronic-63 treatment.

The comparison of tumor-free survival curves of Early and Chronic-63 groups shows that younger mice maintained an anti-tumor immune response for longer time after vaccine stop than elder mice. As reported by Dominguez and colleagues (*Dominguez and Lustgarten, 2008*), elder mice have a higher number of Treg cells than younger ones. However they observed that the depletion of Treg cells in combination with vaccination induced an effective anti-tumor response and developed immune memory only in younger mice. They justify the low efficacy of this combined treatments in elder mice considering that these mice accumulate some defects that, influencing the immune system, reduce the activation of immune response. For this reason the depletion of Treg cells alone might not be sufficient for the complete restoration of immune responses in old tolerant hosts. Therefore Treg cells and other factors could contribute to a more rapid decrease of the protective response in the Chronic group than in the Early group.

In vivo validation of the SimTriplex simulator by testing the vaccination schedules in BALBneuT mice did not fully confirm the expected protective efficacy of the Genetic and Heuristic schedules in terms of tumor-free survival time. Quantifying the protective ability as percentage of prevented tumors compared to untreated mice, *in vivo* and *in silico* results are closer.

As reported in other studies (*Croci et al., 2004*; *Cipriani et al., 2008*) the level of anti-vaccine antibodies at specific time points can predict the ability of a vaccine to prevent tumor onset. This indication was confirmed by the analysis of anti-vaccine antibodies kinetics of the above described *in vivo* experiments. Reaching the safety threshold as soon as possible and maintenance of the antibody level above this threshold seem to be related to vaccine preventive efficacy.

Data produced by *in vivo* validation of the simulator could be used for a second tuning of SimTriplex model. Moreover in order to improve the simulations to better reflect these experimental results, a particular attention should be given to the simulation of precocious vaccine-induced immune responses and to the different immune response in younger and elder mice.

SECTION 2

ANTIMETASTATIC ACTIVITY OF THE TRIPLEX VACCINE

INTRODUCTION

Cancer vaccines: Immunoprevention versus Immunotherapy

Cancer vaccines represent an immunologic approach which can be applied both to immunoprevention and immunotherapy of tumors. The aim of cancer immunotherapy is the eradication of existing tumors. Cancer immunoprevention implements cancer vaccines before tumor onset to induce an immune response that protects the host from carcinogenesis and tumor progression.

The targets of immunotherapeutic cancer vaccines are established malignant tumors. A large neoplastic mass is generally able to grow rapidly and to activate a great number of immunosuppressive strategies, thus it is more resistant to an immunologic attack than a small pre-neoplastic lesion. The activity of several genes is compromised after early genetic alterations and this instability increases with the tumor progression. The selective pressure exerted by the immune system can lead to the selection of tumor clones. Thus a weak immune response could select tumor variants that are less antigenic and less sensitive to immune attack (*Lollini et al., 2006a*). Concerning immune mechanisms, it was shown that the long-term efficacy of vaccines used to prevent carcinogenesis is mediated more by antibodies than by CTL, that are the main actors of cancer immunotherapy (*Nanni et al., 2007*). Consequently a vaccine that is effective in a preventive setting could lose its efficacy in a therapeutic context since the target of the treatment, together with immune effectors required, are different.

Immunopreventive vaccines and clinical trials

The essential distinction between prevention and cure, however, is less striking in the transition from experimental models to clinical practice, as a hypothetical first human implementation of immunopreventive vaccines, phase I and phase II studies, should be performed in cancer patients, i.e. in a therapeutic context rather than preventive one. The use of cancer vaccines with a therapeutic aim in human patients has achieved poor results so far, partial responses are rare and complete responses extremely rare (*Lollini et al., 2006a*; *Finn, 2008*; *Rice et al., 2008*).

Poor therapeutic activity of these vaccines in preclinical studies announced many negative results subsequently obtained in clinical trials. Thus mouse models can help to investigate if an immunopreventive vaccine could be effective also against a pre-existing tumor mass. If a vaccine looks effective, the study should be focused on the immune mechanisms activated against tumor mass and on the comparison with immunopreventive ones.

Triplex vaccine and in vivo therapy

The Triplex vaccine loses progressively its efficacy with the advancement of tumor progression in BALBneuT mice (*Nanni et al., 2007*). It was observed that this vaccine had little or no efficacy at all against incipient mammary carcinomas.

A key step of malignant tumor progression after local growth is metastatic dissemination, which represents a restriction point together with early tumor onset.

AIM

This study was developed to evaluate the therapeutic power of the Triplex cell vaccine against the development of experimental lung metastases.

RESULTS

Therapy of lung metastases with Triplex cell vaccine in tolerant mice

TUBO mammary carcinoma *neu*$^+$ cells (referred to as Neu/H-2^d) were used in this study to induce *neu*$^+$ experimental metastases (Figure 6A) in syngeneic BALBneuT mice. At day 7 after TUBO injection, mice entered the vaccination protocol with Triplex cell vaccine (Neu/H-2^q/IL-12). Control groups included untreated mice (None) and mice treated with single "components" of the Triplex vaccine: recombinant IL-12, mammary carcinoma *neu*$^+$ syngeneic cells (Neu/H-2^d) or allogeneic mammary carcinoma cells (Neu/H-2^q).

The great number of injected cells ($2.5x10^4$ cells) causing a high metastatic burden and the choice of starting the vaccination at day 7 when cells had already established metastatic deposits (Figure 6A) contributed to obtain stringent and realistic preclinical conditions.

As shown in Table 1, the Triplex cell vaccine produced more than 87% reduction of lung nodules in BALBneuT mice compared to untreated mice. The study of semiserial sections showed that the number and the dimensions of lung metastases after 14, 21, and 28 days were lower in vaccinated mice than in untreated mice; moreover, the structure of metastatic lesions was frequently cribriform and less compact than in controls (Figure 6B). All the three stimuli of Triplex vaccine were essential to obtain this good result, since the three other "incomplete" vaccines did not show significant curative ability (Table 1).

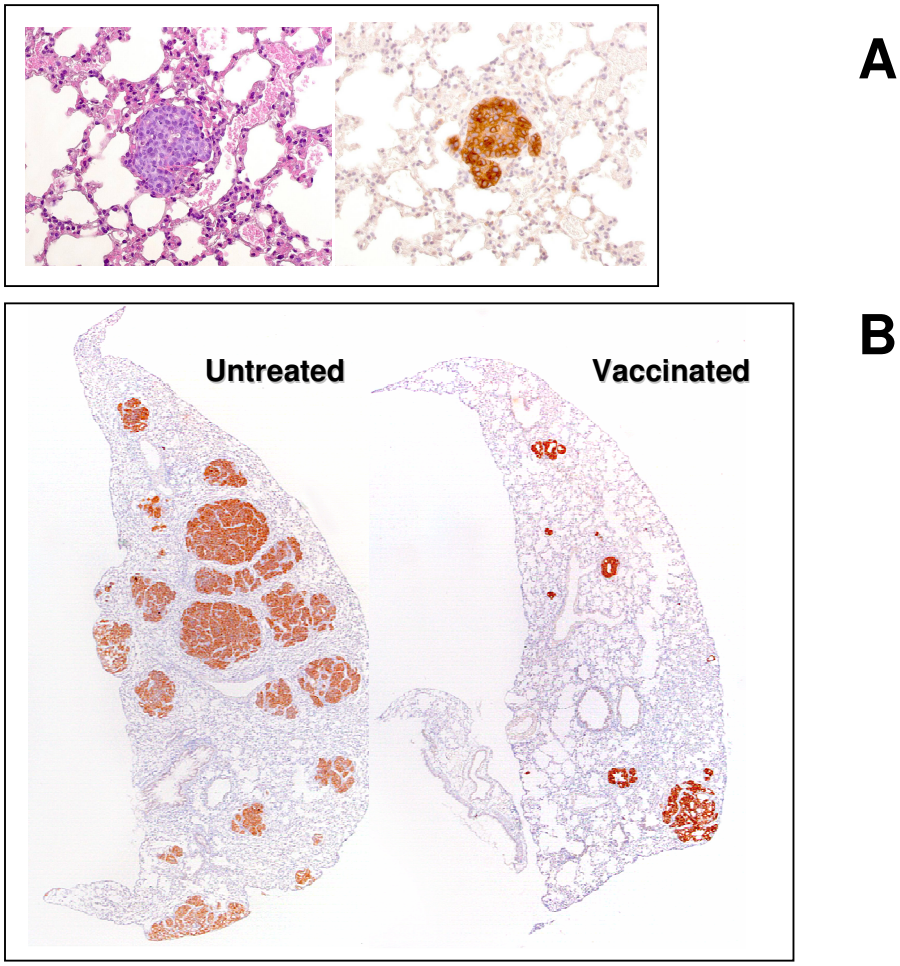


Figure 6. Metastatic development in untreated and vaccinated BALBneuT mice. *A*, mammary carcinoma metastases developing in the lungs of BALBneuT mice seven days after the i.v. injection of TUBO cells (*left panel*, hematoxylin and eosin, 400×, *right panel*, HER-2/neu immunohistochemistry, 400×). *B*, HER-2/neu-stained metastases in the lungs of untreated and vaccinated mice (25×); vaccination started 7 days after metastasis induction, mice were sacrificed 21 days later.

Table 1. Therapeutic vaccination of mice bearing lung micrometastases.

Mice	Start[1] of vaccination	Group	Vaccination	Lung nodules		
				Incidence	Median	Range
BALBneuT	**Day 7**	(a)	None	9/9 (100%)	>200	134->200
		(b)	IL-12	5/5 (100%)	86	42->200
		(c)	Neu/H-2^d	5/5 (100%)	117	89->200
		(d)	Neu/H-2^q	4/4 (100%)	124	59->200
		(e)	Neu/H-2^q/IL-12 (Triplex)	12/12 (100%)	26¶	1-165
	Day 1	(f)	None	10/10 (100%)	>200	80->200
		(g)	IL-12	5/5 (100%)	60$	23-153
		(h)	Neu/H-2^q	5/5 (100%)	81$	76->200
		(i)	Neu/H-2^q/IL-12 (Triplex)	9/10 (90%)	3§	0-27
BALB/c	**Day 7**	(l)	None	10/10 (100%)	>200	47->200
		(m)	Neu/H-2^d	5/5 (100%)	79†	27-97
		(n)	Neu/H-2^q	4/5 (80%)	11†	0-21
		(o)	Neu/H-2^q/IL-12 (Triplex)	1/11 (9%)	0‡	0-4
	Day 1	(r)	None	18/18 (100%)	>200	95->200
		(s)	Neu/H-2^q	0/5 (0%)	0*	0-0
		(t)	Neu/H-2^q/IL-12 (Triplex)	0/15 (0%)	0*	0-0

[1]Days after the i.v. challenge with TUBO cells.

Significance (Wilcoxon rank sum test):

¶ *vs* **a**, **b**, **c** and **d**, $p<0.05$ at least; $ *vs* **f**, $p<0.05$ at least; § *vs* **f**, **g** and **h**, $p<0.01$ at least; † *vs* **l**, $p<0.01$ at least; ‡ *vs* **l**, **m** and **n**, $p<0.01$ at least; * *vs* **r**, $p<0.001$.

Therapy of early lung metastases

Even if the Triplex vaccine showed a high anti-metastatic activity, all vaccinated mice still had sizable metastatic burdens. A possible limiting factor could be the advancement of the metastatic stage. Therefore the beginning of vaccination was anticipated at day 1 after the i.v. injection of Neu/H-2^d cells.

The Triplex cell vaccine produced a stronger antimetastatic effect, giving a 99% reduction of the metastatic development. Under these experimental conditions recombinant IL-12 and Neu/H-2^d cells alone gave a significant antimetastatic effect, but did not reach Triplex vaccine efficacy (Table 1).

Therapy of lung metastases in nontolerant mice

BALB/c mice have the same background of BALBneuT mice but are not tolerant for $p185^{neu}$ antigen, while BALBneuT mice are tolerant for $p185^{neu}$ antigen. Data reported in Table 1 show that the elimination of this limiting factor improves the efficacy of the Triplex cell vaccine. In fact more than 90% of BALB/c mice treated with Triplex vaccine from day 7 did not develop lung nodules. A significant antimetastatic effect was also reported for vaccinations with syngeneic and allogeneic neu^+ mammary cancer vaccines lacking IL-12. The beginning of the vaccination at day 1 produced a complete protection from lung metastases both for the Triplex vaccine and allogeneic cell vaccine lacking IL-12 (Neu/H-2^q).

Depletion of Treg cells enhances antimetastatic efficacy

The stronger therapeutic activity of the Triplex vaccine in nontolerant BALB/c mice confirmed that tolerance is a limiting factor for vaccine efficacy. In order to increase the Triplex cell ability in tolerant BALBneuT mice, host tolerance was manipulated through a transient block of regulatory T cells, since Treg cells contribute to the maintenance of tolerance *in vivo* (*Ambrosino et al., 2006*).

BALBneuT mice bearing lung metastases received multiple i.p. injections of an anti-CD25 mAb named pC61 (see "Materials and Methods"). Such treatment enhanced significantly the efficacy of Triplex cell vaccine (Table 2): vaccination started at day 7 reduced metastatic lung nodules of 99% compared to untreated group, with an efficacy similar to that obtained in BALBneuT mice without Treg depletion and starting the vaccination at day 1.

Table 2. Therapeutic vaccination of BALBneuT mice selectively depleted of Treg cells.

Depletion	Group	Vaccination	Start[1] of vaccination	Lung nodules		
				Incidence	Median	Range
None	(a)	None		12/12 (100%)	190	80->200
None	(b)	Neu/H-2^q/IL-12	Day 7	5/5 (100%)	30	4-68
Treg	(c)	Neu/H-2^q/IL-12	Day 7	4/6 (67%)	3*	0-24
None	(d)	Neu/H-2^q/IL-12	Day 1	9/10 (90%)	3	0-27
Treg	(e)	Neu/H-2^q/IL-12	Day 1	2/6 (33%)	0$^{\#}$	0-11

[1]Days after the i.v. challenge with TUBO cells.

Significance (Wilcoxon rank sum test): * *vs* **b**, $p<0.05$ at least; # *vs* **d** $p<0.05$ at least.

Immune response elicited by the Triplex vaccine

Sera of untreated and treated BALBneuT mice were collected and investigated for the presence of anti-vaccine antibodies, that mainly consist of anti-p185^{neu} antibodies (*Nanni et al., 2001*). Cytofluorometric analysis of these samples showed a high level of antibodies that increased significantly in Treg depleted mice vaccinated with Triplex (Figure 7, upper panel).

Then spleen cells of untreated and vaccinated mice were collected and cultured for six days with different target cells: mammary carcinoma *neu*

negative allogeneic cells (Neu^{neg}/H-2^q), mammary carcinoma *neu*$^+$ syngeneic cells (Neu/H-2^d) and mammary carcinoma *neu*$^+$ allogeneic cells (Neu/H-2^q). The ability of restimulated lymphocytes to secrete IFN-γ and IL-4 in the supernatants was evaluated by ELISA tests. IFN-γ was already evident in spontaneous splenocyte cultures of vaccinated mice (without restimulation) and increased significantly after restimulation with target cells. Cells expressing allogeneic MHC alone or $p185^{neu}$ alone were able to restimulate spleen cells but the combination of these two different stimuli produced the highest level of IFN-γ (Figure 7, middle panel). Finally, IL-4 production was observed both for unstimulated spleen cells and for cells co-cultured with *neu*$^+$ cell lines (Figure 7, lower panel).

CTL assays against *neu*$^+$ syngeneic mammary carcinoma target cells did not reveal any significant activity above background (data not shown).

Immunohistochemical studies were performed in order to analyze local antimetastatic immune responses in the lungs of vaccinated mice. Analysis revealed a low reactive infiltrate at all examined time points. However a slight increase in the presence of $CD4^+$ T cells at the 14^{th} day and of macrophages and granulocytes at the latter time points were observed in vaccinated mice (Figure 8, left panel), whereas no difference was found in the presence of $CD8^+$ T and $B220^+$ B cells at any time point (data not shown). $Foxp3^+$ regulatory T cells were not observed in the lungs of both vaccinated and untreated mice. The vascularization around and inside metastases and the expression and staining intensity of the HER-2/neu product were similar in vaccinated and control mice.

Electron microscopy showed that several macrophages in vaccinated mice were in close contact with tumor cells particularly at the metastasis periphery, where some of them showed aspects of necrosis (Figure 8, right panel).

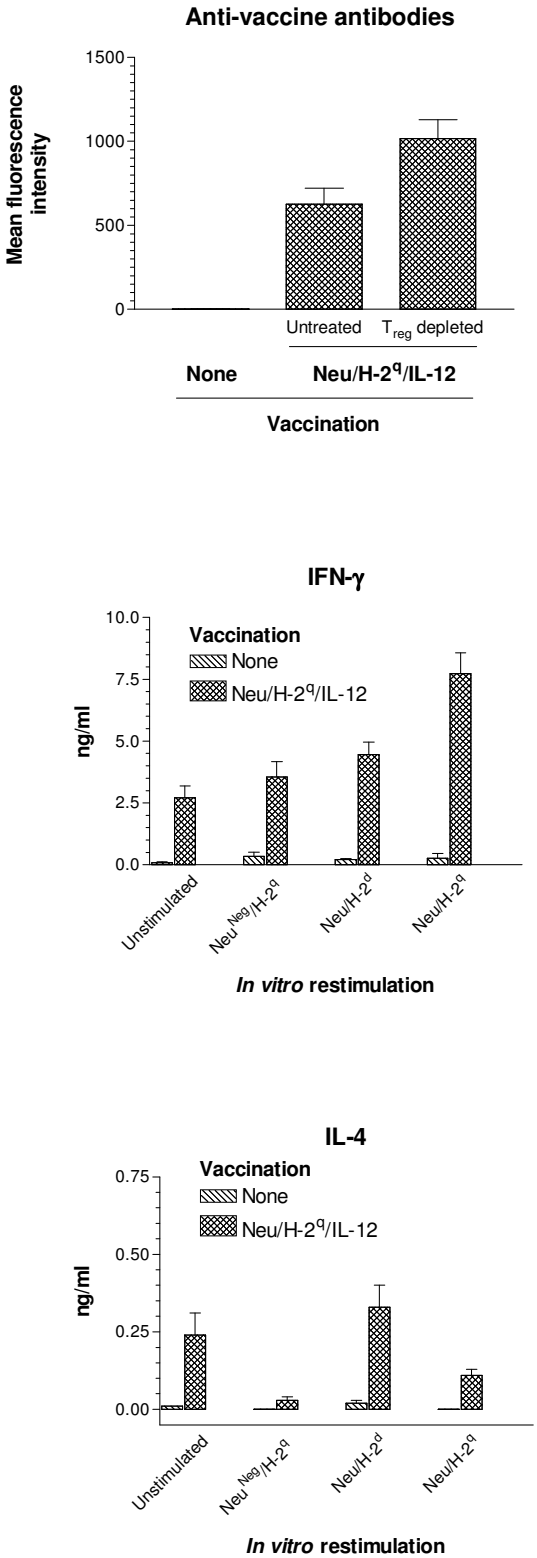
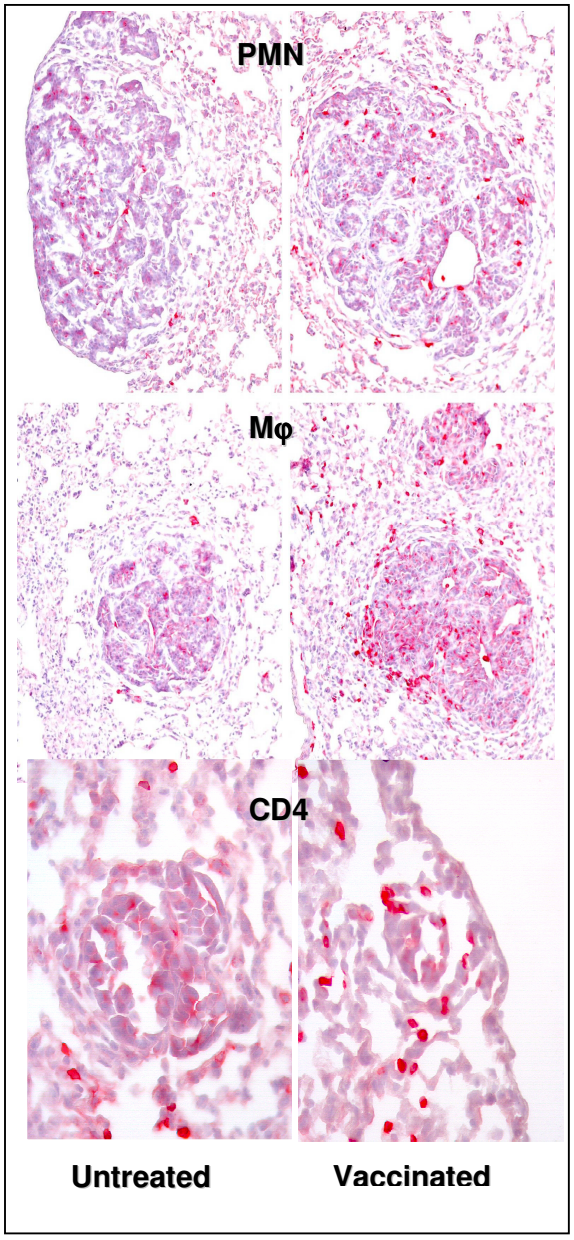


Figure 7. Humoral and cellular immune responses induced by the Triplex cell vaccine (Neu/H-2^q/IL-12) in BALBneuT mice. *Upper*, binding of mice sera (diluted 1:65) to Neu/H-2^q cells as evaluated by cytofuorometric analysis. Each bar represents the mean of 5-10 mice bled after seven vaccine administrations. Statistical significance: vaccinated mice *vs* untreated, $p<0.005$, Treg depleted mice *vs* non-depleted mice, $p<0.05$ (Student's *t* test). *Middle*, release of IFN-γ by splenocytes of untreated and vaccinated mice. Spleen cells were incubated *in vitro* for six days alone or with mitomycin C-treated tumor cells expressing allogeneic MHC glycoproteins (Neu^{neg}/H-2^q), $p185^{neu}$ (Neu/H-2^d), or both (Neu/H-2^q). Supernatants were collected for cytokine ELISA assay and splenocytes were recovered and counted. Each bar represents the mean of 10 mice. Statistical significance: all vaccinated groups *vs* untreated controls, $p<0.001$ at least; restimulation with Neu/H-2^q *vs* unstimulated or other restimulations, $p<0.001$ (Student's *t* test). *Lower*, release of IL-4 by splenocytes of untreated and vaccinated mice, culture conditions as above for IFN-γ. Statistical significance: all vaccinated groups *vs* untreated controls, $p<0.01$ at least; restimulation with Neu/H-2^q *vs* restimulation with Neu/H-2^d, $p<0.01$ (Student's *t* test).



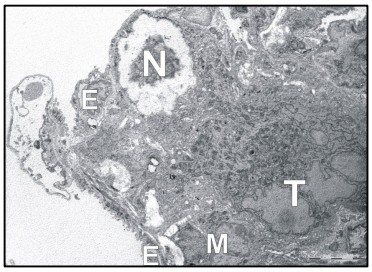


Figure 8. Antimetastatic immune responses induced by vaccination in mice treated 7 days after i.v. tumoral cell injection. *Left panel*, immunohistochemical stainings performed on untreated and vaccinated mice showed in vaccinated mice an increase in the presence of $CD4^+$ cells (200×) 7 days after vaccination and of macrophages and granulocytes 14 days after vaccination (630×). *Right panel*, electron micrograph showing a metastatic aggregate occupying the lumen of a lung capillary lined by endothelial cells (E) and flat protrusions of pneumocyte cytoplasms. A macrophage (M) is in close contact with the epithelial tumoral cell (T). A severely damaged cell with clear aspects of necrosis (N) is visible (2800×).

Antimetastatic immune mechanisms

To study immune mechanisms elicited by the Triplex vaccine, experiments with mice deficient in various immune responses were conducted.

$Rag2^{-/-}/\gamma c^{-/-}$ mice lack B, T and NK populations and their products like antibodies and cytokines. The Triplex vaccine in these mice did not modify both incidence and median number of lung nodules (Table 3).

Table 3. Therapeutic vaccination of immunodeficient mice.

Immune deficit	Group	Vaccination	Lung nodules			
			Incidence		Median	Range
None	(a)	None	10/10	(100%)	>200	47->200
	(b)	Neu/H-2^q/IL-12	1/11	(9%)	0	0-4
$Rag2^{-/-}$; $\gamma c^{-/-}$	(c)	None	4/4	(100%)	>200	>200->200
	(d)	Neu/H-2^q/IL-12	5/5*	(100%)	>200*	>200->200
μMT	(e)	None	11/11	(100%)	185	51->200
	(f)	Neu/H-2^q/IL-12	3/7 #	(43%)	0#	0-2
IFN-$\gamma^{-/-}$	(g)	None	5/5	(100%)	185	86->200
	(h)	Neu/H-2^q/IL-12	6/6*	(100%)	22*	2-65

Significance (Fisher's exact test or the Wilcoxon rank sum test as appropriate):

vs* **b, $p<0.01$ at least; # *vs* **b**, not significant

To analyze the contribution of T cells, $CD4^+$ and $CD8^+$ T populations were depleted in BALBneuT mice by specific anti-CD4 and anti-CD8 antibodies (see "Materials and Methods"). All CD4-depleted vaccinated mice (Table 4) developed lung metastases. The median number of lung nodules was significantly increased compared to vaccinated mice without depletion

confirming that T helper cells have an important role in the antimetastatic immune mechanisms elicited by the vaccine. Depletion of $CD8^+$ cells did not significantly modify Triplex vaccine efficacy. These data are in agreement with the negative results of *in vitro* CTL assays. The third investigated population was NK cells, depleted by anti-asialo GM1 antiserum. The Triplex vaccine efficacy was not modified by NK cell depletion. Therefore NK population did not play a role in mediating the activity of the vaccine (Table 4).

In vitro tests and previous data on immune effectors elicited by Triplex vaccine (*Nanni et al., 2004*) showed that a major contribution of cell-mediated immunity to cancer immunoprevention was through the release of cytokines, in particular IFN-γ and anti-vaccine antibodies production. To investigate if IFN-γ played a causal role in the antimetastatic efficacy, nontolerant IFN-γ deficient mice were vaccinated. In the absence of this cytokine a significant loss of efficacy of the vaccine was found, and all mice developed a sizeable metastatic burden compared to vaccinated nontolerant mice, thus confirming the key role of IFN-γ. On the contrary Triplex vaccine maintained a significant antimetastatic efficacy in nontolerant antibody-deficient mice (μMT) suggesting that the presence of anti-vaccine antibodies was not fundamental for therapeutic efficacy (Table 3).

Table 4. Therapeutic vaccination of BALBneuT mice selectively depleted of immune cell populations.

Depletion	Group	Vaccination	Start[1] of vaccination	Lung nodules		
				Incidence	Median	Range
None	(a)	None		12/12 (100%)	190	80->200
None	(b)	Neu/H-2^q/IL-12	Day 1	4/5 (80%)	4	0-27
CD4	(c)	Neu/H-2^q/IL-12	Day 1	6/6 (100%)	39*	7-121
CD8	(d)	Neu/H-2^q/IL-12	Day 1	5/5 (100%)	11$^{\$}$	1-18
None	(e)	Neu/H-2^q/IL-12	Day 3	5/5 (100%)	36	15-59
NK	(f)	Neu/H-2^q/IL-12	Day 3	5/5 (100%)	41$^{\#}$	14-121

[1]Days after the i.v. challenge with TUBO cells.

Significance (Wilcoxon rank sum test): * *vs* **b**, $p<0.01$ at least; $ *vs* **b**, not significant; # *vs* **e**, not significant.

The results reported above have already been published (*Nanni et al., 2007*).

DISCUSSION

The high preventive efficacy of the Triplex cell vaccine previously reported opened up the prospective of a future translation of the treatment to clinical patients. Early human trials will be necessarily performed in advanced cancer patients, in a therapeutic, rather than preventive set-up. The target of cancer therapeutic approaches could be an established primary tumor as well as metastasis formation. The Triplex cell vaccine activity was investigated, in this thesis, in a preclinical model against the development of experimental lung metastases.

First of all, the vaccine ability was evaluated in BALBneuT mice, immunologically tolerant for the oncoantigen *neu*. The treatment was started seven days after the experimental metastasis induction, i.e. on sizeable and organized metastatic deposits to reinforce this preclinical model. This delay could correspond to some months of metastatic development in humans. The vaccine decreased the metastatic burden more than 87%. Such successful result was further improved by an earlier beginning of the treatment.

The tolerance of BALBneuT immune system for the *neu* oncoantigen surely obstacles the complete eradication of lung metastases by Triplex vaccine. An indirect proof was obtained by the therapy of experimental metastases in nontolerant BALB/c mice since the Triplex vaccine cured almost all mice (> 90%), starting the treatment at day 7, and all mice starting at day 1.

The stronger therapeutic activity of the Triplex vaccine in nontolerant mice suggested that manipulation of peripheral tolerance should enhance antimetastatic efficacy in tolerant mice. Treg cells have been reported to suppress the immune response against tumors and decrease the efficacy of cancer vaccines (*Ambrosino et al., 2006*; *Wei et al., 2008*; *Finn, 2008*). The depletion of Treg cells in BALBneuT mice increased the activity of Triplex cell vaccine since, combining the two treatments, the therapeutic results were of the same order of magnitude as those obtained in nontolerant mice.

The therapeutic activity of Triplex vaccine is supported by various immune mechanisms: among them IFN-γ is the playmaker of the attack against the tumor as demonstrated by *in vivo* and *in vitro* results. Mice knockout for this cytokine were not significantly protected from lung metastases by Triplex vaccine. Moreover the spleen cells derived from vaccinated mice could produce IFN-γ and specific restimulation increased its production level. The key role of IFN-γ is closely related to a T helper response polarized to Th1 immunity. However, *in vitro* tests also showed a significant production of IL-4 in this system, proving that Th2 lymphocytes were activated as well. The importance of $CD4^+$ cells was confirmed by *in vivo* studies in immunodepressed mice, by specific depletion of $CD4^+$ cell population.

High anti-*neu* antibody levels were produced by treated mice, but vaccinated antibody-deficient mice were still protected from lung metastases compared to untreated ones. These data are surprising because mammary cancer prevention by Triplex is completely dependent on antibody production (*Nanni et al., 2004*), and shows that therapy of lung metastases does not depend on anti-*neu* antibodies. The lower therapeutic relevance of the antibody response in metastasis therapy could be explained considering that the time required for the vaccine to induce a strong secondary IgG response was of the same order of magnitude as that of metastasis growth. In the case of cancer prevention, on the contrary, a full-fledged antibody response could reach its upper plateau when fully neoplastic cells are not yet present in the mammary gland (*Di Carlo et al., 1999*; *Nanni et al., 2001*).

In cancer immunoprevention it was observed that the most relevant activity of IFN-γ was the induction of protective antibody responses because vaccine activity completely disappeared both in IFN-γ knockout mice, which no longer produced protective Ig isotypes, and in antibody-deficient mice, which, however, retained the capacity to produce IFN-γ (*Nanni et al., 2004*). In the therapeutic set-up mice lacking anti-HER-2/neu antibodies were protected from

metastatic development, thus other activities of IFN-γ played a more fundamental role than Ig class switch.

Additional vaccine-induced actions were promoted by granulocytes and macrophages infiltrating tumor cell nests in the lungs, therefore the cure of lung metastases took advantage from the peculiar anatomic localization of these metastasis since local inflammatory responses in the lungs play a major role in the defense of host against foreign mechanisms (*Di Carlo et al., 2001*).

On the whole, the Triplex vaccine is an effective treatment not only for the prevention of mammary carcinogenesis but also for the cure of micrometastases. The therapeutic activity of Triplex cell vaccine reported in this thesis is a promising result that opens up the possibility of early clinical testing in a therapeutic, rather than prophylactic, human context.

SECTION 3

A DNA-TRIPLEX VACCINE FOR p53/neu DRIVEN CANCER SYNDROME

INTRODUCTION

Rationale of a DNA Triplex vaccine

The good results of Triplex vaccine as cancer immunopreventive treatment are darkened by several limits. The difficulties to use an allogeneic cell vaccine administered lifelong in human patients are related to logistics (good manufacturing practice-certified cell culture facility, storage and distribution), eligibility (depending on patient's histocompatibility profile) and safety. Although previous studies reported that all the three components of Triplex vaccine are necessary, it was never investigated if they are also sufficient. In fact Triplex cell vaccine likely includes other immune stimuli; some of these molecules could be undesirable and/or unnecessary. Since an optimized vaccine should avoid unnecessary immune stimulation, the so far used form of Triplex cell vaccine could be an obstacle for clinical translation.

The need of easier, simpler and more controlled vaccine formulations could find an answer in DNA vaccines. The possibility to obtain a second-generation Triplex DNA vaccine was therefore investigated.

DNA vaccines

DNA vaccines are a promising strategy being simple to construct, produce and deliver.

DNA vaccines consist of plasmids carrying specific expression cassettes coding for antigenic moieties. Tumor antigens coded for by DNA range from full-length or extracellular domain sequences, required to induce an antibody response, to short MHC class I- or II-binding peptides sequences, to optimize

induction of T-cell responses (*Rice et al., 2008*). Gene expression is mostly driven by the cytomegalovirus immediate early enhancer promoter (CMV IE-EP) and its adjacent intron A sequence, which ensure high transcription efficiency. Other elements include a transcription termination signal and a prokaryotic antibiotic resistance gene (*Draghia-Akli et al., 2008*). An important component of the plasmid can be the presence of unmethylated CpG motifs which can stimulate the innate immunity by binding to Toll-like receptor 9 (TLR9). The activated signaling cascade promotes the production of IFN-α, inflammatory cytokines such as IL-12, and chemokines. IFN-α induces a Th1 response (*Prud'homme, 2005*). Further modifications are being explored to increase performance of vaccines such as the combination with immunostimulatory molecules: chemokines to attract antigen-presenting cells (APC), activating cytokines, co-stimulatory molecules, APC-targeting antibodies and molecules to manipulate antigen presentation and/or processing (*Rice et al., 2008*).

DNA vaccines can be administered by intramuscular, dermal/epidermal, oral, pulmonary or other routes (*Prud'homme, 2005*). The most popular way of delivery is by intramuscular (i.m.) injection. Skeletal muscle is a good target tissue because it is characterized by post-mitotic muscle fibers and high vascularization, moreover muscles are easily accessible and the expression of the target gene is localized (*Prud'homme et al., 2006*). Transfected muscle cells can express antigen that can act as a target for immune effectors. For activation of T cells, antigen must be transferred to a “professional” antigen-presenting cells by cross-presentation; a small rate of DNA is also taken up directly by APC (*Rice et al., 2008*).

DNA vaccination by i.m. route is not usually in large species as effective as in rodents. A major reason for the weak immune response obtained in large animals could be the volume of injection. This parameter is relevant for the production of an hydrostatic pressure which increases transfection and contributes to inflammation by causing a sufficient local damage. Scaling up

from a volume of injections able to induce strong immune responses in rodents, e.g. 50 µl/muscle in mice, to human subjects would be unacceptable (*Rice et al., 2008*).

Several approaches were developed to increase plasmid delivery; among them, the most successful method is the electroporation. This strategy is based on an electrical stimulation of skeletal muscle with a pulse generator that is applied immediately after i.m. injection of DNA. The procedure increases antigen expression presumably by enhancing transfection efficiency thanks to the opening of cell membrane pores (*Draghia-Akli et al., 2008*). Electroporation is accompanied by a transient local tissue injury and inflammation that seem to be essential for effective response to DNA vaccination. The outcome is a dramatic increase of humoral and cellular immune responses (*Fattori et al., 2002*; *Rice et al., 2008*).

At now, some DNA vaccines are already being evaluated in clinical trials, resulting well-tolerated also when combined to electroporation (*Rice et al., 2008*). A pre-treatment with a local anesthetic is used to minimize the discomfort of the procedure (*Draghia-Akli et al., 2008*).

HER-2/neu DNA vaccines

DNA vaccines targeting *HER-2/neu* antigen produced good results in murine transgenic models (*Pupa et al., 2005*; *Wei et al., 2008*; *Cho et al., 2008*). Quaglino and colleagues (*Quaglino et al., 2004*) used, as DNA vaccine, a plasmid coding for the extracellular and transmembrane domains of the product of the HER-2/*neu* oncogene (pNeu) combined with electroporation. I.m. vaccination of BALBneuT mice started at the stage of multifocal *in situ* carcinomas (at 10 weeks of age) and was repeated for four vaccination courses (each course consisted of two administrations) with a 10-week interval between courses. Treated mice were maintained tumor free up to a year of age. This DNA vaccination eliminated existing mammary tumors without inducing

autoimmunity. Mice that also received multiple systemic administration of IL-12 (starting at 7 weeks of age), were protected from tumor onset even when vaccination was started at 16 weeks of age, when mice already developed invasive carcinomas. Moreover only two vaccination courses (at 16 and 23 weeks of age) were sufficient to arrest the tumor progression (*Spadaro et al., 2005*).

AIM

This part of the work was aimed at developing and testing a Triplex-like DNA vaccine (named Tri-DNA) consisting of the combination of three plasmids, each carrying the extracellular and transmembrane portion of HER-2/*neu* (pNeu), the murine IL-12 genes (pIL12) and the D region of the H-2^q (pD^q).

The immune response elicited by Tri-DNA vaccine and its cancer preventive efficacy were compared to those of cell Triplex vaccine in BALBp53neu female mice, a model system more aggressive than BALBneuT female mice.

For this study Tri-DNA and Triplex cell vaccine were administered according to short schedules which could likely allow to outline either better or worse effect of DNA vaccine *versus* cell vaccine.

RESULTS

Plasmid validation

The first step consisted in the demonstration of the ability of the individual plasmids to transfer and to give expression of the transgene carried.

The activity of the plasmid pNeu was reported in previous studies in which this plasmid was used as a single DNA vaccine (*Quaglino et al., 2004*; *Pannellini et al., 2006*; *Cho et al., 2008*): i.m. injection and electroporation of pNeu induced anti-*neu* antibody responses in HER-2/*neu* transgenic mice.

The plasmid carrying murine IL-12 genes was tested by analyzing the level of IL-12 and IFN-γ cytokines in the serum of mice treated with only pIL12. Specifically, a small group of mice received i.m. injections of pIL12 and electroporation with the same conditions chosen for vaccination experiments. Then mice were bled at several time points and the levels of IL-12 and IFN-γ was evidenced by ELISA test. An increase of serum IL-12 after plasmid injection and of IL-12-induced serum IFN-γ was found. The levels of these two cytokines were detectable for some days (Figure 9A). Serum IL-12 or IFN-γ levels were undetectable in untreated mice as well as in mice treated with empty pIRESneo vector (data not shown).

To validate pD^q, a MHC-negative cell line (B78H1) was transiently transfected with this plasmid, coding for H-$2D^q$. After 48 hours H-$2D^q$ expression on cell membrane surface was observed by cytofluorometric analysis (Figure 9B). The ability of pD^q to act as allogeneic MHC was verified through vaccination of BALB/c mice (H-2^d haplotype). Sera of these mice were investigated for the presence of complement-dependent cytotoxic antibodies against H-2^q or H-2^d lymphocytes (obtained from FVB-NeuN#202 or BALB/c mice respectively). Vaccination with pD^q was able to induce specific antibodies with complement-dependent cytotoxic activity towards H-2^q lymphocytes

(Figure 9C). Therefore pD^q plasmid can transfer H-2D^q expression and elicit a specific antibody response.

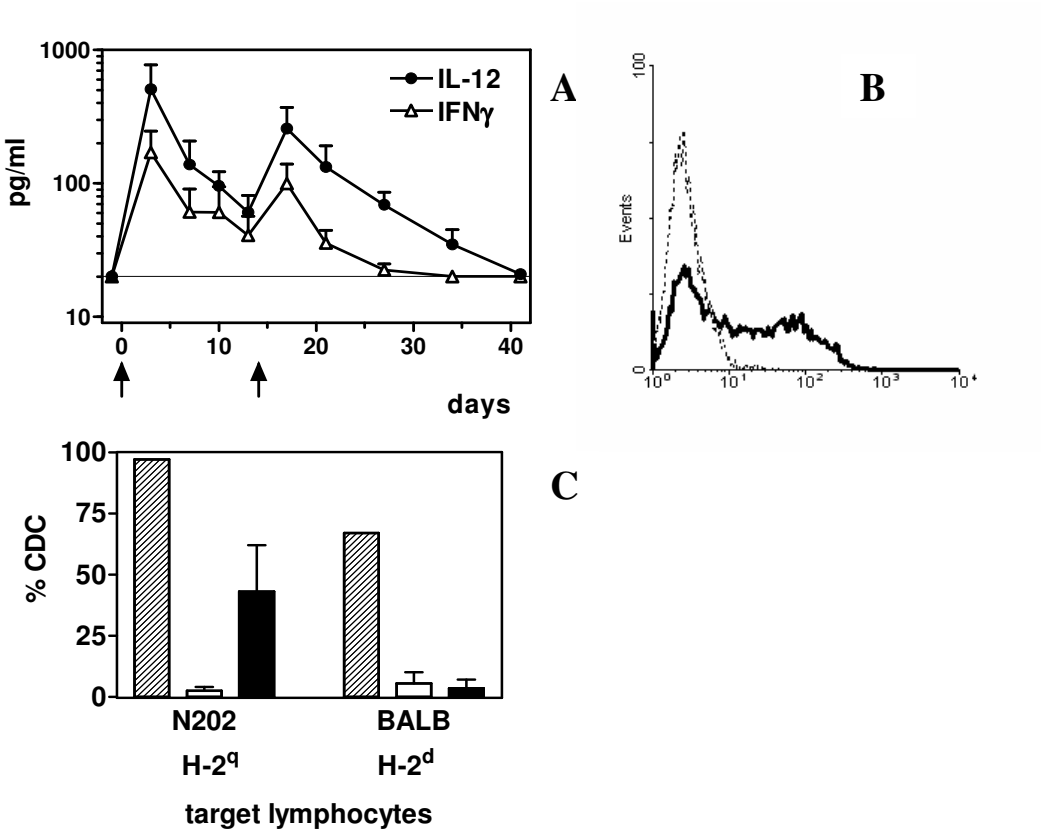


Figure 9. Validation of pIL12 and pD^q vectors. *A*, induction of serum IL-12 (closed circles) and IFN-γ (open triangles) after i.m. injection and electroporation of 50 µg of pIL12 (treatments indicated by arrows). Mean and standard error from 3 mice. *B*, cytofluorometric evaluation of H-2D^q expression in B78H1 cells 48 hours after transfection with pD^q (bold profile). Dotted profile: untreated B78H1 cells. X-axis reports the fluorescence intensity (arbitrary units), Y-axis reports the number of events. C, complement-dependent cytotoxic (CDC) activity against H-2^q or H-2^d lymphocytes by sera of H-2^d mice subjected to i.m. injection and electroporation of 50 µg of pD^q plasmid (closed bars, 2 mice) or untreated controls (open bars, 2 mice). Hatched bars: 28-14-8S positive control serum. Mean and standard error are shown.

Immunoprevention of cancer syndrome through DNA vaccines

BALBp53neu female mice develop salivary gland carcinomas between 13-15 weeks of age and mammary tumors around 19-20 weeks. These mice were treated with Tri-DNA vaccine or cellular Triplex vaccine to compare their effectiveness (Figure 10). The DNA vaccine schedule consisted of four i.m injections and electroporation at 5, 7, 12 and 14 weeks of age. Tri-DNA vaccine, referred to as pNeu+pIL12+pD^{q}, was also compared to other DNA vaccines to evaluate the lack of the allogeneic MHC component (pNeu+pIL12) and of both adjuvants (pNeu). The cellular Triplex vaccine was administered in BALBp53neu female mice for only three 4-week cycles according to the Early schedule which was described in Section 1. Both DNA and cell vaccine stimulation started at 5 weeks of age and ended at 14 weeks of age.

Mice were observed weekly, with recording of the occurrence of the two types of cancer typical of such model, salivary and mammary carcinomas. BALBp53neu females mice, either untreated (*Croci et al., 2004*) or subjected to mock vaccination and electroporation (Figure 10, dotted line) showed a fast and almost simultaneous occurrence of salivary and mammary cancers (with latencies of 13-15 weeks for the former -see also Figure 11A-11D- and slightly longer for the latter). Both cell Triplex vaccine and the three DNA vaccines delayed significantly the mammary and salivary tumor onset. All vaccines had a similar behavior in the prevention of mammary carcinomas moving the median latency from 19-20 to 45-55 weeks of age. For what concerns the salivary carcinogenesis, salivary cancers were fully prevented by Tri-DNA vaccine. Although cell Triplex vaccine was slightly less effective than Tri-DNA vaccine, three treatment cycles were sufficient to prevent almost completely from salivary tumors. Finally, DNA vaccines pNeu and pNeu+pIL12 showed a lower protective ability compared to the complete Tri-DNA vaccine even if with a borderline statistical significance (respectively p=0.074 and p=0.096, Mantel-Haenszel test).

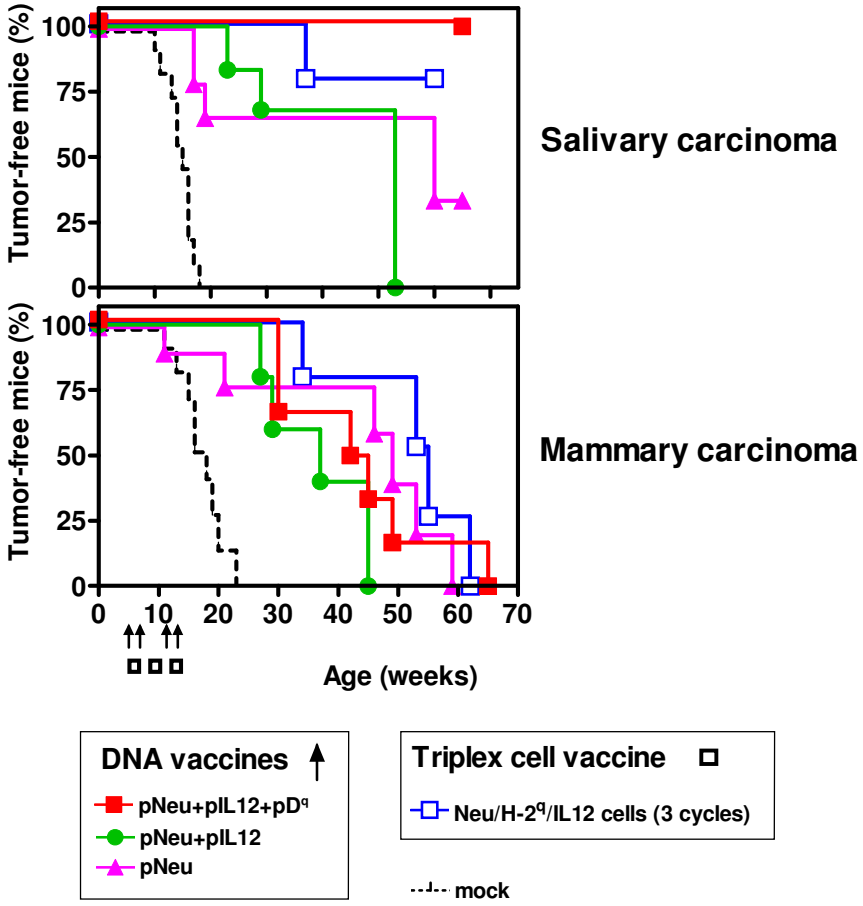


Figure 10. Cancer immunopreventive activity of DNA vaccines and cell Triplex vaccine in BALBp53neu female mice (5-9 mice per group). Vaccination cycles are shown with arrows (DNA vaccines) or boxes (Triplex cell vaccine). Dotted line: mock-electroporated control mice. Significance of differences with Mantel-Haenszel test: any vaccine *vs* mock, $p<0.05$ at least (both salivary and mammary carcinoma); pNeu+pIL12+pD^q *vs* pNeu+pIL12 or pNeu, $p=0.074$ or $p=0.096$ respectively (salivary carcinoma only).

Morphological analysis

Mice vaccinated with Tri-DNA showed normal salivary glands with well-defined ducts and acini that were enclosed in delicate stromal tissue (Figure 11E). On-going inflammatory aspects or inflammation effects (e.g. scars) were not detected. *Neu* expression was not found in salivary glands from Tri-DNA vaccinated mice (Figure 11F), although it was observed in both hyperplastic duct and acinic cells prior to vaccination (Figure 11B and 11D). On the other hand, Tri-DNA vaccinated mice showed several neu^{+}mammary cancer nodules with inflammatory cells in their stroma (Figure 11G and 11H).

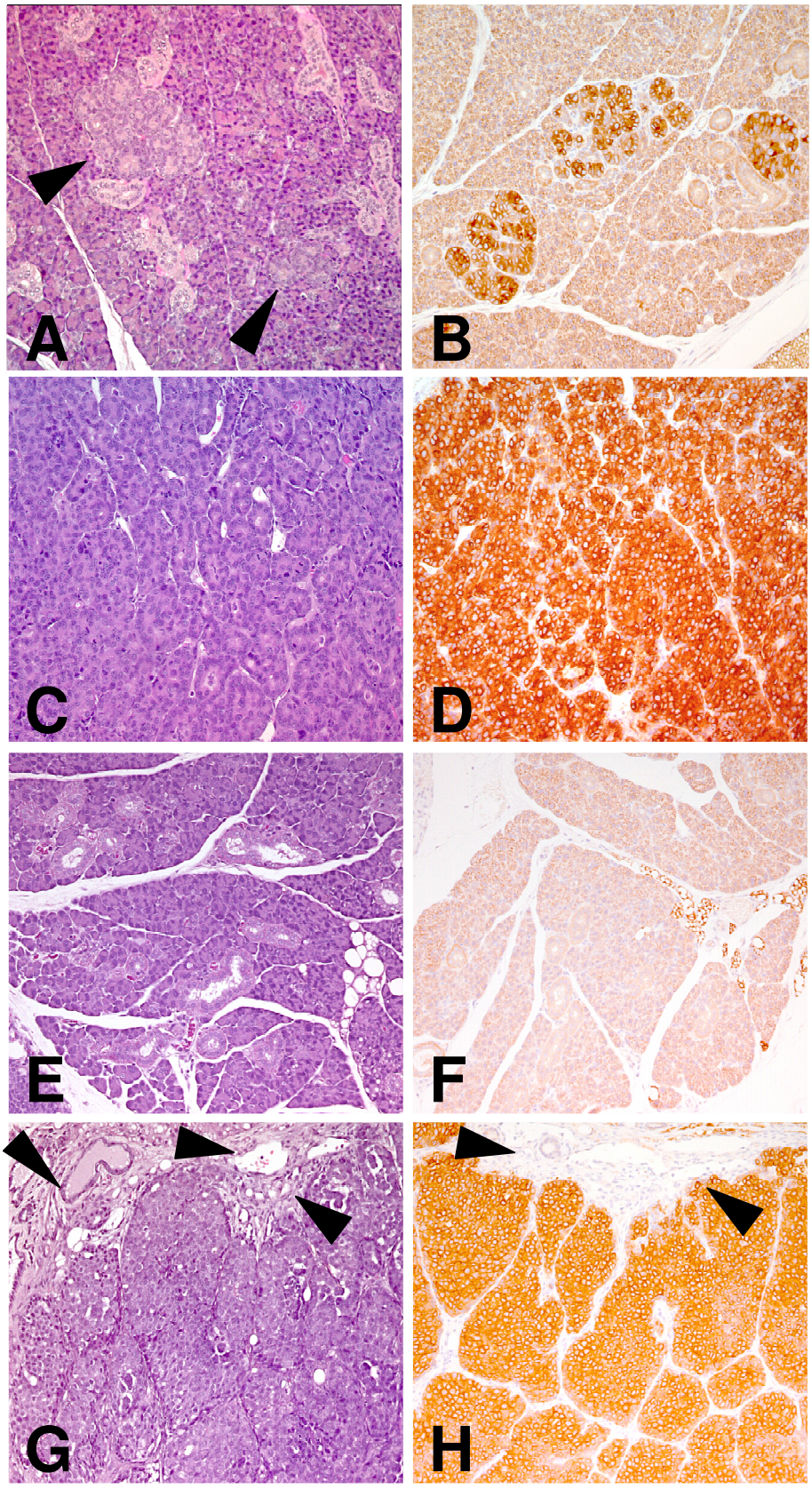

A
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Figure 11. Histology (A, C, E and G) and immunohistochemistry (B, D, F and H) of salivary (A-F) and mammary glands (G-H) of BALBp53neu female mice. The salivary gland obtained from a 5 week-old untreated mouse (A) shows several foci of small duct hyperplasia (arrows) which are intensively positive for *neu* (B). The salivary gland of a 18 week-old untreated mouse is almost completely occupied by a scarcely differentiated *neu*$^+$ carcinoma (C, D). The salivary gland obtained from a 38 week-old Tri-DNA vaccinated mouse is morphologically normal with well defined ducts and acini (E) constituted by cells without expression of *neu* (F) while the corresponding mammary tissue shows invasive carcinoma (G) with cells clearly expressing *neu* on membranes (H). A well vascolarised stroma (G and H, arrows) with inflammatory cells is present at the periphery of the mammary tumor. In F and H within the stroma some false positive cells with macrophage morphological features can be observed.

Cytokine production

Although Tri-DNA and cell Triplex vaccines showed similar preventive efficacy they could drive in different ways the cellular immune responses. The cytokine production by vaccinated lymphocytes can enlighten the immune mechanisms induced by the treatment. Spleen cells of vaccinated and untreated mice at the end of the first vaccination cycle were cultured alone (spontaneous production) or in the presence of proliferation-blocked cell lines able to specifically restimulate *in vitro.* These target cell lines were: mammary carcinoma *neu* negative allogeneic cells (Neu^{neg}/H-2^q), mammary carcinoma *neu*$^+$ syngeneic cells (Neu/H-2^d) and mammary carcinoma *neu*$^+$ allogeneic cells (Neu/H-2^q). Culture supernatants were collected and analyzed for an array of cytokines.

Cell Triplex vaccine induced a very high production of IFN-γ already evident without *in vitro* restimulation, moreover induction of IL-4 together with other Th2 cytokines such as IL-5 and IL-10 was observed (Figure 12). Spleen cells derived from Tri-DNA vaccine group showed a significant IFN-γ production only upon the restimulation with cells presenting allogeneic MHC alone or combined with *neu* expression. This vaccine did not increase significantly the

level of Th2 cytokines. Spleen cells derived from mice treated with pNeu+pIL12 and pNeu alone as well as those from mock-electroporated control mice (data not shown) showed barely detectable spontaneous cytokine production levels. *In vitro* stimulation did not increase IFN-γ or IL-4. All vaccinations produced an increase of GM-CSF after restimulation while TNF-α level did not seem to be different among several treatments.

The production of IFN-γ and IL-4 by $CD4^+$ and $CD8^+$ populations was also studied (Figure 13). $CD4^+$ spleen cells of mice treated with cell Triplex vaccine secreted a high level of IFN-γ both spontaneously and after restimulation. The Tri-DNA induced a more selective and reduced production of IFN-γ by $CD4^+$ cells and only in presence of allogeneic MHC molecules alone or combined with *neu* antigen. Also the production of IFN-γ by $CD8^+$ cells was specifically induced by these two stimuli for both Tri-DNA and cell Triplex (stimulated also by *neu* alone) vaccines. Finally IL-4 was revealed only in the supernatants of $CD4^+$ spleen cells derived from Triplex cell vaccinated mice. The presence of allogeneic molecules in absence of *neu* antigen reduced the rate of IL-4.

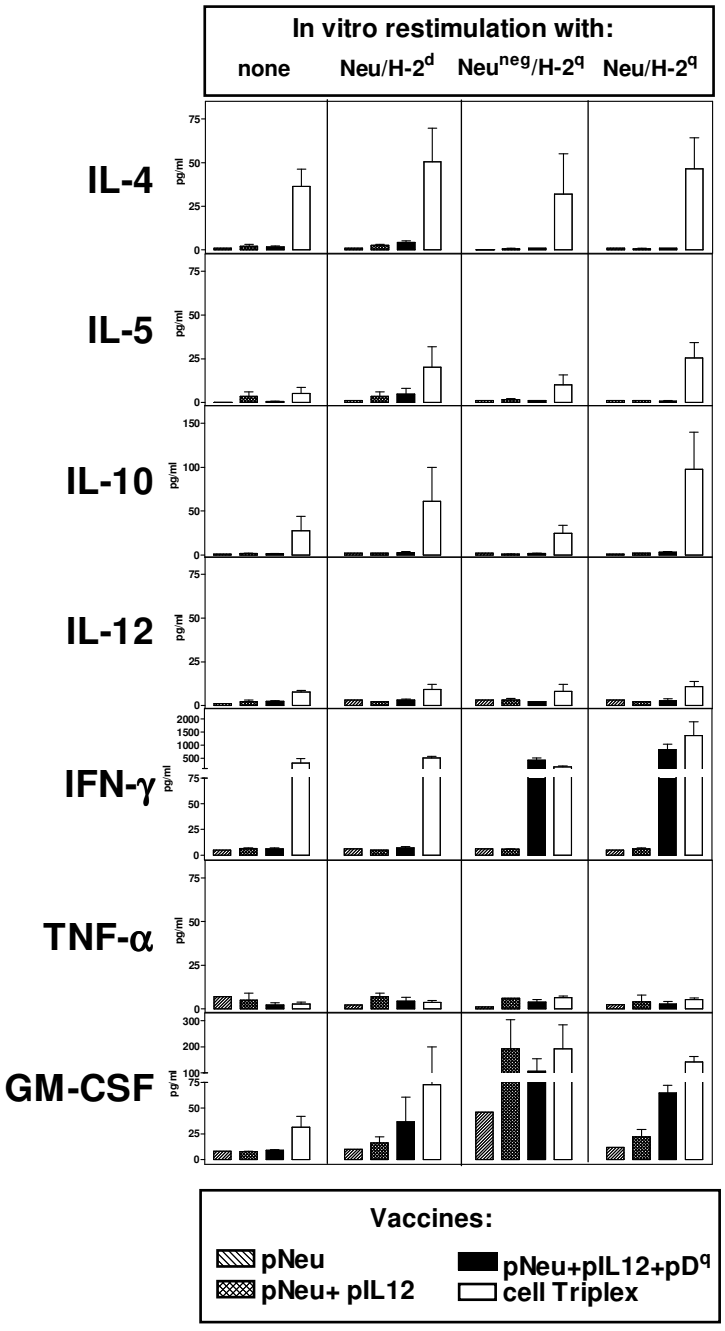

Figure 12. Cytokine production by total unseparated spleen cells collected after the first vaccination cycle and cultured 6 days alone or in the presence of the indicated restimulator cells. Mean and standard error from 2-3 mice per group is shown.

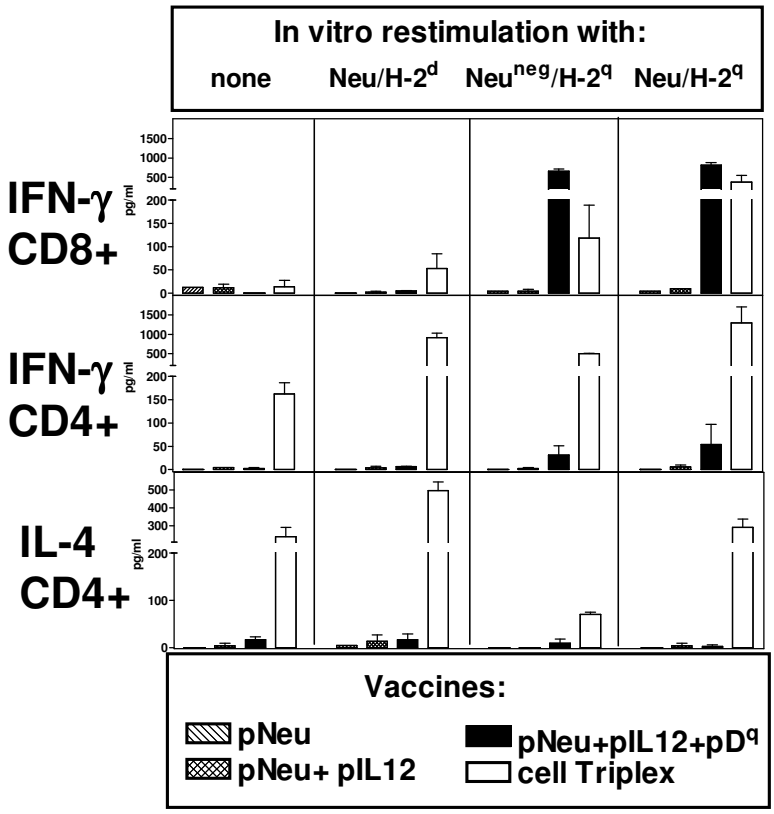

Figure 13. Cytokine production by $CD4^+$ or $CD8^+$ spleen cells collected after the first vaccination cycle and cultured 6 days alone or in the presence of the indicated restimulator cells. No IL-4 production by $CD8^+$ cells was observed (data not shown). Mean and standard error from 2-3 mice per group is shown.

Humoral response

Previous data obtained with cell Triplex vaccine suggested that anti-*neu* antibodies played a key role in the vaccine induced protection. Sera of mice treated with cell Triplex, Tri-DNA, pNeu and pNeu+pIL12 were investigated for the presence of these antibodies by ELISA assay (Figure 14A). Antibodies recognizing *neu* antigen were specifically detected in all vaccine groups. The main differences were found after the first vaccinations since all mice in the

Tri-DNA vaccine group (closed squares) already showed detectable levels of anti-*neu* antibodies, whereas sera of pNeu and pNeu+pIL12 DNA vaccines as well as cell Triplex vaccine revealed some late-responder mice. Then, the level of anti-*neu* antibodies reached at the end of the vaccination protocol was similar for all DNA vaccines, whereas the highest antibody levels were obtained with cell Triplex vaccine (open squares).

The antibody response was detected also by cytofluorometric analysis testing the ability of sera from vaccinated mice to bind syngeneic or allogeneic neu^+ cells (Neu/H-2^d and Neu/H-2^q cells respectively, Figure 14B and 14C). Kinetics and differences of induction of humoral reactivity were similar to those found with ELISA assay. Mock-electroporated control mice did not develop any humoral response, both when tested by ELISA assay and by cytofluorometric analysis (data not shown). The reactivity against Neu/H-2^q cells observed with sera of groups including allogeneic MHC in vaccine formulation obviously comprises anti-histocompatibility antibodies. Anyway a minor contribution to binding is attributable to histocompatibility since such sera tested against a *neu* negative H-$2D^q$ -expressing target cell had a very low binding activity (data not shown). In fact DNA vaccines lacking allogeneic MHC gene (pNeu+pIL12 and pNeu alone) could induce antibodies almost as effectively as the DNA vaccine including the H-$2D^q$ gene.

Finally mice sera were investigated to define the isotype profile of the antibodies binding syngeneic neu^+ cells. The isotype profile showed that all vaccines induced comparable levels of IgG2a antibodies, but only cell Triplex vaccine caused a very high level of IgG3 antibodies, together with a slight increase in IgG1 antibodies (Figure 15).

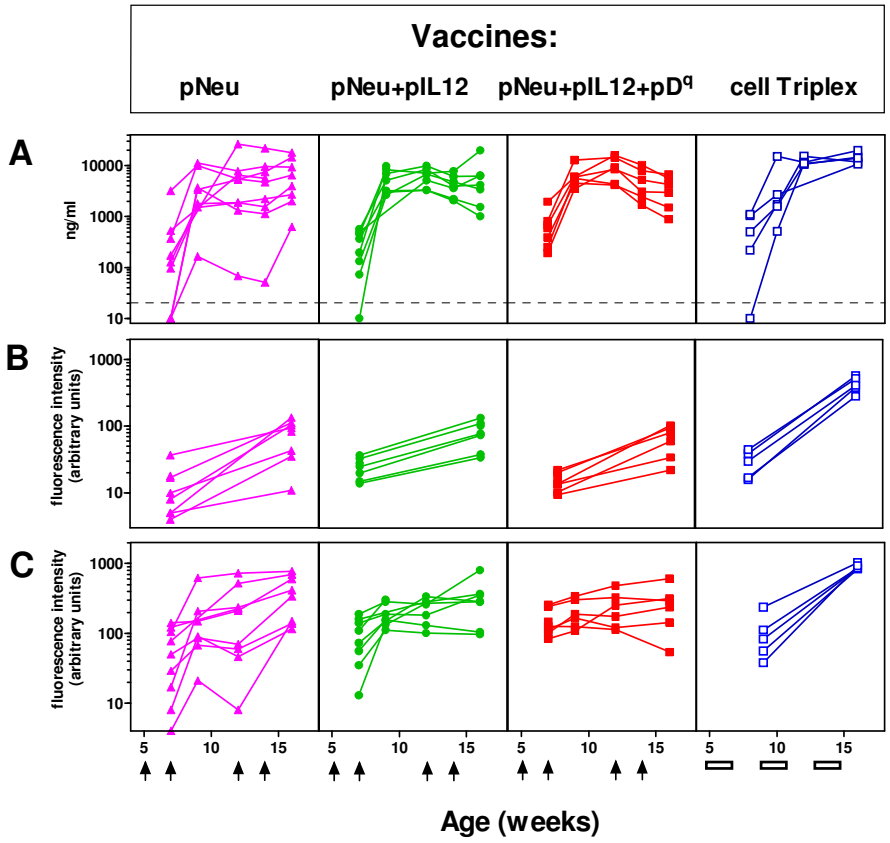

Figure 14. Humoral response of BALBp53neu female mice vaccinated with the indicated DNA vaccines compared to cell Triplex vaccine. Vaccination cycles are shown with arrows (DNA vaccines) or boxes (cell Triplex vaccine). Data from 5-9 individual mice are shown in each panel. *A*, anti-*neu* antibodies, measured by ELISA assay on sera at 1:300 dilution. Dotted line: sensitivity of the test. Significance of differences between groups (non-parametric Wilcoxon test): pNeu+pIL12+pD^q *vs* pNeu+pIL12 or pNeu at 7 wk (1 vaccination), p=0.06 and p=0.04, respectively; any DNA vaccine *vs* cell Triplex at 16 wk, p<0.05. *B*, cytofluorometric analysis of serum binding activity towards Neu/H-2^d cells. Significance of differences between groups (non-parametric Wilcoxon test): pNeu+pIL12+pD^q *vs* pNeu+pIL12 or pNeu at 7 wk, p<0.05; any DNA vaccine *vs* cell Triplex at 16 wk, p<0.01. C, cytofluorometric analysis of serum binding activity towards Neu/H-2^q cells. Significance of differences between groups (non-parametric Wilcoxon test): pNeu+pIL12+pD^q *vs* pNeu at 7 wk, p<0.05; any DNA vaccine *vs* cell Triplex at 16 wk, p<0.01.

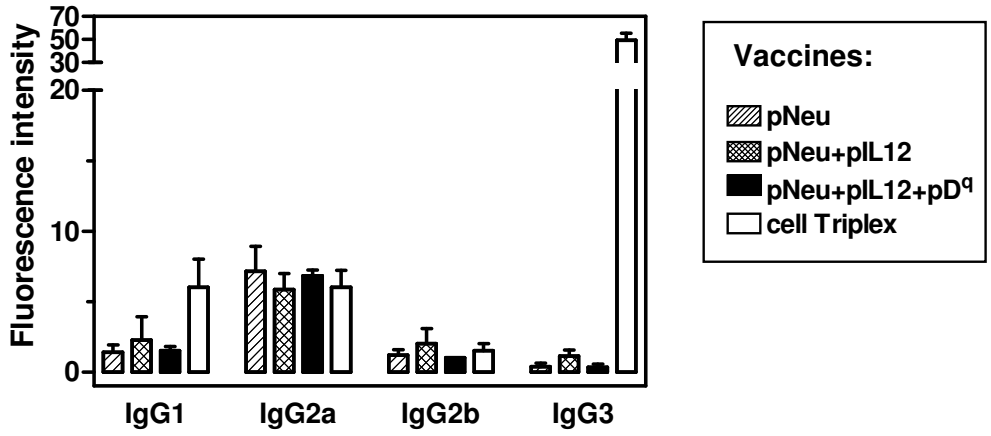

Figure 15. Analysis of antibody isotypes induced by DNA and cell Triplex vaccination. Normalized fluorescence intensity against Neu/H-2^d cells by sera collected from 16-week old mice is shown. Mean and standard error from 5-9 mice per group.

The results reported above have already been published (*De Giovanni et al., 2009b*).

DISCUSSION

The perspective of a future translation of the Triplex vaccine to human patients drove the study of a new generation of Triplex DNA vaccine. The advantage of DNA vaccines for clinical use is closely related to fewer difficulties in vaccine production, greater flexibility and safety compared to cellular vaccines.

The Triplex DNA (Tri-DNA) vaccine combines three plasmids coding for the extracellular and transmembrane portion of the *rat* activated HER-2/*neu* oncoantigen (pNeu), the murine IL-12 genes (pIL12) and the H-2D^q gene (pD^q) respectively. The ability of the vaccine was tested on BALBp53neu female mice, knockout for a p53 allele and transgenic for the rat activated *neu* oncogene. The development of a cancer syndrome with early onset of salivary and mammary tumors gives a more aggressive preclinical cancer progression than in BALBneuT female mice. Therefore the study of vaccines in BALBp53neu mice can highlight positive and negative sides of the treatments.

The aim of *in vivo* studies was to compare two "complete" treatments (with *neu* target antigen and both adjuvants stimuli), Tri-DNA and cell Triplex vaccines. Furthermore the contribution of the two adjuvants was tested through vaccination without plasmid coding for allogeneic MHC or with *neu* plasmid alone. A similar analysis of Triplex components had already been carried out on the cell vaccine in previous studies on BALBneuT mice (*Nanni et al., 2001*; *De Giovanni et al., 2004*) anyway these data are however not free from the possible influence of other cell components which can contribute to the protective ability of the vaccine. Thus the *in vivo* evaluation of Tri-DNA (pNeu+pIL12+pD^q) proved that the presence of only these three stimuli is sufficient for the prevention of early and aggressive carcinogenesis in BALBp53neu female mice.

Tri-DNA and Triplex cell vaccines induced a similar protective response in BALBp53neu mice. The development of mammary carcinoma was delayed by

both treatments moving the median latency from 19-20 to 45-55 weeks of age; the lacking of IL-12 and/or allogeneic MHC molecule D^q did not significantly reduce the protection from mammary cancer. The early onset of salivary tumors was completely prevented at more than one year of age by Tri-DNA and almost completely by cell Triplex vaccine. The absence of one or both adjuvants reduced the efficacy of the prevention from salivary tumors. Although the two different forms of Triplex vaccine achieved similar results, the number of requested injections is surely lower for DNA vaccine and so in a translational perspective less stressing for patients.

The study of immune responses induced by cell Triplex vaccine revealed a powerful Th1 stimulation proved by the high production of IFN-γ, both spontaneous, only for $CD4^+$ cells, and after restimulation. Moreover the presence of relevant levels of IL-4, IL-5 and IL-10 suggested that also Th2 immune response was stimulated by cell Triplex vaccine. In particular the restimulation with *neu*$^+$ syngeneic cells increased the rate of IL-4. On the contrary, Tri-DNA vaccine sustained a highly restricted Th1 response: IFN-γ secretion was detected only after restimulation, mainly by the allorecognition, and the major producers were $CD8^+$ cells.

All studied vaccines induced antibody production. In detail, the various DNA vaccines did not reach remarkably different antibody levels, but the presence of adjuvants in the Tri-DNA vaccine seems to confer a faster and more homogenous response. In the first cycle also cell Triplex vaccine did not induce similar levels of anti-*neu* antibodies as showed by few late-responder mice although at the end of treatment cell Triplex vaccine group reached significantly higher levels of antibodies both binding to syngeneic and binding to allogeneic *neu*$^+$ cells.

The isotypes analysis of antibodies binding syngeneic neu^+ cells confirmed the importance of IgG2a for the activity of all vaccines (*Nanni et al., 2001*; *Nanni et al., 2004*). Cell Triplex vaccine induced higher IgG1 and IgG3, levels compared to DNA vaccines.

Therefore Tri-DNA vaccine was as effective as cell Triplex vaccine. The three stimuli of the Triplex are not only necessary but also sufficient to delay/prevent carcinogenesis in BALBp53neu female mice.

Moreover Tri-DNA induced a more restricted immunostimulation thanks to a controlled antigenicity, in comparison with the cell Triplex vaccine. In fact a cellular vaccine in principle could carry a portfolio of immune stimuli, other than desired, whereas this Tri-DNA vaccine only carries the three genes chosen. In particular an allogeneic cell vaccine carries multiple different MHC genes. In Tri-DNA vaccine a single MHC gene was used as adjuvant instead of a complete haplotype. The MHC gene chosen, H-2D^q, has about 88% homology with the corresponding MHC gene of the host d haplotype, nevertheless it gave a good stimulation as well, as found by the high IFN-γ response by allo-restimulated spleen cells. Such IFN-γ induction could take place even *in vivo* after each vaccination cycle, contributing to the effectiveness of the vaccine.

Monoantigenic anti-*neu* electroporated DNA vaccines have been reported to confer protection against carcinogenesis driven by *neu* alone (*Quaglino et al., 2004*). Carcinogenesis induced by the combination of *neu* and p53 knockout is much more aggressive and hard to prevent (*Croci et al., 2004*; *Pannellini et al., 2006*). Confirming these data, this work proved that Tri-DNA vaccine was more effective than pNeu vaccine in maintaining all mice free from salivary tumors. The main reason of this difference can be found in the faster and homogeneous rise of antibodies induced combining pNeu with two adjuvants plasmids. So the presence of adjuvants can reduce the individual variability of the antibody response induced by DNA vaccines (*Cipriani et al., 2008*) and found in the early stage of vaccination. Since immunoprevention in *neu* transgenic models was reported to be related both to the individual antibody level (*Cipriani et al., 2008*) and to the precociousness of the response induced ((*Croci et al., 2004*; *Pannellini et al., 2006*); our unpublished data, see Section 1), a more homogeneous response induced by Tri-DNA vaccine is desirable.

Differently from salivary carcinogenesis, however, no superior effect by Tri-DNA vaccine was observed for mammary carcinogenesis. Probably, since the onset of salivary carcinoma occurs earlier than mammary carcinoma, an early and necessarily high response is sufficient to arrest definitively the salivary carcinogenesis. On the contrary the arrest of mammary tumor onset requires a more exacting control. In fact, as previously reported in Section 1, a reduction of anti-*neu* antibodies, even after a long time of repeated vaccinations, could be sufficient to allow mammary tissue progressing into tumor.

Tri-DNA vaccine showed a restricted immune response and it is a good attribute for a immunopreventive cancer vaccine. Of note is the absence of IgG3 (corresponding to human IgG2). Murine IgG3 antibodies do not bind FcR, but can activate the complement system through Fc-Fc interactions *(Getahun and Heyman, 2006*). Some data on the involvement of murine IgG3 in autoimmunity have also been reported (*Baudino et al., 2006*). Autoimmunity could be a serious side effect of cancer immunoprevention (*Wei et al., 2008*), since cancer antigens, included HER-2, can be expressed on normal tissues. However in this study, such as in others (*Garcia-Hernandez et al., 2008*), it was not observed any sign of autoimmunity in all vaccinated mice suggesting that the high level of IgG3 antibodies elicited by cell Triplex vaccine could be dispensable but not detrimental to mice health.

In conclusion, DNA vaccines, and in particular Triplex DNA vaccine, resulted as effective as cell Triplex vaccine, exploiting a more restricted immune stimulation.

CONCLUSIONS

This thesis deals with the study of three preclinical conditions applying to the Triplex vaccine.

The preclinical efficacy of Triplex cell vaccine depends on the schedule of treatment. The minimal number of administrations and their distribution are not well defined yet. Anyway, the achieved data suggest that vaccine administration should be frequent in the first weeks of treatment so that a high level of anti-vaccine antibodies can be reached as soon as possible. Moreover long-life periodic boosts are thought to be necessary so that antibody production is kept high. The research for new optimal protocols will go on and first of all *in silico* model shall be improved in order to be closer to *in vivo* conditions.

A second important result was the demonstration of the ability of Triplex cell vaccine to cure experimental lung micrometastases. This successful result opens up the possibility of early clinical testing in a therapeutic, rather than prophylactic, human context. New vaccination schedules, predicted by *in silico* model of metastatic development, may be tested in order to obtain a higher efficacy of the vaccine

Finally the study of a new formulation of Triplex cell vaccine, i.e. Triplex DNA vaccine, showed that DNA vaccine is as effective as cell vaccine, but inducing a more restricted immune stimulation. The combination of multiple plasmids and the high efficacy obtained through electoporation makes the system very handful and flexible. This novel strategy may be effective in the prevention of HER-2 expressing tumors in humans at high risk. This class, even if it is not yet well defined, could include healthy people who have a specific genetic risk of cancer, who have been exposed to an exogenous carcinogen or who bear multifocal pre-neoplastic lesions. Future studies will include the evaluation of Triplex DNA vaccine in a preclinical therapeutic setting.

Other investigations, not reported in this thesis, took into account further aspects potentially relevant for cancer immunoprevention, such as combination with current chemopreventive approaches (*De Giovanni et al., 2009a*), antigen presentation (*Croci et al., 2007*) and the possibility to extend active immunological strategies to prevention of other tumors, such as prostate cancer (*De Giovanni et al., 2007*).

In conclusion, cancer immunoprevention is becoming an attractive goal, although studies have been limited so far to preclinical models. A deeper analysis of such models, with combination of theoretical and technical advancement, the identification of high-risk patients and of suitable target antigens and adjuvants could pave the way to clinical translation in next decades.

MATERIALS AND METHODS

MICE

BALB/cAnNCrlBR (BALB/c) mice were purchased from Charles River Italy. BALBneuT mice (H-2^d haplotype) overexpressing the activated rat HER-2/*neu* oncogene driven by the mouse mammary tumor virus long terminal repeats (MMTV LTR), IFN-γ gene knockout BALB/c mice, and μMT mice (knockout for the immunoglobulin μ chain gene) were bred, maintained, and genetically screened as reported (*Boggio et al., 1998*; *Nanni et al., 2004*). BALBp53neu mice, knockout for the *p53* oncosuppressor gene and transgenic for the activated rat HER-2/*neu* oncogene driven by the MMTV LTR, were bred, maintained, and genetically screened as reported (*Nanni et al., 2003*; *Croci et al., 2004*). $Rag2^{-/-}$;$\gamma c^{-/-}$ breeders were kindly given by the Central Institute for Experimental Animals (Kawasaki, Japan); then mice were bred in the animal facilities of the Cancer Research Section of the Experimental Pathology Department under sterile conditions. Experiments were authorized by the institutional review board of the University of Bologna and done according to Italian and European guidelines.

Individually tagged virgin female mice of specific ages were used: mice of 6 weeks of age for experiments reported in Section 1, mice of 6-9 weeks of age for experiments reported in Section 2 and mice of 5 weeks of age for experiments reported in Section 3.

CELL LINES

Neu$^+$ TT12.E2 cells (in this thesis referred to as Neu/H-2^q cells) and *neu* negative N202.1E cells (in this thesis referred to as Neu^{neg}/H-2^q cells) derived from mammary carcinomas arisen in FVB-NeuN#202 mice (H-2^q haplotype), transgenic for the rat *neu* proto-oncogene (*Nanni et al., 2000*). IL-12 engineered

TT12.E2 cells (here referred to as Neu/H-2^q/IL12) were obtained and described previously (*De Giovanni et al., 2004*).

Neu$^+$ TUBO cells (here referred to as Neu/H-2^d cells) derived from a mammary carcinoma arisen in BALBneuT mouse (*Rovero et al., 2000*).

Cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS; Invitrogen, Milan, Italy) at 37°C in a humidified 5% CO_2 atmosphere.

PLASMIDS

Plasmid pcDNA3 coding for a transmembrane-extracellular domain fragment of rat *HER-2/neu* (in this work referred to as pNeu) has been described previously (*Quaglino et al., 2004*).

Plasmid coding for both murine IL-12 genes (pIL12) was bicistronic pIL12-IRES1neo (*De Giovanni et al., 2004*; *Faggioli et al., 2008*); it was able to induce a high IL-12 expression level when used to obtain stable IL-12 engineered cells (*De Giovanni et al., 2004*). In order to evaluate pIL12 ability to give IL-12 expression *in vivo*, the plasmid was injected i.m. (50 µg) and then the muscle was electroporated (see below for conditions). Empty vector pIRES1neo was used under the same experimental conditions. Sera of treated mice were collected at different time points and investigated for the presence of IL-12 and IFN-γ by ELISA assays (R&D Systems, Minneapolis, MN).

In order to obtain a plasmid coding for an allogeneic MHC gene, the H-$2D^q$ gene was choosen. The H-2^q haplotype was the same carried by the Triplex cell vaccine (H-2^q, FVB/N strain). The construction of pD^q was carried out through the collaboration with Dr. Silvano Ferrini (National Institute for Cancer Research, Genoa, Italy). The H-$2D^q$ cDNA was cloned from different sources (lymphocytes and tumor cells). Briefly, cells were lysed in Trizol (Invitrogen, Milan, Italy) and total RNA was isolated using the RNAeasy system (Qiagen, Chatsworth, CA). The open reading frame of H-$2D^q$ was amplified by RT-PCR from total RNA using the following primers:

5’CGATGGCTCCGCGCACGCTGCTC3’(forward)

5’CAGTCCAGGCAGCTGTCTTCACGC3’(reverse)

H-2D^q cDNA was cloned in the PCR2.1 vector using the TOPO-TA cloning system (Invitrogen), sequenced and then subcloned into the pcDNA3 expression plasmid (Invitrogen): such plasmid will be hereafter referred to as pD^q. Sequencing of different H-2D^q cDNA clones obtained showed an almost complete homology with a reported D^q sequence (*Pullen et al., 1992*), with only two mismatches leading to aminoacid changes (CAG/AGG at codon 69 leading to a Q to R aminoacidic change, and CGC/GCC at codon 104, leading to an A to R change). Due to the consistency of these sequences in different clones, mismatches can be interpreted either as mistakes in the previously published sequence (*Pullen et al., 1992*) or as substrain variations. In order to evaluate the the ability of pD^q to transfer H-2D^q expression a variant of B16 melanoma (B78H1) was transfected with pDq (see “*Gene Transfection*"); moreover sera of mice treated with pDq were investigated for the presence of anti-H-2^q antibodies able to induce complement dependent cytotoxicity (see “*Complement dependent cytotoxicity”*).

Gene transfection

B78H1 is a variant of B16 melanoma. This cell line is a very good transfection recipient and does not express MHC molecules (*Graf, Jr. et al., 1984*; *De Giovanni et al., 1991*). B78H1 cells were seeded at the concentration of 0.25x10^6 cells/well in 6-well plates (Falcon, Oxnard, USA) and let to attach for 24 hours. Then pD^q (2 µg/well) was transfected using Lipofectamine 2000 (Invitrogen, Milan, Italy) at 1:2.5 ratio (DNA µg: Lipofectamine µl) according to the manufacturer’s protocol. Two days after transfection H-2D^q expression was evaluated on harvested cells, through indirect immunofluorescence and flow cytometry by the monoclonal antibody 28-14-8S (*Ozato et al., 1982*) (Cedarlane, Hornby, Canada).

VACCINATIONS AND OTHER MICE TREATMENTS

DNA vaccines and treatment protocol

Plasmid large-scale preparations were set up and purified using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA). DNA was precipitated, dissolved in water at a concentration of 3-5 mg/ml, and stored in monodose aliquots at -20°C for use in immunization protocols. For Tri-DNA vaccine, 50 µg of each plasmid were mixed together and diluted to a final volume of 40 µl per mouse in 0.9% of NaCl and 6 mg/ml polyglutamate (final concentrations). Groups treated with combination of two plasmids or with pNeu alone received 50 µg of each plasmid. Anesthetized mice received the injection of DNA vaccine into the tibial muscles (20 µl each muscle) through a 28-gauge needle syringe and immediately thereafter were subjected to electroporation, consisting of two square-wave 25 ms, 375 V/cm pulses generated by a T830 electroporator (BTX, San Diego,CA). Mice received four vaccinations (5, 7, 12 and 14 weeks of age). Control group received mock vaccinations as above but lacking plasmids. Mice were monitored weekly for mammary or salivary tumor onset.

Cell vaccines

The Triplex cell vaccine consisted in *neu*$^+$ IL-12-engineered allogeneic mammary carcinoma cells (Neu/H-2^q/IL12). The other two used cell vaccines are based on *neu*$^+$ TT12.E2 cells (Neu/H-2^q) and *neu*$^+$ TUBO cells (Neu/H-2^d cells). Before the vaccination, cells were treated with 40 µg/ml of mitomycin C (MitC; Sigma-Aldrich) to block cell proliferation. The evaluation of the surface expression of HER-2/*neu* and class I H-2^q and H-2^d molecules were performed as previously reported (*Nanni et al., 2000*; *Nanni et al., 2001*). Each treatment consisted in i.p. vaccination with $2x10^6$ mitomycin C-treated cells in 0.4 ml PBS. Untreated mice received only 0.4 ml PBS

Prophylactic vaccination protocols

In BALBneuT female mice all vaccination protocols started at 6 weeks of age.

For Chronic-63 and the Early schedules, the vaccination protocol consisted of 4-week cycles: in the first 2 weeks, mice received four twice-weekly i.p. vaccinations followed by 2 weeks of rest. Chronic-63 protocol consisted of 15 vaccination cycles so that mice received in all 60 vaccine doses and the last treatment was administered at the 63rd week of age. Early protocol consisted in only three vaccination cycles, thus the last vaccine administration was at 15 weeks of age.

Heuristic schedule consisted of light vaccination cycles: in the first 2 weeks, mice received two once-weekly i.p. vaccinations followed by 2 weeks of rest. This light vaccination cycle was repeated 15 times and mice received the last injection at 63 weeks of age.

The Genetic protocol consisted in 32 vaccinations irregularly spaced over the time, from 6th weeks to 55th week of age. Considering the day 0 as the first vaccination day, mice were treated at days: 0, 3, 7, 24, 49, 56, 59, 66, 80, 84, 91, 94, 105, 126, 133, 140, 143, 161, 171, 178, 199, 210, 231, 245, 269, 273, 287, 301, 311, 322, 336, 343.

BALBneuT mice, untreated or treated according to the Chronic-63, the Early, the Heuristic and the Genetic protocols, were bled every four weeks starting from the 9th weeks of age. Mice were monitored weekly for mammary tumor onset.

BALBp53neu mice were treated according to a short protocol consisting in three vaccination cycles, as for the Early schedule. The vaccination started at 5 weeks of age. Mice were monitored weekly for mammary or salivary tumor onset.

In silico research of optimizing vaccination protocols for Triplex cell vaccine were kindly performed by the group of Prof. Santo Motta (University of Catania, Catania, Italy).

Lung metastases and therapeutic vaccination

For the induction of lung micrometastases, mice received i.v. $2.5x10^4$ Neu/H-2^d cells. Mice were sacrificed 33 days after cell injection and subjected to an accurate necropsy. Lungs were stained with black India ink to better outline metastases and fixed in Fekete's solution. Lung metastases were counted using a dissection microscope.

Therapeutic vaccination started 1 or 7 days after metastasis induction. Schedule included two weekly administrations of cell vaccines (prepared as described above) repeated until sacrifice. Control groups included untreated mice and mice treated twice weekly with 100 ng/mouse of recombinant IL-12 (kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA).

In vivo cell depletions

All depletion studies were done according to previously standardized protocols (*Comes et al., 2006*). T-cell depletion studies were performed by i.p. injection of anti-CD8 (2.43), anti-CD4 (GK1.5), or anti-CD25 (PC61) rat monoclonal antibodies (mAb), all from the American Type Culture Collection. All mAbs were first administered i.p. 24 hours after micrometastasis induction (i.e., 6 hours before the first vaccination). Treatment with anti-CD4 and anti-CD8 mAbs was then repeated 24 hours after the first vaccination and 6 hours before the second, third, fourth, sixth, and eighth vaccination. The anti-CD25 treatment was repeated 6 hours before the fifth vaccination. The mAb dosages were 200 µg/mouse (anti-CD8) or 500 µg/mouse (anti-CD4 and anti-CD25). For natural killer (NK) depletion, mice received i.v. 0.4 ml of a 1:30 dilution of anti-asialo GM1 antiserum (Wako) 48 hours after micrometastasis induction

(24 hours before the first vaccination); this protocol was found to avoid well-known effects of NK depletion on metastatic seeding.

IMMUNE RESPONSES

Complement dependent cytotoxicity (CDC)

BALB/c mice (H-2^d) were vaccinated at 5 and 7 weeks of age by i.m. injection of pDq plasmid into the tibial muscles (25 µg in 20 µl each muscle) followed by electroporation (with same protocol reported above for DNA vaccination). A week after the second injection, pD^q-vaccinated mice were bled and serum stored at -80°C. Aliquots of 0.5 x 10^6 lymphocytes, collected from BALB/c (H-2^d) and FVB-NeuN#202 (H-2^q) mice, were resuspended in RPMI + 5% FBS containing sera from pD^q vaccinated and from untreated BALB/c mice (1:10 dilution in a final volume of 0.5 ml) and incubated for 30 minutes in ice. Baseline control in RPMI + 5% FBS medium and positive control, consisting in 28-14-8s monoclonal antibody (recognizing both H-2^q and H-2^d epitopes) at 1:10 dilution, were run in parallel. Lymphocytes were then washed once with PBS and incubated with 1:10 dilution of rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada) for 30 minutes in ice. Then lymphocytes were washed again, resuspended in RPMI + 5% FBS and cell number and viability determined by erythrosin dye exclusion test. Percent of cytotoxicity was calculated as: 100 – (cell yield in complement-contaning RPMI + 5% FBS/cell yield with serum)*100.

Mixed lymphocyte-tumor cell cultures (MLTC)

Spleens were collected from treated and control mice, and single cell suspensions were prepared, washed in PBS, and resuspended in RPMI 1640 supplemented with 10% FBS. Total spleen mononuclear cells, or subpopulations ($CD4^+$ or $CD8^+$) purified by magnetic sorting (Miltenyi Biotec,

Bergisch Gladbach, Germany), were cultured ($5x10^5$ cells/ml) for 6 days at 37°C alone or in the presence of proliferation-blocked restimulator cells ($5x10^4$ cells/ml) in RPMI 1640 supplemented with 10% fetal bovine serum and with 20 units/ml of recombinant IL-2. The ratio between lymphocytes and tumor cells was 10:1. Culture supernatants were collected and investigated for the production of murine IL-4 and IFN-γ by ELISA assays (Endogen, Woburn, MA). Moreover, for what concerns experiments in BALBp53neu mice, some supernatants were further evaluated in order to define simultaneously the concentrations of IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN-γ and TNF-α using the Bio-Plex 200 Suspension Array System (Bio-Rad, Milan, Italy). A commercially available premixed multiplex cytokine set was used (Mouse Cytokine Th1/Th2 panel) following manufacturer's instructions and using a high sensitivity setting. Briefly, 50 µl of supernatants and serial dilutions of the cytokine standards were incubated at room temperature for 30 minutes with a mix of different fluorescently dyed beads conjugated with monoclonal antibodies specific for the various cytokines. Afterwards samples were incubated for 30 minutes with 25 µl of detection antibody then with 50 µl of streptavidinphycoerythrin (PE) for 10 minutes. Each step was followed by 3 washings. Finally, samples were resuspended in 125 µl assay buffer and analysed into the Bio-Plex reader. 50 µl volume was sampled from each well and the PE-fluorescent signal of a minimum of 100 beads per cytokine type was evaluated. Bead doublets were excluded from analysis. Data were elaborated with the Bio-Plex Manager Software 5.0. Extrapolating PE-fluorescent signals to a standard curve allowed quantitation of each cytokine in the samples. Experimental range of detection of the various cytokines were the following: IL-4, 2100-0.2 pg/ml; IL-5, 500-0.1 pg/ml; IL-10, 1100-1 pg/ml; IL-12, 1100-1 pg/ml; GM-CSF, 500-2 pg/ml; IFN-γ, 1400-0.3 pg/ml; TNF-α, 3700-3 pg/ml.

Cell-mediated cytotoxicity

Cell-mediated cytotoxicity assays were kindly performed in the Laboratory of Dr. Silvano Ferrini (National Institute for Cancer Research, Genoa, Italy). Although the candidate did not carry out the morphological analysis, methods were reported below for an exhaustive description of the work.

Spleen mononuclear cells of BALBneuT mice which have completed the treatment were restimulated by coculture at a 50:1 ratio with proliferation-blocked Neu/H-2^d cells for 6 days in RPMI 1640 supplemented with 10% fetal bovine serum and with 20 units/ml of recombinant IL-2. The ability of restimulated lymphoblasts to lyse Neu/H-2^d or Neu/H-2^q tumor cells was evaluated by a standard ^{51}Cr release assay, and the percentage of lysis was calculated as described (*Ferrini et al., 1985*; *Di Carlo et al., 2000*).

Antibody response

Mice were routinely bled from a tail vein and sera were stored frozen at -80°C. The production of specific antibodies was then studied both by ELISA assay and/or by indirect immunofluorescence of syngeneic and/or allogeneic *neu*$^+$ cells.

ELISA assay to detect antibodies recognizing rat HER-2 was performed in Maxisorp NUNC 96-well microplates as reported (*Cipriani et al., 2008*). Plates coated with rat *neu* (kindly provided by Dr. Aurisicchio, IRBM, Rome) were incubated overnight with sera at 1:300 dilution, washed, incubated with goat antimouse IgG alkaline phosphatase-conjugated antibody (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, washed again and developed with p-Nytrophenyl phosphate (Sigma). A standard curve with anti-c-ErbB2/Neu mouse monoclonal antibody (clone 7.16.4, Calbiochem) was run in parallel (0.10-250 ng/ml).

For immunofluorescence studies, Neu/H-2^d and Neu/H-2^q cells were incubated with sera at 1:65 dilution for 30 minutes in ice, then washed and incubated with an AlexaFluor 488 F(ab')2 fragment of goat anti-mouse IgG (H-

L) chains antibody (Molecular Probes, Eugene, OR) to evaluate total immunoglobuling binding.

For isotype analysis, the following secondary FITC-conjugated mAbs were used (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. Cytofluorometric analysis was performed through a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Rat HER2/*neu* expression of target cells was evaluated in each set of experiments with anti-c-ErbB2/Neu mouse monoclonal antibody. Data from each sample were normalized over target cells day expression.

MORPHOLOGICAL ANALYSIS

All morphological analysis were kindly performed in the Laboratory of Prof. Piero Musiani (Aging Research Center, Chieti, Italy). Although the candidate did not carry out the morphological analysis, methods were reported below for an exhaustive description of the work.

Therapeutic vaccination: morphological analysis of lungs

Groups of three mice were killed at 7, 14, 21, and 30 days after cell injection. To optimize the detection of microscopic metastases and to ensure systematic uniform and random sampling, lungs were cut transversally to the trachea into 2.0-mm-thick parallel slabs with a random position of the first cut in the first 2.0 mm of the lung, resulting in five to eight slabs per lung. The slabs were then embedded cut surface down. Tissue samples were processed as described previously (*Ambrosino et al., 2006*) for histologic evaluation.

For immunohistochemistry, pyridoxal phosphate–fixed tissues were embedded in OCT and acetone-fixed cryostat sections were incubated for 60 minutes with the following: anti-c-ErbB2/HER-2; anti-proliferating cell nuclear antigen (clone PC10; DakoCytomation); anti-CD4 (Chemicon International);

anti-CD8 (Oxford Biotechnology); anti-Gr1, a cell surface protein mainly expressed by granulocytes (clone RB68C5); anti-CD45R/B220, a subset of mouse CD45 isoforms predominantly expressed on B lymphocytes; anti-CD11b mainly expressed on monocytes-macrophages (BD Pharmingen); anti-Foxp3, nuclear transcription factor specific for regulatory T cells (Treg; clone MF333F, Alexis Italia); and a mix of anti-endothelial cell (CD31, Chemicon International; CD31, clone MEC13.3, and CD105/endoglin, clone MJ7/18, BD Pharmingen) antibodies. After washing, sections were overlaid with biotinylated goat anti-rat and antirabbit Ig (Vector Laboratories) for 30 minutes. Unbound Ig was removed by washing, and slides were incubated with avidin-biotin complex/alkaline phosphatase (DakoCytomation).

For immunofluorescence to detect p185 expression, Alexa Fluor 488–conjugated secondary antibodies were used.

For electron microscopy, lung lobes were fixed for 4 h in 2.5% glutaraldehyde in 0.2 mol/L HEPES buffer (pH 7.4) and processed as reported (*De Giovanni et al., 2004*).

Comparison between Triplex DNA and cell vaccines in BALBp53neu: histology and immunohistochemistry

For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E. For immunohistochemistry, paraffin-embedded sections were immunostained with anti-c-erbB-2 (Dako, Milan, Italy). After washing, sections were overlaid with biotinylated anti-rabbit Ig (Jackson ImmunoResearch Europe, Suffolk, UK) for 30 minutes. Unbound Ig was removed by washing, and slides were incubated with ready to use peroxidase streptavidin (Lab Vision, Bio-Optica Milan, Italy) and diaminobenzidine (Dako).

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