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Preclinical vaccines against mammary carcinoma

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Summary

Vaccines against human breast cancer are an unfulfilled promise. Despite decades of promising preclinical and clinical research, no vaccine is currently available for breast cancer patients. Preclinical research has much to do with this failure, because early mouse models of mammary carcinoma did not mirror the molecular, cellular, antigenic and immunological features of human breast cancer. The advent of HER-2 transgenic mice gave impulse to a new generation of cell and DNA vaccines against mammary carcinoma, that in turn led to the definition of significant antigenic (oncoantigens) and cellular (cancer-initiating cells, preneoplastic lesions, incipient metastases) targets. Future preclinical developments will include the discovery of novel oncoantigens in HER-2-negative mammary carcinoma and the targeting of activated HER-2 molecular variants. Translation to clinically effective vaccines will be fostered not only by new preclinical model systems, but also by the possibility to conduct veterinary vaccination trials in companion animals.

Preclinical models of mammary carcinoma for tumor immunology

Research on mammary carcinoma is inextricably interwoven with the history of preclinical models, starting with the development in the 1920s of the C3H mouse strain, selected for a high incidence of mammary tumors. It is a history that illustrates very well some critical issues of preclinical models in general[1]. On the one side, the C3H model of mammary carcinoma, caused by the mouse mammary tumor virus (MMTV), is no longer regarded as a reliable model for human breast cancer, which is not caused by viruses. On the other side, the discovery of MMTV and the analysis of its oncogenic activity were key steps in the development of molecular oncology, eventually leading to the discovery of various oncogenes.Today MMTV continues to contribute to mammary carcinoma preclinical models, because its (relative) tissue specificity was harnessed to drive the expression of oncogenes and other genes in the mammary gland, and many transgenic mice prone to mammary carcinoma in use today were designed using (non-coding) MMTV sequences[2].

Cell lines

In addition to the viral etiology, MMTV-infected mice have shortcomings as model systems for vaccination studies. Obviously viral antigens are expected to dominate or otherwise distort the immune responses elicited by vaccines, a further difference with human breast cancer. Furthermore, tumor onset and growth required repeated pregnancies and lactation, altogether making MMTV-infected mice a poorly reproducible and cumbersome system for immunological studies[1]. Starting in the 1970s,

immunological research resorted to the use of cell lines derived from mammary carcinomas, for example from occasional spontaneous carcinomas, arising in "retired breeders", i.e. old multiparous females, of MMTV-free strains, like BALB/c[3]. These cell lines were thought to reproduce the low immunogenicity of human tumors, thus giving rise to more faithful preclinical models for vaccination studies. While it is true that cell lines like TS/A were poorly immunogenic, in retrospect they were not exempt from the "viral sin", because in many instances the immunodominant antigens were later shown by molecular studies to be related to endogenous retroviral sequences [4,5].

Genetically modified mice

The advent of genetically modified mice has revolutionized biomedical research, allowing the establishment of preclinical models of human diseases for which no equivalent spontaneous mouse pathology existed or was not a faithful model of human disease, as is the case of mammary carcinoma.

Immunological studies focused on HER-2 transgenic mice, that offered for the first time a preclinical model of high penetrance mammary carcinogenesis caused by an oncogene known to be involved in human breast cancer, and at the same time offered an attractive immunological target. Many different HER-2 transgenic mouse lines were produced over the last 25 years[6] thus it is important to understand their characteristics in relation to human breast cancer[7]. Under this respect it should be noted also that our knowledge of human tumors has evolved in parallel, leading to different perceptions of what can be considered a good animal model. Think for example of point mutations, which for many years were thought to be almost

non-existent in human pathology, whereas next generation sequencing is bringing to light a small but consistent percentage of tumors driven by mutant HER-2[8].

Earlier transgenic mice carried a mutant rat HER-2 (the HER-2 oncogene was originally cloned in rat and called *neu*) transgene controlled by MMTV long terminal repeat (LTR) sequences [2,9] and displayed a very aggressive mammary carcinogenesis, with progressive carcinomas in all ten mammary glands within the first semester of life. The same promoter was then used to drive the wild-type (i.e. non-mutated) rat HER-2 gene in atransgenic model with a milder carcinogenesis, displaying carcinomas in 2-4 mammary glands by one year of age[10].

The following step was the replacement of the rat oncogene with the human ortholog, thus obtaining a transgenic mouse in which anti-human HER-2 antibodies and analogous therapeutic agents could be directly tested[11].Latest developments were driven by the discovery in humans of activated HER-2 variants, such as the Delta16 isoform or the p95 truncation, that were shown to be highly oncogenic in transgenic mice, ultimately giving rise to novel model systems that reproduce the aggressiveness of those based on the mutant rat oncogene[12-14].

Widespread interest in these transgenic models of mammary carcinogenesis led to the developments of countless mouse lines of different genetic backgrounds, bearing different oncogenes, different promoters, and combinations of HER-2 with many other cancer genes[6,7]. We will restrict ourselves to some basic key elements based on our experience for the choice of a system for preclinical vaccine studies.

1. Tumor penetrance is the first element. Many interesting transgenic lines with a low tumor incidence make extremely cumbersome model systems and mandate the use of very large experimental groups. Whenever possible prefer models with a nearly 100% incidence.

2. Tumor latency is another key. Experiments in transgenic mice take a long time in comparison to those with transplanted cell lines, and if your vaccine effectively prevents tumors you will need even longer observation times. Models in which the first tumors appear after one year of age make the life hard for a successful vaccinologist. Apart from practical considerations, it should be also kept in mind that a long tumor latency in a mouse carrying an oncogene means that additional genetic events are required for tumor onset, which could be either an advantage or a disadvantage, depending on the type of research project.

3. Aggressiveness is not to be feared. When we started the study of anti-HER-2 preventive vaccines, we thought that a mouse prone to the onset of invasive carcinomas in all mammary glands was a daunting prospect, however our experience showed that powerful vaccines could completely prevent tumor onset in these mice[15,16], provided that vaccinations started at the right time[17], which brings us to the following point.

4. Timing is of the essence. As transgenic mice reproduce tumor progression, the effectiveness of cancer vaccines is frequently dependent on vaccination schedules taylored on the stages of tumor progression (e.g. atypical hyperplasia, *in* situ carcinoma, angiogenic switch, wtc.) in the model in use. Fortunately, a key advantage of transgenic models of mammary carcinogenesis is the repeatability (with possible variations) of tumor progression.

Human-in-mouse systems

Until recently, the only way to investigate human immune responses to vaccines, short of actually immunize human subjects, was the in vitro study of immune cell reactivity, clearly a suboptimal way to investigate the complexities of in vivo responses. Paradoxically, the advent of highly immunodeficient mouse models has considerably improved not only the study of human tumor biology, but also that of human immunology[18]. Residual immune responses, in particular NK cells, of older immunodeficient mouse models, such as athymic nude mice, considerably impair the survival of implanted human cells, both normal and neoplastic[19,20].

A key development was the knockout of the common gamma subunit of interleukin receptors (Il2rg), that blocks NK development. Mouse models combining T and B cells deficiencies with Il2rg knockout, such asRag2$^{-/-}$;Il2rg$^{-/-}$ (BRG) or NOD-*scid*-gamma (NOG) mice, allow for the first time the study of HER-2+ human breast cancer dissemination, and at the same time can be reconstituted with human stem cells that give rise to a functional immune system[21]. We have recently shown that, in reconstituted BRG mice, the human immune system can respond to vaccines against human HER-2 with the production of specific antibodies and possibly other immune responses that hamper the metastatic spread of human tumor cells[22].

At present the study of vaccines in mice with a reconstituted immune system has various shortcomings, for example human immune responses are clearly suboptimal and incompletely developed. Furthermore, the use of cord blood as the major source of human stem cells rules out the study of autologous immune responses for adult tumors. Current studies clearly show

how far the knockout of just one gene has opened new avenues in preclinical models, thus, even if current models are still imperfect, further improvements are expected to lead to the development of fully human preclinical models for cancer vaccines.

Natural occurring cancers in pet animals

In 2003, the National Cancer Institute's Center for Cancer Research (CCR) launched the Comparative Oncology Program (COP) (https://ccrod.cancer.gov/confluence/display/CCRCOPWeb/Home) to foster the use of naturally occurring cancer in pet animals - primarily dogs and cats – as models of human cancer [23]. Generally, pet's and human tumors have many similarities, including histological appearance, tumor genetics, molecular targets, biological behavior and response to conventional therapies. Moreover, being dogs and cats the favored companions of humans, they share the same environmental exposure to risk factors. Moreover, inclusion of dogs or cats from different breeds in clinical trials provides a cross-sectional value that is often higher than that obtained in studies of inbred laboratory animals, by providing a background genetic diversity similar to that seen in human populations. Noteworthy, the first licensed therapeutic vaccine for the treatment of cancer (ONCEPT™, Merial) is a xenogeneic DNA vaccine against human tyrosinase recently approved for veterinary use against canine malignant melanoma [24], whose initial testing in dogs [25-27] led to its use in human trials [28-30].

Both canine and feline mammary carcinomas can be used to study different aspects of human breast cancer. The percentage of malignant mammary tumors is higher in cats than in dogs. While dogs are considered a

good model of human inflammatory breast cancer, feline mammary carcinoma has been proposed as a model for poor prognosis human breast cancer. Feline mammary cancer is similar to human breast cancer in the age of onset, incidence, histopathologic features, biologic behavior, and pattern of metastasis [31]. In particular, HER-2 overexpressing feline mammary carcinoma is very similar to the human counterpart [32]. Testing of DNA vaccination against HER-2 in cats is ongoing (Dr. Wei-Zen Wei, Karmanos Cancer Center, Detroit, USA; personal communication).

Will the pet lead to a breakthrough in the fight against cancer? The premises are good, but there is still much work to do.

Vaccination strategies and protective immune responses

Vaccines for immunoprevention of mammarycarcinoma

Studies of mammary carcinoma preventive vaccines in HER-2 transgenic mice not only contributed to the notion of non-viral cancer immunoprevention, but also highlighted various important concepts in tumor immunology. We have reviewed this field in the recent past[33-35],therefore here we will focus on general principles relevant to vaccine development, summarizing the most important conclusions.

Effective vaccines were able to completely block the development of mammary carcinoma in HER-2 transgenic mice[15,16,36]. Different vaccine formulations, such as cell-based and DNA vaccines were equally effective[37]. Common vaccinological properties were the use of powerful adjuvants and intensive vaccination schedules. Microscopic analysis revealed that cancer

progression was indefinitely "frozen" at the stage of hyperplasia by vaccination, and that HER-2 expression was greatly down modulated[38].

Immunological studies showed that protective immune responses elicited by vaccines were mainly based on helper T cell cytokines, in particular gamma interferon, and anti-HER-2 antibodies of Th1 isotypes (like IgG2a and IgG2b in the mouse), whereas cytotoxic T cell responses did not play a relevant role[39]. Antibodies were the effectors of long-term protection, and titers after the first cycles of vaccination predicted long-term protection from tumor development[37].

Therapeutic vaccines

Basically most preclinical vaccination experiments targeted "local" mammary carcinomas. Countless experiments were performed over the years using almost any conceivable vaccine design and formulation, and the ensuing publications attest the attainment of several positive results with many different vaccination approaches[40]. A comprehensive review would be at once humongous and useless. We would rather provide a critical appraisal of some key issues related to preclinical models, because the continuing lack of clinical impact *vis-à-vis* the huge amount of positive preclinical results could suggest that past and current preclinical models are part of the problem.

The first thing to stress is that the model system used for preclinical testing is an important variable, and that there are innumerable variations that can directly impact the results. In general one must ask up front how far removed from the human condition is the preclinical model. The trade-off is

between "simple" models that are quite different from clinical situations and cumbersome systems that more closely mirror human pathology and therapy.

The simplest thing to do is to perform a classical vaccination-challenge experiment: tumor-free mice are first vaccinated, and then challenged subcutaneously with a syngeneic mammary carcinoma cell line. At the opposite end of the spectrum is therapeutic vaccination administered to a mouse bearing a spontaneous ("autochthonous") mammary carcinoma or, even better, the same mouse undergoing surgical removal of the primarytumor followed by therapeutic vaccination for prevention or therapy of distant metastases[41].

Being guilty of using, over the years, practically every variation of preclinical models for studying mammary carcinoma vaccines, we can only practice non-directive counselling.

1. Vaccination-challenge experiments are fine to demonstrate principles, but to start a translational approach, a therapeutic set-up, in which vaccines are administered to tumor-bearing mice, would be more realistic.
2. Orthotopic injection of mammary carcinoma is easy. It is well known that growing tumors in the appropriate anatomic site better reproduces human pathophysiology, and injection of mammary carcinoma cells in the mammary fat pad is not difficult, in comparison to the problems posed by other tumor types (think for example of glioblastoma).
3. Metastases are better targets than local tumors. It is quite obvious that preclinical vaccine therapy of local tumors is mostly devoid of translational meaning, because human patients succumb to metastases after surgical removal of the primary tumor. A less

appreciated advantage of preclinical models of metastasis therapy is that metastasis evaluation(by direct count of through imaging techniques) is a highly sensitive and exquisitely quantitative endpoint, superior to volumetric measurements of local tumors. It must be kept in mind that the cure of micrometastasis is at the boundary between prevention and therapy[42]. Metastatic cells newly arrived in a distant organ face much of the hurdles of early neoplastic cells, and are more sensitive to immunotherapies than established tumor masses. In HER-2 transgenic mice we found that the efficacy of an anti-HER-2 vaccine followed a saddle-like curve related to tumor development and progression[35]. Maximal efficacy was obtained in preventive protocols starting before tumor onset, followed by a precipitous loss of activity against established tumors[17,36]. However the same vaccine was again active against metastasis development, demonstrating that preventive oncologists are right when they designate as "tertiary cancer prevention" what medical oncologists call adjuvant therapy[43].

4. Transgenic models of mammary carcinogenesis can be unsuitable for studies of autochthonous metastases[42]. Aggressive mammary carcinogenesis, in some cases leading to the continuing development of independent carcinomas in all mammary glands, can prevent meaningful studies of metastasis therapy in the absence of growing primary tumors.

5. Studying naturally occurring tumors in pets is likely to provide a valuable perspective that is distinct from that generated by studying rodent models alone before first in-human studies[44]. An increasing number of trials is ongoing in various Veterinary Teaching Hospitals, both in Europe and in the USA, and promise to solve many problems of mouse pre-clinical models.

Targeting of cancer stem cells

To date, bulk tumor masses with heterogeneous populations of cancer cells have been used as a source of potential drug or vaccination targets. However, human tumors are composed of heterogeneous cancer cell sub-populations that differ with respect to proliferation, differentiation, and ability to initiate daughter tumors. The slowly dividing cancer initiating cells endowed with stem cell properties, like the capacity to self-renew and to reestablish tumor heterogeneity, appear to be the sub-population responsible for tumor progression, metastatization, resistance to therapy, and tumor recurrence [45].

The notion that cancer stem cells may play a major role in cancer progression has important implications. It may account for most of the difficulties of current treatments in eradicating malignant tumors. Those treatments designed to shrink the bulk of a tumor may fail to eliminate the small fraction of cancer stem cells endowed with chemo- and radio-resistance and immune-evasive features [46]. In some cases, the vaccine-induced response against antigens identified in the bulk of the tumor, may even drive the selection of cancer stem cells and promote tumor growth [47]. Instead, for therapy to be more effective, debulking of differentiated tumors must occur followed by targeting of the remaining surviving often quiescent cancer stem cells. A recent report actually demonstrated that a vaccine done of dendritic cells primed with antigens derived from purified cancer stem cells induced a significant protective antitumor immunity in mouse models [48].

The understanding of the pathways regulating breast cancer stem cell self-renewal, differentiation and tumorigenicity, and the identification of

appropriate drug and vaccination targets may be critical in the development of effective breast cancer therapies[49].

Target antigens

HER-2 is a superior target for mammary carcinoma immunotherapy, both in humans and in preclinical models. Why? In other words, is HER-2 endowed with specific features not shared by other tumor antigens? And can we derive general principles that will help in defining novel tumor antigens on a par with HER-2?

On the experience of cancer immunoprevention in HER-2 transgenic mice, we defined two key antigenic features of HER-2. Firstly, HER-2 was at the same time the driving oncogene of mammary carcinogenesis and the target antigen. In our model systems, HER-2 was necessary for tumor onset, and cell variants with low/absent HER-2 expression (observed only *in vitro*) displayed a simultaneous loss of tumorigenicity[50]. Secondly, down-modulation of class I major histocompatibility complex glycoproteins, an overly common human tumor phenotype[51], was observed also *in vivo* in mammary carcinomas of HER-2 transgenic mice, indicating that T cell recognition of tumor cells could be severely hampered[52].However, surface expression of HER-2 preserved immune recognition by antibodies, which in fact were the main immune effectors of protection from tumor onset[39].

In other words, HER-2 is a good antigen because it is impervious to the two main causes of immunotherapeutic failure, antigen loss and MHC loss. A generalization of these concepts lead to the definition of a novel category of tumor antigens, that we named oncoantigens.

Oncoantigens

In the original definition, we called oncoantigens those tumor antigens that play a causal role in tumor development and are expressed on the cell surface[33]. More recently we revised the original definition to include further categories of promising antigenic targets having in common an oncogenic role[34,35].

Class I oncoantigens coincide with the original definition. The prototypic class I oncoantigen is HER-2, accompanied by receptor tyrosine kinases and many other surface molecules that are indispensable for tumor and metastasis development

Class II oncoantigens include soluble antigenic targets that promote tumor growth directly, for example growth factors, or indirectly, for example angiogenic factors.

Class III oncoantigens are intracellular molecules controlling tumor growth.

In essence the oncoantigen concept affirms that molecules driving cancer are more *persistent* antigenic targets than "passenger" molecular alterations. Cancer progression and selective pressure exerted by therapeutic agents can result in a succession of different drivers over time, therefore the persistence of oncoantigens is to be understood in relative, not absolute terms. However there will always be driving molecules to be targeted in a given stage of tumor development.

From an immunological perspective, the three classes define a hierarchy of effector mechanisms, because class I oncoantigens (membrane)

are targeted both by T cells and by antibodies, class II molecules (soluble) are only bound by antibodies, while class III (intracellular) can be recognized only by T cells in association with MHC molecules. Given the widespread of MHC loss in tumors [53,54], broadly speaking class I (and II) oncoantigens are more attractive, however also class III oncoantigens have been successfully targeted [55,56], and a possible role of antibodies even against these oncoantigens hiddeninside the cell has been recently hypothesized[57,58].

Discovery of novel oncoantigens – Preneoplastic lesions, differentiated tumor cells, cancer stem cells

The most important oncoantigen identified so far for breast cancer is HER-2, and the development of innovative therapeutic options that specifically target HER-2 or other members of the HER family has represented one of the most important achievement in clinical oncology. Similarly, many vaccination strategies against HER-2 have been successfully developed in pre-clinical models and are now under clinical investigation. Nevertheless, only 20-30% of mammary cancers overexpress HER-2; moreover, a prolonged exposition of HER-2 overexpressing tumors to anti-HER-2 treatments, both with antibodies or tyrosine kinase inhibitors, often results in the development of HER-2-negative, therapy-resistant variants [59]. Identification of additional oncoantigens for immune targeting of mammary cancer is thus needed.

Microarray transcription studies are a powerful instrument to identify potential oncoantigens on a genome-wide basis. We have generated a pipeline for oncoantigen identification based on the integration of gene expression data from mammary cancer-prone genetically engineered mice and human mammary cancer [60]. Mammary tissue samples are collected from mice of

various ages, corresponding to different stages of tumor progression, from atypical hyperplasia to invasive cancer; total RNA is extracted and gene expression profiles are generated using genome-wide mouse arrays. Of the transcripts up-regulated going from pre-neoplastic lesions to overt cancer, only those that have an orthologue in humans, low expression in normal human tissues, and a high and homogeneous expression in human cancers are selected. The functional role of the corresponding molecules in fostering the transformed phenotype is investigated. Vaccines against these "putative" oncoantigens are then generated to immunize cancer-prone transgenic mice and assess whether an effective immune response affecting tumor progression can be generated[61].In this way several oncoantigens have been identified [34,61]. Additional oncoantigens generated by specific mRNA isoform usage or represented by aberrant fusion products can now be identified using Next Generation Sequencing technology.

This approach allows the identification of oncoantigens expressed by the bulk of differentiated tumor cells, and by any other cell population in the tumor microenvironment (infiltrating cells, tumor stromal and endothelial cells) whose number increases with tumor progression. On the other hand, it does not allow the identification of the oncoantigens specifically expressed by CSC, whose relative number in the bulk of the tumor cells remains very low.

The surface marker phenotypes of the CSC from different mammary cancer sub-types are still the subject of some debate since significant differences in marker expression within the same sub-type are evident. For this reason CSC isolation on the basis of surface markers alone is quite controversial. Mammary CSC down-regulate cell-cell junctions, display mesenchymal behavior in vitro, and survive and proliferate in anchorage-independent conditions in the form of floating spherical colonies termed

mammospheres [62,63]. CSC from human and mouse mammary tumor specimens, metastases, and cell lines can therefore be isolated in function of their ability to grow as non-adherent spheres [63]. These mammospheres express markers associated with the CSC phenotype, and are able to efficiently generate tumors when injected into syngeneic (murine CSC) or immunodeficient (human CSC) mice. By comparing the transcription profile of the bulk of mammary tumor cells with that of CSC-enriched serial sphere passages of the same tumor cells, a gene signature associated with mammary CSC can be obtained, and potential CSC-specific oncoantigens identified (Cavallo et al., in preparation). We expect that vaccines targeting these CSC-specific oncoantigens will result in a more effective control of clinically evident cancer, recurrences and metastases as compared to vaccines against oncoantigens of the differentiated tumor cell population, whose protective potential is mostly restricted to tumor prevention[33,35,64]. The two kinds of vaccine, against oncoantigens of CSC and of differentiated tumor cells, might have a synergic therapeutic effect.

miRNAs

While several protein-coding genes involved in malignancy have been identified and characterized, less is known for non-coding-genes, such as microRNAs (miRNAs). Nevertheless, miRNA can be of help in understanding the biology of the different mammary cancer subtypes and in identifying novel oncoantigens. Because of their ability to bind to many target mRNA, once their expression is altered, disease could occur through the deregulation of their target gene networks, particularly those leading to cancer. Moreover, miRNA appear to be involved in the maintenance of the CSC phenotype, by connecting stemness and metastasis through regulation of epithelial-to-

mesenchymal transition [65]. The identification of down-regulated miRNA in differentiated tumor cells or in CSC can thus lead to identification of their target genes as potential oncoantigens. Even if the possibility to effectively target in vivo tumor over-expressed miRNA existed, they could hardly be considered a new class of oncoantigens, because they are not immunogenic. Nevertheless tumor-derived, over-expressed miRNAs can be secreted outside of the cell and can be detected in the sera, thus representing a new class of diagnostic and prognostic biomarkers [66]. Their titration can be used to assess disease progression and the effectiveness of vaccination against oncoantigens.

Global miRNA deregulation has been shown in breast cancer [67-70] while specific miRNAs have been associated with clinico-pathological features of breast tumors such as estrogen and progesterone receptor expression [71], tumor grade [68], vascular invasion or proliferation index [72]. Profiling studies have been mainly focused on miRNAs deregulated in primary breast cancer [70,73] or in breast cancer cell lines [74], while characterization of a deregulated miRNA expression profile in different tumor stages would permit to assess miRNA involvement in tumor progression. Using this type of approach, we have recently characterized the deregulated miRNA expression profile during tumor progression in mammary cancer-prone HER-2 transgenic mice. The miRNAs found down regulated during tumor progression and their putative targets are under investigation. miR-135a and miR-135b were found to be up-regulated during tumor progression [75]. While their involvement in breast cancer has never been described, by exploiting data on the expression of miRNAs in human breast cancers [70,73], the over-expression of miR-135b, but not miR-135a, appeared to be associated with poor prognosis. In addition, miR-135b over-expression in basal-like and estrogen-negative human tumor shown by this

meta analysis fits in well with the notion that mammary tumors of our HER-2 trasngenic mice are similar to basal-like and estrogen-negative human mammary carcinomas [76]. Down-modulation of miR-135b in cancer cells from HER-2 transgenic mice revealed its major role in anchorage-independent growth and lung metastasis formation. MID1 and MTCH2 were identified and validated as putative miR-135b targets.

Vaccines and adjuvants

The efficacy of a vaccine rests on its ability to induce an effective antibody and cell-mediated immune response against the target antigen. Once triggered, immunity is kept at an efficacious protective intensity during the aging of the individual, both through natural re-stimulation or vaccine boosts. Two are the components of a vaccine: the antigen and the adjuvant(s). The first one is obviously necessary to direct the induced immune response against the right target, while the second is any substance able to promote antigen recognition and the establishment of the immune memory. Different sources of antigen have been tested. When the antigen source is complex - as in the case of live, irradiated or genetically modified tumor cells, dendritic cells, recombinant viral/bacterial vectors or naked DNA - it normally displays some intrinsic adjuvant activities, while the use of purified proteins or peptides always requires the addition of extrinsic adjuvants. Each vaccine preparation can be given as adjuvanted standalone intervention or combined with cytokines or other immune-modulatory factors to optimize immune system activation.

Proteins and peptides

Protein- and peptide-based antigen vaccines were among the first defined vaccines demonstrating both protective and therapeutic efficacy in animal models [40,77]. Many late-stage clinical trials assessing the efficacy of protein- and peptide-based vaccines have been performed or are currently on going; however, only a few of them have reported consistent rates of objective, long-term clinical responses [78].

Proteins usually contain several MHC restricted epitopes recognized by both cytotoxic and helper T cells, and linear or conformational epitopes that can be recognized by antibodies. Protein vaccines can thus induce a complete adaptive immune response, when taken up and processed by antigen-presenting cells (APCs), but have several demerits in terms of manufacturing and safety controls. To avoid these drawbacks, and as a consequence of the rampant T-cell chauvinisms of the nineties, short synthetic epitopes expected to directly bindMHC molecules, and hence be presented to T cells, have been widely used. The peptides are generally emulsified with Montanide ISA51, a clinical grade of Freund's incomplete adjuvant, prior to administration, or directly pulsed on antigen presenting cells to be used for vaccination [77].

The earlier generations of peptide vaccines, aimed at inducing a cytotoxic T cell response, were composed of one to several MHC class I-restricted peptides of a single MHC-type. Various types of new generation peptide-based vaccines have since been developed. To stimulate both cytotoxic and helper T cell responses, MHC class I- and MHC class II-restricted peptides have been formulated independently and administered at a separate site to the same patients. Alternatively, multi-peptide cocktails have been used. Synthetic long peptides likely to contain both MHC class I and class II epitopes suitable for presentation on several MHC haplotypes

have also been generated. These multi-epitope vaccines can thus be used in a wide range of patients [77]. Most of single- or multi-epitope vaccines are based on the native peptide sequences, with or without modification of the anchor amino acid residues. Some of the latest generation of vaccines however are based on hybrid peptide sequences derived by fusion of peptides from different molecules. The Ii-Key/HER2/neu (776–790) peptide vaccine is an example of hybrid peptide obtained by fusing the Ii-Key 4-mer peptide and the human HER-2 (776–790) helper epitope. The Ii-Key peptide is the shortest active sequence of the Ii protein that catalyses direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing. The Ii-Key/HER2/neu (776–790) hybrid peptide vaccine induces significantly higher antitumor responses as compared with the native HER2/neu (776–790) peptide, even in the absence of an adjuvant [79]. Finally, an attempt has been recently made to generate personalized peptide vaccines, taking into account the pre-existing immunity of patients. Appropriate peptides for vaccination are screened and selected from a panel of vaccine candidates, based on MHC-haplotypes and detection of cytotoxic T cell precursors and IgG in the peripheral blood of each patient before vaccination.

The majority of on going phase I-III clinical trials assessing the safety and efficacy of recombinant peptides in breast carcinoma patients is based on the administration of HER2-derived peptides, either as adjuvanted standalone interventions or combined with additional immunostimulatory agents [78].

Cell vaccines

In principle, cell vaccines expressing a given antigen already contain all the constituents of a good vaccine, with cells themselves acting as complex adjuvants through many activities related also to immunogenic death. In addition to the known target antigen, tumor cells can express many other unknown tumor antigens, resulting in the simultaneous induction of immune responses against a constellation of targets expressed in the tumor. In practice, unmodified tumor cells rarely make good vaccines because on the one side their antigenicity was dampened by immune editing during tumor development, on the other side the immune system is poorly responsive because of immune tolerance and tumor-induced immune suppression.

A major problem in the field of preclinical cancer vaccines is the lack of studies comparing different vaccine designs and vaccination protocols under identical experimental conditions. Fortunately the development of genetically-modified cell vaccines in the 1990s coincided with widespread use of TS/A mouse mammary carcinoma cells as recipients, spearheaded by Guido Forni and co-workers, leading to the establishment of an informal network of researchers that produced a large corpus of comparable experiments[80-82]. The results contributed a considerable understanding to the use of cytokine genes and other immune-related molecules as biological adjuvants, and led to the definition of a small number of genes showing a definite potential in the development of anti-cancer vaccines[82]. These studies provided also evidence of the destructive potential of forced expression of immune-related molecules in tumor cells, which in various instances led to increased tumor or metastasis growth, or to unexpected unwanted effects on the host.

The experience accumulated with earlier gene-modified cell vaccines provided the bases for the development in the following decade of the Triplex

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cell vaccine. To fight the aggressive mammary carcinogenesis driven by the activated HER-2 oncogene we designed a cell vaccine combining three powerful immune stimuli, the HER-2 gene product p185 and two biological adjuvants, interleukin 12 (IL-12), the major cytokine of antigen presentation, and allogeneic class I major histocompatibility complex antigens, which activate many T cell clones. The first Triplex formulation was based on vaccinations with HER-2+, MHC-allogeneic mammary carcinoma cells followed by the administration of recombinant IL-12[15]. To avoid systemic administration of the cytokine we then transduced vaccine cells with IL-12 genes, thus obtaining a single-component cell vaccine[83]. The results obtained with either formulation were similar.

The Triplex vaccine, when administered to young HER-2 transgenic mice was able to block indefinitely mammary carcinogenesis at the stage of atypical hyperplasia, preventing the development of mammary carcinomas and resulting in 100% survival at one year or more of age, when all non-vaccinated mice had already succumbed to tumors[15].

The key elements of the complete protection afforded by the Triplex vaccine were defined through a large series of protocol variations, which were explored not only *in vivo*, butalso using a combination of *in silico* and *in vivo* approaches[84].

1. The Triplex was the minimal combination yielding long-term protection, all subsets of just one or two components significantly prolonged tumor latency, but did not prevent tumor development.
2. To be effective, vaccination had to start early in life, before the onset of malignant tumors.
3. Periodic vaccination boosts were required throughout the life of the mouse to maintain protective immunity, in a situation remindful of

tetanus vaccination in humans, *i.e.* the vaccine elicited long-lasting, but not lifetime immunity.

DNA vaccines

DNA vaccines are simple circles of DNA carrying the sequence coding for the target antigen that once enter mammalian cells result in antigen synthesis, processing and presentation in the MHC-context with induction of both cell- and antibody-mediated immune responses. Their ability to determine a relatively low but persistent in vivo antigen expression may be particularly effective in inducing B-cell affinity maturation. DNA vaccines are stable, relatively inexpensive, have an easy good manufacturing practice production, lack anti-vector immunity, and are extremely versatile. The antigen sequence can be in its native form or modified, alone or together with sequences coding for immune modulators or molecules influencing antigen processing and presentation. They are effective even when administrated without adjuvants, as they carry intrinsic danger signals. Unlike mammalian DNA, plasmids are rich in unmethylated CpG dinucleotides that warn of bacterial infection and activate the innate immune response via Toll-like receptor 9 expressed on APCs. DNA vaccines are commonly delivered intradermal or intra-muscularly by simple injection or through bio-ballistic methods or in vivo electroporation [34]. The latter is one of the most promising current technologies for DNA vaccine delivery [85], that greatly impacts vaccine immunogenicity and efficacy by increasing antigen delivery up to a 1000 fold over naked DNA delivery alone, with improved in vivo immune response magnitude. Recently, a new strategy based on microneedles implantation into the skin of biodegradable polymer films containing DNA

polyplexes and adjuvant molecules, has been tested with promising results [86].

DNA vaccines directed against HER-2 have proven to be successful in the prevention of tumor growth in transplantable tumor models as well as in HER-2 transgenic mice [16,34,36,40].

In HER-2 transgenic mice DNA vaccines based on plasmids coding for the extracellular and transmembrane domain of rat HER-2 (RRT plasmids) effectively and persistently hamper the expansion of incipient tumors. This remarkable protection was correlated with the induction of anti-HER2 antibodies [16]. RRT plasmids efficacy fades away when they are administered to mice bearing advanced tumors[33,35,64]. A significant therapeutic effect was obtained only when RRT vaccination was associated with T regulatory cells depletion [17], which, however, could also increase the risk of autoimmunity [87]. To obtain a stronger immune response and circumvent natural tolerance two new chimeric DNA vaccines (RHuT and HuRT) were constructed, encoding HER-2 extracellular and transmembrane domains composed in part by rat and by human sequences. RHuT encodes a protein in which the 410 NH_2-terminal residues are from the rat HER2 and the remaining residues from human HER-2, while HuRT encodes a protein the first 390 NH_2-terminal residues of which are from the human HER-2 and the remaining part from the rat HER-2. These chimeric plasmids combine the specificity, ensured by homologous portions, and tolerance break, ensured by heterologous portions [36]. We found that this combination effectively primes immune effector cells in tolerant hosts [34,36]. In principle, this strategy of combining heterologous with self moieties can be applied to any oncoantigen that share high level of sequence identity and T cell epitopes to produce a potent DNA vaccine.

A new kind of anti-HER-2 DNA vaccine has recently been developed as part of a strategy aimed at subverting the tumor-induced immunosuppressive circuits that weaken the vaccine induced antitumor response. Most of these circuits are based on abnormal differentiation of APCs which results in decreased production of fully competent APCs and accumulation of immature tolerogenic dendritic cells [88]. This new DNA vaccination strategy combines antigen expression with the silencing of immunosuppressive molecules that are responsible for the tolerogenic behavior of APCs. This double action is associated with two distinct modules; one is the conventional antigen expression cassette, while the other generates short interfering (si)RNAs directed against negative immune regulators, such as IDO or IL-10 [89]. This second module is expected to ensure optimal presentation of the encoded antigen by APCs.

DNA vs cell vaccines

Different vaccination technologies are only rarely compared head-to-head[90].We have developed highly active cell and DNA vaccines against HER-2 in the same model systems[15,16], and we have also taken the opportunity to perform direct comparisons in the prevention of tumor development in transgenic mice[37]. The fundamental result was that both cell and DNA vaccines are equally effective in protecting mice from tumor onset. A preference for DNA vaccines, however, is based on various distinctive features, in particular for what concerns translational value. For example, in our model systems, DNA vaccines required fewer boosts than cell vaccines to maintain long-term protection, furthermore DNA vaccines, being molecularly defined and cell-free, are more suitable for human use. Finally, in the perspective of making vaccines for novel oncoantigens, it is certainly

easier to produce and test a panel of new DNA vaccines than a corresponding cell vaccine endowed with appropriate immunogenicity.

Prime and boost strategies

The successes achieved in vaccination against infectious diseases were the driving force for the generation of anti-cancer vaccines and are still a source of information and ideas for improving the effectiveness of anti-tumor vaccination.

In keeping with the well-known tenet of infective vaccinology that boosting injections are critical for protection, homologous booster immunizations that utilize re-administration of the same vaccine formulation have essentially been used since the initial development of anti-cancer vaccines. An effective vaccine usually requires more than a one-time immunization in the form of a prime-boost. For example, when RRT vaccinations of HER-2 transgenic mice bearing incipient tumors was repeated at 10-week intervals, most of one-year-old mice were still free of palpable tumors [16,36], greatly improving the results of a single cycle of RRT vaccinations.

However the homologous prime-boost approach is not feasible for some types of vaccines like viral vector-based ones, because the immune response induced by the earlier immunization can rapidly clear the vector in subsequent boost immunizations. In these cases prime-boost immunizations have been given with unmatched vaccine delivery methods while using the same antigens, in a heterologous prime-boost format. It is now widely accepted that these heterologous prime-boosts are more immunogenic than

homologous prime-boosts in vaccination against both infectious diseases [91] and cancer [92].

A well-designed heterologous prime-boost approach can expand the scope of immune responses and improve the effectiveness of existing vaccines. The heterologous prime–boost can take various forms; the length of time separating the primary and the following immunizations and the order of prime–boost administrations may be important, although antigen-dependent. In HER-2 transgenic mice priming with RRT plasmids and subsequent boosting with allogeneic (H-2^q) mammary cancer cells expressing rat HER-2 and engineered to release interferon (IFN)-γ were able to arrest mammary tumor progression [38]. The sequential administration of DNA plasmid and an adenoviral vector against HER-2 in different combinations resulted in higher frequencies of antigen-specific antibodies and activated T cells, and higher degree of protection from tumor development than do DNA or recombinant viral vectors alone [93,94].

Vaccines and chemosensitivity

The therapeutic efficacy of vaccination for any human tumor remains controversial because the outcomes from clinical trials are far inferior to that anticipated. The reasons of this general clinical failure of cancer vaccines are still to be elucidated, but the general finding is that vaccines are able to induce an anti-tumor immune response but this is too inefficient to keep pace with rapidly growing, mutating tumors in situ.

The clinical insufficiency of cancer vaccines encourages the examination of synergy between vaccination and other therapies and with chemotherapy in particular. Indeed, certain chemotherapeutic agents have

shown immunomodulatory activities, and several combined approaches have been attempted [95]. For instance, chemotherapy has been proven to enhance the efficacy of tumor cell vaccines by favoring tumor cell death and thus enhancing tumor-antigen cross-presentation in vivo. Drug induced lymphodepletion may induce the production of cytokines favoring homeostatic proliferation, and/or ablate immunosuppression mechanisms. Furthermore, monoclonal antibodies can synergize with chemotherapy by inducing endogenous tumor-specific humoral and cellular immune responses. Moreover, it has been reported that vaccinated patients receiving subsequent chemotherapy exhibited significantly delayed tumor progression and longer survival relative to those receiving vaccinations without subsequent chemotherapy or those receiving chemotherapy alone. Improved clinical outcome appeared dependent on the specific combination of therapeutic vaccination followed by chemotherapy [95,96].

In pre-clinical models we have recently reported that vaccination against antigens expressed by vascular cells can sensitize clinically evident mammary carcinomas to a subsequent chemotherapy treatment [97]. DNA vaccination targeting angiomotin, one of the angiostatin receptors expressed by endothelial cells of angiogenic tissues [98], induced an antibody response that alters the structure and the permeability of tumor vessels, resulting in vessel maturation and stabilization. These antibody-induced vessel alteration was effective both in halting the progression of clinically evident tumors and in making them more susceptible to chemotherapy, thanks to an enhanced tumor perfusion [97]. A similar effect was found in patients treated with a humanized monoclonal antibody neutralizing vascular endothelial growth factor (bevacizumab) [99,100].

Expert Commentary

The enormous amount of successful pre-clinical applications of cancer vaccines has not met a corresponding efficacy when translated into clinic. This fact is not limited to breast cancer, but is a generalized result [101]. These frustratingly slow progresses of anti-cancer vaccination are due to various weaknesses of the pre-clinical testing, such as the target antigen choice (not always the target was an oncoantigen) and the inadequacy of available animal models of human cancer [89]. Genetically engineered mouse models have significantly contributed to our understanding of cancer biology and treatment[18]. They have certainly proven to be better clinical models as compared to "instant cancers" obtained by injecting mice with transplantable tumors, or by bombarding mice with carcinogen doses higher than those that any human will ever encounter[102]. However, they still have significant limitations in modeling human cancer that the recently developed mice with a reconstituted immune system are not expected to easily solve. If we are going to beat cancer, we need a new path to progress [102].

Naturally occurring pet tumors provide meaningful systems to study the complexity of human tumors in a far less artificial way. Using naturally occurring cancer in pet animals could also solve the ethical issues of animal experimentation. While medical research involving animals is sometimes controversial and misunderstood, experimentation in pets with naturally occurring cancer will provide benefits for both man and animals.

The use of pets in translational oncology can hugely accelerate vaccine development for many reasons. When effectiveness of a cancer vaccine has been proven in mice, researchers should move to veterinary trials in pet animals. Whereas there are strict regulations concerning treatments to be used and commercialized for veterinary use, as well as for clinical trials in humans,

there are fewer regulations for phase I/II/III clinical trials before drug use in pets; rather, it is left to the discretion of the owner [103]. Because most pet cancer diagnoses end in death, owners are often eager to enroll their animals in clinical trials that could save their pet's life, and possibly provide the necessary evidence to move a promising vaccine to human clinical trials. Compared with humans, pets have compressed life spans, so the efficacy of a vaccine in improving survival can be determined relatively quickly [44].

In conclusion, we believe that part of the failures of cancer vaccines in humans can be ascribed to the use of unfaithful preclinical models or, more precisely, to the use of models that were inappropriate for the preclinical development of cancer vaccines. The advent of genetically modified mice expressing human oncoantigens, and the possibility to move from experiments in mice to trials in cats and dogs provides a realistic preclinical framework for the development of vaccines against mammary carcinoma endowed with true translational potential.

Five year view

Until now HER-2 has dominated the scene of preclinical vaccine research in mammary carcinoma, not only because it is an excellent oncoantigen, but also because of the lack of promising antigenic targets in HER-2-negative tumors. Search strategies discussed here will lead to the discovery of oncoantigens in triple-negative and other subtypes lacking HER-2 amplification, resulting in the preclinical testing of novel vaccines against mammary carcinomas.

For what concerns the future of anti-HER-2 vaccines, the discovery in human tumors of activated HER-2 variants, such as p95 and Delta16, and the

role of HER-2 in cancer-initiating cells and in the early stages of mammary carcinoma development will refocus the attention of vaccine research from the bulk of tumor cells expressing full length HER-2 to more rare cell populations and molecular variants, possibly representing more significant targets for therapeutic vaccines against mammary carcinoma.

Finally, all preclinical research has shown that anti-HER-2 vaccines can induce therapeutic responses in the adjuvant setting, and the clinical experience with trastuzumab demonstrates that anti-HER-2 antibodies are effective against human breast cancer. Many vaccine trials are ongoing and others will start in the near future. We expect positive clinical results.

Key Issues

- There is no approved vaccine for human breast cancer, despite decades of research. Early mouse models of mammary carcinoma, which do not mirror the immunology of human breast cancer, hampered the development of effective vaccines.
- The advent of HER-2 transgenic mice fostered the design of a novel generation of powerful anti-HER-2 vaccines, and led to the development of novel concepts in tumor immunity
- Different vaccine technologies, designs and protocols yielded excellent protection from HER-2-positive mammary carcinoma in mice. DNA vaccinationemerged as an eminently flexible and translatable technology.
- Highly immunodeficient mice reconstituted with a human immune system can be used to test vaccines against human tumors, however current models do not reconstitute the complexity of human tumor immunology.

- Oncoantigens are defined as tumor antigens causally involved in tumor onset and malignancy. Oncoantigens are optimal vaccine targets for the prevention of tumor onset and metastasis development.

- HER-2 is the prototypical oncoantigen of mammary carcinoma. The definition of search strategies combining preclinical and clinical systems areleading to the discovery of oncoantigens in mammary carcinomas lacking HER-2 overexpression.

- Activated HER-2 isoforms expressed in human breast cancer are a promising target for the development of novel vaccines.

- Preclinical development of innovative vaccines will find an optimal environment in novel genetically modified mice and in veterinary trials in companion animals.

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