Xenogene vaccination in the therapy of cancer.

Xeno-Gene-Vaccination in the Therapy of Cancer

ABSTRACT

The advent of cancer immunotherapy is going to profoundly transform the therapy of cancer. In this context therapeutic cancer vaccines will offer significant opportunities, provided an efficient and robust technology is developed. Targeting tumor associated antigens *via* immunization with homologous immunogens derived from other species, an approach called xeno-vaccination, combined with gene delivery is believed to be a viable strategy. Xeno-gene-vaccination has demonstrated to be more efficient than vaccination with 'self' antigens in rodent models in prophylactic and therapeutic settings against cancer. Depending upon the targeted antigen, the mechanism of action of xeno-vaccines has been shown to depend upon the development of antibody and/cytotoxic T cell responses. More importantly xeno-gene-vaccination has been shown to reproducibly affect cancer growth and to improve survival in veterinary cancer patients, mainly in dogs affected by spontaneous disease. One of these vaccines against dog melanoma has been approved by regulatory authorities in USA. Finally, several xeno-gene-vaccines have been advanced to early Phase I/II human clinical trials where they have shown to be safe, well tolerated and capable to induce detectable immune responses against human tumor antigens. Based on this compendium of results we believe that xeno-gene-vaccination may soon become a well-established weapon in the fight against cancer.

1. INTRODUCTION TO CANCER IMMUNOTHERAPY

Immunotherapy of cancer has been selected by Science Magazine as the scientific breakthrough of the year 2013 [1]. This important recognition stems from a series of clinical achievements of the last 3-4 years which, taken together, demonstrate beyond any skepticism that it is finally possible to harness the power of the immune system to successfully fight cancer. Hence, cancer immunotherapy has finally been elevated to the same rank of other well-established therapeutic modalities.

Cancer immunotherapy is a radical deviation from the classical cancer therapy approaches, represented by radiotherapy, chemotherapy or more recently by the advent of targeted therapies, which directly hit cell intrinsic defects in cancer cells. This new modality targets the tumor only indirectly by potentiating or restoring the ability of the various components of our immune system to recognize cancer cells as different from normal cells and to contrast their growth and propagation. Hence the mechanism of action is radically different and this also may explain the strikingly different kinetics of action of immunotherapeutic agents, which clinicians have empirically learned to observe. Classic anticancer drugs either do not work or if they work, their ability to shrink tumor masses is manifested very rapidly. In contrast very often cancer immunotherapies can take weeks or months to manifest their action and disease progression can be halted for a long time after therapy has been discontinued. Furthermore in these cases it is often possible to observe durable disease stabilizations or even disappearance of the disease for years. For metastatic disease this is an achievement previously impossible. As a matter of fact, a new equilibrium in the body is established wherein the immune system regains the ability to control cancer for a prolonged period of time or even for the rest of the patient's life.

1.1 THREE TYPES OF CANCER IMMUNOTHERAPY

For simplicity we can divide cancer immunotherapy approaches in three distinct types: adoptive T cell transfer, checkpoint inhibitors (usually monoclonal antibodies) and therapeutic cancer vaccines. These three approaches have registered several clinical achievements over the last years. Nowadays monoclonal antibodies acting as checkpoint inhibitors are those that have demonstrated increase in survival in large patients' cohorts.

They are followed by adoptive T cell transfer at a smaller but significant scale. In contrast cancer vaccines, with the exception of a single case, lag behind and still wait for major clinical breakthroughs.

Adoptive T cell transfer, the approach pioneered by Steven Rosenberg at the National Cancer Institute, requires in its original version harvesting T lymphocytes infiltrating the tumors (TILs), their expansion and stimulation in the lab using cocktails of cytokines (mainly IL-2), and their reinfusion back into patients [2]. This approach was mainly used for patients with advanced melanoma where significant clinical responses and durable regressions were observed in advanced disease. Long-term follow-up of patients receiving TILs for metastatic melanoma revealed that a substantial subset of them experienced complete, long-lasting tumor regressions and could be cured. For decades the main limitation of T cell transfer application had been the possibility to use it only when doctors could have access to tumor tissue and only in highly specialized clinical centers. The turning point arrived in 2010 with the introduction of antigen receptor engineering for the generation of the so-called chimeric antigen receptors (CARs) [3]. CARs are recombinant receptors composed of an antibody antigen-binding domain connected with domains from stimulatory receptors, therefore conferring antigen specificity and activation of T cells (called CAR-T cells). They can overcome tolerance by allowing T cells to respond to the cell surface antigen of choice. A large variety of CARs has been generated over the last years, targeting an array of cell surface tumor antigens. With this technology the treatment remains personalized because of the use of patient's own T cells. However, CAR-T cells can be generated without limitations from the peripheral blood and can be genetically modified to endow new specificities capable to recognizing and destroying in theory any type of tumor. Indeed this new approach has demonstrated clinical benefits in children affected by advanced Chronic Lymphocytic Leukemia (CLL), where major clinical remissions after adoptive therapy with T cells expressing a CAR directed against the CD19 surface antigen have been obtained [4]. CAR therapy has become therefore the focus of numerous clinical trials and there is a general expectation that this technology, due to its enormous flexibility and to the increasing standardization of cell transduction technologies, may reach in the near future the same diffusion of bone marrow transplantation.

A deeper understanding of the mechanisms that tumors develop to establish a state of tolerance and avoid their rejection by the immune system paved the way to the development of immunological checkpoint inhibitors. The studies with monoclonal antibodies against the CTLA4 receptor were key at pioneering in this approach that led to the development of one of

the most successful cancer drugs of the last years: ipilimumab [5]. After several decades characterized by unsuccessful clinical trials of melanoma, ipilimumab was the first agent to improve overall survival in a randomized, controlled phase III trial of patients with advanced disease. These results led to the regulatory approval of ipilimumab by FDA in 2011 for the treatment of unresectable or metastatic melanoma. Following this event, significant advances have been made to optimize and improve this therapy by characterizing the spectrum of immune-related adverse events and learning how to mitigate them. Furthermore strong efforts have been directed to discover potential biomarkers of activity, and to identify the potential synergy with other therapeutic agents. CTLA4 is now believed to be just the first example of a large family of targets that work often in non-redundant pathways to put the brakes on the immune system in the control of cancer growth. Targeting them with monoclonal antibodies is now believed to offer a tremendous spectrum of opportunities. Indeed recent phase I trials have established the efficacy and safety of next-generation checkpoint agents, including PD-1 and PD-L1 inhibitors, across multiple tumor types [6,7], and that combined treatment with CTLA-4 and PD-1 inhibitors results in unprecedented objective response rates [8].

The third approach is represented by therapeutic cancer vaccines. These are generally directed against so-called Tumor Associated Antigens (TAAs), namely self-proteins overexpressed in the tumor tissue. The approval by FDA in year 2010 of Sipuleucel-T (Provenge®), a personalized vaccine for hormone refractory prostate cancer patients, was viewed as a paradigm shift and a landmark event for the therapy of cancer [9]. As a matter of fact, in spite of this initial success, the field of therapeutic cancer vaccines has been characterized more by setbacks rather than by successes over the past four years. Several cancer vaccines in advanced phase III clinical development have failed to meet their primary endpoint and their clinical development has been discontinued. Furthermore, Provenge® itself has been experiencing significant hurdles in access to market, due to its complex manufacturing and elevated costs that raise significant reimbursement hurdles [10]. Due to these issues nowadays cancer vaccines are still seeking their final identity. This may be achieved only with the identification of a technology capable of combining together easily scalable and low cost manufacturing with the demonstration of potent and durable immunological responses against TAAs. A major issue is therefore the selection of a robust and scalable technology capable to deliver TAAs in a form that efficiently breaks tolerance and induces generation of potent antitumor responses. In this review we provide evidence in

favor of using xenogeneic TAAs as source of vaccines and of delivering them by nucleic acids as a successful and general approach. In the next two paragraphs we will provide a definition of xeno-vaccination and briefly explain the features and advantages of nucleic acids vaccination respectively.

2. WHAT IS XENO-VACCINATION?

Therapeutic vaccination strategies against cancer are based on the concept that cancer cells, as a consequence of the accumulation of epigenetic and genetic changes, display a gene (and protein) expression pattern significantly different from their natural counterparts. The pool of proteins differentially expressed and their intracellular processing results in the display on the surface of cancer cells of a set of novel or abnormally enriched peptides/epitopes in the context of MHC class I molecules. Hence the resulting surface MHC peptidome of cancer cells as a whole can be considered as a cancer cell specific signature. Vaccination aims at educating the immune system to recognize components of this novel signature, e.g. neo-epitopes or over-displayed epitopes, and to generate effector B or T cell responses against them. Proteins differentially expressed by cancer cells and capable of inducing effector immune responses are generically defined as TAAs. They belong to different groups as reviewed in great detail in [11].

A subset of TAAs is constituted by mutated gene products with or without altered function. Mutations can give rise to a new peptidome that may be immunogenic. Best-known examples are mutated oncogenes such as Ras or Braf. Although our knowledge of mutated genes in cancer and consequently of neo-epitopes is subject to exponentially increase with the advent of next generation sequencing, the limitation of targeting neo-epitopes is that in most cases the frequency of each individual mutation and of the resulting neo-epitope(s) is very low in the patient population. Therefore a vaccine based only on neo-antigens should be considered as an 'extreme' form of personalized therapy.

The majority of TAAs belongs to the categories of differentiation, overexpressed, cancer-testis or universal antigens, which are derived from naturally occurring proteins. This poses significant hurdles in the development of effective immunotherapy because of the occurrence of a condition of immune-tolerance against 'self'. In fact T cells that respond strongly to these antigens are likely to be culled during thymic selection to maintain self-tolerance. Overcoming

this tolerance is, therefore, a major challenge. A strategy that is believed to be helpful in this direction is the use of orthologous proteins or peptides from a different species (xenoantigens) as immunogens. Xenoantigens are believed to act as 'altered self' proteins, i.e. proteins bearing aminoacid changes in one or more epitopes and thus capable of breaking tolerance by inducing B and/or T cell responses cross-reactive (see below) against the endogenous non-mutated TAA.

3. NUCLEIC ACIDS VACCINES

Nucleic acids vaccines are emerging as a robust platform technology for therapeutic cancer vaccination. We have recently reviewed how genetic vaccines either individually or in heterologous prime-boost regimens present all the features for a versatile platform technology that combines easy scale up manufacturing of off-the-shelf products with the desired product stability [12,13]. Furthermore, although pivotal phase III trials aimed at demonstrating increase of survival are still underway, nucleic acids vaccines have already shown clinical evidence of strong immunogenicity and preliminary evidence of clinical activity using surrogate assays.

Genetic vaccines may utilize a wide variety of vectors, such as viral (poxviral, adenoviral, lentiviral, AAV), bacterial (Listeria, Salmonella, etc.) or non-viral vectors. In the latter case they may be constituted by plasmid DNA, amplified DNA fragments or also RNA. For naked nucleic acid vaccines, many chemical or physical methods can be used to potentiate the vaccine immunogenicity, among them intradermal or intramuscular electroporation (electro-gene-transfer) is one of the most promising approach (reviewed by [13]).

One or more expression cassettes carrying (in part or entirely) the coding region of the TAAs of choice are inserted in the vector. In some cases the expression cassettes may be represented by minigenes expressing a string of epitopes selected to be highly immunogenic [14]. Upon in vivo delivery through a variety of routes (i.m., i.d., i.v., intranasal, etc.), the genetic material is uptaken by resident cells and its transcription-translation originates the production of the selected antigen(s) or epitope(s).

The advantages of using gene-based vaccines can be briefly summarized as follows: a) ease of manufacturing; b) induction of local inflammation at the injection site with the production of a pro-immunogenic microenvironment which results in homing and activation of dendritic cells

(DCs) and in their subsequent migration to draining lymph nodes for the priming of immune responses; c) elevated expression and/or uptake of the antigen(s) by professional antigen presenting cells that facilitates epitope display on MHC class I and II molecules; d) simultaneous expression of multiple epitopes from the same or from different target antigens which increase the number of potential targets of the immune response thus reducing the risk of immune escape; e) no need to produce and purify recombinant immunogen(s). All these features taken together make genetic vectors a powerful technology. As we will review in the next sections gene-based vectors have been extensively used for cancer xeno-vaccination.

4. EVIDENCE OF EFFICACY OF XENO-VACCINES IN MOUSE MODELS

The first evidence of a better immunogenicity of a xenoantigen *versus* a self-antigen came from vaccination studies comparing in mice the immunogenicity of the syngeneic melanocyte differentiation antigen gp75 expressed by B16 murine melanoma cells with that of the corresponding human protein expressed by human SK-MEL-19 cells [15]. The authors showed that only vaccination with human gp75 but not mouse gp75 was able to induce antibodies against the mouse gp75 antigen, thus demonstrating that tolerance to 'self' can be more easily broken by administering a 'non self' homologue, and that the resulting antibodies were able to reject tumors. Subsequently in a rat ErbB2 (Her2/neu) mouse model [16] it was shown that vaccination with a segment of the intracellular domain of the human protein could generate strong cross-reactive responses to the rat protein.

In the past 15 years several other studies have been published showing the power of xenogeneic vaccination in rodents; the most relevant ones are schematically summarized in **Table 1**.

In the majority of cases xeno-vaccines have been delivered thanks to the use of genetic vectors, mostly plasmid DNA but also viral vectors, an approach that can be called *xeno-gene-vaccination*. They have used a variety of TAAs, including not only antigens strictly expressed by cancer cells but also by cells of the tumor microenvironment. Furthermore as immunogens not only human genes but also genes derived from other species have been utilized with success, thus demonstrating the generality of this phenomenon. Overall xeno-gene vaccines were able to strongly affect tumor growth through the induction of potent immune responses against self-antigens. Interestingly, depending upon the antigen used and the disease model,

the contribution of CD8+ *versus* CD4+ T cells or *versus* antibody responses was variable. While for TAAs with intracellular localization the role of CD8+ T cells is prominent, in the case of TAAs expressed on the cell membrane the contribution of CD4+ T cells and antibodies might be determinant. This becomes particularly evident when cancer-prone mice transgenic for – and thus tolerant to - oncogenes are used as preclinical models [20,49-51].

A peculiar example is represented by vaccination against ErbB2, a tyrosine kinase overexpressed in 20-30% of breast cancers and in a fraction of other epithelial tumors, all characterized by an aggressive behavior. Since ErbB2 was initially isolated from rat neuroblastomas (neu), various strains of neu-transgenic mice that develop autochthonous mammary tumors have been generated; more recently, mice overexpressing the human ErbB2 (Her2) have also been produced, but not all of them develop tumors [52]. Both neu- and Her2-transgenic mice represent interesting preclinical models, as they are fully tolerant not only to mouse ErbB2, but also to neu and Her2, respectively. The availability of these transgenic mice allowed not only the testing of various types of xenogeneic ErbB2 vaccines [18,19,53,54] but also the comparison of the efficacy of autologous (neu in neu-transgenic mice and Her2 in Her2-transgenic mice) *versus* xenogeneic (neu in Her2-transgenic mice and Her2 in neu-transgenic mice) ErbB2 DNA vaccination [20,49-51]. These comparative studies demonstrated that xenogeneic ErbB2 DNA vaccines are generally superior in activating self-reactive T cells but fail to induce self-reactive antibodies, that were instead induced by autologous vaccination, reflecting the exquisite specificity for the cognate antigen of antibodies induced by ErbB2 DNA vaccines [20,49]. As a consequence, xeno-gene-vaccination was protective against a challenge with CD8+ T cell-sensitive transplantable tumors expressing the self-antigen, but not against spontaneous tumors, that are highly sensitive to antibody-mediated destruction and less sensitive to CD8+ T cells [20,51,55]. These observations should be taken into account for the development of clinical vaccines against ErbB2 and other cell membrane TAAs sensitive to antibody-mediated immunity. Nevertheless, these pre-clinical models are somehow peculiar, in the sense that it may not be excluded that the inability of xenogeneic ErbB2 DNA vaccines to induce self-reactive antibodies in neu- and Her2-transgenic mice might be partly due to the tolerance to the mouse orthologue, as also in wild type mice vaccine-induced antibodies showed high specificity to the cognate antigen [49], while this was not the case in other pre-clinical models of xeno-vaccination [18,56,57]. In a study where the response against mouse ErbB2 was evaluated [19], Her2 DNA vaccination was indeed able to induce antibodies against the mouse

orthologue; it was suggested that these auto-antibodies contribute to the protective anti-tumor response observed in neu-transgenic mice by hampering the development of terminal ductal lobular units of the mammary gland, which in turn reduced the incidence of tumors derived from these structures.

5. CROSS-REACTIVITY, PEPTIDE ANALOGS AND HETEROCLITIC EPITOPES

Prior to discuss the mechanism of action of xeno-gene-vaccination we have to briefly introduce the concept of cross-reactivity and of peptide analogs. Adaptive immunity to pathogens and tumors mainly depends on antigen specific T cells expressing αβ T cell receptors (TCRs) capable of recognizing processed peptides in the context of MHC molecules on the surface of antigen presenting cells. It is estimated that the repertoire of αβ TCRs in the naive T cell pool is less than 10^8. This number is several orders of magnitude smaller than that of potential antigenic peptides that can be encountered during life. This issue is resolved by the ability of each TCR to recognize and be activated by a wealth of peptide-MHC complexes. This phenomenon is called cross-reactivity (reviewed in [58]). Cross-reactivity also explains the finding that, differently from antibody-antigen recognition that is characterized by high affinity binding, TCR/peptide MHC binding affinities are typically several orders of magnitude lower [59].

T cell-mediated immune responses require the activation of CD8+ and CD4+ T lymphocytes. CTLs and their TCRs recognize small peptides presented by MHC-I molecules on the cell surface derived from intracellular antigens via the endogenous antigen processing and presentation pathway [60,61]. Peptides for human CD8+ epitopes range from 7 to 14 amino acids, and typically are 9-10 amino acids in length. TCR recognition of the peptide-MHC class I molecule complexes on the cell surface triggers the cytolytic activity of CTL, resulting in the death of cells presenting the peptide-MHC class I complexes. Similarly, MHC class II epitopes are 13-17 amino acids in length and their recognition by CD4+ T cells induce cytokine/chemokine secretion providing help to effector lymphocytes. It is now firmly established that peptides are able to bind to a given MHC molecule provided that the peptide contains the allele-specific anchor motifs [62,63]. On the T cell side, productive engagement of the TCR with the MHC–peptide complex occurs when critical amino acids are present in certain positions of the peptide [64]. However, it was also demonstrated that residues that are not in TCR contact positions could influence recognition and modulate the functional outcome

of the T cell response [65,66]. Whether T cell activation will take place depends on a number of factors: the affinity of the TCR for the MHC–peptide complex, the density of TCR and MHC–peptide complexes, as well as the expression of co-receptors, adhesion molecules and co-stimulatory molecules on the interacting cells.

As previously described, MHC class I restricted epitope vaccines have been shown to confer protection in animal models. Also, specific CD4+ immunity has also demonstrated significant in cancer immunotherapy. For instance the Ii-Key peptide LRMK, a portion of the MHC class II-associated invariant chain (Ii protein), facilitates the direct charging of peptide epitopes onto MHC class II molecules. Directly linking Ii-Key to MHC class II peptide epitopes greatly enhances their potency in activating CD4+ T-cells. The Ii-Key hybrid AE37, generated by linking LRMK to the known Her2 MHC class II epitope $Her2^{776-790}$, has been shown to generate robust, long lasting Her2-specific immune responses both in patients with breast and prostate cancer (reviewed in [67]).

Epitope-based vaccines offer indeed a number of advantageous features. Peptide vaccines can induce immune responses to subdominant epitopes when there is tolerance to a dominant epitope. The use of peptides as immunogens also minimizes safety risks associated with the use of intact proteins. In order to break tolerance and potentiate immune responses against self-antigens, several efforts have been spent over the years to increase the immunogenicity of subdominant, poorly immunogenic epitopes [68].

Two main strategies have been adopted to modify epitope native sequence in order to increase their immunogenicity: 1) anchor-modified analogs; 2) TCR-engaging heteroclitic peptides.

By virtue of their higher affinity for MHC class I, anchor-modified analogs are able to bind to the MHC groove with a longer half-life thus resulting in a more efficient priming of T-cells which are capable of recognizing the *wild-type* epitopes on the surface of target cells [69]. Prediction and selection of HLA-epitopes rely on the use of specific algorithms that rank all potential sequences within a given protein on the basis of their binding properties to the MHC-I pocket. To date, most studies have targeted peptides restricted by HLA A*0201, the most common human MHC I molecule. As a result, vaccine-oriented approaches, including clinical trials, have used peptides with altered MHC I anchor residues that enhance the stability of the MHC I complex [70]. A key assumption in this strategy is that peptide anchor residue modifications do not transmit major structural changes to the outward-facing TCR

recognition platform or otherwise alter T cell recognition. One of the most studied anchor-modified analogues is CAP1-6D. CAP-1 is a Carcinoembyonic Antigen (CEA) epitope identified based on its ability to bind to HLA-A2 and on its ability to stimulate CTLs from cancer patients immunized with vaccinia-CEA [71] and its analogue CAP1-6D (Asn at position 6 replaced by Asp) generates high efficient CTL clones able to recognize both the analogue and the wild type sequence. Similar properties were described for MART1$^{27–35}$ / Melan-A$^{27–35}$ [72], p53$^{264–272}$ [73] and Her2.369 V2V9 [74]. Other anchor-modified analogues for CEA, Her2 and telomerase reverse transcriptase (TERT) TAAs have been recently described [75] and shown to have enhanced binding properties to multiple MHC haplotypes and to be highly immunogenic compared to native peptides.

The second approach utilizes heteroclitic peptides by replacing TCR-interacting amino acid residues [76]. Unfortunately, these modifications would not apply to all allelic forms of MHC class I molecules. For those modifications that have been found to increase immunogenicity of epitopes without increasing affinity, increased interaction with TCR still remains a hypothesis. As a matter of fact, heteroclitic peptides with improved TCR binding ability are highly difficult to predict. In addition, there is a danger that substitution of TCR contact residues may induce the expansion of T cells with unwanted or irrelevant specificities.

6. WHAT WE HAVE LEARNED ABOUT THE MECHANISM OF ACTION OF XENO-VACCINES

Initial investigations into the mechanism of action of xenogeneic vaccines suggested dependence from heteroclitic CD8+ T cell epitopes between the xenoantigen and the self-antigen. These heteroclitic epitopes overcome tolerance by inducing CD8+ T cell populations that are cross-reactive to both the xenoantigen and to the native antigen as shown in two seminal papers [26,56]. Both these studies dealt, albeit with slightly different approaches, with the same melanoma differentiation antigen gp100. The first study [26] started from the observation that while a recombinant vaccinia virus encoding the mouse (m)gp100 was not immunogenic in C57BL/6 mice, immunization with a similar vector encoding the human (h)gp100 homologue elicited a specific CD8+ T cell response. These CD8+ lymphocytes were cross-reactive against mouse gp100 in vitro and protected mice from established B16 melanoma upon adoptive transfer. These effector T cells were further characterized and it was shown that they recognized a 9-amino acid gp100 epitope (gp100$^{25-33}$), restricted by

MHC Class I H-2D^b, which is different in three positions (amino acids 25 to 27) between the two species. Differences in these three NH_2-terminal amino acids resulted in a 2-log increase in the ability of the (h)gp100$^{25-33}$ to stabilize "empty" H-2D^b molecules on cells and a 3-log increase in its ability to trigger interferon (IFN)-γ release by T cells. No other differences in the two proteins were responsible for the 'xeno-vaccination effect'. The second study [56] employed the plasmid DNA immunization approach. C57BL/6 mice immunized with xenogeneic full-length hgp100 DNA were protected against syngeneic mouse melanoma challenge. In contrast, mice immunized with DNA constructs of (h)gp100 in which the amino acids at positions 25-27 were from the murine sequence or were mutated, thus generating a gp100$^{25-33}$ epitope with weak or no ability to bind H-2D^b, did not reject B16 melanoma. Moreover, mice vaccinated with a minigene construct of (h)gp100$^{25-33}$ rejected B16 melanoma, whereas mice immunized with the (m)gp100$^{25-33}$ minigene did not develop protective tumor immunity. Therefore, in this case of xeno-gene-vaccination, a single (h)gp100 heteroclitic epitope with a higher affinity for MHC class I at minor anchor residues was necessary and sufficient to induce protective tumor immunity. The fortuitous existence of this single peptide homologue resulted in the generation of T cells cross-reactive against 'self'.

Recent findings however suggested that this is not the only possible mechanism of the 'xenogeneic effect' and that this may also be due to the involvement of a heteroclitic MHC Class II epitope. This was shown from studies on yet another melanoma differentiation antigen called Trp2 (tyrosinase-related protein 2). Vaccination of mice with human Trp2 resulted in greater antitumor immunity than vaccination with the murine homologue, providing an additional model for the study of xenoimmunization [21]. Interestingly the protective effect was entirely CD8+ T cell dependent and was due to the development of a cytotoxic response against a peptide (SVYDFFVWL) that is 100% conserved between mice and humans. Hence for some time it has not been clear how breakage of tolerance could be achieved through xenovaccination against a non-heteroclitic MHC class I epitope. This conundrum was resolved more recently in an elegant study by Kianizad et al [77]. These authors showed that the reduced immunogenicity of mouse Trp2 was a consequence of insufficient CD4+ T cell help. They mapped a dominant heteroclitic MHC Class II epitope in human Trp2, which was able to induce cross-reactive CD4+ T cell responses and was able to recapitulate breakage of tolerance against the dominant MHC class I restricted epitope when inserted into the mouse Trp2 gene delivered by adenoviral vector vaccination. These results therefore revealed a novel mechanism by which xeno-antigens can overcome tolerance and

suggest that high affinity CD8+ self-reactive T cells may still be maintained in adult organisms but peripheral tolerance maintains them under check at least in part because of the lack of self-reactive CD4+ T cells. This evidence is also important because the strong involvement of cross-reactive CD4+ helper T cells may be exploited for the induction of high titers of polyclonal antibodies directed against the self-antigen as in the RHuT and HuRT case discussed below.

7. OPTIMIZING XENO-GENE-VACCINES: THE RHuT/HuRT STORY

As discussed previously, in ErbB2-transgenic mice xenogeneic ErbB2 vaccines are generally superior to autologous vaccine in the induction of cell-mediated immunity against the self-antigen (the transgene product), while only autologous ErbB2 vaccines are able to induce antibodies against it. As most ErbB2$^+$ tumors are sensitive to antibody-mediated immunity, at least until they are ErbB2 addicted [78], the ideal anti-ErbB2 DNA vaccine should combine the ability of the autologous vaccine to induce antibodies and that of the xenogeneic vaccine to efficiently induce cell-mediated immunity. To this purpose, hybrid plasmids coding for chimeric rat/human and human/rat extracellular and transmembrane domains of ErbB2 (RHuT and HuRT plasmid, respectively) were generated [51,79]. RHuT encodes a protein in which the 410-NH_2-terminal amino acids are from neu and the remaining residues from Her2, while HuRT encodes a protein in which the 390-NH_2-terminal residues are from Her2 and the remaining from neu. These hybrid plasmids were tested in various strains of wild type, neu- and Her2-transgenic mice [49-51,80]. In summary, the results obtained show that both in wild type and neu-transgenic mice RHuT is superior to the autologous, the fully xenogeneic and the HuRT vaccines in inducing anti-neu antibodies and a protective anti-tumor response against neu+ transplantable and autochthonous tumors. HuRT instead is the most effective in inducing an immune response against Her2 and in hampering the growth of Her2+ transplantable tumors in both wild type and Her2-transgenic mice. In a strain of cancer-prone Her2-transgenic mice [81], RHuT and HuRT were equally effective in hampering the growth of Her2+ autochthonous tumors [51]. In the same strain the protective anti-tumor response induced by HuRT was compared with that of a whole cell vaccine (mitomycin-C-treated Her2-overexpressing human SKOV3 cells) associated with exogenous administration of recombinant murine interleukin-12 (IL-12) [80]. HuRT proved to be superior in inducing anti-Her2 antibodies and in protecting from autochthonous tumor development.

The superior immunogenicity of hybrid plasmids poses the basis on the production of chimeric proteins that are composed of discrete homologous and heterologous portions. The homologous moiety guarantees the specificity of the response in terms of antibody production, while the heterologous moiety provides heteroclitic peptides that ensure overcoming of T cell tolerance. Due to central tolerance, T and B cells recognizing with high-affinity the self-antigen cannot be activated. When the hybrid plasmid is introduced into muscle cells through vaccination, the chimeric protein is produced and becomes available in the microenvironment. DC presentation of peptides derived from the heterologous moiety (heteroclitic peptides) may activate T cells in the draining lymph node. B cells can bind and internalize the chimeric protein through their BCR. This may happen also for self-reactive B cells that recognize the homologous moiety of the chimeric protein through their low-affinity BCR. These B cells can present peptides derived from the heterologous moiety to newly activated CD4+ T follicular cells in the lymph node. In this way they can receive T cell help and undergo the germinal center reaction and maturate toward plasma cells producing high-affinity antibodies against the self-antigen. As a result, a polyclonal antibody response is induced against both the self and the xenogeneic orthologous (**Figure 1**). Moreover, the slight differences in the amino acid sequence and in the structural conformation of the chimeric proteins as compared to neu and Her2 may result in the exposition of subdominant and/or new B cell epitopes that trigger the immune response [51].

The location of the autologous and heterologous moieties on the chimeric protein critically influences the induced immune response. Both in wild type and ErbB2-transgenic mice, the stronger protective response was obtained when the autologous moiety was in the more immunogenic NH_2-terminal part of the ErbB2 molecule [49-51]. Moreover, it has been suggested that amino acids 501 to 687 of the Her2 sequence may activate T regulatory (Treg) cells in H-2^d Her2-transgenic mice. Accordingly, the HuRT plasmid is more effective than Her2 or RHuT in inducing an anti-Her2 immune response in Her2-transgenic mice because it encodes a chimeric protein in which this Treg epitope is replaced by the corresponding neu sequence [49].

On the basis of these data, a clinical trial for testing hybrid ErbB2 plasmids in patients with Her2+ tumors is awaiting the final approval by the Italian authorities. To further support the feasibility of using RHuT and HuRT in human patients new data have been generated by transfecting DCs from patients bearing Her2+ tumors with Her2, neu or the hybrid plasmids and comparing their ability to stimulate autologous T cells (Cavallo et al., manuscript in

preparation). While T cells from healthy donors could be easily stimulated with autologous DCs transfected with any human, rat or hybrid ErbB2 plasmid, only DCs transfected with the hybrid plasmids induced antigen-specific perforin- and IFN-γ-production by T cells from patients with Her2-overexpressing tumors. These Her2-specific T cells were able to hamper tumor growth when adoptively transferred in immunodeficient mice bearing Her2+ SKOV3 tumors. Hybrid plasmids efficacy relied on their ability to circumvent the suppressive effects of tumor-induced Treg cells present in the peripheral blood of cancer patients.

In principle, this hybrid xenogeneic vaccination can be applied for targeting any TAA, provided that a highly homologous ortholog is used for plasmid design.

8. XENO-GENE-VACCINATION IN VETERINARY TRIALS

Client-owned pets, mainly dogs and cats, are emerging as predictive preclinical models in Oncology and, more recently, in Onco-Immunology. Pets and human tumors share many characteristics, including histological phenotype, development of recurrences and metastasis, and often highly comparable gene expression programs [82] and may thus accurately predict tumor behavior and response to immunotherapy in humans. Another advantage of preclinical studies performed in pets is the power to perform trials with a high number of subjects from different breeds, providing a background genetic diversity similar to that seen in human populations.

An important breakthrough in the field of tumor vaccination and in the treatment of canine melanoma was achieved with a DNA vaccine encoding the human tyrosinase (TYR). Merial has recently developed this vaccine in a partnership with the Memorial Sloan-Kettering Center and the Animal Medical Center in New York and has commercialized it under the name Oncept™. It is currently the only veterinary therapeutic tumor vaccine licensed by the USDA, for the treatment of oral and digital melanoma. The licensing followed after a successful clinical trial that resulted in prolonged survival compared to historical control dogs [83,84]. Vaccination with a plasmid encoding murine TYR generated similar results [85]. The plasmid encoding the xenogeneic TYR is administered by a transdermal device and the protocol consists in four biweekly injections followed, in more recent trials [86], by boosts every six months. An antibody response against human TYR was present in three out of nine tested patients, with two of them also positive for antibodies against canine TYR [87]. A correlation

between the antibody response and the clinical response was observed. Of note, TYR is an intracellular protein and hence one would not expect that it could be recognized and targeted by antibodies. One hypothesis is that TYR is expressed at a low-level on the cell-surface of melanoma cells. Recently, it has been discovered that a peptide fragment of TYR is highly presented by MHC class I molecules at the surface of melanoma cells [88].

In 2006 Alexander and coworkers [89] reported tumor control and extended survival time in 35% of melanoma bearing dogs treated with irradiated allogeneic canine melanoma cells transfected with human gp100. However, it is unclear which was the relative contribution of allogeneic antigens expressed by the cells used as vaccine and of the xenoantigen. The delayed type hypersensitivity response against the allogeneic cells correlated with the clinical outcome.

A new xenogeneic DNA vaccine has been recently proposed for the treatment of dogs with oral malignant melanoma expressing the TAA chondroitin sulphate proteoglycan 4 (CSPG4) (Cavallo et al., manuscript in preparation). CSPG4 is an early cell surface progression marker involved in tumour cell proliferation, migration and invasion [90,91] whose expression is similar in human and canine melanoma lesions being about 80% [91] and 60% [92], respectively. The vaccine is a DNA plasmid coding for the human CSPG4 sequence, administered monthly through electroporation. When tested in dogs with surgically resected stage II-III CSPG4-positive oral melanoma, it extended the overall and disease-free survival times of vaccinated as compared to control dogs. All vaccinated dogs developed antibodies against both human and canine CSPG4, showing that xenogeneic vaccination was able to able to overcome host unresponsiveness to the self-antigen (Cavallo et al., manuscript in preparation).

Another xenogeneic approach in dogs is the active vaccination against pro-angiogenic growth factors or their receptors. The safety, anti-tumor and anti-angiogenic effects of vaccination with a xenogeneic (human) vascular endothelial cell growth factor (VEGF) recombinant protein was evaluated in pet dogs with soft tissue sarcoma [93]. The xenogeneic VEGF vaccine was well-tolerated by all dogs and resulted in induction of humoral responses against both human and canine VEGF in animals that remained in the study long enough to receive multiple immunizations. The overall tumor response rate was 30% for all treated dogs in the study.

A further example worth mentioning is a xenogeneic chicken HSP70 (chHSP70) DNA vaccine in an experimental canine transmissible venereal tumor (CTVT) model [94]. This vaccine was very efficient both in a preventive and therapeutic setting when delivered by electroporation.

The CD4+ population of tumor-infiltrating lymphocytes and canine HSP70 (caHSP70)-specific IFN-γ-secreting lymphocytes were significantly increased during tumor regression in vaccinated dogs as compared to control dogs, demonstrating that specific tolerance to caHSP70 has been overcome.
These studies demonstrate the feasibility of xenogeneic vaccination approach in an outbred, highly relevant nonclinical model such as canine cancer patients.

9. HUMAN CLINICAL TRIALS WITH XENO-GENE-VACCINES

A first Phase I trial using a xenoantigen was conducted by Dendreon Corporation in prostate adenocarcinoma patients with rising serum PSA levels and was carried out using DCs pulsed with mouse PAP (Prostatic Acid Phosphatase) recombinant protein [95]. In preclinical studies, only immunization with a xenogeneic PAP (human PAP into rat) was able to generate cytotoxic T lymphocytes and prostate-specific autoimmunity [95]. Twenty-one patients with metastatic prostate cancer (either hormone-sensitive or hormone-refractory) were vaccinated with two monthly doses of pulsed DCs. Vaccine was well tolerated and gave rise to T cell responses against murine PAP in all vaccinated patients. Most importantly, 11 out of 21 patients developed cross-reactive T cell responses against the human PAP, which were associated with the production of a Th1 related pattern of cytokines. Six of the patients that developed autoimmune responses to human PAP underwent disease stabilization. This result was the first to show that xeno-vaccination is able to break tolerance to a self-antigen in humans with a corresponding clinical effect.

Several other xenovaccination clinical trials have been coordinated by the group of Jedd Wolchok at the Memorial Sloan Kettering Clinical Center in New York and focused on either of the two melanoma associated antigens TYR and gp100 delivered as xeno-gene vaccines. In 2007 Wolchok et al. [96] reported a phase I trial with mouse and human TYR DNA in stage III/IV melanoma patients. Eighteen HLA A*0201 patients were randomized in two groups which received six biweekly vaccinations with naked plasmid DNA encoding either mouse or human TYR. In one group patients received the first three injections with mouse TYR DNA followed by three with human TYR DNA. In the other group the schedule was reversed. DNA dose levels were of 100, 500 and 1500 μg. Seven patients (40%) developed CD8+ T cell responses measured either by tetramer or intracellular cytokine staining. However no relationship was found between dose, assigned schedule and T-cell response. In a subsequent study only mouse TYR DNA was injected, this time in combination with DNA electroporation

[97]. Twenty-four patients with different HLA haplotypes were vaccinated with five injections every three weeks with dose levels of 200, 500 or 1500 μg DNA. Only patients vaccinated with the highest dose (6 out of 15, i.e. 40%) developed TYR-reactive T cell responses, showing a trend for a dose-response effect. It is important to notice that this relative high proportion of immune responders was obtained using only a single immunogen, i.e. mouse TYR DNA and not the alternation of two immunogens as in the previous study. Furthermore tetramer responses could be analyzed only in the subset of HLA A*0201 patients, thus potentially underestimating responses to other un-assessed epitopes presented by other HLA types. Interestingly, in one patient epitope spreading occurred with the development of immune responses against the TAA NY-ESO-1 and a clinical effect such as vitiligo. These results strongly suggest that xenogeneic vaccination by DNA electroporation is a promising approach that deserves further assessment in the clinic, possibly in combination with other immunotherapeutic agents [97].

In 2009 Yuan et al. [98] reported data of a Phase I trial of melanoma patients treated with plasmid DNA encoding either mouse or human gp100 and injected i.m. The dose and schedule was identical to the one previously described [96], namely three injections with a first plasmid followed by three with the second plasmid and *vice versa*. 18 patients were assessable for immune responses. Six patients (33%) developed T cell responses measured either as HLA A*0201 - restricted gp100 peptides tetramer positivity or as intracellular cytokine staining. Deep immunological characterization led to conclude that gp100 antigen-specific T cell responses were of the effector memory type. Following this study a second phase I trial was conducted using only mouse gp100 DNA delivered either i.m or by gene gun [99]. The use of gene gun provided the advantage of strongly reducing the dose of plasmid DNA, from the 1000 μg of the naked i.m. injection to only 2 μg of gene gun using gold particles. Eight out of 27 vaccinated patients (30% in total) experienced immune responses against the self-antigen as detected either by tetramer or by intracellular cytokine staining. Of these patients 2 belonged to the i.m. vaccination arm, whereas 6 belonged to the gene gun cohort. Although the numbers are too small to draw conclusions there was a clear trend towards superiority of vaccination using gene gun compared to naked DNA injection.

Finally, very recently another phase I clinical trial has been reported using a DNA vaccine coding for rhesus PSA electroporated i.d. in patients with relapsed prostate cancer [100]. Fifteen patients were vaccinated with five vaccinations every four weeks at DNA doses from 50 to 1600 μg. Again, immune responses monitored as IFNγ ELISPOT were detected in a

significant proportion of patients. Again these clinical findings are extremely promising and warrant further investigation in more advanced clinical trials.

10. CONCLUSIONS

Pre-clinical experiments in mice, veterinary trials and the early Phase I/II human clinical trials have demonstrated that xeno-gene-vaccination is a feasible and promising immunotherapeutic approach that deserves further investigation. Xenoantigens are capable to efficiently trigger a T cell response (involving CD4+ or CD8+ T lymphocytes or both) cross-reactive against 'self' because, embedded into their amino acid sequence, there is a 'natural' source of epitopes with heteroclitic and/or fixed anchor properties.

The presence of these modified peptides is to some extent a serendipitous finding. In other words, a major issue with this approach is that, given a certain species, it is not easy to predict which is going to be the best (most immunogenic) xenoantigen in that species. Are we left, therefore, with the notion that it is impossible to conceive a rational approach to xenovaccination? We believe that the data collected so far allow deducing some rules that can nevertheless be of help in designing an effective approach to xeno-vaccination. First of all, the xenoantigen should share a degree of homology with the self-homologous ranging from 85 to 95%; a lesser homology will probably result in amplification of a T cell response that hardly cross-react with the self antigen, while a greater degree of homology will decrease the probability to have enough heteroclitic epitopes. Another consideration is that the use of discrete portion of the xenoantigen will increase the probability to have heteroclitic epitopes.

The use of DNA vaccines could be particularly advantageous when the target antigen is expressed on the cell membrane, and thus easily reachable by antibodies, since the xenoprotein will be glycosylated by the cells of the host. Finally, the data obtained using hybrid plasmid coding for chimeric proteins, partly xenogeneic and partly homologous, suggest that the concomitant expression of xenogeneic and homologous epitopes maximally exploit the xenogeneic effect, leading to activation of both T and B cells against the self antigen.

EXPERT OPINION

- **Cancer Immunotherapy is becoming a well established therapeutic modality to fight cancer. This is mainly due to the recent clinical success of immunological checkpoint inhibitors and also in part of adoptive transfer of T lymphocytes.**
- **The third option for the immunotherapy, represented by therapeutic cancer vaccination still needs to find its identity.**
- **Xeno-gene-vaccination represents an opportunity to achieve this goal by combining easy scale up manufacturing with the induction of powerful immune responses against 'self' antigens.**
- **In this context, the construction of hybrid plasmids coding for chimeric proteins, partly xenogeneic and partly homologous holds greater potential for the induction of the desired mix of B and T cell response against the 'self' antigen.**
- **Xeno-gene-vaccination is safe, well tolerated and capable to induce immune responses to 'self' tumor antigens in early phase human clinical trials in a high proportion of patients. In dogs with spontaneous tumors several xeno-gene-vaccines have been shown to increase survival and one of them (Oncept®), has been licensed by FDA for the treatment of dog melanoma. Xeno-gene-vaccination holds therefore great promise to become a new weapon for the therapy of human cancer.**
- **In order to accomplish this goal it is mandatory to achieve key proof-of-concept data in advanced Phase II and Phase III clinical trials, which is expected to occur in the next years.**

FIGURE LEGEND

Figure 1. Immunogenicity of hybrid plasmids coding for chimeric protein encompassing homologous and heterologous moieties. **A.** One or two days following DNA electrovaccination, transfected muscle cells (M) overexpress the chimeric protein encoded by the plasmid and the area become markedly infiltrated by macrophages and dendritic cells (DC) that can uptake the chimeric protein shed from the membrane of transfected cells or associated with muscle cell debris. Also B cells can recognize and internalize the chimeric protein. **B.** DCs migrate to the draining lymph node where they can activate T cells specific for peptides of the heterologous moiety and potentially cross-reactive with the homologous counterpart. **C.** The expanded T cells interact and provide helper signals to B cells by recognizing heterologous peptides presented by MHC II molecules on the cell membrane of B cells. The interaction between T and B cells recognizing the heterologous moiety of the chimeric protein leads to germinal center reaction and the production by plasmacells (PC) of antibodies (blue Y) to the heterologous moiety. By contrast, the interaction of expanded T cells with B cells specific for the homologous moiety help to break immune tolerance and lead to the production of antibodies (orange Y) against the self-protein. The various tones of blue and orange represent the polyclonal antibody response generated by the germinal center reaction.

ABBREVIATIONS

TILs	Tumor Infiltrating Lymphocytes
IL-2	Interleukin-2
CARs	Chimeric Antigen Receptors
CLL	Chronic Lymphocytic Leukemia
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
PD-1	Programmed Cell Death Protein -1
PD-L1	Programmed Cell Death Protein - Ligand 1
TAAs	Tumor Associated Antigens
FDA	Food and Drug Administration
MHC	Major Histocompatibility Complex
i.m.	intramuscular
i.d.	intradermal
i.v.	intravenous
DCs	dendritic cells
gp75	glycoprotein 75
gp100	glycoprotein 100
Trp1	tyrosinase related protein 1
Trp2	tyrosinase related protein 2
Erbb2	avian erythroblastosis oncogene 2
Her2	human epidermal growth factor receptor 2
neu	neuroblastoma oncogene
MMP2	matrix metalloprotease 2
VEGF-R2	vascular endothelial growth factor receptor 2
FGF-R1	fibroblast growth factor receptor 1
EGFR	epithelial growth factor receptor
PSMA	prostate specific membrane antigen
TIE-2	tyrosine kinase with immunoglobulin-like and EGF-like domains 2
AFP	alpha fetoprotein
TCR	T cell receptor
CTL	cytotoxic T lymphocytes
HLA	histocompatibility leukocyte antigen
CEA	carcinoembryonic antigen
MART-1	melanoma antigen recognized by T cells – 1
TERT	telomerase reverse transcriptase
RHuT	hybrid plasmid coding for chimeric NH_2-rat/human-COOH ErbB2 protein
HuRT	hybrid plasmid coding for chimeric NH_2-human/rat-COOH ErbB2 protein
IL-12	interleukin-12
BCR	B cell receptor
CSPG4	chondroitin sulphate proteoglycan 4
VEGF	vascular endothelial growth factor
HSP70	heat shock protein 70
PAP	prostatic acid phosphatase
TYR	tyrosinase

REFERENCES

1. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* 342(6165), 1432-1433 (2013).

* **A recent Scienze Magazine Editorial outlining the major clinical breakthroughs of cancer immunotherapy of the last years and the major impact this wil have for cancer patients in the future.**

2. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunological reviews* 257(1), 56-71 (2014).
3. Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. *Cancer discovery* 3(4), 388-398 (2013).
4. Kalos M, Levine BL, Porter DL *et al.* T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science translational medicine* 3(95), 95ra73 (2011).

** **This paper is the first demonstration of clinical efficacy of adoptive therapy of T cells transduced with CAR receptors, reporting high percentage of major objective responses in children affected by chronic lymphocytic leukemia.**

5. Wolchok JD, Hodi FS, Weber JS *et al.* Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. *Annals of the New York Academy of Sciences* 1291, 1-13 (2013).
6. Simeone E, Ascierto PA. Immunomodulating antibodies in the treatment of metastatic melanoma: the experience with anti-CTLA-4, anti-CD137, and anti-PD1. *Journal of immunotoxicology* 9(3), 241-247 (2012).
7. Page DB, Postow MA, Callahan MK, Allison JP, Wolchok JD. Immune modulation in cancer with antibodies. *Annual review of medicine* 65, 185-202 (2014).
8. Wolchok JD, Kluger H, Callahan MK *et al.* Nivolumab plus ipilimumab in advanced melanoma. *The New England journal of medicine* 369(2), 122-133 (2013).
9. Kantoff PW, Higano CS, Shore ND *et al.* Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *The New England journal of medicine* 363(5), 411-422 (2010).
10. Jonsson B, Wilking N. Cancer vaccines and immunotherapeutics: challenges for pricing, reimbursement and market access. *Human vaccines & immunotherapeutics* 8(9), 1360-1363 (2012).
11. Coulie PG, Van Den Eynde BJ, Van Der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nature reviews. Cancer* 14(2), 135-146 (2014).

* **A very recent review which provides an exhaustive historical perspective of the identification of tumor associated antigens (TAAs)**

12. Aurisicchio L, Ciliberto G. Genetic cancer vaccines: current status and perspectives. *Expert opinion on biological therapy* 12(8), 1043-1058 (2012).

* **A comprehensive review describing the principal approaches, both viral and non viral, to gene-based therapeutic cancer vaccines.**

13. Aurisicchio L, Mancini R, Ciliberto G. Cancer vaccination by electro-gene-transfer. *Expert review of vaccines* 12(10), 1127-1137 (2013).
14. Aurisicchio L, Fridman A, Bagchi A, Scarselli E, La Monica N, Ciliberto G. A novel minigene scaffold for therapeutic cancer vaccines. . *Oncoimmunology* 3:e27529(2014).
15. Naftzger C, Takechi Y, Kohda H, Hara I, Vijayasaradhi S, Houghton AN. Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. *Proceedings of the National Academy of Sciences of the United States of America* 93(25), 14809-14814 (1996).

*** **This is the first example of xenovaccination in a murine model and the first demonstration that the human tumor antigen gp75 expressed by human melanoma cells is more immunogenic in mice than the 'self' antigen expressed by murine melanoma.**

16. Disis ML, Shiota FM, Cheever MA. Human HER-2/neu protein immunization circumvents tolerance to rat neu: a vaccine strategy for 'self' tumour antigens. *Immunology* 93(2), 192-199 (1998).
17. Weber LW, Bowne WB, Wolchok JD *et al.* Tumor immunity and autoimmunity induced by immunization with homologous DNA. *The Journal of clinical investigation* 102(6), 1258-1264 (1998).
18. Gallo P, Dharmapuri S, Nuzzo M *et al.* Xenogeneic immunization in mice using HER2 DNA delivered by an adenoviral vector. *International journal of cancer. Journal international du cancer* 113(1), 67-77 (2005).
19. Pupa SM, Iezzi M, Di Carlo E *et al.* Inhibition of mammary carcinoma development in HER-2/neu transgenic mice through induction of autoimmunity by xenogeneic DNA vaccination. *Cancer research* 65(3), 1071-1078 (2005).
20. Jacob J, Radkevich O, Forni G *et al.* Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. *Cellular immunology* 240(2), 96-106 (2006).
21. Bowne WB, Srinivasan R, Wolchok JD *et al.* Coupling and uncoupling of tumor immunity and autoimmunity. *The Journal of experimental medicine* 190(11), 1717-1722 (1999).
22. Steitz J, Bruck J, Steinbrink K, Enk A, Knop J, Tuting T. Genetic immunization of mice with human tyrosinase-related protein 2: implications for the immunotherapy of melanoma. *International journal of cancer. Journal international du cancer* 86(1), 89-94 (2000).
23. Tormo D, Ferrer A, Bosch P *et al.* Therapeutic efficacy of antigen-specific vaccination and toll-like receptor stimulation against established transplanted and autochthonous melanoma in mice. *Cancer research* 66(10), 5427-5435 (2006).
24. Hawkins WG, Gold JS, Dyall R *et al.* Immunization with DNA coding for gp100 results in CD4 T-cell independent antitumor immunity. *Surgery* 128(2), 273-280 (2000).
25. Yamanaka R, Xanthopoulos KG. Induction of antigen-specific immune responses against malignant brain tumors by intramuscular injection of sindbis DNA encoding gp100 and IL-18. *DNA and cell biology* 24(5), 317-324 (2005).
26. Overwijk WW, Tsung A, Irvine KR *et al.* gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *The Journal of experimental medicine* 188(2), 277-286 (1998).

*** **A seminal paper together with (56) demonstrating that xeno-gene-vaccination using a plasmid coding for the human melanoma antigen gp100 is initiated by an heteroclitic high affinity CD8+ T cell epitope which is able to trigger a cross-reactive cytotoxic response against the 'self' epitope.**

27. Su JM, Wei YQ, Tian L *et al.* Active immunogene therapy of cancer with vaccine on the basis of chicken homologous matrix metalloproteinase-2. *Cancer research* 63(3), 600-607 (2003).
28. Lou YY, Wei YQ, Yang L *et al.* Immunogene therapy of tumors with a vaccine based on the ligand-binding domain of chicken homologous integrin beta3. *Immunological investigations* 31(1), 51-69 (2002).
29. Liu JY, Wei YQ, Yang L *et al.* Immunotherapy of tumors with vaccine based on quail homologous vascular endothelial growth factor receptor-2. *Blood* 102(5), 1815-1823 (2003).

30. Wei Y, Sun Y, Song C *et al.* Enhancement of DNA vaccine efficacy by targeting the xenogeneic human chorionic gonadotropin, survivin and vascular endothelial growth factor receptor 2 combined tumor antigen to the major histocompatibility complex class II pathway. *The journal of gene medicine* 14(5), 353-362 (2012).
31. He QM, Wei YQ, Tian L *et al.* Inhibition of tumor growth with a vaccine based on xenogeneic homologous fibroblast growth factor receptor-1 in mice. *The Journal of biological chemistry* 278(24), 21831-21836 (2003).
32. Sioud M, Sorensen D. Generation of an effective anti-tumor immunity after immunization with xenogeneic antigens. *European journal of immunology* 33(1), 38-45 (2003).
33. Lu Y, Wei YQ, Tian L *et al.* Immunogene therapy of tumors with vaccine based on xenogeneic epidermal growth factor receptor. *J Immunol* 170(6), 3162-3170 (2003).
34. Lai MD, Yen MC, Lin CM *et al.* The effects of DNA formulation and administration route on cancer therapeutic efficacy with xenogenic EGFR DNA vaccine in a lung cancer animal model. *Genetic vaccines and therapy* 7, 2 (2009).
35. Tan GH, Wei YQ, Tian L *et al.* Active immunotherapy of tumors with a recombinant xenogeneic endoglin as a model antigen. *European journal of immunology* 34(7), 2012-2021 (2004).
36. Jiao JG, Li YN, Wang H *et al.* A plasmid DNA vaccine encoding the extracellular domain of porcine endoglin induces anti-tumour immune response against self-endoglin-related angiogenesis in two liver cancer models. *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver* 38(8), 578-587 (2006).
37. Ciesielski MJ, Apfel L, Barone TA, Castro CA, Weiss TC, Fenstermaker RA. Antitumor effects of a xenogeneic survivin bone marrow derived dendritic cell vaccine against murine GL261 gliomas. *Cancer immunology, immunotherapy : CII* 55(12), 1491-1503 (2006).
38. Goldberg SM, Bartido SM, Gardner JP *et al.* Comparison of two cancer vaccines targeting tyrosinase: plasmid DNA and recombinant alphavirus replicon particles. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11(22), 8114-8121 (2005).
39. Palomba ML, Roberts WK, Dao T *et al.* CD8+ T-cell-dependent immunity following xenogeneic DNA immunization against CD20 in a tumor challenge model of B-cell lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11(1), 370-379 (2005).
40. Gregor PD, Wolchok JD, Turaga V *et al.* Induction of autoantibodies to syngeneic prostate-specific membrane antigen by xenogeneic vaccination. *International journal of cancer. Journal international du cancer* 116(3), 415-421 (2005).
41. Mincheff M, Zoubak S, Makogonenko Y. Immune responses against PSMA after gene-based vaccination for immunotherapy-A: results from immunizations in animals. *Cancer gene therapy* 13(4), 436-444 (2006).
42. Charalambous A, Oks M, Nchinda G, Yamazaki S, Steinman RM. Dendritic cell targeting of survivin protein in a xenogeneic form elicits strong CD4+ T cell immunity to mouse survivin. *J Immunol* 177(12), 8410-8421 (2006).
43. Luo Y, Wen YJ, Ding ZY *et al.* Immunotherapy of tumors with protein vaccine based on chicken homologous Tie-2. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12(6), 1813-1819 (2006).
44. Harrop R, Ryan MG, Myers KA, Redchenko I, Kingsman SM, Carroll MW. Active treatment of murine tumors with a highly attenuated vaccinia virus expressing the

tumor associated antigen 5T4 (TroVax) is CD4+ T cell dependent and antibody mediated. *Cancer immunology, immunotherapy : CII* 55(9), 1081-1090 (2006).

45. Zhang W, Liu J, Wu Y *et al.* Immunotherapy of hepatocellular carcinoma with a vaccine based on xenogeneic homologous alpha fetoprotein in mice. *Biochemical and biophysical research communications* 376(1), 10-14 (2008).
46. Huebener N, Fest S, Hilt K *et al.* Xenogeneic immunization with human tyrosine hydroxylase DNA vaccines suppresses growth of established neuroblastoma. *Molecular cancer therapeutics* 8(8), 2392-2401 (2009).
47. Xie J, Xiong L, Tao X *et al.* Antitumor effects of murine bone marrow-derived dendritic cells infected with xenogeneic livin alpha recombinant adenoviral vectors against Lewis lung carcinoma. *Lung Cancer* 68(3), 338-345 (2010).
48. Soong RS, Trieu J, Lee SY *et al.* Xenogeneic human p53 DNA vaccination by electroporation breaks immune tolerance to control murine tumors expressing mouse p53. *PloS one* 8(2), e56912 (2013).
49. Jacob JB, Quaglino E, Radkevich-Brown O *et al.* Combining human and rat sequences in her-2 DNA vaccines blunts immune tolerance and drives antitumor immunity. *Cancer research* 70(1), 119-128 (2010).

*** **A seminal paper, together with (51), showing that the use of hybrid plasmids coding for chimeric proteins strongly increase the efficacy of anti-tumor DNA vaccines**

50. Quaglino E, Riccardo F, Macagno M *et al.* Chimeric DNA Vaccines against ErbB2+ Carcinomas: From Mice to Humans. *Cancers* 3(3), 3225-3241 (2011).
51. Quaglino E, Mastini C, Amici A *et al.* A better immune reaction to Erbb-2 tumors is elicited in mice by DNA vaccines encoding rat/human chimeric proteins. *Cancer research* 70(7), 2604-2612 (2010).

*** **A seminal paper, together with (49), showing that the use of hybrid sequences strongly increase the efficacy of anti-tumor DNA vaccines**

52. Quaglino E, Mastini C, Forni G, Cavallo F. ErbB2 transgenic mice: a tool for investigation of the immune prevention and treatment of mammary carcinomas. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* Chapter 20, Unit 20 29 21-20 29-10 (2008).
53. Tegerstedt K, Lindencrona JA, Curcio C *et al.* A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer research* 65(13), 5953-5957 (2005).
54. Triulzi C, Vertuani S, Curcio C *et al.* Antibody-dependent natural killer cell-mediated cytotoxicity engendered by a kinase-inactive human HER2 adenovirus-based vaccination mediates resistance to breast tumors. *Cancer research* 70(19), 7431-7441 (2010).
55. Rolla S, Ria F, Occhipinti S *et al.* Erbb2 DNA vaccine combined with regulatory T cell deletion enhances antibody response and reveals latent low-avidity T cells: potential and limits of its therapeutic efficacy. *J Immunol* 184(11), 6124-6132 (2010).
56. Gold JS, Ferrone CR, Guevara-Patino JA *et al.* A single heteroclitic epitope determines cancer immunity after xenogeneic DNA immunization against a tumor differentiation antigen. *J Immunol* 170(10), 5188-5194 (2003).

*** **A seminal paper together with (26) about the mechanism of action of xeno-gene-vaccination.**

57. Holmgren L, Ambrosino E, Birot O *et al.* A DNA vaccine targeting angiomotin inhibits angiogenesis and suppresses tumor growth. *Proceedings of the National Academy of Sciences of the United States of America* 103(24), 9208-9213 (2006).

58. Petrova G, Ferrante A, Gorski J. Cross-reactivity of T cells and its role in the immune system. *Critical reviews in immunology* 32(4), 349-372 (2012).
* **A detailed review providing mechanistic insights about cross-reactivity in immune responses.**
59. Cole DK, Pumphrey NJ, Boulter JM *et al.* Human TCR-binding affinity is governed by MHC class restriction. *J Immunol* 178(9), 5727-5734 (2007).
60. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* 76(2), 287-299 (1994).
61. Pamer E, Cresswell P. Mechanisms of MHC class I--restricted antigen processing. *Annual review of immunology* 16, 323-358 (1998).
62. Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. *Annual review of immunology* 11, 213-244 (1993).
63. Hammer J, Bono E, Gallazzi F, Belunis C, Nagy Z, Sinigaglia F. Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. *The Journal of experimental medicine* 180(6), 2353-2358 (1994).
64. Evavold BD, Sloan-Lancaster J, Wilson KJ, Rothbard JB, Allen PM. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity* 2(6), 655-663 (1995).
65. Boehncke WH, Takeshita T, Pendleton CD *et al.* The importance of dominant negative effects of amino acid side chain substitution in peptide-MHC molecule interactions and T cell recognition. *J Immunol* 150(2), 331-341 (1993).
66. Kersh GJ, Allen PM. Essential flexibility in the T-cell recognition of antigen. *Nature* 380(6574), 495-498 (1996).
67. Xu M, Kallinteris NL, Von Hofe E. CD4+ T-cell activation for immunotherapy of malignancies using Ii-Key/MHC class II epitope hybrid vaccines. *Vaccine* 30(18), 2805-2810 (2012).
68. Lazoura E, Apostolopoulos V. Insights into peptide-based vaccine design for cancer immunotherapy. *Current medicinal chemistry* 12(13), 1481-1494 (2005).
69. Pinilla-Ibarz J, May RJ, Korontsvit T *et al.* Improved human T-cell responses against synthetic HLA-0201 analog peptides derived from the WT1 oncoprotein. *Leukemia* 20(11), 2025-2033 (2006).
70. Rosenberg SA, Yang JC, Schwartzentruber DJ *et al.* Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nature medicine* 4(3), 321-327 (1998).
71. Kaufman HL, Lenz HJ, Marshall J *et al.* Combination chemotherapy and ALVAC-CEA/B7.1 vaccine in patients with metastatic colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14(15), 4843-4849 (2008).
72. Valmori D, Levy F, Miconnet I *et al.* Induction of potent antitumor CTL responses by recombinant vaccinia encoding a melan-A peptide analogue. *J Immunol* 164(2), 1125-1131 (2000).
73. Hoffmann TK, Loftus DJ, Nakano K *et al.* The ability of variant peptides to reverse the nonresponsiveness of T lymphocytes to the wild-type sequence p53(264-272) epitope. *J Immunol* 168(3), 1338-1347 (2002).
74. Vertuani S, Sette A, Sidney J *et al.* Improved immunogenicity of an immunodominant epitope of the HER-2/neu protooncogene by alterations of MHC contact residues. *J Immunol* 172(6), 3501-3508 (2004).
75. Fridman A, Finnefrock AC, Peruzzi D *et al.* An efficient T-cell epitope discovery strategy using in silico prediction and the iTopia assay platform. *Oncoimmunology* 1(8), 1258-1270 (2012).

76. Parmiani G, Castelli C, Dalerba P *et al.* Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? *Journal of the National Cancer Institute* 94(11), 805-818 (2002).
77. Kianizad K, Marshall LA, Grinshtein N *et al.* Elevated frequencies of self-reactive CD8+ T cells following immunization with a xenoantigen are due to the presence of a heteroclitic CD4+ T-cell helper epitope. *Cancer research* 67(13), 6459-6467 (2007).
*** **This paper identifies an alternative mechanism of action of xenovaccines: in some cases cross-reactive cytotoxic T cell responses can be triggered by a heteroclitic CD4+ T cell epitope.**
78. Wong AL, Lee SC. Mechanisms of Resistance to Trastuzumab and Novel Therapeutic Strategies in HER2-Positive Breast Cancer. *International journal of breast cancer* 2012, 415170 (2012).
79. Marchini C, Kalogris C, Garulli C *et al.* Tailoring DNA Vaccines: Designing Strategies Against HER2-Positive Cancers. *Frontiers in oncology* 3, 122 (2013).
80. De Giovanni C, Nicoletti G, Quaglino E *et al.* Vaccines against human HER2 prevent mammary carcinoma in mice transgenic for human HER-2. *Breast cancer research : BCR* 16(1), R10 (2014).
81. Finkle D, Quan ZR, Asghari V *et al.* HER2-targeted therapy reduces incidence and progression of midlife mammary tumors in female murine mammary tumor virus huHER2-transgenic mice. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10(7), 2499-2511 (2004).
82. Uva P, Aurisicchio L, Watters J *et al.* Comparative expression pathway analysis of human and canine mammary tumors. *BMC genomics* 10, 135 (2009).
** **Using gene expression profiling this paper shows a high degree of similarity between spontaneous dog and human mammary tumors.**
83. Bergman PJ, Camps-Palau MA, Mcknight JA *et al.* Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine* 24(21), 4582-4585 (2006).
*** **This paper describes the steps that led to the demonstration of the clinical efficay of the human tyosinase xeno-gene-vaccine, now licensed as ONCEPT® by USDA.**
84. Bergman PJ, Mcknight J, Novosad A *et al.* Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clinical cancer research : an official journal of the American Association for Cancer Research* 9(4), 1284-1290 (2003).
85. Manley CA, Leibman NF, Wolchok JD *et al.* Xenogeneic murine tyrosinase DNA vaccine for malignant melanoma of the digit of dogs. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine* 25(1), 94-99 (2011).
86. Grosenbaugh DA, Leard AT, Bergman PJ *et al.* Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. *American journal of veterinary research* 72(12), 1631-1638 (2011).
87. Liao JC, Gregor P, Wolchok JD *et al.* Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. *Cancer immunity* 6, 8 (2006).
88. Michaeli Y, Denkberg G, Sinik K *et al.* Expression hierarchy of T cell epitopes from melanoma differentiation antigens: unexpected high level presentation of tyrosinase-HLA-A2 Complexes revealed by peptide-specific, MHC-restricted, TCR-like antibodies. *J Immunol* 182(10), 6328-6341 (2009).
89. Alexander AN, Huelsmeyer MK, Mitzey A *et al.* Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II

clinical trial in canine patients with malignant melanoma. *Cancer immunology, immunotherapy : CII* 55(4), 433-442 (2006).

90. Price MA, Colvin Wanshura LE, Yang J *et al.* CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. *Pigment cell & melanoma research* 24(6), 1148-1157 (2011).
91. Campoli M, Ferrone S, Wang X. Functional and clinical relevance of chondroitin sulfate proteoglycan 4. *Advances in cancer research* 109, 73-121 (2010).
92. Mayayo SL, Prestigio S, Maniscalco L *et al.* Chondroitin sulfate proteoglycan-4: a biomarker and a potential immunotherapeutic target for canine malignant melanoma. *Vet J* 190(2), e26-30 (2011).
93. Kamstock D, Elmslie R, Thamm D, Dow S. Evaluation of a xenogeneic VEGF vaccine in dogs with soft tissue sarcoma. *Cancer immunology, immunotherapy : CII* 56(8), 1299-1309 (2007).
94. Yu WY, Chuang TF, Guichard C *et al.* Chicken HSP70 DNA vaccine inhibits tumor growth in a canine cancer model. *Vaccine* 29(18), 3489-3500 (2011).
95. Fong L, Brockstedt D, Benike C *et al.* Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. *J Immunol* 167(12), 7150-7156 (2001).

*** **The fisrt human clinical trial with a xenoantigen. In this case the antigen was delivered as protein in pulsed dendritic cells.**

96. Wolchok JD, Yuan J, Houghton AN *et al.* Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. *Molecular therapy : the journal of the American Society of Gene Therapy* 15(11), 2044-2050 (2007).
97. Yuan J, Ku GY, Adamow M *et al.* Immunologic responses to xenogeneic tyrosinase DNA vaccine administered by electroporation in patients with malignant melanoma. . *Journal of ImmunoTherapy of Cancer* 1(2013).
98. Yuan J, Ku GY, Gallardo HF *et al.* Safety and immunogenicity of a human and mouse gp100 DNA vaccine in a phase I trial of patients with melanoma. *Cancer immunity* 9, 5 (2009).
99. Ginsberg BA, Gallardo HF, Rasalan TS *et al.* Immunologic response to xenogeneic gp100 DNA in melanoma patients: comparison of particle-mediated epidermal delivery with intramuscular injection. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16(15), 4057-4065 (2010).

** **A Phase I xeno-gene-vaccination using mouse gp100: demonstration that delivery using gold particles achieves the same degree of immunogenicity as naked DNA injection by using a 500-fold lower dose of DNA.**

100. Eriksson F, Totterman T, Maltais AK, Pisa P, Yachnin J. DNA vaccine coding for the rhesus prostate specific antigen delivered by intradermal electroporation in patients with relapsed prostate cancer. *Vaccine* 31(37), 3843-3848 (2013).