Alpha-Enolase (ENO1), a potential target in novel immunotherapies

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1. ABSTRACT

Alpha-enolase (ENO1) is a metabolic enzyme involved in the synthesis of pyruvate. It also acts as a plasminogen receptor and mediates the activation of plasmin and extracellular matrix degradation. In tumor cells, ENO1 is up-regulated and supports the Warburg effect; it is expressed at the cell surface, where it promotes cancer invasion, and is subjected to a specific array of post-translational modifications, namely acetylation, methylation and phosphorylation. ENO1 overexpression and post-translational modifications could be of diagnostic and prognostic value in many cancer types. Information on the biochemical, proteomics and immunological characterization of ENO1, and particularly its ability to trigger a strong specific humoral and cellular immune response, make this ubiquitous protein an interesting tumor target; DNA vaccination with ENO1 in preclinical models efficiently delays the development of very aggressive tumors such as pancreatic cancer. This review aims to analyze the main stages by which the tumor associated antigen (TAA) ENO1 has become a promising target that opens potential avenues for cancer immunotherapy.

2. INTRODUCTION

Tumor immunotherapy is mostly based on the overexpression of tumor associated antigens (TAAs) in cancer, compared to normal tissues and on the ability of the immune system to recognize them and to induce a specific immune response (1). In 1943, the pioneering study of Gross and colleagues proved that tumors induced by oncogenic viruses were rejected through the recognition of tumor antigens, and that chemically-induced tumors were able to immunize mice to recognize a second exposure of the same tumor cells (2). Further studies in the late 70s also demonstrated the presence of tumor antigens in a mouse teratocarcinoma cell line (3) and in spontaneous mouse tumors (4), indicating that tumor antigens were not strictly artifacts induced by chemical treatment and that they are also likely to be present in human tumors (5). As TAAs have been shown to be increasingly important as immunotherapy targets, many groups have looked for not yet characterized or more immunogenic new TAAs, by developing different approaches. Some strategies were based on the reactivity of cytotoxic T lymphocytes (CTL) isolated from cancer patients against the autologous tumor (6-9), while others focused on the antibody response of cancer patients.

3. IMMUNE RESPONSE TO TUMOR ASSOCIATED ANTIGENS IN CANCER PATIENTS

A methodology known as serological analysis of recombinant cDNA expression libraries (SEREX) (10) has been useful to identify several hundreds of TAAs (11, 12). A similar technique, exploiting the presence of antibodies in the sera of cancer patients, coupled with a proteomic approach, is SERological Proteome Analysis (SERPA), which allowed to identify TAAs in many kind of
tumors (13, 14), and in particular against pancreatic ductal adenocarcinoma (PDA), which is one of most aggressive solid tumors (15). Among these antigens, alpha-enolase (ENO1) was identified as a promising TAA due to its ability to induce a humoral and/or cellular immune response in cancer patients (16). Moreover, overexpression of ENO1 at mRNA and protein level was observed in different tumor types including brain, breast, cartilage, cervix, colon, eye, gastric, head and neck, kidney, leukemia, liver, lung, muscle, ovary, pancreas, prostate, skin and testis cancers (17-50) (Table 1).

ENO1 has been shown to induce autoantibody production in many types of cancer patients. More in detail with the SERPA technique has been found the presence of anti-ENO1 antibodies in cholangiocarcinoma, breast cancer, head and neck cancer, leukemia, lung cancer, pancreatic cancer and melanoma patients (39, 42, 50-62). In late stage of lung and breast cancers, anti-ENO1 autoantibodies have been found decreased suggesting that they may serve as a prognostic marker to monitor disease progression (63). An explanation could be that tumor cells reduce the circulating levels of anti-ENO1 antibodies through physical absorption and neutralization with surface-expressed and secreted ENO1, as suggested by in vivo experiment (64).

Interestingly in lung cancer the higher presence of anti-ENO1 antibodies after surgery correlated with a lower hazard ratio and a better progression-free survival (64). A spontaneous immune responses to ENO1 has also manifested in patients with primary and metastatic melanomas (65) (Table 1).

Circulating anti-ENO1 antibodies have been found in several autoimmune disease such as lupus nephritis (66, 67) and autoimmune retinopathy (68) as well as in cancer-associated retinopathy (69, 70). In breast cancer patients with associated retinopathy an increased incidence of autoantibodies in general has been observed (71, 72). Moreover, antibodies against citrullinated ENO1 epitopes were observed in rheumatoid arthritis patients (73, 74). The correlation between cancer and autoimmunity could be due to the production of immunogenic and pro-inflammatory stimuli by tumor cell death and to the resulting activation of the inflammatory process within the tumor microenvironment, which concurs to increase the presentation of self-antigens to the immune system (75).

Almost two-thirds of PDA patients display an antibody response against ENO1, which is absent or present at a very low frequency in healthy donors, non-PDA

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DP: Disease progression, DFS: Disease-free survival, M: Malignancy, OS: Overall survival, PFS: Progression-free survival
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cancer and chronic pancreatic patients (15) suggesting its diagnostic value in PDA (16). Autoantibodies from PDA patients specifically recognize two highly phosphorylated acid isoforms of ENO1 (identified as ENO1.2). ENO1.2 isoforms are up-regulated in PDA compared to normal pancreas and display phosphorylation of serine 419, which is absent in other tumor cell lines (57, 76). Importantly, the presence of autoantibodies against ENO1.2 discriminate PDA patients with normal levels of CA19.9, and complement the diagnostic performance of serum CA19.9, increasing the sensitivity from 62% to 95%. The presence of anti-ENO1.2 antibodies correlate with a longer progression-free survival and a better clinical outcome in PDA patients treated with standard gemcitabine-based chemotherapy (57). In general, phosphorylation of a protein is associated with a higher affinity of its peptides for Major Histocompatibility Complex (MHC) molecules (77), suggesting that peptides from phosphorylated ENO1.2 could be better or more frequently presented by specific MHC complex to T cells. The detection of specific autoantibodies for phosphorylated isoforms of ENO1 indeed correlates with a higher frequency of the allele HLA-DRB1*8 among PDA patients. Furthermore, repeated in vitro stimulation of peripheral blood mononuclear cells (PBMC) from HLA-DRB1*8 healthy subjects with phosphorylated ENO1 peptide (predicted by a bioinformatics algorithm) elicits a significant CD4+ T cell proliferative response compared to the unphosphorylated ENO1 peptide (78). An ENO1 natural antigenic HLA-DRB1*8-restricted peptide from a squamous cell carcinoma cell line (OSC-20), which is specifically recognized by the CD4 cytotoxic T cell line TcOSC-20 has been also identified (79). Many TAAs stimulate an integrated humoral and cellular response by activating both T and B cells (16), and this coordinate response is one of the main effector mechanism exploitable by tumor immunotherapy.

To date, a coordinate specific response to ENO1 has only been demonstrated in head and neck cancer (79, 80), melanoma (65) and pancreatic cancer patients (47, 81-83) (Table 1). PDA patients with anti-ENO1 circulating antibodies display peripheral T cells that secreted IFN-γ when they are activated in vitro with recombinant ENO1 (47). In vitro, ENO1 is also able to elicit specific proliferation and activation of T cells and differentiation of specific CTL from healthy donor PBMC. ENO1-specific CTL spare in vitro normal skin Human Leukocyte Antigen (HLA)-matched fibroblasts from killing, but induce the inhibition of HLA-matched PDA cells in vitro and in vivo (47).

The response of T cells against ENO1 does not always lead to an effector function as, in some circumstances, ENO1-specific T cells display a T regulatory (Treg) phenotype. The presence of ENO1-specific Treg cells in mice with ENO1-overexpressed lung tumors has been demonstrated. In particular, Treg cells isolated from tumors suppressed the proliferation of ENO1-specific CD4+ T cells, and mice bearing ENO1-overexpressed tumors showed a reduced production of anti-ENO1 antibodies (64). These results indicate that the presence of anti-ENO1 antibodies correlates with the anti-tumor effector responses and that these are impaired by anti-ENO1 Treg cells (64). In an extensive ex vivo phenotypic characterization of PDA infiltrating-tumor T cells, it has been found a statistically significant increase in the percentage of ENO1-specific Treg T cell clones (TCC) generated from T cells that infiltrate cancer tissue compared to TCC generated from T cells that infiltrate the healthy pancreatic tissue (82). These Treg TCC also inhibit the proliferation and cytotoxic activity of ENO1-specific effector Th1 and Th17, TCC generated from PDA tissue-infiltrating T cells (82). Th1 and Th17 TCC from PDA tissue-infiltrating T cells have an anti-tumor effector function, and are increased in healthy pancreatic mucosa compared to PDA tissue (82), suggesting that recruitment of anti-tumor Th17 cells to the tumor site is impaired by the immunosuppressive microenvironment, in which Treg cells play an important role (84-86). The presence of ENO1-specific T cells in the peripheral blood of many PDA patients, is triggered by in vitro re-stimulation with recombinant ENO1. Moreover, ENO1-specific TCC generated from PDA patient PBMC display a more pronounced Th1 than Treg phenotype, as observed in the tumor (83). These data suggest that although the recruitment of ENO1-specific T cells from peripheral blood to the tumor is repressed by many immunosuppressive mechanisms (83), their presence in peripheral blood is relevant to prevent metastasis through the removal of cancer-circulating cells (87, 88). Indeed PDA patients with a higher number of ENO-specific TCC from PBMC show significantly longer survival, underlying the importance of ENO1-specific response to improve PDA patient anti-tumor immunity (83).

4. ROLE OF ENO1 IN CANCER

ENO1 is a multi-functional protein, which mainly acts as an enzyme and a plasminogen receptor, thus playing a critical role in cancer proliferation, metastasis and spreading (16, 89). Its enzymatic function is carried out by the conversion reaction of dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in the final step of the glycolytic pathway, while in tumors ENO1 implicated in the maintenance of “aerobic glycolysis” (90). At the surface of cancer cells, ENO1 acts as a plasminogen receptor and, by promoting plasminogen activation into plasmin, a serine-protease involved in extracellular matrix degradation, ENO1 favors cell invasion and metastasis (89, 91). Due to ENO1 upregulation in several tumors and the correlation with shorter overall survival in cancer patients (16, 91), several groups have focused on the study of the perturbation of ENO1 expression in tumors.
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4.1. Metabolic functions of ENO1 in tumors

Reprogramming of metabolism is a well-known mechanism carried out by cancer cells, which demand a high rate of glycolysis to satisfy the increasing requirements for nucleotide, lipid and protein synthesis and to maintain rapid proliferation (92). Although the Tricarboxylic Acid (TCA) cycle and Oxidative Phosphorylation (OXPHOS) would generate more ATP, cancer cells tend to utilize the less efficient method of glycolysis, producing large quantities of pyruvate and lactate, even in the presence of abundant oxygen; this phenomenon is known as “aerobic glycolysis" or the "Warburg effect" (93). The most relevant data concerning the metabolic function of ENO1 in tumor cells are related to its role in maintaining the Warburg effect; inhibition of ENO1 has been shown to increase reactive oxygen species (ROS) that are mainly generated through the sorbitol and NADPH oxidase pathways (94). In particular, ENO1-silenced cells from breast, lung and pancreatic cancers display increased glucose uptake and consequently leads to an excess of intracellular glucose, which is forced towards alternative pathways, such as the pentose phosphate pathway (PPP) and the polyol pathway (PP), with a consequent decrease in lactate levels (94). Similar results in terms of inhibition of glycolysis are obtained after ENO1 silencing in endometrial carcinoma cells (EC): the mRNA level of lactate dehydrogenase A (LDHA) and the protein level of cell glycylisis-associated LDHA is decreased in ENO1-silenced EC as well as the amount of extruded lactate into the media (95). These metabolic changes increase the oxidative stress induced-autophagy, the fatty acid oxidation and the amino acid catabolism, resulting in less growth and senescent phenotype of cancer cells (94). Taken together, these data confirm that ENO1 is essential for maintaining tumor metabolism and suggest that therapies targeting the metabolic function of ENO1 are effective in blocking tumor progression.

4.1.1. Potential approaches for targeting the metabolic function of ENO1

The metabolic switch that occurs in cancer cells may provide promising novel targets for cancer therapy. There is growing evidence to support the potential role of many enzymes, transporters or transcription factors as suitable candidate targets for cancer treatment (96, 97). In particular, there are at least four different approaches: i) inhibition of glycolytic pathway enzymes, ii) inhibition of pentose phosphate pathway enzymes, iii) promotion of the OXPHOS process and iv) attenuation of HIF-1 activity (97). In the glucose metabolic pathway there are multiple therapeutic targets, which could be potential targets for anti-cancer strategies and offer promising clinical potential (98-103). Among this, the inhibition of a key glycolytic enzyme, ENO1 might represent an interesting approach. Chemical enolase inhibitors are sodium fluoride (104), D-tartarate and 3-aminoenolpyruvate 2-phosphate (102, 105), but none of these are appropriate for use in therapeutic protocols. The translational relevance of ENO1 targeting was demonstrated by the use of phosphonoacetylhydroxamic acid (PhAH), a pan-enolase transition-state analogue inhibitor (106), for the treatment of glioblastoma (107), and recently for pancreatic, breast and lung cancers (94). PhAH inhibits enolase both enzymatic activity and proliferation in cancer cells (94, 107). The small molecule, named “ENOBlock” (AP-III-a4), which is the first non-substrate analogue that directly binds to ENO1 and inhibits its activity (108) decreases cancer cell viability under hypoxic conditions. Under normoxic conditions, ENOblock reduces cancer cell invasion/migration in vitro and in vivo without inducing cytotoxicity and synergizes with microtubule destabilizing drugs, suggesting that this ENO inhibitor is suitable for biological assays. In tumors such as Non-Hodgkin’s Lymphomas (NHLs) and breast cancer, inhibition of ENO1 decreased tolerance to hypoxia while increasing sensitivity to radiation therapy, thus indicating that ENO1 may favor chemoresistance (109, 110).

All these ENO1 inhibitors are very attractive candidate compounds for pharmacokinetic and pharmacodynamic studies to assess their potential as anti-cancer drugs, but still require concerted efforts to develop suitable drugs for use in vivo without affecting normal cells.

4.2. Pro-invasive function of ENO1

Overexpression of ENO1 has been correlated with size, disease stage, metastasis and prognosis for many tumors (16, 91). The pro-invasive function of ENO1 is mainly linked to its role as a plasminogen receptor; ENO1 facilitates the binding of elevated concentrations of plasminogen which, after conversion into the serine protease plasmin, promotes extracellular matrix (ECM) degradation. At the cell surface, ENO1 is part of a multi-protein complex including the uPA receptor (uPAR), integrins and cytoskeletal proteins, responsible for adhesion, migration and proliferation (91), while in the cytoplasm, ENO1 interacts with the cytoskeleton to promote migration of tumor cells by providing ATP (111, 112). The spreading and invasion of cancer cells in vivo is strictly related to the high expression of ENO1. ENO1 silencing in tumor cells not only reduces glycolysis but also migration and in vitro invasion, as well as tumorigenesis and metastasis in vivo (18, 48, 94, 95, 113), mirroring the different functions of ENO1 in tumor cells. These effects are mediated by inactivation of PI3K/AKT pathway and its downstream signals including glycolysis, cell cycle progression, and epithelial-mesenchymal transition (EMT)-associated genes in non-small cell lung cancer (113), glioma (18, 113) and endometrial cancer (95).

4.2.1. Potential approaches for targeting the pro-invasive function of ENO1

The prognostic value of high ENO1 expression and its correlation with worse survival has been confirmed...
in several tumors (18, 19, 25, 33, 34, 36, 37, 42, 57) (Table 1). In parallel, the protective role of anti-ENO1 antibodies in cancer patients has been also highlighted, suggesting that their use in cancer therapy could represent a good strategy.

In pancreatic cancer patients, the presence of anti-phosphorylated-ENO1 antibodies correlates with a longer response to therapy as well as overall survival (57). By contrast, in the late-stage of disease in lung and breast cancer patients, there is a marked decrease in basal levels of anti-ENO1 autoantibodies as a common event, which correlates with a better prognosis and longer survival (63). This is due to physical absorption and neutralization of anti-ENO1 Ab to surface-expressed and secreted ENO1, respectively (64). In fact, in vivo adoptive transfer of anti-ENO1 specific antibodies to mice results in accumulation of antibodies in subcutaneous tumors that expressed high levels of ENO1, and a consequent reduction of free circulating anti-ENO1 antibodies. In addition, patients who underwent surgery display an increase of anti-ENO1 Ab, a lower hazard ratio, and better progression-free survival (64). In vivo adoptive transfer of anti-ENO1 antibody in mice previously injected with tumor cells results in a strong inhibition of tumor metastasis in lungs and bone (43). A monoclonal antibody against ENO1 blocks the interaction between plasminogen and ENO1, which leads to the inhibition of in vitro and in vivo migration and invasion (48). However, targeting of the surface ENO1 by the monoclonal antibody (mAb) did not affect in vitro cell proliferation (48). Of note, the in vivo passive immunotherapy using a single administration of Adeno-Associated Virus (AAV)-expressing cDNA coding for anti-ENO1 mAb greatly reduces tumor spreading in the lungs of immunosuppressed mice injected with FDA cells, to a much greater extent than soluble IgG injected twice a week (48).

To define surface molecules targetable by peptides to develop nanocarriers for specific delivery of chemotherapy into tumor cells, ENO1 has been identified as a specific target of a 12-mer peptide (114). An in vitro panning of a phage-displayed peptide library against the colorectal cancer cell line HCT116 identified 36 phages displaying peptides capable of stronger binding compared to the control phage. Of these, three phage clones were subsequently validated in vivo for the increased ability to accumulate at the tumor mass compared to normal organs such as brain, lungs and heart. These three phage clones are highly specific for surface binding of different tumor cell lines as well as HCT116, such as A498 (renal cell carcinoma), B16-F10 (melanoma), H640 (lung carcinoma), HTB-10 (neuroepithelioma), MDA-MB-231 (breast carcinoma), Mia-PaCa-2 (pancreatic adenocarcinoma), PC-3 (prostatic adenocarcinoma) and SKOV3 (ovarian carcinoma). Two phages were chosen for in vitro and in vivo pre-clinical studies (114). Synthetic peptide conjugates were incorporated into liposomes filled with chemotherapeutic drugs and assessed for their in vitro and in vivo cytotoxic ability against colorectal cancer cell line. One peptide in particular was very efficient in delivering drugs into tumor cells and decreasing tumor growth compared to liposomes filled with drugs that were not covered with peptide (114). The LC-MS/MS analysis of the sequences bound by this peptide revealed ENO1 as its target (114). Overall, these data indicate that targeting of ENO1 represents a potential strategy for gene-based therapy, immunotherapy and chemical cancer treatment.

5. ANTI-ENO1 VACCINE AND MECHANISMS OF PROTECTION

Over the last decade, many scientists have invested great efforts in developing approaches for eliciting anti-tumor responses by priming a novel or boosting an existent immune response against tumor cells. These have included tumor-targeting mAbs, oncolytic viruses, dendritic cell (DC)-based therapies, cytokines, immunomodulatory mAbs, pattern recognition receptor (PRR) agonists, peptides, and mRNA- or DNA-based vaccines (5, 115-122). The huge amount of pre-clinical and clinical results led to the approval of their use by the US Food and Drug Administration (FDA) agency and the European Medicines Agency as immunotherapy in cancer patients. The great clinical success of immunotherapy has earned the title "Breakthrough of the Year" in the prestigious Science journal (123).

DNA-based vaccines may represent a suitable and efficient option for immunotherapy. They display several advantages in that they are stable, do not contain viral proteins that could down-regulate the immune system or elicit neutralizing antibodies, and are safe, as several studies have shown that mutations arising from a putative integration event are extremely rare (124). On the other hand, DNA vaccination usually fails to mount a strong immune response and requires additional adjuvant strategies. However, DNA fusion gene vaccine offers the opportunity to include different genes encoding a range of immunostimulatory molecules or short hairpin RNA to switch off suppressive molecules, either into the vaccine vector or by a separate vector (121). A necked plasmid, pVax vector, approved by the FDA for clinical use, was used to express full length human ENO1 and to vaccinate mice that had been genetically engineered to develop autochthonous pancreatic adenocarcinoma (called KC and KPC) (81). The identity of human and mouse ENO1 is up to 95% while the homology is up to 99%, and some CD8-specific epitopes that are shared between human HLA-A02 and mouse H-2b molecule tasks were also found (NetMHC 3.0.). In this setting, KC and KPC mice were vaccinated when Pancreatic Intraepithelial Neoplasia (PanINs) lesions were already present and received a total of three and four rounds of immunization every 3 and 2 weeks, respectively. The ENO1 vaccine induces a specific integrated humoral
and cellular response that efficiently prolonged mouse survival from 48 to 68 weeks of age for KC mice, and from 29 to 35 weeks of age for KPC (81).

There are several protective immunological mechanisms induced by the ENO1 vaccine, namely high levels of anti-ENO1 IgG, activation of specific Th1 and Th17 cells, as well as a large recruitment of CD3 cells into the tumor, and an important decrease of circulating myeloid-derived suppressor cells (MDSC) and both circulating and intra-tumoral Treg cells (125). Notably, the ENO1 vaccine-induced IgG are able to mediate the complement-dependent cytotoxicity of PDA cells, and it was assumed that the cytokines released by activated Th1/Th17 cells promoted the isotype switching necessary to activate the complement. The crucial role of anti-ENO1 antibodies was confirmed by the observation that ENO1-vaccinated mice showed B cells organized in dense aggregates that displayed a distinct structure, the so-called tertiary lymphoid tissue (TLT), which was not found in normal pancreas and only sporadically in PDA of untreated mice or those vaccinated with an empty-vector (126). B cells organized into TLT, namely CD20-TLT, are shown to correlate with a better prognosis and with a greater infiltration of CD8+ T cells in a cohort of 104 PDA patients. To assess the role of tumor infiltrating-CD20+ B cells (CD20-TIL) compared to CD20-TLT, mice orthotopically injected with syngeneic PDA cells were depleted of B cells by a single injection of an anti-CD20 Ab. No TLT is observed in this implantable tumor model, probably because of the absence of a chronic inflammatory response, but CD20-TIL are dramatically reduced in those receiving the Ab. The anti-CD20 treatment induces a significant increase in genes related to T and NK cell recruitment as well as genes involved in lymphoid tissue structure development and CD8+ T cell differentiation and maintenance, suggesting a dual role of B cells in PDA progression (126). ENO1-vaccinated mice not only show more TLT than control mice but also a higher number of tumor-infiltrating CD3 cells but not Treg cells (81).

Another effect elicited by the ENO1-vaccine is the reduction of MDSC. Due to the expression of ENO1 in activated monocytes, its presence on the surface of both human and mouse MDSC has been assessed. In PDA patients, myeloid cells express higher levels of ENO1 and this was further increased by LPS stimulation, as observed on MDSC purified from spleens of tumor-bearing mice (127). An anti-ENO1 mAb is able to limit MDSC adhesion and migration on and through a monolayer of pancreatic endothelial cells, respectively. In addition, the anti-ENO1 Ab reduces the in vivo migration of MDSC from the footpad to the draining lymph node. Antibodies induced by the ENO1-vaccine also limit the infiltration of MDSC into the tumor area of an orthotopic transplantable model of PDA (127). However, the in vitro targeting of surface ENO1 on MDSC does not affect their suppressive function in terms of T cell proliferation, although T cells co-cultured with ENO1-targeted MDSC secrete much more IFN-γ and IL17 and less IL10 and TGF-β compared to those co-cultured with MDSC treated with a control Ab (127). ENO1-targeting does not affect co-stimulatory molecule expression on MDSC, with the exception of CD80 expression, which is up-regulated, but it decreases arginase activity compared to that of control MDSC (127). Overall, these results demonstrate that the ENO1-DNA vaccine elicits an integrated humoral and cellular response to counteract tumor growth, which not only affects the tumor cells themselves but also stromal and reactive cells. Unfortunately, these effects don't last beyond 6 months after immunization, when the mice died; therefore the aim is to combine the vaccination with other strategies to enhance the specific anti-ENO1 integrated response. The ENO1-DNA vaccine, however, is very promising as it efficiently decreased the tumor size in KC mice, which were therapeutically vaccinated when adenocarcinomas were well established, at 8 months of age (81).

6. CONCLUSIONS

The evidence obtained so far demonstrate the role of ENO1 in tumor progression and the concept that ENO1 vaccination effectively induces an integrated immune response, which is able to significantly enhance the survival of genetically-engineered mice (GEM) that spontaneously develop PDA. In addition, blocking ENO1 by a specific monoclonal antibody, or its functional silencing by chemical inhibitors could complement and integrate the mechanisms that are employed, to target ENO1 and counteract cancer progression and spreading. Metabolic inhibition of ENO1 blocks tumor cell proliferation and induces changes that can be perturbed to definitively kill cells (Figure 1). Surface blockade of ENO1 by mAb, instead, may efficiently inhibit PDA spreading and accumulation of myeloid cells in the primary tumor, which can suppress the anti-tumor response (Figure 1). The approach of targeting metabolic and immunological functions in tumors could be further strengthened by combining them with pharmacological inhibitors of immune suppressor cells. For example, recent data have shown that targeting of phosphoinositide-3- kinase (PI3K) γ and δ isoforms is an effective way to unleash the suppressive activity of tumor-associated macrophages (TAM) and regulatory T cells, respectively, thus reinforcing the anticancer immune response (128-130). Inhibitory targeting of PI3Kγ, in fact, stimulates anti-tumor immune responses, leading to improved survival and responsiveness to standard-of-care chemotherapy in animal models of PDA. PI3Kγ selectively drives immunosuppressive transcriptional programming in macrophages, which inhibits adaptive immune responses and promotes tumor cell invasion and desmoplasia in PDA. Inhibition of PI3Kγ in PDA-bearing mice reprograms TAM to stimulate CD8+ T cell-mediated tumor suppression and to inhibit tumor cell invasion, metastasis and desmoplasia (130). These results suggest
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that the combination of the ENO1-DNA vaccine and the PI3Kγ inhibitor enhances the anti-tumor response. Studies are currently ongoing to verify the hypothesis that the targeting of myeloid suppressive cells, via genetic or pharmacological PI3Kγ inhibition synergizes with ENO1-DNA vaccination by inducing the sustained immune response that can effectively counteract PDA. Of note, all these approaches can be easily translated into clinical practice as most inhibitors, as well as the AVV vectors, are already used in the clinic, and the pVAX used for the DNA vaccination has been approved by the FDA.

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**Abbreviations:** Adeno-Associated Virus: AAV; Adenosine Triphosphate: ATP; Alpha-enolase: ENO1; Carbohydrate Antigen 19.9.: CA19.9.; Cytotoxic T lymphocytes: CTL; Dendritic cells: DC; Epithelial-Mesenchymal Transition: EMT; Extracellular Matrix: ECM; genetically engineered mice: GEM; Interferon-g: IFN-g; Interleukin 10: IL10; Interleukin 17: IL17; Human Leukocyte Antigens: HLA; lactate dehydrogenase A: LDHA; Liquid chromatography tandem-mass spectrometry: LC-MS/MS; Hypoxia Inducible Factor 1: HIF-1; Lipopolysaccharide: LPS; Kras-mutated genetically engineered mice that spontaneously develop pancreatic cancer: KC; Kras and p53-mutated genetically engineered mice that spontaneously develop pancreatic cancer: KPC; Myeloid Derived Suppressor Cells: MDSC; Major Histocompatibility Complex: MHC; monoclonal antibody: mAb; reduced Nicotinamide Adenine Dinucleotide Phosphate: NADPH; Oxidative Phosphorylation: OXPHOS; Pancreatic Ductal Adenocarcinoma: PDA; Pancreatic Intraepithelial Neoplasia: PanIN; Peripheral Blood Mononuclear Cells: PBMC; Phosphonoacetohydroxamic acid: PhAH; phosphoinositide-3-kinase: PI3K; Radical Oxygen Species: ROS; Serological Analysis of Recombinant cDNA Expression Libraries: SEREX; SERological Proteome Analysis: SERPA; tertiary lymphoid tissue: TLT; T cell clones: TCC; Th1 cells: T helper 1 cells; Th17 cells: T helper 1 cells; Transforming Growth Factor b: TGF-β; T regulatory cells: Treg cells; Tricarboxylic Acid: TCA; Tumor Associated Antigens: TAAs; Tumor Associated Macrophages: TAM, Urokinase-type Plasminogen Activator (uPA) Receptor: uPAR.

**Key Words:** Alpha-enolase, ENO1, Cancer, Humoral Response, Glycolysis, Invasion, Immunotherapy, Pancreatic Cancer, Plasminogen Receptor, T cell response, Vaccination, TAAs, Metastasis, Review

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