Six-Transmembrane Epithelial Antigen of the Prostate (STEAP): a potential target of immunotherapy in prostate cancer

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Director of Study
Matteo Bellone

External Supervisor
Douglas Noonan
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Abstract

Immunotherapy has been proposed as a complementary or alternative therapy for the treatment of advanced prostate cancer (PC), one of the leading causes of tumor-related mortality in men. From different approaches that have been tested in recent years, it is now becoming more evident that the choice of the appropriate antigen to target is crucial for the best outcome. Generally tumor-associated antigens (TAAs) undergo peripheral tolerance during tumor progression, which dampens the efficacy of vaccination protocols. It can be hypothesized that the kinetic and depth of immune tolerance varies depending on the timing and relative expression of the TAA. These differences may represent a key for successful immunotherapy approaches even in patients with advanced disease.

Aim of my thesis was to investigate the dynamics of CD8⁺ T cells specific for normal tissue antigens over-expressed during the spontaneous tumor development and progression in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, a primary model of human PC.

We have found that Prostate Stem Cell Antigen (PSCA) and Six-Transmembrane Epithelial Antigen of the Prostate (STEAP), two well known PC-associated antigens, behave differently in term of immunological response when compared with the transgenic Tag IV antigen, which drives oncogenesis in TRAMP mice. While a dendritic cells (DC)-based immunization was able to elicit measurable immune responses for all three antigens in young males affected by mouse prostate intraepithelial neoplasia (mPIN), aged mice affected by PC progressively lost immunity against Tag IV and in part for PSCA, but not against STEAP. The findings correlated with the amount of antigens expressed in the prostate, therefore suggesting that tolerance against this type of TAA follows the same rule of that induced for tissue-associated antigens in peripheral tissues: the more the antigen is expressed the more tolerance is profound.

Finally, a combined therapy of allotransplantation and DC-STEAP vaccination effectively reduced tumor burden in TRAMP mice, underlying
how this therapeutic strategy when targeted to a reliable antigen is able to restore cancer immunosurveillance.
1. Introduction

1.1 The Immune System: Innate and Adaptive Immunity

Host defense against microbial infection is mediated by a variety of mechanisms that fall into two categories: innate and adaptive (or acquired). While innate immunity is an evolutionarily ancient and universal form of host defense found in all multicellular organisms, the adaptive immune system exists only in the context of vertebrate physiology.

The epithelium provides both a physical barrier to the entry of microbes and produces a variety of antimicrobial factors. Agents that penetrate the epithelium are recognized by macrophages and related cells possessing “pattern recognition receptors” that recognize key molecules characteristic of many microbial agents. These include several families of molecules, of which the most intensively studied are the Toll-like receptors (TLRs). Each TLR recognizes a distinct substance (or set of substances) associated with microbial agents: TLR4 recognizes lipopolysaccharides; TLR3, double-stranded RNA; and TLR9, unmethylated CpG-containing DNA. Microbial sensors provide a highly efficient means to recognize potential pathogens and in association with the phagocytic activity of macrophages induce antimicrobial systems that aid in the destruction of the pathogen.

The innate immune system also acts to recruit antigen-specific immune responses, not only by attracting cells of the immune system to the site of the infection, but also through the uptake of antigen by dendritic cells (DC) and its transport by these cells to lymphoid tissues where primary immune responses are initiated.

Primary immune responses, or adaptive, start when a foreign antigenic substance interacts with antigen-specific lymphocytes under appropriate circumstances. The response generally consists of the production of antibody molecules specific for the antigenic determinants of the immunogen and of the expansion and differentiation of antigen-specific helper and effector T lymphocytes. The latter include cytokine-producing cells and killer T cells,
capable of lysing infected cells. Generally, the combination of the innate immune response and the primary adaptive response are sufficient to eradicate or to control the infection.

1.2 T Lymphocytes

There are two broad classes of lymphocytes: the B lymphocytes, which are precursors of antibody-secreting cells, and the T (thymus-derived) lymphocytes. T lymphocytes derive from stem cell precursors in hematopoietic tissue, undergo differentiation in the thymus and are then seeded to the peripheral lymphoid tissue and to the recirculating pool of lymphocytes. T cells are subdivided into two distinct classes based on the cell surface receptors they express. The majority of T cells express antigen-binding receptors (TCRs) consisting of \( \alpha \) and \( \beta \) chains. A second group of T cells express receptors made up of \( \gamma \) and \( \delta \) chains. The \( \alpha \beta \) T cells are subdivided into two important sublineages: those that express the coreceptor molecule CD4 (CD4 T cells) and those that express CD8 (CD8 T cells). These cells recognize the antigen in different manners and mediate different types of regulatory and effector functions. CD4 T cells are the major helper cells of the immune system. Their helper function depends both on cell surface molecules such as CD154, induced upon these cells when they are activated, and on the wide array of cytokines they secrete when activated. CD4 T cells tend to differentiate, as a consequence of priming, into cells that principally secrete the cytokines IL-4, IL-13, IL-5, IL-6, and IL-10 (TH2 cells), into cells that mainly produce IFN-\( \gamma \) and lymphotoxin (TH1 cells) or into cells that produce IL-17 and related cytokines (TH17 cells). TH2 cells help B cells to develop into antibody-producing cells, while TH1 cells are effective inducers of cellular immune responses, involving the enhancement of the microbicidal activity of macrophages and the lysis of microorganisms in intracellular vesicular compartments. TH17 cells are efficient recruiters of
granulocytes and other cells of the inflammatory system and play a major role in responses to extracellular bacterial pathogens. T cells mediate important effector functions. Some of these are determined by the patterns of cytokines they secrete. These powerful molecules can be directly toxic to target cells and can mobilize potent inflammatory mechanisms. In addition, T cells, particularly CD8 T cells, can develop into cytotoxic T lymphocytes (CTLs) capable of efficiently lysing target cells that express antigens recognized by the CTLs.

T cells are able to recognize cell-associated molecules and mediate their functions by interacting with antigen-presenting cells (APCs), giving rise to the immunological synapse. The TCR does not recognize antigenic determinants on intact molecule, but it recognizes a complex consisting of a peptide, derived by proteolysis of the antigen, bound into a specialized groove of a class II or class I MHC protein. Indeed, CD4 T cells recognize peptide–class II complexes, whereas the CD8 T cells recognize peptide–class I complexes. The TCR’s ligand (i.e., the peptide–MHC protein complex) is created within the APC. In general, class II MHC molecules bind peptides derived from proteins that have been taken up by the APC through an endocytic process. These endocytosed proteins are fragmented by proteolytic enzymes within the endosomal/lysosomal compartment, and the resulting peptides are loaded into class II MHC that traffic through this compartment. Peptide-loaded class II molecules are then expressed on the surface of the APC where they are available to be bound by CD4 T cells that have TCRs capable of recognizing the expressed cell surface peptide–MHC protein complex. Thus, CD4 T cells are specialized to largely react with antigens derived from extracellular sources.

In contrast, class I MHC molecules are mainly loaded with peptides derived from internally synthesized proteins, such as viral gene products. These peptides are produced from cytosolic proteins by proteolysis within the proteasome and are translocated into the rough endoplasmic reticulum. Such peptides, generally nine amino acids in length, are bound by class I MHC molecules. The complex is brought to the cell surface, where it can be
recognized by CD8 T cells expressing appropriate receptors. This property gives the T cell system, particularly CD8 T cells, the ability to detect cells expressing proteins that are different from, or produced in much larger amounts than, those of cells of host (e.g., viral antigens [whether internal, envelope, or cell surface] or mutant antigens [e.g., active oncogene products]).

This division of class I–binding peptides being derived from internally synthesized proteins and class II–binding peptides from imported proteins is generally correct, but there are important exceptions to this rule that are central for the function of the immune system. The most effective priming of naïve CD8 T cells occurs in response to peptide–MHC-I complexes expressed by DCs, and yet many viruses do not infect these cells but rather target other cell types. It is now recognized that viral antigens produced by infected cells can be taken up by DCs and loaded into class I molecules in a process referred to as cross-presentation.

Once activated, CD8 T cells can exert their cytotoxicity activity. There are two major mechanisms of cytotoxicity. One involves the production by the CTL of perforin, a molecule that can insert into the membrane of target cells and promote the lysis of that cell. Perforin-mediated lysis is enhanced by a series of enzymes produced by activated CTLs, referred to as granzymes. The other mechanism involves the Fas ligand expresses by CTL. The interaction of fas ligand on the surface of the CTL with fas on the surface of the target cell initiates apoptosis in the target cell (1).
1.3 Tumor Immunity: tumor recognition by the cells of the immune system

Tumor development is associated with the acquisition of gene mutations and expression of neoantigens or overexpression of cellular self proteins, which could be target for recognition by the immune system (1). CD8\(^+\) T cells can recognize different non-mutated or mutated antigens.

Non-mutated self antigens

Cancer/testis antigens
The first antigen shown to be recognized by human tumor reactive T cells is MAGE-1. It was isolated by screening a melanoma genomic DNA library derived from the MZ2-MEL cell line with a CTL clone that recognized MZ2-MEL cells (2). MAGE-1 was found to be a nonmutated gene and a member of a large, previously unidentified gene family. The T cell epitope identified using the MAGE-1 reactive CTL clone was recognized in the context of the HLA-A1 restriction element. Several additional members of the MAGE gene family have now been shown to encode T cell epitopes recognized by tumor reactive T cells (3,4). These genes are expressed exclusively in the testes, but not in other normal tissues, and have been termed cancer/testis antigens. Testes fail to express HLA molecules and thus are not recognized by T cells reactive with members of this gene family. Members of the MAGE gene family are expressed in a variety of tumor types, including melanoma, breast, prostate and esophageal cancer.

Another antigen of this family is NY-ESO-1, which was initially identified using the antibodies screening technique (5). It represents a cancer/testis antigen that is unrelated to the MAGE family of genes. The NY-ESO-1 molecule is expressed in approximately 30% of breast, prostate, as well as melanoma tumors. In contrast to other antigens such as tyrosinase and MAGE-1, for which infrequent antibody responses have been observed, 10
out of 12 patients with NY-ESO-1–positive tumors possessed serum antibodies directed against this antigen (6).

**Melanocyte Differentiation Antigens**

Among these antigens there are MART-1 and Melan-A. The genes encoding the melanoma antigen MART-1 (7) and Melan-A (8) were isolated following the screening of melanoma cDNA libraries with an HLA-A2–restricted tumor reactive TIL and a CTL clone derived by in vitro sensitization, respectively. The MART-1 gene encoded a 118 amino acid protein that is expressed in between 80% and 90% of fresh melanomas and cultured melanoma cell lines (9). The majority of melanoma-reactive, HLA-A2–restricted TIL were shown to recognize MART-1, indicating that this is a highly immunodominant antigen (10,11). The MART-1 antigen is representative of a set of gene products, termed melanocyte differentiation antigens (MDAs) that are expressed in melanoma as well as in normal melanocytes present in the skin as well as in the retina.

Other proteins involved in the synthesis of melanin were subsequently found to represent MDAs, including the gp100, tyrosinase, tyrosine-related protein (TRP-1), and TRP-2 gene products.

Epitopes from many of the MDAs have been found to be recognized in the context of restriction elements other than HLA-A2, and in addition, epitopes on these molecules recognized by murine tumor reactive T cells have been identified. Tyrosinase (12,13), TRP-1- and TRP-2-reactive T cells (14-16) have been shown to these gene products in the context of a variety of HLA class I alleles. A peptide epitope recognized by HLA-A2–restricted, TRP-2–reactive T cells, SVYDFFVWL (TRP-2:180-188), also represented a dominant epitope recognized by H-2Kb restricted T cells (17). Adoptive immunotherapy studies carried out using the B16 murine melanoma also demonstrated that this is a tumor rejection antigen (18).
Overexpressed antigens

T cells have been found to recognize also gene products that are expressed at low levels in normal tissues but that are overexpressed in a variety of tumor types. As an example, screening of an autologous renal carcinoma cDNA library with a tumor reactive, HLA-A3–restricted T cell clone resulted in the isolation of fibroblast growth factor-5 (FGF5) (19), a protein that is expressed only at low levels in normal tissues but up-regulated in multiple renal carcinomas as well as prostate and breast carcinomas.

Mutated antigens

A variety of mutated antigens have also been identified as targets of tumor reactive T cells. Mutated gene products that play a role in promoting tumorigenesis may frequently serve as the targets of tumor reactive T cells, as there may have been selective pressure to maintain expression of these products (20-22).

The observations made in murine studies indicating that immunization against an individual tumor does not generally result in cross-protection against multiple tumors has lead to the suggestion that mutant T cell epitopes represent the dominant antigens responsible for tumor rejection (23). The identification of mutated antigens is also difficult due to the problems with identifying the appropriate class I or class II restriction element and may have lead to their being underrepresented in studies attempting to identify human cancer antigens. In addition, the nature of mutated antigens may make them more potent targets, as T cells reactive with these epitopes may not have undergone the same degree of negative selection as those that are reactive with normal, nonmutated self-antigens.
1.4 Immunosurveillance vs Tolerance

Although appropriate T cell activation can lead in some cases to tumor regression, the normal role of the immune system in controlling tumor development is unclear. Burnet and Thomas theorized in the 1950s that the immune system plays an important role in preventing the outgrowth of tumors, a concept that was termed *Immunosurveillance* (24). More recent studies in mice have provided some evidence that the immune system may prevent the outgrowth of tumors bearing highly immunogenic antigens, while tumors that possess mechanisms that prevent the immune system from responding appropriately can grow progressively, a mechanism that has been termed *Immunoediting* (25). Factors that influence tumor cell growth include the immunogenicity of antigens expressed by those cells as well as the sensitivity to host factors that regulate immune responses. Tumors isolated from immunodeficient mice in many cases are rejected following injection into normal mice (26), and enhanced rates of tumor development in response to carcinogen treatment have been observed in mice that lack responsiveness to IFN-γ (27). Expression of pro-inflammatory cytokines such as IFN-γ may be necessary to overcome the effects of soluble inhibitory factors such as TGF-β and IL-10, as well as products expressed in T cells such as CTLA-4 and PD-1 that act to limit normal anti-tumor responses (28,29). Examination of the association between lymphocyte infiltration of tumor sites and patient survival has provided some support for the role of immunosurveillance in controlling tumor growth. The presence of tumor infiltrating lymphocytes (TIL) in a variety of tumor types has been associated with improved survival (30-32). As the presence of a higher ratio of T regulatory cells (Treg) to CD8+ T cells within tumors has been associated with poor prognosis (33-35), further analysis of tumor as well as other sites may be needed to evaluate this issue.

Selective pressure exerted by the immune system can then lead to the outgrowth of tumor variants containing mutations that lead to the loss of recognition by cells of the immune system (*immune escape, Fig. 1*). Relatively
small groups of malignant or premalignant cells that avoid detection by the immune system initially develop random mutations, and pressure exerted by immune cells can then lead to the selective outgrowth of variants that have lost expression of gene products such as HLA class I molecules.

Several mechanisms have been shown to result in the loss or down-regulation of HLA class I expression. β2-microglobulin mutation/deletion results in HLA class I total loss. Defects in components of the antigen-processing machinery that lead to decreased HLA class I expression on the cell surface of human tumors have been described (36). Selective loss of an HLA class I haplotype, locus, or allele has also been observed in human tumor cells (37), as well as the loss of β2-microglobulin (38). While all these mutations could theoretically lead to immune escape, those cells should then be more susceptibility to lysis by NK cells. Recent studies have shown, however, that NK cells express activating receptors such as NKG2D, which bind to stressed-induced ligands (MICA and MICB) that can be up-regulated in a variety of tumors (39). Activation of NK cells through this signaling pathway can overcome the inhibitory effect of HLA class I binding receptors (KIRs). From these data, it is clear that HLA class I–negative tumors should be susceptible to NK killing. Loss or down-regulation of MICA/B expression by actively growing tumors has been suggested as an
escape strategy, even if there is no experimental evidence for this, and actively proliferating tumor cells have been found to express MICA/B independently of cellular stress (39). An alternative explanation for why tumor cells that have lost HLA class I are not destroyed by NK cells may be derived from the activation-inhibition model. NK cells are rapidly activated in the presence of stimulatory factors such as IL-12, IL-2, or type 1 IFNs that are released in response to inflammatory conditions associated with microbial infections. In the tumor microenvironment such stimulatory factors may not be readily available and the cross-talk between DCs and resting NK cells that normally leads to NK cell activation may not occur. In addition, the production of immunomodulatory cytokines such as TGF-β or macrophage migration inhibitory factor (MIF) by tumor cells may directly inhibit NK cell activation and function. Loss of expression of surface antigens can occur independently of the dysregulation of HLA class I expression.

Defective death receptor signaling is another mechanism that may contribute to the survival and proliferation of tumor cells. Recent studies have shown the expression of the caspase-8 inhibitor cFLIP (cellular FLICE inhibitory protein) in various tumors. In these cases, cFLIP may render tumor cells resistant to death receptor–mediated apoptosis and may contribute to immunoresistance to T cells in vivo (40,41). Down-regulation or loss of Fas expression in tumors may also contribute to their resistance to apoptosis. Missense mutations and loss of the Fas gene have been identified in hematological cancers such as multiple myeloma (42) as well as in melanomas (43).

Besides, like most normal tissues, tumor cells usually do not express costimulatory molecules such as B7-1 (CD80), B7-2 (CD86), and CD40. Recognition of tumor antigens by CD4+ and CD8+ T lymphocytes without adequate costimulation will lead to T cell anergy.

Also some cytokines can negatively affect maturation and function of immune cells. Vascular endothelial growth factor (VEGF), which is a cytokine that is produced by most tumors, has been shown in several in vitro studies to
inhibit DC differentiation and maturation through suppression of the transcription factor NF-κB in hematopoietic stem cells (HSCs) (44).

Finally, other immunosuppressive mechanisms involve Treg. These cells are crucial for the maintenance of the peripheral self-tolerance and for the suppression of anti-tumor responses, exerted by different mechanisms, among which the competition for IL-2 with effectors T cells and the secretion of TGF-β play a crucial role (45). Recently, it has been described a new population of CD11b+ IL-4Ra+ cells, known as myeloid derived suppressor cells (MDSC), able to inhibit antigen-activated CD8+ cells, mainly by the production of IL-13 and IFN-γ (46). MDSC may also overexpress both inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1), enzymes involved in the metabolism of arginine. Depletion of arginine from the microenvironment inhibits T cell activation and proliferation, and favors T cell apoptosis (47). Furthermore, iNOS produces nitric oxide (NO), which interferes with IL-2 receptor signaling, leading to cell cycle arrest. Reactive oxygen species (ROS) and peroxynitrites, bioproducts of arginine metabolism, contribute to T cell inhibition.

All together, these phenomena lead to the loss of an effective immune response against tumor antigens, a phenomenon called immune tolerance.
1.5 Cancer Immunotherapy strategies

A wide variety of immunotherapies have been evaluated in model systems and are now being developed for treatment of patients with cancer, which include those that involve direct immunization of patients with a variety of immunogens (active immunotherapy), the adoptive transfer of activated effector cells (adoptive immunotherapy) as well as the passive immunotherapy with monoclonal antibodies and immunomodulating agents.

Active Immunotherapy

Vaccination has been carried out using peptides either in saline or in adjuvants such as incomplete Freund's adjuvant, DCs that have been pulsed with peptides, with naked DNA encoding T cell epitopes, preparations of heat shock proteins prepared from tumor cells, as well as whole tumor cells and tumor lysates that have been pulsed on APCs. In addition, recombinant immunogens have been incorporated into viruses such as vaccinia, bird poxviruses, and adenoviruses that have been used successfully to protect against infectious diseases (48).

Peptides vaccines

Multiple trials have been carried out by administering peptides to patients either in saline or oil-in-water emulsions such as Montanide. Immunization resulted in the expansion of T cells reactive with peptides such as the dominant gp100:280–288 (49) and tyrosinase:369-377 (50) peptides; however, the levels of T cells reactive with these epitopes was generally less than 0.01% of peripheral CD8+ T cells. Similar levels have been observed using recombinant viral vaccines directed against epitopes derived from carcinoembryonic antigen (51) and prostate-specific antigen (52). Prime-boost regimens employing immunization with recombinant viral constructs
followed by boosting with peptide immunization is a strategy that has been found to result in enhanced frequencies of T cells reactive with viral epitopes. Multiple peptides have also been used to vaccinate patients, in attempts to circumvent the escape of antigen loss variants. Evidence has been presented indicating that it is possible to generate T cell responses directed against multiple melanoma antigens (53), and increased frequency of T cells reactive with multiple peptides used for immunization were observed in peripheral blood as well as in sentinel lymph nodes that drained the immunization site (54).

Although the expansion of T cells directed against the immunizing peptide was generally observed in these trials, generally less than 5% of the treated patients demonstrated clinical response (55).

**Whole cells vaccines**

Clinical trials employing whole tumor cells as vaccines were used to activate cells reactive against multiple antigenic targets. Early model studies have demonstrated the ability of autologous tumor cell vaccination to protect mice from subsequent tumor inoculation (56,57). Treatment with a combination of three irradiated allogeneic melanoma cells with BCG (Canvaxin trial) resulted in an overall survival rate of 49%, as opposed to a rate of 37% in patients with melanoma who did not receive the vaccine (58). Further evaluation of this approach in phase III trials, however, showed no significant difference in survival of patients with stage III or IV melanoma receiving this treatment compared to controls receiving BCG alone (59).

Also genetic modified cells have been evaluated for their effectiveness in cancer therapy protocols. In a murine model system, mice that were immunized with B16 melanoma cells transduced with genes encoding 10 cytokines were examined for their resistance to a subsequent inoculation of the wild-type B16 tumor (60). The results indicated that tumors transduced with the GM-CSF gene provided significant protection against B16 tumor
challenge, and a lower level of protection was observed in mice immunized with IL-4 and IL-6 transduced tumors.

**DC vaccines**

Other approaches have used immunization with professional APCs such as autologous DC. One approach has employed immunization of prostate cancer patients with DCs that have been transfected either with mRNA encoding individual tumor antigens or bulk mRNA isolated from tumor cells (61,62). Although expansion of tumor reactive T cells following these treatments has been reported, only sporadic cases of tumor regression were observed in these trials. In one study carried out by the Dendreon Corporation (Seattle, WA), an APC vaccine loaded with an antigen called prostatic acid phosphatase linked to GM-CSF was used to treat men with hormone-refractory prostate cancer (63) and recently FDA approved this formulation.

**Recombinant vaccines**

In some clinical trials recombinant viral constructs or naked DNA encoding particular tumor antigens have been used. In these trials, recombinants containing either full-length gene products or mini-genes containing individual or multiple T cell epitopes were evaluated. The dominance of viral epitopes led to the failure in general to detect enhanced precursor frequencies directed against the tumor antigens.

In studies carried out in a mouse model system, evidence for enhanced immunity was obtained following immunization with a recombinant virus that contained the genes encoding the candidate tumor antigen CEA along with genes encoding LFA-3, ICAM-1, and B7-1, termed TRICOM (64). In a phase I clinical trial, injection of a vaccinia virus TRICOM construct directly into melanoma lesion resulted in one partial response and one complete
response out of the 12 treated patients (65). Larger randomized patient studies will be needed to establish the efficacy of this approach. All these vaccination strategies achieved the goal of increasing the frequencies of CD8+ T cells in the blood of patients. However, only in some cases tumor rejection occurred.

Adoptive Immunotherapy

One explanation for the poor response observed in active vaccines trials could be due to the pre-treatment conditioning and poorly immunogenic vaccine formulations (66).

To overcome the low frequencies of antigen-specific T cells and to expand these cells, in some preclinical and clinical trials it has been used an Adoptive T cells transfer (ACT) therapy. It consists in the infusion of various mature T cell subsets, that can be classified as autologous or allogenic depending on the T cell sources, in order to eliminate tumors and eventually prevent their recurrence (67). Among these, ACT therapy with autologous T cells has been well studied for the treatment of solid tumors, (melanoma) (68).

Adoptive cell transfer (ACT) therapies accomplish T cell stimulation ex vivo by activating and expanding autologous self-/tumor reactive T cells to large numbers that are then transferred back to the patient (69). In the case of melanoma patients, tumor reactive T cells were obtained from tumor infiltrating lymphocytes (TIL) and then re-infused into the patients in combination with IL-2 with success (70).

From retrospective studies, it is now becoming more and more relevant the importance of the subset of T cells transferred to the patient. ACT therapies should be now performed with long-lasting T central memory (T_{CM}) and T stem cell memory (T_{SCM}) cells able of long-term immunosurveillance as well as tumor eradication (71).

Another critical issue in ACT therapies include host preconditioning. In an animal model of melanoma, the transfer of in vitro cultured TCR transgenic
CD8+ cells reactive against a self/tumor antigen gp100 caused substantial tumor regression of s.c. B16 melanoma tumors (72), and the efficacy of this treatment could be improved by prior recipient lymphodepletion (73). This strategy is able to remove endogenous cellular elements that acts as a “sinks” for common gamma chain cytokines (IL-7 and IL-15), capable of improving the efficacy of adoptively transfer tumor-specific CD8+ T cells by enhancing their functional capacity. Lymphodepletion may also reduce the number of CD4+CD25+FoxP3+ Treg cells, reducing the immune tolerance of the microenvironment (74). Lymphodepletion also provides “space” allowing for homeostatic expansion of the infused T cells and DC activation by microbial translocation from gut microflora (75).

To support large-scale trials, alternative ACT treatments have been developed in more recent years. For instance, TCR gene transfer has recently emerged as a potential alternative strategy. PBMCs are modified by the introduction of tumor antigen-specific receptors to improve the efficacy of these cells for ACT therapy (76). For the introduction into the lymphocytes of specific genes, retroviral and lentiviral vectors have been used with success. To reduce the influence of endogenous TCR chains in forming new pairing with the inserted chains (mispairing), murine constant region sequences or cystine residues that favor the pairing between inserted chains have been used (77).

Recently, the generation of chimeric TCR has improved a lot this strategy. In this case antibodies are directly fused to intracellular T-cell signaling chains such as CD3. Thus, chimeric lymphocytes can avoid the limitation of MHC restriction and specifically recognize cell surface TAAs (77). Although this therapy mediated the regression in patients with metastatic melanoma (78), the safety of this approach still needs to be clarified.

**Passive Immunotherapy**

Passive immunotherapy is a strategy aimed at targeting specific tumor antigens or stromal molecules with neutralizing antibodies. The
administration of monoclonal antibodies against tumor antigens in HER2-positive breast cancer (Trastuzumab) (79), B-cell lymphoma (Rituximab) (80), and head and neck, lung and colorectal cancer that expresses the epidermal growth factor receptor (Cetuximab) is clinically effective (81). Efforts are ongoing to produce monoclonal antibodies with new effector functions against known antigens. Among the antibodies directed against other molecules than antigens there is the Bevacizumab, directed against the vascular endothelial growth factor (VEGF) (82), while among the cytokine specific therapies, there is the infusion of IL-2 for the treatment of patients with metastatic melanoma and renal cancer.

Recent studies in tumor immunology have focused on the concept of immune checkpoints, a series of molecules that function to limit an ongoing immune response. Administration of a blocking antibody direct against Cytotoxic T Lymphocyte Antigen 4 (anti-CTLA-4) has been shown to result in enhanced regression in murine tumor model systems when administered in combination with an anti-CD25 antibody and tumor vaccination (83) or with antibodies directed against activating molecules such as 4-1BB (84). CTLA4 blockade has been evaluated in several malignancies, but the most well-developed data come from trials in patients with melanoma, in which the blocking agent is associated with an approximate 10% objective response rate but, also, a significant rate (25–35%) of clinically important immune-related toxicity (85-86). The specific antibody (Ipilimumab) was recently approved by the FDA for the treatment of metastatic melanoma.

Another immunological checkpoint that has been targeted recently in clinical trials is that mediated by the molecule known as programmed cell death 1 (PD1). PD1 was initially identified in a library-based screen of CD8+ T cells undergoing apoptosis (87). Subsequent work identified the ligand for PD1 as B7-H1 (also known as PDL1) (88,89) and showed that the interaction between PD1 and B7-H1 leads to an inhibition of T cell function. In animal studies, PD1 blockade potentiates an antitumor immune response (90-92) and it is now being tested in some clinical trials (93).
1.6 Prostate Cancer Immunology and Immunotherapy

In the western world, prostate cancer is the most common non-cutaneous cancer in men, and it ranks third overall in terms of mortality (behind lung cancer and colon cancer) (94).

As is the case for most types of cancer, the precise etiology of prostate cancer is unknown; however, a great deal of literature supports the hypothesis that both genetic (95) and environmental (96) factors are important. Interestingly, human (97) and animal studies indicate that inflammation might have a role in prostate cancer development, as well as in the progression from organ-confined to metastatic disease (98,99). Like most solid tumors, prostate cancer generally progresses through a series of stages, known as clinical states (100).

In developed countries, many cases of prostate cancer are initially detected by monitoring the levels of Prostate-Specific Antigen (PSA) in the blood. Increased (or changing) levels of PSA prompt a biopsy, and a diagnosis of prostate cancer is based on microscopic evaluation of the biopsy specimen. Diagnosis generally leads to an attempt at local treatment, with either surgery or radiotherapy. For up to 80% of surgically treated men, local treatment is successful in that metastatic disease does not occur within 15 years (101).

When disease does recur, the initial manifestation is often a rising PSA level without radiologically detectable metastases, a clinical state known as biochemical progression (102,103). Men with biochemically recurrent disease can be treated with androgen ablation, through either surgical or chemical castration (104). Eventually, many men with recurring prostate cancer develop metastatic disease, despite androgen ablation. This disease state is known as metastatic, castration-resistant prostate cancer and there are few therapeutic agents for this state of the disease. Among these, the Docetaxel has been approved in Europe. This is also the state in which most immunotherapy approaches have been clinically evaluated. These men have a median survival of ~16 months (105,106).
The goal of most approaches of prostate cancer immunotherapy is to activate and/or increase the population of effector T cells, which can then traffic to evolving tumor and mediate the specific lysis of cancer cells. Several studies identified self antigens overexpressed in human prostate specimens. Among these, not only PSA, but also other antigens have been discovered, such as Prostatic Acidic Phosphatase (PAP), Prostate-Specific Membrane Antigen (PSMA), Prostate Stem Cell Antigen (PSCA), and Six-Transmembrane Epithelial Antigen of the Prostate (STEAP) (107-109). Different approaches of immunization based on these antigens have been used in past years. These can be summarized into active and passive immunotherapy.

**Active Immunotherapy**

**Poxvirus-based vaccines**

Poxvirus vectors were extensively used in different clinical trials of prostate cancer. In vivo, they infect epithelial cells that undergo cell death. Cellular debris, including encoded antigens, are then taken up by nearby immature APCs, which, when appropriately activated, can present these antigens to CD4+ and CD8+ T cells in a pro-inflammatory context. Direct infection of APCs, particularly the Langerhans cells in the skin, is another mechanism by which poxvirus vectors can prime an immune response. For prostate cancer, PSA-targeted vaccinia virus-based immunotherapy has proceeded through several steps, including the incorporation of PSA DNA encoding co-stimulatory molecules (lymphocyte function-associated antigen 3 (LFA3), CD80 and intercellular adhesion molecule 1 (ICAM1); known as TRICOM) into the vaccine (110), as well as optimization of the MHC class II-binding properties of the vaccine antigen (111). The main disadvantage of poxvirus-based vectors results from their propensity to induce a strong antibody response that makes homologous prime-boost regimens ineffective, as the antibody response to viral proteins dominates over the desired response to encoded antigen (112). To circumvent this immunological limitation, a semi-
heterologous prime–boost strategy involving a vaccinia virus prime followed by an analogous fowlpox virus boost (ProstVac VF–TRICOM; Bavarian Nordic) was optimized (112). The clinical development of this agent has been recently reviewed (113), and includes several trials in which ProstVac VF was combined with other conventional or experimental agents (114).

DC-based vaccines

Different clinical trials using dendritic cells have been carried out in recent years. DCs pulsed with Prostate Stem Cell antigen (PSCA) and PSA were used in a Phase I/II clinical trial. This immunization protocol was able to induce an immune response in hormone and chemotherapy-refractory patients. This response correlated with an increase in overall survival (115). The most advanced study regarding the use of DCs to induce an immune response is that referred to Sipuleucel-T (Provenge; Dendreon Inc.) This is a personalized product that is individually manufactured for each patient with prostate cancer (116). First, leukopheresis is carried out, and monocytes are enriched in the leukopheresis product through density–gradient centrifugation. These cells are then incubated with the targeted immunogen, a fusion protein linking granulocyte–macrophage colony-stimulating factor (GM-CSF) to Prostatic Acid Phosphatase (PAP), before intravenous administration. Once infused, these autologous monocytes are thought to mature into functional APCs and to activate PAP-specific CD4+ and CD8+ T cells in treated patients. These activated T cells are then thought to home to tumor lesions, mediating an antitumor response. In this approach, PAP was chosen as the target antigen based on preclinical studies in a rat model that showed that tolerance to PAP in prostate cancer was not mediated by central deletion of PAP-specific T cells, such that PAP-directed vaccination could induce marked T cell infiltration into the prostate gland (117). In terms of clinical development of immunotherapies for prostate cancer, this agent has progressed the furthest: three Phase III studies have been completed (63) and US Food and Drug Administration (FDA) approval was granted in April

**Fig.2. Sipuleucel-T therapy (CancerNetwork).**

-DNA-based vaccines

Different antigen-specific approaches to cancer immunotherapy involved DNA-based vaccines. DNA can be rapidly and precisely synthesized, making it straightforward to target nearly any selected antigen (118). The main disadvantage of DNA-based vectors is their low level of immunogenicity relative to the highly immunogenic viral vectors described above. To improve the outcome, pro-inflammatory molecules — such as herpes simplex virus type 1 tegument protein VP22 (to enhance spreading from transfected cells to DCs) or Toll-like receptor (TLR) agonists (to activate APCs) — have been incorporated into DNA-based vaccines (119), or the vectors have been co-administered with GM-CSF as a nonspecific adjuvant. In this context, GM-CSF was used with the aim of recruiting APCs, particularly DCs, to the vaccine site (120). A recent clinical study (121) highlighted the potential utility
of DNA-based vectors in men with prostate cancer: in a population of men with biochemically recurrent disease given a DNA vaccine encoding PAP, PAP-specific T cell responses were induced, as well as an inhibition of the rate of PSA level increase.

**Whole cells vaccines**

The use of whole cells vaccines for prostate cancer is known as GVAX (BioSante) (122). This formulation includes GM-CSF-transduced tumor cells used as a vaccine. Such cells are injected intradermally; the GM-CSF attracts APCs and T cells to the vaccine site, thereby priming an immune response to tumor antigens. Earlier GVAX trials attempted to engineer a vaccine using autologous tumor cells from individual patients (123), but it was later appreciated that tumor antigens can be cross-presented on patients' APCs (124), so further clinical development focused on allogeneic tumor cell lines of a particular cancer type transduced to secrete GM-CSF. This approach has been developed for several types of cancer, including prostate cancer (125). Prostate GVAX, for example, includes the androgen-sensitive prostate cancer cell line LNCaP, as well as the castration-resistant prostate cancer cell line PC3, and early phase clinical trials suggested that prostate GVAX could induce new antibodies specific for the cell lines injected (126). Similar to Sipuleucel-T, clinical development of prostate GVAX has advanced to the level of randomized Phase III clinical trials. However, for various reasons, these trials have so far not been successful (127), and have been stopped.

**Passive immunotherapy**

Passive immunotherapy aim at targeting specific tumor antigens with neutralizing antibodies. Prostate-specific Membrane Antigen (PSMA) is overexpressed on tumor-associated vasculature, as well as on the cell surface of prostate cancer cells, making this agent potentially applicable to
other types of cancer (128). Early clinical trials of a humanized, PSMA-specific antibody (J591; Cornell Weill Medical College) showed impressive tumor targeting, but few objective clinical responses were noted in the patients with advanced tumors who were included in these studies (129). Similar to monoclonal antibodies developed for the treatment of other types of cancer, the current development of J591 has progressed to a radioisotope-labeled version, with the goal of mediating cancer cell death by localizing a radioactive β-ray emitter close to a patient's tumor mass (130). Several trials involving 177Lu-labelled J591 are currently in progress, including studies combining this agent with conventional cancer therapy.

Among the Immune checkpoint blockade, Ipilimumab has been evaluated in several Phase I and Phase II trials in patients with prostate cancer, and objective clinical responses and decreases in PSA levels have been described (131). Based on those data, a Phase III trial comparing Ipilimumab with a placebo is currently underway in men with castration-resistant metastatic disease who have not responded to prior chemotherapy (clinicaltrial.gov).

PD1 has been less well studied in prostate cancer, although it was found that the CD8+ T cells that infiltrate the prostate gland in men with cancer seem to express PD1 (132). A Phase I clinical trial of a fully human monoclonal antibody targeting PD1 has been completed, with interesting results (133). First, this agent was remarkably well tolerated, with few serious adverse events noted. Second, several objective clinical responses were noted in patients with various types of cancer. Taken together, these data reinforce the relative importance of immune checkpoint blockade in tumor immunotherapy.

In recent years, it is becoming more and more relevant how cancer immunotherapy need to be combined with conventional therapy to achieve maximal patient benefit. Fortunately, many conventional treatments for prostate and other cancers have beneficial immunological effects, making combinatorial trials an attractive proposition. Even chemotherapy, which is
broadly viewed as immunosuppressive, might to some extent boost an antitumor response (134). Recently also the androgen ablation has been revised from a clinical point of view. The immunological effects of androgen ablation are surprising because they involve also the thymus, which is generally not thought of as an androgen-sensitive organ (135). In aged mice, androgen ablation seems to result in regeneration of the normally involuted thymus and in the output of new T cells in peripheral blood (136). Similar effects have been observed in humans were in some trials androgen ablation before prostate cancer surgery results in the infiltration of activated CD4+ T cells into the prostate gland (137). Also recent data showing the induction of new antibody specificities in treated patients support a pro-immunogenic role for androgen ablation (138,139). For these reasons, this therapeutic approach has been evaluated in several clinical trials. An early study tested one dose of vaccinia virus–PSA vaccine (ProstVac) in combination with androgen ablation, finding the combination to be well tolerate (140). In a later randomized study, immune responses to ProstVac were more commonly observed in men who received androgen ablation after active immunotherapy (141), as opposed to receiving androgen ablation before immunotherapy. Taken together, these studies support the notion that combined immune and hormonal therapy might be clinically interesting and worthy of further evaluation.

Also radiotherapy has been revised recently in combination studies. Although the cytotoxic effects of radiation therapy are well-known, recent data support the notion that irradiation of cancer cells can prime an antitumor immune response (142). On a cellular basis, this process seems to involve the uptake of dying tumor cells by APCs (143) and the presentation of tumor antigens to immune cells, as well as the induction of a pro-inflammatory microenvironment by the radiation (144). In patients with prostate cancer, evidence for an immunological effect of radiotherapy is provided by data showing the induction of new antibody specificities following radiotherapy treatment (147). Although the molecular mechanisms for these immunological effects of radiotherapy are complex, recent work has shown
that high mobility group box 1 (HMGB1) released from dying tumor cells can function as a TLR4 agonist, activating APCs in either the tumor parenchyma or in the draining lymph node, and so priming an immune response (142). These immunostimulatory effects are not unique to radiation-induced tumor cell death, but can also be elicited when tumor cells are killed by certain chemotherapy agents. Several preclinical studies support the notion that combining irradiation with immunotherapy can be either additive or synergistic in terms of the antitumor response (145, 146). This concept has been evaluated clinically in a small randomized trial of men undergoing primary radiotherapy for prostate cancer (147); 13 out of 17 patients in the radiotherapy and immunotherapy combination treatment group had a greater than threefold increase in the number of PSA-specific T cells, whereas no increase in the number of PSA-specific T cells was noted in the group that received radiotherapy alone. However, as is the case for combining chemotherapy with immunotherapy, the relative sequencing of agents might be crucial (148).
1.7 Pre-clinical studies in murine model

Murine models represent a unique tool to study in vivo the effects of new vaccination protocols against prostate cancer. The vast majority of immunotherapeutic settings were tested in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model. TRAMP mice are transgenic for the SV40 early genes (large T and t antigens; Tag) expressed under the control of probasin regulatory element (149). While marginal expression of Tag in the thymus causes deletion of high avidity T cell clones, in the periphery Tag is selectively expressed in prostate epithelial cells under the influence of sex hormones. Animals remain Tag-negative and healthy until the puberty [i.e. (wk) 4-5]. In the following weeks all TRAMP mice invariably and progressively develop mouse prostate intraepithelial neoplasia (mPIN; wk 6-12), adenocarcinoma (wk 12-18), and lymph nodes and visceral metastasis (wk 18-30, Fig.3).

![Fig.3. Progression of prostate cancer in TRAMP mice.](image)
TRAMP mice castrated at wk 12, almost invariably (80%) develop a hormone refractory PC within 10-12 wk (149). Thus, cancer development, androgen sensitivity, fine aspects of neo-angiogenesis and metabolic activity in TRAMP mice resemble human PC. In TRAMP mice, the large T encoded antigen (Tag) behaves as a TAA. Disease progression in these mice correlates with induction of tumor-specific peripheral T cells tolerance (150), which recapitulates the tolerance state found in patients with advanced disease. Thus, TRAMP mice have documented progression to invasive carcinoma of epithelial origin and metastasis closely resembling the human pathology.

At 6-7 weeks of age a single vaccination with dendritic cells (DC) pulsed with the Tag-derived immunodominant CTL epitope Tag-IV elicits the induction of Tag-IV-specific CTL. However, TRAMP mice older than 10 weeks fail to respond to DC-mediated vaccination. This suggests that during the course of PC progression, mice become progressively tolerant to Tag (150). In the studies conducted in TRAMP mice different immunogenic strategies have been used. Prostate Stem Cell Antigen (PSCA) DNA followed by an heterologous boost of mPSCA-VRP (virus replicating particles) is able to induces CD4+ and CD8+ T cells that delay prostate cancer growth in a transplantable model (TRAMP-C1 tumor) (151). A similar strategy of priming and boosting was able to delay prostate cancer growth in both young and old TRAMP mice, even if a castration step in old TRAMP mice was necessary to obtain the therapeutic effect (152). Also the vaccination with a plasmid coding for murine PSCA delivered i.m. was able to delay TRAMP-C1 growth in C57BL/6 mice. The immune response obtained in these mice was increased with an adoptive transfer (AT) of splenocytes of immunized WT mice (153). Another antigen that was used in the immunization protocols is Six-Transmembrane Epithelial Antigen of the Prostate (STEAP). STEAP-based vaccination is effective in vivo in prophylactic models. Using CD4 or CD8 knockout mice it was evident how not only CD8 but also CD4 play a relevant role in the immune response against prostate cancer. Unfortunately, a therapeutic protocol based on this approach did not completely control tumor growth (154). DCs pulsed with TRAMP-C1 tumor lysates were used as
immunogen in a preventive setting of vaccination with a priming of recombinant adenoviruses coding for PSCA, STEAP and PSMA (155). These results demonstrated how the prophylactic protocol is the only one able to significantly control tumor growth. STEAP protein was scored by MHC peptide binding algorithms to predict potential STEAP sequences capable of stimulating in vitro naïve CTLs. Different sequences have been tested in vitro for their capacity to stimulate HLA-A2-restricted CTLs (156) or in vivo in HLA-A*0201 transgenic HHD mice (157). Among these, different sequences were able to induce a STEAP-specific immune response and have been proposed for further studies.
1.8 Aim of the thesis

The aim of this thesis project was to evaluate the immune response against prostatic antigens such as PSCA and STEAP in C57BL/6 WT and prostate cancer-prone TRAMP mice, and to compare this response to that obtained with Tag IV immunization. Major goal was to investigate the dynamic of tolerance induction for this two tumor antigens over-expressed during spontaneous tumor development and progression in TRAMP mice. To do that, a dendritic cells (DC)-based vaccination strategy will be used, followed by a comparative gene expression analysis of different TAA, in order to dissect a possible molecular mechanism underlying the different immune response to various antigens.
2. Materials and Methods

2.1 Mice, cell lines and reagents

C57BL/6 mice (H2\(^b\)) were purchased from Charles River (Calco, Italy) and were bred in the Institutional specific pathogen-free animal facility. Heterozygous TRAMP mice on a C57BL/6 background (149) were housed and bred in our animal facility, treated in accordance with the European Union guidelines, and with the approval of the Institutional Ethical Committee. TRAMP mice were typed for Tag expression by PCR-based screening. Isolation of mouse-tail genomic DNA was performed by digestion with NaOH. RMA is a H2\(^b\) Rauscher virus-induced thymoma. TRAMP-C1 cells are a stable cell line derived from murine prostate cancer specimen (158). B6-k0 cells are SV40 transformed kidney cells that express Tag IV (150). For in vitro studies, \(3 \times 10^7\) spleen cells were cultured for 5 days in RPMI medium supplemented with 2mM L-glutamine, 100U/mL Streptomycin/Penicilin, 50\(\mu\)M \(\beta\)-mercaptoethanol, 10\(\mu\)M Hepes, 1mM sodium pyruvate (Invitrogen Life Technology, Milan, Italy) and 10% heat-inactivated fetal bovine serum (FBS, Euroclone, Milan, Italy) at 37°C in a 5% CO\(_2\) atmosphere. For PSCA restimulation, spleen cells were co-culture with K\(^b\) specific PSCA\(_{83-91}\) peptide (NITCCYSDL), while for STEAP\(_{186-193}\) restimulation spleen cells were co-cultured with K\(^b\) specific STEAP peptide (RSYRYKLL). RMA cells were maintained in RPMI medium supplemented with 2mM L-glutamine, 100U/mL Streptomycin/Penicilin, 50\(\mu\)M \(\beta\)-mercaptoethanol, 10\(\mu\)M Hepes, 1mM sodium pyruvate and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO\(_2\) atmosphere. TRAMP-C1 cells were maintained in DMEM medium supplemented with 2mM L-glutamine, 100U/mL Streptomycin/Penicilin and 10% FBS at 37°C in a 5% CO\(_2\) atmosphere.

All antibodies for Flow Cytometry were purchased from BD Pharmingen (San Diego, CA). These antibodies included fluorescein isothiocyanate (FITC)-conjugated anti-CD44 (clone IM7), Peridinin-chlorophyll-protein complex (PerCP) Cy5.5-conjugated anti CD8a (Ly-2, clone 53-6.7), Allophycocyanin
(APC)-conjugated anti-IFN-γ (clone XMG1.2). Staining buffer solution consists on 1x PBS supplemented with 2% FBS and 0.2% sodium azide. Permeabilization buffer solution consists on Staining Buffer solution supplemented with 2% rat serum and 0.5% Saponin (Sigma). Fixation was performed with 2% Formaldehyde (Sigma). Before staining (15 minutes at 4 °C), cells were incubated with FcR blocker (BD Pharmingen) for 10 minutes at room temperature.

2.2 Dendritic Cells (DC) preparation and immunization strategy

DC were prepared and characterized as previously described (159). Briefly, bone marrow cells were seeded into six-well plates at 2x10^6/mL in ISCOVE medium supplemented with 2mM L-glutamine, 100U/mL Streptomycin/Penicillin, 10% FBS, GM-CSF (25 ng/mL), and IL-4 (5 ng/mL; R&D Systems, Minneapolis, MN). Fresh medium was added on day 3 and day 5. On day 7 of the in vitro culture, LPS (1 mg/mL) was added to the cells for the last 8 hours of culture, and non-adherent and loosely adherent cells were collected. DC were pulsed with either PSCA (2 µg/mL) or STEAP (2 µg/mL) during the last hour at 37° C, washed, and resuspended at 2.5x10^6/mL in PBS. Mice were injected i.d. with 5x10^5 DC. Animals were usually sacrificed 7 days after the last vaccination, or different weeks after in the survival experiments.

2.3 Intracellular cytokine production (ICP) assay

Spleen cells were analyzed for PSCA or STEAP-specific IFN-γ release by intracellular staining in vitro. Briefly, splenocytes were cultured in vitro in 10% FBS RPMI medium in the presence of specific peptide. Day-5 cultures were ficolled and blast were stimulated for 4 hours with RMA cells, unpulsed or pulsed with 5 µg/mL of PSCA or STEAP peptide. Brefeldin A (5 µg/mL; Sigma) was added after 1 hour. Cells were then stained with anti-CD44 and
anti-CD8 antibodies for 15 minutes at 4°C, fixed in 2% Formaldehyde for 20 minutes at room temperature, permeabilized with Permeabilization Buffer and further stained for 30 minutes at 4°C with anti-IFN-γ antibody. 100,000 events were collected in a BD FACS Canto (BD Bioscience). Dead cells were excluded electronically by physical parameters.

2.4 Pentamer and Tetramer Staining

For STEAP specific enumeration, splenocytes were stained with PE-labeled Kb/STEAP tetramers (kindly provided by NIH Tetramer Facility, Emory University, GA). Kb/OVA pentamers (Proimmune) were used as a negative control. Pentamer and tetramer staining were performed in combination with fluorochrome-labeled monoclonal antibodies against CD4, CD8, B220, CD11b and the vitality marker To-PRO3.

2.5 In vitro cytotoxicity assay

In vitro cytotoxicity was measured in standard 4h 51Cr-release assay (150). Briefly, targets cells were labeled with 51Cr for 1 hour and then seeded with 5 days blasts for 4 hours in different effector:target ratios. 51Cr release of target cells was always < 25% of maximal 51Cr-release (target cells in 1% SDS). Lytic activity was estimated by 51Cr-release on the medium by killed targets cells.

2.6 In vivo tumor growth

Mice were challenged s.c. in the left flank with 2.5 x 10^6 TRAMP-C1 cells. Tumor size was evaluated by measuring three perpendicular diameters by a caliper. Animals were monitored twice a week and killed when the tumor reached approximately dimension of 15 mm of mean tumor diameter or when they became ulcerated. Splenocytes were harvested, cultured in vitro for 5
days with the relevant peptide and assessed for in vitro intracellular cytokine production (ICP).

2.7 Haematopoietic Cell Transplantation and Donor Lymphocyte Infusion

Mice received a sub lethal dose of total body irradiation (TBI; 600 Rad) and the day after were transplanted by an i.v. injection of \(1 \times 10^7\) viable female bone marrow (BM) cells derived from C57BL/6 CD45.1/CD45.2 mice. BM cell suspensions from donor mice were prepared by flushing the medullary cavity of the humerus, tibia and femur with PBS. Nucleated viable cells were counted in Trypan Blue using a hemocytometer. Cells suspensions were adjusted to a final concentration of \(5 \times 10^7\) cells/mL in PBS and 200µl/mouse were injected i.v. into the lateral tail vein. Donor Lymphocytes Infusion (DLI) consisted on a i.v. injection of \(6 \times 10^7\) splenocytes derived from female C57BL/6 CD45.1 mice. DLI was performed two weeks after BM transplantation.

2.8 Histology and Immunohistochemistry

At the time of mice sacrifice, the urogenital organs were collected, fixed in 4% formalin for 6 hours, then embedded and included in paraffin wax. Sections (5 µm thick) were cut, stained with H&E (Bio-Optica, Milan, Italy), and scored by a phatologist. Sections were also de-paraffinized in xylene, rehydrated in ethanol, immersed in 600 mL 10mM citric acid pH 6.0, heated in microwave, and cooled at room temperature. Endogenous peroxidase was quenched with 3% \(\text{H}_2\text{O}_2\). Slides were incubated with 5% normal goat serum (Vector Labs, Burlingame, CA) and with primary mAb against CD3 (Serotec) 1:200 overnight at cold, followed by incubation with rat anti-mouse (Vector Labs) diluted 1:100. Slides were then incubated with Vactastain Elite ABC (Vector Labs) following the recommended protocol. Peroxidase activity was
visualized using Nova Red chromogen (Vector Labs) and the counterstain was done with Mayer-Hematoxylin. After dehydratation in ethanol and xylene, slides were permanently mounted in Eukitt (Bio-Optica). CD3 sections were digitally scanned (ScanScope, Aperio) and then analyzed with the Spectrum Plus software (Aperio).

2.9 Disease Score

Macroscopic and microscopic specimens were evaluated by a pathologist blinded with respect to the treatment group to which the mice belonged (150). The score of 0 was given to prostates showing complete tumor regression (CR) and the score of 5 was given to invasive adenocarcinoma or metastases. Prostates with areas of CR scattered among acini affected by adenocarcinoma were defined as partial regression (PR). Mice bearing highly aggressive poorly differentiated neuroendocrine tumors were excluded from the study.

2.10 RNA extraction and Real-Time PCR

RNA from prostates of TRAMP mice at different ages was extracted with the RNeasy Mini Kit (Qiagen) and was quantified by measuring the absorbance at 260 nm. The purity of RNA was checked by measuring the ratio of absorbance at 260 and 280 nm, where a ration ranging from 1.8 to 2.0 was taken to be pure. First strand cDNA was generated from 0.5 µg of RNA using the M-MLV RT and M-MLV RT buffer (Invitrogen Life Technologies), according to the manufacturer’s protocol. Real-Time PCR was performed in a total volume of 20 µL using the SYBR Green PCR Master Mix (Applied Biosystems) and 3 µL of cDNA; specific primers for Tag IV (forward: GCTACACTGTTTGTTGCCCA, reverse: CCCCCACATAATTCAAGCAA), PSCA (forward: TCATCTGTGCTGTGCATGAAT, reverse: GCTCACTGCAACCATGAAGA) and STEAP (forward:
GTCACTGATCTCCATGACTGCT, reverse: GTGGGACTGGGAGTCCGT) were obtained from Primm (Milan, Italy). The Real-Time amplifications included 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. To normalize the mRNA expression, house-keeping gene was amplified (L-19, forward: CTGAAGGTCAAAGGAATGTG, reverse: GGACAGAGTCTTGTGATCTC).

2.11 Statistical Analysis

Statistical analyses were performed using the two-tailed Student's t-test or the Log Rank test. Statistical significance: \( p<0.05 \).
3. Results

3.1 DC-based vaccination with TAA and induction of an antigen-specific immune response in C57BL/6 mice

First of all we tested the capacity of the immunodominant cytotoxic T lymphocyte (CTL) peptide epitopes of PSCA (PSCA\textsubscript{83-91}, NITCCYSDL) and STEAP (STEAP\textsubscript{186-193}, RSYRYKLL) to induce an antigen-specific immune response in C57BL/6 WT mice. These epitopes have been previously demonstrated to bind MHC class I (K\textsuperscript{b}) molecules of C57BL/6 mice (151,154). To this aim, DC (0.5x10\textsuperscript{6} cells) pulsed with PSCA\textsubscript{83-91} or STEAP\textsubscript{186-193} (2 \mu g/mL) were injected intradermally in the left flank of 6-8 week-old mice. A cohort of mice was immunized with unpulsed DC as control. Seven days later, mice were analyzed for their capacity to mount a measurable immune response against PSCA or STEAP. Splenocytes from mice vaccinated with either PSCA or STEAP-pulsed DC were restimulated in vitro with the specific peptide. At day 5, blasts were challenged with RMA (a congenic lymphoma cell line) unpulsed or pulsed with the relevant peptide and assessed for intracellular cytokine production (ICP) by flow cytometry (Fig.1, A and B).

Flow cytometry analyses showed a low but consistent number of CD8\textsuperscript{+} T cells producing IFN-\gamma upon challenge with the relevant antigen, but only in mice vaccinated with PSCA or STEAP-pulsed DC, excluding the possibility of in vitro priming. More importantly, the same blasts produced IFN-\gamma when challenged with TRAMP-C1 cells, a cell line obtained from the prostate tumor of a 32-week old TRAMP mouse (158), therefore demonstrating that T cells elicited by the vaccine recognized the naturally processed endogenous antigen.
Fig. 1. C57BL/6 mice vaccinated with either DC-PSCA or DC-STEAP are able to mount an immune response against the relevant epitope. Splenocytes from C57BL/6 mice (n=9) vaccinated with PSCA (A) or STEAP-pulsed DC (B) were restimulated in vitro with 4 μg/mL PSCA or 2 μg/mL STEAP peptide, and 5-day blasts were assessed in vitro for IFN-γ production by an ICP assay. Blasts were challenged with PSCA_{183-193}/STEAP_{186-193} pulsed RMA cells (RMA-PSCA/RMA-STEAP) and stained with fluorochrome-conjugated CD8, CD44 and IFN-γ antibodies before flow cytometry analysis. Cells were gated on live and CD8+ T cells. Each panel is representative of at least 3 independent experiments. Results of the aggregated data (n = 9) are reported at the bottom of panels A and B. Data are expressed as mean ± SD. IFN-γ production in the presence of RMA was subtracted (* p<0.05).
Blasts were also tested for their cytolytic activity in a standard cytotoxicity assay (150). Blasts were able to kill the relevant target in a specific manner, therefore suggesting that they were fully activated CTL (Fig.2, A and B).

**Fig.2.** CTL from C57BL/6 mice vaccinated with either DC-PSCA or DC-STEAP are able to kill relevant targets. (A) 5-days culture blasts were assessed for their cytolytic activity in a standard $^{51}$Cr release assay. Unpulsed (white circles) or pulsed–pulsed (black circles) RMA cells were used as targets. (B) Quantification ($n = 5$) of the cytolytic activity at a 50:1 E:T ratio. Data are expressed as mean ± SD (*** p<0.001).
3.2 Preventive vaccination and delay of prostate cancer growth in a transplantable model

To investigate whether CTL induced by PSCA or STEAP vaccination had also in vivo a functional activity, C57BL/6 mice were vaccinated with peptide-pulsed DC or unpulsed DC as control, and challenged one week later with $2.5 \times 10^6$ TRAMP-C1 cells. Animals were monitored thereafter for tumor appearance and growth. As reported in Fig.3 (A and B), mice vaccinated with peptide-pulsed DC experienced a significant delay in tumor appearance, and survived longer than mice vaccinated with unpulsed DC.

Fig.3. Tumor growth in mice vaccinated with either DC-PSCA or DC-STEAP and challenged with TRAMP-C1. (A) C57BL/6 mice (n=9) were immunized at day 0 with PSCA or STEAP-pulsed DC (2 µg/ml). One week later, mice were subcutaneously challenged with $2.5 \times 10^6$ TRAMP-C1 cells. Tumor size was measured twice per week using a caliper. Statistic analysis of collected data was performed using the Student's t-test: day43, DC W/O versus DC-STEAP p< 0.05. (B) Kaplan Meyer curve (DC-PSCA/DC-STEAP versus DC W/O, n=9/group, p<0.001).
Interestingly, although the two peptides used for vaccination bind equally well the K\(^b\) molecule (151,154), DC-STEAP vaccination showed to be more efficacious in delaying tumor growth (Fig. 3A) and increasing animal overall survival (Fig. 3B; p = 0.0002). In addition, only mice vaccinated with DC-STEAP showed a persistent immunity against the relevant antigen even at the time of sacrifice for tumor overgrowth (Fig. 4).

**Fig. 4.** Specific immune response in mice vaccinated with either DC-PSCA or DC-STEAP and challenged with TRAMP-C1. At the time of sacrifice because of overt tumor growth, splenocytes from PSCA and STEAP-vaccinated mice were collected, restimulated in vitro and assessed for ICP as described in the legend of Fig. 1. Results of the aggregated data (n = 9) are reported at the bottom of panels. Data are expressed as mean ± SD. IFN-\(\gamma\) production in the presence of RMA was subtracted (** p<0.01).
3.3 Persistence of functional STEAP-specific CTL in aged TRAMP mice

Having found that a potential immune response against antigens naturally expressed in the prostate is maintained in adult WT mice, we asked if the same occurred in TRAMP mice that, starting from puberty, progressively develop spontaneous mPIN and prostate cancer (149). Because STEAP gave the best results in WT mice (Fig.4), we focused on this antigen.

We have previously reported that young TRAMP mice (i.e. 6-8 weeks of age) affected by mPIN respond to a DC-based vaccination specific for the TAA Tag IV (150), therefore demonstrating that low-affinity Tag-specific T cells escape central tolerance (172) and reach the periphery, where they can be activated by the vaccine. In the following weeks, likely due to tumor-associated Tag IV over-expression in the prostate, TRAMP mice progressively loose the capacity to respond to the vaccine and enter a state of full tolerance (150). Our hypothesis was that the immune response against all prostate TAA undergoes the same kinetic. Hence, we started by vaccinating young TRAMP mice with DC-STEAP. As shown in Fig.5A, splenocytes from young vaccinated TRAMP males produced IFN-γ in the presence of both STEAP-pulsed RMA and TRAMP-C1 cells.
Fig. 5. Young and aged TRAMP mice equally respond to DC-STEAP vaccination. Eight-week (young) or 15-week old TRAMP males (old) were vaccinated once intradermally with DC-STEAP and sacrificed one week later for immunological analyses. Splenocytes were restimulated in vitro with 2 µg/mL STEAP, and 5 days later, were assessed for ICP and cytolytic activity as described in the legend to Fig. 1. Each panel is representative of at least 3 independent experiments. Results of the aggregated data (n = 9) are reported in each panel. Data are expressed as mean ± SD. IFN-γ production in the presence of RMA was subtracted.
In addition, the same blasts efficiently killed STEAP-pulsed RMA cells (Fig. 6). To our surprise, and at difference with Tag-specific immunity, 15-week old TRAMP mice (age at which TRAMP mice are affected by prostate adenocarcinoma; 150) responded equally well to DC-STEAP.

![Graph showing lytic activity](image)

**Fig. 6.** CTL from DC-STEAP vaccinated TRAMP mice are able to kill relevant targets. (A) 5-days cultured blasts were assessed for their cytolytic activity in a standard $^{51}$Cr release assay. Unpulsed (white circles) or pulsed-pulsed (black circles) RMA cells were used as targets. (B) Quantification ($n = 5$) of the cytolytic activity at a 50:1 E:T ratio. Data are expressed as mean ± SD (** p<0.01; *** p<0.001).

STEAP-vaccinated young and aged TRAMP mice were also challenged subcutaneously with TRAMP-C1 cells and tumor growth was compared with age-matched TRAMP mice vaccinated with unpulsed DC. As shown in Fig. 7, both young and aged TRAMP mice vaccinated with DC-STEAP experienced a delayed tumor growth when compared with age-matched mice vaccinated with unpulsed DC. Despite TRAMP-C1 overgrowth, DC-STEAP vaccinated mice showed a persistent STEAP-specific CTL response, therefore suggesting that growth of neither the transplanted nor the endogenous tumor could induce peripheral tolerance to STEAP.
Fig. 7. Young and aged TRAMP mice vaccinated with DC-STEAP are able to delay TRAMP-C1 tumors and maintain a STEAP-specific immune response. (A) Eight-week or (B) 15-week old TRAMP males were immunized by a single DC-STEAP vaccination. One week later, mice were subcutaneously challenged with $2.5 \times 10^6$ TRAMP-C1 cells. Tumor size was measured twice per week using a caliper. Statistic analysis of collected data was performed using the Student’s t-test: day 47, DC W/O versus DC-STEAP $p<0.05$. Animals were sacrificed at day 47 after tumor challenge, their splenocytes were collected, restimulated in vitro and assessed for ICP as described in the legend of Fig. 1. IFN-γ production in the presence of RMA was subtracted. Results of the aggregated data (n=9) are reported in each panel. Data are expressed as mean ± SD (** $p<0.01$).

To reinforce the hypothesis that STEAP-specific CTL are maintained also in aged TRAMP mice, splenocytes from old TRAMP mice were harvested and stained for STEAP-specific tetramers (Kb/STEAP). The specificity of tetramers was previously tested comparing the staining of CTLs from vaccinated mice with Kb/OVA. STEAP-specific CD8+ T cells were detectable in STEAP-vaccinated TRAMP mice compared to the control group (Fig. 8).
Fig. 8. STEAP-specific CTL are maintained in aged TRAMP mice. K\(^6\)/STEAP tetramer staining of splenocytes from vaccinated TRAMP mice sacrificed 47 days after TRAMP-C1 challenge. Splenocytes were collected, restimulated in vitro stained with fluorochrome-conjugated CD8, CD4, B220, CD11b antibodies before flow cytometry analysis. Cells were gated on live, dump negative (CD4-, B220-, CD11b- cells) and CD8+ T cells. Each panel is representative of at least 3 independent experiments.

Having found a different behavior by Tag IV- and STEAP-specific CTL in aged TRAMP mice, we asked whether in TRAMP mice the immune response against PSCA were more similar to Tag IV or STEAP. Hence, young and aged TRAMP mice were vaccinated with DC-PSCA and assessed for an antigen-specific immunity. As shown in Fig. 9, young and old TRAMP mice mounted a consistent immune response against PSCA, which in aged mice was however lower than that measured against STEAP (compare Figs. 6 and 9).
Fig.9. CTL from DC-PSCA vaccinated TRAMP mice modestly kill relevant targets. (A) 5-days cultured blasts from DC-PSCA vaccinated TRAMP mice were assessed for their cytolytic activity in a standard \(^{51}\)Cr release assay. Unpulsed (white circles) or pulsed–pulsed (black circles) RMA cells were used as targets. (B) Quantification (n = 5) of the cytolytic activity at a 50:1 E:T ratio. Data are expressed as mean ± SD (** p<0.01; *** p<0.001).

All together, these results suggest that during the development and progression of a spontaneous tumor the immune response to TAA may behave differently, and not all tumor-specific CTL undergo full tolerance in TRAMP mice affected by prostate cancer.

3.4 Immune response to the different TAA in aged TRAMP mice correlates with antigen expression in the prostate

We asked if the different behavior of CTL specific for Tag IV, STEAP and PSCA in tumor-bearing TRAMP males could depend on the amount of antigen expressed in the tumor. We hypothesized that the more the antigen was expressed the more profound peripheral tolerance would have been. Hence, prostate specimens from 15-week-old TRAMP mice were assessed for Tag IV, STEAP and PSCA mRNA expression. In parallel, age-matched TRAMP mice were vaccinated with either one of the three antigens and assessed for antigen-specific cytolytic activity. As reported in Fig.10, the expression of STEAP was significantly lower than that of Tag IV and PSCA. Interestingly, antigen expression inversely correlated with CTL activity, and
STEAP, which showed to be the least expressed, was also the one that elicited the highest cytolytic response. This correlation might explain why in STEAP-vaccinated TRAMP mice an antigen-specific immune response persisted for so long.

![Fig.10.](image)

Fig. 10. STEAP expression in the prostates of 15-week-old TRAMP mice inversely correlates with cytolytic activity of STEAP-specific CTL. (A) mRNA expression of TAG IV, STEAP and PSCA in the prostates of TRAMP mice (n=5). (B) Cytolytic activity of CTL from Tag IV, STEAP or PSCA-vaccinated mice challenged with RMA cells pulsed with relevant peptide (for DC-STEAP vaccinated mice) or B6-k0 cells (for DC-Tag IV vaccinated mice). Data are means ± SD of triplicated E/T ratio 50:1 (* p<0.05; ** p<0.01; *** p<0.001).

3.5 The vaccination against STEAP does not prevent development of the autochthonous tumor in TRAMP mice

We (150) and others (168,169) have previously reported that preventive vaccination in TRAMP mice can delay spontaneous tumor progression. We asked if also STEAP could be used as target antigen to induce protective immunity in TRAMP mice. Thus, 7-8-week old TRAMP males were vaccinated once with DC-STEAP and sacrificed 8 week later. As control, a cohort of age-matched TRAMP males was vaccinated with unpulsed DC. At the time of sacrifice, prostates from these mice were harvested and scored by a pathologist as previously described (150). Splenocytes from the same mice were restimulated in vitro and assessed for ICP. Despite the evidence
of a persistent STEAP-specific immune response in DC-STEAP vaccinated mice (Fig. 11B), the disease score in the two groups of TRAMP mice was comparable, therefore demonstrating that the STEAP-specific immune response induced by the vaccine is weaker and less potent than that induced by DC-Tag (150).

![Graph A and B](image)

**Fig.11.** DC-STEAP vaccination does not prevent autochthonous tumor growth. (A) 8-week-old C57BL/6 TRAMP mice (n=9) were immunized at day 0 with STEAP-pulsed DC (2 µg/ml). 8 weeks after the prime, mice were sacrificed and prostates scored by a pathologist. (B) In vitro ICP of splenocytes restimulated in vitro and assessed for ICP as described in the legend of Fig. 1. IFN-γ production in the presence of RMA was subtracted. Results of the aggregated data (n=9) are reported in each panel. Data are expressed as mean ± SD (**p<0.01).

### 3.6 Allotransplantation and DC-STEAP vaccination co-operate for tumor remission in TRAMP mice

We have recently reported that allotransplantation of hematopoietic stem cells (HSCT) and donor lymphocyte infusion (DLI) from female mice, presensitized to male H antigens restores immune competence in the TRAMP model (165). Our protocol in TRAMP mice underlined the ability of minor histocompatibility (H) antigen- and tumor-specific CD8+ T cells to cooperate in PC rejection (165). Post-transplant vaccination played a critical role. Indeed, it prolonged disease-free survival in the case of DLI from donors sensitized against host
recipients (165), and was required for therapeutic efficacy in the case of DLI from unsensitized donors (Hess Michelini R. et al. Manuscript in preparation). We asked whether DC-STEAP vaccination could substitute DC-Tag IV in this therapeutic setting. Hence, 16-17 week-old male TRAMP mice (CD45.2+) were preconditioned by non-myeloablative total body irradiation (TBI, 600 Rad) and transplanted the following day with $1 \times 10^7$ BM cells from (B6.CD45.1+ x B6.CD45.2+) F1 female (HSCT). Two weeks later mice received a donor lymphocyte infusion (DLI) of $6 \times 10^7$ splenocytes from female (fDLI) CD45.1+ congenic donors that were not pre-sensitized against host minor H antigen. Blood samples were taken at different time points after DLI to follow the immune reconstitution. One day after DLI transplanted mice were vaccinated with DC pulsed with either STEAP or unpulsed DC (Fig. 12 depicts a schematic representation of the transplant setting). At the time of sacrifice (42 days after HCT), splenocytes were harvested and assessed for in vitro ICP in the presence of RMA-STEAP. As reported in Fig. 12, in DC-STEAP vaccinated mice a low, but consistent percentage of CD8+ T cells (from donor and host origin) were detectable that produced IFN-γ after antigen challenge.
**Fig. 12. Allotransplantation and DC-STEAP vaccination maintain a STEAP-specific immune response in TRAMP mice.** Sixteen-17 week-old male TRAMP mice (CD45.2+, n=9) were preconditioned by non-myeloablative total body irradiation (TBI, 600 Rad) and transplanted the following day with 1x10^7 BM cells from (B6.CD45.1 x B6.CD45.2) F1 female (HCT). Two weeks later mice received a donor lymphocyte infusion (DLI) of 6x10^7 splenocytes from female (fDLI) CD45.1+ congenic donors. One day after DLI a group of transplanted mice was vaccinated with DC pulsed either with STEAP or unpulsed DC. At the time of sacrifice (42 days after HCT), splenocytes were harvested and assessed for in vitro ICP in the presence of RMA was subtracted. Results of the aggregated data are reported. Data are expressed as mean ± SD (* p<0.05).

Controls TRAMP mice vaccinated with DC-STEAP from previous experiments were compared to TRAMP mice that have received allotransplantation plus vaccination with unpulsed DC (fHCT/fDLI/DC) or plus STEAP peptide (fHCT/fDLI/DC-STEAP). The urogenital apparata (UGA) were recovered, photographed and weighted (*Fig. 13, A-B, respectively).
**Fig. 13.** Allotransplantation and DC-STEAP vaccination impact on UGA morphology in TRAMP mice. Sixteen-week old TRAMP mice vaccinated (DC-STEAP) or transplanted and vaccinated either with unpulsed (fHCT/fDLI/DC) or STEAP-pulsed DC (fHCT/fDLI/DC-STEAP) were sacrificed 1 week after the last vaccination. (A) The size of the UGA of one representative mouse per group is depicted. (B) Individual UGA weights are shown. (*) p<0.05.

UGA were then processed and stained for H&E (Fig. 14, A) and analyzed for Disease Score attribution. (Fig. 14, B). At the Hematoxylin/Eosin staining, prostates tubules from TRAMP mice that received a single vaccination (TRAMP/DC-STEAP) were expanded by a differentiated adenocarcinoma (epithelial proliferation forming cribriform structures, nuclei expansion and hyperchromasia, increase thickness of the fibromuscular wall of tubules and presence of malignant cells in the surrounding stroma). In contrast, the histologic appearance of the UGA of the majority of TRAMP/fHCT/fDLI/DC and TRAMP/fHCT/fDLI/DC-STEAP mice showed evidence of tumor regression and inflammatory infiltrate throughout the organ.
Mice were blindly assigned a DS reflecting the area of residual transformation. The mean DS for TRAMP/DC-STEAP was 3.7 ± 0.5, for TRAMP/fHCT/fDLI/DC was 3.8 ± 0.4, while that for TRAMP/fHCT/fDLI/DC-STEAP group was 2.4 ± 1.4. Among the TRAMP/fHCT/fDLI/DC-STEAP group there was 2 complete regression (CR), 4 partial regression (PR) and 3 non responders (NR).

Fig. 14. Allotransplantation followed by tumor-specific vaccination reduces tumor burden in TRAMP mice. The histology (A) of UGA was analyzed on paraffin-embedded sections by H&E. Panels show 5x magnification. Images from 1 representative prostate for each group. (B) Disease Score was individually assigned on coded samples. Each dot represents the disease score from an individual mouse. The mean disease score between TRAMP/DC-STEAP (n=9) and TRAMP TRAMP/fHCT/fDLI/DC-STEAP (n=9) was statistically different (Student’s t test P<0.05). Samples were also scored for CR, PR or NR, defined as described within the text.
The presence of tumor infiltrate was quantified with immunohistochemistry on paraffin-embedded sections with anti-CD3 monoclonal antibody (Fig. 15, A-B).

![Image of immunohistochemistry results](image)

**Fig. 15. Allotransplantation followed by tumor-specific vaccination recruits CD3⁺ cells in the prostate.** Paraffin-embedded sections of UGA were analyzed by immunohistochemistry with anti-CD3 monoclonal antibody. Sections were digitally scanned and CD3⁺ events electronically quantified. Each dot represents the number of CD3⁺ cells/mm² obtained by the analysis of a representative prostate section of every mouse. The mean infiltrate between TRAMP/DC-STEAP (n=9) and TRAMP/fHCT/fDLI/DC (n=6) or TRAMP/fHCT/fDLI/DC-STEAP (n=9) was statistically different (Student's t test P<0.001).

These results demonstrated how the allotransplantation followed by DLI is sufficient to recruit CD3⁺ T cells in the prostate glands, but only the addition of the STEAP-specific vaccination is able to have a statistically significant impact on the disease score. Hence, transplantation followed by STEAP-specific vaccination circumvented mechanisms of central and...
peripheral tolerance normally hindering protective immunity and favored rejection of established prostate cancer.
4. Discussion

The recent approval from FDA of the first vaccine against hormone-refractory prostate cancer, Sipuleucel-T, highlights how the choice of the antigen and the immunization strategy are crucial for the best outcome. In that case, the choice of the antigen to be used in clinical trials, PAP, was based on the ability of PAP-specific immunization to break peripheral T cell tolerance (117, fda.gov/downloads/biologicsbloodvaccines/.../ucm227998.pdf). However, a clear demonstration either in mice or humans that the therapeutic efficacy of sipuleucel-T correlates with a PAP-specific immune response is still lacking. This might account for the limited success of the trial (63), and underlines the need for a better understanding of the dynamics of the immune response against TAA in cancer patients.

We have reported here that in the reliable TRAMP model, prostate cancer-associated antigens may behave differently in term of immunological response and long-term protection against tumor progression. Firstly, we demonstrated that a DC-based vaccination against two well-known self-antigens, PSCA and STEAP, over-expressed during prostate cancer progression (108,109), can elicit specific CTL that recognize the appropriate target in C57BL/6 WT mice. These preliminary experiments underlined how a single immunization was able to induce a specific response in the treated animals. Besides, PSCA and STEAP-specific CTL were also able to delay TRAMP-C1 growth in WT mice. Notably, while all mice previously vaccinated with either DC-PSCA or DC-STEAP experienced a delay in TRAMP-C1 growth, this was statistically significant only for STEAP-vaccinated mice. Moreover, only DC-STEAP vaccinated mice maintained a STEAP-specific immune response at the time of sacrifice. For that reason we focused our attention on STEAP and tested its efficacy in TRAMP mice. Interestingly, and at difference with Tag (150), tumor-bearing TRAMP mice did not undergo full tolerance against STEAP, and STEAP-vaccinated TRAMP mice showed a delayed growth of subcutaneous TRAMP-C1 tumors, therefore confirming that STEAP-specific CTL remain functional in tumor bearing TRAMP mice.
PSCA showed in TRAMP mice an intermediate behavior: although full tolerance was not reached in tumor-bearing mice, the persistent immune response was weaker than that against STEAP.

Analysis of antigen expression in the prostate of TRAMP mice showed a different expression of the three antigens that inversely correlated with the antigen-specific immune response.

Altogether, these data suggest that tolerance against this type of TAA follows the same rule of that induced for tissue-associated antigens in peripheral tissues: the more the antigen is expressed the more tolerance is profound. This information is fundamental for the choice of the antigen to be used in the clinic.

It is also important to underline the different behavior of Tag and STEAP. Indeed, Tag showed a dynamic of tolerance induction that was comparable to other antigens whose expression was genetically manipulated in TRAMP mice (176-179). Conversely, tolerance to STEAP appears more similar to that found in cancer patients, therefore suggesting that preclinical research should focus more on the latter type of antigens.

Sherman and collaborators (166) investigated in another mouse model the correlation between the amount of antigen expressed in the periphery and both the degree of T cell proliferation in lymph nodes and the rate of tolerance of antigen-specific CD8+ T cells. In their studies in InsHA mice, they showed how homozygous InsHA mice had enhanced activation and proliferation of Kd-restricted HA-specific CD8+ T cells in the pancreatic lymph nodes following HA immunization, but these cells were rapidly deleted. In contrast, in heterozygous mice that have half of the antigen expressed, this deletion was less rapid. Nevertheless, a continuous Ag exposure could also induce clonal deletion or anergy of CD8+ T cells (167). This is what we believe occurs for Tag and PSCA, respectively, in TRAMP mice. The limited over-expression of STEAP even in advanced disease may protect STEAP-specific clones from peripheral anergy/deletion.

Based on these results, DC-STEAP immunization was tested in a prophylactic protocol in TRAMP mice. Despite the presence of STEAP-
specific CTL even months after the priming, this strategy was not sufficient to prevent or arrest the growth of the autochthonous tumor, therefore suggesting that STEAP-specific CTL are qualitatively and quantitatively insufficient to cope with a massive neoplastic transformation of the whole TRAMP prostate. At least at the beginning, the situation in humans is expected to be well different: indeed, human prostate cancer generates from different small foci within the prostate gland. Hence, vaccination against STEAP may prove to be more efficacious in patients with a very early disease. This is a relevant issue for patients who may decide between watchful waiting and radical prostatectomy. Indeed, vaccination might represent a third option.

The fact that STEAP-specific CTL persisted in vaccinated animals demonstrates how they could have escaped most of the immunosuppressive mechanisms that generally dampen the tumor-specific immune response in tumors. We have no clue on the mechanisms by which STEAP-specific T cells escaped tumor-induced tolerance in TRAMP mice. Tumor-specific CTL may encounter an immunosuppressive environment both in the lymph nodes at the time of priming or restimulation, as suggest by Hurwitz AA and collaborators (180), and at the tumor site (181). While the latter should involve all tumor infiltrating CTL, it might be possible that a high amount of antigen presented in the tumor draining lymph nodes rapidly consume the reservoir of antigen-specific CTL that, once activated, deploy to the tumor were they are eliminated. The relatively low expression of STEAP and the low affinity of STEAP-specific CTL may protect them from this consumption. An alternative or complementary explanation is that tumor-specific CTL are activated in many secondary lymphoid organs during tumor progression, with the exception of CTL specific for highly expressed antigens, which are concentrated in tumor draining lymph nodes where most of the immunosuppressive mechanisms are located.

These results also suggested that vaccination targeting even more than one antigen at the time (as for example for GVAX and ProstVac trials) might have limited effects in advanced prostate cancer patients. For that reason, we
tested a combined therapy of allotransplantation and tumor-specific vaccination. The major goal was to eradicate established spontaneous prostate cancer and to induce a long-term immune response. Based on the previous results obtained in TRAMP mice (165), we set-up a non-myeloablative treatment followed by a HCT, fDLI and a STEAP-specific vaccination. The aim of these experiments was also to test if a more realistic tumor antigen when compared to the oncogen-driven Tag IV could have the same therapeutic effects.

The analysis of tumor-specific T cells responses in transplanted mice indicated that T cells of DLI origin were capable of a STEAP-specific response upon infusion and DC-STEAP vaccination in TRAMP mice. It is interesting to note that in this therapeutic setting, also host-derived cells surviving the pre-conditioning maintained the capacity to respond to the vaccine. Hence, combination of non-myeloablative conditioning, allotransplantation and vaccination, by simultaneously providing competent lymphocytes able to respond to the vaccination, appeared to recreate a correct milieu for the generation and the maintenance of an effective protective response.

Minor H-specific T cells could have played a fundamental role in tumor regression. Probably, because of their female origin, they could have reached all the male organs, but particularly the prostate, because of the in situ inflammation and the release of TNF-alpha (174). Here they could have directly recognized H-Y peptides presented by the tumor cells or tumor-associated stromal components, killed prostate cells, favoring the antigen shedding and the activation of tumor-specific T cells. This could have induced a local GVH response and tissue damage, which in turn might have contributed to tumor-specific T cell priming in tumor-draining lymph nodes.

The analysis of the prostates demonstrated how allotransplantation and DC-STEAP vaccination were able to significantly reduce tumor burden (Fig.13-14).

Optimal infiltration of T cells into the tumor mass is a critical issue, based on the fact that defined CD8+ T cells concentrations within the tumor mass are
needed for effective tumor clearance (173). The presence of a high number of CD3+ T cells in the prostates of allotransplantated and vaccinated mice demonstrated how these cells could have played a central role in killing tumor cells. Notably, an increased tumor infiltrate was observed also in mice that received the transplant setting followed by a DC unpulsed vaccination. Most likely, the shedding of tumor antigens in the prostates due to the DLI infusion, activation and subsequent killing of tumor cells could have activated DC and primed new CD3+ T cells.

Taken together, these results demonstrated how tumor eradication requires the concomitance of minor and tumor-specific T cell responses.

Previous studies investigated the possibility of breaking peripheral tolerance in TRAMP mice (168,169). All this works demonstrated how vaccination was able to delay autochthonous prostate cancer only when treatment started when animals were relatively young (10-12 week of age) and thus may not have mimicked the situation found in PC patients and in older TRAMP mice. Instead, in this study the efficacy of the treatment was tested in 16-week-old TRAMP mice, resembling an advanced stage of the disease and most importantly, an antigen, i.e. STEAP, that does not encounter peripheral tolerance was used. It will be important to verify if also DC-STEAP vaccination induces a long-lasting memory response that protect mice from tumor recurrence, as we have seen for DC-Tag (150). Another issue that needs to be addressed is that in humans, donors and recipients are mismatched for more than one minor H antigen. For that reason, experiments using female BALB/b mice as donor are carried out, recapitulating a situation of multiple minor mismatches.

All this work focused the attention on CD8+ T cells, but also other cellular types could have a role in the tumor control after allotransplantation. *Sherman LA* and collaborators demonstrated how CD4+ T cells play a central role in tumor regression, mobilizing effectors CD8+ T cells to peripheral tissues (175). Also NK cells that recognize NKG2D ligands on tumor cells could play a role in reducing prostate cancer progression in TRAMP mice, starting from the observation that TRAMP mice deficient for
NKG2D showed an increased incidence of aggressive tumors (170). Also γδ T cells are involved in prostate cancer progression in the TRAMP model: mice lacking these cells develop large tumors in comparison to normal ones (171). Experiments are ongoing to identify the respective targets of allo-and tumor-specific T cells in transplanted mice (Hess Michelini R et.al. Manuscript in preparation).

Taken together, the results obtained in this thesis demonstrate how STEAP-specific vaccination can induce a long-lasting immune response in TRAMP mice, mainly due to its low expression at the tumor site. While vaccinations targeting antigens like STEAP might be proposed to patients with high probability to develop prostate cancer or for those with a low-risk prostate cancer, a combination of allo-transplantation, DLI and vaccination might be a therapeutic option for patients with castration-resistant and metastatic disease.
5. Bibliography


