UNIVERSITÀ DEGLI STUDI DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA
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Chimeric Antigen Receptor Engineered T cells for adoptive immunotherapy of Prostate Cancer

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"Le difficoltà crescono a misura che ci si avvicini alla meta. Seminare non è così difficile come raccogliere."

W.Goethe

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SUMMARY

Immunology-based interventions have been proposed as a promising curative chance to effectively attack postoperative minimal residual disease and distant metastatic localizations of prostate tumors. In this regard, however, results from clinical trials have shown that a single-approach immunotherapy (antibody-mediated or cell-mediated) might be insufficient to eradicate tumor cells and allows them to survive and adapt to the body's defence mechanisms or to passively administered cures. The aim of my PhD project was the generation of CAR-engineered T cells specific for the human Prostate Specific Membrane Antigen (hPSMA), and their funtional evaluation *in vitro* and *in vivo*.

We exploited the concept that combination of two powerful tools, by endowing T cells with recognition capacity and high specificity of antibodies, can lead to results superior to those obtained by single-approach treatments. To this aim, we developed a CAR containing both the CD3zeta and CD28 signalling moieties fused to a scFv targeting the hPSMA, to engineer human PBMC for the immunotherapy of prostate cancer. As a transfer method, we employed last-generation lentiviral vectors (LV) carrying a synthetic bidirectional promoter, capable of robust and coordinated expression of two transgenes, thus allowing to co-express the CAR in conjunction with a reporter gene (luciferase), to track the transgenic T cell population to the tumor site by *in vivo* optical imaging after adoptive transfer.

Overall, we demonstrated that CAR-expressing LV efficiently transduced short-term activated PBMC that, in turn, were readily stimulated to produce cytokines and exert a relevant cytotoxic activity by engagement with PSMA+ prostate tumor cells.

Then, we exploited our *in vivo* imaging know-how and set up some experiments to precisely define the effect of the Tbodies in tumour-grafted mice and to visualize their effect in a mouse model of prostate cancer. To this end, tumour cells were transduced with a lentiviral vector coding for the reporter gene luciferase. Stably transduced cells were then used for the *in vivo* experiments as described above. For the analysis, mice were anaesthetized and injected with luciferin, the substrate of the enzime luciferase: the catabolyc reaction produced bioluminescence that can be visualized using Ivis Lumina II instrument (Xenogen).

Last, for the setting up of a prostate cancer mouse model, we injected s.c. and i.v. luciferase-transduced PC3-hPSMA cells or PC3-WT as control. Than we evaluated the therapeutic efficacy of the Tbodies after local and systemical injection. Mice were analyzed weekly, using the Ivis Lumina II platform.

Upon *in vivo* transfer in tumor-bearing mice, CAR-engineered T cells survived shortly but were nonetheless capable of inducing striking therapeutic effects, thus supporting the transfer of this approach to clinical settings.

RIASSUNTO

Nell'ambito dell'immunoterapia cellulare adottiva, l'ingegnerizzazione di linfociti T con recettori diretti contro antigeni tumorali rappresenta un'efficace strategia per generare in tempi rapidi un elevato numero di linfociti tumore-specifici. In alternativa al TCR fisiologico, la cellula T può essere ingegnerizzata con recettori chimerici per l'antigene (CAR) costituiti da un dominio di riconoscimento antigenico (derivato da anticorpi monoclonali) fuso a domini di trasduzione del segnale derivati dal complesso TCR: questo tipo di struttura combina la specificità del riconoscimento anticorpale (MHC-indipendente) con le potenzialità anti-tumorali dei linfociti T. L'attività di ricerca svolta nel mio corso di Dottorato si è focalizzata sullo sviluppo di un protocollo per la generazione di linfociti T ingegnerizzati con un CAR di seconda generazione (scFv-CD28-CD3ζ) diretto contro l'antigene hPSMA (Prostate Specific Membrane Antigen) per il trattamento del carcinoma prostatico.
L'utilizzo di vettori lentivirali (LV) come alternativa ai più diffusi vettori oncoretrovirali, consente di trasdurre in modo efficace cellule T scarsamente differenziate, con positive implicazioni sulla loro funzionalità *in vivo*; in questo studio, inoltre, la presenza nel vettore LV di un promotore bidirezionale recentemente descritto ha permesso l'espressione coordinata del CAR e del gene reporter *Firefly Luciferase* con l'obiettivo di monitorare il destino biologico dei linfociti trasferiti adottivamente *in vivo*, mediante imaging di bioluminescenza (BLI).
La popolazione di PBMC ingegnerizzati con recettore chimerico (T-body) è caratterizzata da un'elevata percentuale di espressione del CAR anti-hPSMA (superiore al 50%), da un fenotipo di memoria e dalla capacità di riconoscere e lisare in modo specifico cellule esprimenti l'antigene. In un modello murino di tumore prostatico sottocutaneo i T-body si sono dimostrati efficaci in diversi protocolli terapeutici: co-inoculati con cellule tumorali, inoculati a livello locale, o inoculati per via sistemica in topi portatori di tumori disseminati. Contrariamente il trasferimento adottivo per via sistemica non ha determinato alcun risultato terapeutico nei tumori sottocutanei. Il monitoraggio della distribuzione *in vivo* dei linfociti mediante BLI ha infatti evidenziato una scarsa capacità di sopravvivenza e di *homing* al sito tumorale. I risultati positivi ottenuti in questo lavoro, in particolare lo sviluppo di un recettore chimerico anti-hPSMA di seconda generazione, l'utilizzo di vettori LV e la generazione di T-body funzionali *in vitro* e *in vivo*, costituiscono il razionale per ulteriori studi e per future applicazioni cliniche di questo tipo di approccio in pazienti con carcinoma prostatico; rimane tuttavia fondamentale chiarire le dinamiche di ricircolazione e distribuzione delle cellule T con l'obiettivo di

implementarne la capacità di sopravvivere, ricircolare e raggiungere il sito tumorale, fattori indispensabili per mediare l'effettiva regressione della neoplasia.

INTRODUCTION

1. CANCER IMMUNOTHERAPY

Despite multiple approaches to therapy and prevention, cancer remains a major cause of death worldwide. Most nonsurgical approaches targeting rapidly dividing cells, using radiotherapy or chemotherapy, also affect normal cells and result in side effects that limit treatment. In principle, the exquisite specificity of the immune system could be marshaled to precisely target cancer cells without harming normal tissues (Bergman, 2009). This hope has motivated much research over several decades but has met with only limited success to date. However, the rapid increase in knowledge on the immune system and its regulation have led to a resurgence of interest in immunologic approaches to target and eliminate cancer (Morris et al, 2006).

The relationship between the immune system and human cancer is dynamic and complex. Individual tumors harbor a multitude of somatic gene mutations and epigenetically dysregulated genes, whose products are potentially recognizable as foreign antigens. Proteins used by tumors to promote transformation and tumorigenesis and to establish a malignant phenotype may, in some circumstances, be referred to as tumor-associated antigens (TAA) (Linley et al, 2011; Peskin, 2011). Unique antigens result from point mutations in genes that are expressed ubiquitously. The mutation usually affects the coding region of the gene and is unique to the tumor of an individual patient or restricted to very few patients. Some of these mutations may be implicated in tumor transformation. Such antigens, which are strictly tumor-specific, may play an important role in the natural anti-tumor immune response of individual patients, but most of them cannot be easily used as immunotherapeutic targets because they are not shared by tumors from different patients (Bergman, 2009). On the other hand, shared antigens are present on many independent tumors. They can be further divided into three main groups. One group corresponds to peptides encoded by "cancer-germline" genes, such as Melanoma-associated antigen (MAGE) (Linley et al, 2011), which are expressed in many tumors but not in normal tissues. The only normal cells in which significant expression of such genes has been detected are placental trophoblasts and testicular germ cells. Because these cells do not express MHC class I molecules, gene expression should not result in expression of the antigenic peptides and such antigens can therefore be considered as strictly tumor-specific. The genes encoding such antigens have also been referred to as "cancer-testis" (CT) genes (Kessler et al, 2007). A second group of shared tumor antigens,

named differentiation antigens, are also expressed in the normal tissue of origin of the malignancy. The paradigm is tyrosinase, which is expressed in normal melanocytes and in most melanomas (Burns et al, 2010). Antigens of this group are not tumor-specific, and their use as targets for cancer immunotherapy may result in autoimmunity towards the corresponding normal tissue. In the case of melanocytes, the risk of inducing severe side effects appears minimal, and could be limited to the appearance of vitiligo. More serious concerns about autoimmune side effects apply to carcinoembryonic antigen (CEA) (Shirasu et al, 2010), an oncofetal protein expressed in normal colon epithelium and in most gut carcinomas. Autoimmune toxicity should not be an issue, however, in those conditions where the tissue expressing the antigen is dispensable or even resected by the surgeon in the course of cancer therapy, as would be the case for prostate specific antigen (PSA) (Akhtar et al, 2012). It is much more difficult to make predictions regarding the safety of targeting shared antigens of the third group, which are expressed in a wide variety of normal tissues and overexpressed in tumors (Djavan et al, 2011). Because a minimal amount of peptide is required for the cytotoxic T lymphocyte (CTL) recognition, a low level of expression in normal tissues may mean that autoimmune damage is not incurred. However, this threshold is difficult to define, as is the normal level of expression of those genes for each cell type (Van Der Bruggen et al, 2001). A large series of additional peptides have been described which have not (yet) been included in the tables because formal evidence to fulfill one or several of the aforementioned criteria has not been provided. A number of viruses, such as the Epstein-Barr virus (EBV) and human papilloma virus (HPV), are associated with human malignancies. The antigenic peptides encoded by viral genes have an high potential as targets for immunotherapy.

Shared Antigens		Type of tumor	Normal tissue distribution
Cancer-testis (CT) Ags	BAGE GAGE MAGE NY-ESO-1 SSX	melanoma, lymphoma, lung, bladder, colon and breast carcinomas	spermatocytes/spermatogonia of testis, placenta, ovary cells
Differentiation Ags	Gp100 Melan-A/Mart-1 Tyrosinase PSA CEA Mammaglobin-A	melanoma, prostate cancer, colon and breast carcinomas	melanocytes, epithelial tissues, prostate, colon
Overexpressed Ags	p53 HER-2/neu livin survivin	esophagus, liver, pancreas, colon, breast, ovary, bladder and prostate carcinomas	ubiquitous (low level)
Unique Antigens		**Type of tumor**	**Normal tissue distribution**
Unique Ags	β-catenin-m β-Actin/4/m Myosin/m HSP70-2/m HLA-A2-R17OJ	melanoma, non-small cell lung cancer, renal cancer	N/A
Unique/Shared Antigens		**Type of tumor**	**Normal tissue distribution**
Tumor-associated Carbohydrate Ags	GM2 GD2 GD3 MUC-1 sTn globo-H	melanoma, neuroblastoma, colorectal, lung, breast, ovarian and prostate cancer	epithelial tissues (e.g., renal, intestinal, colorectal)

Table 1. List of the major classes of tumor-associated antigens found in malignancy, including common examples and the types of tumors they are expressed in. Modified by Buonaguro et al, 2010.

Treatment with anticancer drugs is commonly categorized into four different classes: chemotherapy, which involves a large group of cytotoxic drugs that interfere with cell division and DNA synthesis; hormonal therapy, which involves drugs that interfere with growth signalling through hormone receptors on cancer cells; targeted therapy, which consists of a novel group of antibodies and small-molecule kinase inhibitors that specifically target proteins that are involved in growth signalling pathways in cancer cells; and immunotherapy, which aims at increasing anticancer immune responses (Lesterhuis et al, 2011). The concept of cancer immunotherapy goes back as far as the late nineteenth century, when William B. Coley observed tumor shrinkage and even disappearance following the injection of bacterial products in and around tumors. Since then, many observations — such as the rare but well-documented occurrence of spontaneous remissions, the higher incidence of cancer in patients who are immunosuppressed, and the identification of tumor-specific antigens and lymphocytes — have stimulated research on strategies that aim to induce specific antitumor responses (Gulley et al, 2011). Currently, allogeneic bone marrow transplantation and monoclonal antibodies that target tumor cells are two examples of broadly used and efficacious immunotherapies (Lesterhuis et al, 2011).

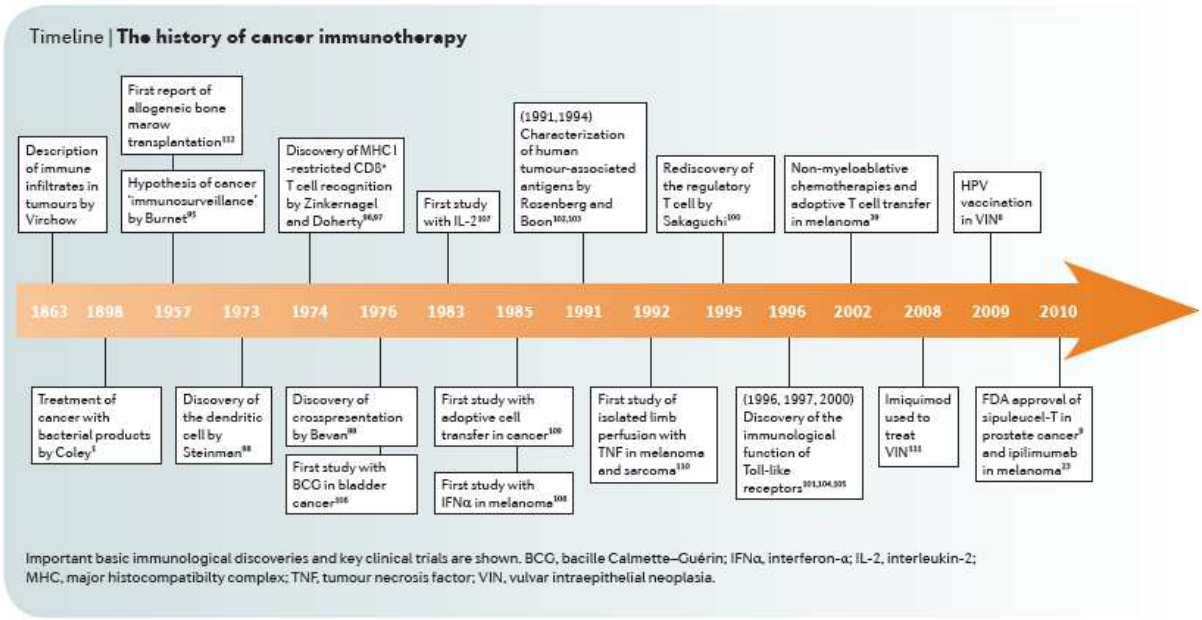


Fig 1. The history of cancer immunotherapy. From Lesterhuis et al, 2011.

Over the past decades, considerable knowledge has been obtained on the components that are relevant in antitumor immune responses and immune escape mechanisms, and CTL response has been identified as the most powerful and effective link in this vast network (Punt et al, 2011). Immunotherapy may be classified into several types, including (1) active immunotherapy, specific stimulation of patient's immune system with vaccines and/or nonspecific stimulation using adjuvants; (2) passive immunotherapy, treatment with exogenously produced antibodies; (3) adoptive immunotherapy, transfer of lymphocytes and/or cytokines; (4) restorative therapy, designed to restore deficiencies in the patient's immune response; and (5) cytomodulatory therapy, meant to enhance the expression of major histocompatibility complex (MHC) molecules on the surface of the tumor cells. Cancer vaccines exemplify active specific immunotherapy, usually combined with adjuvants.

Long considered a simple observer to this battle, the immune system is now being recognized as a crucial player in the outcome of cancer development (Burnet, 1964). Immunity has two seemingly paradoxical effects on cancer. On one hand, immunity prevents against the development of nascent tumors, a concept known as cancer immunosurveillance (Dunn et al, 2004). Indeed, compelling experimental studies in mouse models of cancer together with clinical data from human patients have now unambiguously demonstrated that cancer immunosurveillance functions as an effective extrinsic tumor suppressor mechanism (Zitvogel, 2006). On the other hand, immunity shapes the intrinsic nature of developing tumors through the

immunological pressure afforded by cancer immunosurveillance. This combination of host-protective and tumor-sculpting functions of the immune system throughout tumor development is termed cancer immunoediting (Dunn et al, 2002). Cancer immunoediting refers to a dynamic process comprising of three phases: elimination, equilibrium and escape.

Elimination consists of the classical concept of cancer immunosurveillance (Burnet, 1973), where pre-malignant and early stage malignant cells are directly or indirectly removed by immune cells. Equilibrium is the period of immune-mediated latency after incomplete tumor destruction, and escape refers to the final outgrowth of tumors that have overcome immunological pressure. When the immune system ultimately fails to eliminate all transformed cells, tumors with reduced immunogenicity emerge that are capable of escaping immune destruction and, in some circumstance, harness or alter ensuing inflammatory reactions to their own benefit.

The elimination process encompasses the original concept of cancer immunosurveillance (Zitvogel, 2006). As such, when it is successful in deleting the developing tumor, it represents the complete editing process without progression to the subsequent phases. In the first phase of elimination, once solid tumors reach a certain size, they begin to grow invasively and require an enhanced blood supply that arises as a consequence of the production of stromagenic and angiogenic proteins (Dunn et al, 2004). Invasive growth causes minor disruptions within the surrounding tissue that induce inflammatory signals leading to recruitment of cells of the innate immune system (NKT, NK, $\gamma\delta$ T cells, macrophages and dendritic cells) into the site. Structures on the transformed cells (either expressed as a result of the transformation process itself or induced by the ongoing but limited inflammatory response) are recognized by infiltrating lymphocytes such as NKT, NK or $\gamma\delta$ T cells, which are then stimulated to produce IFN-γ. In the second phase, the IFN-γ that was initially produced may induce a limited amount of tumor death by means of antiproliferative and apopotic mechanisms (Dunn et al, 2002). However, it also induces the production of the chemokines CXCL10 (interferon-inducible protein-10, IP-10), CXCL9 (monokine induced by IFN-γ, MIG) and CXCL11 (interferon-inducible T cell αchemoattractant, I-TAC) from the tumor cells themselves as well as from surrounding normal host tissues (Dunn et al, 2002). At least some of these chemokines have potent angiostatic capacities and thus block the formation of new blood vessels within the tumor, which leads to even more tumor cell death. Tumor cell debris, formed as either a direct or indirect consequence of IFN-γ production at the tumor, is then ingested by local dendritic cells, which home to draining lymph nodes (Rescigno et al, 2007). Chemokines produced during the escalating inflammatory process recruit more NK cells and

macrophages to the site (Dunn et al, 2002). In the third phase, the tumor-infiltrating NK cells and macrophages transactivate one another by reciprocal production of IFN-γ and IL-12, and kill most of the tumor by mechanisms involving tumor necrosis factor−related apoptosis-inducing ligand (TRAIL), perforin and reactive oxygen and nitrogen intermediates (Dunn et al, 2002). In the draining lymph node, the newly immigrated dendritic cells induce tumor-specific $CD4^+$ T helper cells expressing IFN-γ (T_H1 cells) that in turn facilitate the development of tumor-specific $CD8^+$ T cells (Gattinoni et al, 2005). In the fourth phase, tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site, where the cytolytic T lymphocytes destroy the remaining antigen-bearing tumor cells whose immunogenicity has been enhanced by exposure to locally produced IFN-γ. In the equilibrium process, the host immune system and any tumor cell variant that has survived the elimination process, enter into a dynamic equilibrium. In this process, lymphocytes and IFN-γ exert potent selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed containing many genetically unstable and rapidly mutating tumor cells. During this period of Darwinian selection, many of the original escape variants of the tumor cell are destroyed, but new variants arise carrying different mutations that provide them with increased resistance to immune attack. It is likely that equilibrium is the longest of the three processes and may occur over a period of many years (Zitvogel, 2006). In the escape process, surviving tumor variants that have acquired insensitivity to immunologic detection and/or elimination through genetic or epigenetic changes begin to expand in an uncontrolled manner. This results in clinically observable malignant disease that, if left unchecked, results in the death of the host (Charo et al, 2011).

Cancer immunotherapy comprises a variety of treatment approaches, incorporating the tremendous specificity of the adaptive immune system (T cells and antibodies) as well as the diverse and potent cytotoxic weaponry of both adaptive and innate immunity (Rescigno et al, 2007). Immunotherapy strategies include antitumor monoclonal antibodies, cancer vaccines, adoptive transfer of ex vivo activated T and natural killer cells, and administration of antibodies or recombinant proteins that either costimulate immune cells or block immune inhibitory pathways (so-called immune checkpoints) (Topalian et al, 2011).

Three steps are required for effective treatment: there must be sufficient numbers of lymphocytes with avid recognition of tumor antigens; these lymphocytes must reach the tumor, and once there they must be able to destroy the tumor cells. Until the end of the 19th century the possibility that a tumor could be rejected merely by the body's immune defense was no more than a vision (Levy et al, 2010).

Cancer immunotherapy may be classified into passive as well as active strategies, with the latter being specific or nonspecific. Passive or "adoptive" immunotherapy is based on administration of antitumor antibodies or transfer of tumor-reactive lymphocytes. Active immunotherapy is aimed either at eliciting a specific de novo host immune response against selected tumor antigens (Ags) by employing cancer vaccines or at amplifying the existing antitumor immune response by administering nonspecific proinflammatory molecules or adjuvants (Mocellin et al, 2004).
After more than 100 years of preclinical and clinical research in the field, the vision of cancer immunotherapy became real and has, with multiple tools, successfully entered clinical standard practice (Lesterhuis et al, 2011).

2. ANTI-TUMOR VACCINATION

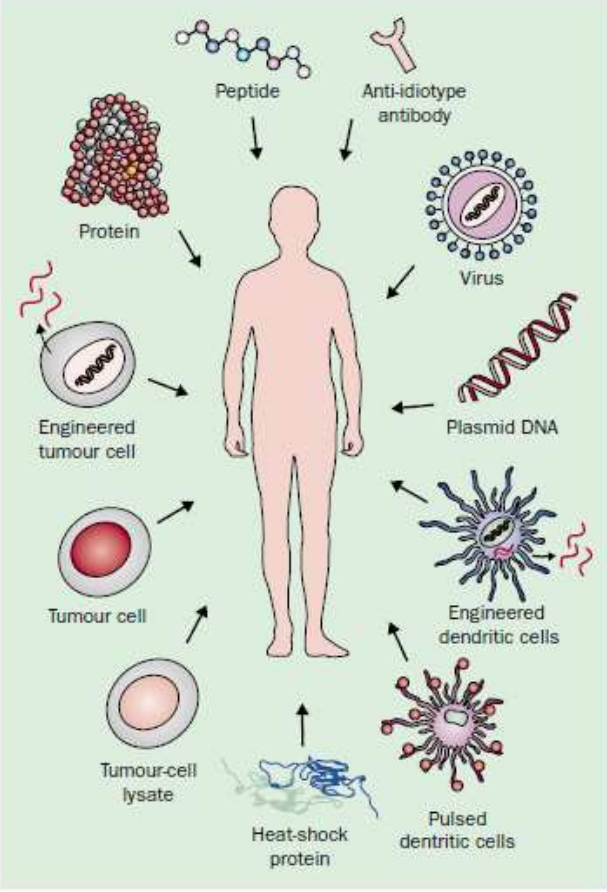


Fig 2. Anti-Tumor Vaccination. From Mocellin et al, 2004.

Vaccine development is one of the most promising and exciting fields in cancer research (Joniau et al 2011); numerous approaches are being studied to developed effective cancer vaccines. The aim of this form of therapy is to teach the patient's immune system to recognize the antigens expressed in tumor cells, but not in normal tissue, to be able to destroy these abnormal cells leaving the normal cells intact (Buonaguro et al, 2011). The molecular identification of tumor-associated antigens has led to new possibilities for the development of effective immunotherapies for patients with cancer (Gerritsen et al, 2011). Although some tumor-associated antigens derive from mutated genes, most are products of non-mutated genes that encode intracellular proteins commonly expressed by autologous cells (Linley et al, 2011). Similar to vaccine development for infectious diseases, this procedure is defined as active specific immunotherapy because the host immune system is activated de novo or is

restimulated to mount an effective tumor specific immune reaction against malignant cells (Klebanoff et al, 2011).

A variety of approaches have been advanced to focus the immune system on the targets (Mocellin et al, 2004), including (1) whole cell, tumor cell lysate, and/or subunit vaccines (autologous, or made from a patient's own tumor tissue; allogeneic, or made from individuals within a species bearing the same type of cancer; or whole cell vaccines from γ-irradiated tumor cell lines with or without immunostimulatory cytokines), (2) DNA vaccines that immunize with syngeneic and/or xenogeneic plasmid DNA designed to elicit antigen-specific humoral and cellular immunity, (3) viral vector-based methodologies designed to deliver genes encoding TAAs and/or immunostimulatory cytokines, (4) DC vaccines, which are commonly loaded or transfected with TAAs, DNA, or RNA from TAAs, or tumor lysates, (5) adoptive cell transfer (the "transfer" of specific populations of immune effector cells to generate a more powerful and focused antitumor immune response), and (6) antibody approaches such as monoclonal antibodies or anti-idiotype antibodies (Campoli et al, 2010). The ideal cancer immunotherapy agent would be able to discriminate between cancer and normal cells (eg, specificity), be potent enough to kill small or large numbers of tumor cells (eg, sensitivity), and lastly be able to prevent recurrence of the tumor (eg, durability) (Linley et al, 2011).

2.1 Current vaccination strategies

***Polyvalent vaccines**:* Whole-cell, Tumor-lysate and Shed-antigens. Live whole tumor cells inactivated by radiation were the first types of antitumor vaccines and have been investigated extensively in humans. Autologous and allogeneic tumor cells have been genetically engineered with cytokines such as granulocyte-macrophage colony-stimulating-factor (GM-CSF) and interleukin 2 to recruit and activate antigen presenting cells (APC) at the vaccination site, thus favouring the uploading of TAA and their presentation to T cells in secondary lymphoid organs. The efficacy of autologous or allogeneic whole-cell tumor vaccines have not been confirmed by phase III trials done in the therapeutic or adjuvant setting. The ability of tumor lysates to induce long-lasting antitumor immunity was first shown in animal studies on viral oncolysis. Subsequent studies showed that tumor cell lines lysed mechanically or enzymatically can also elicit an effective immune response (Aguilar et al, 2011).

On the basis of the encouraging results from animal studies and pilot studies in humans, heat-shock proteins (HSP) are being tested in phase III trials in patients with resected renal-cell carcinoma or with melanoma. These intracellular proteins

act as chaperones for peptides, including peptides derived from TAA. Dendritic cells express a specific receptor for HSP called CD91, whose engagement causes dendritic cells to mature into antigen-presenting cells (Vidal et al, 2012). Therefore, HSP released by necrotic cells function both as an endogenous danger signal and as a vehicle for dendritic cells to cross-present tumor-associated antigens. Heat-shock proteins can be isolated and used as a polyvalent, autologous cancer-vaccine preparation with undefined tumor-associated antigens, thus removing the need to identify the epitopes of TAA that are recognised by cytotoxic T lymphocytes (CTL) (Ferrucci et al, 2011).

***Antigen-defined vaccines**:* Tumor-associated antigens: 1) carbohydrates. Epitopes of tumor-associated antigens include carbohydrates such as glycosphingolipids (eg, gangliosides) and glycoproteins (eg, globoH, sialyl-Tn, Tn-alpha) (Sharma et al, 2010). They are either overexpressed by cancer cells (eg, gangliosides) or are unique tumor-associated antigens originating from the altered glycosylation of mucins associated with tumor progression. In a phase III trial, assessment of the efficacy of a ganglioside-based vaccine in comparison with alpha interferon (IFNα) for treatment of patients with high-risk melanoma showed that IFNα had a survival advantage over vaccination (Davar et al, 2011). 2) recombinant proteins: use of recombinant proteins is appealing because they have been defined molecularly and are produced easily by recombinant technology (Jain et al, 2012). Administration of these proteins leaves the immune system with the task of recognising different HLA class-I-restricted or HLA class-II-restricted peptides and thus overcomes the need to know the sequence of immunogenic epitopes (unlike for peptide-based vaccines) (Fey et al, 2011).

Many studies highlight the presence of tumor-specific antigens on mouse and human tumors that can be recognised by autologous cytotoxic T cells. Most of these antigens are peptides presented to T cells by HLA molecules. Most tumor-associated antigens derived from peptides identified to date are presented in association with HLA class I molecules and are recognised by tumor-specific cytotoxic T cells, although the number of epitopes from tumor-associated antigens presented in association with HLA class II molecules and recognised by helper T lymphocytes is increasing (Topalian et al, 2011). The finding that viral infections can lead to the presentation of viral peptides in association with MHC class I and class II HLA molecules on the surface of infected cells led to active specific immunotherapy strategies in which viruses are used as immunisation vehicles. Viruses such as adenovirus are potentially ideal vectors for the delivery of tumor associated antigens because they directly infect and activate antigen-presenting cells (Low et al, 2008). The microenvironment in which the T cell encounters an

antigen is very important in providing the appropriate costimulatory signals and is mainly determined *in vivo* by cells of the innate immune system (Bear et al, 2011). Danger stimuli such as infectious agents and tissue injury activate antigen-presenting cells, leading to various changes that enhance antigen presentation. Several studies (Kalantari et al, 2011; Palma et al, 2011; Chu et al, 2011; Bauer et al, 2011) have shown that dendritic cells are the most effective antigen-presenting cells for the priming of T cells. Dendritic cells that express high amounts of HLA class I and class II molecules as well as costimulatory molecules can effectively present peptides from tumor-associated antigens to enhance cellular immunity both *in vitro* and *in vivo* (Driessens et al, 2011; Tavare et al, 2011; Zizzari et al, 2011).

DC-based vaccination could be a promising treatment modality for various cancers, but multiple hurdles (reliable biomarkers, vaccine standardization and complicated protocols often resulting in conflicting outcomes, labor intensity, time consumption and high cost) must be cleared before the development of an affordable DC-based vaccination that can be used worldwide (Kalinski et al, 2011).

Consideration of the particular advantages and drawbacks inherent in passive immunotherapy (ie, monoclonal antibodies or T cells) and active immunotherapy (ie, vaccines and checkpoint blockade) should elucidate the best clinical settings for each approach as well as ways in which each can be improved (Brody et al, 2011).

Two advantages of this active immunotherapy approach are that it decreases the likelihood that a tumor cell will be able to alter multiple targets and that the immune system can select novel targets overlooked by the guided process of the passive approach (Bisht et al, 2010). A primary obstacle to immunization is that tumors elude immune responses by appropriating immune inhibitory signals or checkpoints. Increasing recognition of these obstacles has prompted preclinical and clinical studies of checkpoint blockade, whereby the targeting of immune-inhibitory signals can either allow an underlying antitumor immune response to be revealed or increase the efficacy (Lesterhuis et al, 2011).

The adaptive immune system has evolved two sets of genes — B-cell and T-cell receptors (BCRs and TCRs, respectively) — that undergo genetic recombination, allowing their protein products to specifically bind to antigens (Borst et al, 1996). Thus, B and T cells can adapt to specifically recognize, and then eliminate pathogens or host cells bearing these antigens. BCR or TCR targeting a desired antigen can now be engineered into therapeutic products (either monoclonal antibodies or transgenic T cells) and infused into the patient with lymphoma (Molnar et al, 2009). The advantage of such passive immunotherapy is that it does not rely on the patient's immune system—which may be dysfunctional because of tumor burden or prior therapies—to initiate a response. The disadvantage of this approach is that, as the targeted tumor cells mutate, downregulate, or otherwise

alter the targeted antigen, the disease can become resistant to therapy (Brody et al, 2011).

Monoclonal antibodies: Tumor antigen–specific monoclonal antibodies (moAbs) have been successfully implemented into standard treatment regimens for patients with a variety of malignant diseases (Von Mehren et al, 2003). The concept that antibodies could be used for the treatment of malignant disease originated more than a century ago. The first generation of antibody-based therapies were based on the use of TAA–specific allogeneic, autologous, or xenogeneic polyclonal antibodies, which were ill suited as cancer-specific therapies because of their limited or lack of specificity and reproducibility. It was not until the development of the hybridoma technology that antibody-based immunotherapy of malignant diseases became a practical reality (Campoli et al, 2010). The hybridoma technology enabled the production of a large number of human TAA–specific murine monoclonal antibodies (moAbs). Today, TAA-specific moAbs have been established as highly sensitive and reproducible probes in the diagnostic arena as well as in clinically and commercially successful therapies for a variety of malignant diseases (Tartour et al, 2011). Available clinically useful moAbs typically use a combination of mechanisms in directing cytotoxic effects to a tumor cell. Most interact with components of the immune system through Antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and many alter signal transduction within the tumor cell or act to eliminate a critical cell-surface antigen. Monoclonal antibodies can also be used to target payloads (e.g., radioisotopes, drugs or toxins) to directly kill tumor cells or to activate prodrugs specifically within the tumor (antibody-directed enzyme prodrug therapy, ADEPT) (Ludwig et al, 2003). Finally, moAbs can be used synergistically with traditional chemotherapeutic agents, attacking tumors through complementary mechanisms of action that may include anti-tumor immune responses that may have been compromised owing to a chemotherapeutic's cytotoxic side effects on T lymphocytes. Thirty therapeutic MoAbs have been approved around the world, including 24 in the United States, several of which have attained blockbuster status with sales reaching the coveted billion-dollar mark and beyond. Five MoAbs, rituximab (Rituxan), infliximab (Remicade), bevacizumab (Avastin), trastuzumab (Herceptin), and adalimumab (Humira), generated sales of over $4 billion each in 2008, and global sales for this entire sector surpassed $30 billion in that year. There are more than 250 therapeutic MoAbs now undergoing clinical trials, mostly concentrated in the areas of malignancy, autoimmune and inflammatory diseases, and infectious diseases. Beyond these, hundreds more candidates are already at the preclinical stage of development (Yamada et al, 2010).

3. PASSIVE IMMUNOTHERAPY : ADOPTIVE T CELL THERAPY

Passive immunotherapy refers to approaches in which immunologic reagents, such as serum, cells, or cell products, that are thought to have antitumor activity are administered to a tumor-bearing host. Adoptive T cell therapy (ATCT) for cancer is a form of transfusion therapy consisting of the infusion of various mature T cell subsets with the goal of eliminating a tumor and preventing its recurrence (Duong et al, 2011). Allogeneic and autologous sources of T cells derived from several anatomic sites have been tested. Indeed, in the 1970s, Chester Southam and colleagues demonstrated that subcutaneous growth of human tumor autografts to patients bearing advanced cancers was inhibited by the cotransfer of autologous leukocytes in about half of the patients. This finding suggested that lymphocytes with a specific inhibitory effect on the implantation and growth of cancer cells were present in many patients and could be mined as potential candidates for adoptive immunotherapy (Dang et al, 2007; Wrzesinski et al, 2010). The primary advantage of using $CD8^+$ T cells for adoptive therapy, as opposed to other cytolytic cells, such as NK cells, is their ability to specifically target tumor cells through the recognition of differentially expressed tumor proteins presented on the cell surface. Using T cells for adoptive therapy is also attractive due to the long clonal life span of T cells, which allows both therapeutic and immunoprophylactic scenarios to be envisioned. In addition, T cells are well suited for genetic manipulation, which has enabled the evaluation of genetically enhanced or retargeted T cells in pilot clinical trials for cancer as well as other diseases (Gross et al, 1989).

ATCT depends on the ability to optimally select or genetically engineer cells with targeted antigen specificity and then induce the cells to proliferate while preserving their effector function and engraftment and homing abilities (June et al, 2007). Unfortunately, many clinical trials have been carried out with adoptively transferred cells that were propagated in what are now understood to be suboptimal conditions that impair the essential functions of T cells. In addition, until recently, many trials were carried out before the complexity of T cell biology was understood, the important differences in T cell biology between rodents and humans that have emerged in recent years, the observation that T cell populations are heterogeneous and comprise memory cells, effector cells, regulatory cells and the process of immunosenescence (June et al, 2008).

ATCT of Tumor-Infiltrating Lymphocytes (TIL) in combination with lymphodepletion has proven to be an effective treatment for metastatic melanoma patients, with an objective response rate in 50%–70% of the patients (Besser et al, 2010; Rosenberg et al, 1989). It is based on the *ex vivo* expansion and activation of

tumor-specific T lymphocytes extracted from the tumor and their administration back to the patient. (June et al, 2007). In the 1980s, the availability of recombinant IL-2 for *in vitro* use brought the breakthrough of ATCT trials. Using IL-2 as a T cell growth factor in culture promoted the ability to expand human lymphocytes to larger scales and for longer periods (Naldini et al, 2011).

Studies on murine ATCT models (Gattinoni et al, 2005; Wrzesinski et al, 2010) have demonstrated the need for lymphodepletion prior to TIL transfer in order to eliminate suppressive $CD4^+CD25^+$ T-regulatory cells as well as normal endogenous lymphocytes that compete with the transferred cells for homeostatic cytokines such as IL-7 and IL-15. These studies reported a significant correlation between the intensity of lymphodepletion and the *in vivo* antitumor effect of the infused cells.

One of the possibilities for improving ATCT for cancer patients is based on the transfer of genetically modified peripheral T cells instead of TIL (Eshhar et al, 2011). Genetically engineered T cells may overcome several disadvantages of the TIL protocol. The TIL protocol encounters several difficulties, for example, generating TIL cultures is a technical challenge and requires high laboratory skills. Using this approach, patients have to undergo surgery in order to resect tumor tissue for TIL isolation and TIL are a heterogenic cell mixture with unknown antigen specificity (Eshhar et al, 2011). In addition, the endogenous T cell repertoire is not always responsive to defined tumor-associated antigens such as self-antigen due to developed tolerance. These disadvantages led to the investigation of an alternative method producing tumor-reactive T cells (Schroten et al, 2010). The idea is to create T cells (Cartellieri et al, 2010) with desired antigen specificity and thereby to enhance the effectiveness of ATCT. Genetic modification of T cells is based on the generation of tumor-targeted T cells through the genetic transfer of antigen-specific receptors (Berry et al, 2009), which consist of either MHC-restricted T cell receptors (TCR) or non-MHC-restricted synthetic chimeric antigen receptors (CAR) (Gross et al, 1992). The genetic approach is more convenient to the patients as it skips surgery and only requires patient's leukopheresis to obtain peripheral T cells for genetic modification (Hershkovitz et al, 2010).

3.1 Genetic modification using TCR genes

TCR genes isolated from antigen-specific tumor-reactive T cells can be exploited as therapeutic molecules by transfer of genes encoding the TCR-α and -β chains from a donor T cell to a recipient T cell of any specificity (Canderan et al, 2010).

A long-standing problem in the field of TCR gene therapy is that TCR-transduced T cells often have lower avidity than the TCR ''donor'' cell, as wild-type levels of TCR gene expression are rarely achieved in transduced cells (Schmitt et al, 2009).

Moreover, the expression levels of the introduced TCR chains generally decline further when the transduced T cells become quiescent and are no longer being triggered by the antigen, making any persisting memory cells largely ineffective (Zhang et al, 2011). The introduction of a second TCR-α and -β chains into a mature T cell also introduces the significant risk of autoimmunity due to mispairing of the introduced TCR chains with their endogenous counterparts, which not only reduces expression of the desired TCR pair but can create a new TCR with unknown specificity that can potentially cause autoimmunity (Park et al, 2011). Because a high level of TCR surface expression is essential to confer appropriate sensitivity for triggering by cells expressing the target tumor antigen, strategies that enhance TCR-α and -β gene expression levels are an important consideration in TCR gene therapy (Pinthus et al, 2004). Therefore, several strategies have been employed to reduce the likelihood of mixed TCR dimer formation (Zhang et al, 2011). In general, this involves modifying the constant (C) domains of the TCR-α and -β chains to promote the preferential pairing of the introduced TCR chains with each other, while rendering them less likely to successfully pair with endogenous TCR chains (Sadelain et al, 2009). A second approach to minimize TCR chain mispairing is to increase interchain affinity by engineering a second disulfide bond into the extracellular domain of the expressed TCR via introduction of an additional cysteine residue in both the -α and -β C domains (Zhang et al, 2011). A distinct approach has been the development of CD3 independent chimeric antigen receptors, in which the variable domains of the TCR-α and -β chain are fused directly to CD3ζ. These receptors do not pair with endogenous TCR chains, and therefore do not pose a risk of forming potentially self-reactive mixed TCR dimers (Schmitt et al, 2009).

The gene delivery vehicle used to transduce target T cells for gene therapy can influence the safety and function of the TCR expression cassette. The most commonly used vehicles are gammaretroviral (Howland et al, 2010) and lentiviral systems (Bobisse et al, 2009). Retroviruses preferentially integrate near the transcriptional start site of active genes, which can result in insertional mutagenesis with dysregulation of gene expression (Schmitt et al, 2009).

Furthermore, many retroviral constructs, particularly those based on the murine leukemia virus (MLV), have a tendency to undergo transcriptional silencing, particularly in cells not actively cycling. This represents a paradox for establishing a persistent response after TCR gene therapy, because activation through the transgenic gene product (the TCR) is necessary to drive the proliferation that is required to maintain transgene expression (Peinert et al, 2009). Lentiviruses have several advantages over gammaretroviruses, and an increasing number of gene therapy studies are using lentiviral systems for gene delivery. Lentiviruses do not have the same propensity to integrate near transcriptional start sites as

gammaretroviruses, and appear unlikely to lead to oncogenesis. Lentiviral vectors also appear to be less susceptible to transcriptional silencing (Durand et al, 2010).

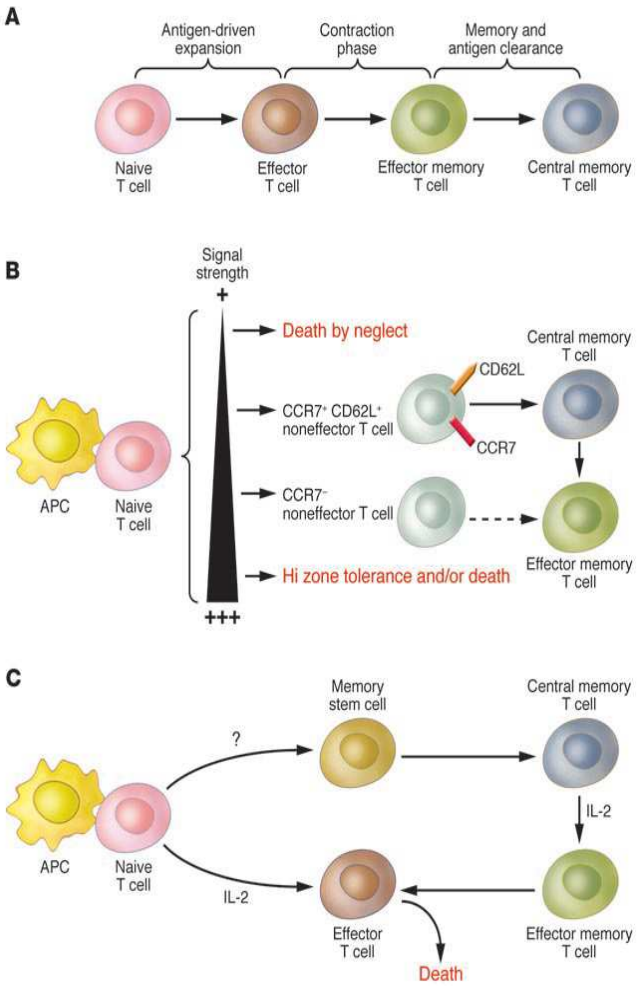


Fig 3. Models of CD8+ T cell differentiation to distinct memory cell subsets. (A) In the linear differentiation model, an autonomous antigen-triggered differentiation process consisting of conversion from naive to effector to TEM cell occurs, followed by the appearance of TCM cells after antigen clearance through a process of dedifferentiation. (B) The signal strength model proposes that naive T cells progress through hierarchical thresholds for proliferation and differentiation as the strength and duration of the interaction with APCs is increased. T cells receiving the weakest signals do not survive, whereas high-intensity signaling causes the development of terminally differentiated effector T cells that cannot survive into the memory phase. The TCM cells, being the least differentiated of the antigenstimulated T cells, retain the developmental options of naive T cells, including their capacity for marked clonal expansion. (C) The memory stem cell model
proposes that the cells within the TCM cell compartment are self renewing and serve as a source of effector T cells .

T cell therapy is often limited by the ability of transferred T cells to expand and persist *in vivo* (Huang et al, 2007; Huang et al, 2006) after transfer, and the intrinsic properties of the T cells from which infused cells are derived contribute to their fate *in vivo*. Conventional CD8 T cells can be divided into naïve T cells (TN) and antigen-experienced memory T cells (TM) (Kerkar et al, 2011). Memory T cells can be further divided into central memory T cell (TCM) and effector memory T cell (TEM) subsets, which have distinct transcriptional programs that dictate homing, phenotype, and function (Sallusto et al., 2004). When TN and TEM cells are stimulated *in vitro*, they expand and differentiate largely into short-lived effector cells, which effectively kill targets, but generally fail to persist for long periods *in vivo*. Although TCM cells also largely expand and differentiate into effector cells in response to *in vitro* stimulation, these effector cells appear to retain some of the beneficial properties of the parent TCM cell from which they were derived (Neeson et al, 2010).

3.2 Chimeric antigen receptors (CARs)

While some efforts have focused on expressing rare, naturally occurring, self- or allo-reactive, tumor-specific TCRs in T cells (Li et al, 2011), many groups have developed instead artificial receptors that are engineered to bind specifically to TAAs (Ramos et al, 2011). These receptors couple a MHC-unrestricted interaction (Eshhar et al, 1997) between a TAA and its recognizing molecule to the activating signal machinery of T cells and, because they combine portions of different molecules, they are usually referred to as chimeric antigen receptors (CARs) (Dotti et al, 2009). Most CARs utilize an antibody-derived antigen-binding motif to recognize antigen. Others utilize receptor or ligand domains as their targeting moiety, such as heregulin or IL13, that bind to their cognate ligand or receptor counterpart. In all of these instances, CARs recognize native cell-surface antigens independently of antigen processing or MHC-restricted presentation. Importantly, CARs therefore do not have to be matched to the patient HLA and can recognize tumors that have downregulated HLA expression (Sadelain et al, 2009). Chimeric antigen receptors, also known as T bodies or chimeric immune receptors, are engineered as cDNA fusion molecules.The encoded fusion receptors are expressed as homodimers at the cell surface, allowing them to be cross-linked upon target antigen engagement. Expression of a CAR thereby allows T cells to be redirected against suitable targets upon the tumor cell surface, although endogenous (TCR-mediated) specificity for processed antigen is also retained. Whilst individual CAR may vary significantly, they generally conform to a classical structure composed of three separate regions, namely the targeting moiety, the spacer region, and the signaling domain (Davies et al, 2010).

4. T-BODY

The term ‘‘T-body’’ was coined in 1993 to define chimeric molecules composed of a specific antigen-binding domain encoding the variable regions of a monoclonal antibody (Eshhar et al, 2008), linked together as a single chain antibody (scFv), and a signaling moiety derived from either the ζ chain of the TCR-CD3 complex or the γ chain of the FceRI receptor (Eshhar et al., 1993). When expressed by T cells, the chimeric molecules bind the specific antigen expressed on the cell surface of the target cells through their antibody-binding moiety, and activate the lytic pathway of the T cells on cross-linking of the chimeric ζ or γ chains that form the receptor endodomains (Gorochov et al, 1992). Many researchers now term these T-bodies "chimeric antigen receptors (CARs)" (Ramos et al, 2011).

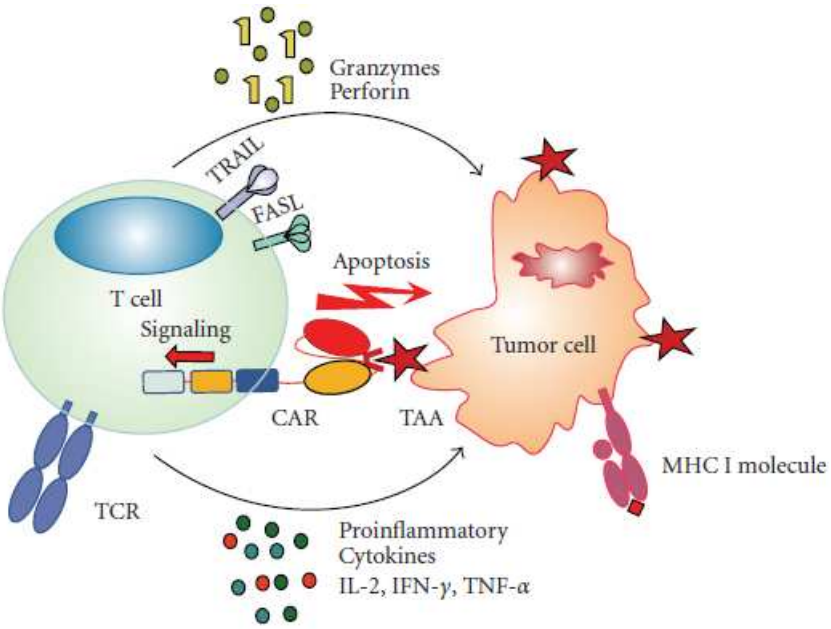


Fig 4. From Cartellieri et al, 2010.

The advantages of CARs over the native antibodies or ligands from which they derive are a consequence of their physical association with effector T cells (Sadelain et al, 2009). Thus, CAR modified T cells can have an active biodistribution (Lanitis et al, 2009), with migration through multiple tissue planes along chemokine gradients, and can recruit the multiple cytotoxic effector mechanisms available to a T cell, rather than the more restricted cytotoxic machinery associated with, for example, the Fc component of an antibody (Chinnasamy et al, 2010). CARs also offer advantages over transfer of native TCRs. Target cell recognition by TCRs is MHC restricted, precluding the design of a "universal" receptor for the treatment of patients with different HLA haplotypes. CARs, by contrast, like monoclonal antibodies, are essentially universal (Davies et al, 2010). Moreover, many tumors downmodulate MHC molecules and/or have dysfunctional antigen-processing machinery so that the target antigenic epitopes for TCR are simply not present. Because CAR-modified T cells bind directly to native proteins expressed on the surface of target cells (May et al, 2011) without the need of antigen processing or MHC-restricted presentation, they are unaffected by this immune evasion strategy. Moreover, CARs can recognize nonprotein antigens, unlike conventional TCRs. The major limitation of CARs versus TCRs is that they are generally unable to recognize antigens that are internal, even when these are processed to peptides presented by HLA molecules (Hwu et al, 1995).

4.1 Design of Chimeric Antigen Receptors

The general design of a CAR (Jena et al, 2010) consists of a binding moiety, an extracellular hinge and spacer element, a transmembrane region, and the signaling endodomain. Commonly, the binding moiety consists of a scFv, comprising the light (VL) and heavy (VH) variable fragments of a TAA-specific monoclonal antibody joined by a flexible linker. Using such scFvs, T cells have been successfully redirected against TAAs expressed at the surface of tumor cells from various malignancies including lymphomas and solid organ tumors (Bridgeman et al, 2010). A major advantage of endowing T cells with non-MHC-restricted, antibody-derived specificity, is that the potential target structures are no longer restricted to protein-derived peptides, but rather comprise every surface molecule on tumor cells including proteins with varying glycosylation patterns and non protein structures like gangliosides or carbohydrate antigens. Thus, the panel of potential tumor-specific targets is enlarged (Dotti et al, 2009).

4.2 First and later generation CARs.

The initially published CARs were designed with a single signaling domain. Several studies employing T cells modified with these so-called first-generation CARs established the feasibility of the approach, but showed very limited clinical benefit (Chicaybam et al, 2010). This has been primarily thought to be due to ineffective or incomplete activation of these cells, leading to a very limited persistence, compared for instance with that of EBV-specific CTLs (Till et al, 2009), which have been detected in circulation up to 15 years after infusion (Nakazawa et al, 2011). To exert its function, a T cell requires binding through its TCR to its cognate (native) antigen presented by an HLA molecule (resulting in the so-called "signal 1"). So as to become fully activated, however, a naïve T cell requires additional stimulatory events prompted by neighboring cells (Tartour et al, 2011). Otherwise, the end result of stimulation through the TCR is T cell apoptosis or anergy. Many of these additionally required pathways have been described, including activating ligands displayed on the surface of the cells presenting the antigen, which bind costimulatory molecules in T cells (leading to the generation of a "signal 2"), and stimulatory cytokines secreted by the same or other nearby cells (sometimes referred to as "signal 3") (Zhao et al, 2009). Examples of these ligands include CD80 and CD86, normally present in activated antigen presenting cells (APCs), which bind the costimulatory CD28 receptor (Tammana et al, 2010), expressed by T cells. As tumor cells often lack expression of the costimulatory ligands involved in physiological activation of T cells, this has been assumed to be the basis for the

modest activation, expansion and persistence of T cells expressing first generation CARs. Additionally, the prolonged expansion period of T cells *in vitro* may also be associated with downregulation of the receptors for those costimulatory ligands, further compounding the problem (Zhong et al, 2010). So as to provide T cells with additional activating signals, more recently developed second-generation CARs have been engineered to include another stimulatory domain (Chmielewski et al, 2011; Hombach et al, 2011; Zhong et al, 2010), usually derived from the intracytoplasmatic portion of costimulatory molecules, such as CD28, CD134/OX40, CD137/4-1BB (Brentjens et al, 2007; Sharma et al, 2010), lymphocyte-specific protein tyrosine kinase (Lck), inducible T-cell co-stimulator (ICOS) and DNA-activation protein 10 (DAP10) (Song et al, 2011). When these costimulatory domains are incorporated in the CAR, its activation, by engagement with the respective antigen, delivers both signal 1 and signal 2 to T cells, bypassing the need for costimulatory ligands and preventing potential anergy or apoptosis resulting from a solitary signal 1. Many *in vitro* and preclinical studies comparing first- and second-generation CARs demonstrate improved function of T cells bearing the latter. Third-generation CARs incorporating three or more stimulatory domains have also been described, but it is unclear whether the strong costimulation potentially obtained will always be advantageous (Chicaybam et al, 2011).

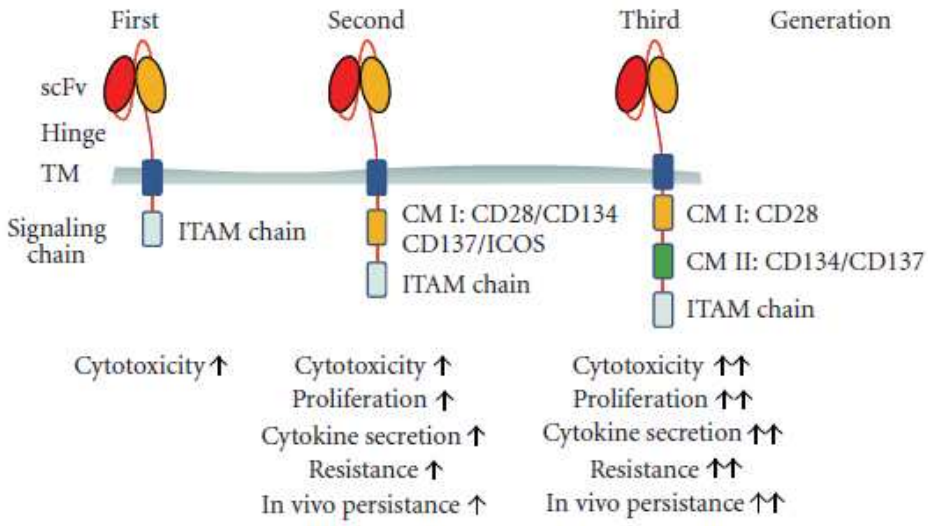


Fig 5. From Cartellieri et al, 2010.

4.3 Clinical trials

A study employing a CAR directed to the alpha-folate receptor (aFR) expressed in ovarian carcinoma demonstrated by PCR analysis that gene-modified T cells were present in the circulation in large numbers for the first 2 days after transfer, but

that they quickly declined to almost undetectable levels 1 month later in most of the 14 patients treated (Chekmasova et al, 2010).

T cells modified with a CAR specific for CD171, a cell adhesion molecule that is overexpressed in metastatic neuroblastoma and that may be involved in progression of the disease, were used for treatment of neuroblastoma patients in another CAR clinical trial. These cells were subjected to polyclonal activation, electroporation with a plasmid encoding the CAR and a marker, and hygromycin selection. No overt toxicities to tissues known to express CD171, such as the CNS, were observed. The persistence of T cells measured by PCR was shorter than 1 week in most patients with bulky disease, but up to 6 weeks in a patient with smaller disease burden (Rosenberg et al, 2008).

In another clinical trial in neuroblastoma, 11 patients were treated with identical numbers of polyclonal OKT3-activated T cells and EBV-specific LCL-stimulated T cells, with both cell populations expressing a GD2-ganglioside-specific CAR. No toxicities were observed and T-cell infusion was associated with tumor regression or necrosis in half of the subjects tested. The modified EBV-specific T cells demonstrated longer survival, of up to 6 weeks.

Several trials addressing treatment of lymphoid malignancies have been reported recently (Kochenderfer et al, 2009). One recent report describes a patient with advanced follicular lymphoma treated with a preparative chemotherapy regimen followed by autologous T cells retrovirally modified to express a CD19-specific CAR; (Kochenderfer et al, 2011; Brentjens et al, 2011; Koya et al, 2009). The patient's tumor underwent significant partial regression and B cells were absent from circulation for at least 39 weeks after T-cell infusion, despite recovery of other blood cell counts. The CD19-CAR transgene was detected in the peripheral blood up to 27 weeks after infusion (Kochenderfer et al, 2009; Kalos et al, 2011).

One of the concerns with any technique that causes permanent genetic changes is that of insertional mutagenesis, especially when there is little to no control over the sites of integration. Although these fears have materialized in studies involving genetic modification of hematopoietic stem cells, in which some children with severe combined immunodeficiency (SCID) who were treated with common gamma chain receptor genes developed leukemia, this has not been seen in any of the preclinical and clinical studies reported so far. That CAR-based gene transfer targets differentiated T cells, which have a lower risk of malignant transformation (Buning et al, 2010), instead of hematopoietic stem cells (Ramos et al, 2010). As with any therapy achieving significant tumor cell destruction, adoptive CAR T cell therapy can in theory lead to release into circulation of nucleic acid catabolites (including urates and phosphates) and intracellular ions (mainly potassium), a condition known as tumor lysis syndrome. In parallel, massive cell activation

resulting from engagement of CARs by tumor antigens could result in release of significant amounts of proinflammatory cytokines into circulation, such as IFN-γ and TNF-α, a situation that has been referred to as cytokine storm. These syndromes are life threatening and, therefore, close monitoring of patients receiving these cells is required. T cells should any untoward effects occur is desirable. Several inducible suicide systems, which could be encoded in *cis* with the CAR gene, have been described (Jena et al, 2010). Strategies involving nucleoside analogues, such as those combining Herpes Simplex Virus thymidine kinase (HSV-tk) with ganciclovir (GCV) and bacterial or yeast cytosine deaminase (CD) with 5-fluoro-cytosine (5-FC) have been used successfully in some cases, especially as a way to eliminate alloreactive T cells infused as part of a hematopoietic stem cell transplant. An inducible caspase-9 (iCasp9) system, which addresses some of the problems of existing approaches, has also been developed. This engineered protein can be activated using a specific chemical inducer of dimerization (CID), a functionally identical analogue of which (AP1903) has been safely tested in a Phase I study in humans (Ramos et al, 2011). The iCasp9 suicide gene is of human origin, and thus should be less likely to induce unwanted immune responses (Hoyos et al, 2010). Transduction of lymphocytes by this gene construct is stable and the cells are quickly and specifically eliminated less than 24 h after exposure to CID. All this suggests that an inducible suicide system could be used for increased safety of CAR-modified T cells (Chicaybam et al, 2011).

5. LENTIVIRAL VECTORS

The major goal of adoptive T cell therapy in patients with cancer is to establish effective antitumor activity that can recognize and destroy malignant cells irrespective of their site in the body (Eshhar et al, 2010). Moreover, this activity should be persistent, so that there will be destruction of resurgent malignant cells, even if these arise from phenotypically distinct tumor progenitor cells that are not themselves effectively targeted. For these benefits to be produced, CAR-modified T cells need to have adequate trafficking to the tumor site, be resistant to tumor-related immunosuppressive factors, and have robust and stable expression of their transgenic CAR (Dotti et al, 2009). Several methods are currently used to express chimeric molecules in T lymphocytes, each with a different profile of efficiency, cost, and complexity (Cheadle et al, 2010). Because T lymphocytes are highly proliferative, most studies of CAR gene transfer have used vectors that integrate into the host cell DNA. Such vectors were typically based on gammaretroviruses or lentiviruses (Jones et al, 2009), but more recent advances in the development of

integrating nonviral vectors are beginning to challenge this monopoly (Almasbak et al, 2011; Nakazawa et al 2011; Zhao et al, 2010).

Lentiviral vectors may offer certain advantages over gammaretroviral vectors (Durand et al, 2011). They can transduce nondividing or minimally proliferating T lymphocytes (June et al, 2008; Naldini et al., 1996; Hu and Pathak, 2000), and the reduced requirement for *ex vivo* activation before transduction may maximize long-term *in vivo* persistence of the transduced cells, by reducing activation-induced cell death or clonal exhaustion (Cavalieri et al, 2003). Compared with gammaretroviruses, lentiviruses also have enhanced cargo capacity, and reduced susceptibility to gene silencing. Although genotoxicity due to insertional mutagenesis may still occur, the frequency is apparently lower as there is a reduced probability of integration into transcriptionally sensitive sites (Montini et al, 2006). Modifications to lentiviruses to make them self-inactivating after integration may further lower this risk (Naldini et al, 2000). Two components are involved in the making of a virus-based gene delivery system: first, the packaging elements encompassing the structural proteins as well as the enzymes necessary to generate an infectious particle, and second the vector itself, which is the genetic material that will be transferred to the target cell (Durand et al, 2011). Biosafety safeguards, one goal of which is to prevent the emergence of replication-competent recombinants (RCRs), can be introduced in designing both of these components. The packaging unit of the first generation of HIV-based vectors comprised all of the HIV-1 proteins except the envelope. A major step towards clinical acceptability was the subsequent demonstration that the fundamental properties of this system were left intact after deletion of four additional viral genes, encoding proteins proven or likely to represent crucial virulence factors: Vpr, Vif, Vpu, and Nef. More recent studies now indicate that the main transactivator of HIV, Tat, is also dispensable for generation of a fully efficient vector. What could be termed the third-generation packaging unit of HIV-1-based vectors thus conserves only three of the nine genes present in the genome of the parental virus: gag, pol, and rev. This eliminates the possibility that a wild-type virus will be reconstituted through recombination (Naldini et al, 2000).

The system would be further improved if the transcriptional elements of HIV were removed from the vector. The modalities of reverse transcription, which generates both U3 regions of an integrated provirus from the 3' end of the viral genome, facilitate this task by allowing the creation of so-called self-inactivating (SIN) vectors (Durand et al, 2011). Self-inactivation relies on the introduction of a deletion in the U3 region of the 3' long terminal repeat (LTR) of the DNA used to produce the vector RNA. During reverse transcription, this deletion is transferred to the 5' LTR of the proviral DNA. If enough sequence is eliminated to abolish the

transcriptional activity of the LTR, the production of full-length vector RNA in transduced cells is abolished. This minimizes the risk that RCRs will emerge (Heslop et al, 2010).

In more detail: the first vector generation was made of three plasmids in which the packaging functions were provided by an Env-coding plasmid and by a packaging plasmid expressing all viral ORFs except Env under the control of a CMV promoter (in which of course the packaging sequence had been removed). The transfer vector was composed of an expression cassette framed by two wild type long terminal repeats (LTRs) and bearing sequences required for viral RNA export in producing cells (the Rev-Responsive Element, RRE), genome packaging and reverse transcription. In the second generation packaging vectors, most accessory genes were eliminated (vif, vpr, vpu and nef) and only Tat and Rev were retained, while in the third generation, Tat was also removed and Rev was provided on a fourth plasmid (third generation vectors are based on four plasmids instead of three) (Naldini et al, 2000). In the case of transfer vectors, a number of modifications contributed to increase the performance of gene transfer, for example the use of post transcriptional regulatory elements that enhance the transgene transcriptional expression as the human hepatitis virus post transcriptional element (HPRE), or the use of heterologous polyadenylation enhancer elements, as those derived from simian virus 40 (SV40) or β-globin, or the use of different internal promoters to express a particular gene (or gene products, as shRNAs) of interest. However, three major modifications have shaped the evolution of transfer vectors from their initial version. The first is the substitution of the 5' U3 viral promoter for a heterologous promoter to allow the Tat-independent transcription of the transfer vector (Durand et al, 2011). The second is a deletion of the enhancer/promoter sequence of the 3' U3. By the gymnastic of reverse transcription, this deleted 3' U3 sequence is copied at both ends of proviral DNA resulting in a provirus that lacks a functional U3 viral promoter. This deletion, at the basis of self-inactivating (SIN) vectors (Zufferey et al, 1998), increases the safety of lentiviral vectors due to the lack of expression of ψ-bearing mRNAs in transduced cells and to the minimization of gene activation in the proximity of the provirus integration site. The third is the inclusion of the central polypurine tract and central termination sequence (cPPT-CTS) that exerts a positive effect on transduction efficiency, despite controversies over its exact function.

Overall, the development of lentiviral vectors, that we have simplified here schematically, seeks to optimize the efficacy of gene transfer, while eliminating the potential dangers due to the use of retroviral vectors.

Expression of multiple transgenes within the same target cells is required for several gene transfer and therapy applications. The most common approach to multiple gene transfer has relied on using internal ribosome entry sites (IRESs). However, several reports have noted important limitations of IRES-based dual gene transfer vectors.

Naldini's group explored a new promoter design (Amendola et al, 2005): they joined a minimal core promoter upstream to an efficient promoter in opposite orientation. The rational of this design was that upstream elements in the efficient promoter, when closely flanked by core promoters on both sides, may drive transcriptional activity in both directions. If such bidirectional activation occurred, expression of both transcripts would be coordinately regulated. They joined a 516-bp fragment from the human phosphoglycerate kinase promoter (PGK) to a minimal core promoter derived from the cytomegalovirus (minCMV) that was previously developed to couple initiation of eukaryotic transcription to tetracycline dependent operators. Then, they flanked the bidirectional promoter with two expression cassettes optimized for LV-mediated gene delivery. The upstream cassette—in antisense orientation relative to the vector LTR—included the constitutive transport element (CTE) of the Mason-Pfizer virus, and a polyadenylation site from the Simian Virus 40 (SV40). The woodchuck hepatitis virus post transcriptional regulatory element (WPRE) and the SIN HIV-1 LTR polyadenylation site. The bidirectional design significantly enhanced transcription from the upstream minimal promoter without affecting downstream expression from the efficient promoter.

Approach	Advantages	Disadvantages	Use in the clinic
Virus-based approaches			
Gammaretrovirus vectors	• Extensive safety profile with two decades of clinical use	• Insertional oncogenesis documented after infusion of engineered HSCs • Poor efficiency for modifying other subsets of lymphocytes such as NK cells • Deliver smaller DNA sequences than lentivirus vectors • Susceptible to silencing, particularly in HSCs • Require induction of cell division for integration	Extensive clinical use
Lentivirus vectors	• High transduction efficiencies • Transduce quiescent T cells	• Higher production costs than for gammaretrovirus vectors • Potential genotoxicity owing to LTR sequences and HIV accessory genes; third-generation self-inactivating vectors have improved safety following removal of these sequences	Early stage clinical trials with engineered T cells using VSV-G pseudotyped lentivirus vectors
Spumavirus vectors or foamy virus vectors	• Non-pathogenic in humans following zoonotic infection • Favourable integration pattern compared with gammaretrovirus and perhaps lentivirus vectors	• Clinical-grade vector manufacturing not yet available	Not yet reached the clinic; foamy virus-mediated insertion of CD18 into HSCs corrected congenital CD18 deficiency in dogs
Ad5–35 vectors	• Recombinant Ad5–35 vectors, which use CD46 as an entry receptor, efficiently infect human T cells and support high-level transgene expression for ~1 week • Potential to introduce plasmids and antigens into T cells to generate T cells that can function as APCs	• Lymphocytes express low levels of the Ad5 receptor coxsackievirus and adenovirus receptor, so are refractory to infection; vector tropism can be modified through manipulation of Ad5 fibre protein	Ongoing clinical trial with modified $CD4^+$ T cells for HIV infection (clinicaltrials.gov identifier NCT00842634).
Lymphotropic herpesvirus (HHV6 and HHV7) vectors	• Infect nearly all humans during childhood, with low pathogenicity • Large payload capacity • Transferred genes expressed efficiently in T cells	• Clinical grade vectors not yet produced	Proposed for use in gene transfer and vaccine therapy
Non-virus-based approaches			
Random integration with electroporation	• Efficient short-term expression of transgenes; rare long-term expression achieved	• For long-term expression, the rare T cells with integrated DNA need to be selected and clonally expanded in culture	Clinical trial with a CD20-specific chimeric immune receptor was successful and resulted in engineered T cells with suboptimal engraftment and antitumour effects
Transposon-based integration	• A larger payload than retrovirus vectors • High-level, persistent transgene expression • Lower cost than for virus-based vectors	• Tests needed to determine the safety and genotoxicity relative to lentivirus vectors	Not yet entered clinical trials

Fig 6. Virus- and non-virus-based approaches for engineering lymphocytes. From June et al, 2009.

6. CANCER IMAGING

The use of small animal models in basic and preclinical sciences constitutes an integral part of testing new pharmaceutical agents prior to commercial translation to clinical practice (Imamura et al, 2011). Whole-body small animal imaging is a particularly elegant and cost-effective experimental platform for the timely validation and commercialization of novel agents from the bench to the bedside. Miniaturized versions of clinical diagnostic modalities, such as Magnetic Resonance Imaging (MRI) (Meier et al, 2011), Computed Tomography (CT), Positron Emission Tomography (PET), and Single Photon Emission Computed Tomography (SPECT) offer deep tissue penetration and high spatial resolution (Thorp-Greenwood et al,

2010). However, in small animal studies, these techniques are more costly and time consuming to implement compared with optical imaging (Kim et al, 2010). Among the optical imaging tools, bioluminescence imaging (BLI) is a promising technique that is especially useful in small animal models and does not require the use of radionuclides with their associated hazards (Gheysens et al, 2010).

6.1 Principles of Bioluminescence

In nature, numerous luminous species exist in more than 700 genera, of which 80% are marine species. Luciferase enzymes have been cloned from both marine (e.g., *Renilla* luciferase) and terrestrial (e.g., firefly and click beetle luciferase) eukaryotic organisms and are commonly used as reporters for *in vitro* and *in vivo* studies (Santos et al, 2009). They emit long wavelengths of bioluminescence (600 nm) in the red and near-infrared regions of the spectrum and are efficiently transmitted through mammalian tissues (Sadikot et al, 2008). These wavelengths can avoid absorbing and scattering environment of mammalian tissues, thus can be efficiently detected outside a small animal's body using BLI. BLI is based on the detection of light emitted by cells that express light-generating enzymes such as luciferase. In bioluminescent reactions, luciferase generates visible light through the oxidation of enzyme-specific substrates such as D-luciferin for terrestrial organisms and coelenterazine for marine organisms. Luciferases from different organisms can be distinguished by their abbreviations: *lux* (bacterial), *luc* (firefly), and *lcf* (dinoflagellate). To track cells *in vivo* by BLI, the cells of interest need to be genetically modified to express luciferase (Klerk et al, 2007). The animal recipient of the cells receives the luciferase substrate either intraperitoneally or intravenously and is placed in a light-tight dark box where luminescence is detected. In a bioluminescent reaction, the generation of light depends on several factors. Firefly luciferase requires ATP, Mg^{2+}, and oxygen to catalyze the oxidation of its substrate D-luciferin and generates CO_2, AMP, inorganic pyrophosphate, oxyluciferin, and a yellow-green light at a wavelength that peaks at 562 nm (Santos et al, 2009).

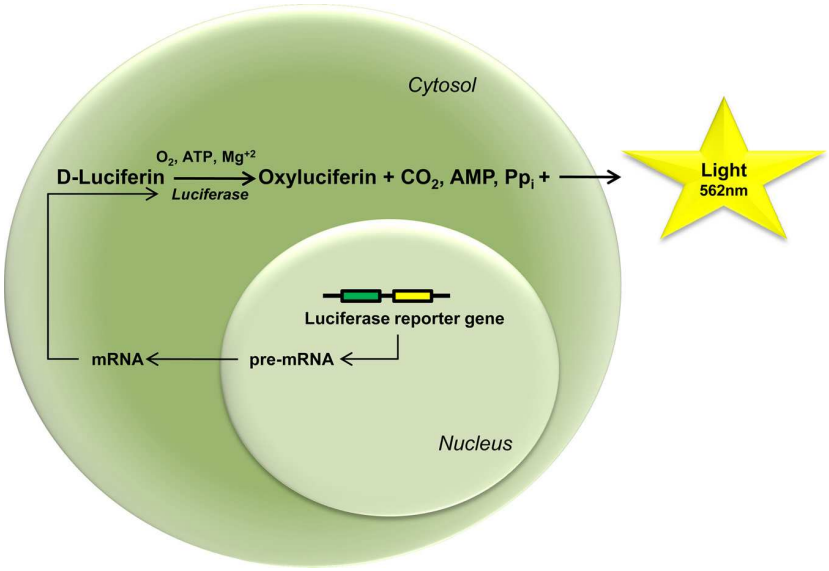


Fig 7. From De Almeida et al, 2011.

By comparison, *Renilla* luciferase catalyzes the oxidative decarboxylation of coelenterazine in the presence of dissolved oxygen to yield oxyluciferin, CO_2, and blue light that peaks at 480 nm. Bioluminescence generated by the luciferase reporter reaction is captured by a cooled charge-coupled device camera (Contag et al, 2002) that can detect very low levels of visible light emitted from internal organs. Charge-coupled device camera-imaged bioluminescence can then be superimposed on photographic images of the mouse to detect quantitatively and repetitively the bioluminescent signal from a given location. Imaging can be conducted 10 to 15 min after intraperitoneal injection of D-luciferin (reporter probe), with relatively stable light emission levels for 30 to 60 min, depending on the experimental conditions. The sensitivity of detection depends on the wavelength of light emission, expression levels of the enzyme in the target cells, the location of the source of bioluminescence in the animal, the efficiency of the collection optics, and the sensitivity of the detector.

BLI is a high throughput technique that can provide unmatched sensitivity because of the absence of endogenous luciferase expression in mammalian cells and the low background luminescence emanating from animals (Patel et al, 2009). BLI can be very useful in evaluating the delivery efficiency of therapeutic genes and their expression levels *in vivo* (Toyoshima et al, 2009). By the use of different cellular promoters to drive the expression of a luminescent reporter gene, BLI allows monitoring of the transplanted cells differentiation status as well as their location and functional characteristics *in vivo*. BLI allows real-time monitoring of survival and homing of therapeutic cells like T-bodies.

The ability to track a small number of tumor cells expressing luciferase has allowed the study of tumor growth, metastasis, and therapeutic responses *in vivo* using BLI (Dobrenkov et al, 2008). Many tumor cell lines that constitutively express luciferase have been developed. In studies characterizing the utility of bioluminescence to track tumor burden, luciferase expression in subcutaneous tumors of mice in a longitudinal study was monitored and the bioluminescent tumor growth profile was similar to that observed with caliber measurements. BLI often has better sensitivity in detecting bone marrow micrometastasis when compared with other noninvasive imaging methods, such as radiography (Santos et al, 2009). In one study, Wetterwald and colleagues found that BLI was sensitive enough to detect small foci of 0.5 mm^3 of bone marrow metastasis of luciferase-expressing human mammary carcinoma cells in mice. The capability to longitudinally track tumor growth and metastasis is useful not only to investigate specific tumor characteristics, but also can be used to better characterize genetically engineered mice with altered tumor suppressor genes (Lim et al, 2009). Luciferase-expressing tumor cell lines inoculated into these engineered mouse models allow the study of various aspects of the oncology disease process that are most relevant to the functions of the specific tumor genes altered and being studied in these mice (Contag et al, 2000). Adenovirus-induced ovarian tumor killing in mice was demonstrated effectively by using bioluminescence (Close et al, 2010). In a study utilizing rats injected with luciferase transfected colon carcinoma cells, the antineoplastic effects of cisplatin were effectively tracked by bioluminescence as concluded by decreased number of light emitting tumor sites and light signal intensity.

6.2 Limitations

BLI has been successfully used to obtain semiquantitative measurements (McCaffrey et al, 2003) of biological processes because of a strong correlation between the number of cells and the bioluminescence signal detected both *in vitro* and *in vivo* (Kagadis et al, 2010). However, a simple quantification of light emission may not provide a true representation of biological processes. This is because the firefly luciferase reaction is a complex interaction of a variety of molecules (eg, ATP, Mg^{2+}, oxygen and luciferin) and because the intensity of the bioluminescence signal depends on multiple factors. In particular, the number of metabolically active luciferase-transfected cells, the concentration of luciferin, ATP and oxygen levels, the spectral emission of bioluminescence probes, and the depth and optical properties of tissues are known to alter the intensity of bioluminescence signal (Virostko et al, 2009). Another issue that should be considered during quantitative BLI is the limited and wavelength-dependent transmission of light through animal

tissues. Light sources closer to the surface of the animal appear brighter compared with deeper sources because of tissue attenuation properties. It is estimated that for every centimeter of depth, there is a 10-fold decrease in bioluminescence signal intensity (Massoud et al, 2003). Mathematical models can be used to predict *in vivo* imaging signal levels and spatial resolution as a function of depth and to help define the requirements for imaging instrumentation. However, the overall low spatial resolution and limited tissue penetrance restrict the use of BLI to small animal studies. Changes in tissue oxygenation can also alter bioluminescence signal. In rat gliosarcoma for instance, bioluminescence signal has been shown to decrease by 50% at 0.2% oxygen (De Almeida et al, 2011). Thus, for reliable BLI measurements, it is important to understand the effects of local niche in which the luciferase expressing cells of interest reside. This is especially the case in cardiovascular studies involving hypoxia (e.g., myocardial infarction). Similarly, BLI quantification has to be carefully interpreted in studies that involve surgical procedures. Changes in tissue thickness because of the presence of inflammation, edema, sutures, and animal growth can alter light absorption and scattering as well as the bioluminescence signal (Sadikot et al, 2008).

6.3 T cell Tracking

Adoptive T-cell therapy (ATCT) has the proven potential of treating established malignancies and is now considered the best available therapy for patients with metastatic melanoma with up to 72% objective clinical responses (Burns et al, 2010). T-cell trafficking and persistence are known to be important factors in clinical responses following ATCT, yet the fate of transferred T cells and the intermediate steps between cell transfer and the outcome of the therapy are not well understood, usually because invasive methods such as biopsies, surgery or sacrificing mice for immunohistology are used (Roszik et al, 2010). These methods are time-consuming, labor-intensive and provide limited data, which consider only one or very few time points per experimental subject. Recent advances in molecular imaging have improved upon imaging techniques such as positron emission tomography, which remains limited by its inability to detect a mass of cells of 3–5 mm in diameter. Bioluminescence imaging (BLI) represents a more sensitive approach that is capable of detecting far fewer cells both *in vitro* and *in vivo* (Patel et al, 2010). It is well established that ATCT is capable of eradicating large tumor burdens. Therefore, major questions remain to be answered with regard to the fate and proliferation dynamics of transferred T cells during ATCT. Among others, these include the spatiotemporal kinetics of these T cells and their expansion rate, as well as the relationship between T-cell dynamics and tumor

rejection (Sweeney et al, 1999). Furthermore, it is expected that dynamic measurement of the trafficking of T cells used in ATCT might explain some observed discrepancies. Recent studies have used mouse or human T cells that were transduced with retroviruses expressing codon-optimized firefly luciferase or co-expressing Gaussia luciferase (Santos et al, 2009) and a T-body to target EG.7 tumor or human acute lymphoblastic leukemia tumor cells in a syngenic or a xenogenic mouse model. These treatments were either ineffective or resulted in a reduced tumor load, but not tumor rejection and therefore could not inform about the dynamic of adoptively transferred T cells during tumor rejection (Zhao et al, 2010). The inability of the transduced T cells to reject tumors can be due to the manipulation during the retroviral transduction process. T-cell differentiation *in vitro* was shown to reduce their ability to reject tumors (Tratour et al, 2011).

Bioluminescent imaging technology provides a mechanism to observe T cell homing and specific CD4+ T cell immune responses *in vivo*, in real-time in individual animals (Hesselink et al, 2007). This technique eliminates the need for large groups of mice terminated at varying time points, reducing animal usage and cost. In addition, this provides a method to efficiently observe and quantitate T cell responses in all tissue sites simultaneously, providing a window on T cell response dynamics not easily achieved using approaches that require tissue disruption for localization and quantitation of T cells (Sweeney et al, 1999).

AIM OF THE PROJECT

Prostate cancer is the second leading cause of cancer death in American men, behind only lung cancer. This type of cancer is an ideal target for immunotherapy for several reasons: i) Prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein and is specifically expressed in prostatic epithelial cells. Moreover is strongly upregulated in prostate cancer and is also present in the neovasculature of other solid tumors. These findings have spurred the development of PSMA-targeted therapies for prostate cancer. ii) The prostate is not an essential organ, and its damage does not compromise the patient's life, for this is an ideal therapeutic target. iii) the sites of metastases (typically lymph nodes and bone) are generally very vascularized, facilitating the penetration of antibodies iv) metastases are typically small and easily achievable v) also the non-invasive evaluation of PSA (Prostate Specific Antigen) serum levels, assists in assessing the response to therapy.

Despite the conditions, passive immunotherapy with monoclonal antibodies directed against prostate antigens and labeled with radioisotopes, has not produced, to date, the expected results. Indeed studies have shown that a single immunotherapeutic approach, the use of cytotoxic cells or monoclonal antibodies, may be insufficient to effectively eradicate the tumor. According to these results, we generated a chimeric receptor, to engineer T cells against hPSMA.

The genetic transfer of antigen receptors is a powerful approach to rapidly generate tumor-specific T lymphocytes to be employed in adoptive cell therapy (ACT) protocols. Differently from the physiological T-cell receptor, chimeric antigen receptors (CARs) encompass antigen recognition moieties, mainly derived from immunoglobulin variable regions, and activating domains derived from the TCR complex. This artificial structure combines the effector functions of T lymphocytes with the ability of antibodies to recognize surface antigens with high specificity and independently of antigen processing or MHC-restricted presentation.

During my Ph.D., I developed a second generation CAR containing both TCR and CD28 signalling moieties fused to a scFv targeting the hPSMA, to engineer human PBMCs for the immunotherapy of prostate cancer. Instead of the most widely used oncoretroviral vectors we propose lentiviral vectors (LV) as an alternative gene transfer method, because of the ability to efficiently transduce even less activated or nondividing T cells. Moreover, the presence of a synthetic bidirectional promoter in the vector design for the coordinate expression of both CAR and Firefly Luciferase, enabled the *in vivo* monitoring of distribution and homing properties of adoptively transferred T cells with bioluminescence imaging (BLI).

Our findings support the feasibility of LV vectors as a valid and safe tool to engineer T cells with chimeric antigen receptors; the anti-tumor efficacy of lymphocytes endowed with second generation CARs constitutes a rationale for further studies and potential clinical application of the approach, but there is still the absolute need to improve persistence, survival and homing properties of infused T cells in order to get positive therapeutic results.

MATERIALS AND METHODS

1. CELL LINES

The following cell lines were used in this study: LNCaP, PC-3 and PC-3-hPSMA (a hPSMA-transfected PC-3 derivative) human prostate carcinoma cell lines; Jurkat, a $CD4^+$ human leukemic cell line; 293T, a human embryonic kidney cell line. Cells were maintained in RPMI 1640 (LNCaP, PC-3, PC-3-hPSMA and Jurkat) or DMEM (293T) medium (Biochrom AG) supplemented with 2 mM L-Glutamine (Cambrex), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Cambrex), 200 U/ml Penicillin, 200 µg/ml Streptomycin (Cambrex) and 10% (v/v) heat-inactivated (56 °C for 30 min) Fetal Bovine Serum (FBS, EuroClone Ltd.), hereafter referred as to complete medium. All cell lines were grown in 25, 75 or 175 cm^2 flask (Falcon, Becton Dickinson) and under standard conditions (95% humidified atmosphere, 5% CO_2 and 37 °C).

2. PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from anonymous healthy donors using Ficoll-Hypaque (GE Healthcare). PBMC were plated at 1×10^6/well in 24-well tissue culture plates and activated for 48 hrs in complete medium with 50 ng/mL of OKT-3 and 300 U/mL of recombinant human interleukin-2 (rhIL-2; Proleukin; Chiron).

3. CAR ANTI h-PSMA GENERATION

The Chimeric Receptor against the hPSMA antigen contains the complete sequence of an anti-hPSMA scFv, which was derived from the anti-hPSMA D2B hybridoma clone generated in Prof. M. Colombatti's laboratory (Verona University). The sequence was cloned inside the multiple cloning site (MCS) of the pSecTag2A vector (Invitrogen) between the restriction sites SfiI and NotI. The MCS of the pSecTag2A plasmid is between two sequences: in 5', the murine Ig kappa light chain leader sequence V-J2-C, and in 3' the Myc Tag sequence. Thus, the leader-ScFv-myc sequence was used for CAR generation. The Leader-ScFv-myc fragment was amplified by PCR and inserted in the pBS SKII construct between the two restriction sites XbaI and HindIII of the previously modified MCS region. The transmembrane/intracellular sequence of the CAR is composed of a portion of the CD28 (bases 438-759) and the intracellular domain of the CD3ζ (227-563 region). The sequences were obtained starting from cDNA of EBV-specific $CD4^+$ T cells, and

than the intracellular, transmembrane and cytoplasmic domains of CD28 were fused to the CD3ζ cytoplasmic domain by PCR.
The obtained fusion product was subsequently digested with the restriction enzimes Hind III and Sal I and cloned inside the previously described pBS SKII vector, downstream the Leader-ScFv-myc fragment. The vector, which codes for the CAR molecule, was then sequenced. The genetic construct obtained was finally subcloned inside the pcDNA3.1 vector (Invitrogen) to check the capacity to drive the membrane expression of the CAR molecule, and into a commercial vector (GeneArt) with the proper restriction sites for the Lentiviral vectors generation.

4. VECTORS

Packaging Vectors (Figure 8)
pMDLg/pRRE: Lentiviral packaging vector coding for the HIV-1 genes gag and pol, without all the accessory genes vif, vpu, vpr and nef; used for the production of recombinant lentivirus.
pRSV-Rev: Lentiviral packaging vector coding for the HIV-1 protein Rev.
pMD2.VSVG: Envelope Vector for the pseudotyping of the viral particles and expressing VSV-g proteins.
pADVantage: Vector that enhances viral production

Transfer vector (Figure 8)
#945.pCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLNGFR.Wpre: SIN vector, HIV derived. The U3 region of the LTR in 3' is deleted. The vector contains a bidirectional promoter; the reporter gene eGFP (enhanced Green Fluorescent Protein) is under the control of a MinCMV promoter, while the DeltaLNGFR gene is under the control of a hPGK promoter. Two additional sequences are present, cPPT and WPRE, that enhance the transgenes expression. All the constructs for the generation of the lentiviral vectors were kindly provided by Dr. Luigi Naldini (DIBIT, Milano).

Other vectors (pcDNA3.1, pMK-minCMV-Luciferase2, pMA-CAR anti-hPSMA)
pcDNA3.1: plasmid coding for the anti-hPSMA CAR and utilized for the eukaryotic cell line transfection.
anti-hPSMA pMA-CAR (o pMA-T-body): commercial vector (GENEART) in which the anti-hPSMA CAR was inserted between the restriction sites RsrII (5') e SalI (3'); subsequently, it was used for cloning the CAR construct into the transfer lentiviral vector, under the control of a hPGK promoter (Figure 9)

pMK-minCMV.Luciferase2: commercial vector (GENEART) in which the gene sequence minCMV.Firefly Luciferase (minCMV.fluc) was inserted between the two restriction sites NheI (5') and EcoRV (3'), for the subsequent cloning into the transfer vector (Figura 9).

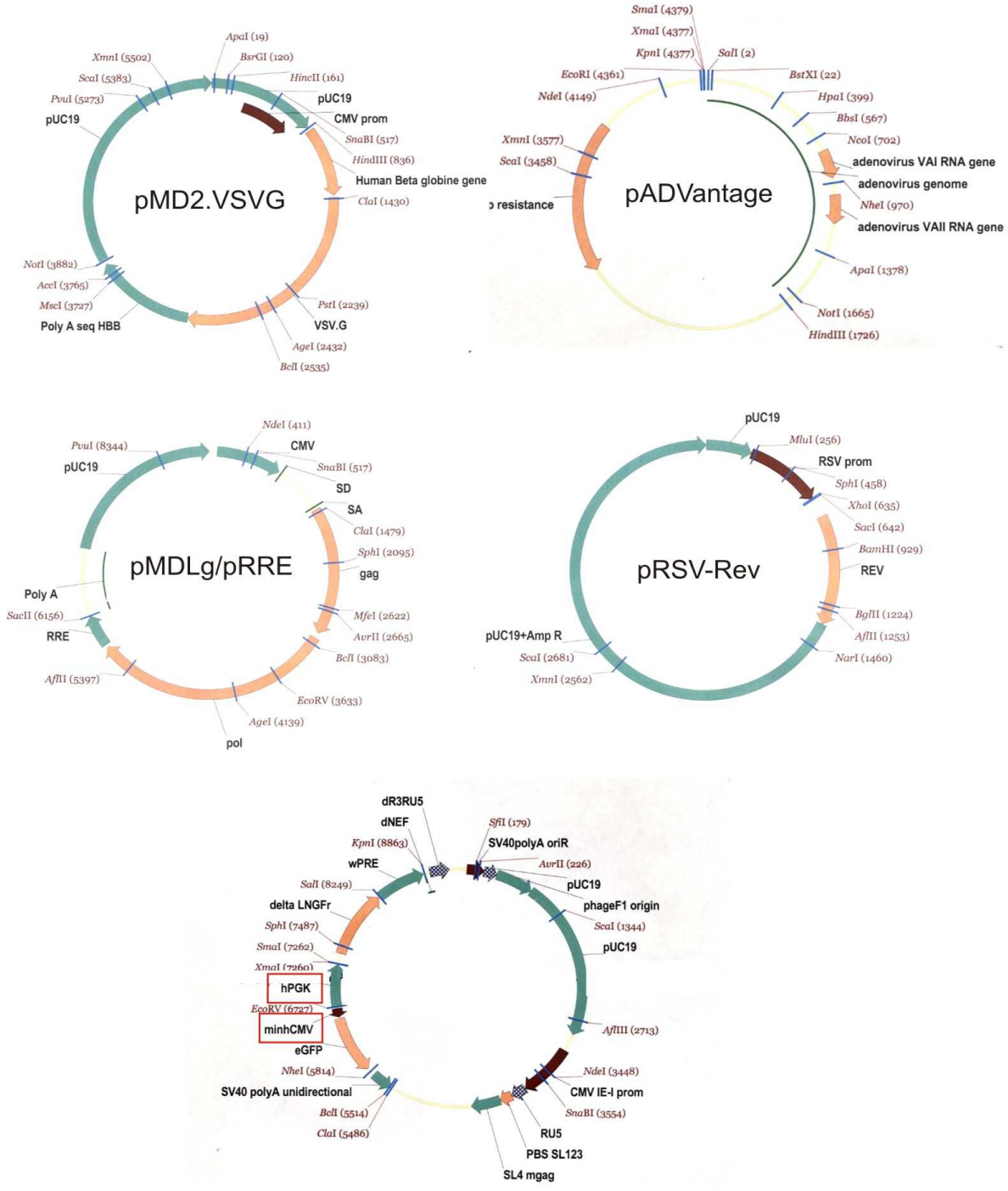


Fig 8. Lentiviral Plasmids used for viral particle generation.

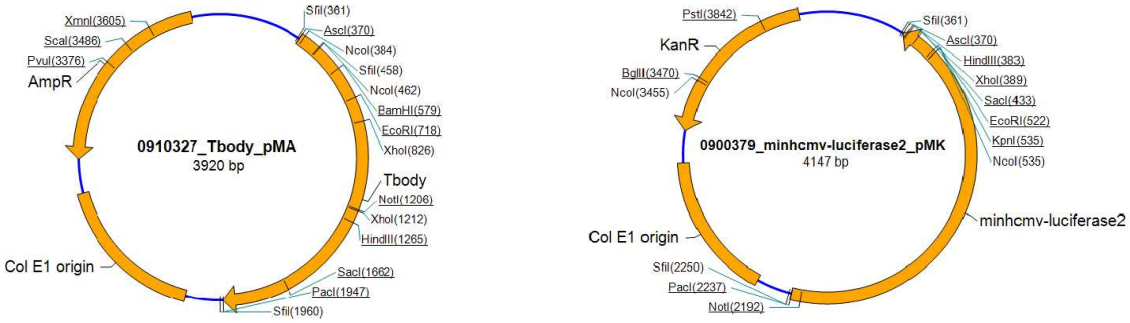


Fig 9. Maps of the commercial plasmids anti-hPSMA pMA-CAR and pMK-minCMV.Luciferase2.

5. ANTI-hPSMA CAR GENE TRANSFER by 293T CELL TRANSFECTION

293T were transfected with pcDNA3.1 coding for anti-hPSMA CAR using the kit Lipofectamine 2000 (Invitrogen). At day 0, 293T cells ($1x10^6$) were seeded in 6-well plates in 2 ml of complete medium; the next day, medium was replaced with OptiMem medium (2 ml) and 500 ul of a DNA-Lipofectamine mix (1: 2, 5, 4 ug DNA and 10 ul Lipofectamine) was added drop by drop. After a 6 hr incubation at 37 °C, the mix was replaced with complete medium.Twenty-four hrs after transfection, cells were detached with DMEM 5mM EDTA and analyzed by flow cytometry to evaluate CAR expression. Pellet of $5x10^5$ cells were stored at -80 °C for the subsequent western blotting analysis.

6. WESTERN BLOTTING

Cellular protein extracts from 293T cells treated with Lipofectamine or transfected with pcDNA3.1-CAR were obtained by lysis of $5x10^5$ cells/sample in loading buffer (2X Loading Buffer (LB): 50 µM Tris HCl pH 6.8; 2% sodium dodecyl sulfate (SDS); 2% β-Mercaptoethanol; 10% glycerol; 0.1-0.05% Bromophenol Blue) at 100 °C for 5 min and successively at r.T. for 5 min. Samples were loaded in a 10% polyacrylamide precast gel (Invitrogen), than loaded 10 µL of SeeBlue Marker as a molecular weight marker (Fermentas). The buffer for the electrophoretic run was: 25 mM Tris HC1; 190 mM glicina; 0.1% SDS; pH 8.5-9. Elettrophoresis conditions were: 2h at 8 mA, at room temperature (r.T.).

Before the transfer (blotting) of the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane (immobilion-P, Millipore), the membrane was renatured in methanol for 1 minute, finally rehydrated in bidistilled water and re-balanced in the transfer buffer (a protein running buffer with 20% of methanol). The blotting was carried out overnight (O.N.) (250 mA at 4 °C). At the end of the transfer, the membrane was washed in TBS 1X (154 mM NaCl (Sigma), 10 mM Tris (Fluka)) Tween 0.1% (polyoxyetylene-sorbitan monolaurate, Sigma) (TBS-T) to

remove any remaining gel and then was incubated 1 hr in 3% milk (Sigma) in TBS-T with constant agitation, to saturate non specific binding sites. After washes with TBS-T, the membrane was incubated 1 hr, in agitation, with an anti-c-myc primary mouse monoclonal antibody (1:1000, Sigma) in TBS-T, followed by incubation with HRP-conjugated goat anti-mouse IgG (Amersham) diluited 1:10000 in milk. At the end of incubation, proteins in membrane were detected using SuperSignal West Pico (Pierce Biotechnology) and analyzed using the BioRad apparatus (Life Science Group).

7. CONSTRUCTION AND GENERATION OF LENTIVIRAL CONSTRUCTS

7.1 minCMV-Luciferase cloning into transfer lentiviral vector.

The gene sequence coding for minCMV-Luciferase was obtained from the pMK-minCMV.Luciferase2 plasmid through digestion with EcoRV and NheI (New England BioLabs), heat inactivation of the reaction, elettrophoretic run of the reaction product and finally band extraction (QIAquick PCR Purification Kit, QIAGEN). The specific band of minCMV-Luciferase was 1852 bp in size. Digestion of the lentiviral transfer

vector#945.pCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLNGFR.Wpre

resulted in two bands of 8370 bp and 913 bp, this latter being relative to minCMV.eGFP; vector was subsequently dephosphorilated by Antarctic Phosphatase (New England BioLabs). minCMV.Luciferase insert and lentiviral vector were incubated in a ligation reaction o.n. at 4 °C with T4 DNA Ligase (New England BioLabs).

The day after, competent bacteria AG1 were transformed with reaction product, the DNA extracted with mini prep protocol (QIAGEN) and then analyzed by enzimatic digestion. The lentiviral vector obtained with the new minCMV.Luciferase insert (10222 bp) generated, after double digestion with EcoRV/NheI, 2 fragments: one of 1852 bp (minCMV.Luciferase) and the second one of 8370 bp. The sequential digestion with restriction enzimes RsrII and SalI instead produced 2 fragments of 9325 bp and 897 bp, this latter being relative to deltaLNGFR.

The vector obtained was identified as: #945pCCL.sin.cPPT.SV40ployA.fluc.minCMV.hPGK.deltaLNGFR.Wpre.

7.2 Anti-hPSMA CAR cloning in transfer lentiviral vectors

The gene sequence coding for the anti-hPSMA CAR was obtained from the plasmid pMA-CAR anti-hPSMA through sequential digestion with restriction enzimes RsrII and SalI (New England BioLabs), heat inactivation of the reaction, elettrophoretic

run of the reaction product and finally band extraction (QIAquick PCR Purification Kit, QIAGEN). The specific band of anti-hPSMA CAR was 1560 bp in size.
Digestion of the lentiviral transfer vector #945.pCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLNGFR.Wpre resulted in two bands of 8386 bp and 897 bp, the second one relative to deltaLNGFR; digestion of #945.pCCL.sin.cPPT.SV40ployA.fluc.minCMV.hPGK.deltaLNGFR.Wpre produced two bands of 9325 bp and 897 bp, the second one relative to deltaLNGFR. Vectors were subsequently dephosphorilated by Antarctic Phosphatase (New England BioLabs). CAR anti-hPSMA insert and relative lentiviral vectors were incubated in a ligation reaction o.n. at 4 °C with T4 DNA Ligase (New England BioLabs). The day after, competent bacteria AG1 were transformed with reaction products, the DNA extracted with mini prep protocol (QIAGEN) and then analyzed by enzimatic digestion. Two different viral constructs were obtained that express CAR anti-hPSMA, and identified as:
vector CAR anti-hPSMA/eGFP: transfer vector coding for CAR and eGFP;
vector CAR anti-hPSMA/fluc: transfer vector coding for CAR and Luciferase.

Table 2 reports the digestion patterns expected for the plasmid used or obtained at the end of cloning.

Plasmid	**Dimension (bp)**	**RsrII/SalI**	**EcoRV/NheI**
#945.pCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLNGFR.Wpre	9283	8386/897	8370/913
#945.pCCL.sin.cPPT.SV40ployA.fluc.minCMV.hPGK.deltaLNGFR.Wpre	10222	9325/897	8370/1852
vettore CAR anti-hPSMA/eGFP	9946	8386/1560	9033/913
vettore CAR anti-hPSMA/fluc	10885	9325/1560	9033/1852
pMA-CAR anti-hPSMA	3920	2360/1560	
pMK-minCMV.Luciferase2	4147		2295/1852

Table 2. Digestion pattern of the vectors utilized.

All plasmids were expanded by *E.coli* bacteria (AG1 strain) transformation and culture; finally, vectors used for production of the viral particles were purified by EndoFree Plasmid Mega Kit (QIAGEN), to obtain pure and concentrated DNA.

7.3 Sequencing

After the cloning, the pattern of digestion of the vectors obtained was controlled and the DNA was sequenced to exclude the presence of mutations before the viral particle production.

Table 3 reports the primers used for cloning.

vector CAR anti-hPSMA/eGFP	vector CAR anti-hPSMA/fluc	
FOR 1: ACCTTCTCAGTCTCTGTC	FOR 1: ACCTTCTCAGTCTCTGTC	FOR A: tcttatcatgtctggatctc
FOR 2: tgagaagagcaatggaac	FOR 2: tgagaagagcaatggaac	FOR B: CGGTTGTTACTTGACTGG
FOR 3: TAGGACGAAGAGAGGAGT	FOR 3: TAGGACGAAGAGAGGAGT	FOR C: CATAGGACCTCTCACACA
FOR 4: TGTGGAGCAAGTGAGAAT	FOR 4: TGTGGAGCAAGTGAGAAT	FOR D: TAATCCTGAAGGCTCCTC
FOR 5: ACTCTGGTCACTGTCTCG	FOR 5: ACTCTGGTCACTGTCTCG	FOR E: GATGAGATGTGACGAACG
FOR 6: ggtgttccgcattctg	FOR 6: ggtgttccgcattctg	FOR F: CTTATGCAGTTGCTCTCC
REV: ACGAGTGATGGAGAT TCG	REV: ACGAGTGATGGAGAT TCG	

Table 3. Primers utilized for the sequencing.

8. VIRAL PRODUCTION

For the production of viral particles, indicated as LV CAR anti-hPSMA/eGFP or LV CAR anti-hPSMA/fluc, a third generation system was used. This system uses 5 plasmid for the transfection of the packaging cells. 293T cells ($9x10^6$) were seeded and grown in T175 cm^2 flask (Falcon, Becton Dickinson) for 24h in complete medium; then, the medium was changed and cells were transfected using calcium phospate transfection method.

The method employed the following DNA concentrations:
9 µg of pMD2.VSVG
12.5 µg of pMDLg/pRRE
6.25 µg of pRSV-Rev
15 µg of pADVantage
32 µg of transfer vector

$CaCl_2$/HBS/DNA precipitate: plasmid DNA, 125 µl of $CaCl_2$ 2,5M (Sigma) and TE/H_2O (2:1) to bring the total volume to 1250 µl. After 5 min at room temperature, the $CaCl_2$/DNA mix was added to the HBS 2X (Sigma) slowly with a

P1000 pipette, mixing gently during the addition and finally added directly to cells by dropping slowly and evenly into medium, trying to cover as much of the plate as possible. After incubation of cells at 37°C/5% CO_2 for 12-14 hrs, medium was removed and replaced with fresh, warm complete medium with the addition of sodium butyrate (1 mM, Sigma), and incubation was continued for 30 hrs prior to assaying. Finally, the medium was collected, centrifuged at 3000 rpm, aliquoted and conserved at -80 °C. Alternatively, the viral surnatant was concentrated by ultracentrifugation at 7200 g for 2 hrs at 4 °C, resuspended in RPMI and conserved at -80 °C.

8.1 Viral particle titration.

The quantity of viral particles present into the viral preparations was estimated with an ELISA system (Kit Innotest™ HIV Ag mAb, INNOGENETICS), and expressed as the amount of $p24^{gag}$ equivalents present in the viral supernatants. The titres obtained in the preparations were about 70-80 µg p24/ml for LV CAR anti-hPSMA/eGFP and of 50-60 µg p24/ml for LV CAR anti-hPSMA/fluc (values in terms of viral production concentrated 100X).

In the case of LV CAR anti-hPSMA/eGFP, titration was also carried out using flow cytometry analysis on 293T cells transduced with serial dilutions of the concentrate viral supernatant. Briefly, at day 0 293T cells (10^5/well) were seeded in 6-well plates (Falcon) and supplemented the day after with viral supernatant at various concentration in 1 ml of complete medium with 8µg/ml protamine sulfate (SIGMA); at day 2, each sample was diluted 1:2 in complete medium and incubated for 72 hrs prior to assaying. For flow cytometry analysis, cells were detached, washed, fixed (PBS 1% Formaldehyde), and incubated 15 min in ice before final resuspension in incubation buffer (0.5 g bovine serum albumin (BSA) in 100mL 1X PBS) for the analysis.

The non concentrated viral preparation titre, expressed in Transducent Units/ml (TU/ml), was calculated using the following formula:

$TU/ml = \%\ eGFP^+ \times (2x10^5)$ cells x virus dilution

For LV CAR anti-hPSMA/eGFP, the average titre obtained was $21x10^6$ TU/ml. Infectivity was calculated as ratio between TU and ng of p24. LV CAR anti-hPSMA/fluc titre was calculated indirectly and was about $14\text{-}15x10^6$ TU/ml.

The multiplicity of infection (MOI) is the ratio of TU to the absolute number of transduced cells. In this study, a MOI 100 was used for transduction of human

PBMC with LV CAR anti-hPSMA/eGFP, while LV CAR anti-hPSMA/fluc was used at 20X concentration.

9. ANTI-hPSMA CAR GENE TRANSFER IN JURKAT T CELLS BY TRANSDUCTION WITH LENTIVIRAL VECTORS

To assess the functionality of LV vectors, $2x10^5$ Jurkat cells were incubated with 1 ml of non concentrated viral supernatant (LV CAR anti-hPSMA/eGFP or LV CAR anti-hPSMA/Luciferase) for 15 hrs in the presence of 8 µg/ml protamine sulfate (SIGMA). The day after, cells were washed and resuspended in complete medium. Non transduced Jurkat cells were used as negative control. Flow cytometry analysis was carried out at different time points post transduction (72 hrs, 7, 14, 28 days, 2 and 3 months) to evaluate CAR and eGFP expression, while Luciferase activity was analyzed by BLI.

10. ANTI-hPSMA CAR GENE TRANSFER IN PRIMARY T CELLS BY TRANSDUCTION WITH LENTIVIRAL VECTORS

T cells cultures that were phenotypically and functionally characterized and then used for studies of in vivo adoptive transfer, have been generated according to the following transduction protocol: $1x10^6$ PBMC were activated for 48 hrs with OKT-3 (50 ng/ml) and IL-2 (300 U/ml); after resuspension with 200 µl of concentrated viral supernatant, PBMC were incubated in 14 ml polystyrene tubes (Falcon) for 1 hr at 37 °C and 5% CO_2, in presence of 40 µg/ml (5X) of protamine sulfate and 500U/ml (5X) of IL-2. After 1 hr, volume was adjusted to 1 ml with complete medium. Thereafter, cells were moved in 24well plates and incubated at 37 °C, 5% CO_2 for 18 hrs (Final MOI: 100 for LV CAR anti-hPSMA/eGFP and 300 for LV CAR anti-hPSMA/fluc). At the end of incubation, supernatant was eliminated and changed with complete medium added with 100U/ml of IL-2. Seventy-two hrs later, PBMC were collected, washed and then analyzed for CAR and eGFP expression by flow cytometry analysis and for luciferase expression by BLI. PBMC transduced with CAR anti h-PSMA are also referred as to T-body-hPSMA/eGFP when expressing eGFP, and T-body-hPSMA/fluc if expressing Luciferase.

11. ANTIGENIC STIMULATION OF THE T LYMPHOCYTES TRANSDUCED WITH ANTI-hPSMA CAR

To expand the transduced T cell population, T-body-hPSMA were restimulated in vitro once a week using irradiated (60 Gy) human prostate carcinoma LNCaP ($hPSMA^+$) tumor cells as stimulator. T cells ($1x10^6$) and LNCaP cells ($1x10^5$) were

co-cultured in 24-well plates in 2 ml of RPMI complete medium added with 100 U/ml of IL-2. Four days after restimulation, transduced T cells were collected, washed, and re-seeded at a concentration of $5x10^5$ cells/ml, with IL-2 (100 U/ml).

12. FLOW CYTOMETRY ANALYSIS

Flow cytometry analysis was carried out with FACSCalibur (Becton Dickinson) and data were analyzed by the CellQuest software (BD Biosciences). Through the various steps of flow cytometry labeling, reagents and centrifugation were maintained at 4 °C. Labeling with antibodies and final cell resuspension before reading were made in flow cytometry buffer (Phosphate Buffered Saline, PBS 1X (Oxoid) with 2% BSA (Sigma) and 0,02% sodium azide, NaN_3 (Sigma)). At the end of labeling and just before the analysis, transduced cells were fixed with PBS 1% Formaldehyde, for 15 min on ice.

12.1 eGFP expression analysis.

eGFP expression in PBMC transduced with the CAR anti-hPSMA/eGFP was assessed directly in cell samples after fixation and resuspension in flow cytometry buffer.

12.2 Assessment of anti-hPSMA CAR expression.

Membrane expression of the CAR was evaluated by flow cytometry taking advantage of recognition of c-myc epitope inserted in the CAR structure. CAR-expressing cells (transfected 293T cells and transduced Jurkat cells or human PBMC) were labeled with the anti c-myc mAb clone 9E10 (SIGMA) or the isotypic control (mouse IgG1, UNLB, Southern Biotech), followed by staining with a secondary antibody conjugated to fluorescein isothiocyanate (Polyclonal Rabbit anti-mouse Ig FITC, Dako). Cells ($2\text{-}5X10^5$/sample) were labeled in ice for 30 min, washed in PBS 1X, fixed and resuspended in flow cytometry buffer for the analysis. Non transduced samples were used as negative controls. For labeling of other surface molecules, specific antibodies were added after the secondary antiserum.

12.3 Evaluation of transduced T cell phenotype.

Phenotype of CAR-transduced T cells, in conjunction or not with c-myc expression analysis, was determined using anti-CD8 APC, anti-CD4 APC, anti-CD62L FITC, anti-CD27 FITC, anti-CD28 FITC, anti-CD57 FITC (all from Biolegend), anti-IL-7Rα PE (CD127, Becton Dickinson), anti-CCR-7 FITC (eBioscience), and the relative isotypic controls purchased from the same companies.

12.4 Flow cytometry analysis of intracytoplasmic IFN-γ production.

IFN-γ production by transduced PBMC was evaluated by intracytoplasmic labeling using the Cytofix/Cytoperm Plus Golgi Stop kit (BD Pharmingen), a FITC-conjugated anti-human IFN-γ mAb and the mouse IgG1 FITC isotypic control (both from IOTest). Analysis was performed 72 hrs post-transduction on T-body-hPSMA/fluc and the citokine production was evaluated in resting conditions (non stimulated T-bodies), and in the presence of PC-3-hPSMA tumor cells (antigenic stimulus), PC-3 cells (not expressing the antigen) and a maximum stimulus, represented by PMA (phorbol, 12-mirystate 13-acetate, 40 ng/ml; Sigma) and Ionomycin (4 µg/ml; Sigma). More specifically, $1x10^6$ T-bodies were seeded in 24-well plates with target cells (1:1 ratio) and incubated for 15 hrs at 37 °C, 5% CO_2 in a final volume of 2ml of complete medium added with monensin (Golgi Stop), an inhibitor of the intracellular transport. After the incubation, cells were washed, resuspended in PBS 1mM EDTA (Sigma) for 15 min at 37 °C 5% CO_2, and finally in 1 ml of PBS 10% FBS to saturate all the aspecific binding sites and incubated for additional 10 min at r.T. After a further wash in PBS, cells were permeabilized (20 min in 250 µl of Cytofix/Cytoperm in ice), washed 2 times in Perm/Wash and finally labeled with the anti-IFN-γ FITC (and the relative isotypic control) for 15 min at r.T. At the end of the labeling, cells were washed two times with PBS and resuspended in flow cytometry buffer for the analysis.

12.5 Evaluation of hPSMA antigen expression in tumor cells.

Expression of hPSMA antigen was evaluated in prostate carcinoma cells, both in culture and *ex vivo* from tumor masses induced by PC-3-hPSMA s.c. injection. In this regard, tumors were explanted, digested 1 hr at 37 °C, 5% CO_2 in a solution containing DNase (270 I.U./ml), Hyaluronidase (70 I.U./ml) and collagenase (200 I.U./ml). At the end of incubation, the suspension obtained was gauze-filtered and washed. Cells ($2\text{-}5x10^5$ cells/sample) were labeled with anti-hPSMA D2B mAb clone or the relative mouse IgG1 isotypic control, followed by staining with PE-conjugated secondary antibody (Goat anti-mouse IgG1 RPE Human adsorbed, Southern Biotech); after washing, cells were resuspended in flow cytometry buffer for the analysis. Alternatively, tumor cells were directly labeled with Alexa680-conjugated D2B mAb.

13. ^{51}Cr RELEASE ASSAY

Lytic activity of PBMC transduced with anti-hPSMA CAR was evauated in a ^{51}Cr-release assay, using PC-3, PC-3-hPSMA and LNCaP cells as targets. Target cells were labeled for 1 hr at 37 °C with 100 µCi of $Na_2{}^{51}CrO_4$ and finally resuspended in

complete medium at the concentration of $2x10^4$/mL. One-hundred µL of each cell suspension (corresponding to 2000 target cells) were seeded in triplicate in a U-bottom 96-well plate, (Bibby Sterlin Ltd) in presence of scalar dilution of effector cells (1:2 dilution). After a 4 hrs incubation at 37 °C, 100 µL of supernatant was collected from each well and radioactivity was assessed using a γ-counter (Cobra Gamma Counting System, Packard Instrument Company). The percentage of specific lysis was calculated using the formula:

$$\% \text{ Specific lysis} = \frac{100\% \times (\text{Sample cpm} - \text{Spontaneous Lysis Control cpm})}{\text{Maximum Lysis Control cpm} - \text{Spontaneous Lysis Control cpm})}$$

14. CONSTRUCTION AND GENERATION OF LUCIFERASE-ENCODING LENTIVIRAL PARTICLES

The commercial vector pGL4.10 (Promega) encoding firefly luciferase (luc2) codon optimised for more efficient expression in mammalian cells was used. The gene was excised by BglII–XbaI digestion and inserted into the transfer plasmid pHR'tripCMV-IRES-tNGFR-SIN (93) to yield pHR'tripCMV-luc2-IRES-tNGFR-SIN. The Fluc gene is driven by a cytomegalovirus (CMV) promoter and followed by the truncated nerve growth factor (tNGFR) gene, separated by an internal ribosomal entry site (IRES). Luciferase-encoding lentiviral vector particles were produced in 293T cells by transient cotransfection of the transfer (pHR'tripCMV-luc2-IRES-tNGFR-SIN), envelope (hCMV-G) and packaging plasmids (p8.74). The vector stock was collected 48 and 72 h post-transfection, and concentrated by ultracentrifugation.

15. GENERATION OF LUCIFERASE-POSITIVE TUMOUR CELL LINES

In order to use bioluminescence *in vivo*, grafted cells need to express the Luciferase enzyme. In this study, cells were transduced with the lentiviral vector coding for luciferase (LV-LUX) described above: $5x10^5$ PC3-hPSMA and PC3-WT cells were harvested and resuspended in 1 ml FBS complete medium with concentrated LV-LUX (3- to 5-fold). Cells were incubated O.N. at 37 °C in the presence of the virus, then the supernatant containing the virions was discarded and fresh medium was added. Seventy-two hrs after infection, $2x10^5$ cells were collected, resuspended in 50 µL PBS and plated in a 96-well black plate (Nunc). Then, 50 µL of D-Luciferin (0.3 mg/ml, Caliper) was added to the cells for 5 min, and the plate was analyzed using IVIS Lumina II.

16. MICE

In vivo experiments were performed using 6 to 8 wk-old male severe combined immunodeficient (SCID) mice, $Rag^{-/-}/\gamma_c^{-/-}$ mice and NOD/SCID mice (n = 5-11 for all groups); animals were purchased from Charles River Laboratories (Calco, Como, Italy) and housed in our specific pathogen-free animal facility at the Department of Oncology and Surgical Sciences, Padua University (Italy). Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (D.L. 116/92 and subsequent implementing circulars), and experimental protocols were approved by the local Ethical Committee of Padua University (CEASA).

17. WINN ASSAY

Winn assay was performed by injecting s.c. a cell suspension of tumor cells (5×10^6/ mouse) alone or mixed with either RPMI or a CAR-transduced T-cell suspension (5×10^6/ animal). Mice were monitored daily to evaluate the growth of the tumor masses and tumor volumes were calculated applying the formula:

$$Tmass = \frac{d^2 \times D}{2},$$

where d and D are the minimum and maximum diameters, respectively.

18. ADOPTIVE TRANSFER OF TRANDUCED LYMPHOCYTES

For therapeutic experiments, tumor-bearing mice were repeatedly treated by i.v. injections with 20×10^6 CAR-transduced T lymphocytes, starting from day 4 after tumor inoculation, and up to a maximum of three injections.
Lux-transfected PC-3-hPSMA and PC3-WT were injected i.v. in NOD/SCID mice ($1x10^5$/mouse) at day 0. At day 3, injected mice were randomly divided into two groups, one receiving 20×10^6 CAR-eGFP transduced T lymphocytes/mouse for 3 times whithin a week. Thereafter, mice were monitored weekly by BLI under gas anesthesia.

In different experiments, PC-3-hPSMA and PC3-WT were injected i.v. in NOD/SCID mice ($1x10^5$/mouse) at day 0. Then, at day 3 part of animals received i.v. 20×10^6 CAR-Lux transduced T lymphocytes/mouse for 3 times whithin a week. All mice were analyzed weekly using IVIS Lumina II. Where indicated, six doses of 30,000

units/dose of rhIL-2 were injected i.p., 6 to 8 h apart, into control and treated tumor-bearing animals.

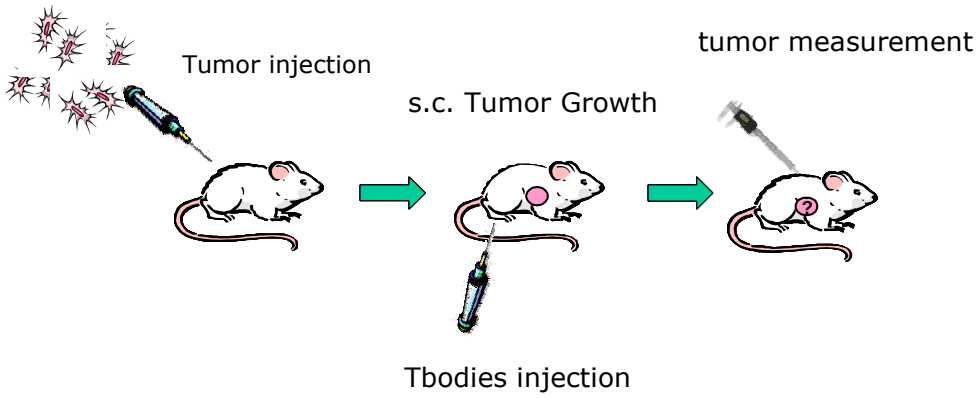


Fig 10. Therapeutic Protocol. Tumor cells s.c. injection and Tbodies adoptive transfer.

Survival curves obtained were analyzed with Log-rank test and p-values calculated using the statistical program MedCalc® (version 10.0.2.0).

The *in vivo* tumour-growth experiments were conducted according to the guidelines of the UK Coordinating Committee in Cancer Research (UKCCCR) (Cancer Metastasis 1989, "UKCCCR guidelines for the welfare of animals in experimental neoplasia")

19. OPTICAL IMAGING (Bioluminescence)

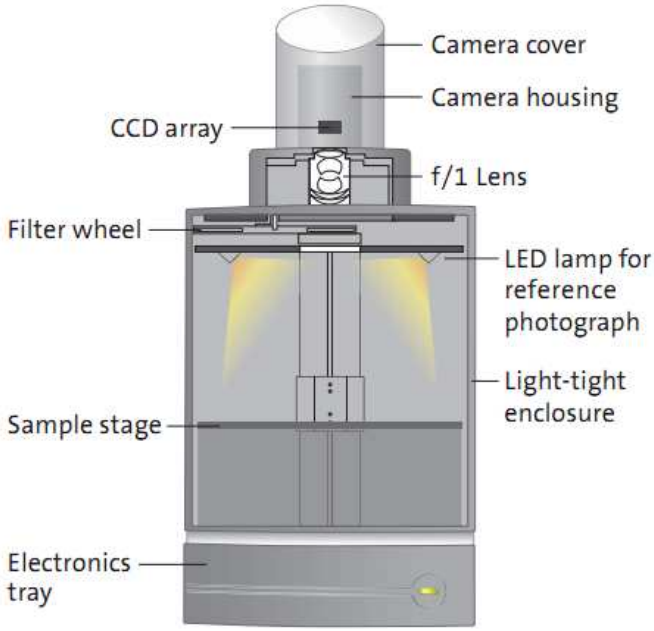
19.1 Bioluminescece (BLI).

In vivo imaging with bioluminescence exploits the catalysis of particular substrates in order to generate photons that can be detected by dedicated instruments. This kind of signal can be useful for monitoring the growth of tumour mass *in vivo* during time and it can be used to set up a more reliable model of pathogenesis *in vivo*. Moreover, it can be very useful in a pre-clinical therapeutic analysis, in order to give precise information on the efficacy of the treatment.

19.2 IVIS Lumina II.

In this study, the IVIS Lumina II (Xenogen/Caliper) was used: the machine consists of a cryogenically cooled charge-coupled device (CCD) and highly sensitive camera that is positioned above the imaging chamber. The animal bed is heated and can

accomodate up to three mice at the same time, with a field of view of 5-12.5 cm. Living Image® Software (Xenogen) (Figure 11) was used to acquire and quantitate the bioluminescence imaging data sets.



INSTRUMENT SPECIFICATIONS

Hardware

Field of View (width x length):	5 cm X 5 cm to 12 cm X 12 cm (variable zoom)
Resolution:	50 to 400 µm (based on zoom lens position)
Sensitivity:	100 photons/s/cm^2/sr
Scan Time:	1 to 5 min
Reconstruction Time:	1 to 30 sec
Scans needed for 1 mouse (Nose to Rump):	1 scan (at farthest position) to 3 scans (at nearest position)
Maximum whole Mice per Scan	3

Fig 11. IVIS Lumina II technical specifications.

19.3 Analysis of cells expressing the enzime Firefly Luciferase.

In order to use bioluminescence in vivo, grafted cells need to express the Luciferase enzyme, an oxidative enzyme that catalyzes the reaction:

$$\text{ATP} + \text{D-luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyiluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

In this study, tumor cells were transduced with the lentiviral vector coding for luciferase (LV-LUX) described above. Cells were incubated O.N. at 37 °C in the presence of the virus, then the supernatant containing the virions was discarded and fresh medium was added; the analysis was performed 72 hrs later. CAR-Luc transduction has been described previously (see section 10). For the analysis, $2x10^5$ cells were collected, resuspended in 50 µL PBS and plated in a 96-well black

plate (Nunc). Then, 50 µL of D-Luciferin (0.3 mg/ml, Caliper) was added to the cells for 10-15 min. For the evaluation of Lux expression, the plate was analyzed using IVIS Lumina II. Platform was positioned in "A" level (FOV 4x4cm), and analyzed at different exposition times, from 1 second to 5 min. The signal intensity was expressed in p/sec/cm^2/sr.

19.4 Animal analysis.

Ten to fifteen min before imaging, a single i.p. injection of 150 mg/kg of D-luciferin (Xenogen/Caliper) in PBS was administered to each mouse. Subsequently, the animals were positioned within the imaging chamber under anesthesia. Three mice were imaged simultaneously with exposure times ranging from 0.5 to 3 min. 12 x 12 cm field of view and low, medium, or high binning levels were applied to maximize sensitivity and spatial resolution. Ventral images were obtained for each animal. The ventral signals were quantified through region of interest (ROI). The resulting signal summations (in units of photons/s) were normalized to the ROI area so that all measurements are given in photons/s/cm^2.

RESULTS

1. GENERATION OF ANTI-hPSMA CAR

We designed a CAR that specifically recognize hPSMA, an integral type 2 membrane protein that is highly and specifically expressed on prostate epithelial cells and strongly upregulated in prostate cancer and metastases. The anti-hPSMA CAR sequence encoded the following components in-frame from 5' to the 3' ends (Fig. 12A): the leader sequence, the single-chain variable fragment (scFv) of the D2B antibody, the myc tag sequence, which allows the rapid and simple detection of the receptor expression, and the CD28 costimulatory molecule linked to the CD3ζ sequence that contains 3 immunoreceptor tyrosine-based activation motif or ITAM for short, which is essential for the signaling capacity of the receptor.

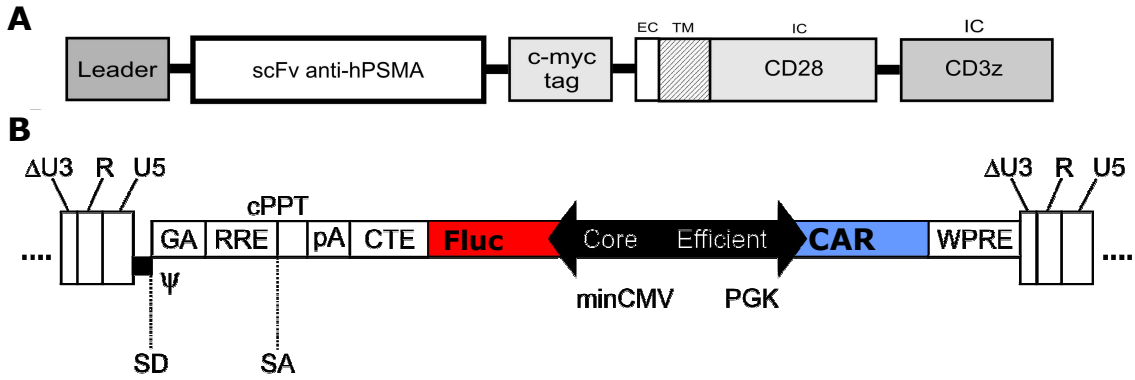


Fig 12. CAR Structure. A) Anti-h-PSMA CAR structure. EC: extracellular domain; TM: transmembrane domain; IC: intracellular domain. B) LV for CAR transduction. HIV-1-derived SIN vector,in which the U3 region of LTR is deleted; it contains a bidirectional promoter and the reporter genes eGFP or Luciferase are under the control of the minCMV promoter, while the CAR gene is under control of the hPGK promoter. The cPPT and WPRE additional sequences are responsible for an increase of transgene expression.

The CD28 sequence, spanning amino acids 114 and 220, include an extracellular portion (114-153 aa), which functions as a spacer between the ScFv and the cell membrane, and a transmembrane portion (154-176 aa), serving as anchorage of the CAR to the membrane. The intracellular portion (177-220 aa) contains the transduction signal domain that was linked to the cytoplasmic region (52-164 aa) of the CD3ζ molecule, which carries the ITAMs regions for the signal transduction.

2. EXPRESSION ANALYSIS OF ANTI-hPSMA CAR BY TRASFECTION

To assess the capacity of the construct to drive the expression of the correct receptor, the CAR sequence was cloned into the pcDNA3.1 vector and the resulting

plasmid was used for transient transfection of 293T cells. At different time points thereafter, expression of the chimeric receptor was assessed by cytofluorimetric analysis and Western blotting using an antibody that recognizes the c-myc tag. Results disclosed that the CAR was correctly mounted to the membrane (Fig. 13, *left panel*) and had the expected molecular weight (about 60 kDa, Fig. 13, *right panel*); CAR expression was already detectable 24 hours post-transfection, to rapidly disappear the next days due to the lack of a selection step.

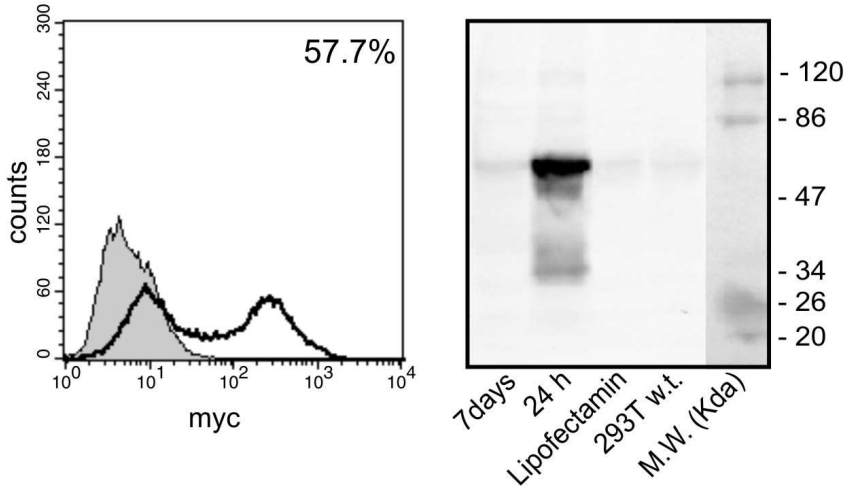


Fig 13. CAR expression in 293T cells transfected with pcDNA3.1. Left panel, Flow cytometry analysis 24 hours after transfection: the panel reports the profile and percentage of cells expressing the CAR (black line); in grey, the non transfected cells. Right panel, Western blotting on the same samples analyzed by flow cytometry; cells were analyzed at day 1 and 7 after transfection and compared with 293T cells untreated or treated with Lipofectamine alone.

3. DEVELOPMENT OF EFFICIENT BIDIRECTIONAL LV FOR ANTI-hPSMA CAR EXPRESSION

LV carrying bidirectional expression cassettes were produced to high titer and infectivity, approaching those obtained with standard vectors. The bidirectional design (Fig. 12B) significantly enhanced transcription from the upstream minimal promoter without affecting downstream expression from the efficient promoter. The bidirectional promoter comprises a part of hPGK promoter, 516 bp, fused to minCMV promoter elements, and is flanked by two expression casettes that allows the contemporary expression of the anti hPSMA CAR and of a reporter gene (eGFP or *Firefly Luciferase*). The first expression cassette includes the costitutive transport element (CTE) of the Mason-Pfizer virus and a poliadenilation site of the simian virus SV40; the second one includes the *woodchuck epatitis virus post transcriptional regulatory element* (WPRE), that allow a better transgene expression, and the poliadenilation site SIN HIV-1 LTR.

For the generation of the bidirectional LV coding for the anti-hPSMA CAR and the reporter genes, several cloning steps and a number of plasmid constructs were

necessary (see Materials and Methods). Finally, two lentiviral transfer vectors were generated: CAR anti-hPSMA/eGFP and CAR anti-hPSMA/fluc. After cloning, the correct digestion profile of the vectors obtained was analyzed (Fig. 14), and sequencing was carried out to exclude insertional mutations.

The Lentiviral vectors obtained were used for the production of non replicative viral particles, pseutotyped with VSV-G protein, by a cotrasfection method that used 5 plasmid (see Materials and Methods). The viral particles obtained were indicated as LV CAR anti-hPSMA/eGFP and LV CAR anti-hPSMA/Luciferase.

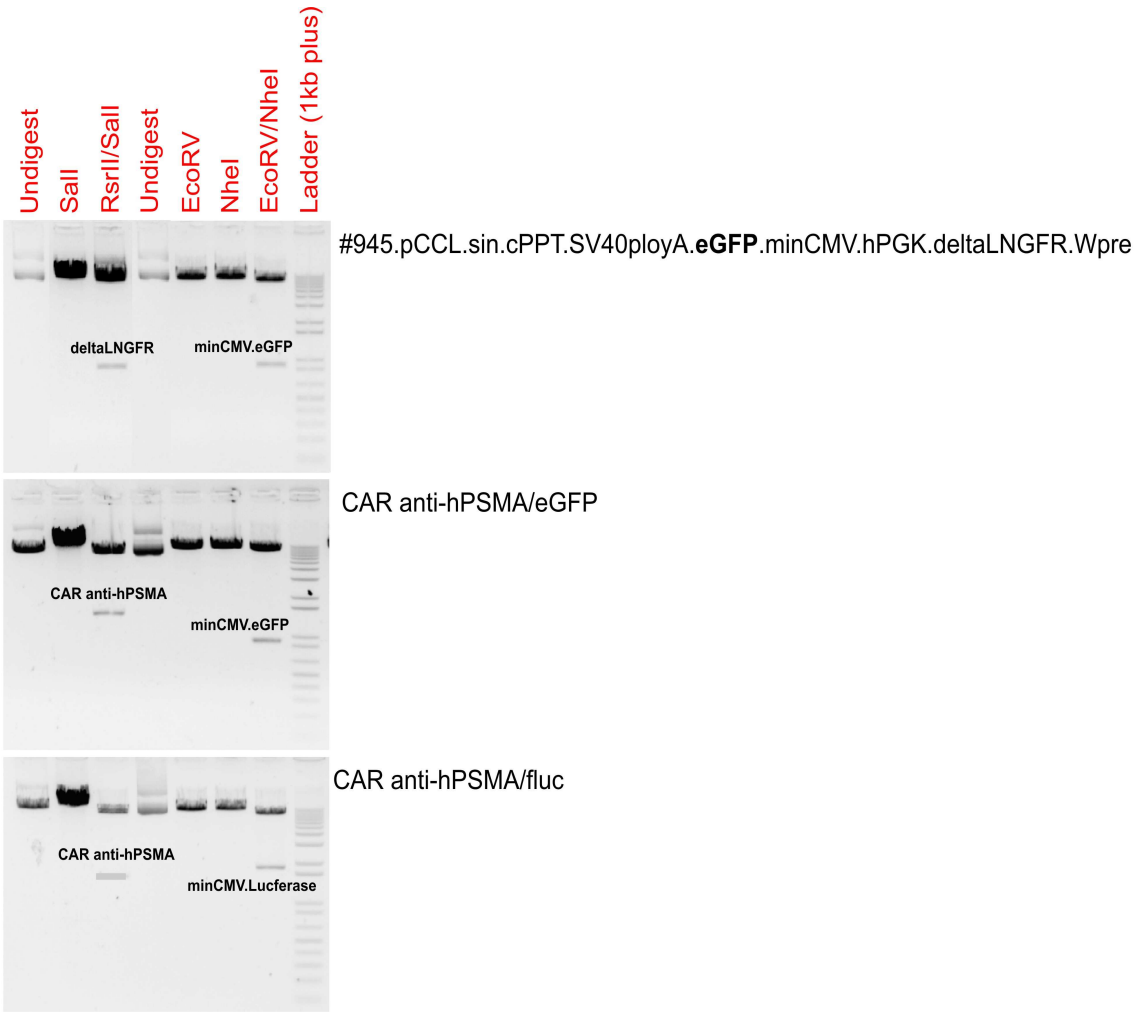


Fig 14. Enzymatic digestion of lentiviral vectors. After the cloning, the lentiviral vectors were digested with RsrII/SalI and EcoRV/NheI that allow to visualize the bands relative to CAR anti-hPSMA and minCMV.GFP or minCMV.Luciferase. As control, the same enzymes were used to digest the original lentiviral vector:
#945.pCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLNGFR.Wpre. For the digestion patterns obtained see table 2 in Materials and methods .

4. ASSESSMENT OF CAR EXPRESSION BY LENTIVIRAL VECTORS

4.1 Jurkat cell line

Jurkat cells were transduced with LV CAR anti hPSMA/eGFP and LV CAR anti hPSMA/fluc, to assess functionality of the vectors. CAR turned out to be highly expressed already 72 hours post-transduction, as demonstrated by the high percentage of c-myc+ cells detected by flow cytometry analysis (Fig 15A). In particular, CAR-expressing cells were more than 90% when transduced with LV CAR anti hPSMA/eGFP and more than 60% when using LV CAR anti hPSMA/fluc. Notably, the bidirectional LV vectors appeared to drive the expression of either the CAR and the reporter genes with a very balanced efficiency, as demonstrated by the high intensity of eGFP signal (Fig. 15B) and the induction of a relevant Luciferase activity (Fig. 15C). A timecourse analysis of CAR expression along a 3-month period, disclosed that Jurkat cells underwent a progressive reduction in the receptor expression, likely due to the lack of a positive selection of the transduced population by antigenic stimulation (Fig. 15D).

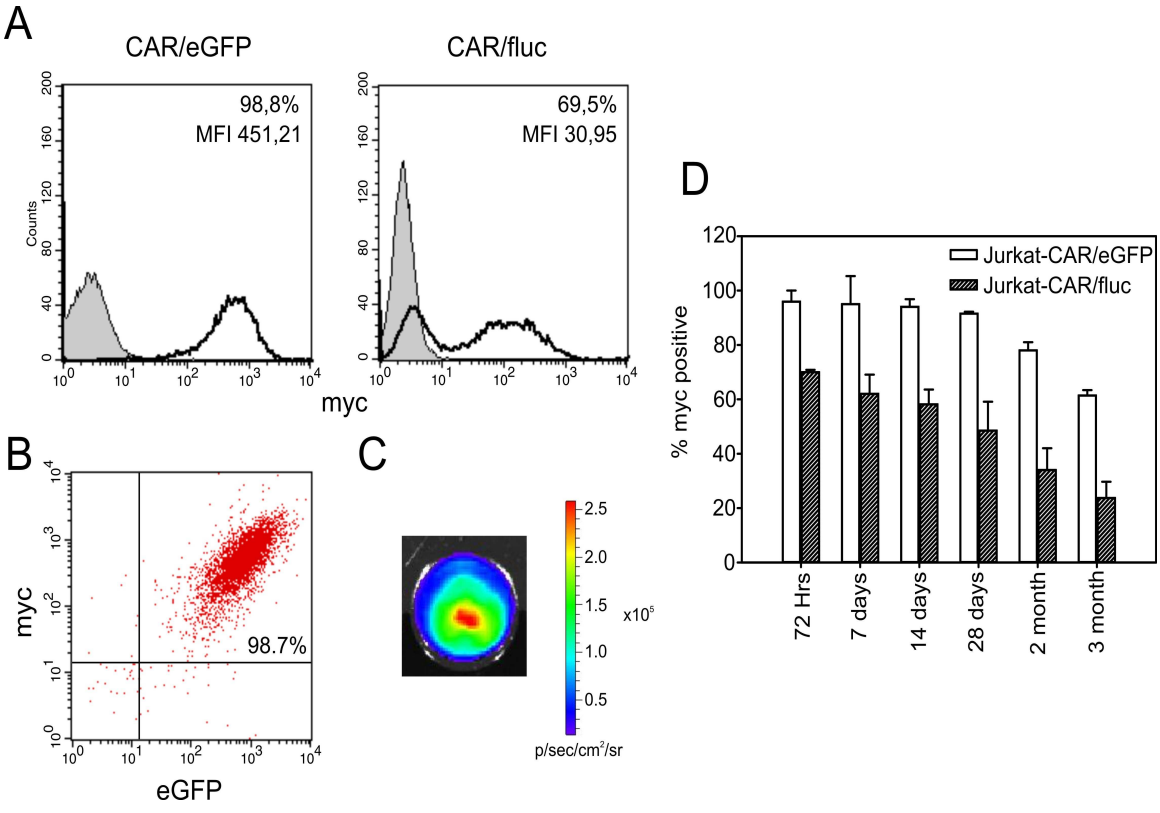


Fig 15. Assessment of LV vector functionality in Jurkat cells. A) CAR expression in CAR/eGFP and CAR/Fluc Jurkat cells; panels show the percentage of c-myc+ cells and relative MFI 72 hours after transduction. B) Cytogram illustrating the coordinated expression of c-myc and eGFP. C) Assessment of Luciferase expression using BLI. D) Timecourse of CAR expression in Jurkat cells transduced with either LV vectors.

4.2 PBMC

For the generation of T cell populations expressing the anti-hPSMA CAR, a rapid expansion protocol was developed. PBMC were activated with OKT-3 and IL-2 at day 0 and transduced with either LV vectors at different time points from stimulation to compare the efficiency of transduction; in particular, infection of PBMC was carried out the same day of activation (Td0), or 48 (Td2) or 72 hours (Td3) later and CAR expression was analyzed 72 hours after transduction. Infection carried out 48 hours after the activation resulted as the best timepoint to obtain a higher percentage of CAR-expressing T cells (Fig. 16A); moreover, this condition was correlated to the maintanance of a less differentiated phenotype, as assessed by the expression of CD27, CD28 and CD62L markers that identify a population with a memory phenotype (Fig. 16B).

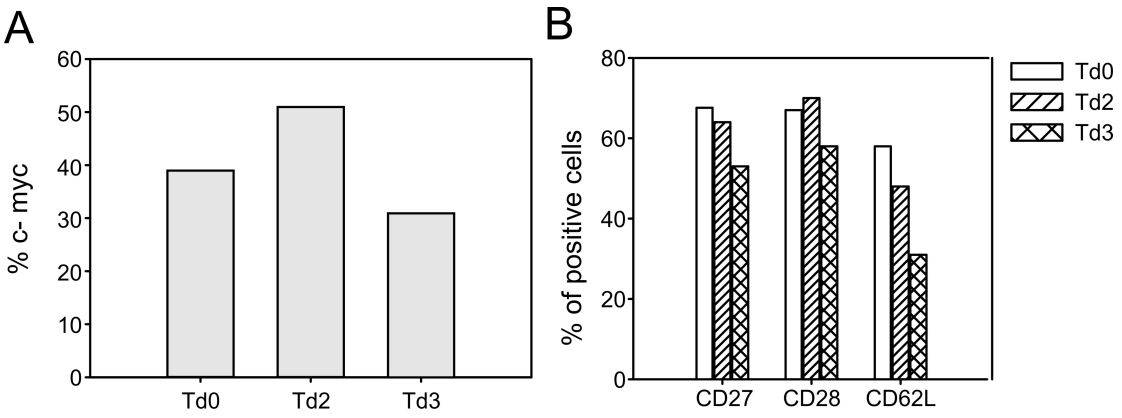


Fig 16. Identification of optimal timing of transduction after T cell activation. PBMCs were activated with OKT-3 and IL-2, and transduced with LV CAR anti-hPSMA/fluc (MOI 300) the same day(Td0), or 48 (Td2) or 72 hours (Td3) post activation. Flow cytometry analysis was carried out 72 hours post infection. Panel A shows the percentage of CAR+ cells while panel B reports the expression of CD27, CD28 and CD62L surface markers.

5. PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF CAR-TRANSDUCED T CELL POPULATIONS

5.1 Phenotypical characterization

To better define the state of differentiation of CAR-transduced T lymphocytes at an early stage post infection and after expansion based on antigenic restimulation, expression of different surface markers (CD62L, CD27, CD28, CCR-7, CD57) was assessed by cytofluorimetric analysis. Seventy-two hours after transduction, the emerging profile was essentially of central memory T cells, as shown by the high expression of CD62L, CD27 and CD28, the presence of CCR-7 positive cells and the low expression of CD57 (Fig. 17A). Following restimulation with the antigen, T cells acquired an effector memory phenotype with the progressive down-

modulation of CD62L, CD28 and CCR-7 and a slight increase in CD57 expression. Interestingly, whereas the subsequent encounter with the antigen led to the positive selection of transduced T cells with the expansion of the CAR-expressing population (Fig. 17B), a striking dicothomy could be observed between the expanding T cell subsets. Indeed, CAR expression was almost equal within the CD4 and CD8 populations immediately after infection, while the antigenic restimulation determined the progressive accumulation of the CD8 subset only that almost completely overcame CD4 T cells by month one after transduction.

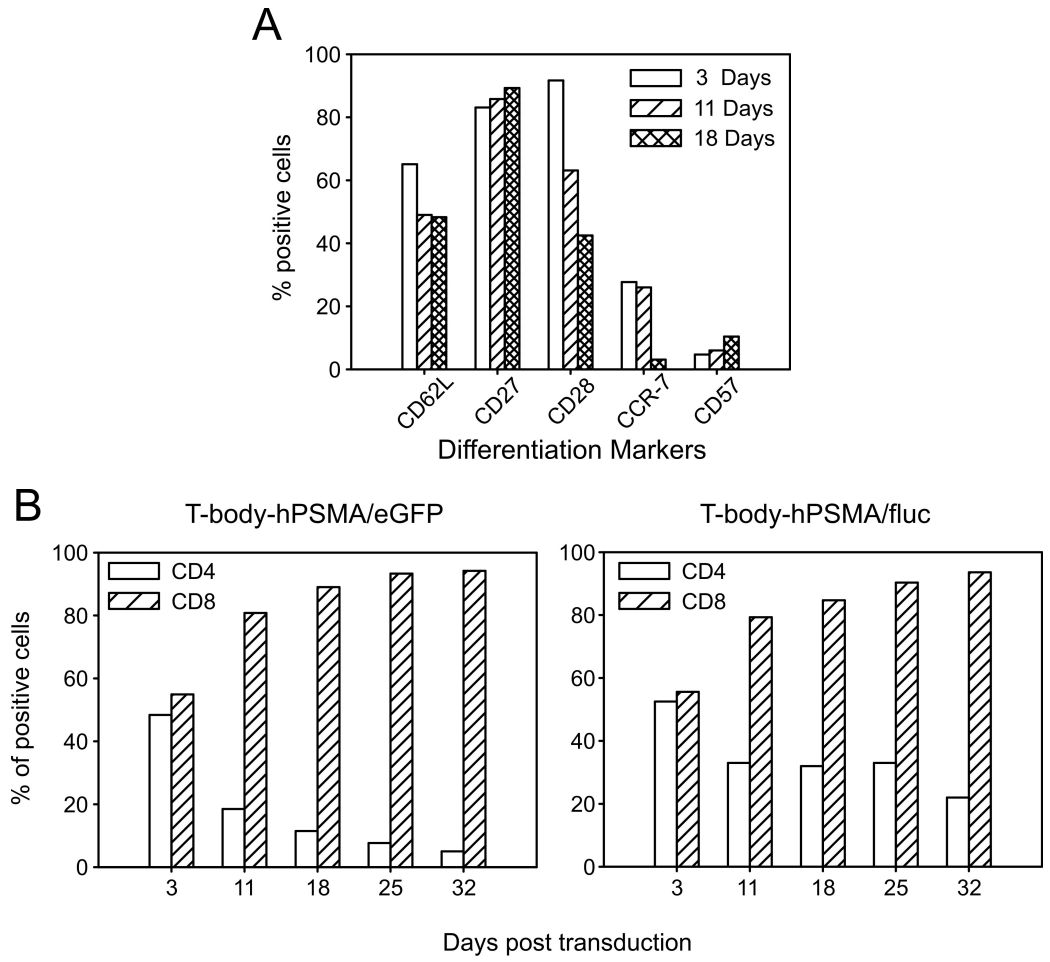


Fig 17. Phenotypical characterization of T-bodies. A) Expression of differentiation surface markers at different time points from transduction. B) CD4 and CD8 phenotype of CAR-expressing T cells at different time points post transduction.

5.2 Functional characterization

CAR expression provided the transduced population with the ability to recognize the hPSMA antigen on the surface of prostate tumor cell lines, and to mediate an high and specific citotoxicity. Indeed, both the Tbody-hPSMA/fluc and Tbody-hPSMA/GFP

populations lysed the hPSMA-transfected PC3 cells sparing the antigen-negative wild-type counterpart; more importantly, the transduced T cells were also capable of recognizing the LnCaP target cells that naturally harbor the hPSMA antigen. Cytotoxicity was already evident, albeit at low levels, 3 days after transduction and increased at maximal levels just after a single round of antigen restimulation, to remain constant thereafter up to 2 months post-infection (Fig. 18).

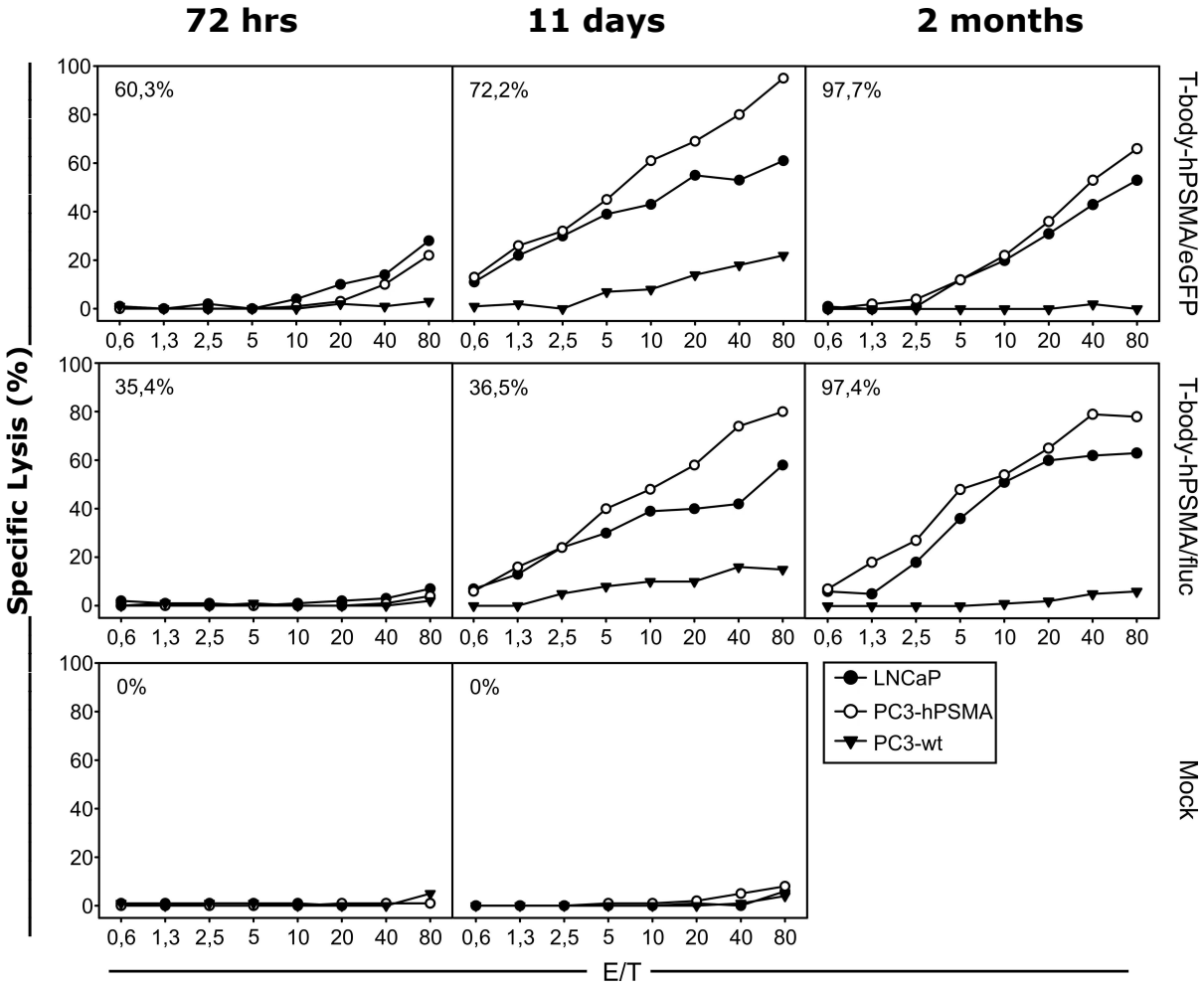


Fig 18. Lytic activity of T-bodies. Cytotoxicity of Mock or CAR-transduced T cells was analyzed 3, 11 and 60 days post-transduction against hPSMA positive or negative PC3 cells, and LNCaP prostate carcinoma cells. Each panel also reports the percentage of c-myc+ cells present in the culture analyzed.

Other than exerting a relevant cytotoxic activity, CAR-transduced T cells also produced IFN-γ in response to hPSMA-expressing tumor cells (Fig. 19). Notwithstanding, cytokine production did not apparently parallel the acquisition of lytic activity, as it took a longer time to be evident, and had also a lower magnitude involving only a fraction of CAR-positive T cells.

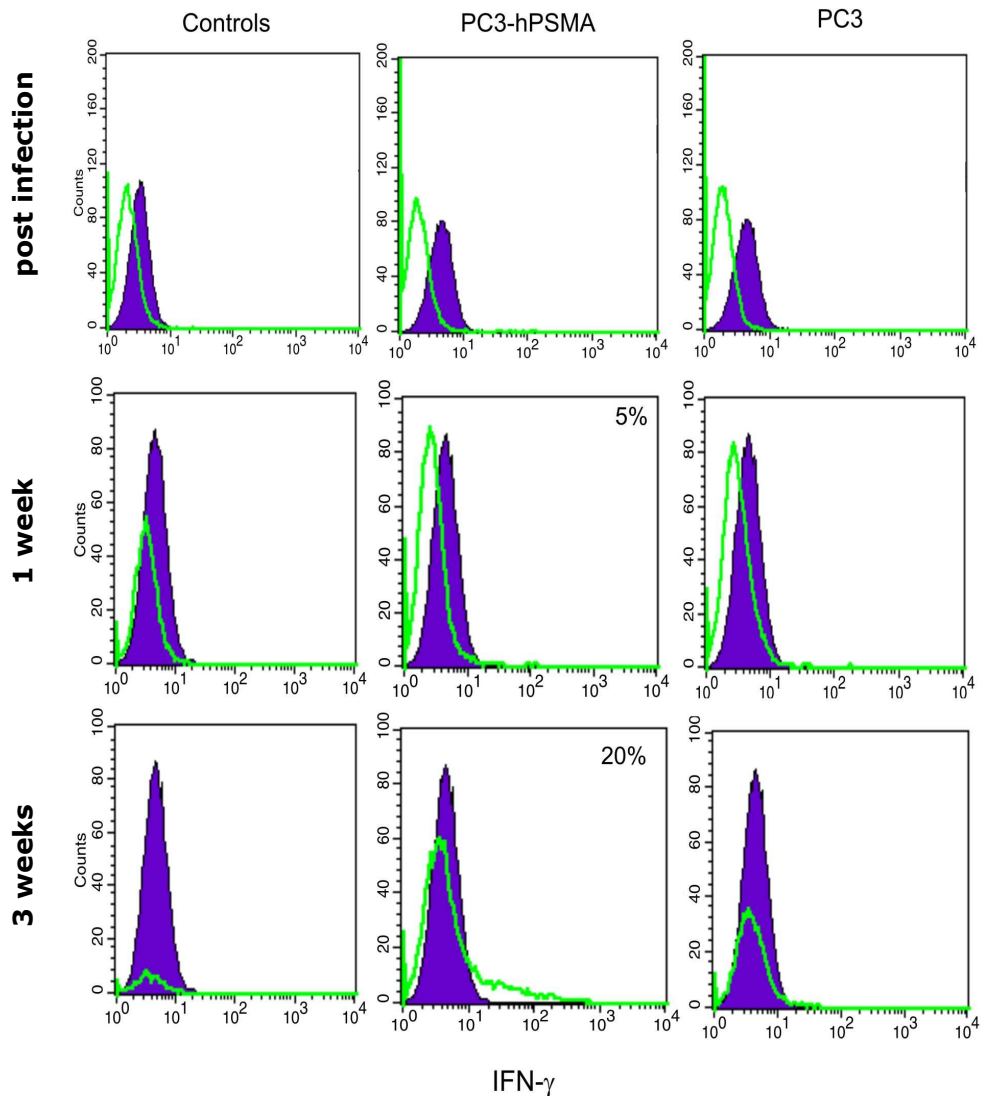


Fig 19. IFN-γ production by T-bodies upon antigen stimulation. IFN-γ production by PBMCs transduced with LV CAR anti-hPSMA/fluc was analyzed at different time points after transduction upon incubation with antigen positive or negative cells, followed by IFN-γ intracellular staining and flow cytometry analysis.

6. EVALUATION OF *IN VIVO* THERAPEUTIC EFFICACY

To assess the ability of CAR-transduced T cells to exert therapeutic activity against prostate carcinoma tumors *in vivo*, a series of experiments was carried out in mice challenged with the rapid proliferating and widely metastatic hPSMA-transfected PC3 cell line.

6.1 Winn assay

In vivo therapeutic efficacy of CAR-transduced T cells was initially evaluated using a Winn assay (Fig. 20), which involves the mixing *in vitro* of the tumor target cells with the effector populations before the subcutaneous inoculation into recipient

mice. More specifically, SCID mice were injected at opposite flanks with either hPSMA positive or negative PC3 cells admixed with CAR-transduced T cells, and tumor growth was monitored thereafter. Moreover, to assess potential difference in terms of therapeutic functionality of T cell cultures at early or late stage of differentiation, T-bodies at 72 hours or 5 weeks post-transduction were studied. Control tumors generated by the injection of either PC3-hPSMA and antigen-negative wild-type counterpart grew in 100% of mice, albeit with difference in growth kinetics, and were already measurable 3 to 7 days post-injection. Conversely, at the site of co-injection of PC3-hPSMA tumor cells and T-bodies no tumor growth could be observed, irrespective of the type of CAR-transduced culture that was transferred (Fig. 20). On the other hand, Tbody treatment did not substantially impact on growth of hPSMA-negative PC3 tumor cells, as a non significant slight delay in growth curve was only observable when the early transduced population was used, thus fully confirming the fine specificity of treatment.

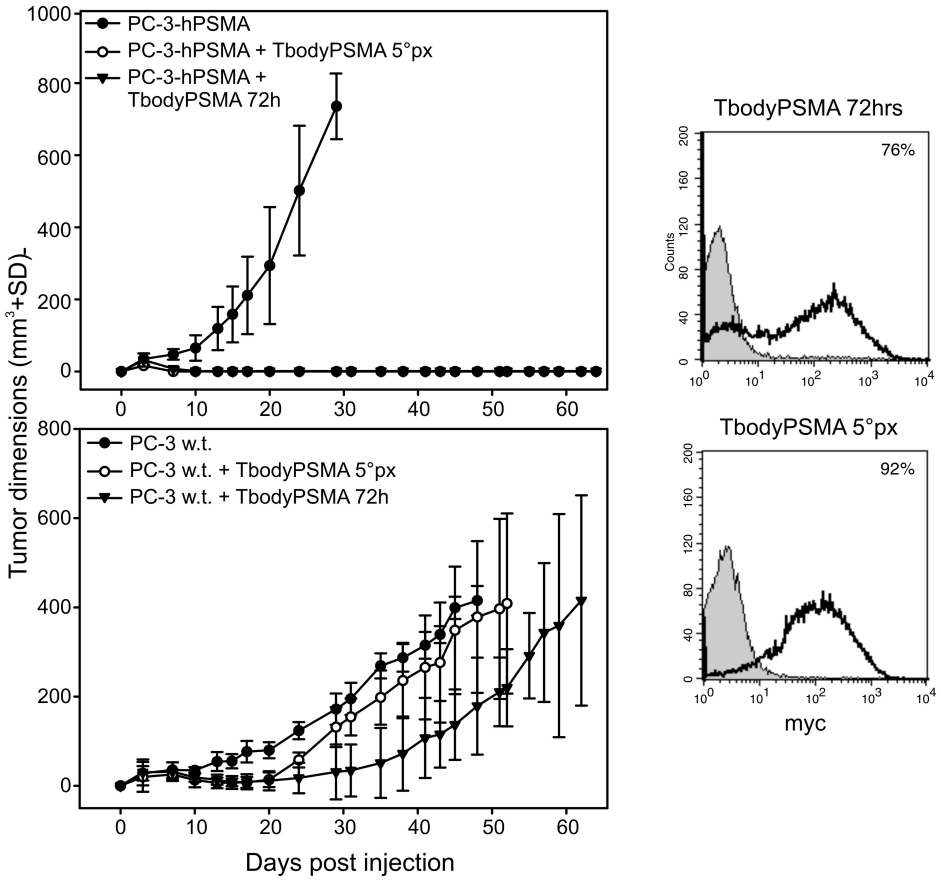


Fig 20. Winn Assay. hPSMA positive or negative PC-3 tumor cells were inoculated at opposite flanks of SCID mice, alone or mixed with Tbody-hPSMA/eGFP at 72 hours or 6 weeks post-transduction in a 1:1 ratio, and tumor growth was monitored over time. Left panels report tumor growth curves of untreated or treated PC-3-hPSMA (upper panel) P = <0,001 and wild-type PC3 (lower panel) prostate carcinoma cells. Cytometry plots of right panels show the percentage of CAR expression in the transduced populations used in the experiments.

6.2 Assessment of therapeutic efficacy of anti-hPSMA Tbody administration against established subcutaneous prostate Tumors

The Winn assay does not recapitulate a real therapeutic setting. To assess the antineoplastic activity of CAR-tranduced T cells against established tumors, SCID mice were injected s.c. with PC3-hPSMA cells and 4 days later, when all mice had large (30 mm^3) vascularized tumors, CAR anti-hPSMA T cells at 72 hrs post-transduction were transferred intratumorally in part of animals. While progressive tumor growth was observed in untreated control mice, Tbody-treated neoplastic masses shrinked up to almost disappear, at least for the first week after treatment. Thereafter, tumors resumed to grow in 50% of treated mice, even though with a strong delay and a slower kinetics (Fig. 21).

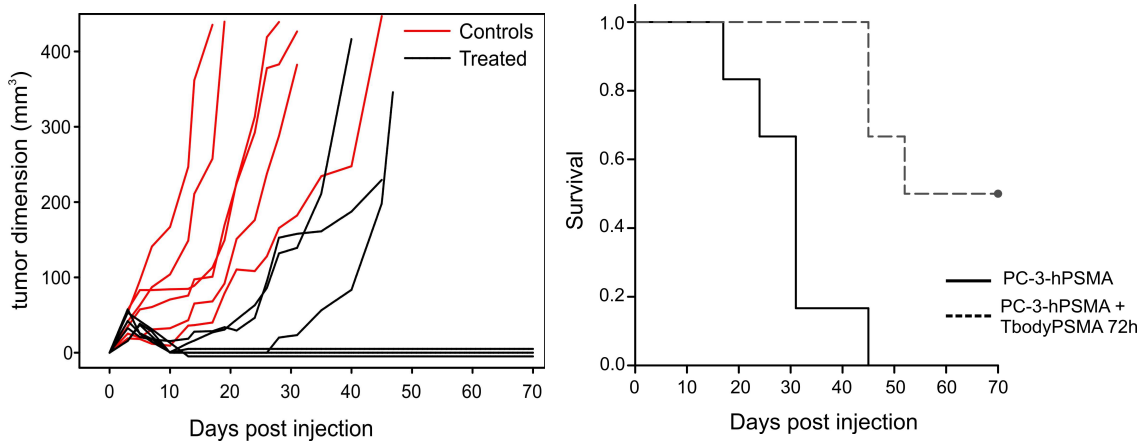


Fig 21. Assessment of Tbody in vivo therapeutic efficacy by loco-regional administration. Tbody-hPSMA at 72 hours after transduction were inoculated intralesionally in SCID mice bearing s.c. 4-day-old PC-3-hPSMA tumors; untreated animals served as control group. Number of mice per group, n = 6. Left panel show tumor volumes. A single injection of CAR-transduced T cells produced the complete regression of the tumor in 50% of treated animals and a statistically significant increase in survival (p=0,0028, right panel).

To rule out that the resumption of tumor growth could be due to the emergence of antigen-loss escape variants induced by the selective pressure of the immunological treatment, untreated and re-grown tumors were explanted and their hPSMA expression was compared to that of tumor cells kept in in vitro culture (Fig. 22). Results showed that *ex vivo* tumors essentially retained hPSMA expression with only a slight decrease in signal intensity.

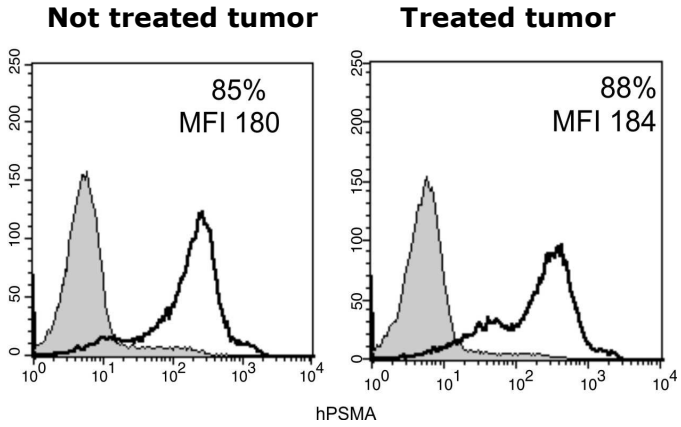


Fig 22. Expression of hPSMA antigen in tumor cells from in vivo explants. The tumors induced by s.c. inoculation of PC-3-hPSMA were explanted and the antigen expression was assessed by flow cytometry analysis. The cytometry plots show representative samples of untreated (left) or treated (right) tumors.

Next, the therapeutic potential of systemic administration of the activated CAR-expressing T cells was evaluated (Fig. 23). A single i.v. administration of either two different CARs populations, Tbody-hPSMA at 72 hrs or 17 weeks post-transduction, failed to exert a therapeutic activity in mice bearing s.c. pre-estabilished tumors.

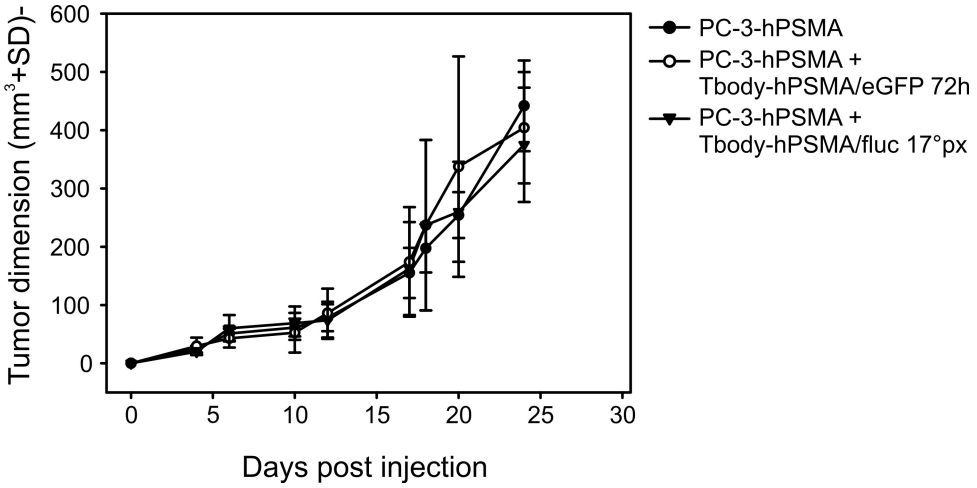


Fig 23. Assessment of Tbody in vivo therapeutic efficacy by systemic administration. Tbody-hPSMA/eGFP at 72 h or 17 weeks post-transduction were inoculated i.v. in mice bearing 4-day-old PC3-hPSMA s.c. tumors . The control group is represented by untreated animals. Number of mice per group n = 6. A single injection of transduced T cells did not induce any significant regression of tumor growth.

One of the 2 Lentiviral vectors developed allows the contemporary expression of the anti-hPSMA CAR and the Firefly Luciferase reporter gene, and was specifically designed to carry out BLI studies aimed at obtaining real-time information about transduced T cell distribution, survival and accumulation into the tumor site. For bioluminescence analysis, a titration study was initially performed to assess the

minimum number of T cells detectable after i.v. administration (Fig. 24A): results showed that less than $1x10^6$ CAR-transduced T cells were hardly detectable. Then, in an attempt to monitor tumor homing of T-bodies, SCID mice bearing subcutaneous PC3-hPSMA masses were injected with Tbody-hPSMA/Fluc and analyzed by BLI at different time points. Unexpectedly, results disclosed a very poor survival of transferred cells, as the BLI signal was already strongly reduced after 5 hours and almost completely disappeared 24 hours after Tbody administration (Fig. 24B).

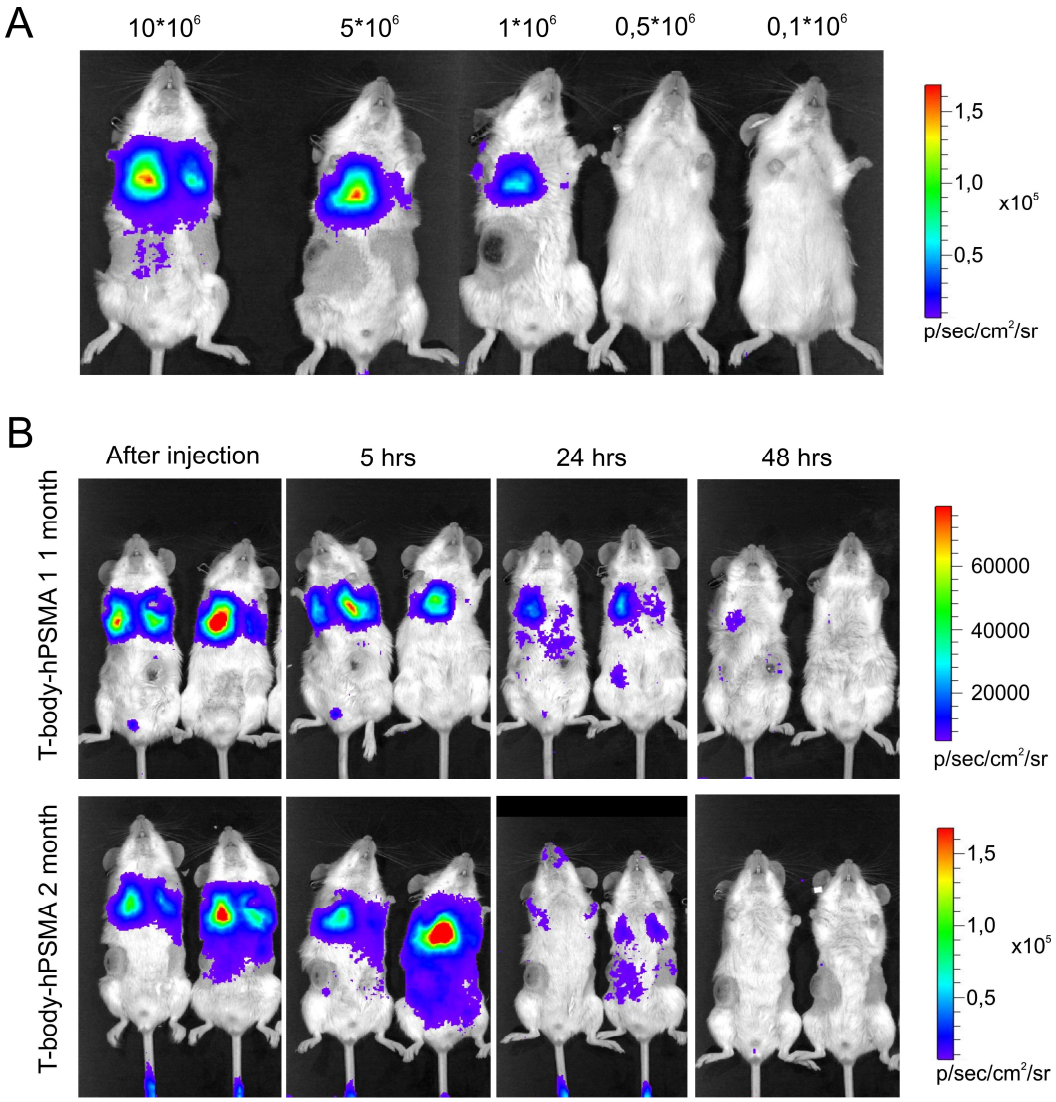


Fig 24. BLI imaging. A) Titration of of the minimum number of Luciferase-expressing T-bodies detectable after systemic transfer. Different amounts of transduced cells were injected i.v. and immediately analyzed by BLI. B) Analysis of survival and distribution of T-bodies. Tbodies-hPSMA/fluc ($10x10^6$/mouse) were inoculated i.v. in tumor-bearing mice and their distribution and survival was assessed at different time points thereafter.

As the poor survival of xenogeneic human T lymphocytes in SCID mice might depend on the presence of NK cells that could destroy the transferred population, additional survival experiments were performed in different mouse strains, namely RAG 2γC and NOD/SCID mice, which lack NK activity. As shown in Figure 25, transferred T-bodies survived very shortly in SCID mice compared to RAG 2γC animals; interestingly, they persisted much longer in NOD/SCID mice, as a clear BLI signal was still evident 24 hours after the administration, and tended also to uniformly distribute throughout the body.

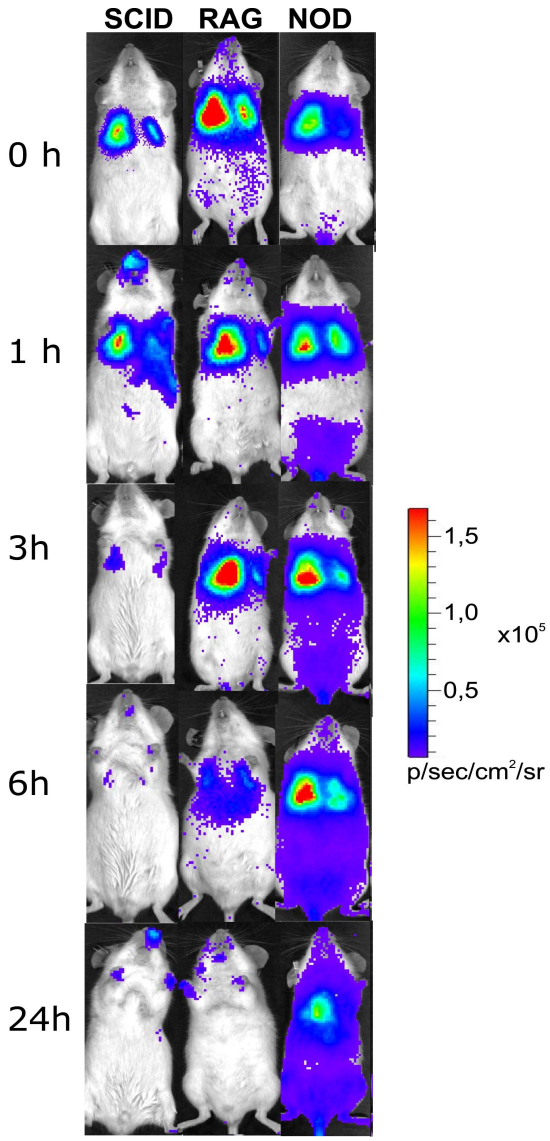


Fig 25. Analysis of survival and distribution of T-bodies in different mouse strains. T-bodies ($2x10^7$/mouse) were injected i.v. in SCID, RAG 2γC and NOD/SCID mice and their survival was comparatively monitored at different time points thereafter by BLI.

6.3 Assessment of therapeutic efficacy of anti-hPSMA Tbody administration against disseminated prostate tumors

The prophylactic and therapeutic studies reported above indicate that injection of CAR T cells can prevent or control advanced subcutaneous tumors, and that such tumor inhibition effect is dependent on CAR specificity, provided that CAR-transduced populations have the chance to interact directly with the neoplastic mass. In fact, no therapeutic effect was observed in mice having pre-estabilished s.c. tumors and receiving adoptive T cell therapy by tail vein, likely due to poor survival of transferred cells and failure to reach the tumor site. Based on biodistribution and survival results in NOD/SCID mice, a new model of disseminated prostate carcinoma was set up that contemplates the i.v. inoculation of Luciferase-expressing hPSMA-PC3 tumor cells in this mouse strain, to monitor neoplastic growth and response to ACT therapy. In a preliminary set of experiments, groups of NOD/SCID mice received bioluminescent hPSMA-PC3 tumor cells and were treated, starting from day 4 after injection, with 1, 2 or 3 inoculi of $2x10^7$ CAR T cells performed every 2 days. Then, BLI monitoring was carried out up to 3 weeks to assess tumor growth and response to treatment. Even though a single injection of CAR-transduced T cells already produced a relevant reduction of neoplastic areas, as assessed by BLI imaging, multiple administrations reinforced the therapeutic effects and further reduced the signal intensity (Fig. 26).

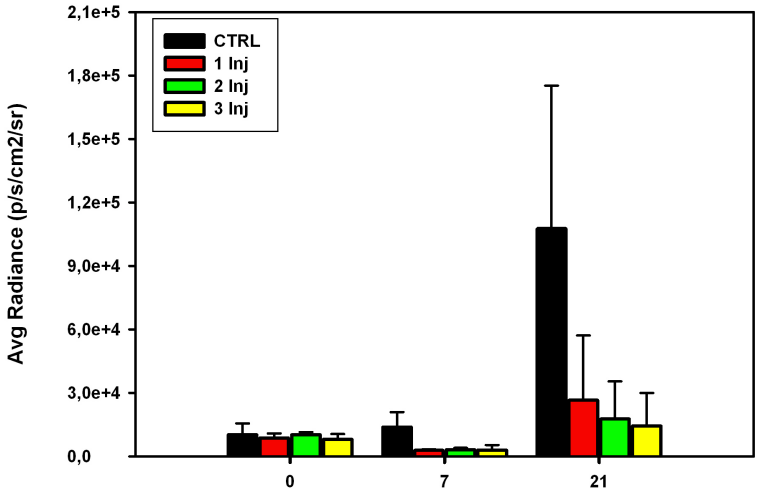


Fig 26. Short-term assessment of Tbody therapeutic efficacy against disseminated prostate carcinoma. One or multiple administrations of CAR T cells ($2x10^7$/treatment) were performed in NOD/SCID mice bearing established bioluminescent hPSMA-PC3 tumors, starting from day 4 and every 2 days; BLI monitoring was carried out immediately after tumor cell injection and a different time points thereafter. Signals emitted by regions of interest (ROI) on total body were measured and data were expressed as photon flux and quantified as photon * sec^{-1} * cm^{-2} * sr^{-1}.

Parallel experiments conducted to evaluate the biological fate of i.v. administered PC3 prostate carcinoma cells, showed that tumors were apparently restrained at lungs for the first 10-15 days and widely disseminated thereafter, producing large metastases in internal organs (data not shown). Therefore, additional experiments were set up to assess the long-term therapeutic efficacy of the ACT approach. To this aim, NOD/SCID mice were injected i.v. with bioluminescent PC3-hPSMA cells and treated for 3 times with $2x10^7$ CAR-transduced T cells, according to the previously described protocol. As reported above, this ACT approach strikingly reduced the BLI signal detectable in treated animals compared to control mice, not only in lungs, which represent the first microcirculatory site entrapping tumor and transferred T cells, but also systemically (Fig. 27). Only after 3 weeks some of the treated mice started to develop bioluminescent lesions, which apparently involved extrapulmonary sites only.

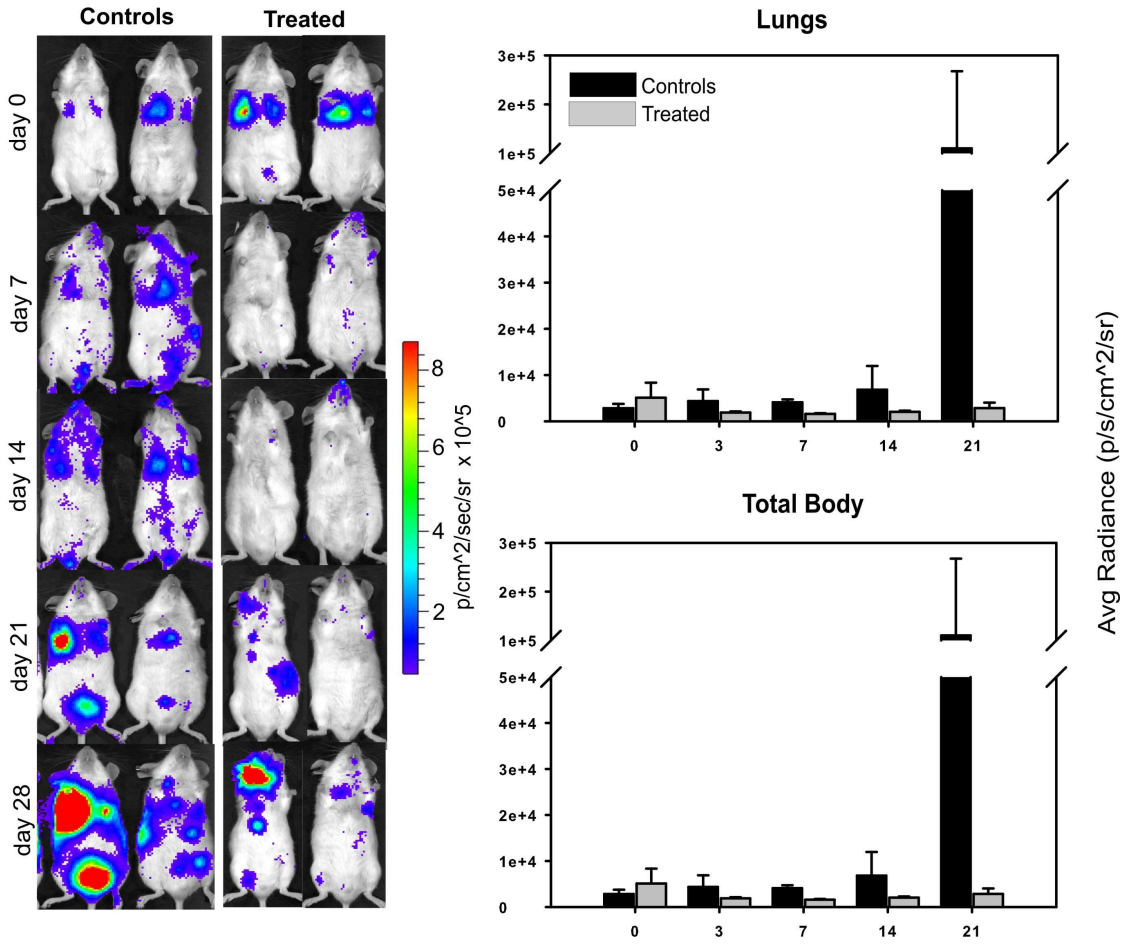


Fig 27. Long-term assessment of Tbody therapeutic efficacy against disseminated prostate carcinoma. Animals (n=6) bearing established bioluminescent hPSMA+ tumors were injected i.v. for 3 times with 2×10^7 CAR-transduced T lymphocytes. Mice not treated (n=6) as control. ACT treatment started 3 days after tumor inoculation and cells were administered every 2 days. Pictures on left show representative animals imaged by BLI at different time points, while right panels report cumulative results of imaging. Signals emitted by regions of interest (ROI), Lungs (upper panel) and Total Body (lower panel) were measured and data were expressed as photon flux and quantified as photon * sec^{-1} * cm^{-2} * sr^{-1}.

More importantly, the transfer of CAR-transduced T cells strongly impacted on long-term survival of treated mice (p<0,001; Fig. 28) and also completely eradicated the neoplasia in more than 60% of animals that were completely disease-free 150 days after tumor induction.

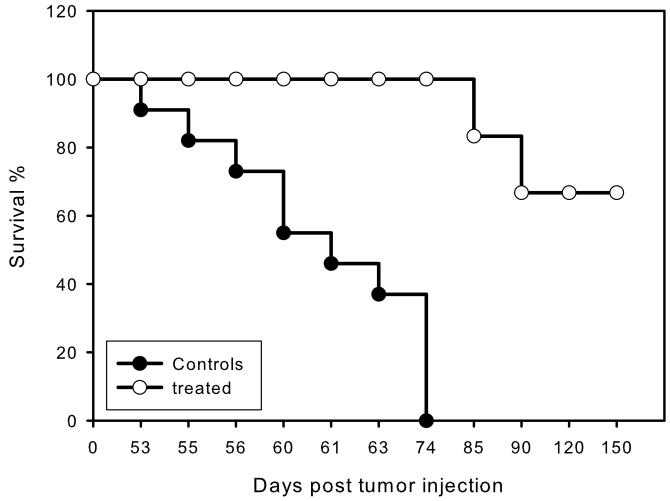


Fig 28. Survival. The mice survival was monitored during the time. Mice not treated was used as controls. Therapeutic activity of CAR-transduced T cells in a mouse bearing disseminated prostate tumors after *in vivo* inoculation. Cumulative Kaplan-Meier survival curves of mice inoculated with Tbodies (LCL group; n = 6; median survival = 130 d) and not treated (n = 6, median survival = 60 d); P = <0,001.

DISCUSSION

The concept of cancer immunotherapy goes back as far as the late nineteenth century, when William B. Coley observed tumor shrinkage and even disappearance following the injection of bacterial products in and around tumors. Since then, many observations — such as the rare but well-documented occurrence of spontaneous remissions, the higher incidence of cancer in patients who are immunosuppressed, and the identification of tumor-specific antigens and lymphocytes — have stimulated research on strategies that aim to induce specific antitumor responses. Currently, allogeneic bone marrow transplantation and monoclonal antibodies that target tumor cells are two examples of broadly used and efficacious immunotherapies. Cancer immunotherapy comprises a variety of treatment approaches, incorporating the tremendous specificity of the adaptive immune system (T cells and antibodies), as well as the diverse and potent cytotoxic weaponry of both adaptive and innate immunity. Immunotherapy strategies include antitumor monoclonal antibodies, cancer vaccines, adoptive transfer of *ex vivo* activated T and NK cells, and administration of antibodies or recombinant proteins that either costimulate immune cells or block immune inhibitory pathways (so-called immune checkpoints). Although clear clinical efficacy has been demonstrated with antitumor antibodies since the late 1990s, other immunotherapies had not been shown to be effective until recently, when a spate of successes established the broad potential of this therapeutic modality. These successes are based on fundamental scientific advances demonstrating the toleragenic nature of cancer and the pivotal role of the tumor immune microenvironment in suppressing antitumor immunity. New therapies based on a sophisticated knowledge of immune-suppressive cells, soluble factors, and signaling pathways are designed to break tolerance and reactivate antitumor immunity to induce potent, long-lasting responses. Preclinical models indicate the importance of a complex integrated immune response in eliminating established tumors and validate the exploration of combinatorial treatment regimens, which are anticipated to be far more effective than monotherapies. Unlike conventional cancer therapies, most immunotherapies are active and dynamic, capable of inducing immune memory to propagate a successful rebalancing of the equilibrium between tumor and host. Over the past decades, considerable knowledge has been obtained on the components that are relevant in antitumor immune responses and immune escape mechanisms, and the cytotoxic T lymphocyte response has been identified as the most powerful and effective link in this vast network. The systematic development and clinical

application of genetically modified T cells is an example of a new class of therapeutics. In this context, gene therapy has been used to overcome one of the major barriers to T-cell therapy of cancer, namely tolerance to desired target tumor-associated antigens. This was achieved by the introduction of chimeric antigen receptors to redirect T-cell specificity to TAA expressed on the cell surface. The prototypical CAR use monoclonal antibody that docks with a designated TAA and this binding event is reproduced by the CAR to trigger desired T-cell activation and effector functions. Multiple early-phase clinical trials are now underway or have been completed to evaluate the safety and feasibility of adoptive transfer of CAR+ T cells (Jena et al, 2010). Engraftment with CARs enables T cells to MHC-independent antigen recognition; thus, major immune escape mechanisms of tumors such as downregulation of MHC molecules are efficiently bypassed. Furthermore, proliferation and survival of modified T cells can be improved by the implementation of a multitude of signaling domains from different immune receptors in a single CAR. The use of CARs to redirect T cells specifically against TAA-expressing tumor cells has a number of theoretical advantages over classical T-cell-based immunotherapies. In particular, in contrast to the long-lasting procedure of *in vitro* selection, characterization, and expansion of T-cell clones with native specificity for MHC tumor peptide complexes, genetic modification of polyclonal T-cell populations allows to generate TAA-specific T cells in one to two weeks.

Prostate cancer is the second leading cause of malignancy-related mortality in males in the Western world. While radical prostatectomy and local radiotherapy are largely successful for patients with localized cancer, available treatments for metastatic prostate carcinoma have demonstrated weak curative efficacy. Immune system-based strategies possess the requisites to successfully attack prostate tumor cells, particularly in metastatic and relapsing disease, where conventional forms of treatment often fail. In this regard, novel biomarkers are nowadays available to specifically direct "immunoweapons" against prostate cancer cells: the PSMA antigen has recently emerged as one of the most promising biomarkers in the diagnosis and treatment of prostate cancer and its clinical relevance is presently being evaluated in several immunotherapy trials. The low expression of PSMA in normal prostate epithelial cells increases several fold in high-grade prostate cancers, in metastatic and in androgen-insensitive prostate carcinoma. The importance of PSMA in cancer detection and therapy is further illustrated by the finding that it is also expressed in tumor neovasculature but it is absent from normal endothelial cells (Gulley et al, 2011). These findings have spurred the development of PSMA-targeted therapies for prostate cancer, including immunotherapy (Djavan et, 2011). Notwithstanding, one of the possible

explanations of the failure of immunotherapy regimens based on single approaches (antibodies, "armed" or otherwise, or vaccination protocols aimed at eliciting cell-mediated responses against tumor cells, or passive administration of immune cells, etc.) is that no single approach could be per se sufficient to attack/eradicate heterogeneous populations of tumor cells simultaneously deploying a wide range of escape mechanisms. To bypass such escape mechanisms, I have decided to take advantage of technological advancements allowing to clone, by recombinant DNA techniques, the antigen-binding domain of a mAb directed to PSMA, which has been demonstrated to retain its specificity and high affinity even as recombinant scFv (M. Colombatti, personal communication). By exploiting such recombinant derivative of anti-PSMA antibody under the format of CAR functional receptor, I envisaged the possibility to redirect T cells against prostate tumors, thus combining the high affinity recognition capacity of antibodies with the powerful effector functions of T lymphocytes.

In this study, a second generation CAR was used that contain the anti-hPSMA ScFv fused to portions of CD28 and CD3ζ. Even though recent advancements in the field have shown that third generation CAR may provide some advantages especially in terms of cytokine production upon antigen stimulation, improvements do not appear substantial (Song et al, 2011). Most prior studies have used retroviral vectors for genetic reprogramming, but Lentiviral vectors may offer certain advantages over gammaretroviral vectors. They can transduce nondividing or minimally proliferating T lymphocytes and the reduced requirement for *ex vivo* activation before transduction may maximize long-term *in vivo* persistence of the transduced cells, by reducing activation-induced cell death or clonal exhaustion. Moreover, the latest generation of LV technology has several built-in safety features that minimize the risk of generating replication-competent wild-type human HIV-1 recombinants. Finally, the self-inactivating LV configuration reduces the likelihood that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed by abolishing the intrinsic promoter/enhancer activity of the HIV-1 LTR, thus minimizing the possibility of oncogene activation and/or inactivation of tumor suppressor genes. These aspects are particular relevant when dealing with the type of T cell to engineer and transfer to achieve optimal therapeutic results (Durand et al, 2011). Much of our current knowledge on the development and character of CD8+ T cells has come from the study of acute viral infections. The dynamic response of antigen specific CD8+ T cells to an acute viral infection has now been resolved at cellular, molecular, and gene-expression levels in mice and to a more limited extent in humans. Briefly, a productive encounter of naïve CD8+ T cells to antigen stimulation follows a prototypical, tri-phasic response: (i) an initial activation phase characterized by a multi-log clonal

expansion of antigen-specific cells and concurrent acquisition of peripheral tissue-homing capabilities, effector cytokine release, and cytolytic activity; (ii) a death phase characterized by a rapid, apoptosis-induced contraction of antigen-specific effector T cells; and (iii) formation of a persistent population of antigen-experienced cells that represent immunologic memory. An analogous response has also been observed among self/tumor-reactive CD8+ T cells activated *in vivo* with a recombinant viral vaccine and exogenous cytokine support. Several key features distinguish the persistent antigen specific cells of the third phase of the immune response described above. These features include an increased precursor frequency compared with naÏve hosts, the capacity for antigen-independent self-renewal through homeostatic proliferation in response to IL-7, IL-15, and possibly IL-21, and the rapid acquisition of effector functions and clonal proliferation upon antigen re-challenge. These attributes, which collectively constitute the hallmarks of immunologic memory, provide the host with long-lived protection from future pathogen encounters. Memory CD8+ T cells are heterogeneous with respect to phenotypic markers, effector functions, and tissue-homing capabilities. One mode of classifying memory T cells is to divide the populations into two broad categories, termed central memory T cells (Tcm) and effector memory T cells (Tem). In this classification schema, Tcm are antigen-experienced cells that constitutively express CD62L and CCR7, two surface molecules necessary for cellular extravasation in high endothelial venules and migration to T cell zones of peripheral lymph nodes. By contrast, Tem are antigen-experienced T cells that have significantly down-regulated these markers and hence have a propensity to populate peripheral tissues, such as the liver and lung, as well as inflammatory sites. Based on these findings, a division of labor among memory T cells was proposed: Tem function as sentinels for immediate protection from a peripheral challenge, while Tcm provide protection from a systemic challenge and can generate a second wave of effector cells. In addition to the ability of Tcm to preferentially migrate to secondary lymph nodes, the capacity to secrete IL-2 has been associated with CD8+ Tcm but not with Tem cells. The question of which of these two T cell memory populations, if either, should be targeted in future immune trials is a subject of considerable interest, and it is now beginning to be addressed *in vivo* in animal models. In both mice and non-human primates, CD8+ Tcm have been shown to be superior mediators of host protection against viral and bacterial challenge compared to Tem cells. The enhanced ability of Tcm cells to confer host protection and tumor treatment has been correlated with their greater proliferative capacity upon antigen-reencounter compared with Tem cells. Ultimately, this potential allows for generation of a larger absolute number of terminally differentiated effector T cells that can infiltrate peripheral sites to mediate antigen clearance. Collectively, data

obtained thus far tend to suggest that Tcm may be more potent on a per cell basis in mediating antigen clearance compared with Tem cells. Nonetheless, further studies are needed to confirm results directly comparing the therapeutic efficacy of Tcm to Tem, particularly in settings of an established disease such as cancer. In this regard, the present study tried to address such question, at least partially, by assessing the therapeutic potentiality of T cells early after CAR transduction or at a later stage of differentiation following several cycles of antigen restimulation, and thus sharing phenotypic features with Tcm and Tem cells, respectively. Notably, while both populations were able to prevent and control s.c. tumor growth when injected directly inside or in close proximity of the neoplastic lesion, and hence having the opportunity to interact physically with target cells, neither of them showed efficacy after systemic administration. This outcome might depend on several aspects and in particular on the biological fate of transferred human lymphocytes, which are transported whithin a xenogeneic microenvironment that could heavily affect their survival, homing capacity and functionality, all aspects that require serial analyses to be assessed. In this regard, conventional studies of cancer growth, responses to therapeutic agents, and host immune responses in small animal models rely upon euthanizing cohorts of animals at multiple time points. This experimental strategy has been the foundation for analyzing cancer pathogenesis and treatment, but there are inherent weaknesses that limit applications of this approach for testing new therapeutic compounds. Analyzing groups of mice at multiple time points after treatment precludes serial studies of disease progression in the same animal. Data from longitudinal studies in the same animal may reveal key information about animal-to-animal variations in pathogenesis or therapeutic efficacy. Furthermore, conventional animal studies require large numbers of animals to generate statistically meaningful data, which in turn necessitates larger amounts of candidate therapeutic agents for initial pre-clinical testing. *In vivo* imaging has emerged as a powerful alternative to these conventional studies of cancer pathogenesis and treatment. Among the many small animal imaging approaches that recently have been developed, BLI is at the forefront of technologies used to study tumors in mouse models, allowing *in vivo* quantification of tumor replication, dissemination, and host immune responses and signaling pathways. Importantly, these processes can be interrogated repetitively in the same animal over the course of hours to weeks, overcoming many of the limitations of conventional assays. In the present study, BLI proved to be a powerful technique for studying longitudinally the biological fate of adoptively transferred T cells in mice, and was instrumental to demonstrate their poor survival after transfer, an aspect likely dependent on immunological characteristics of the immunosuppressed mouse strains used. Indeed, although a potential role of NK

cells in destroying the CAR-transduced T cells could be ruled out by the observation of a quick disappearance of them even the NK-deficient RAG 2γC mice, as compared to the SCID animals, recent evidence from our laboratory identified in the Complement an alternative potential toxic element. In fact, incubation of CAR lymphocytes with serum from either SCID or RAG 2γC mice was followed by C3 and C5 deposition on cell membrane and in vitro lytic activity (data not shown). Since these mouse strains lack immunoglobulins, the alternative way of Complement activation is likely involved due to the the xenogeneic nature of human T cells. Moreover, we must not forget that C3b on plasma membrane acts a potent opsonin recognized by specific receptors on granulocytes and elements of the monocyte/macrophage system, which in turn could be activated for phagocytosis and killing activity. Conversely, NOD/SCID mice are described as having an impaired Complement function; accordingly, transfer of CAR-transduced T lymphocytes in these animals was followed by a longer survival and, more importantly, by a striking therapeutic activity that not only reduced tumor masses but also completely eradicated disseminated prostate carcinomas in more than 60% of treated mice.

Overall, this work demonstrates that CAR engineering is a feasible and efficient strategy for theraputic intervention against prostate carcinoma, thus paving the way to future application in the clinical setting.

ABBREVIATIONS

aa: amminoacids
ACT: adoptive cell therapy
Ag: antigen
APC: Antigen Presenting cell
BLI: Bioluminescence imaging
CAR: Chimeric Antigen Receptor
ChTCR: Chimeric T Cell Receptor
CMV: Cytomegalovirus
cPPT: central polypurine tract
CTE: Constitutive Transport Element
CTL: Cytotoxic Lymphocyte
DC: Dentritic cells
DLI: Donor Lymphocyte Infusion
EBV: Epstein-Barr Virus
FBP: Folate Binding Protein
FBS: Fetal bovin serum
FLI: Fluorescence imaging
fluc: Firefly luciferase
FOV: field of view
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HIV: Human Immunodeficiency Virus
HLA: Human Leukocyte Antigen
hPGK: phospho glycerate kinase gene
HSC: Hematopoietic stem cells
HSV-Tk: Herpes Simplex Thymidine Kinase gene
i.p.: intraperitoneal
IFN-γ: interferon γ
IL: interleukin
IRES: internal ribosome entry sites
ITAM : immunoreceptor tyrosine-based activation motif
i.v.: in tail vein
LTR: long terminal repeat
LV: Lentiviral vector
MoAb: Monoclonal Antibody
MCS: multiple cloning site
MDSC: Myeloid-derived suppressor cells
MHC: Major histocompatibility complex
MoMuLV: Moloney Murine Leukemia Virus
MRI: Magnetic Resonance Imaging
MSCV: Murine Stem Cell Virus
NIR: Near infrared
NK: natural killer cell
NK T: lymphocyte T natural killer
o.n.: overnight
PBL: Peripheral blood lymphocytes
PBMC: Peripheral blood mononuclear cells
PET: Positron Emission Tomography
PSA: Prostate Specific Antigen
PSMA: Prostate Specific Membrane Antigen
PTLD: Post-transplant lymphoproliferative disorder
r.T.: temperatura ambiente
RCR: replication-competent retroviruses
RSV: Human respiratory syncytial virus

Abbreviations

RV: retroviral vectors
s.c.: subcutaneous
scFv: single chain fragment variable
SCID: Severe Combined Immunodeficiency
shRNAs: short hairpin RNAs
SIN: Self inactivating
SPECT: Single Photon Emission Computed Tomography
SPF: specific pathogen free
TAA:Tumor associated antigens
T_{CM}: central memory T cells
TCR: T Cell Receptor
T_{EM}: effector memory T cells
TGF-β: Transforming growth factor β
Th1: T helper 1
TIL: Tumor Infiltrating Lymphocytes
TNF-α: Tumor necrosis factor α
TSA: Tumor specific antigen
WPRE: woodchuck hepatitis virus post-transcriptional regulatory element

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