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Recombinant DNA Technology in Emerging Modalities for Melanoma Immunotherapy

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Additional information is available at the end of the chapter

1. Introduction

The history of immunotherapy of cancer dates back to 1890s when New York surgeon William Coley used Streptococcus and Serratia bacterial extracts to treat cancer. Up to the mid-1930s 'Coley's mixed toxins,'' were used to treat various tumors. Better understanding of the human immune system led to the identification of a number of tumor-associated antigens (TAAs) in the 1980s [1] and development of various immunotherapeutic approaches. Of particular relevance to melanoma immunotherapy was the identification of various antigens expressed specifically in melanocytes and, respectively, in the majority of melanomas. These melanoma-associated antigens include tyrosinase (Tyr), a key enzyme in melanin biosynthesis, tyrosinase-related proteins 1 and 2 (TRP1, TRP2), gp100 (aka pmel17), Melan-a, and MART1. These and several other melanoma-associated antigens formed the basis for the immunologic targeting of the tumor. Up to date, multiple peptide, dendritic cell, adjuvant, lymphocyte, antibody, DNA and virus-based strategies were tested in pre-clinical and clinical studies with varying degrees of success. In recent years, identification of the specific antigenic MHC class I epitopes, advancements in genetic engineering, gene delivery, and cell-based therapeutic approaches allowed development of the novel melanoma-targeting immuno-therapeutics.

2. Genetic engineering of antigen-specific T cells

2.1. Recombinant T cell receptors

Identification of the tumor-reactive T cells among a population of the tumor-infiltrating lymphocytes led to the development of the T cell-based therapies, particularly to the strategy

known as adoptive T cells transfer. This strategy is based on the isolation of the tumor-infiltrating lymphocytes following analysis of their ability to target tumor cells and clonal expansion of tumor-reactive T cells via stimulation of cell proliferation with anti-CD3 and antiCD28 antibodies in the presence of IL-2. Upon obtaining a large quantity ($> 10^8$ cells), these cells are infused back to a tumor-bearing patient along with the lymphodepleting chemotherapy to temporary knock down circulating immunocytes and repetitive administration of the IL-2 (Fig. 1).
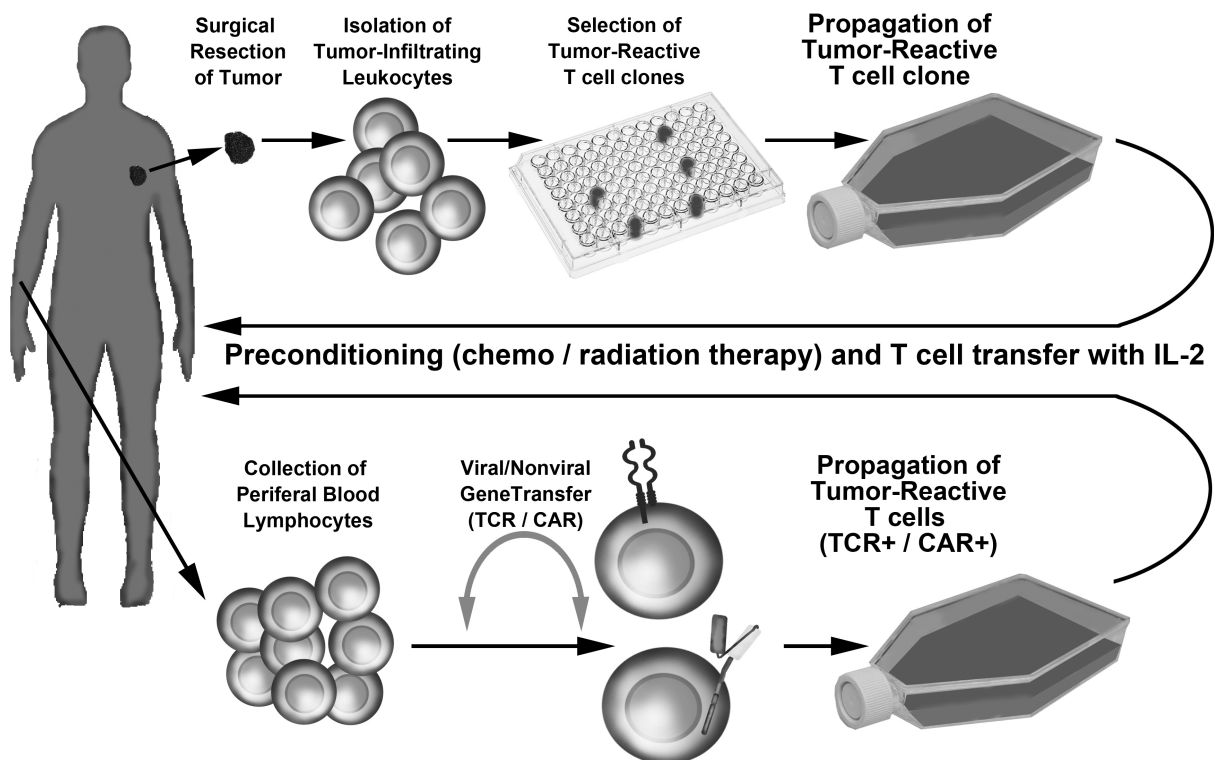

Figure 1. Clinical application of the T cell-mediated tumor immunotherapy. Diagram on the top depicts application of the Tumor-Infiltrating Lymphocytes (TILs). Diagram on the bottom illustrates application of the genetically engineered (TCR and CAR-modified) T cells.

Presently, 87 clinical trials using TIL are completed or on-going. These clinical trials are aimed at treatment of multiple cancers including: Malignant Melanoma, Nasopharyngeal Carcinoma, Hepatocellular Carcinoma, Breast Carcinoma, Leukemia, Lymphoma, Multiple Myeloma, Plasma Cell Neoplasm, Kidney Cancer, Metastatic Colorectal Cancer, Metastatic Gastric Cancer, Metastatic Pancreatic Cancer, Metastatic Hepatocellular Carcinoma, Cervical Cancer, Oropharyngeal Cancer, Vaginal Cancer, Anal Cancer, Penile Cancer, Non-Small Cell Lung Cancer, Brain and Central Nervous System Tumors. Several completed clinical trials on malignant melanoma clearly demonstrated that infusing TILs along with IL-2 and pre-conditioning with reduced-intensity circulating lymphocyte-depleting chemotherapy mediates tumor-targeting immune response in up to 50% of patients [2]. The highest response rate

up to 70 % with up to 30% complete remission lasting for up to 3 years was reported when radiation sensitization was used in conjunction with the transfer of the tumor-reactive TILs.

Despite the success of the pioneering studies at the Surgery Branch of the US National Cancer Institute and the consequent clinical trials, this approach, although holding much promise in treating melanoma, is facing several challenges that limit broad application of the TIL-based immunotherapy. As TILs are isolated from resected tumors, the first and foremost requirement is the eligibility for surgery, which should be conducted, preferably, in the facility equipped for the isolation of TILs, identification and expansion of the tumor-reactive T cells. *Ex vivo* stimulation and propagation of TILs to large quantities required for the effective immuno-therapy is time-consuming, labor-retaining, and expensive. Although recent clinical studies showed that infusion of the minimally cultured TILs without pre-selection for tumor reactivity provide a rather high response rate [3], the search for a better melanoma-targeting strategy is on-going.

Nevertheless, isolation of the individual melanoma-reactive T cell clones allowed the devel-opment of another immunotherapeutic approach – generation of the T cells expressing recombinant antigen specific T cell receptors (TCRs). TCRs are members of the immunoglo-bulin family proteins. Each TCR consists of 2 different membrane-anchored chains that are joined by the disulfide bridges to form heterodimer. About 95% of the T cells are characterized by the expression of the α and β chains, whereas the remaining 5% express γ and δ chains. Respectively, T cells expressing these receptors are often referred to as α/β and γ/δ T cells. Each chain is comprised of the variable and constant regions. The variable domain of both α- and β-chains have three hypervariable regions also known as complementarity determining regions (CDR), however, the β-chain has an additional area of hypervariability that is not involved in antigen binding. TCR α and γ chains are generated within T cells by VJ recombi-nation, whereas β and δ chains by the V(D)J recombination. Currently, the majority of the TILs selected for the ability to target tumors are α/β T cells expressing respective TCR chains that determine T cell specificity to an antigenic peptides presented by the major histocompatability complex (MHC) proteins. Therefore, it was proposed that sequences encoding tumor antigen recognizing TCR chains can be obtained from tumor-reactive T cells and then used for the gene transfer into patient-derived lymphocytes, thereby creating large quantities of tumor-reactive T cells. The first TCRs specific to melanocytic antigens MART-1 and gp-100 were cloned in 1990s. Pioneering clinical studies using human peripheral blood lymphocytes transduced with these TCRs demonstrated melanoma regression in lymphodepleted patients [4] (Fig. 1). Although these and other initial clinical studies demonstrated a feasibility of the recombinant T cells-based approach, they also revealed multiple challenges. For example, the ability of recombinant TCR chains to interact and pair with the endogenous chains could lead to the generation of 4 different TCRs in a single cell (Fig. 2). Chain misparing decreases the expression of the function, tumor-reactive TCRs and therefore reduces T cell-mediated tumor targeting. To overcome misparing, several strategies were proposed. Recent pre-clinical and clinical studies demonstrated that replacement of the human TCR constant region with murine counterpart reduced misparing and allowed enhanced expression of the functional TCRs and improved T cell functional activity [5]. It was also reported that targeted mutagenesis and generation of the additional cystein residues in recombinant α and β chains permitted stronger

pairing of these chains, higher expression of functional TCRs and improved T cell function [6, 7]. Recent studies also showed that targeting of the endogenous chains by siRNA allows higher expression of the functional recombinant TCR. Of particular interest is the proposed approach to encode siRNA along with the TCR chains to concurrently express recombinant and inhibit translation of the endogenous chains [8]. Protein engineering was also employed to improve pairing of the recombinant chains. Thus, substitution of specific amino acids within constant regions of the antigen-specific TCRs supported paring and enhanced functional activity of these receptors [9]. It remains to be determined which of these recombinant DNA-based methods will provide better targeting of melanoma (Fig. 2). Nevertheless, recent studies using chimeric murine-human hybrid highly avid tyrosinase-specific TCR demonstrated a favorable clinical outcome [10].
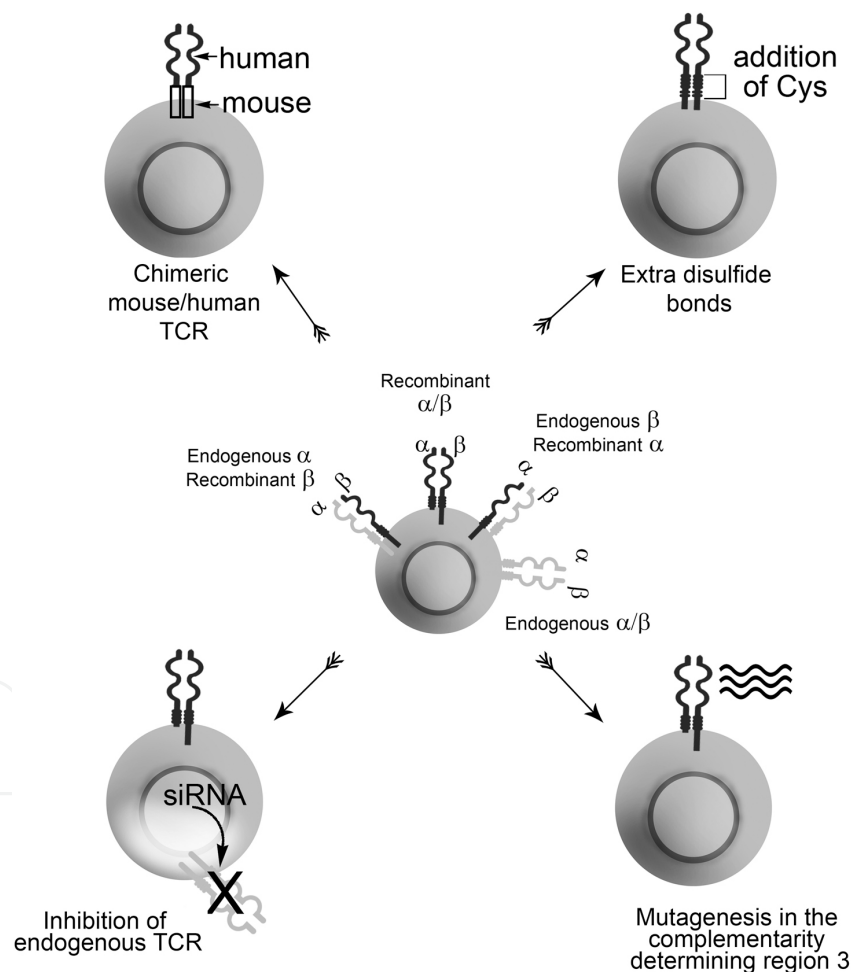

Figure 2. Strategies aimed at the improvement of the recombinant TCR pairing. Expression of the recombinant TCR may lead to the generation of 4 different TCRs within a cell (center). Different strategies designed to improve tumor-specific recombinant TCR pairing and activity include: generation of hybrid molecules containing the constant region from murine TCR, addition of disulfide bonds, alteration of the amino acid sequence within the TCR chains, and siRNA-mediated inhibition of the endogenous TCR gene expression (see text for details).

It is apparent that both α and β chains of the antigen-specific TCR should be expressed in each individual T cell. To date, internal ribosomal entry site (IRES) elements [11], double promoters [12], or co-infection with several viral vectors [13] were used to express several heterologous proteins in cells. However, these methods have their imperfections. For instance, in IRES-mediated co-expression, the upstream protein is usually more strongly transcribed than the downstream protein. Expression of the proteins from two different or biscistronic promoters or the use of multiples viruses also do not provide equal concurrent expression of multiple transgenes. A more promising approach involves the use of the self-processing viral peptide bridges such as 2A or 2A-like peptides described in Picornaviridae [14]. In picornavirus, these sequences share a highly conservative 18 amino acids motif mediating cleavage between C-terminal glycine and N-terminal proline of the 2B peptide. At present 2a and 2A-like sequences are commonly refer to as *cis*-acting hydrolase elements that allows ribosome skipping and cellular expression of multiple, discrete proteins in essentially equimolar quantities derived from a single ORF. To ensure concurrent expression of both α and β chains of the transgenic TCR an A2 sequence is most commonly used for quantitative co-expression of these heterologous proteins.

Transfer of the recombinant TCR genes into the T cells is another somewhat limiting factor for the broad application of the genetically engineered T cells for melanoma immuno-targeting. Currently, for human applications, a gene transfer platform that can mediate stable transfer of the TCR genes is retroviral system [15]. Lentiviral vectors and transposons were also tested [16, 17]. Use of retroviruses provided several advantages including a rather high infectivity and rapid integration of the transgene into host genome. With multiple vector backbones, virus packaging cell lines, and well-established GMP protocols, a retroviral system offers relative simplicity of viral vector construction and production of viruses. Since retroviruses can infect only dividing cells, stimulation of the T cell proliferation must be done prior to the gene transfer. Also, these viruses have limited capacity for the packaging. For instance, high virus titers cannot be obtained with larger retroviral vectors. Although an average size of a viral vector encoding typical α/β TCR is around 7 kb, this limits possible alternative approaches such as inclusion of various regulatory elements or another transgenes that may enhance T cell activation. Use of the viral system also presents certain safety concerns relevant to the random integration of the transgenes into the host genome that may result in the activation of oncogenes or inactivation of tumor suppressors. This may lead to the various adverse invents including development of a lymphoproliferative disease resembling leukemia due, in part, to the integration of the retroviral gene transfer vehicle near an oncogene [18, 19]. Thus far, the development of lymphoma-like symptoms has not been reported in patients treated with recombinant T cells. It is also essential to note that production of the TCR-encoding cGMP virus substantially increases the cost of the treatment with recombinant T cells. On the contrary to the retrovirus-based gene transfer, lentiviruses can infect non-dividing cells and therefore can be used for the gene transfer into quiescent T cells. Although "safe" lentiviral systems are developed to minimize the chance of producing replication-competent virus (eg. ViaraSafe from Cell Biolabs), transduction of patient-derived T cells for the adoptive transfer will always present some degree of risk.

Besides viral approaches, non-viral gene transfer may also be used for the expression of the TCRs in T cells. Recently, a Sleeping Beauty Transposon System was tested for the transduction of the T cells [17]. Sleeping Beauty Transposon System consists of two components - the transposon, composed of inverted terminal repeat sequences (IRs) with desired genetic material in between, and a SB transposase enzyme. Most recently, a number of IRs and hyperactive transposases with increasing enzymatic activities were developed to mediate transposition of transposon-encoding proteins into the genomic DNA [20]. Although transposition of SB transposons appears to be unregulated, it has certain advantages over viral based approaches. For instance, expression of transgenes, TCRs in particular, could be regulated by specific promoters that provide either T cell specific expression (eg. CD3 promoter; [21, 22]), or high level of expression (eg. elongation factor 1 promoter; [23,24]. Promoters may also be selected for further specific applications (discussed below). On the contrary to the viral gene transfer, non-viral systems also permit significantly simpler production of the cGMP-grade material (plasmid DNA) and lesser safety testing. Up to date, the Sleeping Beauty transposon-mediated approach was shown to mediate a long-term stable integration of the T-cell receptor genes targeting melanoma-derived antigen, MART-1, in laboratory settings (Fig. 3b). This system provided 50% efficiency of the TCR integration into the genome of the T cells and sustained functional reactivity of lymphocytes to the antigen-positive melanoma [25].

Other non-viral strategies could be useful in genetic engineering of the T cells. For example, integrase-mediated insertion of the genetic material may provide stable, site-directed integration of the transgenes (TCRs) into T cell genome. This strategy involves integrase from the *Streptomyces* phage ΦC31 that catalyzes unidirectional recombination between attP motifs in phage and attB sites in bacterial genomes. Usually attP and attB sites are cleaved and joined to each other, generating two hybrid sequences (attL and attR) that flank the integrated phage genome. However, ΦC31 integrase can also recognize several endogenous sequences in eukaryotic chromosomes as attP sites and integrate attB-bearing transgenes into them (Fig. 3c). Such pseudo attP sites were found in every mammalian genome with more than 100 ΦC31 integration sites identified in human cells. Thus far, only three preferred sites located in human Xq22.1, 8p22, 19q13.31 loci are commonly used by this enzyme [26, 27]. Therefore, ΦC31-integrase-based system is somewhat similar to the SB transposone system (Fig. 3b, c). Yet, it provides better specificity of the transgene integration. We recently tested whether ΦC31 can efficiently integrate transgenes into the T cells. Our initial data using GFP-encoding reporter plasmid with short (34bp) attB site demonstrated that nucleofection reaction provides rather efficient transduction of the transgene and ΦC31 integrase-encoding plasmids into T cells (Fig. 4a) and stable, integrase-dependent insertion of the reporter into both CD4+ and CD8+ T cells (Fig. 4b). Transduction of the T cells with tyrosinase-specific TCR (described in 10) ligated into the attB-harboring mammalian expression vector also resulted in the sustained expression of this melanoma-specific TCR and the ability of the T cells to target antigen-positive melanoma cells *in vitro* (Fig. 4c)

Collectively, viral and non-viral strategies for the genetic engineering of the T cells expressing melanoma-specific TCRs are suitable for the *ex vivo* production of large quantities (more than 10^8 cells) of the tumor-specific T cells that can be used for the adoptive T cell transfer. However, clinical utility of the non-viral approaches remains to be elucidated. In spite of T cell trans-
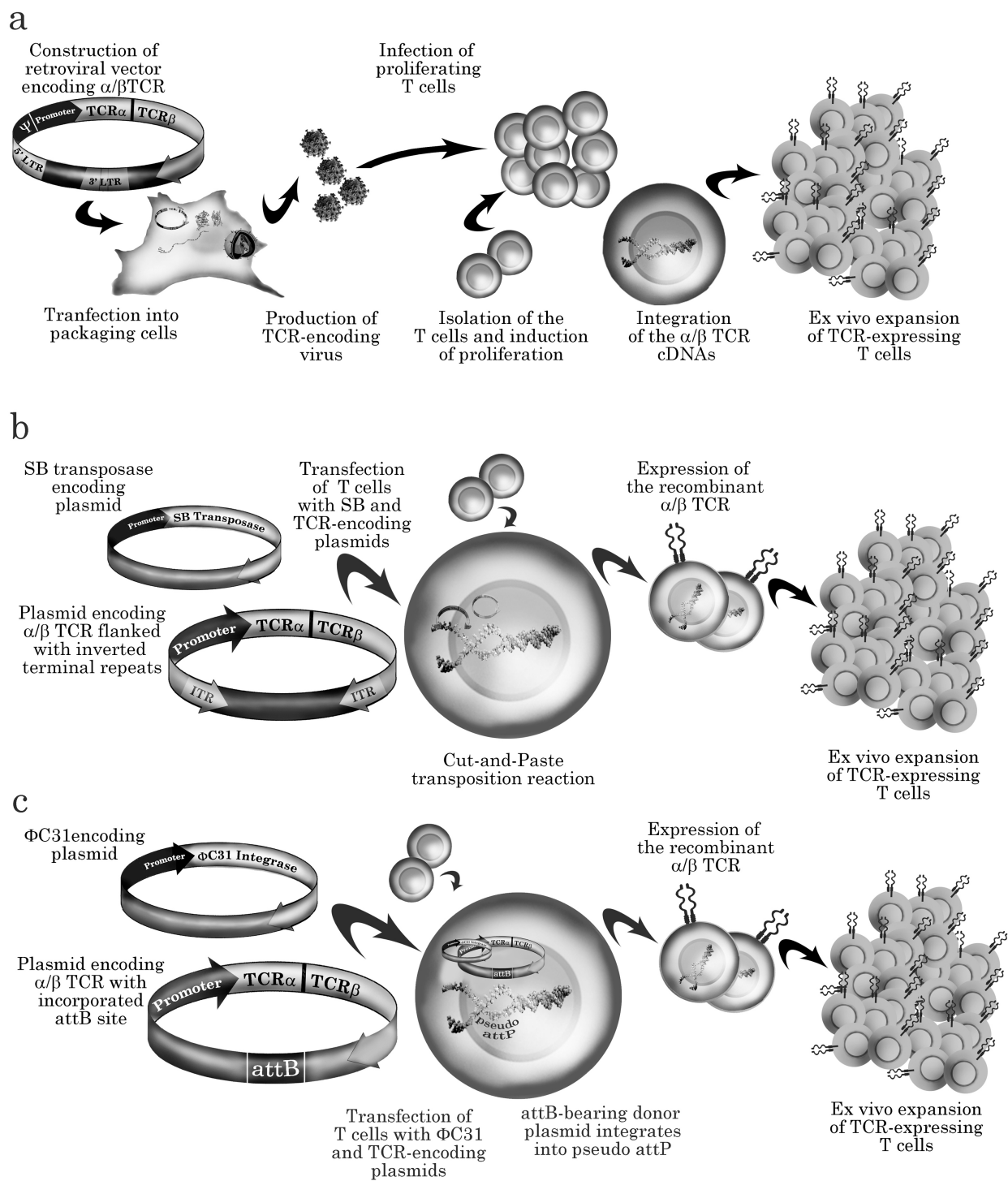
Figure 3. Schematic diagram depicting genetic engineering of the tumor-targeting T cells expressing recombinant TCR. Diagrams depict generation of the recombinant T cells via (a) retrovirus-mediated gene transfer, (b) Sleeping Beauty transposon-mediated gene transposition, and (c) ΦC31 integrase-mediated gene insertion (see text for details).

duction strategy, it is clear that the ability to generate melanoma-specific recombinant T cell receptors allowed significant advancement in the development of the clinically-applicable TCR-based approach for melanoma immunotherapy. Its primary advantages are in the use of
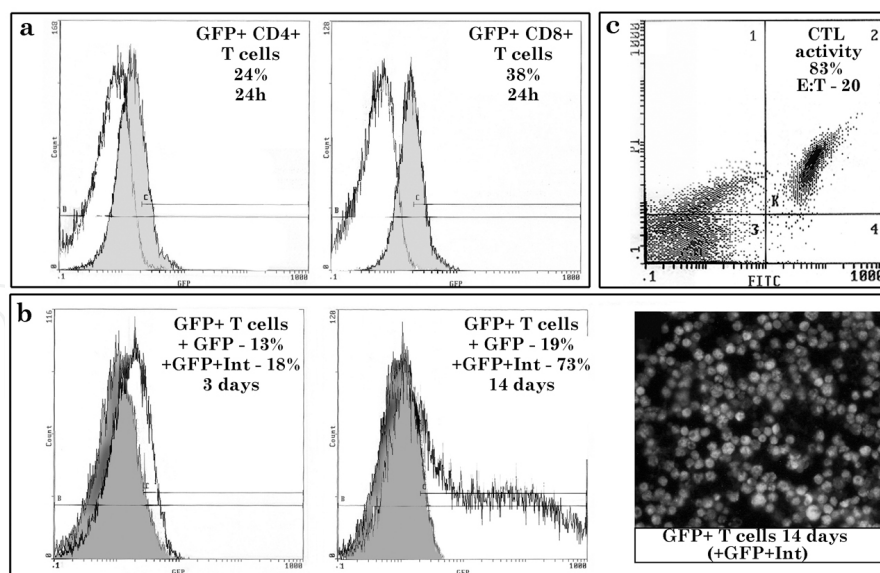
Figure 4. ΦC31 integrase-mediated genetic modification of the T cells. (a) Example showing typical nucleofection reaction of the reporter gene (GFP) into freshly isolated T cells 24 hours post Nucleofection (representative FACS profiles). (b) Analysis of the GFP expression after Nucleofection of the T cells in the presence of absence of the ΦC31 integrase-encoding plasmid (representative profiles and the direct fluorescent microscopy) 3 and 14 days after Nucleofection. (c) FACS-based analysis of the CTL activity of the recombinant T cells generated to express tyrosinase-specific TCR against human melanoma in vitro (representative profile). Times and percentages are indicated in the panels; Shaded Profiles – GFP-positive cells (see text for details).

a natural and a rather well-understood mechanism of the T cell function and the ability to select/generate multiple melanoma-reactive TCRs that can be used alone or in combination. Currently, several melanoma-targeting TCRs specific to tyrosinase, MART-1, and gp100 were cloned. One can envision generation of TCR-encoding cDNA banks that could be utilized for the generation of different melanoma-reacting T cells from the pool of patient-derived T cells to target several TAAs. However, this strategy has several disadvantages including restriction of specific TCRs to one HLA type, dependence from the expression and presentation of an antigen, limited intracellular signaling from the recombinant α/β TCRs, misparing of TCR chains, and the inability to target non-protein tumor antigens.

At present time, about 20 clinical studies involving melanoma-specific T cells expressing recombinant TCR were conducted in US alone (some of them reviewed in [28]). The result of some of the completed trials opened new perspectives for the improvement of the TCR-based strategies. For instance, adoptive transfer of the T cells genetically engineered to express highly avid MART-1-specific TCR has achieved objective clinical responses in a 13% of treated patients [29]. Analysis of CTL-resistant tumor cell revealed that these resistant clones exhibited hyperactivation of the NF-κB survival pathway and overexpression of the antiapoptotic Bcl-2, Bcl-x, Bcl-$_{xL}$, and Mcl-1 genes [30]. These studies suggest that sensitivity of melanoma to the recombinant T cells could be increased by the pharmacological inhibition of the NF-κB pathway and/or Bcl-2 family members. Multiple investigative studies are on-going to further improve recombinant TCR-based approach.

2.2. Chimeric antigen receptors

Independently, an alternative approach involving recombinant DNA technology was developed to generate tumor-targeting T cells. It utilizes fusion of the variable chain of the tumor-antigen-specific antibody, TCR constant region, and intracellular signaling domains. Initially, these structures were called T-bodies [31]. They are comprise of the single-chain antibody (sFv), TCR transmembrane domain and the intracellular signaling domain of the TCR-ζ. One of the first tumor associated antigens targeted by T cells expressing T-bodies was erbB2 (HER2/neu) receptor that is over-expressed in multiple cancers [32]. Later, a more general term – chimeric antigen receptors or CARs emerged. As compared to the TCRs, CARs allow overcoming dependency on HLA type, antigen presentation, and restricted intracellular signaling of the recombinant α/β TCRs. Initial studies with T-bodies (and recombinant TCRs) demonstrated a rather short lifespan of the engineered T cells and the inability of the recombinant receptors to fully support persistence of the T cell. To address this issue, several studies were conducted to identify the most potent CAR structures by testing several signaling molecules involved in T cell activation (Fig. 5). It was demonstrated that fusion of TCR-ζ with the intracellular domain of CD28 can augment cytokine production by CAR-expressing T cells upon encountering antigen and enhance antitumor efficacy [33]. Inclusion of CD134 (OX-40) into CAR structure also led to the elevated tumoricidal activity of the recombinant T cells [34]. Comparative analysis of the different CARs comprised of TCR-ζ signal transduction domain, CD28 and/or CD137 (4-1BB) intracellular domains demonstrated that addition of the CD137 supports T cell function to a greater extent as compared to other constructs [35]. Collectively, addition of these signaling domains to the CAR structure allowed overcoming (to certain extent) inefficient effector function and anergic status of the T cells.
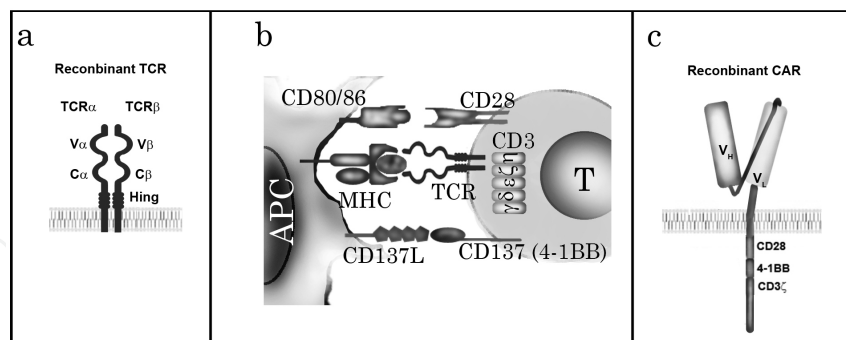

Figure 5. Recombinant TCR and CAR structures. (a) Diagram depicting recombinant TCR structure. (b) Diagram illustrating molecular interactions involved in TCR-mediated pro-proliferation and pro-survival intracellular signaling including engagement of the CD28, CD3, and CD137 (4-1BB). (c) Diagram depicting recombinant CAR structure with CD3ξ, CD137 (4-1BB), and CD28 signaling domains (see text for details).

As recognition of target cells by CAR depends on the antibody, CARs can recognize not only polypeptides but also non-protein molecules such as tumor-associated glycolipids and carbohydrates. However, antibody-mediated binding require surface expression of an antigen and strict selection of TAAs to avoid autoimmune side effects. Also, use of the mouse monoclonal antibody sequences in CAR design may lead to the unwanted immune recognition of

the CAR-expressing T cells and limit long-term clinical use [36, 37]. Nevertheless, existence of a large number of the tumor antigen-specific antibodies and robust anti-tumor response by CAR-expressing T cells suggest great clinical utility of these recombinant molecules. Currently, in US alone there are 18 clinical trials aimed at treatment of various malignancies with CAR-engineered leukocytes, with 16 trials in the recruitment phase. Eight of them are aimed at targeting different B cell malignancies with anti-CD19-CAR. Three trials are intended to test HER2-specific CAR-modified T cells for the treatment of sarcoma, glioblastoma, and advanced Her2-positive lung malignancy.

With regard to melanoma, several CAR designs were tested for the ability to target this malignancy. Thus, recent studies demonstrated that treatment of melanoma xenografts in nude mice using engineered T cells expressing tandem CAR (CD28/TCRζ) specific to ganglio-side GD3 with IL2 supplementation led to complete remissions of the established tumors in 50 % of treated animals [38]. As GD3 is often over-expressed in melanoma, this approach could be potent in eliminating melanoma in human patients.

Another attractive target for the CAR-mediated T cell therapy for melanoma is a high molecular weight melanoma-associated antigen (HMW-MAA) encoded by CSPG4 gene. This is a cell-surface proteoglycan expressed on more than 90% of the tumors. Recent studies on targeting of this antigen using CAR that is comprised of the anti- HMW-MAA antibody chain and intracellular signaling domains of the CD28, CD137, and CD3ζ demonstrated that T cell genetically modified to express this CAR were cytolytic to the HMW-MAA–positive melanoma cells, produced cytokine and proliferate *in vitro* [39]. The potential clinical utility of the CAR-mediated HMW-MAA targeting was emphasized by another recent animal study [40]. Analysis of a few human melanoma biopsies revealed the presence of less than 2% of specific tumor cells co-expressing CD20 and HMW-MAA. Implantation of tumors containing these CD20+ HMW-MAA+ cells into immuno-deficient mice resulted in a rapid growth of tumors. Targeting of these pre-established lesions with T cells expressing either CD20 or HMW-MAA-directed CAR showed elimination of lesions in nearly 90% of treated animals. CD20-specific engineered T cells were unable to eradicated melanoma lesions artificially expressing CD20 suggesting that native expression of the antigen is required for effective targeting. These studies provided additional evidence that direction of the T cells toward HMW-MAA via genetic engineering can permit effective elimination of tumor lesions.

As progression of most tumors including melanoma depends on the microenvironment, T-cell mediated targeting of the microenvironmental components could also be a viable strategy for melanoma immunotherapy. Particularly, tumor survival was shown to be dependent on the *de novo* formation of the intratumoral blood vessels characterised by high levels of the vascular endothelial growth factor receptor 2 (VEGFR2/KDR). Also, a number of studies associated high levels of VEGFR2 expression with various tumor stroma cells including subsets of macrophages, immature monocytes, immature dendritic cells and immuno-suppressive CD4+CD25+ regulatory T cells (Treg) [41-46]. Therefore, it was suggested that targeting of VEGFR2 – positive cells in tumor stroma may provide clinical benefits and tumor regression. In support of this notion, recent studies demonstrated that the direction of the T cells toward VEGFR-2 via CAR provide an effective means to eliminate pre-established experimental melanoma.

Thus, using an animal model, it was shown that after systemic transplantation, anti–VEGFR-2 CAR and IL-12–co-transduced T cells infiltrated the tumors, expanded and persisted within tumor mass leading to tumor regression [47]. The anti-tumor effect was dependent on targeting of IL-12–responsive host cells via activation of anti–VEGFR-2 CAR-T cells and release of IL-12. Based on this data, one clinical trial aimed at the assessment of safety and effectiveness of cell therapy was initiated to treat recurrent and relapsed cancer by using anti-VEGFR2 CAR-modified T cells.

Presently, there is an accumulating body of evidence suggesting clinical utility of the T cell genetically engineered to express melanoma antigen-specific CARs. It is likely that in the near future CAR-mediated targeting of different melanoma antigens will evolve into general practice of cancer immunotherapy.

3. DNA vaccination

Another immunotherapeutic approach directly relevant to recombinant DNA is genetic or DNA vaccination. The original idea of DNA vaccination emanated from the observations that intramuscular injection of DNA encoding influenza A virus protein resulted in the robust activation of the immune responses that protected the host from viral challenge [48]. Generally, DNA-mediated activation of immune response involves multiple processes. First, plasmid DNA should be delivered intracellularly and expressed in the host cells. Next, in most of cases the antigen has to be secreted from the cells and picked up by the dendritic cells (DC), processed and presented in the context of the MHC class II to the CD4+ T helper (Th) cells. Alternatively, if the antigen is expressed directly in the DCs, it could be processed intracellularly and presented via MHC class I molecules, leading to the activation of the CD8+ T cells and induction of the cytotoxic immune responses. Initial studies on DNA vaccination were carried out using an intramuscular route of vaccine administration (Fig. 6). This allowed high levels of antigen expression and secretion from the elongated muscle cells into perimysium, the resident site of the intramuscular DCs. Later, DNA vaccination through the skin was suggested to be superior over the intramuscular route. Skin has evolved as a barrier to prevent the entry of pathogens, with efficient immune surveillance complex including Langerhans cells, dendritic cells, lymphocytes, and other leukocytes. Skin is also rich in lymphatic vasculature network that provides an efficient route for DC and T cell trafficking. Depending on the physical methods of into-skin DNA delivery, DNA-based vaccines can be targeted to specific locations in the skin [49].

The DNA vaccination approach has several advantages over other types of vaccinations: (i) multiple expression vectors coding for different antigen and co-stimulatory molecules can be concurrently delivered into the skin (or the muscle); (ii) the use of cell-type-specific promoters can provide specificity of protein expression; (iii) protein expression from designed plasmids can be controlled by inducible promoters, the use of ubiquitous chromatin opening elements (UCOE), or chemically (e.g. sodium butyrate). Also of note is the relative simplicity and inexpensiveness of the cGMP grade DNA vaccine production and pre-clinical testing. These
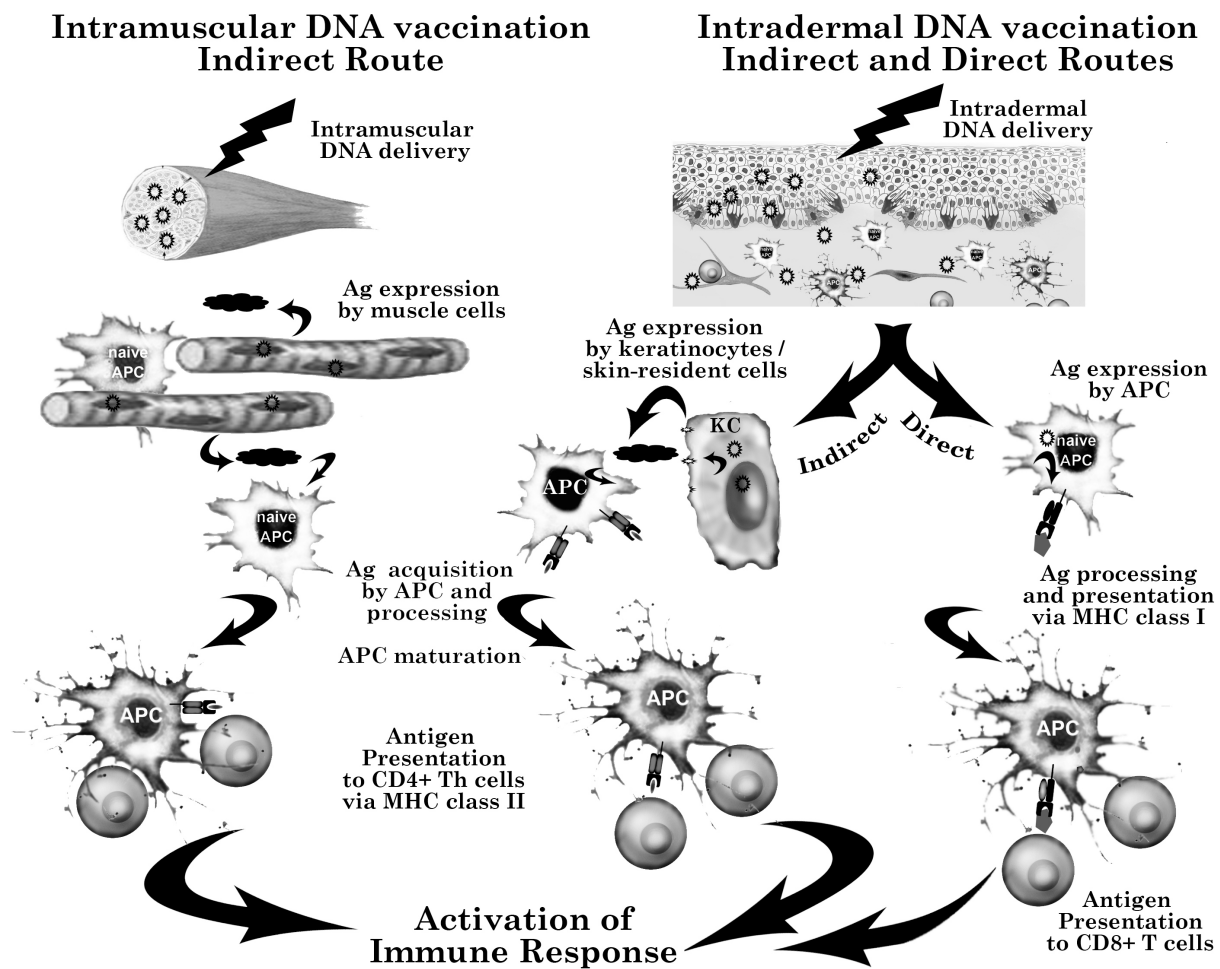
Intramuscular DNA vaccination Indirect Route

Intradermal DNA vaccination Indirect and Direct Routes

Figure 6. Intramuscular and Intradermal DNA vaccination. Intramuscular and intradermal sites are used for DNA vaccination. The former allows high level of antigen (Ag) expression in muscle cells and MHC class II Ag processing and presentation whereas the latter permits expression of the Ag in the Antigen-Presenting Cells (APC) and direct presentation of the antigenic peptides to the CD8+ cytotoxic T cells (see text for details).

attractive characteristics of DNA vaccines have prompted extensive research within the past 10 years.

Multiple studies on pre-clinical animal models of melanoma and other cancers have been conducted. Studies on the canine model of aggressive and metastatic melanoma (stages II-IV) demonstrated that xenogeneic vaccination of dogs with DNA vaccine coding for human tyrosinase led to an excellent clinical response in the majority of vaccinated dogs. A long-term survival of dogs with advanced stage IV disease with bulky lung metastases (on average 400 days) was observed [50]. Vaccinated dogs with stage II/III disease also had long-term survivals (on average 500 days) with no evidence of melanoma on necropsy. Overall, median survival time for all treated dogs was 389 days. Another canine model study [51] showed that xenogeneic DNA vaccination induces melanoma-specific antibody response, which coincides with observed clinical responses. As a result, in 2010 Merial, an animal health company has gained full-licensure from the U.S. Department of Agriculture (USDA) for ONCEPT™ Canine

Melanoma DNA Vaccine. Up to date, ONCEPT is the first and only USDA-approved therapeutic vaccine for the treatment of cancer in either animals or humans. (The first DNA vaccine was licensed by the USDA in 2005 for prevention of West Nile virus infection in horses).

However, presently only a few human clinical trials on DNA vaccination were conducted. One of such study, aimed at the evaluation of the immune response in patients with hormone-refractory prostate cancer showed that DNA vaccination with a prostate-specific antigen (PSA) encoding plasmid given with GM-CSF and IL-2 is safe in doses of up to 900 μg, and that the vaccination can induce cellular and humoral immune responses [52].

Similar to the reference above canine studies, DNA vaccines were shown to be effective in mouse melanoma models when mice were vaccinated with heterologous DNA encoding human melanoma-associated antigen gp-100 [53]. This vaccination regimen was augmented by the GM-CSF and was most effective in the prophylactic setting. It was also effective in suppressing pre-established melanoma. However, vaccinations with autologous mouse melanoma antigens were less successful. Nevertheless, the relative simplicity of modifying recombinant DNA allowed testing of various genetic alterations aimed at breaking the immunologic tolerance and enhancing immune responses to DNA vaccines. For example, concurrent vaccination with DNA encoding several melanoma-specific epitopes can be used. This approach was tested in several studies with different degree of success. As a result, vaccination of mice with $gp100_{25-33}$ and $TRP-2_{181-188}$ encoding minigene was effective in preventing melanoma development [54]. As many of the melanoma MHC class I epitopes were characterized for melanoma including those derived from tyrosinase, TRP1, TRP2, gp-100, MART-1, and MC-1R (some of them shared between mouse and human MHC molecules [55], one can envision generation of an ultimate genetic immunogen capable of targeting several melanoma-associated antigens.

Recombinant DNA technology has also allowed introduction of immuno-augmentation sequences into the DNA vaccine composition. Identified universal pan HLA DR helper binding *e*pitope (PADRE; KXVAAWYLKA) was shown to enhance immunogenicity of both peptide and DNA vaccines [56, 57]. Other studies demonstrated augmentation of melanoma-specific immune responses via direct fusion of the DNA vaccine with the VP22 protein of the herpes simplex virus-1 [58].

Besides introducing immuno-enhancing alteration to the DNA vaccine, other strategies could be employed to enhance DNA vaccination efficacy including addition of the immuno-enhancing molecules to vaccine composition, alteration of the microenvironment at vaccine administration site, and use of the prime-boost immunization regimens. Recent studies demonstrated that antibody-mediated inhibition of the cytotoxic T lymphocyte antigen 4 (CTLA-4) enhances melanoma-specific immune response. This strategy was recently tested in treatment of stage III-IV melanoma and the drug (Ipilimumam) was approved by the FDA as first anti-melanoma immunotherapeutic [59, 60]. CTLA-4 presents its immuno-inhibitory function during activation of the T cells by the antigen-presenting cells. It also inhibits TCR-mediated intracellular signaling in activated T cells and down-modulating T cell mediated immunity. Therefore, it is possible that inhibition of CTLA-4 in conjunction with DNA vaccination may provide significant enhancement of the vaccine-mediated immune response

induction. Although providing CTLA-4 inhibiting antibodies like Ipilimumab along with DNA vaccination is not feasible, other options could be explored. For example, recently characterized genetically engineered lipocalin (LCN2) exhibits a strong cross-species antagonistic activity to CTLA-4 [61]. It is likely that this molecule could be included into DNA vaccine composition to enhance DC-mediated activation of the T cells. Other immuno-modulatory strategies may include addition of CD40 ligand, which was shown to stimulate expression of maturation markers CD80, CD86 and IL-12 in APC [62, 63] and its ability to activate CD8+ T cells and increase cell-mediated immunity [64, 65]. Addition of different cytokines and growth factors including GM-CSF, IL-2, IL12 for stimulation/support of the T cells was also tested in several studies (as exemplified in preceding paragraphs) and could be further explored. Alteration of microenvironment via application chemokines to recruit specific sets of the leukocytes to the vaccine administration site may also provide a favorable milieu for the launch of the effective DNA-vaccine induced immune response [66, 67]. These and many other strategies can be proposed; however, the clinical utility of the DNA vaccination combination with other approaches remains to be determined. Nevertheless, presently in the US alone, 10 clinical studies utilizing xenogenic (mouse) or human DNA vaccines coding for melanoma associated antigens have been completed. In these trials, tyrosinase, gp75, gp100, and TRP2 were used as antigens. Although most of these studies are already completed, currently no study results are posted nor are follow-up reports available on patient survival and characterization of immune response. Nevertheless, DNA vaccination remains to be a promising modality that could provide cost-effective and generic immunotherapy for patients with melanoma and other cancers.

4. Other strategies involving recombinant DNA technology

At the present time, almost every immuno-therapeutic approach utilizes recombinant DNA in one way or another. Understanding of the immuno-regulatory functions of DCs and the molecular mechanisms involved in the capture, processing and presentation of antigens by DCs allowed the development of the DC-based vaccines. Initially, in the mid 1990's, several pre-clinical and clinical studies were conducted using autologous DCs pulsed with melanoma-associated antigens. These studies demonstrated that antigen-loaded DC can trigger active melanoma specific immune responses [68, 69]. However, it became apparent that enforced expression of the antigens in DC rather than loading of these cells with peptides allows for presentation of the tumor-derived antigens via MHC class I complex and priming of the CD8+ T cells to elicit cytotoxic immune response. Moreover, to provide DC specific expression of the antigens, long and short CD11c promoters were characterized and used in several studies [70, 71]. These promoters allow effective and cell type specific expression of the antigens in DCs, as well as more efficient priming and activation of the T cells *in vitro* and *in vivo*. Considering a necessity of the direct interaction of the DC with T lymphocytes, application of T cell recruiting chemokines was also explored recently. These pioneering studies demonstrate that forced expression of the secondary lymphoid chemokine, CCL21, in antigen loaded DCs enhances their ability to recruit and activate T cells [72, 73]. One clinical phase I clinical trial

using CCL21 transduced DCs pulsed with MART-1 and gp100 was completed in 2012. Altogether, a total of 64 clinical trials aimed at targeting of melanoma using dendritic cells are listed. Thirty nine of them are completed with no reports yet available. The majority of these trials in some way utilize recombinant DNA technology.

5. Conclusion

During last decade, various melanoma-specific immunotherapeutics that utilize recombinant DNA have been developed and tested in pre-clinical and clinical studies with varying degrees of clinical success. Many of these approaches, including recombinant TCRs and CARs, have already demonstrated promising clinical results, thus providing us with the hope that in the near future melanoma immunotherapy will become curable for melanoma patients.

Acknowledgements

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References

[1] Knuth, A., Wolfel, T., Klehmann, E., Boon, T. and Meyer zum Buschenfelde, K.H. (1989) Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 2804-2808.

[2] Dudley, M.E., Yang, J.C., Sherry, R., Hughes, M.S., Royal, R., Kammula, U., Robbins, P.F., Huang, J., Citrin, D.E., Leitman, S.F. *et al.* (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol*, 26, 5233-5239.

[3] Dudley, M.E., Gross, C.A., Langhan, M.M., Garcia, M.R., Sherry, R.M., Yang, J.C., Phan, G.Q., Kammula, U.S., Hughes, M.S., Citrin, D.E. *et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16, 6122-6131.

[4] Goff, S.L., Johnson, L.A., Black, M.A., Xu, H., Zheng, Z., Cohen, C.J., Morgan, R.A., Rosenberg, S.A. and Feldman, S.A. Enhanced receptor expression and in vitro effector function of a murine-human hybrid MART-1-reactive T cell receptor following a rapid expansion. *Cancer immunology, immunotherapy : CII*, 59, 1551-1560.

[5] Cohen, C.J., Zhao, Y., Zheng, Z., Rosenberg, S.A. and Morgan, R.A. (2006) Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer research*, 66, 8878-8886.

[6] Cohen, C.J., Li, Y.F., El-Gamil, M., Robbins, P.F., Rosenberg, S.A. and Morgan, R.A. (2007) Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. *Cancer research*, 67, 3898-3903.

[7] Kuball, J., Dossett, M.L., Wolfl, M., Ho, W.Y., Voss, R.H., Fowler, C. and Greenberg, P.D. (2007) Facilitating matched pairing and expression of TCR chains introduced into human T cells. *Blood*, 109, 2331-2338.

[8] Okamoto, S., Mineno, J., Ikeda, H., Fujiwara, H., Yasukawa, M., Shiku, H. and Kato, I. (2009) Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. *Cancer research*, 69, 9003-9011.

[9] Govers, C., Sebestyen, Z., Coccoris, M., Willemsen, R.A. and Debets, R. T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing. *Trends Mol Med*, 16, 77-87.

[10] Frankel, T.L., Burns, W.R., Peng, P.D., Yu, Z., Chinnasamy, D., Wargo, J.A., Zheng, Z., Restifo, N.P., Rosenberg, S.A. and Morgan, R.A. Both CD4 and CD8 T cells mediate equally effective in vivo tumor treatment when engineered with a highly avid TCR targeting tyrosinase. *J Immunol*, 184, 5988-5998.

[11] Douin, V., Bornes, S., Creancier, L., Rochaix, P., Favre, G., Prats, A.C. and Couderc, B. (2004) Use and comparison of different internal ribosomal entry sites (IRES) in tricistronic retroviral vectors. *BMC biotechnology*, 4, 16.

[12] Baron, U., Freundlieb, S., Gossen, M. and Bujard, H. (1995) Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic acids research*, 23, 3605-3606.

[13] Mastakov, M.Y., Baer, K., Kotin, R.M. and During, M.J. (2002) Recombinant adeno-associated virus serotypes 2- and 5-mediated gene transfer in the mammalian brain: quantitative analysis of heparin co-infusion. *Molecular therapy : the journal of the American Society of Gene Therapy*, 5, 371-380.

[14] Ryan, M.D., King, A.M. and Thomas, G.P. (1991) Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *J Gen Virol*, 72 (Pt 11), 2727-2732.

[15] Baum, C., Schambach, A., Bohne, J. and Galla, M. (2006) Retrovirus vectors: toward the plentivirus? *Molecular therapy : the journal of the American Society of Gene Therapy*, 13, 1050-1063.

[16] Frecha, C., Levy, C., Cosset, F.L. and Verhoeyen, E. (2010) Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Molecular therapy : the journal of the American Society of Gene Therapy*, 18, 1748-1757.

[17] Hackett, P.B., Largaespada, D.A. and Cooper, L.J. (2010) A transposon and transposase system for human application. *Molecular therapy : the journal of the American Society of Gene Therapy*, 18, 674-683.

[18] Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M. *et al.* (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *The New England journal of medicine*, 348, 255-256.

[19] Nam, C.H. and Rabbitts, T.H. (2006) The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Molecular therapy : the journal of the American Society of Gene Therapy*, 13, 15-25.

[20] Mates, L. (2011) Rodent transgenesis mediated by a novel hyperactive Sleeping Beauty transposon system. *Methods Mol Biol*, 738, 87-99.

[21] Chatterjee, M., Hedrich, C.M., Rauen, T., Ioannidis, C., Terhorst, C. and Tsokos, G.C. (2012) CD3-T Cell Receptor Co-stimulation through SLAMF3 and SLAMF6 Receptors Enhances RORgammat Recruitment to the IL17A Promoter in Human T Lymphocytes. *The Journal of biological chemistry*, 287, 38168-38177.

[22] Clevers, H., Lonberg, N., Dunlap, S., Lacy, E. and Terhorst, C. (1989) An enhancer located in a CpG-island 3' to the TCR/CD3-epsilon gene confers T lymphocyte-specificity to its promoter. *The EMBO journal*, 8, 2527-2535.

[23] Hahm, S.H., Yi, Y., Lee, D.K., Noh, M.J., Yun, L., Hwang, S. and Lee, K.H. (2004) Construction of retroviral vectors with enhanced efficiency of transgene expression. *Journal of virological methods*, 121, 127-136.

[24] Morita, S., Kojima, T. and Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene therapy*, 7, 1063-1066.

[25] Peng, P.D., Cohen, C.J., Yang, S., Hsu, C., Jones, S., Zhao, Y., Zheng, Z., Rosenberg, S.A. and Morgan, R.A. (2009) Efficient nonviral Sleeping Beauty transposon-based TCR gene transfer to peripheral blood lymphocytes confers antigen-specific antitumor reactivity. *Gene therapy*, 16, 1042-1049.

[26] Sclimenti, C.R., Thyagarajan, B. and Calos, M.P. (2001) Directed evolution of a recombinase for improved genomic integration at a native human sequence. *Nucleic acids research*, 29, 5044-5051.

[27] Groth, A.C., Olivares, E.C., Thyagarajan, B. and Calos, M.P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 5995-6000.

[28] Park, T.S., Rosenberg, S.A. and Morgan, R.A. (2011) Treating cancer with genetically engineered T cells. *Trends in biotechnology*, 29, 550-557.

[29] Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P. *et al.* (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*, 314, 126-129.

[30] Jazirehi, A.R., Baritaki, S., Koya, R.C., Bonavida, B. and Economou, J.S. (2011) Molecular mechanism of MART-1+/A*0201+ human melanoma resistance to specific CTL-killing despite functional tumor-CTL interaction. *Cancer research*, 71, 1406-1417.

[31] Eshhar, Z. (1997) Tumor-specific T-bodies: towards clinical application. *Cancer immunology, immunotherapy : CII*, 45, 131-136.

[32] Moritz, D., Wels, W., Mattern, J. and Groner, B. (1994) Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 4318-4322.

[33] Koehler, H., Kofler, D., Hombach, A. and Abken, H. (2007) CD28 costimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. *Cancer research*, 67, 2265-2273.

[34] Finney, H.M., Akbar, A.N. and Lawson, A.D. (2004) Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J Immunol*, 172, 104-113.

[35] Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D., Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G. *et al.* (2009) Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells

and increased antileukemic efficacy in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy*, 17, 1453-1464.

[36] Kershaw, M.H., Westwood, J.A., Parker, L.L., Wang, G., Eshhar, Z., Mavroukakis, S.A., White, D.E., Wunderlich, J.R., Canevari, S., Rogers-Freezer, L. *et al.* (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12, 6106-6115.

[37] Lamers, C.H., Willemsen, R., van Elzakker, P., van Steenbergen-Langeveld, S., Broertjes, M., Oosterwijk-Wakka, J., Oosterwijk, E., Sleijfer, S., Debets, R. and Gratama, J.W. (2011) Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood*, 117, 72-82.

[38] Lo, A.S., Ma, Q., Liu, D.L. and Junghans, R.P. (2010) Anti-GD3 chimeric sFv-CD28/T-cell receptor zeta designer T cells for treatment of metastatic melanoma and other neuroectodermal tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16, 2769-2780.

[39] Burns, W.R., Zhao, Y., Frankel, T.L., Hinrichs, C.S., Zheng, Z., Xu, H., Feldman, S.A., Ferrone, S., Rosenberg, S.A. and Morgan, R.A. (2010) A high molecular weight melanoma-associated antigen-specific chimeric antigen receptor redirects lymphocytes to target human melanomas. *Cancer research*, 70, 3027-3033.

[40] Schmidt, P., Kopecky, C., Hombach, A., Zigrino, P., Mauch, C. and Abken, H. (2011) Eradication of melanomas by targeted elimination of a minor subset of tumor cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 2474-2479.

[41] Duignan, I.J., Corcoran, E., Pennello, A., Plym, M.J., Amatulli, M., Claros, N., Iacolina, M., Youssoufian, H., Witte, L., Samakoglu, S. *et al.* Pleiotropic stromal effects of vascular endothelial growth factor receptor 2 antibody therapy in renal cell carcinoma models. *Neoplasia (New York, N.Y*, 13, 49-59.

[42] Larrivee, B., Pollet, I. and Karsan, A. (2005) Activation of vascular endothelial growth factor receptor-2 in bone marrow leads to accumulation of myeloid cells: role of granulocyte-macrophage colony-stimulating factor. *J Immunol*, 175, 3015-3024.

[43] Murdoch, C., Muthana, M., Coffelt, S.B. and Lewis, C.E. (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nature reviews*, 8, 618-631.

[44] Suzuki, H., Onishi, H., Wada, J., Yamasaki, A., Tanaka, H., Nakano, K., Morisaki, T. and Katano, M. VEGFR2 is selectively expressed by FOXP3high CD4+ Treg. *European journal of immunology*, 40, 197-203.

[45] Udagawa, T., Puder, M., Wood, M., Schaefer, B.C. and D'Amato, R.J. (2006) Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *Faseb J*, 20, 95-102.

[46] Zhang, N., Fang, Z., Contag, P.R., Purchio, A.F. and West, D.B. (2004) Tracking angiogenesis induced by skin wounding and contact hypersensitivity using a Vegfr2-luciferase transgenic mouse. *Blood*, 103, 617-626.

[47] Chinnasamy, D., Yu, Z., Kerkar, S.P., Zhang, L., Morgan, R.A., Restifo, N.P. and Rosenberg, S.A. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18, 1672-1683.

[48] Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A. *et al.* (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*, 259, 1745-1749.

[49] Alexeev, V. and Uitto, J. (2005) *DNA pharmaceuticals for skin diseases*. Wiley-VCH, Weinheim.

[50] Bergman, P.J., McKnight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., Wulderk, M., Jeffers, Y., Sadelain, M., Hohenhaus, A.E. *et al.* (2003) Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 9, 1284-1290.

[51] Liao, J.C., Gregor, P., Wolchok, J.D., Orlandi, F., Craft, D., Leung, C., Houghton, A.N. and Bergman, P.J. (2006) Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. *Cancer Immun*, 6, 8.

[52] Pavlenko, M., Roos, A.K., Lundqvist, A., Palmborg, A., Miller, A.M., Ozenci, V., Bergman, B., Egevad, L., Hellstrom, M., Kiessling, R. *et al.* (2004) A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. *British journal of cancer*, 91, 688-694.

[53] Rakhmilevich, A.L., Imboden, M., Hao, Z., Macklin, M.D., Roberts, T., Wright, K.M., Albertini, M.R., Yang, N.S. and Sondel, P.M. (2001) Effective particle-mediated vaccination against mouse melanoma by coadministration of plasmid DNA encoding Gp100 and granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res*, 7, 952-961.

[54] Xiang, R., Lode, H.N., Chao, T.H., Ruehlmann, J.M., Dolman, C.S., Rodriguez, F., Whitton, J.L., Overwijk, W.W., Restifo, N.P. and Reisfeld, R.A. (2000) An autologous oral DNA vaccine protects against murine melanoma. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 5492-5497.

[55] Parkhurst, M.R., Fitzgerald, E.B., Southwood, S., Sette, A., Rosenberg, S.A. and Kawakami, Y. (1998) Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer research*, 58, 4895-4901.

[56] Bettahi, I., Dasgupta, G., Renaudet, O., Chentoufi, A.A., Zhang, X., Carpenter, D., Yoon, S., Dumy, P. and BenMohamed, L. (2009) Antitumor activity of a self-adjuvanting glyco-lipopeptide vaccine bearing B cell, CD4+ and CD8+ T cell epitopes. *Cancer immunology, immunotherapy : CII*, 58, 187-200.

[57] del Guercio, M.F., Alexander, J., Kubo, R.T., Arrhenius, T., Maewal, A., Appella, E., Hoffman, S.L., Jones, T., Valmori, D., Sakaguchi, K. *et al.* (1997) Potent immunogenic short linear peptide constructs composed of B cell epitopes and Pan DR T helper epitopes (PADRE) for antibody responses in vivo. *Vaccine*, 15, 441-448.

[58] Engelhorn, M.E., Guevara-Patino, J.A., Merghoub, T., Liu, C., Ferrone, C.R., Rizzuto, G.A., Cymerman, D.H., Posnett, D.N., Houghton, A.N. and Wolchok, J.D. (2008) Mechanisms of immunization against cancer using chimeric antigens. *Molecular therapy : the journal of the American Society of Gene Therapy*, 16, 773-781.

[59] Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine*, 363, 711-723.

[60] Schartz, N.E., Farges, C., Madelaine, I., Bruzzoni, H., Calvo, F., Hoos, A. and Lebbe, C. Complete regression of a previously untreated melanoma brain metastasis with ipilimumab. *Melanoma research*, 20, 247-250.

[61] Schonfeld, D., Matschiner, G., Chatwell, L., Trentmann, S., Gille, H., Hulsmeyer, M., Brown, N., Kaye, P.M., Schlehuber, S., Hohlbaum, A.M. *et al.* (2009) An engineered lipocalin specific for CTLA-4 reveals a combining site with structural and conformational features similar to antibodies. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 8198-8203.

[62] Feder-Mengus, C., Schultz-Thater, E., Oertli, D., Marti, W.R., Heberer, M., Spagnoli, G.C. and Zajac, P. (2005) Nonreplicating recombinant vaccinia virus expressing CD40 ligand enhances APC capacity to stimulate specific CD4+ and CD8+ T cell responses. *Human gene therapy*, 16, 348-360.

[63] Sato, T., Terai, M., Yasuda, R., Watanabe, R., Berd, D., Mastrangelo, M.J. and Hasumi, K. (2004) Combination of monocyte-derived dendritic cells and activated T cells which express CD40 ligand: a new approach to cancer immunotherapy. *Cancer immunology, immunotherapy : CII*, 53, 53-61.

[64] Peter, I., Nawrath, M., Kamarashev, J., Odermatt, B., Mezzacasa, A. and Hemmi, S. (2002) Immunotherapy for murine K1735 melanoma: combinatorial use of recombinant adenovirus expressing CD40L and other immunomodulators. *Cancer gene therapy*, 9, 597-605.

[65] Rousseau, R.F., Biagi, E., Dutour, A., Yvon, E.S., Brown, M.P., Lin, T., Mei, Z., Grilley, B., Popek, E., Heslop, H.E. *et al.* (2006) Immunotherapy of high-risk acute leukemia

with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation. *Blood*, 107, 1332-1341.

[66] Guo, J.H., Fan, M.W., Sun, J.H. and Jia, R. (2009) Fusion of antigen to chemokine CCL20 or CXCL13 strategy to enhance DNA vaccine potency. *International immuno-pharmacology*, 9, 925-930.

[67] Novak, L., Igoucheva, O., Cho, S. and Alexeev, V. (2007) Characterization of the CCL21-mediated melanoma-specific immune responses and in situ melanoma eradi-cation. *Molecular cancer therapeutics*, 6, 1755-1764.

[68] Nestle, F.O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D. (1998) Vaccination of melanoma patients with peptide- or tumor ly-sate-pulsed dendritic cells. *Nature medicine*, 4, 328-332.

[69] 69.Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P. *et al.* (1999) Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *The Journal of experimental medicine*, 190, 1669-1678.

[70] Brocker, T., Riedinger, M. and Karjalainen, K. (1997) Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *The Journal of experimental medicine*, 185, 541-550.

[71] Ni, J., Nolte, B., Arnold, A., Fournier, P. and Schirrmacher, V. (2009) Targeting anti-tumor DNA vaccines to dendritic cells via a short CD11c promoter sequence. *Vaccine*, 27, 5480-5487.

[72] Mule, J.J. (2009) Dendritic cell-based vaccines for pancreatic cancer and melanoma. *Annals of the New York Academy of Sciences*, 1174, 33-40.

[73] Terando, A., Roessler, B. and Mule, J.J. (2004) Chemokine gene modification of hu-man dendritic cell-based tumor vaccines using a recombinant adenoviral vector. *Can-cer gene therapy*, 11, 165-173.