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Isolation and characterization of a TCR specific for a new unconventional NY-ESO-1 epitope

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Have the courage to follow
your heart and intuition.
They somehow already know
what you truly want to become.

Steve Jobs

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Summary

The adoptive transfer of T cells expressing transgenic tumor antigen-specific T cell receptors (TCRs) is a continuously developing immunotherapeutic strategy for patients with advanced tumors, showing promising results in early clinical trials. Nevertheless, there is a strong need to improve efficiency and safety to enhance the clinical benefit for more patients and the identification of suitable therapeutic TCRs remains quite challenging.

T cells expressing a high-affinity TCR recognizing self-antigens on self-major histocompatibility complex (MHC) molecules are mostly eliminated by negative selection in the thymus to avoid autoimmunity. Since tumor antigens are often over-expressed self-antigens, TCRs that develop in a thymic-selected TCR repertoire are largely of low to intermediate affinity. Alternatively, high-avidity T cells recognizing self-antigens can be found in the non-tolerant allogeneic MHC-reactive T cell repertoire that have not encountered the target peptide-MHC complex during thymic selection. Hence, these T cells can serve as sources of high-affinity TCRs that can be utilized to equip patient-derived lymphocytes with receptors mediating the desired anti-tumor specificity.

As previous studies have confirmed that high-avidity T cells specific for different tumor antigens can be isolated from the allogeneic MHC-reactive T cell repertoire using *in vitro* priming procedures, in this project the allogeneic priming approach was performed utilizing NY-ESO-1 as target antigen and the human leukocyte antigen (HLA)-A2 as allogeneic restriction element. Both molecules were introduced as *in vitro* transcribed (*ivt*)RNA into monocyte-derived dendritic cells (DCs) which were then utilized as antigen presenting cells (APCs) for stimulation and expansion of autologous CD8-enriched T cells with the final aim to isolate NY-ESO-1-specific T cells. The innovations of the approach were: i) the utilization of the complete coding region of the antigen to allow presentation of epitopes that were not biased by MHC binding prediction programs; ii) a unique selection process of primed T cells enabling the identification of T cells specific for unknown immunogenic epitopes of NY-ESO-1. This was possible by utilizing modified K562 cells for stimulation while the activation-induced T cell marker CD137 was verified on T cells. Among the isolated T cell clones, the clone 5-271 showed the desired specificity and was further analyzed. The TCR 5-271 sequence was cloned in vectors to enable transgenic expression in recipient T cells. The epitope recognized by the isolated TCR was identified and fine typed. Interestingly, the TCR was specific for a new unconventional peptide derived from the protein encoded by an alternative open reading frame (ORF) of NY-ESO-1 and presented on HLA-A2 molecules. To evaluate the potential for therapeutic use of the isolated TCR, T cells expressing the transgenic TCR were tested for tumor cell recognition, killing capacity,

peptide sensitivity and CD8-dependency. TCR 5-271-transgenic T cells recognized the tested tumor cell lines only after target antigen-loading. Additionally, low peptide sensitivity was observed although the TCR was able to act in a CD8-independent manner.

Zusammenfassung

Der adoptive Transfer von T-Zellen, die einen Tumorantigen spezifischen T-Zellrezeptor (TZR) exprimieren, ist eine sich kontinuierlich weiterentwickelnde Strategie der Immuntherapie für Patienten im fortgeschrittenen Krebsstadium mit vielversprechenden Ergebnissen in frühen klinischen Untersuchungen. Nichtsdestotrotz ist eine Verbesserung der Effizienz und der Sicherheit dringend nötig, um den klinischen Nutzen für mehr Patienten zu erhöhen, und auch die Identifikation von geeigneten therapeutisch nutzbaren TZRs gestaltet sich immer noch schwierig.

T-Zellen, die einen hoch-affinen TZR exprimieren, der Selbst-Antigene auf selbst-Haupthistokompatibilitätskomplexen (MHC) erkennt, werden, um Autoimmunität zu verhindern, meistens durch die negative Selektion im Thymus eliminiert. Da Tumorantigene häufig überexprimierte Selbst-Antigene sind, sind TZRs die im Thymus selektioniert worden sind weitgehend von niedriger Affinität. Alternativ können hoch-avide T-Zellen für Selbst-Antigene in einem nicht-toleranten allogenen MHC-reaktiven T-Zellrepertoire gefunden werden, da diese im Thymus nicht für das Zielpeptid auf dem allogenen MHC selektioniert wurden. Daher können diese T-Zellen als Quelle für hoch-affine TZR dienen, um von Patienten stammende Lymphozyten mit Rezeptoren auszustatten, die die gewünschte Antitumorspezifität vermitteln.

Da frühere Studien bestätigt haben, dass T-Zellen mit hoher Affinität für verschiedene Tumorantigene aus dem allogenen MHC-reaktiven T-Zellrepertoire unter Verwendung von in vitro De-novo-Induktionsverfahren isoliert werden können, wurde in diesem Projekt der allogene De-novo-Induktionsansatz, unter Verwendung von NY-ESO-1 als Zielantigen und des humanen Leukozytenantigen (HLA)-A2 als allogenem Restriktionselement, durchgeführt. Beide Moleküle wurden als in vitro transkribierte (ivt)RNA in von Monozyten-stammende dendritische Zellen (DZ) eingebracht, welche dann als Antigen-präsentierende Zellen (APZ) zur Stimulation und Expansion von autologen CD8-angereicherten T-Zellen verwendet wurden um NY-ESO-1-spezifische T-Zellen zu isolieren. Die Innovationen des Ansatzes waren: i) die Verwendung der vollständigen kodierenden Region des Antigens, um die Präsentation von Epitopen zu ermöglichen die nicht durch Programme zur Vorhersage der MHC-Bindung beeinflusst sind; ii) ein einzigartiger Selektionsprozess der die Identifizierung von T-Zellen ermöglicht, die für unbekannte immunogene Epitope von NY-ESO-1 spezifisch sind. Dies wurde ermöglicht indem modifizierte K562-Zellen zur Stimulation verwendet wurden, während der Aktivierungs-induzierte T-Zell-Marker CD137 auf T-Zellen nachgewiesen wurde. Unter den isolierten T-Zellklonen zeigte der Klon 5-271 die gewünschte Spezifität und wurde weiter analysiert. Die TZR 5-271-Sequenz wurde in

Vektoren kloniert, um eine transgene Expression in Rezipienten-T-Zellen zu ermöglichen. Das erkannte Epitop der isolierten TZRs wurde identifiziert und fein typisiert. Interessanterweise war der TZR spezifisch für ein Peptid, das von einem alternativen offenen Leserahmen (ORF) von NY-ESO-1 kodiert und auf HLA-A2-Molekülen präsentiert wurde. Um das Potential für eine therapeutische Verwendung des isolierten TZR zu bewerten, wurden T-Zellen, die den transgenen TZR exprimieren, auf Tumorzellerkennung und -eliminierung, Peptidsensitivität und CD8-Abhängigkeit getestet. TCR 5-271-transgene T-Zellen erkannten die getesteten Tumorzelllinien nur nach Zielantigen-Beladung. Außerdem wurde eine geringe Peptidempfindlichkeit und Reaktivität beobachtet, obwohl der TCR in der Lage war CD8-unabhängig zu agieren.

1 Introduction

1.1 Tumor immunosurveillance and immunoediting

Tumor development is a multistep process including the accumulation of genetic changes that endow normal somatic cells with unlimited growth capacities and render such newly transformed cells resistant to natural cell death mechanisms [1]. In the last decades, several observations have demonstrated the critical role of the immune system in suppressing tumor growth. Firstly, it has been observed that immunosuppressed patients show an increased risk for developing tumors [2], [3]. Moreover, evidence of spontaneous tumor regression as well as correlations between the presence of lymphocytes in the tumor environment and improved prognosis have been observed for different types of tumors [4]–[6]. Tumor cells can be identified and destroyed by the immune system before they can cause detectable tumor masses. The immune system can therefore prevent tumor progression (immunosurveillance). However, tumors also develop in the presence of a functional immune system. Experiments in mice have shown that tumors derived from immunodeficient mice are more immunogenic than tumors derived from immunocompetent mice [7]. These findings have led to the hypothesis that the immune system not only controls tumor formation by destroying tumor cells, but also influences tumor immunogenicity. This dual role of the immune system is named tumor immunoediting and comprises three phases: elimination, equilibrium and escape [8]. These three phases occur in a sequential order, however in some cases tumor cells can either naturally or influenced by external factors, enter in one of the last two phases without passing through earlier phases. The elimination phase encompasses the original concept of immunosurveillance. Here, innate and adaptive immune systems work together to identify and destroy transformed cells escaping intrinsic controls before the growing tumor becomes clinically observable. Progression to subsequent phases is prevented if the developing tumor is successfully deleted at this stage. In the case of partial tumor elimination, the residual tumor cells can enter in the equilibrium phase during which the immune system prevents tumor outgrowth and shapes the tumor immunogenicity. Tumor cells can remain in this dormancy phase for the entire life, without leading to clinical manifestations. On the other hand, as a consequence of the intrinsic genetic instability of tumor cells and of the sustained immune selection pressure during the equilibrium phase, tumor cells can bypass immune recognition by being poorly immunogenic and can emerge as growing tumors, entering into the escape phase [9]. Different mechanisms can lead to tumor cell escape and consequently to visible tumor growth. Tumor cells can acquire certain characteristics to

either reduce their immune recognition or to increase their resistance to immune effector functions. Loss of immunogenic epitope expression, defects in antigen processing mechanisms, down-regulation of major histocompatibility complex (MHC) molecules, lack of co-stimulation and induction of anti-apoptotic mechanisms are common strategies developed by tumor cells to evade immune responses. Alternatively, the immune system can be impaired by increased tumor-induced immunosuppression within the tumor microenvironment, i.e. production of immunosuppressive cytokines or recruitment of regulatory immune cells that function as immune suppressor cells [10], [11]. The broadened knowledge of the immune system's role in tumor development and the tumor immune escape mechanisms has supported the idea of exploiting the immune system to treat established tumors as an attractive alternative strategy to improve the conventional cancer therapies. Several immunotherapeutic approaches that aim to enhance the effector functions of the immune system and to counteract tumor-induced immunosuppressive mechanisms have been developed in the last years.

1.2 Cancer immunotherapy

Designated "Top Scientific Breakthrough" by Science magazine in 2013, cancer immunotherapy is recently gaining considerable momentum [12]. Immunotherapy has revolutionized the field of conventional chemotherapy and radiation therapy by using the immune system to destroy tumors instead of directly attacking the tumor itself.

First evidence that the immune system can eradicate tumors has been observed in studies of bone marrow transplantation, along with donor-derived lymphocyte infusion (DLI) from healthy donors to patients with hematological malignancies [13]. However, the transfer of allogeneic heterogeneous T cells has been associated frequently with severe adverse effects, designated as graft-versus-host disease (GVHD) [14]. To bypass this safety issue, approaches exploiting autologous T cells have been investigated. The first evidence that autologous T cells can mediate tumor eradication was reported in 1988 by the adoptive transfer of *ex vivo* expanded autologous tumor-infiltrating lymphocytes (TILs) in patients with metastatic melanoma [15]. Nevertheless, only limited responses and short-term persistence of the transferred T cells were observed in treated patients. A critical improvement in TIL therapy was achieved in 2002 showing that a lymphodepleting preparative regimen prior to TIL infusion was able to increase tumor regression as well as the persistence of the transferred T cells [16]. Starting from this finding, autologous TIL therapy has been used successfully to treat patients with metastatic melanoma [17], [18]. Nowadays, the clinical effects observed in advanced melanoma with TIL therapy are mainly

attributed to the presence of T cells recognizing mutated antigens in the transferred T cell populations [19], [20]. Despite encouraging results in melanoma patients, the principal limitation of this approach for broad application is the poor immunogenicity of tumors that impedes the isolation of TILs for many patients and for several tumor types.

To overcome the need for tumor-reactive T cells in tumor samples, multiple therapeutic tumor vaccines have been developed to induce T cells specific for different tumor antigens starting from the autologous T cell repertoire [21], [22]. However, the majority of these vaccinations failed to induce complete remission in patients with advanced tumors. These results might be due to large tumor burdens of vaccinated patients, tumor-mediated immunosuppressive environments, development of tumor escape variants and compromised immune systems of the patients after systemic chemotherapy administration. Moreover, as the majority of vaccines were directed against self-antigens aberrantly expressed in tumors, immunotolerance might have interfered with the induction of effective T cell responses.

An alternative approach that has been developed in the last years encompasses monoclonal antibodies designed for blocking checkpoint molecules involved in inhibitory pathways induced by tumor cells to evade immune controls [23]. The final aim of these checkpoint inhibitors is to favor and restore pre-existing anti-tumor immune responses. Advances in the identification of the molecules involved in immune-suppressive pathways have opened the way to the development of multiple checkpoint inhibitors. The inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on T cells and involved in the down-regulation of the initial stages of T cell activation has been investigated as the first target of checkpoint inhibitors [24]. According to the encouraging prolongation of overall survival observed in the first clinical trials, the anti-CTLA-4 antibody was approved by the US Food and Drug Administration (FDA) in 2011 as therapy for melanoma patients with metastatic disease. Subsequently, antibodies targeting the inhibitory interaction of programmed cell death protein-1 (PD-1) and programmed cell death protein-1 ligand (PD-L1) have been developed and tested in clinical studies with promising and durable clinical responses in several types of solid tumors [25]–[28]. Recently, these antibodies have also received the approval by the FDA as therapy for melanoma, non-small cell lung cancer and renal cell carcinoma. As hypothesized for TIL therapy, the clinical effects observed after treatment with checkpoint inhibitors are supposed to be mainly due to the presence of pre-existing T cells targeting mutated antigens in treated patients [29]–[31]. A combination of either different checkpoint inhibitors or of checkpoint inhibitor with other immunotherapeutic strategies might improve the curative potential of these checkpoint inhibitors and enlarge their range of applications [32].

Since tumor antigens are often over-expressed self-antigens, the clinical efficacy of immunotherapeutic strategies described so far is strongly limited by the tolerance of the autologous immune system to the most relevant tumor antigens. The adoptive transfer of genetically engineered tumor-specific T cells has been developed as an alternative strategy to bypass immunotolerance [33], [34]. This approach provides the possibility to equip autologous T cells with receptors mediating the desired anti-tumor specificity and showing enhanced functionality before transferring them into patients. The final aim is to enable the redirected T cells to effectively recognize and destroy tumors. To note, T cell gene engineering offers the possibility to create T cells with the desired anti-tumor specificity and enhanced functionality by selecting not only the optimal receptor according to specific properties, but also the composition of the genetically modified T cell population that is transferred into patients. T cells undergo progressive differentiation after encountering the antigen and their differentiation state inversely correlates with the capacity of proliferation and persistence [35]. Several clinical studies have shown that the therapeutic efficacy of adoptively transferred T cells is strictly dependent on their ability to expand and persist *in vivo* [18], [36], [37]. In pre-clinical models, it has been observed that the infusion of T cell populations comprising less differentiated cells is characterized by enhanced proliferative capacity, long-term persistence and superior anti-tumor responses [38], [39]. These observations suggest that the composition of the T cell population might drastically influence the success of adoptive T cell therapy. Therefore, the possibility to orchestrate and optimize the composition of the genetically engineered T cell population for the infusion represents an additional advantage of this therapeutic strategy for ensuring maximal clinical efficacy. Once the optimal T cell population for adoptive therapies is selected, its specificity can be re-directed by transferring either a chimeric antigen receptor (CAR) or a conventional αβ T cell receptor (TCR) into the cells.

CARs are recombinant proteins comprising an extracellular target binding module that is usually a single-chain antibody fragment, a transmembrane domain to anchor the receptor into the cell membrane and one or more intracellular TCR-derived signaling domains that transmit activation signals into T cells [40]. As these synthetic receptors show antibody-like specificities, they can recognize surface molecules expressed on target cells in a MHC-independent manner. Consequently, CARs are not affected by immune-escape strategies developed by tumor cells such as down-regulation of MHC molecules or alterations in processing mechanisms. On the other hand, loss of target antigen expression remains a possible mechanism arising in the tumors to evade CAR-mediated T cell responses. Recently, the adoptive transfer of T cells genetically modified with a CAR targeting the B cell lineage restricted CD19 molecule has successfully induced long-lasting remission in patients with hematological malignancies [41]–[43]. Due to these promising results, this

protocol has recently received the approval by the FDA as therapy for the treatment of pediatric patients with acute lymphoblastic leukemia (ALL) and of adult patients with diffuse large B cell lymphoma (DLBCL). Targeting only cell surface molecules, CAR T cell therapy is limited by the number of suitable targets expressed on tumor cells and as a consequence the range of applications is quite restricted. Furthermore, T cells expressing CARs have not shown so far the same encouraging results in solid tumors as in hematological malignancies [44]–[46]. This might be mainly due to the highly immunosuppressive environment surrounding solid tumors.

In contrast, TCRs are heterodimeric protein complexes composed of one α chain and one β chain. The TCR heterodimers are expressed on the cell surface of T cells in association with the CD3 complex, which is comprised of a series of non-polymorphic proteins that serve as the signaling apparatus of the TCR. On the molecular level, TCRα and TCRβ chains are assembled randomly from a multitude of germline encoded gene segments during early T cell development in the thymus. In the process of gene segment rearrangement, the TCRα chain is assembled from one polymorphic variable (TRAV) and joining (TRAJ) gene segment in combination with the monomorphic constant region (TRAC). The recombined part of the TCRβ chain consists of one polymorphic variable (TRBV) and joining (TRBJ) gene segment with an additionally interspersed diversifying (TRBD) gene segment and one of the two constant region (TRBC). During the process of gene segment rearrangement, germline encoded sequences adjacent to the joining regions get modified incidentally. The resulting hypervariable sequences that span the individual V(D)J segment junctions are known as complementarity determining region (CDR) 3. CDR3 regions determine the center of the antigen-binding site of the TCR. The peripheral antigen-binding sites of the TCR, which are mainly in contact with the MHC molecules, consist of CDR1 and CDR2 loops that are encoded within TRAV and TRBV gene segments. More precisely, TCRs recognize peptides derived from intracellular processed proteins that are presented on the surface of antigen presenting cells (APCs) in complex with MHC molecules. In particular, TCRs of $CD8^+$ T cells recognize peptides derived from endogenous (cytosolic) proteins that are presented on MHC class I molecules, whereas TCRs of $CD4^+$ T cells recognize peptides derived from exogenous antigens that have been taken-up by APCs and that are presented on MHC class II molecules. Although with less efficiency, exogenous antigens can be alternatively presented to $CD8^+$ T cells on MHC class I molecules by physiological cross-presentation mechanisms [47]. Importantly, to avoid autoimmunity, T cells expressing TCRs that recognize self-peptides presented on self-MHC molecules with high-affinities leading to the generation of potential auto-reactive T cells are deleted during T cell development in the thymus by negative selection. The resulting self-tolerant MHC-restricted T cell repertoire comprises a wide range of T cells able to react to

foreign antigens, while it usually lacks high-avidity T cells specific for self-antigens [48], [49]. Unlike CAR-based approaches, the transfer of therapeutic TCRs into T cells allows the targeting of any antigen that undergoes intracellular processing and that is presented on MHC molecules of the desired target cells. In the tumor microenvironment, stroma cells can take-up protein fragments released from dying tumor cells and present tumor epitopes on their MHC molecules. Consequently, stroma cells may become susceptible to TCR-mediated recognition. The targeting of tumor embedded cells may represent an important advantage of TCR-based strategies for successful therapy of solid tumors compared to CAR-based therapy [50].

In the last years, several clinical studies have been conducted using TCR-modified T cells for the treatment of several tumor types. In the first published clinical trial, T cells expressing a transgenic TCR targeting the melanoma antigen recognized by T cells (MART-1) were applied to melanoma patients. The therapeutic TCR was isolated from a TIL clone derived from a melanoma patient who showed a nearly complete regression after TIL therapy. However, only modest clinical responses were observed in the treated patients [37]. In a subsequent trial, a TCR specific for MART-1 derived from TILs of the same melanoma patient but showing higher affinity compared to the first TCR tested, was transferred in recipient cells and used to treat advanced melanoma. Although tumor regression was obtained in 30% of the patients treated, on-target/off-tumor toxicity was observed against healthy tissues comprising melanocytes as skin, eyes and ears [51]. Unexpected severe side effects have been reported also in two other studies targeting the MAGE family member A3 (MAGE-A3) by using T cells transduced with patient-derived and affinity-enhanced TCRs. In these cases, the observed toxicity against healthy tissues was due to previously unknown cross-reactivity of the introduced TCRs which cross-recognized self-peptides highly homologous to the actual target epitope (off-target/off-tumor toxicity) [52]–[54]. On the other hand, an affinity-enhanced TCR targeting the cancer/testis antigen NY-ESO-1 have shown promising results in terms of safety and efficacy for the treatment of melanoma, synovial cell sarcoma and myeloma. Clinical responses without any toxicity were observed in 55%, 61% and 80% of the treated patients, respectively [55]–[58]. These studies suggest the powerful curative potential of the adoptive transfer of TCR-engineered T cells. However, the observation of severe side effects has shown the need of further improvements to reduce potential toxicity and to enhance the therapeutic efficacy of TCR-based approaches. The rigorous selection of suitable target antigen as well as of corresponding therapeutic TCRs are essential prerequisites for developing successful TCR gene therapies with minimal toxicity and maximal efficacy.

1.3 Choice of target antigens for TCR-based cancer therapies

Thorough evaluation of the expression profile of the potential target antigen is the first element to be considered for developing TCR-based cancer therapies. An optimal target antigen should be expressed only in tumor cells or its expression in normal tissues should ideally be limited to immune-privileged organs to avoid undesired on-target/off-tumor toxicity reactions. To obtain complete tumor eradication, the target antigen should be present in all tumor cells or at least in the cells that drive tumor outgrowth. To avoid the risk of antigen-loss escape variants and consequent ineffective tumor destruction, a target antigen functionally relevant for tumor survival should be preferably selected. Furthermore, the target antigen should be expressed in a large group of patients and be presented on frequent human leukocyte antigen (HLA) molecules to allow the development of therapies suitable for a broad range of patients. As only very few antigens fulfill all these criteria, the identification of appropriate targets for TCR-based therapies is quite challenging. Nevertheless, potential target antigens for different tumor indications have been identified by evaluating the multitude of different intracellular proteins expressed in tumor cells. Generally, tumor antigens can be classified into three categories: viral oncogenes, tumor-specific antigens and tumor-associated antigens [59], [60]. Tumor antigens produced by oncogenic viruses are ideal antigens for targeting virus-transformed tumor cells, since the expression of viral proteins is restricted to infected cells. However, only 15% of tumors are estimated to be associated with viral infections [61]. The second class of tumor antigens, the so-called tumor-specific antigens (TSAs), comprises tumor aberrations leading to the formation of mutated gene products (neo-antigens) [62]–[65] . Since neo-antigens are often essential for tumor cell survival and accumulate in tumor cells, they are quite interesting targets for TCR gene therapy. However, mutations and corresponding neo-antigens are mainly patient-specific, thereby their use as targets require highly personalized therapeutic approaches. To date, most of the TCR gene therapy approaches focus on tumor-associated antigens (TAAs) [62], [66]. The heterogeneous group of TAAs encompasses cancer/testis antigens (CTAs) (e.g. MAGE-A3 and NY-ESO-1), differentiation antigens (e.g. MART- 1; tyrosinase, melanocyte antigen; CD20, B lymphocyte antigen) and self-proteins over-expressed in tumor cells (e.g. p53, transcription factor; EGFR, epidermal growth factor receptor). One advantage of TAAs is their expression in different types of tumors and consequently their broad range of applications. However, the potential expression of these target antigens in healthy tissue includes the risk of on-target/off-tumor toxicity through unwanted TCR-mediated recognition.

Independently of the category of the tumor antigen, once a tumor antigen-specific TCR is isolated, the specific peptide recognized by the therapeutic TCR needs to be identified and

fine-typed. Although the target antigen shows the desired expression profile restricted to the tumor, alternative peptides highly homologous to the target epitope might be present in the proteome and expressed in healthy tissues. Thus, potential TCR-mediated cross-recognition of these alternative peptides need to be carefully investigated to predict undesired off-target/off-tumor toxicity [67], [68].

1.4 Isolation of tumor antigen-specific T cells and corresponding TCRs

Required characteristics of a therapeutic TCR are efficient tumor cell recognition and optimal affinity for the target tumor antigen in order to be able to discriminate between tumor cells and normal tissues. To date, multiple strategies have been established for the isolation of tumor antigen-specific T cells and corresponding therapeutic TCRs for subsequent genetic transfer into adequate recipient cells.

Therapeutic TCRs can be isolated from *ex vivo* expanded tumor-reactive T cell clones derived from TILs of tumor patients [37], [69], [70]. However, tumors are poorly immunogenic and consequently the frequency of tumor-reactive T cells is very low. The isolation methods using patient material are often hampered by poor cell viability as a result of immune depleting treatments or systemic impacts of advanced disease. Additionally, due to the negative selection in the thymus, TCRs recognizing self-antigens with high-affinity are rarely included in the autologous TCR repertoire. Since the most relevant tumor antigen are over-expressed self-antigen, the isolation of suitable therapeutic TCRs recognizing TAAs from autologous T cell repertoires is extremely laborious and often leads to the identification of TCRs with only sub-optimal efficacy. To improve the clinical efficacy of these autologous TCRs, strategies to increase TCR affinity have been developed and applied to several TCRs [71]–[74]. These systems are powerful and attractive methods to convert low-affinity TCRs derived from an immunotolerant repertoire into high-affinity TCRs able to efficiently recognize tumor cells. However, reduced specificity and increased cross-recognition leading to dramatic side effects have been observed in two clinical trials using affinity-enhanced TCRs targeting MAGE-A3 [52]–[54]. These findings show the need for additional pre-clinical approaches to extensively characterize therapeutic TCRs in terms of potential toxicity prior to clinical applications.

An alternative TCR isolation strategy is based on *in vivo* priming in mice. Since the murine T cell repertoire is not affected by natural negative selection against human antigens, human MHC-transgenic mice can be exploited to isolate murine-derived therapeutic TCRs. These transgenic mice vaccinated with peptides derived from human tumor antigens

represent a source of murine T cells bearing high-affinity TCRs for human peptides presented on human MHC molecules [75]. However, the use of tumor antigen-specific murine TCRs in the clinic is problematic, due to their high degree of immunogenicity in the xenogeneic human host [76]. To overcome this limitation, human transgenic mice carrying the complete human T cell repertoire as well as one human MHC class I allele (HLA-A*02:01) have been generated [77]. In contrast, these mice are deficient in murine TCRαβ as well as in murine MHC class I expression. This *in vivo* system enables the isolation of fully human TCRs from a T cell repertoire unselected for human antigens but restricted to HLA-A*02:01. To date, TCRs specific for different tumor antigens have been successfully isolated using these transgenic mice [77], [78]. However, these mice only expressing HLA-A*02:01, thereby they are suitable only for the isolation of TCRs restricted to this MHC class I allele. The applicability of this *in vivo* system is also limited by the potential presence of peptides highly homologous to the actual human target epitope in the murine peptidome.
Alternatively, as negative selection in the thymus is limited to self-peptides presented on self-MHC molecules, high-avidity T cells recognizing TAAs can be found in the non-tolerant allogeneic MHC (allo)-reactive T cell repertoire that have not encountered the target peptide and the MHC molecules during thymic selection. Therefore, high-avidity T cells recognizing peptides derived from any TAA can be potentially isolated if these peptides are presented on allogeneic MHC molecules [79]. Multiple protocols for *in vitro* induction of allogeneic MHC (allo)-restricted tumor antigen-specific T cells have been developed in the last years using cells derived from healthy donors as starting material (approaches designated as allogeneic priming approaches) [80]–[83]

1.5 Aim of the project

Adoptive transfer of T cells expressing transgenic tumor antigen-specific TCRs is a promising therapy for patients with advanced tumors. Nevertheless, there is an important need to improve efficiency and safety aiming to enhance the clinical benefit for more patients. Additional therapeutic TCRs, able to efficiently recognize tumor target cells without showing undesired cross-recognition and consequently potential toxicity, need to be identified and extensively characterized. Therefore, the aim of this project was to isolate T cells and corresponding TCRs specific for new epitopes derived from the tumor antigen NY-ESO-1 and presented in the context of HLA-A2 molecules by using an *in vitro* allogeneic priming approach.
Previous studies have shown that *in vitro* allogeneic priming approaches enabling the access to the non-tolerant high-affinity allo-restricted T cell repertoire can be used as

efficient procedures to induce functional enhanced TCRs specific for different TAAs [80]–[83]. Our research group established a protocol to isolate allo-restricted tumor antigen-specific T cells using mDCs derived from healthy donors negative for the MHC molecule of interest as APCs [84]. Briefly, mDCs are transfected with *in vitro* transcribed RNA (*ivt*RNA) encoding the foreign MHC molecule together with *ivt*RNA encoding the full-length target antigen and used for stimulation and expansion of autologous T cells. This method is highly flexible since mDCs and T cells can be derived from any healthy donor who does not carry the MHC molecule selected as the allogeneic molecule, any known tumor antigen can be used as target and any allogeneic MHC molecule can be introduced into mDCs. Moreover, the use of *ivt*RNA encoding the full-length tumor antigen allows the potential isolation of T cells specific for any immunogenic peptides derived from the antigen of interest. Due to these advantages, this allogeneic priming protocol was selected for the isolation of NY-ESO-1-specific HLA-A2-restricted T cells in this study.

The HLA-A*02:01 allele was chosen as the restriction element since it is the most frequent MHC class I allele in Caucasian populations and it has already been used for *in vitro* primings to efficiently isolate T cells specific for different TAAs [85]. NY-ESO-1 was selected as the target antigen because it is one of the most promising and immunogenic CTA [86]–[88]. Indeed, NY-ESO-1 is expressed in multiple tumor types like melanoma, synovial sarcoma, lung, esophageal, liver, gastric, prostate and ovarian tumors as well as adult T cell leukemia/lymphoma (ATLL), but it is not detectable in any healthy tissues except for testis. Moreover, natural humoral and cellular immune responses against NY-ESO-1 have been demonstrated in tumor patients [89], [90]. Due to these characteristics, NY-ESO-1 has already been used as the target antigen in different immunotherapeutic strategies. Therapeutic vaccines have proved that NY-ESO-1 is safe for patients and also immunogenic, leading to both $CD4^+$ and $CD8^+$ T cell responses [91]. The adoptive transfer of T cells genetically engineered with an affinity-enhanced HLA-A2-restricted NY-ESO-1-specific TCR in melanoma, synovial cell sarcoma and myeloma patients has shown promising results in terms of safety and efficacy [55]–[58]. To date, several epitopes presented on different MHC class I and II molecules and recognized by $CD8^+$ and $CD4^+$ T cells have been described for NY-ESO-1 [87]. Nevertheless, the peptide NY-ESO-$1_{157-165}$ is the only immunogenic epitope in the context of HLA-A2 molecules identified so far.

Importantly, to enable functional characterization as well as the isolation of individual TCR sequences after a priming procedure, the desired T cells need to be selectively separated as single-cell and expanded. To date, the most common strategy used for this purpose is based on pre-assembled peptide-loaded MHC molecules (multimers) [92]–[94]. With the knowledge of immunogenic epitopes and corresponding MHC alleles, T cells with the defined peptide specificity can be efficiently detected using multimers. However, certain

criteria limit the unrestricted use of multimer-based techniques. First, if presented epitopes of relevant candidate antigens are unknown, peptides for multimer production need to be selected by using epitope prediction tools. The complexity of the multi-step process leading to the formation of the peptide:MHC complex and the limited data regarding MHC-bound peptides, as well as the limited number of experimentally confirmed immunogenic epitopes, make the epitope prediction by algorithms challenging and often unreliable [95], [96]. Currently available software algorithms can fail to accurately predict epitopes that are naturally processed and presented on tumor cells and that can elicit T cell responses. Hence, multimers binding peptides that are not physiologically relevant in tumor cells could be generated leading to unsuccessful isolation of the desired T cells. Second, the availability of multimers is limited by insufficient peptide:MHC complex stability and by sequence-dependent difficulties in MHC protein folding for some HLA molecules. To overcome these limitations, alternative sorting methods have been established that are based on the analysis of e.g. cytokine secretion or surface molecule expression by T cells upon stimulation with APCs carrying the appropriate MHC restriction and target antigen [97]–[101]. Unlike the multimer-based approach, these methods allow the isolation of antigen-specific T cells irrespective of pre-defined specificity. Thus, they represent a powerful system to enable identification of T cells specific for previously unknown immunogenic peptides for a selected target antigen. However, the applicability of these alternative strategies in the context of an allogeneic priming approach is hampered by the high number of allo-restricted T cells that are reactive independently of the presence of the desired tumor target antigen within the priming culture. These unwanted allo-reactive T cells are indistinguishable from the desired tumor-reactive T cells using sorting methods based on either the expression of T cell activation markers or on cytokine release by T cells, limiting the efficiency of these sorting procedures. Improvements are needed to increase the success and the efficiency of these alternative sorting strategies. Thus, an additional aim of this project was to develop an innovative sorting strategy based on the T cell activation-induced marker CD137 in order to allow the isolation of the desired T cells irrespective of pre-defined epitopes also using allogeneic priming approaches.

2 Material

2.1 Equipment

Table 1. Equipment

Product	Source
Balance	Sartorius
C-Chip Neubauer Improved	Carl Roth
Cell Analyzer LSRFortessaTM	BD Bioscience
Cell Sorter FACSAria FusionTM	BD Bioscience
Centrifuge 1-16K	Sigma-Aldrich
Centrifuge Heraeus X3R	Thermo Fisher Scientific
Chemical hood MC6®	Waldner
ChemiDoc™ XRS+ System	Bio-Rad
CoolCell® Cell Freezing Containers	Biocision
Cooling incubator with shaker (bacteria)	Tritec
Electrophoresis chamber and power supply	Bio-Rad
Electroporator Gene Pulser Xcell™	Bio-Rad
Elix® water purification system	Merck
Freezer (-150°C, -80°C)	Panasonic
Freezer (-20°C)	Liebherr
Fridge (4°C)	Liebherr
Ice machine Scotsman AF103 Ice Flaker	Hubbard Systems
Incubator (human cells)	Thermo Fisher Scientific
IncuCyte ZOOM® device	Essen BioScience
Integral water purification system Milli-Q®	Merck
Irradiation device (Xstrahl RS225)	Xstrahl
MACS separator	Miltenyi Biotec
Magnet for cell separation applications	Thermo Fisher Scientific
Micropipettes	Eppendorf
Microscope Primovert	Zeiss
Microwave	Bosch
Multichannel pipettes	Thermo Fisher Scientific
Multiskan™ FC Microplate Photometer	Thermo Fisher Scientific

Product	Source
NanoDrop 2000	Thermo Fisher Scientific
Pipettors	Integra
Sterile laminar flow hood	Thermo Fisher Scientific
Thermocycler	Eppendorf
Thermomixer	Eppendorf
UV-transluminator	Bachofer
Vortex	Scientific Industries
Water bath	VWR

2.2 Consumables

Table 2. Consumables

Product	Source
Conical tubes (15 ml, 50 ml, 200 ml)	BD Falcon Corning
Cryovials (1.8 ml)	Thermo Fisher Scientific
Electroporation cuvettes (0.4 cm, 0.1 cm)	Bio-Rad
Filter (0.2 µm, 0.45 µm)	BD Falcon
LS Column	Miltenyi Biotec
Needles Surflo® winged infusion sets	BD Falcon Termuno
Non-tissue culture plates (6-well, 24-well)	Thermo Fisher Scientific
Nunc-Immuno™ 96 Well Plate	Thermo Fisher Scientific
Nunclon™ Delta flasks	Thermo Fisher Scientific
Parafilm®	Pechiney Plastic Packaging
Pasteur pipettes	Copan
Petri dishes	Thermo Fisher Scientific
Pipettes (5 ml, 10 ml, 25 ml, 50 ml)	Sigma-Aldrich
Round bottom tubes (5 ml)	BD Falcon Corning
Snap-cap tubes (14 ml)	BD Falcon
Syringes (1 ml, 10 ml, 50 ml)	BD Falcon
TipOne® Filter Tips (1-10 µl, 10 -200 µl, 200-1000 µl)	Star Lab
Tips without filter (1-10 µl, 10 -200 µl, 200-1000 µl)	Thermo Fisher Scientific
Tissue culture flasks	CellStar
TPP® tissue culture plates	Sigma-Aldrich
TPP® unit filter (250 ml, 500 ml)	Sigma-Aldrich

Product	Source
Tube with Cell Strainer Cap	BD Falcon Corning
Tubes (0.5 ml, 1.5 ml, 2 ml, 5 ml)	Eppendorf
Tubes with filter blue cap	BD Falcon Corning

2.3 Enzyme, cytokines, reagents

Table 3. Restriction enzymes

Product	Source
AgeI-HF®	New England BioLabs
EcoRI-HF®	New England BioLabs
NotI-HF®	New England BioLabs
SpeI-HF®	New England BioLabs
XbaI	New England BioLabs

HF®: high-fidelity restriction enzyme

Table 4. Cytokines and TLR-ligands

Product	Source
GM-CSF, human	Berlex
Interferon-γ (IFN-γ), human	Boehringer Ingelheim
Interleukin-15 (IL-15), human	PeproTech
Interleukin-1β (IL-1β), human	R&D Systems
Interleukin-2 (IL-2), human	Novartis
Interleukin-4 (IL-4), human	R&D Systems
Interleukin-6 (IL-6), human	R&D Systems
Interleukin-7 (IL-7), human	PeproTech
Prostaglandin E2 (PGE2) , human	Sigma-Aldrich
Tumor necrosis factor-α (TNF-α), human	R&D Systems

Table 5. Enzymes and reagents

Product	Source
100 bp DNA Ladder	Thermo Fischer Scientific
1kb-plus DNA Ladder	Thermo Fischer Scientific
2-Mercaptoethanol	Thermo Fischer Scientific
2-Propanol	Sigma-Aldrich
Agarose Ultra Pure	Thermo Fischer Scientific
Ampicillin	Merck
Bovine serum albumin (BSA)	Sigma-Aldrich
Cyclosporin A (CSA)	Sigma-Aldrich
CutSmart Buffer®	New England BioLabs
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich
Dimethylsulfoxide (DMSO)	Merck
DNA gel loading buffer	Thermo Fischer Scientific
DNA primers	Sigma-Aldrich
Dulbecco's phosphate-buffered saline (DPBS) 1X without calcium and magnesium	Thermo Fischer Scientific
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fischer Scientific
EDTA (0,5 M)	Thermo Fischer Scientific
Ethanol	Merck
Ethidium bromide 0.025%	Carl Roth
FACS™ Flow and Rinse	BD Bioscience
FcR Blocking Reagent, human	Miltenyi Biotec
Fetal Bovine Serum (FBS)	Thermo Fischer Scientific
Ficoll®	Biochrom
Glucose 40%	Braun
Glycerol	Merck
Heparin-Natrium	Braun
HEPES (1M)	Thermo Fischer Scientific
Human serum (HS)	Medigene Immunotherpies GmbH
Human serum albumin (HSA)	Baxalta
IncuCyte® Annexin V Red Reagent	Essen Bioscience
IncuCyte® NucLight Red Lentivirus Reagent	Essen Bioscience
Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fischer Scientific
L-Glutamine (200 nM)	Thermo Fischer Scientific

Product	Source
Luria Broth Base (Miller's LB Broth Base) powder	Thermo Fischer Scientific
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA)	Thermo Fischer Scientific
OKT-3 (anti-CD3 antibody)	Helmholtz Zentrum München
OptEIA™ TMB Substrate	BD bioscience
Paraformaldehyde solution (PFA) 4%	Thermo Fischer Scientific
PBS very low endotoxin (VLE) 1X without calcium and magnesium	Biochrom
Penicillin/Streptomycin	Thermo Fischer Scientific
Phosphoric acid 85%	Merck
Phytohaemagglutinin (PHA)	Remel
Polybrene	Sigma-Aldrich
Puromycin	Invivogen
Retronectin®	Takara
RiboRuler™ High Range RNA Ladder	Thermo Fischer Scientific
RPMI 1640	Thermo Fischer Scientific
RPMI 1640 very low endotoxin (VLE)	Biochrom
Select Agar	Thermo Fischer Scientific
Skim milk powder	Sigma-Aldrich
SOC Medium	Thermo Fischer Scientific
Sodium acetate ($C_2H_3NaO_2$)	Thermo Fischer Scientific
Sodium bicarbonate ($NaHCO_3$)	Merck
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich
Sodium pyruvtae	Thermo Fischer Scientific
T4 DNA Ligase	New England BioLabs
T4 DNA Ligase Reaction Buffer	New England BioLabs
TAE 50X buffer (Tris Acetate EDTA)	Thermo Fischer Scientific
TransIT®-LT1 Transfection Reagent	Mirus
TRI-Reagent®	Sigma-Aldrich
Trypan blue solution 0.4%	Thermo Fischer Scientific
Trypsin-EDTA 0,05%	Thermo Fischer Scientific
Tween® 20	Sigma-Aldrich

2.4 Commercial Analytic systems

Table 6. Commercial Analytic systems

Product	Source
Advantage® 2 PCR Kit	Clontech
$CD8^+$ T cell Isolation Kit	Miltenyi Biotec
Dynabeads® Human T-Activator CD3/CD28	Thermo Fischer Scientific
JETstar Plasmid Purification Kit (Maxi and Mini)	Genomed
mMESSAGE mMACHINE™ T7 Kit	Thermo Fischer Scientific
OptEIA™ Human IFN-γ ELISA Set	BD Biosciences
QIAquick® Gel Extraction Kit	Qiagen
RNeasy Mini Kit	Qiagen
SMARTer™ RACE cDNA Amplification Kit	Clontech
TMB Substrate Reagent Set	BD Bioscience
UltraComp eBeads®	eBioscience

2.5 Cell culture media

Table 7. Cell culture media

Medium	Components	Concentration
DC medium	VLE RPMI 1640	
	Human serum	1.5%
DMEM IV medium	DMEM	
	FBS	10%
	L-Glutamine	2 mM
	MEM NEAA	1 mM
	Sodium pyruvate	1 mM
IMDM IV 20 medium	IMDM	
	FBS	20%
	L-Glutamine	2 mM
	MEM NEAA	1 mM
	Sodium pyruvate	1 mM
LB agar medium	LB medium	
	Select agar	15 g/l
LB medium	dH_2O	
	Luria Broth Base	25 g/l

Medium	Components	Concentration
RPMI VI 20 medium	RPMI 1640	
	FBS	20%
	L-Glutamine	2 mM
	MEM NEAA	1 mM
	Sodium pyruvate	1 mM
RPMI VI medium*	RPMI 1640	
	FBS	10%
	L-Glutamine	2 mM
	MEM NEAA	1 mM
	Sodium pyruvate	1 mM
T cell medium*	RPMI 1640	
	Human serum	10%
	HEPES	10mM
	L-Glutamine	2 mM
	MEM NEAA	1 mM
	Sodium pyruvate	1 mM
	2-Mercaptoethanol	50 µM

* containing 1% Penicillin/Streptomycin when used for cell culture after sorting

2.6 Buffers and solutions

Table 8. Buffers and solutions

Buffer/solution	Components	Concentration
DEPC water	dH_2O	
	DEPC	0.1%
ELISA blocking buffer	DPBS 1X	
	Skim milk powder	1%
ELISA coating buffer	dH_2O	
	$NaHCO_3$	8.4 g/l
	Na_2CO_3	3.56 g/l
ELISA stop solution	dH_2O	93.2 ml
	Phosphoric acid 85%	6.8 ml
ELISA washing buffer	DPBS 1X	
	Tween20	0.05%

2.7 Cell lines, primary cells and bacteria

Table 9. Human cell lines

Name	Origin tissue	Characteristics	Culture conditions	Source
293FT	Embryonic kidney	Packaging cells transformed with the SV40 large T antigen	Adherent DMEM IV	Thermo Fisher Scientific
LAZ 388	EBV transformed B-cells	-	Suspension RMPI IV	Schendel D.
LCL BW	EBV transformed B-cells	HLA-A24-positive	Suspension RMPI IV	Schendel D.
LCL P8	EBV transformed B-cells	HLA-A2-positive HLA-A24-negative	Suspension RMPI IV	Longinotti G.
T2	Lymphoblast	Somatic cell hybrid TAP-deficient cells	Suspension RPMI IV	ATCC

Table 10. Human tumor cell lines

Name	Origin tissue	Disease	Culture conditions	Source/ Reference
AMO-1	Ascitic fluid	Plasmacytoma	Suspension RPMI IV 20	ATCC
EJM	Peritoneal fluid	Multiple myeloma	Adherent/ suspension IMDM IV 20	DSMZ
FM3	Skin	Melanoma	Adherent RPMI IV	Sigma-Aldrich
FM3.29	Skin	Melanoma	Adherent RPMI IV	Sigma-Aldrich
FM6	Skin	Melanoma	Adherent RPMI IV	Sigma-Aldrich
IM9	Peripheral blood/ B lymphoblast	Multiple myeloma	Suspension RPMI IV	ATCC
K562	Bone morrow	Chronic myelogenous leukemia	Suspension RPMI IV	ATCC *Lozzio and Lozzio, 1975*
KMS-12-BM	Bone marrow	Multiple myeloma	Suspension RPMI IV 20	ATCC
KMS-12-PE	Peripheral blood	Multiple myeloma	Suspension RPMI IV 20	ATCC

Name	Origin tissue	Disease	Culture conditions	Source/ Reference
Mel624.38	Skin	Melanoma	Adherent RPMI IV	M. Panelli NCI *Rivoltini et al., 1995*
MelA375	Skin	Malignant melanoma	Adherent RPMI IV	ATCC
MM127	Skin	Melanoma	Adherent RPMI IV	Sigma-Aldrich
MM415	Skin	Melanoma	Adherent RPMI IV	Sigma-Aldrich
MOLP-8	Peripheral blood	Multiple myeloma	Suspension RPMI IV 20	DSMZ
RPMI-8226	Peripheral blood/ B lymphocyte	Plasmacytoma/ myeloma	Suspension RPMI IV	ATCC
SAOS-2	Bone	Osteosarcoma	Suspension RPMI IV	ATCC
SK-Mel23	Skin	Melanoma	Adherent RPMI IV	Schendel D. Memorial Sloan Kettering Cancer Center
U266	B lymphocyte/ Peripheral blood	Myeloma/ Plasmocytoma	Suspension RPMI IV	ATCC

Table 11. Primary cells

Name	Characteristics	Culture conditions	Source
$CD8^+$ T cells	Derived from either PBMC or PBL of healthy donors by MACS enrichment	Suspension T cell medium	Longinotti G.
Clone 10/24	$CD8^+$ T cell clone HLA-A2-restricted HA-1^H-specific	Suspension T cell medium	Sommermeyer D.
Clone 234	$CD4^+$ T cell clone HLA-A24-reactive	Suspension T cell medium	Schendel D.
DCs	Derived from monocytes of a HLA-A2-negative healthy donor	Suspension DC Medium	Longinotti G.
PBL	Derived from PBMC of healthy donors by monocyte plate adherence	Suspension T cell medium	Longinotti G.
PBMC	Derived from human peripheral blood of healthy donors by density gradient centrifugation	Suspension T cell medium	Longinotti G.

Table 12. Bacteria

Name	Characteristics	Genotype	Source
NEB® Turbo Competent *E. coli*	Chemically Competent	*F' proA+B+ lacIq ΔlacZM15/ fhuA2 Δ(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5*	New England BioLabs
One Shot™ TOP10 *E. coli*	Chemically Competent	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG*	Thermo Fisher Scientific
XL1-blue *E.coli*	Electrocompetent	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIq ZΔM15 Tn10 (Tetr)*	Agilent Technologies

2.8 Plasmids, primers, probes

Table 13. Plasmids characteristics

Name	Characteristics	Source
pES12.6	Retroviral self-inactivating (SIN) vector for transduction Elongation factor 1-α (EF1α) internal promoter, cytomegalovirus (CMV) enhancer, 5' LTR of moloney murine leukemia virus (MoMuLV), psi/psi+ packaging signal of MoMuLV, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), self-inactivating (SIN) 3' long terminal repeat (LTR) of MoMuLV, multiple cloning site (MCS), ampicillin resistance cassette	BioNTech Innovative Manufacturing Services/ Medigene Immunotherapies GmbH
pGEM	Plasmid for *ivt*RNA production T7 promoter, polyA116-tail (A116), MCS, ampicillin resistance cassette	Milosevic S.
pMP71	Retroviral vector for transduction [102] Myeloproliferative sarcoma virus (MPSV)-LTR promoter-enhancer sequences and improved UTR derived from the murine embryonic stem cell virus (MESV), MCS, ampicillin resistance cassette	W. Uckert

Table 14. Plasmid generated in this project

Name	Cloning strategy*
pGEM_NY-ESO-1	Traditional cloning
pGEM_NY-ESO-1-pco	Traditional cloning
pGEM_NY-ESO-1-ORF2	Seamless cloning
pGEM_NY-ESO-1-ORF3	Seamless cloning
pGEM_NY-ESO-1-ORF4	Seamless cloning
pGEM_NY-ESO-1-ORF2_1-20 aa_eGFP	Seamless cloning
pGEM_NY-ESO-1-ORF2_11-30 aa_eGFP	Seamless cloning
pGEM_NY-ESO-1-ORF2_21-40 aa_eGFP	Seamless cloning
pGEM_NY-ESO-1-ORF2_31-50 aa_eGFP	Seamless cloning
pGEM_NY-ESO-1-ORF2_41-58 aa_eGFP	Seamless cloning
pGEM_LAGE-1a	Traditional cloning
pGEM_LAGE-1a-pco	Traditional cloning
pGEM_CAMEL	Seamless cloning
pGEM_TCR 1G4-α95:LY	Traditional cloning
pGEM_TCR 5-271	Traditional cloning
pES12.6_TCR 1G4-α95:LY	Traditional cloning
pES12.6_TCR 5-271	Traditional cloning

*Cloning strategies are described in section 3.4.5

Table 15. Plasmids kindly provided by collaborators

Name	Source
pMP71_HLA-A*02:01	Wehner C.
pGEM_HLA-A*02:01	Wehner C.
pMP71_CD86	Milosevic S.
pGEM_HA-1^{H}	Sommermeyer D.
pGEM_eGFP	Wehner C.

Table 16. Plasmids for retroviral transduction

Name	Characteristics	Source
pALF _env 10A1	cDNA encoding the envelope derived from murine leukemia virus (MLV) clone/strain 10A1.	Uckert W.
K83-hCMV_env GALV	cDNA encoding envelope derived from gibbon ape leukemia virus (GALV)	BioNTech Innovative Manufacturing Services
pcDNA3.1_gag/pol	cDNA encoding gag/pol polyprotein derived from MLV	C.Baum

Table 17. Primers RACE-PCR

Primer	Sequence 5' – 3'
5' TRAC	CGGCCACTTTCAGGAGGAGGATTCGGAAC
5' TRBC	CCGTAGAACTGGACTTGACAGCGGAAGTGG
5' nTRAC	CCACAGCACTGTTGCTCTTGAAGTC
5' nTRBC	GCTCAGGCAGTATCTGGAGTCATTGA

Table 18. Primers sequencing

Primer	Sequence 5' – 3'
pGEM FW	TATTACGACTCACTATAGGG
pMP71 FW	GCTCCGCCACTGTCCGAG
pES12.6 FW	CGCAACGGGTTTGCCGCCA
P2A FW	CTGCTGAAACAGGCCGGCG
5' TRAC	CGGCCACTTTCAGGAGGAGGATTCGGAAC
5' TRBC	CCGTAGAACTGGACTTGACAGCGGAAGTGG

Table 19. Probes nanostring nCounter

Probe	Target sequence 5' – 3'	Target region
Probe 1	GTCCGCATGGCGGCGCGGCTTCAGGGCTGAATGG ATGCTGCAGATGCGGGGCCAGGGGGCCGGAGAG CCGCCTGCTTGAGTTCTACCTCGCCATGCCTTT	241-340 nt (NY-ESO-1)
Probe 2	CAACTGCAGCTCTCCATCAGCTCCTGTCTCCAGCA GCTTTCCCTGTTGATGTGGATCACGCAGTGCTTTC TGCCCGTGTTTTTGGCTCAGCCTCCCTCAG	483-582 nt (NY-ESO-1/ LAGE-1a)

2.9 TCR sequences

Table 20. TCR 1G4-α95:LY

	V region	J region	CDR3
TCRα chain	TRAV21*01	TRAJ6*01	CAVRPLYGGSYIPTF
TCRβ chain	TRBV6-5*01	TRBJ2-2*01	CASSYVGNTGELF

HLA-A2-restricted NY-ESO-1/LAGE-1a$_{157-165}$-specific TCR.
Sequence derived from patent WO 2005/113595 A2.

Table 21. TCR 5-271

	V region	J region	CDR3
TCRα chain	TRAV21*01	TRAJ6*01	CAVSPQWGGSYIPTF
TCRβ chain	TRBV6-5*01	TRBJ2-7*01	CASSYLRGGGYEQYF

HLA-A2-restricted NY-ESO-1-ORF2/CAMEL$_{11-18}$-specific TCR.
Sequence identified in this project.

The variable and junctional gene segments are indicated in IMGT nomenclature. TRAV, TCR alpha variable segment; TRAJ, TCR alpha joining segment; TRBV, TCR beta variable segment; TRBJ, TCR beta joining segment.

2.10 Peptides

Table 22. Peptides

Sequence (aa)	X-mer	Antigen/protein	Position	Source
MLMAQEALAFLM	12	NY-ESO-1-ORF2	1-12	Peps4LS GmbH
MLMAQEALAFL	11	NY-ESO-1-ORF2	1-11	Peps4LS GmbH
MLMAQEALA	9	NY-ESO-1-ORF2	1-9	Peps4LS GmbH
MLMAQEAL	8	NY-ESO-1-ORF2	1-8	Peps4LS GmbH
LMAQEALAFL	10	NY-ESO-1-ORF2	2-11	Peps4LS GmbH
FLMAQGAMLAA	11	NY-ESO-1-ORF2	10-20	Peps4LS GmbH
FLMAQGAMLA	10	NY-ESO-1-ORF2	10-19	Peps4LS GmbH
FLMAQGAML	9	NY-ESO-1-ORF2	10-28	Peps4LS GmbH
FLMAQGAM	8	NY-ESO-1-ORF2	10-17	Peps4LS GmbH
LMAQGAML	8	NY-ESO-1-ORF2	11-18	Peps4LS GmbH
LMAQGAMLAA	10	NY-ESO-1-ORF2	11-20	Peps4LS GmbH
ALAFLMAQGAML	12	NY-ESO-1-ORF2	7-18	Peps4LS GmbH
AQGAMLAA	8	NY-ESO-1-ORF2	13-20	Peps4LS GmbH
MAQGAMLAA	9	NY-ESO-1-ORF2	12-20	Peps4LS GmbH

Sequence (aa)	X-mer	Antigen/protein	Position	Source
GLAFLMAQGAML	12	NY-ESO-1-ORF2	7-18 (F7G)	Peps4LS GmbH
AAAFLMAQGAML	12	NY-ESO-1-ORF2	7-18 (L8A)	Peps4LS GmbH
ALGFLMAQGAML	12	NY-ESO-1-ORF2	7-18 (A9G)	Peps4LS GmbH
ALAALMAQGAML	12	NY-ESO-1-ORF2	7-18 (F10A)	Peps4LS GmbH
ALAFAMAQGAML	12	NY-ESO-1-ORF2	7-18 (L11A)	Peps4LS GmbH
ALAFLAAQGAML	12	NY-ESO-1-ORF2	7-18 (M12A)	Peps4LS GmbH
ALAFLMGQGAML	12	NY-ESO-1-ORF2	7-18 (A13G)	Peps4LS GmbH
ALAFLMAAGAML	12	NY-ESO-1-ORF2	7-18 (Q14A)	Peps4LS GmbH
ALAFLMAQAAML	12	NY-ESO-1-ORF2	7-18 (G15A)	Peps4LS GmbH
ALAFLMAQGGML	12	NY-ESO-1-ORF2	7-18 (A16G)	Peps4LS GmbH
ALAFLMAQGAAL	12	NY-ESO-1-ORF2	7-18 (M17A)	Peps4LS GmbH
ALAFLMAQGAMA	12	NY-ESO-1-ORF2	7-18 (L18A)	Peps4LS GmbH
ALMAQGAMLAA	11	NY-ESO-1-ORF2	10-20 (F10A)	Peps4LS GmbH
FAMAQGAMLAA	11	NY-ESO-1-ORF2	10-20 (L11A)	Peps4LS GmbH
FLAAQGAMLAA	11	NY-ESO-1-ORF2	10-20 (M12A)	Peps4LS GmbH
FLMGQGAMLAA	11	NY-ESO-1-ORF2	10-20 (A13G)	Peps4LS GmbH
FLMAAGAMLAA	11	NY-ESO-1-ORF2	10-20 (Q14A)	Peps4LS GmbH
FLMAQAAMLAA	11	NY-ESO-1-ORF2	10-20 (G15A)	Peps4LS GmbH
FLMAQGGMLAA	11	NY-ESO-1-ORF2	10-20 (A16G)	Peps4LS GmbH
FLMAQGAALAA	11	NY-ESO-1-ORF2	10-20 (M17A)	Peps4LS GmbH
FLMAQGAMAAA	11	NY-ESO-1-ORF2	10-20 (A18G)	Peps4LS GmbH
FLMAQGAMLGA	11	NY-ESO-1-ORF2	10-20 (A19G)	Peps4LS GmbH
FLMAQGAMLAG	11	NY-ESO-1-ORF2	10-20 (A20G)	Peps4LS GmbH
ALMAQGAML	9	NY-ESO-1-ORF2	10-18 (F10A)	Peps4LS GmbH
FAMAQGAML	9	NY-ESO-1-ORF2	10-18 (L11A)	Peps4LS GmbH
FLAAQGAML	9	NY-ESO-1-ORF2	10-18 (M12A)	Peps4LS GmbH
FLMGQGAML	9	NY-ESO-1-ORF2	10-18 (A13G)	Peps4LS GmbH
FLMAAGAML	9	NY-ESO-1-ORF2	10-18 (Q14A)	Peps4LS GmbH
FLMAQAAML	9	NY-ESO-1-ORF2	10-18 (G15A)	Peps4LS GmbH
FLMAQGGML	9	NY-ESO-1-ORF2	10-18 (A16G)	Peps4LS GmbH
FLMAQGAAL	9	NY-ESO-1-ORF2	10-18 (M17A)	Peps4LS GmbH
FLMAQGAMA	9	NY-ESO-1-ORF2	10-18 (L18A)	Peps4LS GmbH
SLLMWITQV	9	NY-ESO-1	157-165	ProteoGenix
KVLEYVIKV	9	MAGE-A1	278-286	ProteoGenix

Sequence (aa)	X-mer	Antigen/protein	Position	Source
YMDGTMSQV	9	Tyrosinase	369-377	Peps4LS GmbH
VLHDDLLEA	9	HA-1^{H}	137-145 (H139)	ProteoGenix
VLRDDLLEA	9	HA-1^{R}	137-145 (R139)	Peps4LS GmbH

2.11 Antibodies

Table 23. Antibodies

Specificity*	Fluoro-chrome	Isotype	Clone	Source
CD4	APC	Mouse IgG1κ	RPA-T4	BD
CD8	PB	Mouse IgG1κ	RPA-T8	BD
CD137	PE	Mouse BALB/c IgG1κ	4B4-1	BD
CD86	FITC	Mouse BALB/c IgG1κ	2331(FUN-1)	BD
HLA-A2	APC	Mouse IgG2b, κ	BB7.2	BD
CD4	FITC	Mouse BALB/c IgG1κ	SK3	BD
CD3	PercP	Mouse BALB/c IgG1κ	SK7	BD
CD56	PE	Mouse IgG1κ	CMSSB	eBioscience
CD80	APC-Cy7	Mouse C3H/Bi IgG1κ	L307.4	BD
CD19	PE-Cy7	Mouse IgG1κ	HIB19	eBioscience
CD3	BUV	Mouse IgG1κ	UCHT1	BD
CD14	PB	Mouse IgG2a	M5E2	BD
CD83	APC	Mouse IgG1κ	HB15e	BD
CD86	Alexa700	Mouse BALB/c IgG1κ	2331(FUN-1)	BD
CCR7	PE-vio770	Recombinant human IgG1	REA108	MiltenyiBiotec
HLA-DR	PercP	Mouse BALB/c IgG2a κ	L243	BD
CD274	FITC	Mouse IgG1κ	MIH1	BD
TRBV6-5	PE	Mouse IgG2b	IMMU222	Beckman Coulter

*Anti-human

2.12 Softwares

Table 24. Softwares

Task	Software/online tool
Cloning simulation	Clone Manager
Data analyses	Microsoft Excel Graph Pad Prism
FACS analyses	FlowJo 10
Sequence analyses	Chromas Lite NCBI BLAST IMGT ExPASy
Reference source	NCBI PubMed Mendeley

2.13 Collaborators

Table 25. Collaborators

Name	Company/Institute
Milosevic S.	Medigene Immunotherapies GmbH, Germany
Moosmann A.	Helmholtz Zentrum München, Germany
Schendel D.	Medigene Immunotherapies GmbH, Germany
Sommermeyer D.	Medigene Immunotherapies GmbH, Germany
Uckert W.	Max Delbrück Center, Berlin, Germany
Wehner C.	Medigene Immunotherapies GmbH, Germany
-	BioNTech Innovative Manufacturing Services, Germany
-	Medigene Immunotherapies GmbH, Germany
-	Nanostring Headquarters facility, USA

3 Methods

3.1 Cell-biological methods

To avoid contamination with bacteria, fungi or other organisms, cell cultures were performed under sterile conditions and sterile material, solutions and media were used. Cells were grown in incubators at 37°C, 6.5% CO_2 and 95% humidity in laboratories of S2 standard.

3.1.1 Cell counting

Cell numbers were determined using Neubauer counting chambers. Trypan blue staining was used to allow discrimination of viable and dead cells. Trypan blue is not taken up by viable cell, but passes the cell membrane of dead cells. An aliquot of the cell suspension (10µl) was diluted with trypan blue at a ratio of either 1:2 for samples at low cell concentration or 1:10 for samples at high cell concentration. Using an inverted microscope, the number of viable cells (unstained with trypan blue) in the four big quadrants was counted. The following formula was applied to determine the cell concentration per ml: cell number/ml = mean of cells counted in four big quadrants x dilution factor x chamber factor (10^4).

3.1.2 Cryopreservation of cells

Cells were collected and centrifuged at 350 g for 5 minutes at room temperature. Cell pellets were re-suspended in ice-cold cell freezing medium and frozen in 1 ml aliquots in cryovials. Peripheral blood mononuclear cells (PBMC) and peripheral blood lymphocytes (PBL) were frozen at the cell concentration of 5×10^7 cell/ml, while all the other cells were frozen at cell concentrations ranging between 4×10^6 and 1×10^7 cell/ml. Cryovials were placed into CoolCell® Cell Freezing Containers and stored at -80°C overnight. The following day, the cryovials were transferred into a -150°C freezer for long-term storage.

3.1.3 Cell thawing

Cryovials containing frozen cells were thawed at room temperature. The suspension of each cryovial was transferred to a 15 ml tube containing 10 ml of RPMI VI medium and centrifuged at 350 g for five minutes at room temperature. The supernatant was discarded

and cell pellet resuspended in the appropriate pre-warmed medium. Cells were counted and used for the required applications.

3.1.4 Cultivation of adherent cell lines

Adherent cell lines were cultivated in T25 - T75 cm^2 tissue culture flasks adding 10 - 25 ml of appropriate medium. Approximately every three to four days, confluent cells were passaged either 1:5 or 1:10 depending on the growth rate of the cell line by detaching adherent cells with trypsin/EDTA. Therefore, the medium was removed, cells were washed once with 10 ml of warm DPBS and then incubated with 1 - 3 ml of trypsin/EDTA for three minutes at 37°C. Fresh medium was added into the flask to inactivate trypsin. The desired number of detached cells was resuspended in fresh medium to a final volume of 10 - 25 ml and distributed in new tissue culture flasks

3.1.5 Cultivation of suspension cell lines

Suspension cell lines were cultivated in T25 - T75 cm^2 tissue culture flasks adding 15 - 50 ml of the appropriate medium. Approximately every three to four days, cells were passaged 1:3 or 1:5 depending on the growth rate of the cell line and fresh medium was added.

3.1.6 Cell irradiation

Cells were counted and resuspended at 3 x 10^6 cells/ml in the appropriate medium. According to the total number of cells and the corresponding total volume of medium, cells were seeded into either tissue culture plates or flasks (table 26). After sealing the vessel with Parafilm®, cells were placed into the RS225 irradiation device. For each cell type, irradiation was performed according to the specific protocol shown in table 27.

Table 26. Maximum volume for irradiation

Plate	Maximum volume/well	Flask	Maximum volume/flask
24-well	1 ml	T25	12.5 ml
12-well	2 ml	T75	37.5 ml
6-well	5 ml	T150	50 ml

Table 27. Irradiation conditions

Cell type	Gray (Gy)
K562 cells	100
LCL	100
LAZ 388	100
PBL	50

3.1.7 T cell clone stimulation and expansion

The $CD8^+$ T cell clone 10/24 and $CD4^+$ T cell clone 234 were (re-)stimulated and expanded according to the following protocols.

The $CD8^+$ T cell clone 10/24 was seeded in a 24-well tissue culture plate (1 x 10^6 cells/well) and mixed with an irradiated PBL pool composed of cells of several donors (1 x 10^6 cells/well) as well as with irradiated HLA-A2-positive LCL P8 that were loaded with the peptide recognized by the clone 10/24 (1 x 10^5 cells/well). The final volume per well was 2 ml of T cell medium containing IL-2 (50 IU/ml). Either phytohemagglutinin (PHA 250 ng/ml) or anti-CD3 antibody (OKT-3, 30 ng/ml) was added to the medium. PHA was removed from T cell culture after four days due to its toxicity.

The $CD4^+$ T cell clone 234 was seeded in 24-well tissue culture plates (1 x 10^6 cells/well) and mixed with irradiated HLA-A24-positive LCL BW (3 x 10^5 cells/well) in T cell medium containing IL-2 (50 IU/ml) (2 ml final volume/well).

For both clones, half of the medium was substituted with fresh T cell medium containing IL-2 (50 IU/ml) every two days. When cells were confluent, they were split either in a ratio of 1:2 or 1:3 and fresh T cell medium containing IL-2 (50 IU/ml) was added. T cell clones were restimulated every 2 weeks.

3.1.8 Isolation of human PBMC

Fresh blood from healthy donors was collected after informed consent. PBMC were isolated from blood samples by density gradient centrifugation using a separating solution containing the synthetic copolymer Ficoll®. Syringes for collecting blood were supplemented with 10 IU heparin-sodium/ml of blood. Blood samples were diluted 1:2 with PBS VLE and 35 ml of diluted blood were layered over 15 ml Ficoll® in 50 ml tubes. After centrifugation at 840 x g for 18 minutes without brake, 15 ml of the upper phase were removed and the centrifugation step was repeated. PBMC located in the interphase between plasma and Ficoll® were collected and transferred in new 50 ml tubes (two interphases/50 ml tube). PBS VLE was added to each tube to a final volume of 50 ml. Tubes were centrifuged at 470 x g for ten minutes with brake. Supernatants were discarded and cell pellets were mixed (two cell pellets/50 ml tube) and resuspended in PBS VLE to a final volume of 50 ml. Tubes were centrifuged at 470 x g for ten minutes with brake. Supernatants were discarded and cell pellets were mixed (two cell pellets/50 ml tube) and resuspended in PBS VLE with 0.5% human serum to a final volume of 40 ml. After centrifugation at 130 x g for 15 minutes without brake, the step was repeated. The final cell pellet was resuspended in T cell medium, counted and used freshly in experiments or frozen for long-term storage.

3.1.9 Generation of lymphoblastoid cell lines

The immortalization of human B cells can be achieved *in vitro* by infection with Epstein-Barr-Virus (EBV) which can lead to a transformation of these cells [103]. The lines generated by EBV infection of B cells are called lymphoblastoid cell lines (LCL). For this project, LCL were generated starting from PBMC derived from a HLA-A2-positive healthy donor (see section 3.1.8 for PBMC isolation). The infection was performed using the B95.8 EBV virion-containing supernatant kindly provided by Dr. Moosmann. Freshy isolated PBMC were seeded into a 96-well flat-bottom plate at 5 x 10^5 cells per well in 100 µl of RPMI IV medium containing 1 µg/ml of cyclosporin A (CSA). B95.8 EBV virion-containing supernatant was added to each well (100 µl/well). Only RPMI IV medium containing 1 µg/ml of CSA was added into the negative control wells (100 µl/well). The plate was incubated at 37°C overnight. The following day, half of the medium of each well (100 µl) was exchanged by fresh RPMI IV medium containing CSA (1 µg/ml). The plate was kept in the incubator for the following days. The procedure was repeated every four days. About two or three weeks after infection, each well was inspected for outgrowth of LCL showing spherical aggregates compared to negative control wells. Within the following six weeks, LCL were expanded and finally analyzed by flow cytometry for their purity and quality (staining for B cell marker CD19, T cell marker CD3 and live/dead staining).

3.1.10 Generation of mDCs

To stimulate and expand HLA-A2-allo-restricted NY-ESO-1-specific T cells, mDCs were used as APCs during the priming procedure. Therefore, mDCs were generated *in vitro* using an 8-day protocol and the Jonuleit maturation cocktail [104]. Monocytes were derived from freshly isolated PBMC by plastic adherence. Hence, PBMC were isolated from a healthy HLA-A2-negative donor as previously described (see section 3.1.8). Subsequently, 75 x 10^6 PBMC were resuspended in 15 ml of DC medium and transferred into an 80cm^2 Nunclon® Δ surface cell culture flask. After 25 minutes of incubation at 37°C, the flask was gently shaken and incubated for additional 25 minutes at 37°C. Non-adherent cells were carefully washed away by two washing steps using 15 ml of fresh DC medium. These non-adherent cells represented the 'PBL fraction'. PBL were collected and frozen for other applications. Adherent monocytes were kept in culture overnight adding 15 ml of DC medium into the flask. In order to mature immature DCs (iDCs), on the following day (day 1) as well as after three days (day 3), 100 ng/ml GM-CSF and 20 ng/ml IL-4 were added to the cells. Finally, on day 6, the Jonuleit maturation cocktail was added (table 28). mDCs were harvest on day 8 and used for further experiments. The quality and the purity of the

generated mDCs was evaluated by analyzing the surface expression of typical DC maturation markers by flow cytometry (see section 3.2.1 for staining of cell surface marker).

Table 28. Jonuleit maturation cocktail

Components	Amount
GM-CSF	100 ng/ml
IL-4	20 ng/ml
IL-1β	10 ng/ml
IL-6	15 ng/ml
TNF-α	10 ng/ml
PGE_2	1000 ng/ml

3.1.11 *ivt*RNA electroporation into APCs

APCs were harvested, counted and washed once with RPMI1640 medium without serum (VLE RPMI 1640 in case of mDCs). Cell were adjusted at 2 – 3 × 10^6 cells/200 µl in RPMI1640 medium without serum (VLE RPMI 1640 in case of mDCs) and 200 µl of the cell suspension were transferred into a 0.4 cm pre-cooled electroporation cuvette. Subsequently, either 20 µg of *ivt*RNA or 20 µl of water (negative control) were added into the cuvette (see section 3.4.10 for *ivt*RNA production). The suspension was shortly mixed by pipetting and then quickly electroporated using the Gene Pulser Xcell™ device. Electroporation for each cell type was performed according to the specific conditions shown in table 29. Immediately after electroporation, cells were transferred into the appropriate fresh medium and placed into the incubator. After three hours, cells were counted and resuspended at the adequate concentrations for further applications.

Table 29. Electroporation conditions

Cell type	Protocol	Voltage (volt)	Capacitance (µF)	Time
mDCs	Exponential	150	150	∞
K562 cells	Exponential	300	300	∞
Tumor cell lines	Exponential	300	150	∞
T cells	Time constant	400	-	5ms

3.1.12 Loading of T2 cells with small synthetic peptides

T2 cells were harvested, counted and adjusted to 1 x 10^6 cells/ml using appropriate medium in a 15 ml tube. Subsequently, the required amount of peptide was added to the cells. T2 cells were loaded with either 10^{-5} M or with titrated amounts (ranging from 10^{-5} M to 10^{-12}

M) of the peptide of interest. Cells were incubated for 1h and 30 minutes at 37°C. After incubation, the appropriate medium was added to a final volume of 15 ml. Cells were centrifuged (350 x g for five minutes) and the supernatant was discarded to remove unbound peptides. Cells were resuspended in fresh medium at the required concentration for the subsequent applications.

3.1.13 Isolation of $CD8^+$ T cells

$CD8^+$ T cells were enriched starting either from PBMC or from PBL by the MACS® technology. The $CD8^+$ T cell Isolation kit was selected as means to isolate untouched $CD8^+$ T cells, avoiding direct labeling of $CD8^+$ T cell surface molecules that could interfere with subsequent applications. The MACS separation was performed according to the manufacturer's protocol. Either PBMC or PBL were counted and resuspended in 40 µl of DPBS - 0.5% human serum per 1 x 10^7 total cells into a 15 ml tube. A biotin-antibody cocktail was added to the cells (10 µl per 1 x 10^7 total cells) and well mixed in order to label CD8-negative cells. After incubation of five minutes at 4°C, 30 µl of DPBS - 0.5% human serum and 20 µl of anti-biotin MicroBeads per 1 x 10^7 total cells were added into the tube. The reaction mix was incubated for ten minutes at 4°C. Meanwhile, LS columns were placed into the magnetic MACS separator and rinsed with 3 ml of DPBS - 0.5% human serum. Subsequently, the cell suspension was applied to LS columns. The flow-through containing unlabeled cells represented the enriched $CD8^+$ T cells that were collected into 50 ml tubes. LS columns were washed with 3 ml of DPBS - 0.5% human serum and the flow-through was mixed with the previously collected cells. Enriched $CD8^+$ T cells were counted and resuspended in T cell medium at the required cell concentration for stimulation. Samples before and after enrichment were analyzed by flow cytometry to evaluate the purity of the enriched cell population and the efficiency of the separation process.

3.1.14 Stimulation and expansion of T cells

PBL and CD8-enriched T cells were (re-)stimulated and expanded using Dynabeads® Human T-Activator CD3/CD28. T cells were seeded at 1 x 10^6 cells/ml in a 24-well tissue culture plate in T cell medium with 50 U/ml IL-2. Dynabeads® were resuspended and the desired volume was transferred to a 15 ml tube (25 µl Dynabeads®/1 x 10^6 T cells). An equal volume of DPBS with 0.1 % human serum or at least 1 ml was added. After mixing, the tube was placed on a magnet for one minute and the supernatant was discarded. The tube was removed from the magnet and the washed Dynabeads® were resuspended in T cell medium. The washed Dynabeads® were added to the T cells and the plate was placed

into the incubator. After three or four days, Dynabeads® were removed from the culture. Therefore, T cells were transferred in a 15 ml tube and the tube was placed on the magnet for two minutes. The supernatant containing the cells was collected in a new tube. The remaining Dynabeads® were washed with fresh T cell medium and the procedure on the magnet was repeated. T cells were counted and placed into either a suitable tissue culture plate or into a tissue culture flask at 5 x 10^5 cells/ml. T cells were passaged 1:2 or 1:3 and fresh T cell medium containing IL-2 (50 IU/ml) was added every two days. Restimulation of the cells was performed every 2 weeks.

3.1.15 *De novo* induction of $CD8^+$ T cells using *ivt*RNA-transfected mDCs

mDCs generated *in vitro* from an HLA-A2-negative donor were electroporated simultaneously with *ivt*RNA encoding the foreign HLA-A2 molecule and the full-length NY-ESO-1 antigen (20 µg each *ivt*RNA) to utilize them as APCs for stimulation and expansion of autologous CD8-enriched T cells. As control, *ivt*RNA encoding full-length eGFP was transfected in combination with HLA-A2 *ivt*RNA into mDCs (20 µg each *ivt*RNA). Electroporation was performed as described in section 3.1.11. The expression of HLA-A2 molecules and eGFP was evaluated by flow cytometry 3h after electroporation. On the same day, autologous $CD8^+$ T cells were enriched from freshly isolated PBMC according to the protocol described in section 3.1.13. Co-cultures were initiated 3h after electroporation in 24-well tissue culture plates. The cells were seeded at an effector to target ratio of 10:1 (1 x 10^6 $CD8^+$ T cells and 1 x 10^5 mDCs) in T cell medium containing 5 ng/ml IL-7. Addition of IL-2 (50 IU/ml) was delayed for two days and then added every two days. Cells were expanded for 14 days and subsequently NY-ESO-1-specific T cells were selectively separated using the double sorting strategy (see section 3.2.2.2).

3.1.16 Stimulation and expansion of single-cell-sorted T cells

Single-cell-sorted T cells were stimulated immediately after the double-sorting procedure. Anti-CD3 antibody (30 ng/ml OKT-3), 1 x 10^4 irradiated LAZ 388 cells and 1 x 10^5 irradiated PBL pooled from different donors were added to each well. The final volume per well was 200 µl of T cell medium supplemented with 50 IU/ml IL-2. After 14 days, plates were screened for expanded clones. The expanded clones were tested in the first screening for antigen specificity. Moreover, a fraction of the cells was resuspended in TRI Reagent® for subsequent TCR sequence analysis. A fraction of the cells was restimulated for further expansion according to standard $CD8^+$ T cell clone stimulation and expansion.

3.1.17 Plasmid-DNA transfection into 293FT cells for retrovirus production

Retroviruses needed for subsequent retroviral-mediated transduction were produced by triple plasmid DNA transfection of the packaging 293FT cells. The transgene-encoding DNA retroviral vector and the plasmids encoding the *gag/pol* and the *env* viral genes were introduced into the packaging cells using the TransIT®-LT1 transfection reagent. Therefore, 293FT cells were seeded at 1.5 x 10^6 cells per 100 mm petri dish in 10 ml DMEM IV medium and placed into the incubator overnight. The following day, 470 µl of DMEM medium without serum were mixed with 30 µl of TransIT®-LT1 transfection reagent and incubated for five minutes at room temperature. Subsequently, plasmid DNA was added to the transfection mixture (see table 30). The transfection mix was incubated for 15 minutes at room temperature. The medium of the seeded 293FT cells was replaced by RPMI IV and the transfection mix was added dropwise to the 293FT cells. Petri dishes were incubated at 37°C. After three days, the virus-containing medium was harvested and centrifuged at 200 g for 10 minutes at 32°C. The retroviral supernatant was either used freshly or frozen at -80°C for long-term storage.

Table 30. Amount of plasmid DNA for transfection of 293FT cells

Plasmid DNA encoding	Amount per transfection reaction (µg)
Transgene	4.7
Gag/pol	4.7
Env	3.1

3.1.18 Retroviral-mediated transduction

Retroviral-mediated transduction of T cells and K562 cells was performed using a RetroNectin® reagent to co-localize the virus with the cells in order to enhance the viral transduction efficiency. Transduction was done in two subsequent rounds (infection hits). Therefore, non-tissue culture treated 24-well plates were coated with 10 µg/ml RetroNectin® in 1ml DPBS per well. Plates for the first and the second infection hit were prepared in parallel. Plates were sealed with Parafilm® and kept at 4°C until the next day. The following day, RetroNectin® was replaced with DPBS containing 2% BSA. After incubation for 30 minutes at room temperature, the solution was removed from each well. Retroviral supernatant (either frozen or fresh, see section 3.1.17) was added to the RetroNectin®-coated plates (1 ml/well). If two different retroviral supernatants were used for simultaneous transduction, 1 ml of each retroviral supernatant was added into the well (final volume per well 2 ml). As negative control, only RPMI VI medium was added into RetroNectin®-coated wells. Plates were sealed with Parafilm® and centrifuged for 90 minutes at 3200 g at 32°C.

After centrifugation, the plate for the second infection hit was stored at 4°C until the next day. In contrast, supernatants were removed from the plate for the first infection hit and cells in proliferating phase were seeded into the retrovirus-coated wells (0.5 - 1 x 10^6 T cells/well in 1 ml of T cell medium with 50 IU/ml IL-2, 0.3 – 0.5 x 10^6 K562 cells/well in 1 ml of RPMI IV medium). Cells were also seeded into the negative-control wells. These cells were designated as untransduced cells. The plate was incubated at 37°C. The following day, supernatants were removed from the plate for the second infection hit. Cells were transferred from the plate for the first infection hit to the plate for the second infection hit. Fresh T cell medium was added to the cells (1 ml/well). After three or four days, cells were harvested and seeded at an adequate cell concentration (0.5 x 10^6 T cells/ml; 0.3 x 10^6 K562 cells/ml) either into tissue culture plates or into cell culture flasks in fresh medium (containing 50 IU/ml IL-2 in case of T cells). Transduction rate was verified by flow cytometry, evaluating the surface expression of the introduced molecules. After adequate expansion, cells were harvested, counted and used for further applications.

3.1.19 Lentiviral-mediated transduction

IncuCyte® NucLight Lentivirus Reagent was used for nuclear labelling of T2 cells by homogenous expression of the mKate2 protein (red fluorescent protein). This reagent enabled the generation of stable transduced cell populations using puromycin selection. T2 cells were collected, counted and resuspended at 2 x 10^5 cells/ml in fresh RPMI IV medium. Subsequently, 500 µl of cell suspension were transferred into a 1.5 ml tube. Polybrene (8 µg/ml) and IncuCyte® NucLight Lentivirus Reagent (multiplicity of infection, MOI 3) were added to the cells. The volume of lentiviral supernatant corresponding to MOI 3 was calculated according to the virus titer listed in the manufacturer's datasheet. The sample was incubated for 1h at 37°C and then centrifuged at 400 g for 1h at room temperature. The pellet was resuspended in 2 - 3 ml of fresh RPMI IV medium. Cells were transferred into a 6-well tissue culture plate and placed into the IncuCyte® ZOOM device to monitor the fluorescence expression. Puromycin (1 µg/ml) was added to the plate to select the transduced cells five days after transduction. Red-labelled T2 cells were expanded and kept in culture in RPMI IV medium containing 0.5 µg/ml puromycin. Before performing the functional assays, T2 cells were washed to remove the puromycin.

3.2 Flow cytometry

3.2.1 Staining of cell surface markers

For each staining condition 0.2 – 1 x 10^6 cells were transferred into a 5 ml tube. In case of DC staining, FcR blocking reagent was added to the samples prior the staining to block unwanted binding of antibodies to human Fc receptor-expressing cells. Cells were washed with 1 ml DPBS - 1% FBS buffer and the supernatant removed leaving 100 µl in the tube. To stain the desired surface markers, appropriate antibodies were added and incubated for 30 minutes at 4°C in the dark. After incubation, the tubes were filled with DPBS - 1% FBS ad 1ml, centrifuged (300 g for five minutes) and the supernatants discarded. Cells were resuspended in DPBS - 1% PFA for fixation and stored at 4°C until the flow cytometric analysis was performed using the analyzer BD LSRFortessa™. UltraComp eBeads® were used for fluorescence compensation in case of multicolor flow cytometric analyses. The beads preparation was performed according to manufacturer's instructions.

3.2.2 Fluorescence-activated cell sorting (FACS)

Cell suspensions were stained with fluorochrome-conjugated antibodies as previously described and analyzed for fluorochrome signals. Once the photons emitted by the fluorochromes have been detected, cells were selected according to marker expression. After passing the laser beam, cells were separated using BD FACSAria™ Fusion cell sorter. Cells were sorted applying either “purity” (highest cell yield) or “single cell” (highest purity) mode.

3.2.2.1 Sorting of K562 cells

To generate appropriate APCs for the double sorting procedure and for the screening assays, K562 cells were stably transduced with the HLA-A2 molecule and the CD86 co-stimulatory molecule (see section 3.1.18 for retroviral-mediated transduction). After transduction, the expression of HLA-A2 and CD86 surface molecules was evaluated by surface marker staining with specific antibodies and analyzed by flow cytometry. Subsequently, HLA-A2-positive CD86-positive K562 cells (designated as tgK562) were sorted by FACS to obtain a high purity cell population.

3.2.2.2 CD137-based double-sorting strategy

A 2-step-sorting procedure based on the CD137 activation marker and K562 cells as target cells was performed to selectively separate NY-ESO-1-specific T cells after *de novo* T cell

induction and expansion. In the first step a NY-ESO-1-unspecific stimulation of primed T cells was performed using irradiated water-electroporated tgK562 cells at an E:T ratio of 2:1. Stimulated T cells were stained for CD137 and CD8 cell surface expression 12 h after activation and the CD137-negative CD8-positive fraction was sorted by FACS (CD137-negative cell sorting). In the second step, NY-ESO-1-specific stimulation of the sorted T cells was performed using irradiated NY-ESO-1 *ivt*RNA-transfected tgK562 cells at an E:T ratio of 5:1. After 12 h, stimulated T cells were stained for CD137 and CD8 cell surface expression and the CD137-positive CD8-positive fraction was sorted (CD137-positive cell sorting) as single cells into tissue culture 96-well U-bottom plates by FACS. Single-cell-sorted T cells were stimulated and expanded as described in section 3.1.16.

3.3 Assays for functional analyses

3.3.1 Co-culture of T cells and APCs

The functionality of either TCR-transgenic T cells or T cell clones was investigated by evaluating IFN-γ release after target antigen-specific stimulation as well as by testing the capacity to mediate killing of cells expressing the target antigen. T cells and T cell clones were harvested, counted and used for functional analyses between 10 and 14 days after restimulation.

3.3.1.1 Evaluation of IFN-γ release by T cells

Effector cells and target cells were counted and adjusted according to the desired cell concentration for the co-culture (30000 effector cells/100µl/well, 10000-30000 target cells/100µl/well). The co-cultures for each test condition were performed in duplicates. The effector cells were co-cultured with target cells for 24h at 37°C. After incubation, the co-culture supernatants were harvested and the amount of secreted interferon-gamma (IFN-γ) was assessed by enzyme-linked immunosorbent assay (ELISA) (see section 3.3.2). The residual supernatants were frozen at -20°C for long-term storage.

3.3.1.2 Evaluation of killing mediated by T cells

Killing capacity mediated by TCR-transgenic T cells was evaluated by setting up co-culture experiments with different types of target cells and dyes for live-cell imaging using IncuCyte® ZOOM - Live Cell Analysis System.
In the first approach, red-labelled T2 cells generated as described in section 3.1.19 were used as target cells. Thus, peptides of interest were loaded on red-labelled T2 cells

according to the protocol previously described (see section 3.1.12). Subsequently, loaded red-labelled T2 cells were seeded into 96-well round bottom plates (1 x 10^3 cells/well). The effector cells were added into each well at an effector to target (E:T) ratio of 20:1. The co-cultures for each test condition were performed in triplicates. After setting up the co-cultures, the plates were incubated at room temperature for 30 minutes and then placed into the IncuCyte® ZOOM device. The cells were monitored over 48h by live-cell imaging using IncuCyte® ZOOM - Live Cell Analysis System to evaluate the decrease of the red fluorescence intensity showing T2 cell killing mediated by TCR-transgenic T cells.
In the second approach, tumor cell lines transfected with target antigen *ivt*RNA were used as target cells. The electroporation was performed as described in section see 3.1.11. Tumor cell lines were resuspended in fresh medium and seeded in 96-well flat-bottom-plates (1.5 x 10^4 cells/well) 3h after transfection. After seeding, the cells were incubated at room temperature for 30 minutes to allow an even distribution and settling of the cells and subsequently incubated at 37°C. The following day, half of the medium was removed from the plates. Each well was refilled with 50 µl of the red fluorescent annexin V apoptosis dye (resuspended and pre-diluted according to manufacturer's instructions) and 50 µl of medium containing 5 x 10^4 effector cells. The co-cultures for each test condition were performed in duplicates. After adding the effector cells, the plates were incubated at room temperature for 30 minutes and then placed into the IncuCyte® ZOOM device. To evaluate apoptosis in the co-cultures, the cells were monitored over 48h by live-cell imaging using IncuCyte® ZOOM - Live Cell Analysis System.

3.3.2 IFN-γ ELISA

The measurement of IFN-γ in co-culture supernatants was performed by a sandwich ELISA using the OptEIA™ Human ELISA Set IFN-γ. IFN-γ ELISA was performed according to the manufacturer's protocol. Nunc-Immuno™ MicroWell™ 96-well plates were coated with 50 µl/well capture antibody diluted 1:250 in ELISA coating buffer and incubated at 4°C overnight. The plates were washed five times with 300 µl/well ELISA washing buffer, blocked with 300 µl/well ELISA blocking buffer for 1h at room temperature and washed again three times. Co-culture supernatants as well as serially diluted standard were incubated on the plates for 1h at room temperature (50 µl/well each sample). The plates were washed five times and 100 µl of detection antibody plus peroxidase-conjugated avidin (both diluted 1:250 in blocking buffer) were added into the plates for 1h at room temperature. To remove unbound enzyme, the plates were washed five times. The enzyme substrate was added to the plates (100 µl/well) and incubated for five minutes at room temperature. The reaction was stopped by adding 50 µl/well of ELISA stop solution. The

optical density of the products, that is proportional to the bound cytokines, was detected by a spectrophotometer at 450 nm and 620 nm. The standard curve was calculated according to standard IFN-γ concentration and respective detected optical density. The IFN-γ concentration of the samples tested was interpolated.

3.4 Molecular biological methods

3.4.1 Plasmid DNA digestion

Plasmid DNA was digested with restriction enzymes to enable the cloning of the desired DNA fragments into corresponding expression vectors. The digestion of the plasmid DNA was performed in 1.5 ml tubes in a final volume of 100 µl containing 20 µg of DNA, 10X CutSmart buffer and 40U of the required restriction enzymes. The samples were incubated for 1h at 37°C. The resulting DNA fragments were separated by agarose gel electrophoresis and extracted from the gel as described in section 3.4.2**.**

3.4.2 Agarose gel electrophoresis and extraction of DNA fragments

The separation of DNA fragments was performed in 1% agarose electrophoresis gels with 0.4 µg/mL of ethidium bromide. A DNA ladder (either 1 kb or 100 bp) was included as a size standard during electrophoresis. After 45 minutes at 80 V, DNA bands were exposed to a UV transilluminator and cut from the gels. Subsequently, the QIAquick® Gel Extraction Kit was used for the extraction of DNA fragments from agarose gel according to manufacturer´s instructions.

3.4.3 Ligation of DNA fragments

Digested DNA fragments and linearized recipient plasmids were mixed in 0.5 ml tubes (2 µl linearized recipient plasmid and 6 µl insert). Subsequently, 1µl of T4 ligase buffer (10X) and 1µl of T4 ligase (1 IU) were added into the tubes. The reactions were incubated overnight at 16 °C.

3.4.4 Seamless cloning

The GENEART® Seamless Cloning and Assembly Kit exploits homologous recombination to fuse DNA fragments that share terminal end-homology in an *in vitro* cloning reaction. Briefly, the DNA fragment including the required end sequence homology (20 – 80 ng) was mixed with the linearized recipient plasmid (100 ng), 4 µl of reaction buffer (5X) and water

to a final volume of 18 µl. Subsequently, 2 µl of enzyme mix (10X) were added to the reaction mix. After incubation of 30 minutes at room temperature, the reaction mix was placed on ice and used for bacteria transformation.

3.4.5 Generation of plasmids

Plasmids containing the transgenes of interest were generated using either traditional cloning methods (figure 1A) or using the seamless cloning technology (figure 1B) according to the protocols described in more detail in previous sections. The cloning strategy to generate each single plasmid is listed in section 2.8 table 14.

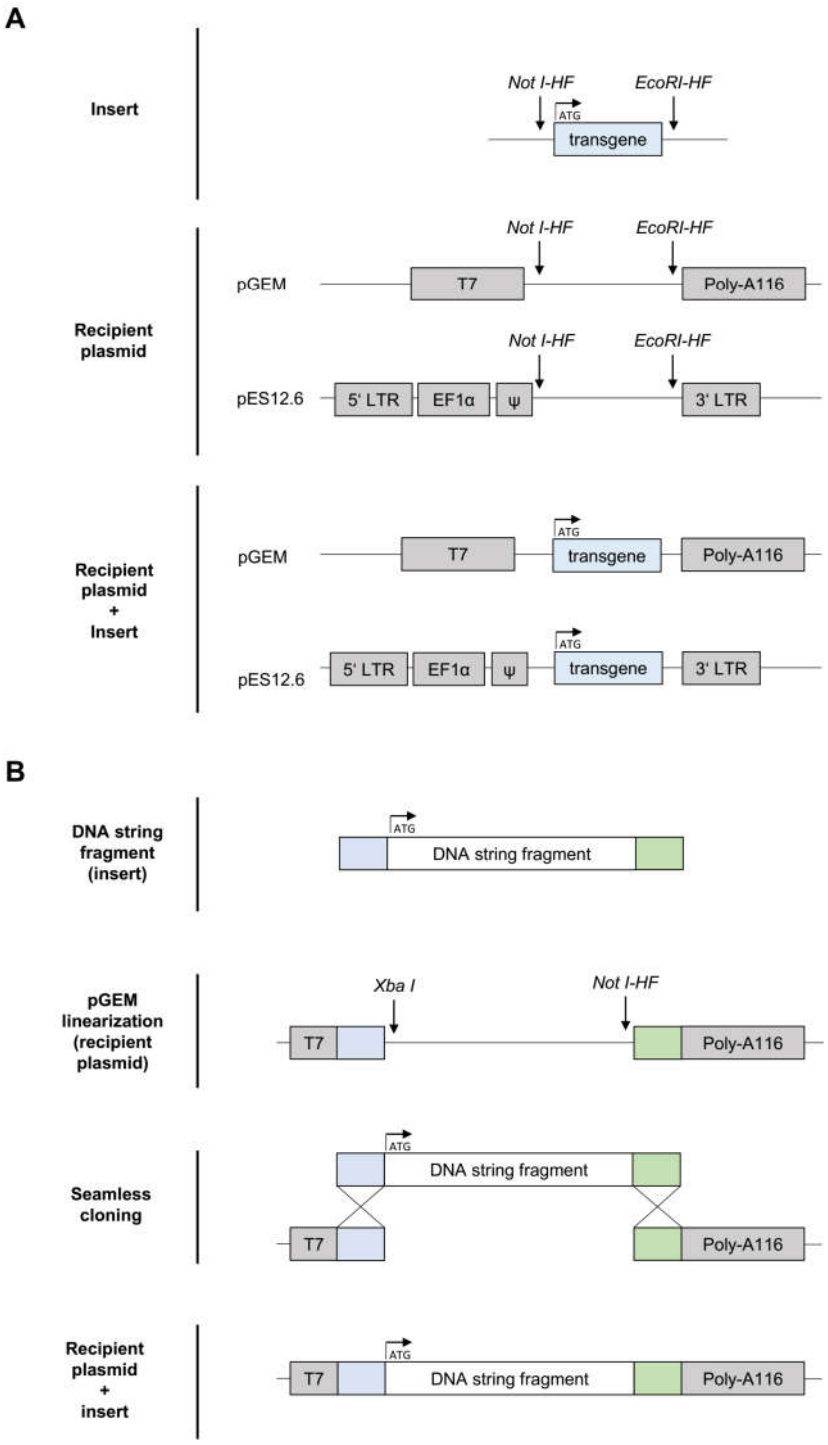


Figure 1. Cloning strategies to generate plasmids containing the transgenes of interest. **(A)** Traditional cloning procedure. The transgene of interest was synthesized and cloned into a shuttle plasmid by Thermo Fisher Scientific. The shuttle plasmid and the appropriate recipient plasmid (either pGEM or pES12.6) were digested with the same combination of restriction enzymes (*NotI-HF*® and *EcoRI-HF*®) and subsequently ligated by a T4 Ligase. **(B)** Seamless cloning procedure. The transgene of interest was produced as DNA string fragment by Thermo Fisher Scientific. The recipient pGEM plasmid was linearized using *XbaI* and *Not I-HF*® restriction enzymes. Subsequently, the GENEART® Seamless Cloning and Assembly Kit was used to fuse the DNA string fragment and the linearized plasmid sharing terminal end-homology (shown in blue and in green) in an *in vitro* cloning reaction.

3.4.6 Bacterial culture conditions

Three different types of bacteria were transformed: electrocompetent XL1-blue *E.coli*, chemically competent One Shot™ TOP10 *E. coli* and chemically competent NEB® Turbo Turbo *E. coli*. Bacteria were cultivated in an incubator at 37°C. For long-term storage, bacterial suspensions were supplemented with glycerol (20%) and stored at -80 °C. Since all the plasmids used for transformation contained an ampicillin resistance cassette, ampicillin was added to the LB medium and to the agar plates (50 µg/ml) for selection of the plasmid-expressing bacteria.

3.4.7 Bacterial transformation

In order to obtain larger amounts of plasmid DNA, competent bacteria had to be transformed with the plasmid DNA of interest and expanded. According to the type of competent bacteria, either chemical transformation or electroporation was performed.

3.4.7.1 Chemical transformation

Chemically competent bacteria were slowly thawed on ice (30 µl bacteria/each transformation). Plasmid DNA was added and mixed with bacteria by tapping gently (3 µl DNA/vial). Cells were incubated on ice for 30 minutes. A heat-shock step was performed by transferring the cells in a water bath at 42°C for 30 seconds. Heat-shock caused the uptake of the DNA by the bacteria. Subsequently, the cells were placed on ice for two minutes. SOC medium was added to the transformation mix. The cells were gently shaken at 37°C for 1h. After incubation, transformation mix was diluted (1:10) in SOC medium, added to the agar plates containing ampicillin and distributed homogeneously. Plates were incubated at 37°C overnight.

3.4.7.2 Electroporation

Electrocompetent bacteria were slowly thawed on ice and transferred in pre-cooled 0.1 cm cuvettes (25 µl bacteria/cuvette). Plasmid DNA was added to the bacteria (1 µl DNA/cuvette) and mixed by tapping gently. The cuvettes were placed into the Gene Pulser Xcell™ device. Electroporation was performed using pre-set protocol for bacteria (1 mm cuvette and 1.8kV). After electroporation, SOC medium was added (1 ml/cuvette) and the samples were incubated at 37°C for 1h in new 1.5 ml tubes. The tubes were centrifuged at 5000 g for ten minutes at room temperature. The supernatants were discarded and the pellets were resuspended in 200 µl of SOC medium. Transformed bacteria were added to

the agar plates with ampicillin and distributed homogeneously. Plates were incubated at 37°C overnight.

3.4.8 Selection and expansion of transformed bacteria

Only bacteria transformed with plasmid DNA encoding resistance to the antibiotic were able to grow and form colonies on selection agar plates containing ampicillin. After overnight growth, colonies were picked from the plates. Each individual colony was inoculated into 5 ml of LB medium containing ampicillin in a 5 ml tube. The tubes were incubated overnight at 37°C with vigorous shaking for efficient growth of transformed bacteria. An aliquot of the transformed bacteria (1 - 2 ml) was used for the isolation of plasmid DNA using JetStar™ 2.0 Plasmid Miniprep Kit according to the manufacturer's instructions. An additional step of bacteria expansion was performed for subsequent DNA isolation using JetStar™ 2.0 Plasmid Maxiprep Kit. An aliquot of the transformed bacteria (200 – 300 µl) was transferred into 200 ml of LB medium with ampicillin and the sample was incubated at 37°C overnight with vigorous shaking for efficient growth of transformed bacteria.

3.4.9 Plasmid DNA extraction from transformed bacteria

For the isolation of high amount of plasmid DNA, the JetStar™ 2.0 Plasmid Purification MaxiPrep Kit was used according to manufacturer´s instructions. The bacteria cultures were centrifuged at 3400 g for ten minutes at room temperature. The supernatant was discarded and the pellet resuspended in 10 ml buffer E1 and transferred to a 50 ml tube. Lysis buffer E2 was added (10 ml) and the collection tube inverted ten times. Thereafter 10 ml precipitation buffer E3 was added and the tube inverted again for ten times. The suspension was centrifuged at 5000 g for ten minutes at room temperature and the supernatant applied to a 70 µm filter onto an equilibrated column. The column was filled up with washing buffer E5 and the flow-through was discarded. The column was placed on top of a 50 ml collection tube and the DNA eluted by adding 15 ml of elution buffer E6. Isopropanol was added (10.5 ml) to the DNA, mixed and centrifuged at 5000 g for 45 minutes at 4°C. The supernatant was discarded and the pellet dehydrated with 1 ml 75% ethanol, air-dried and resuspended in nuclease-free water. The DNA concentration was determined with the Nanodrop.

3.4.10 *In vitro* transcription of single-species cDNA into mRNA

For *in vitro* transcription of single-species cDNA into mRNA (designated as *in vitro* transcribed RNA, *ivt*RNA) the mMESSAGE mMACHINE™ T7 kit was used. For this purpose, 20 µg of pGEM-plasmid DNA containing the T7 promoter were linearized with the

restriction enzyme *SpeI-HF*® overnight at 37°C. The *SpeI-HF*® recognition site is located downstream of a poly-A tail consisting of about 116 adenines. This poly-A tail is meant to increase the stability of *ivt*RNA. The following day, the reaction was stopped and purified by adding 5 µl EDTA (0.5 M), 10 µl sodium acetate (3 M) and 200 µl 100% ethanol. The reaction was incubated at -20 °C for 45 minutes. After centrifugation (15000 x g at 4 °C for 15 minutes), the supernatant was discarded and the DNA pellet was resuspended in 40 µl nuclease-free water. The concentration of the linearized DNA was determined with the Nanodrop. The production of *ivt*RNA was performed using the mMESSAGE mMACHINE™ T7 kit according to the manufacturer's instructions. Briefly, linearized DNA (2 - 3 µg) was mixed with 20 µl NTP/CAP (7-methyl guanosine cap and nucletotides), 4µl Reaction Buffer (10X) and 4µl T7 polymerase. Nuclease-free water was added to a final volume of 40 µl. The reaction mix was incubated for 2h at 37°C. DNAse was added (2 µl/sample) and incubated for 30 minutes at 37°C. Subsequently, the RNeasy® Mini Kit was used to purify the *ivt*RNA according to the manufacturer's instructions. Purified *ivt*RNA was eluted in 40 µl nuclease-free water. The RNA concentration was determined by the Nanodrop and *ivt*RNA was stored at -20°C.

3.4.11 Total RNA isolation

Total RNA was isolated from either T cell clones for TCR sequence analyses by RACE-PCR (see section 3.4.16.1) or from tumor cell lines to evaluate NY-ESO-1 expression by Nanostring nCounter technology (see section 3.4.17). Cells were collected and the pellet was resuspended vigorously in TRI Reagent® to disrupt plasma membranes, inactivate released RNases and homogenize the suspension (1 x 10^6 cells/200 µl of TRI Reagent®). Incubation at room temperature for five minutes allowed the dissociation of nucleoprotein-complexes. Linear acrylamide was added to the sample (10 µl/sample). Subsequently, 40 µl of chloroform were added, the suspensions were vortexed for 15 seconds, incubated at room temperature for ten minutes and then centrifuged at 12000 g for 15 minutes at 4°C. The upper colorless aqueous phase was transferred into a new 1.5 ml tube and 100 µl of isopropanol were added to the sample. The tube was vortexed, incubated at 4°C for ten minutes and then centrifuged at 12000 g for 15 minutes at 4°C. Supernatant was removed and 500 µl of 75% ethanol were added. After centrifugation at 12000 g for ten minutes at 4°C, supernatant was removed and the RNA pellet was air-dried at room temperature for five minutes. Finally, RNA was resuspended in 20 µl of nuclease-free water.

3.4.12 RNA denaturing-agarose gel electrophoresis

To verify the success and the quality of the *ivt*RNA production, an aliquot of the produced *ivt*RNA was loaded on 1% agarose gel (0.4% ethidium bromide, 1X TAE buffer in DEPC-treated water). Therefore, 0.5 µl *ivt*RNA were mixed with 5µl RNA loading buffer (2X) and 4.5 µl nuclease-free water and subsequently heated up to 70 °C for 10 minutes to destroy secondary structures. After incubation, the sample was loaded into the gel. A RNA ladder was included as a size standard for the electrophoresis. After 45 minutes at 80 V, RNA bands were analyzed using an UV transilluminator.

3.4.13 DNA sequencing

The sequencing of double-stranded DNA was performed in collaboration with the external partner MWG Eurofins (Martinsried, Germany). Primers used for sequencing are listed in section 2.8 table 18. The software Chromas lite was used for bioinformatic evaluation of the electropherograms.

3.4.14 Measurement of DNA and RNA concentration

DNA and RNA concentrations were determined using the Nanodrop photometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the samples. A ratio of 2.0 or higher was acceptable for RNA, whereas a ratio of 1.8 or higher was acceptable for DNA.

3.4.15 HLA typing

Fresh blood (3 ml EDTA-blood sample) collected from healthy donors after informed consent were sent to the facility IMGM (Martinsried, Germany) for fine HLA-typing analyses in an anonymous format.

3.4.16 TCR sequence analysis

3.4.16.1 cDNA synthesis and RACE-PCR

The rapid amplification of cDNA ends PCR (RACE-PCR) was used to analyze TCR sequences of single-cell-sorted T cell clones. The ‘RACE-ready’ cDNA was synthetized starting from total RNA using the SMARTer™ RACE cDNA Amplification Kit according to the manufacturer’s protocol (see section 3.4.11 for total RNA isolation).

This kit is based on the joint action of the SMARTScribe Reverse Transcriptase (RT) and SMARTer II A Oligonucleotide. The SMARTScribe RT exhibits terminal transferase activity upon reaching the end of the RNA template, adding 3–5 residues to the 3' end of the first-strand cDNA. The SMARTer oligo contains a terminal stretch of modified bases that anneal to the extended cDNA tail, allowing the oligonucleotide to serve as a template for the SMARTScribe RT. The SMARTScribe RT switches templates from the mRNA molecule to the SMARTer oligo, generating a complete cDNA copy of the original RNA with the additional SMARTer sequence at the end.

The generated first-strand cDNA was used directly for 5' RACE-PCR reactions using the Advantage® 2 PCR kit according to the manufacturer's instructions. To amplify the cDNA encoding TCRα and TCRβ chains, two gene-specific 5' primers were designed to bind the constant region of each TCR chain (5' TRAC and 5' TRBC primers). All TCRα chains have the same constant region, while there are two possible constant regions for the TCRβ chain (Cβ1 or Cβ2). Cβ1 or Cβ2 are very similar, therefore the gene-specific 5' primer for the C region of the TCRβ chain was designed to detect both Cβ1 and Cβ2. Primer sequences are listed in material section 2.8 table 17. Two separated 5' RACE PCR were performed for each sample to identify both TCRα and TCRβ chain sequences (see table 31 for 5' RACE PCR program). One reaction included the 5' primer TRAC and the other included the 5' primer TRBC. The universal primer mix (UPM) binding to the SMARTer sequence at the end of the cDNA was used as 3' primer for both reactions. A second round of amplification was performed (5' nested RACE-PCR) using "nested" 5' primers (see table 32 for 5' nested RACE-PCR program). PCR products were loaded into a 1% agarose gel and separated by electrophoresis. Subsequently, QIAquick® Gel Extraction Kit was used for the extraction of DNA fragments from agarose gel according to manufacturer´s instructions (see section 3.4.2). DNA fragments were sequenced using 5' primer TRAC and 5' primer TRBC.

Table 31. 5' RACE-PCR program

Cycles	Time (seconds)	Temperature (°C)
5	30	94
	180	72
5	30	94
	30	70
	180	72
40	30	94
	30	68
	180	72
_	240	72

Table 32. Nested 5' RACE-PCR program

Cycles	Time (seconds)	Temperature(°C)
	30	94
30	30	68
	180	72
-	240	72

3.4.16.2 TCR reconstruction

Isolated TCRα and TCRβ sequences were reconstructed in adequate plasmids to allow TCR transgenic expression in recipient cells. Therefore, a two-gene expression cassette was designed (figure 2). The TCRβ chain was linked to the TCRα chain by a "self-cleaving" 2A peptide (P2A element) and subsequently the construct was cloned using a traditional cloning procedure into pGEM and pES12.6 recipient plasmids as described in section 3.4.5. The IMGT database was used to identify the specific V regions (TRAV and TRBV) and the joining (J) regions (TRAVJ and TRBVJ) of the TCRα and TCRβ chains.

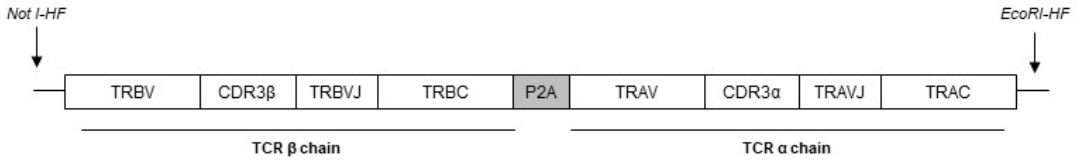


Figure 2. Two-gene cassette designed for TCR reconstruction. The TCRβ chain (TRBV, CDR3β, TRBVJ, TRBC) was linked to the TCRα chain (TRAV, CDR3α, TRAVJ, TRAC) by a P2A element. The TCR construct was synthesized and cloned into a shuttle plasmid by Thermo Fisher Scientific. The shuttle plasmid containing the reconstructed TCR and the appropriate recipient plasmid (pGEM or pES12.6) were digested with the same combination of restriction enzymes (*Not I-HF* and *EcoRI-HF*) and subsequently ligated by T4 Ligase.

3.4.17 Nanostring nCounter

Nanostring nCounter technology is based on digital detection and direct molecular barcoding of target molecules through the use of colour-coded probe pair. The nCounter gene expression assay is designed to provide an ultra-sensitive, reproducible and highly multiplexed method for gene expression profiling. This assay provides a method for direct detection of mRNA molecules without the use of reverse transcription or amplification. The target-specific probe (100 nt) includes a reporter probe (50 nt) and capture probe (50 nt) that hybridize to the target sequences of interest, forming a tripartite complex. The report probe is linked to the colour code signal for digital detection, while the capture probe includes a biotin moiety on the 3' end for target/probe complex immobilization.

First, the probes and the target molecules hybridize in solution forming target/probe complexes. Wash steps are performed for removing excess probes and non-target cellular

transcripts. Second, after washing, the target/probe complexes are insert in a cartridge and immobilized for data collection.

Nanostring nCounter analyses were performed by Nanostring Headquarters facility (Seattle, USA). This technology was utilized to investigate NY-ESO-1/LAGE-1 mRNA expression in several tumor cell lines. Total RNA was isolated from tumor cell lines and utilized as starting material (see section 3.4.11 for total RNA isolation). Two target-specific probes were designed to detect NY-ESO-1 mRNA (see section 2.8 table 19 for probe sequences). The first probe (probe 1) was designed to target a sequence present in NY-ESO-1, but not included in any isoform of the highly homologous tumor antigen LAGE-1 (LAGE-1a and LAGE-1b). In contrast, the second probe (probe 2) was designed to target a region that included the sequence encoding the known immunogenic epitope NY-ESO-1/LAGE-1a$_{157-165}$. Therefore, NY-ESO-1 and LAGE-1a mRNA transcripts comprising the sequence encoding the known epitope were both detected by the second probe.

4 Results

4.1 Isolation of NY-ESO-1-specific CD8⁺ T cells

As an overview, the following steps were performed to induce, expand and separate NY-ESO-1-specific CD8⁺ T cells and corresponding TCRs using an HLA-A2-allogeneic priming approach (figure 3). Mature DCs (mDCs) from an HLA-A2-negative healthy donor were generated *in vitro* within eight days following the Jonuleit maturation protocol [104]. mDCs were transfected with *ivt*RNA encoding the allogeneic HLA-A2 molecule (HLA-A*02:01 allele) and the full-length NY-ESO-1 antigen in order to induce and expand autologous CD8-enriched T cells specific for NY-ESO-1. Subsequently, NY-ESO-1-specific T cells were separated as single cells by FACS using an innovative sorting strategy based on the activation-induced cell surface marker CD137. Two weeks later, the first functional screening was performed to evaluate antigen specificity of expanded T cell clones. TCR sequence of a confirmed NY-ESO-1-specific T cell clone was analyzed and reconstructed for transgenic expression in recipient cells to allow characterization of the properties of the identified TCR. In the following chapters, each step is described in more detail.

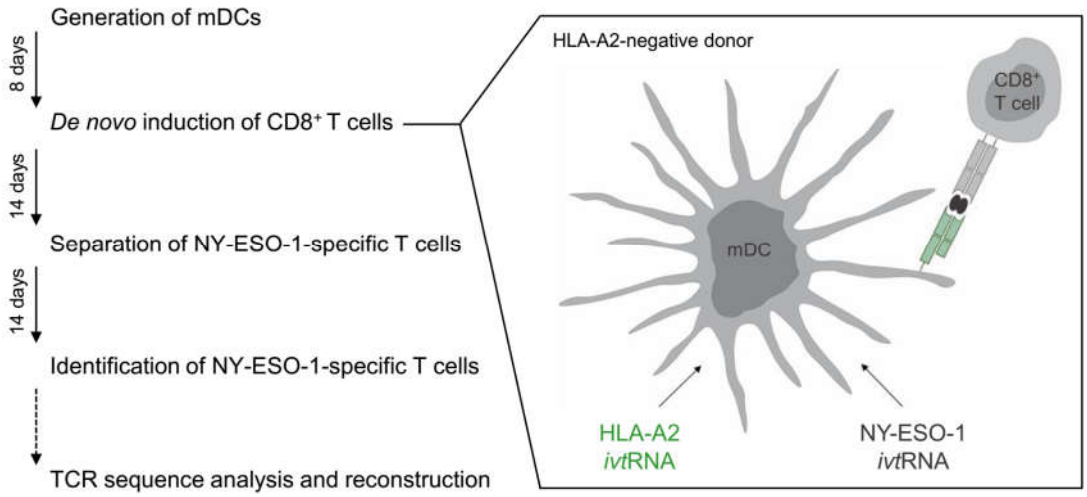


Figure 3. Schematic depiction of the procedure performed to isolate NY-ESO-1-specific T cells and corresponding TCRs. The isolation of NY-ESO-1-specific T cells using an HLA-A2-allogeneic priming approach comprised several steps: mDC generation, *de novo* induction of CD8⁺ T cells, separation of NY-ESO-1-specific T cells, identification of NY-ESO-1-specific T cells, TCR sequence analysis and reconstruction for characterization in a transgenic setting.

4.1.1 Generation of mDCs *in vitro*

mDCs are the most potent APCs for the stimulation of *naïve* T cells [105]. To generate mDCs *in vitro*, monocytes derived from an HLA-A2-negative healthy donor were obtained by plate adherence of freshly isolated PBMC, cultured *in vitro* for eight days and matured by using the Jonuleit cocktail [104]. The mDC phenotype required for efficient T cell stimulation was analyzed by flow cytometry. As shown in figure 4, mDCs showed no expression of the monocyte marker CD14, but high expression of the DC maturation marker CD83. The analyzed B7 family ligands (CD86 and CD80) were highly expressed on mDCs, as well as the inhibitory molecule CD274. The chemokine receptor CCR7 and HLA-DR, stained as representative marker for MHC class II molecules, showed high expression after *in vitro* maturation. CD3 as a marker of T lymphocytes was not detected, confirming that there were not residual autologous T cells in the DC population.

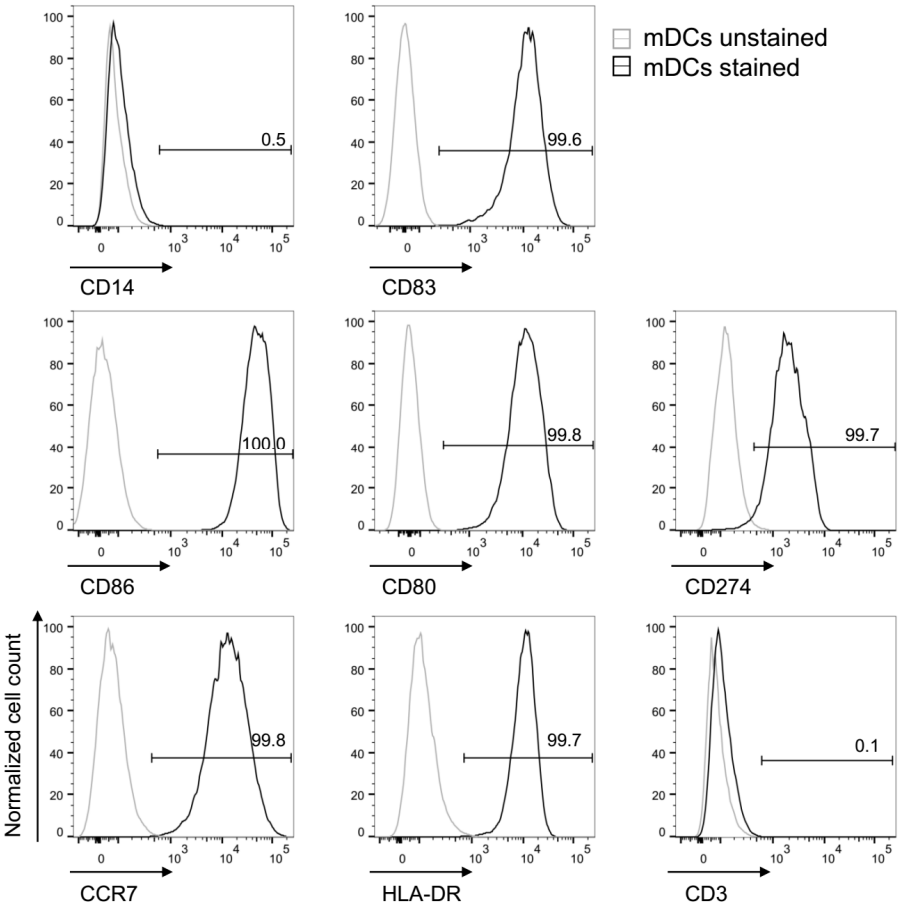


Figure 4. Phenotype of mDCs generated *in vitro*. Cell surface expression of CD14, CD83, CD86, CD80, CD274, CCR7, HLA-DR and CD3 molecules was analyzed by flow cytometry on mDCs generated *in vitro* within eight days using the Jonuleit maturation cocktail. Stained samples are shown in black and corresponding unstained samples in grey. Populations shown are pre-gated on live single cells.

4.1.2 *De novo* induction of CD8$^+$ T cells

mDCs were transfected simultaneously with two *ivt*RNA, one encoding the foreign HLA-A2 molecule and one encoding the full-length NY-ESO-1 antigen. These *ivt*RNA-transfected mDCs were utilized as APCs for induction and expansion of autologous CD8-enriched T cells. As control, *ivt*RNA encoding full-length GFP was transfected in combination with *ivt*RNA encoding the HLA-A2 molecule into the mDCs. Since there were no specific antibodies available for intracellular flow cytometry staining of the NY-ESO-1 protein, HLA-A2- and GFP-transfected mDCs served as an internal control to verify the efficient transfection of two *ivt*RNA simultaneously. Expression of the transgenic HLA-A2 molecules and GFP was analyzed by flow cytometry. As shown in figure 5A, 99.0% of mDCs were positive for HLA-A2 after electroporation with HLA-A2 and NY-ESO-1, proving a good transfection rate. In the control sample, 100% of mDCs were positive for HLA-A2 and 75.0% were double-positive for HLA-A2 and GFP, showing the feasibility of transfecting two *ivt*RNA simultaneously.

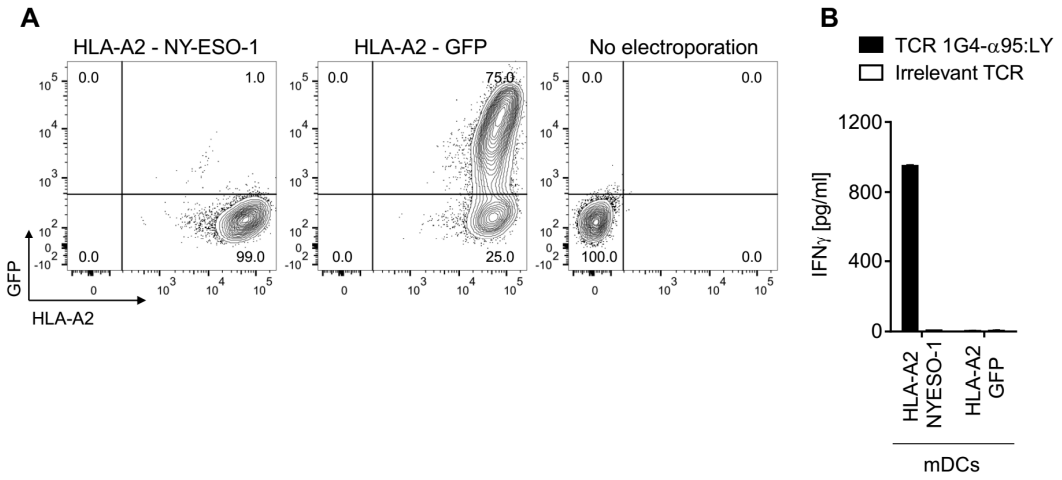


Figure 5. **Evaluation of HLA-A2 and NY-ESO-1 expression in *ivt*RNA-transfected mDCs. (A)** HLA-A2 and GFP expression was detected by flow cytometry after either HLA-A2 and NY-ESO-1 or HLA-A2 and GFP *ivt*RNA electroporation into mDCs generated from an HLA-A2-negative donor. mDCs without electroporation were also stained with anti-HLA-A2 antibody and used as background control. Populations shown are pre-gated on live single cells. **(B)** HLA-A2- and NY-ESO-1-transfected mDCs and HLA-A2- and GFP-transfected mDCs were tested in a co-culture assay to verify their capacity to stimulate NY-ESO-1-specific T cells to release IFN-γ. T cells expressing either TCR 1G4-α95:LY (HLA-A2-restricted NY-ESO-1/LAGE-1a$_{157-165}$-specific TCR) or an irrelevant TCR (negative control) were used as effector cells. IFN-γ release was measured 24h after setting up the co-culture by ELISA. Shown is the mean value of duplicates with standard deviations.

The stimulatory capacity of transfected mDCs and their ability to translate, process and present peptides derived from the introduced NY-ESO-1 *ivt*RNA on foreign HLA-A2 molecules by internal cellular pathways was verified by testing their recognition by T cells. Therefore, *ivt*RNA-transfected mDCs were co-cultured with either transgenic T cells expressing an HLA-A2-restricted NY-ESO-1/LAGE-1a$_{157-165}$-specific TCR (TCR 1G4-α95:LY) or transgenic T cells expressing an HLA-A2-restricted irrelevant TCR serving as negative control [58], [90]. After 24h of incubation, IFN-γ release was analyzed by ELISA (figure 5B). TCR 1G4-α95:LY-transgenic T cells recognized mDCs transfected with HLA-A2 and NY-ESO-1, but not mDCs transfected with HLA-A2 and GFP. T cells expressing the irrelevant TCR were not activated after incubation with any mDCs. These results showed that mDCs generated *in vitro* were able to process and presented a peptide derived from the introduced NY-ESO-1 *ivt*RNA on the HLA-A2 molecules and to stimulate T cells expressing a HLA-A2-restricted NY-ESO-1-specific TCR to release IFN-γ.

The priming was started with as many CD8$^+$ T cells as possible in order to enlarge the starting CD8$^+$ T cell repertoire for the induction of NY-ESO-1 specific T cells. Therefore, autologous CD8$^+$ T cells were enriched by depleting other cell populations in freshly isolated PBMC. To assess the purity of the enriched CD8$^+$ T cell population and the efficiency of the separation process, the expression of CD3 and CD8 molecules was evaluated and compared before and after CD8-enrichment. As shown in figure 6, the sample prior to separation included 20.1% of CD3$^+$CD8$^+$ cells, while after enrichment the population was composed of an increased number of CD3$^+$CD8$^+$ cells (76.1%), confirming a CD3$^+$CD8$^+$ cell enrichment. Subsequently, CD8-enriched T cells were induced and expanded using NY-ESO-1 and HLA-A2 *ivt*RNA-transfected mDCs as stimulator cells for 14 days.

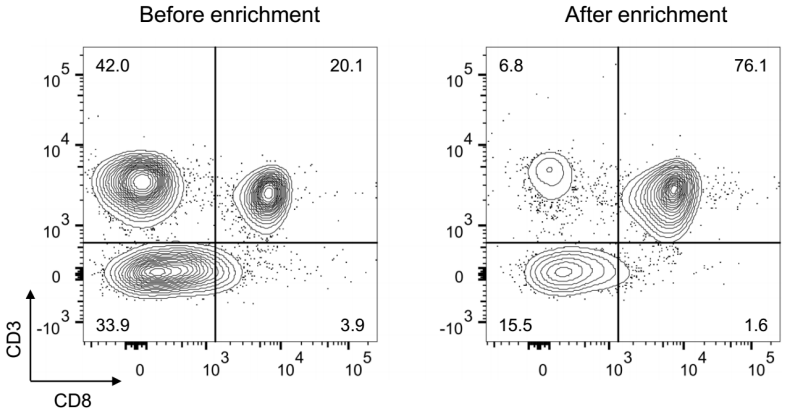


Figure 6. **Frequency of CD3$^+$CD8$^+$ cells before and after CD8-enrichment**. CD8$^+$ T cells were enriched by depleting other cell populations in freshly PBMC isolated from the HLA-A2-negative healthy donor that was selected for performing the priming procedure. The ratio of CD3$^+$CD8$^+$ cells was investigated within the live single cell population by flow cytometry before and after CD8-enrichment.

4.1.3 Separation of NY-ESO-1-specific T cells

NY-ESO-1-specific T cells needed to be selectively separated as single cell and further expanded before performing the first functional screening in order to enable the identification of T cells expressing TCRs specific for NY-ESO-1 within the primed lymphocyte culture. Thus, a sorting method based on the cell surface marker CD137 expressed on T cells upon antigen-specific stimulation was selected to separate NY-ESO-1-specific T cells. CD137-mediated sorting has been shown to be an effective technique for the isolation of $CD8^+$ T cells specific for various tumor antigens [99]–[101]. This strategy allows the isolation of antigen-specific T cells irrespective of known immunogenic epitopes and facilitates the isolation of T cells recognizing all potential immunogenic peptides derived from a selected tumor antigen. Therefore, it represented the best choice to fully exploit the potential of using *ivt*RNA encoding the full-length NY-ESO-1 protein for T cell induction. However, two critical aspects needed to be considered for setting up a CD137-based sorting strategy in the context of an allogeneic priming.

One aspect was the selection of appropriate APCs to be used for NY-ESO-1-specific stimulation to induce CD137 expression on T cells. To address this issue, K562 cells, a tumor cell line endogenously negative for MHC class I and II molecules and for NY-ESO-1, were stably transduced with HLA-A2 molecule and the CD86 co-stimulatory molecule and subsequently transfected with *ivt*RNA encoding the full-length NY-ESO-1. K562 cells expressing CD86 molecules were used to enhance and sustain T cell clone survival after single cell sorting. The advantage of using K562 cells was that these cells allowed the stimulation and consequently isolation of T cells restricted only to the transduced allogeneic HLA-A2 molecules and specific for NY-ESO-1 peptides naturally processed and presented by a tumor cell line. Moreover, being negative for endogenous MHC class I and II molecules, no activation of allo-reactive T cells recognizing foreign MHC molecules other than the introduced HLA-A2 molecules was expected.

The second critical aspect to be considered was that the primed T cell culture contained, as an unavoidable consequence of the HLA-A2-allogeneic priming, a high number of HLA-A2-allo-reactive T cells. These T cells recognize the HLA-A2 molecules independently of NY-ESO-1-derived peptides. Thus, K562 cells expressing HLA-A2 and NY-ESO-1, required for stimulation of the desired NY-ESO-1-specific T cells, will also stimulate the large portion of HLA-A2-allo-reactive T cells to express CD137. An innovative sorting approach, called ‘double-sorting’, based on the CD137 activation marker and K562 stimulator cells, was developed and tested for the first time in this project to reduce the stimulation of HLA-A2-allo-reactive T cells.

4.1.3.1 Generation and evaluation of K562 cells as artificial APCs

K562 cells were stably transduced with the HLA-A2 molecule and the CD86 co-stimulatory molecule to generate artificial APCs (aAPCs) to be used as stimulator cells for CD137-mediated sorting. As shown in figure 7A, the transduction rate of K562 cells was evaluated by flow cytometry and 20.1% of the cells were positive for HLA-A2 and CD86. Transduced K562 cells were sorted by FACS to obtain an HLA-A2$^+$ CD86$^+$ high purity cell population. After one week of *in vitro* expansion, sorted K562 cells were analyzed again for HLA-A2 and CD86 expression that resulted in 95.5% of double-positive cells. These cells are hereafter designated as transgenic K562 cells (tgK562).

To verify the stimulatory capacity of tgK562 cells and to investigate their ability to translate, process and present peptides by internal cellular pathways on transgenic HLA-A2 molecules after electroporation with tumor antigen *ivt*RNA, tgK562 cells were transfected with *ivt*RNA encoding either the full-length NY-ESO-1 or GFP and tested in a co-culture assay. TCR 1G4-α95:LY-transgenic T cells and untransduced T cells were used as effectors. Autologous LCL transfected with *ivt*RNA encoding NY-ESO-1 and T2 cells loaded with the peptide NY-ESO-1/LAGE-1a$_{157-165}$, which is the epitope recognized by TCR 1G4-α95:LY, were used as positive target controls. Autologous LCL transfected with *ivt*RNA encoding GFP and T2 cells loaded with the irrelevant peptide Tyrosinase$_{369-377}$ were used as negative target controls. IFN-γ secretion by T cells was evaluated after 24h of incubation with target cells by ELISA. As shown in figure 7B, NY-ESO-1-transfected tgK562 cells efficiently stimulated TCR 1G4-α95:LY-transgenic T cells to release IFN-γ, demonstrating the capacity of tgK562 cells to process and present peptides for T cell recognition. IFN-γ secretion by TCR 1G4-α95:LY-expressing T cells was also observed after incubation with the positive target controls, whereas no IFN-γ was detected in response to the negative target controls. Untransduced T cells were not activated by any targets used in the assay. According to these findings, tgK562 cells were used as aAPCs in the following experiments.

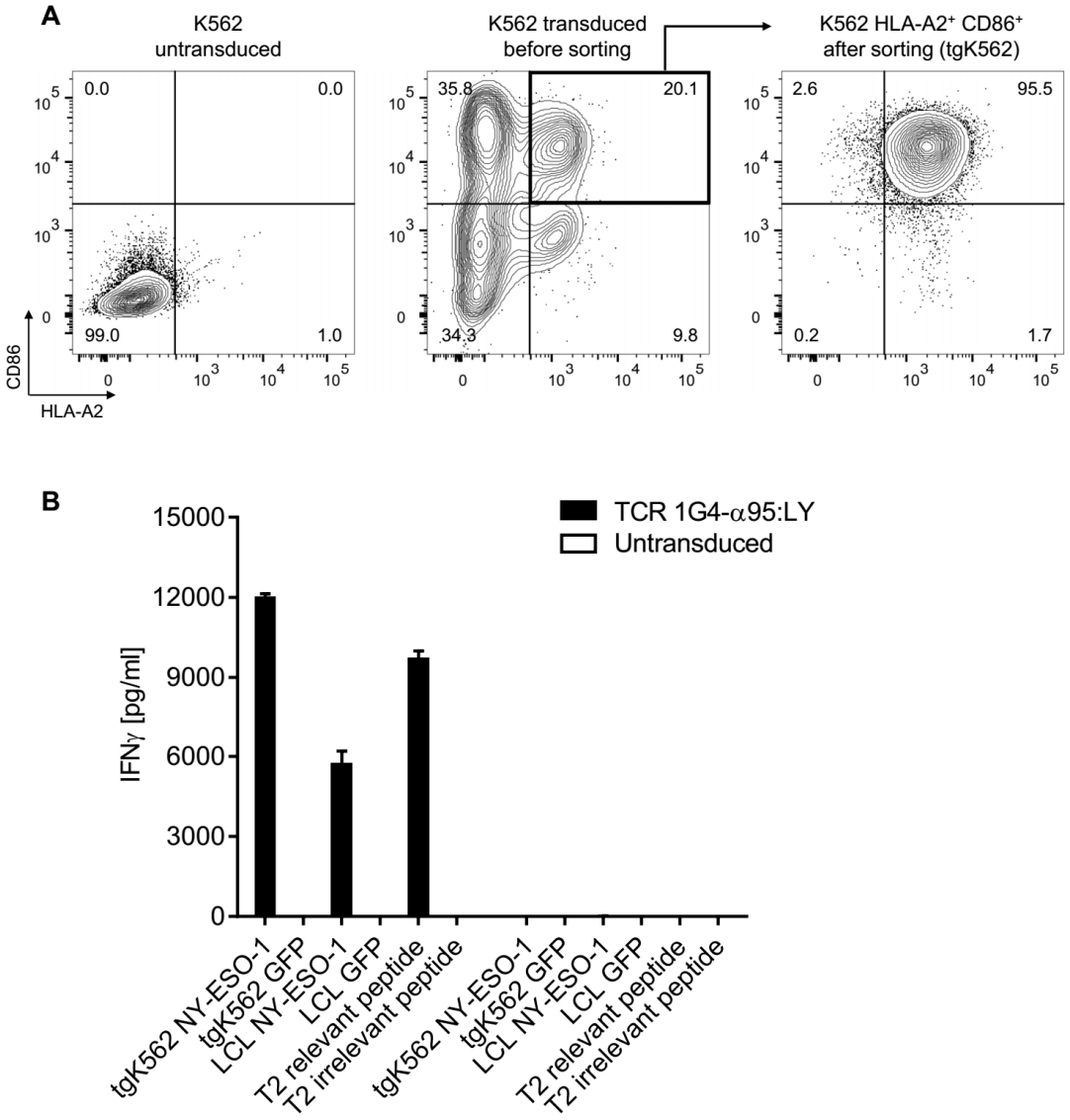


Figure 7. Evaluation of transduction rate and presentation capacity of tgK562 cells. (A) K562 cells were transduced with HLA-A2 molecule and CD86 co-stimulatory molecule to generate aAPCs. The transduction rate was analyzed by flow cytometry and subsequently K562 cells which were double-positive for HLA-A2 and CD86 were sorted by FACS to increase the purity of this population. Untransduced K562 cells were stained with anti-CD86 and anti-HLA-A2 antibodies and used as background control. Populations shown are pre-gated on live single cells. **(B)** After sorting, HLA-A2$^+$ CD86$^+$ K562 cells (tgK562) were transfected with *ivt*RNA encoding either the full-length NY-ESO-1 or GFP and tested in a co-culture assay. TCR 1G4-α95:LY-transduced T cells (HLA-A2-restricted NY-ESO-1/LAGE-1a$_{157-165}$-specific TCR) and untransduced T cells were used as effectors. Autologous LCL transfected with NY-ESO-1 *ivt*RNA and T2 cells loaded with peptide NY-ESO-1/LAGE-1a$_{157-165}$ (epitope recognized by TCR 1G4-α95:LY, relevant peptide) were used as positive target controls, while autologous LCL transfected with GFP *ivt*RNA and T2 cells loaded with an irrelevant peptide (Tyrosinase$_{369-377}$) served as negative target controls. IFN-γ secretion of T cells was evaluated after 24h of incubation with target cells by ELISA. Shown is the mean value of duplicates with standard deviations.

4.1.3.2 Double sorting: 2-step-sorting procedure based on CD137 marker

An innovative 2-step-sorting procedure ('double-sorting') was developed based on the CD137 activation-induced marker and tgK562 cell stimulation in order to isolate NY-ESO-1-specific T cells. This new approach combined the sorting of T cells expressing CD137 upon NY-ESO-1-specific stimulation with a previous step to reduce the isolation of HLA-A2-allo-reactive T cells. As shown in figure 8, in the first step a NY-ESO-1-unspecific stimulation of the primed T cells was performed using irradiated water-electroporated tgK562 cells at an effector to target (E:T) ratio of 2:1 (NY-ESO-1-unspecific stimulation). Kinetic experiments showed that the maximal CD137 expression on T cells was obtained between 12h and 20h after stimulation with tgK562 cells (data not shown). Accordingly, stimulated T cells were stained for CD137 and CD8 cell surface expression 12h after activation and the CD137-negative CD8-positive fraction (44.8%) was sorted by FACS (CD137-negative cell sorting). The aim of the NY-ESO-1-unspecific stimulation and of the CD137-negative cell sorting step was to remove those unwanted T cells which react towards the HLA-A2 molecule independently of the chosen target antigen on tgK562 cells by enriching the CD137-negative fraction including NY-ESO-1-specific T cells. The unwanted CD137-positive fraction was discarded, whereas the CD137-negative fraction was subjected to a second stimulation, this time using irradiated NY-ESO-1 *ivt*RNA-transfected tgK562 cells at an E:T ratio of 5:1 (NY-ESO-1-specific stimulation). After 12h, stimulated T cells were stained for CD137 and CD8 cell surface expression and the CD137-positive CD8-positive fraction was sorted (1.3%) by FACS as single cells and immediately seeded into 96-well culture plates (CD137-positive cell sorting). Single-cell-sorted T cells (600 clones) were expanded for 14 days using irradiated feeder cells, anti-CD3 antibody and IL-2.

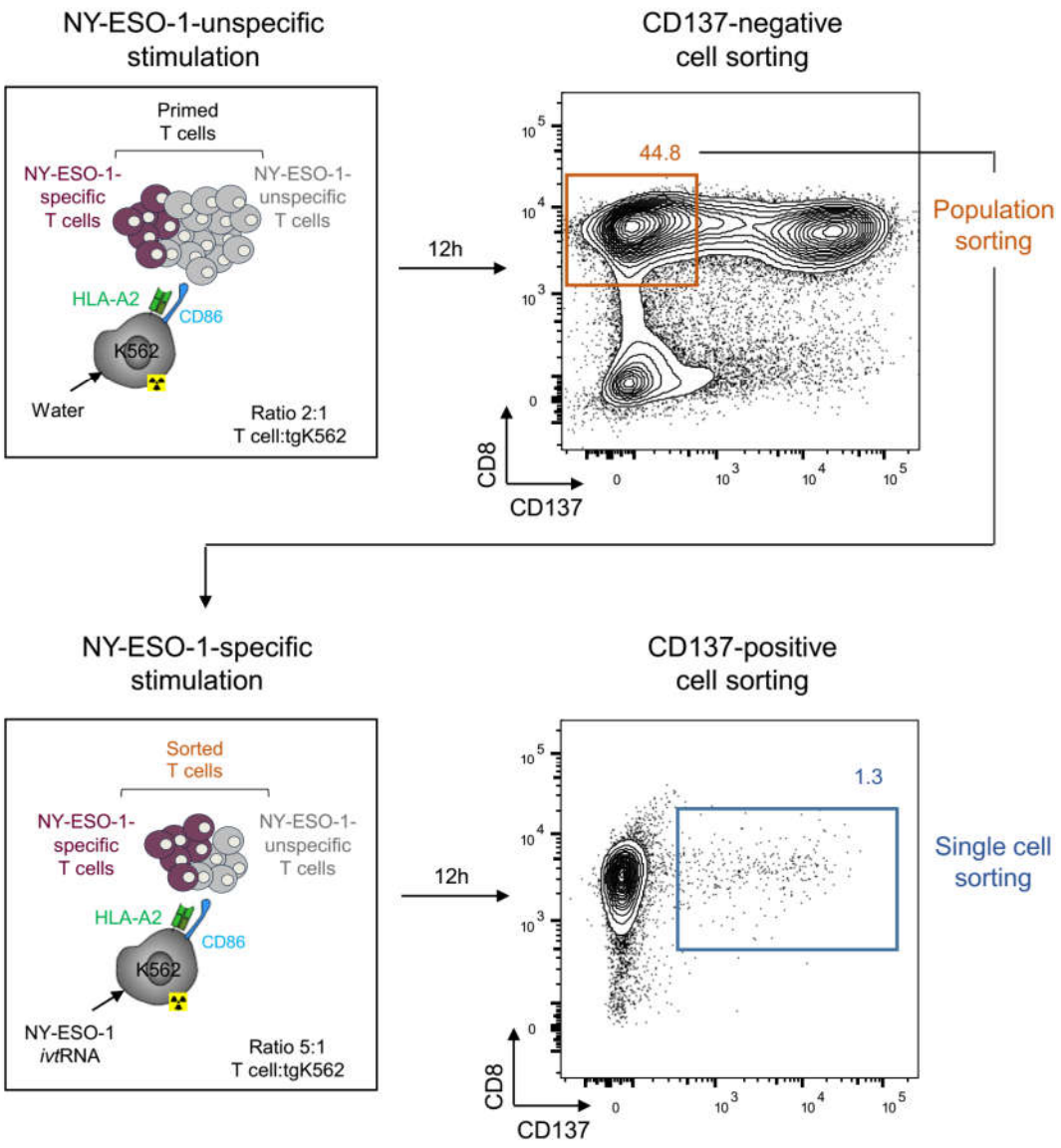


Figure 8. Double-sorting strategy based on the CD137 activation marker and tgK562 stimulator cells. Primed T cells were stimulated using irradiated water-electroporated tgK562 cells at an E:T ratio of 2:1 (NY-ESO-1-unspecific stimulation) and stained after 12h for CD137 and CD8 surface expression. The CD137-negative CD8-positive fraction was sorted by FACS (CD137-negative cell sorting) and subsequently stimulated using irradiated NY-ESO-1 *ivt*RNA-transfected tgK562 cells at an E:T ratio of 5:1 (NY-ESO-1-specific stimulation). CD137 and CD8 staining was performed 12h after stimulation and CD137-positive CD8-positive T cells were sorted by FACS as single cells (CD137-positive cell sorting). Populations shown are pre-gated on live single cells.

4.1.4 Identification of NY-ESO-1-specific T cell clones

Expanded T cell clones (301 expanded clones out of 600 sorted cells) were assessed for IFN-γ release upon stimulation with tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1 (positive target) or GFP (negative target) to identify HLA-A2-restricted NY-ESO-1-specific T cells. Screening results for 15 exemplary clones are shown in figure 9. T cell clones were divided in three groups according to their reaction patterns: clones showing no recognition of any target cell (152 non-reactive clones), clones recognizing HLA-A2 molecules independently of NY-ESO-1 (144 HLA-A2-allo-reactive clones) and clones releasing IFN-γ only after NY-ESO-1-specific stimulation (5 NY-ESO-1-reactive clones). Among the NY-ESO-1-specific clones, T cell clone 5-271 showed the highest amounts of IFN-γ secretion in the presence of NY-ESO-1-presenting tgK562 cells, but no activation after GFP-transfected tgK562 cell stimulation.

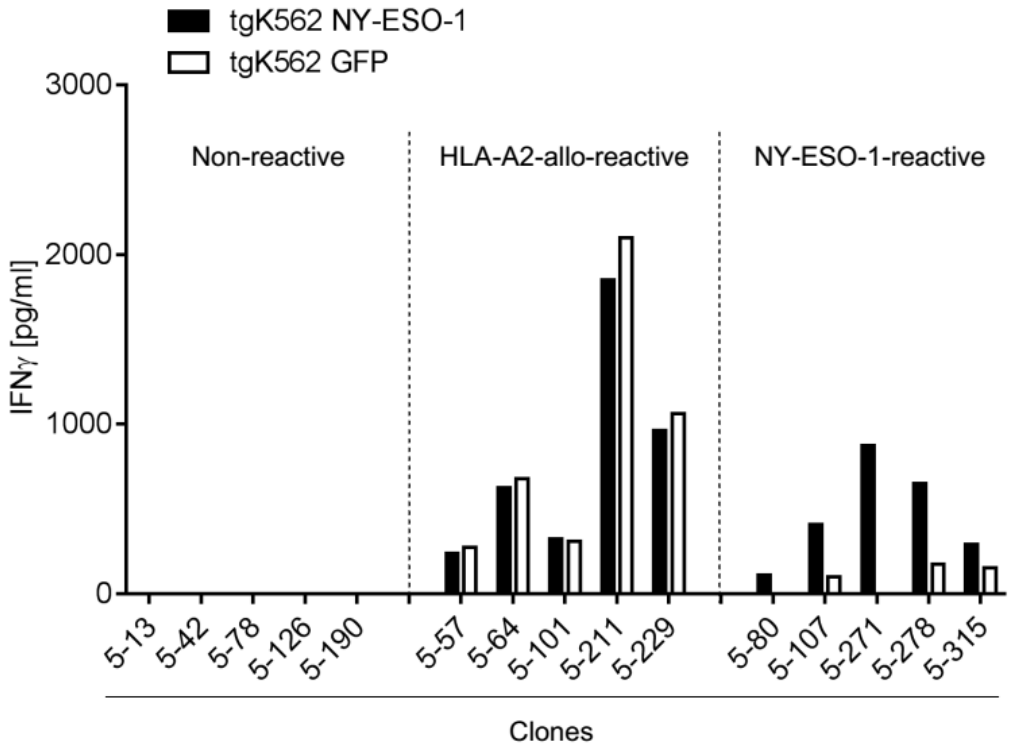


Figure 9. Screening results of expanded clones to identify T cells with NY-ESO-1 specificity. Screening results of 15 exemplary clones sorted by FACS using the CD137-mediated double sorting approach. IFN-γ release by T cell clones was assessed by ELISA after 24h co-culture with tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1 (positive target) or GFP (negative target). T cell clones were divided in three groups according to their reactivity patterns: non-reactive, HLA-A2-allo-reactive and NY-ESO-1-reactive. Data are shown in single for each clone.

4.1.5 TCR 5-271 sequence analysis and reconstruction

The antigen specificity of each T cell clone is defined by its individual TCR. Thus, TCR sequences of the five NY-ESO-1-specific T cell clones were analyzed by RACE-PCR. Unfortunately, due to limited amounts of starting material, the unique TCR sequence was successfully obtained only for the clone 5-271. Sequencing results of TCRα and TCRβ chains are summarized in table 33.

Table 33. TCR sequence analysis of NYESO-1-specific T cell clone 5-271.

TCRα chain	CDR3 region	TCRβ chain	CDR3 region
TRAV21*01 TRAJ6*01	CAVSPQWGGSYIPTF	TRBV6-5*01 TRBJ2-7*01	CASSYLRGGGYEQYF

The variable and junctional gene segments of the TCR 5-271 are indicated in IMGT nomenclature. TRAV, TCR alpha variable segment; TRAJ, TCR alpha joining segment; TRBV, TCR beta variable segment; TRBJ, TCR beta joining segment.

In order to evaluate the expression and the functionality of the identified TCR 5-271 in a transgenic setting and to fully characterize the properties of the TCR, the TCRα and TCRβ sequences were reconstructed in expression vectors (pGEM and pES12.6). For the TCR 5-271 reconstruction, the codon usage in the TCR encoding nucleotide sequences was optimized to enhance protein translation [106]. For both the TCRα and TCRβ chain, critical amino acids of the human constant (C) regions were exchanged for corresponding murine C region counterparts to enforce the pairing of the transgenic TCRα and TCRβ chains over the mixed pairing with the T cell's endogenous TCR [107]. Moreover, one cysteine was introduced into the C regions of each TCR 5-271 chain to allow the formation of an additional disulfide bond between the C regions of the TCR 5-271 α and β chains, aiming to further reduce mixed pairing between endogenous and exogenous TCR chains [108]. To obtain simultaneous and equal expression of TCRα and TCRβ chains, both TCR sequences were inserted into the same vector whereby the TCRβ chain was linked to the TCRα chain by a "self-cleaving" 2A peptide (P2A element) [109]. Subsequently, the construct was cloned into the pGEM and pES12.6 plasmids to achieve transgenic TCR expression in recipient cells by either *ivt*RNA transfection or retroviral transduction.

4.2 Functional characterization of TCR 5-271-expressing T cells

An extensive *in vitro* characterization in terms of specificity and functionality was performed for the isolated and reconstructed HLA-A2-restricted NY-ESO-1-specific TCR 5-271 in a transgenic setting. As a positive control, TCR 1G4-α95:LY was used for all characterization assays. TCR 1G4-α95:LY is a melanoma patient-derived affinity-enhanced TCR that recognizes the peptide NY-ESO-1/LAGE-1a$_{157-165}$ in complex with HLA-A2 molecules. This TCR has been well-characterized by *in vitro* and *in vivo* studies and already tested in clinical trials for synovial cell sarcoma, melanoma, multiple myeloma, ovarian cancer and non-small cell lung cancer with promising results [56]–[58], [90]

4.2.1 TCR 5-271 expression in recipient T cells

Transfer of TCRs into recipient T cells not only serves to fully characterize potential candidate TCRs when the original clone is not available, but also to prove transgenic TCR expression and functionality required for later clinical applicability. The TCR 5-271 and the positive control TCR 1G4-α95:LY were expressed in different types of recipient cells, i.e. effector T cells and CD8-enriched effector T cells. Well-established retroviral transduction and *ivt*RNA electroporation approaches were selected as TCR transfer methods. To assess the successful TCR transfer, the percentage of T cells expressing either TCR 5-271 or TCR 1G4-α95:LY was analyzed by flow cytometry. As both TCRs included the same TRBV chain, the TRBV6-5-specific antibody was used to evaluate the expression of both TCRs. Unmodified T cells were used as control for the endogenous TRBV6-5 chain expression. An example of one representative flow cytometry analysis is shown in figure 10. By subtracting the fraction of endogenously TRBV6-5-expressing T cells (2.9%, untransduced T cells), the percentage of effector cells expressing the transgenic TCR resulted in 43.1% for TCR 5-271 and in 43.6% for TCR 1G4-α95:LY. These data showed that both TCRs were well-expressed after the transfer into recipient T cells.

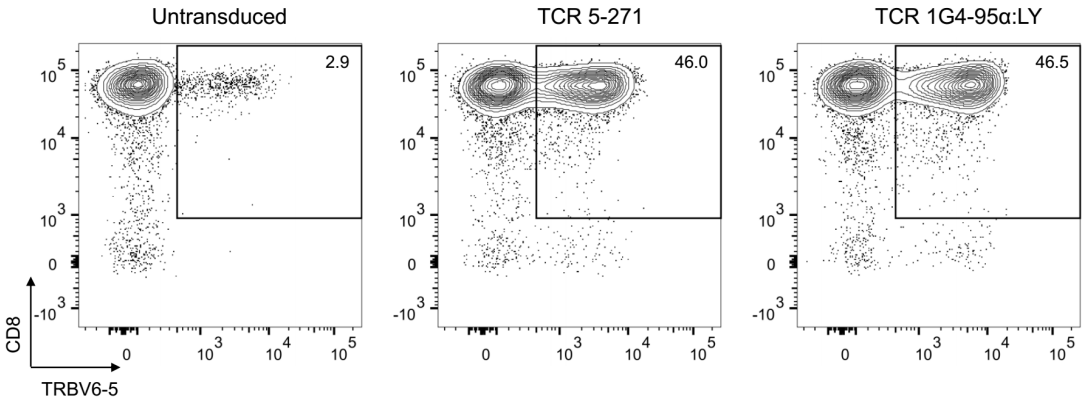


Figure 10. TCR 5-271 surface expression after transfer into recipient T cells. TCR 5-271 and the control TCR 1G4-95α:LY were introduced into recipient T cells by either *ivt*RNA electroporation or retroviral transduction. After TCR transfer, the expression level of the transgenic TCR was determined by FACS using the TRBV6-5 specific antibody. Unmodified recipient T cells were used as control for the endogenous expression of the TRBV6-5 chain. One representative experiment of TCR transfer by retroviral transduction into CD8-enriched T cells is shown. Populations shown are pre-gated on live single cells.

4.2.2 Evaluation of antigen specificity

After TCR transfer and evaluation of TCR expression in recipient T cells, the first step in the TCR evaluation process was to confirm the antigen specificity of TCR 5-271 in the transgenic expression setting. *ivt*RNA encoding TCR 5-271 was introduced into effector T cells derived from a healthy donor. In addition, T cells were also electroporated with *ivt*RNA encoding either TCR 1G4-α95:LY (positive control) or an HLA-A2-allo-reactive TCR (TCR 3-9), the latter was included to assess the expression of the HLA-A2 molecule on targets cells. T cells electroporated with water served as the negative control. Autologous LCL and tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1 or GFP were used as target cells. T2 cells loaded with the TCR 1G4-α95:LY-specific peptide NY-ESO-1/LAGE-1a$_{157-165}$ or the irrelevant peptide MAGE-A1$_{278-286}$ were used as internal target controls for the assay. Moreover, K562 cells untransduced were included as negative target to control the reactivity of the TCR 3-9. After 24h of effector/target co-culture, an ELISA was performed to evaluate IFN-γ secretion by T cells (figure 11). TCR 5-271-transfected T cells recognized LCL and tgK562 cells only when transfected with NY-ESO-1 *ivt*RNA. These findings confirmed the NY-ESO-1 specificity of TCR 5-271 and proved correct TCR 5-271 heterodimer pairing and functionality of the TCRα and TCRβ transgenes in recipient T cells. Interestingly, TCR 5-271 showed a different peptide specificity compared to TCR 1G4-α95:LY because it did not recognize T2 cells loaded with the peptide NY-ESO-1/LAGE-1a$_{157-165}$. TCR 1G4-α95:LY-transfected T cells also recognized LCL and tgK562 cells when they were transfected with NY-ESO-1 *ivt*RNA. Moreover, T2 cells loaded with peptide NY-

ESO-1/LAGE-1a$_{157-165}$ led to the activation of TCR 1G4-α95:LY-transfected T cells. TCR 3-9-transfected T cells secreted IFN-γ after stimulation with all HLA-A2-positive targets used in the assay, confirming the HLA-A2 expression on these cells, whereas they were not activated by wild-type K562 cells that are not expressing HLA-A2 molecules. Water electroporated T cells were not activated in response to any target cells.

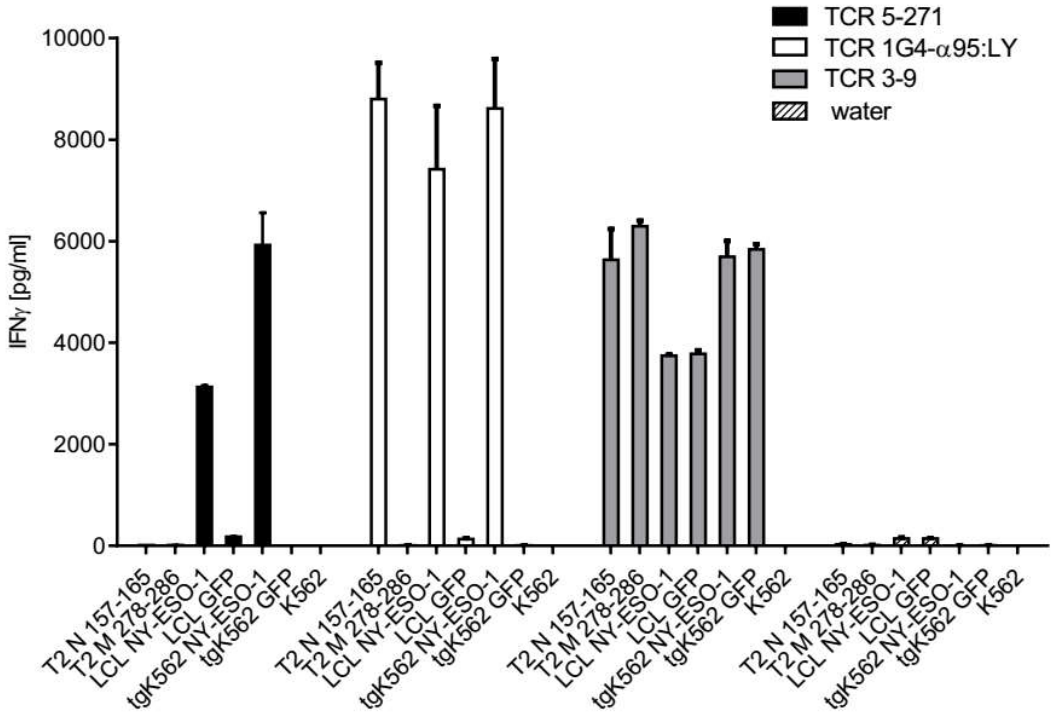


Figure 11. Antigen specificity of TCR 5-271-transgenic T cells. T cells derived from a healthy donor were transfected with *ivt*RNA encoding either TCR 5-271 or TCR 1G4-α95:LY or an HLA-A2-allo-reactive TCR (TCR 3-9). Water-electroporated T cells served as the negative control. Electroporated T cells were incubated with either autologous LCL or tgK562 cells that were transfected with *ivt*RNA encoding either NY-ESO-1 or GFP. Further target cells were T2 cells loaded with either TCR 1G4-α95:LY-specific peptide NY-ESO-1/LAGE-1a$_{157-165}$ (N 157-165) or irrelevant peptide MAGE-A1$_{278-286}$ (M 278-286) and K652 cells not-expressing HLA-A2 molecules (K562). Co-cultures were set-up 3h after electroporation. IFN-γ release of transfected T cells was assessed by ELISA after 24h co-culture. Shown is the mean value of duplicates with standard deviations. This experiment was performed with two different donors. Shown is one representative experiment.

4.2.3 Tumor cell line recognition

To be potentially useful in a clinical setting, it is essential that T cells expressing the candidate TCR are able to specifically recognize tumor cells. In order to select tumor cell lines for testing the recognition mediated by T cells expressing TCR 5-271, the Nanostring nCounter® technology was used to investigate NY-ESO-1 mRNA expression in several tumor cell lines. To note, LAGE-1 is a tumor antigen showing high homology to NY-ESO-1

that comprises two alternatively spliced isoforms (figure 12A). The first isoform is designated as LAGE-1a and encodes a protein of 180 amino acids (aa), while the second one is designated as LAGE-1b and encodes a protein of 210 aa [110]. NY-ESO-1, LAGE-1a and LAGE-1b mRNA transcripts and the corresponding proteins differ in parts of their sequences and in other parts they are identical (identical sequences are shown with an identical pattern in figure 12A). Importantly, the sequence encoding the epitope recognized by TCR 1G4-α95:LY is included both in NY-ESO-1 and in LAGE-1a (NY-ESO-1/LAGE-$1a_{157-165}$). To detect NY-ESO-1 mRNA expression by Nanostring nCounter® technology, two probes were designed. The first probe was designed to target a sequence present in NY-ESO-1, but not included in any isoform of LAGE-1 (probe 1, shown in black in figure 12A). In contrast, the second probe was designed to target a region that included the sequence encoding the immunogenic epitope NY-ESO-1/LAGE-$1a_{157-165}$ (probe 2, shown in grey in figure 12A). Therefore, NY-ESO-1 and LAGE-1a mRNA transcripts comprising the NY-ESO-1/LAGE-$1a_{157-165}$-encoding sequence were both detected by the second probe. Nanostring results for the tumor cell lines showing the highest level of expression for either probe 1 or probe 2 are shown in figure 12B. The analyzed cell lines were all positive for probe 2, while they were either slightly positive or negative for probe 1. Consequently, the tumor cell lines were all positive for LAGE-1a, while only seven out of 15 were also positive for NY-ESO-1 (MelA375, U266, FM6, MOLP-8, EJM, RPMI-8226 and KMS-12-BM). As LAGE-1a is highly homologous to NY-ESO-1 and the exact location of the epitope recognized by TCR 5-271 was unknown, the 15 tumor cell lines were all included in the assay to evaluate tumor recognition mediated by TCR 5-271. According to data derived from the TRON database (data were confirmed by flow cytometry analyses as shown in figure 13B), within the selected tumor cell lines, nine were endogenously positive for HLA-A2, whereas six did not express HLA-A2 molecules.

T cells derived from a healthy donor were transfected with *ivt*RNA encoding either TCR 5-271 or TCR 1G4-α95:LY or TCR 3-9 and used as effector cells in the co-culture assays to assess tumor cell recognition. Additionally, water was electroporated into T cells as the negative control. The tumor cell line SK-Mel23 (endogenously HLA-A2-positive and NY-ESO-1/LAGE-1a-negative) and K562 cells (endogenously HLA-A2-negative and NY-ESO-1/LAGE-1a-negative) were used as the negative controls. To confirm the capacity of TCR-transfected T cells to secrete IFN-γ after specific stimulation, tgK562 cells transfected with either NY-ESO-1 or GFP *ivt*RNA were included as internal target controls. IFN-γ release was evaluated by ELISA 24h after setting up the effector/target co-cultures.

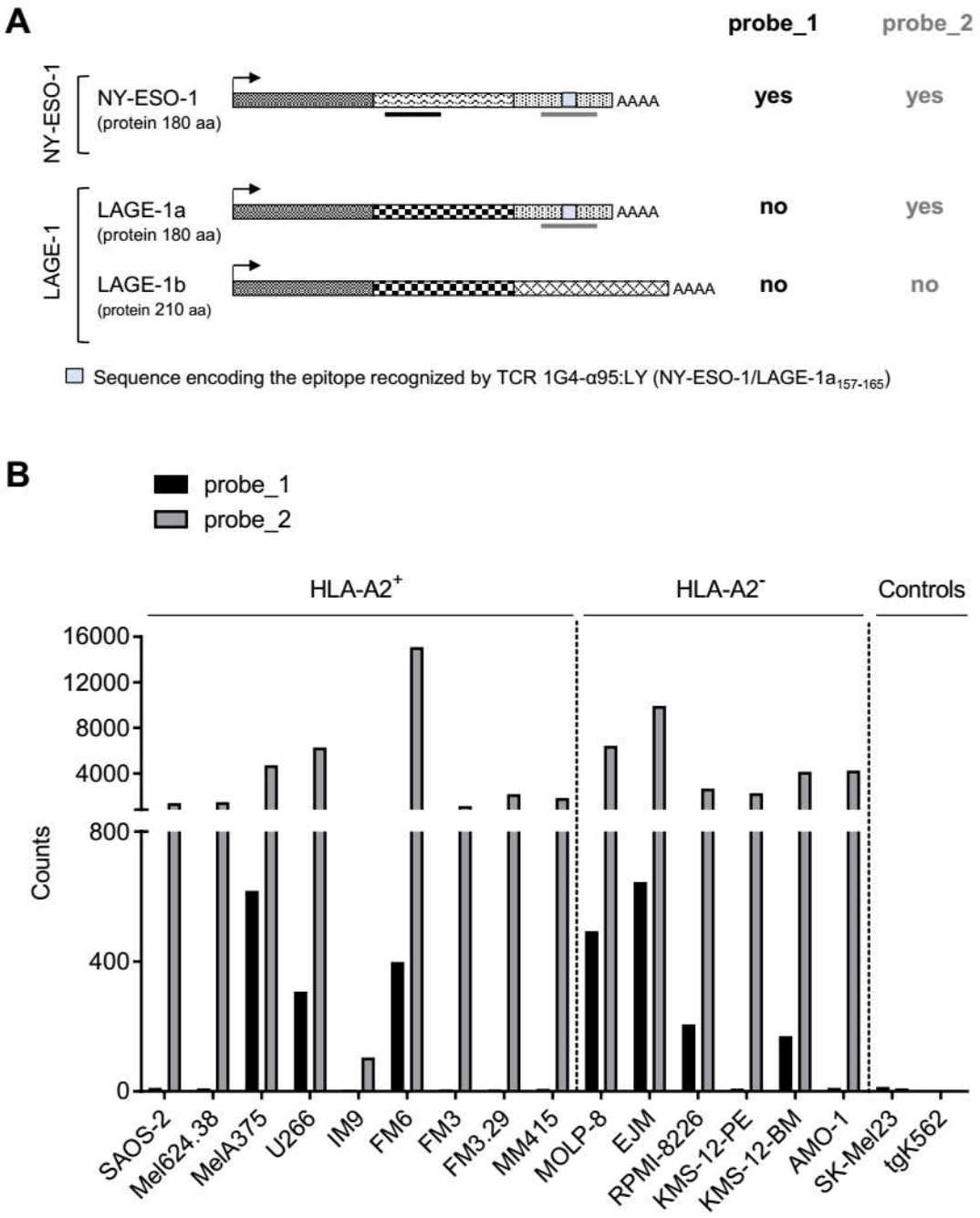


Figure 12. NY-ESO-1/LAGE-1a mRNA expression evaluated by Nanostring nCounter® technology. (A) Schematic depiction of NY-ESO-1 and LAGE-1 mRNA transcripts and of the two probes designed to perform Nanostring nCounter® analyses. Probe 1 (shown in black) targeted a sequence included in NY-ESO-1, but not in any isoform of the highly homologous tumor antigen LAGE-1 (LAGE-1a and LAGE-1b). In contrast, probe 2 (shown in grey) detected a region including the sequence encoding the epitope recognized by TCR 1G4-α95:LY (NY-ESO-1/LAGE-1a$_{157-165}$, shown in blue) which is present in NY-ESO-1 as well as in LAGE-1a transcripts. Identical sequences in mRNA transcripts are shown with an identical pattern. **(B)** Results of NY-ESO-1/LAGE-1a mRNA expression analyses for 15 tumor cell lines (nine HLA-A2$^+$, six HLA-A2$^-$) selected to assess tumor recognition by TCR 5-271-transfected T cells. SK-Mel23 cell line and tgK562 cells (HLA-A2$^+$, NY-ESO-1/LAGE-1$^-$) were included as negative controls. Data obtained using probe 1 (black bars) and probe 2 (grey bars) are shown. Data provided by Nanostring Headquarters facility (Seattle, USA).

As shown in figure 13A (top graph), IFN-γ release of transfected T cells was evaluated after incubation with the nine HLA-A2-positive tumor cell lines. TCR 5-271-transfected T cells did not recognize any tested tumor cell line. Although a slight IFN-γ release was detected after incubation with U266 and IM9 cells, this was due to the reactivity mediated by endogenous TCRs of the T cells and was not related to TCR 5-271-specific recognition since the same level of IFN-γ secretion was detected for T cells electroporated with water. However, TCR 5-271 recognized tgK562 cells expressing NY-ESO-1, proving the capacity of these transfected T cells to release IFN-γ after specific stimulation. In contrast, TCR 1G4-α95:LY-expressing T cells recognized all tested tumor cell lines except for the negative controls. TCR 3-9-transfected T cells released IFN-γ after stimulation with HLA-A2-expressing target cells (FM6, FM3, FM3.29 and MM415 were not analyzed), confirming HLA-A2 expression for the target cell lines.

Since six tumor cell lines were HLA-A2-negative, *ivt*RNA encoding the HLA-A2 molecule was electroporated into these target cells. To verify the capacity of the tumor cell lines to process and present the unknown NY-ESO-1 peptide recognized by TCR 5-271, the same cell lines were also electroporated with *ivt*RNA encoding HLA-A2 in combination with NY-ESO-1. As shown in figure 13A (bottom graph), TCR 5-271-transfected T cells did not recognize any of the HLA-A2-transfected tumor cell line, although they were endogenously positive for NY-ESO-1/LAGE-1a. A slight IFN-γ release was detected after incubation with RPMI-8226 and KM-12-PE cells, which was again due to the reactivity mediated by endogenous TCRs of the T cells and was not related to TCR 5-271-specific recognition since the same IFN-γ secretion was detected for T cells electroporated with water. However, IFN-γ secretion was detected in response to all tumor cell lines electroporated with *ivt*RNA encoding HLA-A2 plus NY-ESO-1, proving that the epitope recognized by TCR 5-271 was efficiently processed and presented by the tested tumor cell lines. In contrast, TCR 1G4-α95:LY-expressing T cells recognized all HLA-A2-transfected tumor cell lines independently of NY-ESO-1 *ivt*RNA transfection and TCR 3-9-transfected T cells released IFN-γ after stimulation with all transfected target cells, confirming HLA-A2 expression after transfection for all samples.

Surface expression of HLA-A2 molecules was analyzed by flow cytometry for all tumor cell lines used in the assays. As shown in figure 13B, the endogenously positive as well as the *ivt*RNA-transfected tumor cell lines were all positive for HLA-A2 molecules.

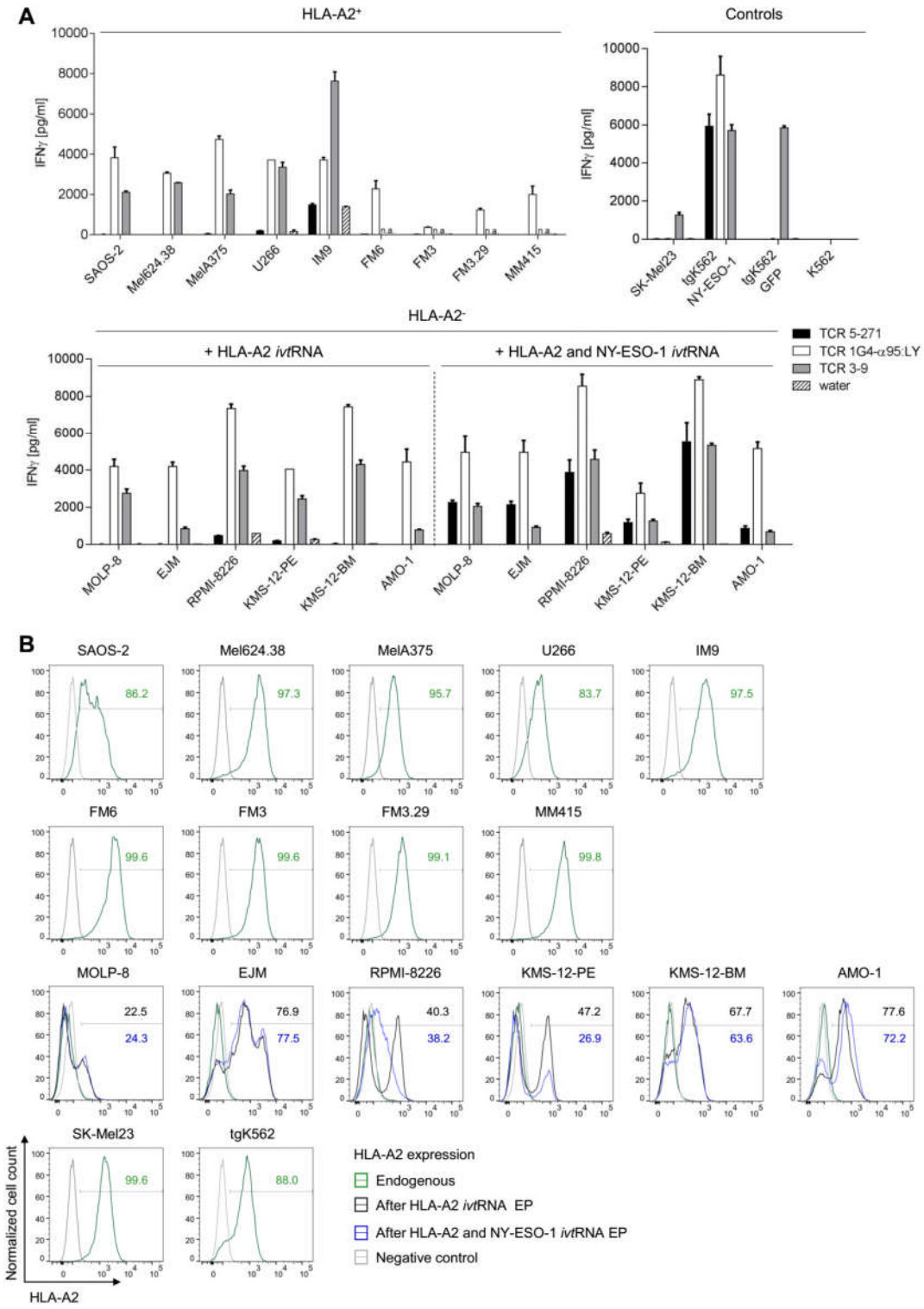


Figure 13. Tumor cell line recognition by TCR 5-271-expressing T cells. (A) T cells derived from a healthy donor were transfected with *ivt*RNA encoding either TCR 5-271 or TCR 1G4-α95:LY or an HLA-A2-allo-reactive TCR (TCR 3-9). T cells electroporated with water were used as a negative control. Electroporated T cells were used as effectors in co-culture assays with 15 tumor cell lines endogenously positive for NY-ESO-1/LAGE-1a according to NanoString data (see figure 12B). Figure legend continues on page 72.

Figure 13. Of the selected lines, nine were endogenously positive for HLA-A2 (top graph), while six were endogenously HLA-A2-negative (bottom graph). Thus, these six cell lines were tested after transfection with *ivt*RNA encoding either HLA-A2 alone (left graph) or in combination with *ivt*RNA encoding NY-ESO-1 (right graph) to verify the capacity of the tumor cell lines to process and present the unknown NY-ESO-1 peptide recognized by TCR 5-271. The SK-Mel23 cell line (HLA-A2$^+$, NY-ESO-1/LAGE-1a$^-$) was used as the negative control. Moreover, tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1 or GFP were included as internal target controls. IFN-γ release of transgenic TCR-expressing T cells was measured by ELISA after 24h co-culture. Shown is the mean value of duplicates with standard deviations. This experiment was performed with two different donors. Shown is one representative experiment. FM6, FM3, FM3.29 and MM415 cell lines were not analyzed (n.a.) for recognition by TCR 3-9-transfected T cells. **(B)** HLA-A2 surface expression was detected by flow cytometry for all tested tumor cell lines. Endogenous expression is shown in green, expression after electroporation (EP) with *ivt*RNA encoding HLA-A2 is shown in black, HLA-A2 expression after EP with *ivt*RNA encoding HLA-A2 and NY-ESO-1 is shown in blue and the negative control (HLA class I-negative K562 cells stained with the HLA-A2-specific antibody) is shown in grey. Populations shown are pre-gated on live single cells.

4.2.4 NY-ESO-1-ORF2 recognition

The wild-type NY-ESO-1 sequence (NY-ESO-1_wt) includes the ORF encoding the full-length NY-ESO-1 protein consisting of 180 aa in the first reading frame (designated as NY-ESO-1-ORF1) and three alternative ORFs in the second and third reading frame encoding proteins consisting of 58 aa, 50 aa and 14 aa (designated as NY-ESO-1-ORF2, -ORF3 and -ORF4, respectively) (figure 14A, left panel). Since the alternative ORFs are located in alternative reading frames compared to NY-ESO-1-ORF1, the corresponding proteins present completely different amino acid sequences compared to the NY-ESO-1-ORF1-encoded protein. After electroporation with *ivt*RNA encoding NY-ESO-1_wt into mDCs, these alternative ORFs might have been translated and the corresponding proteins processed and presented in addition to the full-length NY-ESO-1-ORF1 protein. Consequently, T cells against peptides derived from these alternative NY-ESO-1 proteins might have been induced during the priming process. To verify the antigen specificity of TCR 5-271, a partial-codon-optimized version of NY-ESO-1 (NY-ESO-1_pco) was designed to exclude the alternative ORFs (NY-ESO-1-ORF2, -ORF3 and -ORF4) other than NY-ESO-1-ORF1 and cloned into the pGEM vector (figure 14A, right panel). Additionally, the three alternative NY-ESO-1-ORFs were cloned separately into the pGEM vector. *ivt*RNA was produced for NY-ESO-1-ORF2, -ORF3, -ORF4, NY-ESO-1_pco and NY-ESO-1_wt and then transfected into tgK562 cells. These cells were used to stimulate CD8$^+$ T cells transduced with either TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells were used as a negative control. IFN-γ release was evaluated by ELISA 24h after setting up the co-cultures (figure 14B).

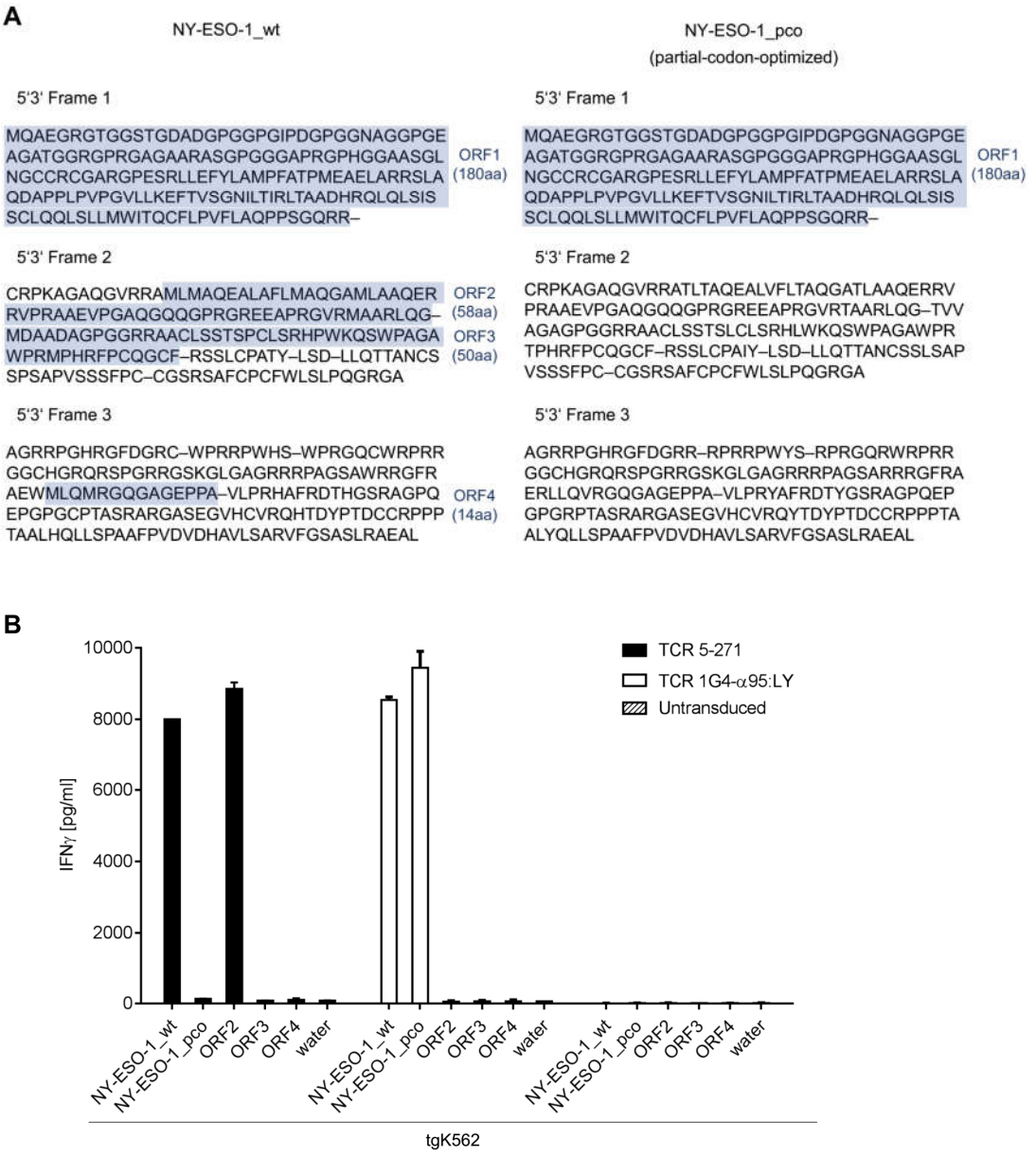


Figure 14. NY-ESO-1-ORF2 recognition by TCR 5-271-transgenic T cells. (A) Schematic depiction of the proteins encoded by alternative ORFs of NY-ESO-1. The wild-type NY-ESO-1 sequence (NY-ESO-1_wt, left panel) includes four different ORFs in the three reading frames encoding proteins of 180 aa (ORF1, 5'3' frame 1), 58 aa (ORF2, 5'3' frame 2), 50 aa (ORF3, 5'3' frame 2) and 14 aa (ORF4, 5'3' frame 3) (ORFs are shown in blue). A partial-codon-optimized (pco) version of NY-ESO-1 (NY-ESO-1_pco, right panel) was designed to eliminate alternative ORFs (ORF2, ORF3 and ORF4) other than ORF1 encoding the full-length NY-ESO-1 protein consisting of 180 aa. Figure wasadapted from the online translate tool ExPASy. **(B)** $CD8^+$ T cells were isolated from a healthy donor and transduced with either TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells served as a negative control. IFN-γ release of TCR-transgenic $CD8^+$ T cells was evaluated after 24h co-culture with tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1_wt, NY-ESO-1_pco, NY-ESO-1-ORF2, -ORF3 or -ORF4. Electroporation of tgK562 cells with water was performed as a negative target control. Shown is the mean value of duplicates with standard deviations. This experiment was performed with two different donors. Shown is one representative experiment.

Interestingly, TCR 5-271-transduced T cells showed IFN-γ secretion after stimulation with tgK562 cells expressing either NY-ESO-1_wt or NY-ESO-1-ORF2, but they were not activated by tgK562 cells transfected with NY-ESO-1_pco or any other NY-ESO-1-ORFs. On the other hand, TCR 1G4-α95:LY-transgenic T cells recognized tgK652 cells transfected with *ivt*RNA encoding NY-ESO-1_wt as well as NY-ESO-1_pco. These results demonstrated that TCR 5-271 was not specific for a peptide derived from the full-length NY-ESO-1-ORF1 protein, but for an epitope present in the 58 aa NY-ESO-1-ORF2 protein. Moreover, they showed that after electroporation with *ivt*RNA encoding NY-ESO-1_wt into tgK562 cells, NY-ESO-1-ORF2 was translated and the corresponding protein processed and presented in addition to the full-length NY-ESO-1-ORF1 protein.

4.2.5 Epitope identification

In order to narrow down the region recognized within the NY-ESO-1-ORF2 protein by TCR 5-271, five constructs were designed encoding 20-mer peptides derived from the NY-ESO-1-ORF2 protein, overlapping by ten aa and linked to GFP (1-20 aa, 11-30 aa, 21-40 aa, 31-50 aa, 41-58 aa) (figure 15A). *ivt*RNA was produced for each construct and used to transfect tgK562 cells. As shown in figure 15B, GFP expression in transfected tgK562 cells was detected for all five constructs, proving successful transfection. A co-culture assay was set up using the transfected tgK562 cells to stimulate $CD8^+$ T cells expressing either TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells served as a negative control. Additionally, *ivt*RNA encoding either NY-ESO-1_wt or NY-ESO-1-ORF2 full-length protein were transfected into tgK562 cells as internal positive controls for the assay and tgK562 cells were electroporated with water as the negative control. IFN-γ ELISA was performed 24h after setting up the co-culture (figure 15C). TCR 5-271-transduced T cells released IFN-γ after incubation with tgK562 cells transfected with *ivt*RNA encoding the 20-mer peptide 1-20 aa of the NY-ESO-1-ORF2 protein, showing that the TCR 5-271 epitope was located in the first 20 aa. *ivt*RNA encoding the other 20-mer peptides of NY-ESO-1-ORF2 (11-30 aa, 21-40 aa, 41-58 aa) did not lead to any activation of TCR 5-271-transduced T cells. As expected, TCR 1G4-α95:LY-transgenic T cells recognized only tgK652 cells transfected with NY-ESO-1_wt.

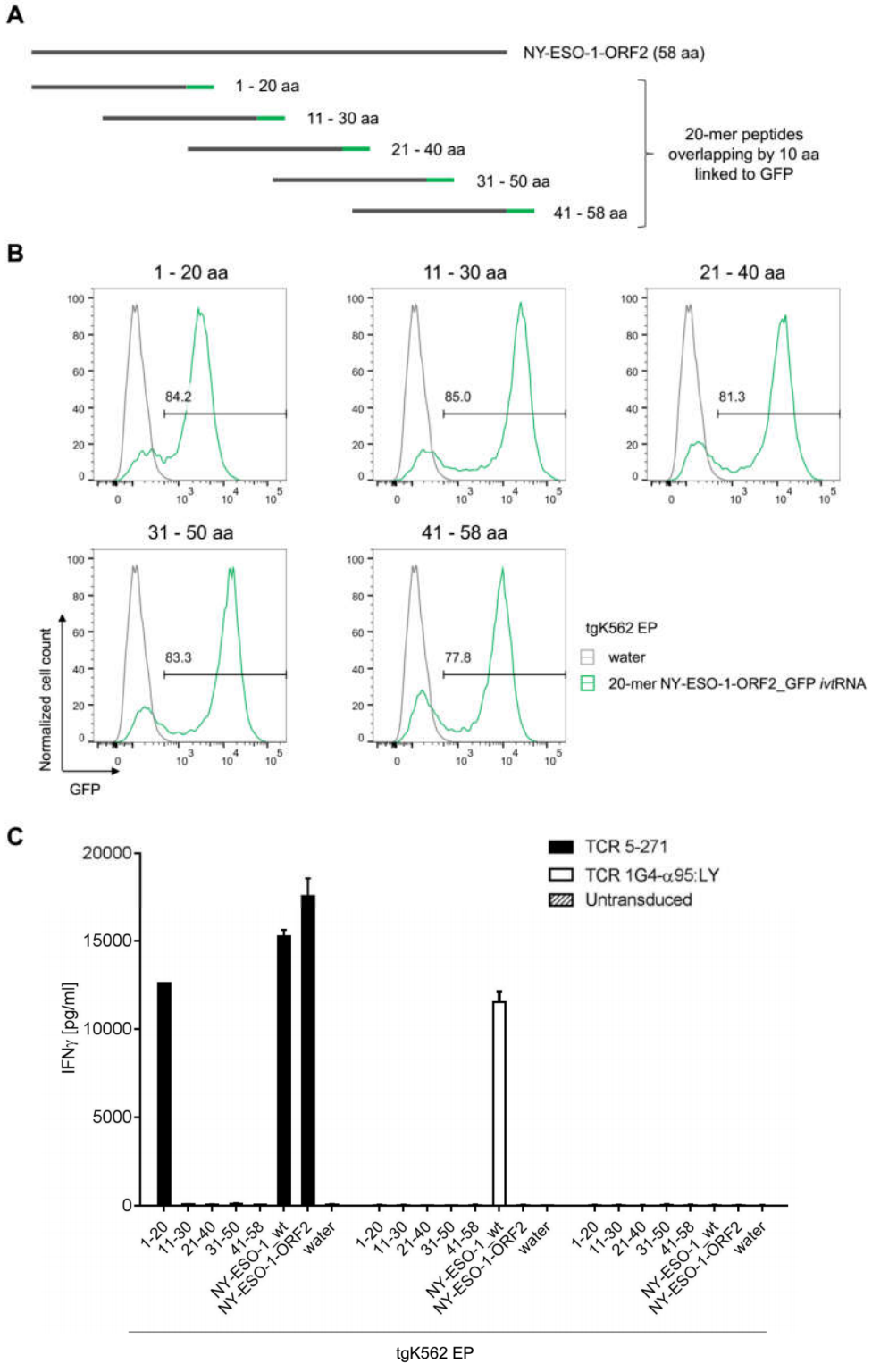


Figure 15. TCR 5-271 epitope identification: NYESO-1-ORF2 first 20 aa. (A) Schematic representation of the five constructs designed to produce *ivt*RNA encoding 20-mer peptides derived from NY-ESO-1-ORF2 protein overlapping by ten aa and linked to GFP (1-20 aa, 11-30 aa, 21-40 aa, 31-50 aa, 41-58 aa). Figure legend continues on page 76.

Figure 15. (B) GFP expression for each construct was detected by flow cytometry after *ivt*RNA electroporation (EP) into tgK562 cells (shown in green). Water-electroporated tgK562 cells were used as the background control (shown in grey). Populations shown are pre-gated on live single cells. **(C)** TCR 5-271- or TCR 1G4-α95:LY-transduced T cells were incubated with tgK562 cells transfected with *ivt*RNA encoding 1-20 aa, 11-30 aa, 21-40 aa, 31-50 aa, 41-58 aa peptides derived from NY-ESO-1-ORF2 protein. In addition, tgK562 cells transfected with *ivt*RNA encoding either NY-ESO-1_wt or NY-ESO-1-ORF2 full-length protein were included as positive controls. Water electroporation into tgK562 cells served as a negative control. IFN-γ ELISA was performed after 24h of co-culture. Shown is the mean value of duplicates with standard deviations. This experiment was performed with two different donors. Shown is one representative experiment.

A group of 14 peptides that bound to HLA-A2 molecules, ranging from 8-mer to 12-mer and covering the first 20 aa of NY-ESO-1-ORF2 protein were selected and subsequently used to assess the core epitope recognized by TCR 5-271 (figure 16A). T2 cells loaded with either these 14 peptides or with an irrelevant peptide (MAGE-A1$_{278-286}$) served as stimulator cells for CD8$^+$ T cells transduced with TCR-5-271 and for untransduced T cells. IFN-γ secretion was determined 24h after setting up the co-cultures by ELISA. To note, whenever no IFN-γ release was observed it was not possible to verify whether the tested peptide sufficiently bound to the HLA-A2 molecules. Therefore, the absence of T cell activation could be due either to the abrogation of the peptide:MHC binding or to the disability of the TCR to interact properly with the peptide:MHC complex in order to lead to T cell activation. TCR 5-271-transduced T cells released IFN-γ in response to T2 cells loaded with six out of the 14 tested peptides (figure 16B). Peptides from 8- to 12-mer mapping from position 7 to position 20 of the NY-ESO-1-ORF2 protein were recognized by TCR 5-271-expressing T cells. As shown in figure 16A, the recognized peptides showed an identical sequence of eight aa, representing the minimum sequence needed for recognition by TCR 5-271 (NY-ESO-1-ORF2$_{11-18}$, LMAQGAML).

A

NY-ESO-1-ORF2 protein first 20 aa

MLMAQEALAFLMAQGAMLAA

peptide #	start	end	X-mer	Peptide sequence
1	1	12	12	MLMAQEALAFLM
2	1	11	11	MLMAQEALAFL
3	1	9	9	MLMAQEALA
4	1	8	8	MLMAQEAL
5	2	11	10	LMAQEALAFL
6	13	20	8	AQGAMLAA
7	12	20	9	MAQGAMLAA
8	11	20	10	LMAQGAMLAA
9	10	20	11	FLMAQGAMLAA
10	10	19	10	FLMAQGAMLA
11	10	18	9	FLMAQGAML
12	10	17	8	FLMAQGAM
13	11	18	8	LMAQGAML
14	7	18	12	ALAFLMAQGAML

B

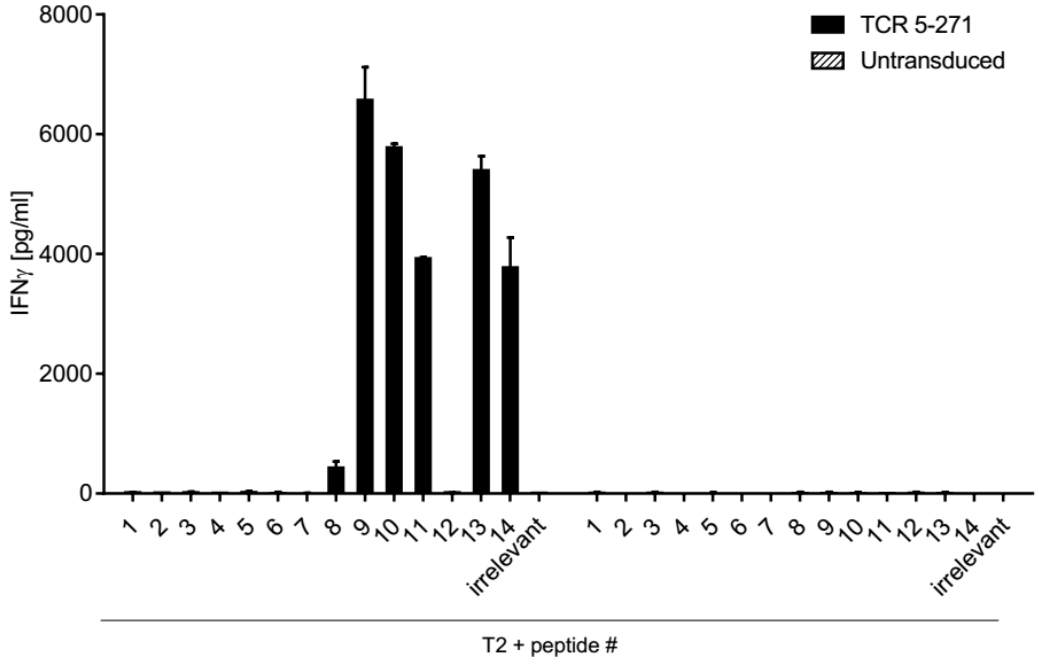


Figure 16. TCR 5-271 core epitope identification: NY-ESO-1-ORF2$_{11\text{-}18,}$ LMAQGAML. (A) Position in the NYESO-1-ORF2-protein (start - end), peptide length (X-mer) and peptide sequence are shown for the 14 peptides selected for the identification of the TCR 5-271 epitope. The six peptides recognized by TCR 5-271-transgenic T cells in the IFN-γ ELISA assay (data shown in figure. 16B) comprised an identical sequence of eight aa (NYESO-1-ORF2$_{11\text{-}18,}$ LMAQGAML) shown in blue. **(B)** CD8^{+} T cells transduced with TCR 5-271 and untransduced cells were stimulated with T2 cells loaded with either the 14 selected peptides or with irrelevant peptide MAGE-A1$_{278\text{-}286}$. IFN-γ release was evaluated 24h after setting up the co-cultures by ELISA. Shown is the mean value of duplicates with standard deviations. This experiment was performed using two different donors. Shown is one representative experiment.

4.2.6 Fine typing of the recognized epitope

The fine typing of an identified epitope is needed to subsequently investigate potential TCR cross-recognition of alternative peptides that contain amino acid exchanges compared to the known epitope sequence [68]. These alternative peptides can be expressed in healthy tissues, presented on corresponding MHC molecules and recognized equally by the transgenic TCR, leading to potential unwanted side effects in clinical applications (designated as off-target toxicity). Therefore, alanine scanning assays were performed to determine the contribution of each aa of the HLA-A2-bound epitope to the interaction with the TCR 5-271. The amino acids included in the 12-mer NY-ESO-1-ORF2$_{7-18}$, 11-mer NY-ESO-1-ORF2$_{10-20}$ and 9-mer NY-ESO-1-ORF2$_{10-18}$ peptides, recognized by TCR 5-271-transgenic T cells in previous experiments, were consecutively replaced by an alanine (or by a glycine where an alanine was already present in the original sequence) (figure 17). T2 cells were loaded with these modified peptides and used as APCs to induce IFN-γ release of either TCR 5-271-transduced T cells or untransduced T cells. The three wild-type peptides were included in the assays as positive controls and the irrelevant peptide MAGE-A1$_{278-286}$ served as the negative control. To note, as discussed before, whenever no IFN-γ release was observed it was impossible to verify whether the tested peptide sufficiently bound to the HLA-A2 molecules. Therefore, the absence of T cell activation could be due either to the abrogation of the peptide:MHC binding or to the disability of the TCR to interact properly with the peptide:MHC complex in order to lead to T cell activation. Replaced amino acids that led to the abrogation of IFN-γ release by T cells were defined as “non-exchangeable amino acids” within the epitope sequence. As shown in figure 17, the phenylalanine (F) at position 4 (P4) in the 12-mer and at P1 in the 11- and 9-mer, as well as the leucine (L) at P5 in the 12-mer and at P2 in the 11- and 9-mer, were identified to be non-exchangeable in the three peptides tested. The substitution of the glycine (G) at P9 in the 12-mer and at P6 in the 11- and 9-mer only affected TCR 5-271 recognition of the 9-mer peptide. All the other aa of the peptides tested could be replaced either by alanine or by glycine without affecting TCR 5-271 recognition.

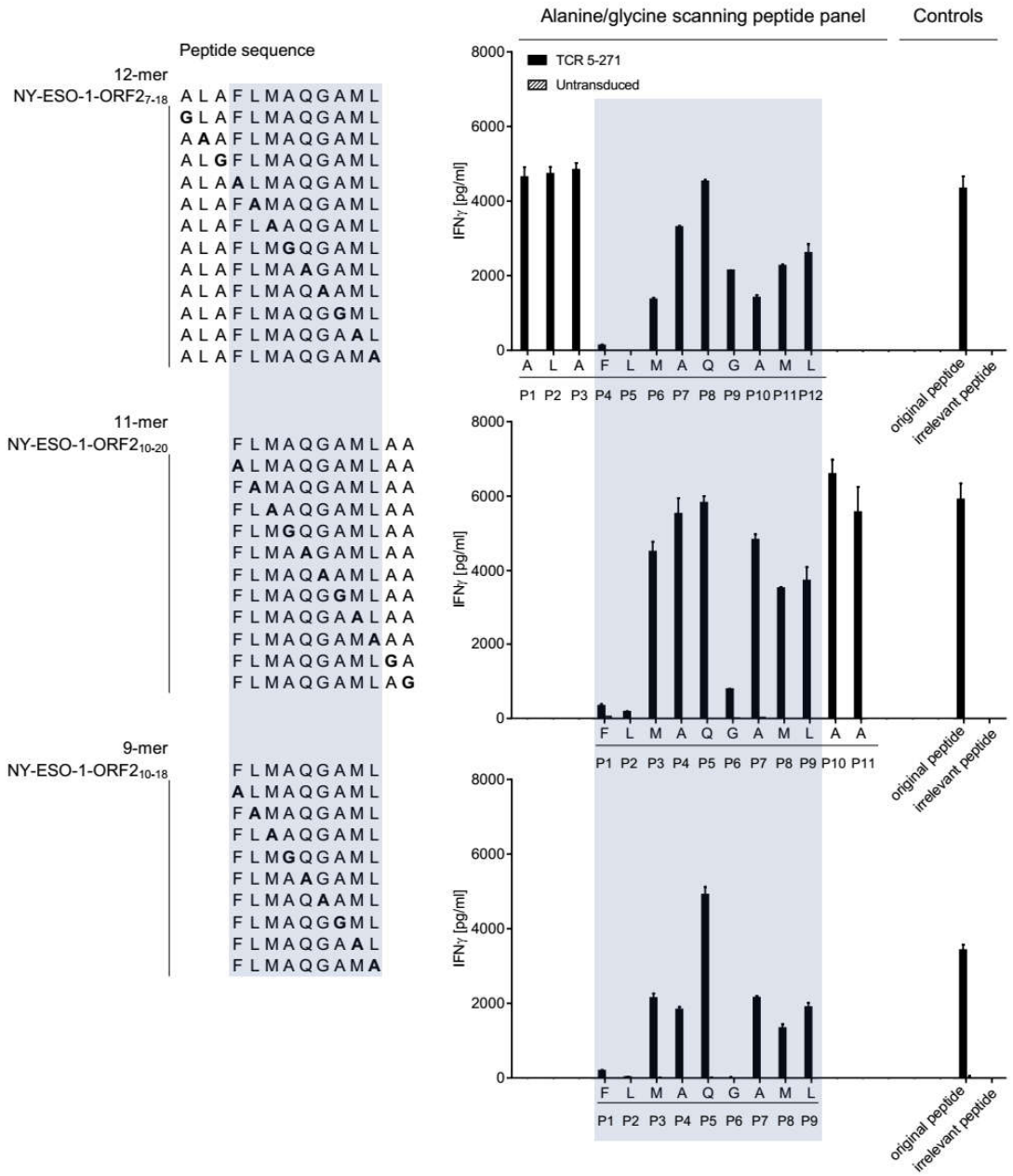


Figure 17. Fine typing of the epitope recognized by TCR 5-271. Alanine scanning analyses were performed for the 12-mer NY-ESO-1-ORF2$_{7-18}$, 11-mer NY-ESO-1-ORF2$_{10-20}$ and 9-mer NY-ESO-1-ORF2$_{10-18}$ peptides recognized by TCR 5-271-transgenic T cells. The amino acids included in these peptides were consecutively replaced by an alanine or by a glycine when an alanine was already present in the original sequence. Peptide tables include the original peptide sequence (first line) and the alanine/glycine scanning peptide panel (exchanged aa are shown in bold). The identical sequence included in the tested peptides is shown in blue. T2 cells were loaded with the modified peptides and used in the co-cultures with either T cells expressing TCR 5-271 or with untransduced T cells to assess IFN-γ release by ELISA after 24h of incubation. The irrelevant MAGE-A1$_{278-286}$ peptide was included as the negative control. Shown is the mean value of duplicates with standard deviations. Each experiment was performed with two different donors. Representatively shown are the results of one donor.

4.2.7 Recognition of LAGE-1 and CAMEL

Both isoforms of the tumor antigen LAGE-1 (highly homologous to NY-ESO-1) include not only the primary ORF in the first reading frame encoding the full-length protein (designated as LAGE-1a-ORF1 and LAGE-1b-ORF1), but also an alternative ORF in the second reading frame (figure 18). This alternative ORF is called LAGE-1-ORF2 and is identical in LAGE-1a and in LAGE-1b. The AUG start codon of the LAGE-1-ORF2 is located 40 base pairs (bp) downstream of the AUG of LAGE-1-ORF1. Translation of LAGE-1-ORF2 leads to the production of a 109 aa protein called CAMEL [111]. Importantly, the N-terminal 54 aa of CAMEL are identical to the N-terminal 54 aa of NY-ESO-1-ORF2 protein. Since the epitope recognized by TCR 5-271 (NY-ESO-1-ORF2$_{11\text{-}18}$) was located in the region shared by NY-ESO-1-ORF2 and CAMEL, the recognition of LAGE-1 and CAMEL by TCR 5-271-transgenic T cells was evaluated. CAMEL, LAGE-1a wild-type (LAGE-1a_wt) and a partial-codon-optimized version of LAGE-1a including only the LAGE-1-ORF1 (LAGE-1a_pco) were cloned into the pGEM vector (figure 19A). *ivt*RNA encoding CAMEL, LAGE-1a_wt or LAGE-1a_pco were transfected into tgK562 cells. In addition, *ivt*RNA encoding NY-ESO-1_wt, NY-ESO-1_pco or NY-ESO-1-ORF2 were transfected into tgK562 cells as positive controls for the assay. Water electroporation of tgK562 cells served as the negative control. Electroporated tgK562 cells were used as stimulator cells for CD8$^+$ T cells transduced with either TCR 5-271 or TCR 1G4-α 95:LY in order to induce IFN-γ release. Untransduced CD8$^+$ T cells were included as a negative control. As shown in figure 19B, T cells expressing TCR 5-271 recognized tgK562 cells transfected with *ivt*RNA encoding NY-ESO-1_wt or NY-ESO-1-ORF2 as well as LAGE-1a_wt or CAMEL. In contrast, they did not release IFN-γ after incubation with NY-ESO-1_pco- and LAGE-1a_pco-transfected tgK562 cells. These results confirmed that the TCR 5-271 was specific for a peptide derived from the proteins encoded by ORF2 of NY-ESO-1 as well as by ORF2 of LAGE-1a. As expected, TCR 1G4-α95:LY-transgenic T cells were activated only in response to tgK562 cells expressing the wt or pco versions of both NY-ESO-1 and LAGE-1a, confirming that TCR 1G4-α95:LY was specific for a peptide derived from proteins encoded by ORF1 of NY-ESO-1 and LAGE-1a. Furthermore, these findings showed that after electroporation with *ivt*RNA encoding LAGE-1_wt into tgK562 cells, LAGE-1-ORF2 was translated and the corresponding CAMEL protein was processed and presented in addition to the full-length LAGE-1a-ORF1 protein. Interestingly, TCR 5-271-transgenic T cells showed better recognition of tgK562 cells expressing NY-ESO-1_wt compared to LAGE-1a_wt-transfected tgK562 cells, whereas TCR 1G4-α95:LY-transgenic T cells were equally activated by tgK562 cells expressing NY-ESO-1_wt or LAGE-1a_wt. This observation could not be explained by differences in

transfection efficiency of NY-ESO-1 and LAGE-1a, and hence one hypothesis was that ORF2 of NY-ESO-1 was more efficiently translated than ORF2 of LAGE-1a.

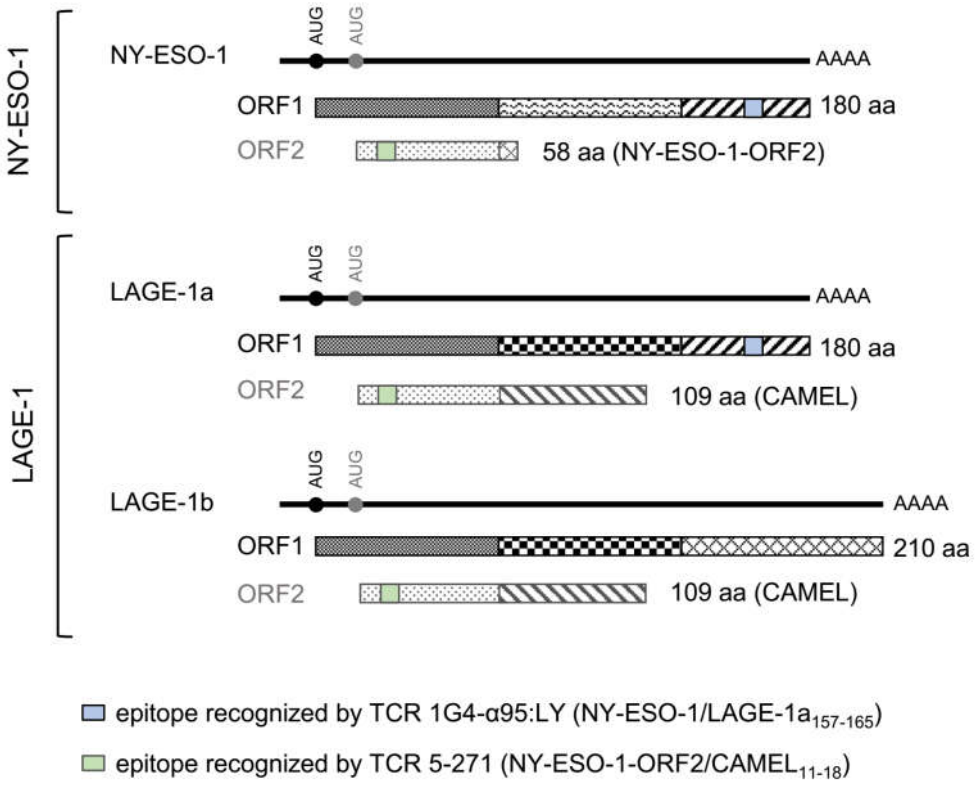


Figure 18. Schematic representation of NY-ESO-1 and LAGE-1 mRNA transcripts and protein products. LAGE-1 mRNA (both isoforms, LAGE-1a and LAGE-1b), as described for NY-ESO-1, comprises not only the ORF in the first frame (LAGE-1-ORF1) encoding the full-length protein (180 aa LAGE-1a and 210 aa LAGE-1b), but also an alternative ORF in the second reading frame (LAGE-1-ORF2, identical for the two LAGE-1 isoforms). Translation of LAGE-1-ORF2 leads to the production of a 109 aa protein called CAMEL. The N-terminal 54 aa of CAMEL are identical to the N-terminal 54 aa of NY-ESO-1-ORF2 protein. Identical sequences in the proteins are shown by identical patterns. The locations of the NY-ESO-1/LAGE-$1_{157-165}$ epitope recognized by TCR 1G4:95:LY and of the NY-ESO-1-ORF2/$CAMEL_{11-18}$ epitope recognized by TCR 5-271 are shown.

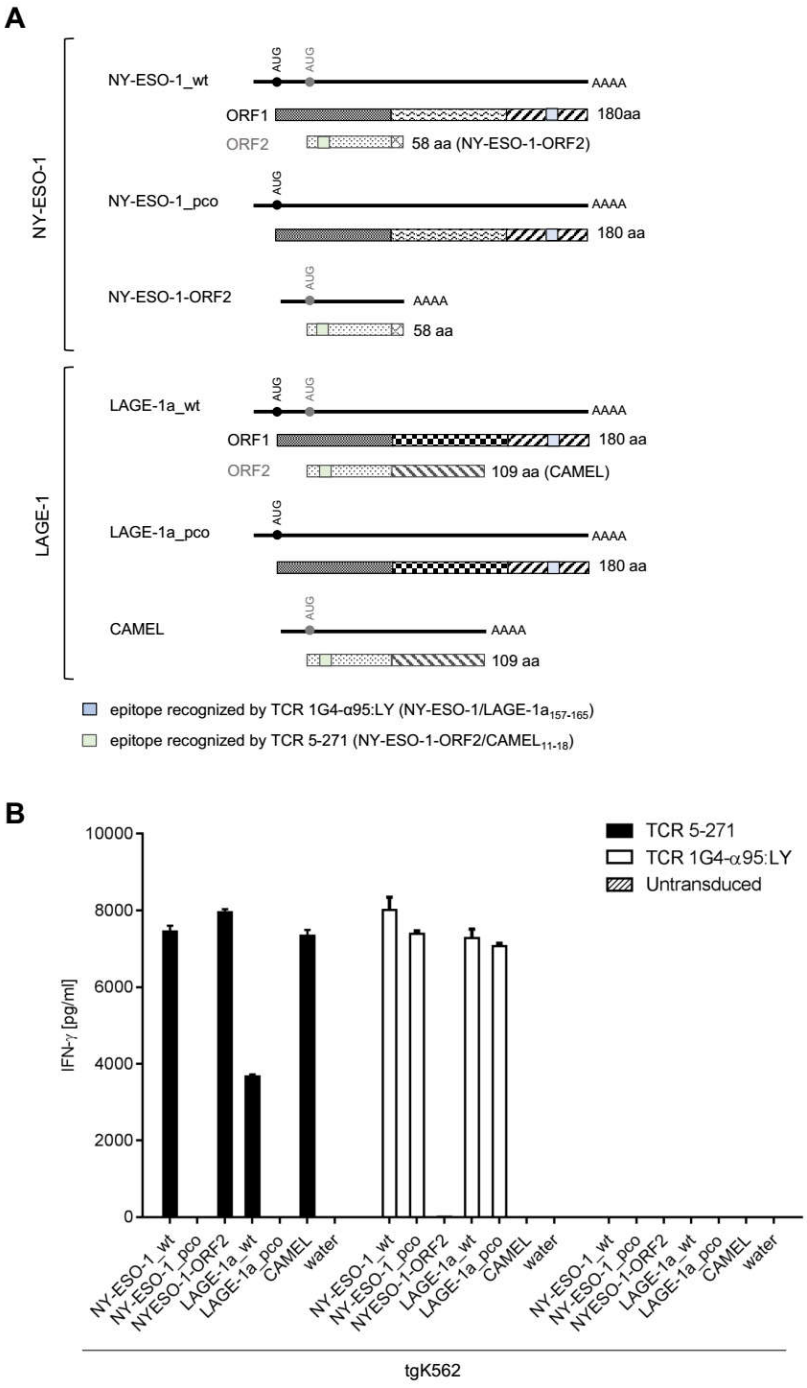


Figure 19. Recognition of LAGE-1 and CAMEL by TCR 5-271-transgenic T cells. (A) Schematic depiction of NY-ESO-1_wt, NY-ESO-1_pco, NY-ESO-1-ORF2, LAGE-1a_wt, LAGE-1a_pco and CAMEL mRNA transcripts and corresponding protein products. Identical sequences in the proteins are shown by identical patterns. The locations of the NY-ESO-1/LAGE-1a $_{157-165}$ epitope recognized by TCR 1G4:95:LY and the NY-ESO-1-ORF2/CAMEL$_{11-18}$ epitope recognized by TCR 5-271 are shown. **(B)** tgK562 cells were transfected with *ivt*RNA encoding NY-ESO-1_wt, NY_ESO-1_pco, NY-ESO-1-ORF2, LAGE-1a_wt, LAGE-1a_pco or CAMEL. tgK562 cells electroporated with water served as negative target controls. Electroporated tgK562 cells were co-cultured with T cells transduced with either TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells were included as negative controls. IFN-γ release was evaluated 24h after T cell stimulation. Shown is the mean value of duplicates with standard deviations. This experiment was performed with two different donors. Shown is one representative experiment.

4.2.8 Recognition of target antigen-transfected tumor cell lines

To further investigate the hypothesis that ORF2 of NY-ESO-1 was more efficiently translated than ORF2 of LAGE-1a, *ivt*RNA encoding either NY-ESO-1_wt or LAGE-1a_wt was transfected in tumor cell lines and the recognition by TCR 5-271-transgenic T cells was evaluated. The NY-ESO-1/LAGE-1a-positive cell lines FM6, MM415, Mel624.38, MelA375 and the NY-ESO-1/LAGE-1a-negative tumor cell lines MM127, SK-Mel23 and gK562 were selected as target cells (see Nanostring analyses for NY-ESO-1/LAGE-1a mRNA expression, figure 12B. Data not shown for the tumor cell line MM127). All the selected tumor cell lines expressed HLA-A2 molecules on the cell surface as confirmed by flow cytometry analysis (figure 20A). In previous experiments, it was shown that the four selected tumor cell lines, endogenously positive for NY-ESO-1/LAGE-1a, were recognized by T cells expressing TCR 1G4-α95:LY. Therefore, an additional internal control was needed to prove that the expected lack or reduction of LAGE-1a recognition by TCR 5-271 was due to reduced ORF2 translation and not due to inefficient transfection. Since selected tumor cell lines did not express the minor histocompatibility antigen HA-1^H, a $CD8^+$ T cell clone specific for HA-1^H (HA-$1_{137\text{-}145(H139)}$) and restricted to HLA-A2 (clone 10/24) was used as an internal control. The tumor cell lines were transfected simultaneously with *ivt*RNA encoding either NY-ESO-1_wt or LAGE-1a_wt in combination with *ivt*RNA encoding HA-1^H. Untransfected and *ivt*RNA-transfected tumor cell lines were used to stimulate IFN-γ release of T cells expressing either TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells served as the negative control. IFN-γ ELISA was performed 24h after setting up the co-culture (figure 20B). As already observed, TCR 5-271-transduced T cells did not recognize any tumor cell lines even if they were endogenously NY-ESO-1/LAGE-1a-positive. However, they secreted IFN-γ when incubated with the same cell lines transfected with NY-ESO-1_wt *ivt*RNA. After incubation with LAGE-1a_wt-transfected cell lines, TCR 5-271-trangenic T cells released either lower amounts of IFN-γ compared to the amount detected by stimulation with NY-ESO-1-transfected cells (FM6, MM415) or did not secrete IFN-γ at all (Mel624.38, MelA375). TCR 1G4-α95:LY-transgenic T cells recognized all tumor cell lines that were endogenously positive for NY-ESO-1/LAGE-1a without the need of additional *ivt*RNA electroporation. Clone 10/24 did not release IFN-γ in response to any of the tested tumor cell lines without *ivt*RNA transfection. On the other hand, T cell activation was observed when clone 10/24 was co-cultured with HA-1^H-transfected cell lines in combination with NY-ESO-1 as well as with LAGE-1a, showing efficient transfections in both cases.

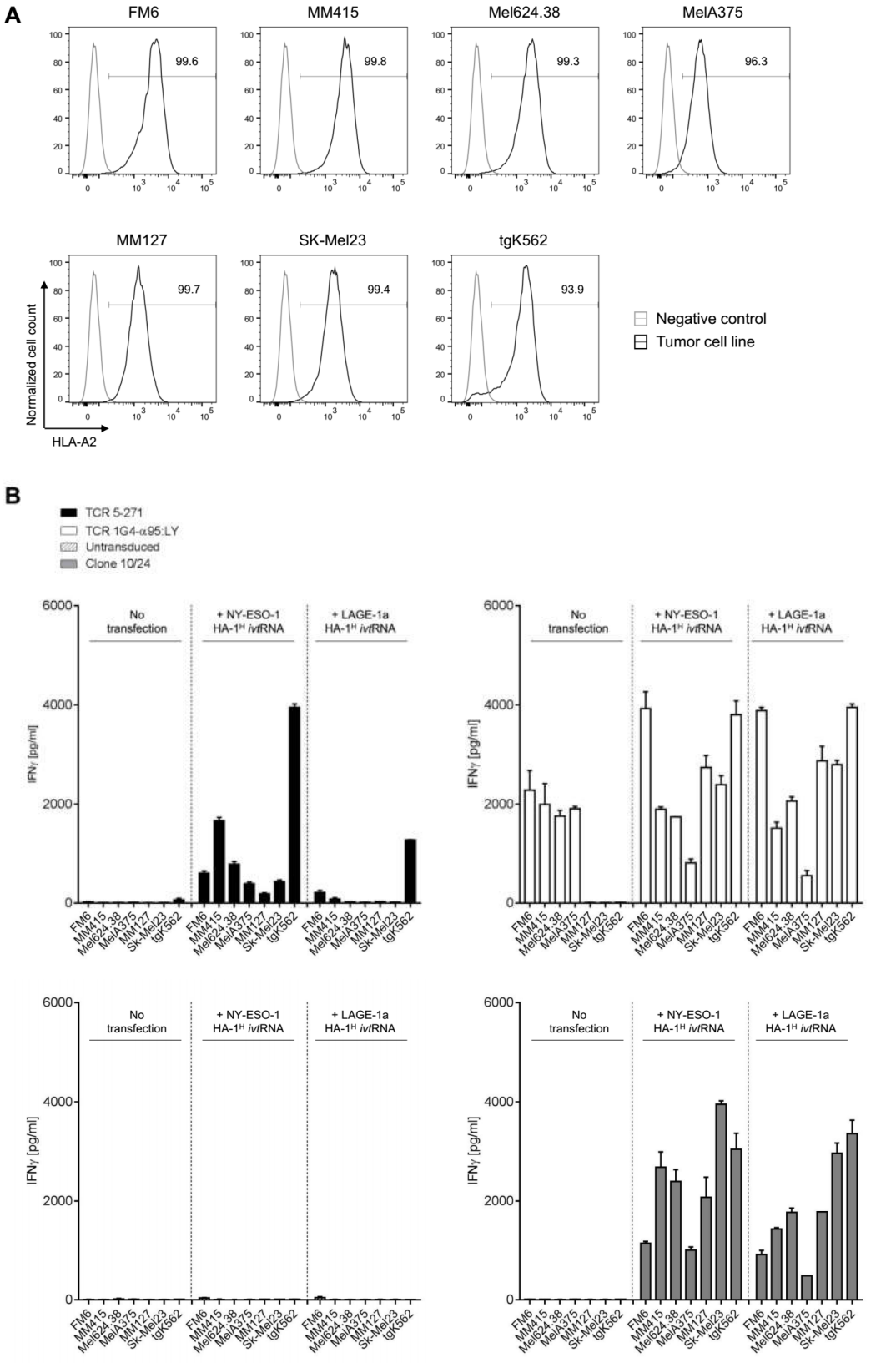


Figure 20. Tumor cell line recognition after either NY-ESO-1_wt- or LAGE-1a_wt *ivt*RNA transfection by TCR 5-271-transgenic T cells. Figure legend continues on page 85.

Figure 20. (A) HLA-A2 expression in FM6, MM415, Mel624.38, MelA375, MM127, SK-Mel23 and tgK562 cell lines was evaluated by flow cytometry. Stained tumor cell lines are shown in black and the negative control (HLA class I-negative K562 cells stained with the HLA-A2-specific antibody) is shown in grey. Populations shown are pre-gated on live single cells. **(B)** The NY-ESO-1/LAGE-1a-positive cell lines FM6, MM415, Mel624.38, MelA375 and the NY-ESO-1/LAGE-1a-negative tumor cell lines MM127, SK-Mel23 and tgK562 were transfected with *ivt*RNA encoding either NY-ESO-1_wt or LAGE-1a_wt in combination with HA-1^H antigen. Untransfected and transfected tumor cell lines were used as target cells in co-culture assays. TCR 5-271-, TCR 1G4-α95:LY-transduced T cells and untransduced T cells served as effector cells. The clone 10/24 was included as an internal effector control recognizing HA-1^H peptide (HA-$1_{137\text{-}145(H139)}$) on HLA-A2 molecules. IFN-γ release was measured by ELISA 24h after setting up the co-cultures. Shown is the mean value of duplicates with standard deviations.

The tumor cell lines MM127, SK-Mel23 and tgK562, endogenously NY-ESO-1/LAGE-1a-negative and HA-1^H-negative, were recognized by TCR 5-271-transduced T cells, by TCR 1G4-α95:LY-transduced T cells and by clone 10/24 only after *ivt*RNA transfection. Interestingly, TCR 1G4-α95:LY-transgenic T cells and clone 10/24 released similar amounts of IFN-γ in response to NY-ESO-1/HA-1^H and to LAGE-1a/HA-1^H stimulation. In contrast, TCR 5-271-transduced T cells showed better recognition when the three cell lines were transfected with NY-ESO-1/HA-1^H compared to LAGE-1a/HA-1^H. All together these results supported the hypothesis that ORF2 of NY-ESO-1 was more efficiently translated than ORF2 of LAGE-1a in tgK562 cells and also in the tested tumor cell lines.

4.2.9 Killing of target epitope-loaded cells

Functionality of TCR-transgenic T cells can be addressed by evaluating IFN-γ release upon target antigen-specific stimulation as well as by testing their capacity to mediate killing of cells expressing the target tumor antigen. Therefore, the killing capacity of TCR 5-271-transgenic T cells was assessed by live-cell imaging. The first experiment was performed to assess the killing of T2 cells after specific peptide loading. T2 cells were stably transduced with a lentiviral vector that enabled nuclear labelling by homogenous expression of the mKate2 protein (red fluorescent protein) without altering cell function and with minimal toxicity. After transduction, T2 cells were analyzed by flow cytometry and 92.1% of the cells expressed mKate2 protein resulting in the red-label (figure 21A). These red-labelled T2 cells were properly detected by the live-cell imaging system (figure 21B).

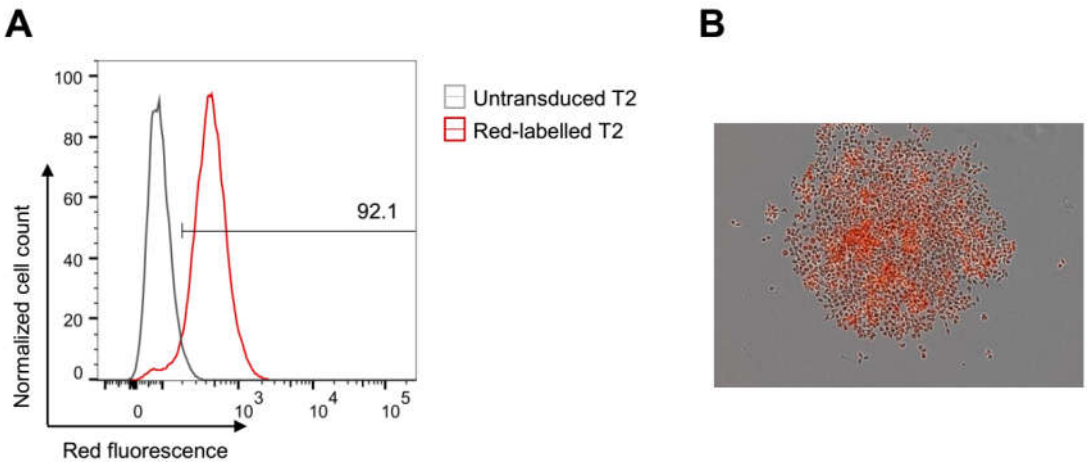


Figure 21. Analyses of T2 cells transduced with a red fluorescent reagent. T2 cells were stably transduced with a red fluorescent reagent for nuclear labelling. **(A)** The transduction rate was analyzed by flow cytometry. Transduced T2 cells are shown in red, while untransduced cells are shown in grey. Populations shown are pre-gated on live single cells. **(B)** Exemplary image of red-labelled T2 cells detected by live-cell imaging.

Red-labelled T2 cells were loaded with either peptide NY-ESO-1-ORF-2_{11-18} (specific peptide for TCR 5-271, irrelevant peptide for TCR 1G4-α95:LY) or with NY-ESO-1/LAGE-$1a_{157-165}$ peptide (specific peptide for TCR 1G4-α95:LY, irrelevant peptide for TCR 5-271). After peptide loading, red-labelled T2 cells were incubated with T cells expressing TCR 5-271 or TCR 1G4-α95:LY. Untransduced T cells served as the negative control. The cells were monitored over 48h by live-cell imaging to evaluate the decrease of the red fluorescence intensity showing T2 cell killing mediated by TCR-transgenic T cells. Images acquired at time point zero (0h) and 24h as well as 48h after setting up the co-cultures are shown in figure 22. A decrease of the red fluorescence intensity was observed only when TCR 5-271-transduced T cells were incubated with peptide NY-ESO-1-ORF-2/CAMEL$_{11-18}$-loaded T2 cells and when TCR 1G4-α95:LY-transduced T cells were added in the co-culture with peptide NY-ESO-1/LAGE-$1a_{157-165}$-loaded T2 cells. In contrast, an increase of the red fluorescence intensity due to proliferation of the labelled T2 cells was detected when TCR 5-271- and TCR 1G4-α95:LY-transduced T cells were incubated with irrelevant peptide-loaded T2 cells. The red fluorescence intensity increased also when untransduced T cells were co-cultured with T2 cells independently of the loaded peptide.

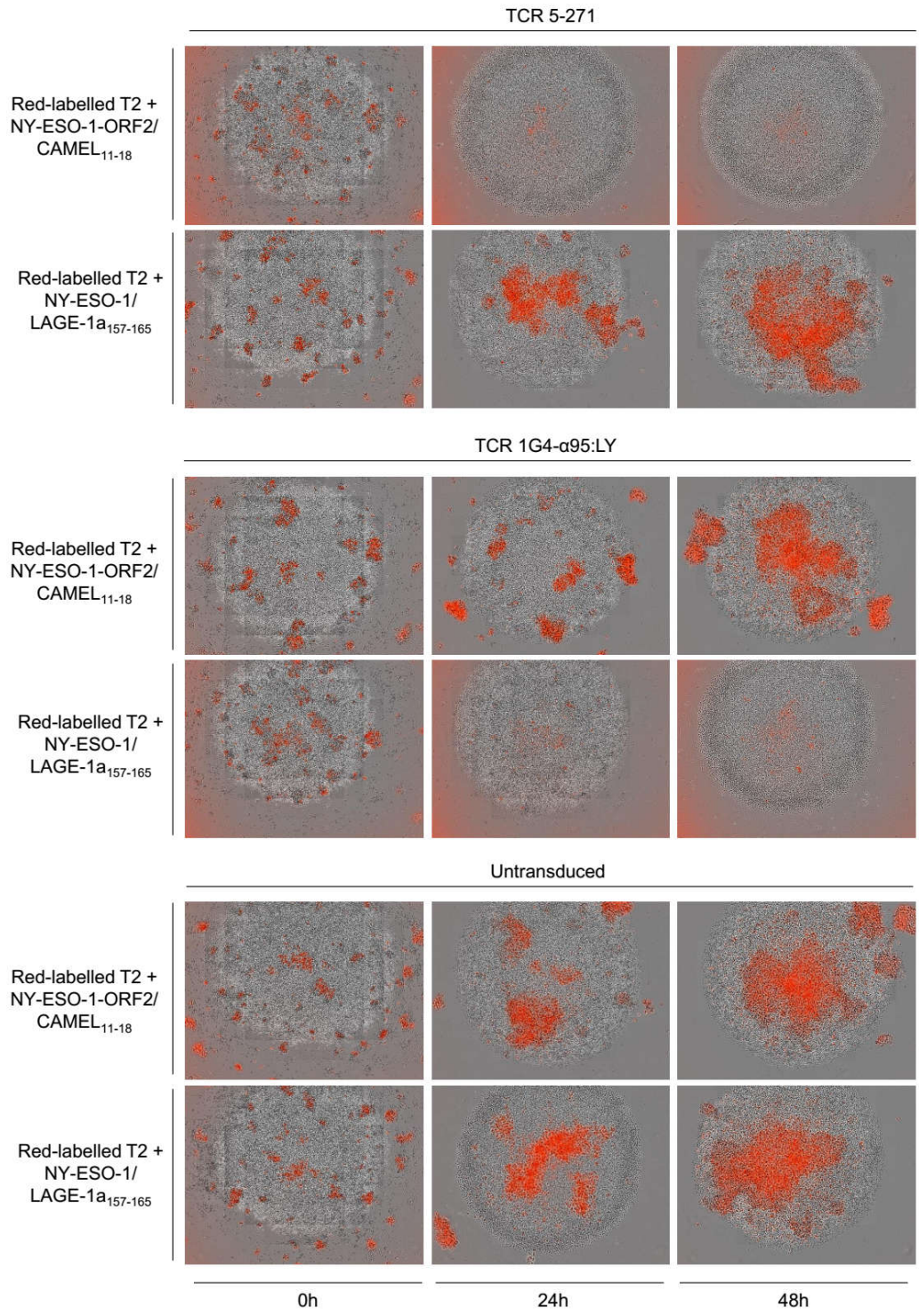
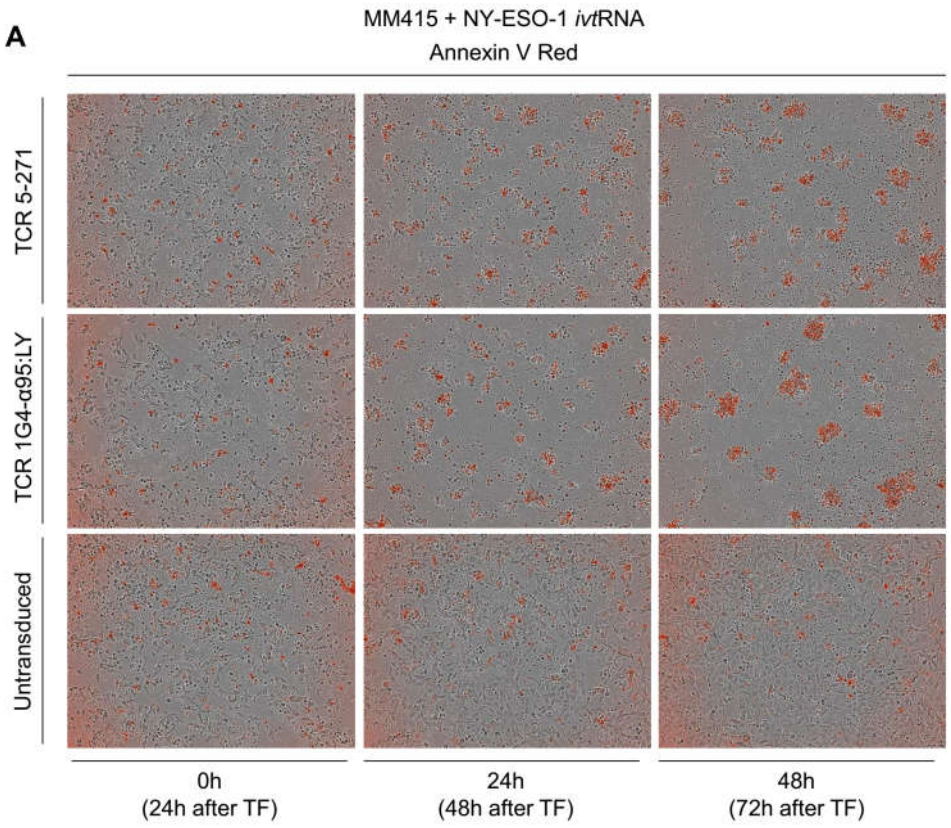
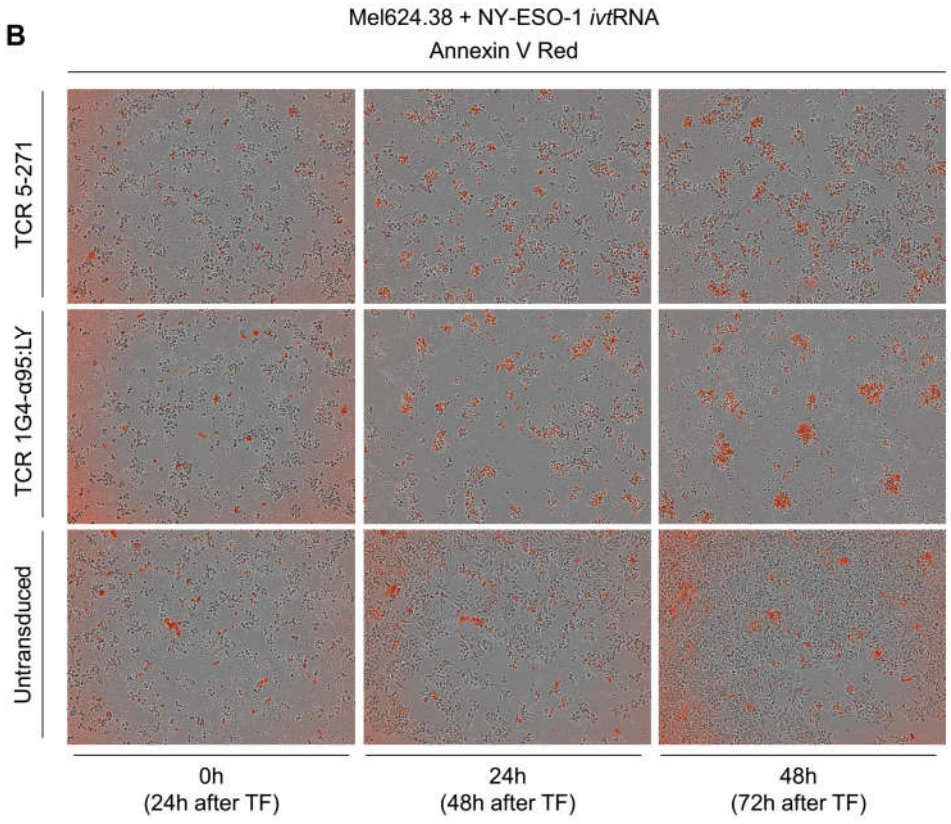


Figure 22. Killing of red-labelled T2 cells loaded with specific peptide by TCR 5-271-transgenic T cells. Red-labelled T2 cells were loaded with either peptide NY-ESO-1-ORF-2_{11-18} (specific peptide for TCR 5-271, irrelevant peptide for TCR 1G4-α95:LY) or with peptide NY-ESO-1/LAGE-$1a_{157-165}$ (specific peptide for TCR 1G4-α95:LY, irrelevant peptide for TCR 5-271). After peptide loading, red-labelled T2 cells were incubated with T cells expressing either TCR 5-271 or TCR 1G4-α95:LY and with untransduced T cells served as negative control (E:T ratio of 40:1). The cells were monitored over a time of 48h using a live-cell imaging system to evaluate the decrease of the red fluorescence intensity showing T2 cell killing mediated by TCR-transgenic T cells. Images acquired at time point zero (0h) and 24h as well as 48h after setting up the co-cultures are shown.

In a second experiment, MM415, Mel624.38 and tgK562 cell lines were selected as target cells and transfected with *ivt*RNA encoding NY-ESO-1_wt to assess the capacity of T cells expressing TCR 5-271 to kill tumor cell lines that processed and presented the TCR 5-271 epitope by internal cellular pathways. T cells transduced with either TCR 5-271 or TCR 1G4-α95:LY were used as effector cells and untransduced T cells were used as the negative control. To evaluate apoptosis in the co-cultures, the Annexin V red reagent was added to the medium and the cells were monitored by live-cell imaging over 48h. Images acquired at time point zero (0h) and 24h as well as 48h after setting up the co-cultures are shown in figure 23. Apoptotic red cells as well as clusters of T cells and target cells were detected for all NY-ESO-1-transfected tumor cell lines after incubation with T cells expressing either TCR 5-271 or TCR 1G4-α95:LY. To note, TCR 1G4-α95:LY-transduced $CD8^+$ T cell seemed to mediate killing with a better efficiency compared to TCR 5-271-transduced T cells. In contrast, growing target cells were observed for all tumor cell lines when untransduced T cells were used as effectors.

In conclusion, these data showed that TCR 5-271-transduced T cells were able to mediate killing of target cells loaded with the specific peptide as well as transfected with NY-ESO-1_wt *ivt*RNA.


A
MM415 + NY-ESO-1 ivtRNA
Annexin V Red
TCR 5-271
TCR 1G4-α95:LY
Untransduced
0h
(24h after TF)
24h
(48h after TF)
48h
(72h after TF)


B
Mel624.38 + NY-ESO-1 ivtRNA
Annexin V Red
TCR 5-271
TCR 1G4-α95:LY
Untransduced
0h
(24h after TF)
24h
(48h after TF)
48h
(72h after TF)

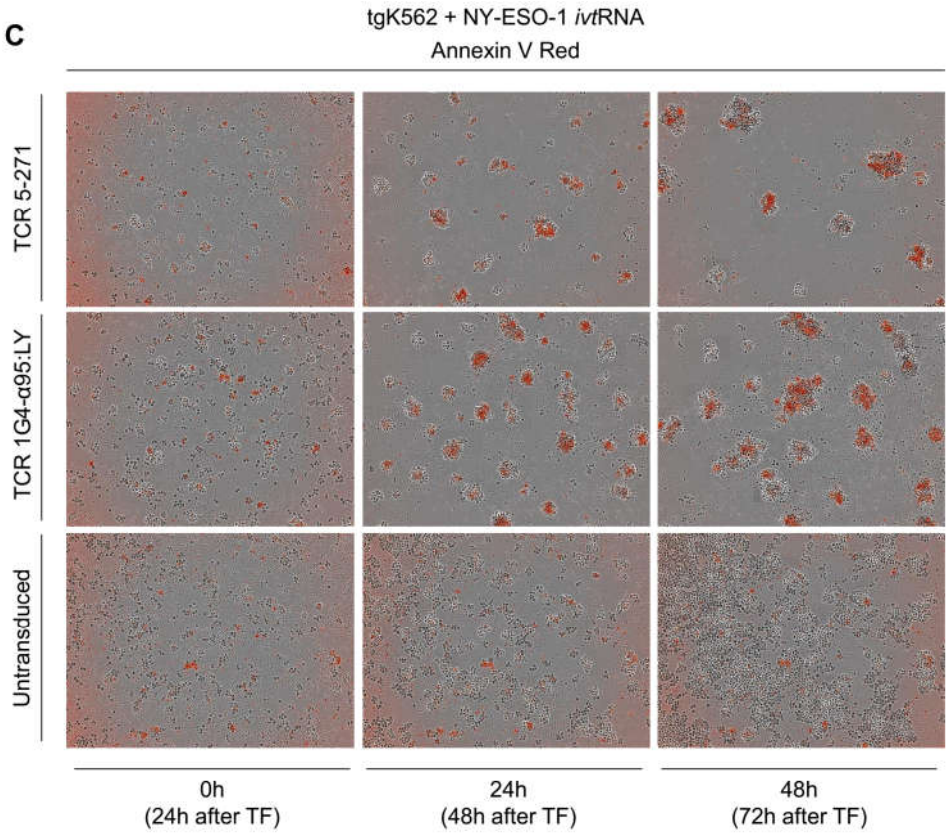


Figure 23. Killing of NY-ESO-1-transfected tumor cell lines by TCR 5-271-transgenic T cells. MM415 **(A)**, Mel624.38 **(B)** and tgK562 **(C)** cell lines were transfected with *ivt*RNA encoding NY-ESO-1_wt. T cells transduced with either TCR 5-271 or TCR 1G4-α95:LY and untransduced T cells were incubated with transfected tumor cell lines 24h after transfection (TF) (E:T ratio of 40:1). Annexin V red reagent was added to the medium for evaluation of the apoptosis over a time of 48h by live-cell imaging. Images acquired at time point zero (0h) and 24h as well as 48h after setting up the co-cultures are shown.

4.2.10 Evaluation of T cell peptide sensitivity

In principle, a therapeutic TCR needs to have high specificity as well as optimal sensitivity for its peptide:MHC complex in order to efficiently mediate tumor killing and to discriminate between tumor cells and normal cells to avoid unwanted side effects. TCR affinity is defined as strength of the monomeric interaction between the TCR and a peptide:MHC complex. Since T cells express more than one TCR molecule, the overall binding strength of all available TCR molecules, called avidity, has to be considered for accurate evaluation in a physiological context. Evaluation of T cell peptide sensitivity, by analyzing IFN-γ secretion by T cells in response to APCs loaded with graded amounts of the target epitope, represents one method commonly used to validate the so called functional avidity of TCR-transgenic T cells. Serial dilutions are used to calculate effective epitope concentrations which induce half-maximal T cell responses, measured by IFN-γ amounts. The lower the amount of specific peptide that is required for half-maximal activation of the T cells, the

higher is the functional avidity and the peptide sensitivity. Importantly, using this method a direct comparison of peptide sensitivity for T cells expressing transgenic TCRs specific for different peptides is impossible because the outcome of the assay is influenced by the stability of the specific target peptide in solution and by the peptide MHC binding affinity. Here, peptide sensitivity of TCR 5-271-expressing $CD8^+$ T cells was assessed by using serial dilutions of either the 9-mer peptide NY-ESO-1-ORF2/$CAMEL_{10-18}$ or of the 8-mer peptide NY-ESO-1-ORF2/$CAMEL_{11-18}$ loaded on T2 cells. Untransduced $CD8^+$ T cells were used as the negative control. IFN-γ release by $CD8^+$ T cells was evaluated 24h after setting up the co-cultures by ELISA (figure 24). Peptide sensitivity of TCR 5-271-expressing T cells were similar in response to titrated amounts of the 8-mer and 9-mer target peptides. TCR 5-271-expressing T cells showed half-maximal IFN-γ release at approximately 10^{-6} M, requiring very high amounts of peptides for activation.

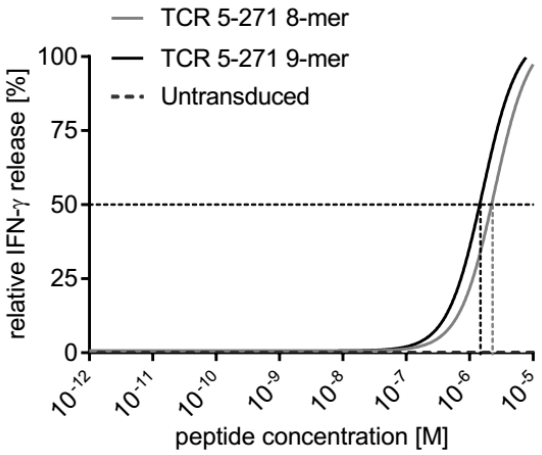


Figure 24. Peptide sensitivity of TCR 5-271-transduced T cells. T2 cells were loaded with titrated amounts of either the peptide NY-ESO-1-ORF2/CAMEL $_{10-18}$ (9-mer) or of the peptide NY-ESO-1-ORF2/CAMEL $_{11-18}$ (8-mer). Loaded T2 cells were co-cultured with TCR 5-271-expressing $CD8^+$ T cells. Untransduced $CD8^+$ T cells served as the negative controls for all conditions. An ELISA was performed after 24h to evaluate IFN-γ release by $CD8^+$ T cells. Dashed lines indicate the peptide concentrations needed to induce the half-maximal IFN-γ secretion. This experiment was performed with two different donors. Shown is one representative experiment.

4.2.11 Evaluation of CD8-dependency

The CD8 co-receptor is known to directly bind to MHC class I molecules and to be critical for the development of $CD8^+$ T cells. CD8 stabilizes the binding of a TCR to the MHC-peptide complex and facilitates early events of the TCR signaling cascade [112]. Nevertheless, it has been shown that some TCRs restricted to MHC class I molecules can induce T cell activation upon antigen encounter without the need of CD8-costimulation [113]. To evaluate the impact of CD8 co-receptor on the functionality of the identified TCR, TCR 5-271 was transferred into a $CD8^+$ T cell clone (clone 10/24) and into a $CD4^+$ T cell clone (clone 234) by retroviral transduction. The same clones were transduced also with TCR 1G4-α95:LY and used as positive controls since it has been shown that this TCR is CD8-independent and functions efficiently in $CD4^+$ T cells [58]. Untransduced T cell clones served as negative controls. As shown in figure 25A, the transduction rates were analyzed by flow cytometry. Transduction was efficient for both TCRs in the $CD8^+$ clone as well in the $CD4^+$ clone. In particular, 29.0% of $CD8^+$ cells expressed TCR 5-271 and 36.0% were TCR 1G4-α95:LY-positive, while 40.5% of $CD4^+$ cells showed TCR 5-271 expression and 42.4% expressed TCR 1G4-α95:LY. Transduced clones were tested in a co-culture assay using tgK562 cells electroporated with either NY-ESO-1_wt *ivt*RNA or with water as target cells and IFN-γ secretion was evaluated after 24h by ELISA. To verify the functionality in terms of IFN-γ release for the $CD8^+$ and $CD4^+$ clones, specific targets recognized by their endogenous TCRs were included in the assay. T2 cells loaded with either the specific peptide recognized by clone 10/24 ($HA\text{-}1_{137\text{-}145(H139)}$) or with an irrelevant peptide ($HA\text{-}1_{137\text{-}145(R139)}$) were used as target controls for the $CD8^+$ clones. HLA-A24-positive and HLA-A24-negative LCL served as control targets for the $CD4^+$ clones since the clone 234 was shown to be HLA-A24-reactive (data unpublished). As shown in figure 25B, $CD8^+$ clone T cells as well as $CD4^+$ clone T cells expressing TCR 5-271 recognized NY-ESO-1-transfected tgK562 cells, showing that the functionality of the transgenic TCR was not dependent on the presence of the CD8 co-receptor. TCR 1G4-α95:LY worked efficiently after transduction in $CD8^+$ clone T cells and in $CD4^+$ clone T cells, confirming its CD8-independency. The functionality of TCR-transduced and untransduced clones was shown by their activation and IFN-γ secretion after stimulation with specific targets for their endogenous TCRs.

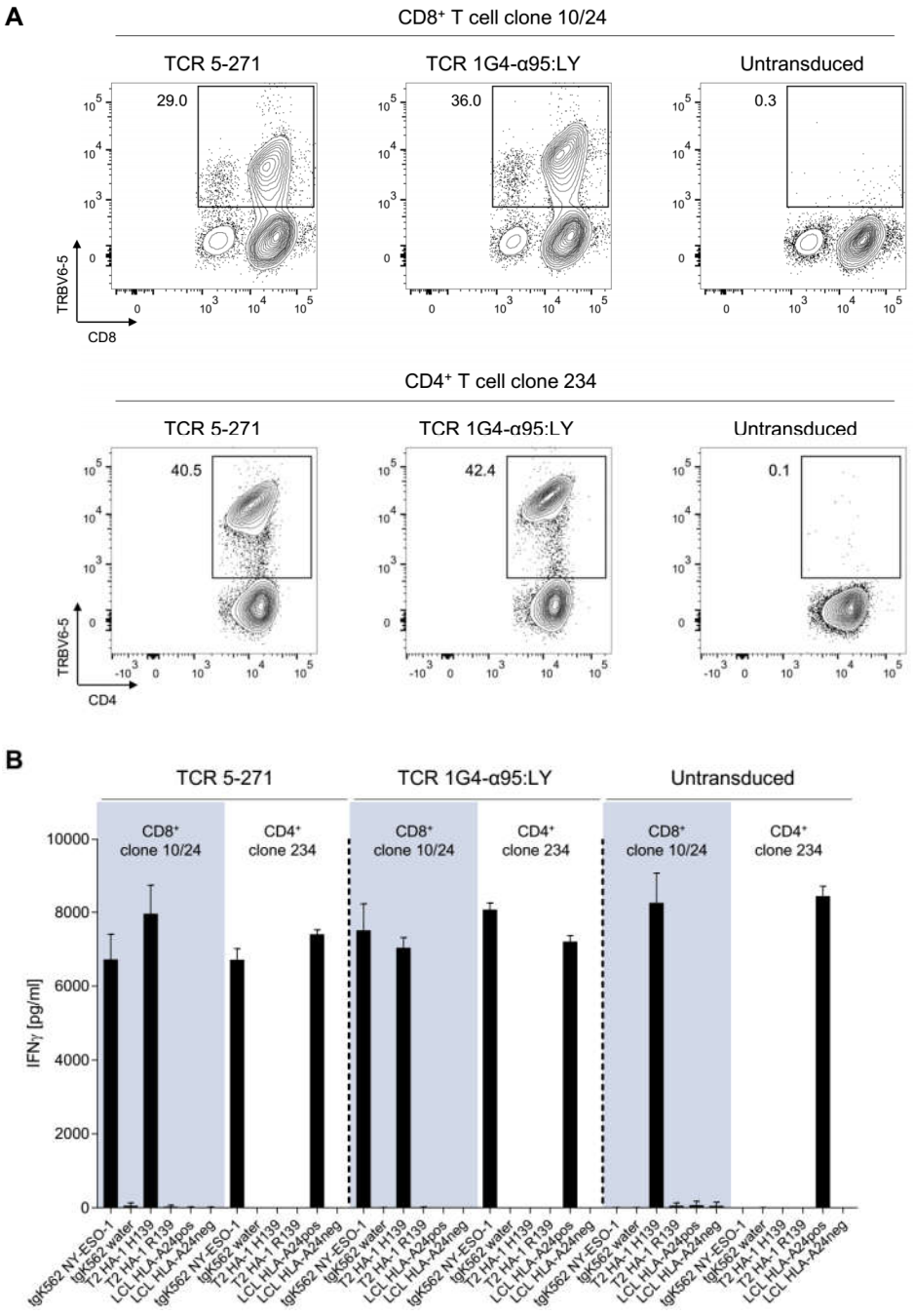


Figure 25. Functionality of TCR 5-271 in CD8⁺ and in CD4⁺ T cells. (A) TCR 5-271 and TCR 1G4-α95:LY were transferred into a CD8⁺ T cell clone (clone 10/24) and into a CD4⁺ T cell clone (clone 234) by retroviral transduction. Transduction rates were analyzed by flow cytometry. Untransduced T cell clones were used as background controls. Populations shown are pre-gated on live single cells. **(B)** Transduced T cell clones were tested in a co-culture assay with tgK562 cells electroporated with either NY-ESO-1_wt *ivt*RNA or water to evaluate the role of the CD8 co-receptor for the functionality of TCR 5-271 and of TCR 1G4-α95:LY. Untransduced clones served as negative controls. T2 cells loaded with the specific peptide recognized by clone 10/24 (HA-$1^{H}_{137\text{-}145(H139)}$, HA-1 H139) or with an irrelevant peptide (HA-$1^{R}_{137\text{-}145(R139)}$, HA-1 R139) were used as target controls for the CD8⁺ clones. HLA-A24-positive and -negative LCL were included as control targets for the CD4⁺ clones since the clone 234 was shown to be HLA-A24-reactive. IFN-γ release was measured 24h after setting up the co-culture. Shown is the mean value of duplicates with standard deviations.

5 Discussion

In this project, a CD8$^+$ T cell clone and the corresponding TCR specific for a new unconventional epitope derived from NY-ESO-1 and LAGE-1 were isolated and characterized. NY-ESO-1-specific T cells were induced using an *in vitro* allogeneic priming approach. An innovative sorting strategy based on the activation-induced T cell marker CD137 and K562 as stimulating cells was developed and tested for the first time in order to enable the isolation of T cells specific for unknown epitopes of NY-ESO-1. The TCR sequence of the isolated NY-ESO-1-reactive clone 5-271 was analyzed and reconstructed for transgenic expression in recipient cells to allow the characterization of the properties of the identified TCR. Interestingly, the recognized epitope turned out to be a peptide derived from the second ORF of NY-ESO-1 and LAGE-1. Tumor cell recognition was observed only in response to antigen-loaded or antigen-transfected tumor cell lines. TCR 5-271-transduced T cells showed low peptide sensitivity, but a CD8-independent functionality.

5.1 Experimental procedure for the isolation of NY-ESO-1-specific CD8$^+$ T cells

5.1.1 Priming approach

In this project, an *in vitro* allogeneic priming approach was performed to stimulate and expand HLA-A2-restricted NY-ESO-1-specific CD8$^+$ T cells. mDCs derived from an HLA-A2-negative healthy donor were transfected with one *ivt*RNA encoding the foreign HLA-A2 molecule and one encoding the full-length NY-ESO-1 antigen and used to stimulate autologous CD8-enriched T cells.

Unlike strategies in autologous settings, the allogeneic priming approach enables the access to the non-tolerant high-affinity allo-reactive TCR repertoire, allowing the potential isolation of high-avidity T cells that recognize peptides derived from the TAA of interest [79]. Due to the excellent capacity of mDCs to induce (prime) antigen-specific T cells from the *naïve* lymphocyte repertoire, priming procedures including *in vitro* mDCs have been successfully established [80], [84]. Accordingly, *in vitro* mDCs were selected as APCs for the priming procedure performed in this project. Protocols using other types of APCs to stimulate tumor antigen-specific T cells from the non-tolerant allo-reactive T cell repertoire of healthy donors have also been utilized by several research groups. Approaches including the T2 cell line (T and B cell hybrid), that has defects in the antigen-processing pathway but

expresses HLA-A2 molecules on its surface, have been developed to isolate tumor antigen-specific HLA-A2-allo-restricted T cells from healthy donors [81], [83], [114]. However, many T cell clones isolated using these non-professional APCs were not able to efficiently recognize tumor cells endogenously positive for the antigen of choice. Other groups have utilized B cells as APCs after they coupled MHC-peptide monomers to the CD20 molecules on the surface [115]. This approach exploits the professional APC capacity of B cells, albeit it is a quite laborious procedure requiring the generation of MHC-peptide monomers for each tumor antigen of interest.

In this thesis, *ivt*RNA transfection was chosen as method to express the desired HLA molecules and tumor antigen into mDCs in order to enable the induction of HLA-A2-restricted NY-ESO-1-specific T cells. In general, MHC epitope loading can be achieved either by external loading of pre-defined peptides or by cell-intrinsic presentation of endogenous peptides by classical MHC presentation pathways [116], [117]. External loading of small synthetic peptides allows only the isolation of T cells with pre-defined specificities. The selection of peptides requires the knowledge of immunogenic epitopes derived from the antigen of interest. Importantly, externally loaded peptides are not subject to post-translational modifications. Therefore, this loading strategy includes the risk of isolating T cells only able to recognize synthetic peptides and not physiologically processed and presented epitopes. In addition, externally loaded peptide:MHC complexes frequently exhibit short half-lives which may hamper successful priming of T cells. All these limitations led to the exclusion of the usage of short peptides to load mDCs as antigen delivery strategy for the isolation of T cells specific for unknown epitopes derived from NY-ESO-1. Electroporation of *ivt*RNA encoding the selected tumor antigen is a strategy for antigen delivery that circumvents most of the disadvantages of peptide loading. Following transfection, *ivt*RNA molecules serve as the template for cell intrinsic translation, adding new antigens to the pool of endogenous proteins. Subsequently, full-length proteins are processed and presented following internal cellular pathways. The use of *ivt*RNA as the antigen source for T cell activation allows any DNA sequence to be used as a transcription template and facilitates the isolation of T cells specific for all potential immunogenic epitopes derived from the tumor antigen of interest. The electroporation of *ivt*RNA leads to a longer presentation of the selected antigen on the cell surface of mDCs, increasing the probability of a successful T cell induction [118], [119]. It has been shown previously that electroporation of mDCs with *ivt*RNA is effective and that mDCs retain their mature phenotype as well as their migratory capacities after electroporation [120], [121]. Moreover, simultaneous transfection of *ivt*RNA encoding MHC class I molecules and TAA into mDCs has been shown to be a reliable method for obtaining co-expression of both proteins [122].

For priming, a high-purity CD8$^+$ enriched T cell population was used from autologous PBMC to enlarge the starting CD8$^+$ T cell repertoire for the induction of NY-ESO-1-specific T cells. Although it is well-known that CD4$^+$ T cells have a pivotal role for T cell priming and for the survival of CD8$^+$ T cells *in vivo*, previous experiments in our group have shown that the presence of CD4$^+$ T cells is not necessary for *in vitro* CD8$^+$ T cell priming and expansion when cytokines, such as IL-7 and IL-2, are added to the priming co-culture (data unpublished). A CD8-untouched cell isolation procedure was used to avoid any unwanted activation of CD8$^+$ T cells mediated by antibody-binding prior to priming procedure.
CD8-enriched T cells were co-cultured with *ivt*RNA-transfected mDCs in the presence of IL-7 and IL-2 in order to sustain the proliferation of CD8$^+$ T cells during the priming procedure. These cytokines are commonly used as supplements for *in vitro* cultivation of T cells to support cell survival and proliferation [123]. Importantly, IL-2 was added to the medium two days after setting up the co-cultures according to the findings of previous studies, where it was observed that T cells reactive to irrelevant antigens can be transiently activated and start to proliferate immediately after induction, leading to a dilution of target-specific T cells [124]. Delaying cytokine addition was shown to help reducing the expansion of these unwanted T cells. Previous reports further showed that high antigen concentrations and repeated stimulations with mDCs could result in the deletion of high-avidity tumor antigen-specific T cells by activation-induced cell death (AICD), affecting the quality of the induced T cells [125], [126]. An effector to target ratio of 10:1 was defined and tested in previous allogeneic priming experiments in our group [84]. This ratio resulted in an efficient induction of high-avidity tumor antigen-specific T cells without causing over-stimulation and death of the desired T cells. Therefore, T cells and mDCs were co-cultured at this standard E:T ratio. In contrast to previous protocols, only one stimulation with mDCs was performed, aiming to improve and optimize the priming procedure. Additional stimulations might induce a massive growth of HLA-A2-allo-reactive T cells reducing the fraction of NY-ESO-1-specific T cells present within the priming culture. Performing only one stimulation might further reduce the risk of AICD and favor survival of the desired NY-ESO-1-specific T cells. Moreover, the entire priming protocol was thereby simplified as the number of mDCs and the time required for the complete procedure were reduced.

5.1.2 Sorting approach

After *de novo* induction, NY-ESO-1-specific T cells needed to be selectively separated from the rest of primed T cells to enable functional characterization as well as isolation of individual TCR sequences. Here, it was established and tested in the context of an allogeneic priming a novel 2-step-sorting strategy independent of the knowledge of

immunogenic epitopes and based on the activation-induced CD137 marker and K562 cell stimulation.

The technology based on multimers is the most common strategy used to selectively isolate tumor antigen-specific T cells [93], [94]. The applicability of multimer technology requires knowledge of immunogenic peptide sequences and corresponding MHC alleles. Therefore, this procedure allows the isolation of T cells only with defined peptide specificities, whereas it lacks the possibility to capture T cells recognizing other peptides generated by using *ivt*RNA encoding the full-length protein as the antigen source. For well-known immunogenic peptides, the multimer technology has been shown to be an efficient sorting method to use with patient samples as well as in combination with *in vitro* and *in vivo* priming protocols [127]–[129]. On the other hand, if less well-characterized antigens are used for T cell priming, peptides for multimer production need to be selected by using epitope prediction tools. Consequently, due to the low reliability of these tools previously discussed, multimers binding peptides that are not naturally processed and presented in tumor cells could be used for sorting, leading to isolation of T cells that are not relevant for clinical application [95], [96]. Furthermore, the availability of multimers is a limiting factor due to possible insufficient peptide:MHC complex stability and to sequence-dependent difficulties in MHC protein folding for some HLA molecules. Due to these limitations, an alternative method based on the analysis of the expression of an activation-induced marker was sought to isolate NY-ESO-1-specific T cells in this project. This strategy allowed to fully exploit the potential of the full-length tumor antigen *ivt*RNA used for T cell induction in the priming procedure.

The sorting strategy required firstly, selecting an appropriate T cell marker which was upregulated strictly and strongly in response to TCR signaling, while the expression absent during the resting phase of T cells prior to stimulation. Accordingly, CD137 was selected as marker for the sorting procedure since it is one of the most promising activation-induced T cell marker described in the literature [99], [100]. CD137 is a member of the TNF receptor family that promotes proliferation and survival of activated T cells [130], [131]. In the clinical setting of allogeneic stem cell transplantation, the evaluation of CD137 expression on donor-derived T cells upon stimulation with allogeneic recipient APCs and the consequent depletion of $CD137^+$ T cells prior to infusion has been proved to be an efficient procedure to reduce alloreactivity [132]. In previous studies, this marker has already been used to efficiently isolate $CD8^+$ T cells specific for different tumor antigens from autologous T cell repertoires [99], [101]. Alternatively, protocols including the analysis of activation-induced cytokine secretion have also been developed by several research groups [97], [98]. Cytokine secretion by T cells upon specific activation is a tightly regulated process, therefore detection of cytokine secretion represents a suitable parameter for the isolation

of tumor antigen-specific T cells. However, such functional assays may reflect only a subset of specifically activated T cells. Due to very heterogeneous cytokine profiles of different T cell subpopulations, functionally diverse cells may not be identified by using a single cytokine, but have to be tracked by a multi-cytokine assays. These assays might result quite laborious and tedious as sorting of a high number of primed T cells need to be performed to meet the rare desired T cells within the population.

Choosing efficient and appropriate APCs to induce CD137 expression on T cells was another crucial step for the sorting strategy. The human erythroleukemic cell line K562 derived from a patient with chronic myelogenous leukemia in blastic crisis was chosen as the source of stimulating cells [133]. K562 cells represent ideal starting cell line to generate cell-based genetically engineered APCs and they have already been used in several protocols for *in vitro* T cell stimulation [134]. K562 cells do not express endogenous MHC class I and II molecules as well as costimulatory and co-inhibitory molecules, other than CD80 at a low level. By contrast, they express ICAM-1 (CD54) and LFA-3 (CD58), which are adhesion molecules required to form an effective immunological synapse [135]. Since they endogenously express β2-microglobulin, the introduction of MHC class I heavy chain molecules alone is sufficient for MHC class I surface expression [136]. Additionally, it has been shown that K562 cells possess fully functional antigen processing and MHC presentation machineries [137], [138]. There were several other advantages to use K562-based aAPCs to induce CD137 expression on T cells compared to either standard APCs, such as autologous mDCs, B cells and LCL, or to other tumor cell lines. Compared to standard APCs, K562 cells enabled stimulation of T cells restricted to the solely desired allogeneic HLA-A2 molecules, excluding potential isolation of T cells restricted to self-MHC molecules which could be present in the priming culture. Additionally, using K652 cells it was possible to isolate T cells specific for NY-ESO-1 peptides that were naturally processed and presented by a tumor cell line, which might be different from the peptide repertoire of mDCs, B cells and LCL. Immune system cells, especially professional APCs, constitutively express immunoproteasome subunits while tumor cells contain nearly only standard proteasome subunits under basal conditions, albeit oxidative stress and proinflammatory cytokines can cause the upregulation of the immunoproteasome components. The standard proteasome and the immunoproteasome are different in their catalytic activity and peptide generation, leading to differences in the peptide repertoire of different cell types [139], [140]. Due to the lack of endogenous MHC molecules in K562 cells, there was no activation of T cells that were reactive to foreign MHC molecules other than the introduced HLA-A2. The activation of high numbers of allo-reactive T cells represented one of the major limitations to use other tumor cell lines expressing up to six different MHC class I molecules. In addition, compared to LCL, K562 cells lack EBV peptides and thus, activation of potentially

primed EBV-specific T cells during analysis of T cell samples was avoided utilizing K562 cells as APCs. Priming of EBV-specific T cells is quite probable if the donor of the priming is EBV-positive, which is the case in about 95% of the world population [141]. Having these advantages, K562 cells were used as a starting cell line to generate aAPCs by the introduction of HLA-A2 molecule and CD86 co-stimulatory molecule by retroviral transduction. Since in previous priming experiments in our group the survival of antigen-specific $CD8^+$ T clones after sorting was extremely low, CD86 co-stimulatory molecule was introduced into K562 cells with the final aim to enhance and sustain T cell clone survival (data unpublished).

An additional critical aspect in developing the sorting strategy based on CD137 was related to the induction of a high number of HLA-A2-allo-reactive T cells within the culture in an HLA-A2-allogeneic priming approach. The T cell repertoire towards an allogeneic HLA molecule has not undergone negative selection. Hence, T cells recognizing allogeneic MHC molecules in combination with self-peptides are still present in the post-thymus selected T cell population [48], [49], [79]. As an unavoidable consequence of the HLA-A2-allogeneic priming, not only are HLA-A2-restricted tumor antigen-specific T cells induced, but also many unwanted HLA-A2-allo-reactive T cells. These HLA-A2-allo-reactive T cells will express CD137 in response to HLA-A2-positive APC stimulation, independently of the presence of the tumor antigen, and it is impossible to distinguish between the desired antigen-specific T cells and HLA-A2-allo-reactive T cells during sorting, but only in subsequent functional screenings at the clonal T cell level. The probability to isolate tumor antigen-specific T cells using an allogeneic priming approach in combination with a sorting method based on CD137 expression on T cells is therefore extremely low. Previous experiments in our group have shown that it is necessary to sort a very high number of clones to be able to catch a tumor antigen-specific clone, limiting the feasibility of the classical CD137-based sorting in combination with allogeneic priming approaches (data unpublished). An innovative 2-step-procedure called ‘double-sorting’ was developed and tested for the first time in this study in order to improve the CD137 based sorting strategy. The innovation within this approach was to perform an allogeneic HLA-A2 and NY-ESO-1-unspecific stimulation of the primed T cells as first step and subsequently use the T cells that do not express CD137 in a next step for HLA-A2 and NY-ESO-1-specific stimulation. For the first NY-ESO-1-unspecific stimulation, irradiated water-electroporated tgK562 cells (endogenously negative for NY-ESO-1) were utilized at a high effector to target ratio of 2:1. The idea of using a high number of K562 cells was to provide sufficient numbers of APCs to activate as many HLA-A2-allo-reactive T cells as possible. At this step, the risk of inducing AICD was not relevant for NY-ESO-1-specific T cells since the activated T cells represented the unwanted cell fraction that was meant to be discarded. The final aim of the

first step was to enrich NY-ESO-1-specific T cells (CD137-negative fraction) by removing T cells that upregulated CD137 independently of NY-ESO-1. To note, the CD137-negative fraction sorted after NY-ESO-1-unspecific stimulation might include not only the desired NY-ESO-1-specific T cells, but also T cells that were either unreactive to HLA-A2 or were HLA-A2-allo-reactive but were not in the cell cycle phase to be activated at the time of sorting. In the second step, for antigen-specific stimulation of the previously sorted T cells, tgK562 cells transfected with *ivt*RNA encoding NY-ESO-1 were used at an effector to target ratio of 5:1. The ratio was higher compared to the number of mDCs used for *de novo* induction (E:T ratio of 10:1) since tgK562 cells are not professional APCs as mDCs.
A high number of single-cell-sorted T cells expanded after 14 days, showing that, in general, T cells were able to survive and expand after two rounds of sorting by FACS. The evaluation of IFN-γ release of the expanded clones revealed T cell clones with different reaction patterns (152 non-reactive, 144 HLA-A2-allo-reactive and 5 NY-ESO-1-reactive). The observation of five potential NY-ESO-1-specific $CD8^+$ T cell clones was the first proof-of-principle for the double-sorting procedure based on CD137 and K562 cells as APCs, demonstrating the feasibility of this approach. However, still a lot of unwanted T cell clones were obtained, showing that the procedure needs to be further optimized.

5.2 Characterization of the properties of the identified TCR 5-271

5.2.1 Antigen specificity: unconventional epitopes

The epitope recognized by the isolated TCR 5-271 was mapped to the protein encoded by ORF2 of NY-ESO-1 and the LAGE-1, supporting findings of previous studies showing the existence of unconventional epitopes encoded by non-primary ORFs for several tumor antigens [142]–[145]. Especially, NY-ESO-1-ORF2 and CAMEL proteins were already shown to contain epitopes recognized by both $CD8^+$ and $CD4^+$ T cells [111], [146]–[149]. Two different peptides derived from NY-ESO-1-ORF2/CAMEL and presented in the context of MHC class I molecules have been identified so far. Wang and colleagues have identified a $CD8^+$ T cell clone derived from TILs of a melanoma patient specific for a peptide derived from NY-ESO-1-ORF2/CAMEL protein and presented on the HLA-A31 molecule (NY-ESO-1-ORF2/CAMEL$_{18-27}$) [149]. An HLA-A2-restricted NY-ESO-1-ORF2/CAMEL-specific (NY-ESO-1-ORF2/CAMEL$_{1-11}$) $CD8^+$ T cell clone was obtained by Aarnoudse and colleagues from PBMC of a melanoma patient using autologous melanoma cells for *in vitro* stimulation in the presence of IL-2 [111]. The isolation of T cells specific for NY-ESO-1-ORF2/CAMEL from melanoma patients proved that epitopes derived from proteins encoded by the alterative ORFs can be naturally processed and presented by tumor cells eliciting T cell

responses. These findings together with the data regarding other antigens demonstrate the immunological significance of epitopes produced unconventionally [150], [151]. The mechanism by which proteins, derived from alternative ORFs, are translated is currently unclear. However, there are several potential explanations for the physiological production of alternative ORF-encoded proteins. One of them is that some ribosomes occasionally bypass the first AUG start codon with a weak kozak sequence (non-optimal adjacent sequences supporting the starting of the translation) and initiate translation at a downstream AUG. This mechanism is designated as leaky scanning [152], [153]. The leaky scanning enables the translation of two different ORFs and the consequent synthesis of two separately initiated proteins from one mRNA transcript. The derived alternative ORF-encoded protein can either be a shorter version of the full-length ORF encoded-protein if the second AUG is in the same reading frame of the first AUG or a completely different protein if the second AUG is in an alternative reading frame. Both in NY-ESO-1 and in LAGE-1 mRNA transcripts, the sequences adjacent to the start codon of the primary ORF are not optimal for supporting the start of translation. Therefore, leaky scanning might happen leading to translation of primary and non-primary ORFs of NY-ESO-1 and LAGE-1. As a consequence, NY-ESO-1-ORF2 and CAMEL proteins can be produced in addition to the full-length NY-ESO-1 and LAGE-1 proteins. Since NY-ESO-1-ORF2 and CAMEL proteins are encoded by ORFs included in alternative reading frames than the primary ORFs, the resulting proteins show a completely different amino acid sequence compared to the conventional ORF1-encoded proteins.

In this project, the cDNA sequence used to generate NY-ESO-1 *ivt*RNA for the priming was not codon-optimized and no kozak sequence was introduced upstream of the tumor antigen. Codon-optimization and addition of a kozak sequence are the most common strategies to increase protein expression of a transgene [106], [152]. However, these strategies were not used in the NY-ESO-1 *ivt*RNA because high amounts of *ivt*RNA were introduced into mDCs to obtain adequate NY-ESO-1 protein expression to efficiently induce NY-ESO-1-specific T cells. Consequently, it was possible that the non-primary ORF was translated, the corresponding protein was processed and non-primary ORF-derived peptides were presented in addition to the primary ORF-derived peptides after NY-ESO-1 *ivt*RNA transfection into mDCs, leading to the potential induction of T cells specific for unconventional peptides in the priming procedure.

5.2.2 Low abundance of alternative ORF-encoded protein in tumor cells

TCRs are only suitable for clinical development if they are able to recognize tumor cells endogenously expressing the target antigen and the MHC molecules. The recognition of

tumor cells by T cells expressing a transgenic tumor antigen-specific TCR requires efficient epitope presentation on MHC molecules by tumor cells. The latter depends on several factors, including the expression level of the tumor antigen in the target cells, the expression level of the MHC molecule on the tumor cell surface, the capacity of tumor cells to process and present the epitope of interest and the binding affinity of the peptide for the MHC molecule [154].

None of the tumor lines tested in the assay were recognized by the TCR 5-271, although NY-ESO-1/LAGE-1a mRNA expression was verified by Nanostring analyses and the surface expression of the HLA-A2 molecules was proved by flow cytometry. Unfortunately, no commercial antibodies were available to detect intracellular expression of the NY-ESO-1-ORF2 and the CAMEL protein. Thus, no information regarding the endogenous expression of ORF2-encoded proteins were available. Nevertheless, the recognition of NY-ESO-1 *ivt*RNA-transfected tgK562 cells and tumor cell lines by TCR 5-271-transgenic T cells showed that in principle ORF2 of NY-ESO-1 can be efficiently translated, the corresponding protein can be efficiently processed and the epitope recognized by TCR 5-271 can be efficiently presented on HLA-A2 molecules by the tested tumor cell lines.

Interestingly, during the characterization assays, it was hypothesized that translation of ORF2 of LAGE-1a was less efficient compared to the translation of ORF2 of NY-ESO-1 after antigen *ivt*RNA electroporation both into tgK562 cells and into other tumor cell lines. According to Nanostring data, the analyzed tumor cell lines endogenously expressed mainly LAGE-1a, but rarely NY-ESO-1 mRNA transcripts. Consequently, the tested tumor cell lines might translate the LAGE-1a-ORF2 with very low efficiency and express very low amounts of the corresponding protein including the target epitope of TCR 5-271. Thus, these tumor cells might not represent the ideal targets to evaluate tumor recognition mediated by TCR 5-271-transgenic T cells. Tumor cell lines expressing endogenously high levels of NY-ESO-1 should be tested to further investigate this hypothesis.

In general, the identification of unconventional epitopes processed and presented by tumor cells might enlarge the repertoire of potential targets for TCR gene therapy strategies. However, one potential limitation is the relatively low abundance with which alternative ORF-encoded products are usually naturally generated compared to primary ORF-derived proteins in tumor cells [150]. Consequently, TCRs specific for these unconventional epitopes might require a very high peptide sensitivity to efficiently recognize tumor cells expressing low amounts of their target peptides and to be considered as potential therapeutic TCRs for clinical use.

5.2.3 Low T cell peptide sensitivity and CD8-independent functionality

The multistep process leading to efficient epitope presentation on MHC molecules by tumor cells is only one of the components that play a critical role in TCR-mediated tumor recognition. The other relevant element is the interaction between the TCR and its specific peptide:MHC complex. The strength and the stability of the interaction between TCR and peptide:MHC complex as well as the ensuing T cell peptide sensitivity are critical to trigger tumor target recognition and effective T cell responses [155], [156].

TCR 5-271-transduced T cells required very high amounts of peptides for activation as determined by the half-maximal IFN-γ secretion in response to titrated amounts of the target peptide. Thus, the isolated TCR 5-271 might not be a very potent TCR, albeit it was isolated using an allogeneic priming approach. This could explain the limited tumor cell recognition mediated by TCR 5-271-transgenic T cells, hence restricting its potential use in clinical application. Strategies to enhance TCR reactivity might be applied to TCR 5-271 with the final aim to improve its functionality without affecting the antigen specificity. Affinity-enhanced TCRs can be obtained by introducing mutations in CDR regions of TCRα and TCRβ chains, aiming to increase the affinity of the TCR for its target peptide:MHC complex (strategy designated as *in vitro* affinity maturation) [71]–[74]. Besides *in vitro* affinity maturation, proximal TCR co-signaling pathways can be manipulated in order to increase TCR sensitivity for stimulation and activation [157]–[159].

Interestingly, the identified TCR 5-271 displayed independency of CD8 co-receptors, showing functionality after the transfer into $CD8^+$ as well as $CD4^+$ T cells. These findings were quite surprising since T cells expressing TCR 5-271 showed low peptide sensitivity. Indeed, functionality in a CD8-independent manner has mainly been observed as characteristic of TCRs showing high-affinity for their target MHC:peptide complex [113]. Nevertheless, each identified TCR needs to be evaluated individually for its CD8 requirement in order to identify exceptions [160]. In principle, CD8-independency represents an advantageous property of a TCR used for T cell engineering, leading to the possibility to generate also tumor reactive MHC class I restricted $CD4^+$ T cells. These modified $CD4^+$ T cells can recognize MHC class I restricted peptides and provide help in anti-tumor reaction and support in terms of proliferation, differentiation and maintenance of $CD8^+$ T cells directly at the tumor site [161], [162].

5.2.4 Fine typing of the recognized epitope

A critical step in the TCR characterization process is the fine typing of the target epitope recognized by the candidate TCR [68]. In this project, the core epitope recognized by the isolated TCR 5-271 was identified and the contribution of each aa of the epitope to the TCR-

mediated recognition was evaluated. Previous studies regarding epitopes presented on HLA-A2 molecules have shown that aa located at the N- and C-terminal end of the peptide are mainly responsible for the interaction with the MHC molecule (anchor residues). By contrast, the interaction with the TCR is principally governed by the central portion of the peptide [163]–[165].

During TCR 5-271 characterization, it was observed that the first and the last leucine (L) of the core epitope (P1 and P8 of the 8-mer peptide LMAQGAML) were essential in determining the target recognition mediated by the isolated TCR. Accordingly, these aa might be anchor residues critical for peptide binding to the HLA-A2 molecule and for the consequent efficient peptide presentation.

The presence of the additional phenylalanine (F) at the N-terminal end of the core epitope might be crucial for further stabilizing the binding between HLA-A2 molecules and the peptide, enabling efficient epitope presentation, particularly under physiological conditions when the peptide is processed and presented by an internal cellular pathway.

Interestingly, the substitution of the glycine (G) in the epitope affected TCR 5-271 recognition in the context of the 9-mer peptide FLMAQGAML, but not in the extended versions of the peptide (11-mer and 12-mer). These results showed that the extended N-terminal or C-terminal ends might alter the accommodation of the peptide in the MHC groove. Consequently, the position and the role of the G in the TCR 5-271-mediated recognition might be different in the extended versions of the peptide.

In conclusion, these findings showed that the modification of single aa in the epitope sequence might either destroy the binding of the peptide with the TCR and/or the MHC molecule abrogating the recognition by the TCR or might not affect the epitope presentation and the TCR recognition. The possibility to modify single aa in the epitope sequence without altering TCR recognition is critical for the analysis of alternative peptides within the human proteome that show homology to the target epitope and might be potentially cross-recognized by T cells expressing the transgenic TCR. In order to determine potentially cross-recognized peptides, N-terminal and C-terminal extended versions of the core epitope need to be also evaluated since they can also trigger T cell stimulation as shown in this study. Other than the alanine scanning assay, tests including combinatorial aa substitutions should be performed to gain more information for each single aa within the epitope. Three-dimensional structure models may also help to fully understand the interaction between peptide, MHC molecule and TCR.

5.3 Outlook

The successful isolation of the T cell clone 5-271, recognizing a new epitope derived from NY-ESO-1-ORF2/CAMEL protein, was the first proof-of-principle study of the innovative double-sorting strategy based on CD137 and K562 cells combined with an HLA-A2-allogeneic priming approach based on mDCs transfected with *ivt*RNA encoding the full-length tumor antigen. Together these features enabled bypassing knowledge of pre-defined epitopes for tumor antigen-specific T cell isolation. However, further improvements can be developed to extend and increase the success and efficiency of the sorting strategy. One possibility could be to use K562 cells transduced only with HLA-A2 molecules without additional co-stimulatory molecules. CD86 was selected as co-stimulatory molecule to enhance and sustain T cell survival after sorting. However, its high expression on K562 cells might also favor low-avidity T cells to upregulate CD137 which are not the desirable ones to be identified for clinical application. Since K562 cells can easily be genetically modified, it would be possible to transduce them with the tumor target antigen to obtain a stable and uniform antigen expression for T cell stimulation. The use of antigen-transduced K562 cells would simplify the double-sorting procedure and it would completely avoid any possible uncontrollable activation of genes in K562 cells due to the electroporation procedure. A 'triple-sorting' approach including two rounds of target antigen-unspecific stimulation might be tested, aiming to further reduce HLA-A2-allo-reactive T cells. Consequently, the entire procedure will take longer and will be even more stressful for the desired T cells. Hence, addition of very low doses of cytokines might be needed during the sorting period to assure T cell fitness and survival. Since studies in our group have shown that the probability to isolate the desired tumor antigen-specific T cells may differ substantially between donors and varies across different tumor target antigens, the double-sorting approach should be tested in multiple priming approaches using several donors and different tumor target antigens to fully evaluate its feasibility and applicability in the context of allogeneic priming protocols. A test system using an established tumor antigen-specific T cell clone might be developed to evaluate the efficiency of the double-sorting procedure. In principle, the concept of 'double-sorting' could be applied to any T cell activation marker, other than CD137, showing expression on the surface of T cells only after TCR engagement.

As observed in this project, the presence of alternative ORFs in alternative reading frames in the wild-type sequence of the selected tumor antigen may reduce the efficiency of the induction of T cells specific for peptides derived from primary ORF-encoded proteins during the priming procedures. Thus, alternative ORFs might be eliminated by partial-codon-optimization of the cDNA sequence encoding the desired tumor target antigen without

altering the primary ORF encoding the protein of interest. Including this concept into the design of the tumor antigen sequences for priming protocols will abolish the induction of T cells specific for peptides derived from low abundant proteins encoded by alternative ORFs. Moreover, it will help to control the priming procedure and to increase its efficiency, allowing the isolation of TCRs specific only for peptides derived from defined proteins.

6 Abbreviations

A	Alanine
aa	Amino acid
ALL	Acute lymphoblastic leukemia
APC	Allophycocyanin
APC-Cy7	Allophycocyanin – cynine 7
APCs	Antigen presenting cells
aAPCs	Artificial antigen presenting cells
ATLL	Adult T cell leukemia/lymphoma
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovines serum albumin
BUV	BD horizon brilliant™ ultraviolet
C	Cysteine
CAMEL	Cytotoxic T lymphocyte-recognized antigen on melanoma
CAR	Chimeric antigen receptor
CCR7	C-C motif chemokine receptor 7
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Complementarity determinig region
CMV	Cytomegalovirus
C region	Constant region
CSA	Cyclosporin A
CTAs	Cancer-testis antigens
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
D	Aspartic acid
DCs	Dendritic cells
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's medium
DLBCL	Diffuse large B cell lymphoma
DLI	Donor-derived lymphocyte infusion
DMSO	Dimethylsulfoxide
DNA	DeoxyriboNucleic Aaid
DPBS	Dulbecco's phosphate-buffered saline
E	Glutamic acid
EBV	Epstein-Barr-Virus
EDTA	Ethylenediaminetetraacetic acid
EF1-α	Elongation factor 1-alpha
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbent assay
Env	Envelope
EP	Electroporation
E:T ratio	Effector to target ratio
F	Phenylalanine
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FW	Forward

G	Glycine
Gag	Group-specific antigen
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft versus host disease
H	Histidine
h	Hours
H_2O	Water
HA-1	Histocompatibility antigen 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HS	Human serum
HSA	Human serum albumin
I	Isoleucine
IFN-γ	Interferon-gamma
Ig	Immunglobulin
IL	Interleukin
iDCs	Immature DCs
IMDM	Iscove's modified dulbecco's media
IMGT	ImMunoGeneTics
IU	International unit
*ivt*RNA	*In vitro* transcribed RNA
K	Lysine
L	Leucine
LAGE-1a	Cancer/testis antigen 2 (CTAG2) isoform a
LAGE-1b	Cancer/testis antigen 2 (CTAG2) isoform b
LCL	Lymphoblastoid cell line
LB	Luria Broth Base
LTR	Long terminal repeat
M	Methionine
MACS	Magnetic-activated cell sorting
MAGE-A1	MAGE Family Member A1
MAGE-A3	MAGE Family Member A3
MART-1	Melanoma antigen recognized by T Cells
MCS	Multiple cloning site
mDCs	Mature dendritic cells
MEM NEAA	Minimum essential medium non-essential amino acids
MESV	Murine embryonic stem cell virus
MHC	Major histocompatibility complex
ml	Milliliter
MoMuLV	Moloney murine leukemia virus
MPSV	Myeloproliferative sarcoma virus
mRNA	Messenger RNA
n.a.	Not analyzed
nt	Nucleotide
NCBI	National center for biotechnology information
NY-ESO1	Cancer/testis antigen 1B (CTAG1B)
ORF	Open reading frame
PB	Pacific Blue
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS VLE	Phosphate-buffered saline very low endotoxin
pco	Partial-codon-optimization
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death protein ligand-1
PE	Phycoerythrin

PE-Cy7	Phycoerythrin – cynine 7
PercP	Peridinin-chlorophyll protein
PFA	Paraformaldehyde solution
PGE_2	Prostaglandin E2
PHA	Phytohaemagglutinin
Pol	Polymerase
Q	Glutamine
R	Arginine
RACE-PCR	Rapid amplification of cDNA ends - polymerase chain reaction
RNA	Ribonucleic acid
RPMI 1640	Rosewell Park Memorial Institute Medium 1640
S	Serine
SIN	Self-inactivating
T	Threonine
TAA	Tumor-associated antigen
TAE	Tris Acetate EDTA
TAP	Transporter associated with antigen processing
TCR	T cell receptor
tgK562	Transgenic K562 cells
TF	Transfection
TILs	Tumor-infiltrating lymphocytes
TNF-α	Tumor necrosis factor-alpha
TRAC	TCR alpha constant segment
TRAJ	TCR alpha joining segment
TRAV	TCR alpha variable segment
TRBC	TCR beta constant segment
TRBJ	TCR beta joining segment
TRBV	TCR beta variable segment
TSA	Tumor-specific antigen
UTR	Untranslated region
UV	Ultra-violet
V	Valine
VLE	Very low endotoxin
W	Tryptophan
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
wt	Wild-type
Y	Tyrosine

7 References

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Affidavit

Longinotti, Giulia

I hereby declare that the submitted thesis, entitled

“Isolation and characterization of a TCR specific for a new unconventional NY-ESO-1 epitope”

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 25.07.2019	Giulia Longinotti
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