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TRUCKs for Metastatic Melanoma: a new motorway for immunotherapy, carrying TCRs and delivering cytokines

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"This is for everybody going through tough times;
Believe me, been there, done that.
But everyday above ground is a great day, remember that."

— Armando Christian Pérez, 2014

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Abstract

Cancer is one of the leading causes of death in developed countries. In solid tumours, such as metastatic melanoma, the tumour microenvironment induces cellular changes to evade the immune response. Immunotherapy has addressed this problem by using cytokines, such as IL-12, or by genetically modifying T-cells. Recently, it has been shown that T-cells can be genetically altered using plasmids in order to recognise antigens and release cytokines upon their recognition, called T-cells Redirected for Universal Cytokine-mediated Killing (TRUCKs). The ideal plasmids should be non-viral and compact, have no antibiotic resistance genes and have persistent expression without incorporating the cell genome. Thus, this thesis aims to develop a TRUCK-like immunotherapy targeting metastatic melanoma using a novel single-vector system that simultaneously expresses DMF5 TCR under the expression of the non-viral S/MAR system and IL-12 under the control of the NFAT binding site as a responsive element of cellular activation.

As a proof of concept, the "one-vector system", a single-vector system expressing both the GFP gene under the control of NFAT and DMF5 TCR, was developed. Its expression was compared with the traditional co-transfection technique using Jurkat 76 cells expressing CD8. Through green fluorescent signal analysis with the Incucyte® SX5 Device, this system demonstrated increased cell activation when cells were artificially stimulated or when in co-culture with target cells expressing MART-1, i.e., MeWo cells or a U87 cell line expressing MART-1. Furthermore, human primary T-cells demonstrated uptake of this system by nucleofection, although there was a decrease on cell viability. However, since only one donor was evaluated, further studies are needed to confirm the results obtained in primary T-cells.

Recently, it was demonstrated that a synthetic promoter named "TATA" presents cell stimulation-dependent gene expression. To evaluate cytokine production between different promoters, an improved version of the Overlap Extension PCR technique was developed for the insertion of promoters into TRUCK-like vectors. Thus, using only PCR primers and following the designed cloning strategy, the TRUCK-like IL-12 vectors were obtained, where the GFP gene was replaced by a promoter, being either the minP (minimal promoter) or the synthetic TATA promoter, and by the sequence corresponding to the cytokine IL-12. For this, a cloning strategy was designed to obtain "nano" versions of the TRUCK IL-12-like vectors, replacing the bacterial backbone and the antibiotic resistance gene by the R6K-RNA-OUT® system developed by NTC©, in collaboration with them.

Overall, in this thesis, it is demonstrated that the one-vector system shows higher gene expression than co-transfection of both expression cassettes. An improved Overlap Extension PCR technique, where only PCR primers are used, is also presented. Thus, two TRUCK-like plasmids were obtained for the evaluation of IL-12 production, depending on the upstream promoter. Finally, a cloning strategy is described to obtain TRUCK-like nanoplasmids, for the potential application of this therapy in the future.

Resumo

O cancro é uma das principais causas de morte nos países desenvolvidos. Em tumores sólidos, como melanoma metastático, o microambiente tumoral induz mudanças celulares para escapar à resposta imune. A imunoterapia tem colmatado este problema utilizando citocinas, como a IL-12, ou modificando geneticamente linfócitos T, para melhorar a resposta imune. Recentemente, foi demonstrado que linfócitos T podem ser geneticamente alterados usando plasmídeos de forma a reconhecer antigénios e libertar citocinas aquando do seu reconhecimento, denominadas de T-cells Redirected for Universal Cytokine-mediated Killing (TRUCKs). Os plasmídeos ideais devem ser não-virais e compactos, não apresentarem genes de resistência a antibióticos e ter expressão persistente sem incorporar o genoma celular. Pelo exposto, esta tese visa desenvolver uma imunoterapia tipo TRUCK dirigida ao melanoma metastático, utilizando um novo sistema de um único vetor que expressa simultaneamente o DMF5 TCR, sob a expressão do sistema não-viral S/MAR, e a IL-12, sob o controlo do local de ligação do NFAT como elemento de ativação celular.

Como prova de conceito, foi desenvolvido o "one-vector system", um sistema de um único vetor que expressa tanto o gene GFP sob o controlo do NFAT como o DMF5 TCR. A sua expressão foi comparada com a técnica tradicional de co-transfeção, usando células Jurkat 76 que exprimem CD8. Através da análise do sinal fluorescente verde com o Dispositivo Incucyte® SX5, este sistema demonstrou uma maior ativação celular quando as células foram estimuladas artificialmente ou quando em co-cultura com células-alvo que expressam MART-1, isto é, células MeWo ou uma linha celular U87 geneticamente modificada para exprimir o epítopo do MART-1. Além disso, linfócitos T primários humanos demonstraram a assimilação deste sistema através de nucleofeção, embora a viabilidade tenha diminuído. No entanto, sendo que apenas um dador foi avaliado, são necessários mais estudos para confirmar os resultados obtidos em linfócitos T primários humanos.

Recentemente, foi demonstrado que um promotor sintético denominado "TATA" apresenta expressão genética dependente da estimulação celular. Para avaliar a produção de citocina entre diferentes promotores, foi desenvolvida uma versão melhorada da técnica de Overlap Extension PCR, para a inserção dos promotores nos vetores tipo TRUCK. Assim, utilizando apenas primers PCR e seguindo a estratégia de clonagem desenhada, foram obtidos os vetores tipo TRUCK IL-12, onde o gene GFP foi substituído por um promotor, sendo ou o minP (minimal promoter) ou o promotor sintético TATA, e pela sequência correspondente à citocina IL-12. Para isso, foi concebida uma estratégia de clonagem para obter versões "nano" dos vetores tipo TRUCK IL-12, substituindo o backbone bacteriano e o gene de resistência a antibióticos pelo sistema R6K-RNA-OUT® desenvolvido pela NTC©, em colaboração com a mesma.

De forma geral, nesta tese, demonstra-se que o one-vector system apresenta maior expressão genética do que a co-transfeção de ambas cassetes de expressão. É, também, apresentada uma técnica melhorada de Overlap Extension PCR, onde apenas são utilizados primers PCR. Assim, foram obtidos dois plasmídeos tipo TRUCK para a avaliação da produção de IL-12, dependendo do

promotor a montante. Finalmente, é descrita uma estratégia de clonagem para a obtenção dos nanoplasmídeos tipo TRUCK, para a potencial aplicação desta terapia no futuro.

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List of Abbreviations

AAV: Adeno-Associated Virus
ACT: Adoptive Cell Therapy
APC: Antigen-Presenting Cell
ApoB: Apolipoprotein B
Bcl: B-Cell Lymphoma Protein
CAR: Chimeric Antigen Receptor
CD: Cluster of Differentiation
CRS: Cytokine Release Syndrome
CTLA: Cytotoxic T-Lymphocyte Antigen
DAPI: 4′,6-diamidino-2-phenylindole
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic acid
E.coli: *Escherichia coli*
ECM: Extracellular Matrix
EF: Elongation Factor
ELISA: Enzyme-Linked Immunosorbent Assay
EMA: European Medicines Agency
FBS: Foetal Bovine Serum
FDA: Federal Drug Administration
GFP: Green Fluorescent Protein
GFU: Green Fluorescence Unit
GP: Glycoprotein
HLA: Human Leukocytic Antigen
IARC: International Agency for Research on Cancer
IFN: Interferon
IL: Interleukin
J76 CD8+: CD8-expressing Jurkat 76 cell line
LB: Lysogeny broth
MART: Melanoma Antigen Recognized by T-cells
MHC: Major Histocompatibility Complex
miniCMV: minimal CytoMegaloVirus promoter
minP: minimal Promoter from the IL-2 gene
mRNA: messenger RNA
NFAT: Nuclear Factor of Activated T-cells
NF-kB: Nuclear Factor κ of activated B cells
NK: Natural Killer
NTC: Nature Technology Corporation
ORF: Open Reading Frame
P2A: Porcine teschovirus-1 derived 2A Peptide
PB: piggyBac
PBMC: Peripheral Blood Mononuclear Cells
PBS: Phosphate-Buffered Saline
PCR: Polymerase Chain Reaction
PD: Programmed Death protein
PE-mTCR: PE anti-mouse TCR β chain
PolyA: Polyadenylation
RNA: Ribonucleic acid
S/MAR: Scaffold/Matrix Attachment Regions
SB: Sleeping Beauty
SEM: Standard Error of Mean
STAT: Signal Transducer and Activator of Transcription
SV40: Simian Virus 40
TATA: synthetic promotor described by Mann *et al.* (2003)
TCR: T-Cell Receptor
TGF: Transforming Growth Factor
TILs: Tumour Infiltrating Lymphocytes
TME: Tumour Microenvironment
TMG: Tandem Mini Gene
TNF: Tumour Necrosis Factor
TRAIL: TNF-Related Apoptosis-Inducing Ligand
TRUCKs: T-cells Redirected for Universal Cytokine-mediated Killing
U87-TMG: U87 cell line that expresses the TMG of the MART-1 epitope
UV: Ultraviolet

Chapter 1 Introduction

1. Cancer Overview
2. Tumour Microenvironment and Immunology
3. Cancer Immunotherapy
4. Genetic Engineering
5. Aim of this project

1. Cancer Overview

Alongside cardiovascular diseases, cancer is one of the biggest causes of death in developed countries [1,2]. It is characterized as the over-proliferation of tumour or cancer cells, which have undergone transformation - the process that leads to the formation of a neoplasm from normal cells and requires the action of tumorigenic agents. It is a relatively lengthy process in most cases, as it involves the acquisition of genomic alterations, especially regarding their metabolism and cell cycle, which confer autonomous growth capacity and selective advantage to the neoplastic cells [3].

In 2011, Hanahan and Weinberg described the "hallmarks" of the cancer cells, which are considered as the gold-standard rules to characterize cancer cells, and they are the following: self-sufficiency of growth stimuli, insensitivity to growth inhibitory signals, evasion of apoptosis, DNA repair deficiency, unlimited replicative potential, and sustained angiogenesis [4].

Cancer can be categorized into liquid or solid tumours [3]. The first type consists in the emergence of cancer cells within blood, lymph nodes or bone marrow. These cells also have the ability to circulate in the organism through the blood flow, without having to change their phenotype [3]. Solid tumours involve the development of cancer cells restricted to an organ system, such as the liver or lungs, creating a tumour microenvironment beneficial for their growth. These cancer cells can acquire the ability of migration, while changing from an epithelial to a mesenchymal phenotype. Metastases are, then, developed due to cancer cell migration through blood flow and posterior invasion of the target tissues [5,6].

The tumour microenvironment and the cell change it instigates will be discussed in subchapter 2.

1.1. Metastatic Melanoma

Melanoma is a solid tumour-producing cancer developed from carcinogenic melanocytes. While these cells are most prevalent in the skin, they are also found in other parts of the body, such as the central nervous system or the eye, and, therefore, melanoma can also emerge in these areas [7,8]. Skin melanoma, specifically metastatic melanoma, will be the main topic of this dissertation.

Melanoma risk factors are classified into two categories: environmental or host related. The first encompasses solar radiation, that is, exposure to ultraviolet (UV) light from sunlight [8,9]. The relationship between sunlight exposure and duration and the development of skin cancer has been extensively studied, which can contribute to melanoma risk stratification [9,10]. The host related risks consist of congenital and acquired melanocytic nevi, pigmentation or complexion differences, familial background, and immunosuppression diseases [8].

A statistical analysis from the International Agency for Research on Cancer (IARC) regarding the estimated age-standardized incidence rates of melanoma of skin in 2020 worldwide [11,12], demonstrated that Australia, New Zealand, and Denmark are the top three countries with the

highest incident rates, which confirms the correlation between the increasing solar radiation exposure and the diminished pigmentation of the population [11,12].

Regarding the immune system, especially T-cells , some studies have been made to understand its role in eliminating metastatic melanoma. It was discovered that CD8+ [(a)] T-cells may destroy tumour cells containing the appropriate tumour-specific antigen, and, in melanoma, Tyrosinase-derived peptides, Melan-A antigen, proteins glycoprotein 100 (gp100), Melanoma-associated Antigen Recognised by T-cell 1 (MART-1), and gp75 are the most used antigens in cancer therapy. These are markers that distinguish the melanocyte lineage alone but are overexpressed in metastatic melanoma.

Focusing on the MART-1 antigen, it is recognised by the majority of HLA-A2 (Human Leukocytic Antigen) restricted T-cells and is identical to the antigen Melan-A [13–16]. It has been reported that MART-1 is widely expressed in this type of cancer, with previous studies evaluating its expression in different development stages [17,18], and it shows a high binding strength when recognised by immune cells. However, it appears that CD8+ T-cells are not truly effective against it, possibly because of resistance mechanisms or poor effector function [3,19–21], further justifying the need for immunological therapy.

2. Tumour Microenvironment and Immunology

There is evidence that there are well-established interactions and communication between tumour cells and stromal cells, as well as the transmission of oncogenic signals between them, promoting carcinogenesis, being referred to as the tumour microenvironment (TME).

The TME is heterogeneous, as it is comprised both of cancer cells, non-cancer cells, such as immune cells or fibroblasts, as well as non-cellular components that make up the Extracellular Matrix (ECM), that cohabit with tumour cells [4,22]. Figure 1 illustrates the components of the TME.

The presence of immune cells with an anti-inflammatory profile in the TME leads to the production and release of several factors, such as cytokines, growth factors, reactive oxygen species and metalloproteinases. These specific cells are known as Tumour-Promoting Inflammatory cells, or Immune Inflammatory Cells [4,23].

Focusing on the tumour's immunology, the TME can be modulated by the constituent cells, for example, the infiltrated immune cells (T and B lymphocytes/cells, macrophages, and Natural Killer (NK) cells), cancer-associated fibroblasts, and vascular cells [4], as it is shown on Figure 1. This heterogeneity nature of the TME provides the best conditions for the tumour's growth, progression, and development [4,24].

Moreover, this peculiar habitat becomes hostile to the cells that do not inhabit it, due to the constant inflammation. The TME is responsible for this aggressive profile, but it is essential for neoplastic progression, and, on the other hand, for immunosuppression. As such, it serves as a

[(a)] CD: Cluster of Differentiation. For instance, "CD8+ T-Cell" translates into "T-Cell positive for CD8 surface receptor".

reservoir of necessary compounds to the tumour's development and, thus, can influence cancer cell infiltration and induce metastasis [3,25].

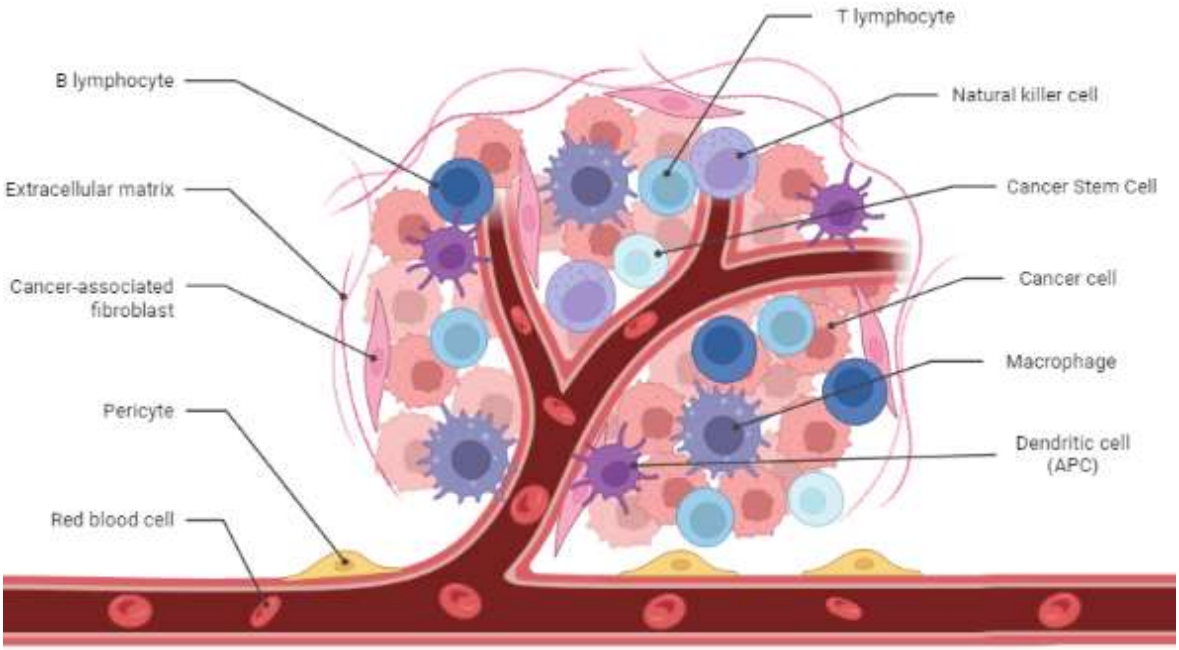

Figure 1 – Tumour Microenvironment (TME) and its constituents.

The TME is extremely heterogeneous. It is composed of different cell types, such as immune cells – lymphocytes, NK cells and macrophages – cancer cells and stromal cells. Moreover, the ECM and the vascular network have a crucial role in creating the environment for cancer development. Adapted from Hanahan & Weinburg (2011) [4]; Created with BioRender.com.

Due to the complexity of the TME, the immune system interacts with it and the cancer cells within, in three major stages: Immunosurveillance and Elimination; Cancer Persistence and Equilibrium; Immunoediting and Escape. These phases will be discussed in the next subchapters.

2.1. Immunosurveillance and Elimination

Tumour cells stimulate the immune response due to their high proliferation, the acidification of the medium and the production of ECM compounds for the construction of their ideal TME [3]. This immune response involves various cell types, such as neutrophils, antigen presenting cells (APCs), macrophages, NK cells, and T-cells, both CD4+ helper T-cells and CD8+ cytotoxic T-cells.

There are also different mechanisms known as immunological checkpoints, which control the immune response, allowing it to adapt its intensity and curb its activity to avoid excessive and unnecessary tissue damage, such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) and CD80, Programmed Death protein 1 (PD-1) and its ligand (PD-L1), that are expressed on the surface of APCs [3,26].

CD8+ cytotoxic T-cells are responsible for the immediate immune response, because once the recognition of the antigen occurs through the T-Cell Receptor (TCR), perforins and granzymes are released into the environment, provoking apoptosis [3]. CD4+ helper T-cells intervene in the activation of CD8+ T-cells, as well as the creation of the memory immune response. Additionally,

CD4+ T-cells can differentiate into several types of T-helper cells, depending on the cytokines and the chemokines produced by the surrounding cells [27].

Once a tumour cell enters into an apoptotic state, its antigens are recognized by CD8+ T-cells and macrophages to trigger the immune response. In addition, the antigens can be phagocyted by APCs, which present them on the surface bound to the Major Histocompatibility Complex (MHC), class I or II, for a faster recognition and immune response [3]. Figure 2 illustrates the MHC-TCR recognition.

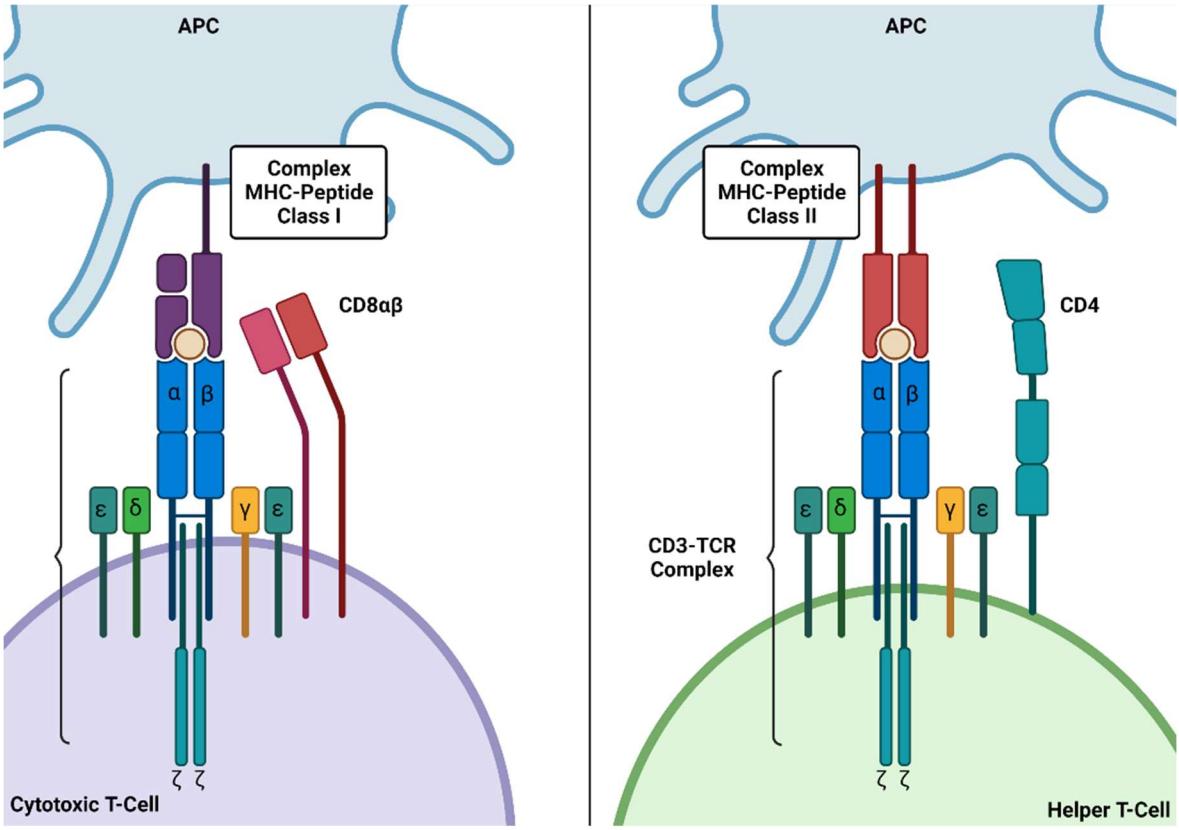

Figure 2 – MHC-peptide complex recognition by T-cells.

On the left, the CD8+ T-cell is represented and, on the right, the CD4+ T-cell. The different MHC present the peptide to the CD3-TCR complex, composed by CD3ζ, CD3γ, CD3δ and CD3ε chains. The T-cell activation is triggered once the co-receptor CD4 or CD8 establish a connection to the MHC-peptide complex. Created with BioRender.com.

In addition to recognising the peptide-MHC assembly through the TCR, T-cells establish additional interactions with the MHC molecule through the CD4 or CD8 molecules that stabilise the binding and allow an effective T cell response [28]. Because this binding is necessary for the T cell to be able to respond effectively, CD4 and CD8 are known as co-receptors. Restrictive binding ensures that CD4+ T-cells respond to MHC-II and CD8+ T-cells respond to MHC-I antigens [3,29].

The TCR, a heterodimeric receptor composed of two chains, α and ß, has short intracytoplasmic chains and is, therefore, not suitable for signalling, so it is aided by the CD3 molecule, present in all T-cells, forming the CD3-TCR complex [30,31] (Figure 2).

As soon as the recognition is done and the consequent activation begins, a complex phosphorylation cascade initiates, which in turn culminates in the production of inflammatory molecules. Apart from phosphorylations, this process implies the exit of calcium from intracellular reserves of the reticulum and, when it binds to a globulin, there is a migration of kinases to the nucleus, and they promote the transcription of genes that encode for the interleukin-2 (IL-2) and its receptor (CD25). This leads to the production of compounds that ensure the survival, proliferation, and differentiation of T-cells. In Figure 3 it is illustrated how this signalling occurs.

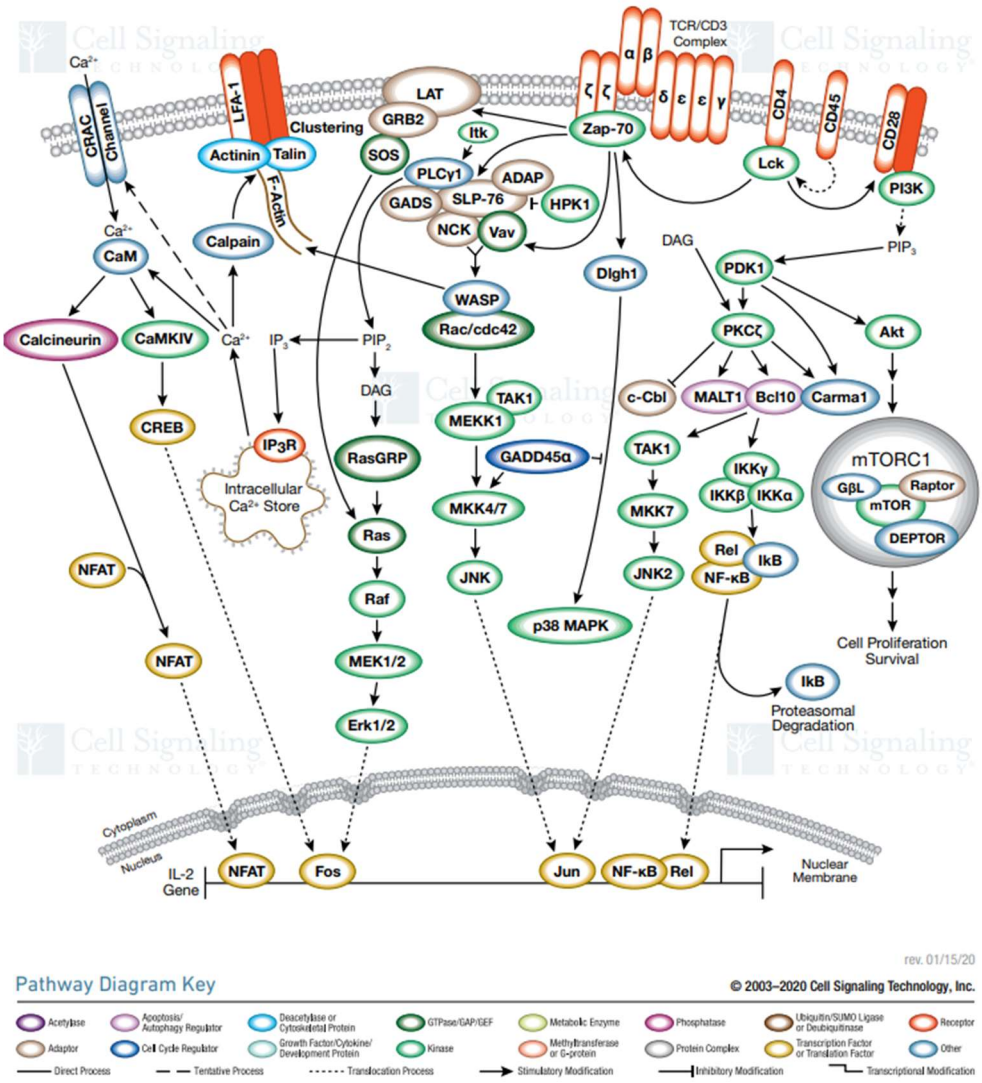

Figure 3 - T-cell Receptor Signalling.

Once the CD3-TCR complex is engaged, the pathways shown are activated. The TCR signalling includes transcription and translation factors, other surface and intercellular receptors, (de)phosphorylations, enzymes, G-proteins, kinases, and other proteins. All these factors contribute to the transcription and translation of IL-2 for the activation of lymphocytes and consequent immune response. Image from Cell Signalling Technology, Inc.

There is an immense variety of compounds that intervene in this activation, and one of the most important is the family of transcription factors known as Nuclear Factor of Activated T-cells (NFAT). Contrary to what the name entails, NFAT is not only expressed by T-cells, but also in

immune and non-immune cells, playing distinct roles throughout the organism [32], and it can also be used as a responsive element [33,34].

As it is shown in Figure 3, the activation of NFAT depends on the activation of calcineurin, which happens due to the production of calcium ions from the TCR engagement [32]. A variety of cytokine genes, such as IL-2, Interferon γ (IFN-γ), and Tumour Necrosis Factor α (TNF-α), are under the NFAT's and the calcineurin's regulation [35,36].

Even though this ensemble is different between each other, all NFAT proteins share a Rel-homology domain, which in turn, grants them to recognise the same DNA binding site (NFAT binding domain) in several genes' regulatory regions [32,36,37].

2.2. Cancer persistence, Immunoediting and Escape

If the tumour cells are not eliminated completely, cancer persistence develops, reaching the equilibrium phase. Here, immune cells are recruited and attack tumour cells. However, there are cancer cells which proliferate at the same rate, and then, escape immunogenic control. This causes tumour dormancy, which justifies a chronic-like treatment during this period.

Due to environmental or immunological pressure, several distinct tumour cell variations may appear, being these cancer cells more resistant to apoptosis as time passes. One variation can eventually manage to bypass the killing process or recruit regulatory immune cells, allowing the cancer cells to propagate unhindered. Thus, the tumour can lose its equilibrium, through genetic instability or immune selection, leading to an increase in the proliferation of tumour cells and preventing the action of the immune system [3].

As a result, tumour cells can evade the immune system through a variety of methods that fall under the categories of immunological tolerance or immunological escape. The release of anti-inflammatory cytokines and chemokines, such as IL-8, IL-10, and Transforming Growth Factor ß (TGF-ß), lead to the recruitment of Regulatory T-cells to the tumour site, making the microenvironment immunosuppressive.

Tumour cells can also induce survival mechanisms and avoid cell death all together, by producing the Nuclear Factor κ of activated B cells (NF-κB) or the B-Cell Lymphoma protein 2 (Bcl-2) [3]. Furthermore, due to several acquired mutations, the tumour cells can obtain a more mesenchymal phenotype, being able to migrate and develop metastasis in other organs.

However, the most impressive mechanism is the reduced immune recognition by immunoediting. This process entails the change of superficial antigens to escape the immune system, such as: the loss of tumor antigens, MHC-I, or co-stimulatory molecules; the overexpression of death receptors such as the TNF-Related Apoptosis-Inducing Ligand (TRAIL) or Fas-Ligand, to induce immune cell apoptosis; and the expression of CTLA4 and PD-1/PD-L1 on the cell surface, to inhibit the immune response. In addition, the presence of the CD47 surface receptor prevents macrophage-mediated phagocytosis [23,38–40].

With these mechanisms of action, the TME becomes immunosuppressive, and the tumour becomes more difficult to eradicate, while it hinders the immune system's response.

3. Cancer Immunotherapy

Given that in certain circumstances the immune system is not enough to fight the tumour cells, radiation and chemotherapy are used as traditional therapies. Chemo- and radiotherapies generate damage signals that will lead to the activation of APCs and the immune system, augmenting the anti-tumoral response. However, toxicity, drug resistance and tumour recurrence reduce these approaches' efficacy [5]. To aid in therapy, cancer immunotherapies have emerged, and they are nowadays a crucial step towards cancer treatment and development of a cure.

Cancer immunotherapy is based on the improvement of the patient immune system, focusing on one or more aspects of the immune response, to combat cancer cells. When battling tumour development and metastasis, immunotherapy can stimulate the immune system, avoid immunosuppression, and stop immune evasion [5,41]. In Figure 4 it is shown the different cancer immunotherapies, based on the review of Meng *et al* (2021) [5].

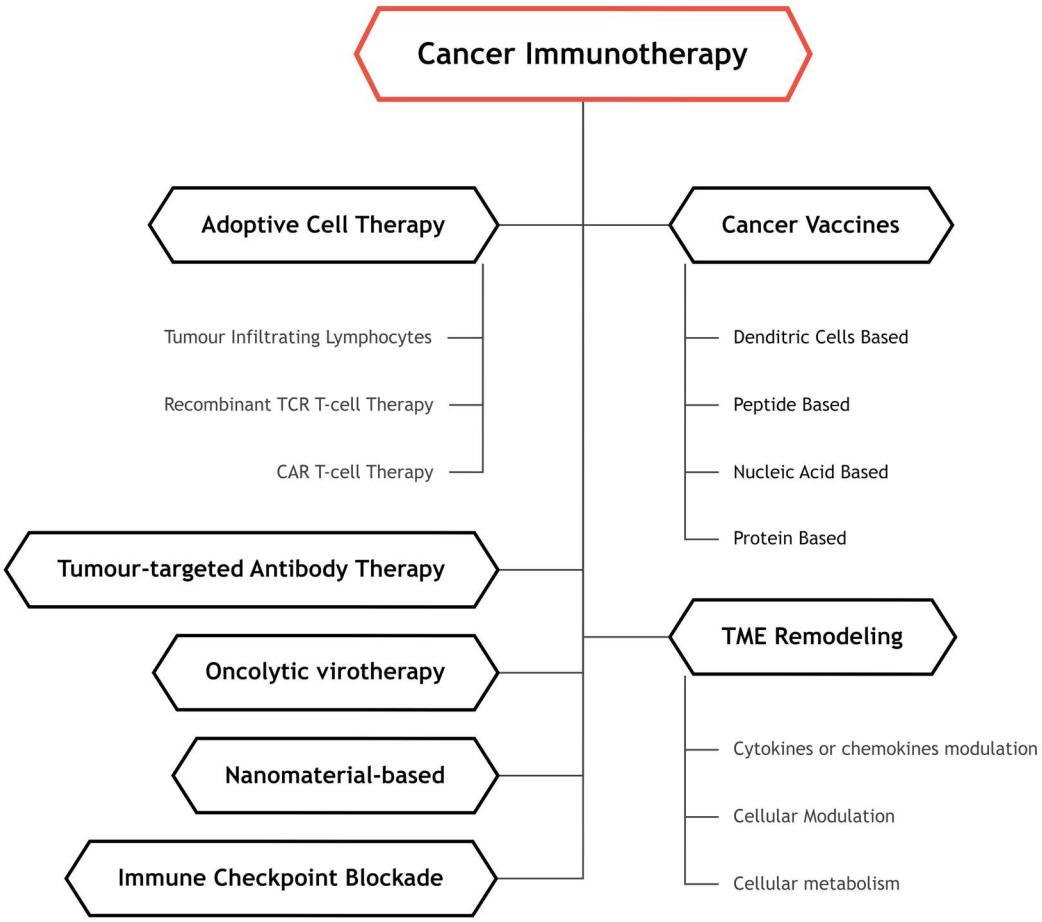

Figure 4 - Different types of Cancer Immunotherapies available.

Based on the review of Meng *et al*. (2021) [5]. Made with Mindomo®.

Although there are several therapies mentioned in Figure 4, only TME Remodelling with cytokines and Adoptive Cell Therapy (ACT) will be addressed, as these are the main therapies for the understanding of this thesis.

3.1. TME Remodelling - Cytokines Modulation

Cytokines, which are proteins or peptides released by different cell types, can control immune response, and intervene in cell growth and differentiation [5,42,43]. Moreover, cytokine secretion can have different effects depending on the microenvironment they are secreted in [44].

Consequently, the immunosuppressive profile of the TME is due to the release of cytokines and the recruitment of anti-inflammatory cells [4,23]. With that in mind, blocking the function of these cytokines or using others with the opposite function can be a feasible option to "resuscitate" the immune system. On the other hand, it was shown that cytokines can cause severe toxicity *in vivo* when administrated systematically [43,45–48].

Within the vast range of existing cytokines, there are several that promote anti-tumour activity and can be used as a monotherapy or a secondary compound to an immunotherapy. Here, there will be a focus on IL-12, as it is mentioned in the literature to be an adjuvant to melanoma immunotherapy [49–52].

3.1.1. Interleukin-12

This cytokine consists of a heterodimeric complex, comprised of two subunits, the α chain (p35 subunit) and the ß chain (p40 subunit), and it is important to note that it cannot be assembled without the expression of both chains [53,54].

When produced by APCs or B cells, IL-12 can induce the differentiation of naive CD8+ T-cells into active CD8+ T-cells and naive CD4+ T-cells into the T helper 1 phenotype [43,55,56]. The latter produces more IL-12, as well as IL-2, IFN-γ, and TNF-α, increasing the pro-inflammatory response. This glycoprotein initiates a signalling cascade with the Signal Transducer and Activator of Transcription 4 (STAT4), enabling the engagement of the immune system and changing the tumour microenvironment into a more inflammatory state [43,57,58].

Tugues *et al.* (2014) discovered that IL-12 triggers immunological memory responses against tumours [59]. It was also confirmed that it augments the cytotoxicity of immune cells, not only CD8+ T-cells but also NK cells [55], which is of considerable importance, especially when developing immunotherapies.

To assess its therapeutic potential *in vivo*, several pre-clinical and clinical experiments were conducted [54,60,61], but, alas, IL-12 is confirmed to have a severe toxic effect when administered systemically [45,62]. Clinical studies have been developed to deliver IL-12 at the tumour site as an attempt to reduce systemic toxicity [63,64], but although the toxicity diminished, their findings are not significant enough to be regarded as a clinical breakthrough.

As a result, some have been focusing on adoptive T-cell therapies with IL-12 being locally released, to boost anti-tumour activity, while minimising harmful consequences [51,65–68].

3.2. Adoptive Cell Therapy (ACT)

Adoptive Cell Therapy (ACT) entails the harvest of the patient's T-cells, which can be genetically modified afterwards, in order to gain the ability to recognise and kill cancer cells that they previously could not [5,69].

Large numbers of lymphocytes, up to 10^{11} cells, from the patient are easily gathered and can be genetically modified, depending on the therapy that will be applied. The effector cells are then cultivated *in vitro*, chosen for the highest recognition of the antigen of interest and administered back to the patient [28,44,69,70].

Compared to other immunotherapies, ACT has a leverage point, as cells are relieved from the *in vivo* inhibitory microenvironment and grown in a more beneficial one for immune response [70]. Additionally, ACT is considered a "living" therapy [70], since the modified cells have the potential to multiply *in vivo* and keep their effector phenotype.

ACT can be divided into three major categories: Tumour Infiltrating Lymphocytes (TILs) Therapy; Recombinant TCR T-cell Therapy; and Chimeric Antigen Receptor (CAR) T-Cell Therapy. The last two therapies entail T-Cell genetic engineering, contrary to TILs therapy.

3.2.1. Tumour Infiltrating Lymphocytes (TILs) Therapy

As the name implies, TILs are T-cells that infiltrate and grow inside the solid tumour. Naturally, TILs make up a heterogenous population, which can be categorized by their phenotype, surface molecules, and functionalities [71] . These populations are usually CD4+ and CD8+ T-cells, with some immature T-cells, meaning that the population obtained is virtually pure [70].

TILs are extracted from a tumour biopsy, cultured, and activated with murine or recombinant IL-2, which promotes their growth [5,72]. Subsequently, the different clones are screened for the target TCR through co-culture assays with the target tumour cells, which can come from either a cryopreserved sample, single cell suspensions, or tumour cell lines [73]. Afterwards, the correct clone is expanded *ex vivo* and the re-administered to the patient, who has suffered lymphodepletion [70].

The first evidence of TILs as a therapy was shown by Rosenberg *et al.* in 1988, where the use of autologous TILs as an ACT could induce tumour regression in individuals with metastatic melanoma [70,74].

Although it is a promising immunotherapy, it has its disadvantages, as it has a lengthy process of cell expansion (around 5 to 6 weeks [70]), can have a limited *in vivo* expansion capacity and it is quite difficult to isolate them from the tumour tissue [44].

3.2.2. Recombinant TCR T-cells Therapy

Over time, genetic engineering emerged, being a great advance for the next-generation ACTs. With the ability to modify immune cells at a genetic level, the expression of the receptor of interest on effector immunological cells became possible, by the insertion of the corresponding transgene, safely and efficiently [28,73,75]. With that, Recombinant TCR T-cells and CAR T-cells therapies were developed. These therapies only differ on the genetic engineering of the T-cell and, thus, on the way they recognize the tumour antigen. CAR T-cells Therapy will be discussed in the next subchapter.

Recombinant TCR T-cells therapy uses the patients' blood T-cells and genetically modifies them, by inserting genes that encode for the conventional αß subunits of the TCR, that recognizes the tumour target antigen [5,24,28,70]. Both subunits will recombine once the cell starts expressing the transfected transgenes, hence the name. Similar to the TILs therapy, the TCR-expressing cells are selected, expanded, and administered to the patients after lymphodepletion, following the conventional adoptive transfer protocols [73,75].

As it is illustrated by Figure 3, in the subchapter "Tumour Microenvironment and Immunology", the TCR binds directly to the MHC-peptide complex expressed on the target cell, and, as such, this therapy is considered MHC-restricted [5,76]. Also, it can have a wider range of targets cells and can be applied in various cancers, like solid tumours with lack of specific surface tumour antigens and only with intracellular antigens [28,44,76].

However, this can be seen as a disadvantage since it depends on the co-stimulation of the TCR-MHC and the co-receptor, and, also, matching between the MHC-peptide and the TCR [44], which may not be achieved within the tumour's heterogeneous nature. Moreover, once the cells get to the TME, they can have their phenotype changed due to the immunosuppressive molecules and to the lack of pro-inflammatory cytokines, compromising the therapy's efficacy.

Even so, TCR T-cells therapy has valuable advantages: it can be used as an "off-the-shelf" product; the cells used are more easily available than TILs; it has the ability to target intracellular antigens; and can have a high engagement with the antigen [44].

According to pre-clinical and clinical studies, the modified T-cells can infiltrate and attack tumours that express the target antigen [75,77,78], but they can also attack healthy cells or tissues that express the same antigen [79,80], causing immune-related toxicity [81]. One example is the study done by Morgan *et al.* (2006) [75], which aimed to treat metastatic melanoma, comparing several TCRs that recognized the MART-1 antigen (DMF4 [75], DMF5 [82] and anti-gp100 TCR [83]) using engineered TCR T-cells. In this clinical setting, 25% of the patients showed partial regression, but more than 80% of patients had temporary toxicities in their skin, eyes, and ears, sites associated with melanocyte production [7,9].

These findings demonstrated that autoimmune diseases mediated by T-cells can occur within immunotherapies, especially in solid tumours that present the same antigen as normal cells, and they can be extremely severe and ultimately result in death. So, it is of immense value to future

immunotherapies to have a compromise between efficacy and safety when targeting tumour-associated antigens [28,71].

3.2.3. CAR T-cells Therapy

As the name suggests, Chimeric Antigen Receptor (CAR) T-cells are T-cells engineered with a synthetic receptor, composed by a fragment of an antibody of interest and the CD3-ζ chain (1st Generation CAR-T cell [72]). It is also possible to add one co-stimulatory domain, as CD28 or CD137 [28,44] (2nd Generation [72]) or to have two co-stimulatory domains simultaneously expressed (3rd Generation [72]). Figure 5 illustrates the differences between a conventional αß TCR and a CAR.

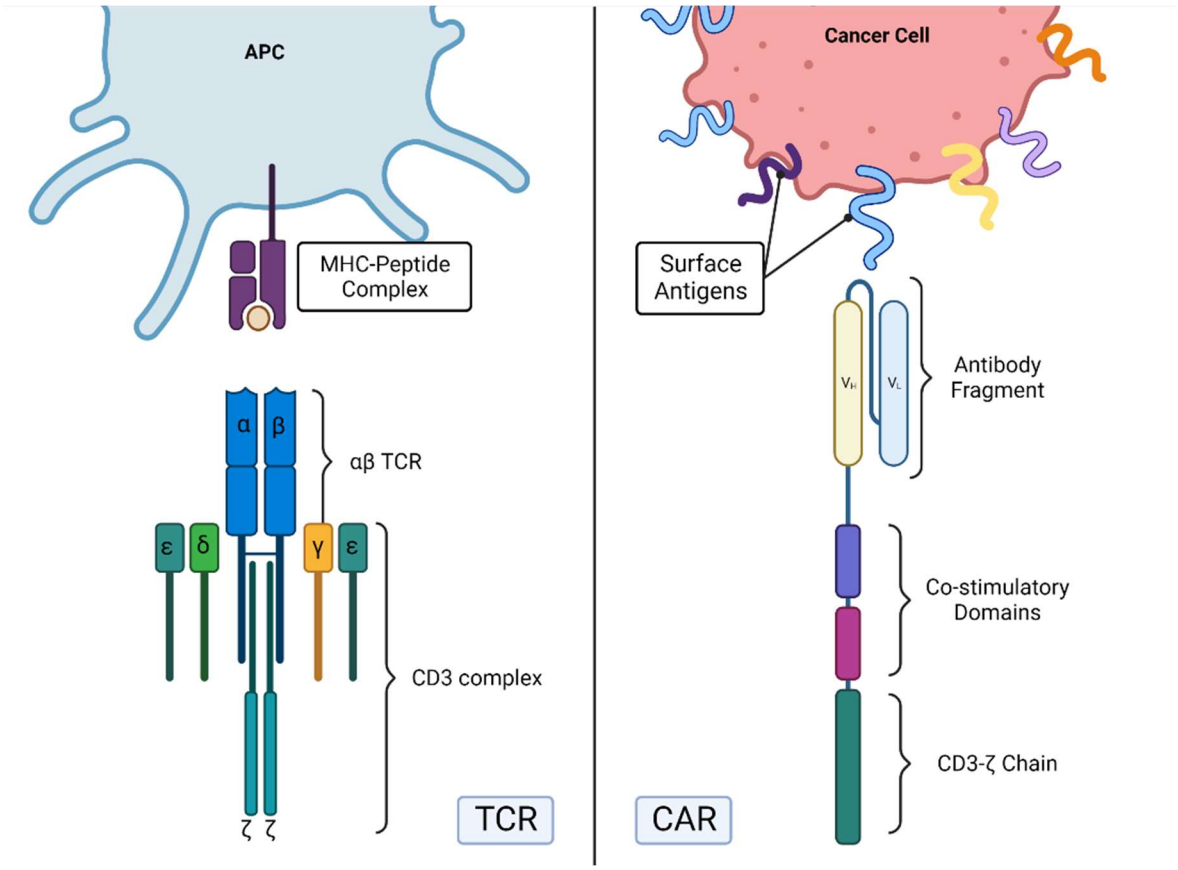

Figure 5 - Comparison between a conventional αß TCR and a 3rd Generation CAR, and the antigens they recognize.

Contrary to TCR which only recognizes antigens presented with a MHC, CARs are able to recognize surface antigens on cancer cells, since a CAR is composed by the heavy and light chains of the antibody of interest. The CD3-ζ domain and the co-stimulatory domains are responsible for the signalling and consequent activation of the T-cell. Adapted from Chen *et al*, 2019 [28]. Created with BioRender.com.

The heavy chain and the light chain of the antibody that binds to the tumour antigen makes up the recognition portion of the CAR, and, in turn, they are connected to the co-stimulatory domains and the CD3-ζ chain [71]. The last two units are responsible of fully triggering the activation of the engineered T-cell, once recognition occurs, and, because of it, the activation can be stronger compared to recombinant TCR T-cells therapy [5,70,73,84]. Similarly, CAR T-cells can be used as an off-the-shelf product and have a high cell availability [44].

Remarkably, CAR engineered T-cells recognise the antigen of interest without depending on MHC-complex expression [60], contrary to TCR engineered T-cells, which is an advantage when immunoediting is triggered and the MHC stops being expressed [85].

Nevertheless, they can only recognize surface tumour antigens [28,70,73], which ultimately limits their use in solid tumours that only express intracellular antigens. Thus, CAR T-cells are mostly used in the treatment of B-cell cancers, such as B-cell acute lymphoblastic leukaemia, where they displayed a high response rate in patients [5,86,87].

However, CAR T-Cell therapy can have serious side effects, notably the development of cytokine release syndrome (CRS) [44,81,88]. CRS is characterized as the exacerbated pro-inflammatory cytokine release, due to the intense activation of CAR T-cells when an antigen is recognized [71,81]. The toxicity caused by this syndrome can provoke fever, tachycardia, respiratory insufficiency, and can be lethal [48,89]. Although it is uncommon, CRS has been described in TCR T-cell therapy [90]. To overcome this issue, antibodies against cytokine receptors have been used in combination with the ACT to prevent the development of CRS [91,92].

3.3. T-cells Redirected for Universal Cytokine-mediated Killing (TRUCKs)

Although ACTs have been reported as successful, there are still obstacles to overcome, especially regarding solid tumour immunotherapy. As already mentioned, with cancer progression, malignant cells acquire mutations that favour them, by losing the surface antigens, and can become invisible to the immune system and escape detection [26].

Likewise, the production of anti-inflammatory molecules and the proliferation of regulatory-like cells, such as regulatory T-cells and anti-inflammatory macrophages, can suppress the pro-inflammatory response and prevent the migration of immune cells into the tumour [26,56]. A promising strategy for the reinforcement of T-cells' activity is the T-cells Redirected for Universal Cytokine-mediated Killing (TRUCKs) therapy, also known as armoured CARs or as the 4th Generation of CAR T-cells [56,84,93,94].

TRUCKs are genetically engineered T-cells that simultaneously express a CAR and transgenic cytokine that enhances anti-tumour killing [56,94,95]. The major objective of this therapy is to recruit more immune cells to the tumour site through pro-inflammatory cytokine build-up without being affected by its heterogeneity and immunosuppressive nature [56,81,84,95]. As Glienke *et al*. (2022) states, "*TRUCKs are used as 'living factories' to produce and deposit substances with anti-tumour activity in the targeted tissue*" [95]. In addition, by exchanging the cytokine for a co-stimulatory domain or an enzyme, the TRUCK develops into a 5th Generation CAR T-cell [85]. Figure 6 illustrates their mode of action.

The cytokines commonly used, such as IL-12 [52,96], IL-15 [97] or IL-18 [95,98], can recruit immune cells, enhance the immune response, and overcome the lack of pro-inflammatory cytokines [93]. Moreover, the cytokine can be released either constitutively, or inducibly, as its expression can

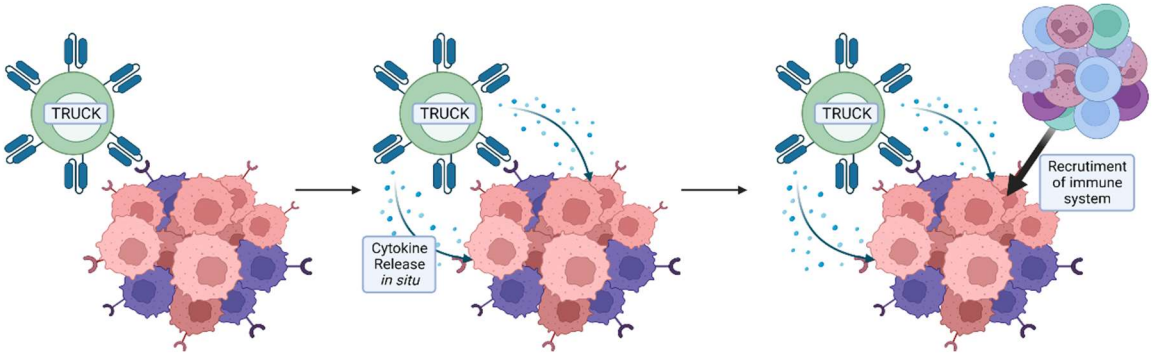

Figure 6 - TRUCKs and their mode of action.

When a TRUCK with an inducible cytokine enters the TME, it can recognise the tumour antigen expressed by cancer cells (1st panel). The TRUCK becomes activated, which in turn triggers the expression of the pro-inflammatory cytokine, being released *in situ* (2nd panel). Thus, the TRUCK recruits more immune cells, such as CD4+ T-cells, NK cells and macrophages, and, thus, increases the immune response where once it was not possible (3rd panel). Adapted from Chmielewski et al, 2014 [94]. Created with BioRender.com.

be under the control of a responsive element, like the NFAT [56,81,99], and can be released upon cell activation [56,84].

Regarding safety issues, inducible release in the targeted tissue in response to CAR signalling is of great value when the chosen cytokine causes toxicity when administered systemically or in a constitutive manner [93], as it is the case with IL-12. TRUCKs with inducible release of transgenic IL-12 have been employed positively in pre-clinical trials [68,100,101], without disclosing significant toxicity. Nevertheless, it is necessary to choose the most appropriate antigen to avoid unspecific binding and consequent toxicity in normal tissues [56].

Previous research has reported the successful construction of inducible-release TRUCKs using recombinant TCR T-cells [52,93,99] or TILs [102,103], some with specificity for metastatic melanoma and with positive preclinical and clinical results [52,102,103]. This shows that TRUCK model is not restricted to CAR T-cells, and there is now a novel range of options capable of improving existing therapies.

4. Genetic Engineering

Once the tumour target is known and the immunotherapy to be applied is chosen, the next step is the construction of T-cells through genetic and cellular engineering. As such, the ideal gene delivery system should have a high transfection rate, thus having a stable and safe gene expression over time [104,105]. However, the effectiveness of gene delivery and its safe and stable expression are some of the main barriers to overcome [106].

Typically, cells are modified by introducing a recombinant plasmid or vector, which is a double-stranded circular DNA molecule that contains the target gene and other components that give rise to gene expression and perform autonomous replication, as it contains sequences that correspond to an origin of replication [107].

Moreover, a vector commonly contains a resistance marker gene, usually to antibiotics like ampicillin or kanamycin, for selection, during bacterial fermentation. When in an environment

with antibiotics, transformed bacteria that grow express the correct vector and, thus, the plasmid is extracted from the same bacteria [107,108].

However, regulatory health companies, such as the Federal Drug Administration (FDA) or the European Medicines Agency (EMA), discourage the use of antibiotics in a clinical setting, due to the high probability of transcriptional inhibition and antibiotic resistance transfer [108-111], meaning that alternatives to antibiotic resistance genes should be chose, when possible.

A promising alternative is the RNA-OUT® technology, developed by Nature Technology Corporation (NTC®), represented in Figure 7. This novel technology is based on an interference RNA sequence and on the use of genetically engineered bacteria, that constitutively express the enzyme levansucrase [110,112,113].

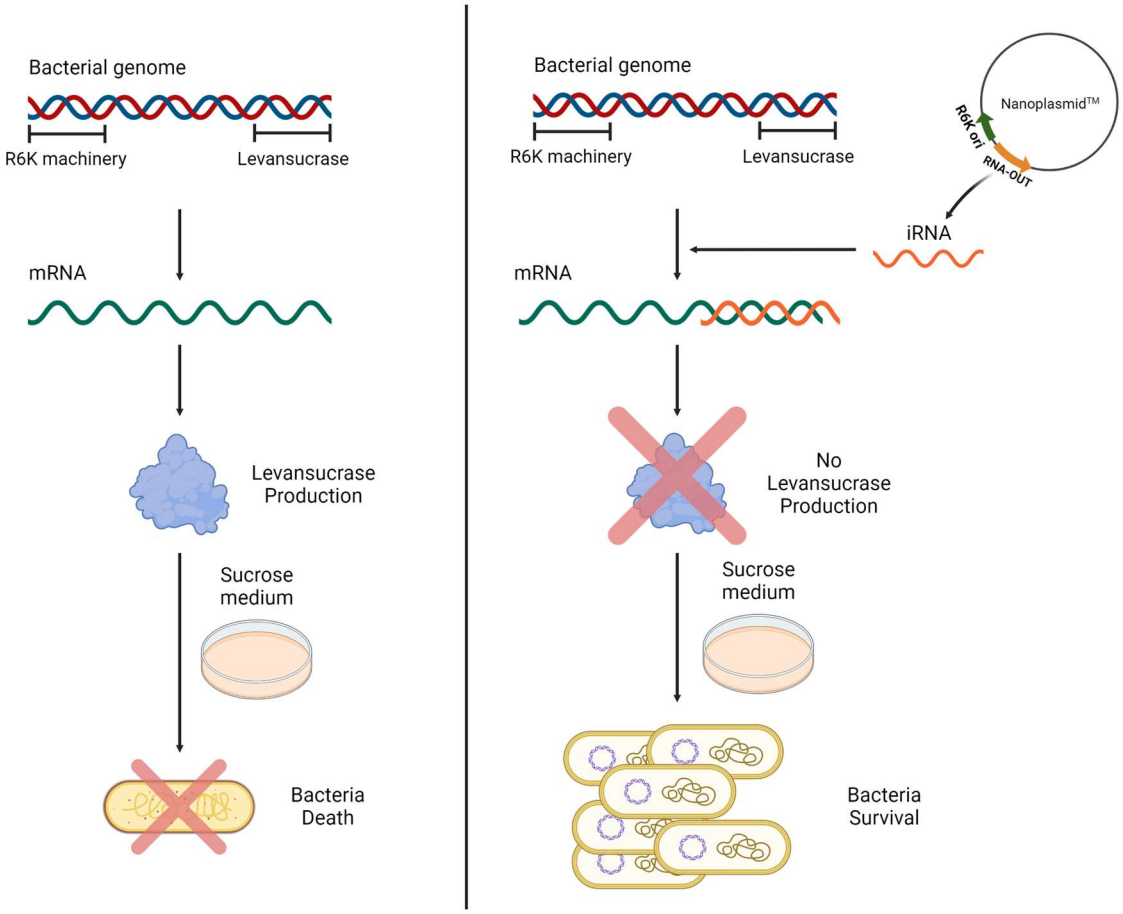

Figure 7 - Bacterial selection using the RNA-OUT technology within the Nanoplasmid™, developed by NTC©.

A genetically engineered strain of *E.coli* leads to the production of a messenger RNA (mRNA) that includes sequences for the translation of R6K machinery and of the enzyme levansucrase. When bacteria without the Nanoplasmid™ are cultured in a medium with sucrose (left panel), the presence of levansucrase leads to cell death. If the bacteria are transformed with the Nanoplasmid™ (right panel), this vector leads to the transcription of an interfering RNA (iRNA) sequence complementary to part of the mRNA produced, stopping enzyme production. Thus, when said bacteria are cultured in sucrose medium, the bacteria grow and proliferate naturally. Adapted from Bozza, 2017 [105]. Created with BioRender.com.

Once the bacteria are plated in sucrose-medium, only the bacteria containing the plasmid of interest survive, as the RNA-OUT® silences the transcription of the levansucrase and, thus, turns the bacteria tolerant to sucrose. As such, this technology enables plasmid selection on sucrose

medium, instead of an antibiotic-containing medium. Luke *et al*. (2009, 2011) has shown high expression rates using this system in comparison to antibiotic-resistant vectors [110,112].

Furthermore, it has been shown that the reduction of vector size increases the efficiency of gene transfection [105,109]. One option is the use of DNA minicircles, which are DNA plasmids that have had their bacterial elements removed and are used in genetically engineered bacteria that express recombinases that replace the bacterial origin [105,114]. However, gene expression decreases when using this system.

To overcome this challenge, NTC© has developed the Nanoplasmid™. The Nanoplasmid™ is an improved bacterial backbone, with less than 500 bp, that merges the RNA-OUT system (Figure 7) with the R6K origin of replication [113,115]. Because of its smaller size, the prokaryotic area is less vulnerable to gene inactivation. When opposed to typical plasmids, RNA-OUT can operate as an enhancer and stimulate gene expression in addition to working as a selection marker [115]. Moreover, the R6K origin needs a customised NTC© *E. coli* K12 strain that produces the replication machinery essential for this cassette. This acts as a safety measure, as nanoplasmids are restricted to replication only within this strain [115]. The use of Nanoplasmids™ has been approved for human therapy, as several clinical trials were already carried out where this new approach has not shown significant negative side effects [115].

T-cells can be engineered with viral or non-viral vector-based gene transfer techniques, i.e., the gene transfer is done with a viral or non-viral system, respectively, and they can result in persistent or transient gene expression. A persistent gene expression requires the integration of the gene into the target cell genome and heritable expression, whereas a transient expression means that the integration does not occur in the genome, instead the gene remains in an episome [3,28]. Once in the nucleus, the expression system should be capable to express the gene without developing genotoxicity, due to insertion of foreign DNA without integrating it.

4.1. Viral Vectors

Viral vector-based systems rely on viral-mediated gene transfer of non-viral DNA [116,117]. Overall, viral systems are easy to manufacture and have a long-term gene expression through integration of the cell genome [71,106,116].

Due to safety concerns, these systems must show lack of genotoxic and immunogenicity, which sometimes can be difficult to achieve [116]. Viral vectors can be divided into retroviruses (gamma and alpha), lentiviruses, adenoviruses, and Adeno-Associated Viruses (AAV) [118,119].

Retroviruses, both gamma and alpha, consist of a lipidic-involucre that surrounds a single-stranded RNA genome with *cis*-acting elements and coding sequences [116,119], and can integrate the genome of dividing cells. Within this system, it is possible to replace the coding regions of the viral genome by the genes of interest, keeping only the regions that encode for the capsid, the involucre and replicating enzymes [116].

Because of their high percentage of successful transduction and considerable transgene expression, gamma retroviral vectors are used in the construction of CAR T-cells and the modification of T-cells [120,121]. However, there has been safety issues about their use, as gamma retroviruses can trigger neoplastic transformation through oncogene activation [116,122]. The separation of coding and regulatory sequences into distinct molecules reduces their remobilization and thus boosts health security [116,123].

Regarding alpha retroviruses, genomic integration is more arbitrary and thus more innocuous compared to gamma retrovirus [116,124]. The ability to create stable cell lines is a great benefit of these vectors, since the use of cell lines reduces the likelihood of recombination and offers the best quality-cost relation [116,124]. Although they have been assessed in cell models [125], alpha retroviral vectors have not been assessed in clinical trials [116,126].

Lentiviral vectors are similar to the gamma retroviral vectors, having a stable integration in the host chromosomal DNA [116] and large cloning ability [127]. However, lentiviruses can infect both actively dividing and non-dividing cells, while retroviruses can only infect dividing cells [28,116,128]. One example of efficiency of lentivirus is their application on anti-CD19 CAR T-cells [116,129]. However, lentiviral systems can also present genotoxicity due to the same reasons mentioned for the retroviruses [116,127].

Adenoviruses are significantly different from retroviruses and lentiviruses, as they do not depend on cell replication nor genome integration for gene expression [128]. Instead, the gene is delivered to the nucleus and kept in an episome [128,130,131]. Consequently, adenoviruses are not adequate for immunotherapy, as they present a transient expression [128,131].

AAVs, similar to adenovirus, do not integrate the host genome and can transduce both dividing and non-dividing cells with high transduction efficiency [127,128]. Curiously, AAVs have long-term persistence of the episome, but it is still not equivalent to permanent gene expression through time [128]. However, they can turn immunogenic in humans, which is considered a major safety concern [127].

Despite viral vectors are growing towards safety improvement, there is still a probability they might cause genotoxicity or mutagenesis, through random integration of viral DNA [28]. Moreover, the protocols currently used for virus vector development are not cost-effective, and thus, a non-viral based vector can be considered a better option for treatment in the near future.

4.2. Non-Viral Vectors

Several non-viral gene delivery techniques have been employed in the recent past, as they are safer than viral options. However, non-viral approaches typically have a lower transfection efficiency compared to viral ones [86,116,128]. Nonetheless, several advances have been made and multiple investigations continue for the improvement of these systems.

The commonly used non-viral system is the transposon/transposase system, such as the Sleeping Beauty (SB) system or the piggyBac (PB) system. This system is characterised by the

translocation of the transposon sequence from one location to another, known as transposition [28,116]. This occurs due to the recognition by transposase of terminal inverted repeats within the transposon sequence, which in turn cuts the segment between those sequences and inserts it into the genome or another DNA molecule, being fully integrated [116,132]. Thus, by inserting the transgene in the transposon cassette and by co-transfection of two plasmids that contain each sequence, long-term stable transgene expression can be achieved [116,133-135].

SB has been used to produce genetically modified T-cells, such as CAR T-cells, showing promising results [28,133,134], and it is now being assessed in clinical trials (NCT00968760, NCT04102436). The PB system is an alternative to the SB system, as it can introduce larger genes into the genome successfully, especially for CAR T-cells [116,136]. However, the PB system is not completely well characterized in human cells, especially regarding its safety [137]. Yet, there are disadvantages: the randomness associated with the genomic integration; the long expansion protocol for their use; and non-optimal transfection efficiency [116,128,135].

A growing approach is the use of messenger RNA (mRNA) for gene transfer and expression. Contrary to both viral and non-viral approaches mentioned, mRNA transfer is not a plasmid-based transfer and does not enter the nucleus for expression, thus considered a cytoplasmic system [116,138,139]. As advantages, mRNA can be transfected into non-dividing cells, does not integrate the genome, and it is extremely easy to make use of [139]. However, the mRNA molecule is not stable enough for a persistent expression with time [116,139], which is not optimal for its use in immunotherapy.

Another promising non-viral system is the use of DNA plasmids that contain Scaffold/Matrix-Associated Regions (S/MAR). S/MARs are DNA sequences present in the eukaryotic genome, that connect the chromatin to the nuclear matrix, to arrange it into loops [140-142]. These sequences are frequently localized in active transcription regions [143,144] and have been shown to be essential for several biological activities such as DNA replication, transcription, repair [140]. By using these regions as a gene delivery system, the S/MARs are able to anchor the sequence of interest and translate it as an episome, thus, not integrating the genome [141,142]. Alas, after transfection, a decrease on the DNA expression was observed over time using these systems [145].

In order to overcome this restriction, the DNA Vector research group has developed nano vectors, that contain S/MARs and the Nanoplasmid™ previously mentioned in Subchapter 4, as a partnership with NTC©. The newly produced vectors can replicate as an episome and provide sustained gene expression, compared to currently used episomal vectors [146-148] and, for that reason, may be a promising technique to be applied in immunotherapy.

5. Aim of this project

ACT is constantly changing in the light of more aggressive and immunosuppressive solid tumours, such as metastatic melanoma. As such, the efficiency of an immunotherapy relies on modified T-cells that migrate, infiltrate, and remain active against cancer cells within the TME. To improve the anti-tumour activity of ACT-cells, T-cells have been engineered not only with receptors of interest but also with other molecules that allow them to resist the unfavourable cancer environment, being TRUCKs one example [98]. Though TRUCKs have been successfully developed using viral systems [98,149], the DNA Vector group has shown recently that CAR-T-cells generated using S/MAR vectors and nanoplasmids (in collaboration with NTC®), can better sustain the genetic content [146,148] and, thus, have the potential to be used for TRUCKs construction.

With that said, the main objective of this thesis was the increase of metastatic melanoma tumour cells killing, by developing an S/MAR vector system that expresses concurrently the anti-MART-1 DMF5-TCR, and the IL-12 cytokine, preceded by six repetitions of the NFAT binding site [150] (NFAT6x) as a responsive element, for the genetic modification of human T-cells. This will be accomplished by using recombinant TCR T-cells therapy and the TRUCKs paradigm simultaneously [56,94], being referred to as "TRUCK-like vectors".

Furthermore, since there is a need to stablish the best method for multi-gene delivery, for the proof-of-concept, two different approaches were compared: a "two-vector" system, where the cells were co-transfected with two vectors, one expressing the TCR and the other the responsive element and the GFP sequence (copepod Green Fluorescent Protein); and a "one-vector" system, where the previously mentioned units were all expressed into a single vector.

Therefore, the specific objectives of this project were to:

1) Design and clone the one-vector system, where the DMF5-TCR, the responsive element NFAT6x and GFP gene are expressed simultaneously.
2) Study and compare, as the proof of concept, the expression of TCR and the GFP intensity of effector T-cells transfected with one-vector system *vs* the two-vector system.
3) Design and clone the IL-12 TRUCK-like vector system, by replacing the GFP reporter gene with both subunits of the human IL-12.
4) Study nucleofection efficiency and TCR expression using J76 CD8+ cells.
5) Design the IL-12 TRUCK-like nano vector, by replacing the bacterial backbone and the antibiotic resistant gene by the R6K-RNA-OUT System.

Chapter 2 Materials and Methods

1. Isolation and Cell Culture of Primary human T-cells
2. Cell Line Culture
3. Construction of Vectors
4. Cell Nucleofection
5. Cell Co-culture
6. Flow Cytometry Analysis
7. Statistical Analysis

1. Isolation and Cell Culture of Primary human T-cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from a buffy coat of a healthy donor, through phase separation with Ficoll® Paque Plus (Merck®), using the company's protocol. After three washes with PBS 1X (Gibco®), to lysate all the erythrocytes, ACK lysis buffer (Gibco®) was added to the PBMCs solution and was incubated for 5 minutes at room temperature. After three more washes with PBS 1X (Gibco®), the PBMCs were cultured with TexMACS medium (Miltenyi Biotec©) supplemented with IL-7 and IL-15 (Miltenyi Biotec©) with a final concentration of 0.1 mg/mL, overnight at 37ºC with 5% CO_2.

On the following day, the primary human CD3+ T-cells were isolated from the PBMCs using the Pan T-cell isolation kit (Miltenyi Biotec©), following the company's protocol. The resulting T-cells were then activated by adding Transact™ (Miltenyi Biotec©) to the medium and cultured in a final concentration of 1×10^6 cells/mL per cm^2. The culture was done with TexMACS medium (Miltenyi Biotec©) supplemented with IL-7 and IL-15 (Miltenyi Biotec©) with a final concentration of 0.1 mg/mL. The cells were incubated at 37ºC and with 5%CO_2, for 3 days before nucleofection.

2. Cell Line Culture

A stable cell line of Jurkat 76 transduced with the $CD8^+$ cell surface marker ("J76 CD8+" cells) was gently provided by Ilka Bartsch. They were kept in culture with RPMI 1640 medium (Sigma-Aldrich®) supplemented with 10% of heat-inactivated FBS (Gibco®) and 1% of Penicillin-Streptomycin solution (5000 U/mL) (Gibco®). MeWo Wild Type ("MeWo") and a stable U87 cell line transfected with a Tandem Mini Gene (TMG) of the epitope ELAGIGILTV of MART-1 and with a puromycin resistance gene ("U87-TMG"), courtesy of Yu-Chan Chih, were maintained in DMEM 1X (Gibco®) supplemented with 10% of heat-inactivated FBS (Gibco®) and 1% of Penicillin-Streptomycin (5000 U/mL) (Gibco®). Puromycin was added as an antibiotic selection marker to the U87-TMG cell line, with a final concentration of 1 µg/mL. All cell cultures were kept in a humidified incubator, at 37ºC and with 5% CO_2.

3. Construction of Vectors

For a better understanding of the cloning strategies, the Figures 24, 25 and 26 are depicted in the Appendix, describing the cloning processes. The Table 1 in the Appendix contains a list of all primer sequences used throughout this work, with their respective application. Enzyme digestion and Sanger DNA Sequencing, according to Eurofins Genomics© principles, were used to confirm all vectors that were obtained. The enzyme digestion agarose gels and the Colony PCR agarose gels are represented from Figure 27 to Figure 34 in the Appendix.

3.1. NFAT6x-GFP-DMF5 Proof-of-Concept Plasmid

The NFAT6x-GFP insert was obtained by Polymerase Chain Reaction (PCR) amplification from the homonymous vector, kindly provided by Alice De Roia, using the CloneAmp™ HiFi PCR Premix (Takara Bio®), the company's provided protocol and the recommended PCR program. To obtain the plasmid's backbone, the coreEF1-MART1 vector, also provided by Alice de Roia, was digested with the FastDigest Restriction Enzyme *Hind*III (Thermo Scientific™) in Green Fast Digest Buffer (2X) (Thermo Scientific™). Both products were gel extracted using the GenElute™ Gel Extraction Kit (Sigma-Aldrich®) and quantified with NanoDrop ND-1000 Spectrophotometer (Thermo Scientific®). Subsequently, the In-Fusion® HD Cloning Reaction (Takara Bio®) was performed following the company's protocol and the resulting mix was transformed in Stellar Competent Bacteria Cells (Takara Bio®), which were then plated in a LB-Ampicillin agar dish and kept at 37ºC overnight. A Colony PCR was done with the CloneAmp™ HiFi PCR Premix (Takara Bio®) to ten or more colonies, with the respective primers (Table 1), and the positive colonies were then incubated in LB liquid medium supplemented with ampicillin (100 µg/mL) at 37ºC overnight with shaking at 200 rpm. The next day, plasmid DNA extraction was performed using the QIAprep Spin Miniprep Kit (Qiagen) and the manufacturer recommended protocol.

3.2. IL-12 Plasmids

The protein sequences for the human IL-12 subunits p35 (α) and p40 (ß) were obtained through UniProtKB, corresponding to the following IDs: P29459 and P29460 [151], respectively. To obtain the DNA sequence, the reverse translation of the protein amino acid sequence was obtained using the Reverse Translation tool of the Bioinformatics website (https://www.bioinformatics.org/sms2/rev_trans.html). Using SnapGene's Software (version 4.0.8), the stop codon in the subunit p35 was removed *in silico* and both subunits' sequences were linked with a Porcine teschovirus-1 derived 2A Peptide (P2A) sequence. Then, the IL-12 subunits were sent to synthesis at Twist Bioscience©. The synthesized sequence was resuspended according to the company's guidelines and went through an adapted Overlap Extension PCR, based on the protocol written by Anna Behle [152]. The followed protocol is described below, and it was created and followed to insert the desired promotor upstream – minimal promotor ("minP") or the synthetic promotor "TATA" described in Mann *et al.* [153] – and the overhangs for the In-Fusion® Reaction. The NFAT6x-GFP-DMF5 vector was digested with FastDigest Restriction Enzyme *Sfi*I (Thermo Scientific™) and *Psp*XI (NEB) in Tango Buffer (10X) (Thermo Scientific™), as the vector backbone. Both products and following processes were handled as previously mentioned.

3.2.1. Adapted Overlap Extension PCR

The protocol is summarized on Figure 18 and all primers used on Table 1. The PCR programmes for each cycle followed the CloneAmp® protocol and had the following layout: one denaturation

step of 5 seconds at 98ºC; an annealing step for 5 seconds using the respective annealing temperature; and an extension step of 5 seconds per kb of insert at 72ºC. These steps were repeated as many times as the disclosed number of cycles. The first PCR programme (Figure 18.1) had 30 cycles of amplification, where the annealing temperature used was calculated considering only the primer part that bonded to the fragment, without considering the overhang. A gel extraction was necessary afterwards. The purified fragment was subjected to a second PCR (Figure 18.2) adding the same primers but in a higher volume (1:1 molar ratio). This PCR occurred during 15 cycles to avoid unspecific binding, and the annealing temperature used throughout was calculated considering the whole primer sequence. Then, the PCR mix was kept at 4ºC until the overhang primers were directly added to the mix and the last PCR was run (Figure 18.3) with the annealing temperature corresponding to the overhang primers, with 30 cycles of amplification. A gel extraction was performed afterwards, to perform purification of the finishing PCR fragments.

3.3. IL-12 Nanoplasmids

Briefly, the Nanoplasmid ™ "NV d-tomato", synthetised by NTC® and kindly provided by Julia Peterson, was amplified by PCR with the primers #11 and #12 (Table 1), with the Nano Backbone, which corresponds to the R6K-RNA-OUT™ system, developed by NTC®. Each IL-12 plasmid, NFAT6x-minP-IL-12-DMF5 and NFAT6x-TATA-IL-12-DMF5, suffered double digestion with FastDigest Restriction Enzymes (Thermo Scientific®) *Hind*III and *Mlu*I, to remove the bacterial backbone and the ampicillin resistance gene. Both products were processed as previously mentioned. Afterwards, the In-Fusion® reactions of each vector were electroporated into the NTC® strain of *E.coli* K12. On the next day, the bacteria were plated in a specific LB agar plate supplemented with sucrose and grown at 30ºC overnight. Colonies were selected and incubated in the specific liquid LB medium, supplemented with sucrose, at 30ºC overnight with shaking at 220 rpm.

4. Cell Nucleofection

The nucleofections were done with the Lonza 4D Nucleofector®, with $2x10^6$ cells and 2 µg of DNA per condition, after washing the cells with Phosphate-Buffered Saline (PBS) solution (1X) (Gibco®) and resuspending them in P3 solution, using 20 µL per condition. The CL-120 pulse code was used for J76 CD8+ cells and the FI-115 pulse code was used for the primary T-cells. Next, the J76 CD8+ cells were incubated in previously warmed RPMI 1640 Medium (Sigma-Aldrich®) supplemented with 10% of heat-inactivated FBS (Gibco®) in a 48-well plate and were kept in a humidified incubator, at 37ºC and with 5% CO_2. The primary T-cells were incubated in previously warmed TexMACS medium (Miltenyi Biotec©) supplemented with IL-7 and IL-15 (Miltenyi Biotec©) with a final concentration of 0.1 mg/mL, at 37ºC and with 5% CO_2. After one day of incubation, the J76 CD8+ cells were analysed through Flow Cytometry, whereas the primary T-cells were analysed two days after nucleofection.

5. Cell Co-culture

Firstly, MeWo and U87-TMG cells were detached from their respective flask with pre-warmed Trypsin and counted with the LUNA™ automated cell counter with Trypan Blue. After, for the co-culture, the MeWo and U87-TMG cells were seeded in a Flat Bottom 96-well plate, in a way where each well had 25 000 cells in 200 µL. As there were 5 conditions (described on Chapter 2 of the Results), there were in total 125 000 target cells. After 4 hours of cell incubation, the nucleofected T-cells, either Jurkat 76 CD8+ cells or primary T-cells, were counted in the same manner for the co-culture assay. The medium was then removed, and the T-cells were transferred on top of the 96-well plate, such that each well had 50 000 cells in 200 µL.

There were three conditions in total: "Unstimulated" (only T-cells), "Stimulated" (Transact™ (Miltenyi Biotec©) added to the T-cell medium) and "On-Target" (T-cells and MeWo or U87-TMG target cells in co-culture). This last condition followed a 2:1 ratio, i.e., this means that there was 50 000 nucleofected T-cells in contact with 25 000 target cells (either MeWo or U87-TMG cells) per well. The respective 96-well plate was placed inside the Incucyte® SX5 Live-Cell Analysis System (Sartorius AG®) for 36 hours, in order to evaluate the green fluorescence intensity of the activated cells, which in turn was located inside of a humidified incubator at 37ºC and 5% CO_2.

6. Flow Cytometry Analysis

After being removed from their culture medium, to assess the viability post-electroporation and after the co-culture, the nucleofected cells were washed three times and resuspended in PBS solution (1X) (Gibco®) supplemented with 1% heat inactivated FBS (Gibco®). When necessary, there was a 30-min staining with PE anti-mouse TCR ß chain (PE-mTCR) antibody (BioLegend®) before adding 4′,6-diamidino-2-phenylindole (DAPI) as a live/dead marker. After flow cytometry data acquisition (LSR Fortessa, BD Sciences), the analysis was performed using FlowJo v8.

7. Statistical Analysis

GraphPad Prism 9 was used to plot and analyse all data shown. Regarding biological replicates, the data is shown with the Standard Error of Mean (SEM). A one-way ANOVA, with a multiple comparisons test, was performed to determine statistical difference, when possible.

Chapter 3 Results and Discussion

1. Development of the proof-of-concept vector
2. Proof-of-concept Experimental Design – Jurkat 76 CD8+ cell line
3. Co-culture of nucleofected J76 CD8+ T-cells
4. Proof-of-concept Experimental Design – Primary human T-cells
5. Co-culture of nucleofected Primary human T-cells
6. Development of the IL-12 TRUCK-like vectors
7. Experimental Design – IL-12 Vectors Nucleofection
8. Design and Cloning of the IL-12 Nanovectors

1. Development of the proof-of-concept vector

As described in the Subchapter 5 of the Introduction, the main objective of this project was to develop a hybrid ACT using the MART-1 antigen, through the combination of the TRUCK model with inducible IL-12 release, and a TCR-recombinant therapy, by taking advantage of the novel S/MAR vectors developed and described by the DNA Vector research group [105,146,148,154]. With that purpose, the DMF5 TCR [82] sequence was used, as well as the IL-12 as the inducible cytokine, mediated by six repetitions of the NFAT binding site from the IL-2 gene [150].

As previously mentioned, the NFAT family plays a key role in the activation of T-cells and consequent immune response. As such, the corresponding binding site can be used as a responsive element, and the use of six repetitions of this binding site for inducible release has already been demonstrated in several studies, not only with reporter genes [35,153,155], but also with cytokines [52,102].

However, before developing the TRUCK-like IL-12 vector, it is important to evaluate if the entire system works, and, for this, the reporter gene copepod GFP was used, referred as GFP from now on, to replace the IL-12 cytokine sequence in the proof-of-concept vector. Theoretically, since the reporter gene is located downstream of the responsive element's sequences, once the T-cell becomes activated due to TCR-MHC engagement, the NFAT binding site becomes active and transcription and translation of the GFP occurs, turning the cell green. Thus, it is safe to conclude that a high green signal intensity emitted from the cell can correlate to a strong cell activation.

Since the TCR and the inducible cytokine need to be co-expressed equally within the cells, it is of great interest to identify the most effective method for their secure development and co-expression. Søndergaard *et al.* (2020) showed that co-transfecting two small plasmids, with between 2-7 kb each, instead of a bigger one, between 9-15 kb, led to better transfection efficiency [156]. Furthermore, as transfection is size-dependent, the reduction of vector size increases the proficiency of gene transfection [105,109]. However, this is not always the case, as in certain circumstances, the single vector with both components has a smaller size than the two vectors when transfected [157]. Moreover, T-cells are extremely sensitive to DNA transfection processes, especially blood-extracted primary cells, so it is important to burden them as little as possible and using only one vector instead of two can be helpful.

S/MAR vectors are mostly being used within the DNA Vector research group, and, as their establishment process is not entirely understood, it is imperative to determine if co-transfection does not result into their segregation. Alice De Roia has generated crucial data in the DNA Vector research group research group, to determine the optimal strategy for safe multi-gene delivery, by comparing two different approaches: the "two-vector" system, that consisted in the co-transfection of two vectors, each with a reporter gene (GFP or d-tomato) and the "one-vector" system, that contained both reporter genes linked by a P2A sequence.

The reporter genes were under the human core Elongation Factor 1α (coreEF-1α) promoter, and all vectors used had the same features, including the coreApoL S/MAR. By transfecting human

Jurkat 76 T-Cells, Alice De Roia studied the transfection efficiency and establishment process through sorting of both approaches and the results obtained are shown in Figure 8.

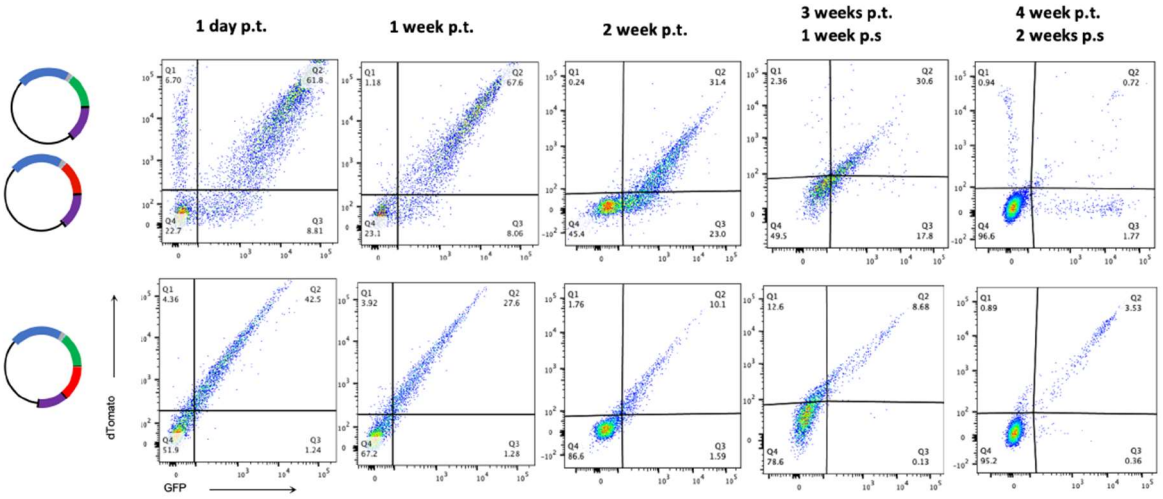

Figure 8 – *Ex vivo* establishment of the two-vector system and the one-vector system. Data generated by Alice De Roia, in the DNA Vector research group, DKFZ.

The human J76 T cell line was transfected and cultured under normal conditions, without any antibiotic selection for one month. Transgene expression was followed for one month and double-positive cells (Q2) were sorted through two and four weeks after the transfection. The first row of results refers to the two-vector system (co-transfection of both vectors, each containing a reporter gene), and the second row to the one-vector system, that contained both reporter transgenes. Data obtained with FlowJo v8. "p.t." – post-transfection; "p.s." – post-sorting.

After one day, the "two-vector" transfected cells showed a higher percentage of double-positive cells, compared to the "one-vector" system, which further confirmed that smaller vectors enhance transfection. The J76 T-Cells were sorted two and four weeks after transfection to decrease the danger of random integration, and they were monitored once a week for a month using Flow Cytometry.

Interestingly, a proportion of double-positive cells were lost in the two-vector system but retained in the one-vector system at the end of the establishment. Alice De Roia noted that this may be explained by the two vectors vying for the same location in the nuclear compartment, and that the one-vector system should be the best method for a downstream application.

To further confirm what was demonstrated by Alice De Roia, two approaches were compared: the "two-vector system" and the "one-vector system", and the corresponding vectors are shown in Figure 9.

The "two-vector system" consists of the co-transfection of two vectors: one with the TCR sequence ("coreEF1-DMF5" vector, Figure 9A) and another with the responsive element, the minimal promoter from the IL-2 gene (minP) and the reporter gene GFP ("NFAT6x-GFP" vector, Figure 9B). The "one-vector system" resulted from the cloning of the PCR fragment obtained from the NFAT6x-GFP vector in the digested coreEF1-DMF5 vector, having both components in one single vector ("NFAT6x-GFP-DMF5" vector, Figure 9C). The cloning strategy is displayed in Figure 24 in the Appendix, and the gel electrophoreses are shown in Figures 27 and 28.

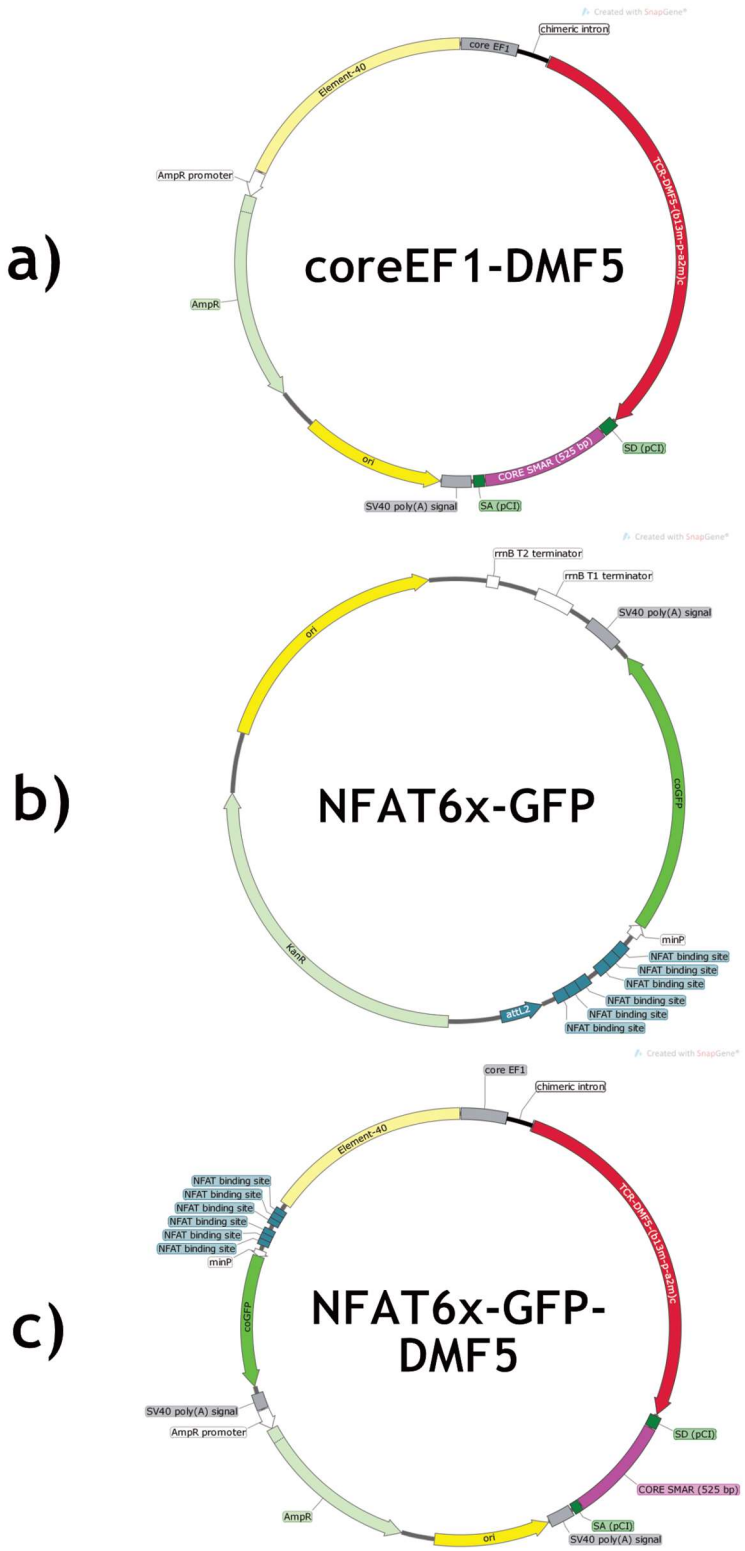

Figure 9 – Vectors used for the Proof-of-Concept Experiment.

A) "coreEF1-DMF5" vector, with 5.7kb. This vector includes the anti-MART-1 DMF5 TCR under the coreEF-1α promoter, with an ampicillin resistance gene and an S/MAR from the core ApoB protein. **B)** "NFAT6x-GFP" vector, with 3.2 kb. It includes the GFP gene under six repetitions of the responsive element NFAT, and a kanamycin resistance gene. The co-transfection of **A)** and **B)** makes up the "two-vector system". **C)** "NFAT6X-GFP-DMF5" vector, or the "one-vector system", with 6.8 kb. It harbours the anti-MART-1 DMF5 under the coreEF-1α promoter, and the GFP gene under the six repetitions of NFAT. It also presents an ampicillin resistance gene and the S/MAR sequence from the core ApoB protein. Vector maps were exported from the respective SnapGene® (v. 4.0.8) files.

Each vector has a polyadenylation (polyA) signal, the Sirian Virus 40 (SV40) polyA signal, after each expression cassette, to regulate gene expression [158,159]. There is also a high-copy-number pUC origin of replication, as well as an antibiotic resistance gene in each construct: an ampicillin resistance gene in the coreEF1-DMF5 and the NFAT6x-GFP-DMF5 vectors and a kanamycin resistance gene in the NFAT6x-GFP vector.

To assemble the TCR properly, it is necessary that the transfected vector express all the TCR chains, as well as the constant chains. To this end, both vector systems contain the α and ß chains of the DMF5 TCR, as well as the α and ß murine TCR constant chains. Moreover, the DMF5 TCR expression is controlled by the coreEF-1α promoter, which is constitutively expressed.

Contrary to other constitutive promoters, such as the minimal cytomegalovirus promoter (miniCMV), the coreEF-1α promoter has shown promising results for episomal gene expression in mammalian cells, revealing high and stable expression of the gene of interest [160].

Between the coreEF-1α and the TCR sequences, a chimeric intron is present. This is an artificial intron, from human ß-globin and immunoglobulin heavy chain genes, which enhances mRNA processing and increases the expression levels of the downstream Open Reading Frame (ORF) [161]. However, the mechanism behind the expression enhancement is not entirely known.

The Element 40 (NCBI: AL929602.3) acts as a genetic insulator, protecting all DNA from possible methylation from heterochromatin-related proteins [162]. It has been shown that when combined with S/MARs, this sequence can provide a long-term and stable gene expression in mammalian cells [162–164].

Since S/MARs are comprised of adenine and thymidine nucleotides, AU-Rich Elements (AREs) are extremely common and can lead to mRNA degradation and lack of gene translation. As shown by Bozza *et al.* (2021), the introduction of splicing sites to flank the S/MAR sequence can produce more stable transcripts [148]. The S/MAR chosen was isolated from the human apolipoprotein B (ApoB) gene cluster, given that Bozza *et al.* (2021) showed that using this system [148].

2. Proof-of-concept Experimental Design – Jurkat 76 CD8+ cell line

To study the one-vector system performance, as well as to compare it to the co-transfection of the two-vector system, the following experiments illustrated in Figure 10 were performed.

To reiterate, for the successful recognition of the MHC-peptide complex, the T-cell needs to express the TCR and the co-receptor simultaneously. In this case, since the MART-1 antigen is recognized by CD8-expressing T-cells, a stable cell line of CD8-expressing Jurkat 76 cells ("J76 CD8+" cells) was used, kindly provided by Ilka Bartsch.

As for the nucleofection, five conditions were studied, being the following: the "Mock" condition, which was comprised of cells that underwent the entire nucleofection process without DNA; as controls, the coreEF1-DMF5 vector and the NFAT6x-GFP vector were nucleofected independently; the one-vector system (NFAT6x-GFP-DMF5) and the two-vector system (co-transfection of the coreEF1-DMF5 and NFAT6x-GFP vectors) were studied. In theory, the condition

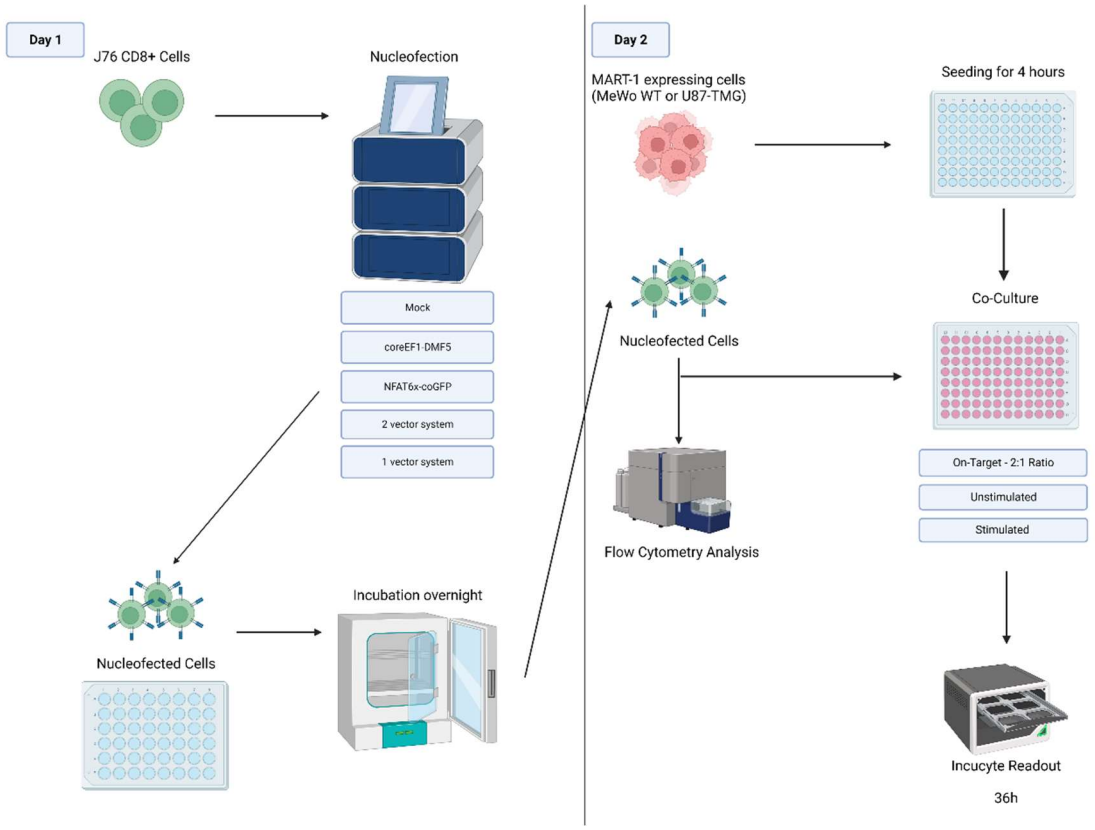

Figure 10 – Experimental Design for the proof-of-concept with Jurkat 76 CD8+ cells.

On the first day (left panel), J76 CD8+ cells were nucleofected with the Lonza 4D device, using the coreEF1-DMF5, NFAT6x-GFP and the NFAT6x-GFP-DMF5 vectors, resulting in the conditions mentioned in the image. The two-vector system is the co-transfection of the coreEF1-DMF5 and the NFAT6x-GFP vectors. Afterwards, the cells were cultured in a 48-well plate and incubated overnight at 37ºC and with 5% CO_2. On the next day (right panel), the MART-1 expressing cells were seeded in a 96-well plate for 4 hours. In parallel, the nucleofected cells were analysed through Flow Cytometry, to study the TCR expression. After the seeding, the T-cells were cultured in three different ways: on top of the target cells – "On Target" – with a 2:1 T-cell:Target cell Ratio; by themselves – "Unstimulated"; and with Transact™ solution – "Stimulated". The cells were then placed inside of the Incucyte System, which is placed inside of an incubator, for 36h to analyse GFP expression and, thus, cell activation. Created with BioRender.com.

nucleofected with the coreEF1-DMF5 vector should express the TCR and not express GFP, whereas the NFAT6x-GFP should not express TCR neither GFP, due to the lack of the surface receptor.

On the following day, the nucleofected cells were subjected to Flow Cytometry Analysis, for evaluation of nucleofection efficiency by analysing the percentage of TCR expression. These results are shown in Figure 11, in the following subchapter.

In parallel, the MART-1 antigen expressing cells, referred as "target cells", were seeded for 4 hours before the co-culture assay. The MeWo cell line and the stable U87-TMG cell line, which was transduced to express the Tandem Mini Gene (TMG) of the MART-1 epitope, were used as target cells. The MeWo cell line derives from human metastatic melanoma skin cells, more specifically from a metastatic lymph node [165]. This cell line can be characterized as adherent fibroblasts that usually overexpress the MART-1 antigen. The U87 cell line is a glioblastoma cell line, which is one of the most aggressive type of brain cancer [166]. This cell line has an epithelial adherent morphology, being similar to MeWo cells, and, interestingly, it has been shown that glioblastoma cells may express antigens from other cancer cell types, in particular the MART-1 antigen [167,168].

Yu-Chan Chih developed a stable MART-1-expressing U87 cell line ("U87-TMG" cells), by transfecting a plasmid encoding a puromycin resistance gene and a tandem mini gene (TMG) of the epitope ELAGIGILTV (epitope ID: 12941 [169]) of MART-1. Yu-Chan Chih kindly provided us these cells, to evaluate the vectors performance in another cell type that also expresses the MART-1 antigen. Therefore, in theory, the recognition of the MART-1 antigen expressed on either of the target cells by the TCR-expressing nucleofected T-cells should lead to TCR activation and, in this case, the cells should express the reporter GFP in their presence.

Three culture environments were studied in this assay (Figure 10): "On-Target", "Unstimulated" and "Stimulated". As the name entails, the "Unstimulated" condition consisted of wells only with T-cells and, thus, the cells are not stimulated. In the "Stimulated" condition, TransAct™ (Miltenyi Biotec), a colloidal polymeric nanomatrix with CD3 and CD28 agonists, was added to the medium, which induced the strong activation and expansion of T-cells. For the "On-Target" condition, T-cells were cultured on top of the target cells, either MeWo or U87-TMG cells, in a 2:1 ratio, i.e., twice the number of nucleofected T-cells than target cells.

Once all cells were in culture, the respective 96-well plate was placed for 36 hours in the Incucyte® SX5 Live-Cell Analysis System (Sartorius AG®), which in turn is located inside of a humidified incubator, allowing cell growth. This system has up to five different fluorescence channels and, by taking pictures of the wells in specific time intervals, it allows the live analysis of the cells' behaviour through the phase channel and the green fluorescent channel. As such, the GFP signal intensity can be obtained, exported, and analysed as a correlation to T-cell activation.

2.1. Nucleofection Efficiency and TCR expression

After one day in culture, the nucleofected cells showed a high viability, as shown in Figure 11, being above 80% in all conditions. This further confirms that the nucleofection process does not affect cell viability.

Regarding the TCR expression, shown in Figure 11 as "mTCR+", the control condition coreEF1-DMF5 condition expresses the DMF5 TCR, as expected, up to 30%. Regarding the two other conditions that should express the TCR, the percentage of cells that expressed it were between 20% (two-vector system) and 40% (one-vector system).

Although the TCR expression of the three conditions aforementioned were statistically different when compared to the Mock condition, the TCR expression is not ideal. Since cells typically begin to express nucleofected constructs after 24 hours, a short cell rest may be the cause of the poor transfection rate and, thus, a longer period of incubation time might have resulted in greater TCR expression.

With this in mind, the same cells were cultured with the target cells a few hours after obtaining these results, which is shown on the next chapter. Nonetheless, it is essential to evaluate more timepoints after nucleofection in the future, to further access the ideal timepoint for co-culture.

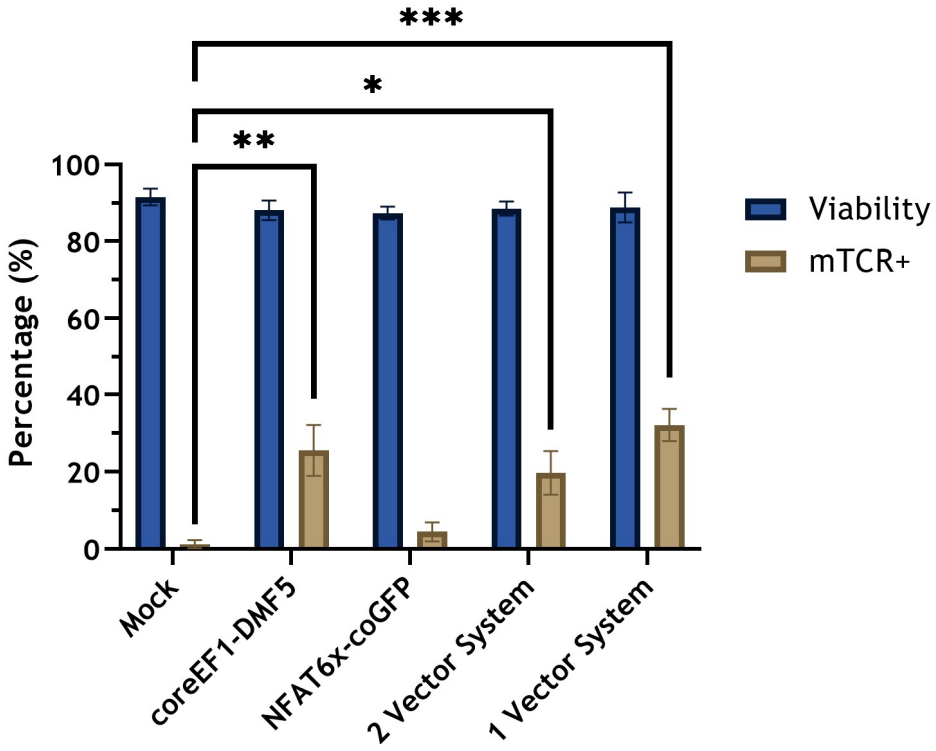

Figure 11 – Nucleofection efficiency after one day post-nucleofection, through Flow Cytometry Analysis, of Jurkat 76 CD8+ cells.

In any condition, the cell viability is above 80%. Regarding the TCR expression (mTCR+), it is under 40% in the conditions that should express the DMF5 TCR (coreEF1-DMF5, two-vector system and one-vector system), showing significant difference when compared to the Mock condition. The data shown was obtained using DAPI and the PE-mTCR marker. The mean of 4 independent experiments, with the respective SEM, is represented for each condition. A one-way ANOVA was performed to the mTCR+ values of the conditions that should express it, with a multiple comparisons test (*** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$). Made with GraphPad Prism 9.

3. Co-culture of nucleofected J76 CD8+ T-cells

After flow cytometry analysis, the nucleofected cells were cultured in the three distinct ways aforementioned in Figure 10 and subsequently placed inside the Incucyte® SX5 Live-Cell Analysis System, to analyse cell activation. Here, the Incucyte® system automatically calculated the average of the green signal intensity after taking 4 photos at various locations inside the same well to cover the entire area. The intensity is shown as "GFP Intensity per μm^2", which is interpreted as the Green Fluorescence Unit (GFU) measured divided by the well area (μm^2). The variation of the green fluorescent intensity with time is presented for each well condition every hour during a period of 36 hours.

3.1. Co-culture with MeWo Cells

The results obtained and the respective SEM from the T-cell culture with MeWo cells (On-target) and the unstimulated and stimulated settings are represented in Figure 12 for 4 independent experiments.

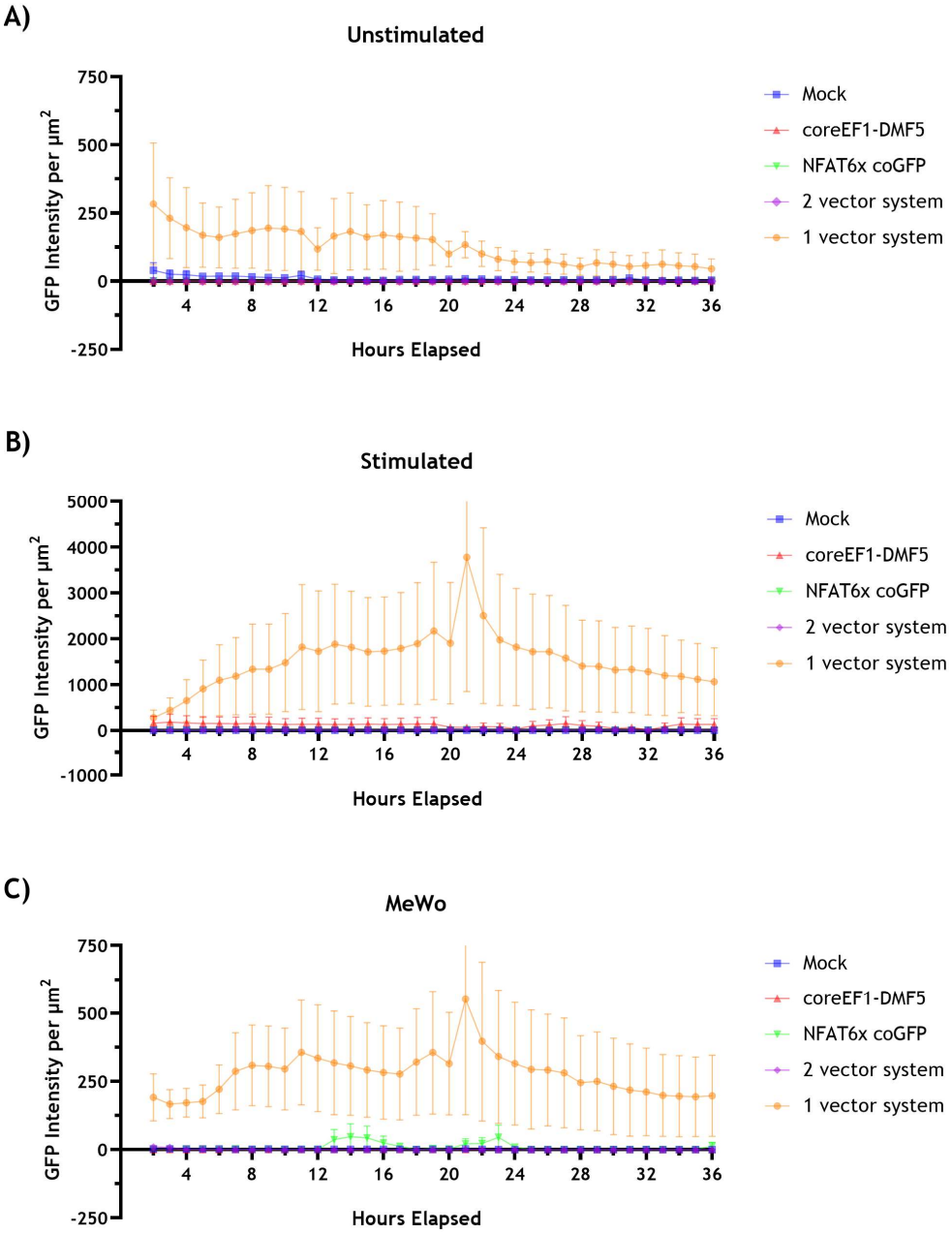

Figure 12 - Green fluorescence Intensity emitted by nucleofected J76 CD8+ T-cells when in culture alone, with MeWo cells or stimulated.

(A-C) Green Fluorescent (GFP) Intensity recorded every hour of each culturing condition (**A**: Unstimulated, **B**: Stimulated, **C:** MeWo Co-culture) using the Incucyte® SX5 Live-Cell Analysis System. When stimulated (B) or when in contact with the MeWo cells (C), the one-vector system nucleofected cells show the highest GFP intensity through time, which translates into the highest cell activation. However, when unstimulated (A), the one-vector system cells show some cell activation. Each point is the mean of 4 independent experiments, with the respective SEM. Made with GraphPad Prism 9.

As expected, in every culture setting (Figures 12.A to 12.C), the Mock and the coreEF1-DMF5 nucleofected cells did not show any fluorescence over time, serving as the negative controls within this assortment of conditions. Moreover, cells in the Unstimulated condition (Figure 12.A) showed low green intensity, and, therefore, revealed low or no cell activation, as anticipated.

However, J76 CD8+ cells nucleofected with the one-vector system exhibited some fluorescent signal that decreased overtime. This could be that the promoter upstream the GFP sequence triggers unspecific GFP protein production.

It could also be that the responsive element is leaky, which can be unlikely, as this specific sequence has been used by other researchers in the past, without showing leakiness [150]. Notwithstanding, it would be of great interest to evaluate the leakiness of the responsive element in the future, by analysing the expression of this green signal for a longer period of time and to use another method to analyse the fluorescence, such as flow cytometry analysis.

Focusing on the Stimulated condition (Figure 12.B), the one-vector system was the only condition that showed intense green signal intensity, reaching up to 2500 GFU/μm^2. This reflects the proper functioning of the one-vector system, as when cells are stimulated, the responsive element becomes activated and there is protein translation. Of note, neither the two-vector system nor the NFAT6x-GFP nucleofected cells demonstrated as high an intensity as the one-vector system, being around 15 GFU/μm^2.

When in contact with MeWo cells (Figure 12.C), the one-vector system nucleofected cells showed the highest activation, and the intensity was constant in several time intervals. However, focusing on the two-vector system, the nucleofected cells did not show any activation, which leads to the conclusion that this system did not work. In the end, the one-vector system had a better performance compared to co-transfection, by showing a higher activation through time.

Both with MeWo cells or when stimulated, the nucleofected T-cells with the one-vector system had a trend of increase in cell activation up to 21 hours of culture, decreasing thereafter. The decline of activation could be due to cellular exhaustion, as cells become increasingly active because of the immune response.

Intriguingly, when cultured with MeWo cells, the activation prompted by MART-1 antigen recognition is not as high as it should be, contrary to the stimulated condition, that shows another order of intensity. These findings rule out the hypothesis of the non-functioning of the responsive element and the reporter gene.

Therefore, the relatively low activation observed with MeWo cells could be explained by a potential low expression of the MART-1 antigen by the MeWo cells used in this assay. Hence, it is imperative to characterize said MeWo cells, through Flow Cytometry using the Alexa Fluor™ 647 Melan-A/MART-1 antibody for example, to determine the MART-1 surface expression.

3.2. Co-culture with U87-TMG cells

The results and the respective SEM obtained from the T-cell culture with the previously described U87-TMG cells, along with the two other culture conditions, studied in 2 independent experiments are represented in Figure 13.

As expected, and similar to the previous set of experiments, the Mock and the coreEF1-DMF5 nucleofected cells did not emitted fluorescence in any culturing condition (Figure 13.A to 13.C).

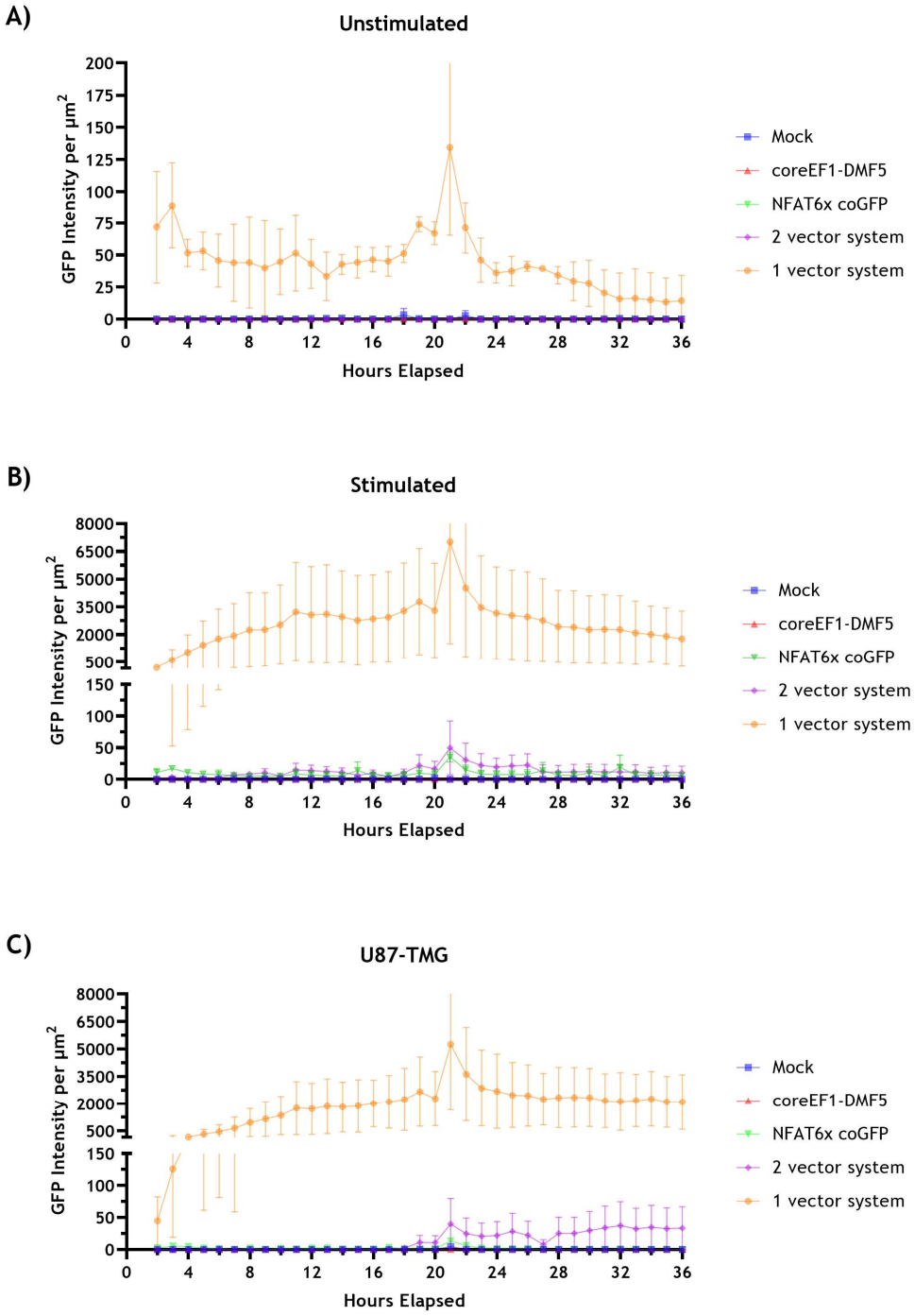

Figure 13 – Green fluorescence Intensity emitted by nucleofected J76 CD8+ T-cells when in culture alone, with U87-TMG cells or stimulated.

(A-C) Green Fluorescent (GFP) Intensity recorded every hour of each culturing condition (**A**: Unstimulated, **B**: Stimulated, C: U87-TMG co-culture) using the Incucyte® SX5 Live-Cell Analysis System. When stimulated (B) or when in contact with the U87-TMG cells (C), the one-vector system nucleofected cells show the highest GFP intensity through time. However, when unstimulated (A), the one-vector system cells showed an arbitrary cell activation. The two-vector system shows some GFP intensity when stimulated (B) and when in contact with U87-TMG cells, although minimal. Each represented value is the mean of 2 independent experiments, with the respective SEM. Made with GraphPad Prism 9.

Regarding the unstimulated cells (Figure 13.A), only the one-vector system cells showed a fluorescent signal, although without a precise pattern. It would be of great interest to use another methodology to measure the activation, to exclude potential unspecific GFP signal.

When stimulated (Figure 13.B), the NFAT6x-GFP and the two-vector system nucleofected cells showed activation, even though discrete, reaching up to 50 GFU/μm^2, higher than what was observed in the previous results. The one-vector system showed the highest fluorescence, surpassing 6500 GFU/μm^2, again corroborating its positive performance.

When in culture with the U87-TMG cells (Figure 13.C), the one-vector system cells showed a significantly higher activation than when cultured with the MeWo cells (Figure 12.C), having almost the same order of intensity to when stimulated (Figure 13.B).

Since the U87-TMG cells are genetically modified to express the MART-1 epitope ELAGIGILTV and are also submitted to a process of puromycin selection, this epitope could be more expressed in the cell surface and have a higher binding strength to the TCR, and, thus, lead to a higher activation. As such, it is also important to characterize the expression of MART-1 on U87-TMG cells and compare their expression levels with the MeWo cells.

Regarding the other conditions, the two-vector system showed some activation when in contact with the U87-TMG cells, but never in the same order of intensity as the one-vector system, either when in culture or when stimulated.

Similar to the previous results shown, when stimulated or in contact with the target cells, the one-vector system nucleofected cells showed a trend of increase in the fluorescence intensity, reaching a peak 21h post co-culture, and then decreasing gradually. This showed that cell activation is time-dependent, which further justifies a treatment like TRUCK immunotherapy, where cell activation and consequent immune response can be “forced” through cytokine release *in situ*.

However, as only two independent experiments were conducted, it is crucial to perform additional ones, to make sure that these results are significant and not coincidental. Moreover, it is also important to have another data output as well, to confirm the results obtained with the Incucyte® SX5 System.

4. Proof-of-concept Experimental Design – Primary human T-cells

To further test the one-vector system against the two-vector system, as well its expression in primary human T-cells, the following experiment displayed on Figure 14 was conducted with one healthy donor.

To obtain the primary T-cells, a buffy coat from a healthy donor was used to isolate PBMCs, which in turn was used to isolate CD3+ T-cells. Although only CD8+ T-cells perform the recognition of the MART-1 antigen as it is MHC-restricted, in the immune environment, CD4+ and CD8+ are highly dependent of one another for the immune response [170], and, thus, it is beneficial to isolate both populations of T-cells.

The primary T-cells were then isolated and activated by adding IL-7 and IL-15 in the culture medium. Once the isolated primary T-cells rested for 3 days, the same conditions used in the previous set of experiments were nucleofected.

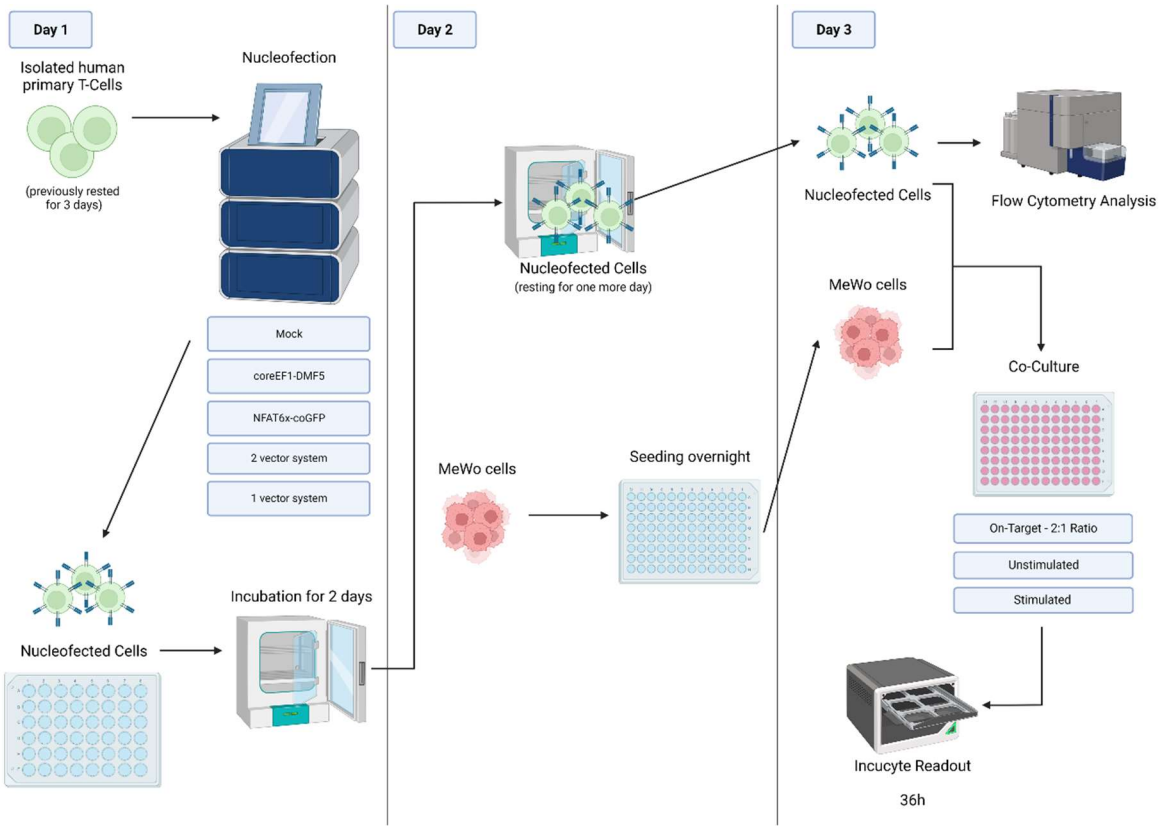

Figure 14 - Experimental Design for the proof-of-concept with Primary T-cells.

On the first day (left panel), previously rested isolated human primary T-cells were nucleofected with the Lonza 4D device, resulting in the conditions mentioned in the image. Afterwards, the cells were cultured in a 48-well plate and then incubated for 2 days at 37ºC and with 5% CO_2. On the second day (middle panel), the MeWo cells were seeded in a 96-well plate. On the last day (right panel), the nucleofected cells were analysed through Flow Cytometry, to study the TCR expression. Afterwards, the T-cells were cultured in three different ways: on top of the target cells – "On Target" – with a 2:1 T-cell:Target cell Ratio; by themselves – "Unstimulated"; and with Transact™ solution – "Stimulated". The cells were then placed inside of the Incucyte System, which in turn is inside of an incubator, for 36h. Created with BioRender.com.

On the following day, the nucleofected cells rested for one more day, contrary to the J76 CD8+ cells. As primary T-cells are directly isolated from blood, they are much more sensitive than cells from a cell line, and, thus, need more time to recover from the nucleofection procedure before doing the co-culture. As such, the Flow Cytometry analysis to evaluate nucleofection efficiency was done two days after the DNA nucleofection process.

Moreover, as the MeWo co-culture results showed less binding than the U87-TMG in the previous experiments, the MeWo cells were seeded one day before the co-culture. This was done so that the cells adhered completely to the well and that they expressed the MART-1 antigen to its fullest. The seeding was done as previously mentioned.

On the third and last day, the cells were co-cultured and submitted to the same methods as described in the previous set of experiments. Due to time constraints, only one donor was used for this experiment and U87-TMG cells were not used.

4.1. Nucleofection Efficiency and TCR expression

In Figure 15, the cell viability and TCR expression of the nucleofected primary T-cells are shown in percentage.

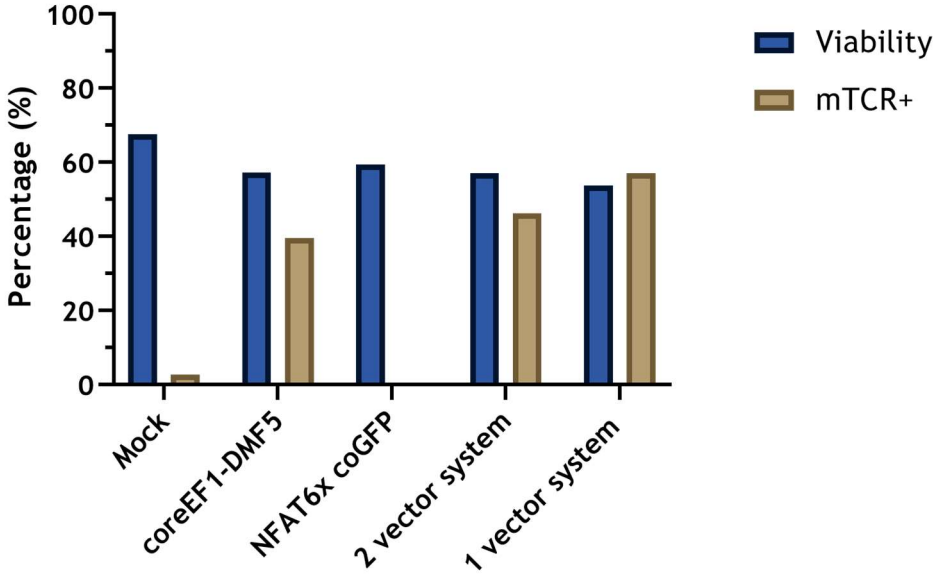

Figure 15 – Nucleofection efficiency after two days post-nucleofection, through Flow Cytometry Analysis, of primary T-cells.

The cell viability is between 50 and 70%, and, regarding the TCR expression (mTCR+), it is between 30 to 60%. The data shown was obtained using DAPI and the PE-mTCR marker. One donor is represented in this data. Made with GraphPad Prism 9.

After two days post-nucleofection, the cells showed a viability between 50% and 70%. This further confirmed the need to leave the cells to rest one more day, as primary cells are extremely sensitive to any kind of DNA transfer process. Likewise, primary T-cells are not as stable out of their own environment as cells from a cell line, which can also explain why the viability is not optimal.

Interestingly, the expression of the DMF5 TCR was higher comparing to the results obtained with the J76 CD8+ cells, falling between 30% and 60%. This could be due to the increased rest time after the nucleofection process as it was mentioned before. As it was still not ideal, it is necessary to evaluate TCR expression in other timepoints after the nucleofection, to determine when is the best timepoint for the co-culture with target cells. Since only one donor was tested, it was not possible to perform a statistical analysis.

5. Co-culture of nucleofected primary human T-cells

After the flow cytometry analysis, the nucleofected primary cells were cultured as done with the J76 CD8+ cells and placed inside the Incucyte® SX5 Live-Cell Analysis System. The signal intensity capture was done the same way as described previously (Figure 16).

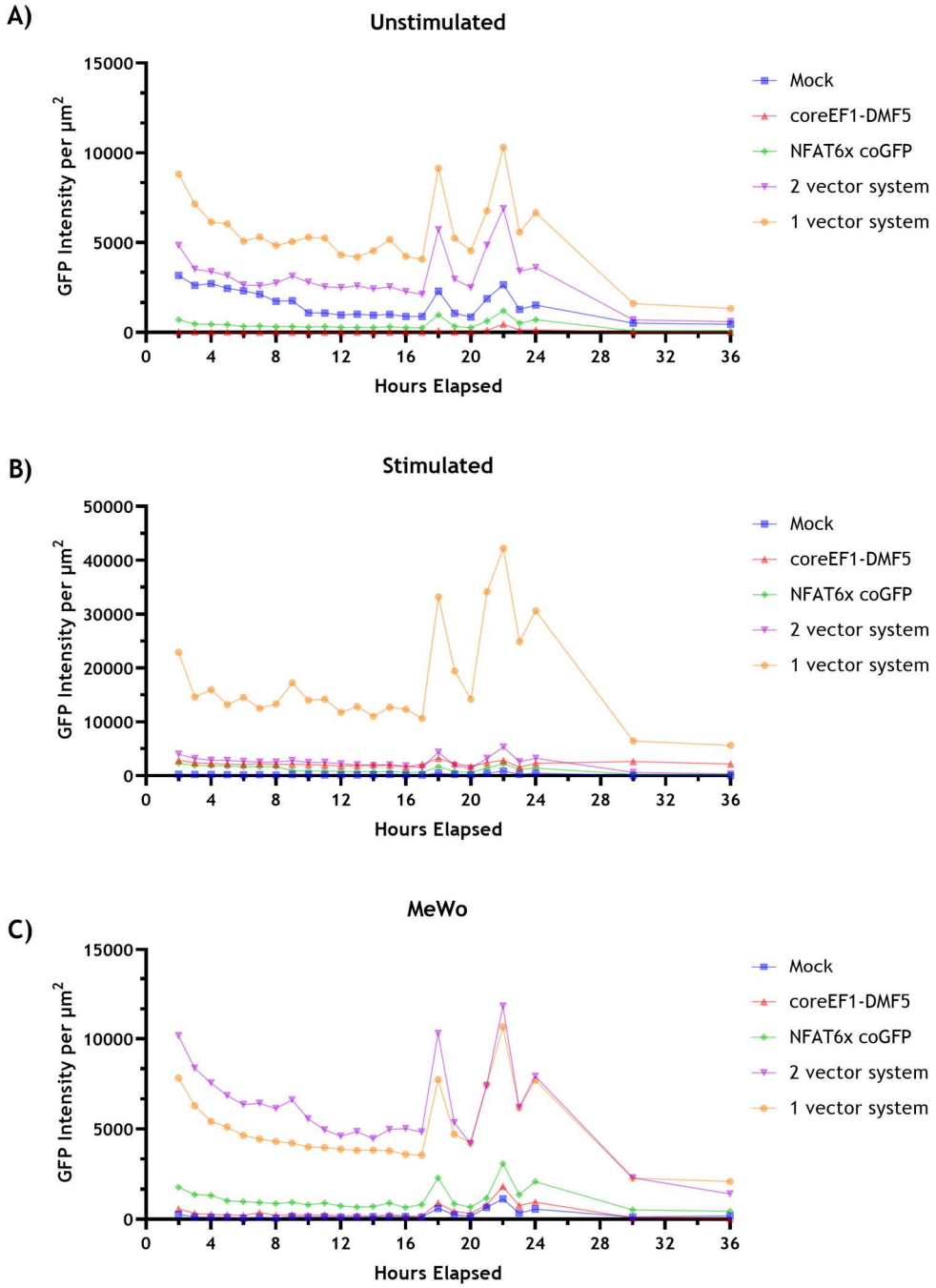

Figure 16 - Green fluorescence Intensity emitted by nucleofected primary T-cells when in culture alone, with MeWo cells or stimulated.

(A-C) Green Fluorescent (GFP) Intensity recorded every hour of each culturing condition (**A**: Unstimulated, **B**: Stimulated, **C:** MeWo Co-culture), using the Incucyte® SX5 Live-Cell Analysis System. When unstimulated (A), almost all cells have some sort of signal. When stimulated (B), the one-vector system cells show the highest activation. However, when in contact with MeWo cells (C), the two-vector system shows a higher signal intensity through time. One donor is represented. Made with GraphPad Prism 9.

Firstly, when analysing the three graphs (Figure 16.A to 16.C), there are two intensity peaks that happened to every nucleofection condition, corresponding to 18 and 22h. This could be an intensity variation caused by the machine internally or externally. With that stated, the results were interpreted regarding the absolute value to one timepoint, and not with time variation.

Focusing on the Unstimulated condition (Figure 16.A), interestingly, the Mock condition showed some intensity, although minimal, following the same pattern as the other conditions. This could be explained by a background signal that the machine emits independently.

On the other hand, as the primary cells showed a non-optimal viability, a signal of this kind could also be self-fluorescence that is emitted and captured by the machine once the cells are going through the process apoptosis. To validate this hypothesis, it would be interesting to use a different coloured dye that marks apoptotic cells, such as the red Caspase 3/7 dye [171].

Regarding the one-vector and two-vector systems, both showed a fluorescent signal, but the first showed a higher intensity when unstimulated. This is not ideal, since the objective of this construct is to have a specific binding to the MART-1 antigen, and this shows a possible unspecific binding.

When stimulated (Figure 16.B), the one-vector system cells showed the highest intensity throughout, whereas the NFAT6x GFP and the two-vector system cells showed some intensity while not comparable, which goes accordingly to the J76 CD8+ experiments.

Though, as the primary cells are activated before the nucleofection and even when in the culture IL-7 and IL-15 are added to the medium, the primary cells may be overly activated beforehand. Thus, it is important to test whether the cytokines used for T-cell activation can have a role in NFAT activation, which can be done by removing the cytokines from the medium a few hours before the co-culture begins.

However, when in contact with the MeWo cells (Figure 16.C), the two-vector system showed a higher intensity, and thus a higher activation, when compared to the one-vector system, which goes against the results obtained with the J76 CD8+ cell line. Nevertheless, considering that these results correspond to a single donor, that the cell viability was not optimal for this type of experiment and that there is the possibility of overly activated primary T-cells, generalisation from these results is not appropriate.

Therefore, it is required to repeat the same experimental set with a larger sample size and to confirm these results with another assay, such as flow cytometry or through image capture and analysis of the fluorescent signal with specific imaging software, such as ImageJ [172].

6. Development of the IL-12 TRUCK-like vectors

Once it was shown that the one-vector system worked in an *in vitro* setting, the following step was to engineer the IL-12 TRUCK-like vectors. As already mentioned during the Introduction Chapter, the cytokine IL-12 has been used already as an adjuvant in metastatic melanoma therapies, with the MART-1 antigen, and in ACTs with inducible release, using the NFAT binding site as a responsive element [52,102,173].

By taking advantage of the platform UniProtKB, the sequences corresponding to both subunits α and ß, with the ID:P29459 and ID:P29460, respectively, of the human IL-12 were linked with a P2A sequence *in silico*. The P2A sequence, when translated in eukaryotic cells, causes the ribosome

to skip that sequence, giving rise to two fragments instead of a continuous one [174]. Thus, in this case, when inside the cell, the translation of this sequence will result into the separate subunits, giving rise to the IL-12 cytokine. This sequence was then sent to Twist Bioscience© to be synthetized, giving rise to the IL-12-P2A sequence shown in Figure 17.

Figure 17 – Human IL-12-P2A sequence.

The subunits α and ß correspond to the IDs P29459 and P29460 respectively, in the UniProtKB platform. The P2A sequence acts as a linker between the two subunits. This linear fragment has 1.7 kb in total. Fragment map was exported from the respective SnapGene® (v. 4.0.8) file.

Mann *et al*. (2020) was able to develop a NFAT-mediated fluorescent system, similar to the one-vector system previously shown, where different promoters, different repetitions of NFAT binding sites, and different reporter genes were compared [153]. Regarding the promoter comparison, Mann *et al*. developed a synthetic promoter, which is referred to as the "TATA-box", or simply "TATA", and compared it to the miniCMV promoter by evaluating the expression of green fluorescent protein [153].

Interestingly, the miniCMV construct showed unspecific protein production, as the cells produced the reporter gene in the absence of stimulation of the NFAT, whereas the TATA synthetic promoter showed a stimulation-dependent production [153]. In the light of these results, and since there was some green signal with the one-vector system nucleofected T-cells when unstimulated, cloning the TATA synthetic promoter and comparing the expression of IL-12 depending on the promoter was followed through.

As for the vector construction segment, an Overlap Extension PCR was used to insert the promoters upstream of the IL-12 α subunit before the In-Fusion® Cloning. The Overlap Extension PCR technique aims to linearly assemble DNA fragments through successive PCR reactions with specific primers and DNA parts, without time-consuming cloning steps [152].

However, since the promoters were smaller than 100 bp, it was not feasible to order synthetized DNA parts solely for this assembly. There was a need to adjust the protocol, and thus, a new Overlap Extension PCR was developed, based on the protocol developed by Anna Behle [152], where only PCR primers were used. The respective diagram of this methodology is shown in Figure 18.

Using SnapGene's software, the primers for the assembly-based cloning were designed *in silico* and are displayed in Table 1 of the Appendix. The first two primers are going to be referred to as "overlap primers" and the other two primers as "overhang primers". The upstream overlap primer contained the promoter in question (minP or TATA) and a few annealed base pairs for the binding, and the downstream overlap primer annealed completely to the human IL-12 sequence. The two overhang primers were used to synthesise the overhangs for the In-Fusion® Reaction.

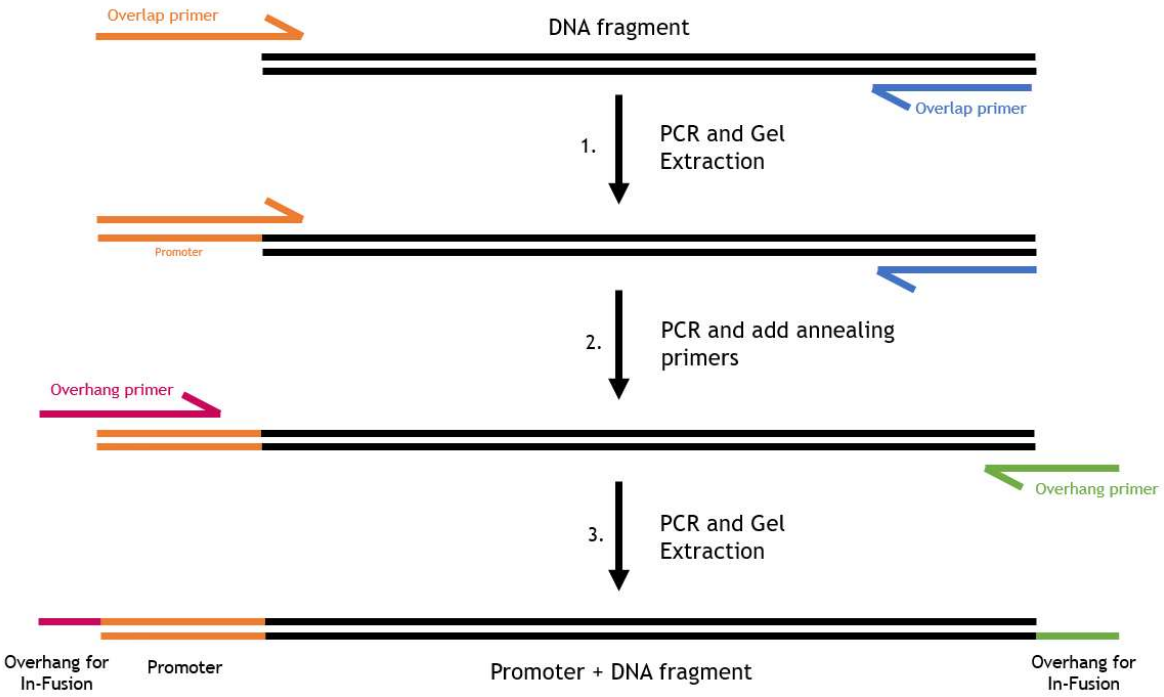

Figure 18 – Adapted Overlap Extension PCR.

The DNA fragment suffered three distinct PCRs, using only PCR primers, in order to add the promoter upstream, as well as overhangs for the In-Fusion® Cloning Reaction. Method developed and adapted from the protocol written by Anna Behle [152].

Once the primers were designed and created, it was time to move on to the PCRs themselves. The first PCR, marked as (1) in Figure 18, aimed to amplify the DNA fragment with the upstream promoter as an overhang, where the annealing temperature used was calculated considering only the primer part that bonded to the fragment, without considering the overhang, as suggested in the original protocol [152]. A gel extraction was necessary to remove all of the secondary structures, primers and other products that naturally occur from this process.

Afterwards, the newly obtained fragment was subjected to a second PCR, represented by (2) in Figure 18, adding the same primers in a higher amount. This resulted in the amplification of the fragment without overhangs and with the promoter fully integrated into the DNA sequence. The last PCR, represented by (3) in Figure 18, was a regular PCR for the In-Fusion® reaction. It was crucial to do a gel extraction again, as this method can give rise to side products, such as the original fragments, secondary structures obtained between primers, or non-specific binding all-together.

Thus, by making use of the protocol of Overlap Extension PCR developed by Anna Behle [152], a novel protocol was attained. The IL-12 fragments with the different promoters were then obtained through this protocol and are portrayed in Figure 19. The gel images portraying these fragments are shown in Figure 30, in the Appendix.

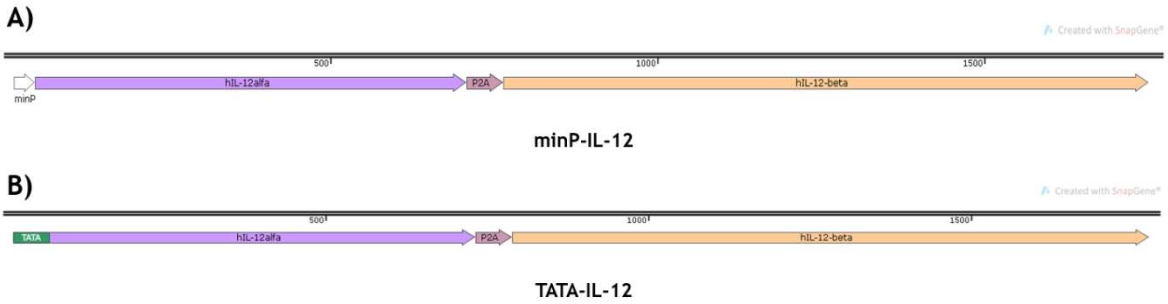

Figure 19 – IL-12-P2A fragments.

A) IL-12-P2A fragment regulated by the minP promoter upstream – "minP-IL-12". **B)** IL-12-P2A fragment regulated by the TATA synthetic promoter upstream [153] – "TATA-IL-12". Both fragments have 1.8kb. Fragment maps were exported from the respective SnapGene® (v. 4.0.8) files.

Once the minP-IL-12 and the TATA-IL-12 fragments were obtained, the NFAT6x-GFP-DMF5 vector was digested with restriction enzymes (Figure 29 in the Appendix) and the fragments were cloned, replacing the minP sequence and the GFP sequence present in the NFAT6x-GFP-DMF5, with the In-Fusion® Reaction fragments of Figure 19.

The positive clones were chosen by Colony PCR, using the primers depicted in Table 1 of the Appendix. The gel images that represent the results of the Colony PCR are shown in the Appendix, in Figures 31 and 33. The cloning is portrayed in Figure 25 in the Appendix, and the vector maps of the resulting constructs are shown in Figure 20.

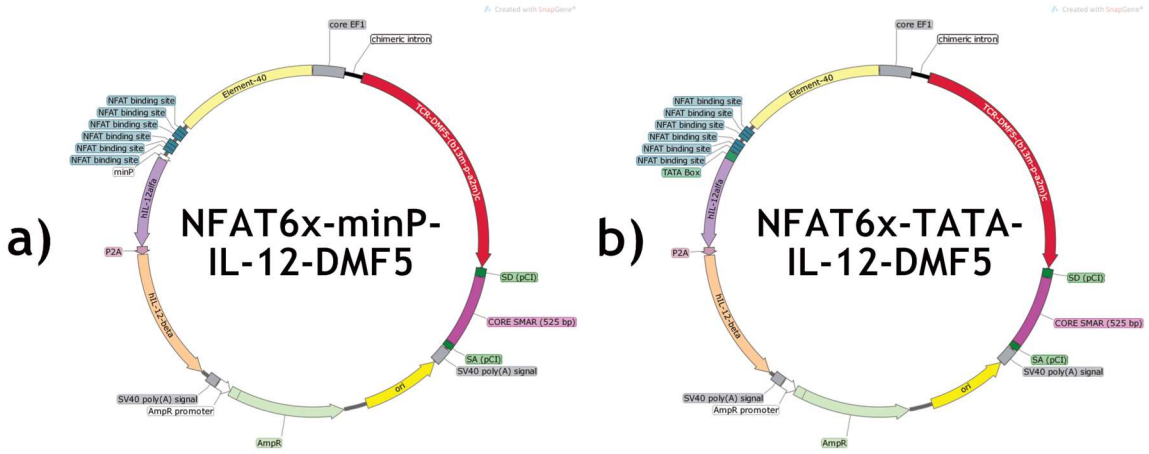

Figure 20 – IL-12 Vector Maps.

A) "NFAT6x-minP-IL-12-DMF5" vector. This vector contains the anti-MART-1 DMF5 TCR under the coreEF-1α promoter, and the IL-12-P2A sequence under the minP promoter and the six repetitions of NFAT. **B)** "NFAT6x-TATA-IL-12-DMF5" vector. It includes the anti-MART-1 DMF5 TCR under the coreEF-1α promoter, and the IL-12-P2A sequence under the synthetic TATA [153] promoter and the six repetitions of NFAT. Both vectors have 7.8kb and present an ampicillin resistance gene and the S/MAR sequence from the core ApoB protein. Vector maps were exported from the respective SnapGene® (v. 4.0.8) files.

When compared to the one-vector system previously mentioned, the obtained vectors only differ in the gene that is being mediated by the NFAT, in this case IL-12, as well as the promoter upstream of the gene, TATA, in the case of the NFAT6x-TATA-IL-12-DMF5 vector. Thus, both vectors include the DMF5 TCR under the coreEF-1α promoter, being constitutively expressed, the

coreApoB S/MAR, between a splicing donor and a splicing acceptor, the ampicillin resistance gene, the origin of replication and the Element 40 for silencing protection.

7. Experimental Design – IL-12 Vectors Nucleofection

Once the IL-12 vectors were obtained, it was important to determine if the vectors were fully functional and were able to be transfected onto cells, without affecting their viability. Thus, the following experiments depicted in Figure 21 were performed.

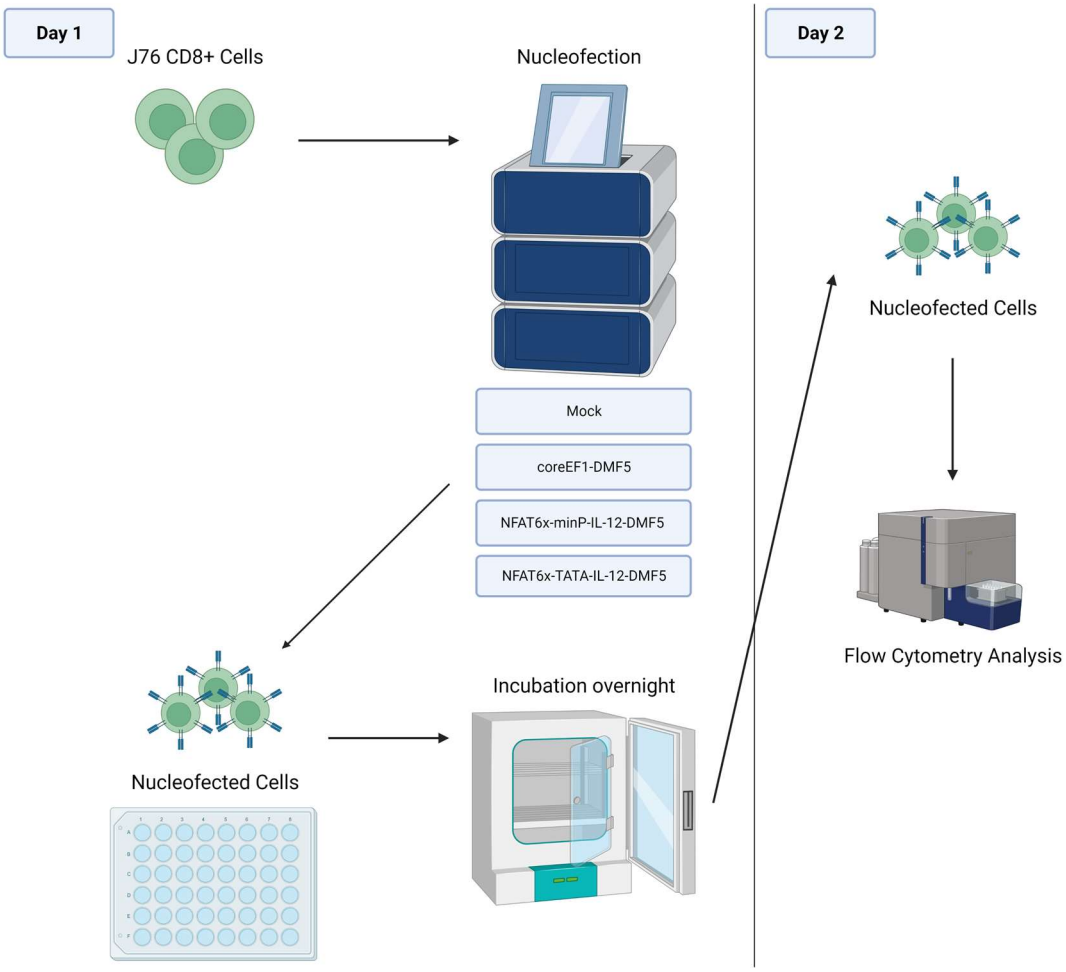

Figure 21 - Experimental Design for the IL-12 vectors with J76 CD8+ cells.

On the first day (left panel), J76 CD8+ cells were nucleofected with the Lonza 4D device, resulting in the conditions mentioned in the image. Afterwards, the cells were cultured in a 48-well plate and then incubated overnight at 37ºC and with 5% CO_2. On the next day (right panel), the nucleofected cells were analysed through Flow Cytometry, to study their TCR expression. Created with BioRender.com.

For this experiment, the J76 CD8+ cells kindly provided by Ilka Bartsch were used. Similar to the experimental designs mentioned before, the Mock condition was comprised of nucleofected cells without DNA, and the following conditions were nucleofected with the homonymic vectors: coreEF1-DMF5, NFAT6x-minP-IL-12-DMF5 and NFAT6x-TATA-IL-12-DMF5. The coreEF1-DMF5 vector was transfected onto cells as a positive control, for TCR expression. Afterwards, the cells were incubated overnight.

The nucleofected cells were submitted to Flow Cytometry Analysis the next day to assess nucleofection efficacy by analysing the percentage of TCR expression of each IL-12 TRUCK-like construct. The Figure 22 depicts the findings, as well as cell viability of each condition.

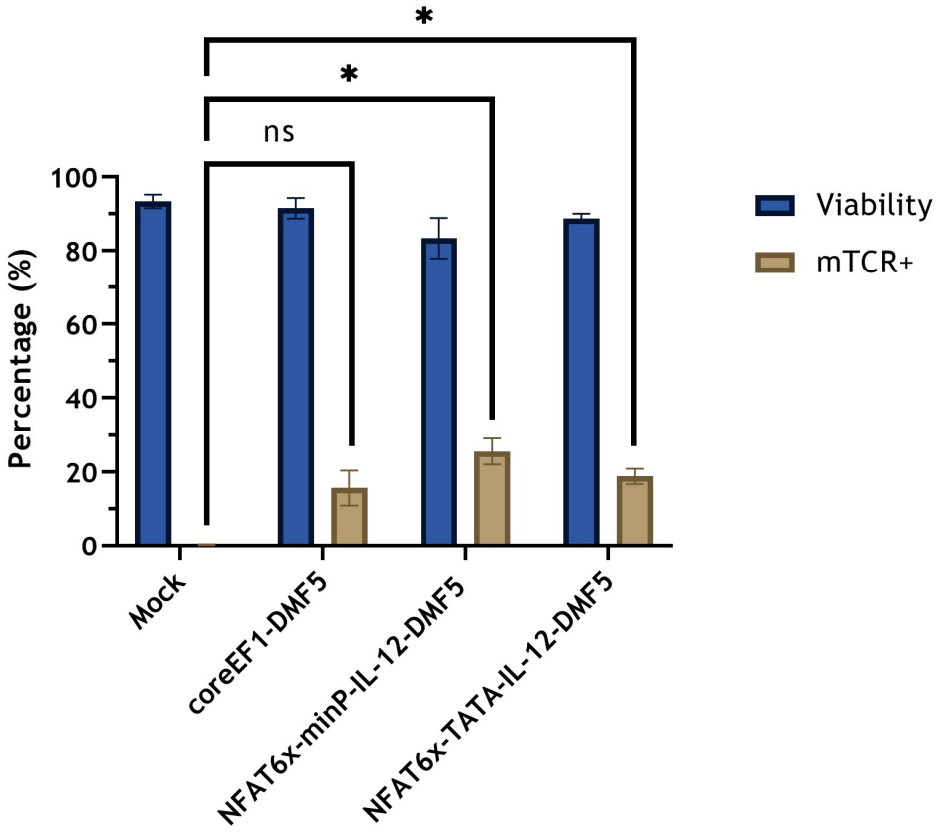

Figure 22 - Nucleofection efficiency after one day post-nucleofection, through Flow Cytometry Analysis, of Jurkat 76 CD8+ cells, with the IL-12 vectors.

The cell viability is above 80% and, regarding the TCR expression (mTCR+), it is under 30% in the conditions that should express the DMF5 TCR (coreEF1-DMF5, NFAT6x-minP-IL-12-DMF5 and NFAT6x-TATA-IL-12-DMF5). The data shown was obtained using DAPI and the PE-mTCR marker. The mean of 2 independent experiments, with the respective SEM, is represented for each condition. A one-way ANOVA was performed, with a multiple comparisons test (* - $p < 0.05$, ns – non-significant). Made with GraphPad Prism 9.

After one day post-nucleofection, the cells demonstrated a viability above 80%, similar to the ones previously nucleofected with the two-vector and the one-vector systems. Again, this further confirms that the nucleofection process performed does not interfere with cell viability.

Although the cell viability was great, the expression of DMF5 TCR was not ideal, falling between 10 and 30%. As mentioned before, since cells typically begin to express nucleofected constructs after 24 hours, a short cell rest may be the cause of the poor transfection rate and, thus, a longer period of incubation time might have resulted in greater TCR expression.

As such, before doing the co-culture assay and the IL-12 ELISA, to evaluate the expression of the IL-12 when cells are activated, it is absolutely necessary to assess more timepoints after nucleofection and determine when is the most optimal timepoint for the co-culture assay.

Nonetheless, these findings show that the IL-12 TRUCK-like vectors are able to be transfected, as both constructs showed statistical difference when compared to the Mock condition, and that T-cells are able to express the TCR within them.

8. Design and cloning of the IL-12 Nanovectors

To restate, due to the high likelihood of transcriptional inhibition and antibiotic resistance transmission, the use of antibiotics and antibiotic resistance genes in a therapeutical setting is discouraged by regulatory health companies.

To avoid their use, several alternatives have been developed, such as the R6K-RNA-OUT™ technology [110], developed by NTC® and commercialised as Nanoplasmid™. This system, already described in the Subchapter 4 of the Introduction, allows the plasmid selection to be done by cloning their host *E.coli* strain in suitable plates as an antibiotic-free medium, with the benefit of having a mini origin, from the R6K plasmid.

Moreover, the DNA Vector research group created S/MAR nanoplasmids, in collaboration with NTC©, and showed that they replicated as an episome, without integrating the genome, and enabled sustained gene expression [146–148]. With all this information, the opportunity to develop a TRUCK-like Nanoplasmid for IL-12 arose.

As an attempt to evaluate the effect that the promoter has on a Nanoplasmid™, the following nano vectors were designed: "NV-NFAT6x-minP-IL-12-DMF5" and "NV-NFAT6x-TATA-IL-12-DMF5". The respective DNA maps are displayed in Figure 23.

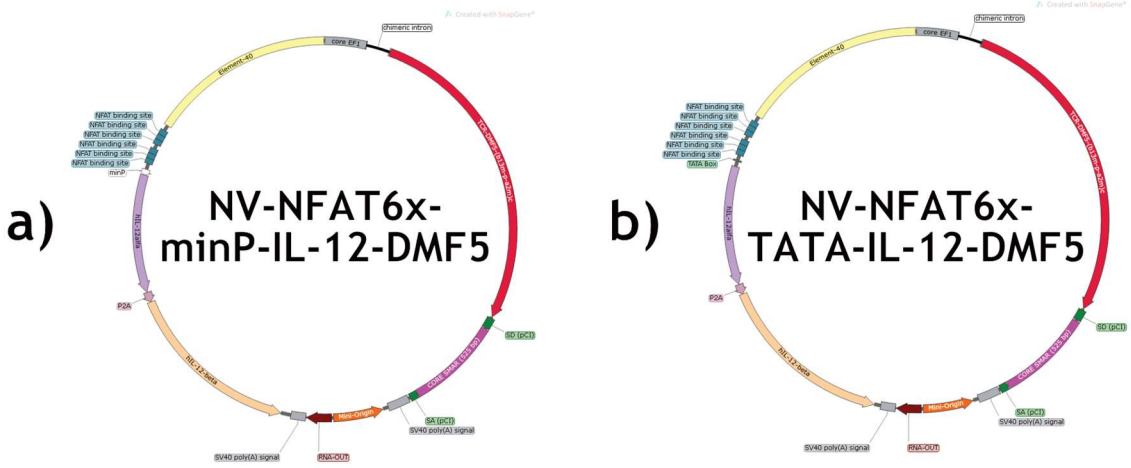

Figure 23 - IL-12 Nanoplasmid Maps.

A) "NV-NFAT6x-minP-IL-12-DMF5" nanovector. It contains the anti-MART-1 DMF5 under the coreEF-1α promoter, and the IL-12-P2A sequence under the minP promoter and the six repetitions of NFAT. **B)** "NV-NFAT6x-TATA-IL-12-DMF5" nanovector. It includes the anti-MART-1 DMF5 under the coreEF-1α promoter, and the IL-12-P2A sequence under the synthetic TATA [153] promoter and the six repetitions of NFAT. Both vectors have 6.5kb and present the S/MAR sequence from the core ApoB protein and the R6K-RNA-OUT™ system from the Nanoplasmid™ created by NTC®. The R6K origin is represented as "Mini-Origin". Vector maps were exported from the respective SnapGene® (v. 4.0.8) files.

For the cloning of the novel IL-12 TRUCK-like nanovectors, both IL-12 vectors were digested to remove the antibiotic resistance gene and the origin of replication, referring to the combination of both sequences as bacterial backbone. Afterwards, the nano backbone was attained through PCR amplification of a Nanoplasmid™ synthetised by NTC® that contained a d-tomato reporter gene regulated by the coreEF-1α promoter. The cloning is depicted in Figure 25 in the Appendix.

However, it was not feasible to perform a Colony PCR in the NTC® host strain of *E. coli* K12, as an unwanted quantity of bacteria and DNA would be lost by dividing the colony into halves. This lengthened the colony screening process. Also due to time constraints, it was not possible to obtain the nanoplasmids at the time this thesis was finished.

Notwithstanding, the cloning design is achievable as the PCR fragment that corresponds to the nano backbone is ready to be cloned into the digested IL-12 vector, either the minP or the TATA-including vector, in order to transform the genetically engineered bacteria and to culture the true positive clones.

Chapter 4 Conclusions

The present thesis set out to develop a novel TRUCK-like immunotherapy against metastatic melanoma, more specifically, the MART-1 antigen, with inducible release of the cytokine IL-12. In order to determine the most adequate multi-gene delivery approach, a novel system for gene expression using the S/MAR sequences was developed, where simultaneous expression of two gene cassettes was achieved. This one-vector system can express both a reporter gene under the control of a cell activation-responsive element, in this case six repetitions of the NFAT binding site, as well as the DMF5 TCR through the S/MAR non-viral expression system – the NFAT6x-GFP-DMF5 vector.

When transfecting J76 CD8+ cells, it was shown that the one-vector system shows a better performance when compared to the co-transfection of the separate gene cassette, referred to as two-vector system. When in contact with U87-TMG cells, the one-vector system showed a stronger antigen recognition of the MART-1 epitope when compared to the recognition of the MART-1 antigen expressed by MeWo cells.

Thus, the one-vector system shows a strong gene expression and it was proven to be a better option when compared to the traditional co-transfection technique. Nonetheless, it is important to carry out more independent experiments with the U87-TMG cells to further confirm these results. Also, it is important to characterize both target cells regarding the expression of MART-1 on their surface.

It was also shown that primary T-cells isolated from a healthy donor are able to take in the one-vector system's genetic information through nucleofection and express the TCR of interest, although the viability decreases with this process. Nevertheless, since this study was limited to only one donor, further research is needed to draw solid conclusions involving gene expression on primary T-cells.

Furthermore, to obtain the IL-12 TRUCK-like vectors, an improved version of the Overlap Extension PCR protocol was devised. This novel procedure aims to synthetize small sequences onto linear DNA fragments only using primers that include said sequences. It also prepares the fragments for downstream In-Fusion Cloning® processes and might be used for other cloning methods, such as Gibson Assembly, by replacing the overhang primers with primers that suit the chosen cloning method.

Herein, through this technique, two TRUCK-like vectors were obtained, where the reporter gene was replaced by the sequences of the IL-12 subunits, linked by a P2A sequence, and the upstream promoter, either the minP promoter – the NFAT6x-minP-IL-12-DMF5 vector - or the TATA synthetic promoter – the NFAT6x-TATA-IL-12-DMF5 vector -, which were cloned to evaluate and compared gene expression on each vector.

By transfecting J76 CD8+ cells, it was shown that both of the IL-12 TRUCK-like vectors are functional and are able to be nucleofected onto cells without any concerns. However, due to time constraints, the assessment of the IL-12 expression between the two vectors was not possible.

In theory, the vector that includes the TATA synthetic promotor should show a more specific IL-12 cytokine expression compared to the one with the minP promoter, based on the results obtained by Mann *et al*. (2020) [153]. Nonetheless, as it was not possible to assess IL-12 production, it is unknown if this hypothesis is correct.

Based on the nanoplasmids developed and described by the DNA Vector research group, in collaboration with NTC©, a cloning strategy was concocted to obtain the "nano" versions of the previously mentioned TRUCK-like vectors – the NV-NFAT6x-minP-IL-12-DMF5 and the NV-NFAT6x-TATA-IL-12-DMF5. For this purpose, the bacterial backbone and the antibiotic resistance gene of each vector will be replaced by the R6K-RNA-OUT® system, amplified from an Nanoplasmid™.

However, it was not possible to obtain the nanoplasmids before the submission of this thesis, as their production is rather lengthy. Whilst this thesis did not study a nanovector's efficiency, it did show that it is feasible to develop a TRUCK-like S/MAR vector with the Nanoplasmid™ characteristics, which is of great value for a clinical setting, as it does not have antibiotic resistance genes.

Overall, this thesis strengthens the idea that TRUCK-based immunotherapies can be applied in the context of metastatic melanoma and can provide a specific activation upon antigen recognition with the NFAT binding site, specifically using the MART-1 model. Moreover, these findings have significant implications in multi-gene delivery, where an "all-in-one" vector shows to be a favourable approach when compared to the traditional co-transfection of two or more genes of interest. Herein, the methods used for the Overlap Extension PCR may be applied in any other context, being of great value for the insertion of small sequences onto bigger linear fragments. Finally, the present thesis lays the groundwork for future experiments in IL-12 TRUCK-like vectors and nanovectors, as well as for future research in cancer immunotherapy.

Chapter 5 Future Perspectives

The findings obtained throughout this thesis show that there are additional studies that should be carried out, in order to completely develop a functioning TRUCK-like plasmid that can be applied to metastatic melanoma, and, hopefully, to other types of solid tumours.

Firstly, the DMF5 TCR expression will be evaluated over 7 to 12 days, through flow cytometry analysis and through quantitative PCR assays, to confirm that the TCR is being expressed persistently through time without genome integration. This will also confirm if the promoter or the responsive element is leaky and if the signal intensity observed in some conditions is from the Incucyte® device or from the construct itself.

Although the results obtained with J76 CD8+ cells showed that the one-vector construct works and can recognise two different cell types expressing the MART-1 antigen, the recognition of MeWo cells was not strong enough to be statistically significant. Therefore, the characterisation of these target cells will be performed, by flow cytometry or through a Western Blot assay, for example, in order to study the MART-1 surface expression. Additionally, there will be two more independent experiments of modified J76 CD8+ cells when in culture with U87-TMG cells to confirm the results presented.

As it was shown, it is possible to nucleofect primary T-cells with the one-vector system, albeit there were insufficient findings to draw firm conclusions about the system's functioning. Thus, the transfection of the one-vector system in primary T-cells isolated from two more donors will be accomplished, in order to compare it to the results obtained with the J76 CD8+ cells. Likewise, three more donors will be used to study the behaviour of modified primary T-cells when in contact with U87-TMG cells.

In parallel, the cloning of the two nanoplasmids will be recommenced, to evaluate gene expression and cell activation based on the nano backbone, comparing to the regular plasmid. Hopefully, a faster method for bacteria screening will be developed in the upcoming future.

Once primary T-cells are nucleofected with the TRUCK-like vectors and nano vectors, it is imperative to evaluate the production of IL-12 as well as the killing ability of the modified cells. As such, an IL-12 ELISA will be conducted and the production of IL-12 will be evaluated maintaining the same conditions of co-culture. Moreover, an IFN-γ ELISA or the Incucyte® Immune Cell Killing Assay will be conducted to evaluate the killing ability of modified cells, specifically of the nucleofected CD8+ T-cells.

It will also be important to study if CD8+ T-cells have the same ability to kill as the CD3+ population when nucleofected with the same construct by comparing isolated human CD8+ T-cells with isolated CD3+ cells from the same donor. A non-nucleofected condition will be studied as well to analyse if the modification of the cells interferes in their killing ability.

As a last step before pre-clinical and clinical trials, the release of IL-12 and the consequent cell behaviour in a 3D tumour-like microenvironment will be studied using human spheroid cultures. The major objective is to study the cell interactions between the modified T-cells with MART-1 expressing cells and the constituents of the TME, i.e., cancer associated fibroblasts or

infiltrated immune cells. Moreover, the study of the microenvironment's modulation through the release of the inducible cytokine is also an objective to be fulfilled in this assay.

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Appendix

1. Vector Cloning

The cloning strategies designed and followed are portrayed as diagrams. All images of vectors and linear sequences were exported from the respective SnapGene® (v. 4.0.8) files. The diagrams here presented were built with the help of Microsoft PowerPoint™ software.

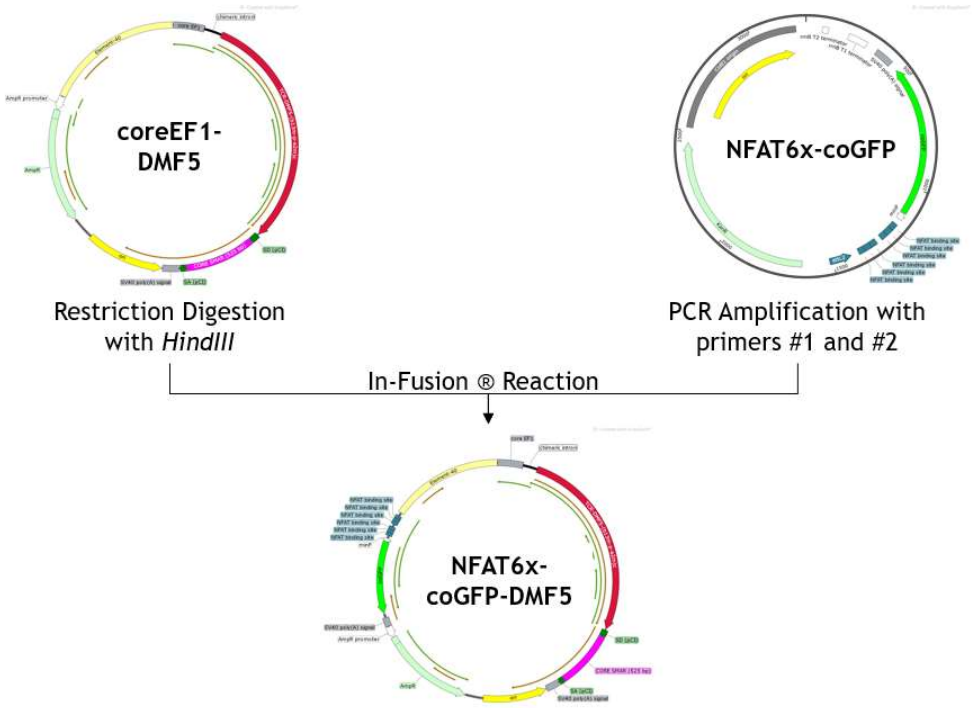

Figure 24 – Cloning of the "one-vector" system plasmid.

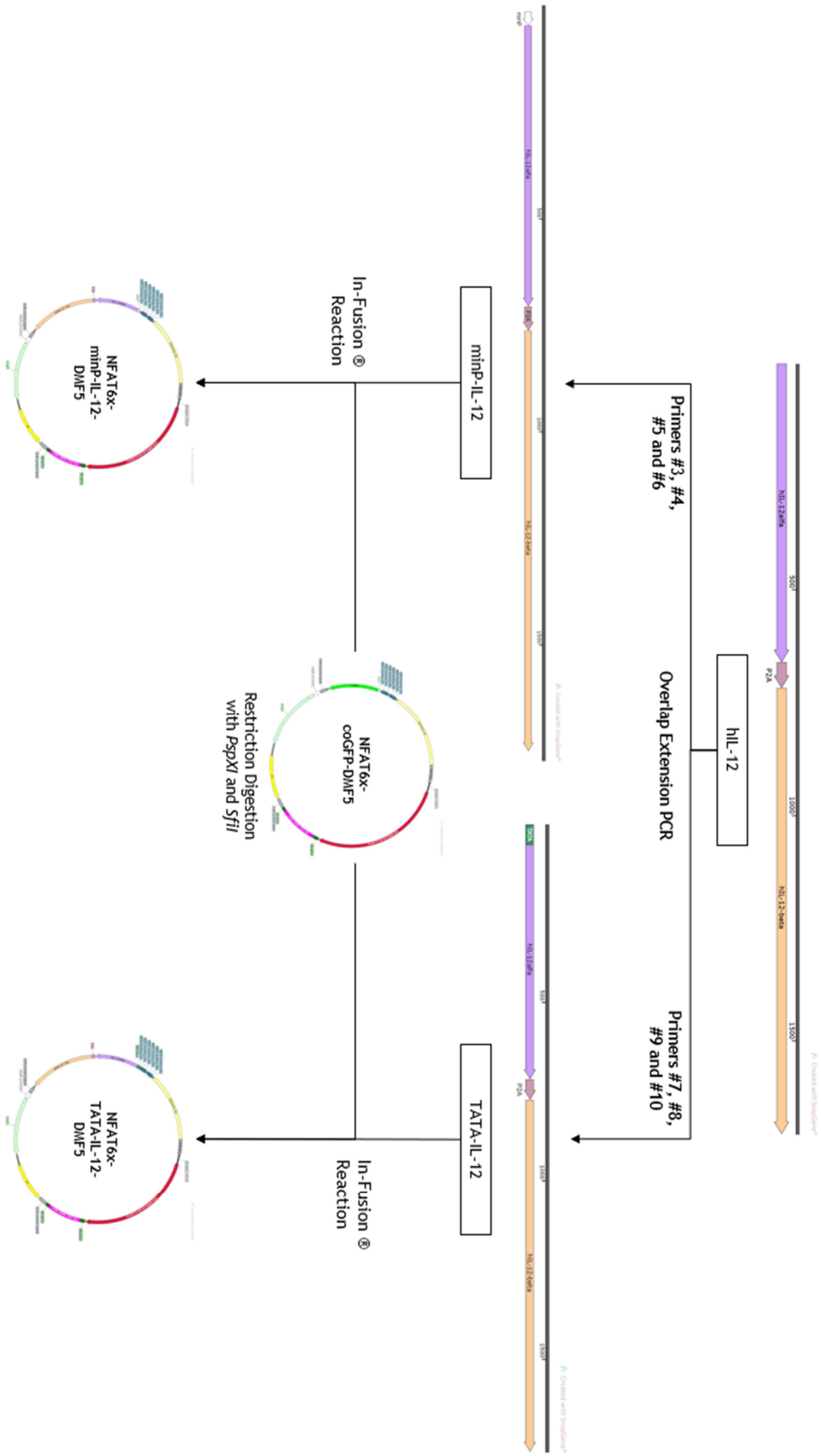

Figure 25 - Overlap Extension PCR of the human IL-12 (hIL-12) to clone the respective promoter upstream. Cloning of the IL-12 plasmids.

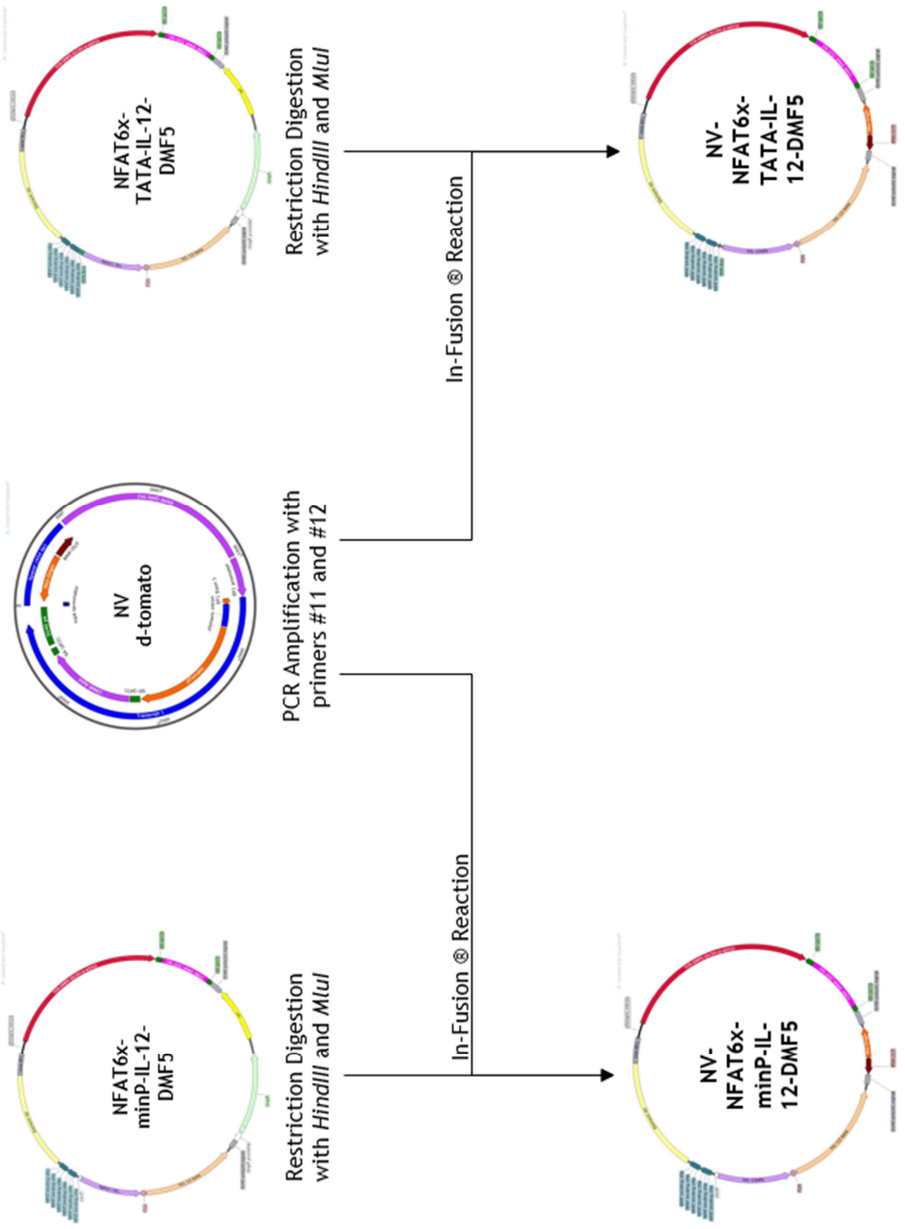

Figure 26 - Cloning of the IL-12 Nanoplasmids.

2. Table of Primers

Below is shown the table of all the primers used throughout this thesis. Every primer was used in a final concentration of 10 μM. For this the sequencing process, all the primers shown here were used for the sequencing of the respective vector.

Table 1 - Table of primers used and the respective application.

Primer Number	Sequence (5'-3')	Application
1	CAGAAGGCGTAGATCTGGTACCTGAGCTCGCTAGCG	PCR to obtain the NFAT6x-GFP insert
2	TCTCATCGAGCTCGAGTCAGGCGAAGGCGATGG	
3	TCTGCAGGACATGTGTAGAGGGTATATAATGG AAGCTCGACTTCCAGATGTGCCCCGCCAGAAG	Overlap Extension PCR for minP-IL12
4	GATAAAAATCATTAGGTACCTTAGCTGCAGGGCACGCT	
5	GCGTAGATCTGGCCTCGGCGGCCTCTGCAGGACATGTGTAGAGGGT	
6	ACTCATCGAGCTCGAGTGATAAAAATCATTAGGTACCTTAGCTGCAGG	
7	CGCGGAGACTCTAGAGGGTATATAATGGATCCCCGGG TACCGAGCTCGAATTCACCATGTGCCCCGCCAGAAGC	Overlap Extension PCR for TATA-IL12
8	TTAGCTGCAGGGCACGCT	
9	ACTCATCGAGCTCGATTAGCTGCAGGGCAC	
10	GCGTAGATCTGGCCTCGCGGAGACTCTAGAG	
11	TAAACAAGTTAAGCTCCTAATGATTTTTATCAAAATCATTAAGTTAAG	PCR to obtain the Nano Backbone
12	ATGTATCTTAACGCGGCCCGCCTAATGAGCG	

3. Gel Electrophoreses

All of the gel electrophoreses done throughout this work are shown here. Each caption describes the respective image.

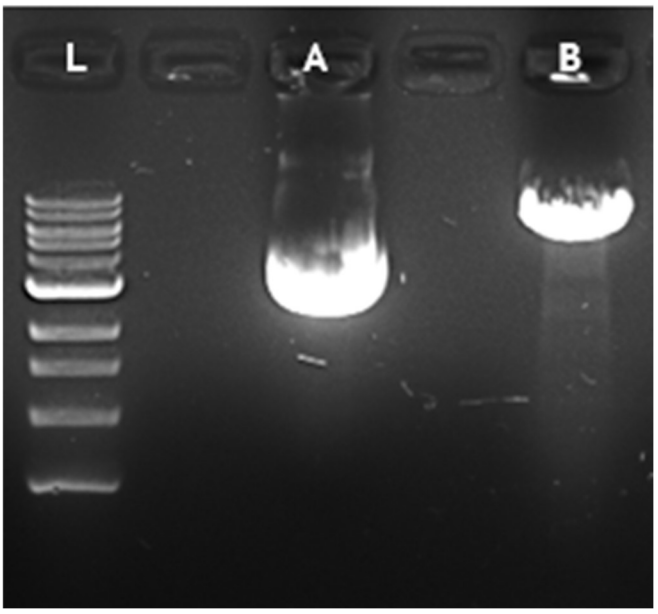

Figure 27 – Gel electrophoresis of the coreEF1-DMF5 vector undigested (A) and digested (B) with *HindIII*.

L: 1kb DNA ladder (NEB). A: coreEF1-DMF5 vector undigested, to verify the quality of the DNA. B: coreEF1-DMF5 vector digested with *HindIII* for the cloning of the NFAT6x-GFP-DMF5 vector.

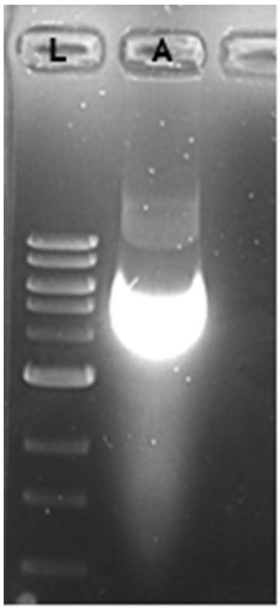

Figure 28 – Gel electrophoresis of NFAT6x-GFP-DMF5 vector.

L: 1kb DNA ladder (NEB). A: NFAT6x-GFP.DMF5 vector undigested, to verify quality of DNA. The strongest band corresponds to the supercoiled form of the vector.

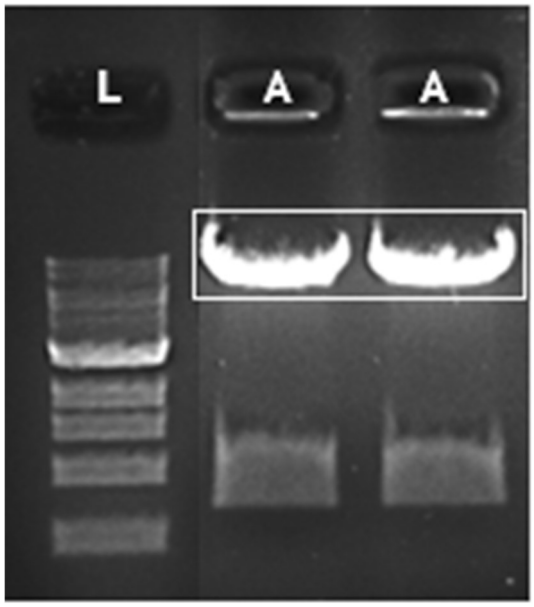

Figure 30 – Gel electrophoresis of the NFAT6x-GFP-DMF5 digested with *PspXI* and *SfiI* for IL-12 plasmid cloning.

L: 1kb DNA ladder (NEB). A: NFAT6x-GFP-DMF5 vector digested with *PspXI* and *SfiI* in Tango Buffer (Thermo Scientific ®). The selected band was gel extracted for the IL-12 plasmid cloning.

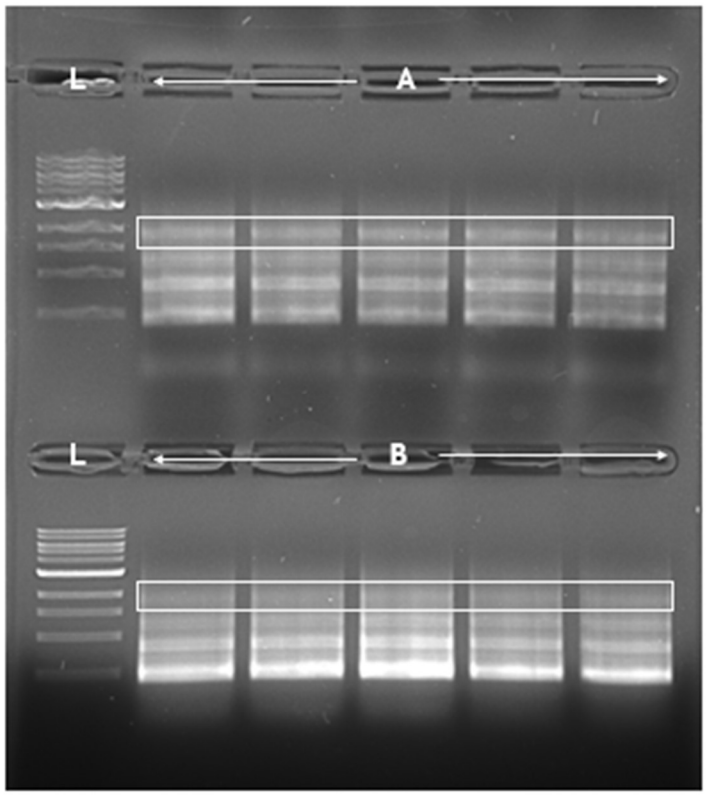

Figure 29 – Overlap Extension PCR of the IL-12 fragment for the insert of the minP (A) or the TATA (B) promoter.

L: 1kb DNA ladder (NEB). A: minP-IL-12 fragment. B: TATA-IL-12 fragment. The selected bands were extracted for the IL-12 plasmid cloning.

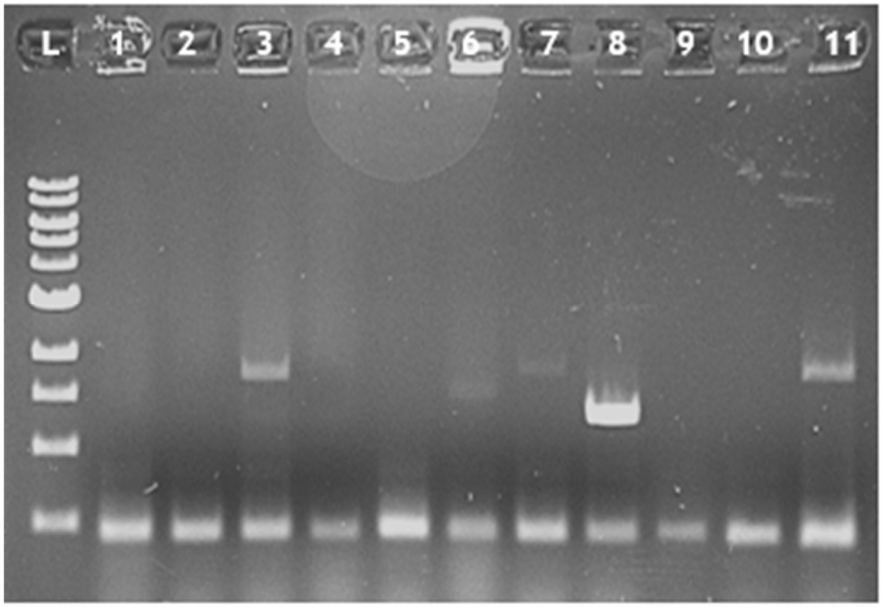

Figure 31 – Gel Electrophoresis of the Colony PCR for the NFAT6x-minP-IL-12-DMF5 clone.

L: 1kb DNA ladder (NEB). Each number from 1 to 11 represents a different colony submitted to the Colony PCR with the primers #5 and #6 (Table 1). The colonies #3, #7, #8 and #10 were cultured for DNA extraction and subsequent control digestion.

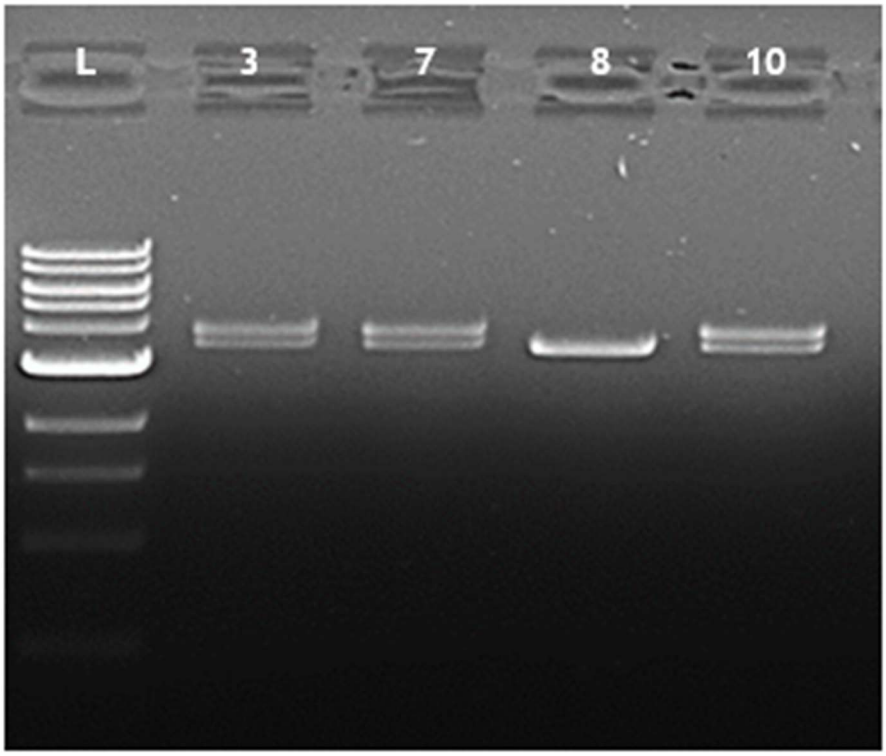

Figure 32 – Gel electrophoresis of the control digestion of the #3, #7, #8 and #10 NFAT6x-minP-IL-12-DMF5 clones.

L: 1kb DNA ladder (NEB). These clones were submitted to control digestion with *BstXI*.

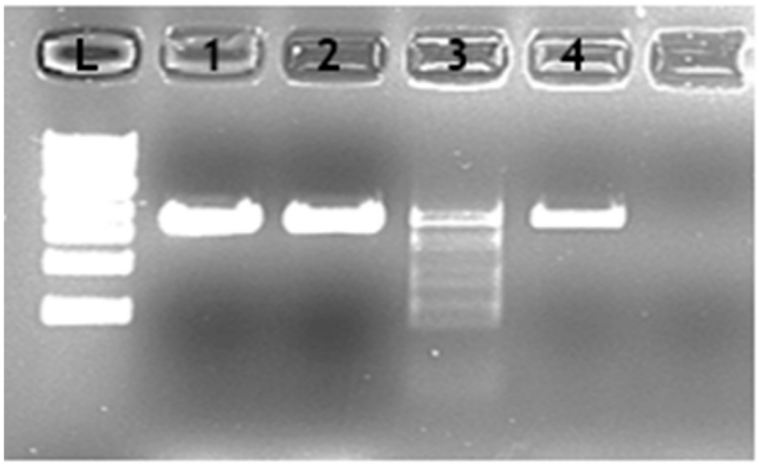

Figure 33 – Gel Electrophoresis of the Colony PCR for the NFAT6x-TATA-IL-12-DMF5 clone.

L: 1kb DNA ladder (NEB). Each number from 1 to 4 represents a different colony submitted to the Colony PCR with the primers #9 and #10. All colonies were cultured for DNA extraction and subsequent control digestion.

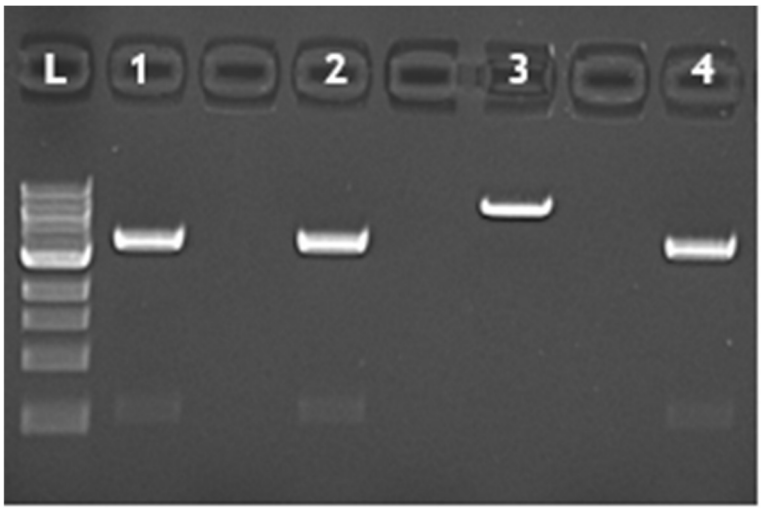

Figure 34 – Gel electrophoresis of the control digestion of the NFAT6x-TATA-IL-12-DMF5 clones.

L: 1kb DNA ladder (NEB). These clones (#1 to #4) were submitted to control digestion with *BstXI*.