THE CREATION OF LONG-LIVED HPA-1A SPECIFIC T-CELL CLONES

THE MEDICAL STUDENT RESEARCH PROGRAM

UNIVERSITY OF TROMSØ, NORWAY

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II Summary

Neonatal alloimmune thrombocytopenia (NAIT) is a disease where the fetus or newborn experience pathological low levels of blood platelets. This is a potentially fatal condition. Antibodies produced by the mother, crossing the placental barrier, targeting the β3-integrins expressed on fetal platelets, drive the disease. The antibodies are initially produced by B-cells, which are dependent on T-cells in order to mount a full response. Therefore, T-cells specific for the same antigen derived from the β3-integrin are believed to be vital in the pathogenesis of the disease. This makes these T-cells an obvious focus of research.

T-cells die in prolonged culture, and in this study three different strategies were used in an effort to obtain specific T-cell clones capable of sustained culture. This would be of great help in future research in our group. Human or murine T-cells with a known specificity were fused with the appropriate immortal partner cell line in order to create hybridoma cells. Further, human T-cells were transfected in order to express ectopic level of telomerase, in order to escape from cell senescence experienced upon multiple cell divisions, due to the shortening of their telomeres.

None of the methods described worked in full. The potential T-cells may serve as useful tools in the ongoing NAIT research in our lab. Either as standards used in the development of reagents capable of identifying specific T-cells isolated from patient sample, or in further detailing the peptide specificity driving the disease.

III Table of contents

I Acknowledgments 2

II Summary 3

III Table of contents 4

1 Introduction 5

1.1 Immunological complications in pregnancy 5

1.2 Neonatal alloimmune thrombocytopenia 6

1.3 Clinical strategies to mitigate low platelets 8

1.4 Prophylactic treatment to negate NAIT 8

1.5 T-cells orchestrate the antibody response 10

1.6 Murine model for use in NAIT-research 11

1.7 T-cell hybridomas 12

1.8 T-cells die during prolonged culture 14

1.9 Rationale and aim of the study 15

2 Material and methods 16

2.1 Cell fusion experiments 16

2.1.1 Materials 16

2.1.2 Methods 18

2.2 hTERT transfection experiments 23

2.2.1 Materials 23

2.2.2 Methods 24

3 Results 26

3.1 Fusion of human HPA-1a specific T-cell clones 26

3.2 Transfection of human T-cell clones with hTERT 29

3.3 Fusion of murine HPA-1a specific T-cell clones 31

4 Discussion 38

4.1 Human HPA-1a specific T-cells hybridomas 39

4.2 Transfection of human T-cell clones with ectopic telomerase 41

4.3 Murine HPA-1a specific T-cell hybridomas 42

5 Future perspectives 46

6 References 47

1 Introduction

1.1 Immunological complications in pregnancy

Peter B. Medawar first questioned the immunological paradox of pregnancy in 1953[1]. Earlier observations detailing the rejection of skin transplants between different human individuals, compared to autologous skin grafts surviving, made him contemplate the immunological response to chimeric antigens. During pregnancy the fetus implants in the uterus creating a semi-allogenic interface between the mother and child, since it shares half of its genes with the mother. At the time, the current immunological understanding dictated that the maternal immune system of the mother would reject the conceptus leading to an abortion. This is clearly not the case in healthy pregnancies. In his seminal paper, Medawar suggested that the lack of rejection was either due to an anatomical barrier separating fetal antigens from the mother, or due to a functional suppression of maternal lymphocytes. Although only partially correct, this has directed the present-day understanding of immunological tolerance.

Today the first hypothesis Medawar suggested has been refuted. Fetal trophoblasts are in direct contact with the maternal blood supply[2]. Although in a modified sense, as the antigen presenting molecules normally causing rapid rejection in human allografts are not expressed[3]. His latter assertion about maternal lymphocytes is considered partially correct. The maternal immune system is evidently not systemically suppressed. For instance, pregnant women mount satisfactory antibody responses to vaccines, and in some cases experience a stronger immune response than non-pregnant women[4]. Thus, it is clear that the pregnant state manipulates the maternal immune system in some regard.

No biological system is perfect. Therefore, it is to be expected that the separation and tolerance of the fetus is incomplete. If so, pathological conditions resulting from maternal

detection of fetal antigens are likely. During the last two trimesters of pregnancy, maternal antibodies are transported over the placental barrier to the fetus, facilitated by the neonatal Fc-receptor[5]. This to protect the newborn while its own immune system matures during the first months of life. Antibodies are potent molecules, known to cause symptoms in several autoimmune diseases. One way is by binding to proteins expressed in self-tissue. This marks the tissue for destruction, and the subsequent interaction with parts of the immune system involved in recognizing and counteracting foreign threats, leads to pathological symptoms. One example is idiopathic thrombocytopenic purpura (ITP), where antibodies bind to the platelets of the patient, leading to abnormal bleeding as platelet numbers decrease.

1.2 Neonatal alloimmune thrombocytopenia

Incidentally, a similar disease to ITP can occur during pregnancy, namely neonatal alloimmune thrombocytopenia (NAIT). Conversely, the difference being that the pathological state arises in the fetus, while the immune system of the mother drives the disease. The low platelet numbers experienced by the child can lead to a wide range of symptoms, from no apparent bruising, to massive intracranial bleeding (ICH) and death[6]. The disease is due to antibodies reactive to a specific protein expressed on the platelets of the fetus. This only occurs when there is a molecularly minute difference in a protein undertaking the same function in both parents. Due to this, an incompatibility transpires in the human platelet antigen (HPA) system[7]. In NAIT, the most common cause is a single nucleotide polymorphism in β3-integrin (GPIIIa). This comprise one half of the GPIIb/GPIIIa complex, forming the fibrinogen receptor on human platelets. The difference appears in an amino acid alteration in position 33, with either a leucine (L33) or proline (P33), hereby referenced as HPA-1a or HPA-1b[8].

For a child to be in danger of low platelet numbers in utero or after parturition, it has to inherit the HPA-1 antigen expressed by its father. Every person carries two alleles of one gene, which in this regards means you can be either of the HPA-1aa, HPA-1ab or HPA-1bb phenotype. The latter is called HPA-1a negative and is always the case in the mother. If not, she would be tolerant towards the HPA-1a peptide and no antibody production would appear. Consequently, the father is either HPA-1aa or HPA-1ab, and the thrombocytopenic child is always HPA-1ab. Therefore, in an incompatible pregnancy where the mother is HPA-1bb, there is a 50% risk of the child expressing the HPA-1a antigen when the father is HPA-1ab, and a 100% chance, if the father is of the HPA-1aa type.

NAIT occurs in about 0.5-1/1000 at-risk pregnancies, defined as the child being HPA-1a positive[9, 10]. Up to 20% of these present with the most feared complication being ICH and possible death or lifelong disability[11]. In total, the HPA-1bb phenotype occurs in roughly 2% of the Caucasian population. One in three also express the human leukocyte antigen HLA-DRB3*01:01. HLA-molecules bind peptide fragments of protein antigens and present these on the cell surface of antigen presenting cells (APC). The identifying end are the T-cells, which will be described in more detail later. Certain HLA-molecules bind certain peptides better, and the increased affinity is thought to mitigate stronger immune responses[12, 13]. In a Norwegian screening study, the aforementioned HLA molecule was found to be present in over 90% of women who produce anti-HPA-1a antibodies in connection with pregnancy[9]. This implies a strong correlation between the two, but only about 35% of HLA-DRB3*01:01 positive mothers get immunized while carrying an HPA-1a positive fetus, and roughly one third will deliver a child with substantial thrombocytopenia[10, 14]. In Norway, there are roughly 60.000 births each year (Statistics Norway). Of these, 2% (2000) are HPA-1a negative and at risk-pregnancies. Roughly one third (400) are born by HLA-DRB3*01:01 positive mothers,

and one third (133) of these are immunized. At last, one third give birth to significantly thrombocytopenic children, totaling roughly 44 cases in Norway each year.

1.3 Clinical strategies to mitigate low platelets

Current treatment options in NAIT are limited[15]. In addition, the natural course of the disease and the subsequent management in future pregnancies is still debated[16]. In the acute setting of very low platelet numbers with symptoms of spontaneous bleeding, random donor platelets can be given. These will transiently raise the platelet numbers of the child, but they are at risk of destruction by the same pathological process driving the disease. In addition, intravenous immunoglobulins (IVIG) can be administered, sometimes paired with immunosuppressant corticosteroids. IVIG are immunoglobulins against random specificities pooled from multiple donors. The precise mechanics for their effectiveness is still a matter of opinion, but are likely to work in multiple ways[17]. As the mother also experience the same antibodies causing NAIT, without evident thrombocytopenia, platelet concentrate prepared from maternal blood can be used in settings where random donor platelets are not enough. Obviously, the antibodies will have to be eradicated prior to administration, which is done by rigorous washing, removing the maternal plasma. Some of these treatment options are laborious and wholly antenatal, and as ICH can occur in utero, sometimes of no use. Therefore, serial cordocentesis and in utero platelet transfusions have been tried with varied success. However, this method is now largely abandoned due to the high procedure-related risks[18].

1.4 Prophylactic treatment to negate NAIT

HPA-1a specific antibodies have been measured in primigravida women[19, 20]. However, in the majority of NAIT cases occur in subsequent pregnancies, indicating that immunization takes place in connection with delivery. These findings, combined with the fact that NAIT can

occur in utero, has prompted investigation in our group of strategies for prophylactic treatments to prevent NAIT. This option can be analogue to anti-D treatment currently used in the clinic to combat hemolytic disease of the newborn (HDN)[21]. In this disease a similar state develops, except maternal antibodies targets the major D antigen on fetal red blood cells, causing acute anemia of the child due to cell lysis. Nevertheless, since the antibodies in HDN are routinely screened for in all pregnancies, seronegative women can be prevented from making antibodies. This is done by administering antibodies targeting the same antigen causing the disease. Immediately, this might appear counterintuitive. Nonetheless, antibodies effectively binding to any red blood cells entering the maternal circulation effectively mask the antigens, greatly reducing the chance of first time exposure and immunization of the maternal immune system.

Further, options to tolerate the immune system are currently also being investigated in our group, hoping to alter the antibody production in such ways that thrombocytopenia does not ensue. One way of doing so is by targeting HPA-1a-specific immune cells. Immune tolerance has been an area of ample research for years. As the finer details have been unraveled, strategies for manipulating this inherent ability of the immune system has appeared. For instance, antibodies of the IgE isotype, specific to various allergens, cause allergies. As they bind to an allergen, antibody coated mast cells degranulate, causing subsequent symptoms after the release of pro-inflammatory molecules. Today, a sublingual immunotherapy against hay fever is routinely used in the clinic, reducing allergic symptoms[22]. By providing the disease triggering antigen in an oral tablet form, the drug piggybacks on the immune modulating effects of the mucosal immune system. In effect, the antibody response is altered, through the interaction with APCs and hay fever peptide specific T-cells. IgG molecules then starts to appear, which binds to the same allergen, causing there to be no antigens left to bind

to IgE coated mast cells, ameliorating the allergic symptoms. Accordingly, it has been proven that a clinically significant manipulation of already established immune responses is possible, opening up for similar strategies in relation to NAIT.

1.5 T-cells orchestrate the antibody response

T-cells are peptide specific, and an integral part of the antibody response. In general, the immune system consists of two arms, both working in tandem to protect the human body[23]. In total, they make up all the different immune cells of the body, with several not discussed here. The innate system is the first responder to invading attackers. It is nonspecific in its defense, with an immediate maximal response. In addition, it does not improve until the next time the body encounters the same pathogen. Therefore, its efficacy over time is hampered due to microbes developing strategies to escape a relatively limited number of protective measures[24]. The second part is the adaptive immune system. In comparison to the innate system, it is capable of an immunological memory[25]. Upon mounting a response to a pathogen, the subsequent reaction will usually be both swifter and stronger, as seen in antibody production.

The main orchestrator of this chain of command is the T-cell, although working together with a number of other different immune cells. These include the professional APCs. Cells such as the dendritic cells (DC) or macrophages, aim to destroy any attackers by consuming their constituents. This in order to present bits of their proteins, split into smaller peptides, on their surface bound to HLA molecules. These in turn are recognized by T-cells by their own surface bound molecule, the T-cell receptor (TCR). This receptor is a heterodimer, made up of two transmembrane polypeptides, designated alpha and beta. In practical terms, there is a TCR capable of recognizing any peptide presented, due to gene rearrangements in the

variable regions of the two receptor chains. The average human therefore demonstrates a TCR repertoire of up to 10^7 different antigen binding sites[26].

B-cells produce antibodies, and later develop into antibody production factories called plasma cells (PC) when fully matured. But in order for that to happen, they need T-cell help[27]. The B-cells express their antigen-specific receptor, aptly named the B-cell receptor (BCR), on their cell surface. This is much like a membrane bound antibody. However, for the B-cell to start producing vast amounts of this molecule secreted in to the periphery, they need an activating signal from a T-cell specific for the same antigen. When an antigen binds to the BCR, the molecular complex formed is internalized, and the protein antigen is broken down into smaller peptides, presented on HLA-molecules. The peptide recognized by the TCR is then not necessarily from the same part of the protein recognized by the BCR. This increases the odds for a successful immune response as the two cells do not have to identify the same part of the antigen.

1.6 Murine model for use in NAIT-research

HPA-1a specific T-cell clones have been isolated from mothers giving birth to thrombocytopenic children[28, 29]. Albeit differences are prevailing between species, comparative immune responses can be studied in mice[30]. A murine model in which the human gene for HLA-DRA/DRB3*01:01 have successfully been introduced is established on a C57BL background[31]. In addition, the same mice express the human co-receptor CD4 on the relevant immune cells. This ensures a proper recognition of the human HLA molecule by transgenic T-cells. We have shown in our group that it is possible to induce DRA/DRB3*01:01-restricted T-cell responses in this specific mouse strain when immunized with relevant peptides or human platelets (data not shown). Thus, the murine cells are a

comparatively easy and suitable source of HPA-1a specific $CD4^+$ T-cells for use in *in vitro* experiments. In addition, the transgenic mouse model as described does not show spontaneous antibody production, developing NAIT upon pregnancy, as the strain does not express the HPA-1a epitope on their equivalent murine β3-integrin.

1.7 T-cell hybridomas

By appreciating the link between the T- and B-cells, we understand that the antibodies are instrumental in the development of NAIT, and that the T-cells are prerequisite for their creation. Therefore, they can serve as a tool for investigations surrounding the HPA-1a specific immune response *in vitro*. T-cells specific for HPA-1a are scarce in blood drawn from immunized women[28]. Consequently, isolation and the following cloning of T-cells from patient samples is time consuming. Additionally, T-cells stimulated and kept in culture have a limited life span, experiencing activation-induced cell death. This is an important checkpoint in avoiding overexpansion of the immune system upon exposure *in vivo*[32]. Hence, a readily available HPA-1a-specific T-cell line is desirable. With this the immune interface centered around the TCR can be studied in greater detail.

In 1973 Kohler and Millstein invented the hybridoma technology, creating immortal B-cells capable of producing monoclonal antibodies[33]. This method was soon adapted to T-cells, creating cell lines retaining their specificity after stimulation with their cognate peptide[34]. Hybridomas are made by fusing a T-cell with a tumor cell line, which does not express TCR on their surface. Further, the tumor cell line, in comparison with the T-cell, is preferably vulnerable to a specific selection medium. The resulting fused cell will then express the TCR and gain the selection resistance from the T-cell. In culture, the end result is that the hybridoma is the sole survivor, as the remaining unfused T-cells die due to the lack of

stimulation needed for healthy cell growth. The resulting cells will usually retain their ability to excrete various cytokines upon stimulation, used to screen the fused population for functional clones. However, both levels and types of cytokines produced cannot be considered physiological.

Different strategies can be employed in order to fuse two cells into one. The well beaten track is by utilizing polyethylene glycol (PEG), inducing cell agglutination and contact, subsequently disrupting the cell membrane which then recombine upon contact with another membrane end. The exact underlying mechanics are however not known[35]. By chance this will be the correct fusion partner. Undesirably, the resulting product can also be made up out of multiple cells, which rarely survives. Depending on the cell ratio, a primary cell fusion is possible, such as a T-cell fusing with one or more different T-cells, resulting in lower fusion efficiency. In total, the process is considered toxic to the cells, and subsequently you need a large number of cells in order to obtain sufficient fusion counts.

Electrofusion is another approach to fusing cells[36]. This technique has previously been utilized in our lab (non-successfully) in order to create B-cell hybridomas secreting monoclonal antibodies specific for HPA-1a. It is a three-step process, were two cells come in to contact by the use of dielectrophoresis, using a high frequency alternating current[37]. The cells line up “like a string of pearls” between the two electrodes, as the cells experience a positive and negative charge at either end. A short high voltage pulse is then applied, permeating the cell membrane, creating pores. Pores of adjacent cells then form small channels, which starts to broaden, ultimately fusing the two cells. Subsequently, the process is stabilized by an alternative voltage. Nonetheless, the fusion process is still not complete as the resultant cell contains two nuclei, making it a heterokaryon. These will only later fuse,

finishing the process. A major advantage of this process over the PEG-strategy is that you need less cells and can fine-tune the fusion parameters for each cell type, resulting in a more efficient fusion process. In one report, electrofusion has been shown to result in 3.8-33 times higher yield of hybridomas per unit number of spleen cells, compared to PEG[38].

1.8 T-cells die during prolonged culture

In 1961 Leonard Hayflick demonstrated that human fetal cells only divide between 40 and 60 times in culture[39]. Prior to this, it was believed that all cells were immortal when kept under ideal conditions, and the new discovery was later termed the Hayflick limit. It was determined that cells enter a senescent phase upon reaching the threshold, initiating apoptosis. This is due to the telomeres, small repetitive non-functional gene segments at the end of DNA, shortening with each cell division and eventually reaching a critical length[40]. The mechanism prevents replication errors due to mutations that will inevitably accumulate in cell populations dividing for prolonged periods. Thus it was no surprise when cancer cells were shown to overexpress the enzyme telomerase which maintains telomere length[41].

T-cells divide up to 25 times upon activation and continued antigen stimulation[42]. Inspired by the tumor pattern of continued cell growth, the proliferative potential of antigen specific T-cells have been extended to more than one year with over 75 generation doublings *in vitro* by selective immortalization[43]. By introducing supraphysiological levels of telomerase induced by viral vectors, the telomeres lengthen upon each successful cell division, thereby preventing apoptosis. In addition, the cells retain their physiological traits with no changes detected in proliferative potential, cytokine secretion or effector function. This in comparison to T-cell hybridoma cells, which are partial tumor cells, presenting variable phenotypes upon

successful fusion. Thus the introduction of telomerase over-expression presents a novel and better opportunity for long term culture of antigen specific T-cells.

1.9 Rationale and aim of the study

With the two different technologies discussed above in mind, the aim of this study is to produce long-lived T-cells with HPA-1a specificity. The rational being that the immune interface between T-cells and antibody producing B-cells is vital to driving the disease. Having readily available HPA-1a specific T-cell clones, to use in various experiments conducted in our lab, would be of great help in the ongoing NAIT-research. Therefore, three different strategies were employed to obtain the desired cells:

1. The fusion of human HPA-1a specific T-cell clones.
2. The over-expression of TERT in human HPA-1a specific T-cell clones.
3. The fusion of murine HPA-1a specific T-cell clones.

2 Materials and methods

2.1 Cell fusion experiments

2.1.1 Materials

Cell lines

The murine fusion partner used was BW5147.G.1.4.OUAR.1 (ATCC CRL-1588), a mutant subline of BW5147, an AKR/J mouse lymphoma, resistant to 1x10^4 M 6-thioguanine and 1x10^3 M ouabain. The human fusion partner utilized was the T-cell leukemia cell line SKW-3 (DSMZ no. ACC 53), a derivative from the KE-37 cell line. The two human HPA-1a specific T-cell clones D8T108 and D7T4 were applied as fusion targets. The cells were isolated in-house from mothers giving birth thrombocytopenic children caused by NAIT[28]. Further, the human B-lymphoblast cell line D4BL4 was previously generated in our lab by viral transformation with Epstein-Barr virus (EBV), using memory IgG+ B-cells (IgM-/IgD-/CD22+) from a donor expressing HLA-DRB3*01:01[28].

hCD4.DR3-DQ2.MHCII$^{-/-}$ Tg Mice

Transgenic mice expressing HLA-DR3, DQ2 and human CD4, in the complete absence of endogenous MHC class II, were obtained from the lab of Prof. James McCluskey, and has previously been described[31]. Mice were bred and housed at the Unit of Comparative Medicine, University of Tromsø, Norway, under pathogen-free conditions.

Cell media

Human T-cells and fusion partner were cultured in IMDM, supplemented with 10% FBS, 2% human HPA-1bb serum (Department of Laboratory Medicine, University Hospital North-Norway, Tromsø) and 1% penicillin-streptomycin. Murine cells and fusion partner were grown in DMEM/F12 GlutaMAX (Cat. #10565018, Gibco). The media (DMEM-10) was supplemented with 10% FBS, 1% penicillin-streptomycin and 50 μM 2-Mercaptoethanol. HAT- and HT-medium was prepared by adding HAT Media Supplement (50×) Hybri-Max or

HT-medium with HT Media Supplement (50×) Hybri-Max (Cat. #H0262 and #H0137, Sigma-Aldrich) to the aforementioned cell media. Final working concentrations were 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine.

Peptides and cell probes

Synthetic peptides were synthesized by Eurogentec, Seraing, Belgium. The 20-mer L33 peptide (VSPMCAWCSDEALPLGSPRC$_{19\text{-}38}$), is derived from the β3-integrin expressed on human platelets. LolP1 (ESWGAVWRIDTPDKLTGPFT$_{191\text{-}210}$), is a 20-mer peptide derived from rye grass pollen. Both anchor to HLA-DRB3*0101 at the underlined amino acid positions[44]. For phytohemagglutinin (PHA) stimulation, PHA-L (Sigma-Aldrich) was used. The monoclonal antibodies used in human hybridoma characterization assays were Anti-TCR PAN α/β-PE (PN A39499, Beckman Coulter), Anti-TCR-V-β9 (PN IM2003, Beckman Coulter), Anti-TCR-Vβ2-PE (PN IM2213) and Anti-hCD4-PE-Alexa Fluor 610 (MHCD0422, Invitrogen). For murine experiments Anti-hCD4-V500 (Cat. #560768, BD Biosciences), Anti-mCD3-PerCP-Cy5.5 (Cat. #551163, BD Pharmingen) and Anti-HLA-DR-PE-Cy7 (Cat. #307616, BioLegend) were used. Further, the non-toxic cell dye CellTrace™ CFSE (Cat. #C34554, ThermoFisher) was used to stain human T-cell clones prior to fusion.

HLA-conjugated beads

Water-soluble biotinylated HLA-DRB3*01:01 molecules, engineered with tethered peptides, have previously been produced in our group using bacolovirus infected insect cells. Either the aforementioned HPA-1a derived peptide L33 was bound to antigen-presenting grove of the HLA-molecule, or the class II-associated invariant chain peptide (CLIP) peptide, which normally resides in the HLA class II peptide grove until fully assembled *in vivo*. The peptide-bound HLA-molecules where then linked to plastic beads, creating multimers, expressing several HLA-molecules on their surface.

2.1.2 Methods

Transfusion of peptide-pulsed DCs to syngeneic mice

In order to immunize the transgenic mice described herein, transfer of syngeneic peptide-pulsed DCs were utilized to elicit a stronger immune response, compared to a peptide based vaccine with adjuvant[45]. In brief, bone marrow was harvested from the femur of two euthanized mice and prepared in a single cell suspension. Red blood cells were lysed using an NH_4Cl-buffer, before the cells were washed once and 2x10^6 cells plated in 100 mm bacteriological petri dishes and incubated at 37°C in a 7.5% CO_2 humidified atmosphere. Thereafter, 10 ml of DMEM-10 together with 1 ng/mL of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF, cat. #554586, BD Pharmingen) was added. This was repeated on day 3. On day 6 and 8, 10 mL of cell media was carefully removed, before the previous step was repeated. On day 10 non-adherent cells were harvested and plated in 24-well plates in media containing 5 ng/mL of lipopolysaccharide (LPS) and 5 μM of L33 peptide. The cells were incubated for 24 hours before the cells were carefully resuspended using a cell scraper and washed twice in PBS.

Maturation of bone marrow derived murine dendritic cells

The peptide-pulsed DCs were kept in 100 μL of PBS at a concentration of 2x10^6 cells prior and prepared on the same day. The cell solution was then transfused back into two mice via the lateral tale vein, with a boost following the same procedure on day 14. On day 19 the mice were sacrificed and the spleens harvested in order to gain access to any primed T-cells.

In vitro expansion of murine HPA-1a specific T-cells

Splenocytes were prepared in single cell suspension using a 100 μm nylon mesh cell strainer (Fisher Scientific). Red blood cells were removed using a NH_4Cl-lysis buffer. The T-cell population was thereafter expanded by stimulation with 5 μM of L33 peptide, together with 10 U/mL human recombinant IL-2.

T-cell hybridoma fusion using PEG

Cell fusion between murine T-cells and the fusion partner BW5147 was carried out using 50% polyethylene glycol/DMSO (PEG/DMSO HybriMax, P7306, Sigma-Aldrich). Both the T-cell clone and fusion partner were spun over density gradient medium (Lymphoprep, Cat. #07801, STEMCELL Technologies), washed and counted prior to fusion. Afterwards two more wash steps were performed using IMDM to remove serum. The cells were then pooled together in a total volume of 100 µL. Fusion was performed in a water bath at 37º C, dripping 100 µL of PEG using a syringe equipped with a 25 G needle tip, into the cell solution over 45 seconds. This was then followed by 100 µL of pre-warmed IMDM dropwise over 30 seconds, before a 1-minute incubation step. The aforementioned step was then repeated 3 times, with doubling of IMDM volumes and incubation time for each round. Finally, cells were suspended in a total volume of 8 mL of IMDM, spun and resuspended in culture medium. The fused cells were plated using limited dilution at a predicted 0.5 hybridomas per well based on average fusion rate. Any growth in culture wells can therefore be considered clonal. Selection was accomplished in HAT-medium for 14 days, before diluting in HT-medium in preparation for transfer to normal cell medium.

T-cell hybridization using electrofusion

The electrofusion system used was an Eppendorf Multiporator, equipped with the electrofusion module (Eppendorf AG, Germany). Cells were washed once in culture medium and twice in ioosmolar buffer (Order #4308 070.536, Eppendorf AG) prior to hybridization. Fusion volume was 500 µL in a hypoosmolar buffer (Order #4308 070.528, Eppendorf AG), in order to enlarge the cell surface facilitating cell fusion. A total of $1x10^6$ cells in a 1:1 ratio were fused. After incubating for 10 minutes in the hypoosmolar buffer, cells were aligned with a 5.0 V 30 seconds alignment period, pulsed 3 times with 30 V for 30 microseconds

each for fusion, before a post-alignment at 5.0 V for 30 seconds was applied. Subsequently cells were washed in culture medium before plated in multi-well plates for culture selection.

Assesment of succesfull T-cell hybridoma clones

In order to analyze surface molecules on cells in a suspension, the most common method is by the use of monoclonal antibodies linked to a fluorescent dye. Once the cells are labeled, they pass through a detection chamber in a single file stream, in a flow cytometer (FACSCanto II, BD Biosciences). Here the cells are lit by a laser emitting a certain wavelength of light. This excites the fluorochrome linked to the antibody, emitting a signal with a different wavelength, which in turn is processed by the flow cytometry apparatus. Based on how the light scatters, cell size and granularity is also assessed, making it possible to examine cell populations in high detail using various plots.

MACS bead selection

In order to isolate and separate cells, indirect magnetic cell labeling using anti-fluorochrome beads were used (MACS Anti-PE MicroBeads, Cat. #120-00-294, Miltenyi Biotec). Cells were first probed using monoclonal antibodies conjugated to a fluorochrome, such as phycoerythrin (PE), targeting the desired cell surface markers. Anti-PE beads were then added, and the labeled cells passed through a separation column placed in a magnet (MACS Separation Columns, Cat. #130-042-401, Miltenyi Biotec). The stained population was then retained, while the remaining cells passed through. The separation column is then removed from the magnet and washed. The previously retained cells are then released in to the eluate. The two cell populations can then be cultured separately for further analysis, and the method repeated if needed.

ELISPOT

The enzyme-linked immunospot (ELISPOT) assay can monitor cell responses, with the ability to identify single positive cells grown in culture upon stimulation with the correct

antigen. This is done by the capture of the desired secreted cytokine on an antigen coated membrane, later detected with an antibody linked to an enzyme, such as horse-radish peroxidase or alkaline phosphatase. A substrate is then added, creating a colored spot in the bottom of the well, marking a reactive cell.

In order to detect IL-2 secreting T-cells, a putative marker for antigen-stimulated T-cells, MultiScreen HTS 96-well microtiter plates with nitrocellulose membrane (Cat. #MSIPS4510, Merck Millipore) were wet with 35% ethanol (30 μL/well) and immediately washed 3 times using PBS (200 μL/well). Following 25 μL of capture anti-IL-2 antibody solution (BD Biosciences) was added to each well and incubated overnight at 4°C wrapped in Parafilm M (Bemis NA). Membranes were then washed 6 times with PBS and subsequently blocked with culture medium for 2 h at 37°C in a 7.5% CO_2 humidified atmosphere. Each well received $1x10^5$ T-cells and $2x10^4$ peptide pulsed APCs, before a final 36-hour incubation step at 37°C, 7.5% CO_2. In order to prepare the plates for analysis, the plates were washed 6 times with PBS/0.01% Tween 20 and once with PBS (200 μL/well). Then 50 μL of 1 μg/mL biotinylated anti-murine IL-2 (BD Biosciences) was added to each well and incubated for 2 hours at 37°C, 7.5% CO_2. The plates were then washed 6 times PBS/0.01% Tween 20 and once with PBS. This in order to remove any remaining unbound antibodies. Streptavidin-alkaline phosphatase conjugate (Cat. # S921, Thermo Fisher Scientific), diluted 1:10 000 with PBS, was then added in 50 μL aliquots to each well before incubating for 45 minutes in room temperature. Another wash step was then performed, 3 times with PBS/0.01% Tween 20 and 4 times with PBS. At last, 50 μL of the alkaline phosphatase substrate BCIP-NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; Moss Biotech) was added to each well and the plates were incubated for 15 minutes in the dark. Plates were then washed in tap water and

dried overnight, before scanned using an Immunospot plate reader (Cellular Technology Ltd.).

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a technique designed for quantifying various substances, including cytokines released by immune cells. It is achieved by the use of ELISA-plates coated with an antibody specific for the antigen to be detected. After the antigen binds, a complex is formed by adding a second enzyme-linked antibody, which upon exposure to a substrate produce a measurable product.

Here ELISA plates were coated with an anti-murine IL-2 capture antibody, a part of an IL-2 antibody kit (BD OptEIA™ Set Mouse IL-2, BD Biosciences, San Diego, CA). The capture antibody was first diluted 1:250 in a coating buffer. Thereafter 100 µL was transferred to each well, before the plates were sealed and incubated overnight at 4°C. Plates where then washed 3 times using an automatic plate washer (ELx405 Microplate Washer, BioTek) (300 µL/well). The plates were then blocked using PBS with 10% FBS (µL/well) at room temperature for 1 hour. Afterwards the previous wash step was repeated. Then 100 µL of cell supernatant harvested from stimulated T-cell hybridoma cultures was added to each well. Plates were again sealed and incubated for 2 hours at room temperature. Another wash step was then performed, increasing to 5 cycles. The detection antibody was then diluted 1:500 in PBS with 10% FBS, and the enzyme reagent streptavidin-horseradish peroxidase conjugate was diluted 1:250 using the detection antibody mixture. The final solution was then added to each the plate (100 µL/well). The plates were again sealed and incubated for 1 hour at room temperature. Continually, a wash step was performed, totaling 7 times. Then 100 µL of the substrate solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (Cat. #T0440, Sigma-Aldrich) was added to each well and incubated in the dark for 30 minutes at

room temperature. The reaction was subsequently stopped by adding 50 μL of 1 M H_3PO_4. Plates where then read at 450 nm within 30 minutes, with λ correction 570 nm, using the Mulstiskan Ascent (Thermo Fisher Scientific) absorbance plate reader.

2.2 hTERT transfection experiments

2.2.1 Materials

Cell lines

In addition to random human donor T-cells, the human T-cell leukemia cell line SKW-3 (DSMZ no. ACC53) and the HPA-1a specific T-cell clone D4T7 were utilized in transfection experiments. The latter isolated from a immunized pregnant woman who gave birth to a child with thrombocytopenia, previously characterized in our lab[28]. For viral packaging, a human embryonic kidney (HEK-293)-derived cell line AmphoPack-293 (Cat. #631505, Clontech Laboratories) was used.

Cell media

AmphoPack-293 cells were cultured in DMEM supplemented with 10% FBS and 1% pencillin-streptomycin. Human T-cell clones and SKW-3 were grown in media as described earlier for fusion experiments.

Retroviral vector plasmids

Two different vectors with gene inserts for human telomerase reverse transcriptase (hTERT) and the fluorescent marker green fluorescent protein (GFP), were used in transfection experiments. pBABEhygroHigheGFPhTERT[46] (Addgene plasmid #28169) and xlox(GFP)hTERT[47] (Addgene plasmid #69809) were purchased from Addgene (Cambridge, MA). Bacteria producing plasmids were grown at optimal conditions and the plasmids isolated using the PureYield Plasmid Midipred System (Promega).

2.2.2 Methods

Isolation of human T-cells

Mononuclear cells were isolated from buffy coats prepared from random human donors collected from healthy volunteers at the Blood Bank at the University Hospital of North Norway. Cells where isolated by density gradient centrifugation using Lymphoprep (Cat. #07801, STEMCELL Technologies). T-cells were subsequently enriched from the isolated cell population by magnetic selection using the Human CD4 T Lymphocyte Enrichment Set-DM (BD Imag) per the instructions of the manufacturer. Cell proliferation was induced with 0.2 μg/mL anti-CD3e (Cat. #MHCD0300-4, ThermoFisher Scientific) and 500 ng/mL IL-15 (Cat. #554712, BD Bioscienses) monoclonal antibodies the day prior to transfection, as the murine leukemia (MuLV) based retroviral vectors used only infect dividing cells.

Retrovirus production

Packaging of the plasmids for retroviral infection were done using AmphoPack-293 cells. The cell line is designed for rapid production of high-titer replication-incompetent retrovirus, either by stable or transient transfection. Infectious particles produced can infect a wide range of mammalian cell types, due to expressing an amphotropic envelope (4070A). Plasmid DNA was transfected in to the packaging cell lines using the non-liposomal reagent FuGENE-6 (Promega), as instructed by the manufacturer. Viral particles were later concentrated by centrifuging filtered supernatant from transfected AmphoPack-293 cells in Amicon Ultra-15 100k filter tubes (Merck Millipore Ltd., Ireland), as previously described[48].

Viral transfection

To facilitate the introduction of the retroviral particles carrying genes for hTERT and the fluorescent protein marker, the transduction reagent ViroMag R/L (OZ Biosciences, France) was employed. These magnetic nanoparticles bind to the viruses. By incubating the cells overnight on a magnetic plate, they help quickly concentrating the particles on to the cell

surface increasing and facilitating cell entry. As the virus dies outside of a suitable host, this potentially increases viral transductions *in vitro*.

Identification of successfully transduced T-cells

In order to assess whether or not the T-cells were successfully transduced the transduced cells were screened using a flow cytometer (FACSCanto II, BD Biosciences), using the FL1 laser known to fluoresce GFP molecules. The cells were then gated for a positive signal compared to untransfected cells of equal type, indicating a successful transduction.

3 Results

3.1 Fusion of human HPA-1a specific T-cell clones

In order to obtain T-cell clones capable of continued growth in culture, the human T-cell leukemia cell line SKW-3 was fused with HPA-1a specific T-cells. Two strategies were employed in order to merge the cells. Electrofusion was tried first, as it is both efficient and fast, providing good hybrid yields. Further it requires less cells, preserves better control over fusion parameters and can be viewed in real time under a microscope. In addition, fusion by PEG was attempted, as it is a tried-and-tested and well-documented technique in our lab.

The electrofusion was carried out combining the fusion partner SKW-3 and the in-house T-cell clone D8T108 with a known specificity for HPA-1a. The D8T108 cells were probed with CFSE prior to fusion, as any positive cells post-fusion would either be T-cells or T-cell hybrids. The cells were then washed and prepared in the appropriate buffer with the correct electrochemical properties. The current induces a dipole within the cells, forcing the cells to line up next to each other in a line between the two poles (Figure 1 and Figure 2). A direct current is applied in short bursts, forcing the cell membranes to join, creating cell hybrids.

There were no successfully growing hybridoma cells after hybridization using electrofusion. Further, no cells were positive for a pan-specific TCR antibody, or expressing the TCR variable chain β2 or β9 from the D8T108 T-cell clone (data not shown).

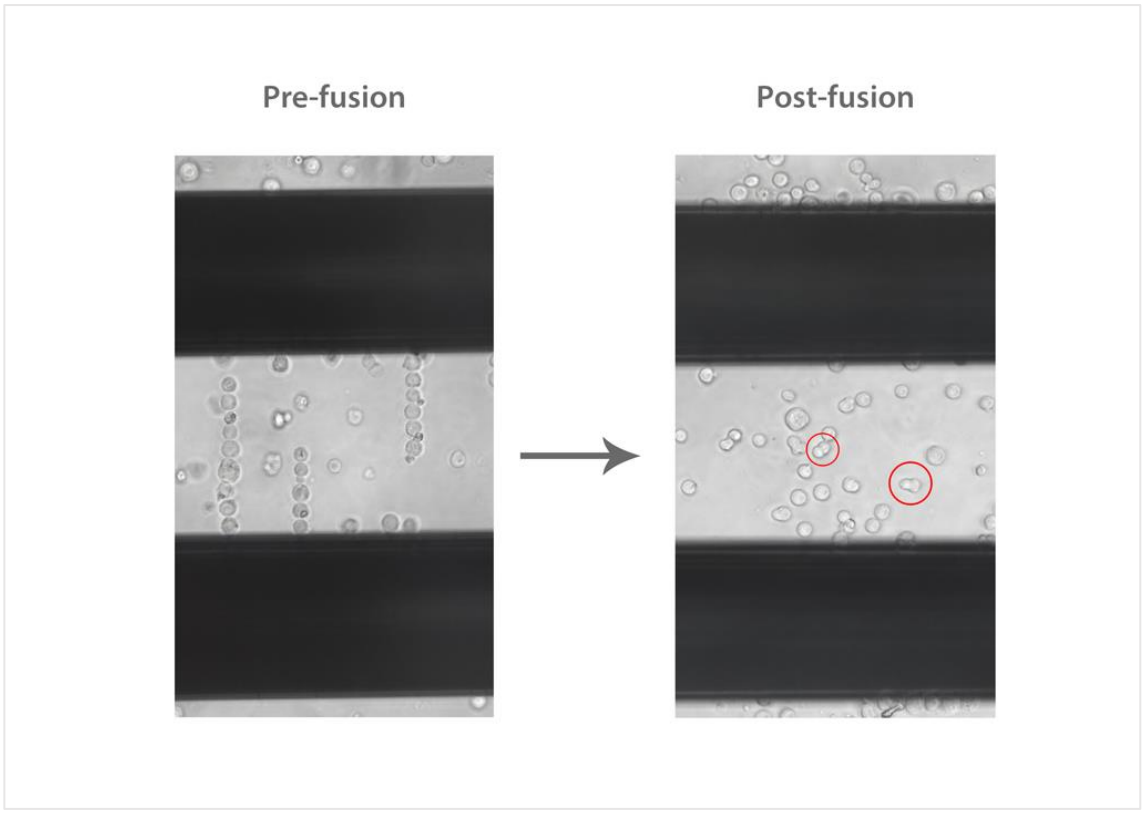

Figure 1: Left: Cells are lined up between two electrodes prior to fusion. Right: Cells can be seen post-fusion, with two joined cells signifying a potential hybridoma, here show within the red rings.

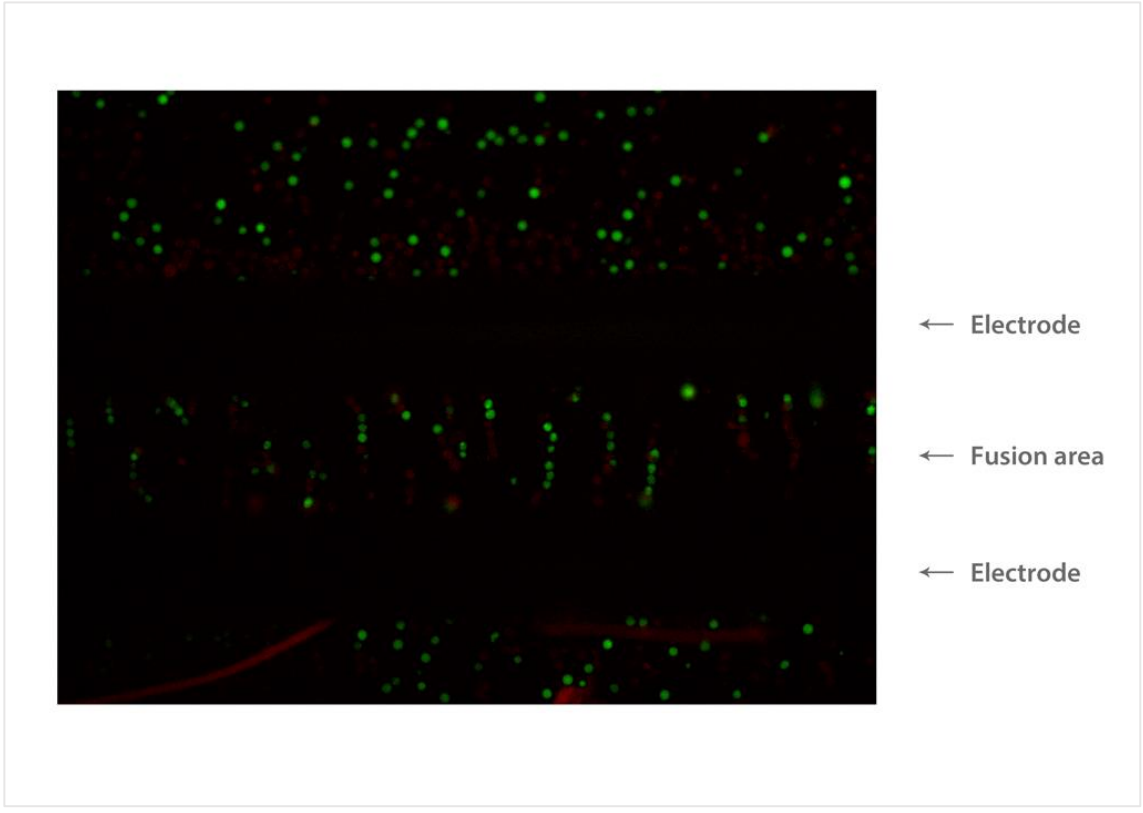

Figure 2: Here, T-cells are probed with a green fluorescent marker, while SKW-3 cells are stained red. The picture is taken after a fusion event.

Since no hybridoma cell lines were derived by electrofusion, we moved on to use the more traditional method with PEG. The HPA-1a specific T-cell clone D7T4 was pooled together with the SKW-3 fusion partner in a 1:3 ratio. Cells were then washed and resuspended, before the addition of PEG and subsequent dilution with serum-free medium to fuse the cells, as detailed in the methods section. Then, a final wash step was performed and cells cultured prior to selection. In order to identify any fused cells, cells were labelled with monoclonal antibodies targeting surface TCR and CD4. Both are known to be expressed by human T-cells, while SKW-3 cells do not express a TCR and express relatively low amounts of CD4. To enrich for potential fused cells, magnetic beads reactive with the PE-fluorochrome conjugated to the prior antibodies were subsequently added, and the cells passed through a magnetic column. Cells were then split in two populations, "adherent" and "flow-through", and cultured for a week observing growth patterns. The process was then repeated, and the two cell populations analyzed using flow cytometry for the expression T-cell specific markers, signifying successful hybridoma growth (Figure 3).

There were significantly less cells in the adherent population after one week of growth, compared to the flow-through, when observed under the microscope. Further no cells were labeled with either anti-CD4 or anti-pan-TCR upon analysis with flow cytometry (Figure 3).

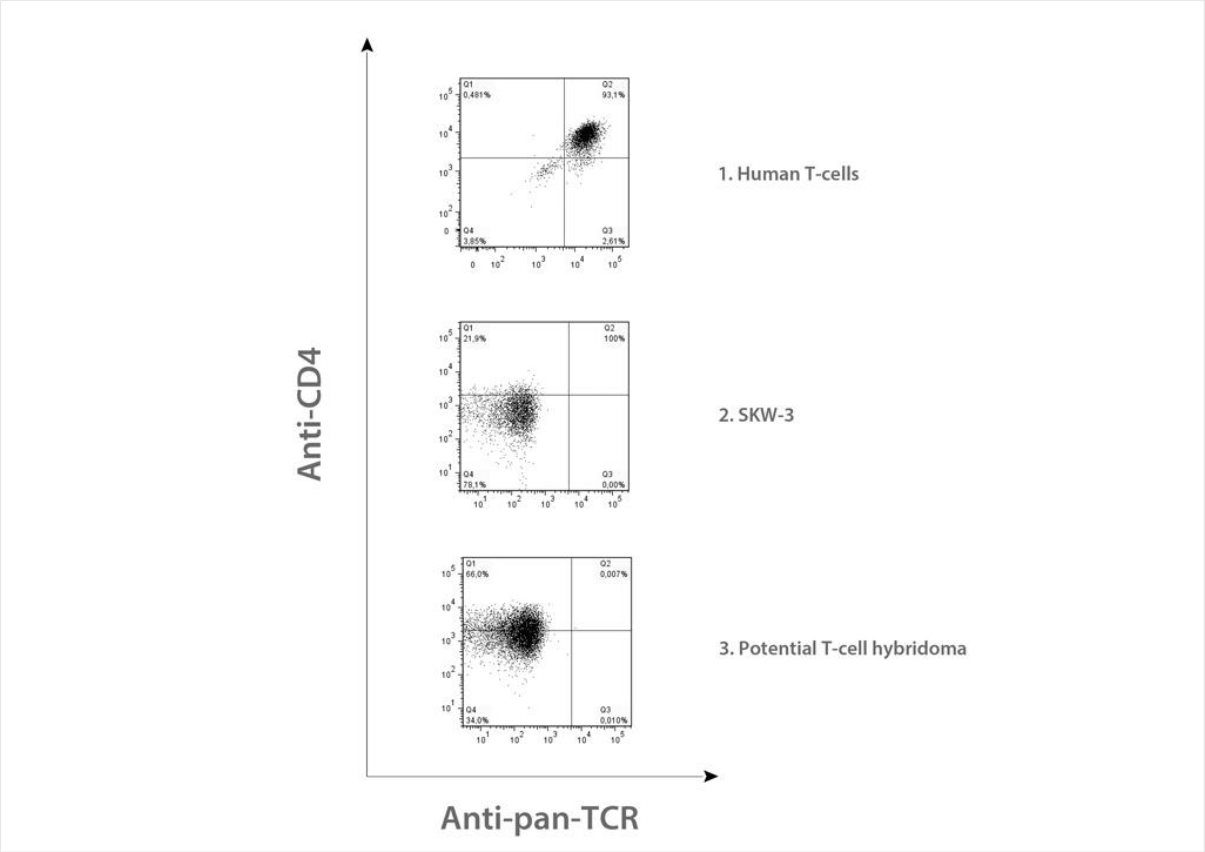

Figure 3: Cells from dusion experiment do not express surface TCR or CD4. Flow cytometry result after electrofusion, representative for severaldusion experiments. No TCR+/CD4+ T-cell hybridoma cells are seen in the upper right quadrant in the potential T-cell hybridoma population.

In total, no hybridoma cell lines were derived by fusion of human HPA-1a specific T-cell clones with the human fusion partner cell line SKW-3.

3.2 Transfection of human T-cell clones with hTERT

T-cells renew their telomeres upon activation in order to sustain several rounds of cell division. However, there is an upper limit to this process. It is however possible to avoid cell senescence by overexpressing human telomerase reverse transcriptase (hTERT), keeping the telomeres at the appropriate length, avoiding apoptosis. The result is T-cells with increased division potential, making it easier to maintain cells for use in experimentation and assays compared to non-transfected T-cells. Using viral transfection this strategy was employed as the previous fusion experiments had proved unproductive.

A plasmid vector (Figure 4) containing the gene for hTERT, was produced in *E. coli* and isolated in bulk, before being transfected into the packaging cell line AmphoPack-293. This created replication-incompetent retroviral particles, later isolated and concentrated by filtration, then introduced into the target cells with the help of magnetic nanoparticles bound to the virus. In addition to hTERT, the vector introduces a green fluorescent protein (GFP) marker. Cells can therefore easily be screened for successful transfections and further cell divisions, by the use of flow cytometry.

Human T-cells isolated from random donors, the human T-cell leukemia cell line SKW-3 and the HPA-1a specific T-cell clone D7T4 were all used in transfection experiments. Two different plasmid vectors containing hTERT and GFP gene inserts were tried. No cells were found to emit a positive signal upon analysis by flow cytometry (Figure 5). Therefore, no cells expressed the green fluorescent protein in combination with hTERT.

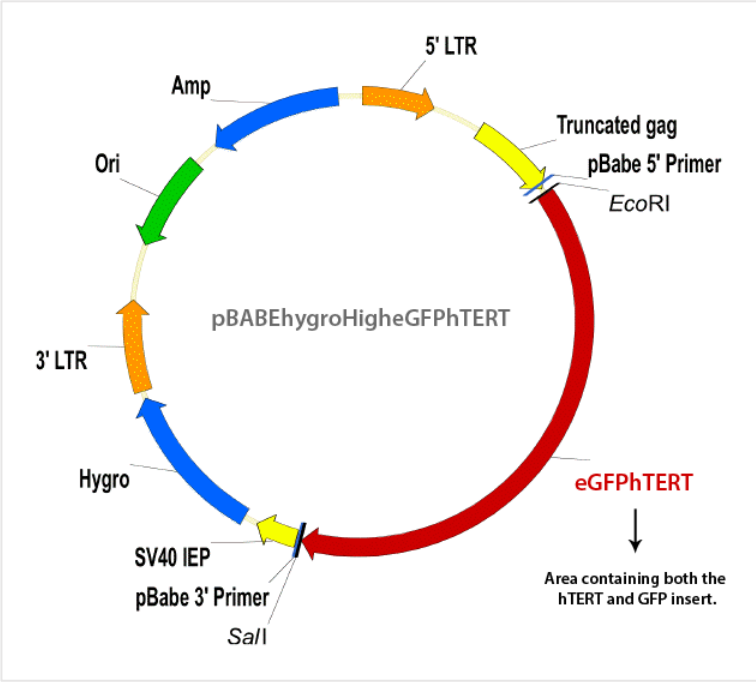

Figure 4: A schematic representation of one of the two plasmid vectors used in transfection experiments.

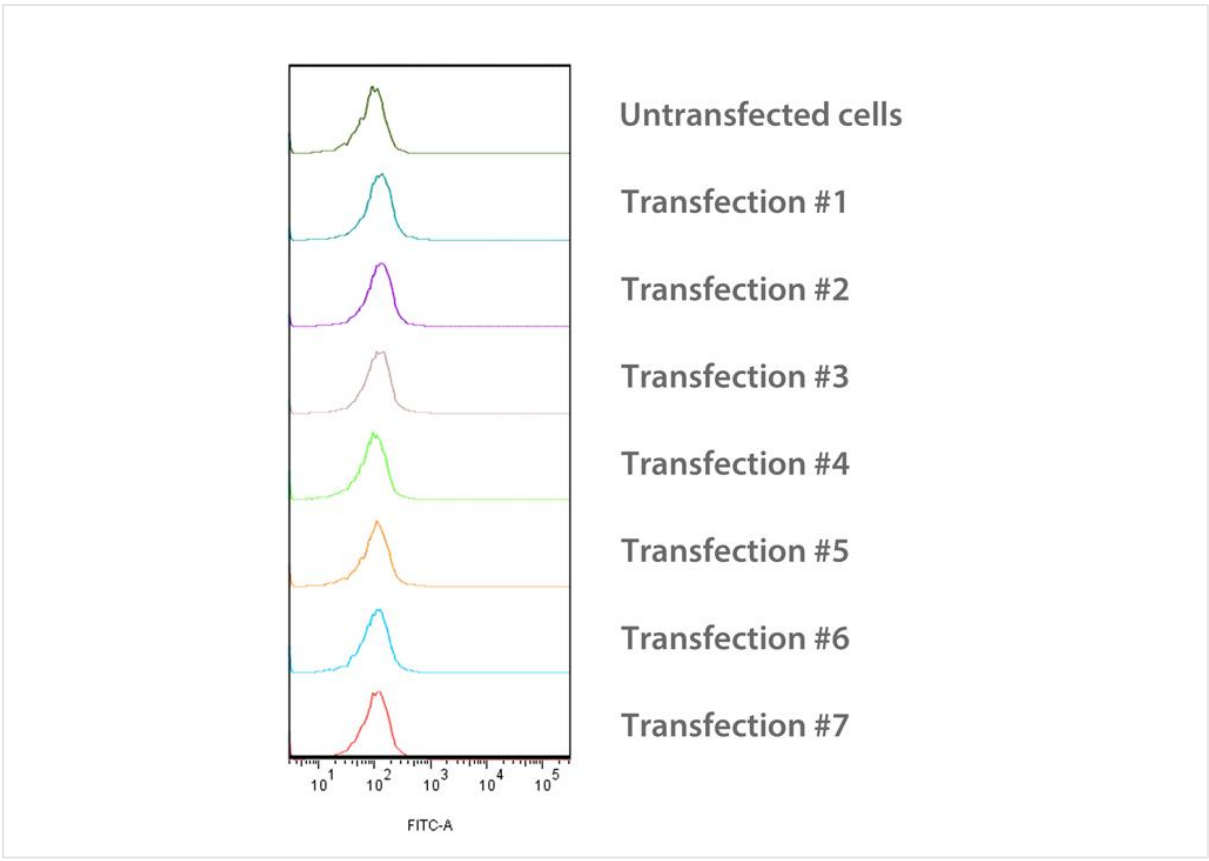

Figure 5: Transfected cells did not express GFP. Flow cytometry analysis of hTERT transfected T-cells, both HPA-1a specific T-cell clones, random donor T-cells and the SKW-3 T-cell leukemia cell line. Analysis of T-cells following multiple different transfection experiments are shown. Cells were analyzed at various time points 1-14 days after transfection. No positive control was available, however the transfected cells are expected to fluoresce a multitude times more than untransfected cells.

3.3 Fusion of murine HPA-1a specific T-cell clones

Since the creation of human T-cell hybrids was unsuccessful, a third option was explored fusing murine T-cells. A transgene mouse model has recently been established in our group. The mice express human HLA-DRB3*01:01, together with human CD4. Combined this enables the study of immune responses on the T-cell receptor level analog to those in humans. Therefore, by immunizing the mice with HPA-1a derived peptides and harvesting the peptide specific T-cells, hybrid T-cell clones can be produced upon fusion with a mouse lymphoma cell line. This technique has a well proven track record, with a large number of published reports detailing peptide specific T-cell hybridoma cell lines[49, 50, 51].

To activate HLA-DRB3*01:01-restricted HPA-1a-specific T cell responses in our transgenic mice, bone marrow derived dendritic cells (DC) were isolated and matured in tissue culture. Further, mature DCs were pulsed with HPA-1a peptide (L33) and used to immunize transgenic mice. Both the two previously mentioned steps were done as described in the methods section. Three weeks following immunization, spleen cells from immunized mice were fused with a mouse myeloma cell line.

Cells were fused using PEG and selected in HAT-medium (as detailed in the methods section), with only potential hybridomas surviving. Ten days after fusion, proliferation was observed by light microscopy in roughly one third of the wells. In wells with growth, cells were analyzed by flow cytometry for the expression of human CD4 (hCD4) and the murine CD3-complex (mCD3), with double positive cells identifying potential functional hybridoma clones; non-fused mouse spleen cells were presumed not to proliferate extensively in the absence of specific antigen-stimulation. The majority of cells analyzed expressed hCD4 (Figure 6). The hCD4 is a co-receptor assisting the T-cells while communicating with endogenous APCs. As murine CD4 analogue binds poorly to human HLA class II, the human version is thought to be required for efficient T-cell response in HLA-transgenic mice[52]. The CD3-complex is a molecular machinery which associates with the TCR. Without it, no stimulation is to be expected and is therefore vital for a useful hybridoma clone.

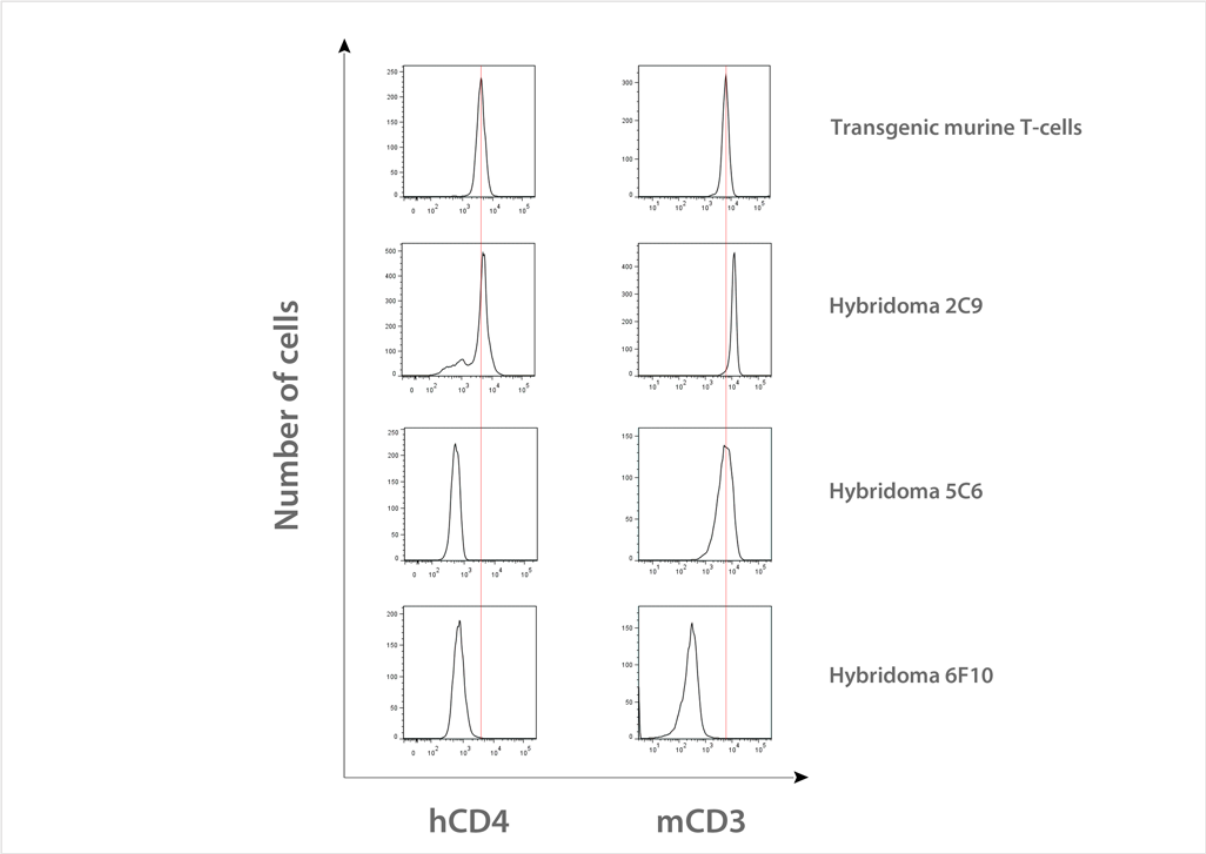

Figure 6: Three of the murine T-cell hybridomas screened for hCD4 and mCD3, showing the three expression patterns 1. hCD4+/mCD3+, 2. hCD4-/mCD3+ and 3. hCD4-/mCD3-.

The next step in the characterization of the T-cell hybridoma lines was to demonstrate epitope specificity. Table 1 shows the response to the peptides L33 and LolP1, presented by syngeneic bone marrow derived DCs matured and pulsed *in vitro*. The cells were co-cultered for 36 hours and the cytokine IL-2 secreted was measured in an ELISPOT assay.

ELISPOT IL-2

L33	LolP1	Negative	PHA	No. of clones
+	+	-	+	10
+	+	-	-	31
-	-	-	+	7
+	+	+	+	5
-	-	-	-	13

Table 1: In total 66 clones were analyzed in an ELISPOT assay after peptide stimulation. A total of 5 response patterns were found in IL-2 secretion, with none being strictly specific for L33 peptide. "+" indicates positive response with cells secreting IL-2 (spots). "-" indicates that no, or only few, IL-2 secreting cells were detected. "Negative": Hybridoma cells stimulated with DC without peptide. PHA: Hybridoma cells stimulated with the T-cell mitogen phytohaemagglutinin (5 µg/mL).

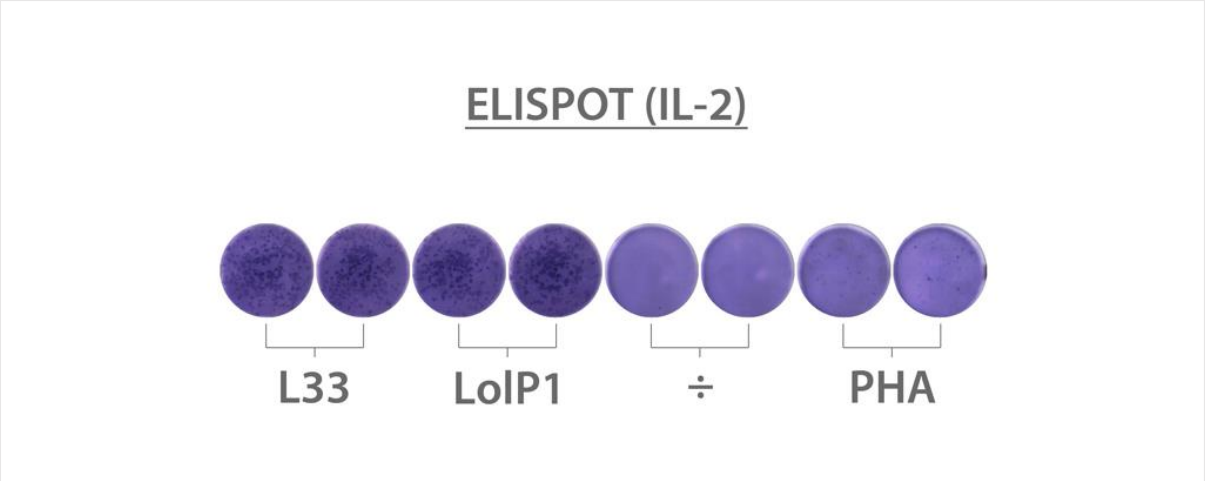

Figure 7: A typical representation of an ELISPOT result. The clone is stimulated by both L33 and LolP1 and PHA. Each spot represents a single cell activated to secrete IL-2. About 1000 hybridoma cells were added to each well.

In order to test to whether or not there was a difference in activation patterns when stimulated by a different type of APC, the human B-cell lymphoblast cell line D4BL4 expressing HLA-DRB3*01:01 was utilized. The D4BL4 cell line is negative for endogenous β3-integrin, in order to minimize any potential background in stimulation assays. The APCs were peptide pulsed in advance and co-cultured with the hybridoma clones. Next, the release of IL-2 in the cell supernatant was measured using an ELISA assay. This in order to quantify the cytokine release upon antigen stimulation, in order to explore whether there was a difference in the magnitude between stimulation with the two peptides L33 and LolP1. Also by ELISA, several different patterns of reactivity were observed (Table 2). Whereas most hybridomas responded to either both or none of the peptides, two hybridoma clones responded weakly to L33 peptide, but not LolP1 peptide, representing possible antigen-specific clones. Further, we compared the magnitude of IL-2 secretion following stimulation with L33 and LolP1 peptides since it is possible that potential HPA-1a-specific hybridomas would respond to antigen-specific stimulation by higher magnitude of IL-2 secretion, which we can measure by ELISA, but not ELISPOT. Figure 8 shows comparisons of IL-2 secretion in response to different stimuli for selected clones. No obvious L33 specificity was observed, although

clone 4G8 (Figure 8) and 5C4 (data not shown) appeared to respond slightly better to L33 compared to LolP1.

ELISA (IL-2)				
L33	**LolP1**	**Negative**	**PHA**	**No. of clones**
+	+	-	+	14
+	+	-	+/-	8
+	+	-	-	10
+	+	+	+	5
+	+	+/-	+	15
-	-	-	-	9
-	-	+/-	-	4
+/-	-	-	+	2

Table 2: In total 96 clones were analyzed in an ELISA assay after peptide stimulation. A total of 28 different response patterns were found in IL-2 secretion, not all are detailed here. This large number was due to certain duplicates analyzed being slightly positive in one of the two wells, measured as at least 3 times the optical density of the blank controls. These are represented with +/-. None of the clones were specific for L33, however 2 clones were slightly positive in one of the wells stimulated with L33.

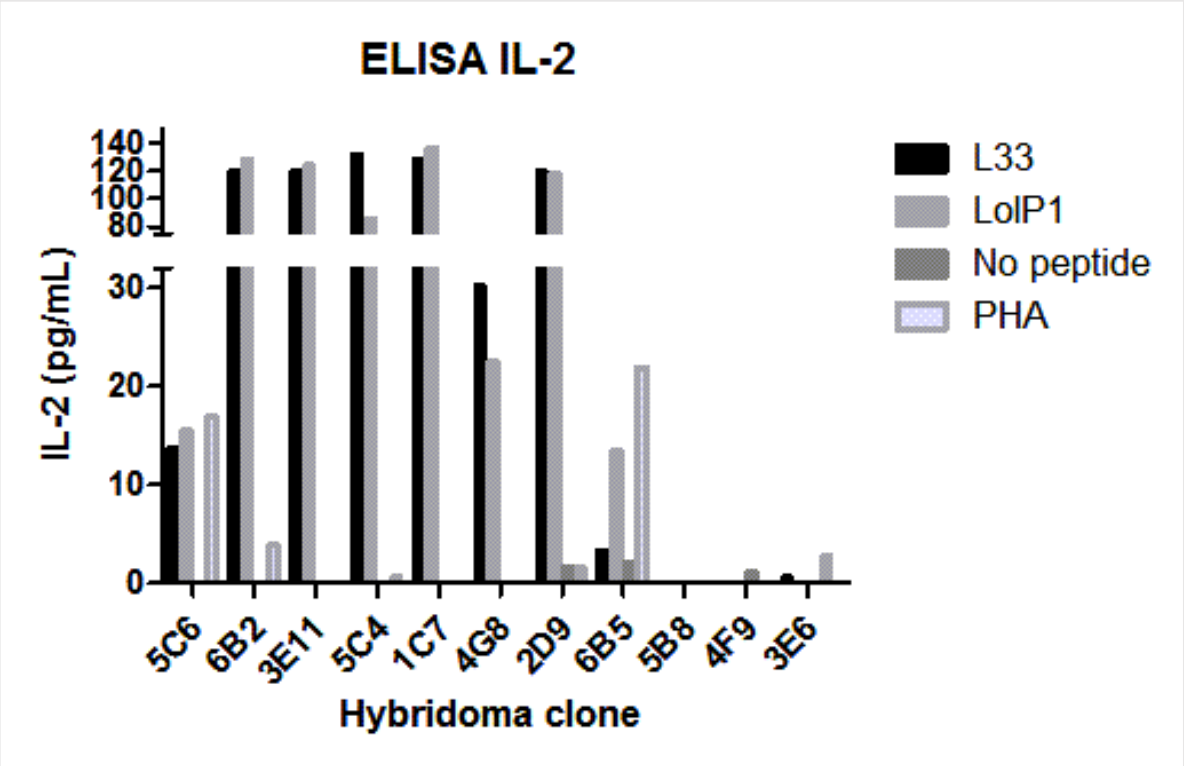

Figure 8: This chart details the cytokine release of a select number of clones upon stimulation with antigen and the pan-specific stimulator PHA, together with a negative control where hybridoma cells were co-cultured with D4BL4 cells alone.

Further a selection of hybridoma clones with unknown specificity was co-cultured with recombinant HLA-DRB3*01:01 molecules conjugated to beads, presenting either L33 or the CLIP-peptide. This to investigate if the cells were stimulated solely by TCR/HLA interaction. This proved futile, as none of the clones incubated together with beads poised a positive signal in an ELISPOT assay (data not shown).

It has previously been shown in our group that L33 specific human T-cell clones express HLA class II molecules, capable of presenting HPA-1a specific antigens[28]. This is hypothesized to help T-cell growth upon activation, as the growing T-cell population quickly outnumber the APCs. In mice this is not the case, as no homologous murine type class II molecules have been shown to be expressed on T-cells[53]. However, as the mice utilized are transgenic for HLA, this option was explored by flow cytometry.

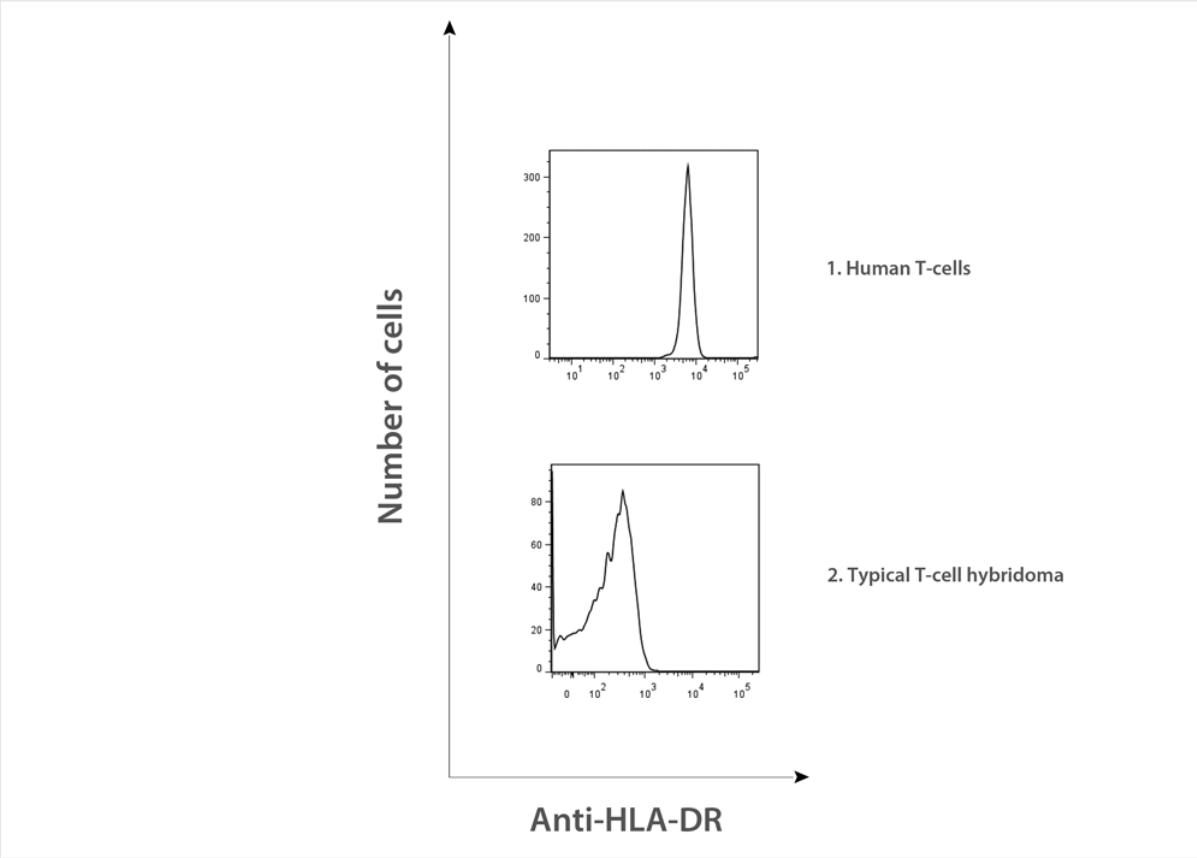

Figure 9: A typical representation of HLA-DR expression on a normal human T-cell, compared to a murine hybridoma derived from an HLA-transgenic mouse. None of the tested hybridomas were found to be HLA-positive on their surface.

In conclusion, no HPA-1a peptide-specific murine T-cell hybridomas were identified. Out of 96 hybridomas analyzed, 5 clones were constituently secreting IL-2, while 9 clones did not secrete IL-2 regardless of stimulation. In addition, 63 clones secreted IL-2 upon stimulation with both L33 and LolP1.

4 Discussion

In this series of studies, the creation of a long-long lived HPA-1a specific T-cell clone was attempted. Both cells of human and murine origin and two different strategies were tried. Firstly, the creation of T-cell hybridomas, which has several advantages compared to normal antigen-reactive T-cells. For instance, they can be propagated without the presence of autologous accessory cells, cytokines or peptides. Further, with a doubling-time of roughly 24 hours, a large numbers of cells can be amassed with ease, in order to study secreted cytokines, membrane-bound receptors and interaction with other cells, such as APCs. Their main disadvantage is that they are cancerous, with the accompanied instability. Secondly, cells displaying increased division potential by the expression of ectopic telomerase are desired due to their comparable response pattern with normal T-cells kept in culture[54]. However, they are dependent on cytokines and antigen-stimulation for continued survival and expansion, making prolonged cell culture laborious.

Easy to procure HPA-1a specific T-cell lines have potential for future use in our group as tools in the development and characterization of HLA class II tetramers. Specifically, four HLA-molecules, loaded with the desired peptide, such as L33, are bound to a streptavidin-molecule with four binding sites, and these tetrameric complexes are finally conjugated to a fluorescent dye for use as reagent for detection of antigen-specific T cells by e.g. flow cytometry. The resulting tetramers are soluble and can be used to rapidly seek out HLA-restricted rare peptide-specific T-cells in human blood samples, as previously shown in influenza A antigen responses[55]. In addition, immortalized T-cell clones can be utilized in cytokine secretion assays. This has previously been helpful in monitoring biological activity of synthetic peptides measuring T-cell mediated immune responses[56].

Neither immortalization through cell hybridization, nor the overexpression of cellular components promising prolonged proliferation succeeded. As the experiments differ in their approach, they will be discussed separately.

4.1 Human HPA-1a specific T-cell hybridomas

The creation of human hybridoma T-cells is desired, preferential to murine T-cell hybridomas, as the resultant cells are less likely to be hampered by cross-species differences. Thus, results originating from human hybrids are expected to be closer to the human biology, increasing the validity of the data gathered.

Over the past decades the reports of human T-cell hybrids are relatively scarce. This is not only due to more hybridoma research being done in mice than humans. The reason is mostly due to the lack of good fusion partners, which is the most decisive factor in successful hybridization attempts in other species[57]. Human hybridomas show low fusion frequencies, together with poor viability and stability of successful clones[58, 59]. Moreover, heterohybridomas created using human T-cells and a murine fusion partner, are genetically unstable, as they preferentially segregate human chromosomes[59, 60].

Electrofusion has been used in previous successful T-cell hybridoma fusion attempts, and promises higher fusion efficiencies, a lower amount of cells needed, faster experiments, together with better reproducibility[59, 61]. We therefore opted for this method first, especially since HPA-1a-specific T-cell clones are in limited supply. However, no method is perfect, and since many parameters, ranging from buffers and pre-fusion cell activation to voltage induced during fusion has to be optimized, this advantage slowly eroded as no successful

fusions were performed. We therefore later opted for PEG-based fusion, which is an established and proven method in our lab[62].

Intraspecies hybridomas are more stable, and in our attempts, we used the SKW-3 cell line, established from a 27 years old man with acute lymphoblastic lymphoma (ALL). This cell line is a derivative of another cell line KE-37, previously used in successful human T-cell hybridoma experiments[63]. SKW-3 cell line does not exhibit susceptibility to HAT-selection. Although mutants that are HAT-sensitive can be induced by irradiation and subsequent culture under the desirable selection conditions, this is time consuming work[63]. Therefore, we tried to circumvent this by labeling the HPA-1a specific clone D8T108 with the cellular dye CFSE, which binds irreversibly to intracellular components. This allows flow cytometry-based separation of newly fused CFSE-labeled T cells and SKW-3 cells from non-fused SKW-3 cells. Thus, we hypothesized that any cell constituents transferred during hybridization would be detectable after fusion. Resultant flow cytometry data showed this was not the case. Coupled with the fact that the cells did not express a TCR, leads us to believe that no hybridomas were produced using electrofusion.

We therefore moved on to use PEG-fusion. As mentioned, this is the more conventional method, both in the literature and in our lab; a B-cell hybridoma secreting anti-HPA-1a-specific antibodies was recently produced in our group by fusing Epstein-Barr virus transformed B-cells from a woman who had had a child affected by FNAIT with a mouse-human heteromyeloma cell line, using PEG in the fusion protocol[62]. This time we chose a different HPA-1a specific T-cell clone D7T4, hoping to negate any potential T-cell clone specific features prohibiting successful hybridization. Further, potential hybrid clones were selected based on the expression of cell surface markers. Both the TCR and CD4 are needed

on a successful hybridoma clone, and the fusion partner SKW-3 only express low amounts of the latter. Therefore, our rationale was that double positive cells would either be still viable primary T-cell clones that would die in prolonged culture, or T-cell hybridomas that would continue to thrive. Cells were found to be double positive upon this method, and were left to grow in culture, before a second selection was performed. However, upon subsequent analysis with flow cytometry, no double positive cells were detected. This made us conclude that the successful hybrids were short lived, and the failure to make a human T-cell hybridoma using PEG-fusion was ultimately down to low fusion efficiency. However, the creation of human T-cell hybridomas have been reported dating back to 1981[64]. Although difficult, it could still be possible in the future after further optimization of our protocol. For example, fusion with our HPA-1a specific T-cells at the various stages of the expansion cycles; it is possible that the activation stages of T-cells plays a role. For instance, the HPA-1a specific clones used in our attempts had been in prolonged culture, already showing loss of proliferative potential upon antigen stimulation.

4.2 Transfection of human T-cell clones with ectopic telomerase

Antigen-specific T-cells derived from immunized individuals can be expanded *in vitro*, but have a finite proliferative life span. Thus, previous efforts has resulted in transduced human T-cells with hTERT-expressing retroviral vectors[65]. This practically immortalizes the cells, as hTERT transduced T-cells can be cultured for an "unlimited" period of time[66]. These cells are then a great resource for the continued study of T-cell interactions, and have even been considered candidates for adoptive immunotherapy[67].

The viral construct used in the expression of ectopic levels of telomerase was linked to a GFP marker. Thus the emission of a fluorescent signal means that the telomerase product is active,

causing lengthening of the telomeres upon cell division. This rescues the cells from senescence. However, no such signal was found in our experiments, meaning no successful transfections were performed. There are several possible reasons for this to happen.

Firstly, viral titers were not measured after production in the AmphoPack-293 cell line. We gambled that since the transfections were easy to measure and perform, this was not needed. Therefore, we do not know if any viral particles were produced. However, we do know that plasmids created in *E. coli* were of satisfactory levels, as analysed using spectrophotometry. One culprit can therefore be in the packaging stage producing the viral particles. Secondly, we opted for a new technique, previously used to concentrate immunoglobulin numbers produced in our lab. There are reports detailing the same technique being used as a quick method to concentrate viral particles[68, 69]. However, viruses are fragile, and it could be that a large number of viral particles were destroyed in this step, reducing transfection efficiency. Thirdly, two different viral constructs were tried, both poised to introduce hTERT and a GFP-marker. No difference was found between the two, making it possible the fatal step lies in the concentration of viral particles, making any transfection attempts futile.

4.3 Murine HPA-1a specific T-cell hybridomas

In comparison with the human experiments, successful mouse clones expressing the desired surface markers TCR and human CD4 were created upon fusion. This was to be expected based on the existing literature. The BW5147 fusion partner is well described, with a propensity to create stable hybridomas, for unknown reasons[70, 71]. However, and more importantly, the clones did not express the desired specificity. No cells showed specific activation upon stimulation with peptide-pulsed APCs using either the HPA-1a derived peptide L33, or the irrelevant LolP1 peptide. Two clones were weakly positive for IL-2

secretion in one of the two wells stimulated with L33 in an ELISA assay. However, it is unlikely that this represents antigen specific clones, as one of them were negative in the ELISPOT assay, and the concentration of IL-2 detected in the ELISA assay was out of the range specified by the manufacturer.

Several clones showed IL-2 release upon stimulation unrelated to the antigen used. Both L33 and LolP1 peptides are known to be presented by the HLA-DRA/DRB3*01:01 molecules, anchoring at the same residues[28]. However, upon co-culture of T-cell hybridomas and APCs that were not peptide-pulsed, the same cells were usually not activated, or showed a very weak response. This can be explained in different ways. First, the argument that the APCs stimulate the T-cell hybridomas in an unspecific manner is the most obvious reason. However, this is countered by the lack of concurrent stimulation in co-cultivation with non-peptide pulsed APCs (ELISA), or only antigen (ELISPOT). Secondly, a single TCR is known to recognize multiple peptides presented by the same HLA-molecule[72]. It is possible that the two very different L33 and LolP1 peptides, upon presentation by the same HLA-DRB3*01:01 molecule, elicit a binding strong enough to activate the hybrid T-cells. It could also be that the enriched T-cell population used in the hybridization with the fusion partner, was monoclonal or oligoclonal. Many of the resulting hybridomas may therefore express the same TCR, and as a consequence, largely the same response pattern. A third option is that the T-cells are stimulated by HLA alone. This model has been proposed as one of two extremes in alloimmunity, where the TCR only recognize the peptide-bound to the HLA-molcule, or the HLA-molecule itself, disregarding the peptide[73]. However, the question can then be raised why the cells were not stimulated by the two different peptides presented to the T-cells on HLA-conjugated beads. However, there was no positive control available at the time, and the technology is still new in our lab, so the results have to be interpreted with caution. A fourth

option is that the hybridomas have a lower activation threshold than normal, and therefore need less potent stimulatory events in order to activate. However, APCs presenting random peptides in the non-peptide pulsed cultures should then likely also activate the cells, as the activating signal is closely tied to antigen specificity.

Murine fusion was attempted with cultured spleen cells in an order to enrich for activated antigen specific cells, predicted to yield a higher fusion rate compared to resting T-cells. It is possible that we enriched for nonspecific cells. For instance, the cells used in the fusion may have had a selection advantage; surviving better *in vitro* than the HPA-1a specific T-cells we were after. In hindsight we may have been more successful in fusing freshly isolated spleen cells.

The nonspecific pattern of recognition has been shown in other normal T-cells. Cytotoxic T-cells have been identified that kill nonspecifically. This has been related to Fas-mediated recognition mechanism, which also has been shown to occur in CD4+ T-cells[74], although we did not measure cytotoxic activity. The fusion partner BW5147 has rearranged TCR alpha and beta chains, which has been shown to pair with donor TCR chains, subsequently expressed on the cell surface[51, 75]. As these TCRs have not been through the same thymic selection as the T-cells enriched from immunized mice, it is possible that they may elicit a strong binding to the HLA-DRA/DRB3*01:01 molecules normally not seen in the transgenic mice, as they would have been weeded out during negative selection. The resultant hybrids are therefore activated by the HLA-binding alone, as discussed above, resulting in a quasi-allogenic situation. Although a possibility, this situation is not likely to occur, since allospecific T-cells occur at a frequency that is quite low. As a last option, the peptide-pulsing itself may activate the APCs, which in turn activate T-cells independent of the TCR.

This would explain the responses observed to APCs pulsed with L33 and LolP1, but not with APCs cultured without peptide.

In conclusion, although no new HPA-1a specific hybridomas were produced, the reactivity of mouse hybridomas to both L33 and LolP1 peptides is interesting, and these hybridomas may in time provide useful information for further development of our murine FNAIT model. A similar response pattern has already been seen in spleen cells isolated from HPA-1a immunized transgenic mice in our lab (data not shown). Therefor this could be an inherent response in T-cells induced under the current immunization protocols.

5 Future perspectives

The project as described has been ambitious. The production of T-cell hybridomas is a difficult procedure, less standardized than the production of B-cell hybridomas. We will continue our efforts to develop both hybridomas and transfected cell lines. One expectation is that the transfected cells may be easier to fuse, expectedly due to both higher numbers and more viable cells.

As discussed above, we may attempt new fusions with freshly isolated mouse spleen cells, since our attempt at enriching HPA-1a specific T-cells in culture, may instead have enriched for the nonspecific phenotype we encountered.

In order for the transfection to work, we need to establish protocols for measuring virus titers in our laboratory. This can be done using plaque-forming assays. Further, a more traditional method of concentrating viral loads using ultracentrifugation can be tried. In addition, a different packaging cell line, such as Phoenix-Ampho, may be utilized.

Finally, any future HPA-1a specific T-cell lines that we develop, will be characterized for their TCR-specificity, cytokine secretion patterns, TCR sequence analysis, HLA-restriction (recognition dependent on the HLA-DRA/DRB3*01:01 molecule) and reactivity with synthetic HLA-DRB3*01:01 molecules, and ultimately, tetramer reagents based on these.

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