Master`s Thesis

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Generation of stem cell-derived hypoimmunogenic β cells that circumvent immune detection

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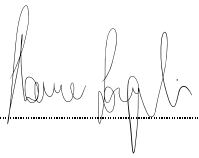

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Kurzfassung

Diabetes Typ 1 ist die häufigste Autoimmunerkrankung bei Kindern mit jährlich 80.000 neuen Patienten weltweit. Die Erkrankung wird durch das selektive Zerstören der pankreatischen β Zellen durch das eigene Immunsystem ausgelöst. Unser Ziel ist es eine Therapie für Diabetes Typ 1 zu entwickeln, welche die Krankheit reversiert und Patienten damit unabhängig von Insulinpräparaten macht. Dafür haben wir insulin-sezernierende glukose-sensitive hypoimmunogene β Zellen entwickelt, welche fähig sind das Immunsystem zu umgehen. Aus diesem Grund haben wir Knock-out Zellen denen HLA-A/B/C fehlt (B2M-KO, HLA-KO) und außerdem PD-L1, CD47 und HLA-G Knock-in Zellen (2in1-KI, 3in1-KI) in β Zellen differenziert. In einem ersten Schritt wurde die Effizienz gemessen, mit welcher diese Stammzellen in β Zellen differenziert werden können. Dafür wurden Proben von allen Genotypen genommen sobald sie das finale Stadium der Differenzierung erreicht hatten und auf die β Zellmarker C-peptid und NKX6.1 getestet. In einem zweiten Schritt wurden die differenzierten β Zellen mit humanen T Zellen co-kultiviert um sie auf ihre Fähigkeit die Immunabwehr zu umgehen zu testen. Die Aktivierung und das Proliferieren von diesen T Zellen wurde sowohl 2 Tagen als auch 7 Tage später ausgewertet.

Wir konnten beweisen, dass es möglich ist immun regulierende Stammzellen in β Zellen zu differenzieren und dass diese fähig sind sowohl die Aktivierung als auch das Proliferieren von T Zellen zu unterbinden. Diese Beobachtungen erlauben die Schlussfolgerung, dass diese Zellen tatsächlich in der Lage sind die Immunabwehr zu umgehen und somit eine Therapie für Typ 1 Diabetes darstellen könnten. Weitere *in vitro* und *in vivo* Experimente, wie das Transplantieren dieser Zellen in ein humanisiertes Mausmodell (hu-SCID) sind nötig um die Frage zu klären ob sie fähig sind Typ 1 Diabetes rückgängig zu machen.

Schlagwörter: Typ 1 Diabetes, hypoimmunogene Stammzellen, B Zellen Differenzierung

Abstract

Type 1 Diabetes is the most common autoimmune disease in children worldwide with an annual occurrence of 80.000 and rising. It results through the selective destruction of pancreatic β cells through the patient`s own immune system. Our aim is to develop a cure for T1D through generating insulin-secreting glucose-sensitive hypoimmunogenic β cells that are able to circumvent the immune detection. For this purpose, we differentiated HLA deficient knock-out cells (B2M-KO, HLA-KO) as well as PD-L1, CD47 and HLA-G immunomodulatory knock-in cells (2in1-KI, 3in1-KI) into SC-β cells. Differentiation efficiency (C-peptide/NKX6.1) was determined every week after entering the final differentiation stage. Finally, SC-β cells were used for immune assays. Human T cells were co-cultured with genome-edited as well as wildtype (HuES8) SC-β cells. Activation as well as proliferation of T cells was measured 48 hours or 7 days later.

We found that it is possible to differentiate immunomodulatory genome-edited cells into SC-β cells and that they are able to prevent activation and proliferation of primary human T cells. Our results suggest that immunomodulatory SC-β cells may eventually circumvent immune detection and therefore could lead to a cure for T1D. Further experiments, such as *in vitro* and *in vivo* GSIS as well as transplantation into a humanized mouse model (hu-SCID) will answer the question if these cells are indeed able to reverse T1D.

Keywords: Type 1 Diabetes, hypoimmunogenic stem cells, β cell differentiation

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1 Introduction

1.1 Type 1 Diabetes

Type 1 diabetes (T1D), one of the two types of diabetes, is an autoimmune disease resulting from the destruction of the insulin-producing β cells in the pancreas leading to insulin deficiency. Although the main function of β cells is to maintain a physiological glucose level in the human body through sensing glucose and releasing insulin, patients suffering from T1D lose more than the control over blood glucose levels. Moreover, T1D can lead to ketoacidosis and hypoglycemia and consequently also to secondary conditions such as heart disease, blindness and kidney failure. T1D patients have a ten-times higher risk of cardiovascular disease, such as myocardial infarction, angina or stroke, than aged-matched non-diabetic individuals[1,2,3,4,5].
Albeit, T1D can be diagnosed at any age, it is the most common chronic disease of childhood. It accounts for 5-10% of all cases of diabetes and is estimated to have an annual occurrence of 80.000 in children with an increasing tendency[1,4].

Admittedly, it is not fully understood why T1D arises. On the one hand, it is associated with different environmental factors such as viral infections, nutritional factors, drugs, toxins, psychological stress, vitamin D and climatic influences and on the other hand with a genetic susceptibility in the Human Leucocyte Antigen (HLA) genes[2,3]. Moreover, large scale genome-wide association studies (GWAS) have identified other potential genetic susceptibility loci and it has been shown that most of the candidate genes are expressed in pancreatic islets regulating pancreatic inflammation and β-cell apoptosis[6]. The abnormal activation of the T cell-mediated immune system leads to the production of autoantibodies against β cell antigens by β cell as well as inflammatory responses within the islets[2]. More than 90% of patients suffering from T1D have one or more of the following autoantibodies: those reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A), and zinc transporter 8 (ZnT8A)[5].

1.2 Management of Type 1 Diabetes

Even though our knowledge about the cause and background of T1D is continuously progressing, patients are still depending on the exogenous supply of insulin for survival. A tight control of blood glucose levels is necessary to reduce the probability of secondary complications such as retinopathy, nephropathy or hypoglycemia[1]. Diabetes management always includes the use of insulin or insulin analogues and mechanical technologies (e.g. insulin pumps and continuous glucose monitors). At the same time, a normal metabolic regulation is difficult to achieve, despite the improved technologies. The levels of glycemic control that are generally achieved in practice nowadays, do not meet the necessary goals.

For example, the risk of hypoglycemia from the use of exogenous insulin is an eligible fear for patients. The primary goal of intervention and the only possible cure of T1D is to improve or prevent the progression of β cell destruction and eventually to replenish the destroyed cells by cells capable of sensing blood glucose levels and secreting insulin in a glucose-dependent manner[1,5]. Different approaches include islets or whole pancreas transplantation from allogeneic or xenogeneic donors, enhancing β cells replication or the differentiation of embryonic stem cells or induced pluripotent stem cells into β cells.

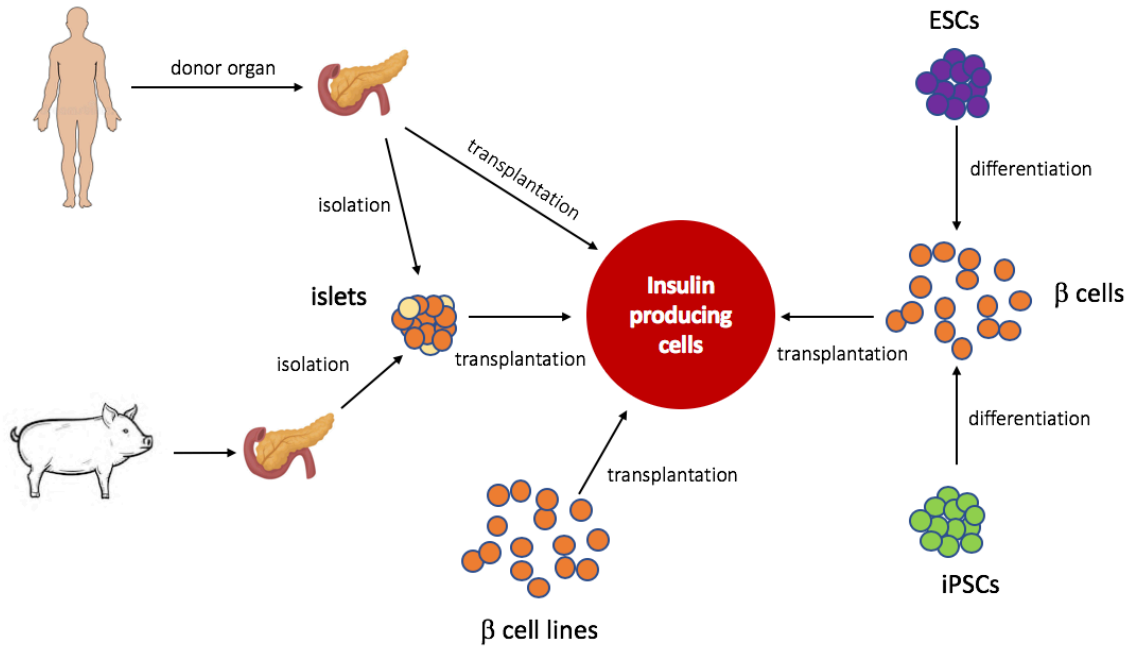

Figure 1. The different β cell replacement therapy approaches. β cell replacement can either be achieved through the use of allogeneic or xenogeneic islets or whole pancreas transplantation. Another option would be to enhance the replication of the patient`s own remaining β cells or generating β cells from hESCs for cell transplantation [7].

At present, the most promising β cell replacement approach is through pancreas or islet transplantation. Figure 1 summarizes the different cell replacement therapies. The first islet isolation and transplantation were done in rats by Ballinger and Lacy in 1972. In 1977, the first islet infusion in humans followed[7]. Today, the procedure is more safe due to the fact that the pancreatic islets are injected into the patient`s portal vein rather than the complicated whole pancreas transplantation[7]. Unfortunately, these procedures have serious limitations such as the shortage of donors (only heart-beating brain-dead donors), and the need for immunosuppressive drugs, which altogether restricts the use of islets and whole pancreas transplantation as a standard procedure[8].

Another option would be the use of xenogeneic pancreatic islets, in particular the use of pig islets. Pig islets are able to substitute human insulin, their islets also regulate blood glucose levels in the same

physiological manner human islets do. It is also possible to genetically modify pigs to make their islets more applicable to humans, and high yields of pig islets can be isolated[7,9]. But pig islet transplantation comes with limitations too. First, the exposure to a zoonosis, the transfer of an infection from the pig to the patient. Second, the risk of an hyperacute immunologic rejection, since pigs express the saccharide glacatose-α1,3-galactose (Gal) to which humans have natural preformed antibodies[7]. To overcome immunogenicity, genetically modified pigs have been developed and islets were encapsulated in a biocompatible membrane and tested on non-human primates (NHP). Clinical trials were performed in Mexico, China, New Zealand and other countries but no group of patients could be rendered insulin independent with such approaches[10].
Others consider treating T1D by stimulating β cell replication of the patient`s own remaining β cell population. One limitation with using the patient's own cells is that β cells are a quiescent population and do not proliferate except for the first years after birth or during pregnancy in humans[7].

A substantial effort is currently made in β cell replacement therapy using stem cell-derived β cells. The capability of embryonic stem cells (ESCs) to develop into any cell type and their ability of self-renewal made them the cell type of choice for this approach[11]. The first to develop a protocol to generate β cells form human ESCs were Baetge *et al.* in 2006. They managed to create a cell population that expressed high levels of proinsulin but could not process it as efficiently as adult islets[12]. Pagliuca *et al.* and Rezania *et al.* both described a directed differentiation protocol *in vitro* creating 20-50% insulin-positive cells from hESCs[13,14]. Both groups could prove graft survival over the course of a few months and restoration of normoglycemia within 2 or 6 weeks[13,14] after transplantation into immunocompromised mice.
Although significant progress was made in finding a cure for T1D, there are still limitations to the use of SC-derived β cells. First is the ethical concern regarding the use and modification of hESCs. Furthermore, remaining undifferentiated cells may give rise to teratoma upon transplantation into patients because of their pluripotency. Moreover, many different cell lines would need to be tested and differentiated to generate ESC lines that provide genetically matched donor cells to patients, preventing graft rejection and lifelong immunosuppression[7].
Induced pluripotent stem cells (iPSCs) are a possibility to bypass the ethical concerns regarding hESCs. In 2006, Takahashi and Yamanaka (winner of the Nobel Prize in 2012 for this discovery) succeeded in reprogramming adult somatic murine cells into iPSCs through the forced expression of the 4 genes OCT4, SOX2, KLF4 and c-Myc. Only one year later they could achieve the same with human cells[15]. Mouse and human iPSCs show the same characteristics as ESCs and are also able to differentiate in any cell type *in vitro*[16]. First attempts in differentiating iPSCs into insulin-secreting β cells were made based on the protocols for ESCs[17]. Unfortunately, the differentiation efficiency was very low, and further protocols focused on increasing it. In 2014, the group of Dr. Douglas Melton, Harvard University, published a protocol that leads to the generation of cells positive for the β cell specific cell markers C-peptide and NKX6.1 from both ESCs and iPSCs with a differentiation efficiency up to 50%[18].

In theory, iPSCs are a great source of autologous cells. Somatic cells can be obtained from a patient, be reprogrammed and transplanted back into the patient without causing immune rejection. However, Zhao *et al.* demonstrated in a mouse model that genetic and epigenetic variations occuring during reprogramming can even result in the rejection of iPSCs[19,20]. Another reason why iPSCs are not a feasible solution is that it would be necessary to generate these cells for each individual patient, making this form of therapy time consuming and expensive. Furthermore, even autologous sc-β will be destroyed by the immune system of T1D patients, because they have autoreactive T cells.

1.3 T cell allorecognition in transplant rejection

Organ transplantation is a common procedure for patients with end-stage cardiac, renal, hepatic and pancreatic failure. But for patients suffering from T1D it is still the only available cure, although the limitations are severe. Not only is an organ transplantation a major surgical procedure but it also requires lifelong immunosuppression to assure graft survival.

Transplants from allogeneic donors are rejected from the recipient because of alloantigens encoded by the major histocompatibility complex (MHC), also called HLA (Human Leucocyte Antigen) in humans. The HLAs consist of two classes: the class I molecules (HLA-A/B/C) and the class II molecules (HLA-DP/DQ/DR), which are responsible for the recognition of antigens by T lymphocytes[21]. The HLA molecules are expressed on the surface of antigen-presenting cell (APCs) such as macrophages or dendritic cells[22]. The HLA-A/B/C molecules are encoded by genes in the HLA region that bind noncovalently to the invariant glycoprotein beta-2-microglobulin (B2M) and are composed of polymorphic heavy-chain glycoproteins[21,22]. On the other hand, HLA-DP/DQ/DR molecules are also encoded by genes in the HLA regions but constitute of two glycoproteins, an α-chain and β-chain[21]. Another major difference between the two types of HLAs is that class I molecules are ubiquitously expressed on almost all cell types, whereas class II molecules are only expressed on specific cell types such as B cells, macrophages, monocytes and dendritic cells. The main function of the HLAs is to bind peptides derived from foreign microorganisms and present them to T cells, which initiates T cell-mediated immune responses[22]. Although many different types of cells can be involved in graft rejection, only T cells seem to be absolutely necessary. Studies on mice showed that the complete absence of T lymphocytes leads to long-term transplant survival due to the lack of the cellular mechanisms to recognize antigens as foreign[22].

There are two distinct pathways involved in the recognition of alloantigens: the direct pathway and the indirect pathway. By the use of the direct pathway, T cells recognize foreign HLA molecules on allogeneic cells and are therefore responsible for the acute immune rejection. In contrast, the indirect pathway recognizes foreign peptides derived from the allogeneic HLA presented by the self-HLA molecules and are consequently responsible for the chronic immune rejection[22,23].

Activation of T cells requires more than one type of signal. The primary signal is delivered through the binding of the T cell receptor (TCR) to the antigen presented on the HLA by antigen presenting cells.

The second signal is a costimulatory signal that is contributed by cell-surface molecules on the APCs[22,24,25]. In fact, it was proven that the lack of a second costimulatory signal leads to T cell unresponsiveness, also called T cell anergy[26]. CD28, CTLA-4, PD1, PD-L1, ICOS and BTLA are members of the family of costimulatory molecules (Figure 2)[27]. Although costimulatory molecules have now been identified on almost all the cell types, they are the most characterized on APCs[27].

One of the most well studied co-regulatory pathways is the B7/CD28/CTLA-4 pathway, which plays an important role in T cell activation and tolerance[28]. The costimulatory signal CD28 is expressed on 80% of human $CD4^+$ T cells and 50% $CD8^+$ T cells but also on other cell types such as bone marrow stromal cells or plasma cells[29,30]. The ligand, B7 consists of two subtypes, B7-1 (CD80) and B7-2 (CD86), which both bind to the costimulatory molecule CD28 and the coinhibitory molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Figure 2). Whereas CD28 is constitutively expressed, CTLA-4 is upregulated after the activation of T cells[28,29]. The binding of B7 to CD28 disseminates a signal which synergizes with the TCR signal to enable T cell clonal expansion through cytokines. It also promotes the cell survival and response of previously activated T cells through the production of interleukin-2 (IL-2) by previously activated T cells through and increase in transcription and mRNA[24,28].

CTLA-4, on the other hand, delivers an inhibitory signal. In the 1990s, is was first demonstrated that CTLA-4Ig, a fusion protein, binds with a higher affinity than CD28 to B7-1 and B7-2 and blocks CD28 from binding, therefore effectively suppressing the TCR- and CD28-mediated signal transduction. Salomon *et al.* established long-term immune tolerance through the use of CTLA-4 and effectively prevented the rejection of a transplanted islet xenograft[24].

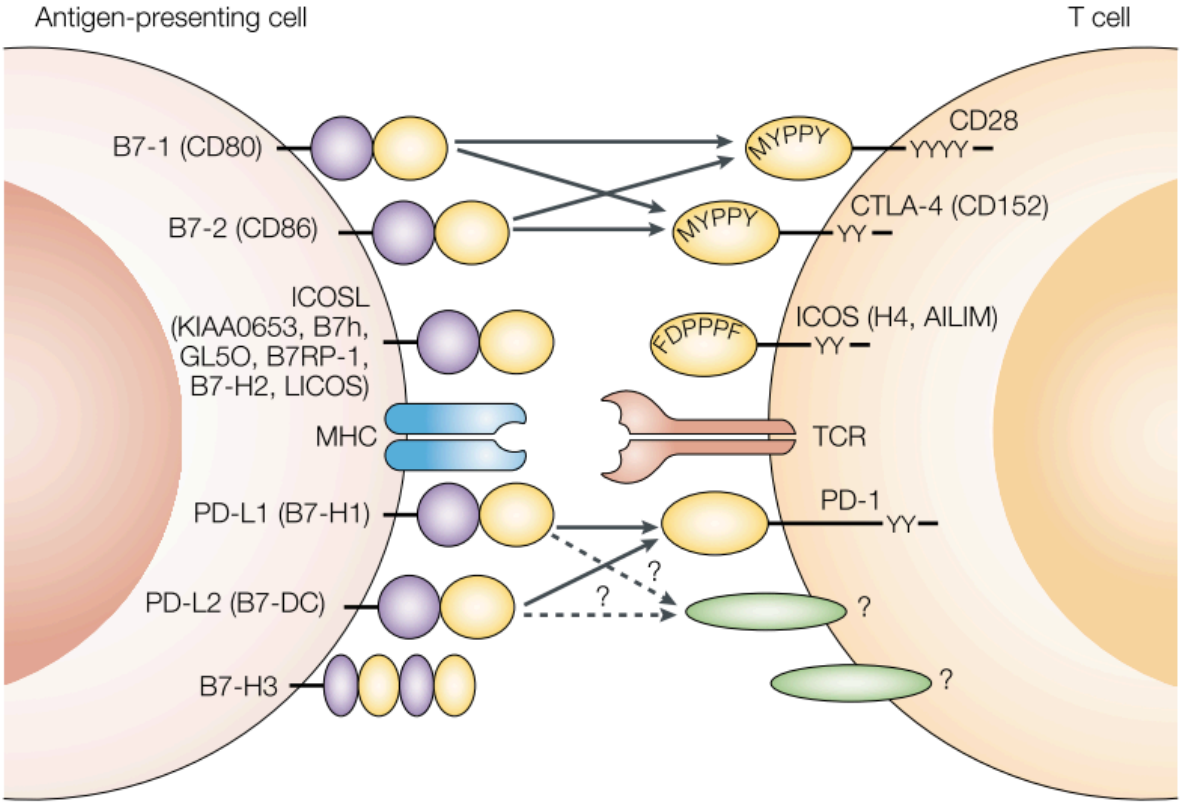

Figure 2. The B7/CD28/CTLA-4 superfamily members. T cell activation requires two distinct signals. First, the binding of the TCR receptor to the antigen presented by the HLA on APCs. The second signal is a costimulatory signal. CD28, CTLA-4, PD1, PD-L1, ICOS and BTLA are members of the coregulatory family. One of the most well characterized pathways is the B7/CD28/CTLA-4 pathway. CD28 and CTLA-4 both bind to the ligands B7-1 and B7-2. CD28 serves as a costimulatory molecule, whereas CTLA-4 poses as a coinhibitory molecule[28].

1.4 Potential cure for Type 1 Diabetes

1.4.1 Hypoimmunogenic ESCs

To circumvent the need for immunosuppression upon transplantation of allogenic hESC-derived cells, it is important to develop a safe immune tolerance strategy. As described in 1.3, immune rejection is of allotransplants is mostly triggered by the HLAs[31]. With the introduction of genome-editing techniques, it is possible to create a HLA I and HLA II knockout, either by directly deleting HLA genes or genes involved in HLA expression such as β2M for class I and MHC class-II transactivator (CIITA) for class II (Figure 3)[32–34]. β2M is the accessory chain of HLA-I, which is necessary for peptide presentation on the cell surface[33]. HESCs lacking the HLA-I genes show reduced sensitivity to T cell or peripheral blood mononuclear cell (PBMC)-mediated killing *in vitro*. Also, *in vivo* after the transplantation of HLA-I-deficient cells into immunocompetent mice, they exhibited longer graft survival compared to wildtype cells[35].

Although the focus in general lies more on the depletion of HLA I, HLA class II can cause $CD4^+$ T helper cell-mediated immune rejection through the activation of B cells, macrophages and other immune cells[36].

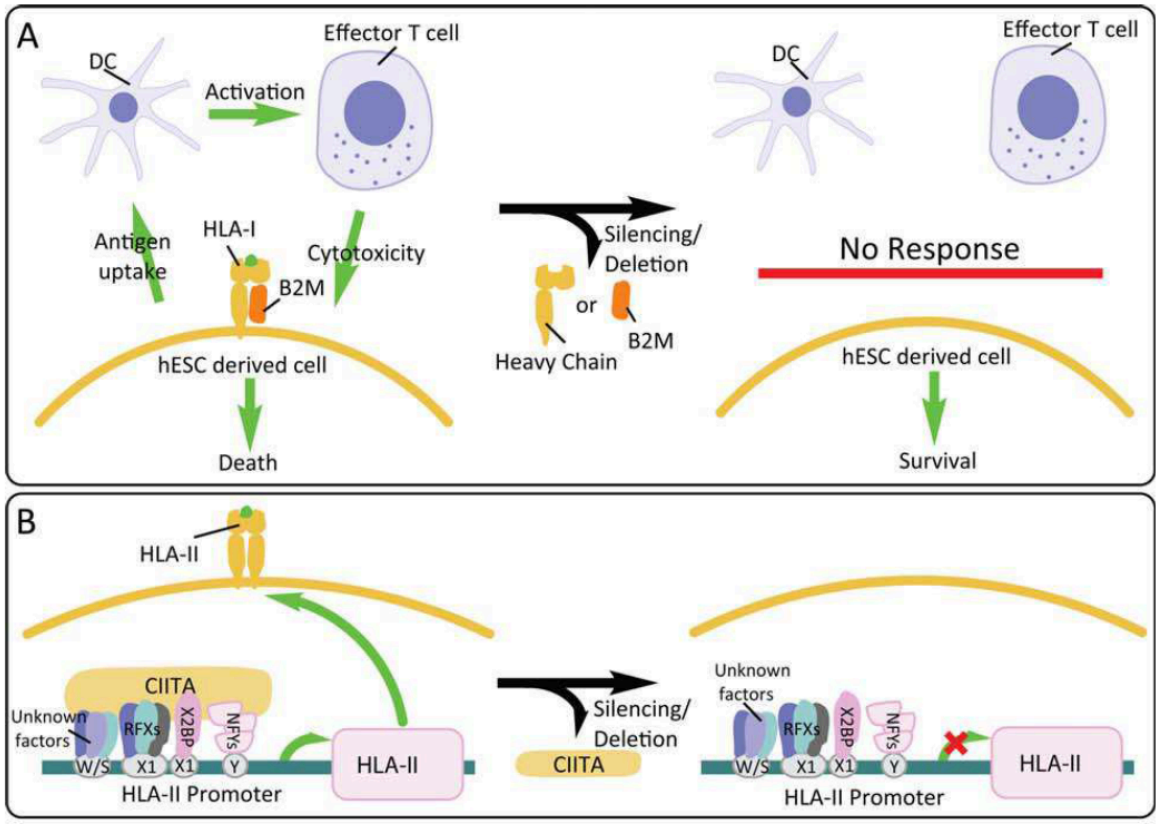

Figure 3. Approaches to prevent immune rejection through the silencing of HLA. A) Inhibition of HLA I expression on the surface by knocking-out the HLA genes or silencing through the disruption of β2M. HLA I is not presented

on the cell surface anymore and therefore becomes invisible to the immune cells of the patient. B) Silencing or deleting the CIITA transactivator, necessary for HLA II expression. HESCs lacking HLA II expression will not show HLA II-dependent immune rejection[31].

The problem that arises from the loss of HLA is the susceptibility to NK cell-mediated cytotoxicity. In 1981, Klas Kärre formulated the missing-self hypothesis, which states that NK cells eliminate other cells that fail to express the major histocompatibility complex class I. Viruses and tumor cells often display low or no expression of MHC I[37,38]. One solution to prevent NK cells from killing the graft cells is the expression of non-classical class I HLA molecules such as HLA-E or HLA-G[39] (Figure 4).
In addition, other strategies for the induction of immunosuppression involve the immune inhibitory molecules CTLA4 and Programmed death-ligand 1(PD-L1). CTLA4-Ig binds with high affinity to B7-1 (CD80) or B7-2 (CD86) and therefore blocks the T cell co-stimulatory pathway, whereas PD-L1 binds to PD-1, which is expressed on activated T cells to induce an inhibitory pathway[40] (Figure 4).

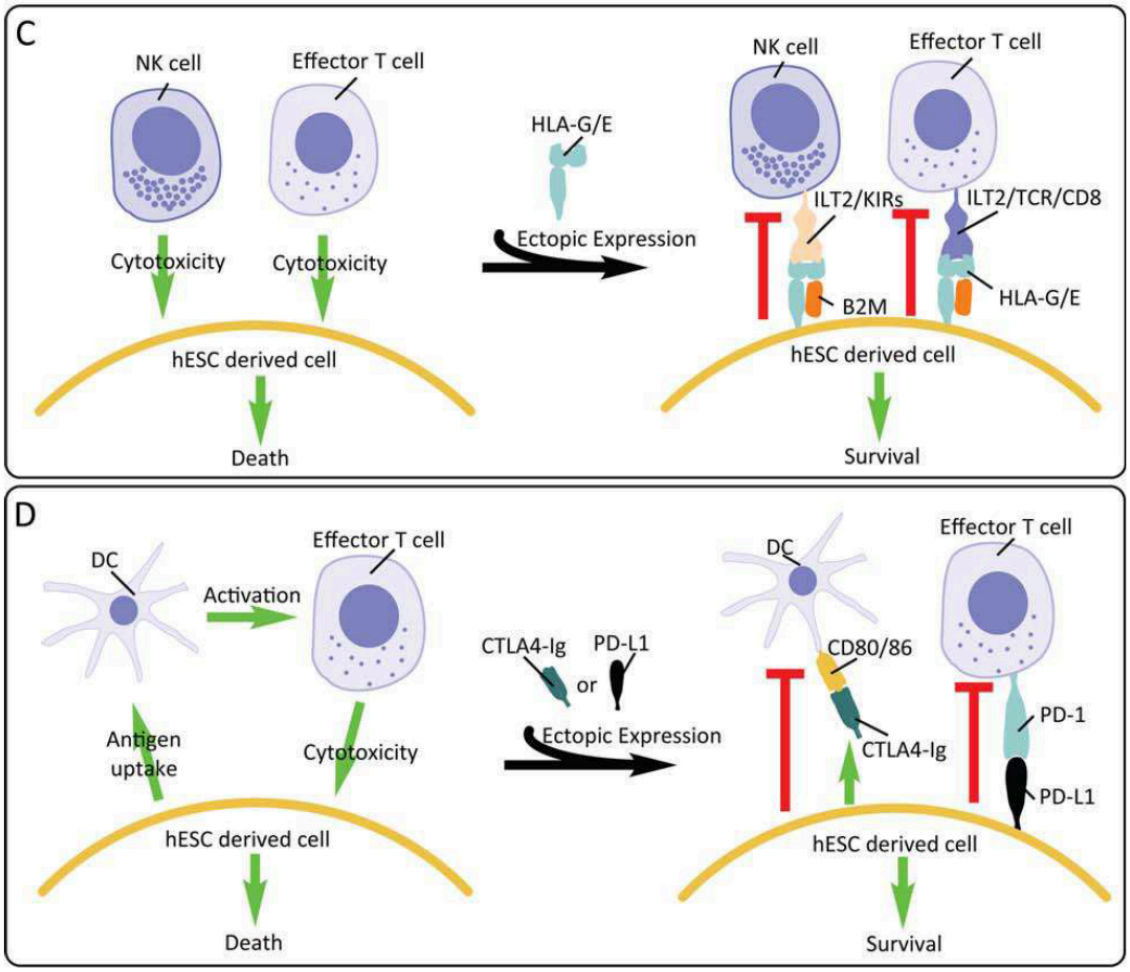

Figure 4. Approaches to prevent immune rejection through immunomodulatory molecules. A) Expression of non-classical HLAs can inhibit NK cell-mediated killing. B) Ectopic expression of the immunosuppressive molecules CTLA4-Ig and PD-L1. CTLA4-Ig binds to CD80/86 to block the activation of T cells. PD-L1 interacts with PD-1 expressed on effector T cells to inhibit their activity[31].

Based on the facts above, the group of Dr. Chad Cowan (Harvard University) has generated different knock-out and knock-in human pluripotent stem cell in HuES8 (Harvard university Embryonic Stem cells 8) cells for further investigation (Figure 5). HuES8 cells are male embryonic stem cells and part of the NIH human embryonic stem cell registry.

One of the created knockout lines (B2M-KO) lacks β2M expression, which is necessary for HLA class I antigen presentation. A second knock-out line (HLA-KO) features deletion of the individual HLA-A/-B/-C genes, as well as the ablation of the master regulator CIITA which results in the loss of HLA class II expression. We hypothesized that cells lacking the HLA I and II molecules will not cause T cell-mediated immune rejection but are still susceptible to other immune cell types. Therefore, the tolerogenic factors, PD-L1, CD47 and HLA-G were introduced into the HLA I- and II-deficient cell line. The cell line called 3in1-KI features a knock-in of all three previously mentioned tolerogenic factors whereas the cell line 2in1-KI only has PD-L1 and CD47 integrated.

The ectopic expression of HLA-G is important in pregnancy, where trophoblasts use it to establish tolerance to the hemiallogeneic fetus[41]. CD47 is the "don`t-eat-me" signal preventing macrophages from engulfing cells[42].

Figure 5. Knock-out and Knock-in human pluripotent stem cell lines generated by the lab of Chad Cowan, Harvard University, compared to wildtype HuES8 cells. KO-B2M is lacking B2M necessary for HLA I surface expression. KO-HLA features deletion of the HLA I and II genes. Whereas, the two knock-in cell lines, in addition to the deletion of HLA I and II, express either the tolerogenic factors PD-L1, CD47 and HLA-G or just PD-L1 and CD47, respectively. (WT) wildtype

1.4.2 β cells differentiation

The group of Prof. Douglas Melton (Harvard University) developed and established a protocol to generate SC-derived β cells.

Already in the nineties, different groups identified signals and genes important for the pancreatic lineage[43,44]. In 2008, Kroon *et al.* showed that pancreatic endoderm derived from ESCs can mature into glucose responsive endocrine cells after transplantation into mice[45]. However, it was unclear if these cells would show the same effect in human patients. Further attempts to create insulin-producing cells from human pancreatic progenitors *in vitro* lead to immature or abnormal phenotypes. These cells were either unable to react to glucose stimuli or unable to release insulin in response to glucose challenge. They did not express specific β cell markers (NKX6.1 or PDX1), expressed other hormones like glucagon or were not able to function properly after transplantation[12,46,47].

In 2014, Pagliuca *et al*. (under the supervision of Prof. Douglas Melton) published a protocol to generate functional human β cells from hPSCs *in vitro* in 4-6 weeks. Therefor, they used sequential modulation of different signaling pathways including Wnt, activin, hedgehog, EGF, TGFβ, thyroid hormone, retinoic acid as wells as γ-secretase inhibition in a 3D cell culture system. They produced glucose-responsive, monohormonal insulin-producing cells, which showed expression of β cell markers and were able to function like pancreatic islets *in vitro* and *in vivo*[18].

For the differentiation of SC-derived β cells (Figure 6), clusters of HuES8 cells or hiPSCs (100-200μm in diameter) were promoted into SOX17$^+$ definitive endoderm (DE) cells and further into PDX1$^+$ pancreatic progenitor (PP1) cells. Further differentiation lead to PDX1$^+$/NKX6.1$^+$ pancreatic progenitors (PP2) before they were either differentiated into INS+ cells along with INS$^+$/Glucagon$^+$ (GCG$^+$) and INS$^+$/SST$^+$ polyhormonal (PH) cells or into NKX6.1$^+$/C-peptide$^+$ (EN) cells and further into functional β cells. Polyhormonal cells resemble human fetal β cells because they do not show glucose-stimulated insulin secretion (GSIS) nor other properties of adult β cells[18]

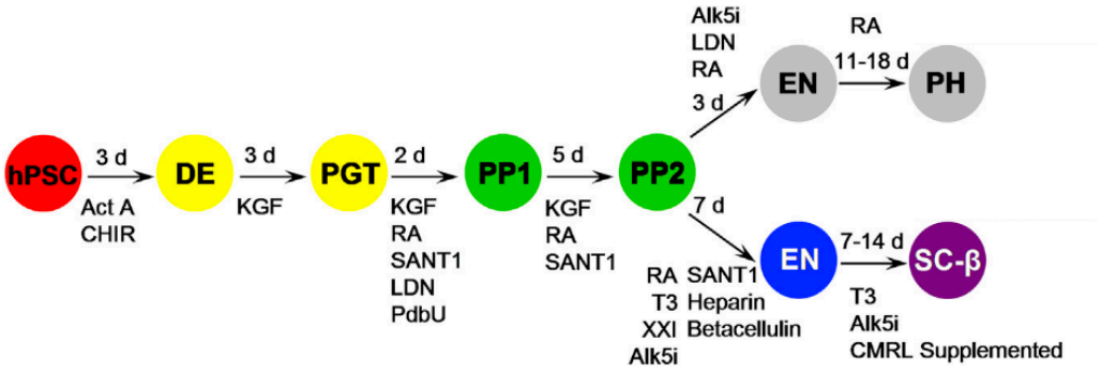

Figure 6. Schematic of directed differentiation from hPSCs into functional β cells through the utilization of different signaling pathways[18]. Human pluripotent stem cells (hPSCs) are converted into definitive endoderm (DE) and further into primitive gut tube (PGT) before they develop into pancreatic progenitors (PP1 and PP2). From this stage, they divide into either polyhormonal cells (PH, C-peptide$^+$/glucagon$^+$) or into stem cell-derived β cells (SC-β, C-peptide$^+$/NKX6.1$^+$).

1.5 Embryonic development of the pancreas

The embryonic pancreatic development has been investigated intensively in rodents but not in humans because of ethical concerns regarding the handling of fetal tissue. In general, the pancreas consists of three different tissue types: the exocrine acinar tissue that produces digestives enzymes, the endocrine cells, also called islets of Langerhans that produce the hormones insulin and glucagon, and the branched ductal tree[48–50]. Figure 7 displays the embryonic development of the pancreas in mice. In humans and mice, the pancreas development begins with the ventral and dorsal buds of the foregut endoderm[48–51]. This process starts in humans at 2 to 3 weeks of fetal age, whereas in mice by embryonic day 8.5-9.5 (E8.5-9.5)[48,51]. Next in the mouse embryonic development of the pancreas the first transition happens where the two buds undergo branching into a ductal tree (E12.5), which in

the end leads to the formation of two primordial pancreas organs. These primitive organs mainly consist of undifferentiated ductal epithelium[48]. During the second developmental transition, the two pancreatic organs fuse into one single organ (E13-14), before the pancreas finally differentiates from the ductal epithelium (E14-5-15-5)[48]. Endocrine cells undergo proliferation and organization into islets-like clusters (E16). At this point in the development, islets are immature and go through remodeling and maturation until 2-3 weeks after birth (third developmental transition)[48].

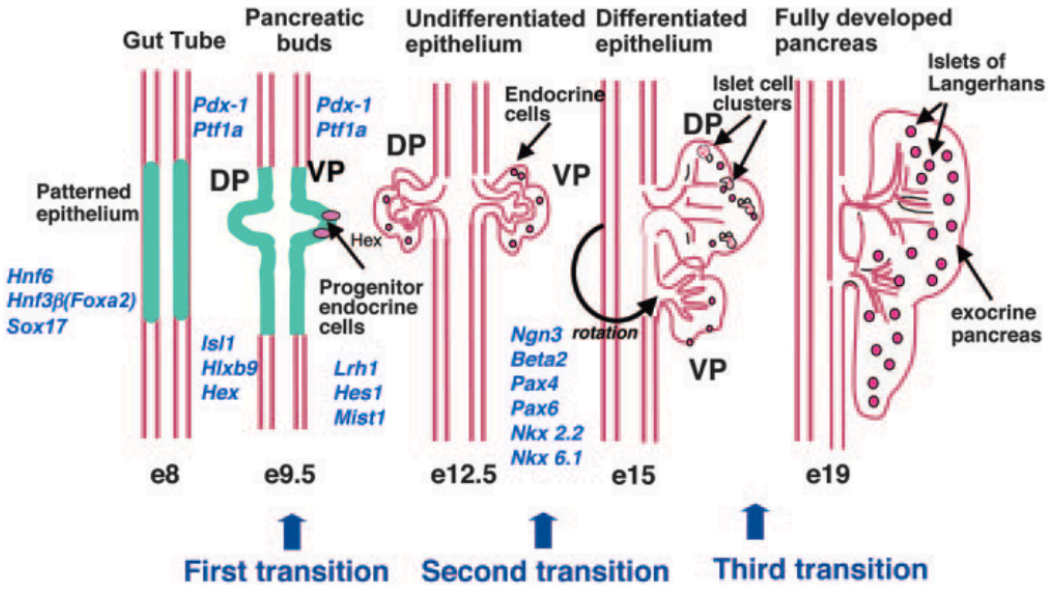

Figure 7. Embryonic development of the pancreas in mice. The development is divided into three transition periods: the pancreas starts to develop two buds from the foregut endoderm. During the first transition period, the ventral and dorsal buds branch and differentiate into two single pancreatic organs. In the second transition period, the two single organs rotate and fuse into one pancreas. During the third developmental transition period, the endocrine cells differentiate into islet-like clusters which mature until 2-3 weeks after birth[48].

In humans as in mice, the two buds express the pancreatic duodenal homeobox 1 (PDX-1$^+$) an early marker for pancreatic development, by excluding sonic hedgehog (SHH)[51,52]. However, during the human embryonic development, after the ventral and dorsal buds have formed, they start branching into the primitive acini, islets and ducts (10-12 weeks)[51]. The ventral and dorsal buds express the transcription factors sex-determining region Y (SRY)-box 9 (SOX9), PDX-1 and GATA binding protein 4 (GATA4)[52]. By week 14-15, vascularized parenchymous lobuli appear already containing small islets. By the second trimester, adult-like islets (NKX6.1$^+$) containing all four types of endocrine cells start to emerge[51].

Scharfmann *et al*. demonstrated the developmental potential of human pancreatic progenitors from the start of the fetal period (8 weeks after conception). Prior to the endocrine differentiation, they transplanted the whole pancreas under the kidney capsule of adult severe combined immunodeficiency (SCID) mice. The transplanted pancreas did grow and contained all cell lineages

including islets[52,53]. Although PDX-1$^+$ pancreatic progenitor displayed a great proliferative potential, cells who underwent differentiation into β cells before being transplanted, showed a reduced rate in proliferation[52,53].

1.6 Cell-based heart regeneration

Cardiovascular diseases remain the major cause of morbidity and mortality worldwide with an increasing tendency over the next decades[54,55]. Although it was proven that the heart muscle does possess some regenerative capabilities, it is still too little to counteract the tissue damage caused by heart diseases such as myocardial infarction. Despite the fact that considerable advances were made in prevention, diagnosis and treatment of cardiac diseases, heart transplantation is still the standard therapy until today[54,55]. Therefore, the main aim in the field of heart regeneration is to find new regenerative strategies to replace the damaged myocardial tissue with functional cardiomyocytes[54].
There are different types of cells available for potential cell transplantation approaches, and most of these cell types exhibit progenitor features. These cell types include embryonic, induced pluripotent, cardiac, bone marrow-derived and skeletal stem cells[54,56].
As described in 1.2, ESCs are not only a promising cell source for potential type I diabetes therapies but also for cell-based heart regeneration. Embryonic stem cells display unlimited self-renewal as well as the potential to differentiate into any cell type of the adult organism, including cardiomyocytes. One technical difficulty is the purity and yield of cardiomyocytes after differentiation. Over the last few years, protocols for cardiomyocyte differentiations have become more efficient and reproducible. Through the manipulation of cardiac-specific signaling pathways (Wnt, Activin/nodal and BMP signaling pathways), it is possible to generate a cell population of up to 80% of cardiomyocytes[57]. Glucose as well as lactate and fatty acids are the main energy sources of cardiomyocytes. Interestingly, fetal and mature cardiomyocytes are able to utilize a different source of energy. In comparison to the adult heart the juvenile cardiomyocytes have a higher capacity for the lactate metabolism. By sorting cells based on their differences in their glucose and lactate metabolism, it is possible to get a purity of cardiomyocytes of up to 99%[56,58].
Kehat *et al.* also demonstrated that hESC-derived cardiomyocytes are able to survive and function as pacemakers in atrioventricular blocked hearts after being transplanted into pigs and guinea pigs[55,59].
To test the applicability of the hypoimmunogenic stem cells to cell types that are of therapeutic interest, beyond sc-β cells, we tested cardiomyocytes. Cardiomyocytes are easily differentiated from ESCs and pose a great interest for new therapies in the context of cell-based heart regeneration.

2 Aims

Type 1 diabetes is the most common autoimmune disease in children worldwide, leading to the destruction of pancreatic β cells by the patient's own immune system. Insulin injection is the only available form of treatment so far, which is still just only an abatement of symptoms. Our ultimate goal is to get a step closer to developing a cell-based cure for type 1 diabetes.
For this purpose, the lab of Chad Cowan (Harvard University) generated universal donor stem cells through the silencing or deletion of the human leucocytes antigens (HLAs) (B2M-KO, HLA-KO) as well as cells which are additionally endowed with immunosuppressive molecules such as PD-L1, CD47 and HLA-G (2in1-KI, 3in1-KI).

One aim of this Master`s thesis was to assess the possibility of differentiating these heavily genome-edited cells into SC-β cells using a well-established protocol by Pagliuca *et al*. and determine the differentiation efficiency and potential bottlenecks.

The second aim was to investigate if these differentiated SC-β cells would be able to circumvent immune detection by primary human immune cells. Accordingly, SC-β cells were co-cultured with human T cells or PBMCs to see if their activation could be avoided and their proliferation inhibited.

3 Material and Methods

3.1 Standard cell culture procedures

Cells were cultured in tissue culture plates and incubated in humidified atmosphere at 37°C and 5% CO_2.

Culture medium for hESCs consisted of the following ingredients:
mTeSR™1 Basal medium (StemCell™ Technologies) supplemented with mTeSR™1 5x Supplement (StemCell™ Technologies) and 1:1000 Rock Inhibitor (StemCell™ Technologies).

Splitting medium for hESCs consisted of the following ingredients:
mTeSR™3D Seed Basal medium (StemCell™ Technologies) supplemented with mTeSR™3D Seed 5x Supplement (StemCell™ Technologies) and 1:1000 Rock Inhibitor.

Feeding medium for hESCs consisted of the following ingredients:
mTeSR™3D Feed Supplement A (StemCell™ Technologies) mixed with mTeSR™3D Feed Supplement B (StemCell™ Technologies).

Freezing medium for hESCs consisted of the following ingredients:
mFreSR™ (StemCell™ Technologies), which is a defined cryopreservation medium for hESCs and hiPSCs.

3.1.1 Thawing of hESCs

Tissue cell culture plates were coated with Corning™ Matrigel™ hESC-Qualified Matrix (Corning) at least 20 minutes prior to thawing. Therefore, an aliquot of Matrigel was thawed in 18ml of cold DMEM/F12 50:50 medium (Dulbecco`s Modification of Eagle`s Medium/ Ham`s F-12 50:50 Mix) (Corning), evenly spread on the tissue culture plates and incubated for at least 20 minutes in the incubator.
All the cells were thawed in a water bath at 37°C and transferred to a 15ml tube containing 5ml Culture medium. Cells were centrifuged at 100g for 2 minutes and the supernatant was aspirated. After resuspending the cell pellet in Culture medium, the cells were transferred to either a 10cm or a 15cm tissue culture plate containing 10ml or 20ml of Culture medium, respectively. The medium was changed every day using Culture medium without Rock inhibitor.

3.1.2 Freezing of cells

The medium was aspirated from the plates and cells were washed once with PBS. In a next step, Gentle Cell Dissociation Reagent (GCDR) (StemCell™ Technologies) was added, and cells were incubated for 5 minutes in the incubator. Afterwards, GCDR was removed and PBS was added. Cells were scraped from the plate using a cell scraper. The cell suspension was transferred to a 50ml tube and tissue culture plates were washed with PBS to collect all the remaining cells. The cell suspension was centrifuged at 100g for 2 minutes, supernatant was aspirated, and the cell pellet was resuspended using Freezing medium. Aliquots were transferred to 1ml cryotubes and slowly frozen at -80°C before they were transferred to liquid nitrogen for long term storage the next day.

3.1.3 Splitting of cells

Cells were grown to 95% confluency before they were passaged, usually every 5-6 days. The medium was aspirated from the plates and cells were washed with PBS. Gentle Cell Dissociation Reagent was added to the plates, and cells were incubated for 5 minutes in the incubator. Afterwards, GCDR was aspirated and PBS was added before cells were scraped from the plate using a cell scraper. The cell suspension was transferred to a 50 ml tube and plates were washed with PBS to collect remaining cells. Cell suspension was centrifuged at 100g for 2 minutes. The supernatant was removed, and cells were resuspended in Culture medium before they were transferred to new plates. Cells were usually split in a ratio of 1:5. The medium was changed every day using Culture medium without the supplement of Rock inhibitor.

3.2 Adaptation of hESCs to Spinner flasks

The medium was aspirated from five 15cm plates with a confluency of 95% each. Plates were washed with PBS once, 10ml of GCDR was added and incubated for 5 minutes at 37°C. Afterwards, the supernatant was removed, 10ml PBS was added, and cells were scraped from the plates using a cell scraper. All cells were collected in a 50ml tube. Plates were washed twice using PBS to collect all the remaining cells. Tubes were centrifuged at 100g for 2 minutes. In a next step, the supernatant was aspirated, and all cells were resuspended in 50ml Splitting medium. The cell number was determined using the Vi-CELL XR Cell Viability Analyser (Beckman Coulter). Therefore, 50µl of the cell suspension was transferred into a cell count tube and 450µl of Splitting medium was added (ratio 1:10). For the adaptation to the Spinner flask, 1 million cells per 1ml were transferred to a 500ml Corning® disposable Spinner flask (polystyrene, non-treated, 500ml, with two sidearms, sterile). Cells were usually seeded in 150-250ml depending on the total cell count. Spinner flasks were incubated at 5% CO_2 and 37°C on a magnetic stirrer with 65rpm.

3.3 Fed-Batch feeding of hESCs in Spinner Flask

Cells adapted to the Spinner flask were fed every day using Feeding medium. Therefore, 0.112 ml of Feeding medium was added per 1ml of cell suspension.

3.4 Splitting of hESCs in Spinner Flask

Gentle Cell Dissociation Reagent was preheated to 37°C in the water bath. The Spinner flask was left under the laminar flow for a few minutes so that cells would settle down on the bottom of the flask. Afterwards, as much medium as possible was aspirated without removing any cells. Cells were transferred into a 50ml tube through a 300μm pluriStrainer (pluriSelect). The Spinner flask was washed multiple times with PBS to collect all the remaining cells. In a next step, the supernatant was aspirated after the cells were allowed to settle at the bottom of the tube. Cells suspension was transferred to a new 50ml tube through a 37μm reversible strainer (StemCell™ Technologies). Important to note is that the pipette should not touch the strainer to exclude the possibility for cell clusters to go through. In this step, cell suspension was pipetted up and down inside the filter to remove as many dead cells and supernatant as possible. The strainer, now containing all the cells, was flipped over onto a new 50ml tube and 30ml GCDR was used to wash cells from the filter. Then, the cell strainer was again flipped over onto a new 50ml tube for further use. Afterwards, cell suspension was incubated at 37°C in the water bath for 6 minutes and then centrifuged at 100g for 2 minutes. During the incubation, cell aggregates are partially dissociated by the GCDR to prepare for the generation of small clumps. During the incubation step, the Spinner flask was washed to be reused using PBS. Spinner flasks were used three times before they were discarded. After the centrifugation, supernatant was aspirated, cells were resuspended in 30ml Splitting medium and cell suspension was again transferred through the 37μm strainer. This time it was important that the pipette touched the filter to make sure cell clusters dispersed into single cells. For cell counting, the 50ml tube was filled to 50ml with Splitting medium, 50μl of cells suspension was mixed with 450μl of Splitting medium and the Vi-CELL XR Cell Viability Analyser was used. 0.6-0.8x10^6 cells per ml were seeded again in the appropriate amount of Splitting medium, and the remaining cells were discarded or frozen.

3.5 Differentiation of hESCs into SC-derived β cells

The cells were split at least three times before starting differentiation and cell clusters needed to have a size of 200-300nm. Differentiation was started with a concentration of 0.8x10^6 cells/ml for the genome-modified cell lines (B2M-/-, HLA-KO, 2in1-KI, 3in1-KI) and with 0.6x10^6 cells/ml for wildtype cells (HuES8). The differentiation protocol is shown in Table 1. For the differentiation, a mix of 12 factors (Activin-A (R&D Systems), Chir99021 (Stemgent), KGF (Peprotech), Rock inhibitor (StemCell™ Technologies), Sant1 (Sigma), RA (Sigma), LDN193189 (Sigma), PdBU (EMD Millipore), XXI (EMD Millipore), Alk5i II (Axxora), T3 (EMD Millipore) and Betacellulin (Thermo Fisher Scientific)) was added

in a specific order over the course of approximately 5 weeks with each stage descending directly into the next one. The whole differentiation protocol lasts 34 days. 23 out of 25 total differentiations were conducted using Single use bio-reactors for 30ml (CSTOF), whereas two differentiations were done in 500ml Spinner flasks.

Table 1. Differentiation protocol for SC-derived β cells

Day of Diff.	Stage	Day	Media	Factors	Dilution	Final conc.
0	Stage 0	1	mTeSR			
1	Stage 1	1	S1	Acitivin-A CHIR99021 Rock Inh	1:100 1:3333 1:1000	100 ng/ml 1.4 mg/ml 10mM
2	Stage 1	2	S1	Activin-A	1:100	100 ng/ml
3	Stage 1	3	N/A	--------	--------	--------
4	Stage 2	1	S2	KGF Rock Inh	1.1000 1:1000	50 ng/ml 10mM
5	Stage 2	2	N/A	--------	--------	--------
6	Stage 2	3	S2	KGF	1:1000	50 ng/ml
7	Stage 3	1	S3	KGF LDN193189 Sant-1 RA PDBU Rock Inh	1:1000 1:5000 1:4000 1:5000 1:2000 1:1000	50 ng/ml 200 nM 0.25 mM 2 mM 500 nM 10 mM
8	Stage 3	2	S3	KGF Sant-1 RA PDBU Rock Inh	1:1000 1:4000 1:5000 1:2000 1:1000	50 ng/ml 0.25 mM 2 mM 500 nM 10 mM
9	Stage 4	1	S3	KGF Sant-1 RA Rock Inh Activin-A	1:1000 1:4000 1:100000 1:1000 1:2000	50 ng/ml 0.25 mM 0.1 mM 10 nM 5 ng/ml
10	Stage 4	2	N/A	--------	--------	--------
11	Stage 4	3	S3	KGF Sant-1 RA Rock Inh Activin-A	1:1000 1:4000 1:100000 1:1000 1:2000	50 ng/ml 0.25 mM 0.1 mM 10 nM 5 ng/ml
12	Stage 4	4	N/A	--------	--------	--------
13	Stage 4	5	S3	KGF Sant-1 RA Rock Inh Activin-A	1:1000 1:4000 1:100000 1:1000 1:2000	50 ng/ml 0.25 mM 0.1 mM 10 nM 5 ng/ml

14	Stage 5	1	BE5	Sant-1 Beta-Cellulin XXI Alk5i T3 RA	1:4000 1:5000 1:10000 1:10000 1:10000 1:100000	0.25 mM 20 ng/ml 1 mM 10 mM 1 mM 0.1 mM
15	Stage 5	2	N/A	--------	--------	--------
16	Stage 5	3	BE5	Sant-1 Beta-Cellulin XXI Alk5i T3 RA	1:4000 1:5000 1:10000 1:10000 1:10000 1:100000	0.25 mM 20 ng/ml 1 mM 10 mM 1 mM 0.1 mM
17	Stage 5	4	N/A	--------	--------	--------
18	Stage 5	5	BE5	Beta-Cellulin XXI Alk5i T3 RA	1:5000 1:10000 1:10000 1:10000 1:400000	20 ng/ml 1 mM 10 mM 1 mM 0.025 mM
19	Stage 5	6	N/A	--------	--------	--------
20	Stage 5	7	BE5	Beta-Cellulin XXI Alk5i T3 RA	1:5000 1:10000 1:10000 1:10000 1:400000	20 ng/ml 1 mM 10 mM 1 mM 0.025 mM
21-34	Stage 6	Odds	S3	--------	--------	--------
	Stage 6	Evens	N/A	--------	--------	--------

In the final stage, the differentiation efficiency was analysed every week by double staining of the cells for C-peptide/NKX6.1 as well as C-peptide/glucagon.

3.6 Quality control

Samples to analyse the differentiation efficiency were obtained every week after entering the final stage (Stage 6) of the differentiation protocol. Accordingly, 5ml of cell suspension was transferred from the spinner flask to a 15ml tube. After 5 minutes, cells settled down to the bottom of the tube and the supernatant was aspirated. Afterwards, 1ml of TripLE™ Express dissociation buffer was added, and cells were incubated for 7 minutes at 37°C in the water bath. To stop the dissociation reaction, 5ml of medium was added to the cells and cells were pipetted up and down a few times. Next, the cell suspension was centrifuged at 100g for 2 minutes before the supernatant was aspirated again. Single cells were resuspended in 500µl 1% PFA and stored at 4°C.

3.6.1 C-peptide/NKX6.1 and C-peptide/Glucagon double staining

Cells fixed in 1% PFA were washed twice with 1% BD Perm/Wash (BD Biosciences), followed by permealizing the cell membrane by incubating them with 1% BD Cytofix/Cytoperm (BD Biosciences) for 30 minutes. Afterwards, cells were again washed twice with 1% Perm/Wash. In a next step, primary antibodies against C-peptide (rat, Cells Signalling Technologies) and NKX6.1 (mouse, Cell Signalling Technologies) or Glucagon (rabbit, Cell Signalling Technologies) were added to the cells in a concentration of 1:100 in Perm/Wash solution and incubated for 1 hour at room temperature. Cells were again washed twice with Perm/wash before the secondary antibodies 488 donkey anti-rat (C-peptide) and Alexa Fluor 647 donkey anti-rabbit (Glucagon) or Alexa Fluor 647 donkey anti-mouse (NKX6.1) were added at a concentration of 250ng/ml in 250 μl for 30 minutes at room temperature. Finally, cells were washed twice with Perm/wash before they were resuspended in 200μl 1% PFA and analysed on the BD LSR II.

3.6.2 HLA-A/B/C staining

To stain for HLA-A/B/C, fixed cells were washed twice with blocking buffer (4% FBS in PBS) before they were incubated in blocking buffer supplemented with 1:1000 human FcR Blocking Reagent (Miltenyi Biotec) for 30 minutes. Afterwards, cells were again washed twice with 4% FBS and PE anti-human HLA-A/B/C antibody (clone: W6/32, Biolegend) was added in blocking buffer at a dilution of 1:100. For the isotype control PE mouse IgG2a (Biolegend) was used. After incubating the cells for 1 hour in the dark, they were washed with 1% FBS, fixed in 1% PFA and stored at 4°C.

3.7 Immunoassays

After 34-41 days of β cell differentiation (Stage 6 day 14-21), cells were used for immunoassays. Therefore, single use bio reactors and spinner flasks containing the cells were placed in the laminar flow hood for β cells to settle down to the bottom of the flask. In a next step, medium was aspirated, and cells were transferred to a 50 ml tube through a 300nm filter. Flasks were washed multiple times with PBS to collect all the remaining cells. After 5 minutes of incubation, β cells settled down on the bottom of the 50 ml tube and supernatant was aspirated again. To achieve a single cell solution, 5ml TripLE dissociation buffer was added, and cells were incubated for 7 minutes in a 37°C water bath. Afterwards, 20 ml of S3 medium was added to stop dissociation reaction. The cell suspension was filtered through a 40μm cell strainer (Falcon). Next, the cell count was measured using the Vi-CELL XR Cell Viability Analyser. Therefore, 50μl of cell suspension was transferred into a counting tube and 450μl of medium was added. Afterwards, tubes were centrifuged at 100g for 2 minutes before the supernatant was removed, and β cells were resuspended to a concentration of 1 Million cells/ml.

3.7.1 Plating of β cells for immunoassays

For the proliferation as well as the activation assay, 100.000 single β cells were plated in either a 48-well plate or a round-bottomed 96 well plate. The cells were cultured in S3 medium containing 1:1000 human Interferon γ (Fisher Scientific). Plates were coated with Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning) at least 20 minutes prior to the cell seeding.

3.7.2 Activation assay

48 hours after plating the β cells, freshly isolated T cells and peripheral blood mononuclear cells (PBMCs) in T cell medium (500ml X-Vivo™10 chemically defined serum-free medium (Lonza) supplemented with 5% human serum heat-inactivated (Fisher Scientific), 5% Fetal Bovine Serum defined (HyClone), 1% Penicillin/Streptomycin (Corning), 1% GlutaMAX™ (gibco by Life Technologies), 1% Non-Essential Amino Acid solution (ATCC) and 20U human IL2 (VWR)) were added in a ratio of 1:1 to each well of the plate. After another 48 hours of incubation, plates were centrifuged at 2000rpm for 5 minutes and 150µl of the supernatant was transferred to a new 96 well plate and frozen at -20°C for further analysis.

The cells were again centrifuged at 2000rpm for 5 minutes before the remaining supernatant was discarded. Cells were then resuspended in 150µl blocking buffer (4% FBS/PBS) and incubated on ice for 30 minutes. Afterwards, 50µl of blocking buffer containing the antibodies Pacific Blue anti-human CD3 (Biolegend), PE/Cy7 anti-human CD4 (Biolegend), PE anti-human CD8 (Biolegend) and Alexa Fluor 647 anti-human CD69 (Biolegend) was added. Cells were again incubated for one hour on ice in the dark. Next, plates were centrifuged, and supernatant was discarded. After washing the plates twice with 150µl PBS, cells were fixed in 1% PFA and stored at 4°C light protected. Plates were analysed using the BD LSR II.

3.7.3 Proliferation assay

48 hours after plating the cells, freshly isolated and CSFE-stained (Cell Trace CSFE Cell Proliferation Kit for Flow Cytometry, Life Technology) T cells or PBMCs were added in a ratio of 1:1. Plates were incubated for 5 or 7 days at 37°C. After 4 days, 500µl T cell medium was added to the plates. At day 5 and day 7, plates were centrifuged at 2000rpm for 5 minutes. 150µl of supernatant of each well was transferred to a new plate and frozen at -20°C for further analysis. Another 400µl of supernatant was discarded before cells were transferred to a new 96-well plate. Next, plates were centrifuged at 2000 rpm for 5 minutes and the remaining supernatant was discarded. Cells were resuspended in 50µl blocking buffer and incubated for 30 minutes. Afterwards, 50µl of blocking buffer containing the antibodies for APC anti-human CD3 (Biolegend), PE/Cy7 anti-human CD4 (Biolegend) and PE anti-human CD8 (Biolegend) were added and plates were incubated for one hour in the dark. In a next step, plates were centrifuged again at 2000rpm for 5 minutes and supernatant was discarded. At last,

cells were washed twice with 1% FBS/PBS, fixed with 1% PFA and stored light protected at 4°C. Plates were analysed using the BD LSR II.

3.8 Isolation of T cells and PBMCs from blood

Blood bags were collected from the Massachusetts General Hospital (MGH) with the patients consent one day prior to cell isolation. First, the blood bag was mixed well to prevent clumping and then cut open. The first few ml were trashed, before 3ml of blood were transferred into a 15ml tube, using separate tubes for T cells and PBMCs. For T cell isolation 150µl of RosetteSep™ Human T cell Enrichment Cocktail (StemCell™ Technologies) were added and tubes were incubated for 20 minutes at room temperature. In the meantime, new 15ml tubes were prepared containing 5ml of Ficoll-Paque™ PLUS (GE Healthcare). After incubation, the blood was diluted with 5ml PBS and very carefully layered on top of Ficoll-Paque™ PLUS. Tubes were centrifuged at 2000rpm for 20 minutes. Important to note is that the brake needs to be turned off on the centrifuge for the different phases not to mix. After the centrifugation, the upper phase was aspirated, leaving approximately 1-2ml. The cell phase which was located between upper PBS and lower Ficoll-Paque™ PLUS phase and contained the T cell and PBMCs respectively, was transferred to a new 50ml tube and washed twice with PBS. Before the second washing step cell count was taken. Finally, T cells and PBMCs were resuspended in a concentration of $10x10^6$/ml in PBS.

3.8.1 CFSE staining

For the CFSE staining the Cell Trace CFSE Cell Proliferation Kit for Flow cytometry from Life Technology was used. Therefore, 5 million T cells and PBMCs were centrifuged and resuspended in 1ml 1µM CSFE solution (1:5000 CSFE dye in PBS). Tubes were incubated for 20 minutes in a 37°C water bath with light protection. Afterwards, 10ml DMEM supplemented with 10% FBS was added and cells were incubated for 5 more minutes in the water bath. During the incubation, the cell count was measured again. At last, cells were centrifuged at 3000rpm for 4 minutes before the medium was aspirated and cells were resuspended in T cell medium to a concentration of $1x10^6$/ml.

3.9 Cytokine assay

For the cytokine profiling of the frozen plates containing the supernatants of all immunoassays, the U-PLEX Biomarker Group 1 (human) Multiplex Assays (MSD U-PLEX Platform) was used.

3.9.1 Preparation of U-PLEX plate

The preparation of the U-PLEX plate involves the coating of the plate with Linker-coupled capture antibodies. It is important that a different Linker is used for each unique biotinylated antibody. First, 200µl of each biotinylated antibody was mixed with 300µl of the assigned Linker, mixed by vortexing

and incubated for 30 minutes at room temperature. In a next step, 200µl Stop solution were added to each tube, again mixed and incubated for 30 minutes at room temperature.
600µl of each U-PLEX-coupled antibody solution was combined together into one single tube and mixed to create the multiplex coating solution. 50µl of multiplex coating solution was now added to each well of the plate. The plate was then sealed with an adhesive plate seal and incubated while shaking over night at 4°C. The next day, the plate was washed 3x with 150µl/well PBS supplemented with 0.05% Tween-20 (PBS-T).

3.9.2 Preparation of Calibrator standards

First, each vial of Calibrator was mixed with 250µl of Diluent 43 to get a 5x concentrated stock solution of each Calibrator, inverted three times and incubated at room temperature for 15 minutes. Each Calibrator was diluted 5-fold to generate the highest point in the standard curve. The Calibrator standards were diluted as shown in Figure 8.

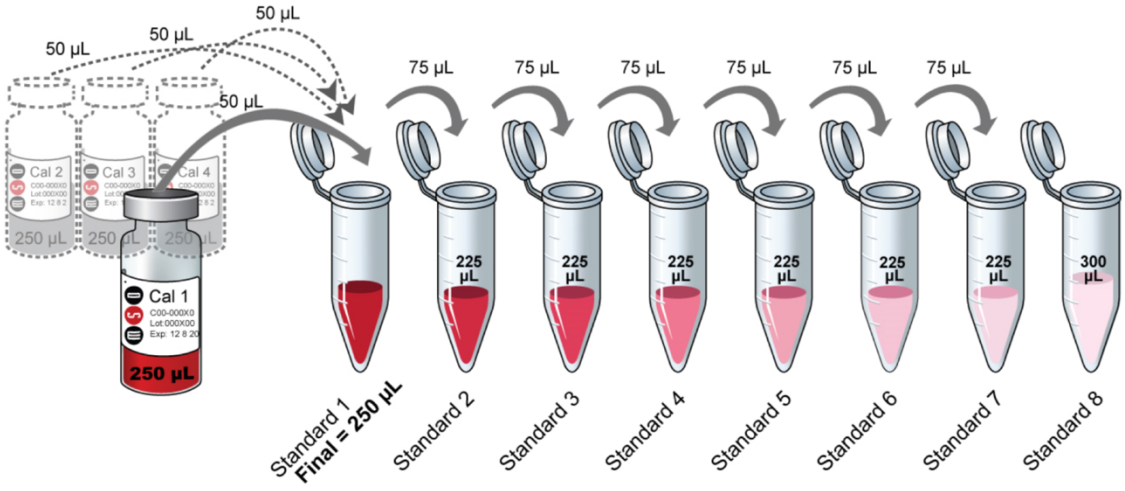

Figure 8. Dilution schema for preparation of Calibrator Standards for U-PLEX Biomarker Group 1 (human) assays[60]

3.9.3 Assay protocol

In a first step, 25µl of Diluent 43 was added to each well, followed by 25µl of the prepared Calibrator standard or sample. The plate was sealed with an adhesive plate seal and incubated at room temperature while shaking at 750rpm. After incubation, the plate was washed 3x with 150µl/well PBS-T. Next, 50µl of detection antibody solution was added to each well, the plate again sealed and incubated for an hour at room temperature while shaking. Afterwards, the plate was again washed 3x with 150µl/well PBS-T before 150µl of 2x Read buffer T was added to each well. Then, the plate was analysed using an MSD instrument. The results were analysed using the Four Parameter Logistic Curve on myassays.com followed by Prism.

3.10 Cardiomyocyte differentiation

Before differentiation was started, cells were grown to 80%-90% confluency. Therefore, plates were coated using Geltrex™ (gibco) at least 1 hour before cells were thawed or split. One aliquot of Geltrex stock was diluted 1:100 with DMEM/F12 medium and equally distributed into the wells of a 12-well plate. StemFlex™ Basal Medium (DMEM/F12(ham) (1:1)) (gibco) was prepared adding 5μM Rock inhibitor. To split cells, old medium was aspirated and 5ml of GCDR was added to the plate. The plate was incubated at 37°C for 6 minutes before the GCDR was again removed. Immediately 5ml of StemFLex supplemented with Rock inhibitor was added to the plate and cells were scraped from the plate using a cell scraper. Cell suspension was collected in a 50ml tube and cell were counted. For the 12 well plate, Geltrex was aspirated and 75.000 cells per well were plated in 2ml StemFlex medium and incubated at 37°C. In the following days, the medium was aspirated and replaced with fresh StemFlex medium (without Rock inhibitor) every day until the cells were confluent and ready for differentiation. On the first day of differentiation, RPMI 1640 medium containing glucose (Thermo Fisher) was mixed with 0.02% B-27 supplement (Thermo Fisher, minus insulin) and placed in a 37°C water bath. Right before starting differentiation, RPMI/B27 medium was mixed with 12mM CHIR99021, 1:1000 ascorbic acid and 1:10.000 Acitivin-A. Old medium was replaced with new medium. Two days later, the medium was aspirated and RPMI/B27 medium with 1:1000 ascorbic acid was added. The following day, 1ml of each well was collected into 50ml tube combined and the same amount of fresh RPMI/B27 as the collected medium was added to the tube. 5mM Wnt inhibitor (WP-4) (stemgent) as well as 1:1000 ascorbic acid and 1:20.000 BMP-4 (stemgent) were added to the combined medium. The remaining medium was aspirated from the plate and replaced with 2ml/well of the combined RPMI/B27 medium. Again, two days later, the medium was removed and RPMI/B27 with 1:1000 ascorbic acid was added. On day 7, RPMI/B27 was prepared with 10μg/ml insulin (Sigma Aldrich) and replaced the old medium. Three days later, the medium was again changed with RPMI/B27 containing insulin. Every 3 days after that medium was changed with RPMI/B26 containing insulin. The cardiac differentiation protocol is summarized in Table 2. The beating of the cardiomyocytes should start at day 12.

Table 2. Differentiation protocol for cardiomyocytes

Day of Diff.	Media	Factors	Conc.
0	StemFlex Basal Medium	Rock inh	5μM
1	RPMI/B27 medium	CHIR99021 Ascorbic acid Acitivin-A	12mM 1:100 1:10000
3	RPMI/B27 medium	Acorbic acid	1:1000
4	50:50 old:new RPMI/B27 medium	WP-4 Ascorbic acid BMP-4	5mM 1:1000 1:20000
6	RPMI/B27 medium	Ascorbic acid	1:1000
7	RPMI/B27 medium	Insulin	10μg/ml
10	RPMI/B27 medium	Insulin	10μg/ml
13	RPMI/B27 medium	Insulin	10μg/ml
16	RPMI/B27 medium	Insulin	10μg/ml
19	RPMI/B27 medium	Insulin	10μg/ml

* green: Start of cardiomyocyte beating

3.10.1 Cardiac Troponin/HLA-A/B/C double staining

Medium was aspirated from plates containing differentiated cardiomyocytes and the cells were washed once with PBS. Cells were dissociated using TripLE dissociation buffer and reaction was stopped with RPMI/B27 medium. Cell suspension was collected in a 50ml tube and centrifuged at 300g for 3 minutes. Afterwards, the supernatant was aspirated, cell pellet was resuspended in eBioscience™ Flow Cytometry Staining Buffer (Invitrogen) and transferred to an Eppendorf tube. Cells were again centrifuged and resuspended in eBioscience™ Flow Cytometry Staining Buffer supplemented with 1:1000 FcR Blocking Reagent. After 30 minutes of incubation at room temperature, tubes were washed twice with eBioscience™ Flow Cytometry Staining Buffer. First, the cells were stained for HLA-A/B/C. Therefore, 4% FBS containing 1:20 PE anti-human HLA-A/B/C antibody was added to the cells, and they were incubated for another 30 minutes at room temperature. In a next step, cells were washed twice before they were fixed with 1% PFA for 20 minutes at room temperature. After fixing, the cell membrane was permealized with 1% BD Cytofix/Cytoperm for 30 minutes. Afterwards, the cells were again washed twice with 1% Perm/Wash. Alexa Fluor 647 anti-Cardiac Troponin T antibody (clone:13-11, BD Pharmingen) in 1% Perm/wash was added for 30 minutes at room temperature before the cells were again washed twice and finally resuspended in 1% PFA.

3.11 Immunoassays using Cardiomyocytes

For both the activation as well as the proliferation assay, the plates containing the cardiomyocytes were used without further sorting the different cell types contained in the plates. Both assays were conducted as described in 3.7.2 and 3.7.3.

3.12 Statistical analysis

All the samples were analysed on a BD LSR II FACS machine. The collected data was analysed using FlowJo v10 (FlowJo, LLC).

Statistics were done utilizing Prism7 (GraphPad Software). For statistical analysis, a parametric t-test, paired was performed with error bars representing SEM.

4 Results

4.1 Reduced growth rate of genome-edited HuES8 cells

Wildtype HuES8 ESCs as well as the knock-out (B2M-KO, HLA-KO) and the knock-in (2in1-KI, 3in1-KI) ESCs were thawed and expanded into 5x15 cm plates before spinner adaptation. After spinner adaptation, ESCs were split at least three times before starting a differentiation. During this procedure it could be observed that genome-edited ESCs did grow slower compared to wildtype HuES8 ESCs. Therefore, we quantified the cell count and survival rate during every expansion step to display the growth rate of genome-edited knock-out and knock-in cell lines compared to wildtype HuES8 cells.

The knock-out, knock-in and wildtype ESCs were split every 3-4 days. If possible, $20x10^6$ cells with a concentration of $1x10^6$ cells/ml were seeded into a spinner flask after every expansion step. The first splitting was performed three days after spinner flask adaptation. The growth rate of all four genome-edited cell lines in suspension is displayed in Figure 9. It could be observed that all the ESCs in general showed a decrease in the cell number when splitting them after three days. Normally, the cell count would double again when the cells were left in maintenance culture for four days. An important influence on cell count may be the passaging number. Especially, the knock-in clones already had a high passaging number (p90-95) before the start of differentiation, which also seemed to correlate with the extended doubling time of the ESCs. Moreover, the knock-out and knock-in ESCs both displayed a lower cell survival during splitting than wildtype cells. For the wildtype HuES8 ESCs the survival rate was at approximately 95-98% (data not shown), whereas the genome-edited ESCs showed a reduced survival rate of 80-87%.

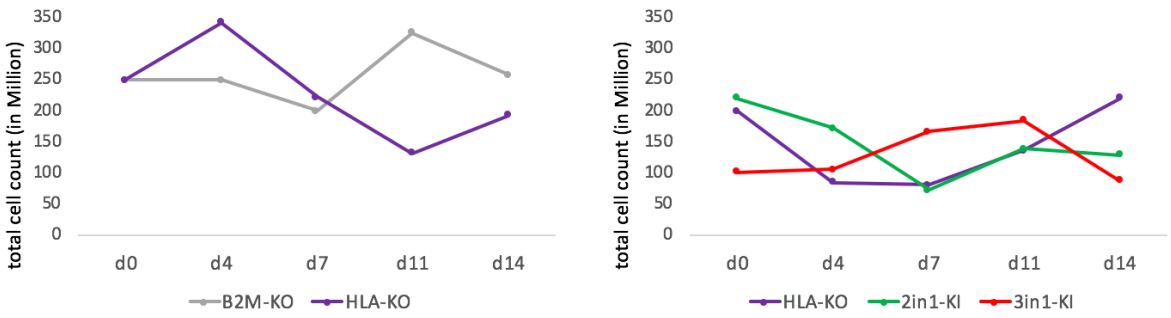

Figure 9. Growth rate of genome-edited ESCs. Genome-edited ESCs show a decrease in cell count 3 days after being split. The cell count recovered when ESCs were propagated in maintenance medium for four days before being split.

4.2 Validation of the absence of HLA-A/B/C in the genome-edited stem cell-derived β cells

The differentiations DJ24 (2in1-KI) and DJ25 (HuES8) were stained with an anti-HLA-A/B/C antibody to confirm the absence of the HLA antigens in the genome-edited cells as well as the presence in wildtype cells. The 2in1-KI was chosen because it is deficient of HLA-A/B/C and additionally has the immunomodulatory molecules PD-L1 and CD47 integrated, but not HLA-G (3in1-KI). In previous experiments, we could see expression of HLA-A/B/C in the 3in1-KI, which could be related to the anti-HLA-A/B/C antibody cross-reacting with HLA-G. The use of the 2in1-KI bypasses this problem. Wildtype SC-derived β cells as well as the 2in1-KI SC-derived β cells were stimulated beforehand with interferon γ for 48 hours. In Figure 10A, the expression of HLA class I in the stimulated and unstimulated 2in1-KI stem cell-derived β cells as well as the wildtype HuES8 SC-derived β cells are compared to an isotype control using histograms. It is visible that interferon γ stimulation leads to an upregulation of the HLA-A/B/C antigens on the surface of the cells. In contrast, the 2in1-KI does not show HLA-A/B/C expression with or without interferon γ stimulation. Figure 10B shows the percentage of HLA class I expression in wildtype HuES8 (w/o interferon γ=3.22%, w/ interferon γ=14.80%) and 2in1-KI SC-β cells (w/o interferon γ=2.01%, w/ interferon γ=2.33%) not stimulated and stimulated in comparison to an isotype control (w/ interferon γ=2,03%). Clearly noticeable again is the upregulation of the HLA-A/B/C expression in HuES8 SC-derived β cells upon stimulation.

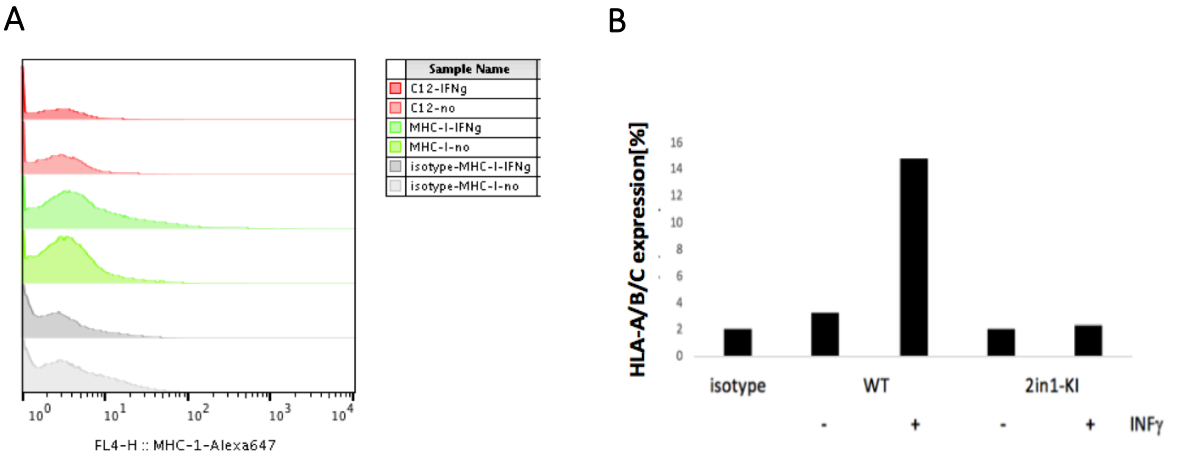

Figure 10. HLA class I expression is upregulated upon stimulation in HuES8-derived β cells compared to 2in1-KI-derived β cells. Wildtype HuES8 β cells as well as 2in1-KI stem cell-derived β cells were either stimulated with interferon γ for 48 hours or kept in maintenance. HuES8 cells show an upregulation of HLA-A/B/C expression upon stimulation. The 2in1-KI cell line does not show HLA-A/B/C expression due to the lack of HLA class I genes. (A) Histogram of the wildtype HuES8 β cells and 2in1-KI β cells as well as an isotype control also stimulated and without stimulation. (B) The upregulation of HLA class I in wildtype HuES8 SC-derived β cells upon stimulation with interferon γ (14.80%) is clearly evident compared to wildtype HuES8 β cells without stimulation (3.22%) and to the 2in1-KI β cells with (2.33%) or without stimulation (2.01%).

4.3 Stem cell-derived β cell differentiation efficiency is lower in KO- and KI-cell lines compared to WT HuES8 SC-derived β cells

The first aim of this thesis was to prove that heavily genome-edited ESCs are able to differentiate into SC-derived β cells. Therefore, both knock-out cell lines (B2M-KO, HLA-KO) as well as the knock-in cell lines (2in1-KI, 3in1-KI) were differentiated into β cells according to a well-established protocol by Pagliuca *et al*. used for differentiation of HuES8 or iPSCs into SC-derived β cells. Differentiation was started with a concentration of $0.8x10^6$ cells/ml for the knock-out and knock-in genotypes, and $0.6x10^6$ cells/ml for the wildtype HuES8 cells. Due to the fact that wildtype HuES8 cells were expanding faster in maintenance medium, a lower starting concentration was used than for the genome-edited ESCs. In total, 25 differentiations were accomplished (DJ1-DJ25): 4 B2M-KO, 7 HLA-KO, 8 2in1-KI, 5 3in1-KI and one wildtype HuES8 (Table 2).

Table 3. Genotypes of the different differentiations (DJ1-DJ25) performed in this study.

Genotype				
B2M-KO	HLA-KO	2in1-KI	3in1-KI	WT
DJ1	DJ5	DJ11	DJ9	DJ24
DJ2	DJ6	DJ12	DJ10	
DJ3	DJ7	DJ13	DJ21	
DJ4	DJ8	DJ14	DJ22	
	DJ15	DJ18	DJ23	
	DJ16	DJ19		
	DJ17	DJ20		
		DJ25		

Differentiations were done in single use bioreactors containing $30x10^6$ cells/bioreactor. Only differentiations DJ24 (HuES8) and DJ25 (2in1-KI) were done in 500ml spinner flasks, starting with $150x10^6$ cells and $200x10^6$ cells in total, respectively. We observed that the genome-edited cell lines did decrease in cell mass over time, especially after entering the final stage (Stage 6). Figure 11 shows the bottom view of two spinner flasks containing the 2in1-KI SC-derived β cells and the wildtype SC-derived β cells at stage 6 (day 14).

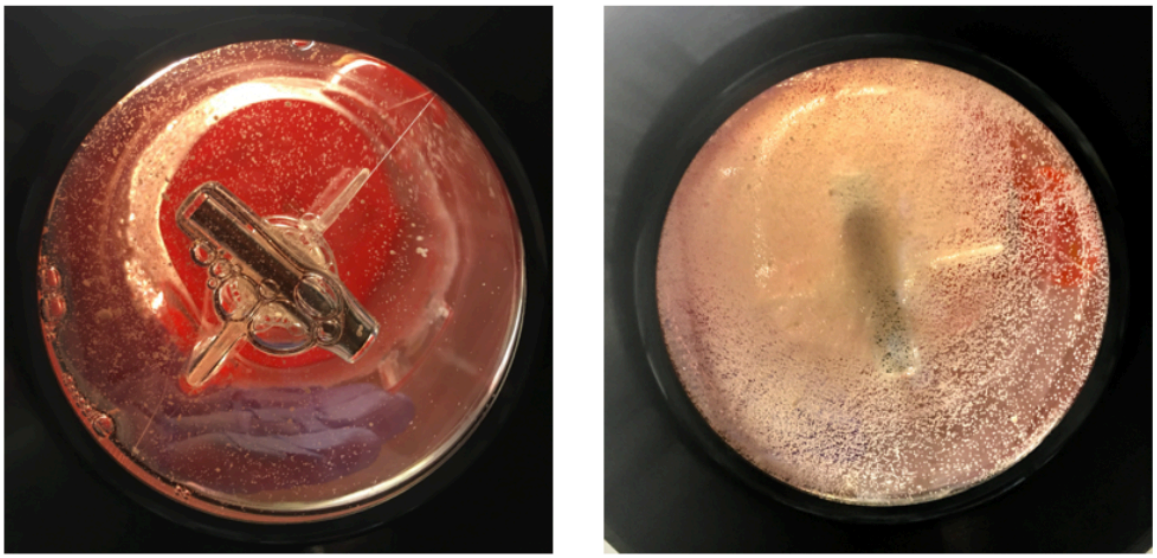

Figure 11. Bottom view of two spinner flask cultures of the 2in1-KI (left) and the wildtype HuES8 (right) SC-derived β cells on day 14 of differentiation in stage 6. The knock-in differentiation culture shows a significantly reduced cell number compared to the wildtype differentiation culture.

After entering the final differentiation stage, samples were obtained every week to check the differentiation efficiency. For this purpose, cells were double-stained for the β cell specific markers C-peptide/NKX6.1 as well as for the polyhormonal markers C-peptide/Glucagon. C-peptide connects the A-chain to the B-chain in proinsulin and can be used as a marker for insulin-producing cells[61]. NKX6.1 is a transcription regulator which is necessary for the development of β cells in the pancreas, whereas glucagon is a hormone which is released by α cells in the pancreas when the insulin concentration in the blood reaches a low[62,63]. Therefore, C-peptide/NKX6.1 is used as a staining for β cells, whereas C-peptide/Glucagon is used to stain for polyhormonal cells. Figure 12 displays the gating strategy for both stainings. After gating on all cells, the next step was to gate only on single cells before dividing cells into four groups: unstained, C-peptide$^+$ cells, NKX6.1$^+$ or Glucagon$^+$ cells and double-stained cells for either C-peptide$^+$/NKX6.1$^+$ or C-peptide$^+$/Glucagon$^+$.

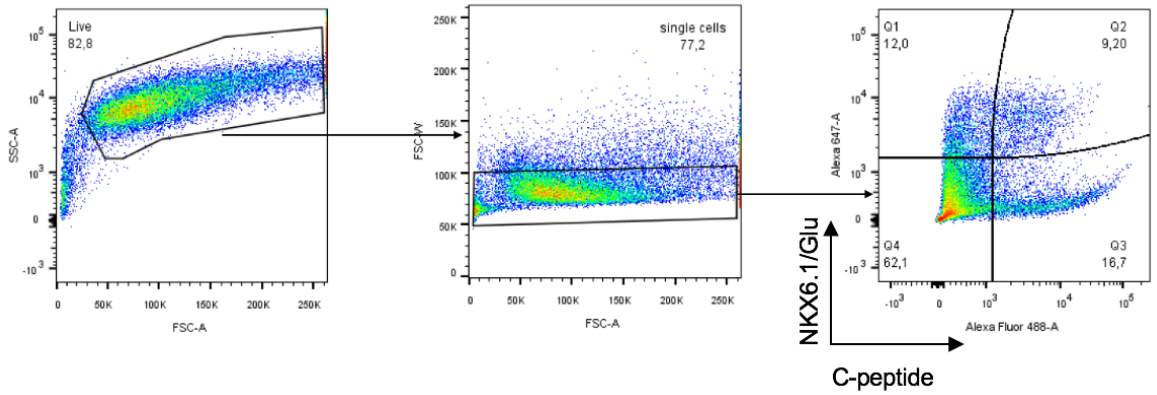

Figure 12. Gating strategy for the double-staining of SC-derived β cells with the specific β cell markers C-peptide/NKX6.1 as well as the polyhormonal cell markers C-peptide/Glucagon. First, dead cells were excluded before it was gated on the single cells. Finally, SC-derived β cells were divided into unstained, only C-peptide$^+$, NKX6.1$^+$ or Glucagon$^+$ as well as double-positive for C-peptide/NKX6.1 or C-peptide/Glucagon.

DJ1-DJ23 were done using a single-use bioreactor, whereas DJ24 and DJ25 were accomplished in a 500ml spinner flasks. Figure 13 demonstrates the differentiation efficiency of heavily genome-edited cells into β cells for all 25 differentiations, gated on C-peptide$^+$/NKX6.1$^+$ cells. Cells for the staining of DJ1-DJ8 were obtained on the first day of the final stage, for DJ9-12 on day7, for DJ13-14 on day 14, for DJ15-23 on day 10 and for DJ24-25 on day 21 after entering the final stage. All cells showed a rather low differentiation efficiency of 0.5%-11%. In comparison, wildtype HuES8-derived β cells differentiated by the Melton lab reached a differentiation rate up to 30%. It is worth mentioning that the wildtype HuES8 β cells (DJ24), differentiated side by side, also displayed an efficiency of only 6%.

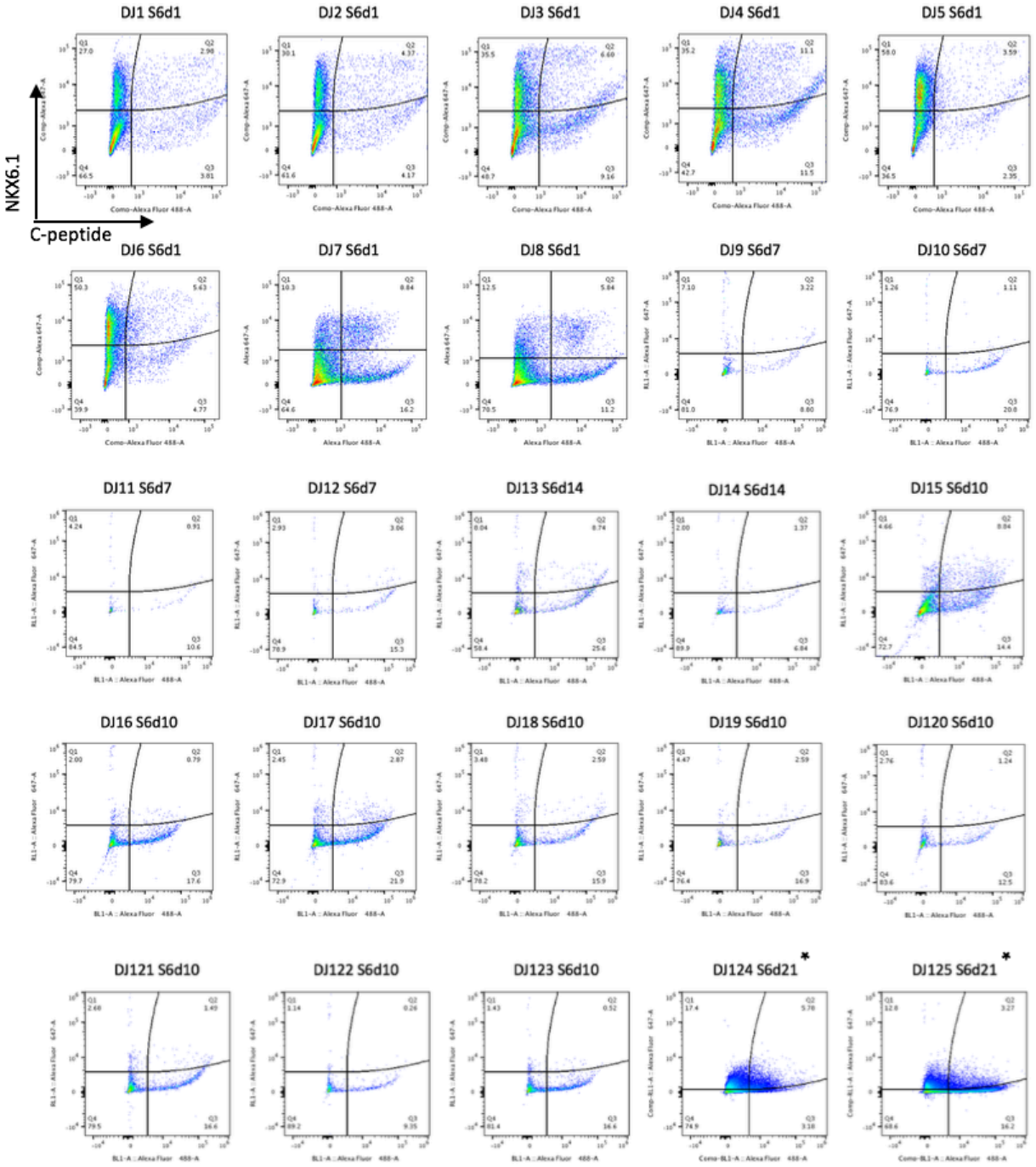

Figure 13. Quality control of the indicated SC-derived β cells differentiations (DJ1-DJ25): C-peptide/NKX6.1. The differentiation efficiency of HLA-KO β cells is lower compared to wildtype HuES8 β cells. β cells were stained for the β cell markers C-peptide and NKX6.1 to check differentiation efficiency of knock-out and knock-in cell lines compared to wildtype cells. All differentiations were done in single-use bioreactors except for (*) DJ24 (2in1-KI) and DJ25 (HuES8), where 500ml Spinner flasks were used.

As mentioned before, C-peptide/Glucagon is used to stain for polyhormonal cells in a cell population. Figure 14 displays the differentiations (DJ1-DJ25) at the same time points as in Figure 13, but the cells

are stained for C-peptide/Glucagon. It is visible that the percentage of polyhormonal cells in each differentiation except in differentiation DJ25 (HuES8) is higher than the β cells population. All the genome-edited cell lines showed a double-positive population of 3%-22%, whereas the wildtype cell differentiation efficiency in this case was under 0.5%.

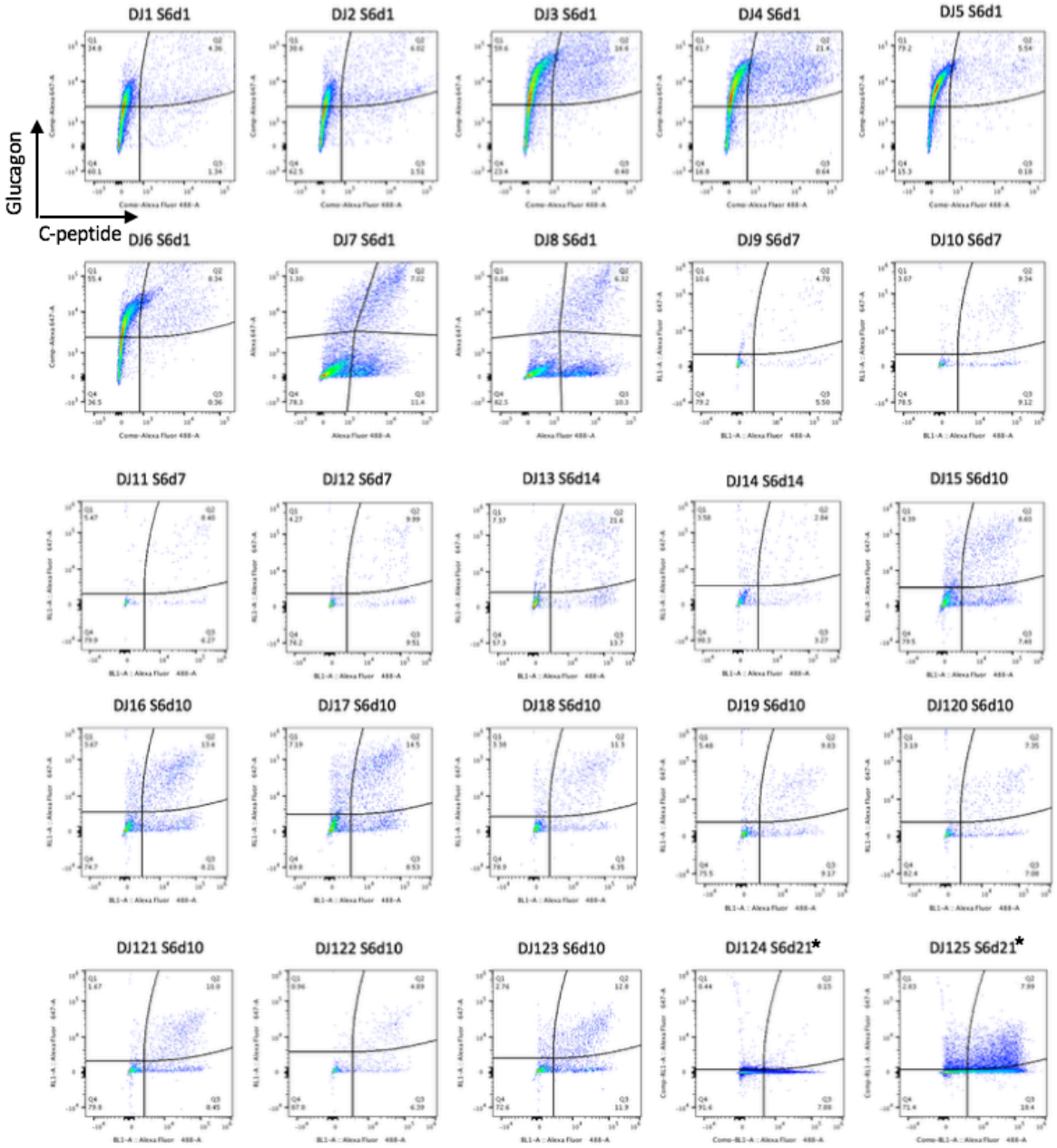

Figure 14. **Quality control of the indicated SC-derived β cells differentiations (DJ1-DJ25): C-peptide/Glucagon.** Differentiated cells (DJ1-DJ25) were stained for C-peptide and Glucagon after entering the final stage to check for polyhormonal cells (C-peptide$^+$/Glucagon$^+$). All differentiations were performed in single-use bioreactors except for (*) DJ24 (2in1-KI) and DJ25 (HuES8), where 500ml spinner flasks were used.

4.4 β cell percentage decreases over time whereas percentage of polyhormonal cells remains constant

As described in chapter 4.3, the differentiation efficiency into β cells was between 0.5%-11%. Reference differentiation efficiencies of HuES8 cells done by the "Foundry" (Melton lab, Harvard University) could reach up to 30% after 14 days in Stage 6. In general, it can be observed that β cells derived from iPSCs or ESCs increase their β cells percentage over time in spinner flask culture. To test if the knock-out as well as the knock-in cell lines also enhance their percentage over time, we took samples every week and stained for C-peptide/NKX6.1 as well as C-peptide/Glucagon. Figure 15 shows the differentiation efficiency for every differentiated cell line over the time course of 14-21 days. It can be observed that the percentage of C-peptide$^+$/NKX6.1$^+$ cells declines over time in both knock-out and knock-in cell lines. Moreover, polyhormonal cells (C-peptide$^+$/Glucagon$^+$) make a higher percentage of the cell population than β cells. In the B2M-KO, the percentage of polyhormonal cells also seem to decrease over time, whereas in the HLA-KO and in both knock-ins the percentage of polyhormonal cells appears to be stable and did not change over the course of three weeks.

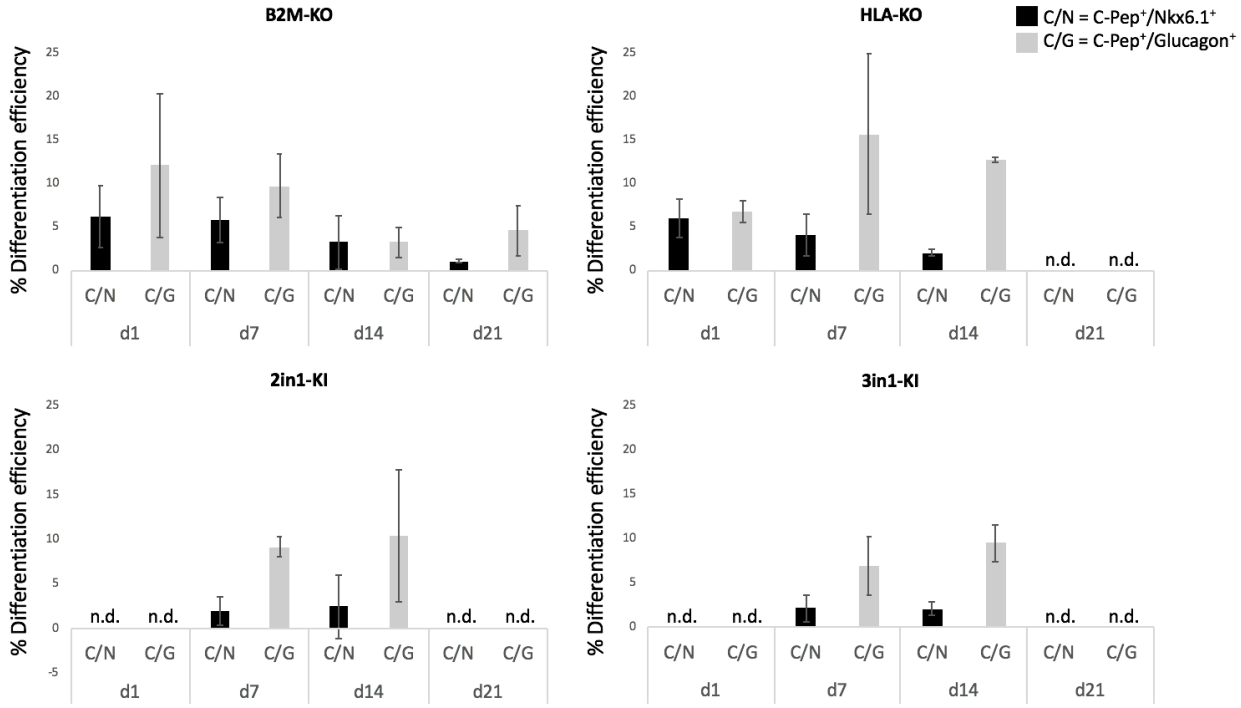

Figure 15. Frequency of SC-derived β cells (black) and polyhormonal cells (grey) over 3 weeks of the final differentiation step in Stage 6 media. Knock-out as well as knock-in cell lines show a decline in differentiation efficiency into β cells over time. Whereas polyhormonal cells are declining in the B2M-KO, they seem to be more stable in the HLA-KO as well as both the 2in1-KI and 3in1-KI over the time of three weeks. Error bars represent standard deviation. n.d.= no data available

To analyze the differentiation efficiency of all cell lines, data obtained from staining β cells for C-peptide/NKX6.1 and C-peptide/Glucagon was combined for every cell line using the average value. Table 4 displays the efficiency for every differentiation performed if available. Beginning from differentiation DJ13 until DJ25, samples were only obtained starting at day 14 after entering the final stage because cell cultures did decline in cell mass and we did not want to sacrifice more cells.

Table 4. DJ1-DJ25 with corresponding genotype and differentiation efficiency after entering stage 6.

		S6d1		S6d7		S6d14		S6d21	
Differentiation	Genotype	%C-pep/Nkx6.1	%C-pep/Glu	%C-pep/Nkx6.1	%C-pep/Glu	%C-pep/Nkx6.1	%C-pep/Glu	%C-pep/Nkx6.1	%C-pep/Glu
DJ1	B2M-/-	2,98%	4,36%	4,32%	5,59%	1,11%	2,08%	1,20%	2,53%
DJ2	B2M-/-	4,37%	6,02%	9,57%	8,78%	5,37%	4,50%	0,93%	6,63%
DJ3	B2M-/-	6,60%	16,60%	4,39%	9,95%	1,91%	13,90%		
DJ4	B2M-/-	11,10%	21,40%	4,93%	14,50%	1,79%	6,39%		
DJ5	HLA-KO	3,59%	5,54%	1,45%	7,09%	1,73%	12,90%		
DJ6	HLA-KO	5,63%	8,34%	2,60%	8,45%	2,37%	12,50%		
DJ7	HLA-KO	8,84%	7,02%	6,60%	25,40%				
DJ8	HLA-KO	5,84%	6,32%	5,58%	21,80%				
DJ9	3in1-KI			3,22%	4,70%				
DJ10	3in1-KI			1,11%	9,34%				
DJ11	2in1-KI			0,91%	8,40%				
DJ12	2in1-KI			3,06%	9,99%				
DJ13	2in1-KI					8,74%	21,60%		
DJ14	2in1-KI					1,37%	2,84%		
DJ15	HLA-KO					8,84%	8,60%		
DJ16	HLA-KO					0,79%	13,40%		
DJ17	HLA-KO					2,87%	14,50%		
DJ18	3in1-KI					2,59%	11,30%		
DJ19	3in1-KI					2,59%	9,83%		
DJ20	3in1-KI					1,24%	7,35%		
DJ21	2in1-KI					1,49%	10,00%		
DJ22	2in1-KI					0,26%	4,89%		
DJ23	2in1-KI					0,52%	12,80%		
DJ24	WT								
DJ25	2in1-KI								

4.5 $CD8^+$ T cells proliferate when engaging wildtype HuES8 SC-derived β cells but not in the presence of hypoimmunogenic SC-derived β cells

The second aim of this Master`s thesis was to determine if the genome-edited SC-derived β cells, featuring the ablation of the HLA-A/B/C genes, are able to circumvent immune detection by primary human immune cells. For this purpose, wildtype HuES8 SC-β cells as well as B2M-KO, HLA-KO and the 2in1- and 3in1-KI-derived β cells were treated with interferon γ 48 hours prior to being co-cultured with freshly isolated CFSE stained human T cells in a ratio of 1:1 for 5 days or 7 days. CFSE dye is used for measuring the number of cellular divisions a cell population did experience. When staining T cells with CFSE, the dye enters the cells and is cleaved by esterases to form a fluorescent aggregate. Upon division of a cell, the fluorescent intensity is halved in each daughter cell (Figure 16A). This process allows to follow the number of the cell divisions a cell population underwent by flow cytometry[64] (Sigma Aldrich) (Figure 16B).

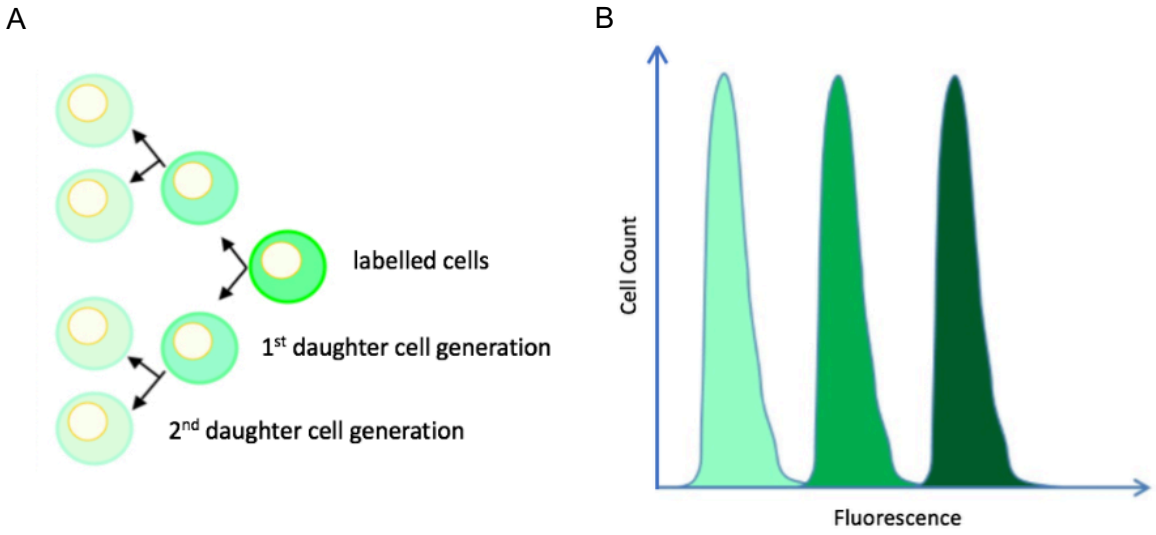

Figure 16. Principle of CFSE dilution as a measure of cell proliferation. The cells are stained with the CFSE dye, which enters the cells and is transformed into the fluorescent compound. (A) Upon division of a cell, the fluorescent dye is passed on to the two daughter cells, which only exhibit half the fluorescent intensity. (B) This process allows the easy tracking of the number of cell divisions of a population of cells by flow cytometry[64]. (Images adapted from[65])

T cells were then stained with fluorophore-coated antibodies against CD3/CD4/CD8 T cells and analysed for the dilution of the CFSE signal. After 7 days, the proliferation assay showed a clear increase in the number of proliferating cytotoxic ($CD8^+$) T cells in the wildtype HuES8 SC-derived β cell co-cultures (12.5% ± 1.11% SEM) compared to the $CD8^+$ T cells co-cultured with the B2M-KO (0.77% ± 0.33% SEM) and HLA-KO (0.60% ± 0.12% SEM) SC-β cells. Figure 17 shows the $CD3^+$, $CD4^+$ and $CD8^+$ T cell compartment for one representative donor (Donor #109). Additionally, there was also increased proliferation in the $CD3^+$ T cell compartment co-cultured with HuES8 SC-β cells (4.47% ± 0.22% SEM), which is understandable given that cytotoxic T cells are part of the $CD3^+$ T cell population. The T helper cells ($CD4^+$) did not show proliferation against either wildtype HuES8 β cells (1.06% ± 0.03% SEM), HLA-KO (1.26% ± 0.31% SEM) or B2M-KO (0.68% ± 0.11% SEM) β cells, similar to the negative control (0.23% ± 0.03% SEM). The positive and negative controls were generated using the same donor. For the negative control, T cells were cultured in T cell medium for 7 days, whereas for the positive control, T cells were stimulated using Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific). After establishing that the proliferation was mainly occurring within the cytotoxic T cell and not the T helper cell compartment, further analysis concentrated on only $CD8^+$ T cells. Our results confirm that cytotoxic T cells display a reduced proliferation against HLA-deficient SC-derived β cells. Similar results were observed when co-culturing PBMCs with HuES8- and HLA-KO-derived β cells (data not shown).

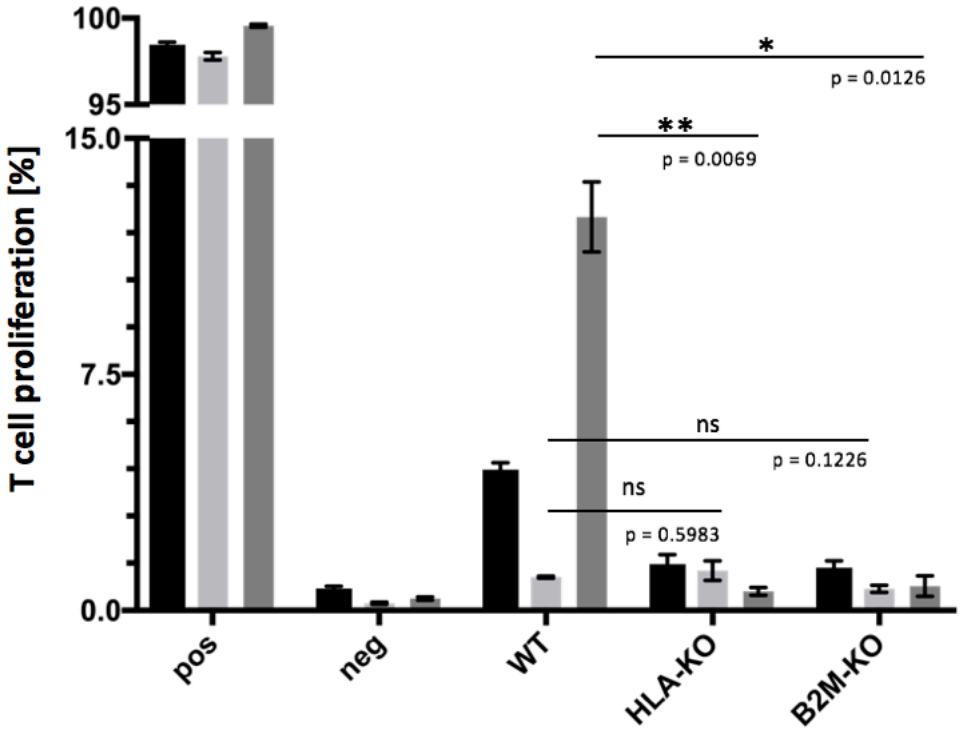

Figure 17. Reduced T cell proliferation against B2M-KO and HLA-KO SC-derived β cells. For this proliferation assay SC-derived β cells were co-cultured with freshly isolated CFSE stained human T cells from one representative donor (Donor #109) for 7 days. Two days prior to the start of the proliferation assay, cells were wqstimulated with interferon γ. Bar graphs displaying the percentage of $CD3^+$ (black), $CD4^+$ (light grey) and $CD8^+$ (dark grey). T cell proliferation against the wildtype HuES8-derived β cells (WT) compared to the T cell proliferation against the HLA-KO and B2M-KO SC-β cells. Positive (pos) as well as negative (neg) controls were used. Only $CD8^+$ T cells co-cultured with wildtype HuES8 SC-β cells (12.5% ± 1.11% SEM) showed proliferation compared to $CD8^+$ T cell against the HLA-KO (0.60% ± 0.12% SEM) and B2M-KO SC-β cells (0.77% ± 0.33% SEM). Due to the fact that $CD8^+$ T cells are part of the $CD3^+$ T cell compartment, they did display proliferation as well against wildtype β cells (4.47% ± 0.22% SEM). Proliferation in the $CD4^+$ T cell compartment could not be observed in either SC-derived β cell line. n=3; T-test parametric, paired. Data are mean ± SEM; n.s.>0.1; *p<0.05; **p<0.01

4.6 T cell proliferation can be observed in co-cultures of different blood donors with wildtype HuES8 SC-β cells but not with HLA-deficient β cells

The proliferation assay was performed in ten biological replicates using 10 different blood donors which three technological replicates for each blood donor. The B2M-KO was utilized in proliferation assays against 4 different blood donors, whereas the HLA-KO was used for 6 assays. For the 2in1-KO, there were 6 proliferation assays performed although the donors #114 and #115 were utilized twice against two different batches of differentiation. For the 3in1-KO, there were three different proliferation assays done.

As expected, the proliferation efficiency showed some donor-to-donor variability. Although the T cell proliferation in response to wildtype HuES8 β cells varied between donors, ranging from 10%-50%, there was no visible response of T cells from any donor to the knock-out and knock-in β cells. Figure 18 displays three independent experiments with four different donors (Donor #109, #110, #114, #115). T cells isolated from donor #109 and #110 showed that there is no proliferation against HLA-KO (#109: 0.60% ± 0.12% SEM; #110: 1.8% ± 0.84% SEM) and B2M-KO (#109: 0.77% ± 0.33% SEM) in the $CD8^+$ T cell compartment, whereas it is clearly visible against wildtype HuES8 β cells (#109: 12.5% ± 1.11% SEM; #110: 42.33% ± 2.28% SEM) although with a different intensity between donors. Similarly, $CD8^+$ T cell from Donor #114 and #115 display the proliferation against wildtype compared to the HLA-KO cells, two independent differentiations of the 2in1-KI and the 3in1-KI cells.

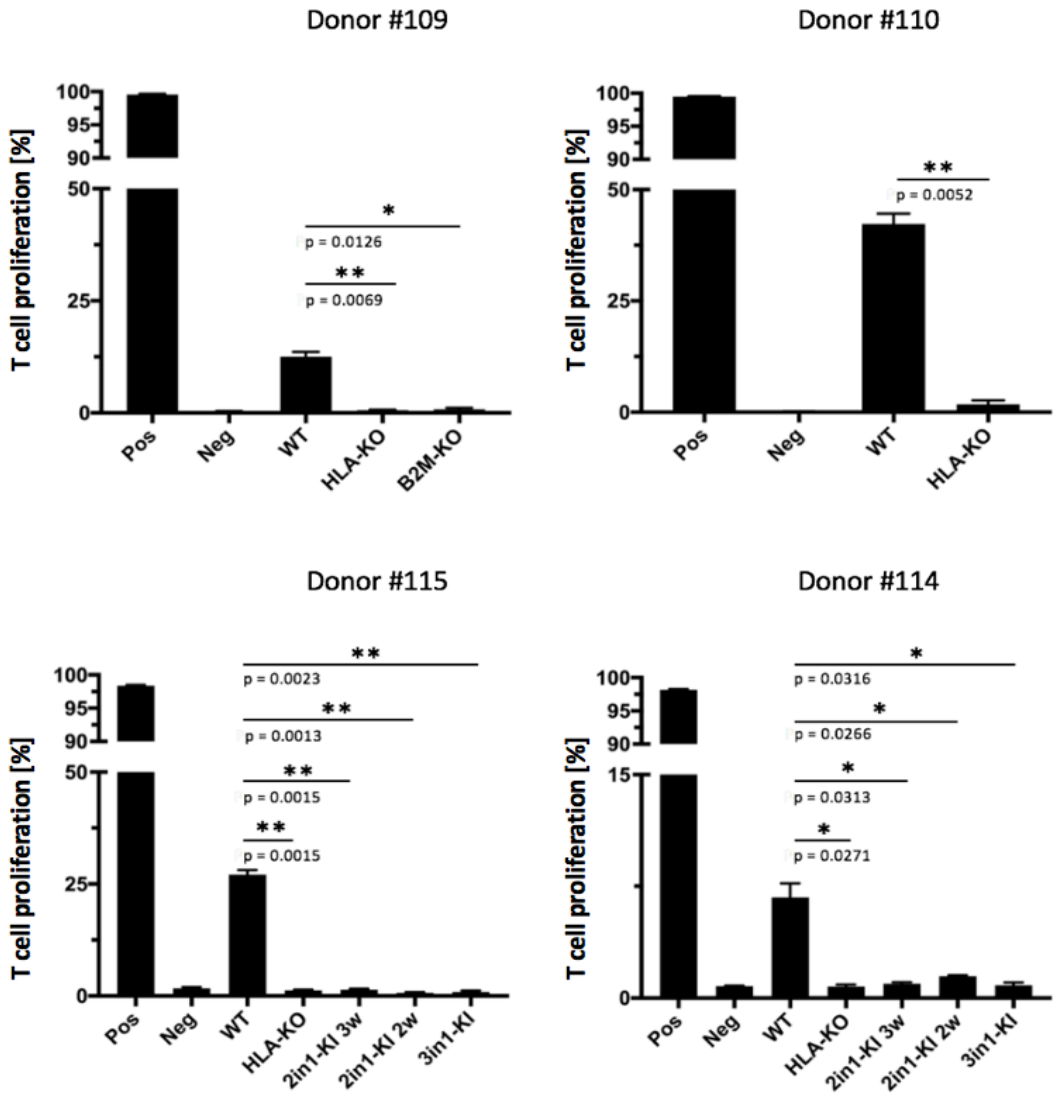

Figure 18. Donor to donor variability is visible in all proliferation immunoassays. Even tough wildtype HuES8 SC-β cells show a variable proliferation after being co-cultured with freshly isolated T cells for 7 days, T cells cultured with the knock-out as well as the knock-in-derived β cells do not show any proliferation. In the top left panel, $CD8^+$ T cells from blood donor #109 present proliferation against the wildtype HuES8 SC-derived β cells (12.5% ± 1.11% SEM) but not against either the HLA-KO (0.60% ± 0.12% SEM) or B2M-KO-derived β cells (0.77% ± 0.33% SEM). $CD8^+$ T cell from blood donor #110 (top right panel) show again proliferation against the HuES8 β cells (42.33% ± 2.28% SEM) but not against the HLA-KO (1.80% ± 0.84% SEM) (right top panel) but with a much higher intensity. The two bottom panels show the proliferation of $CD8^+$ T cells against the wildtype HuES8-derived β cells, the HLA-KO-derived β cells as well as both the knock-in cell lines for two different blood donors (#114, #115). The 2in1-KI is represented twice. For the proliferation against the 2in1-KI two different batches of proliferations were used. One β cell differentiation was in cultured for 3 weeks (2in1-KI 3w) whereas the other was in culture for 2 weeks (2in1-KI 2w). Both proliferation assays displayed proliferation of $CD8^+$ T cells against HuES8 derived β cells (#114: 6.75% ± 0.94% SEM; #115: 27.06% ± 1.02% SEM) but neither against the HLA-KO β cells (#114: 0.77% ± 0.13% SEM; #115: 1.25% ± 0.13% SEM), the 2in1-KI 3w (#114: 0.96% ± 0.11%

SEM; #115: 1.38% ± 1.19% SEM), the 2in1-KI 2w (#114: 1.46% ± 0.06% SEM; #115: 0.71% ± 0.16% SEM) nor the 3in1-KI β cells (#114: 0.86% ± 0.20% SEM; #115: 0.91% ± 0.31% SEM). n=3 for every blood donor; T-test parametric, paired. Data are mean ± SEM; *p<0.05; **p<0.01.

In a next step, we combined the obtained data to prove that even with donor-to-donor variability the proliferation of $CD8^+$ T cells against wildtype HuES8 β cells but not against the genome-edited cell lines is still significant. Figure 19A shows the combined $CD8^+$ T cell proliferation data of 5 different blood donors (#108, #109, #110, #114, #115) with three technological replicates of each donor (n=15) against wildtype HuES8 and HLA-KO β cells. Figure 19B shows the significant proliferation of $CD8^+$ T cells against HuES8 β cells but not against the HLA-KO, 2in1-KI and 3in1-KI β cells displayed. The immunoassay represents three individual blood donors (#113, #114, #115) with 3 biological replicates each (n=9).

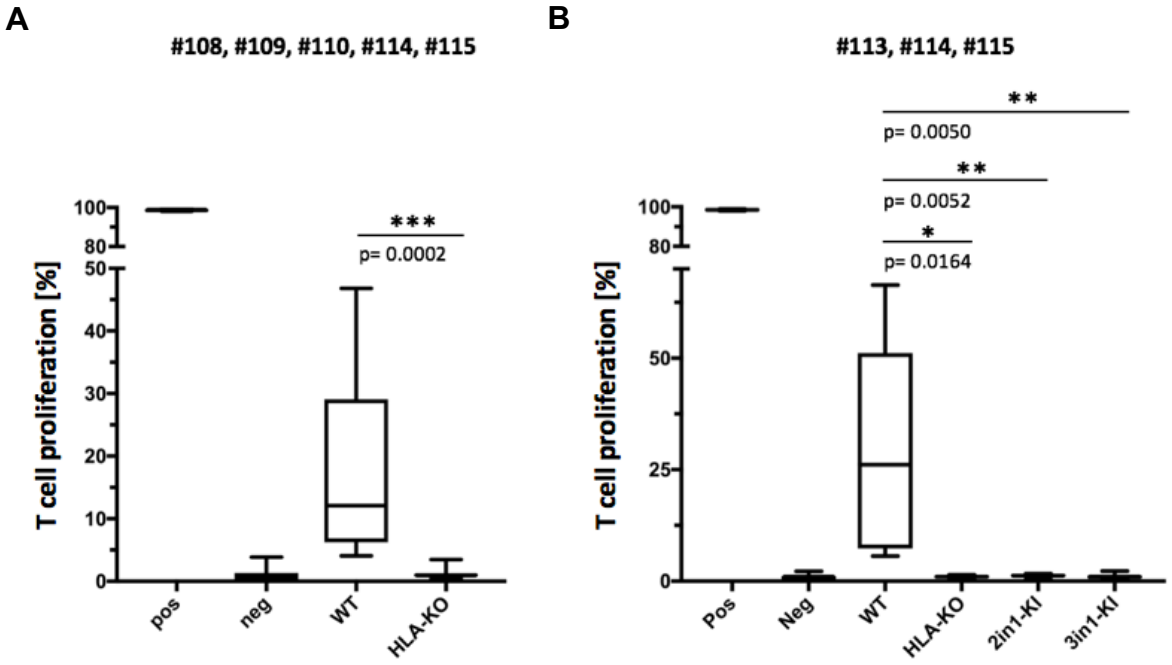

Figure 19. Donor-to-donor variability has no influence on the significance of $CD8^+$ T cell proliferation against wildtype HuES8 β cells. (A) The combined data of $CD8^+$ T cell proliferation of 5 individual blood donors shows compelling proliferation against the HuES8 β cells but not against HLA-KO β cells. n=15; T-test parametric, paired. Data are mean ± SEM; ***p<0.001. (B) Comparison of $CD8^+$ T cell proliferation against wildtype HuES8 β cells and HLA-KO, 2in1-KI, 3in1-KI β cells. n=9; T-test parametric, paired. Data are mean ± SEM; *p<0.05; **p<0.01. In both images, the positive control was achieved co-culturing T cells with Dynabeads Human T-Activator CD3/CD28. After 7 days of incubation, almost all the cells proliferated. For the negative control, T cells were cultured in maintenance medium for 7 days without any stimuli.

4.7 Hypoimmunogenic β cells avoid T cell activation

The activation assay was performed in 4 individual experiments with 8 different blood donors (n=3). Therefore, β cells were co-cultured with freshly isolated T cells for 48 hours before T cells were harvested and stained with the antibodies anti-human CD3, CD4 and CD8 as wells as anti-human CD69, a marker for early activation. Although statistical significance could not be achieved, there is a clear trend visible in the activation. Co-cultures with wildtype HuES8 β cells showed a higher activation of $CD69^+$ T cells than the knock-out or knock-in β cells. Again, donor to donor variability could also be observed in T cell activation against wildtype cells but it did not have any effect on the lack of activation in T cells against the genome-edited cells. Figure 20 displays the expression of CD69 in T cells in two representative experiments with two different blood donors (#107, #113). Image A shows the upregulation of $CD69^+$ T cells from blood donor #107 against wildtype HuES8-derived β cells (40.16% ± 11.45% SEM) as well as against the HLA-KO (5.30% ± 0.80% SEM) and B2M-KO (9.56% ± 1.12% SEM) SC-β cells, whereas image B presents the activation of $CD69^+$ T cells from donor #113 against both knock-in cell lines, 2in1-KI (1.55% ± 0.37% SEM) and 3in-KI (1.65% ± 0.33% SEM), as well as against the HuES8-derived β cells (6.01% ± 1.36% SEM). The positive control was achieved through the activation of T cells with Dynabeads Human T-Activator CD3/CD28. Similar to before, the negative control was generated through culturing freshly isolated T cells in T cells medium for 48 hours without any additional stimuli. A 100% intensity would mean an absolute activation of all the $CD69^+$ T cells.

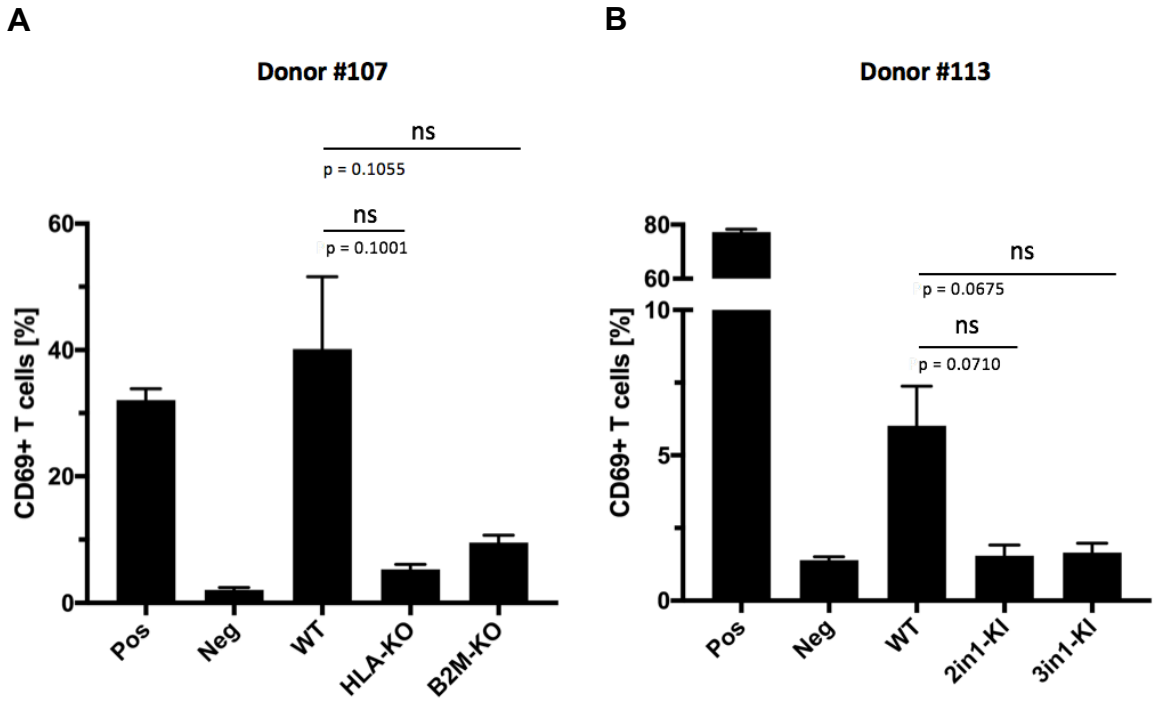

Figure 20. CD69 expression in T cells co-cultured with wildtype HuES8 β cells but not with hypoimmunogenic β cells. Although statistical significance is not reached, there is a clear trend of more pronounced activation of $CD69^+$ T cells when co-cultured with wildtype HuES8 SC-β cells for 48 hours than with the HLA-deficient, genome-edited β cells. (A) $CD69^+$ T cells from blood donor #107 display an almost 8-fold higher intensity against

wildtype β cells (40.16% ± 11.45% SEM) than against HLA-KO β cells (5.30% ± 0.80% SEM) and a 4-fold higher intensity against B2M-KO β cells (9.56% ± 1.12% SEM). n=3; t-test parametric, paired. Data are mean ± SEM; n.s.<0.1 (B) $CD69^+$ T cells from blood donor #113 show an almost 4-fold higher intensity against wildtype β cells (6.01% ± 1.36% SEM) than against the 2in1-KI (1.55% ± 0.37% SEM) as well as the 3in1-KI (1.65% ± 0.33% SEM) β cells. Positive control was again achieved incubating T cells with Dynabeads Human T-Activator CD3/CD28 for 48 hours. For the negative control, T cells were cultured in maintenance medium for 48 hours. n=3; t-test parametric, paired. Data are mean ± SEM; n.s.>0.1

4.8 Cytokine secretion

Cytokines, which includes interferons, chemokines, interleukins, lymphokines and tumor necrosis factors, are small signaling proteins primarily secreted by immune cells. Interferons play an important role in the immune system. Whereas, interferon α and β are secreted by virus-infected cells, interferon γ is secreted by natural killer cells and T cells upon activation[66].

For the cytokine profiling of the frozen plates containing the supernatants of all performed immunoassays, the U-PLEX Biomarker Group 1 (human) Multiplex Assays (MSD U-PLEX Platform) was used. Figure 21 presents the data for the release of interferon γ from T cells co-cultured with HuES8-derived β cells as well as HLA-KO-derived β cells from four different blood donors (#109, #110, #114, #115) combined. For the blood donors #109 and #110, two biological replicates against HuES8-derived β cells and HLA-KO-derived β cells were performed with three technological replicates each. For blood donor #114 and #115, one biological replicate was performed with three technological replicates for each donor. Although all the β cells were stimulated with interferon γ two days prior to starting the immunoassays, T cells against HuES8-derived β cells (14029,76 pg/ml ± 3832,45 pg/ml) show a significant higher release of interferon γ than T cells co-cultured with HLA-KO-derived β cells (5437,10 pg/ml ± 1082,61 pg/ml).

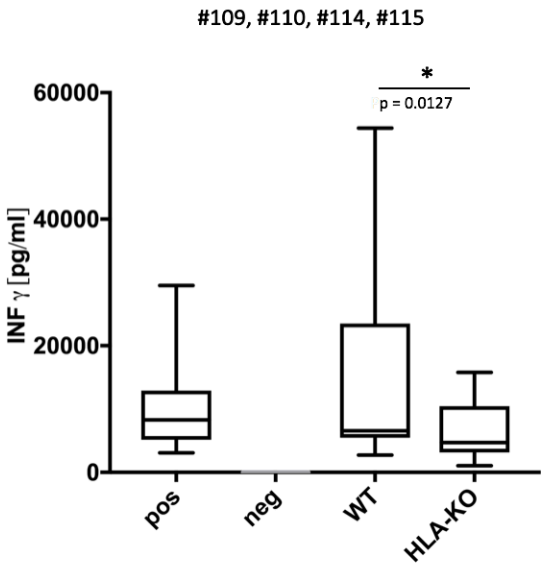

Figure 21. Interferon γ secretion by T cells co-cultured with wildtype HuES8-derived β cells is significantly higher than the secretion by T cells cultured in the presence of HLA-KO-derived β cells. HuES8-derived β cells (14029,76 pg/ml ± 3832,45 pg/ml) enhance the interferon γ release by T cells whereas HLA-KO-derived β cells (5437,10 pg/ml ± 1082,61 pg/ml) do not increase the interferon γ secretion by T cells. n=18; t-test parametric, paired. Data are mean ± SEM; *p<0.05

4.9 HuES8 and HLA-KO show similar differentiation efficiency into cardiomyocytes

Hypoimmunogenic embryonic stem cells do not only pose a promising cell source for T1D therapies but also for other diseases where a loss of cells or tissue occurs. One of the fields where HLA-KO ESCs can be applied to is cell-based heart regeneration. Cardiovascular diseases represent a huge problem worldwide and are the cause for the majority of deaths. Although cardiomyocytes seem to have some ability for regeneration, it is still too little to counteract tissue damage that a myocardial infarction would cause. A successful differentiation of the hypoimmunogenic ESCs into cardiomyocytes could be a major advantage, as it would allow for new approaches in cardiovascular regeneration without the need for immunosuppressive drugs.

To test this hypothesis, HuES8 wildtypes ESCs as well as the HLA-KO ESCs were differentiated into cardiomyocytes as described in 3.10. Only HLA-KO ESCs were used because in the previously described activation and proliferation assays done with β cells it seemed sufficient to circumvent immune detection by only ablating HLA class I. Two days prior to staining for cardiac troponin (cTnT), some cells were additionally stimulated with interferon γ. The interferon γ stimulation was done to induce the expression of HLA-A/B/C. Figure 22 displays similar differentiation efficiencies between wildtype HuES8 cardiomyocytes and HLA-KO cardiomyocytes.

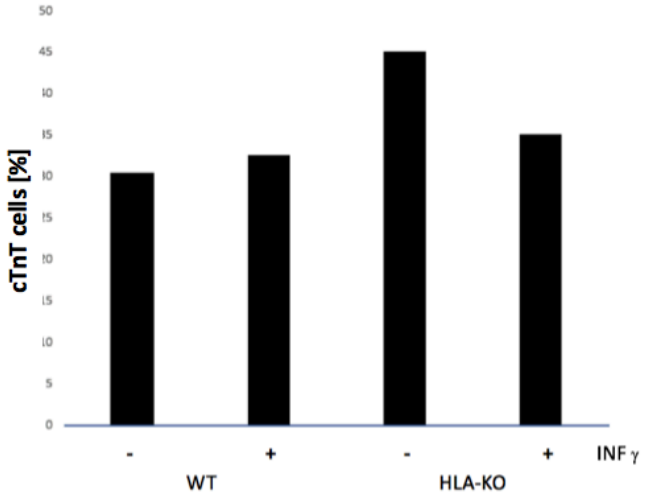

Figure 22. Differentiation efficiency of wildtype HuES8 and HLA-KO ESCs into cardiomyocytes. The differentiation of HuES8 and HLA-KO ESCs shows an efficiency of 30%-45% with no significant difference between the two cell lines. Wildtype as well as HLA-KO cardiomyocytes were stimulated with interferon γ two days prior to cTnT staining to stimulate the expression of HLA-A/B/C on the surface. Interferon γ does not have an influence on the differentiation efficiency.

After gating on cardiomyocytes, the $cTnT^+$ cells were tested for the expression of HLA class I. In Figure 23, wildtype cardiomyocytes stimulated with interferon γ show a significant upregulation of HLA-A/B/C expression compared to HuES8-derived heart cells that were not stimulated, as well as to the HLA-KO-derived cardiomyocytes that were either stimulated and left untreated. This observation confirms that the HLA-KO differentiated into cardiomyocytes also do not express HLA-A/B/C, as it was previously observed for the SC-derived β cells.

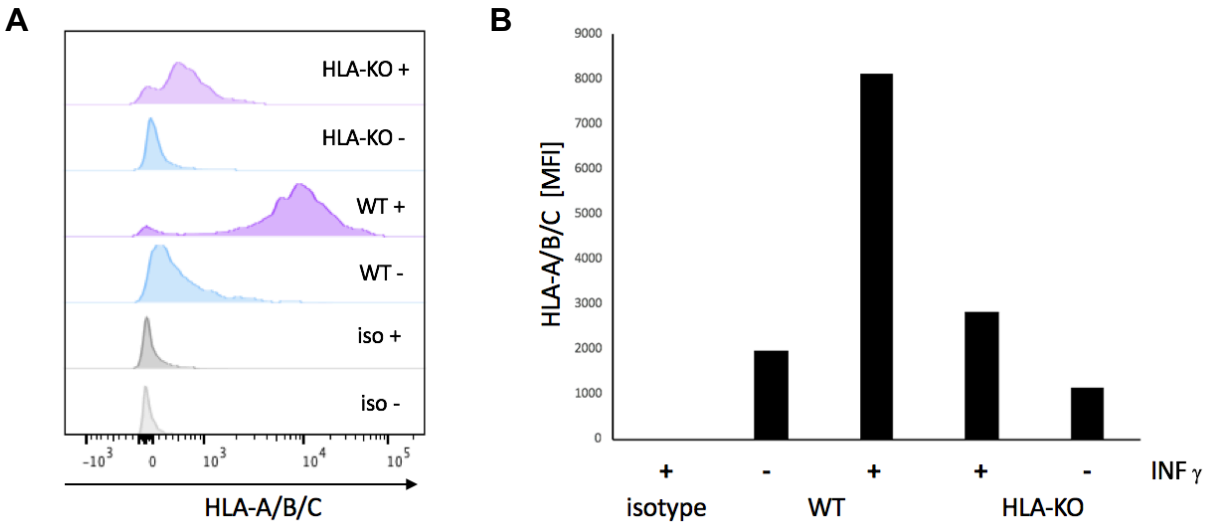

Figure 23. HLA-KO-derived cardiomyocytes do not present and upregulation of HLA-A/B/C upon stimulation with interferon γ. HuES8 wildtype ESCs and HLA-KO ESCs were differentiated into cardiomyocytes (cTnT) and were stimulated with interferon γ two days prior staining for cTnT and HLA class I. (A) Histogram of the unstimulated and stimulated cardiomyocytes. The HLA-KO does not exhibit upregulation of the expression of HLA-A/B/C compared to the wildtype HuES8 cardiomyocytes stimulated with interferon γ as well as an isotype control. (B) Wildtype as well as HLA-KO cardiomyocytes were plotted according to their HLA class I MFI value.

4.10 HLA-deficient cardiomyocytes are able to circumvent immune detection by human T cells

For the hypoimmunogenic ESCs to potentially become a new therapy for cardiovascular diseases, they need to be able to circumvent detection by the recipient`s immune system. Therefore, we co-cultured the differentiated cardiomyocytes with freshly isolated and CFSE stained T cells and incubated them for 7 days to initiate proliferation of the T cells. Two days prior to the co-culture of T cells and cardiomyocytes, cardiomyocytes were stimulated with interferon γ. After one week, the T cells were harvested, and samples were stained for $CD3^+$, $CD4^+$ and $CD8^+$ T cells. In Figure 24, $CD8^+$ T cells are displayed, showing a higher proliferation against HuES8-derived cardiomyocytes (35.27% ± 11.09% SEM) than against the HLA-KO-derived cardiomyocytes (5.32% ± 0.98% SEM), indicating that HLA class I-deficient cardiomyocytes are indeed able to circumvent immune surveillance rejection. The positive control was achieved using CD3/CD28 beads to stimulate T cells, whereas for the negative control T cells were kept in culture without any additionally stimuli.

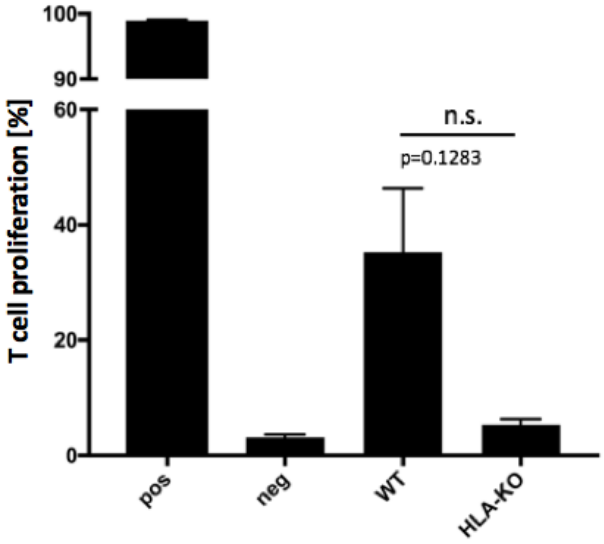

Figure 24. T cells show reduced proliferation against HLA-KO-derived cardiomyocytes. $CD8^+$ T cells form blood donor #122 (n=3) proliferate after being co-cultured with wildtype HuES8-derived cardiomyocytes (35.27% ± 11.09% SEM) but not against the HLA-KO-derived cardiomyocytes (5.32% ± 0.98% SEM) after 7 days of incubation. n=3; T-test parametric, paired. Data are mean ± SEM; n.s.>0.1.

5 Discussion

The purpose of this Master`s thesis was to differentiate heavily genome-edited cells into β cells and test their potential to circumvent immune detection to take a step further in finding a cure for type 1 diabetes. The two knock-out (B2M-KO, HLA-KO) and two knock-in (2in1-KI, 3in1-KI) cell lines were deprived of the HLA class I and II whereas the knock-in cells furthermore had tolerogenic factors integrated. The 2in1-KI expressed PD-L1 as well as CD47, while the 3in1-KI expressed PD-L1, CD47 and HLA-G. In theory, all these cells should have the potential to circumvent immune detection in the patient.

HLA class I and class II expression were prevented in HuES8 ESCs using the CRISPR/Cas9 genome-editing system. While the HLA-B and -C genes are adjacent to each other in humans, the HLA-A gene is located near the telomer. As a consequence, HLA-B and -C (95kb) were knocked out together, while the HLA-A gene (13kb) was excised in a separate step. The HLA II is composed of three polymorphic alleles, HLA-DP/DQ/DR[67], which can be disabled by targeting the master regulator CIITA.
Although knocking out the HLA I genes and preventing HLA II gene expression does eradicate the T cell-mediated adaptive immune rejection, cells are still susceptible to other immune cell types involved in the allogenic immune response. To counteract a cytotoxic response through any residual T cells, the checkpoint inhibitor PD-L1 was knocked-in. PD-L1 interacts with the PD-1 receptor on activated T cells and suppresses a TCR-mediated activation and proliferation[68]. Furthermore, the lack of the HLA genes might lead NK cells to initiate an immune response, which is the reason that one of the immunomodulatory molecules integrated was HLA-G. Finally, macrophages are attracted by cytokines released from other immune cells and will phagocytose foreign cells. Therefore, the “don’t eat me signal” CD47 was introduced as well into the preexisting HLA-KO cells.

5.1 Reduced growth rate and differentiation capacity due to genome-editing procedure?

First, we tested the feasibility for these genome-edited cells to differentiate into β cells. For this purpose, we used a well-established β cell differentiation protocol[18] as described in 3.5 and collected samples every week after entering the final differentiation state (Stage 6) to check for the differentiation efficiency.
It could be observed that the genome-modified cell lines showed a reduced growth rate in both 2D plates as well as in 3D spinner flask culture. Moreover, cells displayed a lower cell survival rate during passaging. While wildtype HuES8 ESCs had a survival rate of 95-98%, genome-edited ESCs only showed 80-87% survival. Because of all these genome engineering and selection steps, the cells already had a high passage number and went through significant cell stress before starting the

differentiation process, which could be an explanation for both the reduced growth rate and also the lower differentiation efficiency.

Of note, DJ24, which is the only wildtype HuES8-derived β cell differentiation performed in parallel also showed a lower differentiation efficiency (6%) when compared to the β cells obtained from the lab of Prof. Douglas Melton, where differentiation efficiency reached up to 30%. Thus, another potential explanation is that the differentiation efficiency was not as high because of some handling mistakes. A handling mistake could be the neglect of a factor or the time points were not maintained strict enough.

Interestingly, in chapters 4.3 and 4.4 we described that the genome-modified cell lines (β2m-KO, HLA-KO, 2in1-KI, 3in1-KI) display a higher efficiency of differentiation into polyhormonal cells (C-peptide$^+$/Glucagon$^+$) than into mature β cells (C-peptide$^+$/NKX6.1$^+$). Moreover, differentiated wildtype HuES8 (DJ24) did not display any fraction of polyhormonal cells. It is possible that the knock-out and knock-in cell lines tend to differentiate into a polyhormonal cell state with the current protocol rather than into β cells. One possible solution could be to differentiate HuES8 as well as all the genome-edited ESCs in parallel and obtain cell samples at the end of every differentiation stage to determine when the protocol fails to work. Pagliuca *et al.* described an induction into definitive endoderm (Stage 1) of over 95% (SOX17$^+$) and over 85% yield into early pancreatic progenitors (PDX1$^+$; Stage 3)[18]. If knock-out and knock-in cells are able to differentiate properly into pancreatic progenitors, a suggestion could be to transplant these cells into mice and see if they are able to accelerate into mature β cell *in vivo*.
The term polyhormonal cells refers to differentiated cells *in vitro* which possess characteristics of fetal and not adult β cells. Based on the fact that the genome-edited differentiated cells exhibit a rise in polyhormonal cells after entering the final differentiation stage, the problem could be in the protocol after reaching stage 6. Normally, *in vitro*-differentiated β cells become more mature over the course of two weeks after entering the final differentiation stage[13,18]. It may be the case that cells differentiate according to the protocol until they reach stage 6 but are not able to transfer from a fetal standing into a more mature one. Then a possible solution could be to screen for additional factors to overcome this hurdle or perform β cell differentiation with the genome-edited ESCs possessing a lower passaging number.
In general, it would also be interesting to start β cell differentiation with genome-edited ESCs that hold earlier passaging numbers and compare the differentiation efficiency to previous differentiations done with higher passaging numbers. If the efficiency increases when cells possess a lower passaging number, then the problem may lie in genome alterations resulting from constant passaging. In general, checking the karyotype of the cells frequently might be recommendable, to exclude growth or other disadvantages.

In conclusion, we succeeded in differentiating heavily genome-edited cells into β cells but with a low differentiation efficiency that will not be enough to use in cell-therapy for T1D.
If a higher differentiation efficiency can be reached than a next step would be to test the glucose responsiveness and the capability to release insulin of differentiated β cells *in vitro* as well as *in vivo*.

5.2 Hypoimmunogenic β cells are able to circumvent immune detection by human immune cells

Human T cells were co-cultured with SC-derived β cells to prove the hypothesis that the genome-edited cells are able to evade the immune detection by immune cells. For this purpose, activation as well as proliferation immunoassays were performed *in vitro*.

To test if T cells proliferate in the presence of our genome-edited β cells, T cells were stained with the dye CSFE to trace proliferation and were co-cultured for one week with sc-derived β cells. Important to note is that β cells were not separated from other cell types present in the culture and all the immunoassays were done with all the cell populations present in the culture after differentiation. For convenience, I will further refer to them as β cells.

It was observable that the immune cells did indeed proliferate in the presence of wildtype HuES8-derived β cells but not in the presence of the hypoimmunogenic β cells, which proves the immunomodulatory quality of these cells.
Another feature that could be observed was that there was visible donor-to-donor variability in T cell reaction against HuES8 β cells. There could be two possible explanations for this occurrence. Either the donor T cells and HuES8 β cells possess an HLA match or there are differences in precursor frequencies. To test if the isolated T cells, which showed a high proliferative response against wildtype HuES8 β cells, had the same HLA type, we stained donor #114 and #115 T cells for HLA-A2, which is the HLA type that is present in HuES8 cells. Indeed, we could observe that the T cells of these two donors were HLA-2A positive (Figure 25), which makes them an HLA match to our wildtype HuES8 cells.

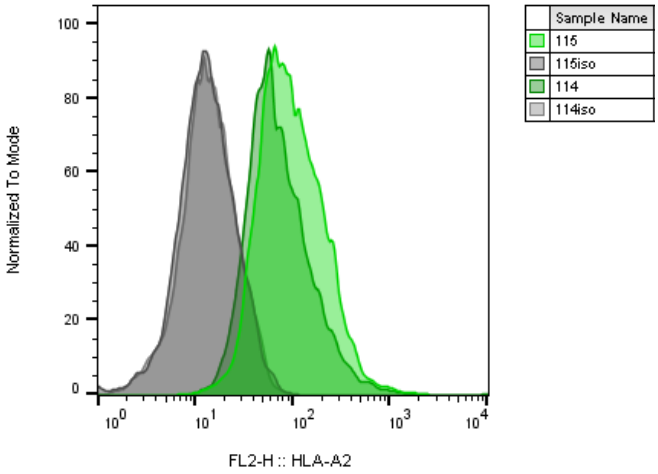

Figure 25. T cells of blood donors #114 and #115 express same HLA type as HuES8 cells. The blood donors #114 and #115 were stained for HLA-2A to screen if T cells and HuES8 cells exhibit the same HLA type. Both donors display a specific staining with an anti-HLA-A2 antibody, when compared to the isotype control. The HLA match is a possible explanation for the high proliferative response of T cells against HuES8-derived β cells.

In all immunoassays performed and described in chapter 4, only $CD8^+$ or cytotoxic T cells expanded or showed upregulation of the expression of CD69 when co-cultured with HuES8 β cells.
$CD8^+$ T cells like $CD4^+$ T cells are generated in the thymus. In contrast to $CD4^+$ T cells, $CD8^+$ T cells respond to peptides presented by the HLA class I molecules, which are expressed on almost all cell types[69]. When $CD8^+$ T cells become activated, they secret different cytokines like TNF-α (tumor necrosis factor-α) and interferon γ, which primarily have anti-tumor and anti-viral effects[69]. The second function of $CD8^+$ T cells is the release of perforins and granzymes. First, perforins attack the membrane of a cell and create a pore, which allows the granzymes to enter the cell and cleave present proteins, effectively leading to apoptosis of the infected cell[69]. Third, activated $CD8^+$ T cells express Fas ligand (FasL) on their cell surfaces, a transmembrane protein that belongs to the TNF family. FasL binds to its receptor Fas on the surface of the infected cell, resulting in the activation of the caspase cascade which in the end also results in the apoptosis of the target cell[69].
In contrast to $CD8^+$ T cells, $CD4^+$ T cells recognize peptides presented on the HLA class II molecules on APCs. In general, $CD4^+$ T cells can be divided into four subsets of cells: T helper cells, Th1 and Th2 cells as wells as Th17 and T regulatory cells (Treg)[70]. $CD4^+$ cells are responsible for the induction of macrophages, the recruitment of granulocytes to the site of infection and for the production of cytokines and chemokines. They also aid in the generation of antibodies by B cells[70].

Because of the characteristic described above, it was predictable that only $CD8^+$ T cells would display a proliferative response against HuES8 β cells. The knock-out as well as knock-in cell lines are not able to express HLA-I molecules, which is the reason that $CD8^+$ T cells only show proliferation when co-

cultured with wildtype HuES8 β cells but not in the presence of hypoimmunogenic β cells. β cells do not express HLA-II on their surface, what would have been a necessity for $CD4^+$ T cells to be activated.

Another hypothesis was that the tolerogenic factors integrated into the 2in1-KI and 3in1-KI cells would lead to an even more observable immune evasion than the HLA-KO alone. To test this hypothesis further experiments are necessary. Proliferation as well as activation assays only performed with T cells alone are not able to answer this question because HLA class I and II are not expressed in any of these cell lines. On the one hand, a NK cells killing assay needs to be performed to test if the integrated HLA-G is sufficient to avert killing by NK cells. On the other hand, a macrophage engulfment assay needs to be done to investigate the influence of CD47 on macrophages.

For the activation assay described in chapter 4.7, freshly isolated human T cells against the genome-edited β cells compared to the wildtype HuES8 β cells did not show a significant activation of T cells but there was a clear trend visible. The activation assay was incubated for 48 hours before T cells were stained for the early activation marker CD69. In the future it would be wise to co-culture T cells with β cells for 5 days and then stain for the expression of CD69 as well as of CD25 as a proxy for T cell activation.

HuES8-derived β cells release significantly more interferon γ into the medium than HLA-KO-derived β cells. T cells from blood donors (#109, #110, #114#, #115) which showed a high proliferative response against HuES8 β cells, also displayed an increase release of interferon γ into the medium. This result was to be expected because one mechanism of activated $CD8^+$ T cells is the release of cytokines against infected or foreign cells.

In conclusion, hypoimmunogenic ESCs differentiated into β cells are able to circumvent immune detection by T cells. Further experiments are necessary to test immune evasion of NK cells as well as macrophages. A next step would also be to transplant differentiated β cells into immunocompromised mice and further into a humanized mouse model (hu-SCID). The final question that needs to be answered is if hypoimmunogenic β cells would be able to reverse T1D in the presence of a human immune system.

6 Supplement

6.1 FACS antibodies

α-HLA-ABC (PE-conjugated), Clone W6/32, Biolegend, Cat#311406

α-CD3 (APC-conjugated), Clone UCHT1, Biolegend, Cat#300412

α-CD3 (Pacific Blue™-conjugated), UCHT1, Biolegend, Cat#300418

α-CD4 (PE/Cy7-conjugated), Clone RPA-T4, Biolegend, Cat#300511

α-CD8 (PE-conjugated), Clone SK1, Biolegend, Cat#344705

α-CD69 (Alexa Fluor® 647-conjugated), Clone FN50, Biolegend, Cat#310918

α-cTnT (Alexa Fluor® 647-conjugated), Clone 13-11, BD Pharmingen, Cat#565744

Isotype 1: Mouse IgG2a, κ Isotype Control (PE-conjugated), Biolegend, Cat#400214

Isotype 2: Mouse BALB/c IgG1, κ Isotype Control (Alexa Fluor® 647-conjugated), BD Pharmingen, Cat#564416

6.2 Immunofluorescence antibodies

α-C-peptide, Cell Signaling Technologies, Cat#4593

α-NKX6.1, Cell Signaling Technologies, Cat#54551

α-Glucagon, Cell Signaling Technologies, Cat#2760

Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 467 conjugate, Life Technologies, Cat#A-31573

Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 467 conjugate, Life Technologies, Cat# A-31571

Donkey anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 488® conjugate, Life Technologies, Cat#A-21208

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List of Abbreviations

APC	Antigen presenting cell
B2M	Beta-2 Microglobulin
CFSE	Carboxyfluorescein succinimidyl ester
C-pep	C-peptide
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
cTnT	Cardiac Troponin
CIITA	class-II transactivator
DE	definitive endoderm
DJ1-25	Differentiation Jacqui 1-25
DMEM	Dulbecco`s Modification of Eagle`s Medium
DMEM/F12 50:50	Dulbecco`s Modification of Eagle`s Medium/ Ham`s F-12 50:50 Mix
ESC	Embryonic stem cell
FBS	Fetal Bovine Serum
Gal	galactose-α1,3-galactose
GATA4	GATA binding protein 4
GCDR	Gentle Cell Dissociation Reagent
Gluc	Glucagon
hESCs	human embryonic stem cells
HLA	Human Leucocyte Antigen
n.d.	no data available
IL2	Interleukin 2
iPSC	inducible pluripotent stem cell
NKX6.1	Homeobox protein NKX6.1
HuES8	Harvard university Embryonic Stem cells 8
hu-SCID	humanized-severe combined immunodeficient mice
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate buffer saline
PD-L1	Programmed Death-Ligand 1
PDX-1	Pancreatic duodenal homeobox-1
PFA	paraformaldehyde
PGT	primitive gut tube
PH	polyhormonal
PP1, PP2	pancreatic progenitor
SC	stem cell
SHH	sonic hedgehog
SRY	sex-determining region Y
SOX9	SRY-box 9 SOX9

TCR	T cell receptor
Th1, Th2, Th17	T helper cells
TNF-α	tumor necrosis factor-α
Treg	T regulatory cell
WT	wildtype