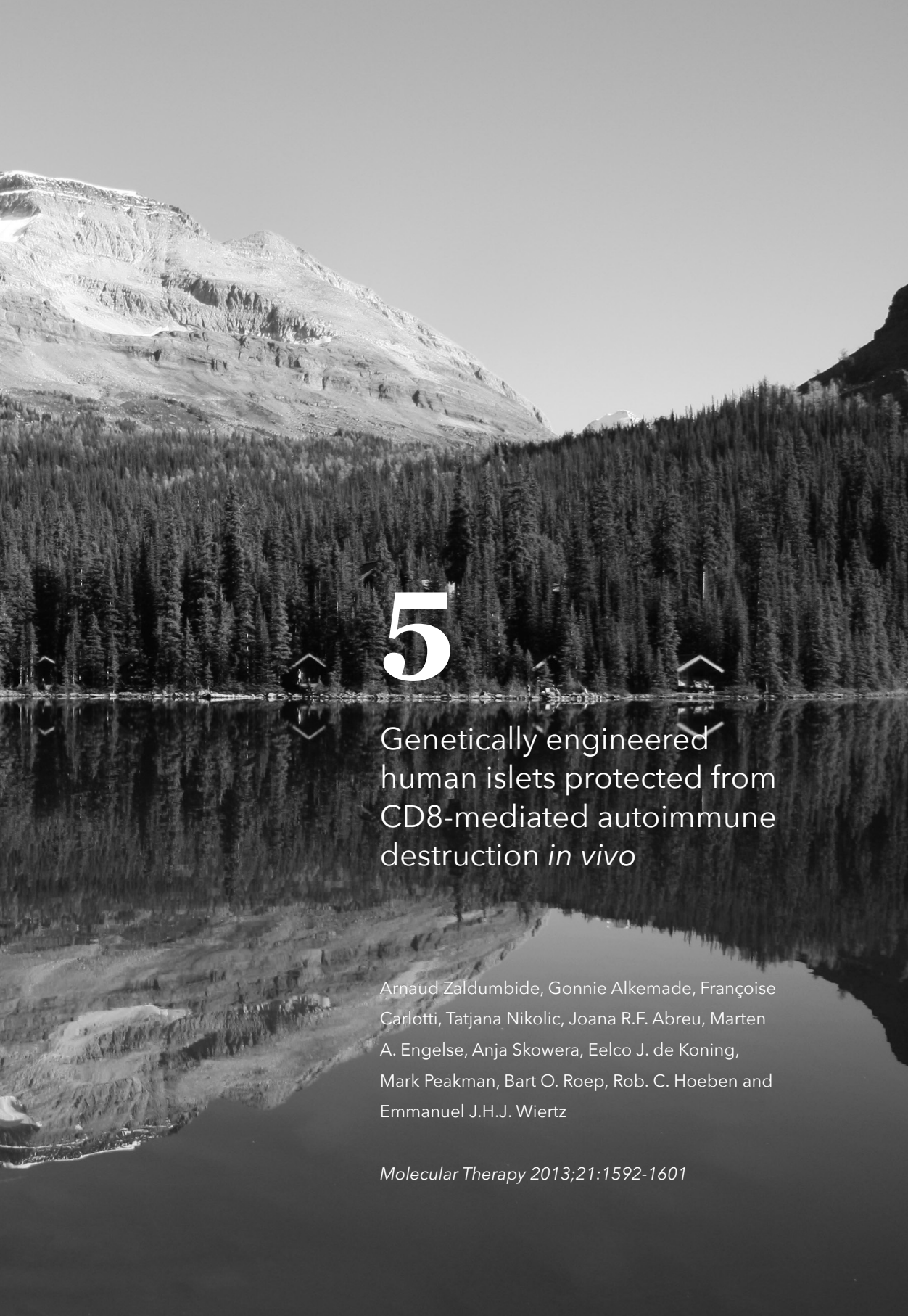

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Genetically engineered human islets protected from CD8-mediated autoimmune destruction *in vivo*

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ABSTRACT

Islet transplantation is a promising therapy for type 1 diabetes, but graft function and survival are compromised by recurrent islet autoimmunity. Immunoprotection of islets will be required to improve clinical outcome. We engineered human beta-cells to express herpesvirus-encoded immune evasion proteins, 'immunevasins'. The capacity of immunevasins to protect beta-cells from autoreactive T-cell killing was evaluated *in vitro* and *in vivo* in humanized mice. Lentiviral vectors were used for efficient genetic modification of primary human beta-cells without impairing their function. Using a novel beta-cell specific reporter gene assay we show that autoreactive cytotoxic CD8+ T-cell clones isolated from patients with recent-onset diabetes selectively destroyed human beta-cells, and that co-expression of the human cytomegalovirus-encoded US2 protein and serine proteinase inhibitor 9 offers highly efficient protection *in vitro*. Moreover, co-implantation of these genetically modified pseudoislets with beta-cell specific cytotoxic T-cells into immunodeficient mice achieves preserved human insulin production and c-peptide secretion. Collectively, our data provide proof of concept that human beta-cells can be efficiently genetically modified to provide protection from killing mediated by autoreactive T-cells and retain their function *in vitro* and *in vivo*.

INTRODUCTION

Type 1 diabetes results from selective and progressive destruction of insulin-producing cells by autoreactive $CD8^+$ T-cells. [1,2] The direct cell-cell contact initiated by T-cell receptors recognizing a beta-cell specific antigenic peptide presented by the MHC class-I (MHC-I) at the surface of the target cell appears to be critical for beta-cell destruction in type 1 diabetes. In humans, during acute insulitis, beta-cells show hyper-expression of MHC-I and selective infiltration by islet-specific autoreactive $CD8^+$ T-cells. [3] Also mice lacking MHC-I expression do not develop diabetes, [4-6] whereas adoptive spleen cell transfer from NOD mice to recipients selectively expressing MHC-I on beta-cells leads to beta-cell destruction. [7,8] In addition, the Fas signaling pathway is not essential in the destruction of beta-cells, [9,10] while disruption of the perforin gene delays the onset of autoimmune diabetes in NOD mice, pointing to the perforin/granzyme pathway as a key effector in beta-cell destruction by cytotoxic T-cells. [11,12]

To date, immunotherapies to cure, prevent, or delay disease onset in humans have been inefficient. [13,14] Options for type 1 diabetes patients to restore normoglycemia are limited to daily insulin injection, combined kidney-pancreas or experimental islet transplantation. However, numerous factors have been reported to potentially affect islet allograft function and survival. [15] Importantly, the close correlation between loss of islet-graft function and frequencies of circulating autoreactive islet-specific T cells observed in transplanted patients, in particular when the donor and the recipient share the same HLA class I haplotypes, points to the importance of recurrent autoimmunity in islet graft failure. [16-19] Along the same line, it appears that, in case of autotransplantation, islet grafts are much less affected, suggesting that persistent attacks from autoimmunity, alloimmunity and possibly the toxicity of immunosuppressive treatments have a major responsibility in graft loss. [20]

An alternative could be the transplantation of genetically immunoprotected beta-cells. We have previously explored the capacity of herpesvirus-derived immunevasins to elude the host immune responses (reviewed in [21-23]). In this study we aim to combine the US2 protein of human cytomegalovirus, known to interfere with antigenic peptide presentation by inducing proteasomal degradation of MHC-I molecules [24], and Serpin-9, a serine protease inhibitor specific for granzyme B activity. [25]

Furthermore, the identification of specific human beta-cell protectants is hindered by the difficulty of studying the dialogue between human insulin-producing cells and the immune system. Even *in vitro*, the cellular heterogeneity of dissociated islet preparations makes the standard release-based assays imperfect to specifically assess beta-cell cytotoxicity. Islet preparations contain a mixture of cell types, including β-cells, α-cells, δ-cells, ε-cells, duct cells, endothelial cells, stem cells, and leukocytes, that all may interfere with the assays to selective assess beta-cell cytotoxicity. [26]

Here we describe the development of a new strategy to measure beta-cell cytotoxicity and protection from autoreactive T-cell-mediated killing. We report that lentivirus

vectors can be used to genetically modify human islet cells. Moreover, using this powerful technology, we demonstrate that downregulation of MHC-I combined with the inhibition of granzyme B activity protects human beta-cells from acute recurrent islet autoimmunity both *in vitro* and *in vivo*.

RESULTS

Primary human islet cells can be transduced efficiently by lentiviral vectors.
Lentiviral vectors are the gene transfer system of choice for stable genetic modification of primary cells. To evaluate their capacity to genetically modify freshly isolated human islets, a vector carrying an enhanced GFP reporter gene under the control of the human cytomegalovirus immediate early promoter was used (LV-CMV-GFP). Seventy-two hours post transduction, islets were inspected for reporter gene expression (Fig. 1a). A MOI of 5 was well tolerated without any apparent signs of virus-induced toxicity. In addition, exposure to the virus did not significantly affect islet function, as exemplified by their capacity to secrete insulin upon glucose stimulation (Fig. 1b). Yet, GFP expression within the islet structures was heterogeneous. Following insulin/DNA staining, whole mount confocal microscopy confirmed that in most islets only cells at the rim of the islets were efficiently transduced, demonstrating that in intact human islets only a limited number of cells are permissive to lentiviral vector transduction (Fig. 1c). To increase the transduction efficiency, human islets were dispersed, and the resulting single-cell suspension was exposed to the lentivirus vector LV-CMV-GFP. Subsequently, pseudoislets were formed by self-aggregation of the transduced cells. Seven days post re-aggregation, the 3D structures were macroscopically similar to islets. The majority of the cells expressed GFP as determined by flow cytometry (Fig. 1d). To evaluate the functionality of genetically modified pseudoislet structures, a static glucose-stimulated insulin secretion test was performed and glucose responsiveness was compared to non-transduced and intact islets. Neither dispersion nor transduction hampered islet functionality, since no differences in insulin release were detected after glucose stimulation (Fig. 1e).

Genetically modified pseudoislets are functional *in vivo*.
To assess the fate of these pseudoislets *in vivo*, non-transduced and LV-CMV-GFP transduced human pseudoislets were transplanted under the kidney capsule of NSG mice. The release of human insulin after intra-peritoneal glucose administration was measured. Assuming that one islet would contain approximately 1000 to 1500 cells, [27] ~3000 islet equivalents (IEQ) were transplanted and compared to $5x10^6$ dispersed/re-aggregated cells. Pseudoislets efficiently released insulin upon glucose challenge, since no significant differences were observed when compared to intact islets (Fig. 2a). In order to define the limitation of the insulin assay used, we transplanted NSG mice with $2.5x10^6$ cells (~1500 pseudoislets) or $5x10^6$ cells (~3000 pseudoislets) and monitored human insulin release. As anticipated, the amount of insulin secreted correlated with the number of transplanted cells (Fig. 2b). Similar results were obtained with a human c-peptide assay (data not shown). To confirm the survival of the insulin-producing cells,

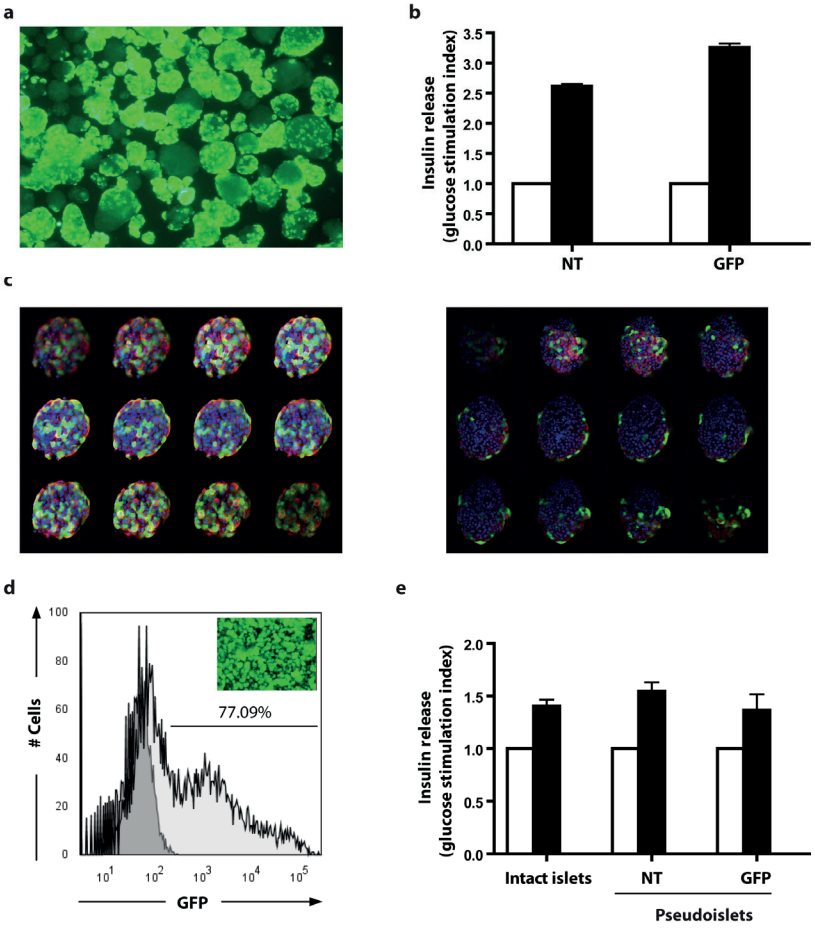


Figure 1 | Genetic modification of human islets *in vitro*.

a) Fluorescent microscopy pictures of freshly isolated human islets transduced with LV-CMV-GFP (MOI=5). GFP expression was assessed by fluorescent microscopy (200x magnification). **b)** Comparative glucose responsiveness of non-transduced islets (NT) and GFP-modified islets (GFP) determined by glucose-stimulated insulin-secretion test. Insulin release data are shown as glucose stimulating index (i.e. insulin-release at low glucose - white bars - is used as reference and set to 1 and used as reference for high glucose (black bars) induction. **c)** Series of optical sections of two representative islets (2μm) taken by confocal microscopy. Insulin is depicted in red, GFP in green and nuclei are visualized by DAPI (blue) staining. While some GFP expression could be observed in the central core of the islet (left panel), in most islets GFP expression was limited to the outer rim of the islet (right panel). **d)** Fluorescent microscopy picture of GFP-modified pseudoislets (MOI=2) and quantification of the GFP positive cells by FACS. Light grey histogram shows GFP-transduced cells and non-transduced dispersed cells are shown in dark grey histogram. Experiments are performed 6-days post-transduction. **e)** Glucose responsiveness of pseudoislets or genetically modified pseudoislets compared to intact islets from the same donor. Similar to panel b, insulin release data are shown as glucose stimulating index. Low glucose (white bars) concentration is set to 1 and used as reference for high glucose (black bars) induction (GFP: green fluorescent protein; MOI: multiplicity of infection; NT: non-transduced).

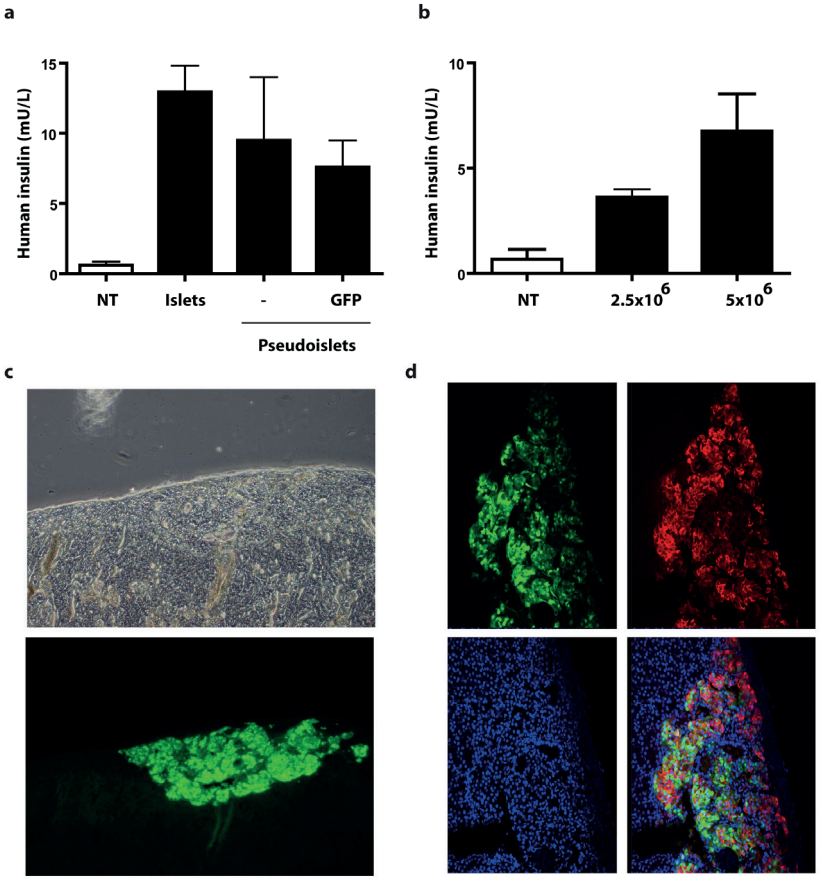


Figure 2 | Function of genetically modified pseudoislets in vivo.
a) Intraperitoneal glucose-tolerance test performed on mice transplanted with 3000 intact islets (n=1) or 5.10^6 GFP modified pseudoislets (n=4) or non-modified islets (n=2). N represents the number of transplanted mice. Results are represented as average of 3 different time points at 4, 11 and 19 days after transplantation. **b)** Similar experiment performed with GFP-modified pseudoislets formed with $2.5.10^6$ cells or 5.10^6 cells (n=2). Non-transplanted mice were used as negative control. **c)** Fluorescent microscopy of the kidney performed after nephrectomy 19 days post transplantation of pseudoislets containg 5.10^6 cells. **d)** Immunostaining of the graft. Insulin is shown in red, GFP in green and nuclei are stained by DAPI in blue. Sections were analyzed by confocal microscopy.

the graft was removed 19-days post-transplantation and analyzed by confocal imaging for GFP fluorescence and, after immunostaining, for insulin reactivity. Macroscopically, a strong and sustained GFP expression was detected at the site of re-implantation (Fig. 2c). Insulin immunostaining demonstrated a large proportion of GFP positive beta-cells (Fig. 2d). From these data we conclude that lentivirus-mediated gene transfer into cells from freshly isolated human islets is feasible, and that the cells can be re-aggregated to form functional pseudoislets.

The human insulin promoter drives beta-cell specific expression in human islet cells.
Next, in order to acquire specific expression of the gene of choice in beta-cells, the CMV promoter was replaced by the human insulin promoter (HIP) (Fig. 3a). To assess HIP promoter specificity, we first compared CMV-GFP lentivirus transduction efficiency in human embryonic kidney cells (HEK) or rat insulinoma cell lines (INS-1E) and confirmed that both cell types can be efficiently modified by lentiviruses (Fig. 3b, upper panel). Secondly, we performed similar experiments using the HIP-GFP lentivirus and detected only few GFP positive HEK cells whereas 25% of the INS-1E expressed GFP (Fig. 3b, lower panel). Finally, we verified HIP specificity and efficiency in human primary cells. One week post transduction, HIP-GFP human pseudoislets were analyzed for GFP expression using confocal microscopy (Fig. 3c). Altogether, these data demonstrate that the HIP promoter facilitates efficient transgene expression and limits this expression to beta-cells.

Autoreactive HLA-A2-restricted PPI-directed CTL clones kill HLA-A2 human islet cells *in vitro*.
To quantify beta-cell death, the GFP cassette was replaced by a destabilized luciferase reporter gene, allowing short half-life luciferase expression specifically in beta-cells (Fig. 3a). To validate this method, we designed an *in vitro* killing assay by incubating HIP-Luc2CP modified human islet cells with autoreactive $CD8^+$ T-cells isolated from a recent onset T1D patient and directed against an epitope located in the signal peptide of the preproinsulin molecule (PPI). [28] CTL killing capacity was validated in a standard chromium release assay using K562 surrogate beta-cells (Figure S1). Using fractions of different purities from the same donor, killing assays were performed with different target/effector ratios (corrected for purity of the fraction). These experiments demonstrate that the luciferase assay is not affected by the quality of the isolated islet fraction (Fig. 4a). Similarly, killing assays performed in parallel with HLA-A2-restricted PPI-directed CTL, incubated with HIP-Luc2CP islet cells from HLA-A3 and HLA-A2 donors, demonstrated that PPI-directed CTL were able to specifically kill HLA-A2 beta-cells, as seen by a massive drop in luciferase activity. When HLA-A3 donor cells were used as targets, no significant decrease in light emission was observed (Fig. 4b). Moreover, when using HLA-A2 restricted pp65CMV-specific CTL, the viability of the HLA-A2 positive beta-cells was not affected (Fig. 4c), which is consistent with the absence of pp65CMV target epitope on human beta cells. This demonstrates that beta-cell death is dependent on the presence of the PPI-specific CTL.

Combined US2/Serpin9 expression protects surrogate beta-cells and human primary beta-cells from PPI-directed CTL killing *in vitro*.
To explore the protective effect of combined MHC-I downregulation and granzyme B inactivation, we generated a bicistronic lentivirus vector encoding the immune-evasion gene US2 and the serine protease inhibitor specific for granzyme B activity (Fig 5a). First, the protective effect was evaluated on surrogate beta-cells generated by overexpression of preproinsulin in K562-A2 cells and then transpose to primary human islets. Insulin mRNA level in surrogate beta-cells was verified by RT-PCR and qPCR (Fig. 5b). Transduction of K562-A2 with a US2-containing lentivirus vector led to decreased HLA class I expression as quantified by flow cytometry (Fig. 5c, lower panel). After transduction of HEK 293T

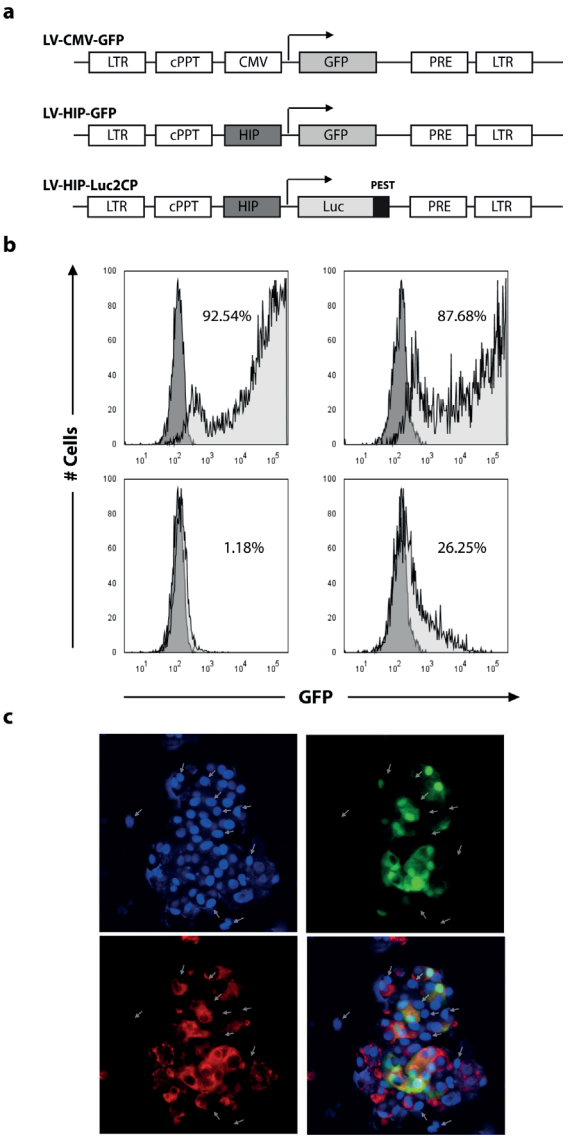


Figure 3 | Human insulin promoter specificity.

a) Schematic representation of the lentivirus constructs used: LV-CMV-GFP; LV-HIP-GFP; LV-HIP-Luc2CP. (LTR: Long terminal Repeat; cPPT: central polypurine tract; PRE: Posttranscriptional Regulatory E (HEK: human embryonic kidney; HIP: human insulin promotor). The arrow indicates the transcription initiation) **b)** Comparative GFP-expression as determined by FACS in HEK 293T cells (left column) and INS1E cells (right column) after transduction with LV-CMV-GFP (MOI=1) (upper panel) or LV-HIP-GFP (MOI=1) (lower panel). Non-transduced cells were used as negative control and shown in dark grey histogram. **c)** Whole mount immunostaining using anti-insulin antibody (red) performed on HIP-GFP transduced pseudoislets. Nuclei were stained by DAPI in blue. White arrows indicate the insulin negative cells.

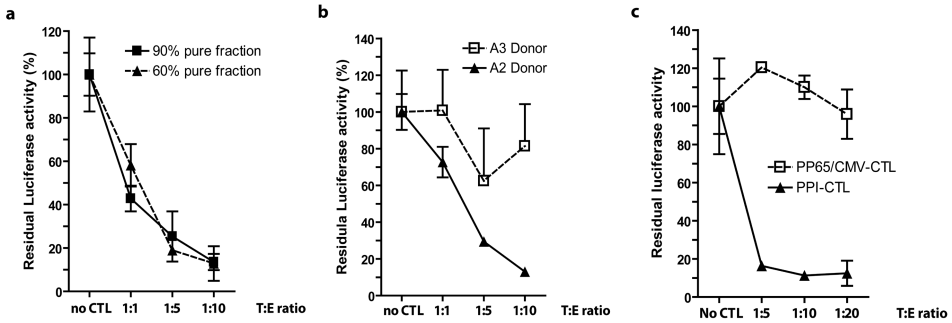
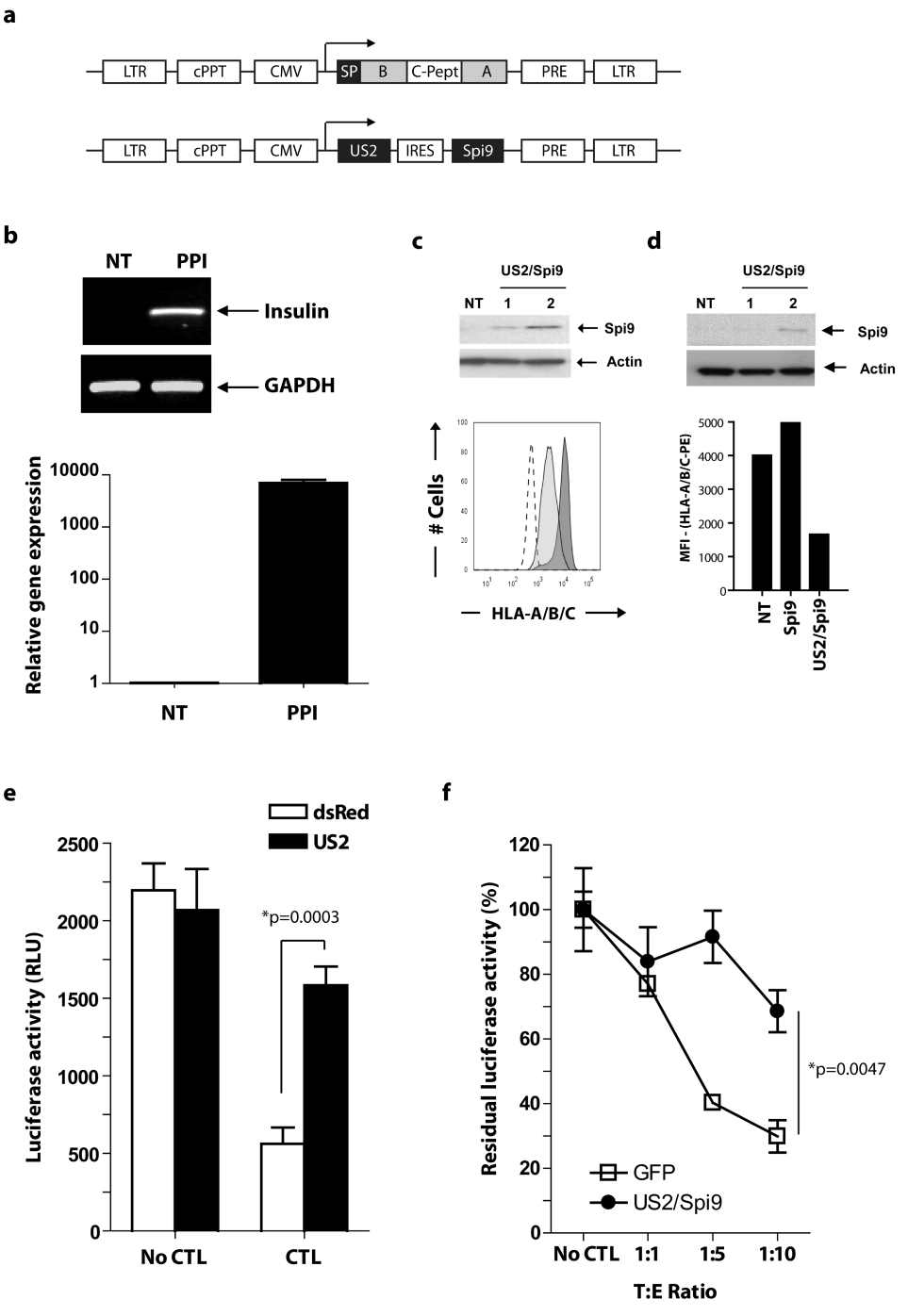


Figure 4 | Autoreactive HLA-A2-specific PPI-directed CTL clones kill HLA-A2 human islet cells *in vitro*.
a) Luciferase killing assay performed on 60% purity (triangle) or 90% pure beta-cell preparations (square) HIP-Luc2CP HLA-A2 human islet cells using PPI-directed CTLs with increasing Target:Effector ratios. Results are shown as residual luciferase activity 48h post coculture. **b)** Luciferase killing assay performed on HLA mismatched (HLA-A3; open square) or matched (HLA-A2; triangle) LV-HIP-Luc2CP-transduced human islet cells using PPI-directed CTLs (with increasing Target:Effector ratios). **c)** Luciferase killing assay performed on HLA-A2 LV-HIP-Luc2CP transduced human islet cells using increasing Target:Effector ratios of pp65-CMV directed CTLs (open squares) or PPI-directed CTLs (triangles). Experiments have been performed in triplicates, normalized to luciferase activity without CTL (set to 100%) and were shown as means of triplicate measurements (± standard deviations). Matched killing assay have been performed and confirmed on HLA-A2 islet cells from 6 different donors (CTL: cytotoxic lymphocyte; PPI: peptire of preproinsulin).

cells at an MOI of 1 or 2, the expression of Serpin-9 was verified by Western blotting (Fig. 5c,upper panel). Similar results were obtained in primary human islets (Fig. 5d). Following co-transduction of surrogate beta-cells with CMV-Luc2CP and CMV-DsRed or CMV-US2 lentiviruses, a killing assay was performed with CTLs against PPI. After 48h of co-culture, massive cell death of DsRed-expressing cells was observed. HLA-A/B/C downregulation mediated by US2 led to protection of the surrogate beta-cells from PPI-specific CTL killing (Fig. 5e). Importantly, US2/Serpin-9 co-expression in primary human islets had no effect on insulin release upon glucose stimulation (supplementary figure S2a) and protected human beta-cells from autoreactive $CD8^+$ T-cells as seen by residual luciferase activity after co-culture when compared to GFP-modified islets (Fig. 5f).

Primary human beta-cells can be efficiently immuno-protected from PPI-directed CTL killing *in vivo*.

As an *in vivo* proof of concept, equal amounts of GFP or US2/Serpin-9 modified pseudoislets (~3000) and PPI-directed CTLs (E:T ratio 1:100) were transplanted under the kidney capsule of NSG mice and human insulin and c-peptide were monitored following intraperitoneal glucose tolerance tests (Fig. 6a,b). In agreement with the *in vitro* results, human insulin or C-peptide secretion by GFP pseudoislets was low following co-transplantation with autoreactive CTL. US2/Spi9 expression had no effect on islets functionality (Supplementary figure S2b) and US2/Serpin-9 expressing cells maintained both insulin (and c-peptide) secretion, to a level similar to the one measured in absence of autoreactive T-cells (Figure 2a and 2b), indicating that US2/Serpin-9 expression does not impact on islet viability *in vivo* and protects beta-cells from autoimmune CTL attack.


a
LTR
cPPT
CMV
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B
C-Pept
A
PRE
LTR
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GAPDH
Relative gene expression
10000
1000
100
10
1
NT
PPI
c
US2/Spi9
NT
1
2
Spi9
Actin
Cells
HLA-A/B/C
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NT
1
2
Spi9
Actin
MFI - (HLA-A/B/C-PE)
5000
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0
NT
Spi9
US2/Spi9
e
dsRed
US2
Luciferase activity (RLU)
2500
2000
1500
1000
500
0
*p=0.0003
No CTL
CTL
f
Residual luciferase activity (%)
120
100
80
60
40
20
0
*p=0.0047
GFP
US2/Spi9
No CTL
1:1
1:5
1:10
T:E Ratio

< Figure 5 | Combined US2/Serpin9 expression protects surrogate beta-cells from PPI-directed CTL killing.
a) Schematic representation of the lentivirus constructs used: LV-CMV-PPI and LV-CMV-US2-bc-Spi9. **b)** Reverse-transcription PCR (upper panel) and reverse-transcription qPCR (lower panel) validating insulin-gene expression in LV-CMV-PPI transduced A2/K562 cells. The GAPDH housekeeping gene was used as internal control. **c)** Serpin-9 expression assessed by western blot using Spi9 antibody following LV-CMV-US2-bc-Spi9 transduction (MOI=1 or MOI=2; non transduced cells –NT- were used as control) of HEK 293T cells (upper panel) and HLA-ABC expression in US2/Spi9 A2/K562 cells assessed by FACS analysis (lower panel). Unstained A2/K562 cells (dashed-line) and HLA-A/B/C stained K562 (dark grey area) were used as controls, HLA-A/B/C surface expression of US2/Spi9 modified A2/K562 cells is shown in light grey area. **d)** Serpin-9 expression and US2 inhibitory effect on HLA-ABC level in human pseudoislets. Following LV-CMV-US2-bc-Spi9 transduction of primary human islet cells (MOI=1 or MOI=2; non transduced cells –NT- were used as control), Serpin-9 expression was assessed by western blot (upper panel). Effect of US2 on HLA-ABC expression in islet cells was determined, following US2/Spi9 transduction MOI=2, by median fluorescent intensity of the HLA-ABC-PE antibody used and compared to non transduced islets (NT) cells or serpin-9 transduced islets (Spi-9) (lower panel). **e)** Luciferase killing assay performed on DsRed LV-CMV-Luc2CP A2/K562 (white bars) or US2/Spi9 LV-CMV-luc2CP-transduced A2/K562 (black bars) using PPI-directed CTLs (Target:Effector 1:5). **f)** Luciferase killing assay performed on LV-HIP-Luc2CP transduced HLA-A2 human islet cells modified by DsRed (opened square) or US2/Spi9 (closed circle) using PPI-directed CTLs. Experiments have been performed in triplicates, normalized to luciferase activity without CTL (set to 100%). Data were shown as means of triplicate measurements (± standard deviations). P value has been calculated by Unpaired Student's t test relative to non protected control.

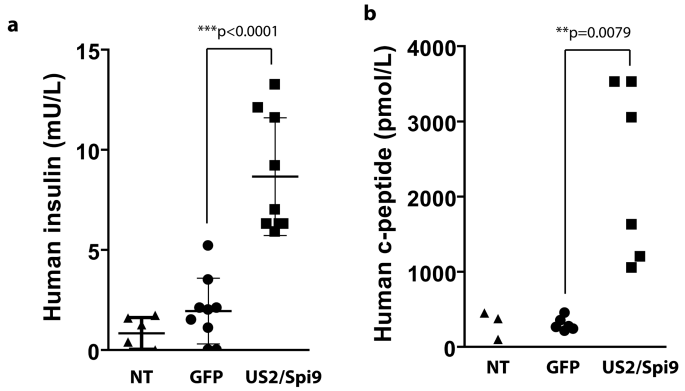

Figure 6 | Immune protection of human islet cells.
a, b) Intraperitoneal glucose-tolerance test performed on mice co-transplanted with 5.10^6 LV-GFP modified pseudoislets (n=3) or LV-US2/Spi9 modified islets (n=3) and PPI-directed CTLs (Target:Effector 100:1). n represents the number of transplanted mice each performed with a different donor. **(a)** Human insulin release values and **(b)** human c-peptide concentration after IPGTT. Three tolerance tests have been performed per mice in the first 3 weeks post transplantation (4, 11 and 19 days). Data are represented as scatter plot showing every glucose tolerance test results (a total of 9 values from bleeding at day 4, 11 and 19 for insulin and a total of 6 values from bleeding at day 11 and 19 for C-peptide). Non-transplanted mice (NT) were used as negative control (n=2). ** is considered to be statistically significant, *** is considered to be extremely statistically significant.

DISCUSSION

Islet transplantation offers a promising approach for restoring endogenous insulin secretion in diabetic patients. However, recurrent islet autoimmunity has been shown to be a major hurdle thwarting the clinical efficacy of beta-cell replacement therapy. Here we propose an alternative with transplantation of genetically immunoprotected pseudo-islets. We demonstrate that third generation lentiviral vectors can be used as efficient gene carriers for protecting primary human beta-cells without affecting their function, thereby confirming earlier studies in intact human islets. [29] As described earlier for rodents, [30] the self-aggregation phenomenon of islet cells offers an attractive opportunity to protect these endocrine "micro-organs" to a large extent prior to transplantation. Although we did not study the precise composition of these pseudoislets in detail, but focused on beta-cells only, our data show that both treatments (viz. dispersion and transduction) are not accompanied by a significant loss in insulin production rate in response to glucose. These data are in line with a recent study aiming at the evaluation of immunoisolation method and showing that pseudo-islets of rat islet cells are superior to primary intact islets in term of survival and function. [31]

The luciferase-based survival assay presented here is an alternative to the currently used traditional tracer-release assays, which have their limitations for monitoring cell cultures (reviewed in [32]). This assay requires a limited number of cells (from 5.000 to 10.000 cells per sample) and the results obtained appeared to be relatively insensitive to variations in the purity of islet preparations. The use of other reporters and substrates (e.g. an amino luciferin derivative specific for caspases 3, 7, 8 or 9) are currently under investigation and may provide new insights into the molecular mechanisms involved in the killing process. [33,34] The assay relies on the specificity of the promoter: the use of a ubiquitously active promoter to drive luciferase-gene expression in assays to measure beta-cell survival was confounded by the increase in luciferase activity due to the expansion of other cell types (data not shown). [26]

Besides systemic immunosuppressive injection or immunomodulatory therapy using educated regulatory T-cells, [35] other strategies to protect or improve insulin secretion of the transplanted beta-cells, for example using encapsulated islets, are currently under investigation. However, the perfect material allowing selective permeability and reduced bioreactivity is not available yet (reviewed in [36]). Similarly to our approach, the direct genetic modification of islets prior to transplantation has also been explored in studies aiming at improving islet function by expression of heme oxygenase (HO-1), downregulation of protein tyrosine phosphatase using siRNA, or prevention of beta-cell death by overexpression of viral proteins (Epstein-Barr virus vIL10, baculovirus p35, and the adenovirus gp19 and RIDa/b). [37-40] However, studies illustrating the feasibility in human islets are rare but efficient thereby lentivirus-mediated expression of cFLIP in as little as 10% of the human islet cells was sufficient to protect these cells from IL-1β, TNF-α, and IFN-γ proinflammatory cytokines treatment. [41]

Herpesviruses have the capacity to establish a lifelong infection of their host, a phenomenon associated with the expression of proteins with potent immunosuppressive properties, eluding $CD8^+$ and $CD4^+$ T-cells, NK-cells and innate immunity. [42,43] Among the different immunevasins, US2 is extremely effective in targeting MHC-I molecules for destruction. Shortly after their synthesis, most of the newly generated MHC class I heavy chains are degraded. [24,44] US2 has been suggested to down-regulate all HLA-A and -G and most HLA-B alleles, whereas several HLA-B alleles and all HLA-C and -E alleles are likely to be insensitive to US2-mediated degradation. Importantly, the fact that US2 does not down-regulate HLA-E may be useful in the context of immune protection, as HLA-E serves as an inhibitor of NK-cells.

We confirmed that US2 expression induces downregulation of class-I in surrogate beta-cells and that its co-expression with serine protease inhibitor 9 protects human islet cells against autoreactive T-cells. The possible risk of increased susceptibility to natural killer (NK) cells associated with MHC-I downregulation could be counteracted with Serpin-9 co-expression by blocking granzyme B activity. [45] The significance of Serpin-9 in protecting beta-cells against PPI autoreactive T-cells has been recently reinforced by a study showing that cytotoxic degranulation is the predominant *modus operandi* of PPI-CD8+ mediated killing. [46]

Environmental factors including proinflammatory cytokines might influence CTL killing capacity and beta-cell survival during transplantation. The clinical transplantation setting where islets are directly infused via the portal vein might complicate the problem. [15] Participation of proinflammatory cytokines, instant blood mediated immune response (IBMIR) and activated complement or beta-cell stress on autoreactive CTL killing capacity and on beta-cell antigenic peptide generation remains to be investigated.

The use of NSG mice allowed us to monitor the human islet survival in mild hyperglycemic environment (basal glucose level is about 1mM higher in mouse than human) to specifically evaluate autoreactive CTL activity without interference from innate immunity and NK activity. [47] The killing assay using human pseudoislets and a human autoreactive T-cell clone isolated from recent onset T1D patient, although not relevant for a long term study of the graft, provides a suitable model to determine the fate of human islet cells in the context of human CD8 autoreactivity. The results prove that these cellular aggregates remained functional and glucose-responsive, indicating that they were capable of resisting autoimmune T-cells.

The lentiviral system used to deliver the protective transgenes is believed to provide a long term expression of US2/Spi9. However cessation of transgene expression, due to promoter silencing, is still possible, and could lead to eradication of the modified cells by immune system. The cytomegalovirus promoter, used here, is very active in finally differentiated cells, like beta-cells but as many viral promoters its expression could be silenced. [48] When translated to the clinic, other promoter, in particular cellular promoters, should be envisaged (Ubiquitin C promoter for example) and included in a specific comparative study.

To summarize, the novelty of our report is three-fold. First we engineered a novel assay to assess specific, autoimmune-mediated destruction of primary human beta-cells, allowing for the definition of selective beta-cell loss *in vitro*. This approach facilitates the creation of a screening platform for identification of new compounds that inhibit the interplay between beta-cells and autoreactive T-cells. Secondly, we designed a preclinical humanized mouse model to allow assessment of the fate of primary human beta-cells in an autoimmune environment. Finally, we showed that lentiviral vectors represent an efficient system for gene transfer into human diploid islet cells that can be subsequently reaggregated into functional pseudoislets. This offers new possibilities for genetic modification as a means of protecting human islet cells against the effect of autoreactive and possibly alloreactive T cells. By targeting two molecular pathways (viz. MHC class I synthesis and the perforin cell death pathway), we could increase the resilience of human beta-cells to acute, antigen-specific autoimmune attack. This study constitutes a proof of concept in mild hyperglycemic condition. The questions of the protective effect in extreme hyperglycemic environment where the increase in insulin production (and by definition the increase of antigenic leader peptide epitope) could exacerbate CTL killing, the scaling up of gene transfer techniques under clinically applicable procedures, the stability of transgene expression, the efficacy of downregulation of different HLA haplotypes and the risk of tumor development remain to be addressed before any translational research. Yet, we can speculate that this type of approach could be successfully extended to protect against any auto- and alloreactivity. A combine approach using other highly potent viral immune evasion proteins could be envisaged to specifically target the HLA type or to increase the level of protection. [21] The next Holy Grail will be the translation of these evasion strategies without the use of viral carrier nor prior disruption of the islet integrity.

MATERIALS AND METHODS

Cells

HEK293T cells were maintained in DMEM + 10% FCS supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml); K562-A2 cells were maintained in IMDM + 10% FCS supplemented with 0,7 mg/ml geneticin (Gibco BRL, Gaithersburg, MD), penicillin (100 units/ml), streptomycin (100 µg/ml). PPI-specific CD8 T cells were cloned previously from a HLA-A201 Type 1 diabetic patient as described previously. [28] For maintenance and expansion, PPI-specific and pp65CMV-directed CTLs were cultured for two weeks with irradiated allogeneic PBMCs, irradiated PPI_{15-24} or CMV_{pp65} peptide-pulsed HLA-A2-expressing EBV-LCL in IMDM supplemented with 10% human serum, 0.5% LeucoA, 0.1 ng/ml recombinant human (rh)-IL12, 10 ng/ml rh-IL7, 25 U/ml rh-IL2 and 5 ng/ml rh-IL15. Cells were frozen in a solution of 20% human pooled serum and 10% DMSO and kept in liquid nitrogen until use. Upon thawing, cells were allowed to rest in IMDM supplemented with 10% human serum, IL-2 (50U/ml) and IL-15 (0.1ng/ml).

DNA constructs

pLV-CMV-US2-bc-Spi9 has been generated from 2 intermediate cloning vectors pLV-CMV-US2-bc-GFP and pLV-CMV-Spi9-bc-GFP. pLV-CMV-US2-bc-GFP has been generated by cloning the US2-bc-GFP cassette from the LZRS-US2-bc-GFP described previously [49] into the pLV-CMV vector. A pJET-Spi9 vector has been generated by cloning a Spi9 PCR fragment into pJET2.1/blunt vector (Fermentas) using the following primers Spi9 Fw5'-TACATAAGGTTACACTAT-3' and Spi9 Rv 5'-AACCCTTGTGTTAAGTAA-3'on human placental cDNA (Agilent technologies). pLV-CMV-Spi9 has been generated by introducing a NotI/XbaI Spi9 containing fragment from pJET-Spi9 into pLV-CMV-bc-GFP. Next, the GFP fragment from pLV-CMV-US2-bc-GFP was removed after NsiI/MscI digestion and blunting reaction, and was replaced by a SmaI/SmaI Spi9 containing fragment from pLV-CMV-Spi9-bc-GFP to generate the final pLV-CMV-US2-bc-Spi9 vector.

The HIP derived constructs have been generated by cloning the human insulin promoter -326/+30 by PCR from human genomic DNA using the following primers FW: 5'-GCGCTCGAGTCTCCTGGTCTAATGTGGAA-3' and Rv: 5'-GCGAAGCTTCTCTTCTGATGCAGCCTGTC-3'. The PCR product containing the XhoI/HindIII linker was then used to replace the CMV promoter in pLV-CMV-GFP to create pLV-HIP-GFP. pLV-HIP-Luc2CP was generated by cloning the HIP-Luc2CP containing fragment from pGL4-HIP-Luc2CP into lentivirus backbone.

Lentiviruses and cell transduction

All vectors are derived from pRRL-cPPT-CMV-IRES-GFP-PRE. Third generation self-inactivating lentivirus vectors were produced as described previously [50]. Lentivirus vectors are quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation, New-York, USA). The infectious titer is derived from the p24 concentration by the conversion that 1ng p24 corresponds to 2500 infectious particles. Whole islets or freshly dispersed islets were seeded in ultralow attachment 6-well plates and maintained in DMEM + 10% FCS + Pen/Strep. Viral supernatants were added to fresh medium supplemented with 8μg/mL polybrene (Sigma), and the cells were incubated overnight. Transduction efficiency was determined by FACS analysis after washing in PBS containing 0.1% BSA and analyzed on a FACS LSRII (BD Pharmingen Inc, San Diego, CA, USA).

Luciferase killing assay

Transduced dispersed islet cells were seeded in U-shape 96-well plates and incubated for 48h with HLA-A2 specific CTLs (directed against pp65CMV- or PPI) in IMDM + 10% human serum supplemented with IL-2 (50U/ml) and IL-15 (0.1ng/ml). After 2 days of co-culture, cells were lysed in Luciferase Lysis buffer (25 mM Tris–HCl, pH 7.8, 2 mM CDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity was determined by luminometry using the Promega Luciferase Assay Reagent. Experiments were performed in triplicate for every target:effector ratio used. Results shown are represented as residual luciferase activity with the luciferase activity of transduced islets incubated without CTL set at 100%.

Western blot

Cells were treated with Luciferase Lysis buffer supplemented with a cocktail of protease inhibitors (Roche). Samples for Western-blot analyses were prepared by boiling protein extracts with Sample buffer (10% glycerol, 2% SDS, 60 mM Tris-Cl (pH 6.7), 2.5% -mercaptoethanol, and 2.5% bromophenol blue from a 1:20-diluted saturated solution) for 5 minutes at 100°C, and analyzed on SDS-PAGE. Proteins were transferred to Immobilon-P (Immobilon-P transfer membrane (polyvinylidene difluoride); Millipore, Etten-Leur, The Netherlands) and visualized by standard protocols with anti-insulin (1:1000, SantaCruz sc14664), and anti-Actin (1:1000 clone C4; ICN Biomedicals, Inc., Zoetermeer, The Netherlands).

Reverse-transcription PCR and real-time PCR

Total cellular RNA was extracted from K562 cells using Trizol. 500 ng RNA was reverse transcribed using Superscript RT II kit (Invitrogen, Karlsruhe, Germany). Expression of the genes of interest was detected using the following primers: Insulin Fw 5′-GCAGCCTTTGTGAACCAACA-3′, Insulin Rv 5′-CGGGTCTTGGGTGTGTAGAAG-3′; GAPDH Fw 5′-ACAGTCAGCCGCATCTTCTT-3′, GAPDH Rv 5′-AATGAAGGGGTCATTGATGG-3′. Real-time PCR was performed in triplicate using the SybrGreen master mix kit (Applied Biosystems) and an Applied Biosystems Step One Plus machine. GAPDH mRNA was used as reference.

Human islet isolation

Human pancreata were harvested from brain-dead organ donors after informed consent was obtained in writing from family members. Leiden University Medical Centre had permission to isolate islets and to use them for scientific research if they are insufficient for clinical islet transplantation, in accordance with national laws and institutional ethical requirements. Human islet isolations were performed in the GMP-facility of the Leiden University Medical Center. Purity of the final islet preparation was assessed by 1 mM Dithizone (Sigma-Aldrich) staining and ranged from 75 and 95%. The purified islets were cultured in CMRL-1066 supplemented with 10% human serum, ciprofloxacin 20 μg/mL, gentamycine 50 μg/mL, L-glutamine 2 nM, fungizone 250 ng/mL, HEPES 10 mM, and nicotinamide 10 mM, and cultured at 37°C in a humidified atmosphere of 5% CO2 for 1–7 days prior to use.

Pseudoislets formation

Dissociation of primary human islets was performed by 5-10 minutes 0.05% trypsin treatment at 37°C. After washing step in DMEM 10% FCS (Invitrogen, Karlsruhe, Germany) containing media, dissociated cells were passed though a 40μm filter and seeded in ultra-low attachment 6-well plates at ~200.000 cells/ml (Corning, NY 14831). Spontaneous re-aggregation of islet cells started to be observed 24h post dissociation. Pseudoislets were maintained 6 days in culture prior reimplantation and medium was refreshed every 2 days.

Mice

6-12 week old male NOD SCID IL-2R-/- mice (NSG) were obtained from the Jackson Laboratory, Europe, and bred in the animal facilities of the LUMC. Animals were

housed under specific pathogen-free conditions. All studies were approved by the local Animal Care Committee.

Human islets/pseudoislets transplantation

3000 islet equivalents and 5 x 10^6 dispersed and reaggregated islet cells, either non-transduced or GFP–transduced, were transplanted under the left kidney capsule, using a polyethylene catheter and precision syringe (Hamilton, Reno, NV). Grafts were removed 19 days post transplantation. Formalin- fixed paraffin-embedded sections were stained for insulin and DNA content. Insulin and DNA staining, and GFP epifluorescence were assessed by confocal microscopy. Evaluation of PPI-specific CTL killing *in vivo* was performed by co-transplantation of 5 x 10^6 reaggregated cells, either GFP or US2/Spi9 transduced, with HLA-A2 PPI-directed cytotoxic T-cells at a 100:1 target:effector ratio. Islets isolated from 3 different donors have been genetically modified by GFP or US2/Spi9 lentiviral vectors and transplanted under the kidney capsule of 3 different NSG mice. Islet functionality was evaluated after IPGTT by human insulin and c-peptide measurement.

Immunostaining and confocal microscopy

Intact islets or pseudoislets were fixed in PBS containing 4% paraformaldehyde. Permeabilization was performed using PBS/1% Triton. Blocking was done with PBS/BSA 3%, and first and secondary antibodies were diluted in PBS/BSA 3%. Anti-Insulin (H86 - Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used at 1:200 and secondary antibody coupled to Alexa 568 was used at a dilution of 1:500. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI). Samples were subjected to optical sectioning using a laser scanning confocal microscope (Zeiss).

Glucose-induced insulin-secretion test

Intact islets or pseudoislets were washed in PBS and transferred to a 8µm pore size transwell plate (Corning® HTS Transwell®-96 well, Sigma-Aldrich) and pre-incubated in a modified Krebs-Ringer Bicarbonate buffer (KRBH) containing 115mM NaCl, 5mM KCl, 24mM NaHCO3, 2.2mM CaCl2, 1mM MgCl2, 20mM HEPES, 2 g/l human serum albumin (Cealb, Sanquin, The Netherlands), pH 7.4 for 2 hours. The transwell plate was then successively transferred for 1 hour to KRBH with 2 mM and 20 mM glucose at 37°C. Insulin concentrations were determined in the supernatants by ELISA (Mercodia, Uppsala, Sweden).

Intraperitoneal glucose tolerance test

For the Intraperitoneal Glucose Tolerance Tests mice were fasted overnight and injected with 2 g/kg Glucose 1 M i.p. Blood was drawn from the tail vein at t=0, t=30 and t=45 min. IPGTT was validated by determination of blood glucose concentrations measured using a glucose meter (Accu Chek, Roche, Basel, Switzerland). Human insulin content and human C-peptide concentrations were determined by ELISA according to the manufacturer's instructions. (Human insulin ELISA 10-113-10 and human C-peptide ELISA 10-1136-01, Mercodia, Uppsala, Sweden).

Statistical analysis

All data were presented as mean ± standard deviation (SD). For Human Insulin and human C-peptide values presented in Figure 6, data were subjected to nonparametric statistical analysis using two-way ANOVA test with Bonferroni correction using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant.

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No potential conflicts of interest relevant to this article were reported.

SUPPLEMENTARY MATERIAL

CTL killing assay

Cytotoxicity was analyzed by a classical chromium release assay. Briefly, following incubation with 100 µl Na-chromate (^{51}Cr, 3.7 MBq) for 1 h, K562-PPI-A2, K562-A2 cell lines were seeded in triplicate at various effector-to-target (E:T) together with CTLs. After 4 h incubation at 37°C in 5% CO_2, supernatants were collected, and the release of ^{51}Cr was measured with a gamma-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and maximum releases were obtained by incubation with medium and 1% triton in PBS, respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 × (experimental release − spontaneous release) / (maximum release − spontaneous release).

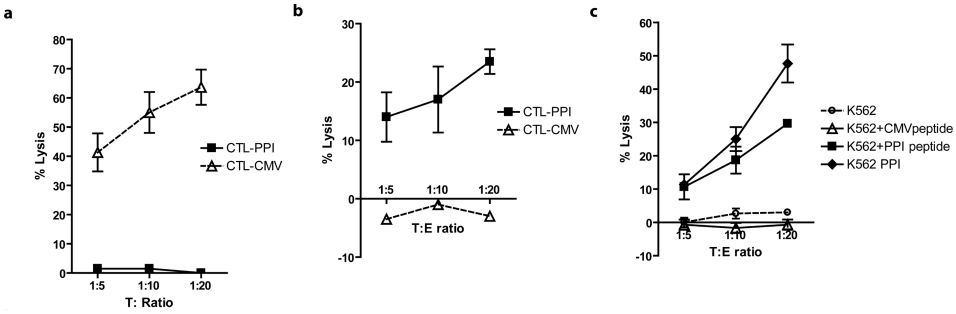

Figure S1 | CTLs killing capacity.

a) Chromium-release assay performed on A2/K562 cells preincubated with 10ng CMV peptide and incubated with CMV-directed CTLs or PPI-directed CTLs. **b)** Similar experiment as seen in a) performed on A2/K562 preincubated with 10 ng PPI purified peptide. **c)** Chromium-release assay performed using PPI directed CTL on A2/K562-A2 or A2/K562 with 10ng PPI peptide or on PPI overexpressing A2/K562 cells.

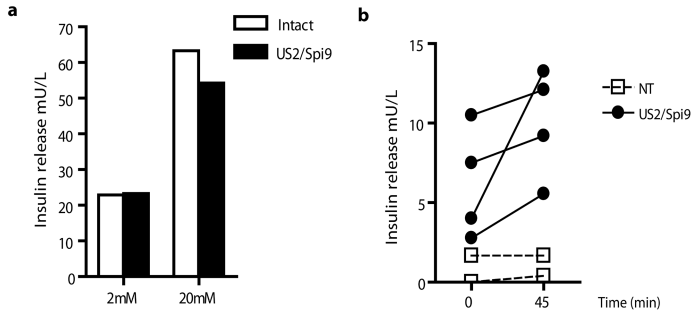

Figure S2 | Effect of US2/Spi9 on islets functionality.

a) Comparative insulin release of intact islets (white bars) and US2/Spi9-modified islets (black bars) determined by glucose-stimulated insulin-secretion test following incubation in 2mM or 20mM glucose. **b)** IPGTT performed on mice co-transplanted with 5.10^6 LV-GFP (n=1) or LV-US2/Spi9 modified pseudoislets (n=2). Non transplanted mice (NT) are used as control (n=1). Blood samples were taken at 4, and 11days post transplantation before the intraperitoneal glucose ionjection (t=0) and 45min after glucose injection (t=45min)

REFERENCES

1. In't Veld P, Lievens D, De Grijse J et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 2007;56:2400-2404.
2. Cnop M, Welsh N, Jonas JC et al. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54 Suppl 2:S97-107
3. Coppieters KT, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60
4. Katz J, Benoist C, Mathis D. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur J Immunol 1993;23:3358-3360
5. Serreze DV, Leiter EH, Christianson GJ et al. Major histocompatibility complex class I-deficient NOD-B2null mice are diabetes and insulitis resistant. Diabetes 1994;43:505-509
6. Wicker LS, Leiter EH, Todd JA et al. Beta 2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. Diabetes 1994;43:500-504
7. Hamilton-Williams EE, Palmer SE, Charlton B et al. Beta cell MHC class I is a late requirement for diabetes. Proc Natl Acad Sci U S A 2003;100:6688-6693
8. Kay TW, Parker JL, Stephens LA et al. RIP-beta 2-microglobulin transgene expression restores insulitis, but not diabetes, in beta 2-microglobulin null nonobese diabetic mice. J Immunol 1992;157:3688-369.
9. Chervonsky AV, Wang Y, Wong FS et al. The role of Fas in autoimmune diabetes. Cell 1997;89:17-24
10. Apostolou I, Hao Z, Rajewsky K et al. Effective destruction of Fas-deficient insulin-producing beta cells in type 1 diabetes. J Exp Med 2003;198:1103-1106
11. Kagi D, Odermatt B, Ohashi PS et al. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J Exp Med 1996;183:2143-2152
12. Thomas HE, Trapani JA, Kay TW. The role of perforin and granzymes in diabetes. Cell Death Differ 2010;17:577-585
13. Roep BO, Atkinson M. Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. Diabetologia 2004;47:1650-1656
14. Roep BO, Atkinson M, Von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 2004;4:989-997
15. Harlan DM, Kenyon NS, Korsgren O et al. Current advances and travails in islet transplantation. Diabetes 2009;58:2175-2184
16. Pinkse GG, Tysma OH, Bergen CA, et al. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci U S A 2005;102:18425-18430

17. Huurman VA, Hilbrands R, Pinkse GG, et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS One 2008;3:e2435

18. Huurman VA, Velthuis JH, Hilbrands R, et al. Allograft-specific cytokine profiles associate with clinical outcome after islet cell transplantation. Am J Transplant 2009;9: 382-388

19. Hilbrands R, Huurman VA, Gillard P et al. Differences in baseline lymphocyte counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes 2009;58:2267-2276

20. Blondet JJ, Carlson AM, Kobayashi T et al. The role of total pancreatectomy and islet autotransplantation for chronic pancreatitis. Surg Clin North Am 2007;87:1477-1501

21. Griffin BD, Verweij MC Wiertz EJ. Herpesviruses and immunity: the art of evasion. Vet Microbiol 2010;143:89-100

22. Horst D, Verweij MC, Davison AJ et al. Viral evasion of T cell immunity: ancient mechanisms offering new applications. Curr Opin Immunol 2011;23:96-103

23. Zaldumbide A, Hoeben RC. How not to be seen: immune-evasion strategies in gene therapy. Gene Ther 2008;15:239-246

24. Wiertz EJ, Tortorella D, Bogyo M et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996;384:432-438

25. Medema JP, De Jong J, Peltenburg LT et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A 2001;9811515-11520

26. Carlotti F, Zaldumbide,A, Loomans CJ, et al. Isolated human islets contain a distinct population of mesenchymal stem cells. Islets 2010;2:164-173

27. Bishop AE, Polak JM. The Anatomy, Organisation and Ultrastructure of the Islets of Langerhans. (Blackwell Science,Oxford,UK, 1997)

28. Skowera A, Ellis RJ, Varela-Calvino R, et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118: 3390-3402

29. Kobinger GP, Deng S, Louboutin JP et al. Transduction of human islets with pseudotyped lentiviral vectors. Hum Gene Ther 2004;15: 211-219

30. Callewaert H, Gysemans C, Cardozo AK et al. Cell loss during pseudoislet formation hampers profound improvements in islet lentiviral transduction efficacy for transplantation purposes. Cell Transplant 2007;16: 527-537

31. O'Sullivan ES, Johnson AS, Omer A et al. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 2010;53: 937-945

32. Jerome KR, Sloan DD, Aubert, M. Measurement of CTL-induced cytotoxicity: the caspase 3 assay. Apoptosis 2003:8:563-571

33. O'Brien MA, Daily WJ, Hesselberth PE, et al. Homogeneous, bioluminescent protease assays: caspase-3 as a model. J Biomol Screen 2005;10:137-148

34. Kanno A, Umezawa Y, Ozawa, T. Detection of apoptosis using cyclic luciferase in living mammals. Methods Mol Biol 2009;574:105-114

35. Kleijwegt FS, Laban S, Duinkerken G et al. Transfer of regulatory properties from tolerogenic to proinflammatory dendritic cells via induced autoreactive regulatory T cells. J Immunol 2011;187:6357-6364

36. O'Sullivan ES, Vegas A, Anderson DG et al. Islets transplanted in immunoisolation devices: a review of the progress and the challenges that remain. Endocr Rev 2011;32: 827-844.

37. Carter JD, Ellett JD, Chen M et al. Viral IL-10-mediated immune regulation in pancreatic islet transplantation. Mol Ther 2005;12:360-368

38. Hollander K, Bar-Chen M, Efrat S. Baculovirus p35 increases pancreatic beta-cell resistance to apoptosis. Biochem Biophys Res Commun 2005;332:550-556

39. Kojaoghlanian T, Joseph A, Follenzi A et al. Lentivectors encoding immunosuppressive proteins genetically engineer pancreatic beta-cells to correct diabetes in allogeneic mice. Gene Ther 2009;16:340-348

40. Lu B, Wu H, Gu P, et al. Improved glucose-stimulated insulin secretion by intra-islet inhibition of protein-tyrosine phosphatase 1B expression in rats fed a high-fat diet. J Endocrinol Invest 2012;35:63-70

41. Fenjves ES, Ochoa MS, Cechin S et al. Protection of human pancreatic islets using a lentiviral vector expressing two genes: cFLIP and GFP. Cell Transplant 2008;17:793-802

42. Wiertz E, Hill A, Tortorella D et al. Cytomegaloviruses use multiple mechanisms to elude the host immune response. Immunol Lett 1997;57:213-216

43. Wiertz EJ, Mukherjee S, Ploegh HL. Viruses use stealth technology to escape from the host immune system. Mol Med Today 1997;3:116-123

44. Barel MT, Pizzato N, Le Bouteiller P et al. Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2- and US11-mediated degradation. Int Immunol 2006;18:173-182

45. Falk CS, Mach M, Schendel DJ et al. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. J Immunol 2002;169:3257-3266

46. Knight RR, Kronenberg D, Zhao M et al. Human beta-Cell Killing by Autoreactive Preproinsulin-Specific CD8 T Cells Is Predominantly Granule-Mediated With the Potency Dependent Upon T-Cell Receptor Avidity. Diabetes 2012;62:205-213

47. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. Nat Rev Immunol 20007;7:118-130

48. Brooks AR, Harkins RN, Wang P, et al. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J Gene Med 2004;6:395-404

49. De La Garza-Rodea AS, Verweij MC, Boersma H et al. Exploitation of herpesvirus immune evasion strategies to modify the immunogenicity of human mesenchymal stem cell transplants. PLoS One 2011;6:e14493

50. Carlotti F, Bazuine M, Kekarainen T et al. Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes. Mol Ther 2004;9:209-217