Biomedical Materials

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Downregulation of beta-microglobulin to diminish T-lymphocyte lysis of non-syngeneic cell sources of engineered heart tissue constructs

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Abstract

The presence of non-autologous major histocompatibility complex class I (MHC-I) molecules on the surface of the grafted cells is one of the main reasons for their rejection in non-syngeneic hosts. We present a straightforward strategy to decrease the presence of MHC-I by shRNA inhibition of beta-2-microglobulin (B2M), a conservative light chain of MHC-I, on the surface of two main cell types that are used to engineer heart tissue constructs.

Engineered heart tissue constructs can be generated by combining mouse WT19 fibroblasts and mouse embryonic stem cell-derived cardiac myocytes (mESC-CM). WT19 fibroblasts were stably transduced with an anti-B2M shRNA, which yielded a cell line with dramatically reduced B2M expression levels (16 ± 11% of mock treated control cell line). Interferon gamma treatment increased the levels of B2M expression by >3-fold in both control and transduced fibroblasts; yet, B2M expression levels still remained very low in the transduced cells. When compared with their unmodified counterparts, transduced fibroblasts caused 5.7-fold lesser activation of cognate T-cells. B2M depletion in mESC-CM was achieved by 72 h transduction with anti-B2M shRNA lentiviral particles. Transduced mESC-CM exhibited regular beating and expressed classical cardiac markers. When compared with their unmodified counterparts, transduced mESC-CM caused 2.5-fold lesser activation of cognate T-cells. *In vivo* assessment of B2M downregulation was performed by analyzing the preferential survival of B2M-downregulated cells in the intraperitoneal cavity of allogeneic mice. Both B2M-downregulated fibroblasts and B2M-downregulated myocytes survived significantly better when compared to their unmodified counterparts (2.01 ± 0.4 and 5.07 ± 1.6 fold increase in survival, respectively). In contrast, when modified WT19 fibroblasts were injected into the intraperitoneal cavity of syngeneic C57Bl/6 mice, no significant survival advantage was observed. Notably, the preferential survival of B2M-downregulated cells persisted in allogeneic hosts with normal levels of natural killer cells, although the effect was lesser in magnitude.

Use of shRNA against beta-2-microglobulin offers a simple and effective approach to minimize immunogenicity of the main cellular components of cardiac tissue constructs in non-syngeneic recipients.

List of abbreviations

MHC-I	major histocompatibility complex class I molecules
MHC-II	major histocompatibility complex class II molecules
mESC-CM	mouse embryonic stem cell-derived cardiac myocytes
ESC	embryonic stem cells
B2M	beta-2-microglobulin
iPSC	induced pluripotent stem cells
EHT	engineered heart tissue
NK	Natural Killer cells
B3Z	H-2K^b-restricted CTL hybridoma
CTL	cytotoxic T lymphocyte
shRNA	short hairpin RNA

1. Introduction

In the age of induced pluripotent stem cells (iPSC), one could argue that obtaining allogeneic stem cell derivatives is becoming obsolete. However, the use of iPSC for tissue repair has a number of limitations. First, iPSC lines vary in their ability to differentiate into certain cell types due to significant epigenetic factors [1–3]. Thus, it may be necessary to derive several iPSC lines from the same patient and to test each line for its differentiation potential. Second, when dealing with genetic diseases, regeneration of damaged organs with a patient's own cells will perpetuate the disease [4]. Third, it is increasingly difficult to generate iPSC from a patient with advanced age [5]. Fourth, the derivation and characterization of iPSC is time intensive, and such a time scale may be inappropriate for treating most diseases. Finally, recent studies have suggested that iPSC-derivatives are targeted by the autologous host's immune system [6], presumably due to genomic alterations acquired during the reprogramming of somatic cells, as well as other poorly understood factors [7].

If the cell sources used to engineer tissue grafts are not autologous, then the host immune system has to be suppressed to prevent graft rejection. However, immunosuppression can lead to alterations in normal graft maturation, toxic side effects, acquisition of unrelated infections, and/or increased graft tumorogenicity [7, 8]. These adverse effects can negate or reduce the beneficial effects of graft implantation [9]. An alternative approach to aggressive immunosuppression of the host is to modify the non-autologous donor cell source, so the cellular graft components are less recognizable to the immune system. In other words, if donor antigens responsible for inducing an immune response are downregulated, then rejection of engineered tissue grafts made with non-autologous cells should diminish. In this study, we tested a hypothesis that the downregulation of major histocompatibility complex one molecules (MHC-I) on the surface of fibroblasts and stem cell-derived cardiac myocytes will diminish their recognition by the immune system of the host both *in vitro* and *in vivo*. Fibroblasts and cardiac myocytes are the two major components of engineered heart tissue (EHT). Many studies, including ours, have shown that both EHTs and stem cell based cardiac grafts can be effective in restoring cardiac function after myocardial infarction [10–12]. However, as of today, studies that use tissue engineering tools to repair injured hearts have only been performed in immunosuppressed or immunocompromised animals. This holds true for studies that employed bulk injection of cardiac progenitors into myocardial scar [11, 13, 14], as well as studies that used EHT constructs to aid cardiac performance [15–17]. Since continuous immunosuppression has a number of disadvantages and side effects, the development of simple and cost effective strategies to decrease the immunogenicity of EHTs made of allogeneic cell components will be a welcome development.

Two major classes of MHC molecules determine the recognition of foreign cells by the host's T-lymphocytes – MHC class I and MHC class II. MHC class I molecules are expressed on most nucleated cells, including cardiac myocytes and fibroblasts. The MHC class I molecule is a heterodimer of a highly polymorphic alpha heavy chain, non-covalently associated with a conserved light chain called beta-2 microglobulin (B2M). The conserved B2M heterodimerizes with the heavy chain to form a functional MHC class I molecules on a cell surface. MHC class I molecules then present endogenously synthesized peptides to host CD8+ T lymphocytes. MHC class II molecules, on the other hand, are mainly expressed by professional antigen-presenting cells, such as dendritic cells, macrophages, and B-lymphocytes. Professional antigen-presenting cells are rare in the heart, and the expression levels of MHC class II molecules in cardiomyocytes and fibroblasts is negligible [18].

We hypothesized that the immunogenicity of two main cell components of cardiac EHTs, fibroblasts and cardiac myocytes, can be significantly decreased by knocking down B2M, the invariable conserved light chain of MHC-I. Specifically, we suggested that shRNA based inhibition of B2M will diminish the presence of functional MHC-I on the cell surface and, as a result, decrease their recognition by T-lymphocytes from allogeneic hosts. We also wanted to show that engineered heart tissue can be formed from stem cell derived cardiomyocytes and any type of fibroblasts—not just cardiac. Non-cardiac fibroblasts will be much easier to obtain from autologous sources, such as the skin, or by scale-up using established fibroblasts lines.

We believe that our findings will be of interest to many groups in the regenerative medicine field, as they seek straightforward strategies to minimize graft rejection in allo- or xenogeneic hosts. Similar approaches can be used for implantation of other tissue types or differentiated cells.

2. Materials and methods

2.1. Cell sources

NIH/3T3 mouse fibroblasts were purchased from ATCC. The WT19 fibroblasts were a gift from Dr. Anne Campbell [19]. WT19 cells are mouse fibroblasts of the H-2^b haplotype obtained from C57Bl/6 mice and are immortalized by transformation with Simian Virus-40 T large antigen. Rat neonatal cardiac fibroblasts were obtained from the pre-plating step during standard isolation of neonatal cardiomyocytes [20]. To insure absence of contamination with rat cardiomyocytes, the adherent neonatal cardiac fibroblasts were passaged in tissue culture-treated dishes two additional times, following the initial isolation. Mouse stem cell derived cardiac myocytes (mESC-CM) were purchased from LONZA (#XCAC-1010N). The mESC-CM were generated from the ES-D3 mouse embryonic stem cell line (ATCC, 129S4/Jae strain) by stable transduction with a

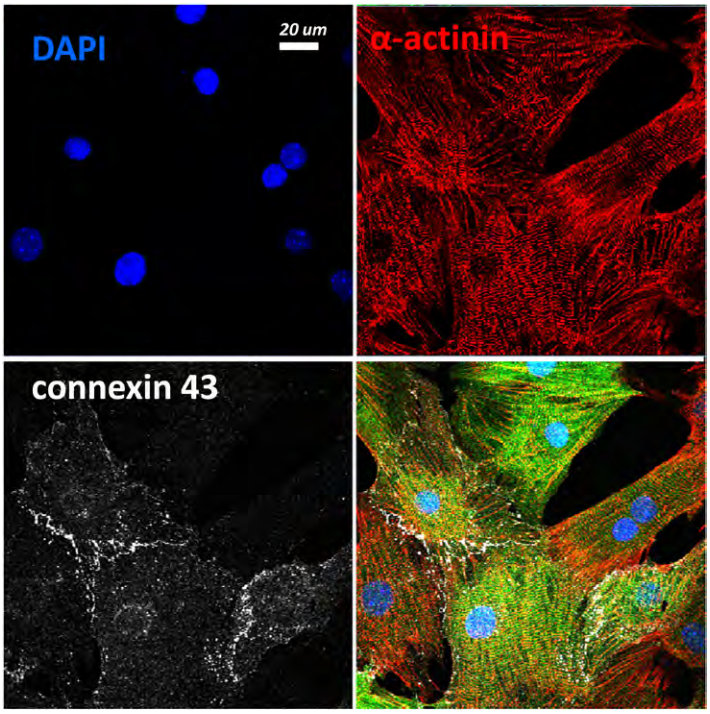

Figure 1. High purity preparations of mouse embryonic stem cell cardiomyocytes (mESC-CM). Use of cardiac-specific promoter driving the expression of both an antibiotic resistance gene and the EGFP cassette enables selection of a high purity population of mESC-CM with >99% purity. A composite image shows nuclei (DAPI), striated sarcomeric alpha-actinin staning (red), connexin 43 staining (white) on the background of endogenous EGFP staining (green).

bicistronic vector [21]. This vector contains a cardiac-specific promoter driving the expression of both an antibiotic resistance gene and the EGFP cassette, ensuring selection of a high purity population of mESC-CM. The mESC-CM were maintained and differentiated according to the manufacturer's instructions. Spontaneously beating mESC-CM layers were used within 3–14 d after cell plating. Where indicated, the cells were treated with 20 ng ml^{-1} recombinant murine interferon gamma (IFNγ (PeproTech, NJ).

2.2. Engineered cardiac tissue fibers

Engineered heart tissue (EHT) in the form of 3D cardiac fibers was created by layering different concentrations of Matrigel (Invitrogen, BD biosciences), as we previously described [22]. To prevent overgrowth, irradiation (20 GY) was used to treat immortalized fibroblast lines before these cells were seeded into fibers. Different ratios of fibroblasts and cardiac myocytes were tested, including 0:1, 1:4, 1:1, 4:1 and 1:0 fibroblast-to-myocyte ratios. 30 μl of undiluted Matrigel matrix was placed in the center of an 18 mm glass coverslip and allowed to solidify for 5 min at 37 °C. A 250 μl suspension of 10^6/ml cells in 12.5% Matrigel was then overlaid on top of the solidified Matrigel pillow. The construct was then allowed to congeal overnight. Cell constructs were then maintained at 37 °C under standard cell culture conditions. The 1:1 ratio produced the best results, yielding reproducible cardiac fibers with a 50–200 μm diameter. The fiber formation process was documented using phase contrast imaging and calcium transients were recorded by loading the fibers with 10 μM Fluo-4, a calcium indicator dye.

2.3. Immunocytochemistry and imaging protocols

Samples were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and blocked overnight in 1% bovine serum albumin. Samples were stained with the following primary antibodies: mouse alpha-actinin (1:800 Sigma), rabbit connexin-43 (1:500 Sigma), followed by goat anti-mouse Cy3 and/or donkey anti-rabbit Cy5 secondary antibodies (1:1000, Jackson ImmunoResearch Labs Inc). Nuclei were counterstained with DAPI (Molecular Probes). To record calcium transients, fibers were loaded with the calcium-sensitive indicator Fluo-4 AM (Invitrogen, 10 μg ml^{-1} for 30 min) and recordings were acquired using a Zeiss LSM 510 confocal imaging system. To visualize different cell types, cells were pre-loaded with either orange or green live intracellular dyes (CellTrackers C34551 & C7025, Invitrogen) according to the manufacturer's instructions.

2.4. Lentiviral transductions

Lentiviral transduction particles were purchased from Sigma Mission (clone #TRCN0000066424). The backbone vector (pLKO.1-Puro) used for the cloning of B2M shRNA contains a puromycin resistance gene. For B2M silencing, the monolayers of WT19 fibroblasts or mESC-CMs were transduced with 3.4×10^5 TU ml^{-1} in the presence of hexadimethrine bromide (polybrene, Sigma Aldrich) at a final concentration of 8 μg ml^{-1}

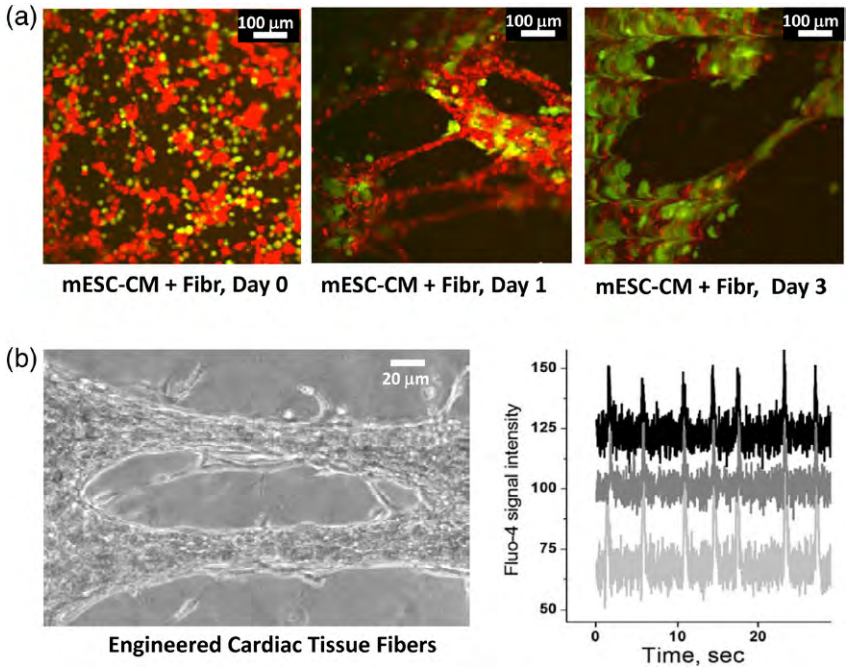

Figure 2. Formation of cardiac fibers from mESC-CM and WT19 fibroblasts. (*a*) A 50:50 mixture of mESC-CM cells (green) and WT19 fibroblasts (red) seeded in a Matrigel pillow led to the formation of regularly contracting engineered cardiac fibers starting on day 3. Cells were preloaded with live cell indicators before seeding. The fibers continued to contract for >10 d after plating. The right panel shows a robust motion artifact associated with repetitive contractions of the fibers throughout acquisition period of a high resolution confocal image. Identical conclusions were derived from $n = 4$ independent mESC-CM and WT19 co-culture experiments. (*b*) Phase contrast image showing the typical appearance of spontaneously beating engineered cardiac fibers made of 1:1 cell mixture of fibroblast and cardiac myocytes. The right panel shows representative traces from one Fluo-4 loaded fiber. Signals were acquired from three different regions of interest, placed at ~200 μm from each other. Each transient corresponds to a single contraction. Synchrony between the individual traces indicates that cardiac myocytes within the construct are electrically well-coupled to each other. When seeded in a Matrigel pillow in the absence of fibroblasts, mESC-CM failed to form 3D cardiac fibers. Instead, mESC-CM formed confluent, flat monolayers indistinguishable in appearance from cells plated on laminin-coated coverslips.

(MOI 1–1.5). Polybrene is a cationic polymer, which was used to increase the efficiency of infection. Media was replaced at 48 h post-transduction. Control (mock) samples were treated identically, but lentiviral particles were omitted.

2.5. Qualitative and quantitative PCR

Genomic DNA was obtained from parental and stably transduced WT19 cells using a Qiagen Purification Kit. DNA was quantitated using a NanoDrop 2000 Spectrophotometer (ThermoScientific), and equal amounts of genomic DNA were used as a template for each clone to prove transgene incorporation. Plasmid DNA encoding the trangene was used as positive control. The presence of incorporated B2M shRNA was determined by using Sigma Mission shRNA Integrants specific primers: Forward -ACAAAATACGTGACGTAGAAA and Reverse—TTTGTTTTTGTAATTCTTTA. For quantitative PCRs, total RNA was isolated using Trizol (Invitrogen) per the manufacturers' instructions. First strand cDNA synthesis was generated using the Affinityscript qPCR cDNA synthesis kit (Stratagene). qPCR was performed on the ABI 7300 (Applied Biosystems) using Brillant SYBR green (Stratagene), and samples were normalized to 18S using the ABI 7300 System Software. The following primers were used for beta2-microglobulin: Forward – GGTGCTTGTCTCACTGACCGGC, Reverse – TTCTCCGGTGGGTGGCGTGA. Primers were ordered from BioSynthesis Inc. (Lewisville, TX).

2.6. T-lymphocyte assay

B3Z cells were a gift from Dr Shastri's Laboratory at the University of California, Berkley [23, 24]. B3Z cells were cultured in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. B3Z is a T-cell hybridoma expressing a T-cell receptor that specifically recognizes the chicken ovalbumin peptide $OVA_{(257–264)}$ (SIINFEKL) in the context of H-2K^{b} haplotype. T-cell hybridoma assays were performed to assess the ability of cells to present a cognate peptide to T-cells. Briefly, confluent monolayers of control and modified cells were loaded with SIINFEKL for 1 h, and the excess peptide was washed away with Tyrode's solution. The same stock solution of B3Z cells was added simultaneously to all samples at 2×10^5 cell ml^{-1} concentrations and co-cultured for 24 h. Co-cultures were washed, fixed, and stained using a beta-galactosidase staining kit (Invitrogen) in accordance with the manufacturer's protocol. The number of activated T-cells, which became visible by

turning blue, were counted within five individual fields of view under 25X magnification by a blind observer. Unloaded cells (without SIINFEKL peptide) served as negative controls.

2.7. In vivo survival assessment of modified versus control cells

Recipient mice (C57Bl/6 and Balb/C, Jackson laboratories, Bar Harbor, Main) were pre-immunized with C57Bl/6 spleenocytes (1×10^7 cells/animal) seven days prior to the transfer of target cells in order to allow for the development of immune response to foreign MHC molecules. Where indicated, mice were depleted of Natural Killer (NK) cells by injection of anti-Asialo GM1 antibody (Wako Pure Chemical Industries, 100 ul/mouse) on day 6 post-immunization [25]. On day 7 post-immunization, mock and MHC-I down-regulated cells were loaded with intracellular dyes (CellTracker, Invitrogen). Control cells were loaded with CellTracker Green (C7025), whereas B2M down-regulated cells were loaded with CellTracker Orange (C34551) as per manufacturer's instructions. The cells were then mixed in a 1:1 ratio and injected into the peritoneum of recipient mice using a 20G needle. Cells were collected 15–18 h post-transfer by intraperitoneal lavage and analyzed by FACS. A total of 5×10^5 cells were collected per mouse for FACS analysis. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at the George Washington University, and in full compliance with federal and international guidelines.

2.8. Statistical analysis

Values are presented as mean ± SEM. Each quantitative or qualitative conclusion was derived from $n \geq 3$ independent experiments. Representative images are shown. A Student's t-test was used to compare the differences between the means with $p < 0.05$ considered significant, unless indicated otherwise.

3. Results

3.1. Cardiomyocytes and fibroblasts: two key components of engineered cardiac tissue

To efficiently form 3D cardiac tissue constructs, both cardiomyocyte and fibroblast cell sources are necessary. This important point is often overlooked, mainly because the most common cell sources used to create cardiac tissue constructs are either preparations of neonatal rat cardiomyocytes or crude mixtures of partially differentiated stem cells. Both sources contain a significant number of residual fibroblasts that rapidly proliferate in culture, thus providing trophic and structural support to cardiac myocytes. Yet, when using purified preparations of cardiomyocytes, such as the ones used in this study (figure 1), the inclusion of fibroblasts becomes a necessity. To verify that engineered heart constructs can be formed using any

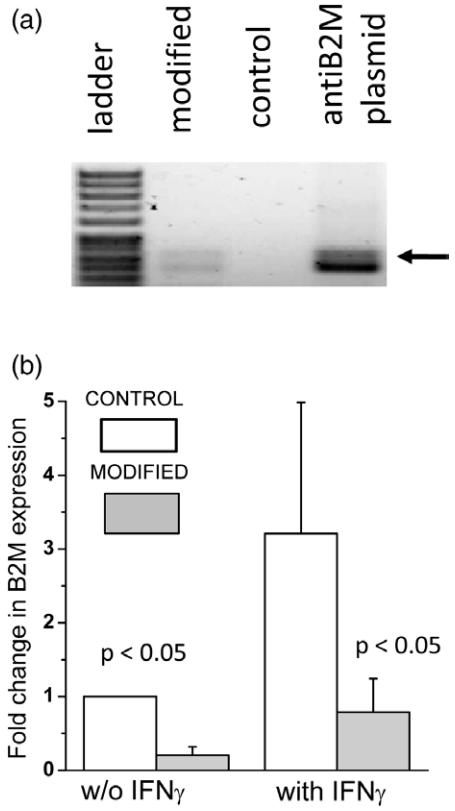

Figure 3. Lentiviral transduction of WT19 fibroblasts leads to a decrease in MHC-I light chain expression. (*a*) Incorporation of shB2M coding sequence was demonstrated by PCR analysis of genomic DNA isolated from parental and shB2M-transduced cells. shB2M plasmid was used as a positive control. (*b*) Levels of B2M expression in control and modified fibroblasts. B2M mRNA expression was significantly downregulated in WT19 fibroblasts compared with the corresponding mock-transduced controls, both with and without IFNγ treatment (48 h).

type of fibroblasts, not just cardiac, we tested three fibroblast sources, including primary rat cardiac fibroblasts and two different immortalized mouse fibroblasts lines, NIH/3T3 and WT19—all of which produced qualitatively similar results. Specifically, when seeded in a 3D environment using a previously described Matrigel pillow approach [22], synchronously beating macroscopic fibers were formed only when fibroblasts and cardiomyocytes were seeded together, with 1:1 seeding ratio yielding the best results. The two cell types began to coalesce after ~2 h in culture, and fiber formation began on day 1 (figure 2(*a*)), fibroblasts pre-loaded with red cell tracker, myocytes with green). The fibers were structurally well developed and contracted rhythmically and synchronously by day 3 of cultivation and for >10 d afterwards. An example of a typical cardiac fiber can be seen in figure 2(*b*), with representative Fluo-4 traces acquired from three different regions of interest (~200 μm distance between). These cardiac fibers exhibited continuous beating with slight variations in frequency; the latter is commonly observed in spontaneous beating, non-paced, cardiomyocytes. Since each calcium transient corresponds to fiber contraction, the fact

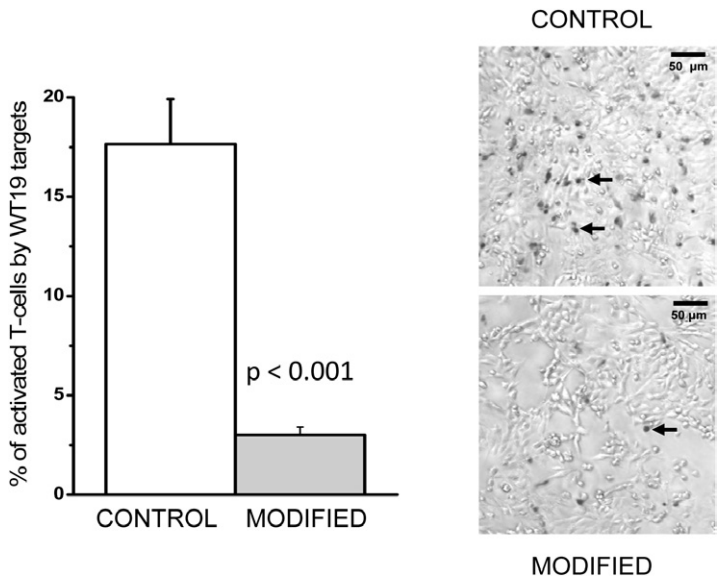

Figure 4. Lentiviral transduction of WT19 fibroblasts leads to decreased *in vitro* activation of T-lymphocytes. Quantification of the activated T cells (dark colored cells) by the control and modified WT19 fibroblasts. Parallel cultures of control and modified WT19 fibroblasts were loaded with SIINFEKL peptide, co-cultured with B3Z hybridoma cells for 24 h, followed by cell count of activated T-cells. On the right is a representative pair of images of activated T cells on the surface of control and modified WT19 fibroblasts. Arrows point to activated T-cells.

that all three traces are in synchrony with each other indicates that the cardiac myocytes within the constructed fiber are well-coupled. These macroscopic fibers continued to be viable and self-contracting for many weeks in culture (>3 weeks). Notably, cell mixtures containing only mESC-CM did not yield any macroscopic fibers. Instead, cardiomyocytes formed an actively contracting flat cell layer on the top of the Matrigel pillow. On the other hand, when seeding cell mixtures contained only fibroblasts, they produced a macroscopic fiber network which was completely motionless (data not shown).

3.2. Downregulation of B2M expression in WT19 fibroblasts

We choose the WT19 fibroblast cell line to test the effectiveness of B2M inhibition strategy because their specific H-2K^b haplotype enables use of the B3Z assay, noted below. The cells were stably transduced with B2M shRNA lentiviral particles and cultured under puromycin pressure for ten generations to ensure stable incorporation of the B2M shRNA gene into the genome (figure 3(*a*)), yielding what we called a modified WT19 cell line. B2M expression was significantly lower in the modified WT19 cells, as compared with control, mock-transduced cells (16 ± 11% from control, $p = 0.003$, figure 3(*b*)). Next we tested IFNγ treatment, which is frequently used to mimic graft inflammation as it upregulates the expression of MHC-I molecules [26]. As expected, B2M expression was upregulated >3-fold in control, mock-transduced cells following IFNγ treatment. However, in modified WT19 fibroblasts, the effects of IFNγ treatment were blunted with B2M expression levels dramatically lower than in the mock-transduced control cell line (24 ± 14% from control, $p = 0.009$, figure 3(*b*)).

3.3. Decrease in the presentation ability of B2M downregulated fibroblasts to T-cell hybridoma (B3Z)

Low levels of B2M mRNA expression detected by qPCR do not necessarily indicate a deficiency in the functional activity of MHC-I molecules on the surface of the cells. To assess the physiological consequences of B2M downregulation, we employed an *in vitro* cytotoxic lymphocyte (CTL) assay. This assay utilizes LacZ expression as an indicator of B3Z T-cell hybridoma activation, which occurs upon recognition of the ovalbumin peptide (SIINFEKL) in the context of a fully assembled MHC-I molecule on the surface of target cells [23]. WT19 fibroblasts are of the H-2K^b haplotype, and therefore, are recognizable by B3Z cells. B3Z cells carry a beta-galactosidase construct driven by a nuclear factor of activated T-cell elements from the interleukin-2 promoter [24], and they develop a colorimetric reaction when a beta-galactosidase substrate is added. B2M downregulated fibroblasts had a significantly lower amount of attached and activated B3Z T-cells compared to the mock-transduced WT19 fibroblasts (17.6 ± 2.2% versus 3.08 ± 0.4%, $p = 0.0004$, figure 4).

3.4. Downregulation of B2M in mESC-CMs

Next, we validated the B2M inhibition strategy in mESC-CMs. Upon terminal differentiation into cardiomyocytes, mESC-CM cease to proliferate. Therefore, we employed a transient (72 h) transduction with a B2M shRNA lentivirus to modify mESC-CM.

Importantly, in all of the independent transduction experiments ($n = 4$), transduced cells expressed standard cardiac protein markers (figure 4(*a*)) and exhibited regular beating (figure 5(b)). As expected, IFNγ treatment dramatically upregulated the expression of B2M in control mESC-CM (40-fold increase, figure 5(*c*)). However, such an effect was blunted in B2M downregulated mESC-CM with B2M expression levels staying significantly lower in both the presence and absence of IFNγ (figure 5(*c*)).

3.5. Decrease in the presentation ability of B2M downregulated mESC-CMs to T-cell hybridoma (B3Z)

As previously mentioned, a reduction in B2M mRNA expression may not necessarily translate into lower levels of functional MHC-I molecules on the surface of the cells. Therefore, to functionally assess the physiological consequences of MHC-I downregulation, we employed the B3Z T-cell hybridoma assay, as described above. Control and modified mESC-CMs were treated with IFNγ for 48 h, loaded with the SIINFEKL peptide and co-cultured with B3Z CD8$^+$ T lymphocytes. Samples with B2M downregulated mESC-CMs had significantly decreased amounts of attached and activated blue B3Z T-cells compared with control mESC-CMs (figure 6).

3.6. Survival of B2M downregulated mESC-CMs and WT19 fibroblasts in live allogeneic hosts

Next, we assessed whether cells with depleted B2M levels exhibit an improved survival rate following *in vivo* injection. A classical method for *in vivo* testing of T-lymphocyte response to an antigen is to inject intravenously syngeneic splenocytes loaded with a particular antigen into a pre-sensitized animal and detect the number of surviving cells in the lymphoid organs of the recipient. However, in our case, we needed to test the reaction of T-lymphocytes to MHC-I molecules that were already present on the surface of the cells to be tested. Cardiomyocytes and fibroblasts are also much larger than splenocytes, and they do not circulate to the spleen in significant amounts when injected via intravenous administration. Therefore, we employed a modified *in vivo* CTL assay in which equal amounts of control and modified cells were injected directly into the peritoneal cavity of pre-sensitized recipient mice (figure 7(*a*)). Animals were pre-immunized using allogeneic splenocytes of an H2b haplotype to allow for generation of an acquired T-cell response prior to cell grafting. The population of NK cells was depleted by anti-Asialo GM1 antibody [25]. The survival rate of B2M-downregulated cells relative to mock-treated controls was determined by FACS-based analysis of cell populations obtained from intraperitoneal lavage. Survival of B2M-downregulated fibroblasts increased 2.01 ± 0.4 fold when compared to parental WT19 fibroblasts. Predictably, no significant survival advantage was seen when B2M-downregulated fibroblasts were injected into syngeneic C57Bl/6 mice (1.32 ± 0.44 as compared with control cells, $p = 0.06$). The survival rate of B2M-downregulated mESC-CMs was 5.07 ± 1.5 fold greater than that of controls (figure 7(*b*)). As expected, modified mESC-CM survived significantly better in NK depleted hosts. However, although it was lesser in magnitude, the effect of preferred *in vivo* survival persisted in NK-naïve

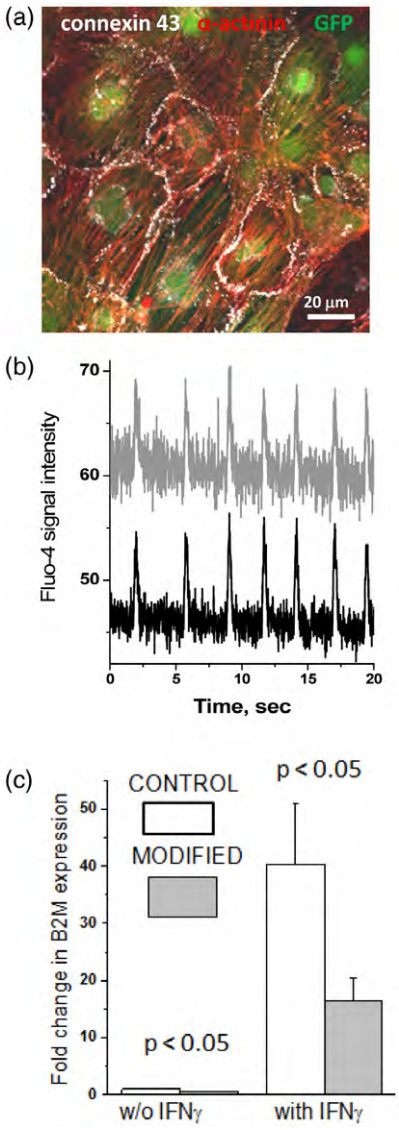

Figure 5. Lentiviral transduction of mESC-CMs leads to a decrease in MHC-I light chain expression. (*a*) Modified mESC-CMs were similar in morphology as control unmodified mESC-CMs, and expressed standard cardiac markers. The representative image shows striated sarcomeric alpha-actinin staning (red) and connexin 43 staining (white) on the background of endogenous EGFP staining (green). (*b*) In all three independent transduction experiments modified ESC-CMs continued to beat spontaneously. The two traces shown were acquired from two different regions of interest within same field of view. The high degree of synchronization is an indicator that different areas of the cell network are well coupled with each other. (*c*) Levels of B2M expression in control and modified mESC-CM, with and without 48 h IFNγ treatment. B2M mRNA expression was significantly downregulated in modified ESC-CMs compared with the corresponding controls, both with and without IFNγ treatment (48 h).

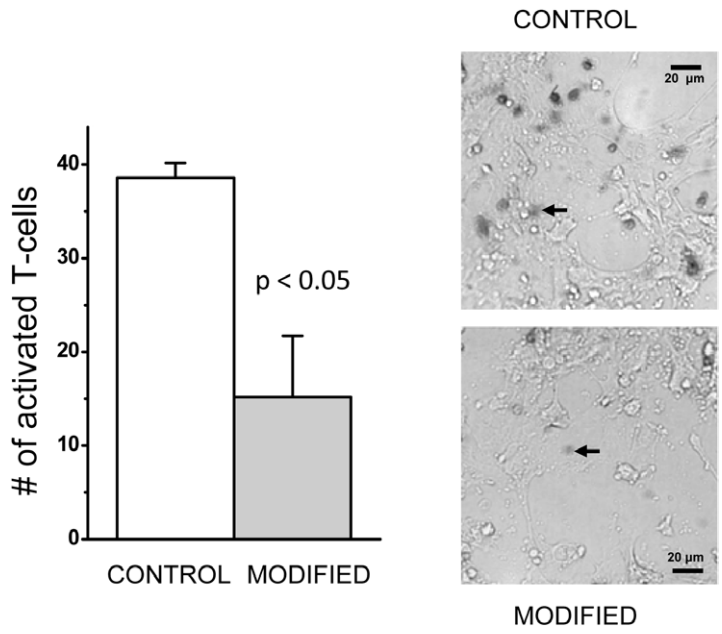

Figure 6. Lentiviral transduction of mESC-CMs leads to decreased *in vitro* activation of T-lymphocytes. Quantification of the number of B3Z T cells activated by mESC-CMs targets. Cells were treated with IFNγ, loaded with SIINFEKL peptide, and co-cultured with B3Z T cells. The relative amounts of activated blue cells on the surface of control and modified mESC-CMs were then compared by counting activated cells from five separate fields of view. On the right, a representative pair of images of shB2M-transduced myocytes loaded with SIINFEKL peptide and exposed to B3Z T cell hybridoma. Arrows point to activated T-cells.

hosts (figure 7(*c*)), 1.98 ± 0.2 fold increase in survival, $p = 0.018$).

4. Discussion

If the amount of functional MHC-I molecules on the surface of a donor cell population decreases, then less antigen fragments (peptide epitopes) are presented to cytotoxic T-cells. This, in turn, makes the donor cell population less susceptible to host $CD8^+$ T-lymphocyte mediated lysis. However, MHC-I molecules also serve as inhibitory receptors on NK cells, and their absence can trigger activation of host NK cells, which would lead to destruction of the donor cell graft. This is one of the potential limitations to using an MHC-I knock-down strategy to hinder graft rejection. Interestingly, stem cell derived cardiomyocytes have limited susceptibility to NK-mediated lysis, even in the case of reduced MHC-I expression [27]. This appears to be due to the lack of NK group 2 member D (NKG2D) and intercellular adhesion molecule 1 (ICAM-1) expression on the stem cell surface. A similar phenomenon was observed in the current study, whereby modified cells survived better than the corresponding controls in both NK-depleted and non-depleted environments.

In addition to MHC-I expression, there are other factors that can affect the long-term survival of allogeneic grafts, including the presence of NK cells, Fas/FasL interaction, expression of multiple different co-stimulatory molecules on the surface of donor cells, Serpin-6 expression, and others [28]. However, several groups have shown successful engraftment and long-term survival of allogeneic ESC-CM into the hearts of athymic mice that lack T-cells. In contrast, no trace of the grafted cells was detected after 6–7 weeks of implanting ESC-CM into immunocompetent animals [29]. Therefore, T cell-mediated lysis of donor ESC-CM appears to be the main route by which these cells are destroyed. By disabling ESC-CM recognition by T-cells, it may be possible to make allogeneic ESC-CM grafts survivable with minimal host immunosuppression.

In many studies, including ours, the short-hairpin RNAs are delivered via a lentiviral-based vector system [30–33]. There are several reasons for this choice of gene delivery. Lentiviruses are known to be able to replicate and integrate into the host DNA independent of the cell cycle [34]. Since ESC-CMs are differentiated cells with limited replication activity, the use of lentiviral vectors is most suitable for the purpose of delivering shRNA. In cases when shRNA based inhibition will be extended to clinical applications, there may be a valid concern regarding the carcinogenicity of introduced lentiviral genes. This problem can be solved by utilizing a self-inactivating lentiviral clone [35]. Cells transduced with such clones cannot produce additional viral particles because the cells do not contain genes for the viral capsid. Furthermore, upon integration into the target cell's genome, the 5′ LTR promoter is inactivated, which prevents replication of the viral sequences.

Human analogs of MHC molecules are called human leukocyte antigens (HLA). In the past, down-regulation of these molecules has been attempted as a means of diminishing the cytotoxicity of the host alloreactive T lymphocytes [36]. Specifically, shRNA targeting of pan-Class I and allele-specific HLA was

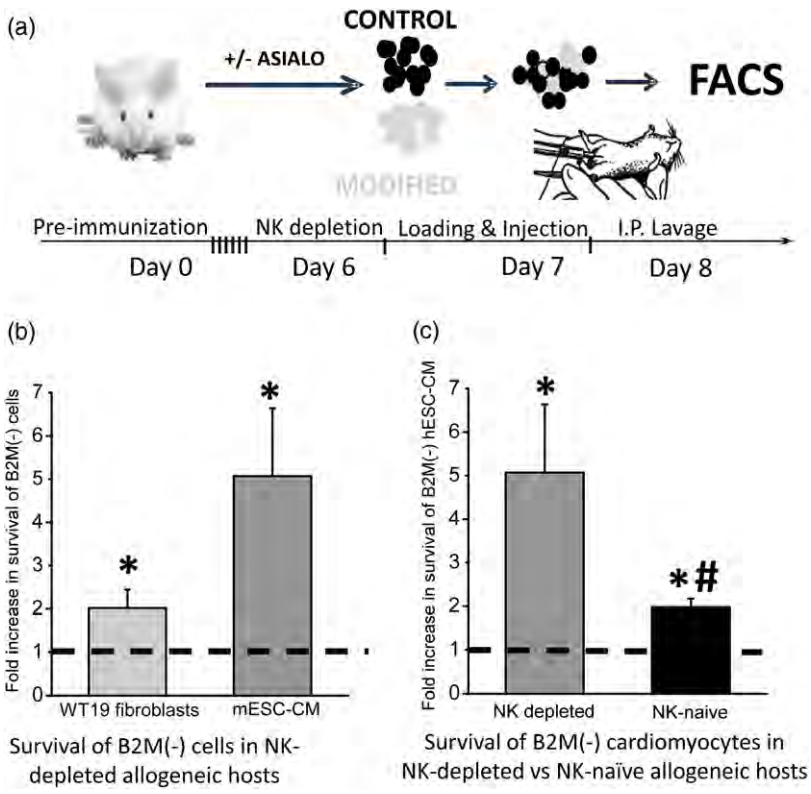

Figure 7. B2M-dowregulated mESC-CM and WT19 fibroblasts demonstrate increased survival in allogeneic recipients. (*a*) Schematic representation of modified CTL assay. Control and modified cells were stained with intravital dye, mixed in 1:1 ratio and injected into the intraperitoneal cavity of recipient mice. After 18 h, cells were collected and the ratio of control (green) cells to B2M-downregulated (orange) cells was determined by FACS after ip lavage. (*b*) The survival of modified cells normalized to the initial injection ratio. Values significantly higher than '1' indicate preferential survival. The modified WT19 fibroblasts and mESC-CM showed an increased rate of survival compared with mock-transduced counterparts (*indicates $p < 0.05$ for each cell type). (*c*) The effect of increased survival of modified mESC-CM persisted in both NK-depleted (five-fold change) and NK-naïve hosts (two-fold change). Asterisk (*) indicates significance in preferential cell survival when compared to initial injection ratio. Symbol # stands for significant increase in survival $p = 0.018$ in NK-depleted versus NK-naïve hosts.

shown to efficiently reduce the surface expression of HLA in HEK293 cells, leading to diminished susceptibility of these cells to alloreactive T lymphocytes, while avoiding MHC-non-restricted killing [33]. Other groups have shown that RNA interference of either B2M or HLA heavy-chain transcripts can suppress HLA class I expression by up to 90% in HeLa cells, B-lymphocytes, peripheral blood monocytes and fibrocarcinoma cells [31, 32]. Similar to our data, this approach was shown to be effective in inflammatory conditions [30, 37] and led to increased *in vivo* survival of B2M depleted cells in allogeneic recipients [30, 32].

While the main interest of our group is in the area of cardiac repair, the described MHC-I inhibition strategy can be used for different types of stem cell-derived populations. We want to emphasize that targeting the invariable light chain seems to be the most efficient approach to disable functional MHC-I molecules, since it hetorodimerizes with many individual heavy chains within the same organism. Therefore, by simply inhibiting beta-2 microglobulin, one can decrease the expression of *all* MHC-I molecules in a given individual. The same strategy can be applied to any somatic or iPS-derived tissue prior to transplantation.

5. Conclusions

The presented data show that downregulation of beta-2 microglobulin by shRNA can successfully decrease the expression of functional MHC-I molecules in both cardiomyocytes and fibroblasts. As a result, an engagement between the presenter cells and the cognate T-cells diminishes *in vitro* and *in vivo*, thus decreasing the overall immunogenicity of the manipulated cells and the cardiac tissue constructs that can be engineered from these modified cells.

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Competing interests section

The authors declare that they have no competing interests.

Authors' contributions section

SI carried out the molecular and cell culture studies, participated in data analysis and provided valuable suggestions for the final version of the manuscript. HD carried out the PCR, FACS and cell culture experiments and performed the statistical analysis. AJ participated in creating and functional analysis of engineered cardiac fibers. NGP helped with PCR and immunostaining protocols and contributed to the writing of the manuscript. ZK and NS conceived of the study, participated in its experimental implementation and co-wrote the manuscript. All authors read and approved the final manuscript.

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