Background—Although human embryonic stem cells (hESC) have enormous potential for cell replacement therapy of heart failure, immune rejection of hESC derivatives inevitably would occur after transplantation. We therefore aimed to generate a hypoantigenic hESC line with improved survival characteristics.

Methods and Results—Using various in vivo, nonischemic, hindlimb xenotransplant models (immunocompetent and defined immunodeficient mouse strains) as well as human in vitro T-cell and natural killer (NK)-cell assays, we revealed a central role for T cells in mediating hESC rejection. The NK-cell susceptibility of hESC in vivo was found to be low, and the NK response to hESC challenge in vitro was negligible. To reduce the antigenicity of hESC, we successfully generated human leukocyte antigen (HLA) I knockdown cells (hESCsiRNA\(^{+IB}\)) using both HLA I RNA interference (siRNA) and intrabody (IB) technology. HLA I expression was \(\sim 99\%\) reduced after 7 days and remained low for weeks. Cellular immune recognition of these hESCsiRNA\(^{+IB}\) was strongly reduced in both xenogeneic and allogeneic settings. Immune rejection was profoundly mitigated after hESCsiRNA\(^{+IB}\) transplantation into immunocompetent mice, and even long-term graft survival was achieved in one third of the animals without any immunosuppression. The survival benefit of hESCsiRNA\(^{+IB}\) was further confirmed under ischemic conditions in a left anterior descending coronary artery ligation model.

Conclusions—HLA I knockdown hESCsiRNA\(^{+IB}\) provoke T-cell ignorance and experience largely mitigated xenogeneic rejection. By generating hypoantigenic hESC lines, the generation of acceptable hESC derivatives may become a practical concept and push cell replacement strategies forward.

Key Words: cells gene therapy immunology

Human embryonic stem cells (hESC) display true pluripotency and have the capacity to propagate indefinitely without senescence. These characteristics make them valuable candidates for cell replacement therapy in the treatment of degenerative diseases, especially heart failure. With transplantation of hESC-derived cardiac progenitor cells that form contractile intracardiac grafts\(^1\) or functional, in vitro-generated engineered heart tissue from hESC,\(^2\) substantial improvements in cardiac function have been reported. However, because of the human leukocyte antigen (HLA) disparity between hESC-derived donor cells and recipients, immune rejection inevitably occurs. Therefore, improvement of rodent cardiac function after acute myocardial infarction so far has only been achieved by transplanting hESC-derived cardiomyocytes into either immunodeficient\(^3\) or heavily immunosuppressed\(^4\) recipients. Immunosuppressive drugs, however, are associated with severe morbidity and may hamper cell engraftment and cell survival.\(^5\)

We and others\(^6\) believe that the best way to overcome immune rejection is to generate hypoimmunogeneic pluripotent cell lines that are barely visible to the immune system and, thereby, can serve as universal cell sources for generating off-the-shelf cellular grafts or tissues. Herein, we present a strategy to generate hypoimmunogeneic hESC by HLA I knockdown. The results demonstrate that HLA I knockdown is both necessary and sufficient to diminish graft rejection and to achieve long-term survival, even in a stringent xenogeneic model.
Methods

Animals
Male BALB/c, SCID Beige (CB17.Cg-Ptkdc<sup>Sck</sup> Lyst<sup>B6</sup>/Crl), nude (Cby.Cg-Fox1<sup>tm1/m</sup>), and periforin<sup>−/−</sup> mice (C57BL/6-Ptpn15<sup>−/−</sup>) were purchased from Charles River Laboratories (Sulzfeld, Germany) and received humane care in compliance with the Guide for the Principles of Laboratory Animals. Myocardial infarctions were induced by ligation of the left anterior descending coronary artery (LAD) through an anterolateral thoracotomy.

ESC Culture and Cell Transplantation
H9 hESC (Wicell; Madison, WI) were maintained on inactivated murine embryonic fibroblast feeder layers (Millipore; Billerica, MA) in hESC medium. The hESC colonies were separated from the murine embryonic fibroblasts by incubation with dispase (Invitrogen; Frederick, MD) and subcultured on feeder-free matrigel-coated (BD Biosciences; San Jose, CA) plates before their use. HLA I surface expression was assessed on a FACS-Calibur system (Becton Dickinson; Heidelberg, Germany) [HLA A/B/C (clone DX17) from BD Biosciences]. Mean fluorescence intensity of HLA I was compared to that of the associated negative isotype control. Normalized expression was calculated as the mean fluorescence intensity of the molecule divided by the mean fluorescence intensity of the isotype control. Therefore, a normalized expression of 1 corresponds to no (0%) molecule expression. Before transplantation, hESC were trypsinized and resuspended in sterile PBS at 1×10<sup>5</sup> cells per 50 μL. The hESC viability was 95% as determined by trypan blue staining. The hESC were transplanted either into the right-side gastrocnemius muscle (nonischemic hindlimb model) or into the border zone of an acute myocardial infarction (LAD ligation model, divided into 4 injections) using a 27-gauge syringe.

Bioluminescence Imaging
Bioluminescence imaging (BLI) was performed using the Xenogen in vivo imaging system (Caliper Life Sciences; Hopkinton, MA) as reported earlier<sup>2</sup> (online-only Data Supplement). The recipient mice were scanned repetitively on days 0, 1, 3, 5, 7, 14, 21, 28, 35, and 42 or until the signal dropped into the background. BLI signals were scanned and analyzing.

Immune Response Assays
Elispot Assays
Allogeneic Elispot assays were done with 5×10<sup>5</sup> hESC and 5×10<sup>5</sup> human peripheral blood mononuclear cells (PBMC) or natural killer (NK) cells (Lanza; Cologne, Germany) according to the manufacturer’s (BD Biosciences) protocol using interferon-γ (IFN-γ) and interleukin-4 (IL-4)-coated plates. Spots were automatically enumerated using an Elispot plate reader (CTL; Shaker Heights, OH) for scanning and analyzing.

Degranulation Assay With NK Cells
Cell-mediated cytotoxicity of human NK cells was evaluated in a CD107a degranulation assay. This assay sensitively detects the surface expression of CD107a as a result of activation-induced degranulation and secretion of the lytic granule contents. NK cells were incubated for 6 hours with FITC-labeled anti-CD107a monoclonal antibody (BD Biosciences). K562, hESC, or hESC<sup>siRNA</sup> were added at ratios of 1:10, and surface expression of CD107a was determined by FACS (BD Biosciences).

HLA I Knockdown Technologies
Both technologies of gene silencing using small interfering RNA (siRNA) at the posttranscriptional level<sup>8</sup> and intrabody (IB) protein knockout at the posttranscriptional level<sup>9</sup> were used separately (hESC<sup>siRNA</sup> and hESC<sup>IB</sup>) or in combination (hESC<sup>siRNA+IB</sup>). For details, see the online-only Data Supplement.

Statistical Analysis
Data are presented as mean±SD, unless otherwise indicated. Comparisons between groups were done by independent sample <i>t</i> tests or ANOVA with Bonferroni post hoc tests where appropriate. Differences were considered significant for <i>P</i>&lt;0.05. Statistical analysis was performed using SPSS for Windows (SPSS Inc; Chicago, IL) software.

Results
hESC Survival After Xenogeneic Transplantation
A total of 1×10<sup>6</sup> hESC were injected into the gastrocnemius muscles of immunocompetent BALB/c or various immunocompromised mouse strains and were followed by BLI (Figure 1A). There was an early decline in cell numbers over the first few days, which occurred in all strains and, therefore, seemed not to be immune mediated. At later time points, the survival curves rapidly diverged (Figure 1B through 1E). The BLI signals dropped into the background in BALB/c mice by day 5, and all cell transplants were rejected. In immunodeficient SCID Beige mice, which lack lymphocytes and NK function, BLI signals steadily increased to regain their initial value by day 21 (Figure 1B). Up to day 80, total BLI flux increased ≈50 times, and macroscopic tumor growth was observed at the site of injection. In nude mice lacking only T cells, 7 of 9 cell transplants survived (Figure 1C), and after 80 days, tumor growth also was observed. Mice with the beige mutation (Figure 1D) and perforin<sup>−/−</sup> mice (Figure 1E) both had defective cytotoxic NK activity but still rejected all cellular hESC transplants at a similar pace compared with immunocompetent BALB/c mice. Adoptive transfer of beige splenocytes into SCID Beige recipients on days −2, 0, 5, and 14 was sufficient to cause hESC rejection in otherwise accepting mice (Figure 1F). When the HLA I-deficient human erythroleukemia cell line K562 was transplanted, all cells were quickly rejected in immunocompetent BALB/c mice but formed tumors in SCID Beige recipients (Figure 1G). The highly NK-susceptible K562 cells were rapidly killed in athymic nude mice devoid of T cells but harboring functional NK cells (Figure 1H). The susceptibility of hESC to Fas-induced cytotoxicity was evaluated. Fas expression was negligible on hESC (1.3±0.1) but could be significantly upregulated by in vitro IFN-γ stimulation (3.9±0.4, <i>P</i>&lt;0.001). Blocking of both NK killing (in beige mice [Figure 1I] or perforin<sup>−/−</sup> mice [Figure 1J]) and Fas-dependent cytotoxicity (using the Fas-blocking antibody ANT-205) did not markedly improve hESC survival.

Allogeneic Cytotoxic Killing of hESC
Human PBMC were incubated with hESC or hESC<sup>siRNA+IB</sup> in vitro (Figure 2A and 2B). Only hESC (<i>P</i>=0.001 and
P=0.002) but not hESC\textsuperscript{siRNA+IB} (P=1.0 and P=0.428) significantly increased the spot frequencies for IFN-γ and IL-4, respectively, compared to resting responder PBMC. 

ELISpots also were then done with NK cells instead of PBMC (Figure 2C). NK-susceptible K562-induced substantial NK-cell activation that was similar to the effect of PMA/ionomycin incubation. Neither hESC (P<0.001 versus K562) nor hESC\textsuperscript{siRNA+IB} (P<0.001 versus K562) induced relevant IFN-γ release. Additionally, only NK cells incubated with K562 but not hESC (P<0.001 versus K562) or hESC\textsuperscript{siRNA+IB} (P<0.001 versus K562) showed increased CD107a surface expression (Figure 2D).

**Generation and Transplantation of Hypoantigeneic hESC**

The siRNA and IB transfection was achieved with an efficacy of \( \approx 74 \pm 12\% \) and \( 82 \pm 11\% \), respectively, and the transduction efficacy of siRNA+IB was \( 88 \pm 9\% \). After 7 days, siRNA and IB significantly lowered HLA I surface expression from \( 11.1 \pm 3.1 \) on hESC to \( 2.6 \pm 0.8 \) (P=0.005) and \( 2.1 \pm 0.7 \) (P=0.004), respectively. Combined use of siRNA and IB even reduced HLA I down to \( 1.1 \pm 0.1 \) (P=0.002) (Figure 3A), which corresponds to \( \approx 99\% \) HLA I reduction. Between days 7 and 42, the lowered HLA I expression remained stable in all 3 groups. After 42 days, HLA I expression in the hESC\textsuperscript{siRNA+IB} group was still significantly...
lower than in either the hESC<sub>siRNA</sub> or the hESC<sub>IB</sub> group ($P=0.003$).

The survival of the 3 generated hESC groups in an immunocompetent host was assessed next. Five days after transplantation into the hindlimb of BALB/c mice, the Elispot assays (Figure 3B) showed that IFN-$\gamma$ spot frequencies were highest for hESC and were significantly reduced with hESC<sub>siRNA</sub> ($P=0.048$), hESC<sub>IB</sub> ($P=0.002$), and hESC<sub>siRNA+IB</sub> ($P=0.002$). Similarly, IL-4 spot frequencies were significantly reduced for hESC<sub>IB</sub> and hESC<sub>siRNA+IB</sub> ($P<0.001$ each). Longitudinal follow-up on cell survival showed that the last detectable BLI signals for hESC<sub>siRNA</sub> and hESC<sub>IB</sub> were found after 10 and 14 days, respectively, and all grafts were rejected (Figure 3C). The rejection of hESC<sub>siRNA+IB</sub>, however, was profoundly mitigated, and the survival was markedly prolonged. In two thirds of the animals, the BLI signals slowly dropped into the background after 28 days, but in one third of the animals, the signals remained stable and showed a slight upward slope after 4 weeks. In these animals carrying long-term surviving grafts, BLI signals were still detectable after 2 months. Thus, with hESC<sub>siRNA+IB</sub>, we were able to achieve long-term cell survival in a stringent xenogeneic setting.

Survival of hESC was further evaluated in a more clinically relevant model of acute myocardial infarction (Figure 4A through 4C). Similar to the results of the hindlimb model, hESC were rapidly rejected within 5 days in BALB/c mice but achieved long-term survival with the development of local teratomas in immunodeficient SCID Beige recipients (Figure 4D). The survival of hESC<sub>siRNA+IB</sub> in immunocompetent BALB/c mice was greatly prolonged compared to hESC, with 2 of 7 animals presenting steady BLI signals beyond 42 days.

**Discussion**

In the present study, we have described the fate of hESC after transplantation into several immunologically well-established mouse models. The survival of hESC in severely immunodeficient SCID Beige recipients exhibiting defective T-cell, B-cell, and NK-cell responses contrasted the rapid cell death in immunocompetent BALB/c mice and underlines the immunologic nature of cell death. Cell stress and injury from needle injection were universally seen in all recipient strains and may be accountable for nonimmunologic early cell losses over the first 5 days. Nude mice lacking T cells but possessing regular NK-cell, B-cell, and antigen-presenting cell activity were unable to reject the majority of hESC transplants. In contrast, in mice with NK-cell impairment because of either the beige mutation or lack of the perforin gene, T-cell-mediated hESC rejection reliably occurred. Additionally, adoptive transfer of beige splenocytes into SCID Beige recipients was sufficient to induce rapid hESC rejection. As previously reported by our group,$^{10}$ T-cell-specific immunosuppression significantly prolongs xenogeneic hESC survival in immunocompetent mice. It has been demonstrated earlier that hESC$^{11}$ or their derivatives$^{12}$ can be recognized by human T cells and that T-cell recognition is strongly facilitated by inflammatory cytokines. The absence of HLA II and costimulatory molecules on hESC excludes direct antigen presentation,$^{13}$ and hESC uniformly escaped rejection in a
humanized mouse model with defective indirect immune recognition pathway.11

Although T cells clearly seem to be required for hESC recognition, other effector cells may participate in the killing. Blocking both perforin- and FasL-dependent killing, 2 major cytotoxic effector mechanisms of T cells, did not prevent hESC rejection. Similarly, allogeneic leukemia cells were shown previously to be rejected in perforin/FasL double-deficient mice with macrophages identified as the effector cell population.14 Komatsu et al15 further demonstrated that T cells can mediate rejection of allogeneic marrow progenitor cells through alternative effector pathways independent of perforin-, FasL-, TWEAK (tumor necrosis factor-like weak inducer of apoptosis)-, and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-dependent cytotoxicity.15 Effector mechanisms involving antibodies16 and complement17 have been suggested to contribute to hESC killing in vivo.

The naturally low HLA I expression of hESC could make them susceptible to NK killing, but in our experiments, only T-cell-deficient animals failed to reject hESC, whereas the lack of NK cells did not prevent vigorous hESC rejection. Other groups using murine ESCs reported substantial18 or negligible19 in vitro NK responses, presumably depending on the experimental conditions. Murine ESCs uniformly have been reported to form teratomas in syngeneic17,20 but not allogeneic17,20 or xenogeneic hosts,20 throwing into question the relevance of NK rejection in immunocompetent hosts. The hESC were shown not to be effectively recognized by human NK cells in vitro, only after additional in vitro NK stimulation.21 The present allogeneic in vitro results confirmed that human NK cells barely responded to hESC challenge and that NK susceptibility did not increase with HLA I knockdown.

Strategies involving the gene transfer of an anti-HLA I single-chain IB9 or plasmids containing siRNA22 recently were introduced to downregulate surface HLA I. Although we could demonstrate in the present study that either technique worked with hESC, only their combination provided the needed level of HLA I knockdown to generate hypoantigenic hESCsiRNA/H11015 IB. To our knowledge, our hESCsiRNA/H11015 IB is the first human cell line to achieve long-term survival in a stringent xenogeneic model without any immunosuppression. The results further demonstrate that the kinetics of immune rejection are similar in the nonischemic hindlimb and ischemic LAD ligation models. The ischemic conditions also may hamper cell engraftment but do not narrow the survival benefit of HLA I knockdown hESCsiRNA/H11015 IB. Differences in the level and endurance of HLA I knockdown still may determine which graft undergoes slow rejection or proceeds

Figure 3. Generation and transplantation of hypoantigenic hESC. Flow cytometry was used to assess hESC HLA I surface expression before and after HLA I knockdown using siRNA, IB, or both. A, Longitudinal analyses demonstrated that once maximal knockdown was achieved after ~7 days, the lowered HLA I expression remained stable for 42 days in all 3 knockdown groups. Data are presented as mean±SEM. *P<0.05 versus hESC, §P<0.05 versus hESCsiRNA, and hESCIB, or hESCsiRNA+IB were transplanted into immunocompetent BALB/c mice. After 5 days, IFN-γ and IL-4 spot frequencies were reduced in the knockdown groups. The last time points of above-background bioluminescence imaging signals were days 10 and 14 for hESCsiRNA and hESCIB, respectively. *P<0.05 versus hESC. B, Two of 6 hESCsiRNA+IB injections became long-term surviving cellular grafts. Data are presented as mean±SEM. HLA, indicates human leukocyte antigen. Other abbreviations as in Figures 1 and 2.
to long-term survival. Future efforts will focus on engineering a reliable level of HLA I knockdown to facilitate routine long-term survival.

In summary, we herein highlight a central role for T cells in the rejection of hESC and demonstrate that hypoantigeneic HLA I knockdown hESCsiRNA-Iβ experience strongly diminished T-cell responses without triggering substantial NK activity. We provide proof of concept that HLA I knockdown on hESC is both necessary and sufficient to achieve long-term graft survival. As progress is made toward the use of hESC therapeutics in human systems, it will be of great interest to determine the effect of HLA I knockdown in an allogeneic human setting.

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Disclosures
None.

References


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HLA I knockdown human embryonic stem cells induce host ignorance and achieve prolonged xenogeneic survival

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Bioluminescence Imaging (BLI)

hESC were transduced with pLenti CMV/TO V5-LUC Puro reporter gene at a multiplicity of infection (MOI) of 10 to stably express Fluc. Viral aliquots were kindly provided by Eric Campeau 1. The Fluc-tagged hESC line is resistant to puromycin and cells were purified using a concentration of 0.2µg/ml puromycin for 5 days followed by plating on feeder layer cells for culturing. BLI was performed using the Xenogen in vivo imaging system (Caliper Life Sciences, Hopkinton, MA, USA) as reported earlier 2. This imaging modality allows for quantitative, longitudinal and non-invasive studies of cell viability and location within the same animals. Mice were anesthetized with 2% isoflurane and D-luciferin (Caliper Life Sciences) was administered i.p. at a dose of 375 mg/kg.

Intrabody technique. E1-deleted human Ad5 recombinant adenovirus containing the anti-human HLA I single-chain intrabody fragment (AdScFv), driven by the cytomegalovirus (CMV) promoter, was constructed, purified and assayed for plaque forming units (pfu) per ml, as described elsewhere 3. The intrabodies recognize a non-polymorphic HLA I epitope. Transduction of cells was performed with a MOI of 150 pfu. To assess transduction efficiency, E1-deleted, human Ad5 recombinant adenovirus encoding for the enhanced green
fluorescent protein (AdvGFP) as reporter construct was generated and served as control constructs. The AdvGFP transduction dilutions were prepared at concentrations of MOI 150.

**siRNA technique.** HLA class I siRNA (Ambion, Darmstadt, Germany), a target-specific 20-25 nt siRNA, was used at a concentration of 20µM. Ten µl of 20µM siRNA solution was incubated with 200µl OptiMEM (Invitrogen) containing 0.3µl DharmaFECT 1 (Thermo Scientific). Then, 1.2ml of prepared siRNA/OptiMEM was added into each hESC well and incubated for 24h. To assess the efficiency of siRNA delivery into cells, siGLO green transfection indicator (Thermo Scientific) localizes to the nucleus. hESC were transfected with siGLO green (20nM), complexed with DharmaFECT 1 transfection reagent (0.1 µl/well), fixed with 3.7% formaldehyde solution and stained with phalloidin-Alexa Fluor-546 as described above.

**Supplemental References**

