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Immunobiology of Fibrin-Based Engineered Heart Tissue

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ABSTRACT

Different tissue-engineering approaches have been developed to induce and promote cardiac regeneration; however, the impact of the immune system and its responses to the various scaffold components of the engineered grafts remains unclear. Fibrin-based engineered heart tissue (EHT) was generated from neonatal Lewis (Lew) rat heart cells and transplanted onto the left ventricular surface of three different rat strains: syngeneic Lew, allogeneic Brown Norway, and immunodeficient Rowett Nude rats. Interferon spot frequency assay results showed similar degrees of systemic immune activation in the syngeneic and allogeneic groups, whereas no systemic immune response was detectable in the immunodeficient group ($p < .001$ vs. syngeneic and allogeneic). Histological analysis revealed much higher local infiltration of CD3- and CD68-positive cells in syngeneic and allogeneic rats than in immunodeficient animals. Enzyme-linked immunospot and immunofluorescence experiments revealed matrix-directed TH1-based rejection in syngeneic recipients without collateral impairment of heart cell survival. Bioluminescence imaging was used for in vivo longitudinal monitoring of transplanted luciferase-positive EHT constructs. Survival was documented in syngeneic and immunodeficient recipients for a period of up to 110 days after transplant, whereas in the allogeneic setting, graft survival was limited to only 14 ± 1 days. EHT strategies using autologous cells are promising approaches for cardiac repair applications. Although fibrin-based scaffold components elicited an immune response in our studies, syngeneic cells carried in the EHT were relatively unaffected. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:625–631*

SIGNIFICANCE

An initial insight into immunological consequences after transplantation of engineered heart tissue was gained through this study. Most important, this study was able to demonstrate cell survival despite rejection of matrix components. Generation of syngeneic human engineered heart tissue, possibly using human induced pluripotent stem cell technology with subsequent directed rejection of matrix components, may be a potential future approach to replace diseased myocardium.

INTRODUCTION

The goal of myocardial tissue engineering is to create three-dimensional tissue constructs with heart-specific morphological and functional properties. Over the past decade, many different cardiac regenerative strategies have been used for heart tissue restoration after myocardial infarction or for drug screening in vitro. The approach of seeding cells onto a preformed biological or synthetic carrier matrix has the advantage of control over the three-dimensional shape of the construct [1, 2]. Stacked matrix-free monolayers generate three-dimensional tissue sheets without the need for potentially immunogenic matrix materials [3–6]. A downside to this approach is that these grafts can be quite fragile, making it difficult to manipulate them during the transplantation procedure. Our laboratory has previously developed engineered heart tissue (EHT) constructs by culturing cardiomyocytes in collagen or fibrin matrices [7–12]. Proof-of-principle preclinical transplant studies showed EHT characteristics similar to native myocardium (morphological and functional), cell survival and vascularization after transplantation, electric and mechanical coupling to host myocardium, and successful cardiac repair [3, 13–16]. Although substantial progress has been made regarding the identification of suitable human cell sources and scaffold components, most preclinical studies require immunodeficient or immunosuppressed

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animals, such that little is known about immune responses to complex cell-matrix EHT after transplantation. Immunocompatibility of EHT with the host organism is an important prerequisite for clinical application and warrants thorough investigation. At present, results have been encouraging from large animal studies using catheter-based transendocardial [17], intracoronary [18], or direct open-chest intramyocardial [19] application routes for delivery of different extracellular matrix materials. Importantly, supportive results from preclinical studies of EHT have led to clinical trials [20].

Several fundamental issues such as mechanical incompatibility, incompatibility of cells with matrix materials, and problems controlling cell distribution within the scaffold require further investigation to further optimize and translate EHT approaches. In this study, we assessed immune responses after transplantation of fibrin-based EHT [7, 21] in a rat model. We used luciferase-positive (luc+) neonatal rat heart cells and determined the differential immunogenicities of EHT matrix and cells. Using different immunological pairings, we were able to distinguish between host responses to matrix versus cellular components of EHT.

Materials and Methods

Animals

Animals are described in the supplemental online data.

Neonatal Rat Heart Cell Surface Molecule Expression

Neonatal rat heart cells (NRHCs) were analyzed for surface molecule expression by flow cytometry with or without preincubation with interferon-γ (IFNγ) 25 ng/ml 48 hours before analysis (PeproTech, Rocky Hill, NJ, https://www.peprotech.com), as described in the supplemental online data and supplemental online Figure 1.

Generation of EHT and Culture Conditions

Manufacture of fibrin-based EHT is described in the supplemental online data.

Animal Surgery

For histological and immunofluorescent imaging analyses and for enzyme-linked immunospot (ELISpot) assays, beating EHT (supplemental online Movie 1) or EHT matrix was transplanted onto the anterolateral wall of the left ventricle of anesthetized, ventilated, recipient Lewis (Lew), Brown Norway (BN), or Rowett Nude (RNU) rats (n = 6 per group). Anesthesia was induced and maintained using isoflurane (2.5%–3.0%, Forane; Abbot GmbH & Co. KG, Wiesbaden-Delkenheim, Germany, http://www.abbott.com). Left anterolateral thoracotomy was followed by pericardectomy. EHT was sutured onto the left ventricular myocardium using 8-0 Prolene sutures (Ethicon GmbH, Johnson & Johnson Medical, Norderstedt, Germany, http://www.ethicon.com). To allow for in vivo bioluminescence imaging (BLI), luc+ EHT were transplanted into the greater omentum of recipient rats through a midline abdominal incision, as described previously [21]. For subsequent ELISpot assays, EHT cell suspension containing 2×10^6 NRHCs was injected into gastrocnemius muscles of Lew, BN, or RNU rats.

Histology

Tissue samples were harvested, fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Sections of 5-μm thickness were cut and stained with hematoxylin (Böhmer hematoxylin; Waldeck, Münster, Germany, http://www.waldeck-ms.de) and eosin (Carl Roth, Karlsruhe, Germany, http://www.carlroth.com). Mononuclear cell infiltration and cardiomyocyte survival in EHT heart grafts were assayed by confocal microscopy using immunofluorescent staining. Details are shown in the supplemental online data.

Enzyme-Linked ImmunoSpot Assay

To evaluate immunogenicity of EHT or EHT matrix in vivo, constructs were transplanted onto the left ventricular myocardium or EHT cell suspension containing 2×10^6 NRHCs was injected into gastrocnemius muscles of Lew, BN, or RNU rats. Spleens of recipient animals were harvested 5 days after transplantation, and 1×10^6 splenocytes per well were used as responder cells; Lew NRHCs treated with mitomycin C (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com) with or without matrix or matrix alone were used as stimulators in ELISpot assays, using IFNγ-coated plates, according to the manufacturer's protocol (BD Biosciences, San Jose, CA, https://www.bdbiosciences.com). Spots were counted after 24 hours of incubation using an automated ELISpot plate reader system (Cellular Technology Limited, Shaker Heights, OH, http://www.immunospot.com).

Bioluminescence Imaging

BLI was used for longitudinal noninvasive in vivo imaging of NRHCs expressing firefly luciferase. D-Luciferin potassium salt (Biosynth AG, Staad, Switzerland, https://www.biosynth.com) dissolved in phosphate-buffered saline pH 7.4 (Gibco, Invitrogen; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) was injected intraperitoneally (375 mg/kg) into anesthetized rats (2% isoflurane) after transplantation of luc+ EHT into the greater omentum. Animals were imaged using the IVIS 200 system (Xenogen, Caliper Life Sciences; PerkinElmer, Waltham, MA, http://www.perkinelmer.com). Region of interest (ROI) bioluminescence was quantified in units of maximum photons per second per square centimeter per steradian. The maximum ROI signal was measured using Living Image 3.1 software (MediaCybernetics, Rockville, MD, http://www.mediacy.com) with repetitive measurements on days 0, 1, and every second day thereafter until signals dropped to background levels or day 110. Results are presented on a logarithmic scale.

Statistical Analysis

Data are presented as mean $\pm$ SD. Comparisons were done by analysis of variance between groups with least significant difference post hoc tests. Probability values of $<.05$ are considered significant. Statistical analysis was performed using the SPSS statistical software package 15.0 for Windows (IBM Corp, Armonk, NY, http://www.ibm.com).

Results

Characterization of Neonatal Rat Heart Cells

Characterization of isolated NRHCs by flow cytometry confirmed the presence of a mixed cell population with detection of molecular markers normally expressed by smooth muscle cells (smooth muscle α-actin), cardiac myocytes (sarcomeric α-actinin), fibroblasts (prolyl-4-hydroxylase), endothelial cells (CD31), and macrophages (CD163) (supplemental online Fig. 1). Based on the

flow cytometry results, the mixed heart cell population expressed the following panel of surface expression markers (expressed as folds of the isotype control): major histocompatibility complex I (MHC I), MHC II, CD40, CD54, CD80, and CD86 molecules. After stimulation with IFNγ, there was a statistically significant upregulation of MHC I ($p < .001$), MHC II ($p < .05$), and CD40 ($p < .05$), whereas the expression levels of the costimulatory molecules CD54, CD80, and CD86 remained largely unchanged (Fig. 1).

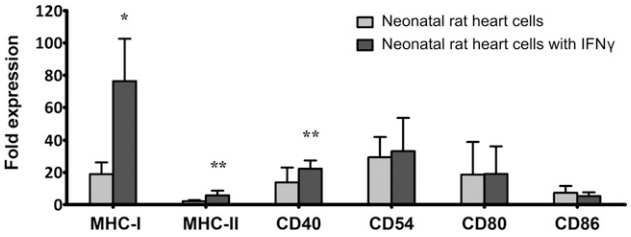

Figure 1. Characterization of neonatal rat heart cell (NRHC) surface molecule expression by flow cytometry. MHC I, MHC II, CD40, CD54, CD80, and CD86 were all detectable on NRHCs. After stimulation with IFNγ, expression of MHC I, MHC II, and CD40 was significantly upregulated, whereas expression of CD54, CD80, and CD86 remained unchanged. The results were expressed as fold-changes versus isotype control (mean $\pm$ SD; NRHCs: MHC I: $n = 5$; MHC II: $n = 7$; CD 40: $n = 9$; CD 54: $n = 8$; CD 80: $n = 6$; CD 86: $n = 8$. NRHCs plus IFNγ: MHC I: $n = 11$; MHC II: $n = 9$; CD 40: $n = 10$; CD 54: $n = 8$; CD 80: $n = 8$; CD 86: $n = 8$). $*p < .001$, $**p < .05$. Abbreviations: IFNγ, interferon γ; MHC, major histocompatibility complex.

Histological Analysis After Transplantation of EHT

Recipient animals were sacrificed 5 days after EHT transplantation, and hearts were fixed for histopathological analyses. Macroscopically, the EHT of all three groups was distinguishable from the left ventricular surface of the heart by hematoxylin and eosin staining, with marked morphological differences among the three recipient groups (Fig. 2). Cells inside EHT were characterized by positive staining for sarcomeric α-actinin and connexin-43. Connexin-43-positive cells were located mostly on the lateral aspects of sarcomeric α-actinin-positive cells. The density and structural organization of sarcomeric α-actinin-positive cells and the presence of connexin-43 seemed best preserved in EHT transplanted in immunodeficient RNU recipients. In this group, the α-actinin-positive cells showed a phenotype resembling the native myocardium with longitudinal orientation of both cells and nuclei. Connexin-43 expression was higher in the area of cell-cell interface, although it was not restricted to the intercalated discs but also was found along the longitudinal cell border. Furthermore, both sarcomeric α-actinin-positive cells and connexin-43 were detectable in Lew recipients in a less organized pattern, albeit at lower density, compared with the RNU group. The EHT transplanted in BN recipients was negative for connexin-43 and showed only diffuse, low-density staining for sarcomeric α-actinin. Dense mononuclear cell infiltrates were observed in both Lew and BN recipients at the graft-host interface, suggesting cellular rejection. Immunofluorescence staining identified CD3+ lymphocytes in EHT constructs transplanted into both Lew and BN recipients but not in the T-cell-deficient RNU group. CD68+ macrophage infiltration was seen in all three recipient types but appeared most pronounced in BN recipients.

Systemic Immune Response After EHT Transplantation

Systemic immune responses after EHT transplantation were quantified by ELISpot assays for IFNγ, a cytokine predominantly expressed by Th1 cells (Fig. 3). Transplantation of EHT into Lew and BN recipients resulted in significantly increased mean spot frequencies compared with immunodeficient RNU recipients (Lew 48.6 $\pm$ 37.6 and BN 43.0 $\pm$ 31.0 vs. RNU 9.5 $\pm$ 5.5 spots; $p < .001$ vs. Lew and BN) (Fig. 3A).

To evaluate whether the immune response was directed against the matrix or the cellular components of the EHT, cell-free EHT matrices and single-cell suspensions were transplanted in the three groups of rats (Fig. 3B). Matrix transplantation induced higher Th1-based rejection in Lew and BN than in RNU recipients (Lew 60.5 $\pm$ 51.4 and BN 64.6 $\pm$ 54.5 vs. RNU 15.9 $\pm$ 5.7 spots; both $p < .05$ vs. RNU). Differences between Lew and BN were not significant. In contrast, cell suspensions induced Th1-mediated rejection only in allogeneic BN recipients (Fig. 3C) (BN 27.9 $\pm$ 19.6 vs. Lew 9.3 $\pm$ 6.6 and RNU 8.4 $\pm$ 7.3; both $p < .001$ vs. BN).

Cell Survival After EHT Transplantation

For monitoring cell survival after transplantation in vivo, EHTs were generated from luc+ Lew rat heart cells ubiquitously expressing firefly luciferase (Fig. 4A, 4B). Signal emission by these cells, detected by a CCD camera, correlated well with cell number both in suspension and in EHT format (cells: $R^2 = .9897$; EHT: $R^2 = .9675$) (Fig. 4C).

Luc+ Lew EHT was transplanted into the greater omentum of Lew, BN, or RNU rats and followed by BLI (Fig. 4D, 4E). In all three recipient types, an initial signal decline was observed. After day 10, curves markedly diverged, with signal intensity dropping below background (1×10^6 photons per second per square centimeter per steradian) in BN recipients by day 14 $\pm$ 2, suggesting graft rejection and loss in the allogeneic recipients. In Lew and RNU recipients, signal intensities were stable after the initial dropoff and remained consistently above a minimum of 2.4×10^6 photons per second between days 26 and 110. No significant differences in signal intensity were observed between Lew and RNU recipients.

In summary, xenogeneic matrix components of EHTs induced an immune response in immunocompetent recipient animals, but the response was selective for the matrix and did not affect survival of cells in a syngeneic context.

Discussion

Cardiovascular cell therapy has the potential to regenerate, replace, or repair diseased myocardium [22]. Successful transplantation of different stem cell types has been demonstrated previously in experimental and clinical studies [23–25]. One of the main problems with the clinical translation of regenerative therapies is the low efficacy seen in clinical trials. This is due to the low engraftment of cells resulting from poor cell retention within the heart and the toxicity of the hypoxic environment of the infarcted myocardium [26]. Extracellular matrix support of transplanted cells is a possible solution that may help overcome this limitation. Cardiac tissue engineering in vitro with the development of EHT creates an organoid that preserves important cell-cell interactions and a three-dimensional structure resembling native heart tissue [27]. Previously, our group and others developed several types of EHT that showed increased structural integration of the grafts with the host myocardium and subsequent functional benefits to the injured heart [15, 28]. Although major

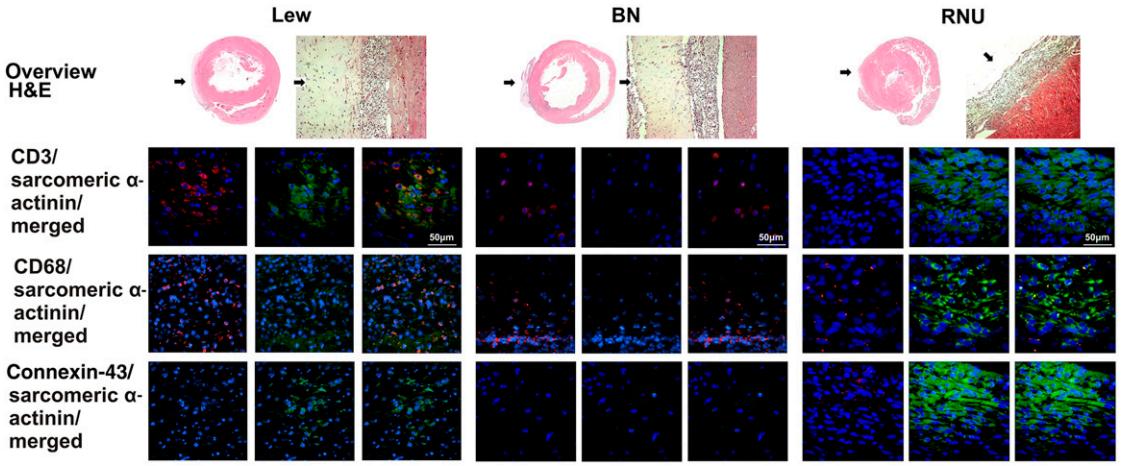

Figure 2. Transplantation of engineered heart tissue (EHT; arrows) onto the anterolateral wall of the left ventricle in Lew, BN, and RNU recipient rats. Representative images of EHT that was macroscopically distinguishable on the left ventricular surface (overview). H&E staining revealed strong infiltration of mononuclear cells in the ventricle-graft interface of Lew and BN recipients. Immunofluorescent staining demonstrated presence of CD3-positive T cells (red) in EHT transplanted into Lew and BN rats but not into RNU rats. CD68-positive macrophages (red) were found in EHT transplanted in all groups. Connexin-43-positive gap junctions (yellow) and α-actinin-positive cardiac myocytes (green) were detectable in EHT transplanted in Lew and RNU rats but not in BN rats. DAPI (4′,6-diamidino-2-phenylindole) nuclear stain (blue). Abbreviations: BN, Brown Norway; H&E, hematoxylin and eosin; Lew, Lewis; RNU, Rowett Nude.

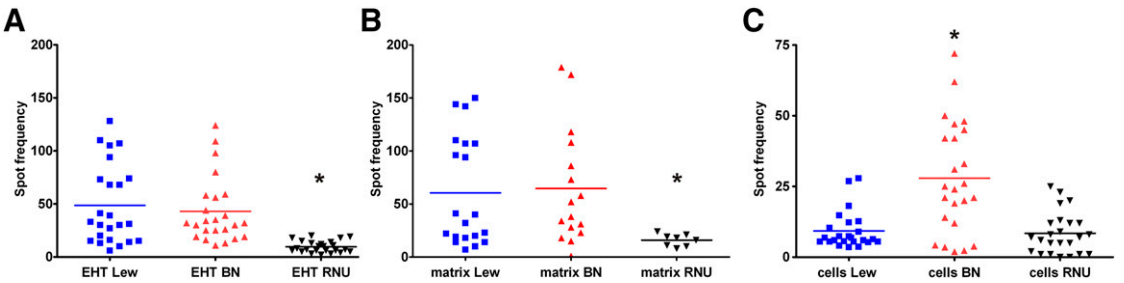

Figure 3. Cellular immune activation on day 5 after EHT transplantation assessed by enzyme-linked immunospot assays. **(A):** After EHT transplantation, interferon γ (INFγ) spot frequencies were significantly higher in Lew and BN recipients compared with RNU recipients (n = 24 wells per group; $p < .001$). **(B):** Separate assessment of cellular immune responses against matrix or cellular components of EHT revealed significantly higher INFγ spot frequencies in both Lew and BN recipients compared with RNU recipients after transplantation of cell-free EHT matrix (n = 24 wells per group; $p = .032$ and $p = .024$, respectively). **(C):** As expected, INFγ spot frequency was significantly higher after injection of neonatal rat heart cells into allogeneic BN recipients compared with syngeneic Lew and immunodeficient RNU recipients (n = 20 wells for Lew, n = 16 wells for BN, n = 8 wells for RNU; $*$, $p < .05$). Abbreviations: BN, Brown Norway; EHT, engineered heart tissue; Lew, Lewis; RNU, Rowett Nude.

progress has been made in regenerative medicine with the identification of suitable human cell sources [29] or biomaterials as scaffolds for the development of EHT [30, 31], the immunological impact of EHT transplantation is a relatively understudied topic, despite the recognized fundamental importance of host responses to implanted scaffold materials [32]. Even albumin, a product from pooled human plasma with a long history of clinical use, can sometimes elicit allergic reactions, including headache, hypotension, urticaria, fever, and chills [33, 34]. Consequently, it is very important to evaluate the immunological impact of different biomaterial matrices used for the generation of cardiac constructs.

With this study, we aimed to characterize the immunological consequences of a particular type of fibrin-based EHT construct (which also contains some residual fibrinogen, Matrigel, horse serum, chick embryo extract, and so forth) (supplemental online data). The particular matrix formulation we used in our experiments is a modification of a collagen-based system [13, 35]. Although collagen is a naturally occurring protein in wide clinical use for years, it is immunogenic: a small percentage of patients treated with bovine collagen implants develop cellular and/or humoral immune responses [36, 37]. In addition, studies have shown that enzyme-digested purified collagen implants can lead to granulomatous-like reactions due to the combination of the bovine collagen with small amounts of altered human collagen [38, 39]. These adverse reactions to collagen, along with the risk of foreign body reaction and occasional necrosis, led us to replace collagen with fibrin for the generation of EHT. Fibrin also facilitates construction and handling of larger numbers of EHT due to its much faster polymerization. Fibrin gels have several advantages as a delivery scaffold for cells [21, 40, 41]. Fibrin gel matrices exhibit biodegradation and resemble the tissue-like mechanical properties of the native heart tissue matrix [7, 35]. Injectable fibrin used as a carrier for various cell types has proven advantageous for cell survival versus cell suspension alone [42–44] and for improving functional hemodynamics [18, 45].

Our flow cytometry cell surface marker analysis of the cellular component of the EHT indicated the presence of cardiomyocytes, smooth muscle cells, fibroblasts, and immune cells such as antigen-presenting macrophages within the NRHC suspension. These results are consistent with our previous work in which we observed a similar composition of cell types in a collagen-based EHT [16].

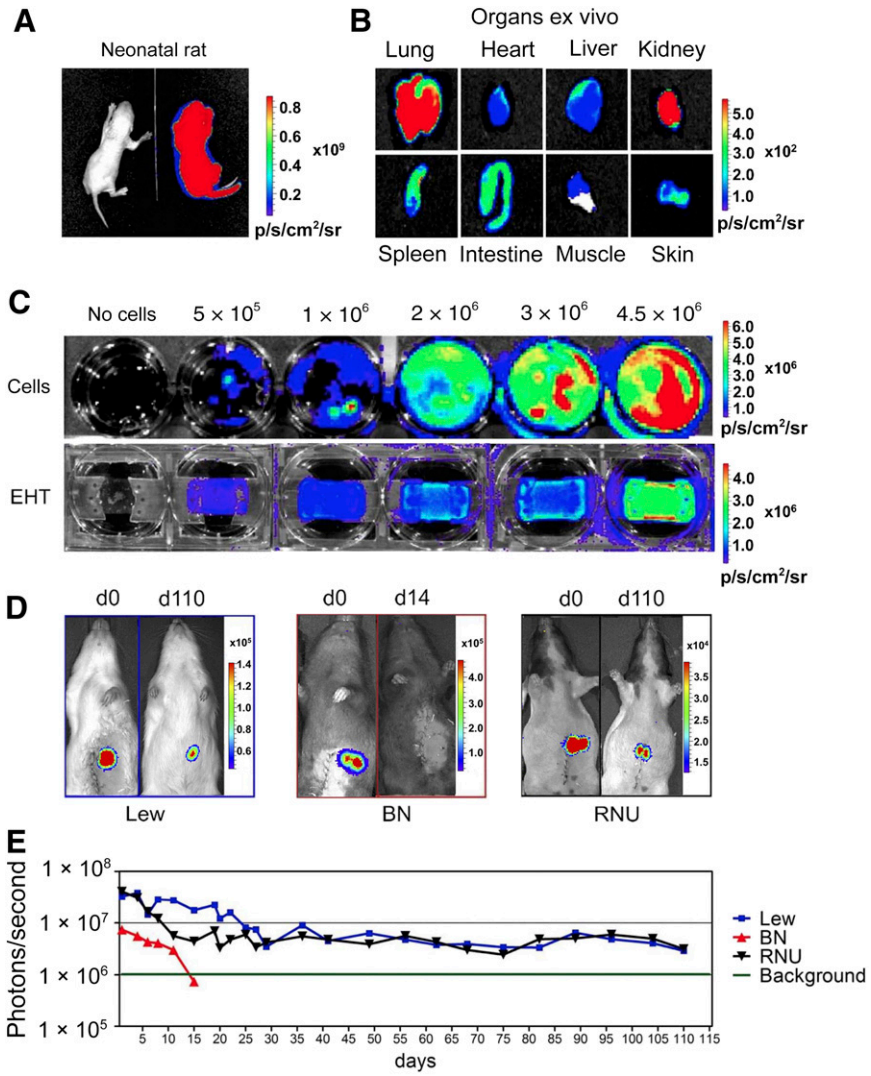

Figure 4. Bioluminescence imaging (BLI) allowed for longitudinal monitoring of graft survival after transplantation. **(A, B):** Neonatal rat heart cells were isolated from neonatal ROSA/luciferase-LEW transgenic rats expressing luciferase ubiquitously. **(C):** Dilution series demonstrated good correlation of cell number in single-cell suspension to luminescent signal intensity (R^2 = .9897) (upper panel) and in EHT format (R^2 = .9675) (lower panel). **(D):** Representative images of BLI after transplantation of EHT into Lew, BN, and RNU recipients at baseline and at long-term follow-up. **(E):** After an initial decrease, signal intensity was stable up to 110 days in Lew and RNU rats but decreased rapidly after transplantation into BN rats and reached background levels at day 14 after implant (n = 6 per group at each time point). Abbreviations: BN, Brown Norway; d, day; EHT, engineered heart tissue; Lew, Lewis; max, maximum; min, minimum; p/s/cm^2/sr, photons per second per square centimeter per steradian; RNU, Rowett Nude.

Histomorphological analyses demonstrated minimal cellular infiltrates into grafted EHT of the immunodeficient RNU group with well-preserved cardiac architecture. This result was expected and is consistent with previous reports on EHT transplantation in immunoincompetent and immunosuppressed recipients [3, 15, 46]. Accordingly, dense cellular graft infiltrates were observed in both immunocompetent Lew and BN recipients, indicating immune responses. However, cardiac tissue structures inside the graft were much better preserved in Lew than BN recipients, indicating that the cellular components of the transplanted EHT were protected in the syngeneic model. These results suggest that the immune responses in syngeneic Lew were directed primarily against the xenogeneic matrix components.

These data were further supported by the ELISpot results for IFNγ, an important mediator of cellular defense predominantly released by Th1 cells. We noticed significant upregulation of MHC I, MHC II, and CD40 surface molecules during an immune response against cell-free EHT matrices (Fig. 3B). Although the matrix components induced T-cell rejection in both Lew and BN animal models, only allogeneic BN recipients rejected the cellular components of EHT.

Furthermore, the long-term in vivo BLI studies confirmed the histology data, in which cellular features were preserved not only in RNU recipients but also in the syngeneic Lew recipient group. A limitation of this approach is that signals detected by BLI cannot be attributed to one specific cell population within the EHT.

An important finding from our BLI studies was that transplantation of EHT into syngeneic Lew recipients preserved the EHT cellular features despite the marked immune response directed against EHT matrix components. Fibrin, a component used to develop these matrices, has been previously proven to stimulate angiogenesis [47, 48] and facilitates differentiation of transplanted mesenchymal stem cells, aiding tissue regeneration [49]. It is also known that fibrin and fibrinogen are major players during the wound-healing process and modulate wound colonization by peripheral blood

mononuclear cells and macrophages [50]. Both fibrin and fibrinogen affect the inflammatory process by facilitating leukocyte adhesion and altering leukocyte and endothelial cell cytokine expression including mediators such as interleukin 8 (IL-8), IL-6, tumor necrosis factor α, and reactive oxygen species [51]. This effect is confirmed by our results and further demonstrates that fibrin and its fragments can modulate immune response [52]. However, fibrin may induce less inflammation and immune response than collagen I, which was originally used for EHT generation [46]. In a previous study, syngeneic cells, embedded in collagen I, were subject to immune rejection, whereas the cells in our syngeneic fibrin-based EHT achieved long-term survival [46]. This led us to conclude that selective rejection of the matrix components can occur without relevant collateral damage to the heart cells within the EHT construct. Consequently, low immunogenic biomaterials including the fibrin gels used in this study could potentially be used as matrices for the delivery of cells into patients to support cell proliferation and engraftment. After complete biodegradation, the EHT scaffold would be fully replaced by the body's own matrix. Another approach is to use fibrin gels along with an anti-inflammatory agent or to inhibit the proinflammatory fragments with antibodies or their signaling pathways to decrease the host's inflammatory response. Some previous animal work has shown that this approach is feasible with the delivery of the anti-inflammatory peptide Bβ15-42. The authors demonstrated that they could prevent the inflammatory process caused by fibrin derivatives during cardiac reperfusion injury [52].

Our study has some limitations. First, EHT monitoring by BLI is feasible only in the greater omentum of rats because of technical constraints. BLI of the heart proved impossible in rats because luminescence signals are largely absorbed by the chest wall; however, using ELISpot assays, we could show that the systemic immune response against EHT transplanted onto the heart or into the greater omentum was similar (data not shown). Second, the assessment of cell survival in engrafted EHT was limited to the overall cell content and did not allow discrimination between different cell types; therefore, no statement can be made about possible changes in the cellular composition of engrafted EHT. The histological data suggest that cardiac myocyte quality was lower in the syngeneic Lew model, arguing for at least some discrete damage to cardiac myocytes related to the immune response against matrix components. Third, EHT transplantation was not performed in an infarct model, and no statements can be made about functional consequences of EHT transplantation.

Potential Implications

In this study, we described the immune response against a complex cell-matrix graft transplanted across different immune barriers. Most important, we showed that grafted syngeneic cells in an EHT survive despite rejection of matrix components. These studies need to be extended using clinically relevant cell sources, with more intense investigation of functional outcome after transplantation and more detailed characterization of the immune response.

Conclusion

Our study demonstrates that immune responses directed against a novel fibrin-based EHT are mediated primarily by a Th1 response. Rejection of xenogeneic EHT matrix containing syngeneic cells is selective for the matrix, without evidence of cell rejection. Degradation of such an immunogenic matrix can be voluntarily controlled by withdrawal of temporary immunosuppression.

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Author Contributions

L.C.: data analysis and interpretation, manuscript writing; S. Schmidt: collection and assembly of data, data analysis and interpretation, manuscript editing; E.N. and T.D.: conception and design, data analysis and interpretation, manuscript writing; L.P. and X.H.: collection and assembly of data, data analysis and interpretation; A.E.: provision of study material; A.H.: provision of study material, data analysis and interpretation, manuscript writing; R.C.R.: conception and design, financial support, manuscript editing; R.E.B. and H.R.: financial support, manuscript editing; T.E.: conception and design, financial support, manuscript editing; S. Schrepfer: conception and design, financial support, data analysis and interpretation, manuscript writing.

Disclosure of Potential Conflicts of Interest

R.E.B. has compensated employment from Stanford University School of Medicine, NorthBay Medical Center. S. Schrepfer has compensated research funding from Rigel, South San Francisco, CA. The other authors indicated no potential conflicts of interest.

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