Scientific Progress Report Migrated - Scientific Progress Report Migrated

Award ID	**16IRG27740007**	Project Title	**Novel Tolerogenic CD34/MSC Di-Chimeric Cell Therapy in Vascularized Composite Allograft and Heart Transplant Models**
Grantee	**Siemionow, Maria**	Institution	**University of Illinois at Chicago**
Award Start	**01/01/2016**	Award End	**12/31/2017**

***1. Please provide a concise summary of progress for each specific aim of your project during this reporting period. If this is your final report, include an overall summary of the project achievements. Note: The suggested length for the Progress Report is approximately 2 pages. You can copy and paste from another document or type responses in the boxes below.**

1. For each aim, describe or summarize the following:
a) Major completed or ongoing activities;
b) Significant results, including major findings, developments, or conclusions (both positive and negative);
c) Discussion of stated goals not met or problems you have encountered and how they were resolved.

The aim of this study was to establish an innovative, less-toxic stem cell based therapy, which will extend allograft survival without the need for life-long immunosuppression. We have developed and tested two alternative therapies of CD90+/CD90+ and CD90+/MSC Di-Chimeric Cells (DCC) for induction of tolerance in Vascularized Composite Allograft (VCA) and Heart transplantation. DCC present both the donor and recipient characteristics and diverse molecular and secretion profiles, which determine their intercellular cross talk, and as such may elicit different tolerogenic properties when applied as the supportive therapy in the context of VCA or solid organ- heart transplantation.
Under Aim 1 of this grant application, we evaluated molecular profile of DCC of hematopoietic CD90+ cells and mesenchymal stem cells (MSC) origin and determined their pro-tolerogenic cytokine mRNA expression profiles following cell fusion. The bone marrow of ACI and Lewis rats was used for the selection of CD90+ HSC and MSC isolation. Next, the parent cell lines were assessed for: cell lines purity, surface markers expression and cell viability. The in vitro T-cell suppression and regulatory T-cell (Treg) induction assays were assessed by flow cytometry. Cytokine mRNA expression profiles were assessed by real time-PCR.
Under Aim 2, we assessed in vivo the tolerogenic effect of the supportive DCC therapy using a new generation of created hematopoietic and MSC Di-Chimeric Cells in VCA and heart allograft transplant models. The outcomes measures included evaluation of VCA and heart allograft survival up to 100 days, assessment of heart allograft histology at day 7 and day 100 post-transplant. At the study end-point, the peripheral blood, lymphoid organs and liver were harvested for assessment of chimerism by flow cytometry. Confirmation of the DCC engraftment combined with ex vivo assessment of cytokine expression and in vivo monitoring of allograft survival would allow determining a preferential DCC therapy for the potential clinical application for tolerance induction in VCA and solid organ transplantation.
Results and accomplishments
Specific Aim 1: To evaluate molecular and secretion profiles of CD90+/CD90+ DCC and CD90+/MSC DCC and to determine pro-tolerogenic cytokine secretion profiles following cell fusion.
1.1 Isolation of CD90+ hematopoietic stem cells: Adjustments were made to the protocol to optimize isolation of CD90+ HSC cells for hematopoietic DCC therapy. Briefly, we used anti-CD90-PE conjugated primary antibody followed by anti-PE microbeads (Miltenyi Biotec) isolation to obtain an

enriched CD90+ population. CD90 HSC were cultured for 7 days, characterized for purity and viability and used for creation of DCC therapies (Figure 1, upper row).

1.2 Isolation of MSC from Lewis rat bone marrow. Using our well-established protocol for MSC isolation and expansion from the bone marrow donor, we have successfully isolated MSC with high purity and yield for creation of the fused DCC cells (Figure 1, lower row).

1.3 Ex vivo creation and characterization of CD90+/CD90+ DCC and CD90+/MSC DCC. Creation of CD90+/CD90+ DCC and CD90+/MSC DCC of ACI and Lewis origin was accomplished by our well-established ex vivo fusion protocol using polyethylene glycol (PEG) technique. Parent cells were stained with PKH26 (red fluorescence) and PKH67 (green fluorescence) (Sigma-Aldrich) membrane dyes prior to cell fusion. After fusion, cells were FACS-sorted based on the presence of double positive-fluorescence (PKH26/PKH67). Fusion of both DCC lines was confirmed by flow cytometry (Figure 2) and confocal microscopy (Figure 3) whereas phenotype for both DCC lines was assessed by flow cytometry (Table 1).

After sorting the fused DCC and their parent cells (controls) were visualized under confocal microscope after DAPI nuclear counterstain. Compared to the parent cells before fusion, the fused DCC cells emitted both PKH26 (red) and PKH67 (green) fluorescence, visualized by overlapping images resulting in orange color (Figure 3). The purity of created DCC was assessed between 85-95% after the fusion procedures.

Confirmation of the fusion procedure of two created DCC lines by phenotype analysis using flow cytometry. Analysis of surface marker expression for all parent cells and two DCC lines was performed and CD90+/CD90+ DCC expressed hematopoietic surface markers specific for CD90+ HSC and CD90+/MSC DCC expressed surface markers specific for both the CD90+ HSC and MSC (Table 1).

Flow cytometry analysis of the phenotype of the adhesive and non-adhesive populations of fused hematopoietic DCC lines at 14-days of culture confirmed high expression of CD90, CD11b/c, ACI rat specific RT1a and Lewis rat specific RT1l MHC surface markers (Figure 4).

Evaluation of allogeneic responses of DCC lines by mixed lymphocyte reactivity assay. Parent cell populations and both DCC lines were cultured in the direct co-culture (ratio 1:1) with Lewis splenocytes, obtained by density gradient centrifugation. Lewis lymphocytes were stained with eFluor 670 proliferation dye prior to co-culture. After 4 days of co-culture, proliferation of cells was analyzed by flow cytometry on the basis of fluorescence changes. Proliferation allogenic ACI lymphocytes was set as the positive control (maximal proliferation) while Lewis lymphocytes in basal media (minimal proliferation) culture served as negative control. Both DCC lines evoked decreased T-cell proliferation compared to the allogenic parent cells (Figure 5).

Evaluation of the regulatory T-cells induction by DCC lines, Lewis splenocytes were stained for CD3/CD4/CD25 expression (BD Bioscience) and CD25 negative T-cell population was sorted by FACS (Moflow Astrios). CD25- lymphocytes were then co-cultured with CD 90+ HSC and MSC derived from the ACI and Lewis rats and with both DCC lines. After 4 days of culture in the specific lympho-proliferative media, cells were harvested and analyzed for expression of CD4, CD25 and FoxP3. The DCC line of HSC/MSC origin induced increased number of regulatory T-cells in vitro, comparable to the results obtained with stimulation of the MSC alone, indicating maintenance of the immunomodulatory characteristics (Figure 6).

Evaluation of the cytokine profiles of both DCC lines of hematopoietic origin (CD90+/CD90+) and of (CD90+/MSC) origin. Cell pellet samples of both DCC lines (CD90+/CD90+ DCC and CD90+/MSC) were collected for real time-PCR analysis following RNA isolation and cDNA synthesis to determine DCC cytokine expression profiles. The level of transcripts IL4 and IFNG was low or undetectable for both CD90+/CD90+ DCC and CD90+/MSC DCC. There were differences in the expression of mRNA coding for cytokines IL-1alpha, IL-10 and TNF-alpha between CD90/CD90+ DCC and CD90+/MSC DCC. The IL10 transcripts were significantly higher in CD90+/CD90+ DCC, suggesting their potential immunomodulatory paracrine role, whereas increased level of TNF-alpha could indicate a potential impact on both the cell apoptosis and stimulation of proliferation, as it is a highly pleiotropic cytokine

involved in hematopoietic cells interactions (Figure 7).
Aim 2. To assess in vivo the tolerogenic effect of the supportive therapy of new generation of CD90+/CD90+ DCC and CD90+/MSC DCC in the VCA and heart transplantation models.
2.1 Assessment of the in vivo tolerogenic effect of CD90+/CD90+ DCC and CD90+/MSC DCC lines in the VCA transplantation model.
We tested tolerogenic effect of both DCC therapies using our well-established VCA model of vascularized skin allograft (groin flap). VCA transplantations were performed between ACI donors and Lewis recipients through transplantation of the vascularized groin flap harvested from the ACI donor and transferred to the groin region of the Lewis recipient (Figure 8). Twenty-four VCA transplants were evaluated in four experimental groups of six animals each: 1) VCA ¿ saline control; 2) VCA- MSC control; 3) VCA -CD90+/CD90+ DCC; 4) VCA CD90+/MSC. VCA recipients in therapy groups received short, 7 day course of immunosuppressive therapy (IS) of anti-TCR alpha/beta monoclonal antibody (250ug/day) and tacrolimus (0.5mg/kg/dose) combined with DCC therapy delivered via intraosseous injection (5 x 10^6 cells) to femur at the completion of VCA transplantation.
Outcomes after VCA transplantation: Monitoring of skin allografts for signs of rejection was performed daily and documented at the day of VCA rejection (Figure 9). VCA survival under 7 day IS of anti-TCR alpha/beta antibody and tacrolimus ranged from 65-100 days for CD90+/CD90+ DCC therapy and from 59-65 days for CD90+/MSC DCC therapy. In contrast saline and MSC controls without IS rejected between 7-10 days.
Total chimerism assessment by flow cytometry for CD90+/CD90+ DCC was above 2.00 % in bone marrow compartment, thymus, and spleen and high level of 9.24% chimerism was found in liver indicating tolerogenic properties of CD90+/CD90+ DCC therapy. In contrast the saline control group no chimerism was detected whereas in MSC control and CD90+/MSC DCC chimerism level was assessed below 1% (Figure 10).
2.2 Assessment of the in vivo tolerogenic effect of CD90+/CD90+ DCC and CD90+/MSC DCC lines in the heart transplantation model.
We have tested DCC therapy in the heterotopic heart transplant model to confirm the tolerogenic effect of DCC in solid organ transplantation scenario. Forty-eight heart transplants were performed across major MHC barrier between ACI (RT1a) donors and Lewis (RT1l) recipients using standard heterotopic heart transplantation procedure to the abdominal cavity of the Lewis recipients (Figure 11). Eight experimental groups were evaluated: 1) heart transplant¿ saline control; 2) heart transplant ¿ MSC control; 3) heart transplant ¿ CD90+/CD90+ DCC therapy; 4) heart transplant CD90+/MSC DCC therapy. Assessments were performed at two study end-points at day 7 and day 100 in the respective study groups.
Outcomes after Heart Allograft transplantation: Daily monitoring of heart allografts was performed by palpation for confirmation of heart beating. All allograft recipients were euthanized at day 100 post-transplant. The gross assessment for each group and representative heart allograft images are presented in Figure 12. Heart allograft survival under 7-day IS of anti-TCR alpha/beta antibody and tacrolimus ranged from 67-100 days for CD90+/MSC DCC therapy and from 57-100 days for CD90+/CD90+ DCC therapy. Heart allograft samples were harvested, embedded, sectioned and stained for H&E. Slides were assessed for the presence of inflammation, fibrosis and rejection (Figure 13). In control groups (MSC or saline delivery) extensive inflammatory cell infiltration was observed at day 7, whereas, inflammation, fibrosis and calcifications were observed at day 100 post-transplant and correlated with allograft rejection. Heart allografts supported with CD90+/CD90+ DCC and CD90+/MSC therapy under 7 day protocol of anti-TCR alpha/beta antibody and Tacrolimus presented reduced inflammatory response, decreased fibrosis and lack of calcifications.
Study Summary and Conclusions
1. We have successfully confirmed, the feasibility of ex vivo creation of two new chimeric cell lines of hematopoietic (CD90+/CD90) and hematopoietic/MSC (CD90+/MSC) origin. Flow cytometry and

confocal microscopy confirmed DCC creation by presence of overlapping PKH26/ PKH67 fluorescence dye. DCC purity was assessed between 85-95% after fusion procedures.
2. Phenotype assessment of the created DCC confirmed markers characteristic for the parent cells. Flow cytometry analysis of the surface marker expression specific for the parent cells before fusion and for both DCC lines after fusion confirmed that CD90+/CD90+ DCC expressed HSC specific markers (CD90, CD45, and CD34) and CD90+/MSC DCC expressed both HSC (CD90, CD45, and CD34) and MSC (CD29, CD73) specific markers. We have also confirmed the maintenance of hematopoietic and mixed (hematopoietic and mesenchymal) phenotype in the cultured DCC cells after fusion.
3. Assessment of allogeneic responses of DCC lines by mixed lymphocyte reactivity (MLR) assay revealed that both DCC lines evoked decreased T-cell proliferation when compared to the allogenic parent cells confirming tolerogenic properties of DCC.
4. Assessment of regulatory T-cells induction by DCC by fluorescence-based lymphocyte reactivity assay revealed that DCC of CD90+/MSC origin induced higher level of regulatory T-cells in vitro, comparable to the MSC controls, indicating maintenance of immunomodulatory characteristics of DCC after fusion.
5. Evaluation of transcriptional levels of cytokines revealed undetectable levels of transcripts IL4 and IFNG. CD90+/CD90+ DCC expressed higher level of IL10 transcripts implying their immunomodulatory properties.
6. Total chimerism assessed at study end-point by FC for CD90+/CD90+ DCC was above 2.00 % in bone marrow compartment, thymus, and spleen and high level of chimerism of 9.24% was found in liver, indicating tolerogenic properties of CD90+/CD90+ DCC therapy. In contrast in the saline control group no chimerism was detected whereas in MSC control and CD90+/MSC DCC chimerism level was assessed below 1%.
7. We have successfully performed allogenic VCA transplants as well as heterotopic heart allograft transplants, and tested survival under novel 7-day IS protocol of anti-TCR alpha/beta antibody and tacrolimus supported by intraosseous delivery of DCC (CD90+/CD90+ or CD90+/MSC). DCC therapy (CD90+/CD90+) significantly extended VCA and heart allograft survival (up to 100 days) under 7- day IS protocol of anti-TCR alpha/beta antibody and tacrolimus combined with DCC. In contrast saline and MSC controls without IS rejected transplants between 7-11 days. Pathology assessment of heart allografts revealed decreased fibrosis and inflammatory response as well as lack of calcifications in DCC therapy groups when compared with controls.
8. Both DCC therapies expressed tolerogenic properties and extended significantly VCA and heart allograft survival under short 7 day IS protocol. These findings are encouraging and further studies are needed to assess the potential clinical application of DCC therapies in VCA and heart transplantation.

***3. Indicate what percent of the project you estimate has been completed (e.g. 30%).**

100

***4. Are there any significant changes or delay in your training program and/or research plan?**

No

***5. Are there any changes in Sponsor (on fellowship or mentored awards)?**

No

***6. Are there any changes in Principal Investigator or collaborators?**

No

***7. Are there any changes in location or facilities?**

No

***13. No Changes.**

Yes

***14. Please describe any significant changes that have occurred, as noted above, or any anticipated changes that represent a significant deviation from the original plan. Briefly discuss the reason(s) for the changes and the implications.**

***15. Expenditures: (not applicable to fellowship awardees.) If you have a low rate of expenditures this fiscal period, please provide a brief explanation.**

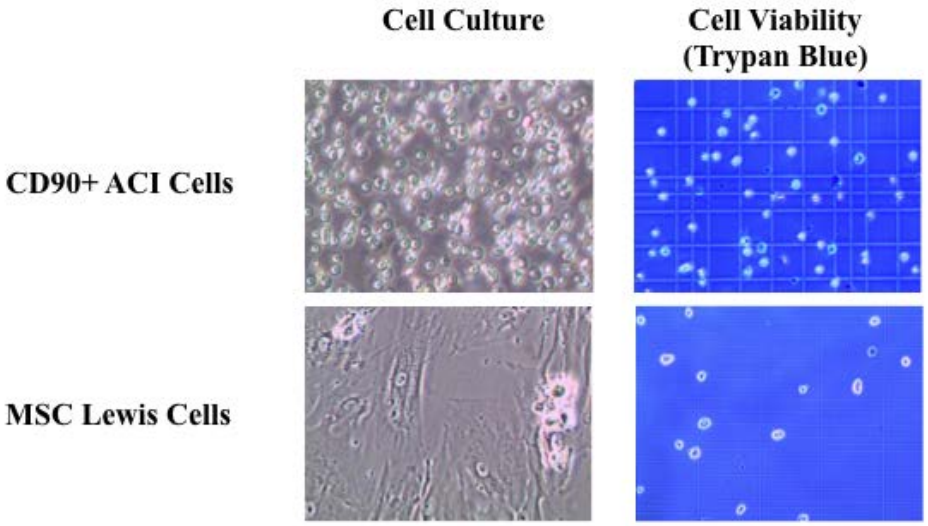


Figure 1. Confirmation of morphology and viability of ACI (RT1^{a}) rat derived CD90^{+} cells and Lewis rat (RT1^{l}) derived mesenchymal stem cells (MSC) after *in vitro* culture.

Upper row: representative images of ACI CD90+ cells in culture (StemSpan SFEM, StemCell Techn.) supplemented with antibiotics (Gibco) and 10% Fetal Bovine Serum (Sigma-Aldrich) at day 7 (left column) and evaluation of viability of CD90+ with trypan blue staining prior the *ex vivo* cell fusion (viability > 85%; right column). Lower row: representative images of Lewis MSC cells in culture (Low glucose DMEM, Corning) supplemented with antibiotics (Gibco) and 10% Fetal Bovine Serum (Sigma-Aldrich) at day 14 (left column) and evaluation of viability of MSC with trypan blue staining prior to *ex vivo* fusion (viability > 95%; right column).

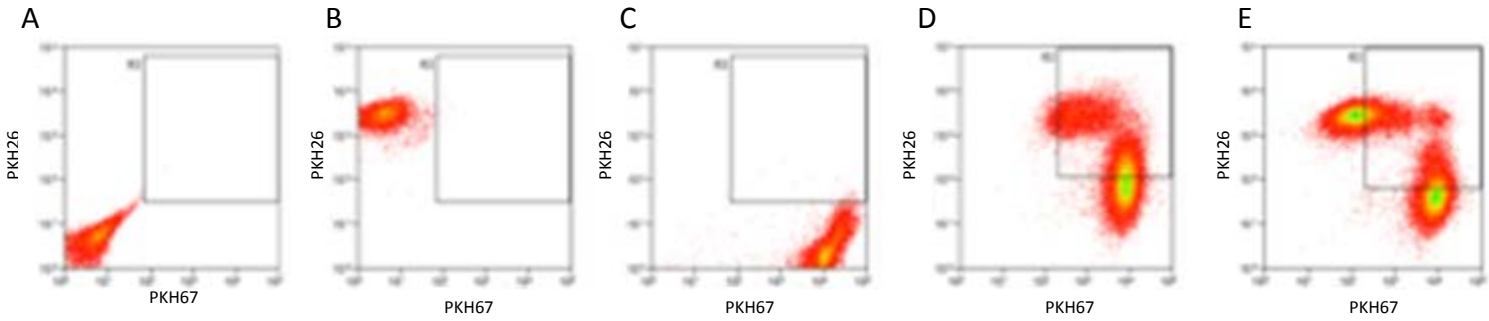


Figure 2. Confirmation of *ex vivo* cell fusion of ACI CD90+ HSC with Lewis CD90+ HSC and ACI CD90+ HSC with Lewis MSC and creation of two Di-Chimeric Cell (DCC) lines: (CD90+/CD90+ and CD90+/MSC) DCC by flow cytometry.

Dot plots present: A) Unstained control, B) ACI CD90+ HSC cell stained with PKH26 membrane dye, C) Lewis CD90+ or Lewis MSC parent cells stained with PKH67 membrane dye. After fusion procedure, created DCC were sorted by FACS sorting (Moflow Astrios) based on the double positive PKH26+/PKH67+ fluorescence (square gate); D) gated DCC ACI CD90+/Lewis CD90+; E) DCC ACI CD90+/Lewis MSC.

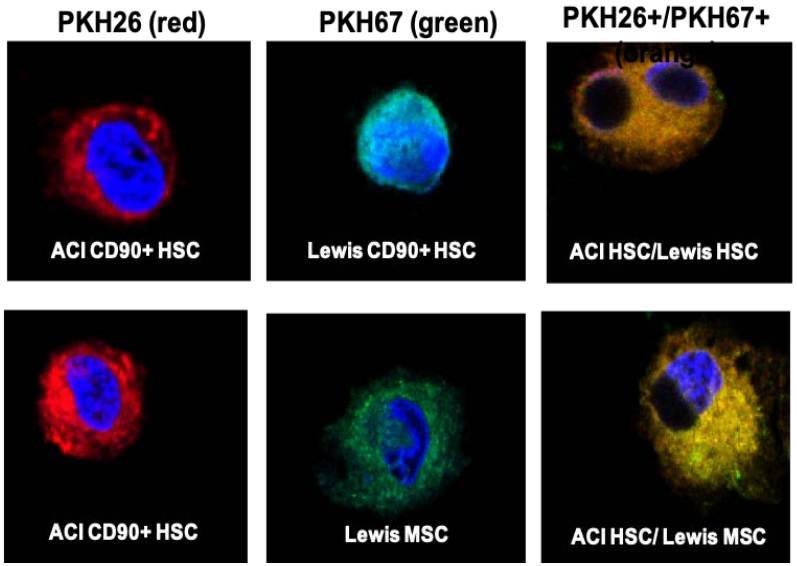


Figure 3. Confirmation of *ex vivo* cell fusion of ACI CD90+ HSC with Lewis CD90+ HSC and ACI CD90+ HSC with Lewis MSC and creation of two Di-Chimeric Cell (DCC) lines: CD90+/CD90+ and CD90+/MSC DCC by confocal microscopy.

ACI CD90+ HSC parent cells were stained with PKH26 (red, Sigma-Aldrich) membrane dye. Lewis CD90+ HSC and Lewis MSC parent cells were stained with PKH67 (green, Sigma-Aldrich). After fusion procedure and FACS sorting based on the double positive PKH26+/PKH67+ fluorescence, the created DCC cells were analyzed for fusion confirmation and purity assessment by confocal microscopy. Fused DCC cells were identified based on overlapping emissions (orange color). Nuclei were counterstained with DAPI (blue). Magnification 400X.

Surface markers (%)	CD90	CD45	CD34	CD29	CD73
ACI CD90+HSC	82,64,92	75,74,92	93,92,95	0.5, 2, 0.7	2.4, 3,5
Lewis CD90+ HSC	76,89,63	95,64,82	91,90,90	1.7, 1, 0.3	0.4,1.2, 4.2
Lewis MSC	66,89,63	2.9, 3.2, 4.1	63,45,52	95,97,93	70,91,73
DCC ACI CD90+ HSC/Lewis CD90+ HSC	63,71,75	85,60,72	80,71,72	2.4,3.5,3.7	1.0, 2,4
DCC ACI CD90+ HSC/Lewis MSC	73,70,77	65,60,63	67,89,76	90,63,87	68,67,74

Table 1. Phenotype analysis of ACI and Lewis parent cells and both created DCC lines (CD90+/CD90+ and CD90+/MSC) for confirmation of phenotype preservation after *ex vivo* cellular fusion by flow cytometry.

All parent cell and DCC lines after cells fusion were stained with anti-rat CD90-APC (BD), CD45-APC-Cy5 (BD), CD34-PE (Abcam), CD29-FITC (BD) and CD73-V450 (BD). Samples were analyzed on BD Fortessa flow cytometer and FlowJo. ACI and Lewis CD90+ HSC parent cells expressed HSC specific markers: CD90, CD45, CD34. Lewis MSC parent cells were positive for CD90, CD29 and CD73. After fusion procedure and sorting based on the double positive PKH26+/PKH67+ fluorescence, DCC ACI HSC CD90+/ Lewis HSC CD90+ maintained HSC specific marker expression (CD90, CD45, CD34). DCC ACI CD90+ HSC/Lewis MSC expressed both HSC and MSC specific markers (CD90, CD45, CD34, CD29 and CD73), double positive CD29+/CD45+ DCC population were higher than 60% (82, 70,71).

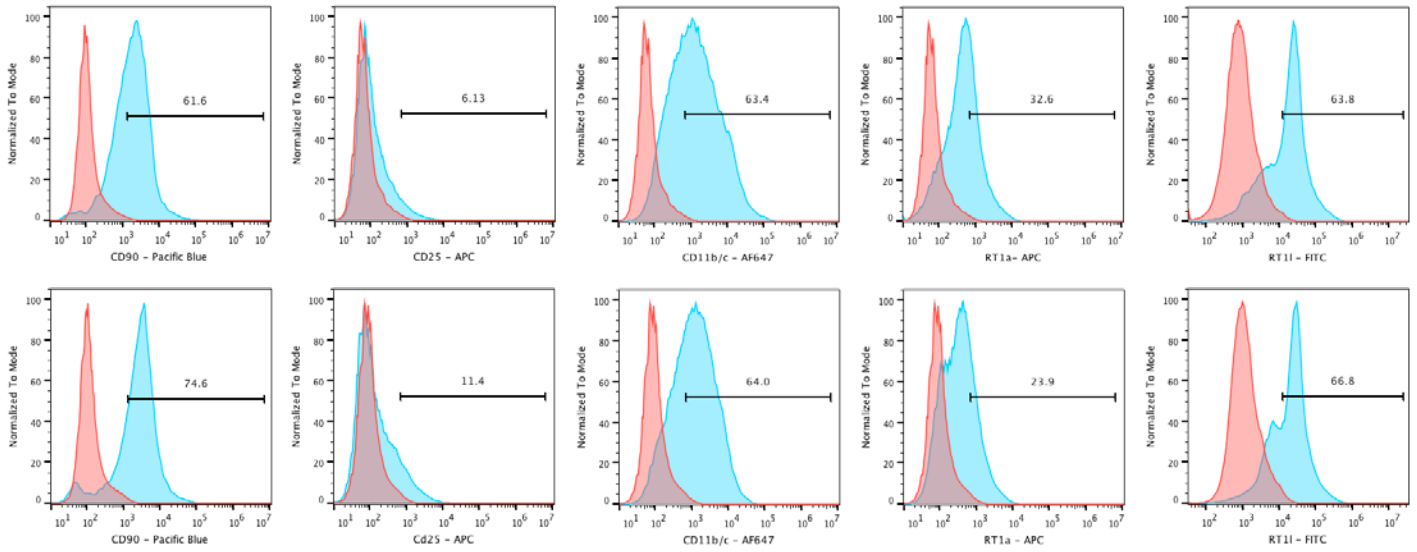


Figure 4. Phenotype analysis of the hematopoietic (HSC) CD90+/CD90+ DCC lines of ACI and Lewis origin after 14 days of *in vitro* culture by flow cytometry.

Two distinct populations of HSC DCC were observed during 14 days of cell culturing: the plastic adherent HSC DCC population (upper panel) and plastic non-adherent HSC DCCs population (lower panel). Cells were assessed for the presence of surface markers: CD90-Pacific Blue, CD25-APC, CD11b/c-AF647 $RT1^a$ – APC and $RT1^l$ – FITC. Samples were analyzed on BD Fortessa flow cytometer and FlowJo. High expression of CD90, $RT1^a$ (ACI donor rat specific marker) and $RT1^l$ (Lewis donor rat specific marker) confirmed maintenance of the hematopoietic - ACI/Lewis mixed phenotype of the CD90+/CD90+ DCC after *ex vivo* cell fusion and cell culture.

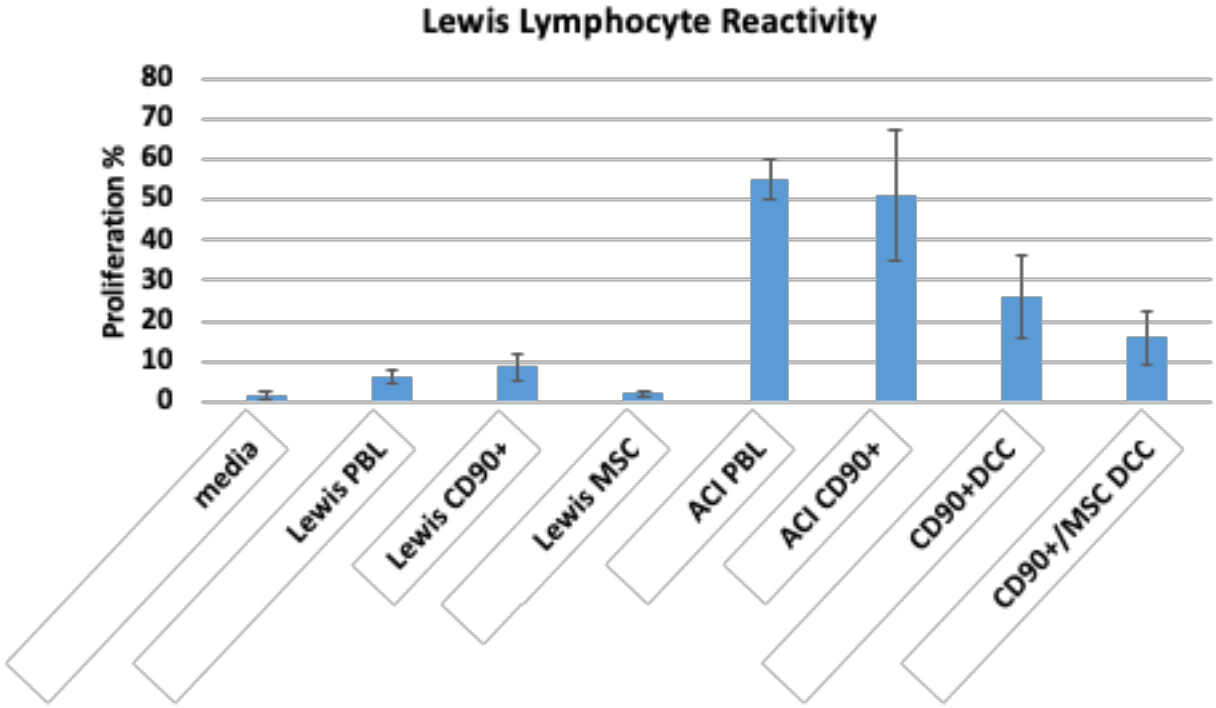


Figure 5. Evaluation of the allogenic response *in vitro* of the ACI and Lewis parent cells and created DCC lines after *ex vivo* cellular fusion by fluorescence-based lymphocyte reactivity assay.

Parent cell populations and both DCC lines were cultured in the direct co-culture (ratio 1:1) with Lewis splenocytes, obtained by density gradient centrifugation. Lewis lymphocytes were stained with eFluor 670 proliferation dye prior to co-culture. After 4 days of co-culture, proliferation of the fluorescence dye was analyzed by Flow Cytometry. Proliferation of the allogenic ACI lymphocytes was set as the positive control (100% proliferation) while culture of the Lewis lymphocytes in basal media (minimal proliferation), which served as the negative control for both DCC lines, evoked decreased T-cell proliferation when compared to the allogenic parent cells co-culture.

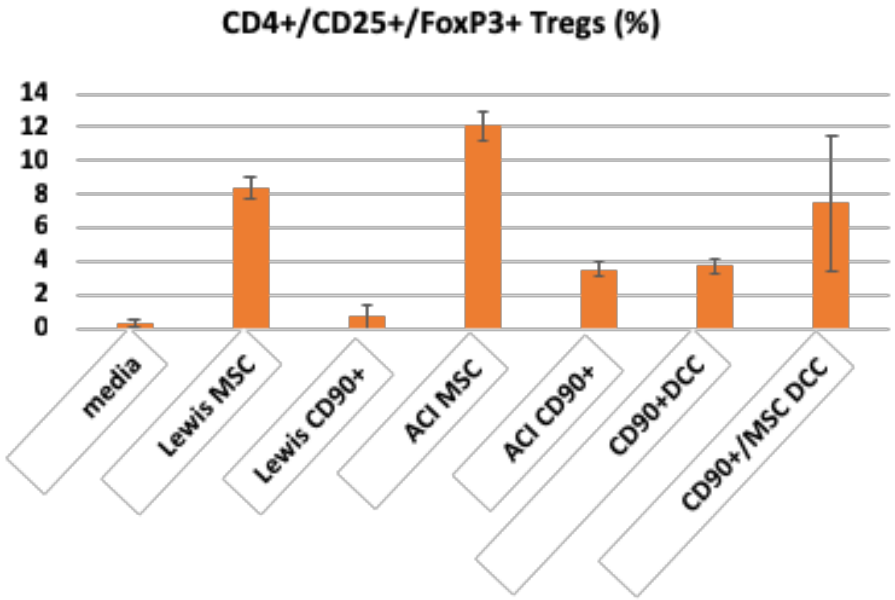


Figure 6. Flow cytometric evaluation of regulatory T-cell (Treg) level during *in vitro* co-cultures of CD25-CD4+ Lewis T-cells with ACI and Lewis parent cells and both DCC lines after *ex vivo* cellular fusion. Lewis splenocytes, after red blood cells lysis and staining with anti-rat CD3-V450, CD4-PE and CD25-APC (BD Bioscience) were sorted based on the CD3+/CD4+/CD25- characteristics. CD25 T-cells were co-cultured in 96 well plates (100.000 cells/well, ratio 1:1) with all parent cell and DCC lines of three fusion. After 5 days of co-culture, samples were harvested and stained with anti-rat FoxP3-FITC, CD3-V450, CD4-PE and CD25-APC (BD Bioscience) and were analyzed to quantify the CD4+/CD25+/FoxP3+cells by BD Fortessa flow cytometer and FlowJo. For negative controls served cells cultured in the basal media. Both ACI and Lewis MSC were inducing elevated levels of Tregs (8.65 and 12%) when compared to the negative controls. DCC of CD90+/MSC origin maintained increased Treg characteristics (7%).

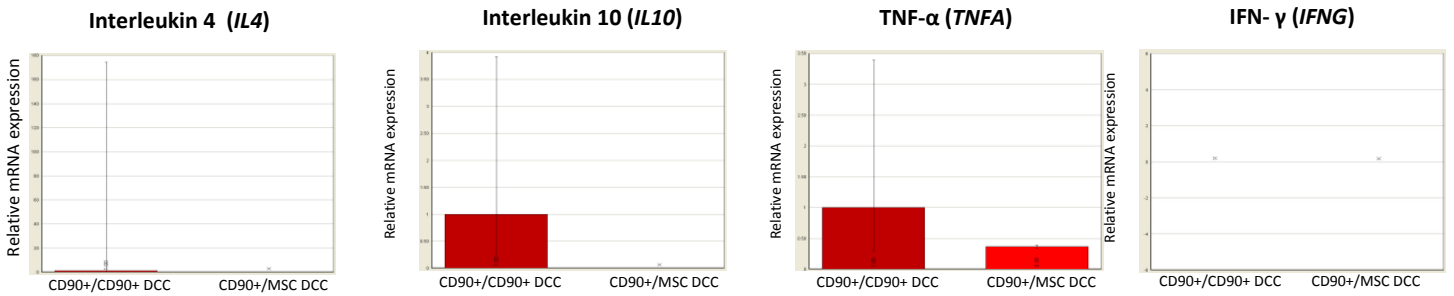


Figure 7. Transcriptional profile of CD90+/CD90 + DCC and CD90+/MSC DCC after fusion for selected cytokine transcripts encoding: Interleukin 4, Interleukin 10, TNF-α and IFN-γ.

Quantitative real-time PCR evaluation of relative expression of transcripts encoding IL-4, IL-10, TNF-α and IFN-γ in cultured (P3) DCC cells: CD90+/CD90+ (dark red) and CD90+/MSC (light red). Cells were harvested via trypsinization (passage 3), RNA was extracted from cell pellets (QIAgen, cat. C74107) and subsequently cDNA synthesis was performed (Applied Biosystems, cat.4368814), following real-time PCR reaction with TaqMan probes and *GAPDH* as a reporter gene for relative delta cT mRNA expression quantification. Bars indicate the SD for n=3 reactions. Evaluation of transcriptional levels of cytokines revealed undetectable levels of *IL4* and *IFNG* in both DCC lines. CD90+/CD90+ DCC were characterized by higher levels of *IL10* expression indicating their immunomodulatory characteristics. Elevated levels of *TNFA* (coding for TNF-α) in CD90+/CD90+ DCC in comparison to CD90+/MSC could imply potential impact on both the cell death and cell proliferation, as it is a highly pleiotropic cytokine involved in hematopoietic cells interactions.

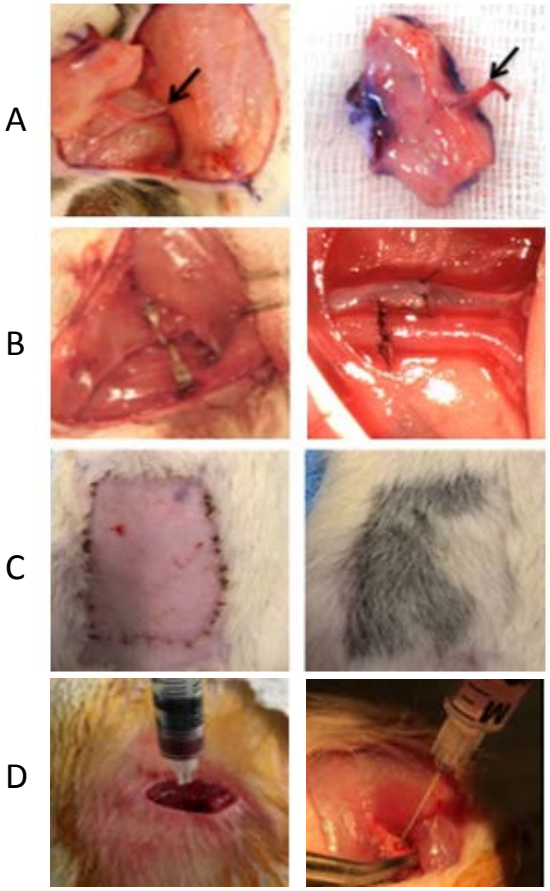


Figure 8. Surgical procedure of vascularized composite allograft (VCA) transplantation between fully MHC-mismatched ACI (RT1^{a}) donor and Lewis (RT1^{l}) recipient, supported by intraosseous delivery of Di-Chimeric Cell (DCC) therapies to the recipient's femoral bone.

Transplantation of the fully MHC mismatched VCA (groin flap) from the ACI (RT1^{a}) donor to the Lewis (RT1^{l}) rat recipient. The VCA was harvested based on the femoral arteries and veins of the ACI donors. Following groin flap harvest from, the ACI donor, the groin flap was transplanted to the groin region of the Lewis recipient and femoral vessels of the donor and recipient were used for vascular anastomosis. (A) *left*: dissected ACI donor vascularized groin flap (4x3 cm) based on the single femoral vessels pedicle; (A) *right*: the donor flap groin prepared for the transplantation; *(B) left*: microsurgical clamps for VCA between donor and recipient rat; *(B) right*: end-to-end anastomosis performed with 10/0 nylon interrupted sutures after surgical clamps; *(C) left*: the transplanted VCA at 7 day follow-up; *(C) right*: the transplanted VCA at 95 day follow-up; (D) *left:* aspiration of bone marrow to create space within the bone marrow compartment of the femur; (D) *right:* intraosseous DCC delivery – injection of DCC suspension into the femur followed by an application of bone wax to prevent cell leakage from the bone.

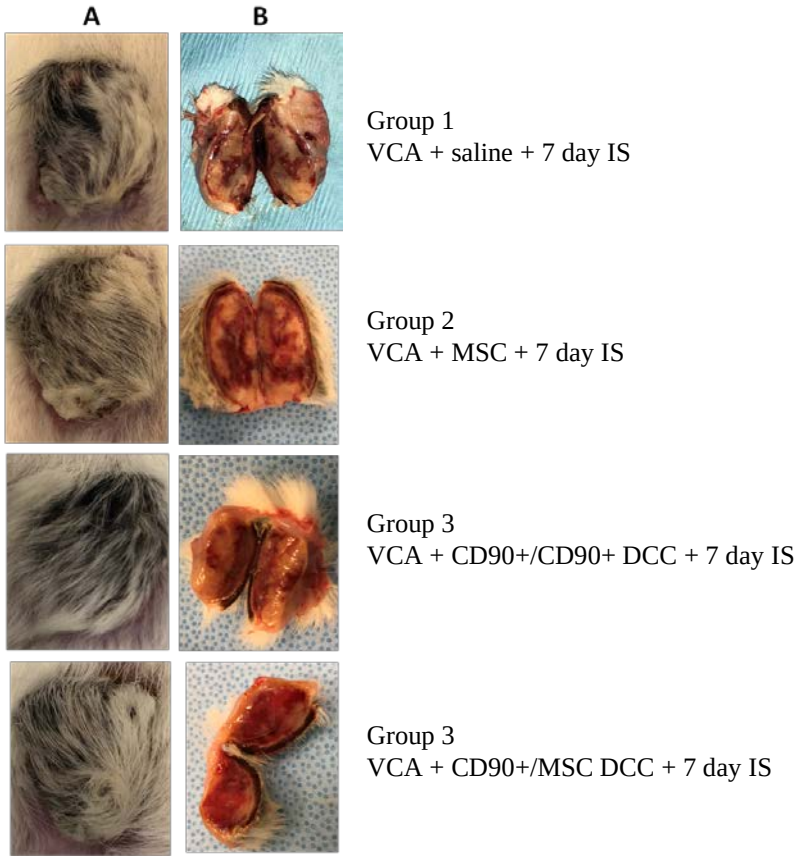


Figure 9. Representative post-transplant images of the fully MHC-mismatched VCA transplants between ACI (RT1^a) donors and Lewis (RT1^l) recipients supported with intraosseous delivery of DCC therapies:
Group 1- saline (control), Group 2- mesenchymal stem cell (MSC, control), Group 3- CD90+/CD90+ DDC therapy and Group 4 - CD90+/MSC DCC therapy. All VCA recipients received a short 7-day immunosuppressive protocol (IS) of anti-αβ TCR monoclonal antibody (250 μg/day) and Tacrolimus (0.5mg/kg dose). Column A- Gross assessment of the long-term survival of transplanted VCA before rejection. Column B- Macroscopic evaluation of the transplanted VCA at the day of rejection representing study end-point.

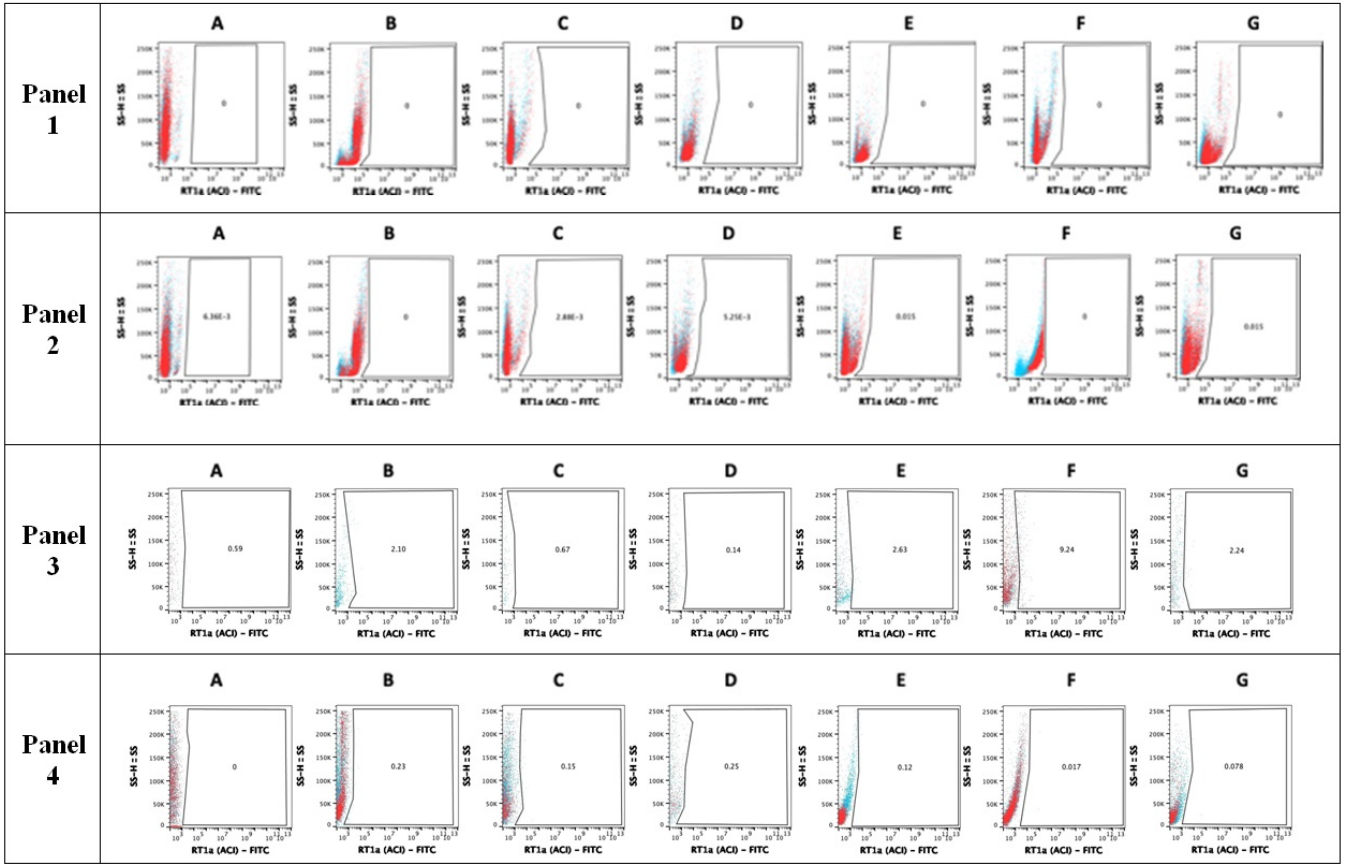


Figure 10. Total chimerism levels in the peripheral blood, bone marrow, lungs, lymph nodes (LN), spleen and liver of VCA allograft recipients at the experimental endpoint.

Representative dot plots of (from left): Panel 1- saline (control), Panel 2- mesenchymal stem cell (MSC, control), Panel 3- CD90+/CD90+ DDC therapy and Panel 4 - CD90+/MSC DCC therapy at 100 days post-transplant. A – Peripheral blood, B – Bone marrow, C – Lung, D – Lymph node, E – Thymus, F – Liver, G – Spleen. The level of total chimerism for CD90+/CD90+ DCC was above 2.00 % in bone marrow compartment, thymus, and spleen and high level of chimerism of 9.24% was found in liver. In contrast the saline control group no chimerism was detected whereas in MSC control and CD90+/MSC DCC chimerism level was assessed below 1%.

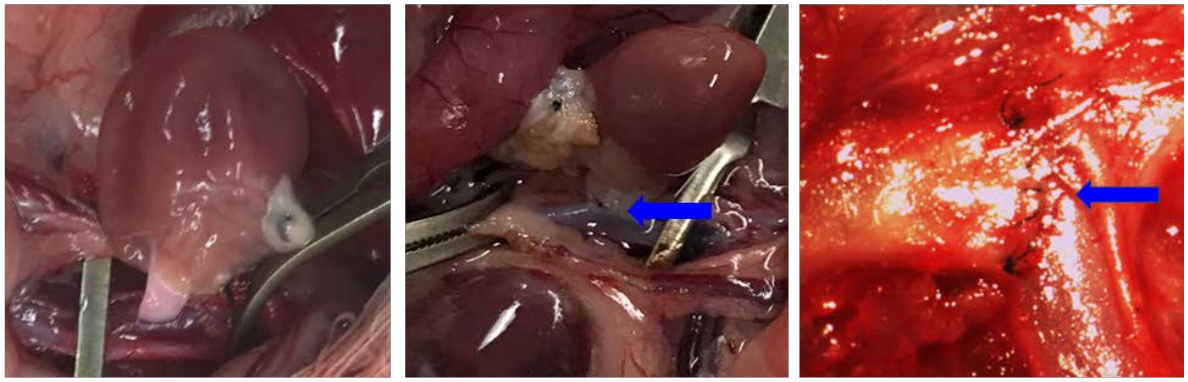
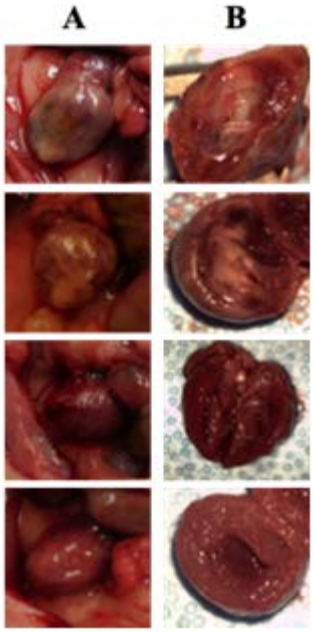


Figure 11. Surgical procedure of heart allograft transplantation between fully MHC-mismatched ACI (RT1^{a}) donor and Lewis (RT1^{l}) recipient, supported by intraosseous delivery of Di-Chimeric Cell (DCC) therapies to the recipient's femoral bone.

Heterotopic heart transplantations were performed between fully MHC mismatched ACI (RT1^{a}) donors and Lewis (RT1^{l}) recipients. Following harvest, the ACI donor heart was placed in ice-cold cardioplegia solution while the major abdominal vessels of the Lewis recipient were clamped. Microsurgical technique of end-to-side anastomosis was performed between the donor's heart aorta and recipient's infrarenal abdominal aorta, followed by anastomosis between the pulmonary artery of the donor's heart and the inferior vena cava of the recipient. Standard microsurgical procedure under 40x microscope magnification using 10/0 nylon suture performed. After completion of anastomoses vascular clamps were removed, and heart allograft was re-perfused. Spontaneous beating of the heart confirmed successful transplantation. From left to right: ACI heart allograft placed in the abdominal cavity of the donor prior to the anastomosis; heart allograft after anastomosis before clamp release; anastomosis site well preserved with beating heart at 100 day follow-up after transplantation.



Group 1
Heart transplant + saline + 7 day IS

Group 2
Heart transplant + MSC + 7 day IS

Group 3
Heart transplant + CD90+/CD90+ DCC + 7 day IS

Group 3
Heart transplant + CD90+/MSC DCC + 7 day IS

Figure 12. Representative post-transplant images of the fully MHC-mismatched heart allograft transplants between ACI ($RT1^a$) donors and Lewis ($RT1^l$) recipients supported with the intraosseous delivery of DCC therapies:
Group 1- saline (control), Group 2- mesenchymal stem cell (MSC, control), Group 3- CD90+/CD90+ DCC therapy and Group 4 - CD90+/MSC DCC therapy at 100 days post-transplant. All heart allograft recipients received a 7 day immunosuppression (IS) of anti-αβ TCR monoclonal antibody (250 μg/day) and Tacrolimus (0.5mg/kg dose). Column A- Gross assessment of the transplanted hearts at 100 day study end-point. Column B- Macroscopic evaluation of cross-sections of the heart allografts presenting the structure of the heart ventricles at 100 day study end-point. In control Groups 1 and 2 extensive fibrosis and calcifications were observed.

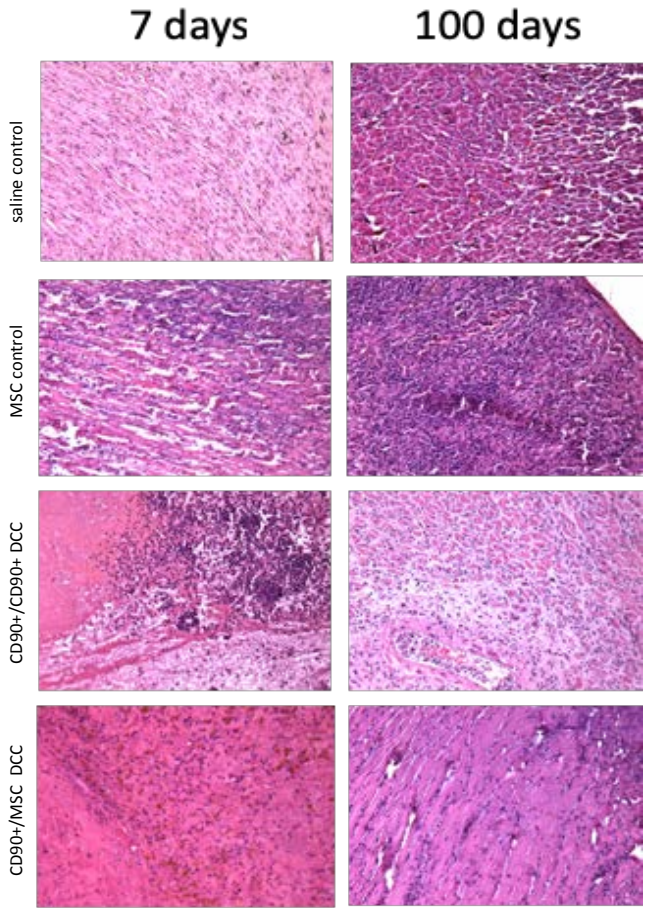


Figure 13. Representative images of histological assessment of heart allografts at day 7 (left panel) and day 100 (right panel) post- transplant.

Representative images of H&E staining: Row 1: Group 1 (control) - heart allografts under 7 day IS protocol of anti-αβ TCR monoclonal antibody (250 µg/day) and Tacrolimus (Tac, 0.5mg/kg dose), Row 2: Group 2 (control)- heart allografts supported with MSC (16x10^6 cells/kg) under 7-day IS protocol of anti-αβ TCR/Tac, Row 3: Group 3 - heart allograft supported with CD90+ DCC therapy 5.0 x 10^6 under 7-day IS protocol of anti-αβ TCR/Tac, Row 4: Group 4 - heart allograft supported with CD90+/MSC DDCC therapy 5.0 x 10^6 under 7-day IS protocol of anti-αβ TCR/Tac. At day 7 post-transplant early inflammatory cell infiltration was observed in the heart allograft control groups. When compared to the controls, reduced inflammatory response was seen in heart allografts supported with DCC therapies (Groups 3 and 4) both at day 7 and day 100 post-transplant (magnification: 10x). At day 7 post-transplant early inflammatory response with lymphocytic infiltration was observed in the heart allograft control groups (saline and MSC groups).