Chapter 3

The Efficacy of Intrathymic Immune Modulation After Allogeneic Tissue Transplantation (Skin and Neonatal Heart) in the Rat

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Abstract

To date, transplant arteriosclerosis (TA), the histologic counterpart of chronic transplant dysfunction, is the most important problem in clinical organ transplantation. Recently we showed in rats that intrathymic (IT) immune modulation substantially prolonged graft survival of MHC-incompatible, heterotopic vascularized cardiac allografts. Although acute rejection was prevented, eventually TA was found to develop. In this study, we analyzed the efficacy of IT immune modulation in two other, more simple, transplant models in which we transplanted tissues instead of whole organs: allogeneic skin transplantation and allogeneic neonatal heart-in-ear transplantation.

Results indicate that IT immune modulation does not prolong graft survival of allogeneic skin grafts. Tissue specific antigens present on the skin grafts may account for this. Graft survival of neonatal cardiac allografts transplanted subcutaneously in the ear-pinnae of recipient rats was significantly prolonged following IT immune modulation. In long-term surviving neonatal allografts, neo-angiogenesis of graft myocardial tissue was observed which was characterized by the appearance of cavernous-like vascular structures. IT immune modulation is effective in inducing prolonged graft survival after allogeneic cardiac tissue transplantation, whereas graft survival of allogeneic skin grafts is not prolonged. In contrast to vascularized cardiac allografts, development of TA was not observed in transplanted neonatal heart tissue, most probably as a result of lack of appropriate vascular structures (functional coronary arteries).
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Introduction

Despite the fact that, to date, clinical organ transplantation has almost become a routine procedure, it is still complicated by the problems associated with the long-term use of systemic immunosuppressive agents. Moreover, the development of chronic transplant dysfunction (CTD) has become the most serious cause of long-term allograft loss\(^1\). Depending on the type of organ grafted (liver, heart, kidney, and lung), three years after transplantation 4 to >50% of the transplants show functional deterioration and histologic changes characteristic of CTD\(^3-5\). The development of CTD seems to be multifactorial, and both alloantigen-dependent and alloantigen-independent factors seem to contribute to the development of CTD. However, the most important risk factor in the pathogenesis of CTD in the clinical setting appears to be histoincompatibility between donor and recipient, leading to alloreactivity directed against the graft\(^6\).

The most characteristic feature of CTD is the development of transplant arteriosclerosis (TA), also referred to as graft arterial disease (GAD). This form of vasculopathy is characterized by perivascular inflammation, thinning of the vascular media (due to media necrosis), focal breaks in the internal elastic lamina (IEL), and a generalized concentric intimal thickening, consisting of vascular smooth muscle (VSM) cells intermingled with T cells and macrophages\(^2\).

Because alloreactivity directed against the graft appears to be the most important contributing factor in the development of CTD, induction of specific hyporesponsiveness to donor antigens would be a possible solution to overcome the problems associated with this pathology. Specific hyporesponsiveness to allografts can be obtained by intrathymic (IT) inoculation of donor-type lymphoid cells (IT immune modulation). IT immune modulation has been shown to be effective to prolong survival of pancreatic islet, cardiac, liver, kidney, and small bowel allografts in rats and mice\(^7-11\). In all of these protocols, a time lag between IT inoculation and the actual transplantation was needed.

Several years ago, we developed a method to induce donor-specific unresponsiveness and transplant a cardiac allograft, simultaneously. Briefly, this protocol consists of intrathymic inoculation of donor-type splenocytes, intraperitoneal (i.p.) injection of rabbit anti-rat lymphocyte serum (ALS) on day 0, and treatment with cyclosporine A (CsA) injected intramuscularly (i.m.) on days 1, 2, and 3 post transplantation\(^12\). Our results show that it is possible to induce functional tolerance (i.e., continuous beating of the graft) after IT immune modulation in the (fully MHC incompatible) PVG to AO rat strain combination. However, we recently showed that although induction of functional tolerance was achieved, IT immune modulation did not prevent the development of TA\(^13\).

Transplantation of a cardiac allograft requires connection of the graft and recipient vasculature via the graft’s pulmonary artery and aorta in order to get perfusion. This is a rather complicated and time-consuming technique and a more simple transplantation model to study IT immune modulation is therefore desirable. In this paper, we investigated whether IT immune modulation could also prevent acute
rejection and induce prolonged graft survival of MHC-incompatible non-vascularized tissue grafts, and therefore allogeneic skin grafting and transplantation of neonatal cardiac tissue was performed. Neonatal cardiac tissue was transplanted subcutaneously in the ear-pinnae of recipient rats. Results showed that IT immune modulation did not prolong survival of MHC-incompatible skin grafts. However, survival time of allogeneic neonatal cardiac tissue transplanted in the ear-pinnae of recipient rats was significantly prolonged. Long-term surviving neonatal allografts were characterized by the appearance of cavernous-like vascular structures. Immunohistochemical analysis revealed that the endothelium of those vessels was of graft origin, suggesting neo-angiogenesis (proliferation of pre-existing vasculature) to be the underlying mechanism of new blood vessel formation. TA was not observed in the neonatal cardiac allografts, most likely due to lack of suitable vessels (functional coronary arteries [CA’s]) for TA to develop in.

In conclusion, also after allogeneic cardiac tissue transplantation IT immune modulation prolongs graft survival. In contrast to vascularized cardiac allografts, TA did not develop in transplanted neonatal cardiac tissue. Nevertheless, contractile activity of cardiac tissue disappeared eventually, most probably as a result of ongoing alloreactivity.

Materials and Methods

Animals
Specific-pathogen free, female PVG (RT-1c) and male AO (RT-1u) rats differing at the MHC-locus were obtained from the Central Animal Facility of the Faculty of Medical Sciences of the University of Groningen. PVG and AO rats were used as donors and recipients, respectively, and were maintained under conventional conditions and fed with regular rat chow and acidified water ad libitum. All transplantations were carried out under halothane inhalation anesthesia.

Skin transplantation
Orthotopic allogeneic skin transplantation was performed by transplanting circular full thickness PVG skin grafts to AO recipients. Both the donors and recipients were 7-10 weeks of age. The dermal side of the skin graft was cleared of all adherent material (blood vessels, muscle etc.) after which the graft was sutured into place with 10 to 14 separate sutures (silk 5-0) on the recipient rat (from which first a circular piece of skin had been excised). The graft was inspected regularly and rejection was defined as macroscopic appearance of graft necrosis.

Neonatal heart-in-ear transplantation
A modified procedure of the transplantation of cardiac tissue into the mouse ear, as originally described by Fulmer et al.14, was used. Briefly, a subcutaneous pouch was prepared on the dorsal surface of the recipient’s (7-10 week old male AO rats) ear-pinna using fine forceps. Subsequently, cardiac grafts obtained from neonatal PVG donors (less than 4 days of age) were rapidly transferred into the pouch using cotton tips. The pouch was closed with 2-3 separate sutures (Monosof 8-0), and the grafts were inspected regularly by visual inspection for pulsatile activity. Rejection was defined as complete cessation
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of visible pulsatile activity.

Vascularized cardiac transplantation
The heart of an age-matched PVG rat (7 to 10 weeks of age) was heterotopically transplanted into the right side of recipient’s neck, as described in detail elsewhere. Graft function was assessed regularly by palpation, and rejection was defined as complete cessation of ventricular contraction.

Intrathymic immune modulation
Intrathymic immune modulation was accomplished by a protocol developed in our lab and described in detail elsewhere. Briefly, partial sternotomy was performed, and 2.5x10^7 PVG splenocytes were injected into the recipient’s (AO) thymus.

Immunosuppression
Immediately after transplantation 1 ml rabbit anti-rat lymphocyte serum (ALS, Accurate Chemical Corp., Westbury, NY) was injected (i.p.). CsA (Sandoz Pharma AG, Basel, Switzerland) was injected i.m. on days 1, 2, and 3 post transplantation (15 mg/kg body weight).

Experimental setup
To investigate the efficacy of IT immune modulation in various tissue transplant models in the rat, allogeneic skin transplantation as well as transplantation of neonatal heart tissue were performed as listed in Table 1. Graft survival of transplanted tissues was compared to the graft survival of vascularized cardiac allografts. Allogeneic skin grafts were transplanted following IT immune modulation (group 3). Control groups consisted of rats receiving no treatment at all (group 1) and rats receiving only ALS and CsA (group 2) after skin grafting. Allogeneic neonatal cardiac allografts were transplanted subcutaneously into a pouch on the dorsal side of the recipient’s ear following IT immune modulation (group 6). Control groups again consisted of rats receiving no treatment at all (group 4), rats receiving only ALS and CsA (group 5), and rats receiving an AO isograft (group 7). Vascularized cardiac allografts were transplanted following IT immune modulation as described previously (group 10). Control groups consisted of rats receiving no treatment at all (group 8), rats receiving only ALS and CsA (group 9), and rats receiving an AO isograft (group 11).

Histological analyses
To analyze vascular structures in long-term surviving neonatal cardiac tissue transplanted in the ear-pinnae of recipient rats, ear tissue including the graft was removed at the time of sacrifice. Tissues were either fixed in Bouin’s fixative and embedded in paraffin or immediately frozen in liquid nitrogen and stored at -80°C for cryostat sections. For general histology, hematoxylin and eosin staining was performed on paraffin cross sections (7 µm). To analyze intimal hyperplasia in long-term surviving neonatal cardiac allografts, an indirect immunohistochemical staining for VSM cell α-actin was performed using mAb asm-1 (Roche Molecular Biochemicals, Almere, The Netherlands). Incubation with the primary antibody was followed by incubation with a second-step horseradish peroxidase-conjugated rabbit anti-mouse antibody (DAKO A/S, Glostrup, Denmark). Subsequently, the chromogen diaminobenzidine (DAB) was applied, fol-
lowed by counterstaining with Mayer’s hematoxylin and coverslipping in Depex mounting medium (BDH Laboratory Supplies, Poole, United Kingdom). Since neonatal cardiac tissue was transplanted subcutaneously without anastomosing the graft vessels to the recipient’s vasculature, we analyzed whether ingrowth of newly formed blood vessels from the periphery of recipient tissue occurs. To discriminate between donor (PVG) and recipient (AO) derived cells and tissues, MHC class I haplotype specific staining was performed on 7-µm cryostat sections as described above using the following mAb’s: OX-18 (pan-class I)\textsuperscript{17}, OX-27 (PVG [RT-1\textsuperscript{c}] class I)\textsuperscript{18} and U9F4 (AO [RT-1\textsuperscript{u}] class I)\textsuperscript{19}. To detect endothelial cells, mAb RECA-1 was used\textsuperscript{20}.

**Statistics**

All data are presented as median survival times (MST) and were evaluated for sta-

### Table 1. Survival of PVG neonatal cardiac tissue, skin grafts, and vascularized cardiac grafts after intrathymic immune modulation in AO recipient rats

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
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<th>P-value\textsuperscript{a}</th>
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<tr>
<td>1</td>
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<td>-</td>
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<td>3</td>
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<td>-</td>
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<td>6</td>
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<td>+</td>
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<td>5</td>
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<tr>
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<td>3</td>
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<td>8</td>
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<tr>
<td>11</td>
<td>3</td>
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<td>-</td>
<td>cardiac isograft</td>
<td>&gt;125, &gt;125, &lt;0.0001\textsuperscript{c}</td>
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Abbreviations: IT, intrathymic inoculation with 2.5x10\textsuperscript{7} PVG splenocytes; ALS, rabbit anti-rat lymphocyte serum; CsA, cyclosporin A; MST, median survival time; NS, not significant; \textsuperscript{a} P<0.05 is considered statistically significant (Student t-test); \textsuperscript{b} compared to the non-treated group; \textsuperscript{c} compared to the ALS + CsA treated group
tistical significance by the Student’s t-test using GraphPad Instat\textsuperscript{tm}, GraphPad Software. Differences with P<0.05 were considered statistically significant.

**Results**

*Graft survival of skin allografts*

To study whether IT immune modulation is also effective in transplant models in which tissues instead of whole organs are transplanted, allogeneic skin transplantation following intrathymic inoculation of donor-type splenocytes and short-term immunosuppression was performed. Table 1 (groups 1-3) summarizes the graft survival rates of the different experimental groups after skin transplantation.

No treatment resulted in rapid rejection of the skin grafts (group 1, MST 8 days), whereas treatment with ALS and CsA resulted in significantly prolonged graft survival after allogeneic skin transplantation (group 2, MST 24 days, P<0.0001) compared to non-treated animals (group 1). Intrathymic inoculation of donor-splenocytes did not result in prolonged graft survival after allogeneic skin transplantation (group 3, MST 24 days) compared to the grafts transplanted in animals treated with ALS and CsA only (group 2) (Figure 1A)

![Figure 1](image-url)
Graft survival of neonatal cardiac tissue

As an alternative tissue transplant model, we transplanted neonatal cardiac allografts in the ear-pinnae of rats, and used the contractile activity of the cardiac tissue as read-out system for the efficacy of the intrathymically induced hyporesponsiveness. Table 1 (groups 4-7) summarizes the graft survival rates of the different experimental groups after vascular heart transplantation. Transplantation of neonatal cardiac AO isografts resulted in indefinite survival in all animals (group 7, MST >125 days). No treatment resulted in rapid rejection of allogeneic skin grafts (group 1, MST 8 days), whereas treatment with ALS and CsA resulted in a slightly prolonged graft survival (group 5, MST 20 days) which did not reach statistical significance compared to the non-treated animals. Intrathymic inoculation of donor splenocytes resulted in a significant prolongation of the graft survival rates of neonatal cardiac allografts transplanted in the ear-pinnae of recipient rats (group 10, MST >125 days, P=0.007). In this group, 2 out of 5 animals rejected their grafts 22 and 81 days after intrathymic inoculation/transplantation, while the other three animals accepted their grafts permanently (Figure 1A).

Graft survival of vascularized cardiac allografts

Table 1 (groups 8-11) summarizes the graft survival rates of the different experimental groups after vascular heart transplantation. IT immune modulation (group 10) resulted in significant prolonged graft survival (P<0.0001) compared to untreated animals (group 8, MST 8 days) and animals treated with ALS and CsA only (group 9, MST 20 days). Figure 1B shows that IT immune modulation is highly successful as the survival rate at 125 days (indefinite survival) is over 90%. AO isografts (group 11) showed indefinite survival (MST >125 days) in all animals, which also differed significantly from the ALS and CsA treated animals (group 9, P<0.0001).

Development of vascular lesions in neonatal cardiac tissue

We previously showed that IT immune modulation does not prevent development of TA after vascular transplantation of cardiac allografts in the PVG to AO rat strain combination13. In this study, we analyzed whether also after subcutaneous transplantation of neonatal cardiac tissue vascular lesions develops in the CA’s of long-term surviving neonatal cardiac tissue. General histology of the various experimental groups (groups 4-7) is shown in Figure 2.

Neonatal AO isografts (group 7) did not show any sign of an inflammatory response to the cardiac tissue. Large blood vessels were abundantly present and apparently newly formed (Figure 2A) since normal, non-transplanted neonatal cardiac tissue does not contain such vascular structures. In contrast to isografts, non-treated allografts (group 4) were characterized by severe parenchymatous infiltration with mononuclear cells. In acutely rejecting grafts, no newly formed blood vessels were observed (Figure 2B). Treatment with only CsA and ALS (group 5) resulted in rejection within 3 weeks, and showed similar histology as the grafts from the non-treated animals (i.e., severe parenchymatous infiltration without newly formed blood vessels) (Figure 2C). Long-term surviving neonatal allografts
Intrathymic immune modulation and tissue transplantation showed neovascularization which was similar to the isografts (Figure 2D). In contrast to the isografts, however, after IT immune modulation also parenchymatous infiltration was present.

We also analyzed whether obliterated CA’s were present in the neonatal cardiac allografts in the IT immune modulated group. Staining for VSM cell α-actin indicated, however, that virtually no CA’s were present anymore (Figure 3G), which was similar to the neonatal AO isografts. Whereas in normal, non-transplanted neonatal cardiac tissue CA’s surrounded by α-actin positive medial VSM cells are clearly present (Figure 3B), CA’s in subcutaneously transplanted cardiac tissue are no longer functional and disappear with time. Since obliteration of the vascular lumen of CA’s with α-actin positive VSM cells is the hallmark of CTD-related histopathology, these results suggest that the neonatal heart-in-ear transplant model is not suitable to study the development of TA.

Although CA’s had completely disappeared, high numbers of apparently newly formed blood vessels were observed. RECA-1 staining confirmed the presence of endothelial cells on the luminal surface of these vessels (Figure 3F). To determine the origin of the blood vessel structures, MHC class I haplotype specific immunohistochemistry was performed. Staining using mAb OX18 (pan MHC class I) con-
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<th>RECA-1</th>
<th>normal</th>
<th>IT immune modulation</th>
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<tbody>
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<td>OX-27</td>
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firmed the presence of MHC class I antigen expression on the EC’s covering the newly formed blood vessels (Figure 3F). Moreover, the cardiac tissue was heavily infiltrated by MHC class I expressing inflammatory cells. Staining with mAb OX27 (donor specific) indicated that the transplanted myocardial tissue itself expressed MHC class I, whereas normal, non-transplanted neonatal cardiac tissue, hardly expressed MHC class I antigens (Figure 3E and J). Moreover, the EC’s covering the newly formed blood vessels express the donor-type MHC class I haplotype (inset Figure 3J). This observation suggests that those newly formed blood vessels are derived from the graft itself, and are not the result of ingrowth from the periphery of recipient-derived blood vessels. This was confirmed by staining with the recipient haplotype MHC class I specific mAb U9F4, which was negative on EC’s of the intragraft blood vessels, whereas positive staining was observed on EC’s of veins and arterioles present in (recipient) ear tissue (not shown). The myocardial tissue was heavily infiltrated with recipient-derived inflammatory cells which were U9F4 positive (Figure 3I). The newly formed blood vessel structures contained recipient-derived inflammatory cells, indicating that the graft and host vasculature were connected (inset Figure 3I). Because of an ongoing inflammatory response in the myocardial tissue, the vascular EC’s have become activated and recipient-derived inflammatory cells adhere to the sticky EC’s (Figure 3F).

**Discussion**

The results obtained after vascularized cardiac transplantation clearly indicate that IT immune modulation induces permanent (>125 days) graft survival in over 90% of the recipient rats in the MHC-incompatible PVG to AO rat strain combination. Most cardiac allografts transplanted after IT immune modulation remained beating for over 450 days, however, the development of transplant arteriosclerosis (TA) was not prevented. In this study we analyzed the efficacy of IT immune modulation after allogeneic non-vascularized tissue transplantation (skin and neonatal heart) which was compared to vascularized cardiac transplantation. In contrast to the data obtained after vas-

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**Figure 3.** (facing page) Immunohistochemical analysis of normal, non-transplanted, neonatal PVG cardiac tissue (A-E) and a PVG allograft (50 days post transplantation) after IT immune modulation (F-J), (A) EC’s in neonatal cardiac tissue. (B) α-actin positive medial VSM cells in neonatal cardiac tissue. (C) hardly any MHC class I expression is observed on non-transplanted neonatal cardiac tissue. (D) and (E) confirmed the absence of MHC class I expression on non-transplanted neonatal cardiac tissue using the MHC class I haplotype specific mAb’s U9F4 (AO specific, negative control) and OX-27 (PVG specific), respectively. Arrowheads in figures A-E indicate a CA present in normal, non-transplanted, cardiac tissue. (F) Newly formed blood vessels contain EC’s as indicated by positive RECA-1 staining. Arrows indicate inflammatory cells adhering to the endothelium. (G) Virtually no α-actin positive VSM cells are surrounding the newly formed blood vessels. (H) Intense MHC class I expression on infiltrating inflammatory cells as well as endothelium. (I) Infiltrating inflammatory cells are of recipient (AO) origin as indicated by positive U9F4 staining. The inset shows recipient-origin (U9F4 positive) of luminal inflammatory cells (arrows), whereas the EC’s are U9F4 negative suggesting graft-origin (arrowhead). (J) Graft-origin of EC’s was confirmed by positive staining using mAb OX-27 (arrowhead), whereas the luminal inflammatory cells are OX-27 negative (arrows).
cularized cardiac allografting, IT immune modulation did not prolong survival of skin allografts in the PVG to AO strain combination compared to control animals treated with ALS and CsA only. These results are essentially the same as those obtained by Nakafusa et al.\textsuperscript{21,22}, who performed skin grafting experiments in the fully MHC disparate Lew (RT-1\textsuperscript{b}) to Buf (RT-1\textsuperscript{b}) strain combination after donor-specific IT immune modulation. Similar results were obtained after kidney transplantation using this protocol. In their study, cardiac allografts showed significantly prolonged graft survival; 88% of the animals accepted their grafts permanently (MST >153 vs. 7 days). Our data and data reported by Nakafusa et al. indicate the presence of tissue-specific non-MHC antigens on kidney and skin allografts, which are presumably lacking on splenocytes and, therefore, may account for organ-specific immunogenicity and graft rejection. We also performed skin grafting experiments with intrathymic inoculation of donor-derived keratinocytes instead of splenocytes. This modification resulted in significant prolongation of skin graft survival; 40% of the animals accepted their skin grafts over 50 days, suggesting that tissue specific antigens indeed might play a role in IT immune modulation\textsuperscript{23}.

We also investigated whether IT immune modulation prevented rejection and induced prolonged graft survival of neonatal cardiac allografts transplanted subcutaneously in the ear-pinnae of recipient rats. After Fulmer et al.\textsuperscript{14} for the first time described the neonatal heart-in-ear transplantation model in mice, several other groups have applied this technique in mice and rats to study the effect of immunosuppressive agents on cardiac allograft survival\textsuperscript{24,25}, the level of cytokine gene expression in rejecting grafts\textsuperscript{26}, and the influence of MHC and non-MHC genes on allograft rejection\textsuperscript{27}.

Our data show that the neonatal heart-in-ear transplantation model is a useful alternative model to study IT immune modulation. The results clearly demonstrate that IT immune modulation significantly prolongs survival of subcutaneously transplanted neonatal cardiac tissue in the PVG to AO strain combination (MST 218 vs. 20 days).

We recently reported that following IT immune modulation vascularized cardiac allografts still develop TA increasing with time\textsuperscript{13}. Development of TA is the hallmark of chronic transplant dysfunction (CTD), which is being recognized as the most important problem in clinical organ transplantation today\textsuperscript{8}.

Development of TA after subcutaneous transplantation of neonatal cardiac tissue is unlikely, since the primary targets for TA, i.e. the CA’s, are nonfunctional after subcutaneous, non-vascularized transplantation. Routine histology as well as immunohistochemical analysis revealed that CA’s were no longer detectable in long-term surviving allografts after IT immune modulation as well as in AO isografts. In contrast to vascularized cardiac allografts, however, neonatal cardiac allografts and isografts showed presence of high numbers of large blood vessels which appeared to be newly formed and had developed from the graft’s own cells. The precise trigger for this formation of cavernous-like vessels is unknown, but ischemia/hypoxia of myocardial tissue might be involved.

So, although new, graft-derived, blood
vessel structures develop in long-term surviving allografts, these vessels are not obliterated by proliferating $\alpha$-actin positive VSM cells. In fact, virtually no VSM cells are present in these vessels at all. Although not clear from the histological analyses, these newly formed blood vessels should have connections with the recipient’s vasculature since recipient-derived inflammatory cells, sticking to the graft-derived EC’s, are clearly present in the lumen of the newly formed vessels. These results suggest that vascular rejection is persisting and, coinciding with the ongoing parenchymatous inflammatory response, may lead to further graft damage and loss of contractile activity eventually.

In conclusion, also after transplantation of allogeneic non-vascularized cardiac tissue, IT immune modulation significantly prolonged graft survival. We propose that since the appropriate CA’s are lacking in long-term surviving cardiac tissue, TA does not develop. Instead, neo-angiogenesis in graft tissue with graft derived blood vessels occurs in which TA is does not develop. Why the newly formed blood vessels do not develop TA in spite of an ongoing local alloresponse remains puzzling, and as such this model may provide information how to prevent the development of TA.

References


