ORIGINAL ARTICLE

Irradiation before and donor splenocyte infusion immediately after transplantation induce tolerance to lung, but not heart allografts in miniature swine

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SUMMARY

Solid organs may differ in their potential to induce and maintain a state of donor-specific tolerance. Previously, we induced stable immunological tolerance in a lung transplantation model in miniature swine. Here, we wished to transfer this established protocol into a heart transplantation model in miniature swine. Heterotopic heart transplantation (HTX) was performed in four and left-sided lung transplantation (LTX) in seven minipigs from gender- and SLA-mismatched donors. All recipients received nonmyeloablative irradiation, donor splenocyte infusion and intravenous pharmacologic immunosuppression for 28 postoperative days. All transplanted hearts were rejected within 95 days. In contrast, four animals of the LTX group developed stable tolerance surviving beyond 500 days, and three further animals rejected 119, 239 and 360 days post-transplantation. In both groups, peripheral blood donor leucocyte chimerism peaked 1 h after reperfusion of the allograft. Importantly, the early chimerism level in the LTX group was significantly higher compared to the HTX group and remained detectable throughout the entire observation period. In conclusion, lungs and hearts vary in their potential to induce a state of tolerance after transplantation in a protocol with pre-operative recipient irradiation and donor splenocyte co-transplantation. This could be due to differential early levels of passenger leucocyte chimerism.

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donor cell chimerism, heterotopic heart transplantation, lung transplantation, tolerance induction

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Introduction

Solid organ transplantation is an effective therapy for patients with various end-stage organ diseases. Across the different organs that are nowadays routinely transplanted, long-term outcome is still hampered by the onset of chronic rejection. The incidence of chronic rejection varies among different organs, with lung

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transplantation taking the infamous lead [1]. This profoundly affects long-term survival and indicates the need for a better understanding of acute and especially chronic rejections and mechanisms leading to improved immunological acceptance.

Induction of immunological tolerance has been the goal of transplantation research for many decades now; however, no protocol has yet been published that would generate tolerance across all organs and had the potential for transfer into clinical practice.

Previously, we were able to show that long-term acceptance of lung allografts can be achieved in an allogeneic miniature swine model by a treatment protocol consisting of whole-body irradiation (1.5 Gy), thymus irradiation (7.0 Gy) and administration of donor splenocytes followed by a course of pharmacological immunosuppression for 28 days [2]. In accordance with previously published data, early donor leucocyte chimerism correlated positively with long-term allograft survival [3,4].

Other groups have previously shown that long-term tolerance towards allografts is achievable in large animal models. Madariarga *et al.* described long-term tolerance in miniature swine receiving a single-lung transplantation and a 12-day course of high-dose tacrolimus, but transferring the same protocol to heart allografts failed. Interestingly, long-term tolerance towards heart allografts was induced when a kidney co-transplantation was performed with the heart, with kidneys known to be tolerance-prone organs in those investigators' previous work [5,6].

In the herein described experiments, we wished to transfer our successful lung tolerance induction protocol, consisting of irradiation, donor splenocyte infusion and a 28-day course of pharmacological immunosuppression, into a heart transplantation model.

Materials and methods

Animals

Miniature pigs (aged 10–15 months) were selected from an outbred specific pathogen-free Göttingen Minipig herd, consisting of eight distinct breeding lines (Ellegaard, Dalmose, Denmark). Animals from different breeding lines were tissue-typed prospectively by a lymphocytotoxic assay. Male donors and female recipients were mismatched for the major histocompatibility complex (MHC) class I DC 80 and H04 haplotypes and for reactivity with the SLA I haplotype d-specific Ab 74-11-10 [7]. SLA II mismatch was confirmed by reverse transcription PCR (RT-PCR) and sequencing of SLA-DQB [8].

All animals received humane care in compliance with the German animal protection legislation, approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the Animal Welfare Service of the Lower Saxony State office for Consumer Protection and Food Safety.

Surgical technique

The surgical technique of left-sided single-lung transplantation in pigs has been described elsewhere [9]. Briefly, lungs were recovered from donor animals after Euro-Collins cold flush perfusion. A permanent vascular access double-lumen 3.2 Quinton atrial catheter was inserted into the right jugular vein of recipient animals. After thoracotomy in the fourth intercostal space, the left lung was removed. The allogeneic lung was transplanted using a telescoping bronchial anastomosis technique with running posterior wall and interrupted anterior wall 4-0 polydioxanone sutures. The venous atrial cuff and the pulmonary artery were anastomosed with running polypropylene sutures. After closure of the thorax and extubation, the animals were put in boxes provided with heating lamps, underfloor heating and drinking water.

Heterotopic heart transplantation was performed as described by J.C. Madsen *et al.* [10]. Briefly, donor hearts were flushed with cold Custodiol (HTK) solution. After separation from the lung, donor hearts were prepared backtable by creation of an atrial septal defect, closure of the pulmonary veins and closure of the venae cavae. Recipients also received a permanent venous catheter through the internal jugular vein. Then, a left flank incision was performed and the infrarenal aorta and inferior vena cava were dissected retroperitoneally. Following administration of 200 IE/kg heparin, both vessels were clamped. End-to-side anastomoses of pulmonary trunk to vena cava and ascending aorta to abdominal aorta were performed with continuous 5/0 polypropylene sutures After de-airing of both ventricles and start of reperfusion, the flank incision was closed and the animals were extubated immediately.

Experimental groups

Animals were assigned to the following treatment groups: LTx: animals received a left-sided single-lung transplantation after preconditioning with irradiation (*s. Irradiation and Immunosuppression*). Additionally,

animals received a donor splenocyte infusion and a 28-day course of immunosuppression following lung transplantation ($n = 7$). Some of the LTx animals were part of a previously reported work [2]. HTx: animals underwent heterotopic heart transplantation ($n = 4$). Pre- and postoperative treatment including irradiation, donor splenocyte infusion and immunosuppression were identical to the LTx group. Animals are summarized in Table 1. The schematic experimental setup is shown in Fig. 1a.

Irradiation and immunosuppression

Irradiation (IRR) consisted of adjusted whole-body irradiation (1.5 Gy total dose) and thymic irradiation in a cervico-sternal field of 6 cm width and 12 cm length (7.0 Gy) using a linear accelerator (Mevatron, Siemens, Munich, Germany) within 12 h before lung transplantation.

Intravenous pharmacologic immunosuppression included 1.5 mg/kg/day methylprednisolone and tacrolimus (Astellas, Tokyo, Japan). The initial tacrolimus dose, given at the time of transplant, was 0.25 mg/kg body weight. Beginning on postoperative day (POD) 2, tacrolimus was administered adjusted to blood trough levels of 16–26 ng/ml. Tacrolimus blood trough levels were monitored using tandem mass spectrometry [11]. Empiric intravenous antibiotic therapy consisted of 2 mg/kg/day Ciprofloxacin (Bayer, Leverkusen, Germany). All immunosuppressives were withdrawn on POD 28.

Splenocyte isolation

Splenocytes were prepared as described previously [2]. Briefly, after sterile harvesting, donor spleens were immediately dispersed in 250 ml Ringer's solution (Berlin-Chemie AG, Berlin Germany) per 50 g spleen. In a closed aseptical system, spleens were disrupted and capsulae residues were retained by a mesh screen. Before administration, the suspension was filtered again through a nylon mesh (100 μm) to remove cell aggregates. From each spleen, a sample for cell count and microbiological screening was withheld.

Rejection monitoring

In the LTx group, animals underwent routine chest X-rays on POD 7, 28, 42, 56, 70, 84, 98 and in 4-weekly intervals and upon showing symptoms (e.g. coughing) thereafter. Chest X-rays were graded using a score from 0 (no pathological findings) to 4 (homogenous infiltration of the left lung, normal right lung) by a blinded

Table 1. Animal characteristics.

Animal # (recipient–donor)	POD of death/sacrifice	Histological rejection grading	No. of SLA I haplotype mismatches	SLA-DQB locus base pairs difference	Splenocyte infusion cells/kg (recipient)	Age (month, recipient–donor
LTx group ($n = 7$)						
93529–93555	239	A3	2 or 1	0	1.08×10^7	12–12
97397–97422	360	A2	2 or 1	9	1.77×10^8	11–11
97204–97356	539	A0	2 or 1	11	3.53×10^8	11–11
92567–93668	884	A0	2 or 1	1	1.67×10^7	16–12
73259–93525	884	A0	2 or 1	3	1.78×10^7	12–12
301938–207515	622	A0	2, 1 or 0	1	3.65×10^9	12–10
206074–207082	119	A4	2 or 1	0	2.7×10^9	12–9
HTx group ($n = 4$)						
300771–205913	65	3R	2	2	1.07×10^7	13–9
300546–302012	70	3R	2	3	8.40×10^6	14–unknown
207414–207350	50	3R	2	1	1.73×10^9	9–9
206248–207130	95	3R	2, 1 or 0	5	8.79×10^8	12–9

Summary of animals: Experimental groups are divided according to the organ transplanted. Recipient and donor animal numbers are listed in the first column. Histological rejection scores of heart (0R–3R) and lung (A0–A3) were graded according to the guidelines of the International Society of Heart and Lung Transplantation. Further, number of SLA haplotype mismatches, splenocyte cell count infusion/kg body weight of the recipient and age of recipient and donor animals are recorded.

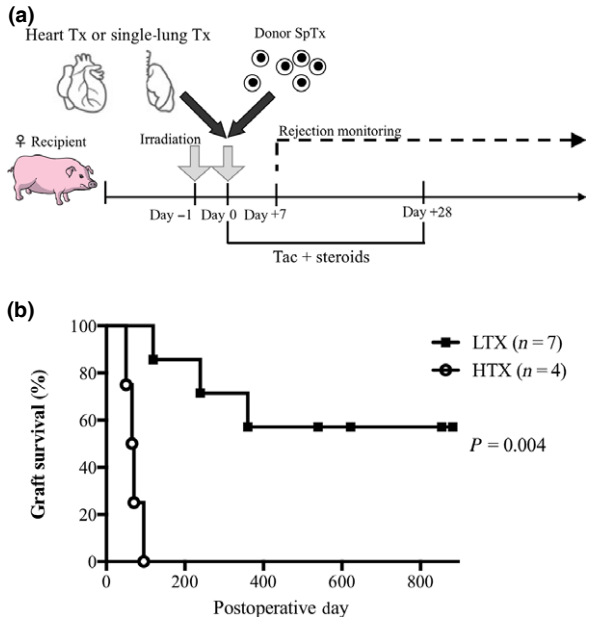

Figure 1 (a) Schematic experimental setup. Miniature swine were gender- and SLA-mismatched. Recipient animals underwent 1.5 Gy whole-body irradiation as well as 7 Gy thymic irradiation on day −1 before transplantation. According to the experimental setup, one group underwent left-sided single-lung transplantation and the other group received a heterotopic heart transplantation. One hour after reperfusion of the allograft, recipient animals received a donor splenocyte infusion from their respective donor animals. Starting on the day of transplantation, recipient animals received tacrolimus and steroids for 28 days. All animals were monitored for rejection with either X-ray and bronchoscopy (LTx) or transabdominal palpation, ultrasound and biopsies (HTx) on a regular schedule; SpTx, Splenocyte infusion; Tac, Tacrolimus. (b) Survival curve. Four animals after heart transplantation rejected their allograft within POD 95. Four animals after lung transplantation accepted their allografts without immunosuppression for >400 days. Three animals rejected their lung allograft on POD 119, 239 and 360, respectively. Survival analysis was performed using log-rank (Mantel cox) test. The survival of both groups is statistically different ($P = 0.004$).

reviewer. The endoscopic appearance of the bronchial mucosa was evaluated, and bronchoalveolar lavages with subsequent differential cell counts were performed. Once the chest X-rays demonstrated a left-sided infiltrate scored 3–4, the animals were sacrificed followed by a full autopsy. Paraffin-embedded lung sections of 5 μm thickness were stained with haematoxylin and eosin and reviewed by a blinded pathologist.

The transplanted hearts were examined daily by transabdominal palpation. Additionally, ultrasound monitoring and needle biopsies (True-Cut-Needle, Magnum®biopsy systems; C.R.Bard GmbH, Karlsruhe, Germany) were performed on POD 7, 14, 28, 42 and 65. Weakening ventricular contraction and thickening of the cardiac septum upon ultrasound examination were indications for allograft rejection, which was confirmed by histopathological examination.

Histological acute or chronic rejection was graded referring to the ISHLT guidelines ranging from A0 to A4 for lungs [12] and 0R–3R for hearts [13].

Isolation of PBMC and flow cytometric analyses

PBMC was isolated from heparinized blood by density gradient centrifugation with Ficoll-PaqueTMPlus (GE Healthcare Biosciences AB, Uppsala, Sweden) and labelled for flow cytometry with the following antibodies: Mouse anti-pig CD25 IgG1 (Cat.No.PGBL25A; VMRD, Pullman, WA, USA), FITC mouse anti-pig CD3 IgG2a BD Pharmingen, Heidelberg, Germany), mouse anti-pig CD21 IgG1 (Clone BB6-11C9.6; Beckman Coulter GmbH, Krefeld, Germany), PE-Cy™5 Streptavidin, PE-conjugated rat anti-mouse IgG1 and FITC-conjugated rat anti-mouse IgG2b (all three BD Pharmingen). Moreover, cells were tagged with mouse anti-pig CD4 74-12-4 IgG2b and mouse anti-pig CD8 76-2-11 IgG2a, which were generated in our own laboratory from hybridoma cell supernatant (ATCC, Manassas, Virginia, USA) as described by Lohse and colleagues before [14]. Cells were analysed on a FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany), and data were interpreted with BD Diva 1.6.3.

Detection of donor leucocyte chimerism

Chimerism in peripheral blood was analysed by quantitative PCR based on the detection of swine male-specific repeat (MSR) DNA present on the Y chromosome [15]. Briefly, DNA was extracted from PBMC using the DNeasy Tissue Kit (Quiagen, Hilden, Germany) and adjusted to a concentration of 100 ng/10 μl water. Genomic DNA was amplified with MSR or control primers listed below. Cycling was performed on an iCycler™ (Bio-Rad, Hercules, CA, USA). SybrGreen® was used for signal induction. Data were analysed using the iCycler™ software. Primers were MSR upper 5′-CCA TCG GCC ATT GTT TTC CTG TTC A-3′, MSR lower 5′-CCT CTG TGC CCA CCT GCT CTC TAC A-3′, S100C upper 5′-ATG CTG GAA GGG ACG GTA ACA ACA-3′ and S100C lower 5′-GCT CAG CTG CTG TCT TTC ACT CGT-3′.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA, USA). All data were reported as means ± standard deviation (SD) or median with interquartile range. For comparisons between groups,

the Mann–Whitney *U*-test or ANOVA for repeated measurements were used, as appropriate. Graft survival was calculated with Kaplan–Meier survival curves and compared using the log-rank test. *P*-values <0.05 were considered statistically significant.

Results

Recipient miniature swine developed long-term immunological tolerance towards lung allografts

Four miniature swine receiving left-sided lung transplantation developed stable immunological tolerance towards their allografts, reflected by graft survival beyond POD 500 (Fig. 1b). Follow-up chest X-rays of these tolerant animals showed well-ventilated lungs throughout the whole postoperative monitoring phase (Fig. 2). After elective sacrifice, histopathological analysis of allograft parenchyma showed no signs of rejection. (Fig. 3a–c) Three of the seven animals (206074, 93529, 97397) were euthanized due to, still rather delayed, rejection on POD 119, 239 and 360, respectively. In addition to clinical symptoms (coughing), chest X-rays revealed complete absence of ventilated parenchyma (Fig. 2). Histopathological analysis confirmed allograft rejection showing severe lymphocyte infiltration and thickening of the interalveolar space. (Fig. 3d–f).

Miniature swine receiving heterotopic heart transplants showed early rejection

Four animals receiving heterotopic heart transplantations rejected their allografts within 95 postoperative days (Fig. 1b). Ultrasound examinations revealed a steady increase of the diameter of the allograft septum. On POD 14, the median diameter of the allograft septum was 8.0 mm (6.35–12.13) increasing to 15.0 mm (15.0–17.23) on POD 65. Interestingly, immediately before rejection, all animals showed a sharp increase of the septal diameter (Fig. 4a). Similar echocardiographic findings were detectable when analysing the thickness of the left ventricular free wall. The diameter of the LV free wall increased over the postoperative monitoring period and showed another distinct increase immediately before final rejection of the allograft (Fig. 4b). Histological analysis showed signs of lymphocytic infiltration, endothelial damage and areas of extensive necrosis in the transplanted hearts (Fig. 5).

Allograft survival was not correlated with tacrolimus blood levels

All animals received tacrolimus for 28 days following transplantation, aiming for blood trough levels of 16–26 ng/ml. Between both experimental groups, no statistical difference in tacrolimus blood trough levels was detectable, although the LTx groups showed a trend towards lower tacrolimus blood levels (Fig. 6a).

Donor chimerism was elevated in lung transplantation recipients

All animals received a donor splenocyte infusion one hour after reperfusion of the lung or heart allografts.

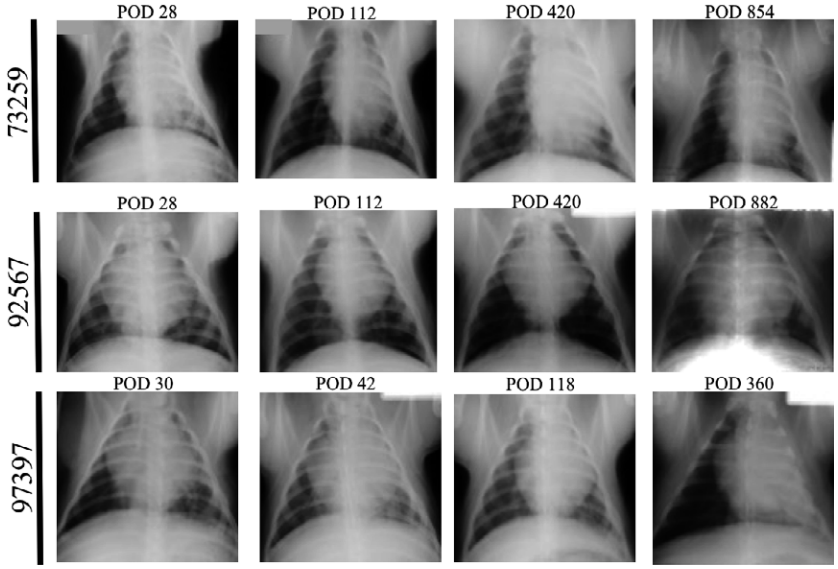

Figure 2 Sequential postoperative chest radiographies of three animals after left-sided lung transplantation. Animal #73259 as well as animal #92567 accepted their lung allograft long-term beyond POD 800. Chest radiographies show well-ventilated bilateral lung parenchyma on POD 28, POD 112, POD 420 and before elective sacrifice on POD 854 and POD 882, respectively. Animal # 97397 showed normal lung parenchyma on POD 30, 42 and 118 but rejected the left lung on POD 360, showing absence of ventilated lung parenchyma on the chest X-Ray on the left side whereas the innate right lung is still normally ventilated.

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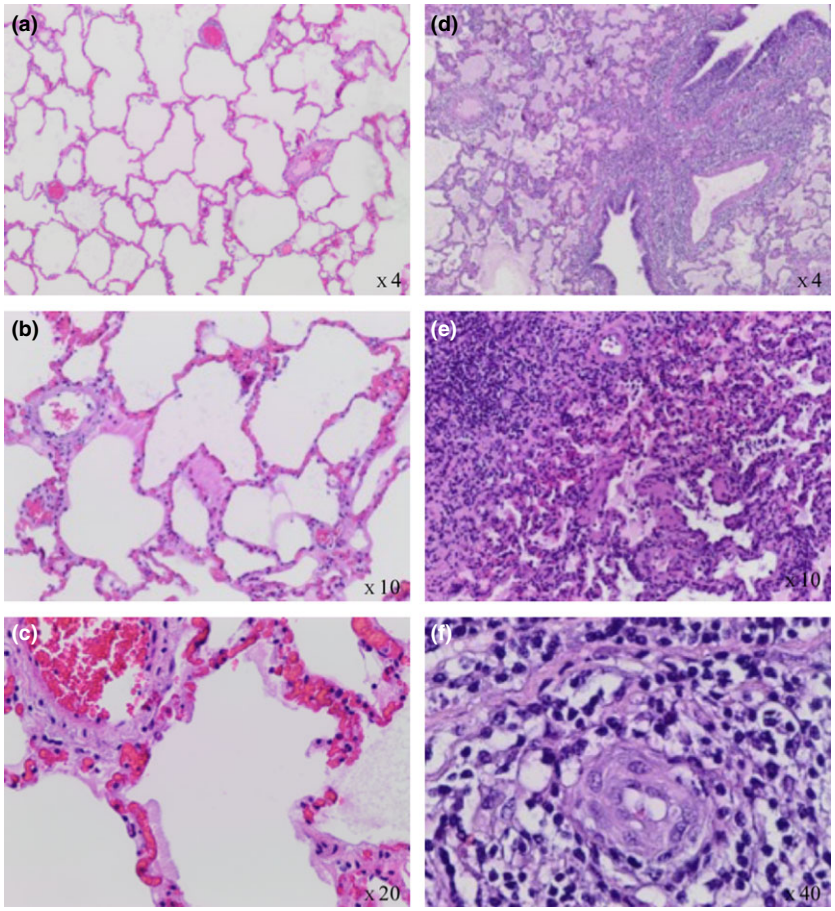

Figure 3 Histology of allograft lungs. All haematoxylin and eosin staining. (a–c) Lung histology of the left allograft of animal #92567 showing normal lung parenchyma on POD 882. No signs of graft rejection are detectable. Alveolar structure as well as small intrapulmonary vessels' histology is preserved. Magnification ×4 (a), ×10 (b) and ×20 (c). (d–f) Lung histology of the left allograft of animal #97397 on POD 360. Lung tissue shows massive lymphocyte infiltration and destruction of alveolar structure, indicating allograft rejection. Magnification ×4 (d), ×10 (e) and ×40 (f).

Cell counts adjusted to the recipient animal body weight varied from 8.79×10^8 to 3.24×10^9 splenocytes per kilogram body weight in the HTx group and 1.08×10^7 to 3.65×10^9 in the LTx group, and the number of cells depended on the cells procurable from the respective donor spleens.

Donor leucocyte chimerism in peripheral blood was significantly higher in the LTx group compared to the HTx group beginning one hour after reperfusion and remained higher until POD 28. Donor chimerism, as measured by the percentage of male DNA detectable in the female recipient animal, converged to nearly zero in the HTx group already on POD 7 (Fig. 6b). Interestingly, the relative increase of male DNA in the LTx group one hour after allograft reperfusion is 2.4 times higher than in the HTx group. This might be of special interest, as blood sampling for this analysis was performed even before donor splenocyte infusion was started. The absolute number of donor splenocytes transferred showed no significant correlation to allograft survival in the individual animals (data not shown).

LTx animals show higher percentages of lymphocytes in peripheral blood

Differential blood cell counts from peripheral blood showed no significant differences in total numbers of leucocytes, red cells and platelets between both groups during the entire follow-up up to 100 days after transplantation. However, animals in the LTX group revealed a significantly higher percentage of lymphocytes among all leucocytes as compared to HTX animals (Fig. 7a). Interestingly, further analysis for $CD8^+$ lymphocytes showed no significant difference between both groups while pharmacological immunosuppression was still administered. However, after cessation of tacrolimus administration, HTx animals expressed significantly higher percentages of cytotoxic $CD8^+$ lymphocytes as compared to LTx animals, suggesting a pro-effector lymphocyte phenotype (Fig. 7b). Similarly, LTx animals show a trend towards a higher percentage of $CD4^+CD25^{high}$ lymphocytes in peripheral blood as compared to HTx animals, suggesting a proregulatory cell milieu after cessation of pharmacological immunosuppression (Fig. 7c).

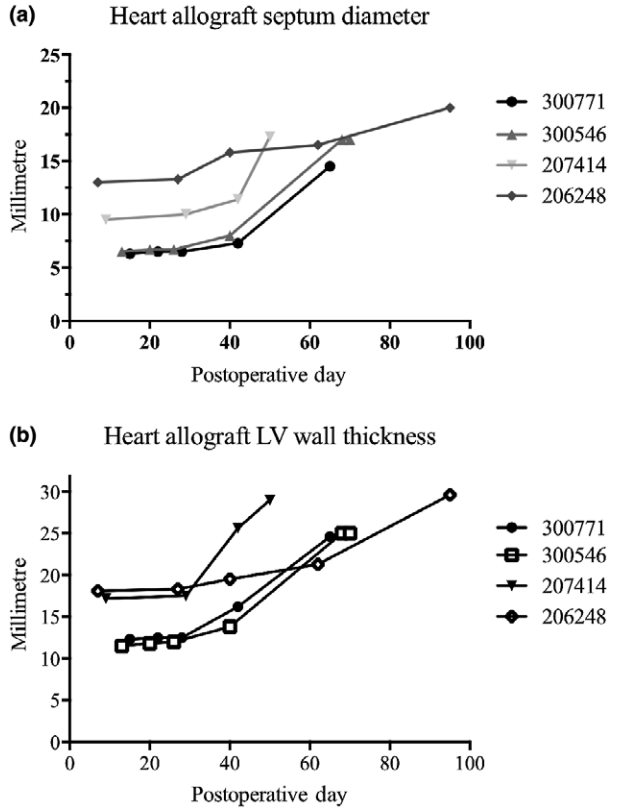

Figure 4 Echocardiography data from animals after heterotopic heart transplantation. (a) Diameter of interventricular heart allograft septa of all animals. Similar to the LV wall is a distinct increase of the diameter detectable over time, especially immediately before allograft rejection. The dynamic of diameter increase is similar in all animals. (b) Left ventricular wall diameter of all animals showing an increase over time, especially immediately before allograft rejection, is a sharp increase detectable.

Tolerant pigs showed hyporeactivity towards donor antigens

Mixed leucocyte reaction assays were performed for serial monitoring of immune responses towards the respective donor. LTx recipients that showed no signs of rejection at any point after transplantation also remained hyporesponsive towards their donors in MLR assays, although still showing increased stimulation indices towards a positive control. In turn, animals showing allograft rejection, either heart or lung, demonstrated elevated donor responsiveness in MLR assays at the time of rejection.

Discussion

Achieving long-term tolerance of recipients towards allografts remains the holy grail of transplantation medicine. Solid organs may become accepted by an allogeneic recipient immune system [16]. However, not all organs are alike and, kidney and especially liver grafts are usually easily tolerated than lung or heart allografts [17]. From experimental models, a multitude of protocols for tolerance induction have been published over the past four decades, with the vast majority being studied in small animal models only, lacking reproducibility in large animals, let alone humans. Also, success rates and survival times differed largely across the various solid organs [18–23].

Kawai showed long-term allograft survival and donor-specific tolerance in a kidney transplantation model in cynomolgus monkeys by applying a preconditioning regime including thymic irradiation, cyclophosphamide and anti-CD2 monoclonal antibody treatment followed by a donor bone marrow transplantation immediately after kidney transplantation, creating a state of mixed hematopoietic chimerism in the recipient. This protocol was successful not only in a large animal model, but also in a small clinical trial [24,25].

We have previously shown that donor-specific long-term tolerance can be induced in miniature swine towards lung allografts applying a protocol of whole-body and thymus irradiation, donor splenocyte infusion and a 28-day course of pharmacological immunosuppression. This protocol induced tolerance for >800 days post-transplant in the majority of animals [2].

To investigate whether this approach would also lead to acceptance of a heart allograft, the same induction protocol was applied to a group of miniature swine receiving heterotopic heart transplantations. These hearts were not accepted by their MHC-mismatched recipients receiving the perioperative treatment that led to long-term tolerance in lung allografts. Therefore, in our model, heart allografts appear comparatively resistant to tolerance induction.

One assumed mechanism leading to long-term tolerance in our previous experimental approaches was the detection of early postoperative peripheral blood donor leucocyte chimerism, caused by passenger leucocytes, that was correlated with later allograft survival [3,4]. In particular, the magnitude of early donor leucocyte chimerism was correlated with later long-term allograft survival [3]. In the herein presented data, lung allografts revealed a 2.4-fold higher percentage of circulating donor mononuclear cells in peripheral blood one hour after reperfusion of the graft as compared to heart recipients, even before additional donor splenocytes were transfused. This early passenger leucocyte chimerism is, presumably, the result of a 'washout' effect from

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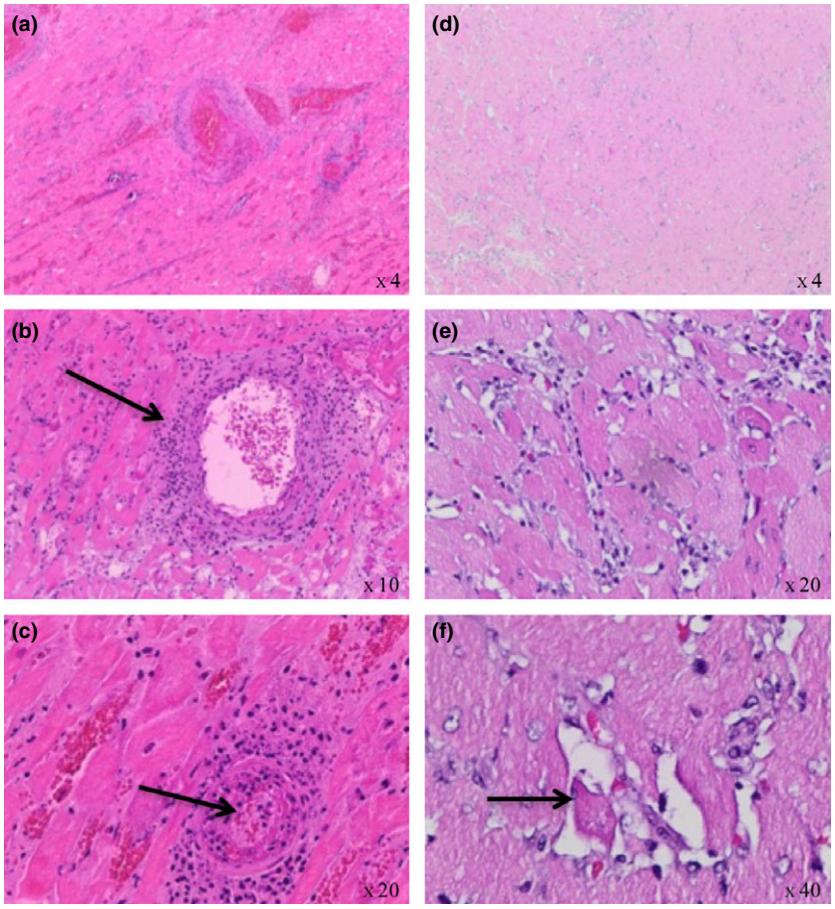

Figure 5 Histology of heart allograft of animal #207414 and #300771. All haematoxylin and eosin staining. Histological analysis reveals massive lymphocyte infiltration of the myocardium, indicating severe allograft rejection (a–f). In detail, larger vessels show massive infiltration of adventitia, intima and media (→b) whereas smaller vessels show occlusion due to cell infiltration (→c). Cardiomyocytes are infiltrated by lymphocytes (e), partially leading to cell necrosis (→f). Magnification ×4 (a, d), ×10 (b), ×20 (c, e) and ×40 (f).

the donor lung. Donor splenocytes augment this effect, leading to an overall higher chimerism in lung recipients as compared to heart recipients. This donor leucocyte chimerism remained elevated as compared to the heart transplant cohort throughout the entire monitoring phase. This indicates that donor leucocyte chimerism may play a mechanistic role in the induction, and potentially, maintenance phase of tolerance in organ transplantation.

Other groups have likewise described the importance of this, often transient, leucocyte chimerism and its role for the onset of operational tolerance towards allografts, in recent years [26–29].

The aforementioned clinical cohort of human kidney transplant recipients maintaining graft tolerance after cessation of pharmacological immunosuppression had previously received a pre-operative donor bone marrow co-transplantation leading to a state of mixed hematopoietic chimerism [24].

In a rodent model, Hayashi *et al.* [30] were able to induce tolerance towards skin grafts by administering donor splenocytes, creating a state of mixed chimerism. This work demonstrated that mixed chimerism is achievable without donor bone marrow transplantation by just transferring isolated spleen cells composed mainly of B cells, T cells and monocytes/macrophages.

In our study, animals that had received a lung transplantation demonstrated higher numbers of circulating donor leucocytes in peripheral blood than those that had received a heart transplantation at all time points postoperatively. Assuming that lung allografts contain higher numbers of leucocytes, partly in bronchus-associated lymphatic tissue, but partly also in the alveolar septal interstitial space, than hearts that are known to bear only small numbers of passenger leucocytes, per se, the higher early postreperfusion leucocyte chimerism in peripheral blood may be simply an effect of numbers.

Tonsho *et al.* were able to identify similar pathways in a non-human primate model of lung transplantation, in which long-term graft acceptance was achieved. Three of four primates became tolerant towards their donor lungs after receiving a delayed tolerance induction protocol. In accordance with results from our experiments, tolerant animals revealed a donor-specific hematopoietic chimerism in some animals >300 days

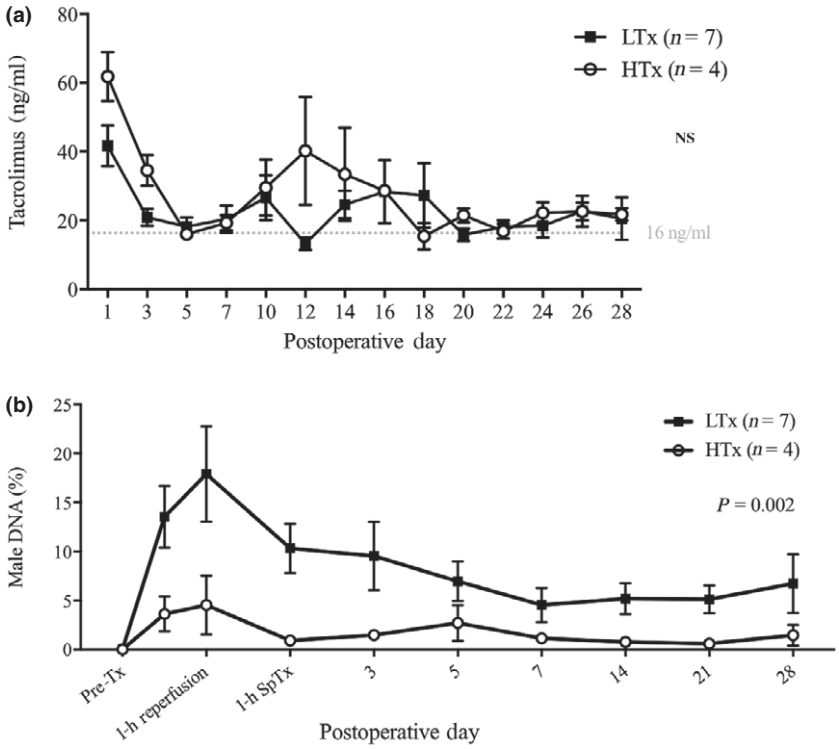

Figure 6 (a) Tacrolimus blood trough levels of all animals after transplantation displayed until POD 28, when pharmacological immunosuppression was ceased. Tacrolimus blood trough level was monitored using tandem mass spectrometry and was adjusted accordingly. All animals show a sufficient immunosuppression aiming for to blood trough levels of 16–26 ng/ml. No statistically significant difference is detectable between Htx and Ltx group (P = n.s., ANOVA for repeated measurements). (b) Donor leucocyte chimerism was analysed in peripheral blood samples by real-time quantitative PCR, detecting swine male-specific repeat DNA present on the Y chromosome. Animals of the LTx group reveal a significant higher percentage of donor chimerism until POD 28 (P = 0.002, ANOVA for repeated measurements). The highest peak of detectable male DNA in both groups is one hour after allograft reperfusion. In animals after heterotopic heart transplantation, male DNA is already close to zero (<1%) on POD 7, still decreasing until POD 28. In the LTx group, male DNA is still detectable (>5%) on POD 28.

after transplantation, thus strengthening the importance of this mechanism for tolerance induction [31].

The second observation in our experiments is differing cell phenotypes in both groups after cessation of pharmacological immunosuppression. While heart transplant recipient animals possess higher numbers of pro-effector lymphocyte phenotypes, lung transplant recipients show a trend towards proregulatory cell types. These findings may further underline the importance of protolerogenic regulatory lymphocytes for the onset of allograft tolerance, which has widely been recognized in rodent, large animal as well as human studies [32–34].

Future work should focus on protocols for the induction of leucocyte chimerism and thus, potentially, tolerance, that are, however, feasible for clinical application. Therefore, the reduction of side effects of conditioning protocols as well as the feasibility for widespread application, that are more recently the real hurdles of clinical application, should become priorities in transplantation tolerance research.

Limitations of the study

Several limitations of this study are evident. First, using outbred minipigs from eight different breeding lines, only approximately 75% of the SLA class I haplotypes could be typed. This could be a limitation; however, nonrejecting animals included into this study were genotyped for the SLA class II DQ alleles. This analysis revealed between one and eleven different base pairs in donor–recipient combinations, rendering it very unlikely to account the findings to accidental SLA matching. Second, irradiation of recipient animals may provoke unspecific immunosuppression causing immunological ignorance, rather than tolerance. Third, the varying number of splenocytes infused per animal may affect the extent and duration of peripheral chimerism, thus influencing the onset of tolerance. At last, the applied tolerance induction protocol remains difficult to apply in humans for concerns regarding long-term side effects of irradiation. Translation into clinical practice could

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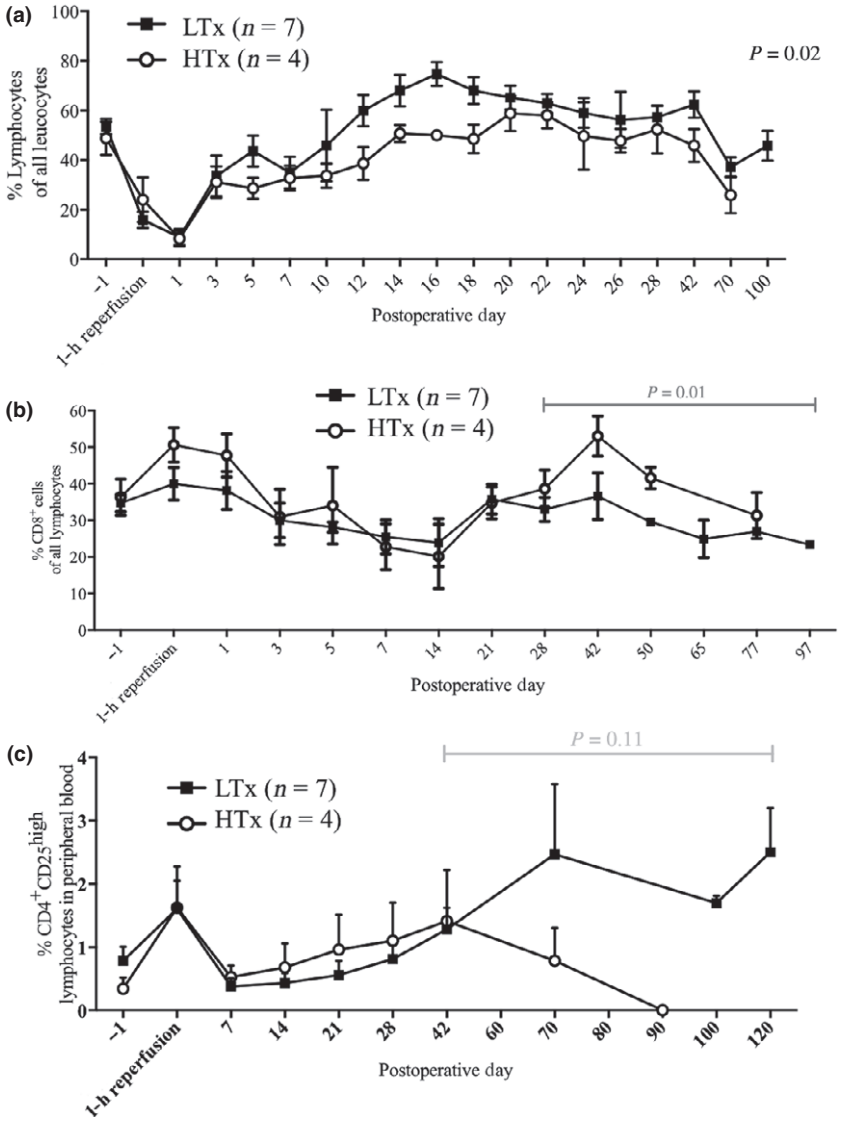

Figure 7 (a) Illustration of the postoperative course of lymphocytes measured as percentage of all leucocytes in peripheral blood in both experimental groups. Animals after lung transplantation remain to have higher percentage of lymphocytes in peripheral blood within the first 100 days after transplantation as compared to animals undergoing heterotopic heart transplantation ($P = 0.02$, ANOVA for repeated measurements). (b) Flow cytometric analysis of $CD8^+$ cytotoxic lymphocytes within the first 100 days after transplantation. In the time interval in which pharmacologic immunosuppression was administered, no significant difference in $CD8^+$ cells is detectable. However, when analysing the immunosuppressive-free time interval, animals following heart transplantation reveal significantly higher percentage of $CD8^+$ lymphocytes as compared to animals after lung transplantation ($P = 0.01$, ANOVA for repeated measurements), suggesting prorejective cell composition. (c) Flow cytometric analysis of $CD4^+CD25^{high}$ lymphocytes in peripheral blood within the first 120 days after transplantation. After cessation of pharmacological immunosuppression, LTx animals show a trend towards a higher percentage of $CD4^+CD25^{high}$ lymphocytes as compared to HTx animals ($P = 0.11$, ANOVA for repeated measurements), suggesting a protolerogenic cell milieu.

instead include pharmaceutical or antibody-mediated lymphocyte depletion prior to donor-specific alloantigen transfusion, necessitating further experimental work.

Conclusion

In conclusion, achieving donor-specific tolerance via a protocol consisting of low-dose whole-body and thymus irradiation as well as donor splenocyte co-transplantation, followed by a 28-day course of pharmacological immunosuppression, is effective for lung but not heart allografts in miniature swine. Passenger leucocytes, increased in lung allograft recipients as compared to heart allograft recipients, in both numbers and longevity, may play an important role in the induction of immunological tolerance.

Authorship

WS: Performed research, collected data, analysed data and wrote the manuscript. GB: Performed research, collected data, analysed data and wrote the manuscript. KJ: Performed research, collected data, analysed data and revised manuscript. MA, AKK, JS, KH, TS, AR: Performed research and revised manuscript. JG, JHK, DJ, AH: Contributed to experiments and revised manuscript. MS, GW: Designed research, performed research and revised manuscript.

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Conflict of interest

The authors have declared no conflicts of interest.

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