Effects of Lung Cotransplantation on Cardiac Allograft Tolerance Across a Full Major Histocompatibility Complex Barrier in Miniature Swine

M. L. L. Madariaga, **P. J. Spencer**, **S. G. Michel**, **G. M. La Muraglia II**, **M. J. O'Neil**, **E. C. Mannon**, **C. Leblang**, **I. A. Rosales**, **R. B. Colvin**, **D. H. Sachs**, **J. S. Allan**[†], and **J. C. Madsen**[†,*]

Center for Transplantation Science, Department of Surgery, Massachusetts General Hospital, Boston, MA

Abstract

A 12-day course of high-dose tacrolimus induces tolerance of major histocompatibility complex–mismatched lung allografts in miniature swine but does not induce tolerance of heart allografts unless a kidney is cotransplanted. To determine whether lungs share with kidneys the ability to induce cardiac allograft tolerance, we investigated heart–lung co-transplantation using the same induction protocol. Hearts (n = 3), heart–kidneys (n=3), lungs (n=6), and hearts–lungs (n=3) were transplanted into fully major histocompatibility complex–mismatched recipients treated with high-dose tacrolimus for 12 days. Serial biopsy samples were used to evaluate rejection, and *in vitro* assays were used to detect donor responsiveness. All heart–kidney recipients and five of six lung recipients demonstrated long-term graft survival for longer than 272 days, while all heart recipients rejected their allografts within 35 days. Tolerant recipients remained free of alloantibody and showed persistent donor-specific unresponsiveness by cell-mediated lympholysis/mixed-lymphocyte reaction. In contrast, heart–lung recipients demonstrated rejection of both allografts (days 47, 55, and 202) and antidonor responsiveness *in vitro*. In contrast to kidneys, lung cotransplantation leads to rejection of both heart and lung allografts, indicating that lungs do not have the same tolerogenic capacity as kidneys. We conclude that cells or cell products present in kidney, but not heart or lung allografts, have a unique capacity to confer unresponsiveness on cotransplanted organs, most likely by amplifying host regulatory mechanisms.

Introduction

Achieving immunologic tolerance has been a long-standing goal in solid organ transplantation. However, different organs demonstrate varying propensities for tolerance, even when transplanted under the same induction regimen. For example, kidneys and livers seem to be more tolerogenic, with cases of spontaneous kidney or liver acceptance occurring

*Corresponding author: Joren C. Madsen, jcmadsen@partners.org.
†Authors contributed equally to this work.

at appreciable rates in murine recipients (1,2) and operational tolerance in human transplant recipients (3,4). In contrast, cardiac allografts rarely exhibit spontaneous tolerance (5).

Using the preclinical, large-animal model of major histocompatibility complex (MHC)-inbred miniature swine (6), we previously showed that isolated, class I–disparate hearts transplanted without immunosuppression all rejected within 8 days (7). The addition of a 12-day course of calcineurin inhibition prolonged class I and full MHC-mismatched heart allograft survival slightly; however, all grafts rejected by postoperative day (POD) 55 (8,9). In contrast, calcineurin inhibition can lead to tolerance of class I and full MHC-mismatched kidney allografts (10,11), as well as full MHC-mismatched lung allografts (12).

Recently, we found that kidney cotransplantation under a 12-day course of tacrolimus is able to induce tolerance of heart allografts across a full MHC mismatch (8,9), reminiscent of the "liver effect" first described in 1969 (13). However, the mechanism underlying kidney-induced cardiac allograft tolerance (KICAT) is unknown. Mechanistic requirements appear to include the thymus (14), a radiosensitive kidney cell population (15), T regulatory cells (16,17), and MHC-matching between heart and kidney parenchyma (18).

To determine whether lung cotransplantation could induce tolerance of fully mismatched heart allografts in an analogous fashion, we conducted a series of heart–lung transplants using the same tolerance induction regimen used in the KICAT experiments.

Materials and Methods

Animals

Transplant donors and recipients were selected from our herd of partially inbred miniature swine (age, 3–6 months; weight, 15–50 kg). The immunogenetic characteristics of this herd have been described previously (6). Donor organs were transplanted into recipients to achieve a two-haplotype full MHC (SLA in swine) class I and class II mismatch [e.g., SLA^{cc} (class I^c/II^c) into SLA^{dd} (class I^d/II^d)]. Group 1 animals underwent heart transplantation, group 2 animals underwent heart–kidney transplantation, group 3 animals underwent lung transplantation, and group 4 animals underwent heart–lung transplantation. All recipients demonstrated significant *in vitro* antidonor activity by cell-mediated lympholysis (CML) and/or mixed-lymphocyte reaction (MLR) before organ transplantation. The study was approved by our Institutional Animal Care and Use Committee. All animal care and procedures were in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council and updated and published regularly by the National Academy Press.

Surgery

The surgical procedures used for heterotopic heart transplantation, combined heart–kidney transplantation, orthotopic lung transplantation, and skin grafting have been described in detail previously (7,19,20). For combined heart–kidney transplantation, the recipients underwent bilateral nephrectomy. The aorta and inferior vena cava were used for end-to-side arterial and venous anastomoses for both the heart and kidney, with the heart placed at least

1 cm caudad to the kidney. The kidney transplantation was completed by performing a vesicoureteral anastomosis. For combined heart–lung transplantation, the recipients first underwent left orthotopic lung transplantation before heterotopic heart transplantation. In brief, after induction of general anesthesia, a left thoracotomy was performed and the recipient hilar structures were isolated. Heparin was administered (200 units/kg) and a pneumonectomy was performed. The donor lung was harvested via median sternotomy. Heparin was administered (400 units/kg). The pulmonary artery was cannulated and flushed *in situ* with 2 L of Perfadex (XVIVO Perfusion AB, Göteborg, Sweden) containing prostaglandin E1 (500 μg/L). The lung was prepared surgically and immediately transplanted. A thoracostomy tube was placed to evacuate the pleural space and was removed on POD 2.

Two indwelling Silastic central venous catheters were placed surgically into the external or internal jugular veins in all recipients. The catheters facilitated tacrolimus administration and frequent blood sampling for *in vitro* assays and for monitoring of renal function and whole blood tacrolimus levels.

Skin grafting was performed by placing split-thickness skin grafts on the dorsum of long-term tolerant recipients. Animals received fresh (self) or frozen (donor, third party) skin grafts that were harvested by using a dermatome. Graft beds were prepared with a single pass of the dermatome. An occlusive compression dressing was applied and removed on POD 4 after skin grafting. Day of rejection was defined as the point at which the skin graft became necrotic, as confirmed by biopsy.

Rejection monitoring

Kidney function was monitored by serial serum creatinine levels. Heart function was monitored by daily palpation and electrocardiogram using the AliveCor Veterinary Heart Monitor (AliveCor, Inc, San Francisco, CA). Lung function was monitored by clinical examination of breath sounds and chest radiographs. Routine biopsies were performed on all transplant recipients via flank incisions (for heart–kidneys) or mini-thoracotomies (for lungs) at predetermined time intervals (PODs 20–30, 50–60, 90–100) or whenever there was clinical suspicion for rejection. Cardiac allograft rejection (heart survival time) was defined by either loss of a ventricular impulse on palpation and/or QRS-wave amplitude of less than 0.3 mV and/or the lack of ventricular contraction on echocardiography (21). Renal allograft rejection was defined as sustained rise in serum creatinine level to greater than 10 mg/dL and/or uremia. Allograft rejection was confirmed histologically in all cases.

Immunosuppression

Tacrolimus (Haorui Pharma-Chem Inc, Irvine, CA) was mixed and administered as an intravenous suspension according to the specifications of the manufacturer. Tacrolimus was given as a continuous infusion at a dose of 0.10 to 0.20 mg/kg (adjusted to maintain a whole blood level of 30 to 50 ng/mL) for 12 consecutive days, starting on the day of transplantation (POD 0).

Histopathological examination

Core needle biopsies were performed on cardiac allografts. Wedge biopsies were performed on lung allografts. Acute rejection was scored according to the guidelines promulgated by the International Society for Heart and Lung Transplantation (ISHLT) (22,23) by a transplant pathologist without knowledge of the functional status of the graft. Wedge biopsies were performed on kidney allografts. Acute rejection in the kidney allograft was scored according to the Banff classification (24) by a transplant pathologist without knowledge of the functional status of the graft.

Preparation of peripheral mononuclear cells

Freshly heparinized whole blood was diluted approximately 1:2 with Hanks' balanced salt solution (HBSS; Gibco BRL, Grand Island, NY), and the mononuclear cells were obtained by means of gradient centrifugation with Histopaque (Sigma, St. Louis, MO). The mononuclear cells were washed once with HBSS, and contaminating red cells were lysed with ammonium chloride potassium lysing buffer (BioWhittaker, Inc, Walkersville, MD). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were kept at 4°C until used in cellular assays.

Cell-mediated lympholysis

Cell-mediated lympholysis (CML) assays with porcine cells have been described previously (25). Briefly, lymphocyte cultures containing 4×10^6/mL responder and 4×10^6/mL stimulator peripheral blood mononuclear cells (PBMCs) (irradiated with 2500 cGy) were incubated for 6 days at 37°C in 5% carbon dioxide and 100% humidity in CML media. Bulk cultures were harvested, and effectors were tested for cytotoxic activity on ^{51}Cr-labeled (Amersham, Arlington Heights, IL) lymphoblast targets generated from phytohemagglutinin (M-form;Life Technologies, Gaithersburg, MD) stimulation. Effector cells were incubated for 5.5 hours with target cells at effector:target ratios of 100:1, 50:1, 25:1, and 12.5:1. Two target cells were tested in each assay: (a) PBMCs matched by MHC to the donor and (b) third-party PBMCs. Supernatants were then harvested using the Skatron collection system (Skatron, Sterling, VA), and ^{51}Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as a percentage of specific lysis of counts per minute (cpm) and calculated as follows:

$$\text{Percentage of specific lysis} = \{(\text{experimental release [cpm]} - \text{spon taneous release [cpm]})/(\text{maximum release [cpm]} - \text{spon taneous release [cpm]})\} \times 100$$

Mixed-lymphocyte reaction

Mixed-lymphocyte reaction (MLR) assays with porcine cells have been described previously (25). Briefly, cultures containing 4×10^6 responder and 4×10^6 irradiated (2500 cGy) stimulator PBMCs were incubated in 200 μL of media in 96-well flat-bottomed plates (Costar Corning, Lowell, MA) for 5 days at 37°C in 5% CO_2 and 100% humidity. After the 5-day incubation, 1 μCi of [^{3}H]thymidine was added to each well, followed by an additional 5-hour incubation under the same conditions. [^{3}H]Thymidine incorporation was determined

in triplicate samples by beta-scintillation counting. Absolute counts were compensated for background and then expressed as stimulation indices (SI), calculated as SI = average cpm for a responder– stimulator pair per cpm of the same responder stimulated by an autologous stimulator.

Assessment of alloantibody

The presence of antidonor immunoglobulin (IgM and IgG) in the serum of experimental swine was examined by indirect flow cytometry using a Becton Dickinson FACScalibur (Sunnyvale, CA) to determine the MHC-binding specificity of the antibody. Fluorescein isothiocyanate (FITC)-labeled goat anti-swine IgM or IgG polyclonal antibodies were used as secondary reagents (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD). For staining, 1×10^6 cells per tube of donor-type PBMCs were resuspended in 100 μL HBSS containing 0.1% bovine serum albumin and 0.05% sodium azide and incubated for 30 minutes at 4°C with 10 μL of undiluted, decomplemented test sera. After two washes, a saturating concentration of FITC-labeled goat anti-swine IgM or IgG was added and incubated for 30 minutes at 4°C. After a final wash, cells were analyzed by means of flow cytometry with propidium iodide gating to exclude dead cells. Both normal pig serum and pretransplant sera from each experimental animal were used as controls for specific binding.

Statistical analysis

Graft survival times were compared using a two-tailed Mann– Whitney U test. Differences in graft survival time were deemed significant when $p < 0.05$.

Results

Lung cotransplantation, in contrast to kidney cotransplantation, was unable to induce long-term acceptance of heart allografts

To determine whether lung cotransplantation imparts the same tolerogenic effect as kidney cotransplantation, three animals underwent combined heart–lung transplantation across a full MHC mismatch (group 4, Table 1). Two animals (nos. 22008 and 22334) lost their heart impulse by POD 55 and showed evidence of severe heart rejection (Figures 1 and 2). One animal (no. 22063) maintained its heart impulse and had only mild to moderate acute cellular rejection on serial biopsy samples (Figures 1 and 2). However, on POD 202, this animal showed evidence of chronic rejection with cardiac allograft vasculopathy and endothelialitis (Figures 1 and 2). In comparison, previously published results of combined heart–kidney transplantation across a full MHC mismatch demonstrated long-term rejection-free survival of heart allografts (group 2, Table 1), while transplantation of isolated hearts across a full MHC mismatch in the same study resulted in heart rejection within 35 days (group 1, Table 1) (9).

Induction of tolerance to lung allografts is disrupted in the setting of combined heart–lung transplantation

We have previously shown (12) that a short course of tacrolimus induced long-term tolerance of lung allografts across a full MHC mismatch in five of six recipients (group 3, Table 1). However, when hearts and lungs were transplanted into the same recipient across a

full MHC mismatch, lung allografts demonstrated severe rejection (group 4, Table 1, Figures 1 and 2). Animal no. 22063 was graded as having mild rejection of the lung on POD 202; however, gross appearance of the lung at this time was notable for friable, nonaerated tissue (Figures 1 and 2). Of note, as previously reported (9), when hearts and kidneys were transplanted into the same recipient across a full MHC mismatch, both hearts and kidneys remained free of rejection (group 2, Table 1).

Heart–lung transplant recipients showed antidonor responsiveness in vitro

To assess the ability of lung cotransplantation to induce donor-specific unresponsiveness, MLR and CML assays were performed before and after heart–lung transplantation. Two of three heart–lung recipients showed persistent antidonor responsiveness by MLR (nos. 22334 and 22063, Figure 3). The remaining heart–lung recipient (no. 22008) showed loss of donor-specific responsiveness by MLR, but antidonor response was regained by day of allograft rejection (Figure 3). Two of three heart–lung recipients showed donor-specific hyporesponsiveness by CML at the time of graft rejection (nos. 22008 and 22334); the remaining heart–lung recipient (no. 22063) showed donor-specific responsiveness by CML postoperatively (Figure 4). In contrast, previous studies showed persistent loss of donor-specific responsiveness in MLR and CML assays in all heart–kidney recipients (group 2, Table 1) (9). In another earlier study, five of six lung transplant recipients demonstrated donor-specific hyporesponsiveness postoperatively; animal no. 15616 demonstrated antidonor responsiveness on day of lung rejection (POD 103, group 3, Table 1) (12). Of note, one heart recipient (no. 21109) maintained donor responsiveness by MLR and CML assays, while the other two heart recipients developed donor-specific hyporesponsiveness (group 1, Table 1) (9).

Circulating alloantibody is detectable in select heart and heart–lung recipients after heart rejection

To determine whether rejection of heart–lung allografts led to alloantibody formation, flow cytometry analysis of antidonor antibodies was performed. IgG alloantibody was elevated in one of three heart–lung recipients and one of three heart recipients (nos. 22008 and 21109, Figure 5, Table 1) (9). In contrast, in previous studies, heart– kidney recipients never showed detectable levels of circulating IgM and IgG alloantibody (9). Alloantibody data for lung recipients are not available (12).

Donor skin graft survival is not prolonged in a heart–lung recipient

To test the immune competence of heart–lung recipients, animal no. 22063 underwent skin grafting on POD 140. The donor skin graft (class I[d], class II[c]) rejected in 20 days and the challenge skin graft (class I[d], class II[a]) rejected in 10 days (data not shown). In contrast, a donor skin graft remained intact for 95 days on long-term tolerant heart– kidney recipient no. 21019 (9). Animal no. 22063 demonstrated rejection of heart and lung allografts 42 days after donor skin graft rejection (Figure 1). In contrast, heart and kidney allografts from animal no. 21019 remained free of rejection despite donor skin rejection (9).

Discussion

We have previously shown that tolerance of both kidney (10,11) and lung (12,26) allografts can be induced in MHC-inbred miniature swine using a short course of high-dose calcineurin inhibition. However, we have been unable to induce tolerance of heart transplants despite using similar tolerance induction strategies (7,8). Taking advantage of the tolerogenic properties of kidney allografts, we previously demonstrated that kidney cotransplantation could induce tolerance of heart allografts across a full MHC mismatch (9). To date, the mechanism underlying this "kidney effect" is unknown.

To investigate whether the mechanism underlying KICAT is unique to the kidney or is also a property of the lung, we evaluated whether lung cotransplantation could provide a similar tolerogenic influence on the heart. The data presented here show that, in contrast to kidney cotransplantation, which results in indefinite heart allograft survival, lung cotransplantation failed to protect cotransplanted heart allografts. Although the sample size is too small to determine whether the difference in graft survival of hearts in heart recipients versus heart–lung recipients is statistically significant, it is clear that the lung cannot provide the same tolerogenic effect as the kidney. Indeed, both heart and lung allografts in heart–lung recipients demonstrate decreased survival of both grafts compared with heart–kidney recipients or lung recipients.

It is intriguing to consider what unique cell or cell product present in the kidney but not (or to a lesser extent) heart or lungs allografts is able to confer tolerance on heart allografts. The tolerogenic capacity of the kidney has been attributed to its ability to induce immune regulation (27) [reviewed in (37)]. It is hypothesized that regulatory T cells generated by the kidney migrate to the thymus and/or to the heart allograft, providing immune regulation. Indeed, renal tubular epithelial cells are known to induce FOXP3^{+} regulatory T cells via transforming growth factor-β signaling (28), and renal endothelial cells express indoleamine 2,3-dioxygenase, an enzyme that leads to T-cell suppression, via interferon-γ signaling (29). Histological analysis of tolerant kidney grafts also demonstrate the presence of "T reg-rich organized lymphoid structures," defined as nodular infiltrates rich in FOXP3^{+} -expressing cells and plasmacytoid dendritic cells associated with the vasculature of the kidney (17). Similar regulatory T cell–rich organized lymphoid structures have also been identified in tolerant mouse lung allografts (30). However, in contrast to the T-cell "acceptance reaction" that occurs in kidneys (31), the immune network of the lung, being a barrier organ, may favor alloreactivity. After lung transplantation, alveolar macrophages upregulate signaling molecules such as CD40 and CD80 to enable more-efficient antigen presentation and activate T cells (32). Nonhematopoietic cells in the lung such as vascular endothelium and airway epithelium express MHC class II constitutively (33) and can also serve as potent antigen presenting cells (34,35).

Interestingly, tolerance of lungs was achieved in the setting of lung transplantation (12) but not in the setting of combined heart–lung transplantation. This suggests that the proinflammatory milieu of thoracoabdominal surgery (which involves a longer operation in two body cavities) and the presence of a rejecting heart graft overcame the tolerogenic potential of lung allografts. The ability of one rejecting organ to promote rejection of

another was previously demonstrated when heart rejection abrogated tolerance of kidneys in recipients of full MHC-mismatched heart and kidney allografts that were also fully MHC mismatched to each other (18). The lung may be more susceptible than the kidney to proinflammatory changes accompanying heart transplantation, as rejection of both heart and lungs occurred when both donor organs were MHC matched. Alternatively, the cardiac allografts may establish an immunologic milieu particularly conducive for rejection, and the lung allografts may be more vulnerable to this than the kidney allografts. Importantly, the mechanism of KICAT cannot be solely dependent on increased antigen load, as neither double heart transplantation (36) nor combined heart–lung transplantation leads to heart tolerance.

The results of our CML, MLR, and alloantibody assays demonstrated that the relationship between in vitro antidonor responsiveness and *in vivo* graft outcome was imperfect. For instance, despite *in vitro* evidence of preserved antidonor responsiveness (by both CML and MLR), one of the lung–heart recipients (no. 22063) failed to develop severe acute cellular rejection. While this recipient ultimately developed cardiac allograft vasculopathy, it did not develop more severe acute cellular rejection, which is interesting because it was the only recipient in this group to maintain antidonor responsiveness by CML assay throughout the entire experiment. This disparity could be explained by the contributions of regulatory mechanisms *in vivo* that are not recapitulated *in vitro*. Of note, however, all tolerant heart–kidney recipients and lung recipients consistently show loss of donor-specific responsiveness *in vitro*.

We recognize that the use of historical data could affect our results because of the potential for subtle changes in the operator, the procedure, or the genetics of the herd over time. However, the same surgeons were involved in all the transplants using the same procedures, and the breeding of our swine is carefully controlled to mitigate genetic drift. Finally, the same swine strains (SLA^{cc} and SLA^{dd}) were used as donor and recipients in most experiments. We have not found that the direction of the transplant, SLA^{cc} into SLA^{dd} or the reverse, affects the outcome of heart, lung, or kidney transplantation.

In conclusion, we find that lung cotransplantation, in contrast to kidney cotransplantation, is unable to induce tolerance of heart allografts, suggesting that kidneys possess a unique and organ-specific ability to confer tolerance. Further work will address how kidney allografts are able to foster an immunological environment that allows tolerance induction of resistant organs such as the heart.

Acknowledgments

Abbreviations

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CML	cell-mediated lympholysis
ISHLT	International Society for Heart and Lung Transplantation
KICAT	kidney-induced cardiac allograft tolerance
MHC	major histocompatibility complex
MLR	mixed-lymphocyte reaction
PBMC	peripheral blood mono-nuclear cell
POD	postoperative day

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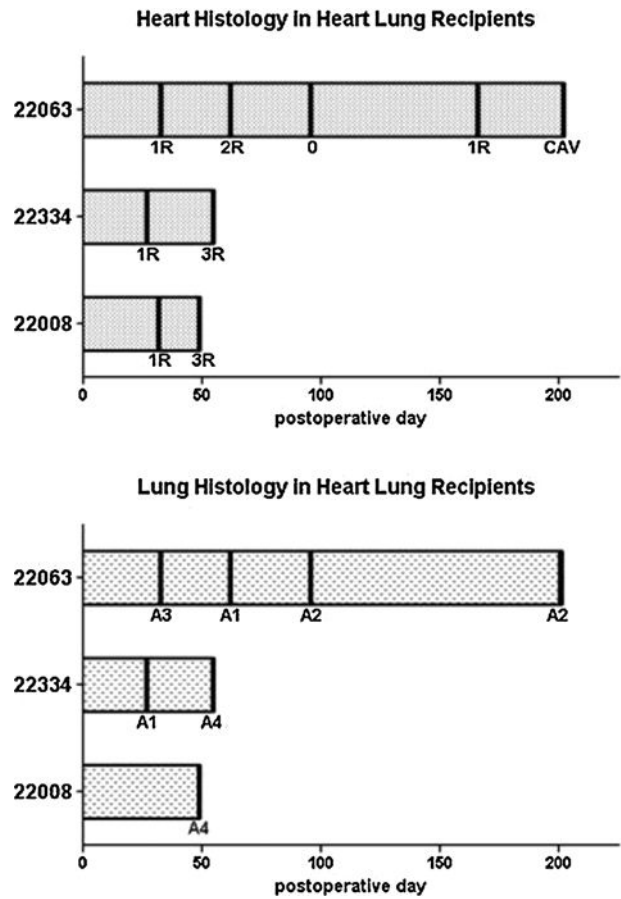
Figure 1. Pathological grading of heart and lung biopsies

Serial biopsies of heart (top) and lung (bottom) allografts were graded according to the ISHLT grading system.
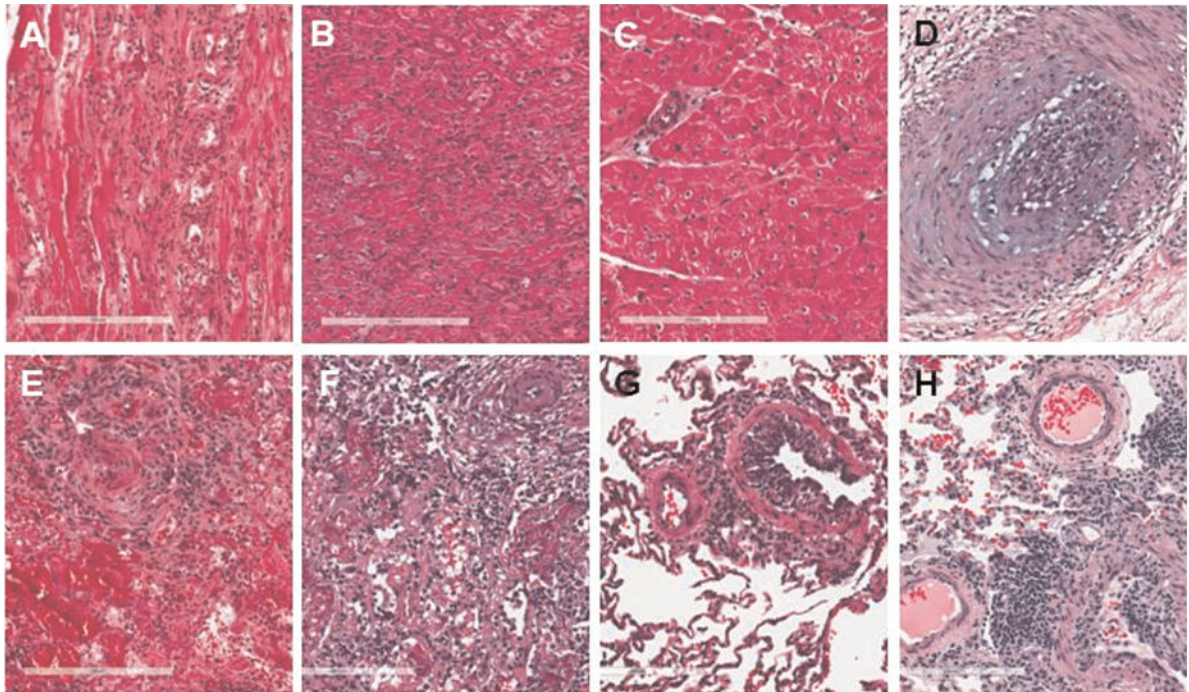
Figure 2. Histology of representative heart and lung biopsies

(A and E) On POD 49, animal no. 22008 showed severe rejection of the heart (3R) and severe rejection of the lung (A4B2C×D1). (B and F) On POD 55, animal no. 22334 showed severe rejection of the heart (3R) with diffuse myocyte necrosis and severe rejection of the lung (A4B×C×D×) with superimposed pneumonia. (C and G) On POD 97, animal no. 22063 showed no rejection of the heart (0) and mild rejection of the lung (A2B1C0D0). (D and H) On POD 202, animal no. 22063 showed cardiac allograft vasculopathy with endothelialitis of the heart and mild rejection of the lung (A2B1C×D×). POD, postoperative day.
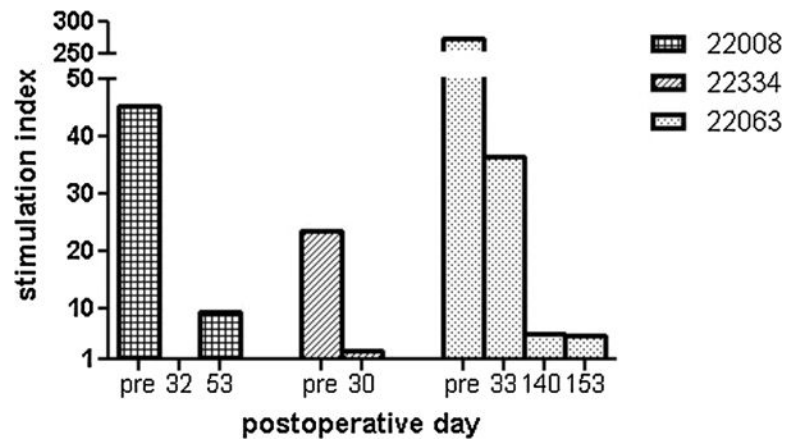
Figure 3. MLR assays from heart–lung recipients

SIs to donor-type PBMCs are plotted as a function of postoperative day for each animal.

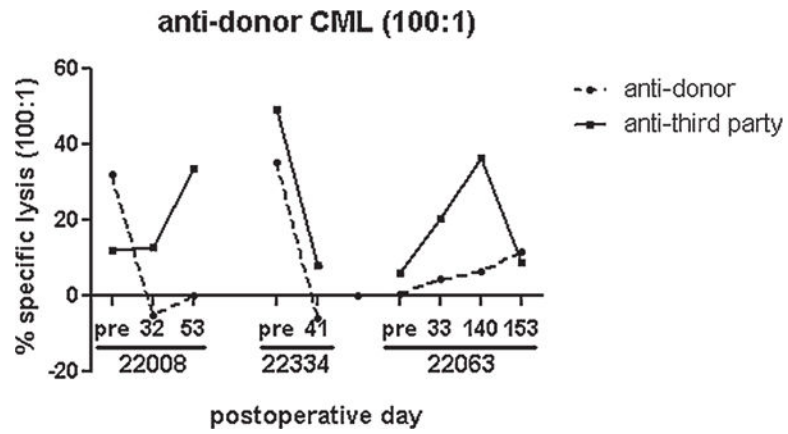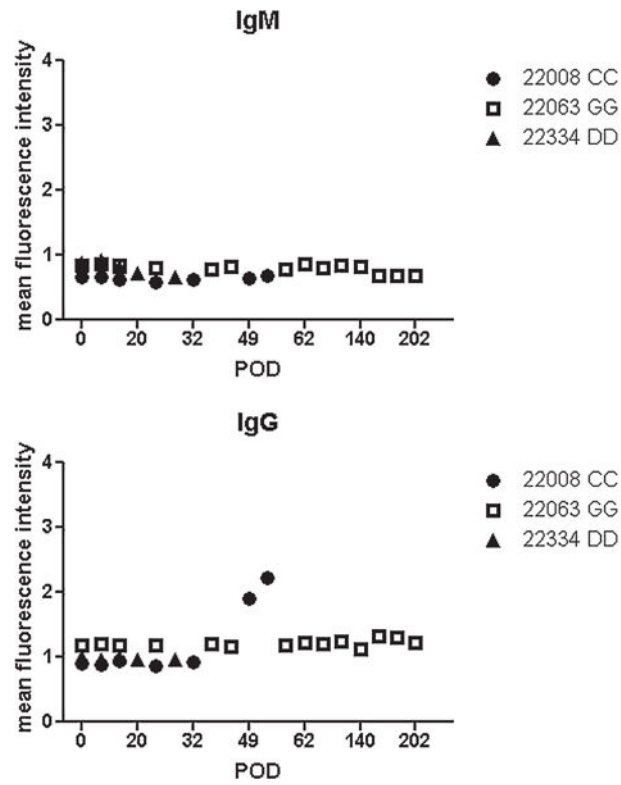MLR, mixed-lymphocyte reaction; PBMCs, peripheral blood mononuclear cells.

Figure 4. CML assays from heart–lung recipients

Percentage specific lysis at 100:1 effector: target ratio is plotted as a function of POD.

Responses against donor-type targets (dotted line) and third-party Yorkshire targets (solid line) are shown for each animal. POD, postoperative day.

Figure 5. Alloantibody response

Levels of circulating antidonor IgM (left) and IgG (right) alloantibody were measured by flow cytometry in heart–lung recipients. Data were normalized to the mean fluorescence intensity of negative control values to plot normalized mean fluorescence intensity as a function of POD. POD, postoperative day.

Table 1

Histology and survival of allografts in recipients of MHC-mismatched transplants treated with 12 days of tacrolimus

Group	Organ(s) transplanted	Animal no.	Full MHC mismatches (class I/class II)		Heart graft survival	Final heart pathology[*]	Final kidney pathology[†]	Lung graft survival	Final lung pathology[‡]
			Recipient	Donor					
1[§]	Heart	21109	CC	DD	35	3R	—	—	—
		21189	CC	DD	32	3R	—	—	—
		21257	CC	DD	18	3R	—	—	—
2[§]	Heart–kidney	21019	CC	DD	>295	0	0	—	—
		21018	CC	DD	>284	0	0	—	—
		20977	CC	DD	>272	0	0	—	—
3[‖]	Lung	15515	DD	AA	—	—	—	>515	A2
		15616	DD	CC	—	—	—	103	A4
		15841	DD	AA	—	—	—	>429	A2
		15843	DD	CC	—	—	—	>481	0
		15895	DD	AA	—	—	—	>389	0
		16052	DD	CC	—	—	—	>438	0
4	Heart–lung	22008	CC	DD	47	3R	—	47	A4
		22334	DD	CC	55	3R	—	55	A4
		22063	CD	DC	>202	CAV	—	202	A2

CAV, cardiac allograft vasculopathy; ACR, acute cellular rejection.

[*] Grading of rejection from 0 (no rejection) to 3R (severe rejection) based on ISHLT scoring system (22).

[†] Grading of rejection from 0 (no rejection) to ACR3 (severe rejection) based on Banff scoring system (24).

[‡] Grading of rejection from 0 (no rejection) to A4 (severe rejection) based on ISHLT scoring system (23).

[§] Group 1 and 2 animals previously published (9).

[‖] Group 3 animals previously published (12).