Transplant Immunology 65 (2021) 101350

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Research Article

Delayed non-myeloablative irradiation to induce long-term allograft acceptance in a large animal lung transplantation model☆

Karolin S. Hacker[a,b,1], Katharina Jansson[a,b,1], Jeanette Pichler[a,b], Jawad Salman[a], Murat Avsar[a], Thierry Siemeni[a], Ann-Kathrin Knöfel[a,b], Klaus Höffler[a], Jens Gottlieb[b,c], Jörg Frühauf[d], Martin Werner[d], Reza Poyanmehr[a], Danny Jonigk[e], Michael S. Balzer[f], Marion Hewicker-Trautwein[g], Axel Haverich[a,b], Wiebke Sommer[a,b,2], Gregor Warnecke[a,b,*,2]

[a] Division of Cardiac, Thoracic, Transplantation and Vascular Surgery, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[b] German Centre for Lung Research, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[c] Department of Respiratory Medicine, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[d] Department of Radiation Therapy and Oncology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[e] Institute for Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[f] Department of Nephrology and Hypertension, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[g] Department of Pathology, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany

ARTICLE INFO

Keywords:
Preconditioning regime
Irradiation
Tolerance
Lung transplantation
Transplantation tolerance
Porcine

ABSTRACT

We previously induced long-term allograft acceptance in an allogeneic lung transplantation (LTx) model in miniature swine using perioperative non-myeloablative irradiation (IRR) combined with infusion of donor specific alloantigen. In order to improve clinical applicability, we delayed induction with irradiation in this study. Left sided single LTx was performed in minipigs. Group 1 received non-myeloablative irradiation (7Gy thymus and 1.5Gy whole body IRR) before LTx and a perioperative donor specific splenocyte infusion (SpTx). Group 2 received perioperative SpTx but delayed IRR three days after LTx. Group 3 was exposed to delayed IRR without SpTx. Whereas 4 out of 7 animals from the non-delayed group never rejected their grafts and were electively sacrificed on postoperative day (POD) +500, all animals from group 2 rejected their grafts before POD 108. In group 3, 3 out of 8 animals developed long-term allograft acceptance. In all groups, donor leukocyte chimerism peaked up to 20% in peripheral blood one hour after reperfusion of the lung. Group 1 maintained prolonged chimerism beyond POD 7, whereas chimerism levels in groups 2 and 3 decreased continuously thereafter. Delayed irradiation has the potential to improve long-term graft survival, yet not as efficient as a perioperative conditioning protocol.

1. Introduction

Lung transplantation is an established treatment option for patients with end stage lung failure [1]. However, mandatory lifelong immunosuppressive therapy causes severe side effects including infectious complications, malignancies and renal failure [2,3]. Chronic allograft rejection manifests as chronic lung allograft dysfunction (CLAD) in lung transplantation and continues to limit outcomes beyond the first year after transplantation [4]. Treatment protocols creating immunological tolerance would therefore make permanent immunosuppression dispensable, making this approach attractive for the field of lung transplantation [5]. To induce a persistent state of tolerance, intricate preconditioning regimes of the respective organ recipient appears inevitable. Avsar et al. have shown that irradiation alone and the

☆ This study was supported by grants from the Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research (DZL), Hannover, Germany.Astellas, Osaka, Japan, kindly donated Tacrolimus.

* Corresponding author at: Division of Cardiac, Thoracic, Transplantation and Vascular Surgery, Hannover Medical School, Carl-Neuberg-Str.1, 30623 Hannover, Germany.
E-mail address: warnecke.gregor@mh-hannover.de (G. Warnecke).

[1] Karolin S. Hacker and Katharina Jansson are co-authors.
[2] Wiebke Sommer and Gregor Warnecke are shared last authors.

https://doi.org/10.1016/j.trim.2020.101350
Received 17 April 2020; Received in revised form 26 October 2020; Accepted 26 October 2020
Available online 27 October 2020

administration of a splenocyte infusion did not lead to acceptable results [6]. In our porcine allogeneic lung transplantation model, preoperative low dose total body irradiation combined with a 28-day immunosuppression led to stable allograft acceptance in 3/5 of the animals [7]. Combining irradiation with a perioperative splenocyte infusion (SpTx) obtained either from the respective donor or even from a third party animal further improved the incidence of long-term allograft acceptance, whereas SpTx alone failed to prolong allograft survival [6]. Moreover, we found that a certain threshold dose of irradiation had to be exceeded to reach sufficient lymphocyte depletion, which is mandatory to allow donor cells to coexist in the recipient [8]. The required irradiation dose of 1,5 Gy whole body irradiation (WBI) in combination with 7 Gy thymic irradiation (TI) was tolerated reasonably well by the animals. However, the physical condition of patients awaiting a lung transplantation might be more critical as compared to healthy animals.

2. Hypothesis

We hypothesized, that delayed irradiation would lead to prolonged allograft survival in our large animal lung transplantation model. Postponing induction would offer the opportunity, to decide by the individual patients postoperative medical condition whether irradiation is indicated or not and could translate into improved clinical applicability of our protocol.

3. Materials and methods

3.1. Animals

22 Göttingen minipigs (Table 1), originating from an outbred, specific-pathogen-free (SPF) herd consisting of 9 different breeding lines, were obtained from Ellegaard (Dalmose, Denmark). Animals were prospectively tissue-typed by a lymphocytotoxic assay. MHC I mismatch was accomplished for haplotypes DC45 and W12 as well as haplotype d-specific mAb 74–11-10. In long-term surviving animals, MHC II incompatibility was confirmed via reverse transcriptase (RT) PCR and subsequent sequencing of the swine leukocyte antigen (SLA) DQB gene (data not shown). 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.

3.2. Surgical technique

The surgical technique of left-sided single lung transplantation has been described in detail before [9]. After thoracotomy in the fourth intercostal space, left lungs were procured from male, porcine donors after Celsior cold flush perfusion (Celsior, Genzyme/Sanofi, Cambridge, UK). Allogeneic lung transplantation was performed using an end-to end 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 weaning off mechanical ventilation, the animals were put in boxes provided with heating lamps, underfloor heating and drinking water.

3.3. Experimental groups

According to the treatment protocol, animals were assigned to three different groups. The schematic experimental setup is shown in Fig. 1. An overview of the animals and the respective protocol is listed in Table 1.

1) IRR preOP, SpTx POD 0 group ($n = 7$): Animals were exposed to irradiation (IRR, 7 Gy thymus and 1.5 Gy whole body IRR) within 12

Table 1
Summary of animals and protocols.

Animal no. (recipient-donor)	POD of death / sacrifice	Protocol	Histologic rejection grading	Chest x-ray score	No. of SLA-I haplotype mismatches	Splenocyte infusion cells/kg (recipient)	Weight (kg, recipient-donor)	Age (month, recipient-donor)	Allokidney histology
		IRR preOP, SpTx POD 0 (n = 7)							
97204–97356[a]	539		A0	L1	2 or 1	3.53×10^8	19.0–24.0	11–11	Necrosis[b]
97397–97422[a]	360		A2	L4	2 or 1	1.77×10^8	15.2–21.4	11–11	
93529–93555[a]	239		A3	L4	2 or 1	1.08×10^7	18.0–19.2	12–12	
92567–93668[a]	884		A0	L0	2 or 1	1.67×10^7	23.0–19.6	16–12	Necrosis[b]
73259–93525[a]	884		A1	L0	2 or 1	1.78×10^7	16.9–18.8	12–12	i0, t2, v0[b]
301983–207515[a]	622		A0	L1	2 or 1	3.65×10^9	19.2–22.7	12–10	i3, t3, v0-1[b]
206074–207082[a]	119		A4	L4	2 or 1	2.7×10^9	22.3–25.5	12–9	
		SpTx POD 0, IRR POD 3 (n = 3)							
219264–316269	65		A3	L3	2 or 1	4.35×10^7	20.0–21.4	11–11	
219773–219713	108		A3	L3	2, 1 or 0	6.58×10^6	22.8–22.0	12–12	
219789–220177	105		A3	L3	2, 1 or 0	3.23×10^5	23.2–18.4	11–11	
		IRR POD 3 (n = 8)							
219222–291309	226		A3	L3	2 or 1		22.0–25.6	11–11	
317615–317027	896		A0	L0	2, 1 or 0		23.0–22.5	11–12	i3,ti3,v3, PTC3[b]
220975–318514	715		A0	L0	2 or 1		29.0–27.0	13–13	i3, t3, ti3, v3[b]
224158–323353	73		d.h.i.	L3	2, 1 or 0		31.0–30.1	13–14	
224087–323977	71		A2	L3	2 or 1		28.6–26.0	14–12	
323501–223936	134		A3	L2	2 or 1		27.4–24.5	14–14	
226422–226750	157		A4	L3	2 or 1		26.6–26.8	13–12	
226654–226725	617		A0	L0	2 or 1		27.6–24.2	13–12	Banff Kat. 2, Typ III[b]

[a] Animals were previously published [7,8,14], d.h.i. =diffuse hemorrhagic infarction.
[b] Kidney histology according to BANFF 07 classification [6,10–12].

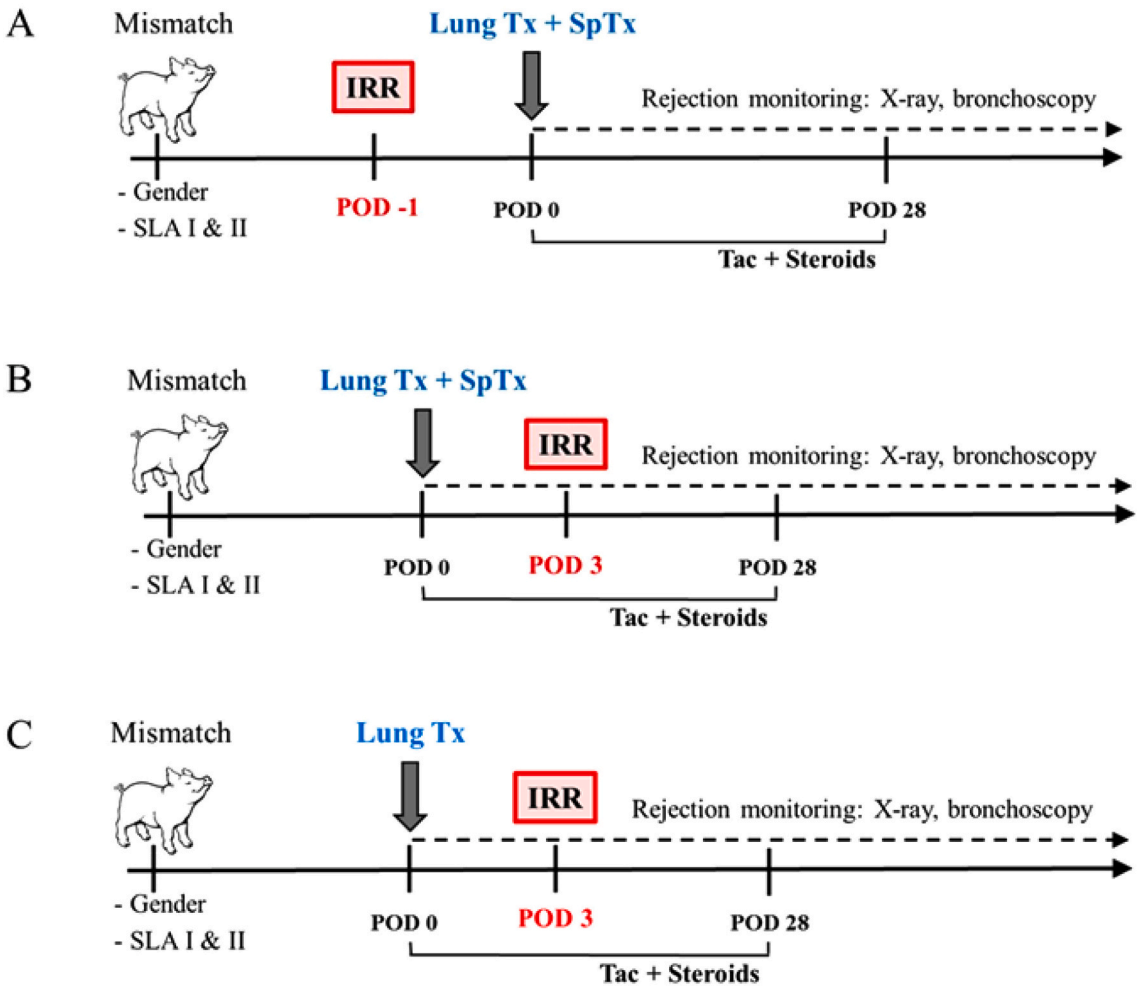

Fig. 1. Experimental setup. Left sided single LTx (Lungtransplantation) from MHC mismatched male donors was performed in outbred female minipigs. All groups were maintained for 28 days with tacrolimus (Tac) and methylprednisolone (Steroids). Thereafter, allograft survival was monitored by bronchoscopy and sequential chest x-rays. (A) Group 1 ($n = 7$) received non-myeloablative irradiation (7 Gy thymus and 1.5 Gy whole body IRR) 12 h before LTx and a perioperative donor specific splenocyte infusion (SpTx). (B) Group 2 ($n = 3$) received perioperative SpTx and delayed IRR three days after LTx. (C) Group 3 ($n = 8$) was exposed to delayed IRR without SpTx.

h before transplantation. They additionally received donor specific SpTx on the day of transplantation (Fig. 1A) This group has previously been published [6,8,10].

2) SpTx POD 0, IRR POD 3 group ($n = 3$): Animals received donor specific SpTx on the day of transplantation and underwent IRR (7 Gy thymus and 1.5 Gy whole body IRR) three days after transplantation (Fig. 1B).
3) IRR POD 3 group ($n = 8$): Animals were exposed to IRR (7 Gy thymus and 1.5 Gy whole body IRR) three days after transplantation without SpTx (Fig. 1C).

In all groups, IRR comprised whole body irradiation with 1.5 Gy and thymic irradiation with 7.0 Gy in a cervico-sternal field of 6 cm width x 12 cm length, applied with a linear accelerator (Mevatron, Siemens, Munich, Germany). Immunosuppressive therapy consisted of Tacrolimus (Fujisawa Pharmaceutics, Japan) adjusted to 16–26 ng/ml blood trough levels and 1.5 mg/kg/d methylprednisolone. Ciprofloxacin (Bayer, Germany) was administered at a dose of 2 mg/kg/d as intravenous antibiotic prophylaxis. All immunosuppressive drugs were withdrawn on postoperative day (POD) 28.

3.4. Preparation of SpTx

Donor spleens were mechanically dissected, placed in PBS and incubated with DNAse and collagenase (both Sigma, St. Louis, MO, USA). The mixture was filtered using a mesh to retain residual splenic capsule and connective tissue. Splenocytes were then isolated by density gradient centrifugation and filtered through a nylon mesh (100 μm) to remove cell aggregates. After washing, the suspension was administered intravenously immediately after reperfusion of the transplanted lung. Characterization and composition of the SpTx has been described before [6]. Cell numbers ranged between 3,23 × 105 and 3,65 × 10^9 splenocytes per infusion (Table 1).

3.5. Rejection monitoring

Chest radiographies and bronchoscopies were performed on POD 7, 21, 28, 42, 70,120 and as clinically necessary. For the chest radiographies, a score from L0 (no pathological findings) to L4 (homogenous infiltration of the left lung and normal right lung) was applied by a blinded reviewer.

In case of clinical deterioration indicating graft rejection, animals were sacrificed and a full necropsy was performed including histological sampling of the allograft. Histological signs of rejection were classified following the International Society for Heart and Lung Transplantation guidelines ranging from A0 to A4 [13].

Rejection was defined as a strictly left-sided infiltrate of the chest radiograph scored 2 or higher in combination with a grade A2-A4

histologic rejection in the absence of infection.

3.6. Donor leukocyte chimerism

Chimerism in peripheral blood was analysed by quantitative PCR of the swine male specific repeat (MSR) DNA present on the Y-chromosome [14]. Briefly, blood DNA was extracted from isolated PBMC using the DNeasy Tissue Kit (Qiagen, Venlo, NL). Genomic DNA was amplified with MSR or control primers listed below. Cycling was performed on an iCycler™ (BioRad, Hercules, CA, USA). SybrGreen® (also BioRad) was used for signal induction. Fluorescence was measured every two cycles, and DNA was relatively quantified. Data was 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′.

3.7. Secondary kidney transplantation from third-party donors

Animals showing long-term allograft acceptance beyond POD +500 after lung transplantation underwent secondary orthotopic kidney transplantation from SLA mismatched third-party donors to confirm alloantigen specificity of the induced long-term allograft acceptance. Briefly, the right kidney was dissected, flushed with Custodiol® (HTK) solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) and procured from donors weighing 20–30 kg (Göttingen minipig).

Thereafter, kidneys were orthotopically implanted into the respective recipients. All recipients showed spontaneous urine production intraoperatively.

Kidney allograft function was monitored by duplex sonography and fine needle biopsy was performed on POD 3 to detect ischemia–reperfusion injury and surgery-related complications. Intravenous antibiotic treatment was maintained for 28 days without any immunosuppressive therapy. Recipients were euthanized 28 days after kidney transplantation. Allokidneys were histologically graded according to the Banff working classification [12].

3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). For comparisons between the groups, the Mann-Whitney-*U* test and for survival analyses the log-rank test was used. *P*-values less than 0.05 were considered significant. Results are presented as means ± standard error of the mean (SEM).

4. Results

4.1. Delayed irradiation with donor antigen exposure does not lead to persistent graft survival

All animals from group 2 rejected their grafts before postoperative day 108 (Fig. 2). Chest radiographs were scored L3 with a histological grading of A3 (Table 1, Fig. 1). Peripheral blood lymphocyte cell counts initially decreased post transplantation but recovered until the day of irradiation (POD 3). Following irradiation, leucocyte count decreased again and slowly recovered after withdrawal of immunosuppression on POD 28. Platelets slightly decreased after surgery. Between POD 7 and 14, platelet counts deteriorated below basal level but recovered to a normal level after POD 21 (Fig. 3). Peripheral blood leukocyte chimerism peaked >30% donor cells one hour after reperfusion but was not detectable beyond POD 1 (Fig. 5).

4.2. Stable allograft acceptance is achieved with a combination of preoperative irradiation and donor antigen transfusion

In group 1, as previously published [6,8,10], mean survival was 504 days and four out of seven animals developed stable long-term allograft acceptance (Fig. 2). Four long-term survivors (97204, 92567, 73259, 301983) underwent secondary kidney transplantation from third-party donors on POD +500. All kidneys showed normal perfusion and diuretic function instantly after transplantation, but histological analysis of the allokidneys on POD 28 (day of elective sacrifice) revealed high grade interstitial parenchymal inflammation and tubulitis. In two animals, signs of necrosis were found (Table 1). Lungs of long-term survivors were histologically graded A0-A1 corresponding to a chest x-ray score between L0-1. The other three animals rejected their grafts between POD 119 and 360 with a chest x-ray score of L4 and histological findings ranging from A2-A4. In these animals lymphocyte counts were decreased immediately after surgery and remained at a lower level as compared to animals showing long-term allograft acceptance. This group also displayed an earlier decrease of platelets compared to other groups, yet platelet counts began to rise again from POD 21 onwards (Fig. 3). Chimerism peaked at 22% 1 h post reperfusion and started to proliferate again after POD 28. In comparison, chimerism in other groups decreased after an initial peak one hour after reperfusion and was <1% beyond day 42 (Fig. 5).

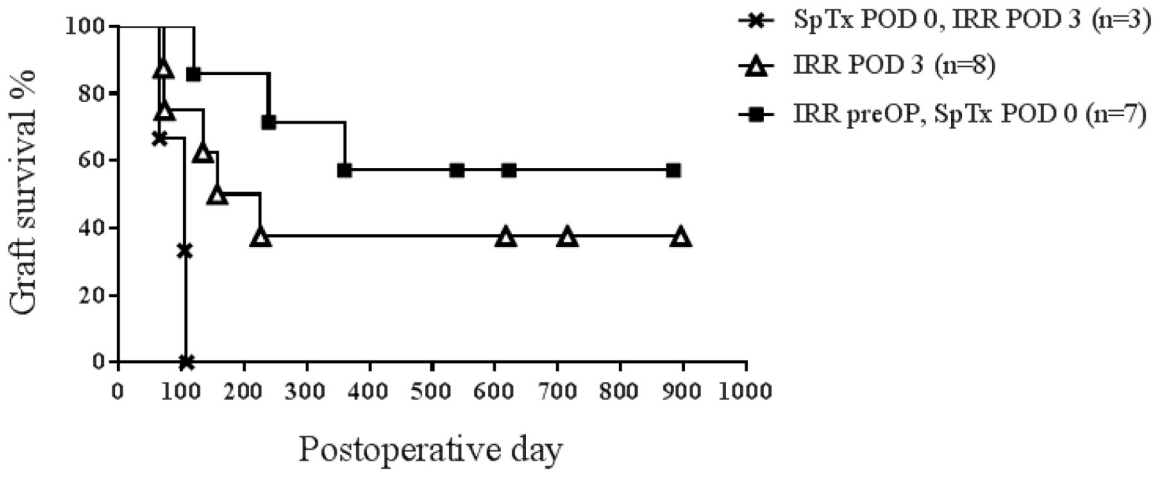

Fig. 2. Kaplan-Meyer survival curve. Per the Log-rank (Mantel-Cox) test, survival differed significantly between group 2 and 3 (p* = 0,0371). No significance was found between group 2 and group 3 (*p* = 0,3007).

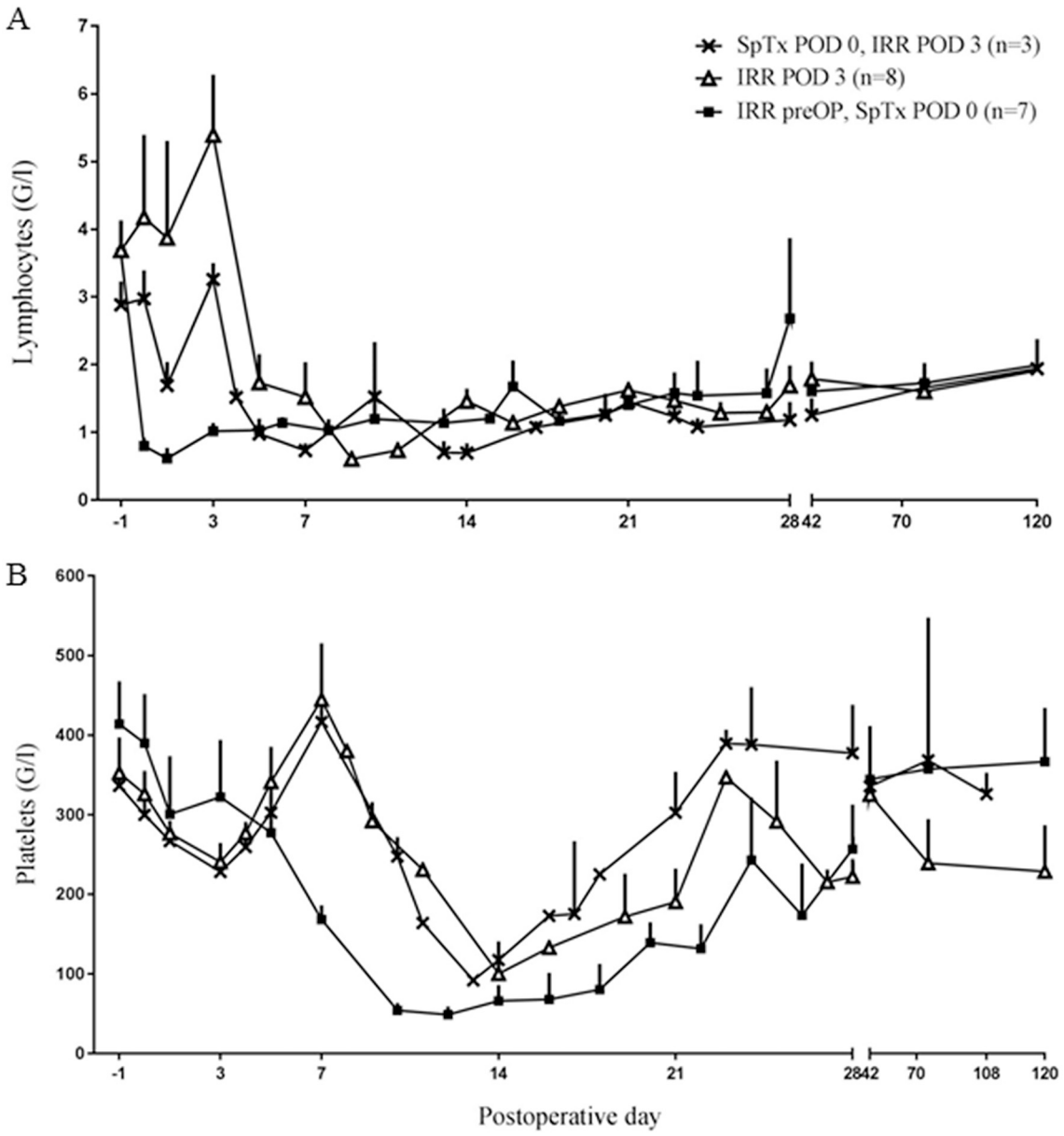

Fig. 3. Lymphocytes (A) and platelets (B) from the differential blood cell counts. Lymphocyte depletion was observed in all groups but in group 1 the depletion starts earlier. This group showed a strong decrease of platelets during the period between POD 5 and 42. The platelets in the delayed IRR groups were nearly identical but also decrease between POD 7 and 28.

4.3. Delayed irradiation leads to acceptable allograft acceptance but compromises long-term donor chimerism

In the delayed irradiation group 3, 3 out of 8 animals developed long-term allograft acceptance ($p = 0.30$ vs group 1, $p = 0.04$ vs group 2, Fig. 2). Three animals without signs of allograft rejection (317,615, 220,975, 226,654) also underwent third-party kidney transplantation on POD +600. The pigs were euthanized after secondary kidney transplantation on POD 8, 9 and on POD 21, respectively, due to clinical deterioration. All three animals showed severe inflammation of the renal allograft parenchyma with severe tubulitis and intimal arteritis. The chest x-ray score and histological grading of these 3 animals showed no signs of graft rejection in the allolungs at time of euthanization (Table 1, Fig. 4).

Lymphocyte cell counts increased to a maximum of 5.26 G/l before IRR on POD 3. After irradiation, lymphocyte count decreased similar to the other groups, recovery was detectable beyond day 28 (Fig. 3A). Platelet counts decreased below basal level immediately after surgery. Starting on POD 3, cell numbers increased to a maximum, but then decreased again reaching a nadir on POD 14. Thereafter, platelet counts returned to normal (Fig. 3B).

In this group, chimerism peaked up to 20% immediately after reperfusion and decreased gradually until POD 28 to a level close to zero (Fig. 5).

4.4. Achieved allograft acceptance remains donor-specific

Animals developing stable allograft acceptance underwent third-party orthotopic kidney transplantation. As evident in duplex sonography (either increased resistive indices in kidney segmental and interlobar arteries or absent perfusion) and nephropathological grading (Table 1), all animals rejected kidney allografts within the first 28 days without immunosuppression, suggesting donor-specific lung allograft acceptance.

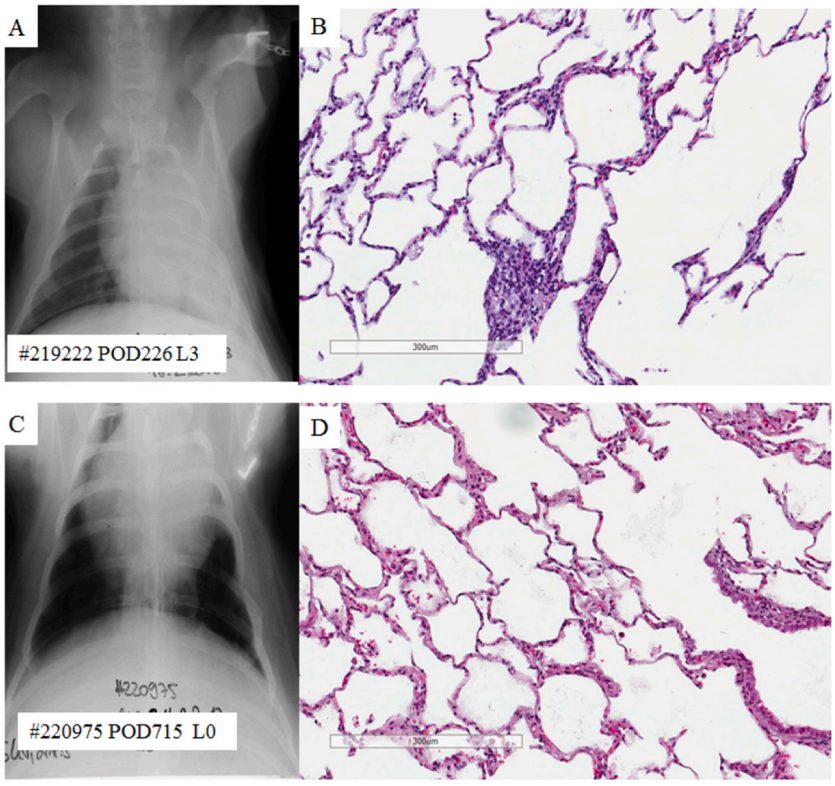

Fig. 4. Chest radiographies of two representative animals from group 3 with associated lung histology (haematoxylin and eosin staining). One animal had rejected the allograft (219222) and one is a long-term survivor (220975). (A) Animal 219,222 rejected the left lung on POD 226. The left side of the lung showed absence of ventilated lung parenchyma whereas the innate left lung was well-ventilated. (B) The lung histology of animal 219,222 showed a moderate acute rejection with dense perivascular mononuclear cell infiltrates and a high-grade small airway inflammation. (C) Animal 220975 accepted the lung long-term beyond POD 500 (radiography from POD 715). (D) Histology of lung biopsy after elective sacrificed animal on POD 715 with no signs of rejection.

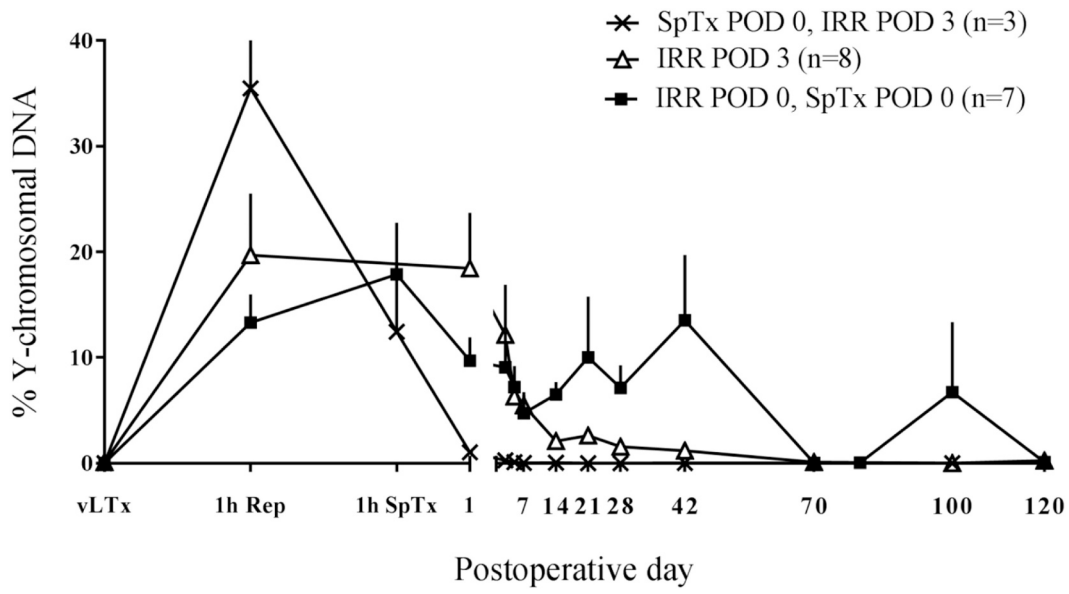

Fig. 5. Donor cell chimerism in peripheral blood. In all groups, donor cell chimerism peaked up to 20% instantly after reperfusion of the lung. Levels in groups SpTx POD 0, IRR POD 3 and IRR POD 3 constantly decreased thereafter; however, group IRR POD 3 fell slower. Chimerism in group IRR preOp, SpTx POD 0 started rising again from POD 7 onwards ($p^* = 0.0123$).

5. Discussion

Our study demonstrates the potential of delayed non-myeloablative irradiation to induce long-term allograft survival in a large animal model. Delayed irradiation was introduced to improve the clinical feasibility of our already established preoperative conditioning protocol [6–8,10,15]. The results of this protocol, which includes preoperative irradiation as well as perioperative donor splenocyte transfusion, are promising. However, transfer to a clinically applicable setting remains difficult. The main drawback is preoperative irradiation, given the imminent surgery and its unforeseeable timing. Alternatively, performing the irradiation three days post transplantation leads to easier logistics and many patients are at this point in a stable situation within a normal ward setting with low risk of bleeding or other severe complications.

In previous experiments, pigs were either irradiated alone or treated with a splenocyte infusion alone before lung transplantation, however, this did not lead to favorable results. In animals receiving a splenocyte

infusion, all recipients rejected the allolung until POD 121. In the group receiving early irradiation, only one animal survived long-term [6].

Therefore, we hypothesized that delayed irradiation on POD 3 with or without donor splenocyte infusion protocol would lead to stable allograft acceptance, thus creating a more clinically applicable protocol. Therefore we created two groups that were exposed to non-myeloablative irradiation on POD 3. Encouraged by our successful protocols from the past, one group received donor splenocytes on POD 0.

Our core idea of tolerance induction is based on a mixed chimerism approach, which includes exposure of the recipient's immune system to a maximum of donor specific antigen during a short phase of subtotal bone marrow depletion, leading donor-derived cells to settle and proliferate in the generated hematopoietic niche.

However, delayed irradiation in combination with perioperative donor splenocyte infusion did not show a beneficial effect on survival, all animals showed acute cellular rejection of the allografts within 108 days. In the group undergoing delayed irradiation without donor splenocyte transfer, allograft survival was markedly better with 3/8 animals developing stable allograft acceptance until elective euthanization +500 days after transplantation, these results being superior to previously published results on animals receiving total body irradiation prior to lung transplantation. Especially in direct comparison to early irradiation, the potential of delayed irradiation becomes more evident [6,16].

In order to prove donor-specificity of the achieved allograft tolerance, animals without signs of lung allograft rejection +500 days underwent orthotopic kidney transplantation using a third-party donor. All kidneys were rejected within 28 days (226654, 317615 and 220975 had to be euthanized earlier due to poor general condition) without signs of lung allograft rejection (Table 1), suggesting donor specific allograft acceptance of the initial lung graft while maintaining overall immune competence.

Irradiation had significant effects on peripheral blood cell counts. Lymphocytes decreased in all groups following irradiation with recovery once immunosuppression was stopped. As expected, depletion in group 3 was postponed with lymphocytes initially increasing after lung transplantation and a rapid decrease after IRR on POD 3. This phenomenon was also observed in other cell lines. Interestingly, group 1 and the group 3 receiving irradiation alone on POD 3, showed a prolonged phase of lymphocyte depletion as compared to the group 2, suggesting that lymphocyte depletion is beneficial for graft survival. Previous studies also showed, that allograft acceptance is positively correlated with lymphocyte depletion, indicating that "space" had to be created within the leukocyte compartment and/or bone marrow of the recipient, promoting donor cell chimerism [6,8,16,17]. In our experiments, donor chimerism peaked one hour after reperfusion of the allo-lung across all experimental groups, suggesting that donor cells are "washed out" of the lung. In group 2, chimerism levels rapidly decreased thereafter, being undetectable by POD 5 (Fig. 5). This may be due to the relatively low cell numbers infused, since the number of cells obtained from the donor in this group was lower than the cell number in group 1 (Table 1). In a human kidney transplantation study, Hutchinson et al. showed that a higher donor cell number tended to improve kidney function [18]. Therefore, the obtained cell number in group 2 may not have been sufficient, explaining the rapid decrease of male donor cells. Group 1 did not develop a comparable high peak of chimerism as groups 2 and 3 did, however, this group showed a very early increase in chimerism. This may be due to the previous irradiation, which presumably led to a hematopoietic niche for the infused cells. In contrast, chimerism levels in group 3 decreased gradually and Y-linked donor DNA was still detectable on POD 42. Animals irradiated before LTx with donor antigen infusion on POD 0 developed long-term peripheral blood macrochimerism with ~10% of nucleated cells in peripheral blood being of donor origin on POD 28 even further increasing to ~15% on POD 42. Donor chimerism remained stable up to +500 days in some animals, suggesting a beneficial milieu for long-term allograft acceptance. These results are consistent with graft survival, thus supporting the concept of long-term tolerance by mixed-hematopoietic chimerism.

The underlying mechanism for the results in this study might be that delayed irradiation leads to depletion of both, host and donor antigen presenting cells, thus preventing persistent donor chimerism.

Hayashi et al. [19] achieved donor specific immune tolerance and chimerism by administration of donor splenocyte infusions without donor bone marrow transplantation in a rodent model. However, our study showed that splenocyte infusion leads to an increased graft survival only with prior irradiation. If, as in group 2, a splenocyte dose was administered before IRR, this even had a negative effect on the lung transplant's survival, suggesting deleterious sensitization of the host to donor antigen.

Other experiments in miniature pigs are consistent with the hypothesis that permanent mixed chimerism without thymus irradiation was only possible if an immunotoxin was used for T-cell depletion, which probably enabled the depletion of thymus T-cells directly by the toxin and not by the usual antibody -mediated mechanisms [20,21].

Similar observations were also made in mice subjected to tolerance with tandem donor bone marrow transplantation (BMT) using a short-duration non-myeloablative conditioning regimen and PTCy. The mice showed a permanent acceptance of the pulmonary allograft with higher levels of donor chimerism and lymphocyte responses [22].

Our understanding of transplant acceptance is consistent with the hypothesis that the complete depletion of mature, donor-reactive T cells must first happen as a result of the preparative regime. Although IRR in a delayed manner has the potential to induce long-term graft survival in the absence of elevated donor antigen exposure, such as SPTX, before IRR in our experimental protocol. Therefore, delayed irradiation is detrimental for the favorable effect of peritransplant DST observed in group 1.

It would be interesting to examine whether delayed irradiation with splenocyte infusion at the same delayed time leads to good graft survival with donor cell chimerism. The only difficulty is the easy and practical conservation of the SpTx-infusion without major cell loss.

6. Limitations of the model

Using Minipigs from eight different breeding lines might be a limitation given that the SLA variability could be decreased, since only 75% of the SLA class I haplotypes were known. Another limitation is that irradiation could produce a non-specific immunosuppression.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

Acknowledgments

The authors thank Birte Kristensen for performing the SLA-typing and Karin Peschel, Astrid Diers-Ketterkat, Natalie Frank and Petra Ziehme for their invaluable technical assistance. Astellas, Osaka, Japan kindly donated Tacrolimus. This study was supported by grants from the Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research (DZL), Hannover, Germany.

References

[1] J.M. Grinyo, Why is organ transplantation clinically important? Cold Spring Harb. Perspect. Med. 3 (6) (2013 Jun 1) https://doi.org/10.1101/cshperspect.a014985.
[2] J.L. Scheffert, K. Raza, Immunosuppression in lung transplantation, J. Thorac. Dis. 6 (8) (2014 Aug) 1039–1053.
[3] N. Nair, E. Gongora, M.R. Mehra, Long-term immunosuppression and malignancy in thoracic transplantation: where is the balance? J. Heart Lung Transplant. 33 (5) (2014 May) 461–467.
[4] I. Al-Githmi, N. Batawil, N. Shigemura, M. Hsin, T.W. Lee, G.W. He, et al., Bronchiolitis obliterans following lung transplantation, Eur. J. Cardiothorac. Surg. 30 (6) (2006 Dec) 846–851.

[5] D.L. DeMeo, L.C. Ginns, Lung transplantation at the turn of the century, Annu. Rev. Med. 52 (2001) 185–201.
[6] M. Avsar, K. Jansson, W. Sommer, B. Kruse, S. Thissen, K. Dreckmann, et al., Augmentation of transient donor cell chimerism and alloantigen-specific regulation of lung transplants in miniature swine, Am. J. Transplant. 16 (5) (2016 May) 1371–1382.
[7] G. Warnecke, M. Avsar, M. Morancho, C. Peters, S. Thissen, B. Kruse, et al., Preoperative low-dose irradiation promotes long-term allograft acceptance and induces regulatory T cells in a porcine model of pulmonary transplantation, Transplantation 82 (1) (2006 Jul 15) 93–101.
[8] K. Jansson, K. Dreckmann, W. Sommer, M. Avsar, J. Salman, T. Siemeni, et al., Splenocyte infusion and whole-body irradiation for induction of peripheral tolerance in porcine lung transplantation: modifications of the preconditioning regime for improved clinical feasibility, Transplant. Direct 3 (7) (2017 Jun 6), e170.
[9] M. Struber, J.M. Hohlfeld, T. Kofidis, G. Warnecke, J. Niedermeyer, S.P. Sommer, et al., Surfactant function in lung transplantation after 24 hours of ischemia: advantage of retrograde flush perfusion for preservation, J. Thorac. Cardiovasc. Surg. 123 (1) (2002 Jan) 98–103.
[10] W. Sommer, G. Buechler, K. Jansson, M. Avsar, A.K. Knofel, J. Salman, et al., Irradiation before and donor splenocyte infusion immediately after transplantation induce tolerance to lung, but not heart allografts in miniature swine, Transpl. Int. 30 (4) (2017 Apr) 420–431.
[11] K. Solez, International standardization of criteria for histologic diagnosis of chronic rejection in renal allografts, Clin. Transpl. 8 (3 Pt 2) (1994 Jun) 345–350.
[12] K. Solez, R.B. Colvin, L.C. Racusen, M. Haas, B. Sis, M. Mengel, et al., Banff 07 classification of renal allograft pathology: updates and future directions, Am. J. Transplant. 8 (4) (2008 Apr) 753–760.
[13] R.D. Yusen, L.B. Edwards, A.Y. Kucheryavaya, C. Benden, A.I. Dipchand, F. Dobbels, et al., The registry of the International Society for Heart and Lung Transplantation: thirty-first adult lung and heart-lung transplant report—2014; focus theme: retransplantation, J. Heart Lung Transplant. 33 (10) (2014 Oct) 1009–1024.
[14] R.W. Gruessner, B.K. Levay-Young, R.E. Nakhleh, J.D. Shearer, M. Dunning, C. M. Nelson, et al., Portal donor-specific blood transfusion and mycophenolate mofetil allow steroid avoidance and tacrolimus dose reduction with sustained levels of chimerism in a pig model of intestinal transplantation, Transplantation 77 (10) (2004 May 27) 1500–1506.
[15] G. Warnecke, J.A. Hutchinson, P. Riquelme, B. Kruse, S. Thissen, M. Avsar, et al., Postoperative intravenous infusion of donor-derived transplant acceptance-inducing cells as an adjunct immunosuppressive therapy in a porcine pulmonary allograft model, Transpl. Int. 22 (3) (2009 Mar) 332–341.
[16] G. Warnecke, M. Avsar, M. Morancho, C. Peters, S. Thissen, B. Kruse, et al., Preoperative low-dose irradiation promotes long-term allograft acceptance and induces regulatory T cells in a porcine model of pulmonary transplantation, Transplantation 82 (1) (2006 Jul 15) 93–101.
[17] B. Kruse, S. Thissen, G. Warnecke, M. Avsar, J. Gottlieb, J.M. Hohlfeld, et al., Correlation of donor leukocyte chimerism with pulmonary allograft survival after immunosuppressive drug withdrawal in a porcine model, Transplantation 87 (10) (2009 May 27) 1468–1477.
[18] J.A. Hutchinson, P. Riquelme, B.G. Brem-Exner, M. Schulze, M. Matthäi, L. Renders, et al., Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation, Transpl. Int. 21 (8) (2008 Aug) 728–741.
[19] Y. Hayashi, S. Yamazaki, A. Kanamoto, T. Takayama, Splenocytes can replace chimeric cells and maintain allograft tolerance, Transplantation 84 (9) (2007 Nov 15) 1168–1173.
[20] C.A. Huang, K. Yamada, M.C. Murphy, A. Shimizu, R.B. Colvin, D.M. Neville Jr., et al., In vivo T cell depletion in miniature swine using the swine CD3 immunotoxin, pCD3-CRM9, Transplantation 68 (6) (1999 Sep 27) 855–860.
[21] C.A. Huang, Y. Fuchimoto, R. Scheier-Dolberg, M.C. Murphy, D.M. Neville Jr., D. H. Sachs, Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model, J. Clin. Invest. 105 (2) (2000 Jan) 173–181.
[22] J.M. Dodd-O, S. Ganguly, A. Vulic, A. Panoskaltsis-Mortari, J.F. McDyer, L. Luznik, Induction of major histocompatibility complex-mismatched mouse lung allograft acceptance with combined donor bone marrow: lung transplant using a 12-hour Nonmyeloablative conditioning regimen, Transplantation 100 (12) (2016 Dec) e140–e146.