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Abbreviations

Heart transplantation (HTx)

Nonhuman primate (NHP)

α1,3-galactosyltransferase-knockout (GTKO)

Endothelial protein C receptor (EPCR)

C-reactive protein (CRP)

Interleukin-4 (IL-4)

Type 2 T helper cell (Th2)

Interleukin-1 beta (IL-1β)

Tumor necrosis factor-alpha (TNF-α)

Interleukin-1 (IL-1)

Interleukin-6 (IL-6)

Interleukin-5 (IL-5)

High mobility group box-1 (HMGB1)

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Immunosuppressive (IS)

Interleukin-10 (IL-10)

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Human CD46 (hCD46)

Human thrombomodulin (hTM)

Central venous catheter (CVC)

Left atrium (LA)

Right atrium (RA)

Inferior vena cava (IVC)

Pulmonary artery (PA)

Data Sciences International (DSI)

Mycophenolate mofetil (MMF)

Quantitative real-time polymerase chain reaction (qPCR)

Relative quantification (RQ)

White blood cell (WBC)

Creatine kinase (CK)

Threshold cycles (Ct)

1 Introduction

Over the last few decades, heart transplantation (HTx) has become the preferred therapy for end-stage heart failure patients who have tried conventional medical therapy or other surgeries without conditions improved. Although mechanical devices, including left ventricular assist devices, greatly improved patient survival, complications like infections, bleeding and thrombotic complications remain. As another train of thought, regenerative medicine has a good success for other tissues, but for heart failure stem cell technology is still at the primary stage. As a matter of fact, more significant numbers of patients remain who would benefit from heart transplantation if donor organs were available. The field of xenotransplantation has experienced substantial progress and is considered as a potential solution to this problem.

1.1 History of cardiac xenotransplantation

The first heart xenotransplantation in humans ever performed was by James Hardy. He had been considering heart allotransplantation since he was inspired by the good outcome of some patients transplanted with chimpanzee kidneys. In 1964, Hardy decided to accomplish the first clinical cardiac transplantation and chose to acquire some chimpanzees as possible organ donors. He proposed a recipient who was in a semi-comatose state with extensive atheromatous vascular disease, for which he had taken amputations of both legs. Because the patient was deteriorating rapidly, Hardy made a decision to implant the chimpanzee heart. The heart turned out not to be strong enough to support the circulation and eventually failed within two hours[1]. However, because the consent form for the operation did not mention that an animal heart might be used, Hardy's transplant surgery received opposition from the public and medical peers, so he did not perform any further attempt.

In 1967, on the basis of the experimental research of Cooper [2], the procedure of clinical orthotopic cardiac allotransplantation was first established by Christiaan Barnard [3]. He later also developed a technique of heterotopic heart xenotransplantation, using a chimpanzee and a baboon as donors [4]. The baboon heart failed rapidly after surgery, but the chimpanzee heart supported the patient who was in postcardiotomy shock after cardiopulmonary bypass for four days.

In 1984, the paediatric cardiac surgeon Leonard Bailey transplanted a baboon heart orthotopically into an infant girl (Baby Fae) that was born with hypoplastic left heart syndrome [5]. The operation was technically successful, but the patient died 20 days later because of heart rejection. Although cyclosporine was administered which was potent and new at that time, the immunosuppressive therapy was inadequate to prevent xenograft rejection. Furthermore, the baboon heart was ABO-incompatible with the infant, which might have added to the seriousness of rejection across the species barrier. This well-known case drew the public's attention to the shortage of accessible human organs for babies.

Donald Ross and Denton Cooley, two outstanding cardiac surgeons, transplanted pig and sheep hearts into patients who were about to die [6, 7]. At that time, Ross did not anticipate hyperacute rejection that occurred a few minutes after he performed heterotopic cardiac transplantation. Cooley’s sheep heart in further transplantation attempts endured the same destiny.

From these early experiences, researchers have gained a lot of experience with non-primate mammalian heart and nonhuman primate (NHP) heart transplantation.

1.2 The immunobiological barriers of cardiac xenotransplantation

Advances in immunosuppressive (IS) therapy have now made cardiac transplantation the gold standard treatment for heart failure treatment. However, cardiac xenotransplantation could be a solution to the lack of donor organs. Although primates are known to be phylogenetically closer to humans, pigs are now considered a more appropriate donor for several reasons[8]: They have organs comparable in size and function to those of humans, maintain a high level of reproductive efficiency, mature very quickly, and are easy to raise in microbiologically-controlled environments with little ethical controversy. Moreover, pigs can be genetically modified[9].

Many experimental studies have been conducted with pigs as donors focusing on rejection. In fact, there are three types of rejection after xenotransplantation: hyperacute, acute and chronic rejection.

1.2.1 Hyperacute rejection

Hyperacute rejection is the first immunologic barrier for transplantation between human or NHP and pig. It starts immediately when the pig coronary arteries are perfused by primate blood. The graft is destroyed within 24 hours, but often even within the first hour: primate blood contains "natural" anti-pig antibodies that bind the vascular endothelial cells of the pig heart and activate the complement cascade. This leads to immediate injury of the endothelium, which causes thrombosis in vessels and edema that disrupts the function of the endothelium and heart within minutes [10].

The rejection is caused by the interaction between a carbohydrate epitope, galactose-α1,3-galactose (Gal), from porcine endothelial cells and antibodies present in the primate blood[11]. During neonatal life of all primates, these antibodies develop and are probably a reaction to micro-organisms that colonize the gastrointestinal tract [12, 13]. This response is similar to that in ABO-incompatible allotransplantation rejections [14].

It is possible to reduce this immune reaction by removing naturally preformed antibodies [15] and inhibiting the reaction of the complement system [16]. Dalmasso and White suggested a different approach to overcome hyperacute rejection: They proposed genetic modifications as a strategy to express human complement inhibiting proteins on porcine endothelial cells [17, 18]. This was achieved in the 1990s by a number of groups, creating the first genetically engineered pigs for xenotransplantation experiments [8, 18]. In 2001, pigs were genetically modified to express human complement-regulatory protein CD46 [19]. When the important role of Gal was established, it was suggested that the elimination or knock-out of the Gal expression in pigs was a solution to prevent hyperacute rejection. [20, 21]. In 2003, this important achievement was done by suppressing the gene for coding α1,3-galactosyltransferase [22]. The production of genetically engineered pigs is very helpful in avoiding the occurrence of hyperacute rejection.

1.2.2 Acute humoral rejection

Following transplantation, binding of recipient antibodies to endothelium and complement activation promote coagulation, inflammation, and immunity[23]. This triggers acute humoral rejection - also called acute vascular rejection - which is similar to hyperacute rejection but takes place over several days or weeks.

With the development of genetically modified pigs as donors both of these barriers have now been overcome. On one hand, pigs that are genetically edited for complement regulatory proteins (CRPs) were produced [24]. GTKO pigs prevent rejection by expressing human CRPs. On the other hand, the main target for primate antibodies has been removed in GTKO pigs [22]. The combination of GTKO and human CRPs has more successfully prevented early postoperative failure of pig donor organs. [25]. As compared to hyperacute rejection, there are also some immune cells involved in acute humoral rejection such as lymphocytes, macrophages, natural killer cells, and neutrophils [26, 27].

1.2.3 Acute cellular rejection

Antigens from the graft activate an immune response of the recipient, stimulating T-cells to attack graft heart cells, thus triggering acute cellular rejection [28, 29]. Acute cellular rejection usually occurs in the first 3 to 6 months after transplantion.

Intense cellular graft infiltration is common and occurs early after heart allotransplantation, but it was rarely described after heart xenotransplantation. Several studies proposed that T-cell response should be more vigorous in the transplanted xenograft [30-32].

1.2.4 Chronic rejection

Chronic rejection may occur within months or even years after transplantation, and is diagnosed clinically by progressing graft failure. Chronic rejection is assumed to develop as a consequence of the combined effects of different factors. It is suggested that the role of T-cells and antibodies destroys vascular endothelial cells which is the initiating factor of chronic rejection. At present, the specific mechanisms of chronic rejection after xenotransplant are unclear.

1.2.5 Coagulation dysfunction

Even if graft rejection is prevented, another barrier to the success of heart xenotransplantation is abnormal coagulation within graft vessels. Thrombotic microangiopathy plays an important part in the failure of the graft [33-35]. Fibrin deposition and thrombocyte aggregation lead to thrombosis and eventually processes to ischemic necrosis injury [33, 34, 36].

Thrombotic microangiopathy is believed to result from activation of the graft's endothelium cells by deposition of antibody and complement fraction. When vascular endothelial cells are activated, they transfer from an anticoagulant state to a procoagulant state [37]. Several molecular incompatibilities between anticoagulants on endothelial cells of pigs and primates are known; incompatible anticoagulants cannot inhibit the activation of coagulation factors, which contributes to the dysregulation of coagulation between graft and recipient [38].

It is difficult to overcome coagulation dysfunction after xenotransplantation by pharmacotherapy. Genetic engineers made efforts to insert further "anticoagulant" or "antithrombotic" transgenes into genetically modified pigs. These include CD39 and endothelial protein C receptor resulting in extended graft survival [39].

An effective genetical modification is the expression of human thrombomodulin on porcine endothelial cells which has an inhibitory effect on the coagulation system by binding to primate thrombin [40]. Byrne has proved that increased immunosuppression therapy is effective in inhibiting thrombotic microangiopathy development [41].

1.3 Inflammatory response

There is convincing evidence that inflammatory responses to pig grafts play an important role in the failure of the graft after xenotransplantation [42]. Inflammatory responses promote coagulation dysfunction after xenotransplantation [42, 43]. Even though the manifestation of the inflammatory response in xenotransplantation is not well explained, but it can be associated with the increase of white blood cells, especially neutrophil [42]. Also, an elevation of C-reactive protein (CRP) is a clinically significant inflammatory marker [44]. Li's study monitoring CRP in NHP recipients with pig grafts proved tocilizumab reduce the inflammatory response after xenotransplantation [45].

Inflammatory cytokines are molecules which work as inflammatory mediators. They are excreted from different cell types and can be measured in the blood. Changes in cytokine production in the tissue can be quantified by gene expression analysis. The following cytokines are involved in inflammation:

1.3.1 IL-4

Interleukin-4 (IL-4) is an anti-inflammatory cytokine of the type 2 T helper cell (Th2) subtype, secreted by immune cells, including lymphocytes and mast cells. Cytokine IL-4 has been shown to be involved in the cardiac fibrosis process, which leads to reduced myocardial compliance and ultimately a cardiac failure; Interleukin-1 beta (IL-1β) is also associated with this progress [46, 47]. The pro-inflammatory response of tumor necrosis factor-alpha (TNF-α), Interleukin-1(IL-1), and Interleukin-6(IL-6) are also inhibited by IL-4[48].

1.3.2 IL-5

As IL-4, interleukin-5 (IL-5) is also produced mainly by activated Th2 and mast cells, their genes are both located on the same chromosome within a cytokine gene cluster. IL-5 is a cytokine that plays a central role in eosinophil development, activation and survival[49]. Proteins released by eosinophil may influence coronary atherosclerosis independent from the IL-1 and IL-6 pathways [50]. IL-5 is also an essential cytokine associated with human myocarditis [51]. Braun's study revealed that IL-5 and eosinophils are related to the promotion of rejection after cardiac allotransplantation [52].

1.3.3 IL-6

Interleukin-6 (IL-6) is created by a multitude of cells including fibroblasts, monocytes, T-cells, B-cells, endothelial cells and mesangial cells [53]. IL-6 is an interleukin that functions as both pro-inflammatory and anti-inflammatory cytokine[54]. Many articles have already explained the key roles of IL-6 in inflammation and allotransplantation [55, 56]. It has been shown that excessive production of IL-6 stimulates B cells to generate antibodies which eventually results in graft failure in transplantation [57]. Both Ezzelarab's and Gao's studies suggested that IL-6 might promote the occurrence of coagulation dysregulation and inflammation response after xenotransplantation [42] [58]. Ezzelarab also indicated that IL-6 was increased without immunosuppressive treatment in pig-to-baboon heart xenotransplantation [42].

Li's experiments in rat-to-mouse cardiac xenotransplantation indicated that inhibition of IL-6 using an anti-high mobility group box-1 (HMGB1) antibody might prolong the survival of cardiac xenografts [59]. In the pig-to-monkey islet model, Min observed that blockage of IL-6 signalling could not prolong the survival of porcine pancreatic

islets in monkeys (treated with tocilizumab) but decreased levels of CRP [60]. Iwase showed that tocilizumab therapy decreased CRP in baboons with a porcine artery patch or heart transplant [61]. Tocilizumab therapy also extended survival time of kidney xenografts of transgenic pigs [62].

1.3.4 IL-8

Interleukin-8 (IL-8) is a chemokine created by macrophages, epithelial cells, and endothelial cells. IL-8 is an important pro-inflammatory chemokine that plays a primary role in the activation of neutrophils during inflammation response. French‘s study, in both ex vivo pig lung perfusion model and in-vivo pig-to-baboon lung transplantation experiments, showed that IL-8 activated human neutrophils and increased their adherence to pig aortic endothelial cells[63]. In this same trial, pig IL-8 increased in the ex vivo model but not in the vivo experiments, whereas in the human and baboon models IL-8 was similarly elevated. Ezzelarab reported that baboon IL-8 levels were increased after pig-to-baboon artery patch transplantation, but no elevation was observed after pig heart or kidney xenotransplantation [42]. In addition, IL-8 was elevated with no immunosuppressive (IS) treatment in artery patch recipients, but IL-8 was reduced with IS therapy. Iwase showed post-transplant levels of IL-8 were elevated when no anti-inflammatory agent was administered [64].

1.3.5 IL-10

Interleukin-10 (IL-10) is known to be produced by monocytes, T cells and B cells. It is a pleiotropic anti-inflammatory cytokine. Kaur’s [65] study indicated that a decrease in IL-10 correlates with depressed cardiac function. Krishnamurthy et al. demonstrated that IL-10 not only has as an anti-inflammatory effect but also improves left ventricular function by inhibiting cardiac fibrosis and remodeling [66]. Other researchers, however, did not find an association between genotypes of IL-10 and rejection or heart failure after transplants [67].

1.3.6 TNF-α

Investigations have shown that TNF-α is a key pro-inflammatory cytokine and an essential part of the innate immune system. It was produced by immune cells, such as activated macrophages and lymphocytes, endothelial and epithelial cells, smooth muscle cells, and heart myocytes. TNF-α can be released from the myocardium when the heart experiences pressure or volume overload [68]. TNF-α leads

to hypertrophy and cardiac enlargement when it is chronically overexpressed in the myocardium[69]. Numbers of researches indicated that TNF-α in cardiac transplant recipients was elevated without histological evidence for rejection [70, 71]. Xu reported that rejecting porcine hearts secreted TNF-α in a pig-to-baboon orthotopic cardiac xenotransplant model [72]. Additionally, Ashton-Chess demonstrated that upregulated TNF-α expression was observed in both non-immunosuppressed baboons and those treated with immunoadsorption after transplantation of hCD55-transgenic pig hearts [73]. In both kidney and islet xenotransplantation models, blockade of TNF-α increased the postoperative survival time of xenografts [62, 74].

1.3.7 IL-1α

Interleukin-1 alpha (IL-1α) is a potent inflammatory cytokine of the IL-1 family involved in various immune responses. It is also known as hematopoietin 1 and produced by macrophages, neutrophils, epithelial cells, and endothelial cells. IL-1α is constitutively present in most healthy cells as a precursor protein and released after an injury like alarm bell[75]. Dead cardiomyocytes release IL-1α that can trigger a post-infarction inflammatory response [76].

1.3.8 IL-1β

IL-1β is a pro-inflammatory cytokine produced by many cells, such as macrophages, NK cells, monocytes, and neutrophils, myeloid cells, fibroblasts, and endothelial cells. IL-1β was found to be increased postoperatively after xenotransplantation in vitro models [58]. Moreover, it has been shown that IL-1β correlated with the complete range of organ ischemia/reperfusion injury, from acute rejection to chronic allograft dysfunction [77-79]. One of the early studies of transplant inflammation discovered that there was a significant rise in IL-1β expression from cardiac allografts with rejection compared to allograft controls [80].

1.3.9 Pro- and anti-inflammatory cytokines

The appropriate concept of anti-inflammatory cytokines is a group of molecules that regulates the cytokine response to inhibit IL-1, TNF-α synthesis. The interplay between pro- and anti-inflammatory cytokines characterizes inflammation. The classification of cytokines is listed in table 1. Pro-inflammatory cytokines assist with infection resistance and can lead to systemic inflammation[81]. In some in vitro studies related to xenotransplantation, all of the cytokines/chemokines, IL-6, IL-1β,

and TNF-α, are likely to stimulate inflammation and coagulation in response to a xenograft [58]. An increase of cytokines after xenotransplantation was observed in the absence of immunosuppressive therapy but not when immunosuppressive agents were administered[42]. Ezzelarab[82] indicated that the effect of immunosuppressive therapy on anti-inflammatory cytokines in xenotransplant recipients was unclear and required further inquiry.

1.3.10 Acute and chronic inflammatory cytokines

It is possible to divide inflammatory cytokines into two groups (table 1): those engaged in acute inflammation and those responsible for chronic inflammation. IL-1, TNF-a, IL-6, and IL-8 play an important role in acute inflammation. The chronic inflammation cytokines group can be further devided into cytokines that mediate humoral reactions like IL-4, IL-5, and IL-6, as well as those that mediate cellular reactions like IL-1, IL-10, and TNF-a. Some cytokines, like IL-1, contribute considerably to both chronic and acute inflammation.

Table 1 Classification of inflammatory cytokines

pro-inflammatory	anti-inflammatory	acute inflammatory	chronic inflammatory
IL-1α	IL-4	IL-1α	IL-1α
IL-1β	IL-5	IL-1β	IL-1β
IL-6	IL-6	IL-6	IL-4
IL-8	IL-10	IL-8	IL-5
TNF-α		TNF-α	IL-6
			IL-10
			TNF-α

2 Materials and methods

2.1 Heart xenotransplantation

2.1.1 Animals

In this study, pigs of the breeds large white/landrace (*Sus scrofa*) that had a homozygous α1,3-galactosyltransferase-knockout (GTKO) and were heterozygous transgenic for human CD46 (hCD46) and human thrombomodulin (hTM) were used as organ donors (age 7-12 weeks, weight 8-23 kg, n=16). Captive-bred baboons served as organ recipients (age 4-12 years, weight 11-25 kg, n=16). The body weights of donor and recipient were matched to ensure similar heart sizes at the time of transplantation. Seven days before transplantation, physical examination and transthoracic echocardiography were performed to examine the cardiac function and exclude congenital anomalies, such as aortic insufficiency.

The pigs originated from the Institute for Molecular Animal Breeding and Biotechnology, Gene Center, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany), the baboons from the Germany Primate Centre (DPZ), Göttingen, Germany. Their health condition was examined several times before and after they were transferred to the Walter-Brendel-Centre where the experiments were performed. Blood samples were taken for analysis when they arrived. The baboons were held together in one animal facility and regularly received dry food, fresh fruit, vegetables, and drinking water at their free disposal. Postoperatively, each of baboons was kept in a big cage specifically designed and constructed to allow post-operative treatment.

Before transplantation, the recipient baboon was sedated by intramuscular administration of 0.3-0.5 mg/kg midazolam (Midazolam-ratiopharm; ratiopharm GmbH, Germany) and 6-8 mg/kg ketamine hydrochloride (Ketavet 100 mg/mL; Pfizer Deutschland GmbH, Germany) [83]. A peripheral venous line was inserted for the preoperative medical treatment (see 2.1.3) and blood sampling. Baboons underwent a physical examination for signs of contraindications for surgery.

Two days before cardiac transplantation, the baboon received a central venous catheter (CVC), which was implanted into the left jugular vein and tunnelled

subcutaneously to the side of the thoracic cage. Postoperatively, a tethering system with a baboon jacket and swivel was used to hide and protect the CVC and the infusion system from damage by the animal. The infusion lines connected to CVC were passed via a swivel through the roof of the cage. In this way, continuous administration of medication was possible.

2.1.2 Surgical procedures

On the day of the operation, general anaesthesia of donor pig was induced by an intravenous bolus of 20 mg propofol and 0.05 mg fentanyl. To maintain anaesthesia, propofol (0.12 mg kg^{-1} min^{-1}) and bolus administrations of fentanyl (2.5 µg kg^{-1}, repeated every 30 min) were used. After medial thoracotomy and systemic heparinization (500 IU kg^{-1}), the ascending aorta was exposed and cannulated. The heart was emptied by closing of the superior and inferior vena cava. Ischemic and non-ischemic organ preservation methods were used:

1. For experiments PAV1 to PAV4, 20 ml kg^{-1} crystalloid cardioplegic solution (histidine-tryptophan-ketogluterate (custodial HTK, Dr. Franz Köhler Chemie) or University of Wisconsin (Belzer's UW) solution) at 4° C was infused into the coronary arteries via the ascending aorta. The right and left atrium appendices were opened for reducing the pressure. The heart was then harvested, submerged in the cold cardioplegic solution.

2. For experiments PAV5 to PAV16, an oxygenated 8°C cold albumin-containing hyperoncotic cardioplegic nutrition solution with hormones and erythrocytes preservation medium (based on Steen Solution™, a sterile, non-pyrogenic physiological salt solution containing human serum albumin and dextran 40) was infused into the coronary arteries via the ascending aorta, following a protocol described by Steen et al [84]. The heart was then excised and transferred into a heart preservation system (consisting of a pressure- and flow-controlled roller pump, an O_2/CO_2 exchanger, a leukocyte filter, an arterial filter and a cooler/heater unit), where it was continuously perfused with preservation medium, an oxygenated albumin-containing hyperoncotic cardioplegic nutrition solution with hormones and erythrocytes. In this group, intermittent perfusion of the heart was continued during implantation.

Meanwhile, general anesthesia of the recipient baboon was induced by an intravenous bolus of 2.0-2.5 mg/kg propofol (Propofol®-Lipuro 2%; Melsungen, Germany) and 8 µg/kg fentanyl (Fentanyl-Janssen 0.5 mg; Neuss, Germany). Blood pressure, heart rate, and electrocardiography were monitored. Endotracheal intubation was performed with a cuffed endotracheal tube (5.0-7.0 mm diameter; Mallinckrodt, Athlone, Ireland). Anaesthesia was maintained with continuous infusion of propofol at 0.16±0.06 mg/kg/min [83].

After medial thoracotomy, heparin (500 IU kg^{-1}; Ratiopharm) for heparinization was given and the heart-lung machine was connected to both venae cavae and the ascending aorta. The ascending aorta was cross-clamped, the heart of the recipient was excised at the atrial levels, and both large vessels were cut. The heart of the porcine was transplanted using the method of Shumway and Lower [85]: The donor graft was implanted beginning with the left atrium (LA) anastomosis. The donor right atrium (RA) was opened by incising it from the inferior vena cava until the base of the right atrial appendage; then the anastomosis of the RA was established. The donor and recipient pulmonary artery (PA) were linked together with an anastomosis. Using the same end-to-end anastomosis method, the donor heart's ascending aorta was connected by incising to the recipient's aorta.

At the end of the procedure, a telemetric monitoring system (Data Sciences International, DSI) was implanted. The telemetry system was used for postoperative assessment of graft function and recipient hemodynamic monitoring (see 2.1.4).

2.1.3 Perioperative treatment

Immunosuppressive (IS) regimen for all recipient animals included an induction therapy which comprised anti-CD20 antibody (mabthera, Roche Pharma) and ATG (thymoglobuline, Sanofi-Aventis). Maintenance IS therapy consisted of methylprednisolone (urbasone soluble, Sanofi-Aventis), mycophenolate mofetil (CellCept, Roche) and either an anti-CD40 antibody (mouse/rhesus chimeric IgG4 clone 2C10R4, NIH Non-human Primate Reagent Resource, Mass Biologicals; courtesy of K. Reimann) or humanized anti-CD40L PASylated Fab (XL-Protein and Wacker-Chemie).

Anti-inflammatory therapy included TNF inhibitor (Enbrel, Pfizer), IL-1-receptor antagonist (Kineret, Swedish Orphan Biovitrum), and IL-6-receptor antagonist (RoActemra, Roche).

Additive treatments included unfractionated heparin (heparin-natrium-25000-Ratiopharm, Ratiopharm), ganciclovir (cymevene, Roche), C1 esterase inhibitor (bioinert, CSL Behring), epoetin beta (neorecormon 5000IU, Roche P), acetylsalicylic acid (aspirin, Bayer Vital), and cefuroxime (cefuroxim, Hikma).

Antihypertensive medication including enalapril (Enahexal, Holzkirchen) and metoprolol (Beloc, AstraZeneca) was administered postoperatively, aiming at mean arterial pressures of 80 mmHg and heart rates of 100 beats per minute). Additionally, PAV10 to PAV16 received temsirolimus as daily short intravenous infusions, aiming at rapamycin trough levels of 5-10ng/ml.

2.1.4 Postoperative monitoring

The health condition of the animal was monitored daily by clinical parameters such as the animal's behavior, appetite, urination, defecation, and respiration rate. Any abnormalities and changes in clinical manifestations were recorded. The following parameters were assessed and continuously monitored by DSI: systolic, diastolic and mean pressure of left ventricle, electrocardiography (ECG), systolic and diastolic pressure of the aorta, heart rate derived from pressure waves of the left ventricle, heart rate derived from ECG, and body temperature.

At least once a week, the baboon was sedated for a clinical examination and an assessment of the function and growth of the porcine graft by echocardiography. Furthermore, postoperative pericardial or pleural effusion could be diagnosed non-invasively by ultrasound.

Blood was sampled via the CVC and analyzed in the Department of Clinical Chemistry of the University Hospital of Munich. Serum chemistry, haematology, coagulation, serology, complement, therapeutic drug blood concentration was measured regularly. Inflammatory markers were: WBC count, neutrophils count, lymphocytes count, CRP, IL-6. The serum concentration of cardiac injury markers (Troponin T, CK) was measured to identify myocardial damage.

2.1.5 Endpoints of the study and sample collection

The primary endpoint was the postoperative survival of the recipient baboons after pig-to-baboon cardiac xenotransplantation. If graft function and general condition of the recipient deteriorated (see 2.1.4), the animals were euthanized in general anaesthesia by anaesthetic overdose according to governmental regulations. The recipients were also euthanized after reaching a survival time of 90 days (PAV1-PAV14) and six months (PAV15, PAV16), respectively, according to study protocol.

The hearts were explanted immediately after euthanization. Both left and right ventricle myocardium were sliced into 5*5mm small squares and immediately placed in liquid nitrogen. Long-term preservation was achieved by storage at -80°. As negative controls, three non-transplanted hearts from age- and weight-matched pigs were used. Samples were collected using the same methods as described for transplanted animals.

2.2 RNA Extraction

2.2.1 Extraction methods

Total RNA was isolated from 32 frozen samples of left and right ventricle myocardium of each post mortem heart using the RNeasy Mini Kit (Qiagen). A maximum amount of 30 mg of frozen tissue was processed. The samples to be processed were placed into lysis tubes (Analytik Jena) with 500µl Buffer RLT (Qiagen) for disruption and homogenization: Homogenization was done twice for 30 seconds using a SpeedMill PLUS (Analytik Jena) and cooling on ice for 1 hour after homogenization according to the instructions. After homogenization, the lysate was centrifuged at 14,500 ×g for 3 min. The supernatant was carefully transferred by pipetting to a new RNase-free micro centrifuge tube. 500µl of 70% ethanol was added to the lysate and mixed immediately. 700 µl of the sample, including any precipitate, was transferred to an RNeasy spin column, placed in a 2 ml collection tube and centrifuged at ≥8000 x g for 15 s. 700 µl buffer RW1 (containing a guanidine salt and ethanol, used as a washing buffer, Qiagen) was added to the RNeasy spin column and centrifuged at ≥8000 x g (≥10,000 rpm) for 15 s to wash the spin column membrane. 500 µl buffer RPE (a mild washing buffer to remove traces of salts, Qiagen) were added to the RNeasy spin column and centrifuged for 15 s at ≥8000 x g. To wash the spin column membrane, 500 µl buffer RPE was added to the RNeasy spin column. The RNeasy spin column

was moved in a new 2 ml collection tube and discarded the old collection tube with any flow-through. The spin column was moved in a new 1.5 ml collection tube, 30µl RNase-free water was added directly on the spin column membrane and the collection tube was centrifuged for at ≥8000 x g for 1 min to elute the RNA.

2.2.2 Quantitation and storage of isolated RNA

RNA concentration and purity were assessed by ultraviolet spectroscopy method. 1 µL of the RNA solution was analyzed with a Nanodrop 2000c spectrophotometer (ThermoScientific). A260 and A280 readings were used to determine the concentration (µg/µL) and assess purity (A260:A280). The concentration of each RNA sample was measured and used to calculate volumes of 1µg RNA. Accurate volumes of 1µg RNA solution were transferred into 200 µl tubes for storage (-80°C).

2.3 Reverse transcription

RNA was reverse-transcripted to cDNA using the Thermo Scientific Maxima H Minus Reverse Transcriptase kit (Thermo Fisher). 1 µg of RNA solution was diluted by nuclease-free water to reach a total volume of 16 µL. Incubated at 65°C for 15 minutes. 4 µL Maxima H Minus cDNA synthesis master mix was added for a final volume of 20 µl. The tubes were placed in the Applied Biosystems 2720 Thermal Cycler (Thermo Scientific) for synthesis using a protocol of 25°C for 10 minutes, 50°C for 15 minutes, 85°C for 5 minutes, and finally 4°C until removal from the machine. The cDNA was then diluted in 100 µL with sterile nuclease-free water and stored at -20°C.

2.4 Quantitative real-time PCR (qPCR)

2.4.1 Primers

Eight inflammatory genes (IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, IL-1α, and IL-1β) were investigated for gene expression analysis by real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the data. Primer sequences for all genes, lengths of sequences, melting temperature (Tm) of the primers, and guanine-cytosine content (GC content) of sequences are shown in Table 2.1. All primer sequences were obtained from Eurofins Scientific. Although we used pig specific primers for qPCR analysis, cross-reaction between pig and baboon cannot be completely eliminated.

Table 2.1 Primers used in qPCR

Primer Pair	Sequence (5′→3′)	Length (bp)	GC content	TM (°C)	Amplified Gene
F.GAPDH S.scofra	TTCCACGGCACAGTCAAGGC	20	60%	61.4	GAPDH
R.GAPDH S.scofra	GCAGGTCAGGTCCACAAC	18	61.1%	58.2	GAPDH
IL-4 Ex1 F1	GCAACTTCGTCCACGGACAC	20	60%	61.4	IL-4
IL-4 Ex3 R1	TTTGCCATGCTGCTCAGGTT	20	50%	57.3	IL-4
IL-5 Ex1 F1	GCCTACGTTAGTGCCATTGCTG	22	54.5%	62.1	IL-5
IL-5 Ex4 R1	TCTCCATCTTTCCCCTCCACA	21	52.4%	59.8	IL-5
IL-6 Ex4 F1	ATCACCACCGGTCTTGTGGA	20	55%	59.4	IL-6
IL-6 Ex5 R1	CCTCAGGCTGAACTGCAGGA	20	60%	61.4	IL-6
IL-10 Ex1 F1	CCTTCGGCCCAGTGAAGAGT	20	60%	61.4	IL-10
IL-10 Ex4 R1	CGGCCTTGCTCTTGTTTTCA	20	50%	57.3	IL-10
IL-8 Ex1 F1	CCAAACTGGCTGTTGCCTTC	20	55%	59.4	IL-8
IL-8 Ex3 R1	CTGCACCCACTTTTCCTTGG	20	55%	59.4	IL-8
TNF-α Ex1 F1	GGCCCCCAGAAGGAAGAGTT	20	60%	61.4	TNF-α
TNF-α Ex4 R1	ATGCGGCTGATGGTGTGAGT	20	55%	59.4	TNF-α
IL-1α Ex5 F1	TTCGAGACCCGTCAGGTCAA	20	55%	59.4	IL-1α
IL-1α Ex7 R1	ATGGGCGGCTGATTTGAAGT	20	50%	57.3	IL-1α
IL-1β Ex3 F1	TGATGGCCCCAAAGAGATGA	20	50%	57.3	IL-1β
IL-1β Ex5 R1	GCACGTTGGCATCACAGACA	20	55%	59.4	IL-1β

2.4.2 Quantitative real-time polymerase chain reaction (qPCR)

QPCR amplifications were performed on each cDNA sample in triplicate within wells of a 96-well PCR plate. A negative control reaction containing no cDNA was included to determine if there was any DNA contamination of the reactions. A PowerUp™ SYBR™ Green Master Mix (Thermal fisher) containing the following reagents were prepared for each primer:

Table 2.2 Composition of master mix

Component	Volume
SYBR® Green qPCR Master Mixes	5µl
Nuclease-free H2O	2µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
Total volume	8 µl

2µl of cDNA was added to each well of a 96 well PCR plate to create a final reaction volume of 10µl. The plate was sealed with an adhesive cover, then centrifuged briefly to spin down the contents and eliminate any air bubbles. qPCR was performed in "standard cycling - mode using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) set as following:

Table 2.3 qPCR steps

Step	Temperature	Duration	Cycles
UDG activation	50°C	2 minutes	Hold
DNA polymerase	95°C	2 minutes	Hold
Denature	95°C	15 seconds	40
Anneal/extend	60°C	1 minute	40

A default dissociation step (melt curve stage) was performed directly after the real-time PCR run. The following settings were used:

Table 2.5 Dissociation curve conditions (melt curve stage)

Step	Ramp rate	Temperature	Time
1	1.6°C/second	95°C	15 seconds

2	1.6°C/second	60°C	1 minute
3	0.15°C/second	95°C	15 seconds

Table 2.6 qPCR instrument set up

Set up for instruments	
Experiment type	Standard curve
Reagent	SYBR™ Green reagents
Reporter	SYBR™
Quencher	None
Ramp speed	Standard
Melt curve ramp increment	Continuous

2.5 Statistical analysis

For calculation of the baseline and threshold cycles (Ct) for the amplification curves, the instrument software (Steponeplus) was used.

The threshold cycle (Ct) is the cycle number at which the fluorescence generated within a reaction crosses the threshold line, usually set sufficiently above background fluorescence (Applied Biosystems). The Ct is a relative measure of the target concentration in the PCR reaction; high Ct indicates a low concentration of template, whereas low Ct indicates a high concentration of target molecules in the sample.

If the gene did not amplify during a qPCR reaction all three times, the Ct value of such an experimental reaction was set to 0, indicating that there was no expression of this gene in the respective experiment.

Relative expression was quantified using the 'Comparative Quantitation' method or $2^{-\Delta\Delta Ct}$ method[86], similar to the previously published ΔΔCt method[87]. ΔΔCt enables the dynamic amplification efficiency of each run to be determined by taking into account the take-off point, similar to the Ct (Applied Biosystems). The software automatically calculates the take-off point for each amplified target. Relative expression was quantified as follows:

To calculate the ΔCt value or normalized value (i.e., relative expression to GAPDH), the means of triplicate Ct values for GAPDH were subtracted from the means of triplicate Ct values for each target using formula (1):

(1) ΔCt = Ct TARGET – Ct GAPDH.

To calculate the ΔΔCt value, the ΔCt value of the control sample was subtracted from the ΔCt of the experimental sample using formula (2):

(2) ΔΔCt =ΔCt CONTROL - ΔCt SAMPLE

Relative quantification (RQ) is calculated by using formula (3):

(3) $RQ=2^{-\Delta\Delta Ct}$

ΔΔCt presents the size and direction of the gene expression change. A ΔΔCt < 0 indicates an overexpression of the target gene as compared to the control, whereas a ΔΔCt > 0 indicates an underexpression. ΔΔCt was used for correlation with other linear parameters, such as laboratory markers and postoperative survival.

RQ is the relative fold change; an RQ of 2 describes an 2-fold increase of the target gene as compared to control. If RQ < 1, the gene was down-regulated. If RQ > 1, the gene was up-regulated. RQ was used for comparison between different experiments and genes.

Data analysis including statistics and graphs were performed with Microsoft office GraphPad Prism 8.0 software. Due to the small number of experiments normal distribution of data was assumed. The statistical significance of RQ or fold-change was calculated using the Student’s t-test. A P value of less than 0.05 was considered statistically significant. The calculation of ΔΔCt and RQ of control heart was used in the same methods.

3. Result

3.1 Postoperative survival

Survival times of 16 xenotransplanted baboons are shown in Figure 3.1. The longest survival was 195 days (PAV16). The shortest survival was one day (PAV2, PAV4, and PAV6). The median survival of baboons was 61.3 days. The maximum survival time was only 30 days in the first 8 experiments; the longest survival time was seen in the last two experiments.

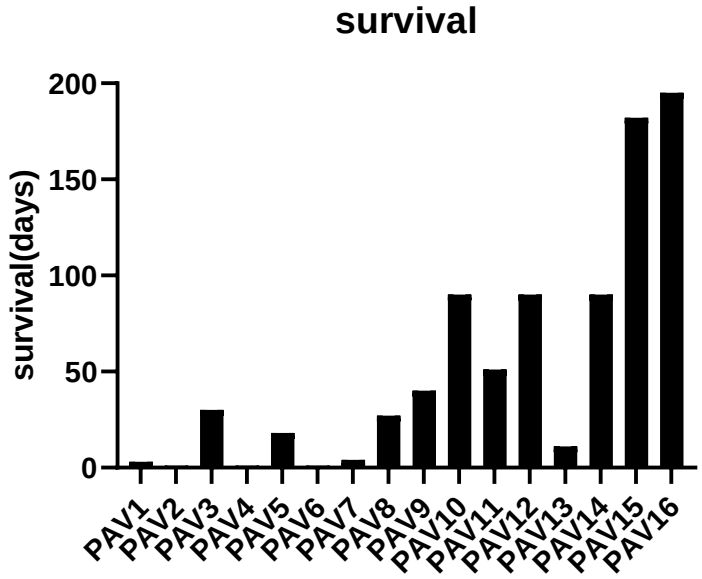

Figure 3.1 Postoperative survival after orthotopic cardiac xenotransplantation

3.2 Inflammatory markers in peripheral blood

Laboratory results of CRP, IL6, white blood cell count, lymphocytes and neutrophils are shown in Figure 3.2. On the last day of the experiments, the mean CRP concentration was 2.1 ± 2.1 mg/dl. High levels of CRP were observed in PAV 1 (7.5 mg/l) and PAV 11 (6.1 mg/l), all other recipient baboons had a normal CRP concentration (＜5 mg/l). Mean serum concentration of IL-6 was 275.6 ± 474pg/ml (Figure 3.2.b). The normal range of IL-6 in human adults is 5-15 pg/ml.

Mean level of white blood cell (WBC) count was 8.1 ± 6 $\times 10^9$/L (Figure 3.2.c), absolute neutrophil count was 5.8 ± 5 $\times 10^9$/L (Figure 3.2.d), absolute lymphocyte count was 1 ± 0.9 $\times 10^9$/L (Figure 3.2.e). Blood cell counts were all in the normal

human ranges (WBC count: 4.5-11.0 × 10^9/L; Neutrophils count: 2.0-7.0×10^9/l; lymphocytes count:1.0-3.0×10^9/l). PAV1, PAV4, PAV6, PAV7, and PAV 8 had abnormally high levels of WBC count and Neutrophils count. PAV3 and PAV14 had abnormally low levels of both. There were differences between the first 9 and last 7 experiments in WBC count (p=0.01) and Neutrophils count (p=0.008).

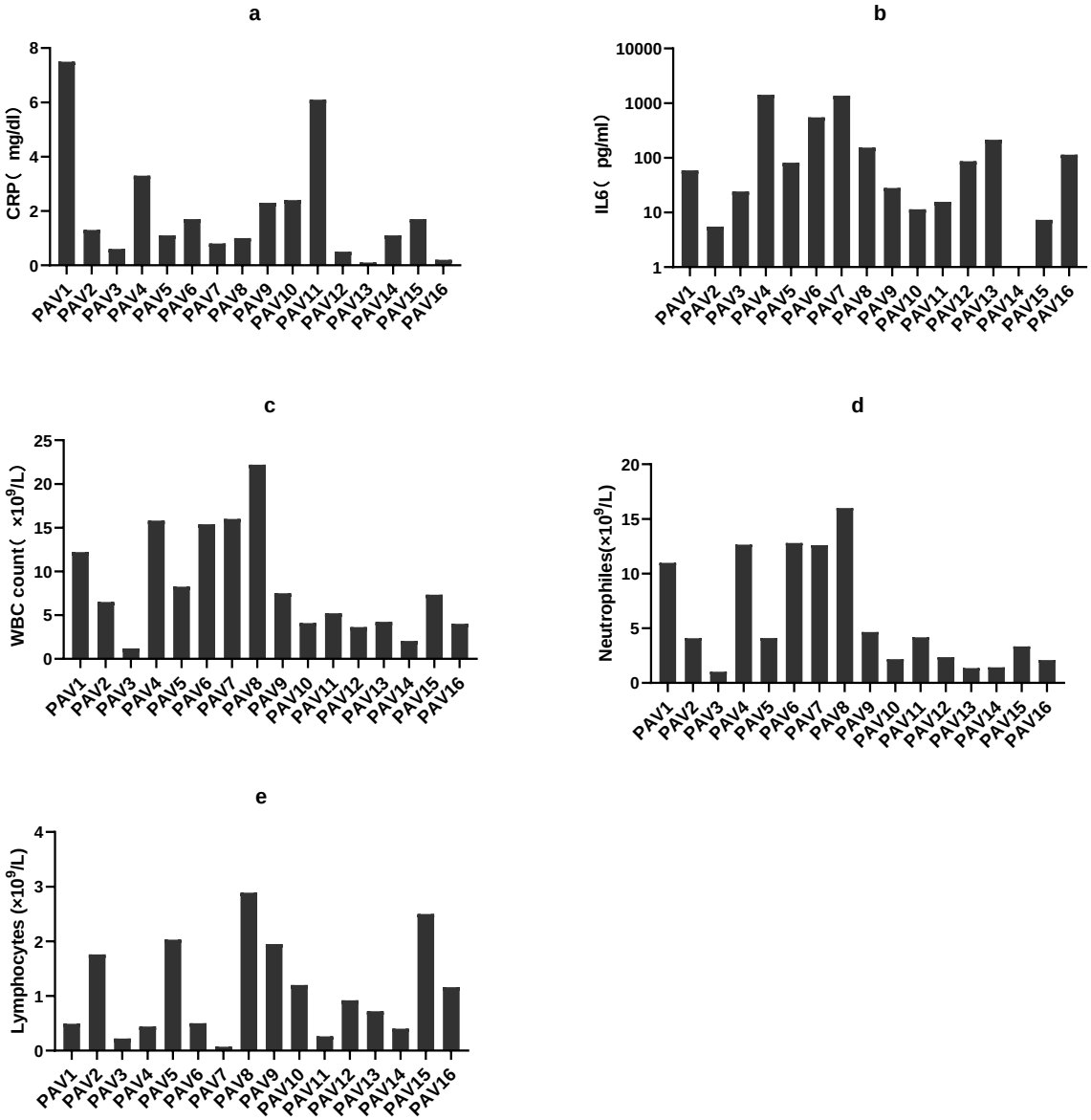

Figure 3.2 Laboratory results of xenotransplant recipients on the last day of the experiment. Serum concentration of CRP (a), IL6 (b), WBC count (c), neutrophil count (d), lymphocyte count (e).

3.3 Markers of myocardial damage in peripheral blood

Mean creatine kinase (CK) concentration was 6599 ± 9708 IU/L (Figure 3.3.a), which was higher than the normal range (5 to 25 IU/L). There was a difference between the first 9 and the last 7 experiments in serum CK values (p=0.027). The mean serum concentration of Troponin T was 2.53 ± 5.79 ng/L (Figure 3.3.b). The normal range is less than 0.015 ng/L.

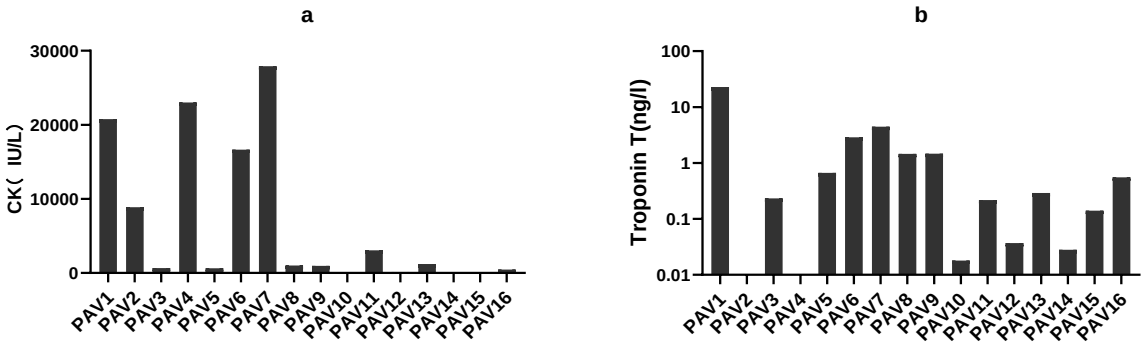

Figure 3.3 Laboratory results of xenotransplant recipients on the last day of the experiment. The serum concentration of CK (a) and troponin T (b)

3.3 Inflammatory gene expression in the graft

RQ of all measured inflammatory genes (IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, IL-1α, and IL-1β) are shown in Figure 3.4. Comparative analysis of gene expression (Figure 3.5) between left and right ventricle revealed no significant difference in all experiments (P＞0.05). The average RQ of left and right ventricle was calculated to present fold changes in each graft gene expression (Figure 3.6).

In figure 3.7, the gene expression level of each experiment is depicted. There were no statistically significant difference of IL-4 (1.11 ± 2.46, p=0.861), IL-5 (14.11 ± 31.05, p=0.102), IL-6 (19.10 ± 49.95, p=0.158), IL-10 (1.37 ± 2.53, p=0.248), TNF-α (6.63 ± 12.84, p=0.089), IL-1α (1.38 ± 2.44, p=0.538) and IL-1β (1.59 ± 2.50, p=0.285). Only IL-8 (4.28 ± 5.54, p=0.025) was upregulated among all 8 inflammatory genes (Figure3.6).

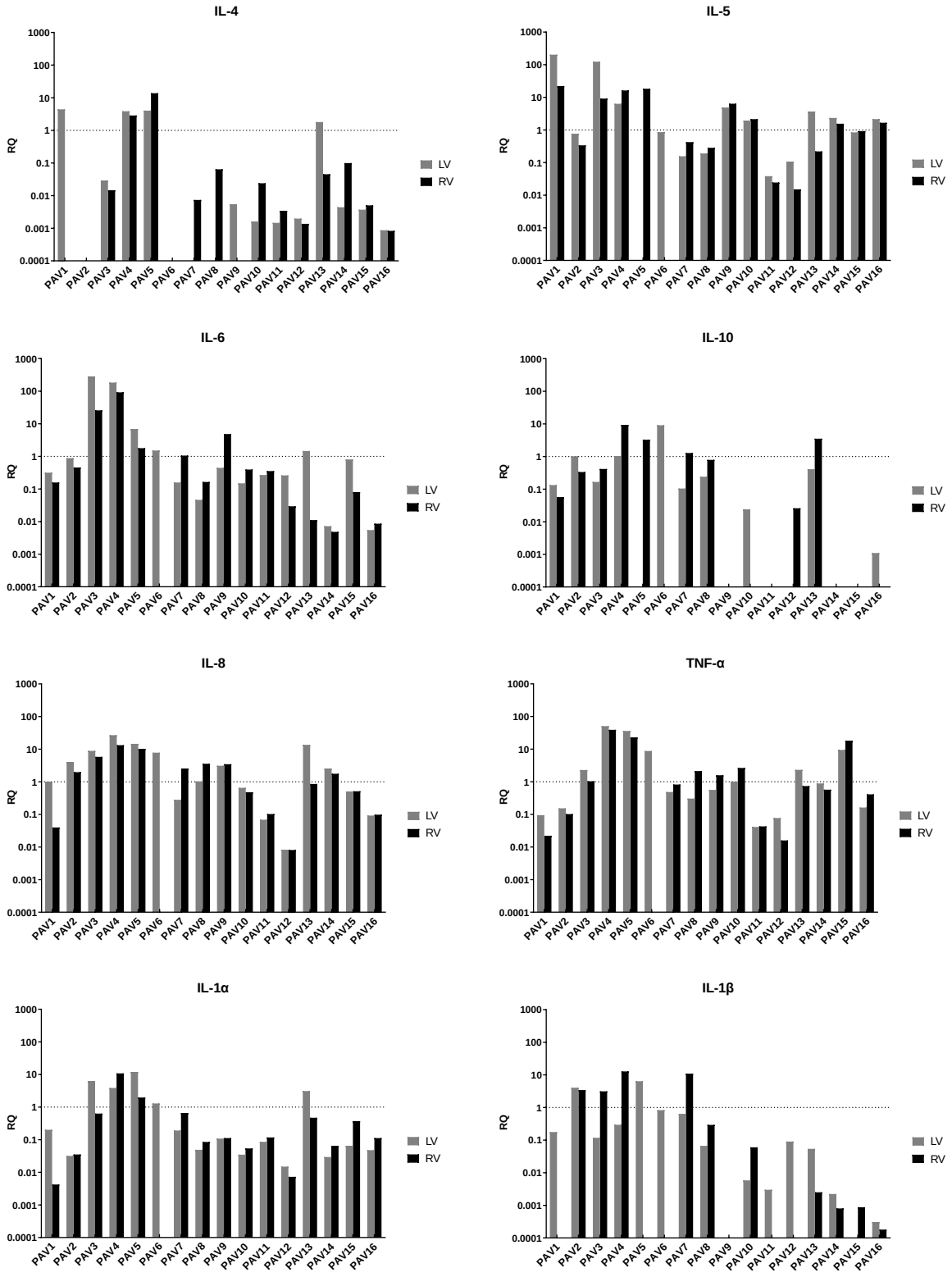

Figure 3.4 Quantification of 8 different gene expression of left and right ventricle (left is grey; the right is black) in the graft. Absence of column indicates no gene expression.

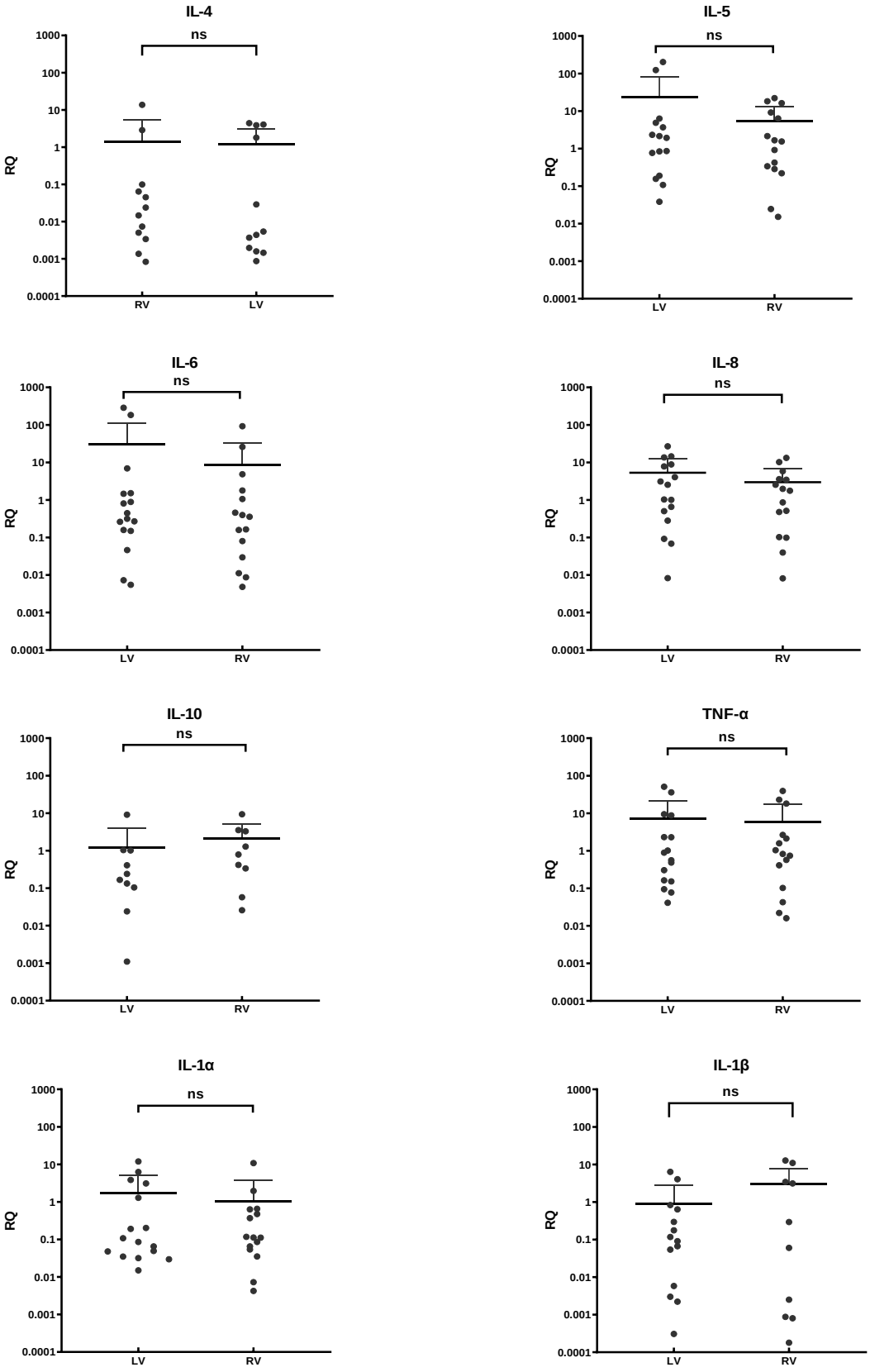

Figure 3.5 Gene expression of the graft after cardiac xenotransplantation. There were no significant differences between the left and right ventricles.

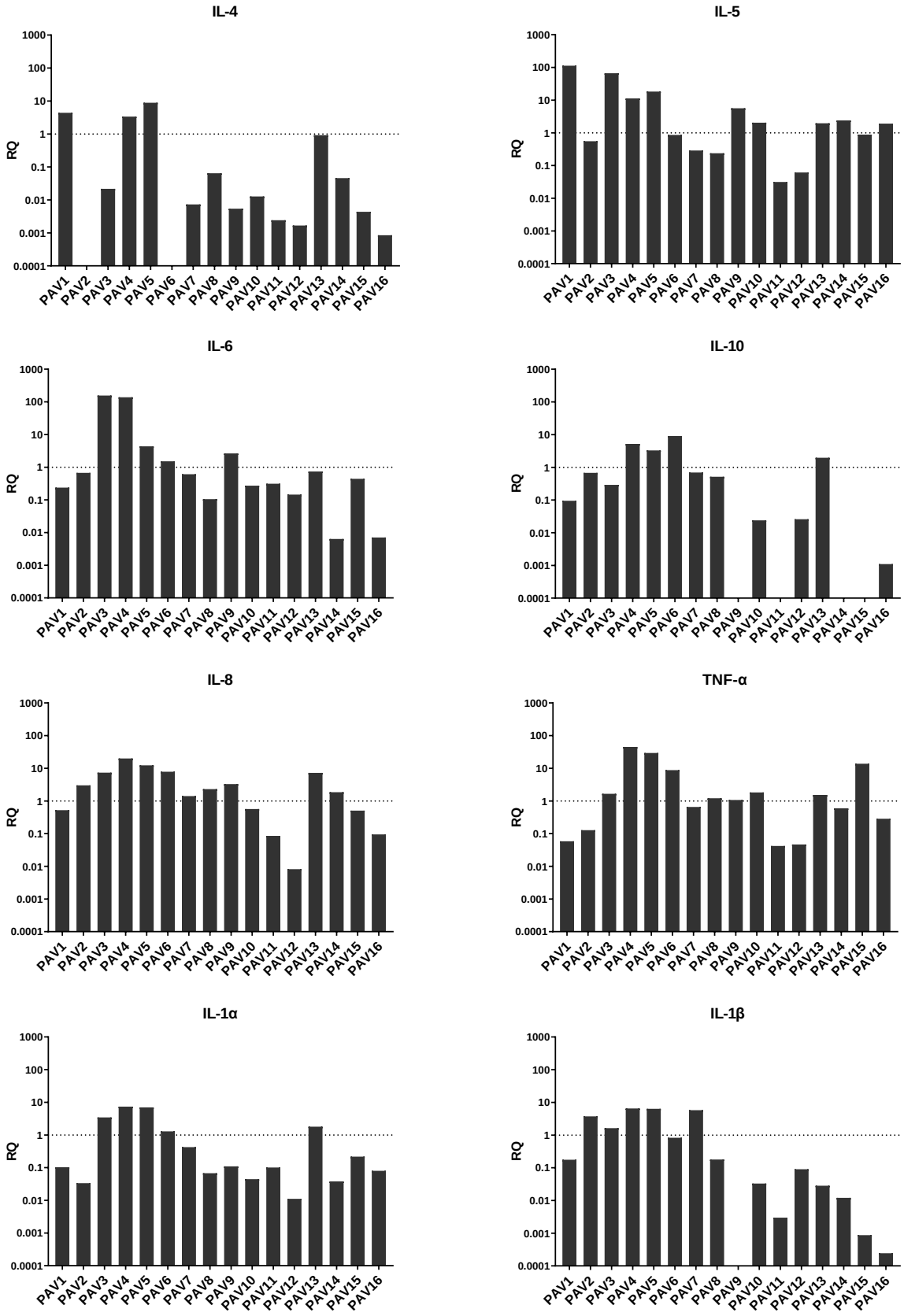

Figure 3.6 Gene expression of the graft after cardiac xenotransplantation. Mean RQ of each gene plotted. Absence of column indicates no gene expression.

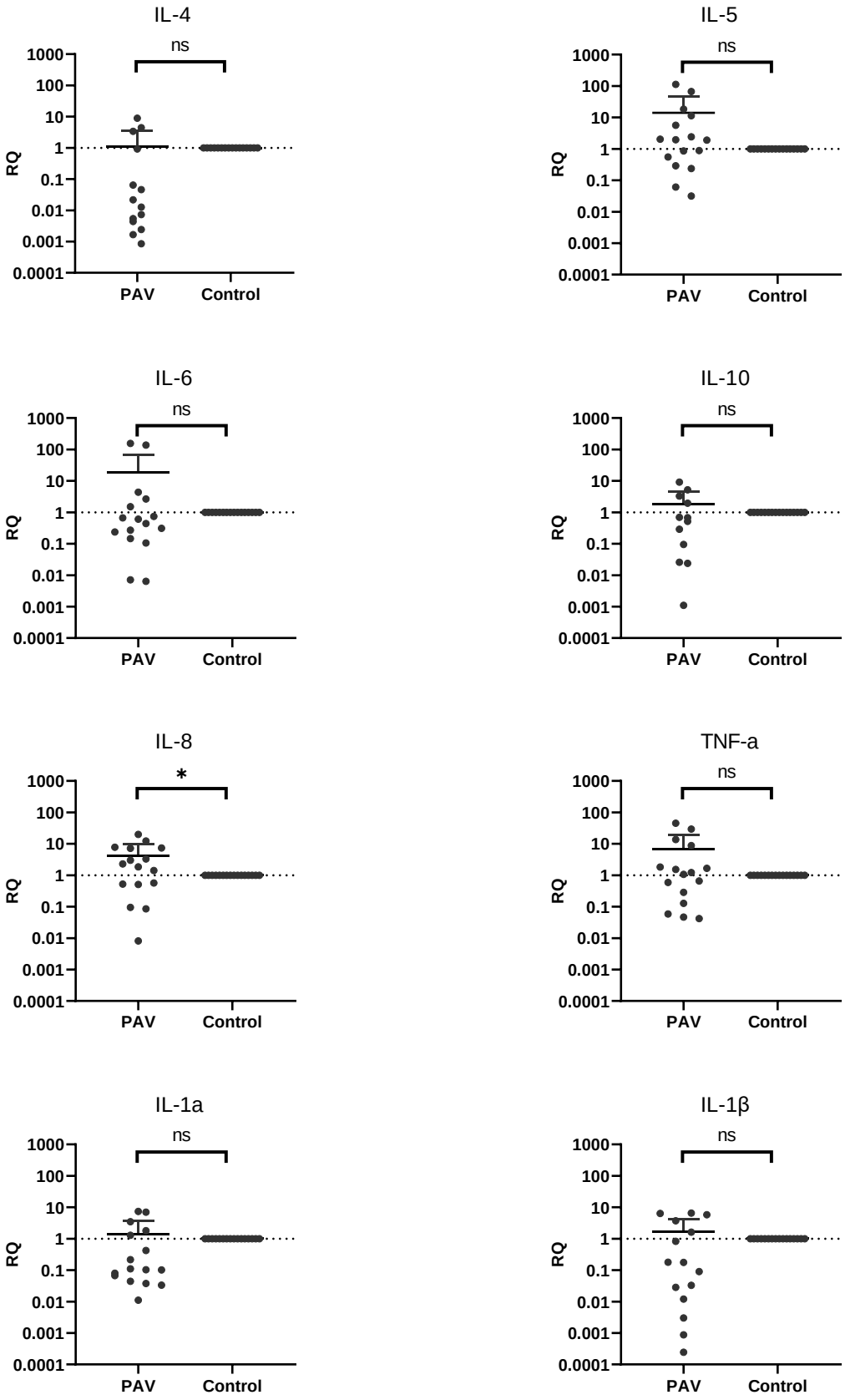

Figure 3.7 Gene expression of the graft after cardiac xenotransplantation. Comparison to control for each gene.

The colour map (Figure 3.8) displays the fold changes of each gene in each experiment more clearly. The mean fold change of gene expression was calculated for each experiment (Figure 3.9). In most experiments there were no significant changes (Figure 3.10): PAV1 (14.79 ± 39.59, p=0.35), PAV2 (1.10 ± 1.44, p=0.85), PAV3 (29.56 ± 55.68, p=0.17), PAV4 (29.60 ± 45.86, p=0.10), PAV6 (3.77 ± 4.01, p=0.07), PAV7 (1.24 ± 1.89, p=0.73), PAV8 (0.59 ± 0.79, p=0.16), PAV9 (2.12 ± 2.17, p=0.43), PAV10 (0.60 ± 0.85, p=0.21), PAV13 (2.02 ± 2.21, p=0.21), PAV14 (0.71 ± 1.01, p=0.28), PAV15 (2.26 ± 5.08, p=0.57). PAV5 (11.24 ± 8.80, p=0.0053) was the only experiment in which the mean of all observed gene expressions was upregulated. PAV11 (0.08 ± 0.11, p<0.0001), PAV12 (0.05 ± 0.05, p<0.0001) and PAV16 (0.30 ± 0.66, p= 0.0092) were three experiments, in which the mean of all gene expressions was downregulated.

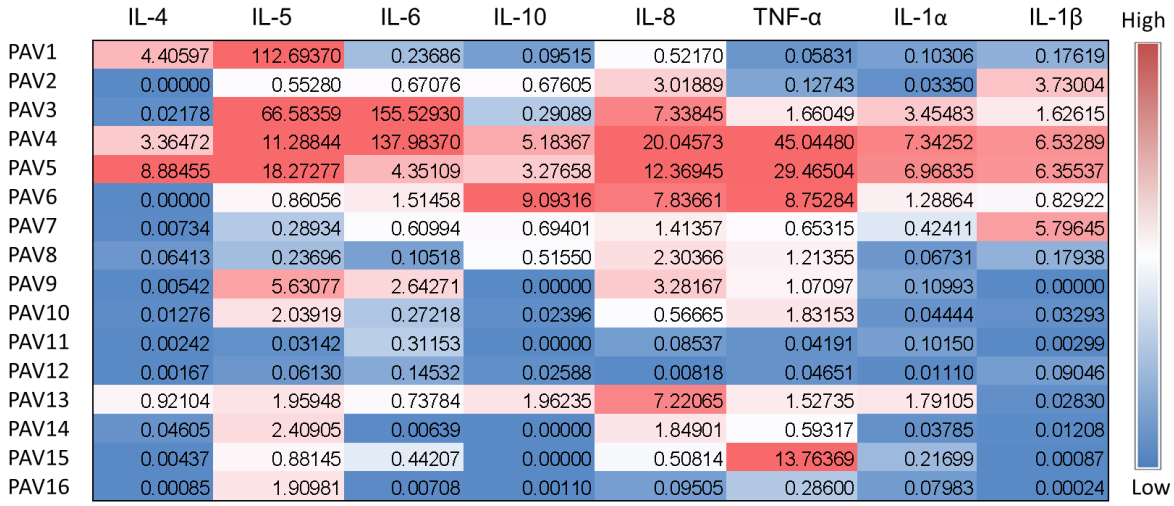

Figure 3.8 Colour map of gene expression. Red colour indicates gene upregulation, blue colour gene downregulation.

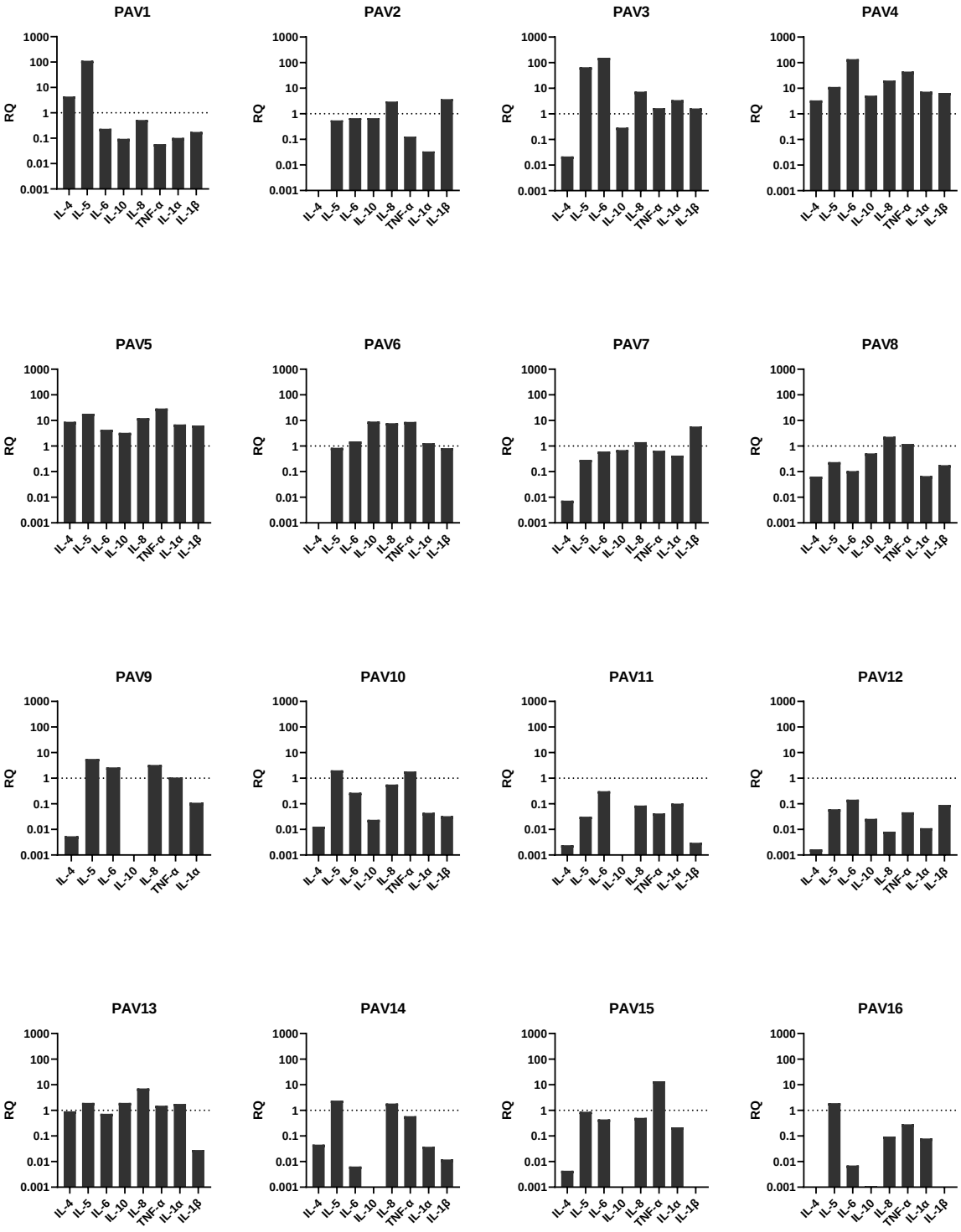

Figure 3.9 Gene expression of the graft after cardiac xenotransplantation. Mean RQ of each experiment plotted. Absence of column indicates no gene expression.

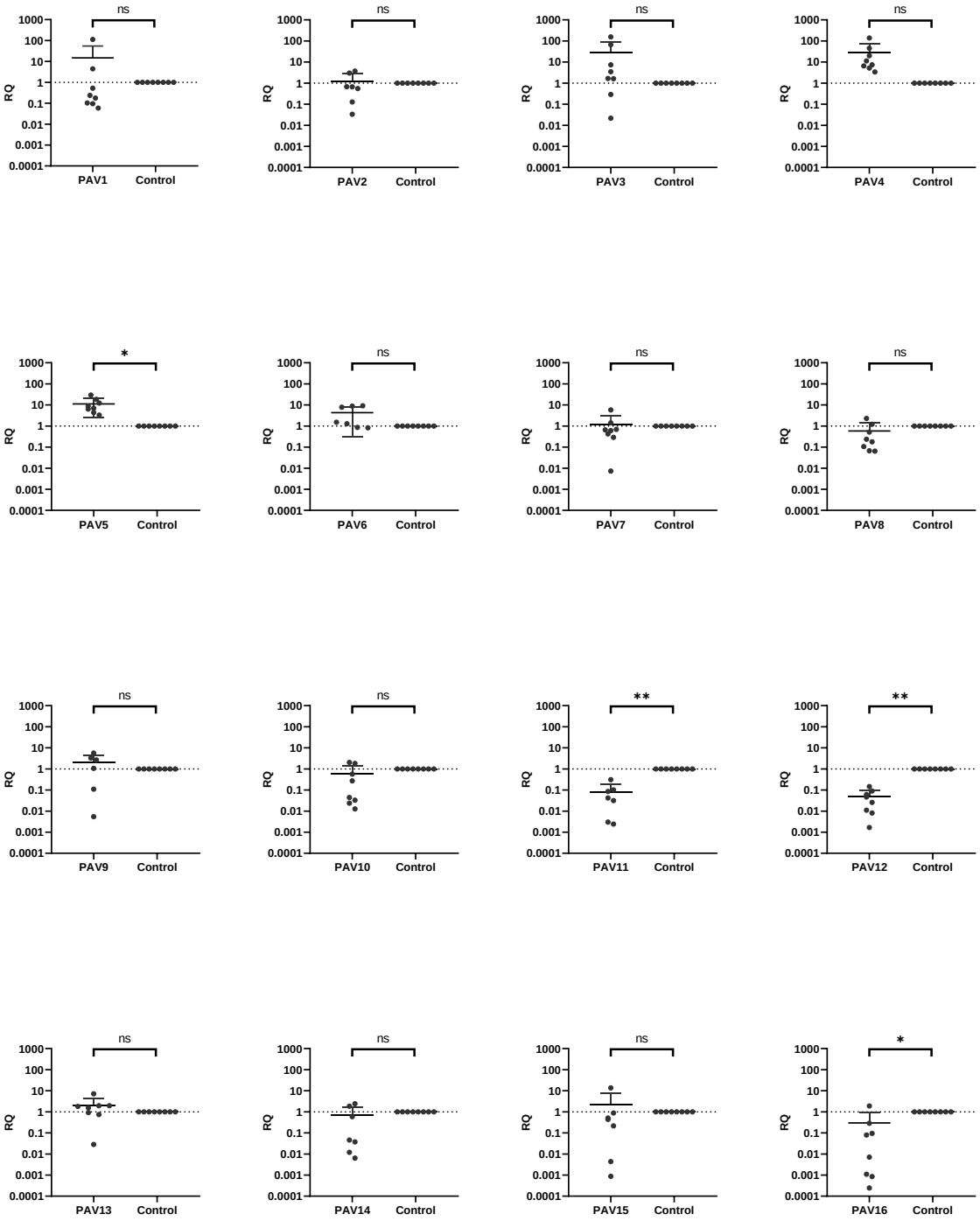

Figure 3.10 Gene expression of the graft after cardiac xenotransplantation. Comparison to control for each experiment. (*$p<0.05$, **$p<0.001$)

3.4 Correlation between gene expression and survival

There was a significant correlation between ΔΔCt of all genes and postoperative survival (figure 3.11). There were significant correlations between survival time and ΔΔCt of IL-4 (r=-0.52, *p*=0.048), IL-10 (r=-0.58, *p*=0.037) and IL-1β (r=-0.65, *p*=0.007) (figure 3.12).

Grouped by more or less than 40 days of survival, the differences of these experiments were examined by t-test. Except for IL-5 and TNF-α, significant differences were found in the other 6 genes (Figure 3.13): IL-1β (0.94±5.69，-7.89±3.22， *p*=0.004), IL-4 (0.144±7.45，-7.55±2.16，*p*=0.021), IL-6 (1.72±5.11，-3.32±2.96，*p*=0.034), IL-8 (3.12±4.89，-1.89±2.96，*p*=0.029), IL-10 (1.30±5.22，-6.99±2.93，*p*=0.026), and IL1-α (0.39±5.18，-4.14±1.32，*p*=0.041).

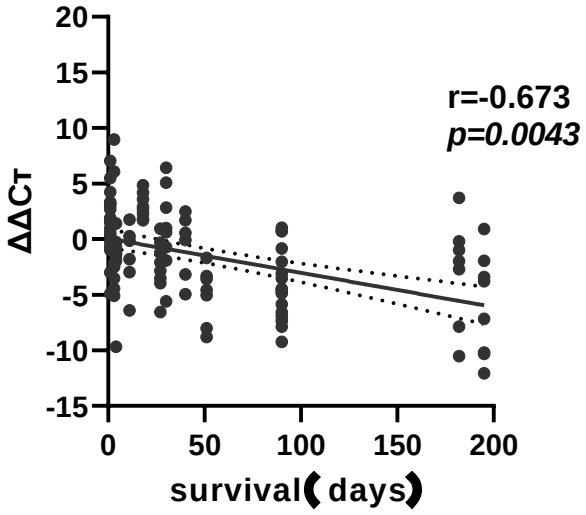

Figure 3.11 Correlation between all gene expression ΔΔCt and survival.

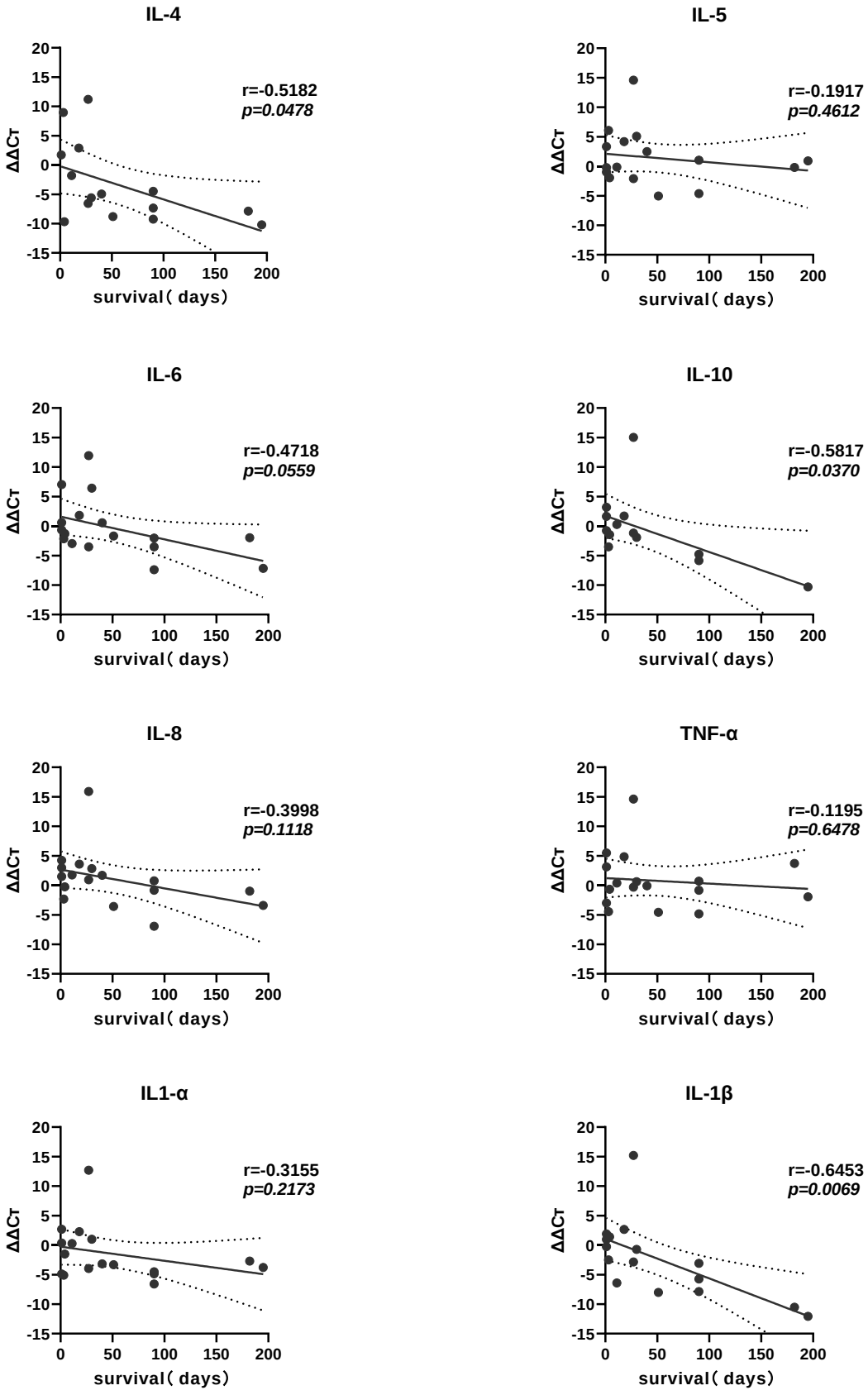

Figure 3.12 Correlation between each gene expression ΔΔCt and survival.

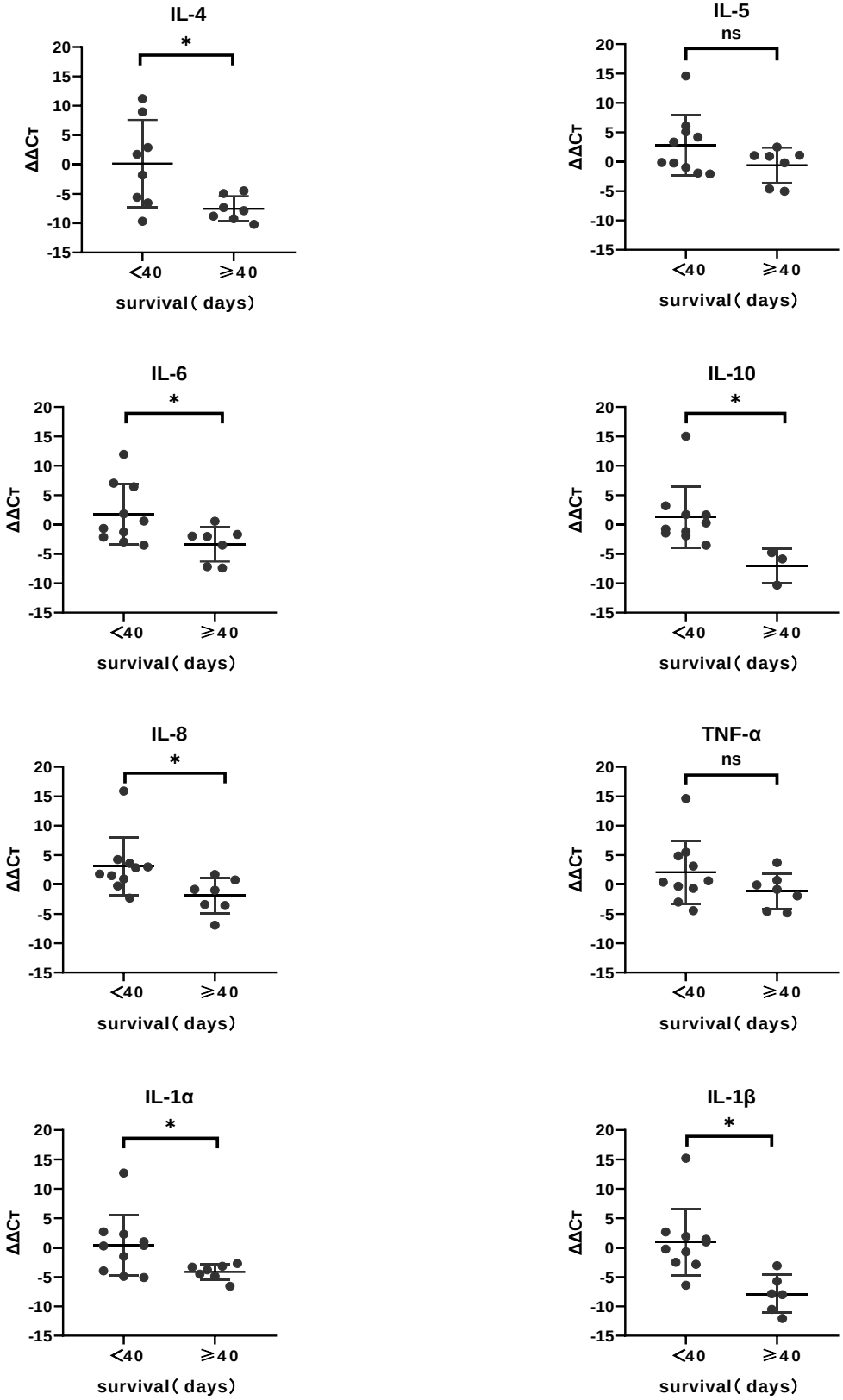

Figure 3.13 Gene expression of the graft after cardiac xenotransplantation. Groups differ by survival of more or less than 40 days. (*p＜0.05)

3.5 Correlation between gene expression and laboratory markers

Correlation of gene expression with laboratory results was tested, there were two significant results. IL-5 gene expression strongly correlated with troponin T (r = 0.8059; *p* =0.0005) (Figure 3.14.a). IL-1β fold change correlated with IL-6 serum concentration (r = 0.6581; *p* =0.0105) (Figure 3.14.b).

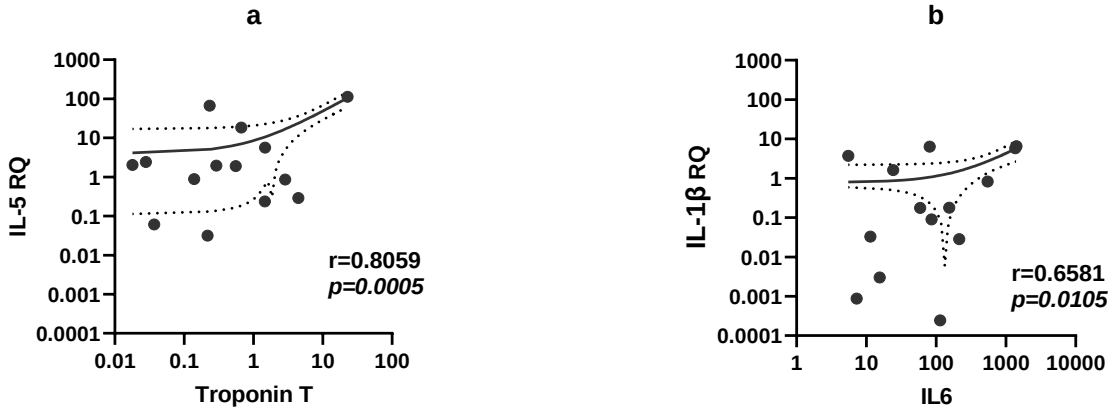

Figure 3.14 Correlation between IL-5 gene expression and serum concentration of troponin T (a); the correlation between IL-1βgene expression and serum concentration of IL-6(b).

Table 3.1 Correlation coefficient r of lab results and gene expression.

r	CRP	CK	Troponin T	WBC	IL6	Neutrophils	Lymphocyte
IL-4	0.237	0.221	0.361	0.199	0.069	0.174	0.106
IL-5	0.538	0.266	**0.806**	-0.009	-0.160	0.100	-0.275
IL-6	-0.024	0.182	-0.113	-0.025	0.346	0.037	-0.336
IL-10	0.031	0.364	-0.103	0.389	0.422	0.404	-0.187
IL-8	-0.080	0.303	-0.176	0.265	0.518	0.251	-0.119
TNF- α	0.051	0.283	-0.153	0.305	0.500	0.261	0.074
IL-1 α	-0.063	0.193	-0.149	0.141	0.405	0.115	-0.087
IL-1 β	-0.100	0.539	-0.014	0.345	**0.658**	0.320	-0.066

Table 3.2 Correlation *p* of lab results and gene expression.

p	CRP	CK	Troponin T	WBC	IL6	Neutrophils	Lymphocyte
IL-4	0.414	0.448	0.226	0.494	0.824	0.552	0.719
IL-5	0.032	0.319	**0.0005**	0.975	0.568	0.714	0.302
IL-6	0.931	0.501	0.700	0.926	0.207	0.891	0.204
IL-10	0.923	0.245	0.777	0.212	0.172	0.193	0.561
IL-8	0.768	0.254	0.546	0.322	0.048	0.349	0.660
TNF- α	0.851	0.289	0.600	0.250	0.058	0.328	0.787
IL-1 α	0.818	0.474	0.611	0.602	0.134	0.671	0.748
IL-1 β	0.722	0.038	0.965	0.208	**0.011**	0.244	0.815

3.6 Pro-inflammatory and anti-inflammatory

There was no statistically significant difference between all pro- and anti-inflammatory cytokine gene expressions together (Figure 3.15). There were significant correlations between ΔΔCt of both pro- and anti-inflammatory genes and days of survival (Figure 3.16).

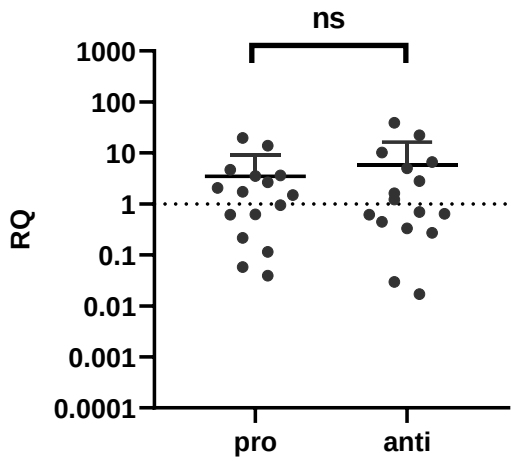

Figure 3.15 No significant difference was found between pro-inflammatory and anti-inflammatory gene expression.

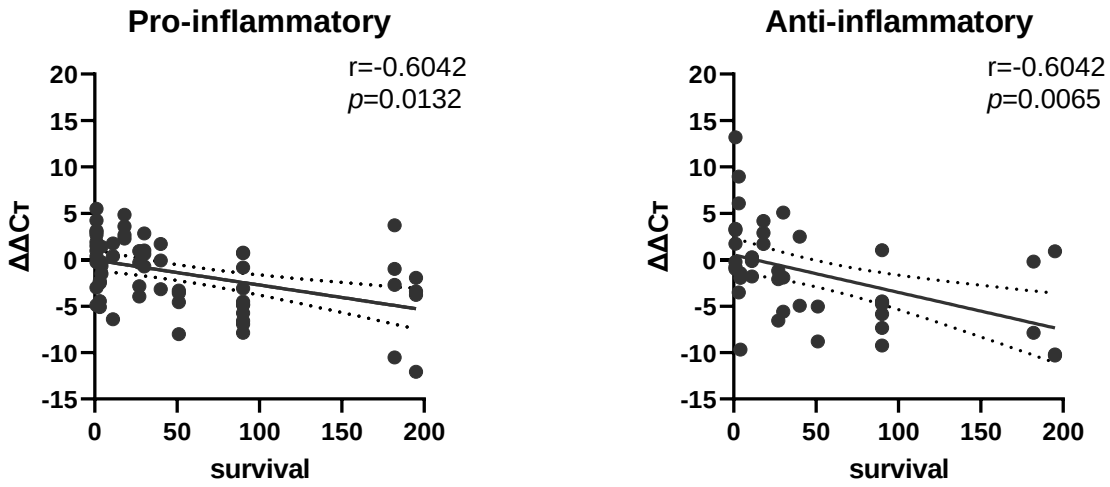

Figure 3.16 Correlation between survival and pro-inflammatory and anti-inflammatory gene expression.

3.7 Acute-inflammatory and chronic-inflammatory

There was no significant difference between acute and chronic inflammatory cytokines (Figure 3.17). Gene expression of both acute inflammatory (r=-0.604, *p*=0.035) and chronic inflammatory (r=-0.604, *p*=0.007) cytokines correlated with survival time (Figure 3.18).

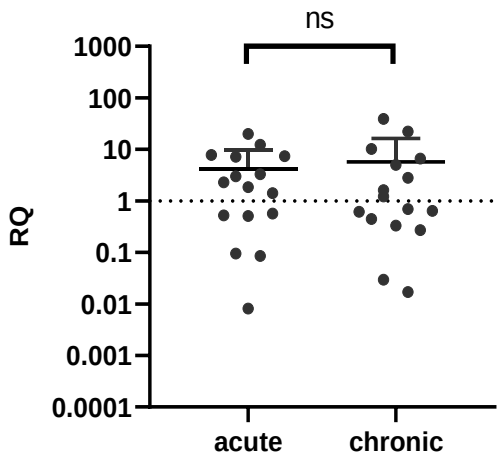

Figure 3.17 No significant difference was found between the acute-inflammatory and chronic-inflammatory gene expression.

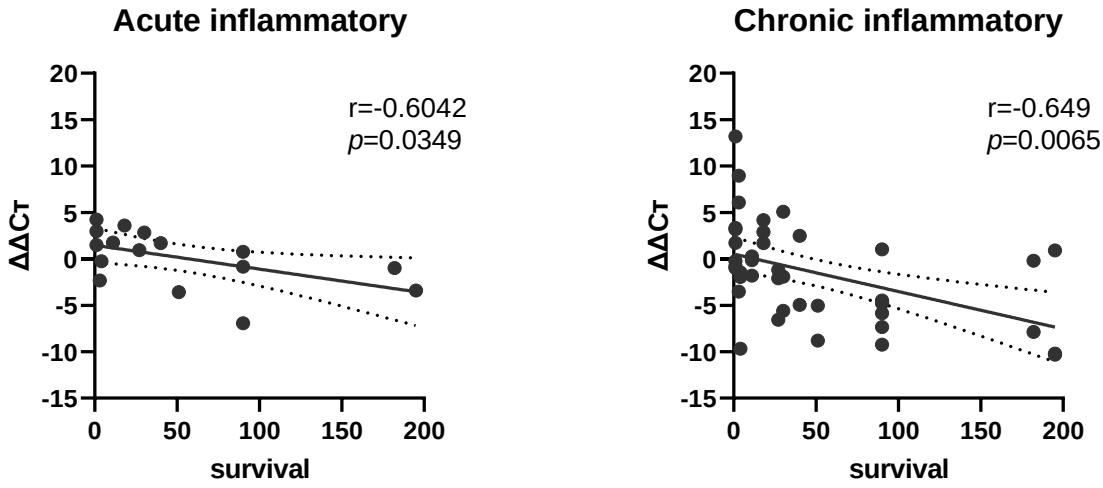

Figure 3.18 Correlation between acute inflammation and chronic inflammation gene expression and survival.

3.8 Heart preservation

Grouped by different heart preservation methods during transplantation, IL-5(47.78±52.06， 2.882±5.091， *p*=0.0068) and IL-6(73.61±84.77， 0.9288±1.320，

p=0.0063) gene expression were significantly different (Figure 3.19). No difference in RQ was found in other inflammatory genes between these groups.

3.9 Temsirolimus treatment

Temsirolimus was administrated in experiments from PAV10 to PAV16. Comparing these experiments, the expression of the inflammatory gene IL-1β (3.153±2.789，0.02398±0.03214，p=0.0112) was significantly reduced. In contrast, there was no significant difference in fold change of other genes before and after using temsirolimus (Figure 3.20).

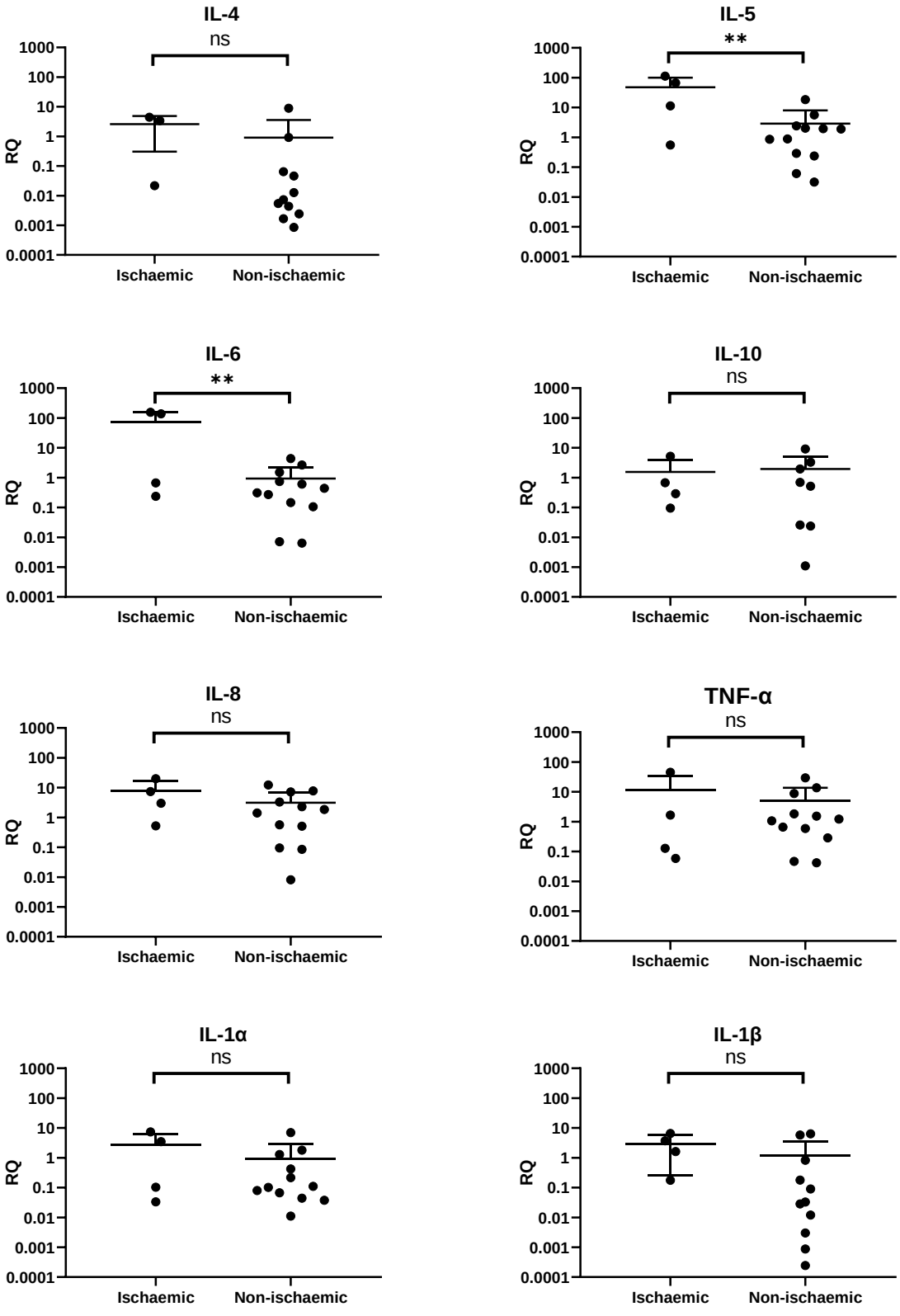

Figure 3.19 Experiments grouped by ischemic or non-ischemic heart preservation, significant differences were found in IL-5 and IL-6 gene expression. (**p＜0.01)

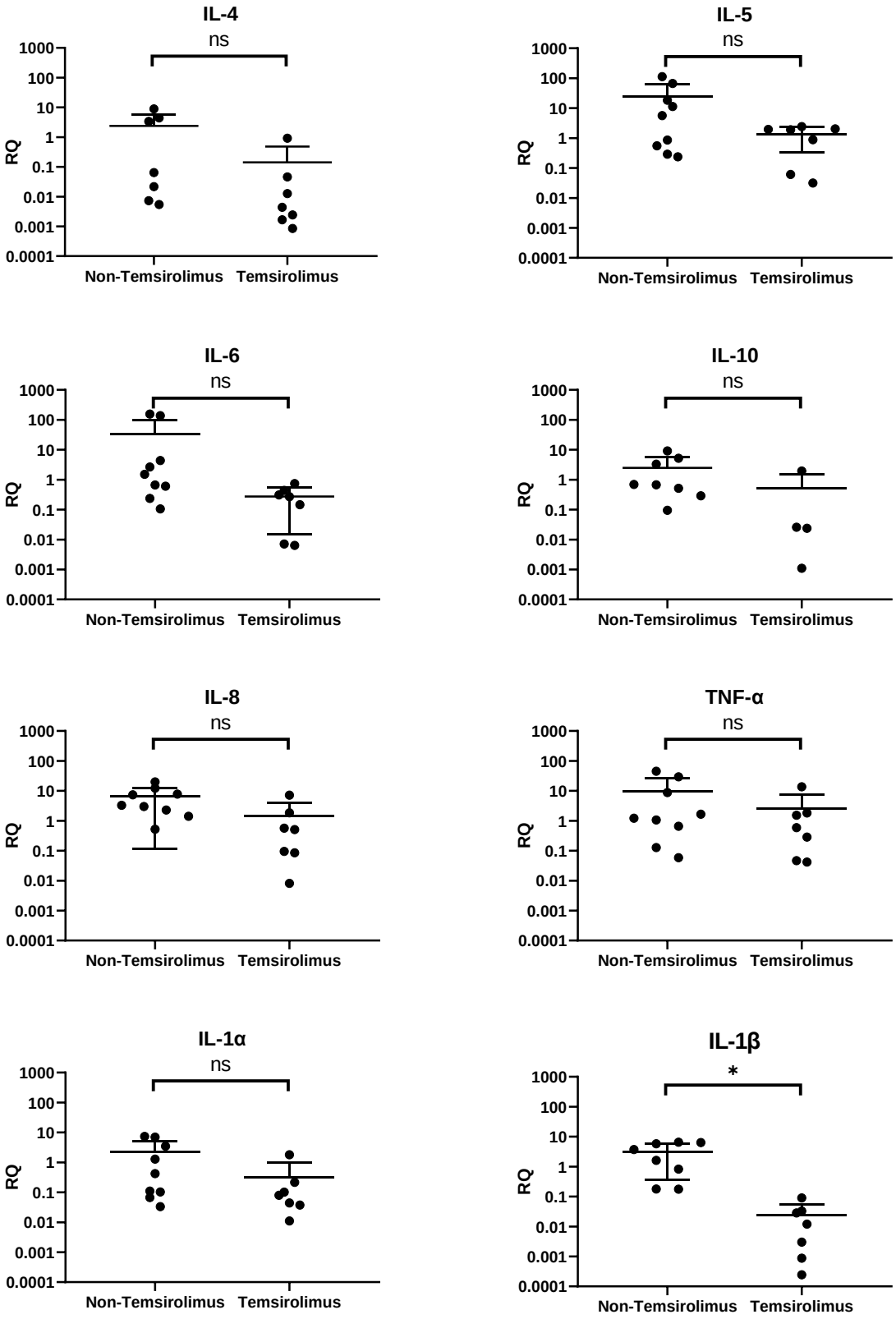

Figure 3.20 Only IL-1β gene expression was found a significant difference between non-temsirolimus and temsirolimus groups. (*p＜0.05）

4. Discussion

Some of the results from our pig-to-baboon orthotopic heart transplantation experiments have been published elsewhere, especially with respect to the overall survival of the recipients and the specific experimental model [88]. Briefly, the application of non-ischemic heart preservation method during the transplantation resulted in the first milestone of our experimental progress, preventing early perioperative graft failure. Xenograft overgrowth, which was shown to cause graft failure within the first postoperative weeks, could be inhibited mainly by adding another immunosuppressive agent, temsirolimus, to the original treatment protocol, with addition of an antihypertensive treatment regimen and the fast tapering down of corticosteroids. Thus, consistent survival times of more than 90 days – in two cases more than 6 months – could be achieved. These results not only bring xenotransplantation closer to the clinical application but may also have beneficial impact on human allotransplantation.

4.1 Systemic inflammatory markers and cardiac markers

Just as researchers gradually overcome the problem of graft rejection and other barriers, the inflammatory response after surgery turns into one of the puzzles that need to be solved. Taking together clinical and experimental results, we are aware of the inflammatory response that is triggered after cardiac xenotransplantation [64]. There is increasing evidence of a sustained state of systemic inflammation following xenotransplantation of pig-to-nonhuman primate [89].

CRP is the most common systemic inflammatory marker measured in clinical practice. In only two experiments the serum CRP was elevated on the last day of the study, whereas CRP was within normal ranges in all other experiments. These observations indicate that in most experiments no major systemic inflammation was present at that time. In the case of CRP elevation, it is unclear whether the development of the inflammatory response was caused by a primary xeno-immune response or a secondary response to a surgical injury.

WBC count is, like CRP, an important clinical marker of inflammation. White blood cells, including lymphocytes and neutrophils, recognize and destroy invading pathogens, such as bacteria. A high level of WBC may imply a bacterial infection but

also a state of stress, trauma or allergy. In our recipients, however, the absolute WBC, Lymphocyte and neutrophil counts on the last day were normal, except for PAV4, PAV6, PAV7, and PAV8. In the longest surviving animals PAV10, PAV12, PAV15 and PAV16 WBC counts were normal. We can conclude that there was no systematic inflammation in these xenograft recipients before euthanasia, especially no strong response in the peripheral blood. Another possible explanation is that we administrated full IS regimen in last 7 experiments, which inflammatory cells were probably suppressed in these recipients.

IL-6 is a multifunctional cytokine secreted by various cells, such as T cells, B cells, monocytes, fibroblasts, endothelial cells, and cardiac myocytes[90]. This means that IL-6 in peripheral blood may come from the donor's heart or from the recipient itself. We used human test reagents in the laboratory examination, which detect IL-6 of both species in serum. Therefore, we cannot rely on the level of IL-6 serum concentration to discriminate between the inflammatory response of the whole body or the porcine heart itself. However, the IL-6 gene expression values of the samples taken from myocardial tissue should preferably represent the level of the inflammatory response of the xenograft itself. In addition, we did not find any correlation between IL-6 RQ and serum concentration (see result 3.5), which supports this hypothesis. In future research, test kits specific for pigs should be used to search for a correlation between pig IL-6 in the serum and gene expression in the porcine heart.

When we correlated the fold change of gene expression and laboratory results, only two pairs showed significant correlations. IL-5 and Troponin T have a positive correlation (r= 0.806, *p*= 0.001). Troponin T is a cardiac-specific molecule produced after myocardial cell injury and released into the systemic circulation. In a previous study, a correlation between troponin T and systemic inflammation markers in patients with critical illness has been reported [91]. One possible explanation is a troponin T release from cardiomyocytes during systemic inflammation. However, we did not find any associations with other inflammation markers, such as IL-6. Because both troponin and inflammatory markers have a concentration curve over postoperative survival time in the body, it is possibly inaccurate to only use the results at the end of the experiment to describe the relationship between them. For a

more profound analysis, continuous assessment of serum concentrations and sequential biopsies for gene expression analysis would be necessary.

We calculated the correlation of the serum concentration of IL-6 with the expression of each inflammatory gene. Only IL-1β expression proved a positive association with IL-6 serum concentration on the last day of experiments. This indirectly indicates that in our experiments the systemic inflammation of the recipient animal is not caused by the heart. IL-1β and IL-6 both play important roles in inflammation [92, 93]. The correlation between heart IL-1β and serum IL-6 is not certain to be caused by the same inflammatory stimuli. In addition, we have not found any relationship between IL-1β and other systemic inflammation makers.

4.2 Inflammatory gene expression in the graft

It is worth noting that there were no differences between the left and right ventricles. Likewise, we didn't find any differences in the gene expression in samples from the left or right ventricle. We calculated mean values of left and right ventricles to represent the fold change of the genes in our samples.

With regard to fold changes of the different genes (Figure 3.5), only IL-8 was up-regulated, all other 7 genes were normally expressed. We can conclude that the pig-to-baboon xenotransplanted heart does not show a severe inflammatory response at the end of the experiment. One reason may be the administration of anti-inflammatory agents beneficial to prevent or control inflammation. In this respect, the IS based on CD40/CD40L costimulation blockade in combination with genetic modification of the donor's heart inhibited a relevant inflammation.

In Ezzelarab's studies, interestingly, blood IL-8 levels were not elevated after cardiac xenotransplantation [42, 89]. Their results show IL-8 levels were elevated with no IS and reduced with IS. Their other study indicated that post-transplant levels of IL-8 were elevated when no anti-inflammatory agents were administered [64]. In our experiments, we have administrated IS and anti-inflammatory agents, but IL-8 was still upregulated. One reason for these contrasting results may be that we measured the expression of IL-8 directly in heart tissue, which may represent the inflammatory response of the heart itself. Ezzelarab et al detected IL-8 in the blood, which could be a manifestation of systemic inflammation.

IL-8 is a potent pro-inflammatory chemokine that plays a primary role in recruiting and activating neutrophils in a variety of contexts during inflammation. Research by French et al implies that porcine and human IL-8 activate human neutrophils and boost adhesion to endothelial pig aortic cells [63]. In their experiments, Reparixin (IL-8/ CXCL8 receptor blocker) was used to inhibit the IL-8 pathway in lung xenotransplantation experiments. To our knowledge, there are no studies considering this aspect of heart xenotransplantation. Our work implicates that IL-8 is a possible factor in mediating xenograft inflammation worth more thorough research.

In 12 of 16 experiments, there was a statistically significant difference between gene expression results from xenotransplanted porcine heart and those from non-transplanted hearts. PAV5 was the only with significantly up-regulated genes. PAV5 was an early experiment when the non-ischemic heart preservation method just had been implemented and no temsirolimus was administrated. PAV11, PAV12, and PAV16 had down-regulated gene expressions. For these experiments non-ischemic heart preservation methods were used and both CK and troponin T were in normal ranges which proved their xenografts were well preserved. It seems probable that the combination of an optimized IS regimen with the administration of anti-inflammatory agents in these experiments was instrumental for achieving the results mentioned above.

4.3 Postoperative survival

According to our results, gene expression of inflammatory cytokines was negatively correlated with survival time. That means that these genes were overexpressed in baboons with short survival and less expressed in those with longer survival. Especially for IL-1β, the lowest ΔΔCt was measured in the two longest surviving animals: -10.53, day 182 and -12.06, day 195.

In order to compare the data, we arbitrarily grouped the experiments by survival time, less than 40 days or 40 days and more. The ΔΔCt of inflammatory genes was compared between the two groups. IL-4, IL-6, IL-8, IL-10, IL-1α, and IL-1β expression was significantly different between these groups. These results indicate that inflammatory responses, both pro-inflammatory and anti-inflammatory, were gradually reduced. These inflammatory cytokines play a key role in the defense and

repair mechanisms following surgical trauma. In other words, the inflammatory reaction gradually decreased as the postoperative rehabilitation progressed.

However, we still observed a tendency to a gradual decrease of inflammatory gene expressions after undergoing perioperative recovery. This is possibly related to the sufficient use of anti-inflammatory agents and IS therapy. Some previously findings indicate that regulating inflammatory reactions in recipients of xenografts may require extra anti-inflammatory treatment that may be crucial for the long-term survival of xenograft [64, 94]. However, at the end of the experiment, we did not observe elevated systemic or graft inflammation.

4.4 Pro- and anti-inflammatory, acute and chronic inflammatory

A sensitive balance between pro- and anti-inflammatory cytokines is preserved in a healthy condition. Such homeostasis, however, is interrupted by the surplus elevation of pro-inflammatory cytokines in stress states such as trauma [64]. When excess pro-inflammatory cytokines are released, anti-inflammatory cytokines are secreted eventually to restore the equilibrium. Our results show that pro- and anti-inflammatory gene expressions are similar at the end of the experiment. As described above, both pro- and anti-inflammatory responses gradually decreased as recovery progressed. We believe it may be related to the administrated of IS regimens and anti-inflammatory agents.

In cardiac xenotransplantation, the advancement of IS regimens and genetical modifications have improved outcomes over recent years. Many studies have emphasized that the strategy of anti-inflammatory agents is increasingly important for postoperative survival [64, 89]. But before we draw any conclusion, administration of IS and other treatments cannot be ignored. To study this more thoroughly, single variable research would be a good choice. For example, monitoring inflammatory response while changing the anti-inflammatory drug or dose in a xenograft in which immunological tolerance has been obtained. In preclinical baboon experiments this approach is difficult to realize and other models might be needed.

No statistical difference was found between acute and chronic inflammatory-related gene expressions. This might be because our recipient animals did not have severe systemic or local inflammation. We also found a correlation with survival days, which

indicates that acute inflammation and chronic inflammatory response decrease with postoperative survival time. Therefore, the early postoperative inflammatory response is most likely due to surgical trauma or acute immune response and gradually decreases with treatment and rehabilitation.

4.5 Heart preservation

The different heart preservation methods during the surgery had a significant influence on IL-5 and IL-6 expression. Gene expression for both cytokines was lower in the group of non-ischaemic perfused hearts. Myocardial ischemia is known to induce IL-6 production in human patients [95]. Similar to other inflammatory reactions, CD4+ TH1 cells may play a key role in the pathogenesis of ischemia through releasing pro-inflammatory cytokines, whereas CD4+ TH2 cells may play a protective role through anti-inflammatory cytokines such as IL-5 [96]. In our study, the samples were taken from the animal on the last day of survival. However, survival in the ischemic group was very short (PAV2, PAV4, and PAV6 were survival one day) and upregulation during the transplantation process can only be seen in this group. In further experiments, the immediate inflammatory gene expression could be measured by myocardial biopsies to prove the difference between the two different heart preservation methods.

4.6 Temsirolimus treatment

Starting with PAV 10, temsirolimus was added to maintenance immunosuppression. Temsirolimus mitigates myocardial hypertrophy in our heart xenotransplantation model [88]. In our experiments, temsirolimus reduced IL-1β expression, whereas no influence on other inflammatory genes was observed. Jia et al demonstrated that temsirolimus is able to inhibit the production and secretion of IL-1β in mice [97]. As mentioned above, IL-1β was positively correlated with survival time, thus the role of temsirolimus in this experiment may also be related to inhibition of IL-1β, not just the control of heart overgrowth.

4.7 Conclusion

Inflammation may play an important role in prolonging the survival time after cardiac xenotransplantation. We did not find intense systemic inflammation caused by inflammatory processes in the graft of 16 recipient animals. IL-8 was up-regulated

after transplantation, and likely plays a potent role in mediating xenograft inflammation. IL-4, IL-10 and IL-1β expression had a significant correlation with longer postoperative survival time. Especially IL-1β may be used as a target to reduce inflammation and increase survival. This study highlights the utility of gene expression analysis to monitor inflammation after xenotransplantation and to identify new potential markers that contribute to cardiac xenotransplantation research.

Summary

Inflammatory cytokines have important effects on organ transplantation. However, their role in cardiac xenotransplantation remains elusive. The aim of this study was to explore inflammatory gene expression associated with an orthotopic cardiac xenotransplantation model with genetically modified pigs as organ donors.

16 genetically modified pig hearts (GTKO, hCD46, hTM) were orthotopically transplanted into baboons. Ischemic (n=4) or non-ischemic (n=12) heart preservation methods were performed during operation. Immunosuppression was based on steroids, MMF, and CD40/CD40L blockade. Treatment for growth inhibition including temsirolimus was administrated in seven experiments. IL6, C-reactive protein (CRP), creatinine kinase (CK), troponin T concentration and WBC (including neutrophils and lymphocytes) count were measured at the end of the experiments.

Snap frozen myocardial samples were collected from the grafts immediately post mortem. The total RNA was extracted from 20~30mg of tissue and reverse transcripted to cDNA. The expression of associated inflammatory genes Interleukin 1 alpha (IL-1α), IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10 and tumor necrosis factor α (TNF-α) were measured using quantitative real-time polymerase chain reaction (qPCR). Gene expression was compared to control, not transplanted pig hearts, and relative gene expression was analysed.

The mean survival of baboons was 61.3 [1-195] days. On the last day of the experiments, the mean serum concentration of IL6 was 1475 ± 4819 pg/ml, the CRP concentration was 2.1 ± 2.1 mg/dl, CK concentration was 6599 ± 9708 IU/L. IL-8 was upregulated as compared to control. Comparative analysis of gene expression between left and right ventricle for each experiment revealed no significant difference. There were significant correlations between survival days and ΔΔCt of IL-4, IL-10, and IL-1β. Gene expressions of IL-4, IL-6, IL-8, IL-10, IL-1α, and IL-1β showed significant differences between groups of more and less than 40 days of survival. A strong correlation was found between IL-5 and troponin T. IL-1β fold change and IL-6 serum concentration also have a positive correlation. Grouped by different heart preservation methods during transplantation, IL-5 and IL-6 gene expression were

significantly different. The expression of IL-1β was significantly reduced by using temsirolimus.

Non-ischaemic heart preservation method and administration of temsirolimus significantly reduced inflammation in xenotransplant heart. Correlation analysis indicated that a significant correlation between inflammatory cytokines gene expression and postoperative survival time. Inflammatory processes in the graft did not lead to measurable systemic inflammation.

Zusammenfassung

Inflammatorische Zytokine haben wichtige Auswirkungen auf Organtransplantationen. Dennoch bleibt ihre Rolle in der Xenotransplantation von Herzen unklar. Das Ziel dieser Studie war es, die in einem orthotopen Xenotransplantationsmodell mit genetisch modifizierten Schweinen als Organspender assoziierte inflammatorische Genexpression zu untersuchen.

16 genetisch modifizierte Schweineherzen (GTKO, hCD46, hTM) wurden orthotop in Paviane transplantiert. Die Spenderherzen wurden während der Operation entweder ischämisch (n=4) oder nicht-ischämisch (n=12) präserviert. Die Immunsuppression basierte auf Steroiden, MMF und CD40-/CD40L- Blockade. In sieben der 16 Experimenten wurde zusätzlich das Wachstum des Spenderherzens mittels Temsirolimus gehemmt. Die Konzentration von IL-6, C-reaktivem Protein (CRP), Kreatinkinase (CK), Troponin T und die Anzahl weißer Blutzellen (inklusive Neutrophilen und Lymphozyten) wurden am Ende der Experimente gemessen.

Unmittelbar post mortem wurden gefriergetrocknete Myokardproben entnommen. RNA wurde aus 20-30mg großen Gewebeproben extrahiert und durch reverse Transkription in cDNA umgeschrieben. Die Expression der inflammatorischen Gene Interleukin 1 alpha (IL-1α), IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10 und Tumornekrosefaktor α (TNF-α) wurden mittels quantitativer Polymerase-Kettenreaktion (qPCR) gemessen. Als Kontrolle dienten nicht-transplantierte Schweineherzen. Die ermittelte Genexpression wurde mit der Kontrolle verglichen und die relative Genexpression analysiert.

Die mittlere Überlebensdauer der Paviane war 61.3 [1-195] Tage. Am letzten Tag der Experimente betrugen die Serumkonzentrationen von IL6 1475 ± 4819 pg/ml, von CRP 2.1 ± 2.1 mg/dl und von CK 6599 ± 9708 IU/L. IL-8 war im Vergleich zur Kontrolle hochreguliert. Vergleichende Analysen der Genexpression des linken und rechten Ventrikels für jedes Experiment zeigten keine signifikanten Unterschiede. Die Überlebenszeit korrelierte signifikant mit $\Delta\Delta$Ct von IL- 4, IL-10 und IL-1β. Die Genexpressionen von IL-4, IL-6, IL-8, IL-10, IL-1α und IL-1β waren signifikant unterschiedlich zwischen den Gruppen mit mehr und weniger als 40

Überlebenstagen. Es zeigte sich eine starke Korrelation zwischen IL-5 und Troponin T. Ebenfalls positiv korrelierte die IL-6 Serumkonzentration mit der Fold Change von IL-1β. Gruppiert nach unterschiedlichen Organpräservationsmethoden während der Transplantation zeigte sich ein signifikanter Unterschied der Genexpression von IL-5 und IL-6. Die Expression von IL-1β wurde durch den Gebrauch von Temsirolimus signifikant reduziert.

Die nicht-ischämische Methode der Herzpräservation und der Einsatz von Temsirolimus reduzierten die Inflammation in xenotransplantierten Herzen signifikant. Korrelationsanalysen ergaben eine signifikante Korrelation zwischen inflammatorischen Zytokinen und postoperativer Überlebenszeit. Inflammatorische Prozesse im Graft führen nicht zu einer messbaren systemischen Inflammation.

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