Medizinische Fakultät

der

Universität Duisburg-Essen

Aus der Klinik für

Nephrologie

Characterization of the Fischer to Lewis Rat Renal Transplant Model

Inaugural Dissertation

zur

Erlangung des Doktorgrades der Medizin

Durch die Medizinische Fakultät

Der Universität Duisburg-Essen

Vorgelegt von

Yang Dai

Aus Hubei, P.R. China

2022

DOI: 10.17185/duepublico/76484
URN: urn:nbn:de:hbz:465-20220913-085036-2

Dekan: Herr Univ.-Prof. Dr. med. J. Buer

1. Gutachter/in: Herr Univ.-Prof. Dr. med. B. Wilde

2. Gutachter/in: Frau Prof. Dr. med. A. Gallinat

Tag der mündlichen Prüfung: 6. Juli 2022

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1 INTRODUCTION

1.1 Organ transplantation

Organ transplantation is one biggest achievement in modern medicine. Since Dr. Thomas Murray's first successful kidney transplant between identical twins in 1954, it has become an acceptable treatment for organ failure within one century. Currently, organ transplantation is still the most effective way to extend the life of patients with end-stage organ failure. According to the data from the "Global Observation on Donation and Transplantation" (GODT), the number of solid organ transplantations increased from 6.82 to 22.04 per million population worldwide, and from 17.58 to 42.7 per million population in Europe between 2000 and 2020. Meanwhile, the number of kidney transplantations increased from 3.82 to 13.89 per million population worldwide, and 7.75 to 25.88 per million population in Europe, making it the most frequently transplanted solid organ

The challenge of renal transplantation nowadays is to extend graft survival and optimize graft function. Cyclosporine discovered in 1976 and applied in clinical routine since the 80s, changed the prognosis of organ transplantation fundamentally resulting in increased one-year survival in kidney transplant recipients reaching 95% minimizing the episodes of early acute rejections (Bezinover & Saner, 2019). Latest immunosuppressive agents (tacrolimus, cyclosporine, sirolimis, everolimus and mycophenolic acid) gradually prolonged graft survival time with fewer side effects (Wong et al., 2017). With the application of new drugs and therapies, the graft loss rate has declined year by year. The latest patient one-year survival rates were 97.4%, 90.5%, 89.8%, and 86.0% in kidney, heart, liver, and lung transplantation. However, the ten-year survival rate remained steady at 50% in kidney transplantation(Hart et al., 2020). Late graft loss is one of the main problems in the field of renal transplantation, leading to repeated transplantation and the recipient's death. Chronic rejection, humoral rejection, and drug toxicity are major reasons for late graft loss (Jevnikar & Mannon, 2008; Sun & Yang, 2013).

1.2 Static cold storage and cold ischemia time of the renal graft

In clinical practice, static cold storage is the most frequently used organ preservation method. Moreover, cold ischemia time (CIT) is the most frequently mentioned technical parameter in organ transplantation. Although the cold preservation time of organs has been gradually extended with technological advancement, graft damage still builds up over time. Previous studies have suggested that longer CIT will increase the risk of early period post-transplantation graft failure (Helanterä et al., 2020; Rodrigues et al., 2021). In kidney transplantation, a prolonged CIT is an independent risk factor for delayed graft function, acute rejection, and poor graft survival (Perico et al., 2004). Therefore, the current strategy is to reduce CIT as much as possible.

1.3 Graft rejection in renal transplantation

Graft loss or dysfunction is mainly caused by immune responses directed against the graft, i.e. graft rejection. Two main types of rejection have been distinguished: T-cell mediated rejection (TCMR) and antibody-mediated rejection (ABMR). Both types of rejection can occur alone or simultaneously in parallel (Halloran et al., 2016). Clinically graft rejection is classified into hyperacute rejection (minutes to hours), acute rejection (anytime after transplantation, usually days to months) and chronic rejection (months to years).

Hyperacute rejection is highly related to the presence of pre-formed antibodies such as blood group antibodies and anti-donor antibodies. Cross-match tests are usually performed prior to transplantation, and transplantations are usually performed according to blood group compatibility. Therefore, hyperacute rejection is extremely rare. Acute rejection is mainly identified as TCMR and is well treated with modern immunosuppressive agents. Chronic rejection usually develops as ABMR at a later time point and leads to irreversible allograft failure. Contrary to acute rejection, an effective treatment is not available (Böhmig et al., 2019). In clinical practice, acute and chronic rejection can occur alone or in combination. With the application of new

immunosuppressive agents and therapies, the frequency of TCMR gradually decreases over time after transplantation and is very rare ten years after transplantation under ongoing immunosuppressive therapy. ABMR gradually becomes more common over time after transplantation (Halloran et al., 2015). ABMR is the main cause of late graft failure and significantly affects the long-term survival of patients and grafts.

1.4 Donor specific antibodies of renal transplantation

Donor specific antibodies (DSA) are defined as antibodies induced by antigens only present in the graft donor but not in the recipient. DSA could develop from polymorphic molecules (for example, major histocompatibility complex molecules) and non-polymorphic molecules (for example, vimentin). Major histocompatibility complex (MHC) related DSA represent the major group of DSA, mediate ABMR and play an important role in the development of ABMR (McCaughan & Tinckam, 2018; Nakamura et al., 2019). In addition, non-MHC related DSA, including minor histocompatibility antigens, major histocompatibility complex class I-related chain A and B, vimentin, angiotensin II type I receptor can have an adverse effect on long-term allograft outcomes (Clatworthy et al., 2010; Sánchez-Fueyo & Dazzi, 2021). At the same time, due to the polymorphism of the MHC gene, DSA shows a wide variability.

Serum DSA detection methods can be roughly divided into donor lymphocytes dependent and donor lymphocytes independent methods (Nakamura et al., 2019). Donor lymphocytes independent methods need refined MHC antigens which can only cover some of the common polymorphisms. Donor lymphocytes dependent methods present intact antigens, even including non-MHC antigens. However, this method requires a continuous supply of donor lymphocytes, which puts a great limit on application and standardization. Donor spleen obtained during organ harvesting is often used as a source of donor lymphocytes, especially in cadaveric donors.

DSA mediated rejection is one kind of ABMR involving complement pathways and immune cells. DSA binding to donor-derived antigens activates the C1 complex and the classical complement pathway, ending up with the formation of the membrane attack complex and damage to the allograft (Stegall et al., 2012). DSA also active NK cells, T cells, neutrophils and eosinophils in an Fc-dependent manner damaging allograft (Hirohashi et al., 2012; Loupy & Lefaucheur, 2018).

DSA mediated rejection is the leading cause of graft loss, but the severity also depends on antigen classes, antibody classes and antibody titer. There are five classes of immunoglobulins (IgA, IgD, IgE, IgG, IgM). DSA of IgG and IgM classes are studied intensively and considered the most important antibodies in ABMR. DSA IgM antibodies occur in the early and late period post-transplantation and are also highly related to decreased survival in kidney and heart transplantation (Stastny et al., 2009). DSA IgG are formed after class switch from IgM to IgG, and belong to different IgG subclasses (IgG1, IgG2, IgG3, IgG4). These subclasses have different complement-fixing abilities. In human, IgG3 and IgG1 complement-fixing abilities are much stronger than IgG2 and IgG4. However, poor allograft prognosis correlates with all types of DSA IgG subclasses (Cicciarelli et al., 2013; Khovanova et al., 2015; Pernin et al., 2020). In the rat, IgG subclasses (IgG1, IgG2a, IgG2b, IgG2c) of DSA are quite different from the human situation. Complement-fixing abilities of rat IgG2b and IgG1 are much stronger than IgG2c and IgG2a. IgG2b is the strongest IgG subclass to mediate complement-dependent cell-mediated cytotoxicity (Miyatake et al., 1998). Marquina et al. reported allograft specific antibody (ASA) IgG1 and IgG2c elevations 2 weeks post-transplant and dramatic decreases within 5 weeks, IgG2a peaked at 5 weeks and remained high at 10 weeks, IgG2b remained high until 10 weeks after increasing at week 2. (Marquina et al., 2017). In contrast, in rats with enhanced immune tolerance, IgG2a dominates the IgG response (Cuturi et al., 1994; Ding et al., 2008; Minami et al., 2006). In rats in which donor-specific tolerance was induced, IgG2b and IgM were absent (Singer et al., 2001).

In our study, DSA was detected using splenocytes of Fischer inbred rats but not the original donor rats. The antibodies we measured were slightly different from strictly defined DSA. Therefore the term allograft specific antibody (ASA) was used when describing rat DSA in our study.

1.5 DSA-producing plasma cells

DSA is produced by donor-specific and matured B cells. Maturation occurs through antigen encounter and sensitization. Sensitization may happen even before transplantation through pregnancy, blood transfusion and even cross-reactivity to pathogens (Alberú et al., 2007). The distribution of DSA-producing plasma cells throughout the recipient's body is not clear yet, but clinical data indicate that the graft, bone marrow and spleen are the main locations where DSA- producing plasma cells can be found. Plasma cell infiltration was observed directly in the bone marrow (Radbruch et al., 2006) and the graft (Filippone & Farber, 2020). Meanwhile, the splenectomy can significantly alleviate rejection in case ABMR occurs (Kaplan et al., 2007; Orandi et al., 2014).

The B cell activating factor (BAFF) is an important cytokine to support the survival and maturation of B lymphocytes (Schweighoffer et al., 2013). Therefore, the occurrence and maintenance of ABMR depends on BAFF. Furthermore, clinical studies also suggest that BAFF can be used as a molecular marker for predicting ABMR and that increased serum BAFF levels prompt ABMR (Irure-Ventura et al., 2020; Wang et al., 2019). Anti-BAFF treatment is effective in the rat renal transplantation model and led to reduced B cell numbers in allografts and spleen. In anti-BAFF treated rats, plasma cells are diminished, and ASA levels are reduced (Steines et al., 2020).

1.6 Regulatory B cells

Besides the ability to produce antibodies, B cells also have the functions of antigen presentation and regulate immunity, including T-cell immunity. B cells can produce a

range of pro-inflammatory and anti-inflammatory cytokines to promote and suppress immune responses. B cells that produce anti-inflammatory cytokines are called Regulatory B cells (Bregs). There is no unique phenotype attributed to Bregs. Therefore, IL-10 expression is mostly used to identify Bregs (Mauri & Bosma, 2012). The spleen is the largest reservoir for Bregs, representing about 1% of total spleen B cells (DiLillo et al., 2010). Moreover, a rise up to 3%-5% after antigen exposure is described in the literature (Matsushita et al., 2008). Bregs are considered to play an important role in rejection and immune tolerance based on immunosuppressive effects (Liu et al., 2020; Shen et al., 2021). Luo et al. found that IL-10+ Bregs decreased in patients who suffered antibody-mediated rejection(Luo et al., 2020). Kimura et al. successfully induced immune tolerance in a mouse skin transplant model by transfer regulatory B cells. (Kimura et al., 2020)

1.7 Rat kidney transplantation model

The animal model is an important research platform in the medical field. Transplantation is one of the fields that highly rely on animal experiments due to its traumatic and ethical issues. The rat kidney transplantation model used in this study was first described in 1965 (Fisher & Lee, 1965) and is still widely used. Fischer-344 to Lewis rat kidney transplantation model can simulate acute and chronic ABMR resembling the phenotypic and molecular features seen in human ABMR (Grau et al., 2016; Joosten et al., 2005).

1.8 Aims of study

In this study, we aimed to investigate the association between cold ischemia, ASA, and B-cell immunity in the rat kidney transplant model. In order to achieve this goal, the following research questions were studied:

1. Do allograft specific antibodies evolve in the Fischer to Lewis rat renal transplant model?

2. How is the kinetics of allograft specific antibodies over time, and is there a relation to the cold ischemia time of the graft?
3. How are specific anti-inflammatory B-cell responses and pro-humoral B-cell responses characterized in this animal model?

2 MATERIALS AND METHODS

2.1 Rat kidney transplant model

The rat model of kidney transplantation is well established in the scientific field and was used in this study. Fischer to Lewis allograft transplantations and Lewis to Lewis isograft transplantations were performed in our experimental series.

The kidneys from inbred rats were harvested and stored after cold perfusion with HTK (Histidine-tryptophan-ketoglutarate, Custodiol). After static cold storage with a cold ischemia time (CIT) ranging from 0 to 12 hours, the donor kidneys were orthotopically transplanted to Lewis rats after left side native uni-lateral nephrectomy. Ten days after transplantation, the recipients underwent right-side native nephrectomy to remove the remaining native kidney.

Male rats weighing 260g to 365g were obtained from Charles River Deutschland (Sulzfeld, Germany). All rats received care in compliance with the Principles of Laboratory Animal Care, and the experimental protocol was approved by the local Animal Care and Research Committee. Surgery was performed in pain-free conditions and deep anesthesia. 0.05mg/kg bodyweight buprenorphine (1:10 diluted in saline) injection s.c. was given to control pain. In addition, 2-3% isoflurane in 1.6 L/min oxygen was used for anesthesia induction, and 1-2% isoflurane in 1.6 L/min oxygen for maintenance.

After initial flushing with cold preservation solution (HTK), the donor's kidney was stored on crushed ice. All recipient rats received cyclosporine at a dosage of 1.5mg/kg body weight for ten days from the day of transplantation.

In this study, the following model settings were applied:

(1) Donors and recipients

Two kinds of combinations were used in this study. In the allograft groups, Fischer rats served as donors and Lewis rats were recipients. In the isograft group, Lewis rats served as donors and recipients.

(2) Cold ischemia time

In this study, the donor organs underwent static cold storage to a varying degree, and the following cold ischemia times were tested: 0 hours, 4 hours, 8 hours, 12 hours. 0 hours means that transplantation took place immediately after donor organ harvest. However, also under these conditions, the preservation solution had to be used for a short period of static cold storage during harvest and surgical procedures.

(3) Survival time

In this study, three survival end-points were considered, 30 days, 12 weeks and 28 weeks post-transplant. For rats with extended cold ischemia time, long-term survival was reduced and limited to 30 days and 12 weeks post-transplant.

2.1.1 Surgery instruments and sutures

A set of general surgery instruments was used for abdominal incisions and sutures, including one tissue forceps, one straight tissue scissor, and one needle holder. A set of microsurgical instruments was used to dissect and suture vessels and ureters, including one straight 0.1mm tip forceps, one curved 0.15mm tip forceps, one 8mm straight spring-type micro scissors, one microneedle holder, and two neurovascular micro clamps. A microsurgical microscope was used with 10x and 15x magnifications. The anastomoses were performed with non-absorbable Nylon 10/0 DR4. For ligations, 7/0 nylon was used. The abdominal wound was closed with resorbable suture 5/0 DSM 13, and the perinephric fat was fixed with Nylon 8/0 DR6. For organ flushing, we used a metal cannula of 1.3mm diameter. Furthermore, Bipolar coagulator was used to stop slight bleeding. A heating plate was used to maintain rat body temperature(Figure 1).

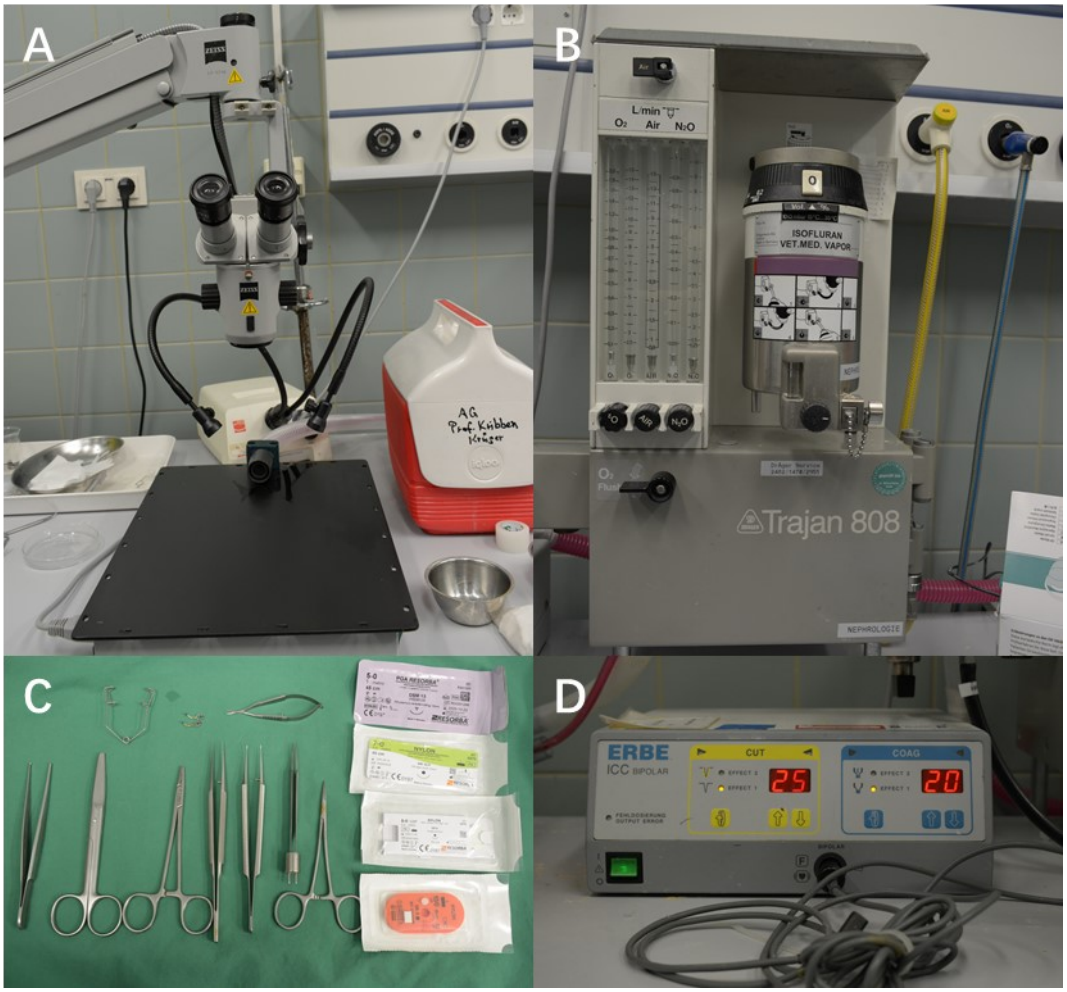


Figure 1: Surgery instruments and sutures

(A) Microsurgical microscope and Electric heating plate. (B) Anesthetic machine. (C) Surgery instruments and Sutures. (D) Bipolar coagulator

2.1.2 Preparation for surgery

Surgery was performed under clean conditions but not strictly sterile. Rats were shaved, and the operation area was sterilized with alcohol-based skin disinfection. Instruments were disinfected with 70% ethanol. The surgeon wore surgical masks and gloves. After anesthesia, rats were fixed supine and heparinized with 100 IU heparin (diluted in normal saline) injected through the tail vein. A cold preservation solution was prepared for donor rats and hung 1.2 meters higher than the operating plate. For recipient rats, a heating plate was used to prevent hypothermia.

2.1.3 Donor organ harvest

After preparation and anesthesia, a median incision laparotomy from the pubic symphysis to the xiphoid was performed with scissors (Figure 2A). Slight bleedings were coagulated by the bipolar coagulator to prevent bleeding out. The stomach, spleen and intestinal canal were covered with saline moistened gauze and everted to the right side of the rat. The ureter was located and protected by moistened gauze before dissection.

The aorta was dissected apart from the fat tissue. Three pre-set ligations were placed around the aorta; one was above the right kidney artery, the other two were below the left kidney artery and 5mm apart (Figure 2B). The renal artery and vein were separated and skeletonized. Sometimes the inferior suprarenal artery arose from the left renal artery (Figure 2C). In this condition, the inferior suprarenal artery was ligated. In order to solve the angiospasm of the renal artery after dissection, drops of lidocaine were applied. A clamp was applied to the aorta below the renal bifurcation. The abdominal aorta was cut between the two lower pre-set ligation to insert the cannula connected with cold preservation solution, and the cannula was fixed with the ligation. Then the top ligation was tightened, the clamp was released, and the cannula was opened quickly to minimize the warm cold ischemia. After perfusion started, the rat was sacrificed by a heart cut. With the preservation solution running through, the kidneys start to turn pale (Figure 2D). After the kidneys turned totally pale, the renal vein was cut close to the first branch. The ureter was cut 2cm away from the renal hilum and dissociated from periureteral fat (Figure 2E). The left kidney was resected en bloc with the cannula fixed with the aorta, and the right kidney was harvested with a dissociation directly on the renal hilum. The left donor kidney was stored in HTK solution on melting ice (Figure 2F). The right kidney was treated the same way and served as the control kidney not being transplanted. A timer was set to record cold ischemia time.

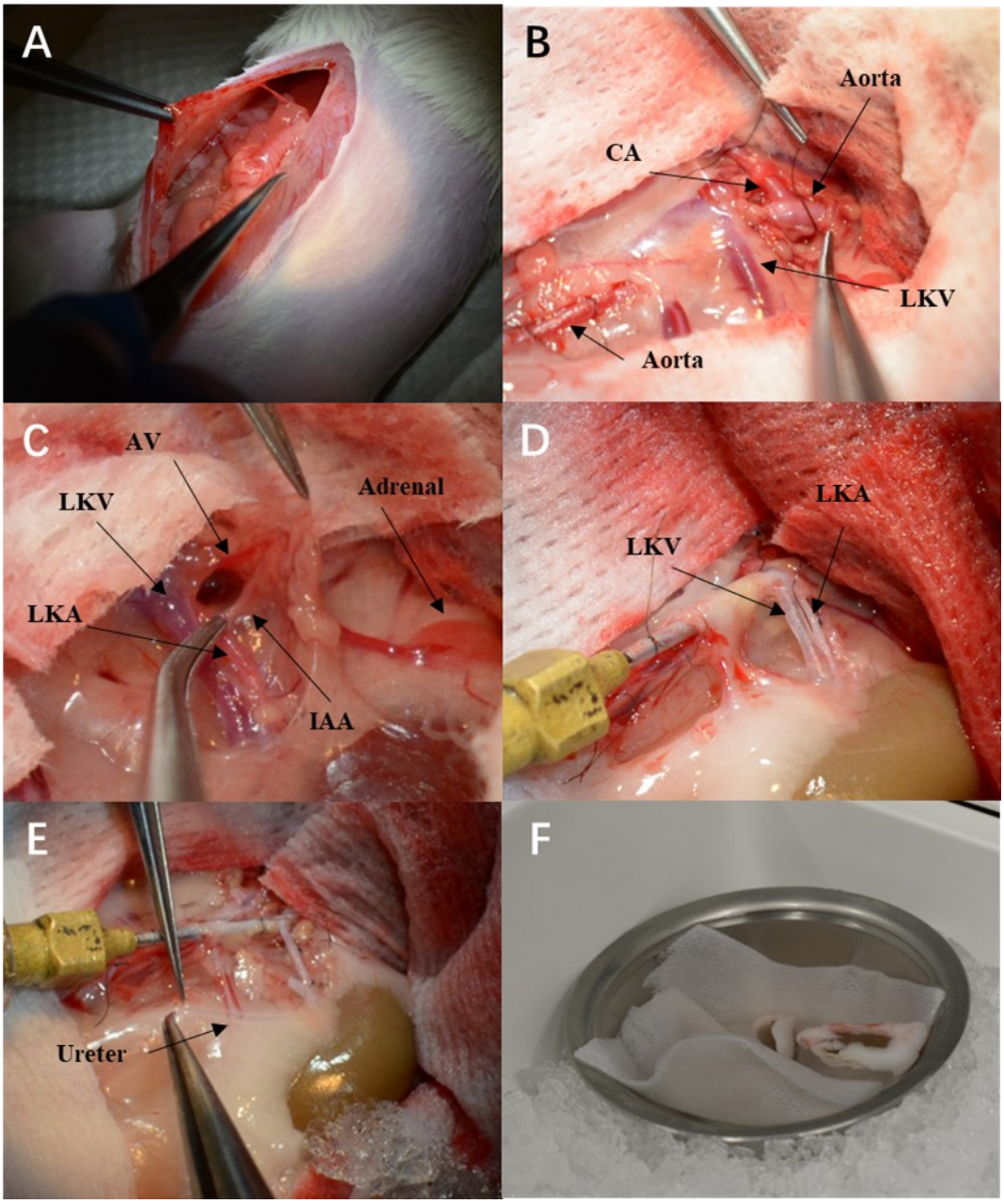


Figure 2: Organ Harvest

(A) Median incision laparotomy. (B) Pre-set ligations around the aorta. (C) Renal vessels and inferior suprarenal artery and vein. (D) Perfusion with HTK. (E) Dissect Ureter. (F) Allografts static cold storage. CA: Celiac Trunk; LKV: Left Kidney Vein; LKA: Left Kidney Artery; AV: Adrenal Vein; IAA: Inferior Adrenal Artery.

2.1.4 Orthotopic renal transplantation

Cold saline was used at the end of cold storage to flush out preservation solution by the renal artery. The renal artery was cut close to the aorta.

After preparation and anesthesia, a transverse incision was made from the left anterior axillary line to the right linea pararectalis, 1 cm below the costal arch (Figure 3A). Small bleedings were coagulated by the bipolar coagulator to prevent bleeding out. The incisions were covered with saline moistened gauze. The stomach, spleen and intestinal canal were covered with saline moistened gauze and everted to the right side of the rat. The liver was pushed upwards with moistened gauze to expose the left kidney. Warm saline was periodically added to the gauze over organs during surgery to prevent dehydration. Saline was injected subcutaneously if necessary.

The renal hilum was dissected, the renal artery and vein were separated and skeletonized (Figure 3B). The fascia around the renal artery was removed to expose adventitia. When vessels preparation was completed, vascular clamps were applied to the renal artery and vein away from the renal hilum, artery first and vein next. A clean-cut was made at the close to the renal hilum. The lumens of blood vessels were immediately washed with 1% heparinized saline to prevent blood clots. The adventitia was cut off at the end of the renal artery to provide a fine tip for an end-to-end suture. The ureter was cut at the level of the inferior renal pole. Finally, the left kidney was removed with perirenal fascia (Figure 3C). The renal allograft was covered with cold saline moistened gauze and placed into the abdomen to align the vascular ends with a small gap, ensuring a mild tension during anastomosis.

The continuous suture was used for both renal artery and vein. The artery was sutured before the vein due to anatomical position. The continuous suture started with a suture and a knot at the caudal side of the vessel. Then the vessel was turned over by pulling the suture line under the vessel to the cranial side. With gradual release of the suture line, the

vessel flipped back automatically and proved an excellent vision to suture over the vessel. In this position, the continuous suture was made from the cranial side to the caudal side until the first knot. After that, another knot was made to end the continuous arterial suture, but not necessary for the vein. The tension of the suture line was essential. Over-pulling the suture line may cause stenosis of the vessel. During anastomosis, heparinized saline was used to prevent the vessels from drying out and visualizing the lumen.

After anastomosis, the clamp on the vein was first removed, then the artery was opened (Figure 3D). Small bleedings were controlled with slight compression with gauze for a few minutes. If the bleeding was not stopped, the bleeding point was located and sutured. The kidney turned pink at the beginning with blood perfusion and turned back to normal later (Figure 3E). If the kidney turned dark or purple later, it indicated angiospasm or vessel stenosis. Lidocaine was used on the vessels and the kidney to solve angiospasm.

The ureter was anastomosed with six to eight single sutures evenly dispersed over the ureter (Figure 3F). The dorsal side was sutured first, and a long tail was kept to flip the ureter for additional sutures. During ureter anastomosis, saline moistened gauze was covered over renal vessels and kidney, and check kidney color periodicity.

The vessels were finally checked, small bleedings were coagulated, and the blood clots were removed. The kidney was fixed to the abdominal wall by suturing perinephric fat and peritoneal fat with 8-0 nylon. Internal organs were put back into the abdominal cavity. Then, the abdominal incision was closed by the absorbable, continuous sutures. The peritoneum and the abdominal wall muscles were closed as the first layer. The skin was closed as the second layer. The isoflurane was turned off as soon as the sutures were finished. The rat was placed on a heating plate with oxygen insufflation until body movement appeared. Then the rat transferred to a cage on a heating plate. The heating support lasted for a few hours until the rat was brought back to the animal facility. The

rat was checked and injected with Cyclosporin (1.5 mg/kg s.c.) every day until 10 days post-surgery. Daily post-operative checkups include weighing, judging appearance and activity. 10 days post-surgery, the rat underwent right side native nephrectomy.

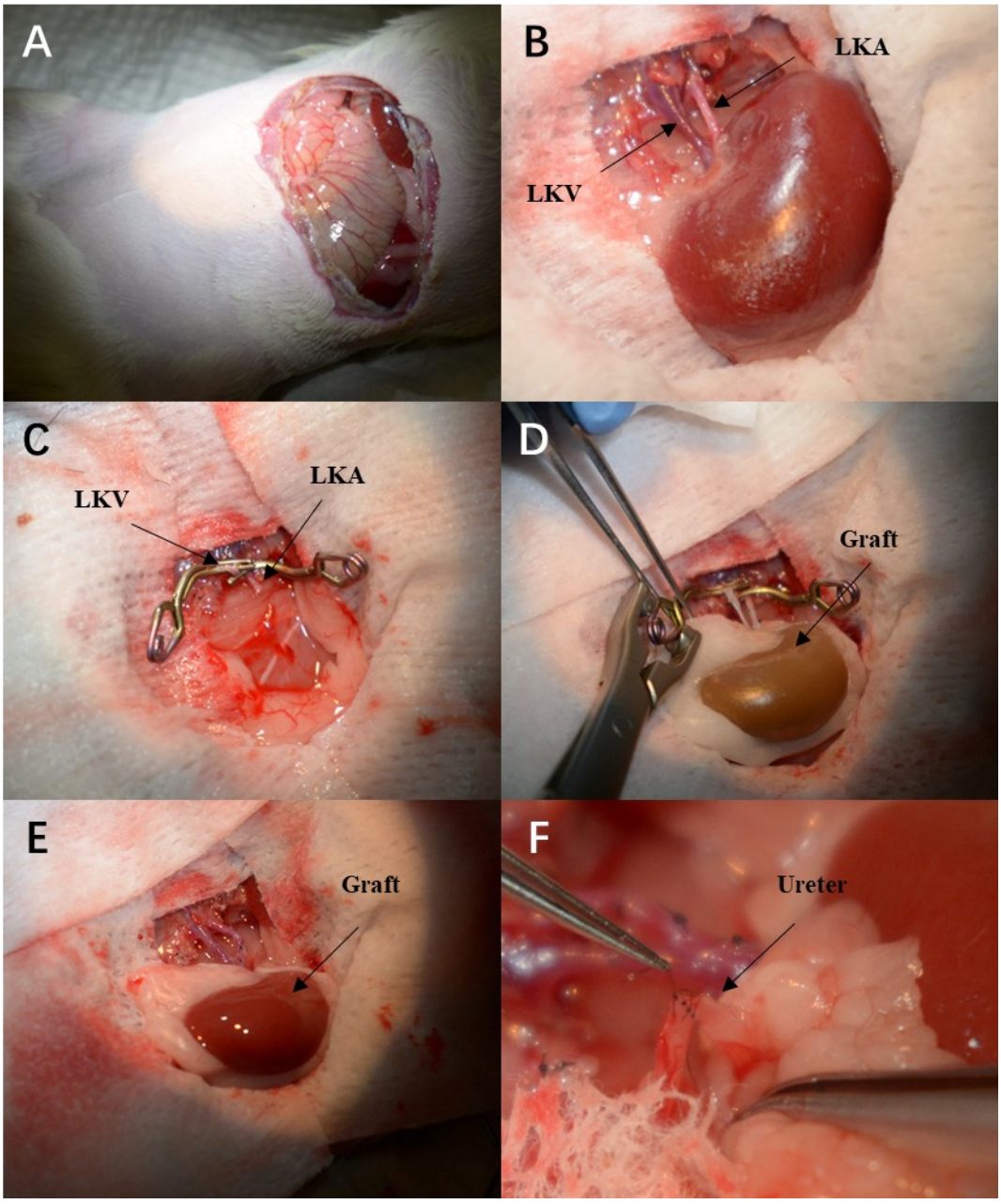


Figure 3: Kidney transplantation

(A) Transverse incision laparotomy. (B) Dissected renal hilum. (C) Apply clamps and remove the left kidney. (D) Remove clamps after vascular anastomosis. (E) Reperfused kidney. (F) Ureter anastomosis. LKV: Left Kidney Vein; LKA: Left Kidney Artery.

2.1.5 Right side nephrectomy of the native remaining kidney

Right side nephrectomy of the recipient was carried out later to overcome initial allograft complications such as delayed graft function (DGF). After preparations for surgery, a cut through the old incision to the linea pararectalis was done. The internal organs were pulled out and covered with moistened gauze. Then, the internal organs were pushed to the right side to expose the allograft. Color, hardness and the expansion of the ureter were checked. If the kidney appeared pale and hard, a lack of arterial perfusion was assumed. Rats with perfusion issues of the graft or with obstruction of the ureter were removed from the experiment due to technical failers. Then the internal organs were pushed to the left side to expose the right kidney. The right renal vessels and ureter were dissected and double ligated. After the right renal hilus was cut off, the right kidney was removed with perinephric fats. The kidney bed was washed to remove blood clots. Small bleedings were carefully coagulated. Then the internal organs were put back in order, and the incision was closed with the peritoneum and skin sutured separately.

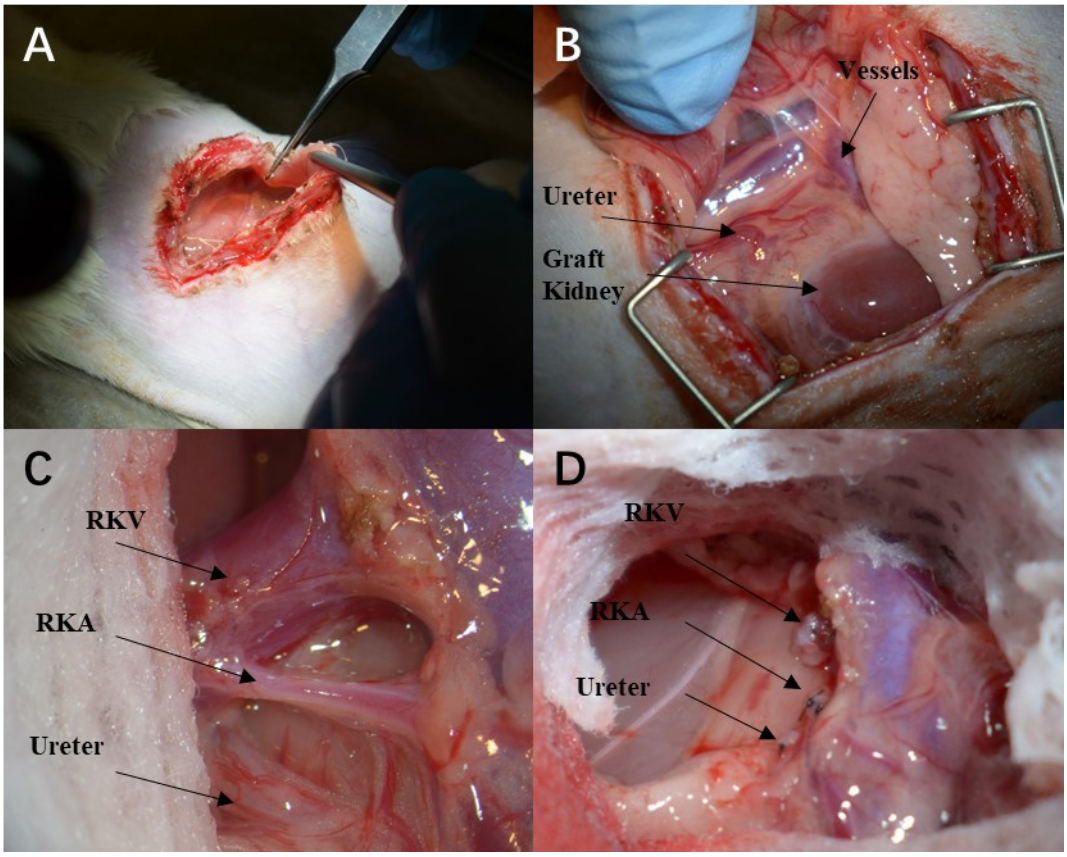


Figure 4: Right side nephrectomy

(A) Transscar laparotomy. (B) Expose and check the transplanted kidney. (C) Expose right renal vessels and ureter. (D) Ligate and remove the right kidney. RKV: Right Kidney Vein; RKA: Right Kidney Artery.

2.1.6 Post-operative management and follow up

The rats had free access to clean water and foods, and were carefully monitored in the early days post-operation. In the first 30 days post-operation, the body weight was recorded every day. After that, the body weight was measured once a week. The rats received buprenorphine as the pain killer at least twice post-surgery and in addition if any sign of pain was found.

Urine was collected with a metabolic cage when reaching the end-point. Then, the rats were culled in deep narcosis, and samples like whole blood, renal allografts and spleen

were harvested. Blood and urine samples were centrifugated at 2500 RCF for 10 minutes; the supernatants were stored at – 20°C.

2.2 Assessment of renal allograft function and damage

At the end-point of the experiment, the rats were put into the metabolic cage to collect urine. Then the rats were culled in deep narcosis, and the blood was collected from the aorta. The blood and urine samples were centrifuged at 2500 RCF for 10 minutes, and the supernatants were collected and stored at -20 ℃. Blood urea nitrogen (BUN) and creatinine were determined from serum in the central laboratory by standard methods to assess kidney function, and urinary albumin was determined by enzyme-link immunosorbent analysis (ELISA) to assess kidney damage.

2.3 Determination of allograft-specific antibodies by FACS

Spleen T cells from Fischer rats were used as the source for donor antigens, capable of binding DSA in serum from Lewis recipients. However, the Fischer inbred rats we used were not the original donor rats. The antibodies we measured were slightly different from strictly defined DSA. Therefore the term allograft specific antibodies (ASA) was used when describing rat DSA in our study. ASA binding to Fischer rat splenocytes were measured by flow cytometry with fluorochrome-labeled antibodies. The fluorescence intensity was considered linear to the ASA concentration (Huang et al., 2014; Kohei et al., 2013; Marquina et al., 2017; Reese et al., 2021).

2.3.1 Fluorochrome labeled antibodies

This study investigated two ASA, IgG and IgM, as well as the subclasses IgG2a and IgG2b. Fluorochrome-labeled anti-rat immunoglobulin antibodies were used to detect ASA bound to splenocytes. In order to eliminate the interference of non-selective antibody binding, ASA binding was determined on gated living T cells (7-AAD ^{low} $CD3^{+}$

cells). Therefore, three different fluorochrome-labeled antibodies were used in combination.

Antibody agent for one test as follows:

Antibody Classes detected	Antibody agent	Dosage
IgG	Fluorescein (FITC) AffiniPure Fab Fragment Donkey Anti-Rat IgG (H+L)	10µl
	Allophycocyanin (APC) anti-rat CD3	2µl
	7-AAD Viability Staining Solution	1µl
IgM	FITC anti-rat IgM	1µl
	APC anti-rat CD3	2µl
	7-AAD Viability Staining Solution	1µl
IgG2a	Phycoerythrin (PE) anti-rat IgG2a	0.5µl
	APC anti-rat CD3	2µl
	7-AAD Viability Staining Solution	1µl
IgG2b	Alexa fluor 647 anti-rat IgG2b	0.5µl
	FITC anti-rat CD3	0.5µl
	7-AAD Viability Staining Solution	1µl

Table 1: Antibody agent used for ASA measurement.

2.3.2 Splenocyte isolation from Fischer rats

The spleens were harvested the same way described in chapter 2.1.3 (donor organ harvest) from Fischer rats. The spleens were harvested together with allograft kidneys. The spleens were stored in 5 ml RPMI 1640 Medium on melting ice after harvest. After transfer to a clean bench, the spleens were pushed through 40 µm cell strainers with 50ml RPMI 1640 Medium to get single-cell suspensions of splenocytes. Splenocytes were then spun down at 400g for 10 minutes, with full brake at room temperature. The splenocytes were then resuspended in red blood cell lysing buffer for 10 minutes at room temperature to lyse red blood cells. The lysing procedure was stopped by a wash with PBS, and the splenocytes were spun down again at 400g for 5 minutes and resuspended in 50 ml of PBS. The splenocytes went through a 40 µm filter again and spun down at 400g for 10 minutes.

Splenocytes were resuspended in cell culture medium at 2 Mio/ml and incubated at 37 °C, under 5% CO_2 atmosphere overnight for further steps.

2.3.3 Flow cytometric measurement

Splenocytes were counted after overnight culture, collected and spun down at 400g for 10 minutes. The splenocytes were washed and re-suspended in PBS at 9 Mio/ml and stored on ice. The 50 µl splenocytes suspension was incubated with the same amount of recipients' serum for 60 minutes at room temperature. As the control, 50 µl splenocytes suspension was incubated with the same amount of PBS. After washing with PBS twice, splenocytes were incubated with fluorochrome-labeled antibodies for another 30 minutes at 4 °C. Splenocytes were washed PBS twice again after and resuspended in 400µl PBS for flow cytometry. A Navios Flow Cytometer by Beckman Coulter Life Sciences was used.

2.3.4 Flow cytometric data analysis

Kaluza Analysis Software Version 2.1.00001.20653 was used to analyze the flow cytometric results. For all groups, lymphocytes were selected through FSC and SSC characteristics, and living T cells were selected from the lymphocyte gate being $CD3^+$ and negative for 7-AAD. The mean fluorescence intensity (MFI) of the anti-rat Ig antibodies was determined in the condition in which serum of the recipient was used and in the control condition. The MFI fold change was calculated from the condition with the serum of the recipient (MFI_{Ig}) and the control (MFI_{PBS}):

$$MFI\ Fold\ change = \frac{MFI_{Ig}}{MFI_{PBS}}$$

MFI_{Ig}: Mean fluorescence intensity of anti-rat IgG, anti-rat IgM, anti-rat IgG2a and anti-rat IgG2b with recipient serum groups. MFI_{PBS}: Mean fluorescence intensity of the same fluorochrome-labeled antibody with paired PBS condition.

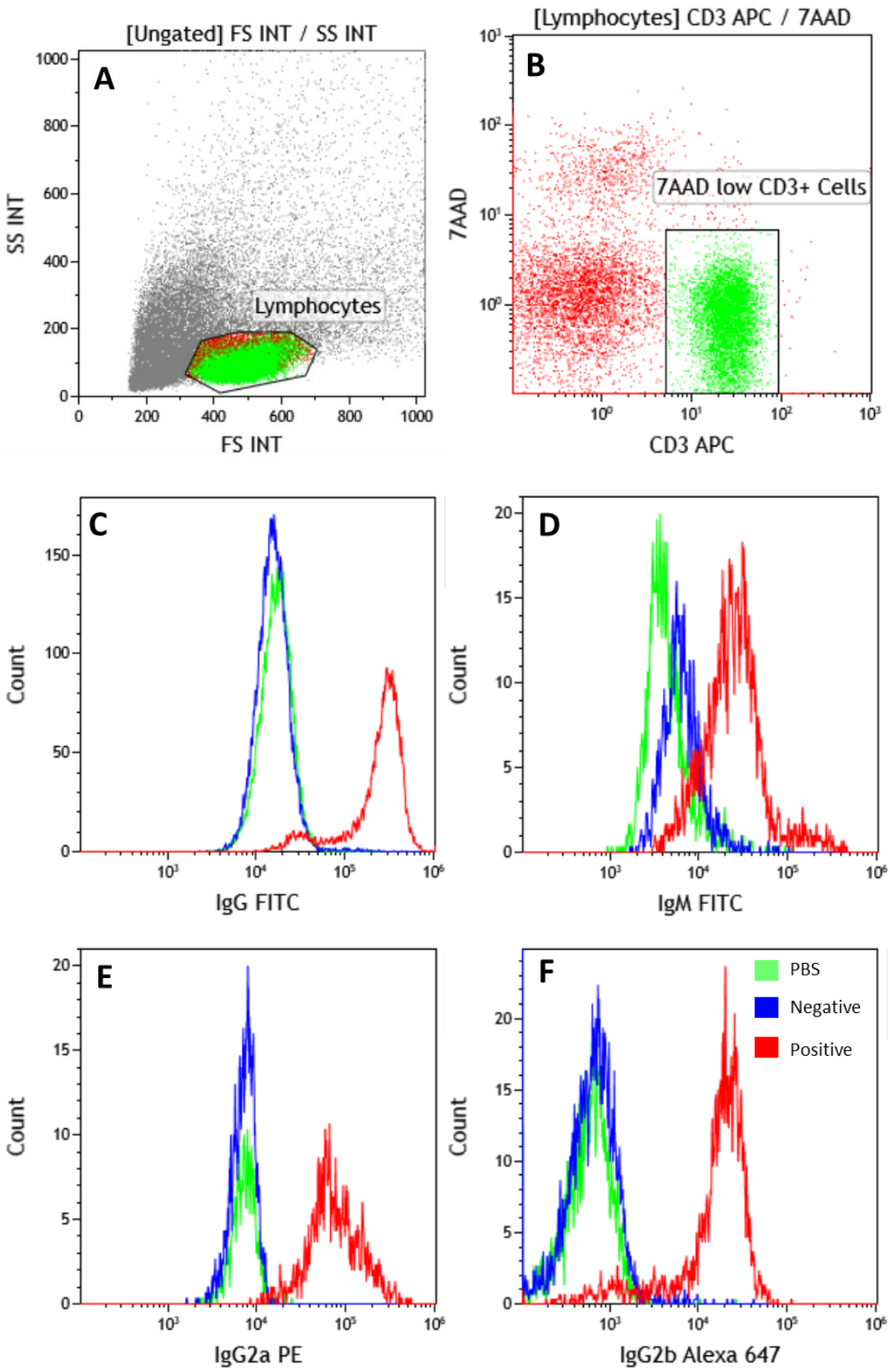


Figure 5: Gating Strategy and analysis of ASA level.

(A) Lymphocyte Gate (B) Gate on vital T-cells (C) PBS control, negative and positive fluorescence intensity of ASA IgG. (D) PBS control, negative and positive fluorescence intensity of ASA IgM. (E) PBS control, negative and positive fluorescence intensity of ASA IgG2a. (F) PBS control, negative and positive fluorescence intensity of ASA IgG2b.

2.4 Determination of the B cell-activating factor

The B cell-activating factor (BAFF) was measured from sera of recipients by ELISA with a commercially available ELISA Kit (Abbexa).

2.5 Assessment of regulatory B cells

Splenic B cells were purified and cultured in vitro. IL-10 was detected in the cell culture supernatant as the marker for the Breg.

2.5.1 Spleen B cell Isolation

Spleens were harvested when rats reached the end-points of experiments, and B-cells were positively selected by magnetic isolation. The harvested spleens were stored in RPMI 1640 Medium on ice and then transferred to a clean bench. The spleens were pushed through 40 μm cell strainers with 50ml RPMI 1640 Medium to single-cell suspensions of splenocytes. The splenocytes were spun down and resuspended in Buffer-I (PBS, 0.5%BSA, 2mM EDTA), then filtered through 30 μm pre-separation filters. The splenocytes were counted, and 50 Mio of them were resuspended in 400 μl Buffer I in a 15 ml falcon tube. Another 100 μl of anti-rat CD45RA microbeads were added to the 15 ml falcon tube and incubated for 20 minutes at 4 ℃. After being washed twice with Buffer I, the splenocytes were transferred to LS COLUMN in the magnetic field. The columns were rinsed with 3 ml of Buffer I thrice in the magnetic field. Then the columns were filled with another 5 ml of Buffer I, removed from the magnetic field immediately and put onto a new 15ml falcon tube. The buffers in columns were flushed out by pushing the

plunger. The target cells were washed twice with the cell culture medium and re-suspend in another 2 ml culture medium. The target cells were counted for further treatment.

2.5.2 B cell cultures

96-well U-bottom plates were used for cell culture. 1×10^5 isolated splenic B cells were added per well with the following conditions: None, CpG, CpG + IL-2, CpG + IL-21, CpG + IL-2 + IL21. The control condition (None) contained B cells in 200 µl of plain culture medium. In the CpG condition, 0.5 µl CpG oligodeoxynucleotides (CpG ODN D-SL01, stock: 4.17 mg/ml, final concentration 10.4 µg/ml) was added to 200 µl of culture medium. In the CpG + IL-2 condition, rat IL-2 (final concentration 50 ng/ml) and 0.5 µl CpG ODN D-SL01 were added in combination to the culture medium. In the CpG + IL-21 condition, rat IL-21 (final concentration 50 ng/ml) and 0.5 µl CpG ODN D-SL01 were added to the culture medium. In the CpG+ IL-2 + IL21 condition, rat IL-2 rat, IL-21 and CpG ODN D-SL01 were combined.

Splenic B cells were incubated at 37 ℃, 5% CO2 incubator for five days. After five days, the supernatants were collected and frozen in a -20 ℃ refrigerator until further determination.

2.5.3 IL-10 measurement

IL-10 concentration was measured by ELISA in cell culture supernatants. The commercially available rat IL-10 Quantikine ELISA kit from R&D Systems was used.

2.6 Reagents and consumables

Chemicals/Consumables	Manufacturer
Fischer rats	F344/DuCrl, Charles River, Germany
Lewis's rats	LEW/DuCrl, Charles River, Germany
HTK solution	Custodiol®, Germany

Isoflurane	Isothesia®, Henry Schein Vet GmbH, Germany
Buprenorphine	Temegesic®, Germany
Lidocaine 1%	Braun, Germany
Cyclosporine	Sandimmun, Novartis, Germany
0.9% saline	Fresenius, Germany
Heparin 25000 I.E/5ml	LEO, GmbH, Germany
Incision disinfection	Octenisept®, Germany
Skin disinfection	Cutasept®, Germany
Nylon 10/0 DR4	Resorba®Medical GmbH, Germany
7/0 nylon DR10F	Resorba®Medical GmbH, Germany
PGA Resorb 5/0 DSM	Resorba®Medical GmbH, Germany
Nylon 8/0 DR6	Resorba®Medical GmbH, Germany
Syringe 50 ml	Braun, Germany
Syringe 5 ml	TERUMO®; German
Syringe 1ml	BD plastipak™, Germany
Needle (27Gx3/4mm)	BD Elipse, Germany
BD connecta	BD, Germany
Perfusion line	Sendal, Germany
RPMI Medium 1640 (1x) + GlutaMAX TM -I	Gibco® by life technologies TM
MEM NEAA (100x)	Gibco® by life technologies TM
Sodium Pyruvate 100mM (100x)	Gibco® by life technologies TM
Pen Strep	Gibco® by life technologies TM
Fetal Bovine Serum	Biowest
Aqua 1000ml	B. Braun Melsungen AG
Pre-Separation Filter, 30µm	Miltenyi Biotec
LS Column	Miltenyi Biotec
Falcon Cell Strainer 40µm Nylon	Corning Incorporated
CD45RA MicroBeads rat	Miltenyi Biotec
CpG ODN D-SL01	InvivoGen
Rat IL-2 research grade	Miltenyi Biotec
Recom blinant Rat IL-21 Protein	Novus Biologicals
96 Well U-Bottom Plate	Corning Incorporated
Flow Clean Cleaning Agent	Beckman Coulter
IsoFlow Sheath Fluid TM	Beckman Coulter
Turk's Solution	Sigma-Aldrich
DPBS (1x) 1000ml	Gibco® by life technologies TM
7-AAD Viability Staining Solution	Biolegend
APC anti-rat CD3	Biolegend
FITC anti-rat CD3	Biolegend

Fluorescein(FITC)-conjugated AffiniPure Fab Fragment Donkey Anti-Rat IgG (H+L)	Jackson Immuno Research
FITC anti-rat IgM	Biolegend
PE anti-rat IgG2a	Biolegend
Alexa Fluor® 647 anti-rat IgG2b	Biolegend
Safe-Lock Tubes 1.5mL	Eppendorf, Germany
Safe-Lock Tubes 2.0mL	Eppendorf, Germany
100ul Ultra Point Graduated Filter Tip	Star Lab, Germany
200ul Ultra Point Graduated Filter Tip	Star Lab, Germany
1000ul Ultra Point Graduated Filter Tip	Star Lab, Germany
Eppendorf ep TIPS 0.1-10 µl Box	Eppendorf, Hamburg
Pipette Tip 200 µl	Sarstedt
Pipette Tip 1000 µl	Sarstedt
Tube 15ml	Sarstedt AG & Co
Tube 50ml	Sarstedt AG & Co
nitrocellulosis membrane 0.2um	Amersham™ Protran®, MERCK, Germany
Albumin Fraktion V	Carl Roth, Germany
Ethylenediamineteraacetic acid disodium salt dihydrate	Sigma-Aldrich
Ammonium chloride	Sigma-Aldrich
Potassium hydrogen carbonate	Sigma-Aldrich
Rat Albumin ELISA kit	ALPCO®, American
Rat IL-10 ELISA kit	R&D Systems ®, American

2.7 Apparatus

Instrument/Apparatus	**Manufacturer**
Anesthesia machine	Dräger Trajan 808, QMS medical technology, Germany
straight fine forceps	BD 331R, jeweler's type; Germany
straight spring type microsissors	OC 499R
microneedle holders	FD 285R
neurovascular micro clamps	MICROMED, Germany
microsurgical microscope	OPMI 1-FR, Carl Zeiss, Germany

electro-coagulator	ERBE ICC BIPOLAR, Germany
heating plate	MEDAX GmbH; Germany
Flow cytometer (Navios TM)	Beckman Coulter, Germany
Navios Software Version 1.3 Build 0001	Beckman Coulter, Germany
Kaluza Flow Cytometry Analysis Version 2.1.00001.20653	Beckman Coulter, Germany
Heracell 150i CO2 Incubator	Thermo Electron Corporation
GLW L46 Vortex	Oehmenlabortechnik, Germany
Rotina 35R centrifuge	Hettich, Germany
HeraeusMegafuge 16R centrifuge	Thermo Scientific, USA
Microbiological Safety Cabinet	Thermo Scientific, USA
Finnpipettevariabel 0,2– 2 µ	Thermo Scientific, USA
Finnpipettevariabel 0,5 – 10 µl	Eppendorf, Germany
Finnpipettevariabel 5 – 50 µl	Thermo Scientific, USA
Finnpipettevariabel 20 – 200 µl	Thermo Scientific, USA
Finnpipettevariabel 200 – 1000 µl	Thermo Scientific, USA
Carl Zeiss Microscope Image.A1	Carl Zeiss Microimaging GmbH
Systec VX150	Systec Gmb hours Labor Systemtechnik
Cover glass	Thermo Scientific, USA
Hemocytometer	Hect-assistant, Germany

2.8 Statistical Analysis

Data are expressed as mean ±SEM. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). Kaplan-Meier method was used to draw survival curves and survival analysis. Statistical analysis for significance and comparison of two groups was performed with the *Mann-Whitney-U-Test*. ANOVA analyzed the statistical difference between mean values of more than two groups with Bonferroni's post hoc test. Statistical significance was set at $P < 0.05$.

3 RESULTS

In our study, isograft transplantation (Lewis to Lewis transplantation, L-L) and allograft transplantation (Fischer to Lewis transplantation, F-L) were investigated. Four different times for the length of static cold storage were used: 0 hours, 4 hours, 8 hours and 12 hours. 30 days post-transplantation (short-term) and 12 weeks (long-term) post-transplantation were used as the end-points in the rat model. 28 weeks post-transplantation was also considered for specific experimental series. Survival rates of L-L and F-L transplanted rats were statistically assessed with the Log-rank test.

3.1 Impact of cold ischemia time on survival after rat renal transplantation

In the short-term group, the survival rate decreased with increasing CIT but without reaching statistical significance (Figure 6). The 30-day survival rates of rats with 0 hours CIT and 4 hours CIT were 100% for both F-L and L-L transplantation. With 8 hours of CIT, the survival rate of L-L transplanted rats was also 100% (n = 10), and the survival rate of F-L transplanted rats was 95% (n = 20, L-L vs. F-L, $P = 0.470$, Figure 6C). When the CIT reached 12 hours, the 30-day survival rate of L-L transplanted rats was 94.4% (n = 18) as compared to 70.8% of F-L transplanted rats (n = 24, L-L vs. F-L, P = 0.064, Figure 6D).

In the long-term group, the survival rate decreased more obviously with 8 hours and 12 hours of CIT (Figure 7). The 12-week survival rates of rats with a CIT of 0 hours and 4 hours were 100% for both F-L and L-L transplantation. With 8 hours of CIT, the survival rate of L-L transplanted rats was 80% (n = 5), and the survival rate of F-L transplanted rats was also 80% (n = 15, $P = 0.970$, Figure 7C). With 12 hours of CIT, the 12-week survival rate was 41.7% in the L-L transplanted rats (n = 12) and 42.1% of the F-L

transplanted rats (n = 19), being not significantly different (L-L vs. F-L, *P* =0.810, Figure 7D).

Thus, longer CIT reduced short-term and long-term survival in both allogeneic and syngeneic transplantation.

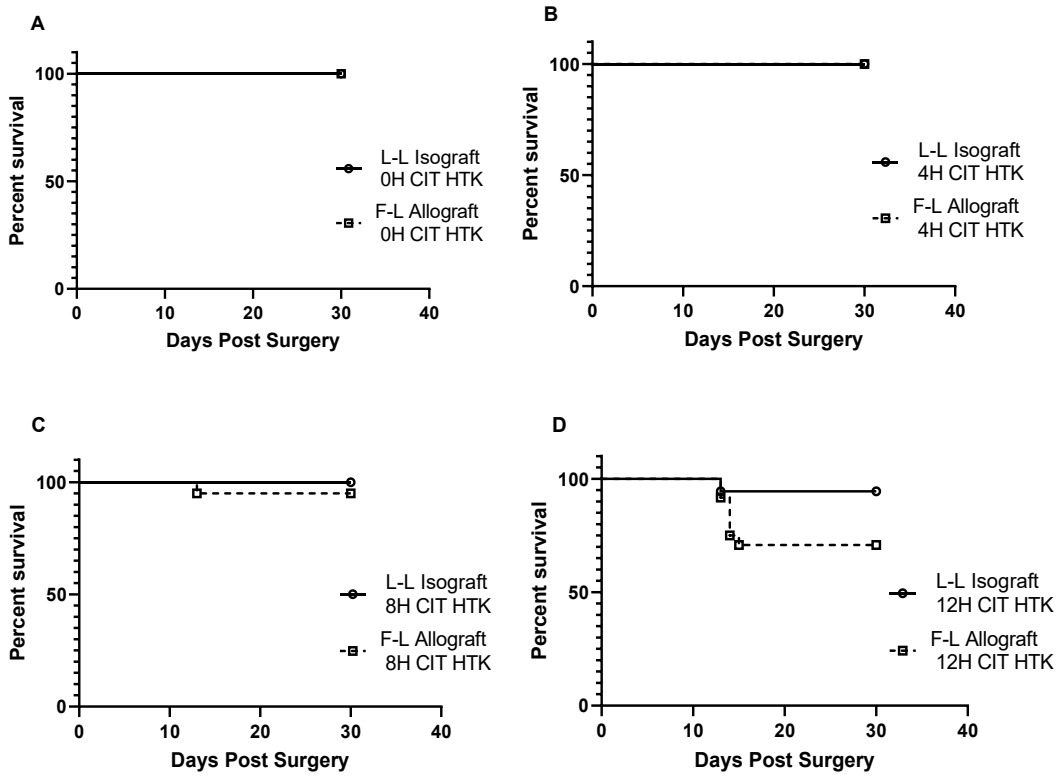


Figure 6: Short-term survival rate after iso- and allo-graft transplantation.

Survival analysis of recipients at 30 days after kidney transplantation with different CIT after static cold storage with HTK solution. (A) Showing survival rate with no CIT in F-L (n =10) and L-L (n =8) transplantation. (B) Showing survival rate with 4 hours of CIT in F-L (n =15) and L-L (n =7) transplantation. (C) Showing survival rate with 8 hours of CIT in F-L (n =20) and L-L (n =10) transplantation. (D) Showing survival rate with 12 hours of CIT in F-L (n =24) and L-L (n =18) transplantation. CIT: Cold ischemia time. H: hours.

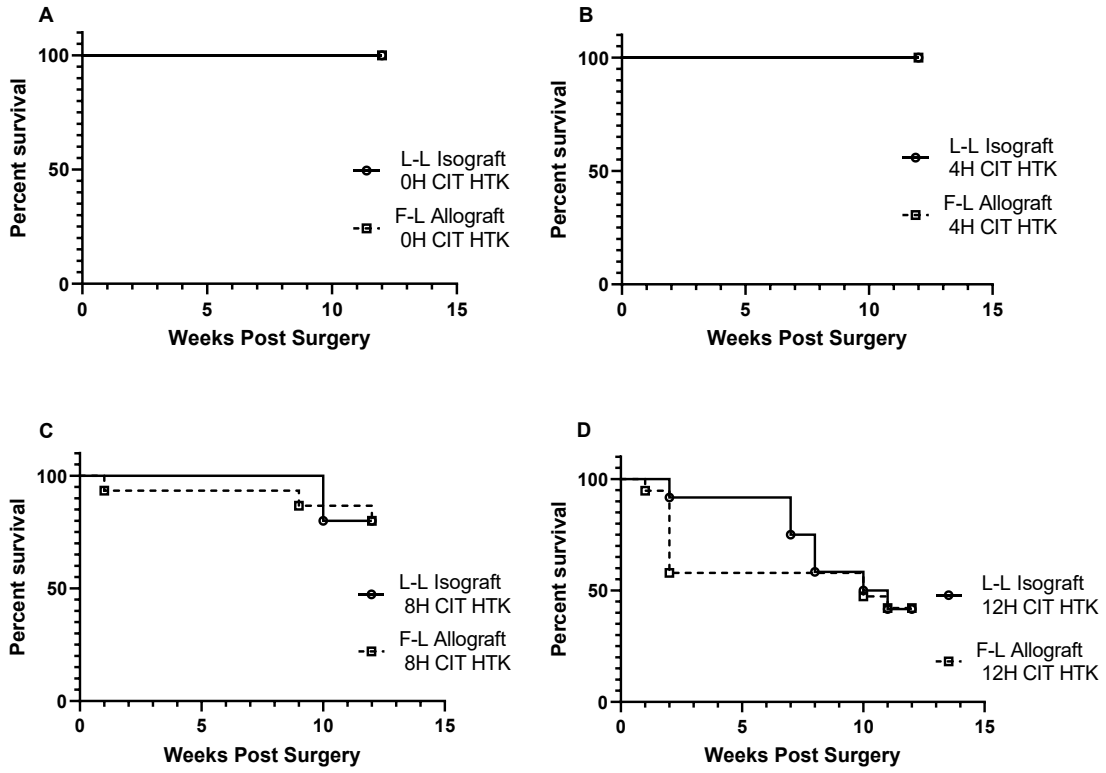


Figure 7：Long-term survival rate after iso- and allo-graft transplantation.

Survival analysis of recipients at 12 weeks after kidney transplantation with different CIT after static cold storage in HTK solution. (A) Showing survival rate with no CIT in F-L (n =10) and L-L (n =6) transplantation. (B) Showing survival rate with 4 hours of CIT in F-L (n =9) and L-L (n =6) transplantation. (C) Showing survival rate with 8 hours of CIT in F-L (n =15) and L-L (n =5) transplantation. (D) Showing survival rate with 12 hours of CIT in F-L (n =19) and L-L (n =12) transplantation. CIT: Cold ischemia time. H: hours

3.2 Function and damage of the renal graft after transplantation

3.2.1 Renal graft function decreased with longer cold ischemia time

BUN and creatinine were detected from serum to determine renal graft function. In order to investigate short-term and long-term groups separately, serum samples were harvested at fixed time points: 30 days and 12 weeks after transplantation.

In the short-term group (30 days of survival pot-transplantation), F-L transplanted rats had higher BUN levels than L-L transplanted rats in all CIT groups (Figure 8). The mean BUN levels of F-L transplanted rats increased with prolonging of CIT, from 24.11 mg/dL with 0 hours of CIT to 75.04 mg/dL with 12 hours of CIT (0 hours vs. 8 hours $P = 0.038$, 0 hours vs. 12 hours $P = 0.008$, 4 hours vs. 8 hours $P = 0.031$). Likewise, the mean BUN levels of L-L transplanted rats increased with prolonging of CIT, from 24.11 mg/dL with 0 hours of CIT to 48.64 mg/dL with 8 hours of CIT. At 12 hours of CIT, BUN was 48.26 mg/dL (0 hours vs. 8 hours $P = 0.023$, 0 hours vs. 12 hours $P = 0.026$). Similar to BUN results, F-L transplanted rats had higher serum creatinine levels than L-L transplanted rats in all CIT groups (Figure 10, F-L vs. L-L CIT 0 hours $P = 0.001$, CIT 4 hours $P = 0.066$, CIT 8 hours $P = 0.687$, CIT 12 hours $P = 0.145$). The mean serum creatinine levels of F-L transplanted rats increased with prolonging of CIT, from 0.38 mg/dL with 0 hours of CIT to 1.54 mg/dL with 12 hours of CIT (0 hours vs. 12 hours $P = 0.013$). Likewise, the mean serum creatinine levels of L-L transplanted rats increased with prolonging of CIT, from 0.28 mg/dL with 0 hours CIT to 0.68 mg/dL with 12 hours of CIT (0 hours vs. 12 hours $P = 0.011$).

In the long-term group (12 weeks of survival post-transplantation), BUN levels were not significantly different between F-L and L-L transplanted rats (Figure 9). With a CIT of 8 hours, F-L transplanted rats tended to have higher BUN levels than the L-L rats. Interestingly, at a CIT of 12 hours, BUN levels tended to be lower in F-L transplanted rats than in L-L rats. (F-L vs. L-L, 0 hours $P = 0.069$, 4 hours $P = 0.576$, 8 hours $P = 0.302$, 12 hours $P = 0.406$). Unexpectedly, though not statistically significant, BUN levels tended to be lower in the F-L rats at a CIT of 12 hours than at a CIT of 8 hours. (85.04 mg/dl vs. 61.2 mg/dl, p=0.921). Meanwhile, the BUN levels L-L transplanted rats increased with longer duration of CIT, from 21.53 mg/dL at 0 hours of CIT to 75.05 mg/dL at 12 hours of CIT (0 hours vs. 12 hours $P = 0.001$, 4 hours vs. 12 hours $P < 0.001$, 4 hours vs. 12

hours P = 0.012). The serum creatinine levels mirrored the results obtained by BUN measurements. (Figure 11).

In summary, allogeneic transplantation led to worse graft function than isogeneic transplantation, except for the long-term group with 12 hours of CIT. Longer CIT led to a worse graft function than shorter CIT in both the allogeneic and isogeneic settings.

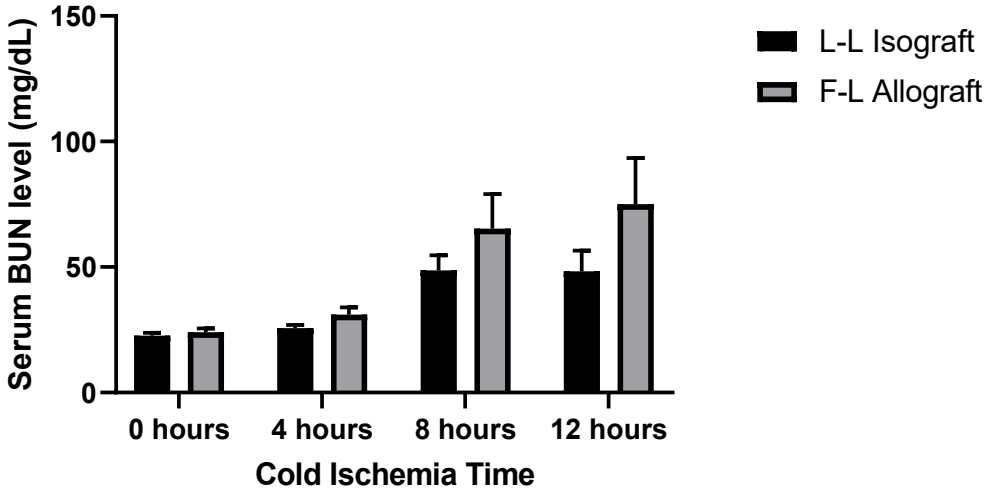


Figure 8: Serum BUN levels of iso- and allograft rat renal transplantation at 30 Days post-transplantation.

BUN levels from F-L and L-L transplanted rats were measured 30 days post-transplantation stratified by CIT (F-L: CIT 0 hours n = 7, CIT 4 hours n = 6, CIT 8 hours n = 5, CIT 12 hours n=5; L-L: CIT 0 hours n = 5, CIT 4 hours n =3, CIT 8 hours n =5, CIT 12 hours n=5). BUN levels of the allograft group were 24.11 ±1.46 mg/dL, 31.00 ±2.92 mg/dL, 65.36 ±13.69 mg/dL and 75.04 ±18.39 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. BUN levels of the isograft group were 22.66 ±1.12 mg/dL, 25.7 ±1.15 mg/dL, 48.64 ±6.08 mg/dL and 48.26 ±8.33 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. CIT: Cold ischemia time.

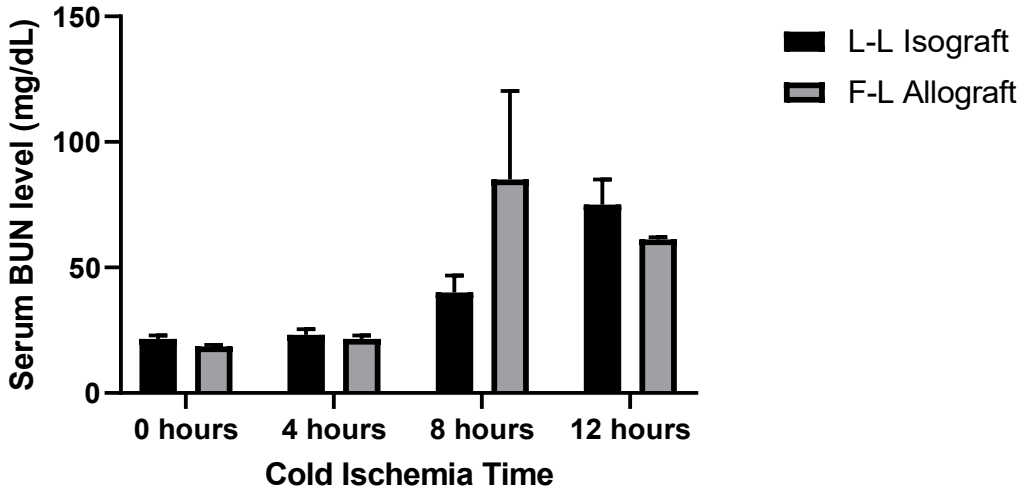


Figure 9: Serum BUN levels of iso- and allograft rat renal transplantation at 12 weeks post-transplantation.

BUN levels from F-L and L-L transplanted rats were measured at 12 weeks post-transplantation stratified by CIT (F-L CIT 0 hours n = 5, CIT 4 hours n = 4, CIT 8 hours n = 5, CIT 12 hours n = 2; L-L CIT 0 hours n = 3, CIT 4 hours n =4, CIT 8 hours n =4, CIT 12 hours n = 4). BUN levels in the allograft group were 18.50 ±0.671 mg/dL, 21.57 ±1.34 mg/dL, 85.04 ±35.22 mg/dL and 61.20 ±0.80 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. BUN levels in the isograft group were 21.53 ±1.43 mg/dL, 25.7 ±1.15 mg/dL, 40.07 ±6.79 mg/dL and and 75.05 ±9.96 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. CIT: Cold ischemia time.

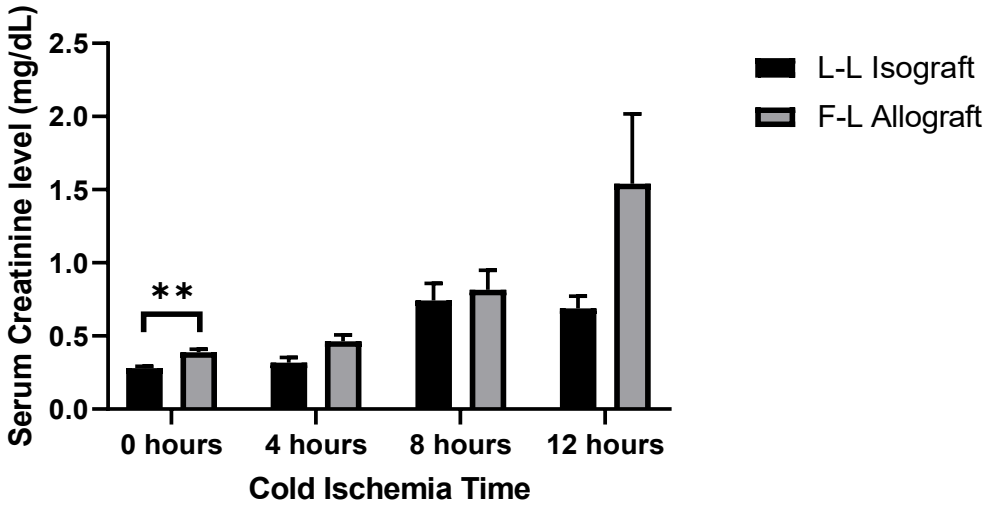


Figure 10: Serum creatinine levels of iso- and allograft rat renal transplantation at 30 days post-transplantation.

Serum creatinine levels from F-L and L-L transplanted rats were measured at 30 days post-transplantation stratified by CIT (F-L CIT 0 hours n = 7, CIT 4 hours n = 6, CIT 8 hours n = 5, CIT 12 hours n = 6; L-L CIT 0 hours n = 5, CIT 4 hours n =3, CIT 8 hours n =5, CIT 12 hours n = 5). Creatinine levels in the allograft group were 0.389 ±0.020 mg/dL, 0.463 ±0.043 mg/dL, 0.816 ±0.133 mg/dL and 1.540 ±0.479 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Creatinine levels in the isograft group were 0.280 ±0.012 mg/dL, 0.317 ±0.037 mg/dL, 0.742 ±0.117 mg/dL and 0.688 ±0.085 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. **P< 0.01. CIT: Cold ischemia time.

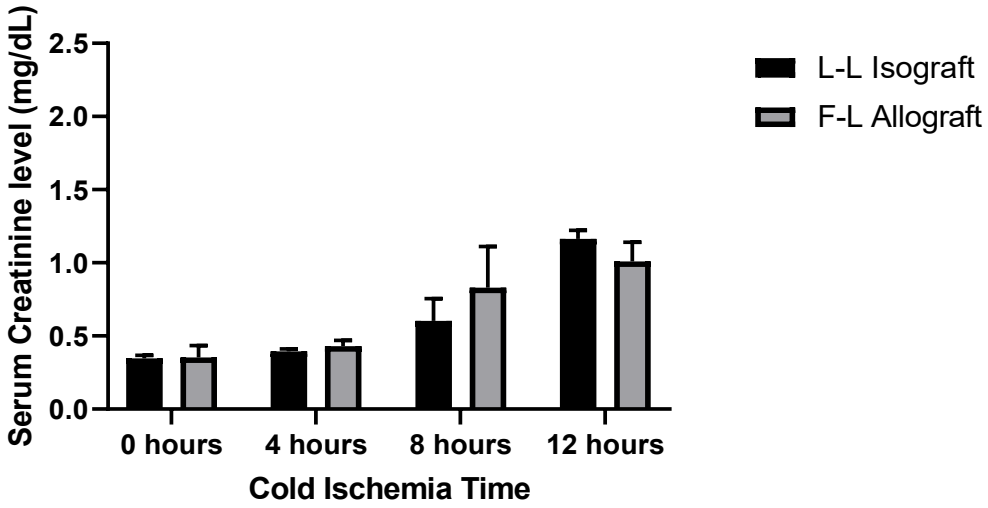


Figure 11: Serum creatinine levels of iso- and allograft rat renal transplantation at 12 weeks post-transplantation.

Creatinine levels from F-L and L-L transplanted rats were measured at 12 weeks post-transplantation stratified by CIT (F-L CIT 0 hours n = 5, CIT 4 hours n = 4, CIT 8 hours n = 4, CIT 12 hours n = 2; L-L CIT 0 hours n = 3, CIT 4 hours n =4, CIT 8 hours n =4, CIT 12 hours n = 4). Creatinine levels in the allograft group were 0.354 ±0.08 mg/dL, 0.430 ±0.041 mg/dL, 0.830 ±0.282 mg/dL and 1.010 ±0.130 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Creatinine levels in the isograft group were 0.347 ±0.02 mg/dL, 0.395 ±0.016 mg/dL, 0.603 ±0.153 mg/dL and 1.163 ±0.060 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. CIT: Cold ischemia time.

3.2.2 Renal graft damage depends on cold ischemia time

Urinary albumin excretion is a sensitive indicator of renal damage. The urine samples were collected in metabolic cages. Urinary albumin concentration was normalized to the creatinine concentration in the urine and is given as albumin to creatinine ratio. Urinary parameters were determined at 30 days after transplantation (short-term group) and 12 weeks after transplantation (long-term group).

In the short-term group, the calculated 24 hours urine volumes increased with longer CIT for both F-L and L-L transplanted rats, except for the 12 hours CIT condition of L-L transplanted rats (Figure 12). In F-L transplanted rats, the calculated mean 24 hours urine volume increased from 25.11 mL with 0 hours CIT to 49.21 mL with 12 hours CIT (0 hours vs. 12 hours $P = 0.014$). In L-L transplanted rats, the calculated mean 24 hours urine volume increased from 10.96 mL with 0 hours CIT to 33.80 mL with 12 hours CIT (0 hours vs. 12 hours $P = 0.002$). F-L transplanted rats had a higher urine volume than L-L transplanted rats (F-L vs. L-L 0 hours $P = 0.001$, 4 hours $P = 0.003$, 8 hours $P = 0.258$, 12 hours $P = 0.105$).

The long-term group harvested 12 weeks after transplantation also showed a similar result (Figure 13), F-L transplanted rats had higher urine volume, except for the 12 hours CIT condition (F-L vs. L-L: CIT 0 hours $P = 0.204$, CIT 4 hours $P = 0.050$, CIT 8 hours $P = 0.064$, CIT 12 hours $P = 0.090$). For L-L transplanted rats in the long-term group, the calculated mean 24 hours urine volume increased from 9.58 mL with 0 hours of CIT to 38.07 mL with 12 hours of CIT (0 hours vs. 12 hours $P < 0.001$). For F-L transplanted rats in the long-term group, the calculated mean 24 hours urine volume increased from 15.86 mL with 0 hours of CIT to 36.93 mL with 8 hours of CIT. Surprisingly, at a CIT of 12 hours, the urine volume decreased slightly to 14.29 mL.

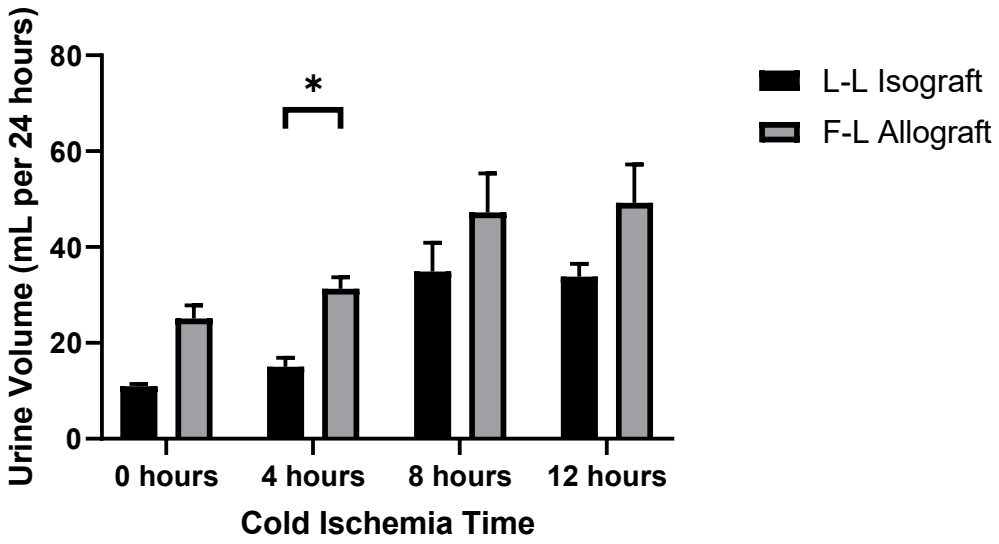


Figure 12: Calculated urine volume in iso- and allograft rat renal transplantation at 30 days post-transplantation.

The calculated 24 hours urine volume of F-L and L-L transplanted rats was measured at 30 days post-transplantation stratified by CIT (F-L CIT 0 hours n = 7, CIT 4 hours n = 9, CIT 8 hours n = 5, CIT 12 hours n = 5; L-L CIT 0 hours n = 5, CIT 4 hours n =3, CIT 8 hours n =5, CIT 12 hours n = 5). The calculated 24 hours urine volume of the allograft group were 25.11 ±2.71 mL, 31.31 ±2.36 mL, 47.21 ±8.17 mL and 49.21 ±8.02 mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. The calculated 24 hours urine volume of the isograft group were 10.96 ±0.47 mL, 15.05 ±1.85 mL, 34.87 ±6.01 mL and 33.80 ±2.66 mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. *P< 0.05. CIT: Cold ischemia time. CIT: Cold ischemia time.

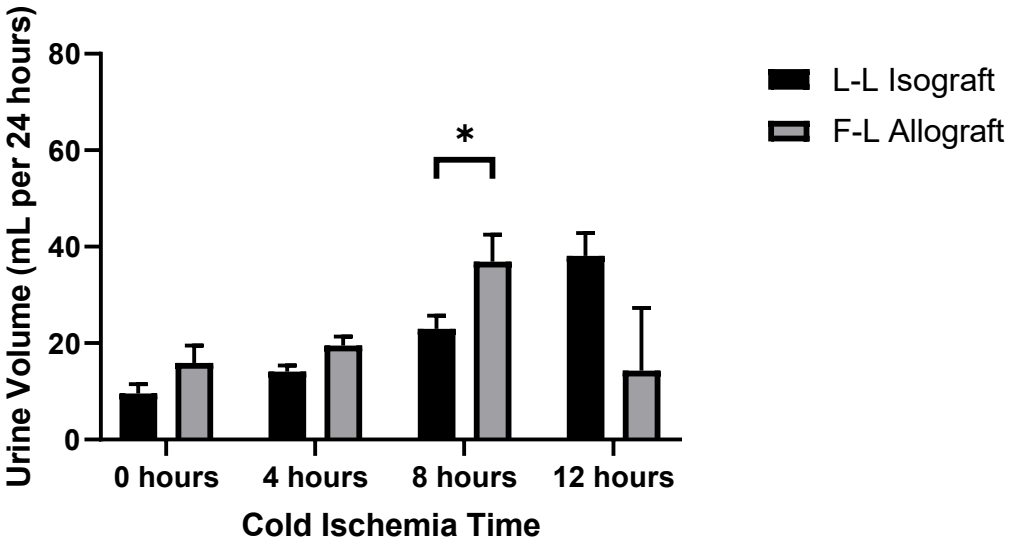


Figure 13: Calculated urine volume of iso- and allograft rat renal transplantation at 12 weeks post-transplantation.

The calculated 24 hours urine volume of F-L and L-L transplanted rats were measured at 12 weeks post-transplantation stratified by CIT (F-L CIT 0 hours n = 5, CIT 4 hours n = 4, CIT 8 hours n = 4, CIT 12 hours n = 2; L-L CIT 0 hours n = 4, CIT 4 hours n = 4, CIT 8 hours n =4, CIT 12 hours n = 4). The calculated 24 hours urine volume of the allograft group were 15.86 ±3.66 mL, 19.52 ±1.82 mL, 36.93 ±5.54 mL and 14.29 ±13.03 mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. The calculated 24 hours urine volume of the isograft group were 9.58 ±1.94 mL, 14.11 ±1.25 mL, 22.94 ±2.73 mL and 38.07 ±4.77 mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. Data are depicted as mean ±SEM. *P< 0.05. CIT: Cold ischemia time.

In the short-term group, the mean urinary albumin concentrations of F-L transplanted rats were 0.14 ±0.06 mg/mL, 3.18 ±3.03 mg/mL, 23.30 ±6.37 mg/mL and 17.94 ±11.66 mg/mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively; the mean urinary albumin concentrations of L-L transplanted rats were 0.15 ±0.03 mg/mL, 0.42 ±0.04 mg/mL, 25.31 ±7.13 mg/mL and 100.35 mg/mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively (Figure 14A). In L-L transplanted rats, the urinary albumin

concentration increased with extended CIT (0 hours vs. 12 hours $P = 0.006$). Likewise, in F-L transplanted rats, the urinary albumin concentration increased from 0 hours to 8 hours of CIT. Unexpectedly, urinary albumin concentration decreased at a CIT of 12 hours compared to the condition with a CIT of 0 hours (Figure 14A).

Urinary creatinine results in the short-term group are shown in Figure 14B. L-L transplanted rats had higher urinary creatinine concentrations than F-L transplanted rats in call conditions (F-L vs. L-L, CIT 0 hours $P < 0.001$, CIT 4 hours $P < 0.001$, CIT 8 hours $P = 0.023$, CIT 12 hours $P = 0.075$). The mean urinary creatinine concentrations of F-L transplanted rats were 36.27 ±2.57 mg/dL, 28.43 ±1.65 mg/dL, 14.02 ±1.68 mg/dL and 13.12 ±2.07 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT (0 hours vs. 4 hours $P = 0.036$, 0 hours vs. 8 hours $P < 0.001$, 0 hours vs. 12 hours $P < 0.001$, 4 hours vs. 8 hours $P < 0.001$, 4 hours vs. 12 hours $P < 0.001$); Meanwhile, the mean urinary creatinine concentrations of L-L transplanted rats were 80.88 ±3.08 mg/dL, 66.20 ±5.65 mg/dL, 24.72 ±3.42 mg/dL and 20.44 ±2.92 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT (0 hours vs. 8 hours $P < 0.001$, 0 hours vs. 12 hours $P < 0.001$, 4 hours vs. 8 hours $P < 0.001$, 4 hours vs. 12 hours $P < 0.001$).

Furthermore, the urinary albumin to urinary creatinine ratio was calculated (Figure 14C). The ratios were non-significantly higher in F-L transplanted rats with 0 hours, 4 hours and 8 hours CIT, but lower with 12 hours CIT (Figure 14C, F-L vs. L-L, CIT 0 hours $P = 0.437$, CIT 4 hours $P = 0.517$, CIT 8 hours $P = 0.407$, CIT 12 hours $P = 0.156$). The mean urinary albumin creatinine ratios of F-L transplanted rats were 14.25 ±13.78, 12.13 ±11.51, 171.75 ±40.56 and 174.21 ±132.37 with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. The mean urinary albumin creatinine ratios of L-L transplanted rats were 0.18 ±0.04, 0.64 ±0.08, 122.33 ±39.45 and 531.01 ±185.46 with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively.

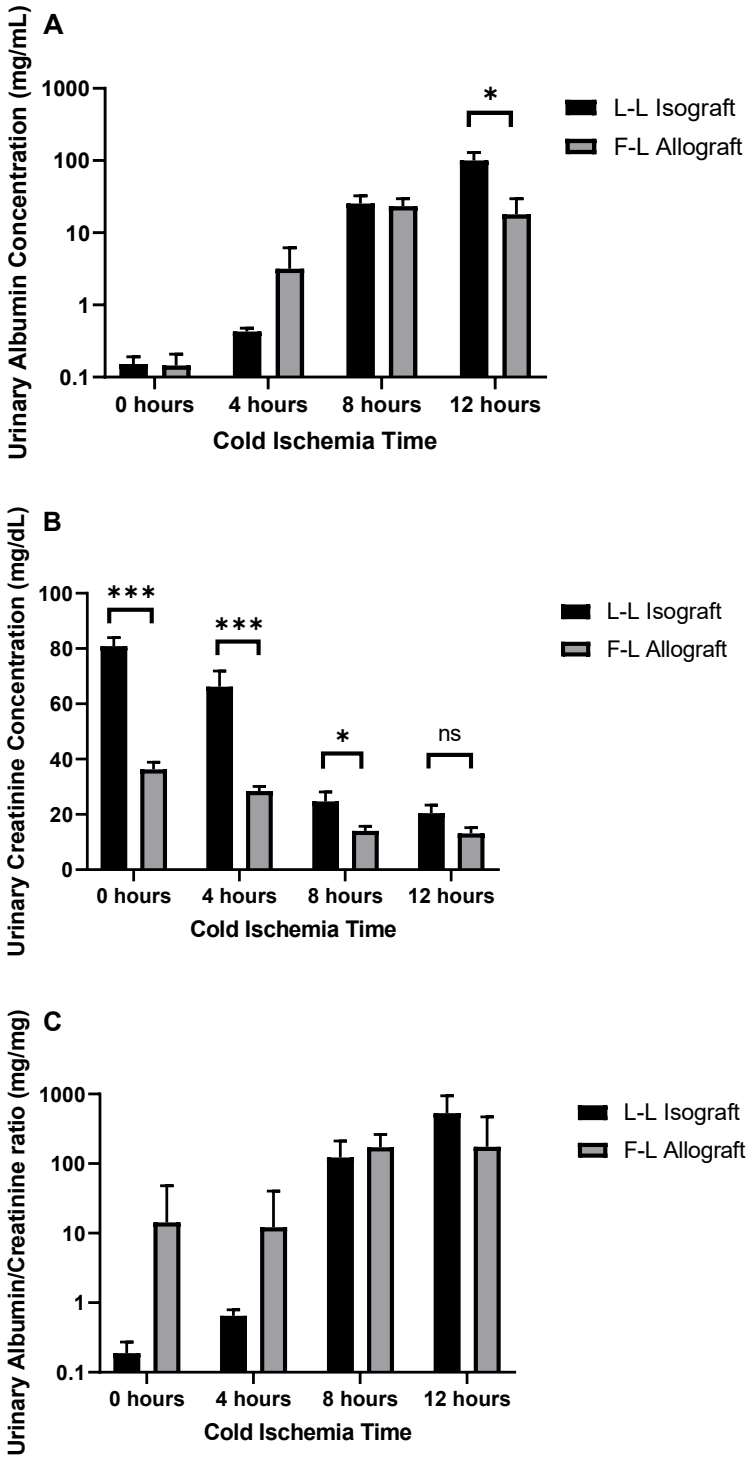


Figure 14: Urinary albumin and creatinine levels of iso- and allo-graft rat renal transplantation at 30 days post-transplantation.

Urinary albumin to creatinine ratio from F-L and L-L transplanted rats were measured at 30 days post-transplantation stratified by CIT (F-L CIT 0 hours n = 6, CIT 4 hours n = 6,

CIT 8 hours n = 5, CIT 12 hours n = 5; L-L CIT 0 hours n = 4, CIT 4 hours n =3, CIT 8 hours n =5, CIT 12 hours n = 5). (A) The urinary albumin concentration depending on CIT is shown. (B) The urinary creatinine concentration is shown stratified by CIT. (C) The urinary albumin to urinary creatinine is depicted for the different experimental groups stratified by CIT. Data are depicted as mean ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001. CIT: Cold ischemia time.

In long-term group, the mean urinary albumin concentrations of F-L transplanted rats were 0.55 ±0.20 mg/mL, 0.27 ±0.10 mg/mL, 1.96 ±0.85 mg/mL and 40.50 ±7.97 mg/mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively; the mean urinary albumin concentrations of L-L transplanted rats were 0.41 ±0.10 mg/mL, 29.59 ±7.67 mg/mL, 49.69 ±24.85 mg/mL and 45.44 ±7.02 mg/mL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively (Figure 15A). Urinary albumin concentrations of L-L transplanted rats increased with CIT from 0 hours to 8 hours CIT, reached the maximum at 8 hours of CIT, and slightly decreased at the CIT of 12 hours. As for F-L transplanted rats, the urinary albumin concentration decreased from 0 hours to 4 hours of CIT and increased from 4 hours to 12 hours CIT (0 hours vs. 12 hours $P < 0.001$, 4 hours vs. 12 hours $P < 0.001$, 8 hours vs. 12 hours $P < 0.001$). F-L transplanted rats tended towards a lower urinary albumin concentration than L-L transplanted rats except for a CIT of 0 hours (F-L vs. L-L, CIT 0 hours $P = 0.582$, CIT 4 hours $P = 0.008$, CIT 8 hours $P = 0.103$, CIT 12 hours $P = 0.692$).

In the long-term group (Figure 15B), L-L transplanted rats had higher urinary creatinine concentrations than F-L transplanted rats in all experimental CIT conditions (0 hours $P = 0.011$, 4 hours $P = 0.003$, 8shours $P = 0.015$, 12 hours $P = 0.100$). The mean urinary creatinine concentrations of F-L transplanted rats were 33.23 ±1.74 mg/dL, 49.41 ±3.93 mg/dL, 13.90 ±2.90 mg/dL and 8.95 ±0.55 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. The mean urinary creatinine concentrations of L-L

transplanted rats were 90.72 ±12.32 mg/dL, 94.50 ±15.40 mg/dL, 38.37 ±10.77 mg/dL and 18.50 ±2.97 mg/dL with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively.

Furthermore, the urinary albumin to urinary creatinine ratio was calculated for the long-term survival group (Figure 15C). The mean urinary albumin creatinine ratios of F-L transplanted rats were 0.25 ±0.02, 0.49 ±0.18, 26.25 ±20.72 and 459.82 ±117.32 with 0 hours, 4 hours, 8 hours and 12 hours of CIT, respectively. The rato increased with the extension of CIT (0 hours vs. 12 hours $P < 0.001$, 4 hours vs. 12 hours $P < 0.001$, 8 hours vs. 12 hours $P < 0.001$); the mean albumin creatinine ratios of L-L transplanted rats were 0.49 ±0.17, 32.60 ±10.45, 217.42 ±146.83 and 280.61 ±74.21 with 0 hours, 4 hours, 8 hours and 12 hours of CIT, also increased with the prolonging of CIT. The urinary albumin to creatinine ratios were higher in L-L transplanted rats at 0 hours, 4 hours and 8 hours of CIT, but lower at 12 hours CIT as compared to the F-L transplanted rats (F-L vs. L-L, CIT 0 hours P = 0.241, CIT 4 hours P = 0.007, CIT 8 hours P = 0.244, CIT 12 hours P = 0.247).

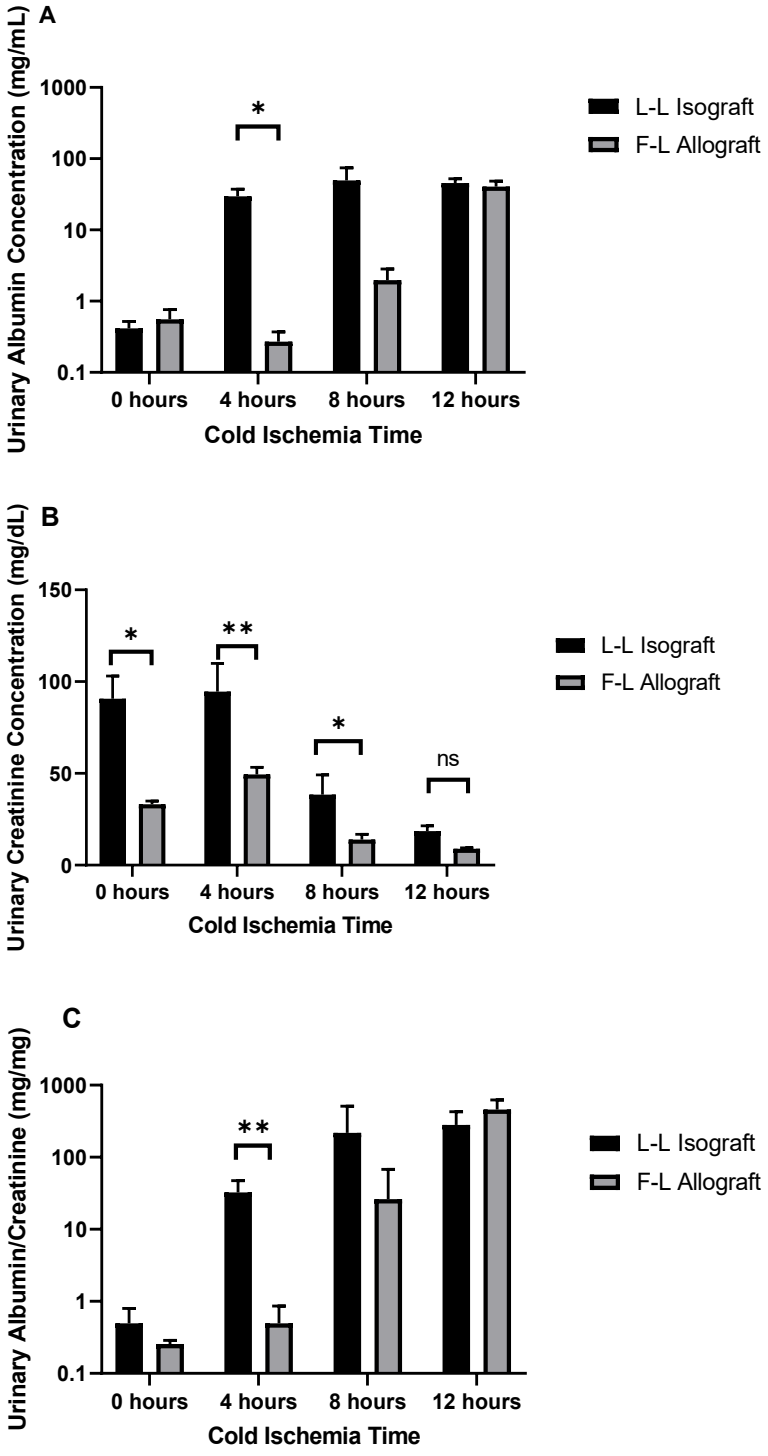


Figure 15: Urinary albumin and creatinine levels of iso- and allo-graft rat renal transplantation at 12 weeks post-transplantation.

Urinary albumin to creatinine ratio from F-L and L-L transplanted rats were measured at 12 weeks post-transplantation stratified by CIT (F-L CIT 0 hours n = 5, CIT 4 hours n =

4, CIT 8 hours n = 4, CIT 12 hours n = 2; L-L CIT 0 hours n = 4, CIT 4 hours n =4, CIT 8 hours n =4, CIT 12 hours n = 4). (A) The urinary albumin concentration depending on CIT is shown. (B) The urinary creatinine concentration is shown stratified by CIT. (C) The urinary albumin to urinary creatinine is depicted for the different experimental groups stratified by CIT. Data are depicted as mean ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001. CIT: Cold ischemia time.

In summary, renal function decreased in both the iso- and allograft condition with longer cold ischemia time. Likewise, enhanced renal damage as measured by urinary albumin excretion was correlated with longer cold ischemia time. In both iso- and allograft conditions, renal damage and functional impairment of the renal graft were detected.

3.3 Determination of allograft specific Antibodies in the rat renal transplantation model

Allograft specific antibody (ASA) levels in serum are one direct biomarker for antibody-mediated rejection. Two classes of immunoglobulins, IgG and IgM, were studied in this study. Moreover, two subclasses of IgG: IgG2a and IgG2b, were studied in addition. Blood samples were harvested at 30 days, 12 weeks or 28 weeks post-transplantation. IgG, IgM, IgG2a and IgG2b ASA serum levels were measured using Fischer splenocytes; ASA bound to splenocytes were detected with anti-rat Ig antibodies and measured by flow cytometer. As the control, PBS instead of serum-incubated splenocytes were used. The mean fluorescence intensity of ASA on splenic 7-AAD^{low} CD3^{+} cells was taken to minimize bias by non-selective binding. The fold change of MFI (MFI of serum incubated splenocytes divided by MFI of PBS incubated splenocytes) was calculated and used to depict ASA levels.

3.3.1 Serum ASA levels in rat renal transplantation 30 days post-transplantation stratified by CIT

ASA levels in the short-term group(rats 30 days post-transplantation) were measured, and experimental groups were stratified by CIT (Figure 16). Serum levels from ASA IgG, IgM, IgG2a and IgG2b were presented separately.

Serum IgG-ASA levels in the short-term group are shown in Figure 16A. IgG-ASA were nearly undetectable in L-L transplanted rats independent of CIT (IgG ASA MFI fold change:0 hours of CIT 0.84 ±0.06, 4 hours of CIT 0.84 ±0.05, 8 hours of CIT 0.88 ±0.11, 12 hours of CIT 0.63 ±0.02), as expected. Meanwhile, Serum IgG-ASA of F-L transplanted rats were highest at lowest CIT but were detectable in all other CIT groups (IgG ASA MFI fold change: 0 hours of CIT 8.90 ±2.17, 4 hours of CIT 6.90 ±1.33, 8 hours of CIT 2.38 ±0.92, 12 hours of CIT 3.84 ±0.95). Serum IgG-ASA were higher in F-L transplanted rats than in L-L transplanted rats (F-L vs. L-L CIT 0 hours $P = 0.003$, CIT 4 hours $P = 0.012$, CIT 8 hours $P = 0.156$, CIT 12 hours $P = 0.021$).

IgM-ASA serum levels are shown in Figure 16B. Serum IgM-ASA were detectable on a low level in L-L transplanted rats independent of CIT (IgM ASA MFI fold change: 0 hours of CIT 2.34 ±0.75, 4 hours of CIT 2.02 ±0.10, 8 hours of CIT 3.78 ±0.57, 12 hours of CIT 2.20 ±0.10). Serum IgM-ASA of F-L transplanted rats were highest at 12 hours of CIT (IgM ASA MFI fold change: 12 hours of CIT 9.55 ± 1.58, vs. 0 hours $P = 0.0248$, vs. 4 hours $P = 0.006$, vs. 8 hours $P = 0.022$, Figure 16B), and were lower at 0, 4, 8 hours of CIT (IgM ASA MFI fold change: 0 hours of CIT 3.82 ±0.79, 4 hours of CIT 2.47 ±0.25, 8 hours of CIT 4.18 ±0.49). Serum IgM-ASA were significantly higher in F-L transplanted rats than in L-L transplanted rats at 12 hours of CIT ($P = 0.039$). No significant difference was detected at 0, 4, 8 hours of CIT comparing F-L transplantation to L-L transplantation (F-L vs. L-L 0 hours $P = 0.293$, 4 hours $P = 0.277$, 8 hours $P = 0.648$).

IgG-ASA subclasses were specifically measured and are shown in Figure 16. As expected, IgG2a-ASA were nearly undetectable in L-L transplanted rats independent of CIT (IgG2a ASA MFI fold change: 0 hours of CIT 1.00 ±0.01, 4 hours of CIT1.00 ±0.01, 8 hours of CIT1.03 ±0.01, 12 hours of CIT1.02 ±0.01). Serum IgG2a-ASA of F-L transplanted rats were highest at 12 hours of CIT (vs. 0 hours $P = 0.044$, vs. 4 hours $P = 0.029$, vs. 8 hours $P = 0.053$), and nearly undetected at the other CIT (IgG2a ASA MFI fold change: 0 hours of CIT 1.15 ±0.06, 4 hours of CIT 1.02 ±0.03, 8 hours of CIT 1.35 ±0.19, 12 hours of CIT2.85 ±0.53). F-L transplanted rats had significantly higher mean serum IgG2a-ASA than L-L transplanted rats at a CIT of 12 hours (F-L vs. L-L 0 hours of CIT $P = 0.171$, 4 hours of CIT $P = 0.651$, 8 hours of CIT $P = 0.327$, 12 hours of CIT $P = 0.019$).

Serum IgG2b-ASA levels were also measured and are shown in Figure 16D. IgG2b-ASA levels in L-L transplanted rats were nearly undetectable (IgG2b ASA MFI fold change: 0 hours of CIT 1.28 ±0.02, 4 hours of CIT 1.32 ±0.14.13, 8 hours of CIT 1.17 ±0.01, 12 hours of CIT 1.23 ±0.02). In contrast, serum IgG2b-ASA in F-L transplanted rats were highest at the lowest CIT and were also detectable in all other CIT groups (IgG2b ASA MFI fold change: 0 hours of CIT 91.17 ±55.27, 4 hours of CIT 49.03 ±27.13, 8 hours of CIT 24.70 ±15.76, 12 hours of CIT 40.61 ±10.56). F-L transplanted rats had higher levels of serum IgG2b-ASA than L-L transplanted rats (F-L vs. L-L 0 hours of CIT $P = 0.067$, 4 hours of CIT $P = 0.052$, 8 hours of CIT $P = 0.067$, 12 hours of CIT $P = 0.005$).

Thus, ASA were detectable in serum already 30 days after allogeneic transplantation and were predominantly of the IgG2b subclass. Serum ASA IgG levels were not further enhanced by the extension of CIT. In contrast, serum ASA IgM levels were highest at the longest CIT in the allogeneic setting.

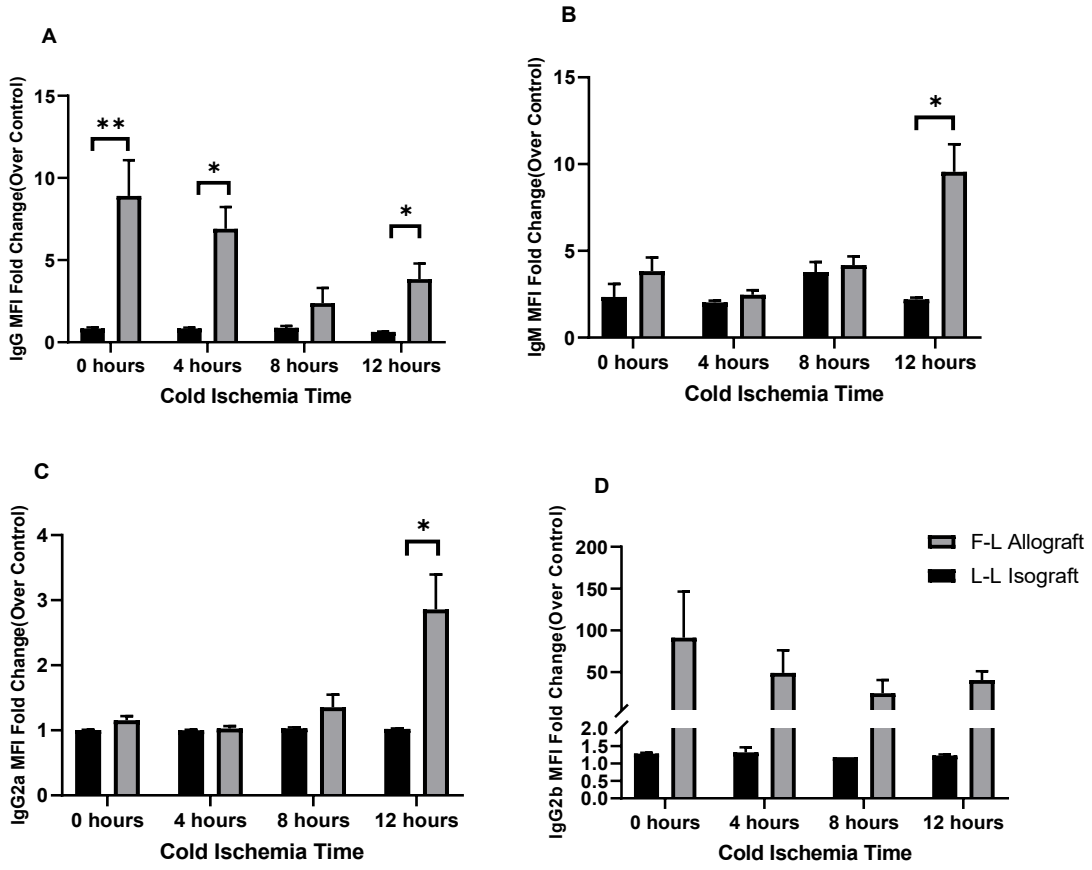


Figure 16:Serum ASA levels in iso- and allo-graft rat renal transplantation stratified by CIT at 30 days post-transplantation.

The ASA serum levels from F-L and L-L transplanted rats were measured at 30 days post-transplantation stratified by CIT (F-L, CIT 0 hours n =3; CIT 4 hours n =3, CIT 8 hours n =5; CIT 12 hours n =5; L-L, CIT 0 hours n =2; CIT 4 hours n =2, CIT 8 hours n =2; CIT 12 hours n =2). The serum levels of ASA were measured by flow cytometry and are given as fold change of mean fluorescence intensity over control (MFI). (A) ASA IgG serum levels are depicted for the experimental groups stratified by CIT. (B) ASA Serum IgM levels are depicted for the experimental groups stratified by CIT. (C) ASA serum IgG2a levels are depicted for the experimental groups stratified by CIT. (D) ASA serum IgG2b levels are depicted for the experimental groups stratified by CIT. Data are depicted as mean ±SEM. * $P < 0.05$. ** $P < 0.01$. ASA: Allograft specific antibodies. MFI: Mean fluorescence intensity. CIT: Cold ischemia time.

3.3.2 Serum ASA levels in rat renal transplantation after 12 weeks post-transplantation stratified by CIT

ASA levels in the long-term group (12 weeks post-transplantation) were measured, and experimental groups were stratified by CIT. Serum levels for ASA IgG, IgM, IgG2a and IgG2b were shown (Figure 17).

IgG-ASA were nearly undetectable in L-L transplanted rats independent of CIT (Figure 17A, IgG ASA MFI fold change: 0 hours of CIT 1.20 ±0.01, 4 hours of CIT 1.16 ±0.05, 8 hours of CIT 1.07 ±0.02, 12 hours of CIT 0.99 ±0.08), as expected. Serum IgG-ASA were detected in F-L transplanted rats (IgG ASA MFI fold change: 0 hours of CIT 8.21 ±1.06, 4 hours of CIT 4.06 ±0.27, 8 hours of CIT 3.15 ± 0.97, 12 hours of CIT 1.48 ±0.18), and gradually declined with longer CIT (0 hours vs. 8 hours $P = 0.013$, 0 hours vs. 12 hours $P = 0.025$). Serum IgG-ASA in F-L transplanted rats were much higher than in L-L transplanted rats (F-L vs. L-L 0 hours of CIT $P = 0.011$, 4 hours of CIT $P = 0.009$, 8 hours of CIT $P = 0.339$, 12 hours of CIT $P = 0.142$).

Serum IgM-ASA were detectable on a low level in L-L transplanted rats independent of CIT (Figure 17B, IgM ASA MFI fold change: 0 hours of CIT 2.26 ±0.37, 4 hours of CIT 2.18 ±0.07, 8 hours of CIT 2.73 ±0.18, 12 hours of CIT 2.43 ±0.18). Serum IgM-ASA of F-L transplanted rats were highest at 12 hours of CIT (IgM ASA MFI fold change: 0 hours of CIT 3.54 ±1.41, 4 hours of CIT 1.92 ±0.02, 8 hours of CIT 2.59 ±0.19, 12 hours of CIT 5.10 ±0.18). Serum IgM-ASA was higher in F-L transplanted rats than in L-L transplanted rats at 0 hours and 12 hours CIT (F-L vs. L-L 0 hours of CIT $P = 0.410$, 12 hours of CIT $P = 0.041$).

IgG-ASA subclasses were specifically measured and are shown in Figure 17. As expected, IgG2a-ASA were nearly undetectable in L-L transplanted rats (IgG2a ASA MFI fold

change: 0 hours of CIT 1.01 ±0.01, 4 hours of CIT 1.01 ±0.01, 8 hours of CIT 1.01 ±0.01, 12 hours of CIT 1.04±0.04). Serum IgG2a-ASA were detected on a low level in F-L transplanted rats (IgG2a ASA MFI fold change: 0 hours of CIT 1.63 ±0.21, 4 hours of CIT 1.56 ±0.53, 8 hours of CIT 1.79 ±0.48, 12 hours of CIT 1.01±0.01). F-L transplanted rats had higher mean serum IgG2a-ASA than L-L transplanted rats (F-L vs. L-L 0 hours of CIT $P = 0.141$, 4 hours of CIT $P = 0.409$, 8 hours of CIT $P = 0.461$, 12 hours of CIT $P = 0.638$).

Serum IgG2b-ASA were also measured and are shown in Figure 17D. Like the short-term group, serum IgG2b-ASA levels in L-L transplanted rats were nearly undetectable (IgG2b ASA MFI fold change: 0 hours of CIT 1.25 ±0.06, 4 hours of CIT 1.37 ±0.21, 8 hours of CIT 1.51 ±0.08, 12 hours of CIT 1.52 ±0.31). In contrast, serum IgG2b-ASA levels in F-L transplanted rats were detectable in all CIT groups but were highest at the lowest CIT (IgG2b ASA MFI fold change: 0 hours of CIT 127.03 ±19.91, 4 hours of CIT 60.15 ±5.72, 8 hours of CIT 28.88 ±12.51, 12 hours of CIT 66.10 ±48.12). F-L transplanted rats had higher levels of serum IgG2b-ASA than L-L transplanted rats (F-L vs. L-L 0 hours of CIT $P = 0.003$, 4 hours of CIT $P < 0.001$, 8 hours of CIT $P = 0.224$, 12 hours of CIT $P = 0.168$).

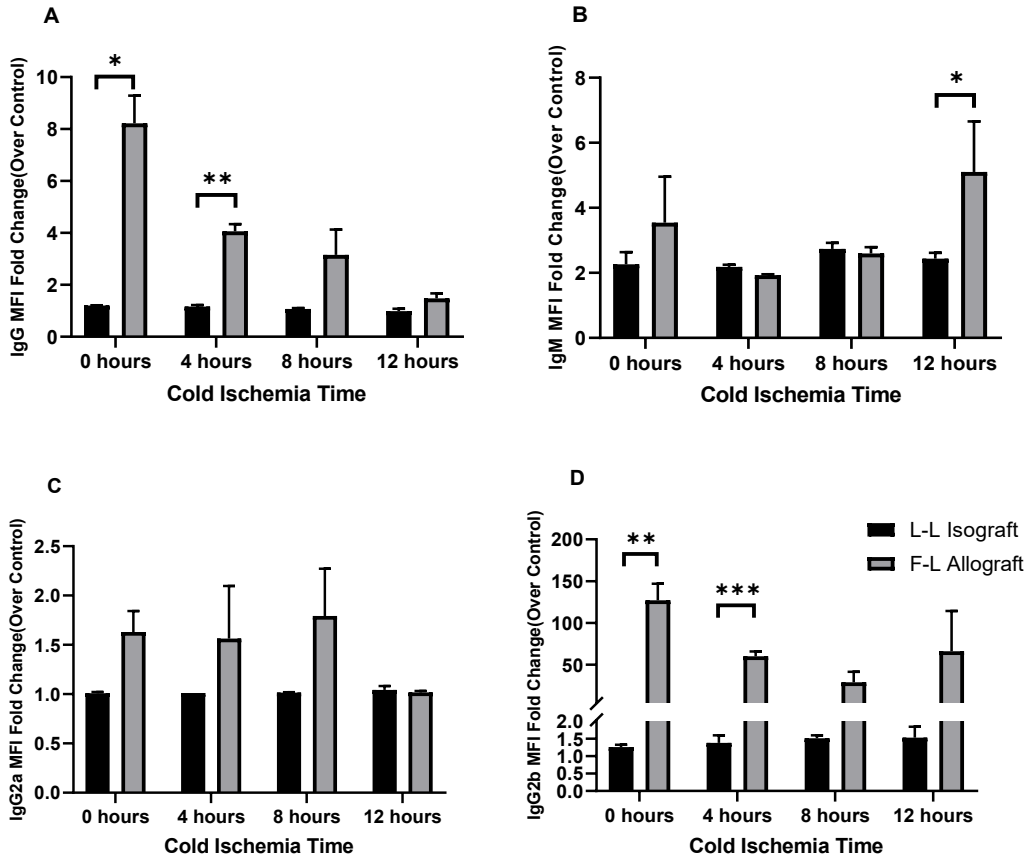


Figure 17: Serum ASA levels in iso- and allo-graft rat renal transplantation stratified by CIT at 12 weeks post-transplantation.

The ASA serum levels from F-L and L-L transplanted rats were measured at 12 weeks post-transplantation stratified by CIT (F-L, CIT 0 hours n =5; CIT 4 hours n =2; CIT 8 hours n =9; CIT 12 hours n =2; L-L, CIT 0 hours n =2; CIT 4 hours n =2; CIT 8 hours n =2; CIT 12 hours n =2). The serum levels of ASA were measured by flow cytometry and are given as fold change of mean fluorescence intensity over control (MFI). (A) ASA IgG serum levels are depicted for the experimental groups stratified by CIT (B) ASA Serum IgM levels, are depicted for the experimental groups stratified by CIT (C) ASA serum IgG2a levels are depicted for the experimental groups stratified by CIT. (D) ASA serum IgG2b levels are depicted for the experimental groups stratified by CIT. Data are depicted as mean ±SEM. * $P < 0.05$. ** $P < 0.01$. ASA: Allograft specific antibodies. MFI: Mean fluorescence intensity. CIT: Cold ischemia time.

3.3.3 ASA levels in rat renal transplantation at 28 weeks post-transplantation

ASA levels of rats reaching 28 weeks of survival after transplantation were measured for the allograft experimental series only. Rats were stratified according to CIT (Figure 18).

At 0 hours of CIT, the serum IgG-ASA levels were slightly but not significantly higher at 28 weeks post-transplantation as compared to 30 days post-transplantation in the allograft group (IgG ASA, fold change of MFI: 30 days 6.23 ±2.95 vs. 28 weeks 8.13 ±2.61, P = 0.695). There was no significant difference at 12 weeks and 28 weeks post-transplantation in the allograft group (IgG ASA MFI fold change: 12 weeks 8.21 ±1.06 vs. 28 weeks 8.13 ±2.61, P = 0.981). At 8 hours of CIT, the serum IgG-ASA levels were lower with a longer time of survival (IgG ASA, fold change of MFI: 30 days 4.24 ±1.99, 12 weeks 2.87 ±0.91, 28 weeks 0.74 ± 0.04, 30 days vs. 28 weeks P = 0.035).

The serum IgM-ASA levels decreased with a longer time of survival. There were no significant differences comparing serum IgM ASA levels overtime at 0 hours of CIT (IgM ASA, fold change of MFI: 30 days 3.82 ±0.79, 12 weeks 3.54 ±1.41, 28 weeks 2.51 ± 0.33, 30 days vs. 12 weeks P = 0.962, 30 days vs. 28 weeks P = 0.393, 12 weeks vs. 28 weeks P = 0.443). At 8 hours of CIT, a similar pattern was observed (30 days 4.18 ±0.49, 12 weeks 2.61 ±0.17, 28 weeks 2.28 ± 0.08, 30 days vs. 12 weeks P = 0.189, 30 days vs. 28 weeks P = 0.221, 12 weeks vs. 28 weeks P = 0.941).

The serum IgG2a-ASA levels slightly increased with the extended time of survival at 0 hours of CIT, though this was not statistically significant (IgG2a ASA, fold change of MFI: 30 days 1.15 ±0.06, 12 weeks 1.63 ±0.21, 28 weeks 1.78 ± 0.36, 30 days vs. 12 weeks P = 0.763, 30 days vs. 28 weeks P = 0.575, 12 weeks vs. 28 weeks P = 0.951). At 8 hours of CIT, the IgG2a-ASA serum levels were comparable over time(IgG2a ASA, fold change of MFI: 30 days 1.35 ±0.19, 12 weeks 1.78 ±0.42, 28 weeks 1.39 ± 0.33, 30

days vs. 12 weeks $P = 0.718$, 30 days vs. 28 weeks $P = 0.998$, 12 weeks vs. 28 weeks $P = 0.799$).

The IgG2b-ASA serum levels were lowest at 28 weeks post-transplantation (Figure 18). Interestingly, 8 hours of CIT resulted in much lower levels of IgG2b ASA than 0 hours of CIT at 28 weeks after transplantation (IgG2b ASA MFI fold change, 0 hours of CIT 73.83 ± 27.91 vs. 8 hours of CIT 3.14 ± 1.99, $P = 0.449$).

In summary, ASA evolved in the allogeneic setting only and serum ASA were predominantly of the IgG2b subclass. ASA remained detectable in serum over 28 weeks after transplantation at stable levels in conditions without CIT. ASA IgG serum levels seemed to decline over time at extended periods of CIT.

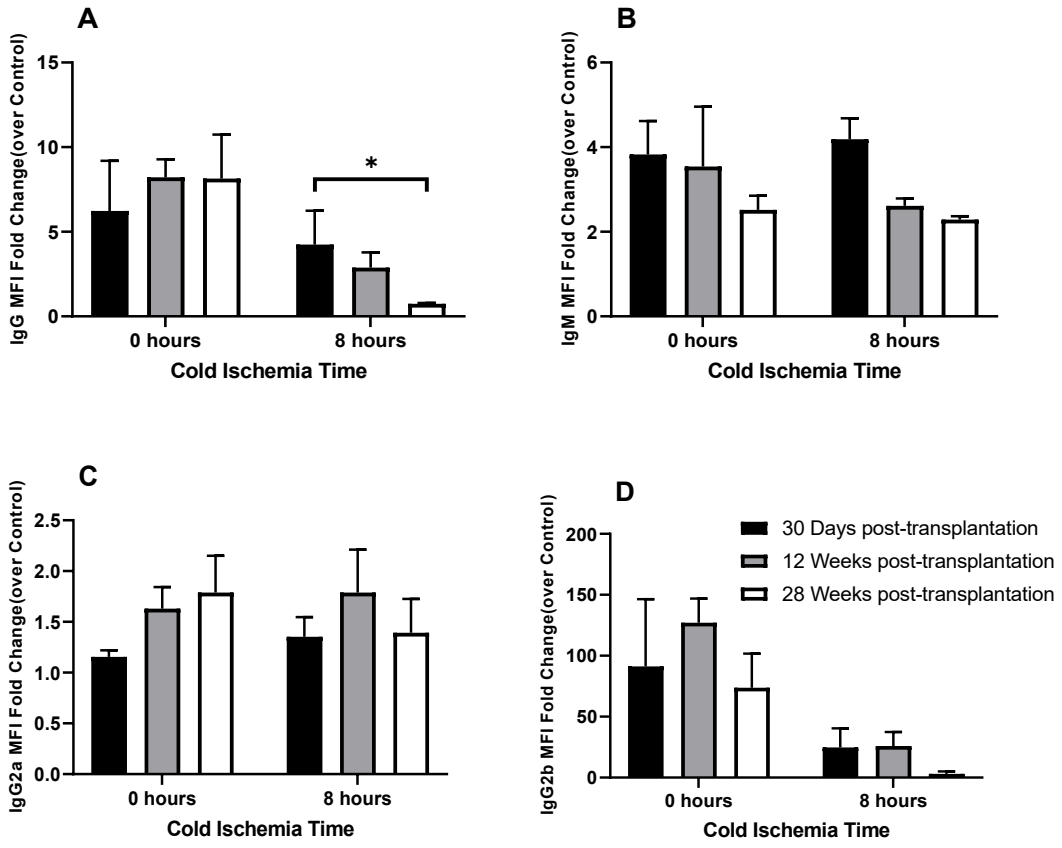


Figure 18: Serum ASA levels in allo-graft rat renal transplantation stratified by survival time and cold ischemia time.

The ASA levels from F-L transplanted rats were measured at 30 days, 12 weeks and 28 weeks post-transplantation. For each time point, two different lengths of CIT were assessed (0 hours of CIT: 30 Days n =3, 12Weeks n =5, 28 Weeks n =8; 8 hours of CIT: 30 Days n =5, 12 Weeks n =9, 28 Weeks n =3). The serum levels of ASA were measured by flow cytometry and are given as fold change of mean fluorescence intensity over control (MFI). (A) ASA IgG serum levels are depicted for a CIT of 0 and 8 hours stratified by survival time. (B) ASA IgM serum levels are depicted for each experimental group. (C) ASA IgG2a serum levels are depicted for each experimental group. (D) ASA IgG2b serum levels are given for each experimental group. Data are depicted as mean ±SEM. *P< 0.05. ASA: Allograft specific antibodies. MFI: Mean fluorescence intensity. CIT: Cold ischemia time.

3.4 Characteristics of B-cell immunity in the rat renal transplantation model

The formation of ASA depends on B cell differentiation, plasma cell maturation and affinity maturation. Thus, serum levels of B cell activating factor (BAFF) as the major driver of B cell differentiation and plasma cell formation were studied. In addition, regulatory B cells were investigated. This specific B cell population can inhibit the differentiation of B cells into plasma cells.

3.4.1 B cell-activating factor (BAFF) serum levels in the rat renal transplantation model

BAFF is a TNF-like cytokine that supports the survival and differentiation of B cells at distinct developmental stages. BAFF supports plasma cell survival and maturation, with differential impacts on IgM and IgG-producing populations. BAFF was measured from sera of rats after renal transplantation by ELISA.

In general, BAFF levels were lowest at 0 hours of CIT and higher when CIT was extended. However, there was no strict correlation of BAFF levels and CIT at 30 days post-transplantation (serum BAFF level, 0 hours of CIT 247.00 ±165.21 pg/mL, 4 hours of CIT 772.66 ±135.07 pg/mL, 8 hours of CIT 534.80 ±136.87 pg/mL, 12 hours of CIT 292.20 ±147.54 pg/mL, $P = 0.073$). In contrast, BAFF levels varied in the long-term survival group, and the BAFF levels at 12 hours of CIT were significantly higher than at 4 hours and 8 hours of CIT (serum BAFF levels, 0 hours of CIT 254.40 ±155.79 pg/mL, 4 hours of CIT 73.33 ±73.33 pg/mL, 8 hours of CIT 38.66 ±25.86 pg/mL, 12 hours of CIT 604.00 ±177.00 pg/mL; 4 hours of CIT vs. 12 hours of CIT $P = 0.023$, 8 hours of CIT vs. 12 hours of CIT $P = 0.015$). BAFF levels were not different at 0 hours of CIT comparing short-term and long-term survival groups ($P = 0.975$). However, BAFF levels in the short-term group were significantly higher at 4 hours or 8 hours of CIT than in the long-term group with the respective CIT (4 hours of CIT $P < 0.001$, 8 hours of CIT $P < 0.001$). However, with 12 hours of CIT, rats in the long-term group had higher BAFF levels than in the short-term group (P = 0.290). Thus, BAFF was detectable in all rats after allogeneic transplantation and no clear relation to CIT or time after transplantation was seen.

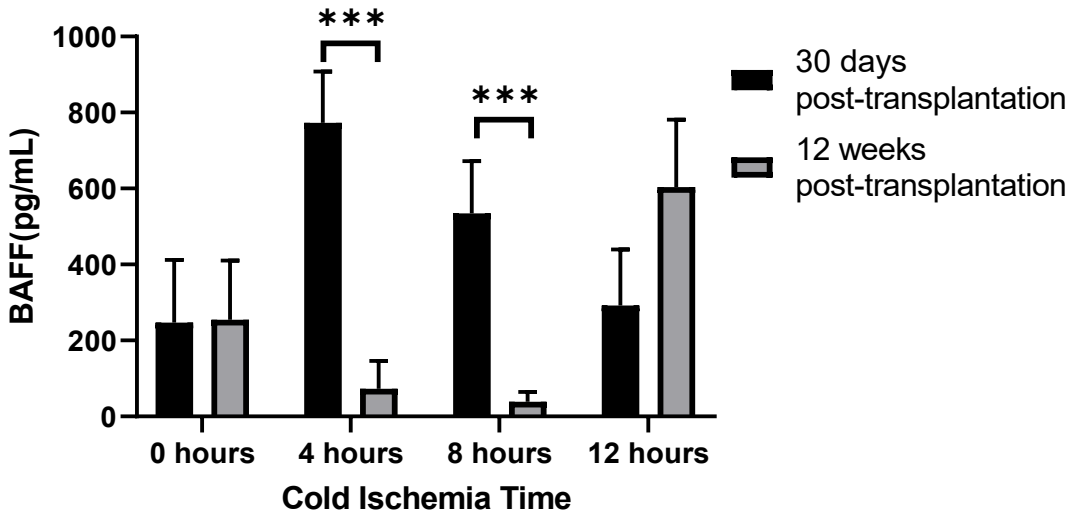


Figure 19: Serum BAFF levels after allo-graft rat renal transplantation stratified by survival time and cold ischemia time.

Serum BAFF levels of F-L transplanted rats were measured at 30 days post-transplantation (short-term group, 0 hours of CIT n = 7, 4 hours of CIT n = 6, 8 hours of CIT n = 5, 12 hours of CIT n = 5) and at 12 weeks post-transplantation (0 hours of CIT n = 5, 4 hours of CIT n = 9, 8 hours of CIT n = 9, 12 hours of CIT n = 2) stratified by CIT. Data are depicted as mean ±SEM. *** $P < 0.001$. BAFF: B cell-activating factor. CIT: Cold ischemia time.

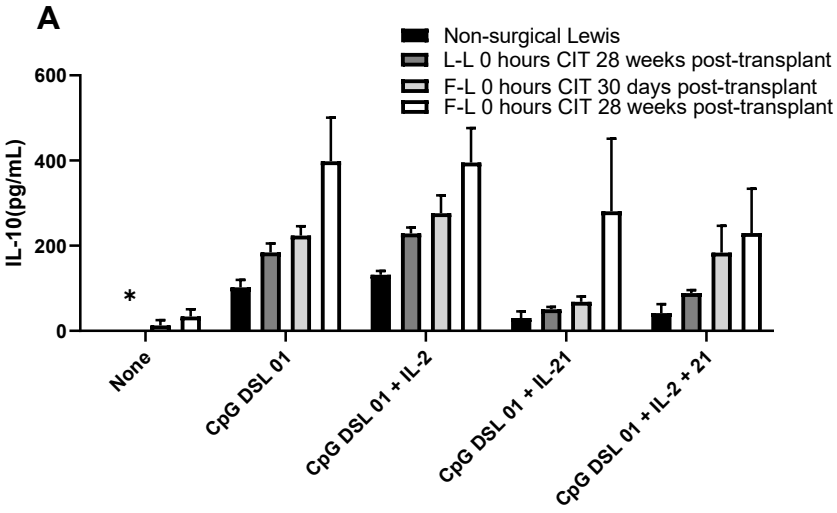
3.4.2 Regulatory B cells in the rat renal transplant model

Regulatory B cells (Breg) were identified by the signature cytokine IL-10. B cells were purified from the spleen of rats after renal transplantation. B cells were then stimulated and cultured with CpG in the presence of IL-2 or IL-21. IL-10 levels in cell culture supernatants were detected then by ELISA. Lewis rats without surgery served as controls. Splenic B cells from F-L transplanted rats were harvested 30 days and 28 weeks after transplantation. B cells stimulated with CpG + IL-2 led to the highest IL-10 levels in each experimental group. Thus, this condition was chosen for further analysis to compare the experimental groups.

IL-10 levels for rats with 0 hours of CIT were determined 30 days and 28 weeks post-transplantation (Figure 20A). With 0 hours of CIT, IL-10 levels in allograft rats 28 weeks post-transplantation tended to be higher than 30 days post-transplantation (IL-10 levels, 28 weeks 395.00 ± 81.01 pg/mL vs. 30 days 276.00 ± 42.32 pg/mL, $P = 0.262$). Meanwhile, IL-10 levels in allograft rats tended to be higher than in isograft rats and Lewis control rats (IL-10 levels, 28 weeks post-transplant, isograft rats 0 hours of CIT: 228.66 ± 14.14 pg/mL, non-surgical Lewis control rats: 131.66 ± 8.83 pg/mL, allograft rats 28 weeks vs. isograft rats 28 weeks $P = 0.113$, allograft rats 30 days vs. Lewis control $P = 0.028$, allograft rats 28 weeks vs. Lewis control $P = 0.031$).

IL-10 levels for allograft rats with 4 hours of CIT were also measured 30 days post-transplant (Figure 20B). IL-10 levels in allograft rats with 4 hours of CIT tended to be higher than with 0 hours of CIT 30 days post-transplant (IL-10 levels, 4 hours of CIT 357.66 ± 53.67 pg/mL vs. 0 hours of CIT 276.00 ± 42.32 pg/mL, $P = 0.298$).

In summary, IL-10 secreting Breg were enhanced after allogeneic transplantation. IL-10 secretion seemed to increase over time after transplantation and tended to be higher when the graft was exposed to prolonged cold ischemia.



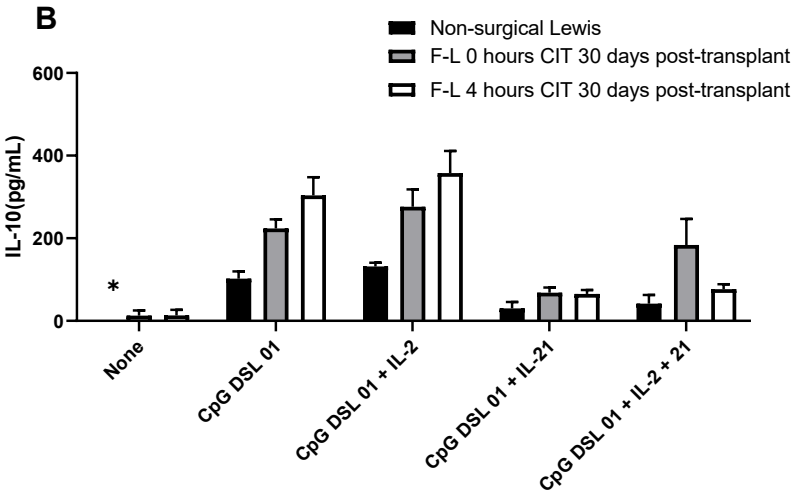


Figure 20: IL-10 levels in B cell culture supernatants in iso- and allo-graft rat renal transplantation.

Splenic B cells were isolated and purified from untreated Lewis rats, L-L transplanted rats and F-L transplanted rats. IL-10 levels were measured from the splenic B cells culture supernatants after five days of culture with CpG, IL-2 and IL-21 in vitro. Lewis rats (n =3) as control. (A) IL-10 levels are depicted for a CIT of 0 hours (allograft 30 days n =3, allograft 28 weeks n =3, isograft 28 weeks n = 3). (B) IL-10 levels of Breg harvested 30 days after allogeneic transplantation are shown (0 hours of CIT n =3, 4 hours of CIT n =3). Data are depicted as mean ±SEM. CIT: Cold ischemia time. * IL-10 was undetected.

4 DISCUSSION

Organ transplantation is currently the most effective treatment for end-stage organ failure and offers a satisfying early survival rate. The main problem at present is to extend graft survival. DSA-mediate chronic ABMR is one of the main causes of late graft loss and there is no efficacious treatment available. In this study, we aimed to investigate the association between cold ischemia of the graft, kinetics of ASA, and B-cell immunity in the rat kidney transplant model.

To carry out this study, we established a rat kidney transplantation model. Fischer to Lewis kidney transplants (F-L transplant, allograft) and Lewis to Lewis transplants (L-L transplant, isograft) were performed. 0 hours, 4 hours, 8 hours, and 12 hours of CIT were studied in both models. Samples were harvested 30 days post-transplantation (short-term group) and 12 weeks post-transplantation (long-term group); for selected groups, the survival period was extended to 28 weeks.

The survival rate over time decreased with longer CIT in both the allograft and isograft groups. Noris et al. performed both F-L and L-L transplants in rats but only with 0 hours of CIT and reported no deaths 12 weeks after transplantation (Noris et al., 2001). Consistent with these findings, the survival rates for a CIT of 0 and 4 hours, were 100% for both allograft and isograft transplants at 12 weeks post-transplant in our study. Herrero-Fresneda studied the effect of cold ischemia in the F-L model. The authors found a decreased survival of only 53% 12 weeks post-transplant at a CIT of five hours versus 100% without CIT (Herrero-Fresneda et al., 2005). Ripoll et al. investigated the effect of short CIT (2.5 hours) in an allogeneic and isogeneic setting (Ripoll et al., 2012). For this purpose, renal grafts from Brown Norway (BN) rats were transplanted to Wistar-Agouti (WA) rats for the allogeneic setting or from WA donors to WA recipients for the isogeneic setting. Interestingly, cold ischemia reduced survival significantly in the allogeneic setting but not in the isogeneic setting. Dragun et al. investigated the L-L transplant model

with an extended period of warm ischemia and found a progressive decline of survival rate over 20 weeks (Dragun et al., 1998). Thus, our findings that longer CIT impairs survival after renal transplantation are consistent with the literature. However, we did not find any significant difference in survival rates between allograft and isograft transplantation. Baker et al. reported rat strain differences in resistance to ischemia, and Basile et al. even used chromosome substitution to prove this genetic difference could affect the resistance to kidney injury following ischemia-reperfusion (Baker et al., 2000; Basile et al., 2013). It can be hypothesized that renal grafts of Lewis donors are more susceptible to ischemia-reperfusion injury than renal grafts of Fischer donors. This may explain why survival in the allogeneic setting (Fischer renal graft) with the additional immunological damage of the graft was not worse than in the isogeneic setting (Lewis renal graft). Likewise, at a CIT less than 8 hours or 12 hours, renal graft function was comparable in the allogeneic and the isogeneic condition.

BUN and creatinine were detected from serum to evaluate graft function. We found that longer CIT were associated with higher serum BUN and serum creatinine levels. Qiu et al. reported consistent findings in a mouse renal transplant model showing that prolonged CIT resulted in elevated levels of BUN and serum creatinine (Qiu et al., 2020). Furthermore, allogeneic transplantation was associated either higher serum BUN than in the isogeneic transplantation setting at a moderate CIT of eight hours. This difference was reported in multiple previous articles involving F-L and L-L transplant models (Hullett et al., 2005; Pan et al., 2003; Ramirez-Bajo et al., 2020; Zou et al., 2017).

The urinary albumin-to-creatinine ratio was detected to evaluate graft damage. Longer CIT led to a higher urinary albumin-to-creatinine ratio in both the allogeneic and syngeneic context. Tullius et al. used the F-L transplanted model and found increased proteinuria with higher CIT and higher donor age (Tullius et al., 2000). Takada et al. reported increased proteinuria with high CIT even in an isogeneic context using the L-L

transplanted model (Takada et al., 1997). Rovira et al. used the F-L and L-L rat renal transplant model and reported no difference in proteinuria at 4 weeks post-transplantation with a CIT of 0 hours; at 12 weeks post-transplantation, allograft rats had higher proteinuria than isograft with 0 hours of CIT(Rovira et al., 2018). Ramirez-Bajo et al. reported higher proteinuria in F-L transplanted rats than in L-L transplanted rats at 4 and 12 weeks post-transplant (Ramirez-Bajo et al., 2020). Apart from our study, there is no other study comparing allograft and isograft damage with a CIT longer than one hour.

In our study, serum samples were harvested from allograft and isograft rats that reached the endpoints of survival. Then, allograft specific antibodies (ASA) were detected by flow cytometry. ASA were found positive in allograft rats and negative in isograft rats, regardless of short-term (30 days post-transplant) or long-term (12 weeks post-transplant) survival. This is consistent with the results reported by Vogelbacher et al. Vogelbacher et al. found ASA positive at weeks 3 and 12 post-transplant in the F-L kidney transplant model (Vogelbacher et al., 2010).

Kohei et al. studied ASA changes in Brown Norway to Lewis rat skin transplant model with flow cytometry and reported that IgM peaked on day 7 after transplantation and disappeared after 3 weeks post-transplant, while IgG peaked after 14 days and lasted for more than 60 days (Kohei et al., 2013). Huang et al. used flow cytometry to prove that the IgG2b subclass of ASA was found positive 4 weeks after exposure to donor-specific antigens in Brown Norway to Lewis rat transplant model while IgG2a and IgM were negative (Huang et al., 2014). This is consistent with our results on ASA in the short-term survival group in which ASA were predominantly of IgG isotype belonging to IgG2b subclass.

Meanwhile, Reese et al. also used flow cytometry to study serum ASA levels in the F-L rat transplant model over 12 weeks. The authors found that serum ASA were of the IgG,

IgG2a and IgG2b isotype. However, ASA IgM were not detectable (Reese et al., 2021). This is inconsistent with our findings in the long-term survival group in which ASA IgM were positive However, there is no previous study comparing ASA levels over different CIT. Our study is the first to compare serum ASA levels both short-term and long-term groups stratified by CIT.

Our results indicate that CIT of the transplant may affect serum ASA levels, and this association was not reported previously. At extended CIT, a decline of ASA was seen. Kohei et al. used skin graft pre-sensitized rats to prove kidney grafts could adsorb ASA from serum. Serum ASA IgG levels decreased 2 hours after kidney transplantation in Brown Norway to Lewis renal transplant model (Kohei et al., 2013). Zhang et al. studied a rat to mouse kidney transplant model and reported that dense deposition of IgG and IgM could be found in glomeruli, vessel walls, and in the interstitial renal compartment (Zhang et al., 2000). Joosten et al. also studied the deposition of ASA in the F-L kidney transplanted model by immunofluorescence. The study of Joosten et al. further indicated that subclasses of ASA distribute differently in kidney graft: IgG and trace amount IgM were found in the glomerular basement membrane; IgG2a was only observed in the tubular basement membrane; IgA, IgG2b, and IgG2c were completely negative in all kidney compartments (Joosten et al., 2002). Moreover, Dragun et al. studied vascular injury in rat renal isograft transplantation, and immunohistochemistry showed that ED-1^+ cell, VLA-4^+ cell, MHC class II-positive cell and ED-2^+ dendritic macrophage cell influx increases dramatically if grafts were exposed to a CIT over 6 hours. At the same time, VCAM-1 staining increased, indicating that a CIT exceeding 6 hours led to progressive vascular injury resulting in severe structural damage (Dragun et al., 2001). Longer CIT may lead to higher antigen exposure within the renal graft. This could result in increased binding of ASA to the renal graft and declining serum ASA levels. Further evaluation of graft immunopathology is required in our experimental series to confirm

that ASA are deposited in the renal graft and that uptake of ASA by the graft reduces the ASA serum levels.

We also investigated the B cell-activating factor (BAFF), an important factor in the differentiation and maturation of plasma cells. A clinical study by Wang et al. indicated that DSA were found only in transplant patients with high serum BAFF (> 573.14 pg/mL) (Wang et al., 2019). In another study, Wilson et al. studied the effect of BAFF blockade on ASA in the murine kidney transplant model and reported that BAFF blockade could significantly decrease ASA IgG and IgM levels by reduction of B-lymphocyte subsets in both spleen and bone marrow (Wilson et al., 2019). In our study, the BAFF levels were measured after allograft transplantation at day 30 and week 12 post-transplantation. Schuster et al. reported that BAFF levels in patient serum gradually increased over 14 days, 12 weeks and 1-year post kidney transplant (Schuster et al., 2021). This is consistent with the results of our study; BAFF levels were higher in the long-term group than in the short-term group at a CIT of 12 hours. In contrast, at a CIT of 4 hours or 8 hours BAFF levels were much lower in the long-term survival group than in the short-term group.

IL-10 producing regulatory B cells were also studied in our project. Splenic B cells were collected, purified and stimulated with CpG and IL-2 followed by determination of IL-10 secretion to assess regulatory B cells function. Selected experimental groups were assayed for regulatory B-cells in rats 30 days post-transplantation (allograft 0 hours and 4 hours of CIT) and 28 weeks post-transplantation (allograft 0 hours of CIT, isograft 0 hours of CIT). In a clinical study, Laguna-Goya et al. detected IL-10^{+} Breg in fresh blood samples of kidney transplant patients and reported a significant decrease of IL-10^{+} Breg in the first 12 weeks post kidney transplant. The Breg remained low during the first year (Laguna-Goya et al., 2020). Cherukuri et al. proved that $CD24^{hi}$ $CD38^{hi}$ Breg are enriched for IL-10 expressing Breg. The authors reported that circulating Bregs from stable patients and healthy controls can be detected at similar frequency, absolute number and

IL-10 expression. However, in patients with rejection, the absolute number of Breg and IL-10 expression of Breg was decreased (Cherukuri et al., 2014). Shabir et al. also performed a 1-year observation of $CD24^{hi}$ $CD38^{hi}$ Breg in kidney transplantation patients. The authors reported that Bregs decreased in the first 90 days, followed by a slight increase and finally stabilized after 6 months post-transplant (Shabir et al., 2015). In contrast, our data showed increasing IL-10 secretion by Breg over time and IL-10 secretion was highest 28 weeks post-transplant. In addition, CIT mildly enhanced IL-10 secretion by Breg in our study. The relation of CIT and Breg has not been studied before.

In conclusion, the Fischer to Lewis transplantation model is suitable to study the mechanisms behind allograft specific antibody formation in renal transplantation. ASA formation is accompanied by enhanced secretion of IL-10 by Breg.

5 SUMMARY

Antibody-mediated rejection (ABMR) is the major barrier to prolong graft survival after renal transplantation. It was the main aims of our study were to investigate whether allograft specific antibodies evolve in the Fischer to Lewis rat renal transplant model, to study the kinetics of allograft specific antibodies over time, its relation to cold ischemia of the graft and to characterize anti-inflammatory as well as pro-humoral B-cell responses in this animal model. For this purpose, the rat Fischer to Lewis renal allograft transplantation model and Lewis to Lewis renal isograft transplantation model was used. Grafts were transplanted to recipients after a specific time of static cold storage. Rats were harvested 30 days, 12 weeks and in selected conditions 28 weeks post-transplant. Survival analysis, analysis of graft function and analysis of graft damage were performed at the specific time points. Allograft specific antibodies, serum B cell activating factor (BAFF) and splenic regulatory B-cells were assessed in parallel for the specific time points.

For both allograft and isograft rats, the survival rate decreased with the extension of cold ischemia time. Graft function and damage were worse with longer cold ischemia time in both the allogeneic and the isogeneic setting. Allograft specific antibodies were detected in the allogeneic setting only already 30 days after transplantation and remained detectable 12 and 28 weeks after transplantation. The antibodies were predominantly of the IgG isotype belonging to the IgG2b subclass. The serum levels of B cell activating factor levels were enhanced in the allogeneic setting. Anti-humoral regulatory B cells showed enhanced IL-10 secretion in the allogeneic setting. IL-10 secretion was increasing over time after transplantation and was highest at 28 weeks post-transplant.

In conclusion, the Fischer to Lewis transplantation model is a suitable model to study the pathophysiological mechanisms of humoral-induced allograft damage resembling antibody-mediated rejection.

6 ZUSAMMENFASSUNG

Die humorale Abstoßung ist der wichtigste limitierende Faktor für das Langzeitüberleben nach Nierentransplantation. Ziel dieser Untersuchung war es, das Auftreten von allospezifischen Antikörpern nach Nierentransplantation in einem experimentellen Ratten-Nierentransplantationsmodell zu charakterisieren. Die Kinetik der humoralen allospezifischen Immunantwort, die Korrelation zur kalten Ischämiezeit des Transplantats sowie pro-humorale und anti-inflammatorische Immunantworten wurden untersucht. Zu diesem Zweck wurden allogene und syngene Nierentransplantationen durchgeführt. Für den allogenen Ansatz wurden Nierentransplantate aus Fischer-Ratten in Lewis-Ratten und für den syngenen Ansatz Nierentransplantate aus Lewis-Ratten in Lewis-Ratten transplantiert. Das Transplantat wurde vor Transplantation unterschiedlichen, spezifizierten Kaltischämiezeiten ausgesetzt. Die Beobachtungszeit endete nach 30 Tagen, 12 Wochen oder in ausgewählten Experimenten nach 28 Wochen. Es wurde eine Überlebensanalyse, Untersuchung der Transplantatfunktion und des Transplantatschadens durchgeführt. Allospezifische Antikörper, das pro-humorale Zytokin „B-Zell-aktivierender Faktor“ und anti-inflammatorische regulatorische B-Zellen wurden bestimmt. Die Überlebenszeit war mit längerer Kaltischämiezeit bei syngener und allogener Transplantation verkürzt. Die Transplantafunktion war schlechter und der Transplantatschäden war größer mit zunehmender Kaltischämiezeit bei syngener und allogener Transplantation. Allospezifische Antikörper waren nur nach allogener Transplantation und bereits 30 Tage nach Transplantation nachweisbar. Die Antikörper persistierten nach 12 und 28 Wochen. Die Antikörper waren dem Isotyp IgG2b zuzuordnen. Die Konzentrationen des pro-humoralen Zytokins B-Zell-aktivierender Faktor waren erhöht nach allogener Transplantation. Anti-humorale regulatorische B-Zellen sezernierten vermehrt und mit der Zeit nach Transplantation zunehmend IL-10.
Zusammenfassend ist das Fischer-Lewis Nierentransplantationsmodell geeignet um die pathophysiologischen Mechanismen eines humoral-induzierten Transplantatschadens, entsprechend einer antikörper-vermittelten Abstoßung, zu untersuchen.

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8 DIRECTORIES

8.1 Figures

8.2 Tables

8.3 Abbreviations

CIT	Cold ischemia time
TCMR	T-cell mediated rejections
ABMR	Antibody-mediated rejections
CNIs	Calcineurin inhibitors
DSA	Donor specific antibodies
ASA	Allograft specific antibody
MHC	Major histocompatibility complex
Ig	Immunoglobulin
BAFF	B cell activating factor
Bregs	Regulatory B cells
IL	Interleukin
HTK	Histidine-tryptophan-ketoglutarate
DGF	Delayed graft function
BUN	Blood urea nitrogen
ELISA	Enzyme-link immunosorbent Assay
FACS	Flow cytometer
FITC	Fluorescein
APC	Allophycocyanin
PE	Phycoerythrin
FSC	Forward scatter

SSC	Side scatter
MFI	Mean fluorescence intensity
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid
CpG ODN	CpG oligodeoxynucleotides
F-L	Fischer to Lewis
L-L	Lewis to Lewis
CsA	Cyclosporin A

9 ACKNOWLEDGEMENTS

This thesis was written in the Nephrology Laboratory of the University Hospital Essen. I spend a pleasant four years of research time here, which is a valuable experience and treasure in my life. With the help of my supervisor, colleagues, and friends, I successfully completed my research. And I would like to express my sincere gratitude to everyone who helped me during the study.

First and foremost, I would like to show my deepest gratitude to my supervisor, Prof. Dr. med. Benjamin Wilde, a responsible, resourceful and respectable scholar, offered me a precious opportunity to do scientific research in his laboratory and provided me with valuable guidance in every step of my research and study. He has designed this whole project and given me guidance in experimental techniques. He has also given me care and support in my spiritual and daily life. Without his inspiring guidance, impressive kindness and patience, I could not have finished my thesis.

I gratefully acknowledge the enthusiastic supervision of PD Dr. med Sebastian Dolff and Dr. Robert Langer for their guidance and help during my time here.

I appreciate Ms. Barbara Nilewski-Kühl, Ms. Tanja Hinkeldein, Ms. Simone Leyting, and Mrs. Thesing Annete for their help and support in my study.

I am thankful to my colleagues in our lab, who gave me much help during my work, especially Ming Sun, Xiaokui Zeng, Xin Ma, Yunqing Zhi, Zhuohang Li, Shenming Li, De Luo, Baotong Zhou, for their kindly collaboration and well support in the experimental phase of my work. I also thank Mr. Lennart Hess, Ms. Gudrun Stolz, et al. for their support and companionship, giving me a happy and comfortable life abroad.

Last but not least, I would like to thank my family. Their support and encouragement were ultimately what made this thesis possible. My parents deserve my sincere gratitude and love for their dedication and support.

10 CURRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.