ORIGINAL ARTICLE

Influence of antithymocyte globulin treatment of brain-dead organ donor on inflammatory response in cardiac grafts: an experimental study in mice

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SUMMARY

The expression of proinflammatory cytokines in donor hearts after antithymocyte globulin (ATG) treatment given prior to organ removal was evaluated to analyze changes in inflammatory response. Adult female OF-1 mice were randomized into brain death (BD) groups (BD Control, BD ATG) with or without treatment, and Controls (Control, ATG). BD induction was performed through gradual inflation of an intracranial positioned balloon catheter. At the end of a 6-h observation period, ATG (1 mg/kg BW) was given intravenously. After 45 min, the donor hearts were removed. Proinflammatory markers IL-2 and IL-6 were examined using ELISA and immunohistochemistry staining. After single administration of ATG, the inflammatory reaction in the myocardium showed a significant reduction in IL-2 expression (BD Control vs. BD ATG, $P = 0.033$). Our investigation showed expected increase in proinflammatory mediators after BD. This increase was abolished by single infusion of ATG, indicated by significant reduction in IL-2 levels in the myocardium. We observed a reduction of IL-6 deposition in media cells in ATG-treated specimens. Further research is necessary to evaluate the role of ATG in donor management considering a potentially positive effect of ATG on IL-2-directed inflammatory response and possible reduction of IL-6-mediated vascular changes.

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Introduction

Heart transplantation depends on grafts of brain-dead human donors. Brain death (BD) induces various pathophysiological processes that interfere with organ systems prior to procurement. Numerous experimental and clinical studies have demonstrated that this exposure leads to a worse outcome after transplantation and to a decreased long-term graft survival [1–4]. BD is considered as the first trigger for graft impairment as it leads to a systemic increase in proinflammatory mediators [5]. Thereby, the inflammatory response influences organ function and quality preceding transplantation [3,6,7]. Hormonal and metabolic imbalances, catecholamine storm with related vasoconstriction, rising neuropeptide levels and circulating mediators released by ischemia in the brain tissue have been identified as impetus trigger mechanisms for this proinflammatory response in the brain-dead donor [5].

A second detrimental factor that affects the quality of grafts is ischemia/reperfusion injury (IRI). This injury comprises the initial hypotensive and vasoconstrictive reactions after BD as well as *in situ* warm ischemia [8] and cold ischemia during procurement and transport. The subsequent reperfusion increases this damage to cells, tissues and organs [9,10]. Both mechanisms result in an increased expression of proinflammatory molecules and adhesion molecules and in an elevated leukocyte infiltration of the grafts [11]. Thereby, organ quality is affected prior to transplantation and as well as short- and long-term survival of donor hearts [3,12,13].

Antithymocyte globulin (ATG), a potent immunosuppressive agent that contains T-cell-specific antibodies and antibodies against activated B cells, adhesion molecules, monocytes, natural killer cells and transduction molecules, would be capable of reducing the inflammatory response in the brain-dead organ donor through a massive depletion of peripheral blood lymphocytes and an antibody-mediated reduction of the activity of the above-mentioned cells and molecules [14–16]. Cicora *et al.* showed less interstitial edema in rat donor hearts after treatment with rabbit ATG right after induction of BD [17]. ATG has a protective effect in the context of IRI, which makes it an applicable agent in the management of potential brain-dead organ donors. IRI studies have shown less damage to the endothelium and a reduction in fibrin layers, adherent thrombocytes, and the leukocyte infiltrate after ATG administration [18–20]. The aim of this study was to investigate the influence of ATG therapy on the inflammatory response in donor hearts of brain-dead mice.

Materials and methods

Study population and experimental groups

Adult female OF-1 mice (weight 35 ± 5 g) were randomized and divided into two treatment groups ATG (n = 3, no BD induction but ATG therapy) and BD ATG (n = 6, BD induction with ATG therapy); and two control groups Control (n = 6, no BD induction nor ATG treatment) and BD Control (n = 6, BD induction without ATG therapy; Table 1).

The timeline of our experimental protocol is shown in Fig. 1. In the BD ATG and BD Control groups, gradual BD induction was performed, followed by a 6-h observation period. With ATG and Control groups, this observation period began after borehole excavation, without placement of balloon catheter. At the end of the observation period, a bolus of ATG was administered intravenously to the tail vein. The control groups without treatment received 0.5 ml of a saline solution intravenously; here, the experiments were concluded after 45 min of treatment, and the hearts of the animals were removed.

Animal care

The animals were housed with a 12-/12-h day/night cycle in air-conditioned rooms (22 ± 1 °C) and were given free access to water and standard mice chow. The

Table 1. Experimental groups.

Group	Brain death	ATG treatment
Control (n = 6)		
BD (n = 6)	•	
ATG (n = 3)		•
BD ATG (n = 6)	•	•

ATG, antithymocyte globulin; BD, brain death.

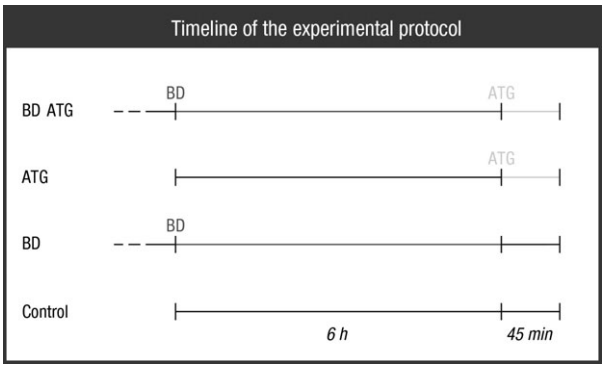

Figure 1 Timeline of experimental protocol.

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local committee for ethics and animal trials approved all experiments. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication Vol 25, No. 28 revised 1996).

Experimental procedure

The animals received an intraperitoneal combination of 100 mg/kg of ketamine (Ketasol aniMedica, Senden-Bösensell, Germany) and 12 mg/kg of xylazin (Rompun Bayer Austria, Vienna, Austria) for anesthesia induction and an intravenous combination of 3.3 mg/kg/min of propofol (Propofol "Fresenius") and 2.5 mg/kg/min of ketamine (Ketasol) for the maintenance of anesthesia. After anesthesia, tracheotomy and intubation were conducted, and the animals were mechanically ventilated (tidal volume 150 ± 25 μl; rate 125/min) with room air (Minivent; Hugo Sachs Elektronik, March, Germany) and fixed in prone position. Body temperature was measured by a rectal thermo probe and maintained at 37 °C with a heating pad.

Gradual brain death induction

The procedure has been described in detail and published by us [21]. In short, after a midline incision on the scalp, the epicranial muscles and periosteum were resected, and a 2-mm cannulation hole was drilled using a 12-gauge needle through the cranial bone 4–5 mm lateral to the sagittal suture. A self-designed balloon catheter was inserted intracranially through the borehole. Additional apertures were drilled for monitoring the central activity with EEG electrodes, and a micro-tip catheter (MillarSP407; Millar, Houston, TX, USA) was used for measuring the intracranial pressure. Recordings were registered continuously by an online data acquisition system. The BD induction was performed by graded inflation of the balloon catheter. A volume of 20 μl of a saline solution was injected every five minute under registration of cerebral activity by EEG and ICP monitoring. The major criterion for BD was a flat-line EEG. Further criteria for verifying BD were the cessation of spontaneous respirations and maximally dilated and fixed pupils. The balloon was kept inflated during the entire 6-h follow-up in the BD groups.

After occurrence of BD, hemodynamic support was maintained through supplying of fluids; no other supportive, vasoactive substances were administered. In the sham-operated controls, the skull was opened in the same way as in the other groups, but no balloon catheter was inserted.

Registration of cerebral electrical activity

Two electrodes were inserted into the epidural space through the skull. One electrode was placed in the frontal lobe, and one was placed in the occipital lobe. A third grounding-electrode was attached to a lower extremity. Recordings were performed prior to the inflation of the intracranial balloon, during inflation and continuously through the entire experiment.

Antithymocyte globulin treatment

Following the 6-h observation period, mouse-specific ATG (Genzyme Corp., Cambridge, MA, USA) was administered through venous access at a dose of 1 mg/kg of bodyweight (mg/kg BW). After 45 min, the hearts of the animals were removed and either cooled in liquid nitrogen and stored for molecular biological analysis at a temperature of −80 °C or perfused and fixed in 4% formalin for immunohistochemical assessment.

Enzyme-linked immunosorbent assay

For quantitative detection of cytokines interleukin-2 (IL-2) and interleukin-6 (IL-6) in the myocardial samples of the individual experimental groups, sandwich ELISA protocol was performed with mouse IL-2 and mouse IL-6 ELISA kits from Bender MedSystems GmbH (Vienna, Austria). The experiments were performed according to the manufacturer's protocol.

Immunohistochemistry

After excision of the hearts, the grafts were perfused and fixed with 4% buffered paraformaldehyde solution containing 0.1% glutaraldehyde and embedded in paraffin. IL-2 (H33, Sc-7896 Santa Cruz Biotechnology Inc. (Dallas, Texas, USA)) and IL-6 (M19, Sc-1265-R Santa Cruz Biotechnology) were assessed by immunohistochemistry with kits from Vector Laboratories Inc. (Burlingame, California, USA).

Reactivity of interleukins, IL-2 and IL-6, was scored as 0–, none; 1+, minimal; 2+, mild; 3+, moderate, and assessed by a blinded pathologist.

Statistical analysis

The data are presented as the means ± standard deviation (SD). One-way ANOVA, Bonferroni's *t*-test and Student's *t*-test were applied to compare the changes in the different groups. Values for immunohistochemistry were

compared using an overall Fisher exact test for categorical variables. For all the statistical procedures, SPSS Inc. (Chicago, Illinois, USA) statistical analysis software (version 19.0 for Windows) was used. Statistical significance was considered at $P < 0.05$.

Results

Different clinical stages could be discriminated by the electroencephalographic findings. At stage I, slow delta waves were observed from the anesthesia. During BD induction, stage II, faster delta and theta waves were registered. A flat-line EEG marked the time of BD (Table 2). All animals were hemodynamically stable at baseline and during the preparation. In the BD groups (BD Control, BD ATG), inflation of the intracranial balloon catheter induced a Cushing reflex with an increase in heart rate (418.29 ± 38.22 bpm). Changes were observed within the first 15 min after BD; the heart rate decreased to 373.71 ± 30.54 bpm and remained close to baseline values until the end of the experiments. No differences were observed between the BD and control groups. The intracranial pressure values were 27.86 ± 15.23 mmHg 10 min before, 56.27 ± 28.25 mmHg 5 min before and 111.0 ± 35.49 mmHg with the onset of BD. The average balloon volume to induce BD in OF-1 mice gradually was 76 ± 12.65 µl.

None of the animals died during the first three hour after BD induction. Cardiovascular failure was observed in two animals in the BD groups during the 6-h observation period, which resulted in a cardiac survival of 86% when BD was induced.

ELISA

The measurements of IL-2 (Fig. 2a) and IL-6 (Fig. 2b) levels in the myocardium were compared for the individual experimental groups Control (no treatment or BD induction), ATG (control group which underwent therapy but no BD induction), BD Control (brain-dead animals that did not undergo therapy), and BD ATG (brain-dead animals that were given therapy). Our investigations showed increase in IL-2 after BD (624 ± 215 pg/ml Control vs. 650 ± 153 pg/ml BD Control, $P = 1$; 325 ± 93 pg/ml ATG vs. 364 ± 95 pg/ml BD ATG, $P = 1$; Fig. 2a).

IL-2 concentration in the myocardium after BD was significantly lower in the ATG treatment group than in the BD group without treatment (650 ± 153 pg/ml BD Control vs. 364 ± 95 pg/ml BD ATG, $P = 0.033$; Fig. 2a). There was no difference in the hearts without BD.

IL-6 levels were increased in the control groups, with significant increase in the ATG groups (1452 ± 220 pg/ml Control vs. 1041 ± 351 pg/ml BD Control, $P = 1$; 1457 ± 240 pg/ml ATG vs. 637 ± 158 pg/ml BD ATG, $P = 0.031$; Fig. 2b). Treatment with ATG caused a tendency toward lower IL-6 concentrations in the myocardium after BD (1041 ± 351 pg/ml BD Control vs. 637 ± 158 pg/ml BD ATG, $P = 0.365$; Fig. 2b). There was no difference in the hearts without BD.

Table 2. Clinical stage and electroencephalogram.

Stage	Experimental status	Cerebral electrical activity
I	Anesthesia	Slow delta waves
II	Brain death induction	Faster delta and theta waves
III	Brain death	Flat-line EEG

Immunohistochemistry

The effect of BD only showed minor changes in IL-2 *in situ* expression (1.25 ± 0.5 Control vs. 1.67 ± 0.5 BD Control, $P = 0.486$; 1.25 ± 0.5 ATG vs. 1.2 ± 0.48 BD ATG, $P = 1$). In the BD groups, ATG administration displayed lower values in immunohistochemistry for IL-2 (1.67 ± 0.58 BD Control vs. 1.2 ± 0.45 BD ATG, $P = 0.464$).

Similarly, the IL-6 *in situ* expression did not reach statistical significance after BD (1.75 ± 0.5 Control vs. 2 ± 0.00 BD Control, $P = 1$; 1.25 ± 0.5 ATG vs. 1.4 ± 0.55 BD ATG, $P = 1$; Fig. 3). In the BD groups, ATG administration displayed lower values in immunohistochemistry for IL-6 (2 ± 0.00 BD Control vs. 1.4 ± 0.45 BD ATG, $P = 0.196$).

Finally, we saw a reduction in IL-6 deposition in media cells in ATG-treated specimens (Fig. 3).

Discussion

To investigate a possible therapy approach of reducing inflammation, we evaluated the expression of proinflammatory cytokines in donor hearts after ATG treatment prior to organ removal. According to our well-established mouse model of BD, an intracranial positioned balloon catheter was gradually inflated, and intracranial pressure was increased under electroencephalographic monitoring until the occurrence of BD [21]. We were able to detect different clinical stages by changes in the EEG waveforms based on techniques formerly employed and similar to the findings in a feline model [22]. The Cushing reflex occurred after inflation of the intracranial balloon

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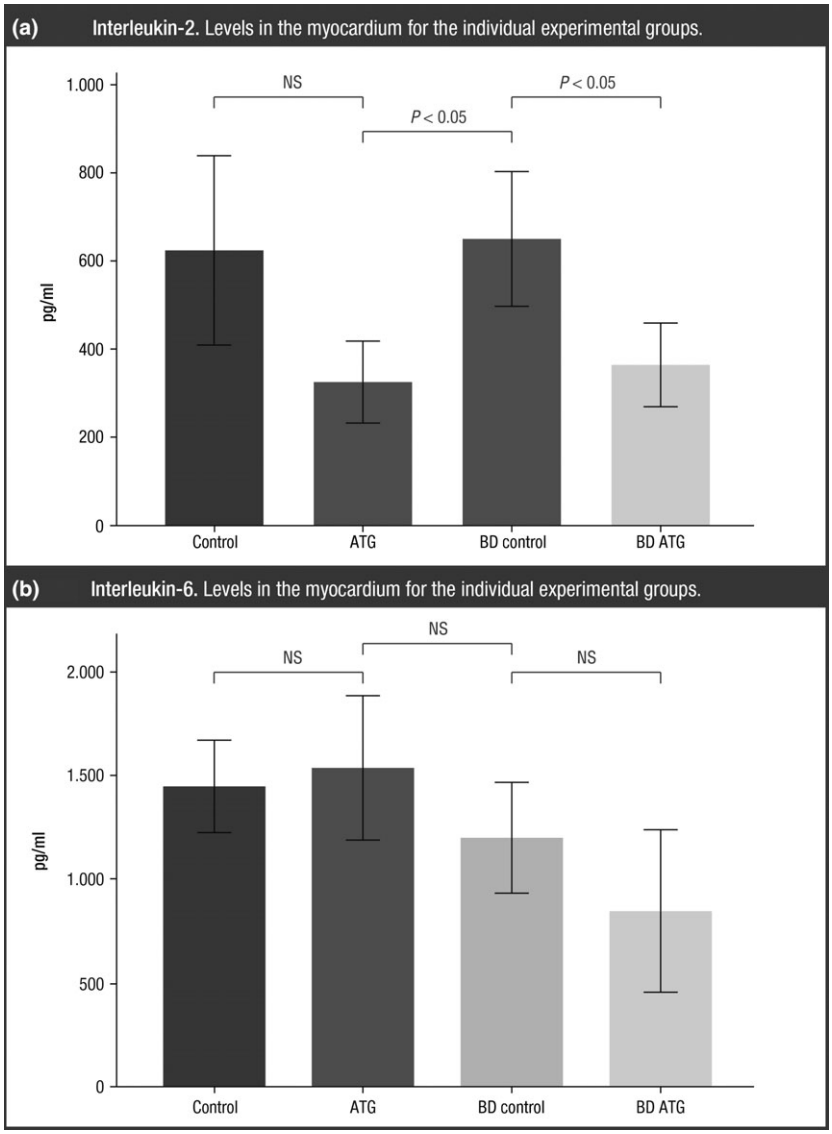

Figure 2 (a) Interleukin-2 and (b) interleukin-6. Levels in myocardium for the individual experimental groups.

catheter. The changes were transient and only observed within the first 15 min after BD. The EEG waveforms decreased and remained close to baseline values until the end of experiments; no differences were observed among the BD and control groups.

After a single administration of ATG, examinations in the brain-dead mouse concerning inflammatory reaction in the myocardium showed a significant reduction of interleukin-2 concentration values measured in the excised hearts. The measurements in the control groups displayed lower cytokine concentrations without statistical significance.

The only anti-inflammatory donor therapy in use is treatment with steroids, which causes an inhibition of the inflammatory response with cytokine levels comparable to those found in living donors [23], a reduced rejection response of these grafts [24] and an improvement in function after transplantation [25,26]. The donor therapy we selected in this study, ATG a much more potent immunosuppressive by lymphocyte depletion through complement-dependent lysis and cell activation [27], is capable of significantly reducing the concentration of the proinflammatory cytokine IL-2 in the myocardium after the occurrence of BD. ATG aims to reduce the inflammatory response in the donor. A reduction we were able to demonstrate in our examinations of donor hearts with the help of a realistic model, including an extended observation period and a brief duration of therapy according to a real transplant setting. In T cells, B-lymphocytes, and natural killer cells, IL-2 triggers a signaling cascade that results in activation and clonal expansion of these cells. IL-2 is released by T cells and acts in autocrine fashion.

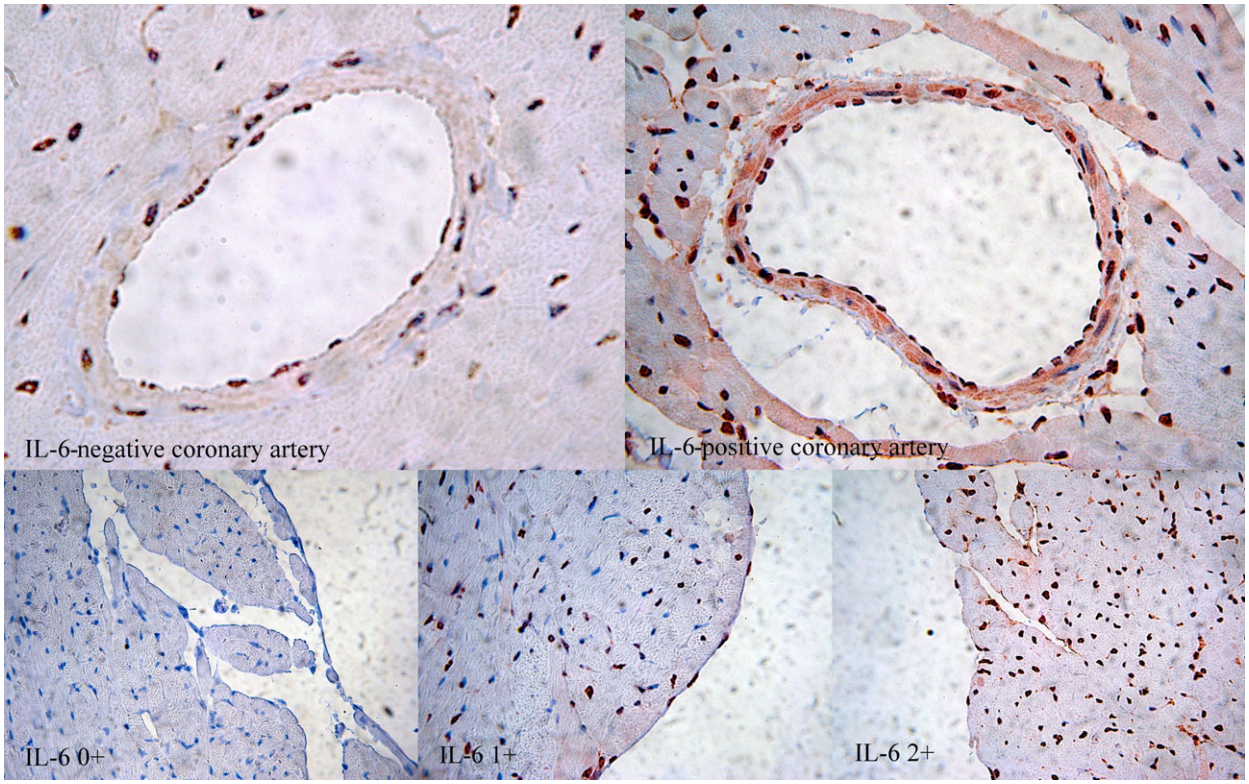

Figure 3 Immunohistochemistry of interleukin-6.

Furthermore, IL-2 is one of the most important signaling molecules. IL-2 effects have been proven in different studies, and influence in immune homeostasis has been demonstrated [28].

There is no established dosing standard for ATG administration after donor BD. Our study is the first study to investigate reduction of inflammatory response after BD at a low dose of 1 mg/kg BW ATG in healthy animals. Aim of our study was to achieve a reduction of inflammatory response in cardiac tissue with a low single dose. In a similar study by Floerchinger *et al.* [29], they applied 25 mg/kg BW ATG as single dose with significant reduction of immune responses subsequent to BD. As there are no outcome studies with different dosing strategies, this is a first approach with a low-dose regime aiming to avoid adverse effects.

Well-studied side effects of ATG are first dose syndrome, anaphylaxis, and serum sickness, which were not encountered during our trial. In a case–control study, De Pietri *et al.* [30] showed that ATG infusion resulted in increased body temperature, worsening of hemostasis, metabolic and hemodynamic imbalance, and higher volume of blood products needed when ATG is administered intra-operatively during liver transplantation.

Monocytes, macrophages, endothelial cells, and fibroblasts release IL-6 upon stimulation by TNF-α and IL-1β. In addition to its proinflammatory effects, an anti-inflammatory effect is presumed. The anti-inflammatory effect hypothetically occurs by direct inhibition and through induction of TNF-α antagonists [31,32]. IL-6 induces formation of acute-phase proteins in the liver and, similarly to IL-1β and TNF-α, has a pyrogenic effect. Administration of IL-6 causes fever, but does not result in septic shock in contrast to IL-2 [33].

Studies in the context of experimental IRI in cynomolgus monkeys showed significant differences in IL-6 concentrations between groups that underwent ATG therapy and those without 30 min after therapy application [34]. In humans, circulating cytokines were examined after infusion of lipopolysaccharides, and the highest values for IL-6 were registered 3–4 h after induction of inflammation [35,36]; in experimental animal studies, these differences were found after 2 h [37,38]. In another experimental model, the onset of hypertrophy and fibrosis, that both lead to chronic rejection, was prevented by neutralization of IL-6. Disruption of already established tolerance in a transplant setting by IL-6 has been demonstrated [39]. The concentration of IL-6 in the myocardium registered in our study might be caused by a peak of this cytokine occurring too early, or to general fluctuations in tissue concentration, which are caused by administration of different anesthetics [40,41]. In an animal

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model, Gomez *et al.* showed that IL-6 is significantly increased in injured arteries compared to controls. Highest levels were measured 3 h after injury, with first increase 1 h postinjury and remaining elevated for 24 h [42]. We noted decreased IL-6 presence in the media tissue of coronary arteries of ATG pretreated grafts. Similar results were demonstrated by Beiras *et al.*, showing decreased expression of interleukins and positive immunostaining predominantly in the control groups, not treated with ATG, localized in endothelial cells and perivascular structures[34].

It has long been demonstrated that circulating IL-6 plays a pivotal role in early atherosclerosis and therefore is relevant to understand cardiovascular disease [43]. Proinflammatory cytokines such as IL-6 induce monocyte migration and are relevant for vascular smooth muscle cell proliferation and apoptosis. Gomez *et al.* showed that after balloon injury in iliac arteries, IL-6 was mostly localized in media cells of injured vessels, which is comparable to our findings [42]. The results in our animal model might indicate that by reduction of inflammatory response, ATG reduces IL-6 infiltration of the tunica media and therefore could lead to a positive effect on remodeling of the vascular wall. These findings may be important in relation to possible measures to repress future cardiac allograft vasculopathy. Further research regarding media activation in this particular setting involving inflammation will be necessary.

We decided on a small number of animals in the treated control group (ATG) due to the fact that we expected a less severe inflammatory reaction caused by the surgical procedure.

The therapeutic time frame to improve organ quality prior to transplantation stretches from BD diagnosis, cold storage and transport, to arrival at the recipient and implantation of the graft. Various experimental studies attempt to circumvent this time limitation by beginning therapy early – before occurrence of BD – but when applied to the potential donor this is, in our view, potentially unethical. We defined a 6-h observation period before onset of therapy as a realistic time frame for performing BD diagnostics and donor evaluation, in regard to a far higher metabolism in mice compared to human donors. Another advantage of our therapy approach is a maintained cellular metabolism in the brain-dead donor. After organ removal and cooling, therapy is only possible in a rather restricted manner due to changes within membrane permeability and disruption of cellular transport mechanisms.

Conclusion

The success of organ transplantation is decisively dependent on organ quality and immunogenicity which are extremely affected by the event of BD. ATG therapy in the brain-dead donor reduces inflammatory response and leukocyte infiltration in the cardiac graft in a reproducible mouse model. Further research is necessary to evaluate the role of ATG in donor management considering a potentially positive effect of ATG on IL-2-directed inflammatory response with inherent implications in the clinical setting and possible reduction of IL-6-mediated vascular changes.

Authorship

JK: performed research, analyzed the data and wrote the manuscript. GKM: designed the study, performed research, analyzed the data, and wrote the manuscript. KA: analyzed the data. PM: performed research. GS and AW: contributed to the literature review and manuscript drafting. AZ and BKP: designed the study.

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Conflict of interest

The authors have declared no conflicts of interest.

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