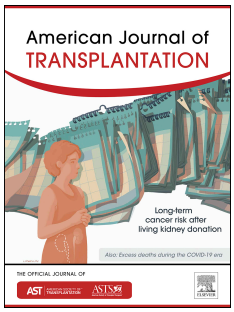

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BEST PRACTICES OF HEART TRANSPLANTATION IN MICE

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ABSTRACT

Heart transplantation in mice has served as a reliable in vivo model in transplant research worldwide for more than half a century. It is not only useful for addressing cardiac graft-specific questions but also provides mechanistic insights and therapeutic strategies that have broad impact across all solid organ transplants. Compared to other mouse models of solid organ transplantation, such as kidney, lung, or small intestine transplants, the surgical techniques to perform mouse heart transplantation (mHT) are relatively easy to master, and the graft heartbeat offers a simple means to evaluate transplant viability. However, as with other in vivo mouse models, mHT has distinct strengths and limitations. Multiple factors can influence the accuracy and reproducibility of the results, including microsurgical techniques and microsurgeons' skills, post-op monitoring methodologies, mouse strain combinations, sex/age. As innovative biotechnologies continue to emerge, the future holds many opportunities for preclinical research utilizing the mHT model. It is therefore imperative to provide the field with optimized mHT protocols and maintain standard reporting requirements. This minireview provides a concise summary and recommendations for standardized practices to ensure the accuracy, reproducibility, and translational value of findings generated from mHT model.

INTRODUCTION

Heart transplantation (HT) in mice is the model of choice for mechanistic studies to address fundamental biological and immunological questions relevant to transplantation. Mechanistic insights gained from these studies have improved our understanding of transplant genetics and alloimmunity and served as blueprints for the development and screening of novel therapeutic strategies, which have led to improved clinical practice and transplant outcome.

While many important discoveries from the mouse HT (mHT) model have contributed to advancing the field, concerns can arise about consistency and reproducibility, especially when results are compared across studies and laboratories, as findings generated from the mHT are influenced by microsurgeons' skills, types of microsurgical techniques, post-op monitoring, mouse strain combinations, sex/age, and environmental parameters. This minireview provides a concise summary and recommendations of best practices to improve the reproducibility and translational value of insights generated from mHT.

1. TECHNIQUES

The use of mice to model human HT (hHT) dates back to the 1960s, when advances in microsurgery permitted the technical success of transplanting a heart into several experimental animals[1] and the monumental first successful hHT was reported[2]. Since orthotopic HT is technically not feasible in mice due to their size, the heart graft is placed heterotopically in a different location, usually the abdomen, or neck of the recipient. With the rich source of genetic well-defined inbred mouse strains and need for sophisticated mechanistic studies, several different surgical techniques have been developed (supplementary appendix (S)-Table I), along with various assessments to determine severity and endpoint of allograft rejection.

1.1. Non-vascularized HT.

Before a vascularized HT model was available, a surgical technique of non-vascularized HT was developed by Fulmer et al.[3] in 1961 and further improved by Judd and Trentin[4] to explore immunologic and physiologic impact specific to HT. In this model, a neonatal murine heart graft is implanted subcutaneously into a pouch formed in the pinna of the recipient's ear without vascular anastomoses and is evaluated by assessing the presence of autonomous beating, as determined by visual inspection or electrocardiography (ECG), and by histological evaluation. The

transplanted fetal heart can beat >100 days post-transplant, and the heart can produce an expected ventricular depolarization detected by ECG[5]. The simplicity of the intrapinna neonatal HT technique has made it a useful model for researchers to investigate basic immunological questions[6, 7]. However, the translatability of the non-vascularized model is limited as it does not allow the study of donor endothelial cell (EC)/host immune interactions. Donor vascular ECs are the first targets of ischemia/reperfusion injury and alloimmune responses. Cold storage and implantation during transplant procedures result in EC activation and endothelium damage leading to upregulation of cell surface major histocompatibility complex (MHC) class II antigens[8]. Activated ECs act as semi-professional antigen-presenting cells (APCs) to recruit and prime recipient immune cells, such as circulating memory T cells, which exacerbate early allograft rejection[9, 10]. Moreover, continuous immune insult on ECs leads to development of transplant vasculopathy, a hallmark of chronic rejection. Vascularized HT permits dissection of the role of ECs in early ischemia/reperfusion injury (IRI) as well as the development of acute and chronic rejection.

1.2. Donor procurement.

Most published studies use hearts from a normal living donor for mHT with minimum IRI. Recently, hearts from brain death (BD) or cardiac death (CD) donors or hearts with prolonged cold storage times (CST) were used in mHT models to simulate clinical transplant settings using DBD or DCD. The process leading to BD or CD causes extensive hormonal alterations[11]. EC activation, hemodynamic changes, and intense inflammatory responses[12], which may influence organ quality prior to organ procurement, exacerbating the early graft injury and allograft rejection[13-15]. Exploration of HT IRI utilizing these donor hearts helps to better understand the processes that ensue early post-transplantation.

1.3. Hemodynamics of the heterotopic HT.

The heterotopic HT model alters hemodynamics of heart circulation. The blood supply of the recipient's arteries (e.g. abdominal descending aorta) flows through the graft coronary arteries → capillaries → the coronary veins → the coronary sinus (CS) → the right atrium (RA) → the right ventricle (RV), and then drains out through the donor pulmonary artery to the recipient's venous system (e.g. IVC), bypassing the left chambers. Thus, the oxygen supply of cardiac walls is ensured by the coronary circulation, while only partial chambers of the heart are being perfused.

Consequently, the graft does neither maintain physiological cardiac output nor pump against physiological pressure. These physiologic alterations contribute to the challenges in studying CAV, as noted in the Considerations section.

1.4. Vascularized mHT Techniques.

The surgical procedures for heart procurement and transplant are simpler, requiring a relatively shorter learning curve, comparing to other vascularized transplants, and the operation can be completed within one hour by an experienced microsurgeon. Various microsurgical techniques are employed creating different heterotopic HT models (S-Table I). Key features of these models are summarized below.

i. The standard abdominal mHT. To overcome the limitations associated with non-vascularized model, Drs Corry, Winn, and Russell pioneered the abdominal vascularized HT in mice in 1973[16], which was adapted from the rat HT model introduced by Abbott et al[1]. Since then, many efforts have been made to simplify or refine the technique[17-21], with several modified techniques of vascular anastomosis reported[22-24]. Nonetheless, the original Corry's technique seems be the standard for HT and has been the most used.

ii. Cervical mHT. Techniques for cervical mHT were first described in 1991 by Matsuura et al[25] and Chen et al.[26]. Reperfusion was established by connection between carotid and jugular vein using suturing[26] or cuff techniques[25, 27]. The cuff technique is the preferred method for the cervical mHT model[28]. This technique permits to transplant a second heart into the same recipient, which is a well-accepted approach to determine antigenic specificity of induced tolerance. It is noted that due to space constraints, connective tissue adhesions may develop surrounding the heart graft[29]. To reduce adhesions, care should be taken to minimize excessive bleeding and soft tissue damage.

iii. Re-transplantation of a previously grafted heart. Re-transplanting a heart graft into a different recipient offers a tool to further investigate the role of graft-associated factors in the maintenance of tolerance or development of chronic rejection. Li et al[30] detailed a procedure in which a heart graft is removed along with its vasculature and re-transplanted into a naïve recipient using the abdominal HT technique. In addition, re-transplantation can be performed using the cervical HT as described by Wang et al.[31]. Despite technical challenges, these techniques have been successfully employed in numerous studies.

iv. Groin site mHT. Recently, Li et al.[32] described a groin-site mHT using a cuff technique, in which the donor aorta and pulmonary artery are anastomosed to the truncated femoral vessels of the recipient. The rejection pattern of groin HTs is consistent with the typical survival time of abdominal HTs. Additionally, enlarged popliteal lymph nodes (LNs) can be observed on the grafted side. While pros and cons remain to be further determined, this model may be ideal to study the role of draining LNs in the rejection process in a vascularized organ transplant setting.

1.5. Post-operative monitoring.

The graft is monitored for the strength of heart contraction/beating by direct abdominal manual palpation, daily within the first week post-transplantation, followed by at least three times per week onwards. ECG, echocardiogram, and micro-MRI have been used as complementary tests to monitor graft size, function, and perfusion[33-35]. However, their utility is limited by requirement for extensive training, expensive equipment, sedation protocols that can influence hemodynamics, and proximity to other visceral organs that can complicate readings[36]. Thus, the direct abdominal palpation by experienced investigators, despite subjectivity, remains the most practical method for determining the endpoint of graft failure.

To minimize subjectivity, a scoring system as a non-invasive assessment of graft health is recommended to semi-quantify the changes in the strength of heartbeats[21, 37]. The endpoint of rejection can be defined as two consecutive measurements of a questionable weak beat (between 1+ to 0) or complete cessation of a palpable beat, confirmed by two to three independent individuals. Because acutely rejected hearts initially become larger likely due to congestion and edema and then smaller with fibrosis, changes in the size of the graft as assessed by palpation as well as heart rate can also be informative.

1.6. MHC disparity and other factors.

MHC disparity between donor and recipient mouse strains determines the severity and types of allograft rejection (reviewed elsewhere[38, 39]). Unlike mouse kidney transplants, most MHC fully mismatched HT undergo robust acute rejection with complete cessation of heartbeat in 7-10 days, whereas single class I/II MHC mismatched hearts are chronically rejected in 30-60 days. HT in single minor histocompatibility antigen mismatch models (e.g. H-Y, Bm12 to B6) can continue

beating beyond 60 days, despite detectable alloimmune responses and vascular changes, e.g. vasculopathy, suggestive of chronic allograft rejection[40].

Mouse strain, sex, and age can significantly impact experimental results and data interpretations, thus allowing exploration of distinct mechanistic questions. For instance, BALB/c mice tend to develop more robust Th2 responses, while B6 mice are more Th1-prone. Young (2-3 months) male heart recipients exhibit prolonged graft survival times compared to young female recipients, but this difference is lost in older (>18 months) mice[41]. In contrast, donor sex had little influence on heart graft survival[42]. Moreover, different microbiota between transplant hosts can differentially tune alloimmune responses that may contribute variability in cardiac transplant outcomes between institutions or animal facilities. Therefore, it is imperative to take careful consideration of appropriate sex- and age-matching as well as environmental factors to minimize experimental variations and biases.

2. TERMINOLOGY/ CLASSIFICATION OF REJECTION

In addition to functional assessment through palpation or imaging modalities in keeping with clinical practice, histopathology is still the gold standard for assessment of ischemic injury, rejection grade, and chronic allograft vasculopathy.

2.1. Ischemia reperfusion injury (IRI).

IRI is an unavoidable event in the transplantation process. As such, the mHT model has been used extensively to explore IRI mechanisms and the efficacy of therapeutic interventions. The severity of IRI is linked to the later development of acute and chronic rejection and thus the study of IRI specifically in the heart transplant model is important. While the concept of IRI has been studied in myocardial infarction models, the pathophysiology of heart transplant IRI is far more complex. Beyond the scope of alloimmunity, heart transplant IRI is also influenced by donor injuries (brain death and donation after cardiac death), cold storage (static and pulsatile), and global ischemia, injuries that are not modeled in the transient focal ischemia seen in myocardial infarct models, and injuries that often occur in donor graft prior to transplantation. Since these injuries likely set the graft up for early damage which can contribute to exacerbating long-term outcomes, standardization of methods to measure, quantify and extend the rigor and reproducibility of the assessment of heart transplant IRI will enable a framework allowing for comparisons between studies.

IRI is a multifactorial process which involves the generation of reactive oxygen species (ROS), activation of the complement system, endothelial dysfunction, and infiltration of neutrophils and other immune cells. These processes contribute to cell death, increased vascular permeability, and inflammation, which compromise graft function. Thus, utilizing a domain approach for qualitatively/quantitatively assessing IRI will provide important mechanistic insights and enable standardization of outcomes. Recent studies in lung injury have led to the development of standardized criteria for assessment of injury. The American Thoracic Society Guidelines center around the investigation of four domains; 1. Function, 2. Injury (histological and serological measures), 3. Inflammation (immune cell infiltrates and cytokine levels), and 4. Barrier function (EC activation and barrier integrity)[43]. While these have not been applied directly to HT, the principles investigated are important to heart transplant IRI.

Histopathological assessment of injury has been used by many to dissect mechanism and therapeutic efficacy. Several studies employ composite scoring schemas, where histopathological evidence of injury in the epicardium, myocardium, and endocardium are scored from 0-3, and a further 0-3 score is given to inflammation. The score can be presented cumulatively from 0 to 12 or individually[15, 44]. Pairing this approach with serological measures, such as cardiac enzyme troponin I or lactate dehydrogenase, can aid interpretation and provide an index of cardiac damage[45]. Immunohistochemical studies on immune cell infiltrates and flow cytometry of dissociated tissues are useful to investigate quantity and activation status of immune cell infiltrates, endothelial activation, injury, and edema. Additional techniques such as Evans blue or FITC-dextran graft accumulation, can provide important quantitative information regarding endothelial barrier integrity *in-situ* as done in other organ ischemia systems[46, 47].

2.2. Acute cellular rejection (ACR).

The rodent HT literature has utilized a variety of histopathological scoring schema to assess rejection. To more thoroughly align with the clinical scenario, and thus aid translation, many studies have employed the International Society of Heart and Lung Transplantation (ISHLT) grading criteria[48-50]. While clinically the ISHLT grading criteria have been criticized since their inception for limitations in both reliability and accuracy, much of this is due to sample adequacy. These critiques are somewhat assuaged in the context of rodent transplantation where the whole heart is available for assessment. The histopathological diagnosis of ACR involves evaluating

myocardial tissue for hallmark features which include interstitial and perivascular infiltration by mononuclear inflammatory cells, predominantly T lymphocytes, along with associated myocyte injury. The ISHLT grading system can be used to categorize the severity of rejection, ranging from 0R (no rejection) to 3R (severe rejection) **(**SA-Table II**).** This system has been utilized extensively to grade and mechanistically dissect the impact of therapeutics, largely in the context of major mismatch models of cardiac transplant rejection[48-50].

2.3. Antibody-mediated rejection (AMR).

In addition to ACR, AMR can be a significant clinical problem. As such, rodent models of cardiac AMR have been developed and include passive transfer of alloantibody, skin transplant sensitization, C3H to BALB/c[51], and A/J (H-2^a) to C57BL/6 CCR5$^{-/-}$ models[52]. In keeping with ACR, the histopathological features of AMR are defined in the ISHLT criteria and include evidence of microvascular inflammation, defined by the presence of capillaries often with accumulation of neutrophils and mononuclear cells, endothelial cell swelling and damage, interstitial edema and hemorrhage, and, in severe cases, thrombotic microangiopathy or myocardial necrosis. Additionally, a hallmark feature is the presence of capillary C4d deposition determined by immunohistochemistry. Central to the classification of AMR is the presence of donor-specific antibodies (DSA) and thus in addition to histopathological features, the presence of circulating DSA should be investigated as has been described[51, 52].

2.3. Chronic Rejection.

Heart transplants that are partially MHC-matched (e.g. bm12 to B6 [53, 54]) or are minor histocompatibility antigen-mismatched (e.g. minor H antigens[55, 56]) with the host do not develop robust ACR or AMR and instead develop features indicative of chronic rejection (CR). In addition, full MHC-mismatched allografts with short-term immunosuppression often display features of CR[57, 58]. These models provide valuable tools to investigate mechanisms of CR. The histopathological quantitative assessment of chronic allograft vasculopathy (CAV) involves evaluating changes in the structure and composition of graft vasculature. The principal feature of CAV is luminal occlusion. Histomorphometric analyses are used to determine intimal thickening, luminal occlusion, and changes in medial smooth muscle remodeling. These can be performed using standard hematoxylin and eosin stains but are often supplemented with elastic van Gieson

tinctorial stains in which staining the elastic fibers helps define the border between media and intima[57, 59].

3. EFFECTS OF IMMUNOSUPPRESSION

The conventional immunosuppressive (cIS) drugs prescribed to humans have been used in mice often at similar or higher doses, but usually as short-term or transient monotherapies or in combination with experimental drugs to test their synergistic effect (*S*-Table III and IV**).** As monotherapies, tacrolimus (TAC), CsA, rapamycin, mycophenolate mofetil (MMF), or methylprednisolone (MP) induce very modest prolongation of graft survival in MHC mismatched mHT models, except perhaps at the highest doses of TAC.

Long-term graft acceptance is of course a goal in the field, but graft persistence does not necessarily equate transplantation tolerance. Long-term graft acceptance may be achieved with a combination of cIS drugs if therapy is continued. In contrast, tolerance refers to graft maintenance after cessation of IS. *Prope* ('close to' in Latin) tolerance describes such a state of graft acceptance, whether this is achieved through graft accommodation, physical shielding of the graft from the host's immune response, or actual immunological tolerance of alloreactive T cells. The designation of donor-specific tolerance, in mouse models, is reserved to animals that not only retain their graft after IS cessation, but also spontaneously accept a secondary donor-matched cardiac allograft in the absence of immunosuppression but reject a secondary third-party allograft.

Tolerance has not been observed in mice treated with cIS, but costimulation blockade has gained traction in m HT for its promise to induce donor-specific transplantation tolerance. CTLA4-Ig binds APCs on B7 family members, the ligands of the main costimulatory receptor CD28 on T cells; anti-CD154 binds CD154 (also called CD40L) on recently TCR-stimulated T cells to prevent its engagement to both CD40 and CD11b receptors on interacting cells[60]. CTLA4-Ig and anti-CD154 in monotherapy have not been successful at inducing donor-specific transplantation tolerance in mice although they can drive long-term graft acceptance. However, when in combination with donor-specific transfusion (DST), anti-CD154 can induce donor-specific tolerance[61]. Anti-CD154 had lost favor in the clinic as its first trial resulted in thromboembolic adverse events due to cross-linking of CD40L and FcγRs on human platelets but has now been modified to avoid these complications, and clinical trials with two humanized anti-CD154

antibodies are slated to start in kidney transplantation. Thus, anti-CD154 plus DST provides a useful tool for mechanistic studies of tolerance induction/maintenance.

4. BENEFITS OF THE MOUSE MODEL

The mHT model combined with a rapidly growing number of research tools opens broad avenues for mechanistic studies. The parameters of immune responses to a heterotopic mHT approximate clinical situations of IRI, donor antigen release and delivery to recipient secondary lymphoid organs, immune cell activation and trafficking into the graft, and interactions between recipient immune system, and graft vasculature and parenchyma.

4.1. Allorecognition.

After recognizing the importance of MHC antigens for heart allografts, the concept of direct and indirect T cell allorecognition was proposed and experimentally confirmed in mouse skin allograft recipients, recipients sensitized by allogeneic cells, and in human transplant patients[62]. However, the relevance of these pathways to graft tissue injury was formally tested using mHT models. Combining elegant approaches and newly generated TCR transgenic mouse strains, several groups demonstrated the potential of CD4 or CD8 T cells primed via direct or indirect pathways to reject vascularized mHT[63-66]. The remaining questions of how direct CD8 T cell responses are sustained in the apparent absence of donor APCs were later resolved by the discovery of the semi-direct allorecognition pathway in which intact donor MHC molecules are acquired by recipient dendritic cells[67, 68]. More recently, the concept of extracellular vesicles, such as exosomes, was introduced to explain the process of intact MHC acquisition by recipient APCs[69, 70]. The controlled environment provides unparalleled opportunities for targeted mechanistic studies.

4.2. Contribution of cardiac-specific autoantigens in rejection.

Another important discovery made in a mHT model was the importance of T cells and antibodies reactive to self-antigens released and presented in a pro-inflammatory milieu. In 1999, Fedoseyeva and colleagues reported that mHT elicits T cell responses to cardiac myosin that in turn contribute to the rejection process[71]. Since then, many self-antigens eliciting cellular and humoral autoimmunity were described in experimental and clinical transplantation, including

extracellular matrix and cell surface proteins, nuclear antigens and apoptotic cells[72-76]. While some of these antigens are graft tissue-specific, many are ubiquitously expressed and their relevance to graft rejection and sensitivity to treatment strategies continue to be explored using the mHT model.

4.3. Differential roles of T cell subsets.

The widespread use of the mHT model coincided with the discovery of T cell differentiation pathways into Th1 and Th2 effector subsets, with a later addition of Th17, Treg, Tfh cells, etc. Initially, allograft rejection was associated with type 1 T cell responses characterized by signature cytokines IFNγ and TNFα[77]. Nevertheless, it rapidly became apparent that other conventional T cell subsets can also initiate allograft rejection employing distinct effector mechanisms. In particular, Th2 and Th17 cells were reported to mediate acute or chronic graft tissue injury in a number of studies, whereas Tfh cells were implicated in alloantibody generation[78-81]. On the other hand, the graft-prolonging properties of Tregs and the benefits of allospecific CAR Tregs were also described in a mHT model using recipients of HLA-A2-expressing hearts[82, 83].

As mHT rejection is typically T cell mediated, it is a relevant model for studying the role of T cell memory in transplantation. Alloreactive memory T cells in pathogen-free transplant recipients can be induced by direct exposure to alloantigens, infections via heterologous immunity, homeostatic expansion or simply by adoptive transfer of relevant subsets. Applying these approaches in mHT models enabled important insights into the impact of memory T cells on various aspects of anti-donor immune response, their resistance to therapies, effector functions, and pathways critical for their generation and reactivation[84-94].

A classic example of mHT model discoveries that led to changes in clinical practice is the introduction of Belatacept. In the seminal 1996 study, Larsen et al. reported the efficacy of CTLA4-Ig and anti-CD154 monoclonal antibody in significantly extending mouse heart and skin allograft survival in the absence of other immunosuppression[95]. Another critical insight from this manuscript is the fact that calcineurin inhibitors interfere with the effects of costimulatory blockade. The report "launched a thousand ships" seeking to understand the mechanisms of CTLA-4 action in mice and translate the approach to organ transplantation in non-human primates and humans. The collective endeavor led to the approval of Belatacept for the treatment of acute rejection in kidney transplantation both in the United States and Europe in 2011 (reviewed in [96]).

Interestingly, when increased incidence of acute renal graft rejection episodes in recipients treated with Belatacept was revealed by clinical studies, this led investigators back to the drawing board of experimentation in mice. Several important lines of investigation are ongoing to understand the mechanisms of and prevent CTLA4-Ig-resistant rejection, focusing on the role of graft IRI, inflammation, memory responses and specific lymphocyte subsets[97, 98]. Alternative or additional opportunities of targeting other costimulatory pathways are also being explored in mHT models (reviewed in [99, 100]).

5. CONSIDERATIONS

While mHT has proven to be an invaluable tool for the transplant research the field, some considerations should be factored into experimental designs and data interpretation. These, like all model systems, arise principally from biological discrepancies between species, the controlled nature of experimental conditions, and specific technical and physiological constraints, and provide opportunities for model refinement and additional mechanistic investigations.

5.1 Immunobiology.

Although mice and humans share many conserved pathways in innate and adaptive immunity, there can be differences in immune cell subsets, cytokine profiles, immunoglobulin isotypes, receptor expression, and signalling cascades. For example, T cell subsets differ not only in their frequency but also in their repertoire diversity and memory phenotypes[101]. Some aspects of AMR such as the interaction of DSAs with complement pathways and the resulting endothelial cell damage are less prominent in mice[102]. Mice possess different IgG isotypes to humans (IgG1, IgG2a/c, IgG2b, IgG3), with varying capacities to fix complement and engage Fc receptors on effector cells, such that IgG isotypes should be carefully assessed. Genetic engineering approaches attempt to bridge some of these gaps. For instance, allogeneic mHT in $CCR5^{-/-}$ mice develops robust AMR reminiscent of hHT[52]. Additionally, humanized mice, generated by engrafting immunodeficient mouse strains with human haematopoietic stem cells or by introducing human HLA class I and II molecules, can model human-like T cell alloresponses and B cell repertoires[103].

5.2. Homogeneity.

Human transplant recipients live in a complex microbial world, hosting thousands of bacterial species and being constantly exposed to environmental pathogens. In contrast, laboratory mice are raised in specific pathogen-free (SPF) conditions, experiencing a limited microbiota. Their immune systems consequently lack the breadth and depth of memory T cell subsets found in humans, who have typically encountered multiple infections, vaccinations, and environmental antigens. By some estimates, wild or pet-store mice harbour a significantly more diverse microbiome, shaping T cell function, TCR diversity, and mucosal immunity[104]. Such differences can affect T cell alloreactivity and graft durability. Introducing pre-formed memory T cells or environmental complexity by co-housing laboratory mice with wild-derived mice or altering their microbiome composition has been shown to bring murine immunophenotypes closer to human profiles[105].

5.3. Physiological alterations.

Heterotopic HT introduces significant physiological differences compared to human orthotopic transplants that are exposed to physiological pressure loads, pulsatile flow, and normal coronary circulation patterns. Importantly, heterotopic transplanted hearts in mice do not contribute to systemic circulation; instead, these transplants rely on coronary perfusion for oxygen supply. The altered hemodynamics can also exaggerate IRI, potentially accelerating acute rejection processes in a manner that may not fully mirror human physiology. The observation that orthotopic heart xenografts in primates survive longer than heterotopic grafts in the same species combination supports this hypothesis[106]. In addition, vascular damage due to rejection may lead to formation of a thrombus in the left ventricle (LV), due to a retrograde blood flow from the abdominal aorta via the ascending aorta to the LV. Taken together, these unique features should be taken into consideration when interpreting the studies using nonfunctional, heterotopic mHT models.

6. FUTURE PERSPECTIVES

For decades, heterotopic mHT has remained a reliable workhorse for transplant immunologists despite its limitations. A large body of accumulated data in a variety of strain combinations is critical for testing specific mechanistic questions and identifying new pathways for potential therapeutic intervention. Given the tremendous progress in transplant immunology achieved by experiments in mice, the mHT model should not be dismissed based on an overly simplistic

argument that “mice are not humans”. Instead, to ensure the relevance of data from mHT to clinical transplantation, specific features of the model must be carefully considered during experimental design and data interpretation. Exploring innovative techniques and biomarkers for early detection of cardiac allograft rejection, using models enriched for memory lymphocytes, or employing genetically modified models to better mimic human conditions, including CAV and AMR, are key priorities to expand the usefulness of current models. Manipulating the microbiota diversity of laboratory mice through co-housing or select challenges offers promising avenues to enhance the clinical relevance of preclinical findings. Furthermore, developing uniform criteria analogous to the Banff classification including graded scales of cellular infiltration, fibrosis, and vascular changes, would likely facilitate reproducibility, enable meaningful comparisons across studies, and reduce the translational gap.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: