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Review article

Models of immunogenicity in preclinical assessment of tissue engineered heart valves☆

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ABSTRACT

Tissue engineered heart valves may one day offer an exciting alternative to traditional valve prostheses. Methods of construction vary, from decellularised animal tissue to synthetic hydrogels, but the goal is the same: the creation of a 'living valve' populated with autologous cells that may persist indefinitely upon implantation. Previous failed attempts in humans have highlighted the difficulty in predicting how a novel heart valve will perform *in vivo*. A significant hurdle in bringing these prostheses to market is understanding the immune reaction in the short and long term. With respect to innate immunity, the chronic remodelling of a tissue engineered implant by macrophages remains poorly understood. Also unclear are the mechanisms behind unknown antigens and their effect on the adaptive immune system. No silver bullet exists, rather researchers must draw upon a number of *in vitro* and *in vivo* models to fully elucidate the effect a host will exert on the graft. This review details the methods by which the immunogenicity of tissue engineered heart valves may be investigated and reveals areas that would benefit from more research.

Statement of significance

Both academic and private institutions around the world are committed to the creation of a valve prosthesis that will perform safely upon implantation. To date, however, no truly non-immunogenic valves have emerged. This review highlights the importance of preclinical immunogenicity assessment, and summarizes the available techniques used *in vitro* and *in vivo* to elucidate the immune response. To the authors knowledge, this is the first review that details the immune testing regimen specific to a TEHV candidate.

1. Introduction

Over 300,000 heart valve replacements are performed annually – a number expected to triple by 2050 [1,2]. For most patients, the choice of prosthesis is between a mechanical or tissue valve. Mechanical valves, though durable, require lifelong anticoagulation to prevent thrombosis [3]. Tissue valves, usually made of glutaraldehyde-fixed bovine pericardium, carry less thrombotic risk but instead are subject to immune-mediated degradation, leading to their calcification and failure in as little as 10 years [4]. Both are inert and without growth potential, necessitating multiple re-operations in younger patients as they outgrow their prostheses. In the last two decades, researchers have endeavoured to create a tissue engineered heart valve (TEHV): a valve prosthesis capable of supporting repopulation by autologous cells, and able to persist indefinitely through constructive remodelling [5–7].

Such a valve must have near-physiological mechanical and haemodynamic properties, resist immune-mediated rejection, and support at population of living cells able to secrete extracellular

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matrix (ECM), thus replacing the graft with neotissue over time. By far the biggest hurdle in the creation of TEHVs is limiting the destructive forces of the host's immune response [8]. Some immune response is favourable, indeed necessary, to encourage remodelling of the ECM [5], but excessive response can lead to acute rejection in the short term, and chronic degradation in the longer term. The fine line between encouraging cell migration and avoiding catastrophic immune cell influx must be fully elucidated before any TEHV makes it to market.

ISO standard 5840 governs the testing regimen of traditional valve prostheses. The full course of preclinical assessment includes mechanics, haemodynamics, thrombogenicity, and durability [9,10], but the existing standard does not take into account important outcomes of novel bioactive scaffolds. To date, no true TEHVs have been approved for use [42,123]. Proposed 'living valves' raise new questions about how cell-based TEHVs can be declared safe. This review will examine the preclinical models used to evaluate the immunogenicity of TEHVs.

2. Different valves, different challenges

Tissue engineers employ a variety of processes and materials to build TEHVs. Common approaches include 1) the use of readily available animal ('xenogeneic') tissue in a decellularisation protocol, which removes antigenic cellular material from the graft, leaving behind an extracellular matrix (ECM) scaffold, and 2) the use of synthetic materials that can be moulded, electrospun, or bioprinted into the desired shape [7,11]. The choice of immunogenicity evaluation modality will be dependant on the material of construction, as each of the above materials have unique challenges with respect to *in vivo* immune response.

2.1. Xenogeneic TEHVs

Structural proteins of the ECM are highly conserved between species, allowing xenogeneic tissue to be used for scaffold production with reduced likelihood of an immune response [12–14]. There are, however, several xenogeneic matrix components that are strongly immunogenic in humans. These antigens can be subdivided into hydrophilic (water soluble) and lipophilic (water insoluble). This classification is useful as the two classes can be detected independently, and emerging evidence suggests that lipophilic antigens are more strongly immunogenic [14,15]. Of particular interest here are antigens that stimulate the humoral arm of the adaptive immune system – namely those reactive to pre-existing circulating antibodies. The carbohydrate epitope α-1,3-galactose (α-Gal), N-glycol neuraminic acid (Neu5Gc), and major histocompatibility complexes (MHCs) are the most well studied [16–19], but the full complement of xenogeneic antigens is yet to be elucidated. α-Gal represents a particular challenge, as humans do not express α-Gal, and have high levels of circulating anti-Gal antibodies due to the expression of this epitope on intestinal microflora [20].

Also of importance are antigens capable of stimulating the innate immune system. Damage associated molecular patterns (DAMPs) are recognised by pattern recognition receptors (notably, toll-like receptors) on the surface of macrophages and dendritic cells, and stimulate an immune response [5,13,21–23]. While the decellularisation process aims to reduce DAMP concentrations to acceptable limits, complete removal is impossible. DNA fragments and other cellular componentry are highly immunogenic, and researchers have employed enzymatic treatments to reduce these antigens. Too rough a treatment, however, can result in damaged ECM proteins, which themselves are DAMPs. Decellularisation protocols walk a fine line between adequate removal of cell-associated antigens and preservation of matrix proteins: too much of either can result in a deleterious immune reaction.

Nowhere is the need to elucidate the immune response to xenogeneic TEHVs more evident than in the failed trials of two decellularised heart valve prostheses: Synergraft and Matrix P. Simon et al. reported the first fatal outcomes of Synergraft^{TM}, a decellularised porcine valve, after 4 Ross operations in children [24]. As early as day 2, explants revealed "severe foreign body type reaction" characterised by neutrophils, granulocytes, and fibrous encapsulation of the graft, ultimately weakening the valve to the point of rupture. The authors suggest that incomplete decellularisation and inherent antigenicity of the collagen scaffold are responsible. The Synergraft process has been applied to allografts with good mid-term results [25], suggesting that the xenogeneic tissue requires more rigorous treatment to achieve biocompatibility. In the case of Matrix P [26–29], a decellularised porcine pulmonary root, initial trials in adults undergoing the Ross procedure were promising [26,27], but analysis of long-term data revealed the prostheses had become regurgitant [29]. In children, Matrix P fared worse. In a trial of 16 Ross procedures in young patients, 6 of 16 grafts required replacement within 15 months [28]. Explant analysis revealed a foreign body reaction characterised by "massive fibro-proliferative response", presence of multi-nucleate giant cells, and granulomatous tissue formation. In both instances, pre-clinical testing failed to foresee the negative outcomes these valves experienced. These failures are a lesson to would-be valve graft manufacturers: the immune reaction to TEHVs, both short- and long-term, must be understood prior to implantation.

2.2. Hydrogel TEHVs

In order to steer away from the immunogenicity of animal tissue, researchers have turned to scaffolds made of natural and synthetic polymers. Using hydrogels gives greater control over the potentially immunogenic components of the matrix [23]. Hydrogel TEHVs aim to be porous, resorbable structures that can function as a valve upon implantation while being replaced by neotissue over time. Natural polymers such a collagen and fibrin have low antigenicity *in vivo*, but the difficulty of replicating the delicate microarchitecture of native valves mean these valves often have poor strength and mechanical properties [30]. Synthetic hydrogels offer researchers finer control over scaffold stiffness and confer improved mechanical properties.

3. The immune response to biomaterials

The immune response to biomaterials has been extensively reviewed elsewhere [5,13,14,23,31], but to frame the outcomes of interest with regard to TEHVs, a brief overview will be included here (Fig. 1). Upon implantation, biomaterials induce a myriad of time-specific responses of both the innate and adaptive immune system. In the very short term (minutes to hours), circulating proteins and platelets adsorb to the TEHV surface [13,31]. At this stage, agglutination of circulating xeno-reactive antibodies (*e.g.* anti-Gal) can cause hyper-acute rejection of decellularised valves (see Fig. 2) [5,13,14,16]. The day-weeks period is characterised by cellular influx: initially by neutrophils, then transitioning to mononuclear cells. Monocytes in particular are key in determining the acute outcome of a TEHV: once recruited by chemoattractant cytokines (*e.g.* CCL2), these cells are capable of differentiating into dendritic cells or macrophages depending on the chemical milieu at the implant site [23]. Dendritic cells present antigens to T-cells and stimulate an adaptive immune response to an antigenic biomaterial, resulting in the formation of lymphoid follicles, where subsequent anti-graft B cell proliferation occurs [32]. Ideally, an implanted TEHV will persist without stimulating an adaptive immune response at

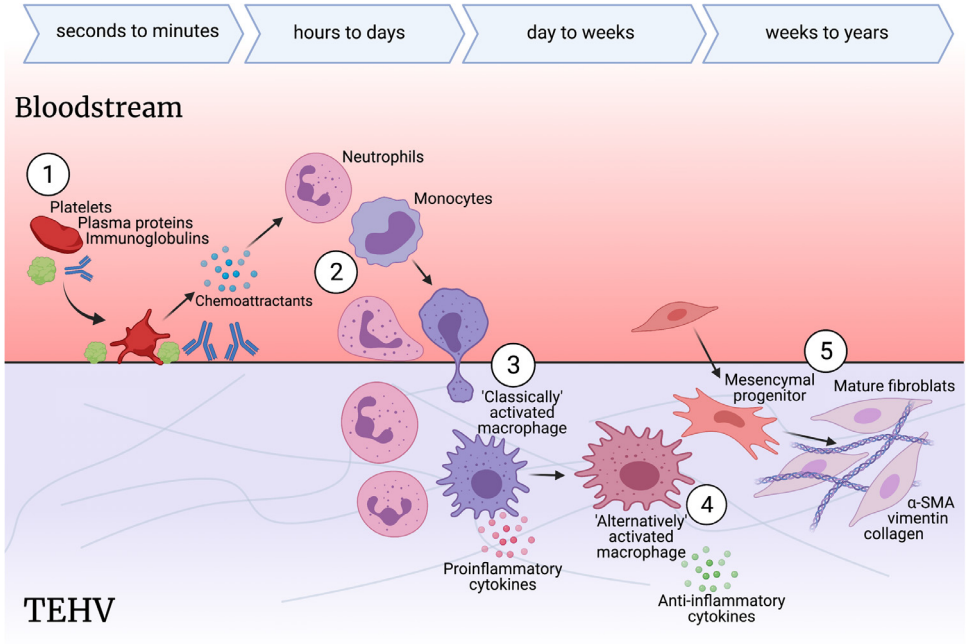

Fig. 1. Depicts the ideal response to a TEHV upon implantation. In the hyperacute term (1), circulating proteins immediately adsorb to the scaffold surface, and serve as the substrate for the cell mediated response thereafter. An ideal TEHV will contain minimal antigenic material and avoid adsorption of existing immunoglobulins. Chemokines released by adhered platelets attract circulating granulocytes and monocytes (2). A non-immunogenic TEHV will not induce massive neutrophil influx and consequent foreign body response. Invading monocytes differentiate into macrophages (3), initially to a pro-inflammatory classically activated subtype. Ideally the macrophage population shifts towards an anti-inflammatory phenotype in the chronic term, associated with wound healing and production of anti-inflammatory cytokines, eventuating in the migration of mesenchymal progenitor cells (5), either from the bloodstream or the surrounding tissues. A TEHV should foster the differentiation of these progenitor cells into a valvular interstitial cell-like phenotype, expressing α-SMA, vimentin, and producing new matrix. A TEHV of this nature can, in theory, persist indefinitely through this constructive remodelling. A comprehensive description of the immune reaction to biomaterials can be found at [31].

all, and instead be remodelled by the cellular component of the innate system only (*i.e.* macrophages) [23]. In practice, however, highly immunogenic biomaterials may be subject to acute rejection, mediated by an adaptive immune response to xenoantigens or bio-incompatible synthetic material [20,23]. Adverse adaptive responses are depicted in Fig. 2.

The chronic outcomes of TEHVs are dependant on the material of construction. Ideally, a "constructive remodelling" response occurs, involving alternatively-activated M2 macrophages, production of new matrix, culminating in the migration of mesenchymal progenitor cells [5] and their subsequent differentiation into a valvular interstitial cell phenotype [33]. A condensed schematic of an ideal constructive response is outlined in Fig. 1.

An adverse chronic response, "maladaptive remodelling", is characterised by pro-inflammatory, classically activated M1 macrophages, the formation of multi-nucleate foreign body giant cells (FBGCs), and granulomatous infiltration [5,34], such as that observed in Matrix P [28]. FBGCs arise from the fusion of monocyte-derived macrophages under certain pro-inflammatory stimuli, critically IL-4 and IL-13 [31]. Degradative enzymes and reactive oxygen species released from FBGCs are responsible for degrading foreign material, and the release of pro-inflammatory cytokines prolong the acute inflammation into the chronic term. The outcomes of chronic inflammation include the laydown of fibrous scar tissue in the form of a 'capsule' [35], persistence of a pro-inflammatory biochemical environment [5], proliferation of FBGCs, ultimately interfering with the mechanical properties of a TEHV to the point of failure. This effect is depicted in Fig. 2.

The complex behaviour of macrophages in response to biomaterials is not yet fully understood. 'Classically activated' M1 macrophages are implicated in the initial 'pro-inflammatory' phase of a response, phagocytosis, and cell recruitment. 'Alternatively-activated' M2 macrophages arise later, and are associated with anti-inflammatory cytokines and ECM production [38]. Physiology of M2 macrophages is the subject of much current research; some authors further split M2s into subtypes: M2a, M2b, and M2c, with each with unique roles [13,39]. The reviewers must insert a caution here: the M1/M2 paradigm is not a clear-cut polarisation, rather a continuum of cell populations, with cells capable of expressing features of both M1 and M2 [20,21,31,38]. These subtypes are plastic, variable, and capable of expressing all macrophage-associated markers [39]. Macrophage function is highly dependant on the nature of the biomaterial, and researchers have consistently found that macrophages respond in a 'mixed' fashion [36,40].

The ultimate goal of TEHV production with respect to the immune response is a material that curbs the adverse immediate responses of circulating antibodies, granulocytes, and the cells of the adaptive immune system (dendritic cells, T cells), while also providing a niche that supports the differentiation of invading monocytes to M2-type macrophages. It is of utmost importance that preclinical testing investigates the innate and adaptive immune response to a TEHV candidate.

4. *In vitro* assessment

Principles of animal ethics suggest that *in vitro* testing on a TEHV must occur prior to implantation in an animal [41]. Blum et al. note that the bulk of pre-clinical TEHV assessment jumps directly to large animals without consideration of *in vitro* or small animal studies [42]. This paucity of "mechanistic studies" leads this reviewer to include references to research performed on 'biomaterials' in general, not specifically on TEHVs as the findings are relevant, nonetheless.

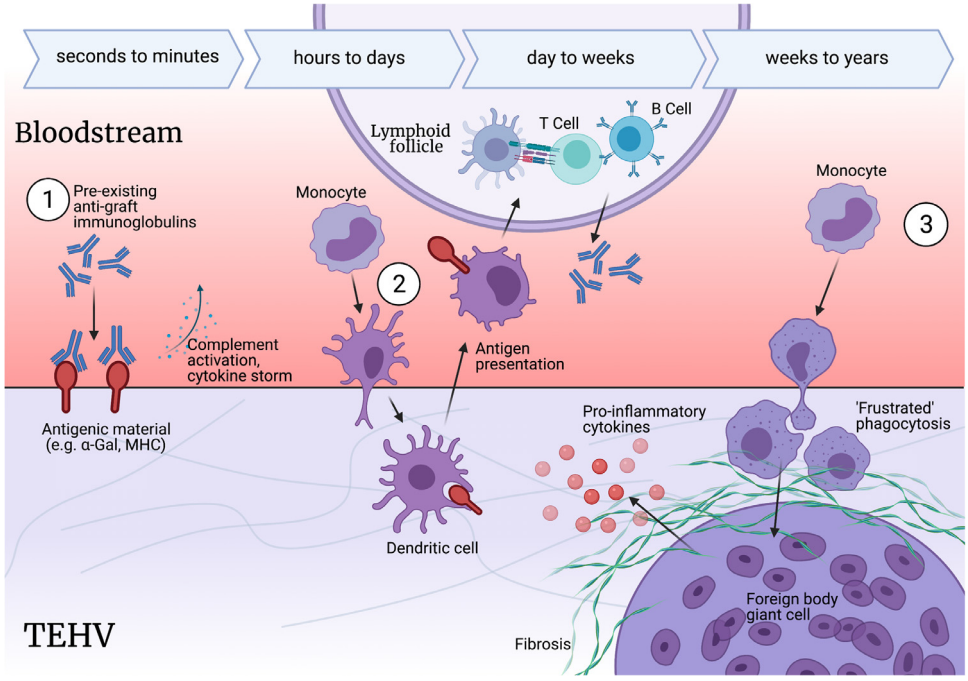

Fig. 2. Depicts three distinct adverse immune scenarios that a TEHV may experience. 1) Hyperacute rejection, whereby pre-existing graft-reactive antibodies bind antigens on the surface of the TEHV, resulting in a cascade of pro-inflammatory reactions. This hyperacute rejection is theorised to be the likely process in α-Gal mediated rejection [16,36]. 2) The adaptive cellular response is initiated by monocyte-derived dendritic cells becoming antigen presenting cells. These cells travel to lymphoid follicles and mount an adaptive immune response against the TEHV via anti-graft antibodies. Note that the finer details of T cell and B cell activation have been omitted for brevity, and readers are directed to more extensive reviews, such as [37]. Lymphocyte activation is responsible for acute rejection. Fusing of macrophages to from a FBGC at 3) is typical of a chronic inflammatory response. In this schematic, the prolonged pro-inflammatory environment leads to fibrous infiltration and release of degradative biomolecules, ultimately ruining the mechanical properties of the TEHV.

4.1. Xenoantigens in decellularised tissue

Benchtop immunogenicity testing begins by identifying known antigens, the most infamous being the α-Gal epitope. The 'M86' anti-Gal monoclonal antibody can be used to identify α-Gal histologically [17], or used to quantify the epitope in tissue homogenate by ELISA [17,43,44] or Western blot [15,32]. MHC class I and II are also strongly immunogenic, and quantification of these residual antigens is similarly achieved [15,32,44].

Foreign DNA is highly immunogenic. Crapo et al. outline standards for the successful removal of nucleic material from decellularised tissue as [11]:

- <50 ng dsDNA per mg of dry tissue
- <200 base pair DNA fragment length
- No visible nuclear material in sectioned tissue stained with H&E or DAPI

It is important to note, however, that the frequently cited 'cut off' of 50 ng/mg remains unvalidated [35]. DNA is commonly extracted from prepared matrices using commercially available kits, *e.g.* the DNeasy kit (Qiagen) [44–48]. Extracted DNA is then quantified using PicoGreen kit (Invitrogen) [36,49–51] or NanoDrop™ (Thermo Scientific)[45–47]. NanoDrop has a published detection limit of 2 ng/mL [52], but in reality contamination of the extract with RNA and other co-purified biomolecules renders this method too insensitive for use [53]. PicoGreen has a published detection limit of 25pg/mL [54], and, as with other fluorescent intercalating dyes, are adequately sensitive for quantifying total DNA from scaffold extracts. DNA base pair length is determined by gel electrophoresis [11]. These methods have been applied to commercially available decellularised tissues, revealing trace amounts of DNA in many test articles, though the concentrations were <2 ng/mg. The authors concluded that these levels would not induce a clinically significant response [55].

4.2. Cell viability/cytotoxicity assays

Scaffolds must be able to support migrating cells, from the initial influx to the long-term migration of mesenchymal progenitors. Methodologies vary, but the basic principle is to expose a population of cells to the biomaterial extract, and observe cell status by microscopy or colour-changing reagent [56]. This process is useful for synthetic scaffold manufacturers, who are able to assess cytotoxicity of novel chemicals, and to xenogeneic valve makers who can assess the toxicity of chemical residues from the decellularisation protocol [14].

Kluin et al. demonstrated their modified polycarbonate TEHV was non-cytotoxic using murine fibroblasts in an MTT assay [57]. Gong et al. assert that instead of MTT, a 'destructive assay', a resazurin-based assay can be used to repeatedly track cell viability without destroying the sample [58]. They found that resazurin (alamarBlue™ – Thermo Fisher) reliably assessed cell viability on polylactic acid scaffolds when compared to MTT. PCR after repeated assays of the same sample showed that resazurin did not significantly alter gene expression when compared to untreated controls, provided incubation times were kept short. Spatial distribution of cytotoxicity can be achieved with Thermo Fisher's LIVE/DEAD™ kit, where two fluorescent dyes resolve live and dead cells within a TEHV matrix [59,60]. A robust example of cell viability can be found at [15], where the authors applied green fluorescent protein (eGFP) labelled human mesenchymal stem cells to a decellularised scaffold, and tracked the cell proliferation by histology and alamarBlue assay over 5 days.

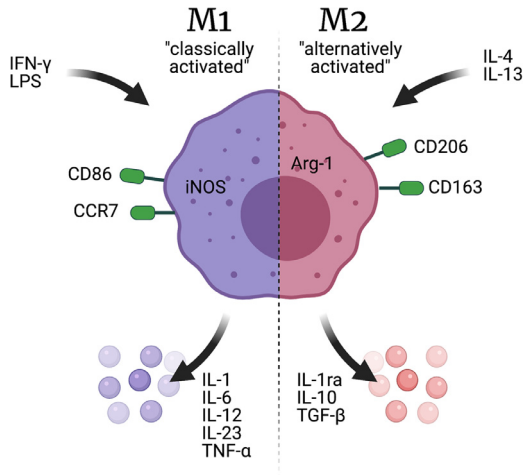

Fig. 3. A condensed description of markers chosen by researchers to identify M1 and M2 macrophage subtypes in immunohistochemistry and proteomics.

4.3. Monocytes and macrophages

The phenotype of macrophages in the scaffold directly affects the migration of mesenchymal progenitor cells [21], influences the rate of scaffold degradation, and therefore determines long term success of the TEHV [5]. Researchers can expose scaffolds to macrophages and identify phenotypic changes in gene and protein secretion by PCR and ELISA respectively. As aforementioned, M1/M2 polarity is not concrete, but it can help paint a picture of the immunostimulating properties of a graft. Commonly selected markers of interest related to the M1/M2 polarisation states are outlined in Fig. 3. Readers are directed to reviews of macrophage expression for a more robust outline [13,39,61].

Histological analysis of scaffolds can reveal pattern of degradation over time. Wissing et al. investigated the effect of scaffold microarchitecture on the degradative function of macrophages. THP1 monocytes (an immortalised cell line) were exposed to polycaprolactone-based scaffolds with different structural properties [40]. While the different scaffolds did indeed show variable degradation rates histologically, expression of pro- and anti-inflammatory genes/cytokines associated with M1 and M2s did not significantly differ between scaffolds. The authors concluded the effect of microarchitecture on macrophages could not be neatly explained by the M1/M2 paradigm alone. VeDepo et al. cultured bone marrow mononuclear cells (BMNCs) on decellularised human valves in a pulsatile bioreactor and, upon immunohistochemistry (IHC) and quantitative PCR, found similarly variable results [62]. BMNCs readily migrated into the matrix, and over the time their gene expression transitioned from a typical mononuclear pattern (CD34+, CD68+, CD54+) to a mesenchymal cell pattern (α-SMA+, VIM+), indicating the scaffold supported proliferation of the mesenchymal fraction of the BMNCs. A caveat to the *in vitro* use of macrophages is that cell lines do not faithfully represent native cell function *in vivo*. While they are certainly much cheaper and easier to use than animals, transcriptome analysis of cell line macrophages reveal scant overlap with expression of those *in vivo* [63].

5. *In vivo* assessment

The complexity of the immune system cannot be replicated in a dish, and so TEHVs must undergo testing *in vivo* [34,64]. There are many animal models for testing biomaterials and choosing the appropriate one will depend on the research question [65]. Small animals are a cheap and easy way to assess chronic immune response, whereas larger animals allow site-specific investigation of biocompatibility. Choosing a time point to harvest an implanted TEHV will also influence the outcome: the hyperacute reaction occurs within minutes, while remodelling of the matrix takes months. Animal models of the immune system are just that – models – and can never faithfully replicate human immunity with complete accuracy [66,67]. Some animals represent a closer approximation (*e.g.* non-human primates), while others with dissimilar immune sytems (*e.g.* mice) may be chosen for their ease of use. In order to lessen the gap between the animal model and the human immune system it tries to replicate, a number of transgenic animals have been developed. Details of the various models are included in Table 1.

Qualitative data of the immune reaction to TEHVs is relatively simple to achieve histologically. Acute phase cellular influx (neutrophils, lymphocytes) have a distinctive appearance upon H&E staining [44]. Integrity of the matrix and pattern of neotissue can be identified with a number of stains: Masson's trichrome [36,57,60,71,73,93–95] and picrosirius red [15,32,57,95,96] are commonly used for collagen, the latter being particularly useful for identifying thickness of collagen bundles under polarised light [97]. Elastin Van Gieson (EVG) is able to resolve elastin fibres [60,71,73,93,98]. Semi-quantitative methodologies are achieved by 'scoring' histological sections for parameters such as cellular infiltrate and inflammation [99–101]. Such methodologies are robust when performed by blinded researchers with experience in the field (*e.g.* veterinary pathologists [15,32]), but they can also be misrepresentative [102]. For example, quantifying 'cellularity' without distinguishing between neutrophils and macrophages may masque the type of response occurring. More robust characterisation of immune cell infiltration requires multiple IHC labels. Commonly selected markers are included in Table 2.

In vivo models provide the opportunity for serum sampling to identify immune markers over a range of time points without sacrificing the animal. Of interest to the decellularisation field are the antibodies against unknown graft-specific antigens. A qualitative look at anti-graft immunoglobulins in serum can be achieved by incubating TEHVs with serum sampled from an animal post-implant [43]. For quantification, a number of authors use homogenised TEHV extract immobilised on an ELISA plate. Both Wong and Helder in their respective rabbit [32] and sheep [43] studies found significant increase in anti-graft IgM and IgG post implantation. Interestingly, in Daly et al.'s non-human primate implantation of decellularised porcine submucosa, there was no increase in anti-graft IgM or IgG post-implant [36]. Dalgliesh et al. generated anti-graft antibodies by injecting rabbits with homogenised native bovine pericardium. They used the serum generated to quantify residual antigenic proteins extracted from their decellularised matrix by Western blot, and compared this result to the antibody titre in rabbits implanted with the matrix [15]. They found that the presence of lipophilic antigens in particular were correlated with an increase in graft specific antibodies over a number of time-points up to 56 days post-implant. Importantly, the authors found that reducing lipophilic antigens by >92% correlates with in a *in vivo* anti-graft humoral response comparable to the response to a glutaraldehyde-fixed, 'industry standard' pericardial scaffold.

5.1. Small animal model

Small animals are widely used for *in vivo* biomaterial assessment for their low cost, ready availability, ease of handling, and well-defined immune parameters. These models are generally used for assessment of chronic changes to TEHVs implanted in an ec-

Table 1
Details four of the most common *in vivo* models of TEHV immunogenicity assessment.

Parameter	Rodent	Sheep	Pig	Non-human primate (NHPs)
Initial cost and upkeep (GBP), adapted from: [68]	£5–40, low cost of upkeep	£250, moderate cost of upkeep, special facilities required for housing [65]	£250, moderate cost of upkeep, special facilities required for housing [65]	£300–2000, high cost of upkeep, special facilities required due to social needs of primates [69]
Anatomical and physiological similarity	Not suitable for *in situ* study due to dissimilar anatomy [68]. Especially suitable for ectopic studies (*e.g.* subcutaneous implantation)	Good [68]. Some authors describe reduced platelet activity [70]. Elevated calcium metabolism allows 'worst-case scenario' calcification model [71,72]	Good [68]. Coronary ostia more closely resemble human valve compared to sheep [73]	Best [10]; absence of α-Gal epitope allows assessment of xenogeneic tissue [71]
Immune similarity	Relatively dissimilar, readers are directed to [66] for a comprehensive review with respect to mice. Notably, differences in toll-like receptors [74], and inflammatory cytokines [75], amongst others. Pabst reports mice share ~10% immune homology [76]. Not α-Gal naive	Relatively similar. Differences exist in leucocyte activation [77]. Not α-Gal naïve.	Good. Pabst reports 80% of immune parameters mimic those in humans [76]. Importantly, macrophage polarisation is more faithfully reflected in pigs when compared to rodents [78], as are pattern-recognition receptors [78]. Not α-Gal naïve.	Best. Genetic similarity to humans confers the best immune similarity [79]. Old world monkeys are α-Gal naïve [80], and circulating anti-Gal antibodies represent 1% of the total immunoglobulin fraction of these species [81]. Importantly, all NHPs are Neu5Gc positive, while humans are negative [82]
Transgenic models available? (KO = knockout)	Yes: Gal KO mice [83,84], Neu5Gc KO mice [85]	None found	Yes: Gal KO pigs [86,87], Gal/Neu5Gc double KO pigs [19,88], Triple KO pigs available, including Gal/Neu5Gc/Sd [89], Gal/Neu5Gc/CD46 [90] Note these animals are yet to be used as models in immunogenicity assessment	None found
Somatic growth rate	N/A	Suitable in adult sheep. Juvenile lambs may be used for a growing model [91]	Unsuitable; high somatic growth rate causes size mismatch [10]. Mini pig breeds may be used [73]	Suitable
Ease of surgical manipulation	Good, requires basic equipment	Good, requires more specialist equipment	Prone to postoperative infection; temperamental under anaesthesia [92]	Specialist equipment and training required [69]
Suitable outcomes	Chronic outcomes (*e.g.* remodelling, macrophage polarity) [34]	Haemodynamics; hyper-acute, acute, and chronic immune response	Haemodynamics; hyper-acute, acute, and chronic immune response	Haemodynamics; hyper-acute, acute, and chronic immune response

topic (non-cardiac) location. Serum sampling for graft-specific antibodies and inflammatory cytokines also provide a window on immune response in the longer term [34].

Ectopic models do present limitations: the absence of a blood-graft interface and the immobility of an implant in small animal models prevent these studies from making concrete conclusions about a graft's immunogenicity *in situ*.

Rats are the most common small animal used in the assessment of TEHVs [21,44,59,60,95,103]. Common approaches are the subcutaneous implantation and abdominal wall repair model. Well-designed rat experiments will account for variability by controlling for rat strain, age, gender, genetic and physiological status [34]. The subcutaneous approach is usually performed by opening a small pocket on the dorsum, implanting a test article, then harvesting the tissue at a timepoint specific to the immune parameter of interest. Owing to the mobility of rodent skin, grafts must be immobilised to prevent 'rolling-up'. Khorramirouz et al. immobilised decellularised pericardium in a rat model using either sutures or a PCL-based frame [103], and found both methods to be effective when compared to a freely mobile graft. Interestingly, the frame resulted in the least inflammation while the sutured graft had an increased degradation rate.

Harvested tissue can be assessed histologically, by IHC or gene expression analysis of recruited cells. Liu et al. assessed the immunogenicity of porcine valve tissue decellularised with several different protocols after implantation rats [44]. H&E showed an initial neutrophil influx that peaked on day 3 and had resolved by day 14. Interestingly, sodium dodecyl sulphate (SDS) treated tissue resulted in significantly fewer neutrophils compared to Triton X100 treated grafts. CD68 staining showed macrophage presence increasing from day 14 to day 28 in all test articles, and again, the SDS-treated tissue showed a more muted response compared to the other treatments. CD4+ and CD8+ lymphocytes were present 14 in all implants initially, and decreased by day 28, suggesting a resolution of the acute adaptive immune response. Dai et al. created modified TEHVs by coating decellularised porcine valves in a hydrogel ± stromal-derived growth factor 1α (SDF-1) and implanting subcutaneously in rats [60]. CD68 staining revealed prominent macrophage presence throughout the decellularised graft, while in the hydrogel-coated grafts, the macrophages were confined to the periphery. Further staining with iNOS and CD206 for M1 and M2 macrophages respectively showed that the SDF-1 hydrogel coated scaffold induced a greater anti-inflammatory response than the decellularised graft. The SDF-1 hydrogel also supported a significantly larger population of CD90+ mesenchymal cells than the unloaded hydrogel, suggesting SDF-1 promoted constructive remodelling. Wong et al. utilised a sub-pannicular rabbit model for the assessment of their step-wise antigen removal of bovine pericardium [32]. Their optimised protocol yielded a scaffold that performed well under semi-quantitative scoring of local cellular re-

Table 2
Contains the commonly used immunohistochemical markers used to investigate the immune response to biomaterials implanted *in vivo*. Markers of mesenchymal and endothelial cells have been included, as they feature in the chronic remodelling of grafts.

	Description	Species	Reference
Leukocytes			
CD3	T cell co-receptor	Rat	[59,60,103]
		Rabbit	[15,32]
CD4	T cell co-receptor	Rat	[44]
CD8	T cell co-receptor	Rat	[44]
		Sheep	[43]
CD45	Common leucocyte antigen	Sheep	[57,91,96,100,104]
Pan-macrophage			
CD68	Mononuclear cell associated glycoprotein	Rat	[21,44,60]
		Sheep	[105]
		Non-human primate	[106]
HAM-56	Monoclonal antibody to macrophages	Non-human primate	[106]
MAC-387	Monoclonal antibody to macrophages	Rabbit	[15,32]
		Non-human primate	[71]
F4/80	Monoclonal antibody to macrophages	Mouse	[84]
M1 macrophage			
CD197 (CCR7)	C-C chemokine receptor 7	Rat	[21]
		Pig	[32]
		Sheep	[72,107]
iNOS	Induced nitric oxide synthase	Rat	[60]
		Sheep	[72]
M2 macrophages			
Arg-1	Arginase	Sheep	[72]
CD200R	CD200 receptor	Sheep	[72]
CD163	Haemoglobin scavenger receptor	Rat	[59,103]
		Pig	[32]
		Sheep	[72,107]
CD206	Mannose receptor	Rat	[21,60]
Mesenchymal cells			
α-SMA	α smooth muscle actin	Rat	[44,59,103]
		Mouse	[94]
		Sheep	[57,91,96,98,100,104,105,107–109]
		Pig	[32]
		Non-human primate	[71,106]
VIM	Vimentin	Rat	[59]
		Sheep	[57,96,98,100,104,108,109]
Endothelial			
CD31	Platelet endothelial cell adhesion factor 1	Rat	[59,103]
		Mouse	[94]
		Sheep	[57,91,98,100,105,107–109]
		Pig	[32]
		Non-human primate	[71,106]
eNOS	Endothelial nitric oxide synthase	Sheep	[104]
		Non-human primate	[106]
vWF	Von Willebrand factor	Sheep	[104,107,109]
		Non-human primate	[106]
Progenitor cells			
CD34	Haematopoietic cell associated glycoprotein	Rat	[44,59,103]
		Non-human primate	[106]
CD90	Thymocyte antigen	Rat	[60]

sponse, utilising MAC-387 and CD3 staining for macrophages and T-cells respectively.

The abdominal wall repair model is a tool for assessing 2D biomaterials. Unlike the subcutaneous model, implants in this model bear tension, and so provides the additional benefit of assessing the scaffold's immunogenicity while subject to mechanical forces [110]. Brown et al. investigated 14 commercially available biomaterials in an abdominal wall repair in rats and assessed each for the differential macrophage response [21]. Sections were scored semi-quantitatively by blinded investigators and revealed 3 'groups': group 1 scaffolds showed a chronic inflammation, foreign body-type response, with the highest amount of CD68+ macrophages; group 2 scaffolds avoided chronic inflammation but did not substantially remodel; and group 3 scaffolds showed beneficial constructive remodelling, with the greatest amount of CD206+ M2-type macrophages. Both the absolute number of M2s and the M1:M2 ratio were found to be significantly correlated to histological score at day 14 and 35.

Rodents are selected for their ease of handling, despite the relative dissimilarity of their immune parameters [66]. To overcome this, transgenic rodents (usually mice) may be created to better approximate the human immune system. An α-Gal KO mouse model is produced by 'knocking out' the alpha1,3-galactosyltransferase enzyme. Lim et al. used this model to evaluate the effect of α-

galactosidase in the decellularisation of bovine pericardium [111]. They found that 1) the enzymatic treatment qualitatively decreased the presence of α-Gal and 2) the post-implant response of α-Gal KO mice included a sharp increase in anti-Gal antibodies in the control group without enzymatic treatment – indicative of a 'human-like' anti-Gal response. Other transgenic mice (such as those lacking Neu5Gc [85]) also exist for the purpose of evaluating novel biomaterials. However, transgenic mouse models are still immunologically distinct, and extrapolation of results out to humans is unwise [66,77,112].

5.2. Large animals

The next step in a TEHVs journey from bench to bedside is in a large animal. Unlike immunogenicity assessment in rodents, TEHVs are implanted in the analogous anatomical location, where site-specific interactions with the immune system can be investigated. In particular, investigation of hyperacute changes (seconds-minutes) at the blood-graft interface can reveal the pattern of protein adsorption and cellular adherence. ISO 5840 mandates site-specific testing of heart valve prostheses, and while no single species is specified, the bulk of preclinical testing is done in sheep [10].

5.2.1. Sheep

Sheep are by far the most common large animal model for evaluating TEHV biomaterials [10,43,57,72,91,92,96,98–100,104,105,107,109,113–115]. Their cardiovascular anatomy, haemodynamics, and ease of surgical handling provide a good model for the human heart [64,92]. The model is versatile in that juvenile lambs can be used as a model for conduit growth in paediatric cases [91,105], while adult sheep, with their elevated calcium metabolism, may be used as a model for 'worst-case scenario' of graft calcification in older patients [6,72,104,116]. Kluin et al. implanted polycaprolactone (PCL) based synthetic TEHVs in the pulmonary position and found neutrophil influx occurred at 2 and 6 months but had resolved by 1 year. IHC showed persistent presence of CD45+ leukocytes over the entire course [57]. Contrastingly, van Rijswijk et al. implanted TEHVs made of CorMatrix^{TM} decellularised porcine submucosa in the pulmonary positions of sheep [101], and found "an intense inflammatory reaction", characterised by persistence of neutrophils, lymphocytic infiltration, macrophages, and giant cells. Histological analysis of the CorMatrix^{TM} scaffold prior to implantation revealed the likely culprit was incomplete removal of cellular material during decellularisation. Dekker et al. have validated an exhaustive panel of sheep-specific antibodies for use in the preclinical assessment of TEHVs [72]. Of the 47 antibodies used, 14 pertain to different aspects of the inflammatory response. As well as pan-leucocyte and lymphocyte markers, the authors paid careful attention to markers of M1 and M2 macrophages, some of which are included in Table 2. Motta et al. quantified the macrophage response to their TEHV by staining for CCR7 and CD163, markers for M1s and M2s respectively, then comparing these values to the total number of cells. They found that the M1/M2 ratio, as well as absolute macrophage number, correlated with TEHV remodelling outcomes [107]. Quantitative gene expression analysis of TEHVs explanted from sheep provides a window into the specific immune-mediated processes occurring within the valve. Fioretta et al. profiled the gene expression of explanted synthetic TEHVs after 24-weeks in the pulmonary position of sheep [100]. They found that pre-seeding the TEHV with mononuclear cells resulted in upregulation of inflammatory marker TGF-β and pro-calcific markers BMP-2 and B-GLAP. Most striking was the variability in expression of anti-inflammatory markers IL-10 and IL-4, not only between different valves of the same group, but also between different leaflets of the same valve. This variability may be interpreted as evidence of a 'mixed' macrophage response and reinforces the difficulty of classifying macrophages as simply M1 or M2.

5.2.2. Pigs

Porcine cardiovascular anatomy is remarkably similar to humans, and they therefore serve as a good option for short-term studies of valve prostheses [64,68,92,117]. Platelet activity in pigs is a closer approximation of humans than the sheep model, meaning pigs can be used to test haemodynamics [73]. However, their rapid growth, temperamentality under anaesthesia, and susceptibility to post-operative infection make standard pig breeds unsuitable for long term studies [10,68,73,92]. Rapid somatic growth in particular causes implanted grafts to quickly become size mismatched, leading researchers to explore the use of smaller pig breeds. Gallo et al. establish the Vietnamese pot-bellied pig as a model for TEHV evaluation, asserting that the reduced growth and human-like immune system make this animal model more suitable and cost effective than standard pigs [73]. Their rapid growth, however, does not prevent pigs from being used for ectopic studies, such as the carotid patch angioplasty performed by Wong et al. on scaffolds treated with hydrophilic and lipophilic antigen removal protocols [32]. This procedure includes the benefit of a blood-graft interface, where other small animal ectopic studies do not. The authors utilised CCR7 and CD163 stains for M1 and M2 macrophages respectively, demonstrating an improved M1/M2 ratio in their optimised scaffold, indicative of a more favourable remodelling response. Pabst reports that 80% of immune parameters measured in pigs resemble those in humans [76]. Indeed, this similarity of porcine immunity has made them a prime candidate for vaccine research in the past, meaning a large volume of the cellular componentry, surface markers, etc. are well characterised [118].

A variety of transgenic pigs have been made available, primarily to serve as a tissue source for bioengineers. Initially, the α-Gal epitope was removed from porcine matrices by knockout of the alpha1,3-galactosyltransferase enzyme [86], and the resulting tissue has been used for cardiac [119] and renal transplantation [120]. Fang and colleagues confirmed that α-Gal KO pigs produce anti-Gal antibodies – a response analogous to humans, indicating that these pigs may serve as appropriate immune models for α-Gal naivety [121]. N-glycol neuraminic acid (Neu5Gc) is another non-human epitope of concern to tissue engineers, and this too has been knocked out of α-Gal-KO pigs in the hope of yielding a non-immunogenic tissue source [19]. These double KO pigs have been even further modified to form 'triple KO' pigs, included in Table 1. Despite the improvements the transgenic pigs offer, it seems, that the issues in housing, anaesthesia, and operating on pigs has kept them out of preclinical evaluation of TEHV immunogenicity.

5.2.3. Non-human primates

Non-human primates (NHPs) are phylogenetically the closest of the animal models to humans. While their near-identical immune systems, cardiovascular anatomy, and electrophysiology makes them the ideal candidate for TEHV evaluation, their routine use in pre-clinical assessment is hindered by the exorbitant cost of upkeep and stringent ethical requirements [10,68]. Old world NHPs are of particular interest in immunological assessment due to their lack of α-Gal [8,80]. Weber et al. implanted synthetic TEHVs made from polyglycolic-acid (PGA) mesh into the pulmonary position of baboons in two separate studies. In the first instance [106], TEHVs were seeded with BMNCs, and in the second [71], the BMNCs were removed by decellularisation. At 4 weeks immunological elements were evaluated with transmission electron microscopy (TEM), histology, and IHC. The BMNC-seeded TEHVs showed evidence of an acute-phase immune reaction, with fibrin deposition and leucocyte adherence, while the decellularised grafts did not show ad-

herent cells. Histology in both instances demonstrated cellular infiltration, though the absence of neutrophils indicates avoidance of acute rejection. The cellular infiltrate was α-SMA+ suggesting migration of mesenchymal cells. CD68 and MAC-387 immunostaining showed resident macrophages, though no further characterisation into subtypes was made. NHPs have also been used to evaluate immunogenicity of TEHV-candidate biomaterials in an ectopic setting. Daly et al. investigated the immune response of African green monkeys to decellularised small intestinal submucosa (SIS) from wild type and α-Gal knockout-out pigs in an abdominal wall repair model [36]. Outcomes of interest included serum anti-Gal antibodies, serum anti-porcine antibodies, and a gene expression analysis of the explants with particular emphasis on genes implicated in M1/M2 macrophage polarity. The α-Gal+ SIS resulted in a significant increase in anti-Gal antibodies compared to the knockout SIS, but interestingly, this increase made no difference in the implant survival or gene expression. They conclude that 1) the α-Gal epitope does indeed increase production of anti-Gal antibodies, but 2) remodelling response and biocompatibility is independent of this effect. While α-Gal has garnered the most attention from the tissue engineering sphere, the Neu5Gc antigen may also represent an antigenic challenge. NHPs are, however, Neu5Gc positive, and so wild-type animals cannot be used to model the human immune response to this antigen [82]. Furthermore, when evaluating decellularised tissue from gene edited sources (mentioned in Section 5.2.2.), NHPs may demonstrate an adverse response to grafts owing to pre-existing antibodies to 'triple knockout' matrices [122].

While non-human primates represent the gold standard in immunogenicity testing, their significant cost places them at the very end of any pre-clinical assessment regime.

6. Discussion

Thorough preclinical testing of the immune response to TEHVs is absolutely essential prior to human trials. Past failures of prostheses have highlighted the gaps in scientific understanding of how the immune system responds to biomaterials in the short and long term. A startling feature of the literature reviewed in this article is the variability in testing methods. As noted by Blum [42], many researchers jump directly to large animal models without rigorous testing *in vitro* or in smaller animals. The mechanisms by which macrophages and mesenchymal progenitor cells migrate and differentiate within a TEHV are highly predictive of long-term success of the prosthesis, and so must be fully elucidated prior to implantation. Acute phase immunogenicity is relatively well understood, and researchers are able to use a variety of *in vitro* and *in vivo* models to determine how a TEHV will respond immediately upon implantation. Predicting the long-term survival of the graft, however, remains tricky. More research is needed into the chemical and mechanical cues that drive wound healing so that TEHVs may be designed to remodel favourably. Central to this difficulty is the nuanced and poorly understood behaviour of macrophages over time. A metric that appears to have favourable predictive value of macrophage response is the M1/M2 ratio. Several authors have found this parameter to be correlated with positive remodelling outcomes [21,32].

Here, the reviewers offer a panel of key immune outcomes that could serve as the basis for a complete assessment of preclinical immunogenicity. This panel is by no means exhaustive, but rather serves as a 'springboard' for developing a rigorous testing schedule for a TEHV candidate.

1) **Quantification of known antigens**

TEHVs made from decellularised animal tissue contain an amount of antigenic material that can be quantified using benchtop assays. α-Gal is of particular concern as humans are 'primed' against this epitope due to circulating anti-Gal antibodies that may cause hyperacute rejection. MHC molecules and residual DNA can provoke acute rejection *in vivo*, and also must be reduced to acceptable limits by the decellularisation protocol. Many of these antigens have cheap, well-validated methods of quantification. Unknown graft-specific antigens can also be quantified by use of serum generated in an *in vivo* model. Emerging research demonstrates that avoidance of acute adaptive immune rejection can be achieved if >92% of lipophilic antigens are removed.

2) ***In vitro* macrophage response**

Macrophages that take up residence in the TEHV control the degradation rate of the graft and so are pivotal for long term survival. *In vitro* assays whereby macrophages are exposed to the biomaterial allow researchers to predict how the graft will behave *in vivo*. Gene expression analysis and cytokine quantification of conditioned media can reveal the position on the M1/M2 spectrum the cells take. The literature demonstrates that the M1/M2 classification is hazy and by no means represents a perfect picture. Standardisation of the markers used to identify these cell subtypes would enhance the reproducibility of these assays. Microscopy of the exposed graft can reveal the degradation rate of the biomaterial, which is of particular use for synthetic TEHVs as the chemical makeup of these materials can be tweaked to yield improved degradation characteristics.

3) **Ectopic implantation in a small animal model**

Small animals are an effective way of assessing *in vivo* immune responses to biomaterials, in particular the chronic inflammation and tissue remodelling. The low cost, ease of housing, and well-defined genetics of these animals makes it easy to conduct large, adequately powered studies compared to larger animals. Serum sampling of live animals can track systemic inflammation over time. Explanted tissue at defined time points allows researchers insight into the acute and chronic influences of the animal's immune system on a graft. Current literature is quite variable with respect to the research outcomes chosen, and standardisation of IHC and gene expression markers would benefit this area.

4) ***In situ* implantation in a large animal**

ISO 5840 mandates *in situ* testing in a large animal for traditional protheses, but in the case of TEHVs, where controlled cell migration is required, outcomes of interest need to be expanded to include key immune parameters. In the acute phase, adsorption of leukocytes and immunoglobulins will determine hyperacute rejection. The α-Gal naivety of non-human primates make them the gold standard for this type of investigation, although the literature is unclear if residual α-Gal in decellularised scaffolds is truly of serious concern. A qualitative picture of leucocyte-mediated acute rejection is simply achieved by histology. More robust investigation of the adaptive response involves quantification of graft-specific antibodies over time. Chronic rejection and remodelling thereafter is somewhat murkier: a focus on macrophage subtype by IHC and gene expression can paint a picture of how the graft will survive long term. Studies must run for an appropriate amount of time to gauge this effect, with many extending up to a year. More research is needed to reveal markers predictive of a constructive or maladaptive

macrophage response. Finally, the migration of mesenchymal progenitors, their consequent differentiation into a valvular interstitial cell type, and laydown of neotissue must be elucidated.

7. Conclusion

Tissue engineered heart valves offer an exciting improvement to traditional prostheses. Failures of TEHVs in the past are borne of insufficient understanding of the immune response to these novel biomaterials. Several immune parameters are well understood, while others remain elusive. More research into the cellular mechanisms of chronic macrophage-driven changes to TEHV scaffolds is needed. Ultimately, preclinical immunogenicity testing remains a significant hurdle in bringing TEHVs to clinical practice.

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Declaration of competing interest

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.

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