The host immune response to tissue-engineered organs: current problems and future directions

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Abstract

As the global health burden of chronic disease increases, end-stage organ failure has become a costly and intractable problem. De novo organ creation is one of the long-term goals of the medical community. One of the promising avenues is that of tissue engineering: the use of biomaterials to create cells, structures, or even whole organs. Tissue engineering has emerged from its nascent stage, with several proof-of-principle trials performed across various tissue types. As tissue engineering moves from the realm of case trials to broader clinical study, three major questions have emerged: 1) Can the production of biological scaffolds be scaled up accordingly to meet current and future demands without generating an unfavourable immune response? 2) Are biological scaffolds plus or minus the inclusion of cells replaced by scar tissue or native functional tissue? 3) Can tissue-engineered organs be grown in children and adolescents given the different immune profile of children? In this review, we highlight current research in the immunological response to tissue engineered biomaterials, cells, and whole organs and attempt to provide the answers to these questions.

Introduction

End-stage organ failure is one of the most devastating and costly problems facing modern medicine [1]. An ever increasing number of individuals join the organ donor register, but there is still a dearth of supply [2]. In 1989, there were 17,917 patients in the United States registered on the combined active and suspended solid organ transplant waiting lists. By 2015, this number had risen to 79,062 active patients with a further 44,334 on the temporary suspension list to total 123,396 individuals [3]. Approximately 21 patients die each day waiting for a transplant that will never happen due to the national shortage of available organs [4]. While solid organ transplantation has been in place for over half a century, the fortunate recipients are relegated to lifelong immune suppression with the associated increased morbidity and mortality. The gold-standard solution of “off-the-shelf” organs that are non-immunogenic and have the viability of the host has yet to be achieved. Tissue engineering has long been a much-heralded tool that could, in theory, provide organs for those unable to receive conventional allotransplants.

Tissue engineering was pioneered in 1933 when Bisceglie [5] first demonstrated that mouse tumour cells, when encased in a polymer in the abdominal cavity of the pig, did not mount an immune response. When Macchiarini and colleagues performed proof-of-principle studies in pigs, the prospect of human organ tissue engineering trials resurfaced [6]. Pioneered in the airway with tracheal transplantation, studies have progressed to investigate tissue-engineered transplants in humans involving the nose, genital tract small bowel, lung, urethra and liver, among others [7-13]. These

tissue engineered constructs may comprise scaffolds only, cells only, or both combinations of scaffolds and cells. Scaffolds may be allogenic, xenogenic, or synthetic in origin and can be seeded with autologous cells. The nascent days of this technology have been challenging but also rewarding.

All of the patients that have undergone these procedures have received transplants on compassionate grounds as a palliative measure in an attempt to extend or significantly improve quality of life (Table 1). In light of the published case trials on the background of the publication of a five-year follow-up to the first tissue engineered transplant, we have reached a turning point for tissue engineering organs [14]. Grafts are generally safe and well tolerated, but their utility has yet to be demonstrated on a larger scale. The first clinical trial of this technique is due to begin in 2015 (RegenVox study).

Nevertheless, the early studies reveal host immune response to the graft has profound effects on the viability and durability of the graft. Grafts may be immunogenic (capable of triggering an immune response), antigenic (capable of binding and interacting with the host but not necessarily causing an immune response), or both. How immunogenic a graft is depends on a variety of factors including graft complexity, insolubility, and protein content. Furthermore, the “dose” of the graft, how many grafts are required, the route in which the graft is delivered, the location of the graft, and any knock on treatment that is required all affect the extent and type of immune response that occurs. Finally, the immune response is not all deleterious; immune

modulation and graft remodelling may be valuable tools for improving graft outcomes.

The immune response to the graft is mediated by the innate and adaptive immune systems. The innate immune system is the omnipresent first line of immune response. Phagocytic white blood cells, dendritic cells, Natural Killer (NK) cells, and plasma proteins identify microbes immediately at the site of infection. The adaptive immune response responds to pathogens that have overcome the innate immune system and, in doing so, develops “memory” of the microbe. The adaptive system has two arms: the cell-mediated and humoral immune responses. The cell-mediated response involves peptide-induced T cell activation, giving rise to helper T cells and cytotoxic T cells. Helper T cells secrete products that aid macrophages and B cells whereas cytotoxic T cells are actively involved in the destruction of pathogens. The humoral response, in contrast, relies on circulating antigens to trigger maturation of B cells into antibody secreting plasma cells. Antibody secreting plasma cells are specific for a certain microbe, “marking” their target for destruction by phagocytes. The inappropriate identification of a graft as “foreign” leads to graft rejection, graft versus host disease, and graft failure. Current research is focused on both identifying the fate of grafts as well as developing novel strategies of harnessing the host immune response to tolerate the graft.

Ref	Author	Year	Patients	Transplant Type	Mortality	Graft-related?
Trachea						
[15]	Macchiarini	2008	1 Adult	Allogenic scaffold + autologous cells	0	N/A
[16]	Omori	2008	4 Adults	Synthetic scaffold + collagen coating	0	N/A
[17]	Jungebluth	2011	1 Adult	Synthetic scaffold + autologous cells	NR	NR
[18]	Delaere	2012	5 Adults	Allogenic scaffold + autologous cells	NR	NR
[19]	Elliott	2012	1 Child	Allogenic scaffold + autologous cells	0	N/A
[20]	Berg	2013	1 Adult	Allogenic scaffold + autologous cells	1	No
Oesophagus						
[21]	Ohki	2013	9 Adults	Autologous epithelial cells	0	N/A
Blood vessels/ vascular grafts						
[22]	Mertsching	2009	1 Adult	Xenogenic scaffold + autologous cells	0	N/A
[23]	Hibino	2010	25 Children	Autologous bone marrow + autologous cells	0	N/A
[12]	Olausson	2012	1 Child	Allogenic scaffold + autologous cells	0	N/A
[24]	Olausson	2014	2 Children	Allogenic scaffold + autologous cells	0	N/A
Bladder						
[9]	Atala	2006	7 Adults	Synthetic scaffold + autologous cells	0	N/A
[11]	Raya-Rivera	2011	5 Children	Synthetic scaffold + autologous cells	0	N/A
Vagina						
[8]	Raya-Rivera	2014	4 Children	Allogenic scaffold + autologous cells	0	N/A
Muscle						
[25]	Mase	2010	1 Adult	Synthetic scaffold	0	N/A
[26]	Sicari	2014	5 Adults	Xenogenic scaffold + autologous cells	0	N/A
Cartilage						
[27]	Brittberg	1994	23 Adults	Autologous chondrocytes	0	N/A
[28]	Almqvist	2009	21 Adults	Synthetic scaffold + allogenic chondrocytes	0	N/A
[29]	Lim	2009	Unknown; ongoing	Mesenchymal stem cells	NR	NR
Nose						
[13]	Fulco	2014	5 Adults	Autologous condrocytes	0	N/A
Heart valves						
[30]	Cebotari	2006	2 Children	PV allografts + autologous cells	0	N/A

[31]	Dohmen	2011	11 Adults	PV allograft + autologous cells	0	N/A
[32]	Kneib	2012	12 Adults	6 AVs + 5 PV allografts +/- autologous cells	0	N/A
Bone						
[33]	Vacanti	2001	1 Adult	Synthetic scaffold + autologous cells	0	N/A
Skin						
[34]	O'Connor	1981	2 Adults	Autologous skin	0	N/A
Cornea						
[35]	Pellegrini	1997	2 Adults	Autologous cells	0	N/A

Table 1: Key cases of tissue engineering transplants in adults and pediatrics. N/A: Not applicable. NR: Not reported.

Tissue engineering therefore presents important questions regarding the role of the host immune response. 1) Can the production of biological scaffolds be scaled up accordingly to meet current and future demands without generating an unfavourable host immune response? (discussed here in section 1) 2) Are biological scaffolds plus or minus the inclusion of cells replaced by scar tissue or native quality tissue? (discussed here in sections 2 and 3) 3) Can tissue-engineered organs be grown in children and adolescents in spite of the specific immune profile of children? (discussed here in section 4) [36]

The purpose of this review is to discuss the role of the host immune system in confounding or promoting the advancement of organ tissue engineering and the areas of research that are promising.

1 The immune response to synthetic, biological, and xenogenic biomaterials

Whole organ tissue engineering requires the use of scaffolds on which to seed cells for soft tissue regeneration. Scaffolds can be constructed *de novo* using synthetic or biological materials or derived from allogenic and xenogenic tissues. The choice of materials is increasingly complex; Figure 1 demonstrates the current possibilities for skeletal muscle reconstruction alone. Although the choice of biomaterials has been reviewed elsewhere [37], here we provide an overview of the immunological profile of the biomaterials used in scaffolds (Table 2).

Figure 1: Schematic overview of scaffold materials used for skeletal muscle reconstruction in tissue engineering (Reproduced with permission from [38])

1.1 Synthetic biomaterials

Synthetic biomaterials are widely available and already have many uses within medicine. Examples include hydrogel, plastic, polystyrene, and gold [39]. The advantages of synthetic materials are clear: they are largely inert, mass producible, and can be tailored to meet the specific requirements of the organ in question. Their hydrolytic properties mean that even their degradation profile can be controlled and manipulated [40]. Unfortunately, synthetic biomaterials are among the most immunogenic biomaterials, strongly activating the innate immune system. Recent research has demonstrated that, through their effects on toll like receptors, synthetics trigger pathogen associated molecular proteins and local inflammasomes, causing widespread damage at the graft site [41]. One potential tool to circumvent the immune profile of synthetics is to use peptides or other

biologicals to coat the surface of synthetic biomaterials [42]. An even more sophisticated approach would be to coat a synthetic material with a modulatory extracellular matrix in order to modulate the host response [41].

1.2 Biological *de novo* biomaterials

Biological biomaterials that can be constructed *de novo* into scaffolds include collagen [42], fibrinogen, hyaluronic acid, GAGs, hydroxyapatite, chitosan, silk [43] and starch [44]. De novo biological materials are promising because they are readily available, cost-effective, and able to be produced in large numbers. Concerns include their intrinsic biophysical properties (tensile strength, contractility, etc), degradation profiles, sterilisation cost, and pathogenic potential. Studies of starch-based scaffolds implanted subcutaneously and intramuscularly suggest good integration of the materials in the host independent of the tissue location [44]. Silk-based biomaterials provoke an untransformed CD14+ human monocyte response characterized by IL-1β (an inflammatory cytokine) and IL-6 (an acute phase reactant) but not IL-10 (anti-inflammatory) gene expression and protein production [43]. The macrophage response to silk is mostly mediated by the sericin protein [45]. Large silk biomaterials fail to induce peripheral T cell activation, an interesting finding that merits further study. Current hypotheses include a role for expression markers that down-regulate T cell responsiveness [43]. Silk could therefore find several implementations: as a surgical device to replace conventional mesh, as a cloak for other biomaterials, or as a research device for further elucidating T cell downregulation.

1.3 Xenogenic biomaterials

An ideal biomaterial would have adequate biomechanical properties, be readily available, promote a favourable immune response, and present a low risk for infectious disease transmission [22]. The use of xenogenic material is therefore particularly suitable. However, the immunogenicity of xenogenic biomaterials is still the primary drawback to their use. Heart valves, the ubiquitous usage of a xenogenic biomaterial, are still subject to immune mediated degradation in an area that has a minimal immune response [46]. The Gal epitope has been the most studied in this context. For example, pig to primate liver transplantation with livers transgenic for human CD55 (which mediates complement activation) or α-1,3-galactosyltransferase knockout was associated with extended survival [47, 48]. Although modifying the Gal epitope may mitigate acute rejection, there is still a delayed form of antibody-mediated rejection. There are most likely to be non-Gal based xenoantigens, although these have yet to be fully identified [49, 50]. Ideally, a xenogenic scaffold could be decellularized and modified to the point where acute and chronic rejection were minimized.

One potential route to the use of xenogenic biomaterial is through immunoisolation: when the foreign material is immunologically isolated from the surrounding tissues [51-53]. There are two mechanisms by which immunoisolation can occur: encapsulation and immunocloaking. Lim and Sun pioneered this technique in 1980 when they found that encapsulated islets correct diabetes in rats [54]. Modern encapsulation techniques use extravascular (membrane/ hollow fibres) or intravascular (like dialysis)

techniques in order to contain islets. These capsules permit oxygen, nutrient, and molecular exchange to a degree but prevent large immune molecules from passing [55]. Immunocloaking is an alternative technique in which a natural nanofilm is injected prior to transplantation in order to camouflage antigens [53]. For example, Brasile et al used a nano-barrier membrane to cover canine kidneys in a renal transplant model. No immune suppression was required, and the mean rejection time was 30 days versus 6 days untreated [56]. Although both of these mechanisms are promising, they still require some degree of immune suppression. Using silk as a cloak would be an interesting avenue for further exploration. However, it is still not clear what catastrophic results would occur if and when degradation of the protective coating began.

Biomaterial	**Synthetic**	**De novo Biological**	**Xenogenic**	**Allogenic**
Advantages	Inert, mass producible, modifiable, degradation profile known, cost	Readily available, mass producible, cost	Readily available, mimics native tissue, cost, producible	Mimics native tissue
Disadvantages	Immune response	Immune response, sterilisation cost, biomechanical properties	Immune response, pathogenic potential	Immune response, pathogenic potential, cost
Innate immune response	+++	+	+++	+
Adaptive immune response		+	++	+
Overcome Immunity	Peptide cloaking	Sericin protein modulation; Identify	Immunocloaking; immunoisolation	Recellularisation with autologous cells

	expression markers that down-regulate adaptive immune repsonse	Harness M1/M2 response and T cell class switching

2 The immune response to allogenic biomaterials

2.1 Decellularisation and immunogenicity

Allogenic biomaterials are those derived from another host that have been modified in order to function as a graft. Allogenic materials are advantageous because they maintain tissue composition and architecture and are very similar to the native tissues they aim to replace. Hypothetically, allogenic materials can be harnessed to both remodel and regenerate a host tissue while circumventing the immune response [57, 58]. Considerable research has focused on creating non-immunogenic allogenic materials. One of the first steps in “treating” an allogenic biomaterial is to decellularize the graft. The ultimate aim of decellularisation techniques is the removal of antigens that elicit an adverse immune response while preserving scaffold (i.e., ECM) integrity. This integrity is typically measured through collagen, elastin, s-glycosaminoglycan, and growth factor content, as well as structural and ultrastructural integrity. Cryofixation with glutaraldehyde and preservation and fixation [30] been attempted but generated non-viable grafts.

One early method, pioneered by Yates et al, removes 98.2% of nucleated cells and 98.7% of soluble protein in bone via wash and centrifugation steps [59]. Decellularisation methods have developed since then with a wide variety

of results both in terms of functionality and structural content [60-62]. It is important to note that the two do not entirely correlate [63]. However, in a recent study, rabbit cricoarytenoid dorsalis muscles were harvested and analysed using different methods: histochemical, immunohistochemical, and molecular. Latrunculin B, potassium iodide, potassium chloride, and deoxyribonuclease all led to total DNA clearance, decreased MHC II expression, and preservation of structural integrity [57]. Most protocols are prohibitive due to the time (28 days) required to produce the scaffold and the resultant financial costs. Most recently, Lange et al have described an enzyme/detergent protocol which, when combined with vacuum technology, can significantly reduce the time required to create clinically suitable airway scaffolds [64]. However, this method is not necessarily applicable to all tissue sources and tissue types of biological scaffolds as these techniques have been primarily focused on airway structures. Developing an appropriate test for the immunogenicity or antigenicity of a scaffold will be important as the levels of MHC I and II and the structural integrity alone are not predictive.

Decellularised scaffolds can also modulate the immune response of their hosts with decreased T cell proliferation and a shift towards the M2 macrophage phenotype [58, 65]. For allogenic and xenogenic materials, the tissue remnants after decellularization may still provoke an innate immune response. For example, Damage Associated Molecular Proteins (DAMPs) may still be present after decellularization. These DAMPs are not only found in the native tissue but they are also actively secreted during cell necrosis and by macrophages that respond to the acute tissue injury. DAMPs upregulate

HMGB1, which mediates the proinflammatory response through increased chemokine and TLR4 mRNA expression [66]. Counterintuitively, minimizing this initial macrophage response by inhibiting a well-characterized DAMP, HMGB1, led to an increase in the proinflammatory response, cell death, and chemokine expression [66]. There is therefore a potential for DAMPs to be manipulated as bioinductive molecules within an ECM scaffold.

Decellularization also does not, however, fully remove MHC I and II [67]. Interestingly, Haykal et al noted that decellularization delays leukocyte involvement but leads to cartilage degradation [67]. Macrophages are very plastic and can switch from M1 to M2 in response to the environment [68, 69]. In theory, the injury caused by inserting a decellularized scaffold can modulate the macrophage response to an immunotolerant, injury response M2 macrophage type [70, 71] (See Section 4.1). Macrophage repolarization could lead to site-appropriate remodelling [72, 73], and is therefore a valuable direction of future study.

The immune response to decellularised scaffolds has raised questions and offered solutions for harnessing the immune response to scaffolds (See Section 4.1). Recellularising grafts with autologous cells alters the immune response to these grafts (See Section 2.2).

2.2 Recellularisation and immunogenicity

Once scaffolds have been decellularized, recellularization with appropriate cells is the ultimate goal. Which cells to use and when to use them are questions that have been addressed elsewhere [74]. Recently, one group has

grown three individual scaffolds: the epithelial (physical barrier), fibroblast (ECM production), and dendritic cell (immune sensing) layers. The epithelial layer in particular was grown at the air-liquid interface for four weeks leading to a functional barrier and transepithelial electrical resistance mediated by tight junctions [75]. As regards the immune response towards these new cells, cellularization provides an important opportunity to modulate the immune response.

Stem cells can be loaded with the required genes prior to incubation. For example, Holladay et al found that, when stem cells were transfected with IL-10 before being loaded onto a collagen scaffold, there was a significant improvement in the survival of the cells [76]. Such techniques should be approached with caution. For example, although some factors, such as FGF, activate the key regenerative processes of inflammation, wound response, and chemotaxis, they also provoke a strong immune response [77]. Incorporating the use of external factors in promoting cellularization and graft survival is still in its nascent stages. In one of the human cases, a 12-year old boy with congenital tracheal stenosis was transplanted with a scaffold seeded with bone marrow mesenchymal stem cells and granulocyte-colony stimulating factor with recombinant erythropoietin and transforming growth factor beta applied. A subsequent strong neutrophil response at 8 weeks generated neutrophil extracellular traps, which considerably affected mucosal clearance [19]. Therefore, current efforts focus on the use of autologous cells rather than stem cells.

Using autologous cells prior to implantation initially requires a bioreactor. Ideally, the bioreactor presents a similar environment to the host optimal for cell growth and integration [41]. For example, lymph nodes have been assessed in mice as scaffolds. The lymph nodes were processed using sodium dodecyl sulfate detergent, and the matrices were repopulated with splenocytes, implanted in submuscular pockets in the host, and harvested 14 days later. They were then implanted in the renal capsule of syngenic or allogenic mice recipients and analysed. The result was successful *in vivo* cell delivery with no significant antigenic response [78]. The greatest concern with the applicability of such a method is that the renal capsule itself is a privileged site that induces tolerance [79]. A further concern with such a method is the need to house the tissue in a bioreactor as, when tried in a human patient without use of a bioreactor, the graft did not have biomechanical strength until 18 months [80].

A human study of allogenic trachea tissue engineering used the patient's forearm as a bioreactor [81]. The authors have subsequently employed this method in five patients with similar results in four of them: with withdrawal of immunosuppression, the patients tolerated the grafts well [18]. In one patient however, the graft was not immune tolerant at withdrawal of immunosuppression. The host immune response led to the appropriate resoprtion of the donor mucosa. However, the donor cartilage was afforded considerable immunoprotection only by the immunosuppression [82]. The requirement for immunosuppression would be largely unsuitable for the transplant and neonatal populations. For the moment, using the host is not a

feasible option for an immunosuppression-free graft but provide a potential and viable alternative for the future.

External bioreactors are currently the preferred option for growing autologous cells as they remove the potential for host morbidity and could, in theory, be made rapid and cost-effective. Bioreactors do have to, however, provide an environment that improves mass transfer, allows perfusion of vascular structures, and provides biocompartmentation as needed [83, 84]. The development of the rotating bed bioreactor leads to high rates of mass transfer and improved oxygenation of the tissue due to the shear stress [85, 86]. Simultaneous transmural and axial flow within the material increases both the mechanical strength of the scaffold and the vascular development of the seeded cells [87, 88]. In theory, cell culture in the bioreactor and inclusion of factors could minimize the immune response and maximise viability of the graft due to the greater theoretical control afforded by an extrinsic method. Autologous cells could in theory also be grown to the scale required for whole organ engineering.

3 Immunological outcomes for tissue engineered organs

3.1 Lessons from case studies

Functional organ replacement is the ultimate aim of tissue engineering. Tissue engineering to date has seen major successes in using scaffolds alone or scaffolds plus cells to reconstruct body tissues. Functional organ replacement is a future endeavour, but one that is no longer enshrined in mythology [89].

Successful attempts to create in vitro organ models include the production of the heart [90], liver [91, 92], lung [93], and recently the kidney [94].

Proof-of-principle trials have demonstrated both that there is minimal humoral immune reaction towards decellularized tissues and that recellularization is effective with no neoplastic element [21, 36, 95]. Thus, Macchiarini and colleagues performed the first tissue-engineered allogenic tracheal transplant in 2008. There were no signs of anti-donor antibodies or inflammation at follow up [15]. Gonfiotti et al have recently published a five-year follow up of a subsequent case of tissue engineered airway transplantation [36, 95]. The tissue-engineered trachea was open, well vascularised with respiratory epithelium, had normal ciliary function, and normal mucus clearance. However, the patient developed stenosis in the native trachea close to the proximal anastomotic site. Certainly, identifying ways to minimize scarring given the local immune response to the changing environment is an important avenue of research [14]. These results are not reproducible: in another trial in which a 76 year old patient with non-resectable tracheal stenosis was given a tissue-engineered transplant [20]. The patient passed away shortly thereafter from a cardiac arrest secondary to severe stenosis of the coronary arteries. However, at autopsy, the anastomoses were intact, the submucosa had reformed, there was one layer of squamous epithelium, neovascularization was occurring (lumina of capillaries and red platelets were visible), and the seeded chondrocytes were intact.

Xenogenic human case studies have also been performed. Vaginal organs were engineered using allogenic cells and xenogenic scaffolds. In these patients, the scaffolds were derived from decellularized porcine small intestine submucosa. However, the patients' own epithelial and muscle cells were cultured, expanded, and seeded onto these biomaterials. At yearly biopsy, the vaginal structure comprised three layers: epithelium, matrix, and muscle. Furthermore, the patients reported improved sexual function. No significant detrimental immune response was recorded [8]. Xenogenic materials, such as used in this study, may prove advantageous as they are more readily producible and when "cloaked" with autologous cells, appear to be afforded some immunoprotection.

The concept of immunocloaking is also being explored in developing functional pancreatic islet cells for the treatment-resistant Diabetes [96, 97]. An initial investigation into islet cell transplantation provided reproducible success in the short-term [98]. However, long-term insulin independence has not been achieved [99]. Several solutions have been suggested including the inclusion of laminin and collagen IV, microencapsulation, and immunocloaking. Conrad et al bioengineered a scaffold based on decellularized pancreas extracellular matrix and mesenchymal stem cell / islet cell co-culture, generating functional endocrine tissue and reversing the diabetic state [89, 100]. Therefore, protecting the graft from the host immune response is a key predictor of success in case trials.

Creating blood vessels is vital not only for cardiovascular uses but also to develop a blood supply for more complicated grafts at other sites. The growth of blood vessels using bio-engineered scaffolds is progressing. Olaussen et al were able to transplant the first tissue engineered allogenic vessels into two pediatric patients. These grafts did not have any antibodies to Major Histocompatibility Complex I or II at follow up and remained patent with good response to pressure [24]. Acellular heart cadaveric extracellular matrix has been assessed in mice, but leads to clots even when transplanted with anticoagulation. By relining vascular conduits with rat aortic endothelial cells, a less thrombogenic left ventricle with better contractility was created. The trial arms that involved recellularizing the brachiocephalic artery and the inferior vena cava and the brachiocephalic artery led to improved rat aortic endothelial cell proliferation and von Willebrand Factor and nitric oxide synthase expression. The expression of these factors decreased thrombogenicity, thereby improving graft outcome [101]. Further rat models have also managed to reseed decellularized rat hearts with cardiac endothelial cells. By 8 days post-seeding, under physiological load, the hearts could generate pump function equivalent to 2% of adult function in a modified working heart preparation [90]. These studies suggest the potential for functional myocardial tissue replacement. However, the need for additional factors raises questions about how such factors could be incorporated as these factors are, in themselves, immunogenic.

An observational first-in-human trial of five patients demonstrated the efficacy of engineered autologous tissue grafts for nasal reconstruction following

tumour resection. The selected patients possessed a greater than 50% alar subunit defect after non-melanoma excision on the alar lobule. The procedure for cellularisation of the graft involved expanding, seeding, and culturing autologous chondrocytes in serum onto collagen type I and II over four weeks. These grafts were remodelled into fibromuscular fatty structures similar to the tissue at the site of implantation. There were no adverse events reported following implantation and patient satisfaction was high at 1 year. However, no cartilage was present at 6 months. In goats, however, engineered nasal cartilage grafts implanted in the knee remain cartilaginous. What factors, if any, are necessary to maintain and develop these grafts are still unknown [13]. It may not even be necessary to maintain the cartilaginous graft if the remodelling into the scar provides the necessary properties as in this case.

3.2 Innate immune response to biological scaffolds

Even as trials are providing insight as to what is occurring macroscopically, there is a growing body of evidence to suggest that the innate and adaptive immune response can both promote and undermine the viability of these grafts. The innate immune response to biological scaffolds can lead to early failure of the graft but, even in the absence of early graft failure, may also have knock-on effects for graft function and viability long term. The initial injury of placing the graft provokes an inflammatory response. The terminal biochemistry of the graft surface then modulates the serum proteins that adsorb to it. Biomaterial adherent macrophage apoptosis is also increased by hydrophilic substrates *in vivo*. Hydrophilic substrates also decrease monocyte and macrophage adhesion and fusion used in an *in vivo* rate cage implant

system [102]. For example, integrins bind less if the molecule is hydrophilic and anionic [41]. Passive modulation of the biomaterial surface properties may limit macrophage adhesion, activation, and fusion to foreign body giant cells [41].

Promotion of a long-term constructive macrophage phenotype is vital in achieving graft tolerance [103]. The classical macrophage type, M1, is IL-12^{high}, IL-23^{high}, IL-10^{low} and produce IL-1β, IL-6, and TNFα. Through the action of these CCR7+ CD80+ and CD86+ macrophages, Th1 inflammation and infection is induced. These macrophages cause inflammatory extracellular matrix destruction and injury. Matrix degradation can be extremely valuable in tissue sites as its functionality is gone once appropriate cellular architecture has been restored. However, in some contexts matrix maintenance is fundamental to the suitability of the graft, particularly in structures that carry a lot of load (such as bone) or that are prone to dangerous collapse (such as the airway). This is in contrast to the M2 macrophage subtype, which is IL-12^{low}, IL-23^{high}, and IL-10^{high}. These macrophages are CD163+, CD206+, and ArgI+ and lead to tissue repair through activation and in conjunction with the Th2 subtype [104]. Thus, some of the scar formation that was found in clinical trials may reflect a valuable form of matrix degradation. The question then becomes, how do we modulate this macrophage phenotype?

Xenogenic tissue culture models have provided some insight into the macrophage innate immune response. In porcine acellular bladder matrix

combined with human urinary tissue, there was a time-dependent infiltration by CD8+CD80- cells accompanied by maturation to a CD163 phenotype. PPARγ signalling predominated in the polarization of macrophages from M1 (CD80+) towards M2 (CD163+) [105]. One of the problems with the use of a porcine model is that there is a dearth of markers for porcine cells. Another study in mammalian ECM found that degradation from ECM bioscaffolds promotes M2 macrophage polarization *in vitro* leading to the migration and myogensis of smooth muscle progenitors from solubilized small intestine submucosa. The secretome of this constructive graft was similar to that of IL-4 polarized M2 macrophages [106].

3.3 Adaptive immune response to biological scaffolds

Proof of principle studies in humans have already demonstrated that the humoral immune response does not destroy tissue-engineered biological scaffolds. However, there may still be a cellular immune response. Indeed, the Th1 response is associated with classical acute graft rejection [73, 107, 108]. Ideally, a scaffold should have delayed degradation time, decreased sensitized T cell proliferation, and improved survival of donor-derived cells [58]. Measuring the survival and functionality of the graft *in vivo* is difficult. *In vitro* studies have demonstrated that graft survival is associated with decreased IL-2, IFNγ and increased IL-10 levels, Furthermore, in tolerant grafts, the factors IL-4, TLR2, an TLR4 and their gene expression have decreased inversely proportional to recellularization of grafts while TGF-B1 is proportional [109]. Although being able to analyse the graft is important, the real question is how to modulate the T cell response away from Th1 effector

function and towards a Th2 tolerant phenotype by harnessing the macrophage M2 phenotype [69].

Transplant studies offer valuable insight into the adaptive immune response to grafts. Transplant recipients must take immunosuppression drugs, which contribute to graft failure and are toxic to the host. Ever since [110] demonstrated that there was specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporins, researchers have been trying to come up with a way to conduct transplants without immunosuppression. Central tolerance is achieved by depleting reactive T cells in the thymus. *In utero* transplantation and non-myeloablative bone marrow transplantation leading to mixed chimerism have both been attempted in order to induce central tolerance [111, 112]. The latter has been demonstrated in a proof of principle study [111]. As regards peripheral tolerance, strategies have included co-stimulatory blockade by manipulating regulatory T cells or through tolerogenic dendritic cells [113, 114].

In vitro studies have shown that decellularized scaffolds can circumvent the cell-mediated immune response and modulate this host response towards a favourable phenotype [57, 58, 115, 116]. The ONE study is currently assessing the roles of Treg cells, regulatory macrophages, and tolerogenic dendritic cells in redirecting the host response towards an M2 macrophage and Th2 lymphocyte phenotype in human transplantation [117-119]. This study will have profound implications for how immune tolerance to biological scaffolds is established and promoted especially in future trials [120].

4 Immune response to biomaterials in pediatric patients

Pediatric patients represent an important population. Structural abnormalities in children that cause debilitating or life-threatening outcomes require surgery. Pediatric patients have different requirements to adults: surgery should preferably be one time only and any resulting graft should in theory survive the length of the adult lifespan. 3% of newborns have congenital malformations including 2% with bronchopulmonary problems and tracheal problems (including agenesis, tracheomalacia, and bronchomalacia) [121]. Congenital anomalies further affect the heart with 10,000 children requiring surgery each year. Finally, genitourinary tract problems affect 4% of livebirths, with gynaecological problems in particular [122, 123]. There are differences in the immune profile of children that have implications on the design and implementation of tissue engineered organs. A recent five-year follow-up of a tissue-engineered trachea transplanted into a 10-year old child using a decellularized autologous graft with allogenic cells has confirmed that the scaffold demonstrated long-term viability. However, there were initial interventions, and a method to achieve this replacement with lower morbidity and cost is warranted [124].

5.1 Profile of pediatric innate and adaptive immunity

In the newborn, innate immune cells mount a different cytokine response to pathogens as compared to adults. IL-12, IFN1, and IFNγ decrease whereas

IL-1β, IL-6, IL-23, and IL-10 increase [125]. Innate immunity of the newborn is polarized towards a high ratio of IL-6/TNFα production [126]. Serum collected from the newborn has increased IL-6/TNFα ratios as compared to cord blood. The cause of the hyperactive innate immune response may be due to negative regulation of the adaptive arm via IL-10. Type I IFNs feedback on IL-10 production as well as regulation IL-1β pro-inflammation by counter-regulating the IL-1R antagonist. IL-6 production is then due to both neonatal cellular (monocyte) and humoral (serum) factors. This production is then associated with elevations of IL-6 inducible reactants CRP and LPS-binding protein. [127]. An observational study of pre-term infants who developed sepsis noted that these infants had decreased monocyte class II antigen, again suggesting that the adaptive immune system is delayed [128]. This has led to the hypothesis that there is a later set point for coupling adaptive and innate immunity [128-130]. Physiologically this is logical: fetal T cells are highly responsive in cell culture to *in vitro* antigen stimulation, however, there are limited numbers of T cells as the newborn immune system must tolerate the mother's for birth. Minimizing or modulating the innate immune response, particularly to synthetic biomaterials, will be important in ensuring graft survival in pediatric patients.

Many *in vivo* and *in vitro* studies to date have described the deficiencies or immune deviation among T cells, B cells, and APCs in neonates [131]. There is limited IL-2 production and deficient proliferation by human neonatal T cells. Adult-like Th1 can be achieved and has been demonstrated in response to antigen exposure [132, 133]. It is not known how skewed this response is to

the Th2 phenotype. Mouse CD4+ T cells of fetal origin mount Th-2 skewed responses in an antigen dose-dependent manner [134]. The transfer of neonatal TCR transgenic CD4+ T cells into adoptive neonatal hosts leads to the development of both Th1 and Th2 cell primary effector function after immunization. After re-exposure to antigen *in vivo*, neonatal Th1 but not Th2 cells undergo apoptosis. This process can be inhibited by IL-4R or IL-13R specific blocking antibodies [135]. One study in pigs found that younger animals have site-appropriate tissue remodelling of small intestine submucosa extracellular matrix as compared to scaffolds from older animals. Furthermore, this remodelling was consistent with a dominant M2 and Th2 response, suggesting that tolerance was easier to achieve [136]. How the immune profile of a pediatric or neonatal patient will present towards a tissue-engineered biomaterial is unclear. However, the upregulation of the innate immune response and downregulation of the adaptive suggests that initial immunoprotection say through cloaking could be particularly valuable as subsequent degradation would be more likely to produce a tolerogenic graft.

4.2 Cell replacement and immune desensitisation

Cell replacement following immune desensitisation has been proposed as a therapy for CNS diseases, particularly in neonates. The immune response mounted against stem cells often hampers in vivo investigations. Current research has recognised the need for immune modulation in order to carry out these transplantations in animal models [137]. Two recent reports have demonstrated conflicting results with neonatal tolerance to xenografts in rats.

The first found that human embryonic stem cell-derived mesenchymal stem cells survived as long as eight weeks and with a dampened CD4+ inflammatory response when the rats were immune desensitised prior to the transplantation [138]. The conflicting study employed the same method but used human glial-restricted precursor cells from the foetus [139]. A third study demonstrated that the method was reproducible using the same embryonic stem cell-derived mesenchymal stem cells as the first study in rat joint cartilage [140]. However, when reproduced in three strains of neonatal immune-intact mice, using two different brain transplant regimes and three independent stem cell types, implanted cells were rapidly rejected [141]. The efficacy of immune desensitisation has yet to be fully determined, however this is still a promising technique for avoiding some of the cell-related immune response.

4.3 Lessons from organ transplantation in pediatrics

Tolerant pediatric transplants have unique T and B cell profiles. CD4+CD25+ Treg cells have been implicated in the development of neonatal tolerance to transplantation antigens [131]. In a mouse model, CD4+CD25+ Treg cells block CD8+ Treg cells which themselves downregulate the Th2 cell-mediated pathology in a neonatal transplantation model [142]. For those human pediatric patients who develop a tolerant transplant profile, studies have demonstrated a unique adaptive immunophenotype. For example, in liver transplant, tolerant patients have increased numbers of naïve CD4+RA+ and CD4+CD197+RA+ T cells and fewer CD4+CD197+RA- T cells. Furthermore, there were more inducible CD4+CD25+ T cells. These tolerant patients also

had fewer CD19+CD127+ B cells and increased numbers of CD27-CD38+, IgD+, and unswitched memory CD27+ IgD+ IgM+ B cells [143]. How this switch occurs is still unknown but may relate to environmental or other antigenic factors. Harnessing and identifying this switch is vital in modulating a pediatric immune response response.

Immunodepletion has been a much-researched technique for decreasing the antigenicity of the transplant. For example, high-risk acute leukaemia patients sometimes require transplants that cannot be granted by a fully matched family donor. The advantages of T cell depletion are decreased graft versus host disease, decreased toxicity to the organ, lessened need for immunosuppression, and lower early transplant-related mortality. Trials have shown that, with a high dose of T cell-depleted haematopoietic progenitor cells, no suppression is required to control graft rejection or graft versus host disease [144]. The event free survival is similar but there are important implications of immunodepletion. There may be delayed immune reconstitution, viral reactivation and other infectious complications, decreased graft versus leukaemia effect, an increased incidence of graft failure, and increased risk of Epstein Barr Virus associated lympho-proliferative disorder.

Immunodepletion also reveals how T cell tolerance may occur. T cell receptor Vδ2 γδ T cells are implicated in host defence whereas Vδ1 γδ T cells have a role in modulating the immune response [145, 146]. Studies have role for γδ T cell reconstitution in improving event-free survival. For example, infection increases in the presence of low numbers of Vδ2 γδ T cells. There is also a

significant impact of the maximum number of CD3+, CD4+, and CD8+ T cells and donor source on the Vδ2 γδ T cell recovery [147]. When Vδ1/Vδ2 ratio was assessed in a liver transplant cohort, it was found that, when the ratio was the highest, patients were more likely to be graft tolerant. Furthermore, the Vδ1 gene had a complementarity-determining region 3 sequence (100% homologous) among all tolerant patients and dominant in 6 out of 9 patients. This clone was also found in some of the normal livers but none of the rejected organs [148]. Grafts could in theory be modified or selected for favourable T cell phenotype. One could imagine a bioreactor in which the host immune response was mimicked and the graft manipulated using genetic and environmental means to promote a tolerant response.

Conclusions

Organ tissue engineering is still in its earlier days, but the questions have now changed from whether or not it is possible, to how to fully harness its potential. One of the greatest stumbling blocks to the use of xenogenic, allogenic, synthetic or *de novo* biological tissues is the continued ability of the graft to provoke an immune response in the host. The immunogenicity of grafts has implications both for the scale of production that may be achieved and also for the outcomes of grafts once transplanted into the host. Additionally, the pediatric population has a robust innate immune system but a fledgling adaptive immune response, which could present significant problems with the initial scarring and graft remodelling. One solution to solving the immune problem is to effectively cloak grafts in immune-neutral substances such as

the extracellular matrix or peptides. Additionally, studies are finding techniques to suppress or harness the immune system to promote graft tolerance. Through the ONE study as well as further investigation into macrophage and T cell class switching, harnessing the immune response could overcome the major immunological barriers to whole organ tissue engineering. The questions (and beginnings of answers) presented here will continue to provide challenging questions for clinicians and researchers looking to implement and advance tissue engineering.

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