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Regulation of decellularized matrix mediated immune response

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The substantially growing gap between suitable donors and patients waiting for new organ transplantation has compelled tissue engineers to look for suitable patient-specific alternatives. Lately, a decellularized extracellular matrix (dECM), obtained primarily from either discarded human tissues/organs or other species, has shown great promise in the constrained availability of high-quality donor tissues. In this review, we have addressed critical gaps and often-ignored aspects of understanding the innate and adaptive immune response to the dECM. Firstly, although most of the studies claim preservation of the ECM ultrastructure, almost all methods employed for decellularization would inevitably cause a certain degree of disruption to the ECM ultrastructure and modulation in secondary conformations, which may elicit a distinct immunogenic response. Secondly, it is still a major challenge to find ways to conserve the native biochemical, structural and biomechanical cues by making a judicious decision regarding the choice of decellularization agents/techniques. We have critically analyzed various decellularization protocols and tried to find answers on various aspects such as whether the secondary structural conformation of dECM proteins would be preserved after decellularization. Thirdly, to keep the dECM ultrastructure as close to the native ECM we have raised the question "How good is good enough?" Even residual cellular antigens or nucleic acid fragments may elicit antigenicity leading to a low-grade immune response. A combinative knowledge of macrophage plasticity in the decellularized tissue and limits of decellularization will help achieve the native ultrastructure. Lastly, we have shifted our focus on the scientific basis of the presently accepted criteria for decellularization, and the effect on immune response concerning the interaction between the decellularized extracellular matrix and macrophages with the subsequent influence of T-cell activation. Amalgamating suitable decellularization approaches, sufficient knowledge of macrophage plasticity and elucidation of molecular pathways together will help fabricate functional immune informed decellularized tissues *in vitro* that will have substantial implications for efficient clinical translation and prediction for *in vivo* reprogramming and tissue regeneration.

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1. Introduction

End-stage or multi-organ failure is one of the critical and growing healthcare problems encountered by our modern society. Conventional therapeutic approaches fail to trigger tissue regeneration when body tissues or organs are severely affected by disease, trauma or cancer, and congenital defects. Hence, current clinical options utilize transplantation of organs harvested from either live or recently deceased donors. But this approach is constrained by the limited availability of high-quality donor tissues.[1] The substantially growing gap between suitable donors and patients waiting for a new organ makes the situation even more challenging. Furthermore, issues related to biocompatibility and bio-functionality often result in transplant rejection, thus making even the highly updated transplantation procedures unsuccessful.[2]

A decellularized extracellular matrix (dECM) has lately been used successfully as an alternative to the constrained availability of donor organs and has been used to recreate different types of tissues and organs, such as the skeletal muscle,[3] blood vessel,[4,5] heart valve,[6,7] cornea,[8] urinary bladder,[9] skin,[10] *etc*. For example, a decellularized scaffold derived from the bovine small intestinal submucosal ECM has been successfully used for the repairing of a critical-sized full-thickness skin defect in a rodent model.[11] The primary aim of the decellularization process is the removal of the cellular and nuclear contents while maintaining the inherent ultrastructure and biochemical constitution of the native ECM.[12,13] The dECM should also permit the autologous cells or pluripotent progenitor cells of a patient to adhere and proliferate, in order to eventually develop a patient-specific functional tissue.

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Alternatively, only the dECM scaffold (without any cells seeded) can be implanted directly into a patient's body at the site of the defect, in anticipation that the chemical composition and architecture of the dECM may augment the exploitation of the regenerative capacity of the patient.[14] Hence, the harvested tissue must be properly decellularized since the presence of any residual content such as xenogeneic/allogeneic DNA and cell membrane antigenic epitopes exposed post-decellularization could trigger an adverse immune response. Furthermore, these implanted scaffolds may begin to degrade slowly at the site of implantation, with a concomitant release of biologically active peptides, which in turn may influence other cellular processes such as angiogenesis, cell proliferation, migration, differentiation, and mononuclear cell infiltration.[15,16] Advanced decellularization strategies would also widen the use of the dECM for numerous applications such as bioinks for 3D bioprinting, drug screening, and as an *in vitro* disease model in a patient-specific manner apart from organ transplantation (Fig. 1). Even after rigorous research done by the decellularization community, this field is still grappled with various limitations, which restricts the clinical translational of the dECM. Firstly, the primary aim of decellularization is to preserve the ECM ultrastructure. However, it is arduous to completely evade the damage or denaturation of ECM proteins. Secondly, although a large number of decellularization protocols exists, the so-called optimal method specific for a particular organ is mostly decided by the 'hit and trial' method. Thirdly, there is still a large gap in the proper understanding of the innate and acquired immune response to the xenogeneic and allogenic biologic scaffolds and the role played by macrophages or other immune cells in dECM degradation and remodeling. Although the results obtained from *in vitro* and *in vivo* studies are used to understand the processes of inflammation and immune response, extrapolating these results in humans would be inappropriate. Thus, there is an utmost need to gain deep mechanistic insights into the entire process of decellularization which would allow tissue engineers to more efficiently regenerate patient-specific tissues and organs.

In our previous study, we demonstrated efficient decellularization of the goat cornea by perfusing Triton X-100 through them *via* a perfusion bioreactor that not only aided in effective removal of the cells but also retained the overall ECM ultrastructure. The controlled flow of the detergent persuaded the cells to undergo apoptosis, instead of necrosis[17] (Fig. 2). This indeed was a unique finding since the elimination of cells by following the necrotic pathway can significantly elevate inflammation and the possibility of graft rejection, since, in necrosis leakage of intracellular constituents takes place due to the permeable cell membrane whereas, in apoptosis, cells undergo programmed cell death.[18] In contrast, decellularization owing to apoptosis is associated with the discharge of Prostaglandin E2 along with the activation of effector caspases, which has been related to tissue regeneration.[19] Furthermore, in a follow-up study, we elucidated that by adopting a slow perfusion based decellularization strategy, the ECM ultrastructure can be retained to some extent, but it was difficult to completely evade the denaturation of the collagen secondary conformation.[20] But, when we implanted these decellularized corneas into the rabbit stroma it triggered inflammation. Interestingly, after crosslinking the decellularized corneas with chondroitin sulfate seamless graft integration, cellular infiltration, and diminished inter-species reaction were observed. Therefore, compared to decellularized corneas the one crosslinked with chondroitin sulfate helped in resolving the inflammation and immune response. In spite of successfully decellularizing many tissues/organs, the *in vitro* manipulation of the dECM and *in vivo* responses raised uncertainties and encour-

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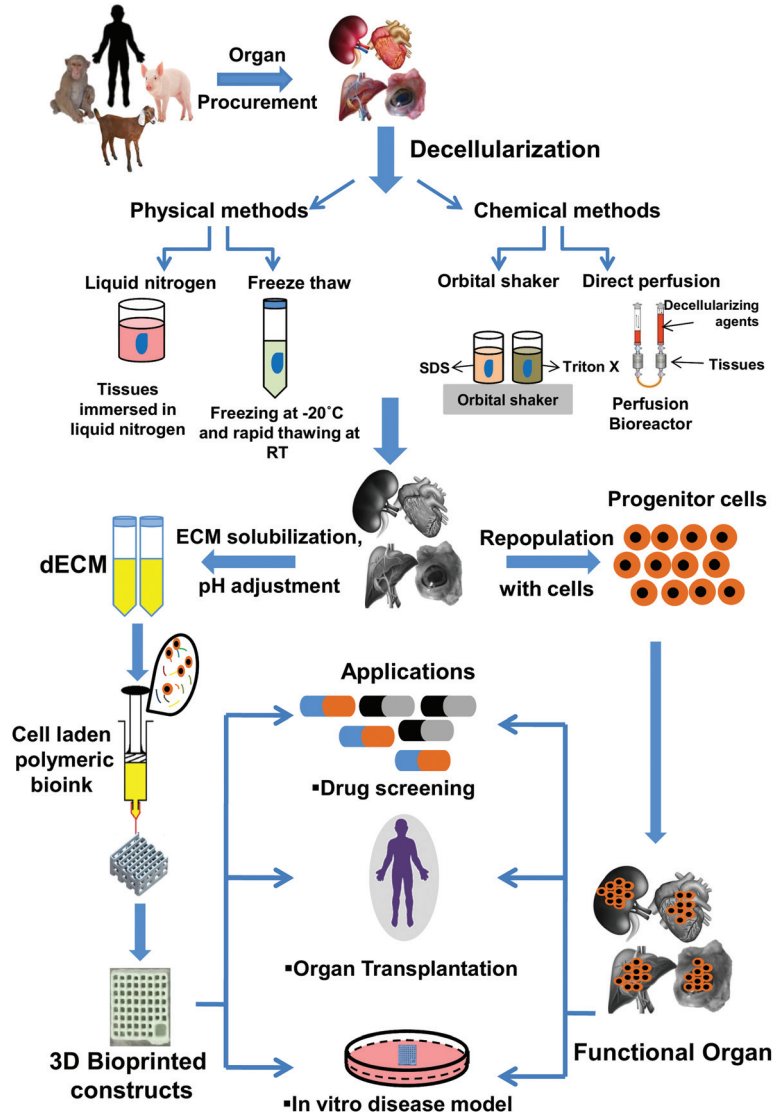

Fig. 1 Multifactorial approaches of decellularization and its potential applications in tissue engineering or as an *in vitro* diseased tissue model.

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aged us to take a re-look at the decellularization protocols. The application of immune-suppressor drugs to prevent host immune system-mediated rejection may not be always possible due to an already weakened immune system and the threat of unwanted secondary bacterial infections. To overcome this limitation, *in vivo* reprogramming through an immune informed dECM would be an essential prerequisite. This can only become possible when the dECM allows the proliferation of the desired cells over it for a prolonged period of time with stealth behavior to the resident and monocyte driven macrophages *in vivo* after implantation. In that way, it would result in a patient-specific bioengineered ECM with no immunogenicity.

These studies encouraged us to achieve deep mechanistic insight into the process of decellularization and the effect of immune response on the dECM. With these aims in this review, we have attempted to highlight some unaddressed

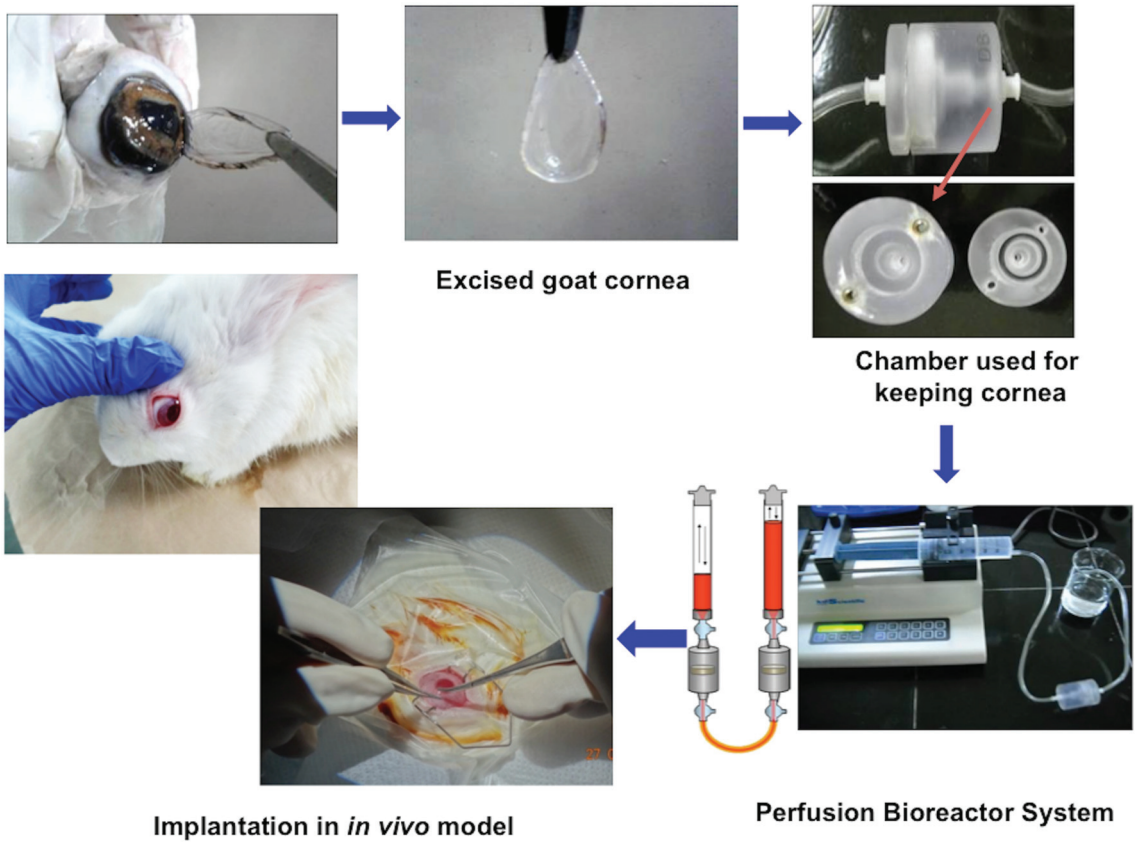

Fig. 2 Decellularization by perfusion using a syringe pump: TRITON X-100 at 0.1% was perfused at three different flow rates: 10 μl min^{-1}, 50 μl min^{-1}, and 100 μl min^{-1} continuously for 10 h using the syringe pump at room temperature.

areas of decellularization and its effect on the *in vivo* reprogramming mechanism. The foremost question is, can the decellularization process impart changes in the secondary conformation of the collagen fibrils even when the overall ECM ultrastructure seems to be preserved histologically? This can be a serious matter of concern, as modulation of the secondary conformation may expose the hidden antigenic motifs, which may thus elicit an immune response. Secondly, instead of using a single decellularization method, can strategically planned studies employing multiple agents enable us to achieve more efficient decellularization? Thirdly, we have tried to identify, "How good is good enough"? Do we really need to remove all cellular and DNA fragments from the decellularized matrix? What is the scientific basis of presently accepted criteria? Lastly, we have shifted our focus to the process of macrophage polarization and T-cell interaction on the dECM along with the effect of xenogeneic scaffolds on the ECM surface. The behavior of healthy monocytes on the dECM surface may help gain valuable insights into *in vitro* reprogramming and thus would help predict the *in vivo* reprogramming of the transplanted dECM. Importantly, these insights will lead to an improved tissue regeneration strategy, only when we comprehend exactly how these techniques can modulate the expression of various transcription factors and multiple signaling pathways.

2. Effective decellularization and its effect on the ECM ultrastructure

With the aim to remove all nuclear and cellular contents from a tissue or organ whilst retaining the ECM architecture, a number of decellularization protocols and agents were employed that vary according to the type of tissue and organ involved. Several factors such as the thickness, cell density, anatomical complexity, *etc*. can influence the process of cell removal. Therefore, the protocol undertaken for efficient decellularization should be tuned with respect to the tissue intended for treatment. Secondly, it is often overlooked that the decellularizing agents and methods may cause a subtle alteration of the native ECM ultrastructure. Therefore, care should be taken to identify and minimize the effects of these deleterious agents. In this context, researchers have utilized a variety of methods which include physical (high hydrostatic pressure, freeze–thaw, *etc*.), chemical (detergents, acids, bases, and alcohols) and biological (*e.g.* enzymatic) treatments (Table 1).

However, a single method may be insufficient to achieve adequate decellularization and, therefore, a combination of agents and techniques should be employed to obtain an efficient decellularized matrix. In order to gain profound mechanistic insight into the various processing methods

Table 1 Schematic representation of the agents and techniques used for decellularization

Category	Agent/technique		Mode of action	Advantages/disadvantages	Ref.
Chemical strategy	Surfactant-based	Sodium dodecyl sulfate (SDS)	-Solubilizes nuclear and cytoplasmic membranes -Disrupts the protein–protein interactions	-Aids in the complete removal of cellular contents -Disrupts the triple-helical collagen structure -Causes swelling of the elastin network	1, 12, 24 and 25
		Triton X	-Disrupts the DNA protein, lipid–lipid and lipid–protein interactions	-Eliminates cellular contents from thick tissues -Reduces collagen and GAG contents	25 and 37
		Sodium deoxycholate	-Denatures the protein interaction	-Used in combination with other detergents -Requires washing to reduce immune response after decellularization	21, 24 and 35
		CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate	-Acts as both an ionic and nonionic detergent	-Used for the decellularization of thinner tissues -Should be used at a balanced pH	48, 55 and 170
	Acid and bases	Peracetic acid (PAA)	-Commonly used as a disinfectant	-The least effect on the ECM ultrastructure -Disrupts collagen fibrillary arrangement	39
		Ethylenediaminetetraacetic acid (EDTA)	-Acts as a chelating agent sequester divalent metallic ions from the ECM	-Used in combination with other enzymes such as trypsin	44 and 45
Biological strategy	Enzyme-based	Trypsin	-Disrupts protein–protein interactions and hydrolyzes protein	-Cleaves proteins on the carboxyl terminus of arginine/lysine -Disrupts elastin and collagen	37, 43 and 52
		Nucleases	-Cleaves nucleic acid sequences	-Added when detergents fail in eliminating residual DNA	47–49
		Phospholipases A2	Causes hydrolytic cleavage of the phospholipids present in cells	-Hydrolyzes the ester bond in phospholipids -Depletes GAG content	56, 171 and 172
Mechanical strategy		High hydrostatic pressure (HHP)	Cause cell denaturation by an increase in pressure	-Uses greater than 600 MPa pressure -Maintains the GAG and collagen structure -Expensive technique	58 and 59
		Freeze–thaw	Freezing temperatures (−80 °C) and biological temperatures (~37 °C) are alternately used	-Maintains the proteins present in the ECM -Stabilizes mechanical properties	61, 173 and 174

undertaken we would address the effect of various decellularization agents and methods on the native ECM ultrastructure.

2.1. Ionic and non-ionic detergent mediated decellularization

Among the chemical agents employed, detergents such as sodium dodecyl sulfate (SDS), Triton X-100 and sodium deoxycholate are primarily used for decellularization. However, almost invariably these chemicals denature and/or modulate the triple helical structure of collagen fibrils of the tissues, which ultimately results in decreased mechanical strength of the dECM.[21–23]

SDS, an anionic detergent, is employed for the extraction of proteins and efficiently denatures proteins by disrupting the protein–protein interactions.[24] In comparison with other detergents, it aids in the complete removal of the cellular, antigenic contents and the removal of the cytoplasmic proteins such as vimentin.[25] SDS contains a hydrophilic head group and hydrophobic tails, so that at suitable temperature and concentration when dissolved in water, it self-assembles into spherical micellar structures. The resemblance in molecular structures causes the penetration of SDS into the phospholipid bilayer, resulting in the disruption of the cell membrane.[26] Therefore it functions by breaking the bonds between the cellular membrane and cytoplasmic proteins.[27] In addition, it reduces the soluble collagen content present in the cells or induces alteration in the molecular framework of the collagen to the point of insolubility.[28] Kim *et al.*[29] carried out decellularization of heart valve leaflets using three methods. In comparison with freeze–thaw and Triton X-100 based methods, a fruitful result was achieved with NaCl-SDS in terms of both decellularization and repopulation of the acellular leaflets with endothelial cells. Costa *et al.* demonstrated a reduction in the immune response in decellularized human homografts by employing 0.1% SDS, which was further accompanied by improved hemodynamic conduct until 18 months after clinical use.[30] In contrast to the above results, a matter of concern while using SDS is the triggering of the immune response that has been addressed in our previous study.[17] We observed that treating goat cornea with as low as 0.1% and 0.5% SDS could clear the cellular and nuclear remnants resulting in a residual DNA content that was less than 50 ng. However, the ATR-FTIR and Raman Spectroscopy studies elucidated the detrimental effect of SDS on the ECM ultrastructure, which may potentially evoke an immune response.[17] The triggering of the immune response may be attributed to the fact that the application of SDS exposes the C terminal, helical and N terminal telopeptides of the collagen layer which are the central determinants (hidden antigenic sites within the collagen). This exposure results in the production of IgG molecules from the host cells which makes the ECM marked for the initiation of phagocytosis and giant cell encapsulation. But augmentation of humoral and T lymphocyte-mediated reaction by distorted collagen layers may be avoided by complete residual telopeptide removal and appropriate glutaraldehyde crosslinking.[31] Furthermore, due diligence is required in the decellularization procedure,

washing and crosslinking to avoid collagen-mediated immunogenicity.

A reduction in the glycosaminoglycan (GAG) content along with the elimination of growth factors, for example, the vascular endothelial growth factor, was observed in tissues treated with SDS,[32] which can influence the biochemical signaling controlling the cellular function.[33] Xenogenic scaffolds were found to have cytotoxic effects when treated with SDS, which was due to the presence of residual SDS within the decellularized ECM and its perpetual outflow from the treated tissues.[34] Therefore, it is essential to wash the tissue rigorously in order to maintain the viability of the decellularized matrix.[24] Phosphate buffer saline (PBS) is used for the washing of cells treated with many of the surfactants. However, SDS provides additional drawbacks due to the difficulty faced in the requirement of extensive washing steps post-decellularization.

The detrimental effects of SDS on the structural and signaling proteins can be overcome using another ionic detergent sodium deoxycholate. Scaffolds procured by this detergent have been reported to be cytocompatible as the metabolic activity of the cells seeded on sodium deoxycholate decellularized matrices was higher in comparison with the one decellularized by SDS.[1] Moreover, it eliminates the cellular constituents by denaturing and disrupting the protein interaction. This was validated by Syed *et al.*[24] in a comparative study where the ECM biomaterials obtained from small intestinal submucosa (SIS) when decellularized with sodium deoxycholate were found to be more metabolically active than cells cultured on the SDS treated dECM which remained unchanged even when subjected to a prolonged washing period of 6 h. A study conducted by Zhou *et al.*[23] demonstrated that sodium deoxycholate enabled preservation of collagen and elastin structures present in valve leaflets and did not elicit an immune response in comparison with SDS and trypsin-EDTA. However, as for SDS a substantial extent of washing is required post-decellularization in order to remove the DNA which may be left behind due to agglutination on the tissue surface that may contribute to the immune response.[35]

In an attempt to avoid issues concerned with the removal of residual SDS and sodium deoxycholate from whole organs decellularized by the perfusion of detergents, Gilpin *et al.*[36] and Syed *et al.*[24] employed Triton X-100. In fact, Triton X-100 is not only easy to wash but is also an effective decellularizing agent and is comparatively less harsh than SDS due to its non-ionic nature. It eliminates the cellular remnants from thick tissues in cases where enzymes and osmotic approaches fail such as in valve conduits.[37] It results in the loss of the ECM protein by disrupting the DNA–protein, lipid–lipid and lipid–protein interactions while at the same time maintaining the native protein structure.[19] The loss of the ECM protein is followed by a reduction in an immune reaction *in vivo*; however, it leads to the elimination of certain GAG moieties.[37] A similar trend was observed in our previous study where perfusion of 0.1% Triton X-100 through the native goat cornea not only resulted in the efficient elimination of cellular and nuclear remnants but also had minimal detrimental effects on the collagen conformation. It is noteworthy that the exclusive finding of our study[17] was that the ultrastructure of Descemet's and Bowman's membrane was preserved which was apparent from H&E staining. Vavken *et al.* reported that on post-treatment with Triton X-100, porcine decellularized tissue exhibited successful repopulation with human ACL fibroblast cells which displayed not only an increased procollagen content but also a stable DNA content.[38] Although Triton X-100 resulted in efficient decellularization, it caused a decrease in the collagen and GAG contents[25] which ultimately affected the thickness and integrity of the dECM. Taken together, ionic and non-ionic detergents are widely used for achieving effective decellularization either alone or when combined together by denaturing proteins, disrupting DNA–protein interactions, lipids, and lipoproteins. Consequently, these chemicals invariably result in the impairment of the ECM architecture, resulting in damage to the collagens, growth factors. The focus should be on minimizing the debilitating effects of such chemicals as much as possible.

2.2. Multivariate roles of acids and bases in decellularization

Though most studies have reported the use of detergents as an efficient decellularizing agent, exploring other techniques could prove beneficial over the existing ones. In this regard, the effects of acids and bases have been investigated in the denaturation of nucleic acids and hydrolysis of the cytoplasmic constituents. They bring about the solubilization of cellular membranes by utilizing charged particles.[1] To name some, peracetic acid, EDTA, sodium hydroxide, and calcium hydroxide are widely used to achieve effective decellularization.

Peracetic acid (PAA) is commonly used as a disinfectant that aids in the expulsion of the cellular and nuclear remnants with the least effect on the ECM ultrastructure.[39,40] Treatment with peracetic acid has been reported to retain the collagenous structure in an intact form akin to the native tissue[41] and has no deleterious effect on sulfated GAGs (sGAG) compared to the previously described detergents[42] and has been used to successfully decellularize small intestinal submucosa (SIS)[24] and the urinary bladder.[39] Bases, such as sodium sulfide, calcium hydroxide, and sodium hydroxide, play a vital role during the initial stages of decellularization.[32,43] For instance, sodium hydroxide disrupts the cross-linked collagen and also cleaves the collagen fibrils which in turn causes a decrease in the mechanical properties of the ECM along with the complete eradication of growth factors such as the fibroblast growth factor.[32] Some studies have directly tried to sequester divalent metallic ions from the cell adhesion sites of the ECM[44] by the application of chelating agents such as ethylene diamine tetraacetic acid (EDTA) which ultimately results in cellular disintegration.[45] Although it is incapable of removing the cellular contents alone and even when agitated,[46] when combined with some enzymes such as trypsin[47] or other detergents[32,37,48] it works efficiently in eliminating the cellular and nuclear contents while maintaining the essential ECM constituents. Grauss *et al.*[49] carried out the decellularization of porcine pulmonary valves with 0.02% EDTA along with 0.05% trypsin for a

period of 24 h with continuous agitation that resulted in the effective elimination of the cellular contents; however, decellularization with 0.05% trypsin and other detergents led to incomplete removal of the cellular contents and produced non-viable cells. Hence, acids and bases function to solubilize cellular components and modify nucleic acids, therefore, disrupting cells. But, being non-selective in nature allows them to modify the ECM components, specifically GAGs and growth factors.

2.3. Enzymatic digestion of the ECM ultrastructure

Enzyme based techniques when supplemented with detergents can further improve the decellularization efficiency and therefore check the negative consequences offered by the earlier mentioned simpler techniques. Enzyme-based techniques are popular means and serve as a successful first step in tissue decellularization in situations where the ECM and cell walls come up with undesirable contaminants in a cell/tissue extract and thus, in turn, conserve the collagen ultrastructure, but cause disruption of the extracellular constituents of various tissues.[50] The serine protease trypsin cleaves the proteins adherent to the cells on the carboxyl terminus of arginine/lysine residues which results in the separation of cellular contents from the tissue surface.[43] Its enzymatic activity is found to be highest at pH 8 and at a temperature of 37 °C. It has been reported to be more destructive to elastin and collagen in comparison with other detergents such as SDS and eliminates the cellular contents very slowly; however, it is efficient enough to conserve the GAG contents present in the tissue.[47,49,51] Meyer *et al.* reported that 0.5% trypsin, when used for 48 h, resulted in the destruction of the collagen and elastin contents of the aortic valve ECM.[37] Similar findings have also been reported by Grauss *et al.*[22] However, a contrasting result was obtained upon decellularization of porcine adipose tissue with 0.02% trypsin causing a minimal change in the tissue ultrastructure.[52] One of the drawbacks of using trypsin as a decellularizing agent is that its efficacy gets reduced by 60% after 12 h; however, the rate of elimination of cellular contents increases remarkably following the addition of enzyme for the decellularization process. This can somewhat be overcome by the introduction of some protease inhibitors such as aprotinin, leupeptin or phenylmethylsulfonyl fluoride.[43] Interestingly in cases where detergents fail to effectively remove the residual DNA, nucleases (DNases and RNases) are added, which aid in cellular denaturation by cleaving the nucleic acid sequences followed by subsequent removal of the nucleotides.[47,48,53] For instance, the addition of DNase (20 mg mL^{-1}) and RNase (0.2 mg mL^{-1}) for an extra period of 1 h boosted the decellularization of aortic valves when a 24 h SDS treatment alone failed to produce acellular scaffolds.[49] Nucleases such as endonucleases work by cleaving the internal bonds present in ribonucleotide or deoxyribonucleotide chains; on the other hand, exonucleases hydrolyze the bonds present at the terminal of the chains thus resulting in the breakdown of RNA or DNA. It becomes cumbersome to eliminate the endonucleases from the tissues. As a result, it hampers the repopulation of cells and can elicit an immune response post-transplantation.[54] Application of serum abolishes the requirement of the elimination of nuclear remnants as low as 2.5% from the decellularized tissue.[55] Apart from the above-mentioned enzymes, some studies have utilized the phospholipase A2 that carries out the hydrolysis of the ester bond at the sn-2 location (that influences the physiological functioning of cells) of phospholipids which can produce free fatty acids such as arachidonic acid and lysophospholipids. Lynch *et al.*[56] successfully hydrolyzed the phospholipids present in the corneal tissue, thus preserving the proteoglycans and the collagen architecture; however it resulted in the depletion of the GAG content. Nevertheless, when combined with sodium deoxycholate for the limbal reconstruction of porcine corneas it resulted in the re-epithelialization of the transplanted grafts within 3 to 8 days in a rabbit partial limbal defect model.[57] Conclusively, enzymes can persist in the tissues in remarkable quantities that may evoke a good amount of immune response. They can even result in the elimination of laminin, collagen, elastin, fibronectin, and GAGs as well as cause disruption of the ECM ultrastructure. However, they play a key role in targeting the peptide bonds which helps in adhering of the cells to the ECM.

2.4. Effect of physical and miscellaneous agents

The drawback associated with the chemical and enzymatic methods is that they are less predictable[19] and hence result in poor reproducibility, followed by an alteration in the ECM ultrastructure.[21] These shortcomings can be overcome by introducing physical or mechanical decellularizing agents such as freeze–thaw and high hydrostatic pressure, which would not only ease the prediction of the decellularization process but would also aid in efficient denaturation of the cellular constituents.[19]

Hashimoto *et al.* used a high pressure of 980 MPa for a period of 10 minutes at 10–30 °C for the decellularization of the porcine cornea.[58] A similar strategy was applied to the decellularization of porcine blood vessels.[59] The high hydrostatic pressure (HHP) method has been used as an alternative in order to get rid of or minimize the time of exposure of detergents which are harsh to the tissues during the decellularization process[59] and a pressure of more than 600 MPa is applied for the disruption of the cell membrane. In the study conducted by Hashimoto *et al.*[58] and Funamoto *et al.*,[59] although HHP leads to the disruption of the cells, it failed to remove the DNA contents. As a result, DNase I was added to the wash the solution during decellularization to aid in the disintegration of the DNA fragments to avert any immune response. In order to preserve the native ECM ultrastructure, the freezing phase during the HHP process should be circumvented. Moreover, the pressurization effect has been reported to be detrimental to collagen and elastin fibers that results in the modification of the mechanical properties.[59] However, although the HHP method aids in efficient disruption of the bacterial and viral membranes through its short exposure time, it is obligatory to adopt a substantial washing protocol following this treatment.

Another widely used physical technique involves the freeze–thaw method which although efficiently aids in the disruption of cells present in the tissues and organs but requires a successive number of steps to completely evade the intracellular and membranous remnants from the tissues.[60] In our previous study, we reported that although freeze–thaw resulted in the complete elimination of the residual cellular remnants of the native goat cornea, it failed to preserve the ECM ultrastructure.[17] A freeze–thaw cycle induces denaturation of the tissue ultrastructure but to a small extent, and as a result, the ECM mechanical properties are preserved. 5% trehalose, which is a widely used cryoprotectant, can be used to lessen the detrimental effect of freeze–thaw in the ultrastructure of the tissues.[61] Immune reactions such as leukocyte invasion can be minimized in ECM scaffolds by employing one freeze–thaw cycle.[62] However, decellularization processes might involve more than one freeze–thaw cycle[63,64] which aids in preserving the residual ECM proteins from tissue.[65] Therefore this process should be applied in cases where negligible denaturation of the ECM ultrastructure is acceptable in the final product.[43,46] Cornelison *et al.* treated *ex vivo* nerve tissue with camptothecin, a chemical which selectively inhibits the nuclear enzyme DNA topoisomerase type I. As a result, it induces active caspase-3 expression (an early-stage marker of apoptosis) and DNA fragmentation (a stage marker of apoptosis). Camptothecin-treated nerves could preserve the anatomical architecture and retain collagen, GAG. *In vitro* cytocompatibility was assessed by culturing Schwann cells on decellularized nerve constructs. Upon subcutaneous implantation in rats, these constructs were immunologically tolerated.[66]

2.5. Effect of supercritical carbon dioxide ($scCO_2$)

The use of supercritical carbon dioxide is a relatively unexplored technique for decellularization. It has the unique properties of being non-toxic, non-flammable, and relatively inert and having mild critical temperature along with desirable solvent properties that increase its viability at physiological temperatures.[67] A study carried out by Sawada *et al.*[68] exhibited efficient elimination of DNA and cellular contents but also resulted in substantial removal of volatile substances during treatment, specifically the elimination of water. Scaffold embrittlement and dehydration causing hardening of the tissue affected the scaffold viability, thus preventing further progress. This was overcome by pre-saturating $scCO_2$ with water which reduced tissue dehydration to a large extent.[67] In another study, Zambon *et al.*[69] carried out the decellularization of pig esophagus by combining ethanol with $scCO_2$ treatment. This novel method resulted in the achievement of a dried acellular matrix suitable for the regeneration of the esophagus and was successful in maintaining the ECM ultrastructure of the native tissue.

Thus, conclusively, each of the above-mentioned techniques and agents has its own pros and cons. For example, although the physical methods are less deleterious to the tissue ultrastructure, they cannot fulfill the demands and requirements in relation to immunogenicity. At the same time, one should note that the particular concentration of the same detergent might have variable effects on different tissues. The majority of the studies have so far used these techniques singly. We believe that instead of using a single decellularization method, strategically planned studies employing a combination of different agents and techniques[63,70] can enable us to achieve more efficient decellularization. It is important to note that although this combinative approach requires added chemicals along with an elongated processing time in comparison with the single treatment methods, optimization of the individual parameter is essential to be suitable for a specific tissue type. Brown *et al.*[52] carried out multistep decellularization on porcine adipose tissue. The tissue was treated with trypsin/EDTA along with the detergents Triton X-100 and sodium deoxycholate. This was followed by sterilization with ethanol and peracetic acid and the residual polar contents were solubilized with *n*-propanol. The entire multistep treatment comprised a total of 16 steps, following which complete cell, lipid, and DNA contents were reported. Although this process resulted in the preservation of collagen type IV and laminin fibers, collagen I fibers got distorted along with a decrease in the levels of the vascular endothelial growth factor and transforming growth factor and contrasting results were obtained upon employing two-variable buffers, the first one supplemented with 100 mM KCl and 5 mM $MgCl_2$ along with 100 mM dithiothreitol and the second one with 0.5% SDS with the aim to solubilize the cell membranes. Furthermore, the tissue was subjected to hyaluronidase treatment followed by alternating freeze–thaw cycles and nuclease digestion. Although the method was successful in eliminating a major part of the cellular and nuclear contents, an increase in porosity resulted in lowering of much essential mechanical properties.[71] Taken together we speculate that effort should be made to minimize the variability in the decellularization procedure due to its multiple applications and rational approach with respect to specific tissue anatomy or vasculatures. Changes in the concentration and incubation time of decellularizing agents are the main variable factor that needs more attention from the research community to establish a standard combinative approach that has a strategically beneficial effect on the decellularization process.

3. How good is good enough?

Several decellularized matrices are commercially available and approved by the FDA (Table 2). But limitations of the process of decellularization and the cellular remnant mediated immune response and rejection are still a long-debated topic. Though a vast amount of literature claims effective decellularization along with complete removal of dsDNA and nuclear contents, it is almost inevitable that all the decellularization strategies may inflict a certain extent of ECM damage and disruption of the collagen triple helical structure along with the elimination of matrix-embedded growth factors. Effective decellularization could be achieved by optimizing the balance

Table 2 An overview of the commercially available decellularized tissue

Commercial name	Manufacturer	Tissue source	Application	Ref.
AlloDerm	Life Cell Corp., Texas, USA	Human dermis	Palatal connective tissue in root coverage grafting and soft tissue ridge augmentation, breast reconstruction	175 and 176
Allomax	Davo Inc., Rhode Island, USA	Human dermis	Breast reconstruction	177
Allopatch HD	ConMed, Utica, New York	Human dermis	Treatment of chronic non-healing wounds such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers	178
Axoguard	Axogen, Florida, USA	Porcine submucosa	Nerve connector and nerve protector	179
Collagen repair patch	Zimmer Inc., Indiana, USA	Porcine dermis	Soft tissue repair	180
Dermagraft	Organogenesis Inc., Canton, USA	Human fibroblasts	Neuropathic and diabetic foot ulcer	181
DermACELL	LifeNet Health, Virginia, USA	Human dermis	Chronic non-healing wounds such as diabetic foot ulcers and skin burn injuries	182
FlexHD	Ethicon, USA	Human dermis	Breast reconstruction	183
Glyaderm	EuroSkin Bank, Beverwick Netherlands	Human dermis	Dermal substitute for a full-thickness wound	184
PerioDerm	Musculoskeletal Transplantation Foundation, Edison, New Jersey	Human dermis	Augmentation of soft tissue for soft tissue defects	185
SureDerm	Biowel Sciences, Seoul, Korea	Human dermis	Soft tissue	186

between the elimination of the residue of cellular constituents, dsDNA and nuclear content and the damage that is caused to the native ECM. On the other hand, mild and inadequate decellularization may also cause problems related to cytocompatibility and thus can elicit an immune response in the host *in vivo*[72] and also upon repopulation with cells.[73,74] In general, the research community has accepted the following norms that tissue must fulfill the following minimum eligibility criteria for it to satisfy the purpose of decellularization:[19]

(1) The content of dsDNA should be <50 ng mg^{-1} of the dry weight of the ECM. But there are reports where implantation and successful integration of commercially available ECM scaffolds have been corroborated even with a higher concentration of DNA (≥60 ng mg^{-1}).[75]

(2) The length of the DNA fragment should be <200 bp.[73,76]

(3) Staining with DAPI (4′,6-diamidino-2-phenylindole) or H&E should reveal the complete absence of the nuclear material in the dECM.

These general criteria were proposed by Prof. Badylak and others, and eventually validated by various researchers. However, these guidelines are not sacrosanct but can differ based on tissue types. Although, almost every decellularization based study assessment of the treated matrix is carried out by following the above-mentioned general criteria, to the best of our knowledge a clear scientific basis for the selection of the optimized criteria is still lacking. Some previous studies have mentioned that the selection of dsDNA fragments is related to the programmed cell death process, *i.e.* apoptosis.[77] This process involves the fragmentation of cellular and nuclear components upon condensation followed by engulfment by the resident macrophages and monocyte-derived macrophages through phagocytic cells.[78] CD14+ monocyte-derived macrophages represent the basic immunological clues that can be utilized to derive tissue macrophages and polarized toward different phenotypic conversions. This results in the formation of 180 bp units of the nucleosome by the fragmentation of the chromosomal DNA[79] which is brought about by caspase-activated DNase (CAD). Apart from CAD, degradation of DNA into 50–200 kb fragments is also brought about by endonuclease.[80] However, no degradation occurs in the absence of CAD, suggesting that other nucleases do not participate in the process or else have a limited role to play.[81] Phagocytosis plays a key role by recognizing the surface residual content and thus engulfs them to avert the liberation of their intracellular contents which could thus elicit an immune response. Interestingly another valid reason for the fragmentation into a length of about 300 bp is the binding of toll-like receptors to the soluble DNA which aids in breaking down the nucleotides that can further be used by the cells.[82,83] Gilbert *et al.* conducted a comparative study to investigate the DNA content and the length of its fragment produced *in vitro* and in the commercially obtainable ECM scaffold material.[84] A DNA fragment of <300 bp in length was found to be present in the biological scaffold material that was investigated, except GraftJacket, which is a collagen matrix derived from human dermal tissue and is directly integrated into the body. Any remnant DNA content is subjected to enzymatic breakdown similar to the other ECM constituents.[84] Another important factor that must be taken into consideration is that the segment of DNA which is left behind in the biological scaffold material might be a constituent such as the detrimental nucleic acid derived from viruses and prions. However, such DNA contents would be nominal. In this context, peracetic acid, one of the widely used decellularizing agents, has the ability to get rid of such viral contents.[85]

Therefore, it is strongly suggested that the residual DNA content should be carefully investigated as an important marker of decellularization, for the determination of the presence of any unwanted leftover cellular remnants which may be harmful upon implantation.[84] Contrastingly, the presence of residual RNA is often not considered due to its rapid denaturation tendency; as a result, the predominance of RNA in the

process of decellularization is very less in comparison with that of DNA. Hence, the DNA content can be determined by staining the tissue specimen with DAPI or Hoechst stain which has the unique property of binding with the AT residues of the minor groove of DNA.[84,86,87] Propidium iodide and Pico green assays are also used for the determination of the DNA content.[88] Besides verifying the components that have been eliminated it becomes a prerequisite to also determine the desirable ECM constituents that have been preserved after decellularization which includes growth factors, collagen structure, fibronectin, and elastin. There is a surprising paucity of studies on residual DNA and nucleotide content that interacts with resident macrophages. Although the above-mentioned guidelines are generally followed by the decellularized community they are not sacrosanct, and may vary from species to species and organ to organ. An enhanced understanding of the macrophage directed phagocytic uptake of remnant DNA of various lengths *in vitro* would help in gaining deep mechanistic insights into the biocompatibility of the decellularized tissue.

4. Characterization and identification of the decellularized ECM and exposed antigenic motif

Validation and characterization of the tissues or organs post-decellularization still hold a great challenge. A delicate balance should be maintained between the preservation of the essential constituents and removal of the unwanted components which have the potential to evoke an immune response when implanted *in vivo*. In this context, a number of characterization techniques have been explored for the evaluation and characterization of various decellularized scaffolds.

One of the extensively used methods for the analysis of the ECM structure of the decellularized tissue or organ is mass spectroscopy[89,90] due to its ability to detect an enormous amount of protein in a single run.[91] A variety of mass spectroscopic techniques are employed for the characterization of decellularized tissues such as Matrix-Assisted Laser Desorption Ionization/Time of Flight (MALDI/TOF),[92] Liquid Chromatography-Mass Spectrometry (LC-MS),[90] and Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS).[89] Among these, the most commonly used method known so far for peptide recognition is the 'shotgun' method employing the LC/MS/MS technique. In tandem mass spectroscopy (LC-MS/MS) a PAGE gel, often a 2D-gel, is used where the protein samples are loaded and allowed to run on the gel after which the resultant gel is extracted followed by purification using liquid chromatography.[93] The proteome of decellularized VF mucosa was characterized by Welham *et al.* using LC-MS/MS where the 66 spots of protein species were resolved using 2D-SDS-PAGE and 73 distinct proteins were assigned from the spots present in the gel employing LC-MS-MS runs.[94] One of the unique findings of this method is the localization of proteins in the histological sections of tissues contributing to the impartial visualization of the spatial organization of the biomolecules that can be determined by matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS).[95] In a study carried out by Groseclose *et al.*[96] and Casadonte *et al.*[97] that employed the MALDI IMS strategy whose workflow was similar to LC-MS/MS, tryptic peptides, which are disclosed upon selective split opening of ECM proteins and are confined within the tissue, were released by spraying trypsin on tissues to aid in peptide ionization which localizes at various positions on the tissue that are further recorded using a mass spectrometer.[96,97] However, this process fails to eliminate some collagen and elastin sequences.[98] The tedious steps required before processing and solubilization in LC/MS/MS and MALDI/ToF could be overcome by Time of Flight Secondary Ion Mass Spectroscopy (ToF-SIMS). Currently, ToF-SIMS is being used extensively for the characterization and analysis of the decellularized matrices/materials to be implanted and it helps overcome the tedious steps involved in LC/MS/MS and MALDI/ToF.[89,99,100] White *et al.* employed this technique to identify cellular and nuclear fragments present in the biological scaffolds as well as choline, phosphocholine, and glycerophosphocholine ions which play an essential role in maintaining the structural ultrastructure of native and various detergent treated porcine urinary bladder matrices.[101] Nonetheless, the failure of this process lies in the overlay of multiple proteins in the mass spectra obtained from decellularized matrices which consist of a large number of proteins.[89,102] The main limitation lies in the fact that the presence of proteins and their identification depend on the analysis of the fragment containing amino acids which does not provide information about the actual amount of proteins present.[91] Moreover, it is not capable of elucidating the threshold quantity of the residual cellular contents present in the decellularized matrix which is capable of eliciting an immune response post-implantation.[84,103] In addition, it fails to provide information on the secondary conformations of the proteins whose determination plays a key role in any decellularization protocol.[104]

This problem was overcome by the use of Circular Dichroism (CD), an extensively used spectroscopic approach that monitors the alterations in the conformations of the proteins, to determine the random coil (unordered) and alpha-helix/beta-sheet (ordered) states of the protein structure.[104,105] Hashimoto *et al.*[106] employed CD spectroscopy to determine the effect of the HHP method on the triple helical structure of collagen present in porcine cornea post-decellularization and showed preservation of the triple helical structure of collagen. In a similar study, Park *et al.* studied the effect on the thermal denaturation of collagen using CD spectroscopy for the determination of the denaturation temperature of a collagen triple helix. The obtained results provided an important perspective on the breakdown and restoration of the structure of the ECM.[107] However, in spite of its wide applications, its use is limited by some factors such as the poor quality of reference structures used[108] and the inconsistency of the reference CD

database. Moreover, different databases show a variable CD spectrum and the proteins that are commercially available for reference might not be of high purity.[109,110] Instability in the accuracy of the instrument used for determining the reference and the CD spectra further limits its use.[111]

To overcome such constraints several studies have used FTIR (Fourier transform infrared spectroscopy) for the evaluation of the chemical constitution of the extracellular matrices of various decellularized scaffolds.[112–114] This process can thus overcome the limitations associated with histology, such as the discrepancy associated with the staining solution and qualitative elucidation of stains. In fact, we employed ATR-FTIR to determine the alterations that occurred in the collagen secondary structure post-decellularization[17] and it was further used for the confirmation of the crosslinking of the decellularized collagen I with chondroitin sulfate.[8] Although there are several techniques such as scanning electron microscopy (SEM) and 3D X-ray microscopy (X-ray μCT) for the architectural mapping of the bio-scaffolds, they are unable to detect the subtle modifications that take place in the ultrastructure of the matrix which restricts their further utilization.[115] In this regard, the traditional FTIR technique has been utilized to evaluate the constitution of the extracellular matrix of the scaffold–cell interface and it has successfully mapped the distribution of the protein or minerals deposited throughout the multi-tissue. An advanced form of this FTIR, popularly known as synchrotron radiation-based FTIR (SR-FTIR), has the advantage that it can be utilized for the quantitative mapping of the decellularized scaffold. It enables detection of the changes in the dECM microstructure at a high resolution in comparison with the traditional methods such as SEM, X-ray μCT and conventional FTIR. Zhou *et al.*[115] used modified synchrotron radiation-based Fourier transform infrared microspectroscopy (SR-FTIR) for the quantitative mapping of fibrocartilage bio-scaffolds which were decellularized and the results so obtained were compared with the histology staining results and the biochemical assay. The beauty of this method is that it allows the detection of the composition of the sample tissue and evaluation of the presence of various biological constituents such as collagen and proteoglycan present in the dECM, thus providing significant knowledge about the architecture of the decellularized scaffolds.[115]

5. Innate and adaptive immune response to a decellularized matrix

5.1. Effect of decellularization on the ECM architecture and α-gal epitope

The ECM is composed of a constellation of highly insoluble proteins which confers an appropriate mechanical architecture that is necessary to grow the desired cell population on its surface for tissue remodeling. Each of the individual components and molecular structures of the proteins in the dECM elicits a specific cytokine response by host cells after implantation. It modulates their functions which are essential for various physiological and pathological steps, such as initiation of inflammation, or cell migration into inflamed tissues and differentiation of progenitor cells in the dECM towards tissue regeneration.[12] Increasing pieces of evidence suggest that an alteration in the architecture of functional protein, glycoprotein, and glycosaminoglycan in the native ECM is inevitable after all the decellularization or proteolytic digestion processes. The hidden antigenic motifs of the ECM protein (α1β1 integrin, laminin, aggrecan, versican, collagen types I and IV, and hyaluronan) always remains integrated with the ECM.[116,117] It helps maintain the secondary immunity, B cell differentiation, antibody production, and chemokine receptor (CXCR)-1 and 2-mediated neutrophil attraction, and facilitate antigen presentation under normal physiological conditions with the help of elastin binding protein.[13,118] Decellularization mediated changes in the ECM ultrastructure activate a group of potential matrikines such as collagen IV (7S domain/α3 chain), integrin (entactin/nidogen) and the C terminal end of globular laminin (α1 & α5 chains); so the xenoantigenicity of the dECM needs to be checked properly to avoid jeopardizing the graft specific host immune response (Fig. 3). Well-directed research to find a glutaraldehyde like chemically crosslinking substance which can crosslink to the xenogeneic decellularized tissue to avoid the hyperacute and acute immune response after implantation is a future need. It has already been reported that all these matrikines play diverse roles in stimulating cell migration, neutrophil chemotaxis and initiation of different inflammatory crosstalk through elastin binding protein, CXCR1, and CXCR2.[119] The kappa-elastin and VGVAPG peptides represent the receptor-binding sites in the elastin moiety after decellularization and are involved in monocyte/polymorphonuclear leukocyte activation, T-lymphocyte survival and MHC-1/MHC-2 complex formation. The 25/45 kDa N terminal domain, 15 amino acid peptides, thrombospondin-1, anastelin, and osteonectin/BM-40 are a group of matrikines that are generated from a glycoprotein and have specific immunomodulator, anti-tumorigenic, anti-angiogenic, and heparin binding properties. All these ECM proteins play a well-defined role in the production of immune informed decellularized tissue, to support *in vivo* reprogramming. Therefore, a well-directed study with a specific matrikine checkpoint will help address this issue and some new criteria could be helpful in the identification of an ideal non-immunogenic decellularized tissue that would be functional after the implantation. Appropriate selection criteria are a prerequisite to identify the antigenic motif or matrikine like peptides on xenogeneic decellularized tissue to avoid the chances of interspecies reaction upon clinical transplantation.[120,121]

The components of the dECM can change the plasticity of adherent macrophages, for example, versican can recruit and activate pro-tumorigenic immune cells such as M2 macrophages in the implanted site, thereby promoting inflammation and immunomodulation for cancer progression. These changes in macrophage plasticity ultimately lead to an increased and consistent release of IL-10 rather than IL-12 and initiate the changes in the plasticity of T helper cells.

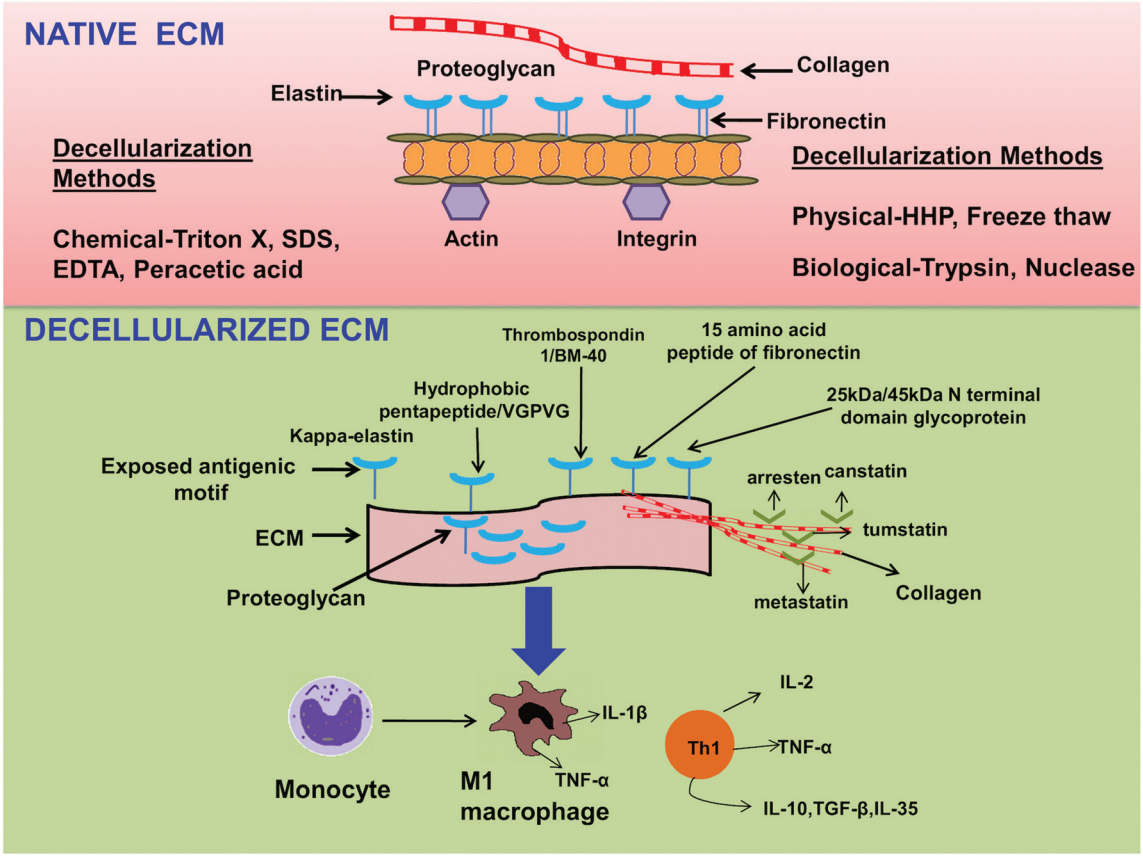

Fig. 3 Different processes of decellularization and their effects on various ECM protein (collagen, proteoglycan, fibronectin, elastin, actin, and integrin) ultrastructures. A pictorial representation of various antigenic motifs and fragments (kappa-elastin, thrombospondin, BM-40, arresten, canstatin, tumstatin, and metastatin) on the decellularized ECM which may act as matrikines with the host cell, to cause changes in the plasticity of healthy monocytes and generate a distinct immunological response. Arrestens can regulate signal transduction at G protein-coupled receptors. Canstatin inhibits angiogenesis. Tumstatin acts as both an antiangiogenic and proapoptotic agent.

Complete removal of MMPs through decellularization may inhibit the crucial link between the ECM and inflammation. Hence, optimized decellularization strategies can lead to specific ECM peptide fragments in the ECM, which can regulate cell migration, leading to integration with host tissues, and can be exploited therapeutically to modulate the pro-inflammatory signal mediated immune response.

Transplantation of organs from one species to another, such as from pigs to humans, is accompanied by the occurrence of hyper-acute rejection due to the presence of the alpha (1,3) epitope which is a terminal carbohydrate formed by the enzyme alpha(α) 1,3-galactosyltransferase.[122] The potential of the α-gal epitope to trigger an immune response is a matter of concern especially upon transplantation of decellularized xenograft tissue because it creates basically hyperacute and acute immune rejection of xenogeneic decellularized tissue.[123,124] The α-gal epitope is present abundantly on the cell surface of nearly all species except humans and Cercopithecoidea (old world monkeys); however, the anti-gal antibody is the major natural antibody in humans.[125] Interestingly, the reason for the non-expression of the α-gal epitope in humans and old-world monkeys is basically two-fold. The first reason is the occurrence of two frameshift mutations at the gene coding for 1,3-galactosyltransferase. The second reason is the abundant production of anti-gal antibodies (Ab) which comprise 1–3% of the total circulating immunoglobulins[126,127] along with the release of IgG, IgM, and IgA. These circulating antibodies and the precipitated IgG on the decellularized surface cause the rejection of the xenograft by activating the classical complement and humoral immune system by activating the monocyte, granulocyte, lymphocyte, polymorphonuclear leucocyte, and T-cell response.[125,128]

The process of decellularization is supposed to eliminate the α-gal epitope from the cell surface. However, its complete eradication from the ECM derived scaffolds needs to be ensured.[124] A number of studies have investigated the presence of α-gal epitope in xenogeneic biological scaffolds, for example in the porcine anterior cruciate ligament (ACL), cartilage[129] and porcine SIS-ECM.[130] Each of them has been reported to be positive for the gal epitope. Even after fixing bioprosthetic substitutes in glutaraldehyde, the α-gal evokes an immune response upon implantation of the prosthesis *in vivo*.[131,132] An elevation in the levels of anti-gal and IgM was observed in

patients accepting porcine bioprosthetic valves ten days post-surgery, which might lead to degradation and calcification, thus eventually resulting in graft rejection mainly in younger recipients.[131]

Numerous studies have established methods to diminish the effect of the α-gal antigen. For instance, knocking out the gene for α 1,3-galactosyltransferase from pig and mice could eliminate the effect of α gal.[133] To validate the point of whether deleting the gene for α-gal (1,3) eliminates all the gal carbohydrate, Milland *et al.*[133] designed monoclonal antibodies specific to the α 1,3-gal epitope. Upon the characterization of the cell lines, those transfected with α-gal (1,3) were stained and those without α 1,3GT remain unchanged. Interestingly, α 1,3GT knockout mice showed positive staining with similar monoclonal antibodies.[133] Furthermore, xenografts such as the heart[134] and kidney[135] that are produced by the same strategy evoked an immune response followed by rejection within a period of 6 months and 1 month, respectively. This study concluded the fact that even after deleting the gene for α-gal (1,3) a small amount (less than 2%) of the antigen is expressed by α 1,3GT knock out pigs. Apart from this, other strategies involve the application of α-galactosidase to the xenogeneic tissue[136,137] and the administration of the decellularization protocol. Gonçalves *et al.*[136] showed that among various detergents only SDS could eliminate the xenoantigens from the tissues. This was further confirmed by Wu *et al.*[138] and they used a comparative method for the decellularization of the porcine annulus fibrosus. Among all the methods employed SDS was able to eliminate the α-gal antigen as evidenced by quantification before and after treatment. Solubilization-based specific antigen removal may be the future strategy to remove the specific antigenic motif by exploiting its physicochemical properties to produce immunologically accepted decellularized tissue. Different types of reducing agents (urea, ethanol and dioxane aqueous solution) and salts have already been used to get rid of antigen from decellularized tissue. A list of amino acids and peptides are successfully dissolved by substitution in an amino acid chain and decreasing hydrophobic interactions in the non-polar group.[139] So an appropriate combination of decellularization approaches that are specific for identified antigen removal will be the future direction of reduced immunogenicity in a decellularized scaffold.

A number of recent studies have shed light into the fact that circulating macrophages may elicit a requisite M2 phenotypic response upon activation by the xenogenic ECM, thus resulting in an anti-inflammatory or constructive remodeling reaction to the scaffold or the xenograft.[139] Therefore, the absence of α-gal epitopes in the dECM tissue would safeguard the implantation of the xenograft in humans, thus lowering the probability of immune rejection.

5.2. M1 *vs.* M2 macrophage phenotyping crosstalk

Macrophages are the major checkpoint of a healthy immune system which has a strong and long-lasting impact on graft integration and rejection. Bone marrow-derived precursor cells give birth to nascent monocytes in the fresh bloodstream that circulate for a couple of days before they finally migrate into the specific tissue and mature.[140,141] Several cell types, plasma proteins, platelets, neutrophils, mononuclear phagocytes, parenchymal cells, and extracellular fluid constituents play a key role in the tissue response of early interactive events. Previously, it was hypothesized that macrophages were entirely produced from circulating monocytes. But emerging evidence showed that the tissue-resident macrophages sustain their local populations by rapid proliferation during injury depending upon the nature of the ECM because during morphogenesis the native ECM supports cell motility, proliferation, and differentiation.[142] The underlying mechanism by which a decellularized ECM maintains the balance between dynamically different pro-inflammatory and anti-inflammatory macrophages is still not clear.[143,144]

We identified the role of monocytes and their subsequent differentiation into macrophages post-implantation with decellularization of goat cornea in rabbits.[8] The rationale behind this attempt targeted three objectives, firstly to retain the native ECM architecture with the collagen conformation, peptidoglycans, and GAGs intact using controlled fluid flow using a perfusion bioreactor.[145] Secondly, several studies demonstrated that the fate of the decellularized scaffold depends on the nature of the monocyte driven macrophage (MDM) present on the surface of the dECM. These polarized macrophages are often referred to as M1 or M2 cells by studying the expression of only limited CD markers. MDM mediated secretion of cytokines and chemical mediators are regulated by two phenotypic and functional polarization stages, M1 and M2, which direct towards two diverse mechanisms, inflammatory and wound healing responses.[146] A broad range of CD markers or molecular markers needs to be studied to understand how the decellularized ECM can modulate monocyte differentiation. Thirdly, it would help create crosstalk between host cellular enzymes and the ECM to augment the regeneration capacity.[147] Our major focus was to correlate *in vitro* graft-specific assessment of MDM development with actual *in vivo* response in another species.

Through a series of studies, we have tried to maintain a balance between the remodeling rate of the dECM and the regeneration rate of the host tissue matrix. Although we observed monocyte differentiation onto the dECM surface, it did not appear to penetrate the ECM even after 1–14 days of culture and the surface was not degraded, as was evident through scanning electron microscopy at different time points[148] (Fig. 4a). We observed a specific elongated morphology of M2 macrophages with the identification of large and flattened multinucleated giant cells. We have used fresh monocytes, macrophages, and a PMA (phorbol 12-myristate-13-acetate) induced group to get a clear idea about the ability of the decellularized matrix to evoke any changes at the cellular level.[8] Healthy monocytes with a cellular diameter of <10 μm and a round and spherical morphology (no cytoplasmic projection) were identified on the normal corneal surface after 7 days of culture (Fig. 4b, A). We identified the

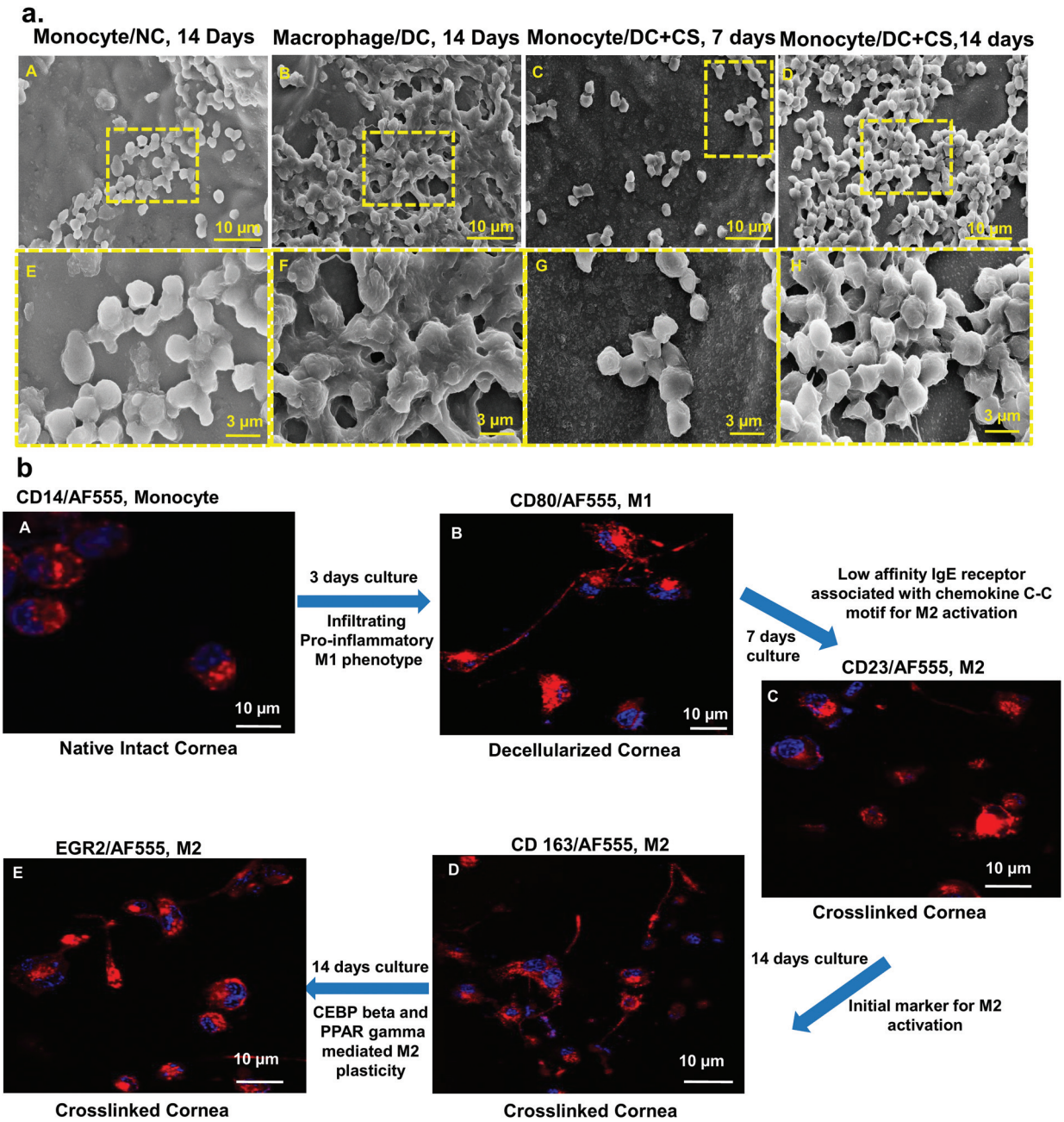

Fig. 4 Scanning electron microscopy of cultured monocytes on the surface of native goat cornea (A – 14 days), decellularized (B – 14 days) and crosslinked with chondroitin sulfate (CS) (C – 7 days and D – 14 days), E, F, G and H are enlarged view of A, B, C and D respectively. (b) Macrophage polarization on the surface of native goat cornea (A – CD14/monocytes), decellularized (B – CD80/M1pro-inflammatory) and crosslinked with CS (C – CD23/M2-chemokine activation, D – CD163/M2-initial marker, and E – EGR2/M2-CEBP/PPAR).

M1 population by CD80 staining on the decellularized surface after 7 days of culturing with a specific amoeboid morphology (Fig. 4b, B). It showed that dECM mediated polarization of the MDM into a specific M1 subtype elicited an inflammatory response. In contrast, the native corneal tissue did not evoke any changes in monocytes at the cellular level even after 14 days incubation and the differentiation would only be possible after treatment with IL-4, IL-10, GM-CSF or PMA.[149] The decellularized and crosslinked ECM exhibited generation of the M2 subtype from the MDM and these changes in plasticity were evident with different cell surface markers (CD23, CD163, and EGR2) upon immunostaining with a large bipolar spindeloid morphology.[150] After the implantation of decellularized tissue, the initial presence of the M1 macrophage is essential for the primary inflammatory response. But we observed that the dECM for 14 days culture with human monocytes revealed alteration in the plasticity and genetic phenotype. The M1 macrophage population is replaced by the M2 macrophage

after 7 days of culture, which plays an important role in tissue remodeling. This M2 subtype has four different subclasses (M2a, M2b, M2c, and M2d), which are often ignored and considered as one single group. These specific groups have immense importance with respect to wound healing, immune regulation, and tissue remodeling along with their involvement in the polarization of Th2. The M2a subtype classically represents the wound healing one and it can be polarized with the help of IL-4 and IL-13. The M2b subtype basically draws attention in this group due to its dual role in protection and pathogenesis, and as a result, its identification and pathophysiology on the decellularized tissue surface are still unclear. The M2c subtype shows its potential in the healing procedure by the secretion of large amounts of IL-10 and TGF. Hence we believe that it is inappropriate to generalize the macrophage phenotype into major subclasses (M1 and M2). A specific well-directed study to distinguish phenotypic differences of macrophages in the dECM surface will help predict the behavior of the dECM. M1 macrophages are known to be involved in rheumatoid arthritis, macular degeneration, intervertebral disc degeneration, and fibrous encapsulation through the release of different pro-angiogenic factors and with the help of some multinucleated fused cells to perform "frustrated phagocytosis". A series of studies suggests that the M1 macrophage initiates the vascularization through a pro-inflammatory signal in the decellularized tissue through high levels of IL-12, IL-23, IL-1β, IL-6 and TNFα within 1–6 days of transplantation,[151] whereas, the M2 subtype inhibits the granuloma or fibrous encapsulation by increasing IL-10 release with fast iron transport for positive tissue remodeling. Thus, we believe that there is an urgent need to elucidate macrophage polarization and differentiation on intact dECM and/or chemically decorated dECM surfaces, and to gain new knowledge of immunogenicity with respect to regenerative scaffolds to increase tissue regeneration and thus decrease graft rejection.[8] Regenerative medicine and *in vivo* reprogramming of decellularized tissue are a dual approach that has received recent attention in the scientific community. The involvement of the innate immune system with a differential macrophage population in this context is highly responsible for tissue reconstruction and bone marrow-derived monocyte migration.[7,152] It ultimately controls the supply of the inflammatory substrate over the implanted dECM, which helps reduce inflammation and degradation with a supportive role in constructive tissue remodeling. A constellation of factors such as growth factors, cryptic peptides, receptor–ligand binding, protease, surface topography, porosity, and cell–cell and cell–ECM interactions play a vital role in the remodeling of the vascular network, synthesis of the ECM and in decellularized tissue after implantation.[146,153] Each specified factor has the ability to alter the immune response and an extended duration of the M1 macrophage has been observed in the decellularized ECM surface followed by encapsulation due to poor tissue remodeling. Through several studies, it has been hypothesized that a successful ECM derived scaffold will facilitate the macrophage plasticity in a smooth way so that it can complete the transition from M1 to M2 within stipulated time periods (7–14 days).[154,155] Delivery of inflammatory (heat shock protein 70/SDF-1 and CXCL-12/PGE2) or anti-inflammatory molecules, a pro-resolving mediator, an inhibitor of TNF/NFkB, an anti-inflammatory cytokine, siRNA/miRNA, and an extracellular vesicle is a variable way that has been followed to avoid any unwanted host-specific reaction after implantation. But the difference in the plasticity of macrophages and involvement of their intermediate subtypes (M2a, M2b, and M2c) in the transition process of immune modulation are still unclear.[156,157] Several studies have been directed on the two major subtypes (M1 and M2) to identify the immune regulation on the decellularized ECM surface but the interaction of specific subtypes with the previously mentioned factors of the decellularized ECM is still not clear.[119,158] So, to get proper control over the immune system, more detailed well-directed research in immune modulation in tissue regeneration is a future need. If we have a basic understanding of the host cell interaction and immune response, then we can build appropriate immune informed decellularized tissue that can control the tissue remodeling and initiate *in vivo* reprogramming in a favorable way.[159,160]

5.3. Regulation of Th1 and Th2 lymphocyte pathophysiology

After implantation of the dECM in the human body, other than monocyte driven macrophages, lymphocytes may appear at the site of inflammation, which further recognizes the antigenic fragments on the dECM surface, leading to the activation of macrophages and dendritic cells. Lymphocytes play a key role in the T and B cell-mediated adaptive response by interacting with the major histocompatibility complex (MHC) family which denotes twenty different polymorphic genes that are expressed on the surface of a specific antigen and are identified by the receptors on the T cell surface.[161] T-cells are able to identify the difference between the peptides of host cells (integrins, laminin, aggrecan, and versican) and the peptides on antigen-presenting cells. MHC class I molecules represent the peptide on the antigen surface that replicates simultaneously and those proteins present in the cytosolic fraction of the cells, to activate cytotoxic CD8+ T cells.[162] The difference in the amino acid sequence of the heavy chain makes the peptide-binding groove of MHC class II molecules distinctively different from that of the MHC I molecules. The MHC group of molecules confirms the presence of exogenous antigenic peptides on the dECM by CD28-B7 APC (Adenomatous Polyposis Coli) protein interactions. Hence its involvement in the immune response through the endocytic vesicles of phagocytic cells may have a crucial effect on helper CD4+ T cells.[163] After activation, CD4+ Th1 cells and cytotoxic CD8+ T cells migrate to the decellularized tissue, where they can activate resident and monocyte driven macrophages to combat the antigenic motif present after extensive decellularization.[164] Accumulation of T cells is directly correlated with the expression of MCP-1, GM-CSF, and TNF-alpha, and the production of the chemokine/monokine interferon-γ-inducible protein-10 (IP-10)/interferon-γ (MIG). Additional co-stimulatory interactions with specific cytokines such as IL-1, IL-2, IL-4,

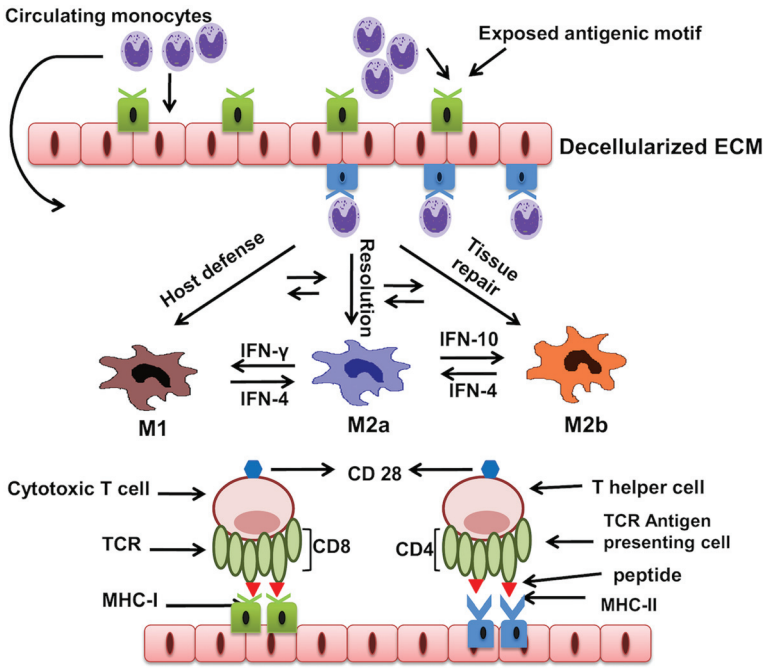

Fig. 5 Macrophage polarization and T-lymphocyte interaction with the decellularized ECM to support cell proliferation, development, and differentiation. ECM architecture regulates the communication between macrophages and T cells through different cryptic and bioactive peptides, which have a chemotactic effect on adjacent cells. Changes in the plasticity of the lymphocyte and macrophage to the Th2/pro healing macrophage (M2) promote cell differentiation and ECM remodeling, and support *in vivo* reprogramming.

IL-5, and IL-10 on the decellularized surface are required upon the interaction between CD80 or CD86 on the APC peptide and CD28 on the T-cell surface.[165] T-cells have the capability to identify between the xenogeneic and allogenic decellularized surfaces. In our study, we have already observed the transition in the plasticity of healthy monocytes on the decellularized surface. In the early 3 days culture, there was a consistent expression of proinflammatory M1 macrophages (CD80) which indicated the presence of cytotoxic T-helper cells (CD3+ and CD8+) in the decellularized and crosslinked ECM. After 7 days of culture, the population of M1 macrophages decreased with an increase in the pro-remodeling M2 (CD23, CD163, and EGR2) subtype that releases IL-4, IL-5, and IL-10 for a favorable Th2 phenotype (Fig. 5). In contrast, without chemical modification, the decellularized tissue showed the presence of an increasing number of M1 macrophages after 7 days of culture with cytotoxic T-cells throughout the study.[166,167] Characteristics of activation of T-cells and B-cells include expression of specific cell surface markers and production of the classic activation cytokines IL-2 and IFN-γ which induce MHC expression in APC. T cells undergo clonal expansion by proliferation and up- or down-regulation of their effector function. Potentially this argument could suggest that the effects of different decellularization techniques, source of decellularized tissue, alteration in the ECM protein ultrastructure, and crosslinking are crucial parameters that ultimately decide the involvement of T-helper cells in the immune response and pro-regenerative capability of polarized macrophages to predict future immune concerns.[168,169]

6. Conclusion

The native mammalian ECM represents the ultimate microenvironmental niche essential for functional cellular growth and tissue remodeling. The vital biochemical and architectural cues provided by the ECM help in controlling the cellular function and phenotype essential for development, homeostasis or remodeling. Although, substantial advancement has been made in the development of a suitable decellularized ECM in the past few decades, clinical translation of the resultant dECM is still a big challenge. So far, a major focus is on the reduction of antigenicity, and removal of cell membrane fragments, residual DNA or nucleic acid contents. The extent to which a balance between the preservation of the native ECM ultrastructure and removal of cellular contents needs to be maintained is still debatable. Even when the ECM gross ultrastructure is preserved by employing a suitable decellularization protocol, almost all the decellularization processes can impart changes in the secondary collagen conformation and expose the 'hidden or unrecognized' sites *i.e.* the cryptic domains. The mechanism by which the residual collagen fragments post-decellularization may contribute to cytocompatibility and give rise to an adverse immune response in the recipient body is still not clearly understood. Deep mechanistic insight into the host immune response to the dECM would aid in the successful transplantation of a biocompatible patient-specific decellularized ECM. The altered native ECM protein ultrastructure and/or ECM fragments may open many cryptic antigenic domains on its surface which may activate the MHC complex

thus leading to T cell and NK cell activation. It is highly essential to understand the role of the adaptive and innate immune responses by predicting the behavior of dendritic cells, macrophages, neutrophils, and T and B cells on the dECM surface. The predictive plasticity and behavior of the host macrophage on the decellularized tissue surface will help gain detailed information regarding the immunological response.

Furthermore, there should be improved understanding about the ability of the dECM to alter the immune balance and activation of specific signaling pathways which may initiate *in vivo* regeneration. A detailed understanding of the role of fragments of ECM components (matrikines, such as tumstatin, arresten, canstatin, *etc.*) in triggering immune activation, cell proliferation, or modulation of vascularization, autocrine remodeling of the surrounding ECM will aid in determining the extent of the decellularization protocol, and ultimately provide with an ideal ECM template capable of *in vivo* reprogramming. An improved understanding of the conjugation of small molecule drugs, cytokines, chemokines and their interaction with ECM dynamics and macrophage plasticity will predict the tissue regeneration ability of the dECM. These will help in building an appropriate host-specific immune informed future decellularized ECM that would have immense clinical translational significance.

Conflicts of interest

All the authors declare no potential conflict of interest.

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