Progress in Vocal Fold Regenerative Biomaterials: An Immunological Perspective

Patrick T. Coburn, Xuan Li, Jianyu Li, Yo Kishimoto, and Nicole Y. K. Li-Jessen*

1. Introduction

The vocal folds are functional organs located in the larynx that play a critical role in our daily breathing, speech, and swallowing functions. A key structural feature of the vocal folds is the delicate mucosa, which consists of a thin, layered epithelium and underlying extracellular matrix (ECM)-rich lamina propria. Irreversible changes to the vocal fold mucosa, such as scarring and atrophy, require regenerative medicine approaches to promote controlled regrowth of the mucosa. Various biomaterial systems have been engineered with an emphasis on stimulating extracellular matrix production from local fibroblasts. Simultaneously, it is imperative to minimize the foreign body response and associated inflammation that can hinder biomaterial–tissue integration. Biomaterial designs have become increasingly focused on actively harnessing the immune system to accelerate and optimize tissue regeneration. An array of biomaterial parameters have been reported to effectively modulate local immune cells, such as macrophages, to initiate tissue repair and restore the function of the vocal folds. This perspective article, the unique immunological profile of the vocal folds is first reviewed. Key physical and chemical biomaterial properties relevant to immunomodulation are then highlighted and discussed. A further examination of the physicochemical properties of recent vocal fold biomaterials follows to generate deeper insights into corresponding immune-related outcomes. Finally, a perspective will be offered on the opportunity of integrating material-driven immunomodulatory strategies into future vocal fold tissue engineering therapies.
material. The innate system constitutes the initial response that serves to provide a rapid, nonspecific inflammatory reaction. Host plasma components including body proteins, ions, lipids, and sugars seep through the surrounding blood vessels and rapidly adsorb on the biomaterial surface. In tandem, local interstitial macrophages and dendritic cells (DCs) sense and recognize compositional characteristics of the biomaterial as danger-associated molecular patterns (DAMPs). These “danger signals” elicit an immediate response that activates and recruits circulatory neutrophils and monocytes to the implantation site. Monocytes are stimulated to differentiate into macrophages that are able to bind to, and phagocytize, foreign materials. Depending on the biocompatibility and immunogenicity of the material, macrophages may fuse to form multinucleated, foreign body giant cells (FBGCs) that surround the material. Subsequent fibroblast recruitment and upregulated collagen production may then occur that can ultimately culminate in fibrous encapsulation of the material. If the initial innate response is not controlled, the acute inflammatory reaction may exacerbate to chronic inflammation and fibrotic activity.

DCs function as “sentinels” of the immune system and play a role in bridging between the nonspecific innate response and antigen-specific, adaptive immunity. The DC response to biomaterials has been far less characterized compared with that of pathogen invasion (see comprehensive reviews in other studies). Monocyte-derived DCs, immature DCs, and Langerhans cells that reside in local interstitial tissues are thought to first infiltrate the biomaterial implantation site to expose themselves to the foreign material itself or associated DAMP. Regenerative biomaterials applied to soft tissues (including the vocal folds) have a tendency to be biodegradable to help facilitate remodeling via cellular infiltration and ECM synthesis. When activated, immature DCs encounter these materials; they can capture antigens released during the degradation process and transport them to local lymph nodes. Depending on the maturation process, DCs may extract peptides from the biomaterial-associated antigens, load them onto the major histocompatibility class II complexes, and present the resulting epitopes on the cell surface. Mature DCs also upregulate the expression of costimulatory molecules (CD40, CD80, CD86) and release proinflammatory cytokines (e.g., IL-12), which in turn activate and mediate B- and T-lymphocytes for adaptive immunity and immune memory.

Patently, innate and adaptive immunity plays a critical role in determining the clinical success of healthcare biomaterials. Being able to create a biomaterial with the competency to integrate with, and subsequently repair, host tissue without inducing an adverse immune response remains a key challenge for regenerative medicine. Recent advances in tissue engineering have furthered the concept of actively controlling and directing the host immune response toward a proregenerative, nonfibrotic outcome. To that end, the development of bioinstructive materials is proposed to exert intrinsic or extrinsic cues, either biochemical or physical, that activate or suppress specific immune cell functions throughout the tissue healing process.

Innovations of bioinstructive, immune-centric materials are yet to be fully explored for vocal fold regenerative biomaterials. This perspective paper aims to address the viability of materials with immunomodulatory properties advancing the field of vocal fold tissue engineering. The unique immunological profile of the vocal folds was first reviewed. Key chemical, physical, and mechanical properties of biomaterials relevant to immunological modulation were then explored. Physicochemical properties of recent vocal fold biomaterials were then examined to synthesize deeper insights into their reported immune-related outcomes. Finally, a perspective on the opportunity of integrating the immune system into the vocal fold tissue engineering strategies was provided.

2. Immunological Profile of the Vocal Folds

Located at the upper junction of the respiratory airway and gastrointestinal tracts, the larynx serves an important checkpoint of mucosal immunity for any inhaled and ingested materials. Specific to the glottic region of the larynx, the epithelium and lamina propria of the vocal folds displays distinctive immunological architecture (Figure 1). The epithelium comprises 5–10 layers of stratified squamous cells. In healthy vocal folds, a subset of DCs known as Langerhans cells have been identified in the suprabasal layer of the squamous epithelium although the corresponding cell density was noticeably lower than other tissues such as the epidermis. Although immunoglobulin (Ig)-A, IgG, and IgE and lactoferrin were profusely detected in laryngeal secretions, Ig-secretory glandular cells were only found in those of pseudostatified columnar epithelium covering supraglottic and subglottic regions but not of the glottic region in vocally healthy adults. When the vocal folds undergo benign or malignant changes, a notable increase in Langerhans cells, T-lymphocytes, and IgA secretory components has been reported within the epithelium.

Regarding the lamina propria, a certain number of local immune cells reside in the interstitial space next to blood capillaries and lymph microvessels throughout the human body. For instance, the trafficking of resident DCs and effector-memory T-lymphocytes from local interstitial tissue to the draining lymph node is a key mechanism to maintain immune tolerance. Despite the presence of extensive capillary and lymphatic networks in the vocal folds, resident immune cell populations were scarcely documented at the glottic region in vocally healthy adults. Two remote studies may, however, provide some insights on this subject matter. A human fetus study showed that tissue-resident DCs, T-lymphocytes, and B-lymphocytes were observed in the mucosa covering subglottic and supraglottic regions but not of the glottic region. A recent human adult study quantified the average frequencies of T-regulatory and T-lymphocytes in tissue biopsies from the supraglottic region of the larynx as 1.63% and 23.65%, respectively, in vocally healthy adults. Further studies are warranted to quantify tissue-resident DCs and lymphocytes at the glottic region as well as track their antigen trafficking between the vocal fold lamina propria and draining lymph nodes to understand the maintenance of immune homeostasis in vocally healthy adults.
In contrast, macrophages, fibroblasts, and myofibroblasts were the most commonly reported cell populations residing in the vocal fold lamina propria. In healthy adults, macrophages and myofibroblasts were predominantly found in the superficial lamina propria (SLP) of the vocal folds. Fibroblasts were abundantly populated across all layers of the lamina propria, with the highest number found in the deep lamina propria (DLP). Upon injury to the lamina propria, rapid neutrophil and monocyte recruitment from peripheral capillaries occurs, as demonstrated across experimental models of mice, rats, rabbits, and pigs. Li-Jessen’s team applied multicolor flow cytometry to characterize temporal trajectories of neutrophils (CD45⁺ His48⁺), macrophages (CD106⁺ CD44H⁺), fibroblasts (CD29⁺ CD105⁺ CD106⁺), and endothelial cells (CD29⁺ CD44H⁺ CD106⁺) in surgically injured rat vocal folds. In particular, nonpolarized, basal macrophages were expected to dominate the local macrophage population of the vocal folds. Following injury, M1 polarization would be significantly upregulated during the initial, proinflammatory response. However, to effectively resolve inflammation and promote healing, M2 polarization would be required to succeed M1 as the dominant local population of macrophages. Such speculations of vocal fold macrophage polarizations were observed in the animal study and later confirmed by Kishimoto’s research team. Also, fibroblast–myofibroblast transdifferentiation was observed in the same animal study, emulating in vitro studies, subjecting vocal fold fibroblasts to growth factor stimulations from Thibeault’s and Kishimoto’s research teams, respectively. Furthermore, a functional study by Li-Jessen’s team revealed that macrophages and fibroblasts were major cell sources of high-mobility group protein 1 (HMGB1), a typical DAMP cytokine, within injured vocal fold mucosa. The translocation of vocal fold HMGB1 from nuclear to extracellular space was related to the early accumulation of proinflammatory cytokines (IL-1β and TNF-α) in the wounded mucosa.

All told, various intrinsic or extrinsic signals in the vocal fold mucosal microenvironment can modulate the phenotype of vocal fold macrophages and fibroblasts to direct their functionality from proinflammatory toward reparative activity or vice versa. When local danger signals become considerable, immunocompetent cells are recruited rapidly from local blood capillaries and lymphatic microvessels as well as likely from adjacent subglottic and supraglottic regions to the vocal fold mucosa. Investigations on tracking resident DCs, T-, and B-lymphocytes as well as the role of the adaptive immune system in healthy and injured vocal fold mucosae are essential for further development of immunocentric biomaterials therapeutics for vocal fold regeneration.
3. Key Biomaterial Properties Relevant to Immunomodulation in Tissue Repair

The physicochemical properties of biomaterial scaffolds offer an opportunity to engineer a local microenvironment conducive to inflammatory resolution and prorogenerative activities (Table 1, Figure 2). A broad variety of chemical (e.g., wettability, charge), physical (e.g., porosity, surface roughness), and mechanical (e.g., stiffness, viscoelasticity) parameters have been reported to engage with, and influence, immune cells such as neutrophils, macrophages, DCs, and T-lymphocytes.[20,21,54–56] However, while these general observations can provide some clues for predicting immunological outcomes of biomaterial implantation, it is critical to consider that immune responses are often highly specific for a given material and tissue.[57–59] As such, the observed effects can vary dramatically across material composition and host tissue type. Alongside material-driven strategies, cell and drug immunomodulatory therapies have also been actively pursued in the field of regenerative medicine, although this is beyond the scope of the current perspective.

3.1. Chemical Material Parameters

3.1.1. Crosslinking

Chemical and physical crosslinking methods are available to enhance biomaterial mechanical integrity and control its degradation profile. The products of crosslinking are either covalent bonds that are strong and permanent or physical counterparts such as ionic and hydrophobic associations that are reversible and transient.[60] Chemical crosslinking relies upon chemical strategies to crosslink polymer chains or decellularized ECM (dECM) particles into hydrogels with prolonged residency and enhanced mechanical properties. Often, chemical crosslinking reagents are required that may be cytotoxic and potentially invoke an elevated immune response. For example, a tris-hydroxymethyl phosphine (THP) crosslinker applied to resilin hydrogels induced chronic inflammatory reactions compared with noncrosslinked controls when applied subcutaneously to mice in vivo.[61] In addition, chemical crosslinkers can impact cell functionality. For example, although calcium is a common crosslinker for alginate hydrogels, calcium has a significant effect upon multiple immune cells. Ca\(^{2+}\) signaling is involved in DC cytokine secretion, maturation marker expression, and phagocytosis.[62,63] Furthermore, neutrophils require Ca\(^{2+}\) for degranulation and T-lymphocytes require Ca\(^{2+}\) signaling for IL-2 and IL-4 production.[64] To this end, increasing focus has been on self-crosslinking polymers such as chitosan and click reaction strategies that are independent of biomolecules and biological processes.[65,66]

The avoidance of potentially cytotoxic chemical crosslinking agents has contributed to the rise in the popularity of physically crosslinked materials.[67] For instance, γ-irradiation was applied to crosslink a β-glucan hydrogel without the need for crosslinking agents. Compared with noncrosslinked controls, γ-irradiated β-glucan hydrogels displayed significantly reduced inflammatory cell infiltration, granuloma formation, and hyperemia in vivo when tested both subcutaneously in mice and in paralyzed rabbit vocal folds.[68] The reduced inflammation may be attributed to crosslinking, preventing rapid hydrogel resorption into the surrounding tissue and thus limiting inflammatory cell recruitment.

3.1.2. Chemical Moieties

Immune cells are reliant on protein adsorption to facilitate anchorage and interaction with a material surface such as through integrin linkages. The conformation and density of adsorbed proteins are critical factors for immune cell recruitment, surface adhesion, and inflammatory activity following biomaterial implantation.[69]

The chemical composition of a material, in particular, surface moieties, can strongly influence protein adsorption and subsequent immune cell activities. In general, surface oxygen correlates with immune passivation, whereas surface carbon triggers immune activation.[70] Moieties such as amino (–NH\(_2\)), hydroxyl (–OH), and carboxyl (–COOH) groups were shown to increase surface hydrophilicity and reduce protein adsorption.[71] For hydrophilic and neutral surfaces, neutrophils and macrophages were found to display reduced recruitment, adhesion, proinflammatory cytokine production, and FBGC formation.[72,73]

Conversely, methyl groups (–CH\(_3\)) are associated with increased hydrophobicity. Hydrophobic materials are recognized as DAMPs by the human immune system, which triggers increased immune cell recruitment and activation alongside prolonged inflammation.[74–76] For instance, neutrophil recruitment and lifespan are increased in the presence of hydrophobic materials. This elevates the potential for chronic inflammation and fibrotic activity as neutrophil apoptosis is a controlling factor for M2 recruitment that triggers inflammatory resolution and tissue healing.[77,78] Hydrophobic surfaces may also trigger DCs, leading to functional maturation, greater antigen internalization, and increased expression of costimulatory molecules such as CD86 and MHC-II.[79]

3.1.3. Degradation Profile

Biomaterial degradation must balance maintaining scaffold integrity for structural guidance and biomechanical cues, while also facilitating material—tissue integration via cellular infiltration and remodeling. Degradation byproducts are important considerations as the cytotoxicity of the bulk, intact material may differ significantly from the wear particles produced during immune cell-mediated breakdown.[80] For example, polyethylene glycol (PEG) has been proposed as a biomaterial for multiple applications and is considered nontoxic.[81,82] However, the ethylene glycol monomers of PEG generated during degradation are toxic to many tissues, highlighting the potential downstream immune implications and wider, nonlocalized host immunological effects of a biomaterial.[83] Antibodies against PEG have been reported in healthy individuals and patients recently.[84] In addition to the cytotoxicity of the degradation byproduct itself, subsequent inflammatory activity is also size dependent. For example, particles ≤50 nm have been found to induce increased neutrophil recruitment and phagocytosis, alongside a decrease in M2 macrophage presence.[85] Larger particles may also be
Table 1. Immune cell responses to biomaterial properties.

| Parameter                  | Neutrophil                                      | Macrophage                                      | DC                                                | [T]-lymphocyte |
|----------------------------|------------------------------------------------|------------------------------------------------|--------------------------------------------------|---------------|
| Surface Chemistry          | COOH: ↓ MMP-9 secretion\[^{194}\] (immobilized gold nanoparticles) | COOH: ↓ TNF-α, IL-6, IL-1β\[^{194}\] (immobilized gold nanoparticles) | OH/COOH/NH₂-odorate maturation\[^{195}\] | Not reported |
|                            | Hydrophobic: ↓ recruitment, lifespan\[^{55}\] | Hydrophilic/neutral: ↓ cell density, adhesion, FBGC formation IL-10 ↓ IL-6, IL-8, IL-1β (≤72 h)\[^{196}\] (modified PET surfaces) | Hydrophobic: ↓ maturation, antigen internalization, CD86 & MHC-II expression\[^{79}\] (PLA-based microparticles) | Negative charge: ↓ proliferation\[^{197}\] (Carbon nanotubes) |
| Surface Wettability/Charge | Hydrophilic: ↓ recruitment ↓ ROS\[^{198}\] | Hydrophobic/cationic surfaces: ↓ adhesion, FBGC fusion\[^{79}\] (modified PET surfaces) | Low-MW HA (1.3–5.3 kDa): ↓ IL-1β, TNF-α, IL-12\[^{102,103}\] | Low-MW HA (1.5–5.3 kDa): ↓ proliferation\[^{102,103}\] (HA fragments) |
| Molecular Weight           | Low-MW HA: ↓ recruitment\[^{195}\] (HA fragments) | Low-MW HA: M1, proinflammatory response | High-MW HA: M2, prohealing response\[^{108,109}\] (HA fragments) | M2, proinflammatory resolution\[^{102,103}\] (HA fragments) |
| Porosity                   | Synthetic, nonporous materials: | 30–40 μm pore size: ↑ M2 | | Not reported |
|                            | ↓ recruitment, NETs, inflammation\[^{20}\] (polydioxanone electrospun fibers) | | | |
|                            | Nonporous: ↑ M1\[^{194}\] (PMMA hydrogel) | | | |
|                            | 34 μm pore size: ↓ fibrosis, thinner fibrous capsule (vs. 160 μm pore size/ nonporous)\[^{194}\] (pHEMA scaffolds) | | | |
|                            | 3 μm pore size: ↓ TNF-α, IL-6, IL-1β/\[^{136}\] thicker fibrous capsule (vs. nonporous)\[^{136}\] (ePTFE materials) | | | |
| Stiffness                  | ↑ Stiffness (5–100 kPa range): ↓ cell spreading, motility during chemokinesis and chemotaxis\[^{191}\] (polyacrylamide gels) | ↑ Stiffness (11–232 kPa range): ↑ M1 population\[^{114}\] (polyacrylamide gels) | ↑ Stiffness (2–50 kPa range) modulates podosome formation via CCR7, CD83, C-type lectin, β2-integrin expression\[^{20}\] (polyacrylamide gels) | ↑ Stiffness (10–200 kPa): ↓ IL-2 secretion, stronger cell attachment ↓ activation\[^{20}\] (polyacrylamide gels) |
|                            | ↑ Stiffness (0.2–32 kPa range): NETs, TNF-α, IL-6, IL-1β\[^{20}\] (PDMS surfaces) | ↑ Stiffness (130–840 kPa range): ↓ cell spreading, activation, F-actin, integrins, FBR severity\[^{20}\] (PEG-based hydrogels) | | |
| Topography                 | ↑ Roughness: ↑ recruitment, adhesion, activation, IL-1β\[^{20}\] (PS-PEO microparticles) | Rougher particles: ↓ phagocytosis\[^{20}\] (PS-PEO microparticles) | ↑ Roughness: ↓ activation, maturation\[^{20}\] (teflon, PS, PEN, PMMA surfaces) | Not reported |
|                            | ↑ Roughness: MMP-9\[^{194}\] (immobilized gold nanoparticles) | | | |
|                            | Angular edges: ↑ infiltration, adhesion\[^{20}\] (alginate/PCL/PS spheres) | Angular edges: ↓ acute response\[^{20}\] (alginate/PCL/poly(styrene spheres) | | |

\[^{194}\] ROS, reactive oxygen species; NET, neutrophil extracellular trap; MW, molecular weight; M1, proinflammatory macrophage; M2, anti-inflammatory macrophage; FBR, foreign body response; FBGC, foreign body giant cell; MHC, major histocompatibility complex; HA, hyaluronic acid; PS-PEO, polystyrene-poly(ethylene oxide); PCL, poly(caprolactone); PS, poly(styrene); PET, poly(ethylene terephthalate); PCBMA, poly(carboxybetaine methacrylate); PMMA, poly(methyl methacrylate); pHEMA, poly(2-hydroxyethyl methacrylate); ePTFE, expanded poly(tetrafluoroethylene); PEG, poly(ethylene glycol); PLGA, poly(lactic-co-glycolic acid); PLA, poly(lactic acid); PDMS, polydimethylsiloxane; PEN, poly(ethylene naphthalate). The approximated trends displayed will not necessarily apply across different tissues and materials due to the specific nature of the immune response.
Figure 2. Effects of biomaterial parameters on immune cells. a) Chemical material parameters (crosslinking, moieties, charge, degradation profile) that can influence immune cell behavior. b) Physical material parameters that can influence immune cell infiltration or surface binding activities. c) Mechanical material properties are critical in replicating the native tissue microenvironment to drive appropriate mechanosensing by immune and tissue cells. d) Immune cells can respond to different material parameters through the release of cytokines and chemokines to trigger cell signaling pathways. Following protein adsorption, activated neutrophils release NETs to start a signaling cascade. Neutrophil apoptosis attracts macrophages to the site with the capacity to differentiate to either a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. Macrophage activity can activate DCs that interact with naïve T-cells to stimulate their activation and subsequent recruitment to the biomaterial site. Of critical importance, the specific nature of the immune response is tissue and material dependent; the effects indicated earlier are generalized trends that are not universally applicable in all cases.
problematic, with particles ≥3 μm suggested to clog capillaries and undergo slow phagocytosis leading to extended tissue residency.[70]

Another important aspect of degradation is the potential for biomaterial migration away from the site of implantation. Biomaterials can travel significant distances from the original implantation site, such as vocal fold biomaterials migrating to cervical or retropharyngeal lymph nodes.[86,87] Migration hinders biomaterial long-term efficacy and performance and can lead to significant health complications or failure of the implant (Figure 3).

3.2. Physical Material Parameters

3.2.1. Porosity

Biomaterial–immune cell interactions can be influenced by porosity components such as the size, density, and spacing of the pores. For example, immune cell adherence to material surfaces can be modulated through spatial confinement to control actin polymerization, chromatin compaction, and epigenetic alterations.[88,89] Increasing material porosity in polydioxanone scaffolds led to a shift toward M2 populations and proregenerative environments for in vitro cultures of bone marrow-derived macrophages.[90] Fiber alignment and thickness may also influence cellular infiltration and inflammatory activity. For instance, when implanted subcutaneously in rats, a polycaprolactone electrospun scaffold containing aligned fibers with a 3 mm gap facilitated increased macrophage infiltration and thus reduced FBGC formation compared with unexpanded scaffolds.[91]

Most synthetic, nonporous materials induce a chronic inflammatory response characterized by the excessive macrophage and FBGC presence, neutrophil extracellular trap (NET) extrusion, and fibrotic encapsulation.[92,93] Increasing the pore size can help reduce the fibrosis associated with synthetic materials.[104] A PMMA hydrogel inserted into rat myocardium demonstrated a reduced fibrotic response alongside increased M2 macrophage presence for implants with 30–40 μm pores compared with nonporous controls.[94]

In contrast, natural materials with a high surface-area-to-volume ratio (high porosity) and limited processing do not generally induce fibrotic responses. Such materials tend to promote inflammatory resolution and regeneration as characterized by increased macrophage-to-FBGC ratios.[92,95–98] In general, ECM-derived scaffolds fit this definition, offering porous, highly interconnected materials that facilitate cell infiltration and remodeling to prevent frustrated phagocytosis and chronic inflammation.[99] That being said, porous HA hydrogels with an initial elastic moduli of ~2 kPa were associated with chronic inflammation when tested in murine myocardium.[99] This response could be related to the production of low−molecular−weight fragments generated during degradation that can induce increased neutrophil recruitment, M1 presence, DC activation, T-lymphocyte proliferation, and proinflammatory cytokines (e.g., IL-1β, TNF-α, IL-12).[100–103]

Related to vocal fold biomaterials specifically, the phenotype and ECM production of vocal fold fibroblasts encapsulated within PEG-based hydrogels were found to correlate with the porosity and elastic moduli of the material in vitro.[104] Decreasing the mesh size of the hydrogels from 27 to 9 μm induced a greater shift to myofibroblast-like phenotypes alongside reduced collagen deposition.

3.2.2. Surface Roughness

To influence cells directly, surface features ranging from nanosize up to tens of micrometers have been suggested for cell modulation strategies.[105,106] The impact of surface roughness can vary considerably by cell type with a multitude of effects reported for neutrophil, DC, and macrophage activity. Neutrophil adherences to roughened polystyrene surfaces implanted subcutaneously in rats were associated with increased cell death and production of reactive oxygen species compared with smooth surfaces.[107] On rougher (>4 μm) titanium surfaces, DCs have displayed activated, mature phenotypes characterized by increased CD86 and MHC-II expression.[108] Mature DCs upregulate the inflammatory response, delay healing, and ultimately lengthen the biomaterial-related immune response.[113] Macrophage activity has also been reported to increase in the presence of roughened surfaces. Rough surfaces are prone to the release of particulate debris that, upon ingestion by macrophages, triggers perpetual exhaustive phagocytosis, inflammatory cytokine release, and lymphocyte proliferation.[109,110]

The effect of surface topography has been effectively demonstrated with silicone in the context of breast implants. Implants with an average surface roughness of 4 μm were associated with the mildest inflammation and foreign body response (FBR) compared with rougher (15, 30, 90 μm) surfaces when tested in the mammary fat pads of mice or rabbits.[111] The fibrous capsule generated for the 4 μm breast implant was also significantly thinner than that of smooth implants at both 3 and 6 weeks postimplantation. Furthermore, in comparison with smooth implants, higher incidences of breast implant-associated anaplastic large cell lymphoma were reported in human patients who received implants with >300 μm roughness.[112]
3.3. Mechanical Material Parameters

3.3.1. Stiffness

Innate immune cells rely upon adhesive interactions mediated via integrins to bind to the proteins adsorbed on the biomaterial surface upon implantation. Varying the stiffness of a biomaterial can influence the strength of the integrin linkages present and modulate subsequent immune cell activities. For example, macrophage polarization was found to correlate with stiffness, with larger populations of M1 and M2 macrophages, respectively, observed for stiffer (323 kPa) versus softer (≤88 kPa) polyacrylamide gels. Increased M1 populations for the stiffer gels may have been related to stronger integrin linkages, leading to increased macrophage adhesion and fusion. Stiffness may also modulate the activities of tissue repair cells with increased stiffness, reducing substrate movement, thus enabling greater loading of the integrin–actin linkage, and strengthening focal adhesions and cell attachment. For example, fibroblasts in contact with soft hydrogels (≤1 kPa) displayed limited focal adhesion maturation, cytoskeleton assembly, and cell spreading. In comparison, stiffer hydrogels induced extended focal adhesions and fibroblast spreading, increased tyrosine phosphorylation of FAK and paxillin expression, and a well-defined cytoskeleton. These values also demonstrate that the response to stiffness is not universal and the stiffness variances that cells respond to can vary significantly across materials, moduli, and cell types.

In the context of vocal folds, stiffness was found to exert a synergistic effect on macrophage and fibroblast activities. Li-Jessen’s team increased the stiffness of a glycol-chitosan hydrogel (1.11–7.86 kPa) by varying glycol concentrations, resulting in elevated production of the anti-inflammatory cytokine IL-10 in cocultures of THP-1 macrophages and immortalized vocal fold fibroblasts. Given the important role of IL-10 for stimulating inflammatory macrophage polarization, this finding suggests that biomaterial stiffness could be utilized to ultimately promote nonfibrotic fibroblast phenotypes within the vocal fold mucosa.

3.3.2. Viscoelasticity

Tuning biomaterial viscoelasticity could help provide appropriate biomechanical cues and increased control over cellular spatial confinement. Spatial confinement limits cell volume and morphological changes and has been associated with decreased inflammatory activity and reduced proinflammatory macrophage activation. Fibroblasts seeded on viscoelastic 2D alginate gels have demonstrated robust focal adhesions and stress fibers when compared with soft, elastic controls. Gels fabricated with faster stress relaxation, enhanced creep, or higher loss moduli have also been associated with changes in cellular behavior when used for 3D cell culture. Fibroblasts, myoblasts, and mesenchymal stromal cells (MSCs) displayed increased cell spreading, focal adhesion maturation, and proliferation when cultured in PEG, PEG–alginate, RGD–alginate, and HA–collagen gels.

Elastic scaffolds fabricated from polyethylene or PEG were associated with increased neutrophil recruitment and accumulation up to 3 weeks postimplantation in mice compared with natural ECM implants. The polyethylene/PEG implants also displayed decreased expression of CD206, a marker associated with M2 macrophages. The lack of viscoelasticity in the polyethylene/PEG materials used may be a contributing factor for the increased and sustained presence of inflammatory immune cells.

4. Immunological Evaluation of Biomaterials for Vocal Fold Regeneration

A literature search was performed on PubMed, Google Scholar, and Embase of peer-reviewed articles published between 2010 and 2021 to evaluate the complex interplay between biomaterial parameters and vocal fold immunology. Based on the search, only five original studies met our search criteria, specifically, that the proposed biomaterials were tested in 1) defected or injured vocal fold mucosa; 2) in vivo animal models with immunological evaluation; and 3) the absence of exogenous cells, growth factors, and other compounds (Table 2).

4.1. Association Between Chemical Properties and Immunological Outcomes

Natural materials such as HA and dECM were common components for the vocal fold biomaterials currently under development (Table 2). The innate bioactive properties and cell adhesion sites of natural materials are believed to facilitate material–tissue integration in the local microenvironment. However, natural materials do have the potential to induce inflammatory activity via immunogenic impurities or antigens. For instance, most dECM-based vocal fold biomaterials are animal sourced (e.g., bovine, porcine, etc.), which may increase the potential for xenogeneic immune responses following in vivo implantation.

In addition, natural materials often need to be functionalized through substitution with chemical groups that permit crosslinking to produce materials with better gelation kinetics and mechanical stability. For instance, a HA–gelatin vocal fold hydrogel used the low-immunogenic, hydrophilic crosslinker PEGDA to enhance the mechanical properties of the gel system. Hydrophilic components present on PEGDA, such as hydroxyl (–OH) groups, may drive anti-inflammatory activities in macrophages related to the increase in dysopsonin surface deposition (e.g., albumin) that upregulates anti-inflammatory M2-related pathways.

To date, reported HA- and dECM-based vocal fold biomaterials were all noted as hydrophilic, displaying prominent amino, hydroxyl, and carboxyl groups. All materials, either with, or without, chemical crosslinkers, displayed foreign body reactions that varied in severity during animal testing. Reactions ranged from early granulocyte infiltration to the eventual formation of fibrous tissue and may be attributable to the injection procedure or an innate immune response related to the material. Although most animals appeared to survive the duration of these studies, the concern remains whether human recipients could tolerate such biomaterial-induced reactions for extended time periods while maintaining necessary daily vocal fold functions of communication, swallowing, and breathing.
Table 2. Immunological properties of biomaterials designed for vocal fold engineering.

| Materiala | Crosslinker | Surface | Structural properties | Mechanical properties | Animal model | Timepoints | Material residency | Immunological Findings | Authors (year) |
|-----------|-------------|---------|-----------------------|-----------------------|--------------|------------|-------------------|----------------------|---------------------|
| HA-Gelatin | PEGDA (chemical crosslinking) | Hydrophilic | Particle size: 1300 nm | E: 22 kPa G': 70 Pa (1 Hz), 90 Pa (10 Hz) G': < 1 Pa (1 Hz), 5 Pa (10 Hz) | Rat | 3, 14, 28 days | N/D | ✓ | Absent | Absent | Coppoolse et al. (2014) |
| RLPAM/HA-SH | Thiol-acylamide bonding (chemical crosslinking) | Hydrophilic | Gel point: ≈ 40 min | G' = 1570 ± 500 Pa (0.1–100 Hz) | Rabbit | 5 days | Complete degradation | ✓ | ✓ | Absent | King et al. (2019) |
| Bovine vocal fold ECM | N/U | Hydrophilic | Porosity: 90.49 ± 4.33% Average pore size: 2.2 μm | N/D | Rat | 3 days, 7 days, 1 month, 3 months | Complete degradation (3 months) | ✓ | ✓ | Absent | Xu et al. (2019) |
| Porcine bladder ECM | N/U | Hydrophilic | N/D | N/D | N/D | Canine | 6 months | Complete degradation | N/D | Absent | Absent | Kitamura et al. (2016) |

a) HA, hyaluronic acid; PEGDA, poly(ethylene glycol) diacrylate; E, elastic modulus; G', storage shear modulus; G", loss shear modulus; N/D, no data; N/U, none used.

5. What Is Next for Vocal Fold Regenerative Biomaterials?

With technical advances in biomaterial fabrication, current trends have been steered toward biomaterial designs that recapitulate the native microstructure of the organ. The design of emerging, advanced, and injectable biomaterials that recapture the native vocal fold architecture (e.g., porosity, elasticity, stiffness) serve as critical foundation to achieving patient-specific, regenerative solutions. This is particularly evident in the development of vocal fold regenerative biomaterials that can more closely imitate the native vocal fold tissue mechanics.

Most vocal fold biomaterials were designed as injectables, as such, certain physical properties such as viscoelasticity and gelation time are already constrained by clinical application. Further, the stiffness of human vocal fold tissue requires for phonation, Young's modulus of human vocal fold tissue has been recorded as ranging between 2.45 and 29.4 kPa. This phonatory function, stiffness, is notably important for vocal fold physical properties, as it is fundamental to appropriate physiological standards. In addition, stiffness and Young's modulus are already constrained by this clinical application. A further task would be to comprehend the range of stiffness, as ranging between 2.45 and 29.4 kPa, to be successfully incorporated into the scaffold volume. Of the materials reviewed, only one (bovine vocal fold ECM) was characterized for its porosity.

Contributing factors to that observation are speculated to be reduced lung and vascular cell penetration due to the scaffold volume. Of the materials reviewed, only one (bovine vocal fold ECM) was characterized for its porosity and the mass transport of nutrients and oxygen throughout the scaffold. Reduced oxygen and nutrient diffusion throughout the scaffold volume facilitates vascularization up to a diameter of 400 μm. This is a critical factor for preventing frustrated phagocytosis and minimizing the risk of chronic inflammation. That being said, the scaffold volume may also be reduced, leading to limited endothelial cell penetration required for vascularization up to a diameter of 400 μm. This is a critical factor for preventing frustrated phagocytosis and minimizing the risk of chronic inflammation.

Materials with increased pore size (>100 μm) are associated with increased porosity. In general, biomaterials that are nonporous or have small pore sizes (≤100 μm) have been shown to be less efficient in delivering cells and fibers. However, biomaterials with increased porosity (>100 μm) have been shown to facilitate improved cell penetration and subsequent remodeling. Generally, biomaterials that are nonporous or have small pore sizes (≤100 μm) have been shown to be less efficient in delivering cells and fibers. However, biomaterials with increased porosity (>100 μm) have been shown to facilitate improved cell penetration and subsequent remodeling.
critical for the clinical translation of these therapeutic products. The fabrication of highly complex biomaterials is expensive and challenging to scale-up production. Overengineering materials can also impede the innate processes of cells and tissues. One modern approach is to utilize the physicochemical properties of biomaterials to actively engage with, and modulate, the material-associated immune response. Such immune-centric biomaterials, ideally, should be capable of mitigating the foreign body reaction, priming T-lymphocytes, accelerating vascularization, and ECM remodeling for functional tissue regeneration.\[^{121,139–141}\]

### 5.1. Promise of ECM-Based Biomaterials to Be Bioinstructive

Natural ECM products retain native proteins and bioactive molecules that can promote vascularization and tissue remodeling. However, pure ECM-based biomaterials suffer from weak mechanical integrity and inconsistent gelation. Frequent issues contributing to the mechanical weakness of ECM-based biomaterials include 1) relatively low density of crosslinks within the matrix; 2) lack of toughening and energy dissipative mechanisms for mechanical toughness; and 3) limited percolation of the matrix where excess network imperfections (e.g., dangling chains, widely spaced dECM particles, etc.) exist. Remedies for the first two issues have focused on the density and types of crosslinks used to form ECM-based biomaterials. Higher crosslinking densities produce stiffer and stronger biomaterials. While crosslinker concentration is readily tunable, the reaction efficiency is a key factor constraining the crosslinking density of existing ECM-based biomaterials. Widely used strategies, such as carbodiimide chemistry, are notable for their limited reaction efficiency. Other chemistries, especially those developed for tissue adhesion and expected to be applicable to ECM components (e.g., proteins, polysaccharides, etc.), offer potential alternatives to explore.\[^{142}\] In addition, a combination of crosslinking strategies warrants investigation in the light of the development of tough hydrogels.\[^{143,144}\] Specifically, the incorporation of physical crosslinks, such as ionic crosslinks, capable of energy dissipation and self-healing into covalently crosslinked networks is anticipated to substantially enhance the mechanical performance of ECM-based biomaterials. Regarding the network percolation, the shape of dECM particles is an understudied design factor. Finally, harnessing cellular activities particularly matrix deposition is another direction to pursue that could overcome current design flaws associated with dECM biomaterials.

### 5.2. Cell-Based Therapies

Although the discussion within the present review has focused on acellular material strategies for immunomodulation, incorporating various cell types into biomaterials has been proposed to create a more conductive microenvironment for mucosal repair.\[^{145–147}\] Despite their advantages, cell-laden biomaterials have certain practical and immunological challenges not present for acellular therapies. For example, sourcing primary autologous (or even allogenic) vocal fold fibroblasts and epithelial cells is difficult due to the small size of the vocal folds and accessibility issues.\[^{148,149}\] As such, other cell sources such as MSCs derived from bone marrow\[^{150–152}\] or adipose\[^{153}\] tissue and induced pluripotent stem cells (iPSCs)\[^{154}\] have been sought for defective vocal fold treatments.

MSCs have been shown to display fibroblast-like behavior in the vocal fold microenvironment.\[^{155}\] Likewise, Thibeault’s team has shown success in differentiating iPSCs into vocal fold epithelial cells.\[^{156}\] However, limitations exist for the use of stem cells. Inefficient target differentiation of MSCs and their niche activity for tissue regeneration remain major challenges for their application clinically.\[^{156–158}\] For iPSCs, in addition to spontaneous differentiation challenges, there can also be retention issues at the site of injection.\[^{154}\]

From an immunological perspective, nonautologous cells present foreign antigens that will trigger the host’s immune system. In particular, these antigens will induce an adaptive immune response involving T- and B-cell activation that ultimately culminates in the production of memory cells. Memory cells present a significant obstacle to future injections using the same cell material therapy. Furthermore, the majority of cellular biomaterials have been applied for testing in xenogenic\[^{159–162}\] or allogenic\[^{156,160}\] models, which can further exacerbate the inflammatory response, potentially masking the efficacy of the therapy.

### 5.3. New Experimental Platforms for Immunological Evaluations of Regenerative Biomaterials

Rats, rabbits, and canines are widely used for vocal fold biomaterial evaluation due to similarities in their vocal fold anatomies to that of humans. However, from an immunological perspective, the predictive power of animal models to reflect human biological processes remains inadequate. For instance, although over 85% of protein-coding regions were found identical between human and mice,\[^{163}\] those 15% differences in DNA sequence and related gene regulation machineries are expected to result in a differentiated immune response between the two species.\[^{162}\] With the advances of genetic engineering such as CRISPR-Cas9, various laboratories have begun to genomically humanize rodents that may better represent, for instance, the reaction of the human immune system to viral infection and wound injury.\[^{163–167}\]

At the same time, recent innovations in science, technology, and ethics have intensified the debate around animal models and whether they represent the most efficient preclinical testing model.\[^{168,169}\] Biomedical research continues to be challenged by complex, multifactorial diseases such as cancer, cardiovascular diseases, infectious diseases, and neurodegenerative disorders that require flexible, clinically relevant experimental models to explore the biology of, and potential therapies for, such conditions. One major criticism of animal models (and traditional in vitro models) is that they frequently fail to replicate human pathophysiology responses.\[^{170,171}\] A further limitation of these models is the inherently low-throughput assessment techniques available for use.\[^{172}\]

To overcome the long-standing challenges associated with animal models and traditional cell culture, complex 3D culture models such as organ-on-a-chip\[^{173}\] and organoids\[^{174}\] have been developed. Over the past decade alone, these models have utilized biomechanical cues including stretching forces (e.g.,
Conflict of Interest
The authors declare no conflict of interest.

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lung-on-a-chip[175,176] or improved spatial complexity and vascularization (e.g., brain organoids)[177] in an effort to enhance the physiological relevance of cell culture microenvironments. Organ-on-a-chip models have already been widely applied for high-throughput drug testing and disease modeling applications.[176,178–181] In the evaluation of biomaterials, an organ-on-a-chip model was developed to investigate the FBR to titanium beads via circulating monocytes through an endothelial cell-lined channel.[182] In reality, many biomaterial properties are codependent and interrelated. For example, the crosslinker concentration of a hydrogel has the capacity to vary multiple material properties including stiffness, pore size, and degradation profile, each of which can impact cellular activities in different ways. Decoupling the biological impact of different material properties can be experimentally challenging. In addition, vocal folds have unique anatomical features and an immunological profile difficult to recapitulate using existing in vitro models with restricted cell types and study length. As such, computer simulations are used to explore a much wider parameter space and longer timescale than what would be costly, or perhaps impossible, with in vitro, animal, or clinical models. Computational simulations can help decipher how individual and combined material properties will determine specific vocal fold cell activities and long-term mucosal tissue growth. For instance, continuum mechanics and computational fluid dynamics techniques were widely used to simulate the biomechanical and aeroacoustic mechanisms of vocal fold oscillation as a function of its ECM property and biomaterial location.[2,183,184] Agent-based computational models were also developed to numerically simulate the cellular and molecular biology of vocal folds with respect to injury and repair.[44,185–187] One future direction is to couple these physical and biological models into a multiscale computing platform to enable the systematic investigation of how individual biomaterial parameters affect vocal fold immunological and repair processes across microscopic (cell activity), mesoscopic (ECM microstructure), and macroscopic levels (tissue mechanics). In silico models can then be applied to effectively narrow the biomaterial options for immune-centric design. Further development and evolution of computer simulations will likely become a standard, widely used approach alongside conventional in vitro and in vivo approaches for regenerative biomaterial design and evaluation.
Patrick Coburn graduated from the University of Manchester where he received his M.Eng. in biomedical materials science (2015). He is currently a Ph.D. candidate at McGill University in the laboratory of Dr. Li-Jessen. His current research interests include the immune response to vocal fold biomaterials and the application of microfluidic technology to study the impact of air pollution on the vocal folds.

Xuan Li is a Ph.D. candidate at McGill University in the laboratory of Dr. Jianyu Li. She received her B.Eng. in microelectromechanical systems engineering at Northwestern Polytechnical University (2016) and later gained her M.Eng. in instrument science and technology at the University of Science and Technology of China (2019).

Jianyu Li is an assistant professor within the Department of Mechanical Engineering at McGill University. He obtained his B.Eng. in chemical engineering at Zhejiang University (2010), received his Ph.D. from Harvard University (2015), and completed his postdoctoral training in bioengineering at Wyss Institute (2017). The research of Professor Li’s group is interdisciplinary in nature, focusing on the interface between mechanics, materials, and biomedical engineering. Fundamentally, his research focuses on biomaterial innovation for enabling technologies and therapies to improve health via principles of mechanics, chemistry, physics, biomimetics, and biology.

Yo Kishimoto is an associate professor of Department of Otolaryngology-Head and Neck Surgery, Kyoto University, Japan. His research interests include vocal fold mucosal biology and immunology and regenerative medicine of the upper airway organs. He graduated from the faculty of medicine, Kyoto University, in 2001, and finished residency training as an ENT. He received his Ph.D. in medicine from Kyoto University in 2011 and completed a postdoctoral fellowship at University of Wisconsin-Madison during 2009–2013.

Nicole Li-Jessen is an associate professor at McGill University. A speech language pathologist and computer biologist by training, she obtained her clinical degree at the University of Hong Kong. She received her Ph.D. from the University of Pittsburgh and completed her postdoctoral training in tissue engineering at the University of Wisconsin-Madison. Her laboratory focuses on advancing personalized medicine in voice and upper airway disorders through the development of point-of-care diagnostics and regenerative biomaterials. Her team integrates in vitro, in vivo, and in silico methods to tailor therapeutic approaches for vocal fold repair and regeneration.