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Review article

Biomaterial-enhanced treg cell immunotherapy: A promising approach for transplant medicine and autoimmune disease treatment

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ABSTRACT

Regulatory T cells (Tregs) are crucial for preserving tolerance in the body, rendering Treg immunotherapy a promising treatment option for both organ transplants and autoimmune diseases. Presently, organ transplant recipients must undergo lifelong immunosuppression to prevent allograft rejection, while autoimmune disorders lack definitive cures. In the last years, there has been notable advancement in comprehending the biology of both antigen-specific and polyclonal Tregs. Clinical trials involving Tregs have demonstrated their safety and effectiveness. To maximize the efficacy of Treg immunotherapy, it is essential for these cells to migrate to specific target tissues, maintain stability within local organs, bolster their suppressive capabilities, and ensure their intended function's longevity. In pursuit of these goals, the utilization of biomaterials emerges as an attractive supportive strategy for Treg immunotherapy in addressing these challenges. As a result, the prospect of employing biomaterial-enhanced Treg immunotherapy holds tremendous promise as a treatment option for organ transplant recipients and individuals grappling with autoimmune diseases in the near future. This paper introduces strategies based on biomaterial-assisted Treg immunotherapy to enhance transplant medicine and autoimmune treatments.

1. Introduction

The orchestration of immune regulation relies heavily on a specialized subset of T cells known as regulatory T cells (Tregs), which have emerged as pivotal guardians of immune homeostasis and prevent autoimmunity. Tregs operate by actively suppressing self- and allo-antigen-reactive immune cells, thus averting autoimmune responses and preserving transplantation tolerance [1–4]. Their diverse origins and regulatory mechanisms, encompassing both thymus-derived Tregs (tTregs) and peripherally induced or induced Tregs (pTregs/iTregs), contribute to their remarkable versatility in immune regulation. Tregs exhibit distinct developmental pathways, with tTregs differentiating within the thymus under robust T cell receptor (TCR) signaling, while pTregs/iTregs arise from naïve conventional $CD4^+$ T cells (Tconvs) in peripheral tissues upon antigen stimulation and exposure to specific Treg-associated cytokines like interleukin-2 (IL-2) and transforming growth factor-beta (TGF-β). These intricacies underline the complexity of Treg biology and its fundamental role in maintaining immune tolerance [5,6].

In recent years, the growing field of biomaterial-based strategies has revolutionized the manipulation of immune cells for therapeutic purposes. Biomaterials, derived from natural or synthetic sources, present versatile platforms for modulating immune responses due to their biodegradability and biocompatibility. Leveraging these properties, researchers have developed various biomaterial-based approaches to provoke Tregs, offering promising avenues for treating autoimmune diseases and improving outcomes in transplantation medicine. Recent advancements in biomaterial-based strategies have opened novel avenues for harnessing Tregs in immunotherapy. By exploiting biomaterial properties, researchers can manipulate Treg function, enhance their proliferation, and improve their therapeutic efficacy. These innovative approaches hold immense potential for restoring immune balance and

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revolutionizing treatment modalities for a range of pathological conditions [7,8].

In this review, we provide insights into the diverse origins and regulatory mechanisms of Tregs, emphasizing their critical role in immune regulation. Additionally, we delve comprehensively into the applications of biomaterials in provoking Tregs, discussing their potential to enhance therapeutic interventions in autoimmune diseases and transplantation medicine. Through a comprehensive examination of recent advances, challenges, and future perspectives, we aim to shed light on the evolving field of biomaterial-based Treg modulation and its profound implications for clinical practice.

1.1. Outlook of regulatory T cells

Since the discovery of suppressor T cells in 1969, the field of regulatory T cells (Tregs) has experienced remarkable growth [9]. In 1995, Sakaguchi et al. demonstrated that a specific subset of $CD4^+$ T cells expressing the IL-2 receptor α-chain (CD25) could prevent autoimmunity. These Tregs, commonly marked by CD4+/CD25high/Foxp3+, make up approximately 5–10 percent of $CD4^+$ T cells in the bloodstream and are responsible for maintaining tolerance in humans [1–4]. They actively maintain immunological transplantation tolerance and self-tolerance, by inhibiting self- and allo-antigen-reactive immune cells. Tregs play a pivotal role in preventing the activation and proliferation of self-reactive T cells that manage to escape elimination in the thymus. The development and function of Tregs are controlled by the transcription factor forkhead box P3 (Foxp3). Defects in this process can result in inflammatory and autoimmune disorders in both humans and mice [10–12]. Furthermore, the presence of the CD127 (IL-7 receptor) exhibits an inverse relationship with Foxp3 expression and the suppressive capabilities of Tregs. Consequently, Tregs are now defined as Foxp3+/CD127low/CD25high/CD4+ cells [13].

The thymus is a critical organ for generating Tregs, and their differentiation depends on the duration and strength of T cell receptor (TCR) signaling after exposure to self-antigens. In the thymus, immature single-positive CD4 T cells receive TCR signals of varying strength, resulting in the determination of their fate. Strong TCR signals allow cells to avoid deletion and commit to differentiation into Tregs, which are referred to as thymus-derived Tregs (tTregs) [5,6]. The alternate pathway to generate Tregs involves the differentiation from naïve conventional CD4+/Foxp3- T cells (Tconvs) in peripheral tissues (*in vivo*), such as in gut, adipose tissues, skin, lung, and central nervous system (CNS) or outside the body (*in vitro* or *ex vivo*) upon exposure to antigen stimulation alongside specific Treg-associated cytokines, such as interleukin-2 (IL-2) and transforming growth factor-beta (TGF-β). When Foxp3+ Tregs are generated *in vivo*, they are called peripherally induced Tregs (pTregs), and when produced *in vitro* or *ex vivo*, they are termed induced Tregs (iTregs).

Differentiating between tTregs and pTregs/iTregs poses a challenge. To discern these subsets, markers such as the membrane protein neuropilin-1 (Nrp1), transcription factor Ikzf2 (Helios), RAR-related orphan receptor gamma (RORγt), and GATA Binding Protein 3 (GATA3) have been commonly suggested. Most tTregs are Helios+/Nrp1-/GATA3+, while pTregs/iTregs often show Helios-/Nrp1+/RORγt + expression [14–16]. Additional factors, such as thymic stromal lymphopoietin (TSLP), T cell-specific DNA-binding protein (TCF-1), ICOS/ICOSL, Wnt/β-catenin signaling pathway, and NFAT/AP1, also play roles in controlling the transcriptional process of human tTreg differentiation [17–21]. The expression of Foxp3 relies on the presence of γ-chain cytokines such as IL-17, IL-2, and IL-15, and the suppression of the mTOR/Akt/PI3K signaling pathway. This pathway is essential for Foxp3 expression, and consequently, for the development and suppressive function of tTregs [18,22].

The differentiation of pTregs is primarily induced by exposure to foreign antigens, including those encountered in the respiratory and gastrointestinal tracts. They also play a role in maternal-fetal tolerance and tolerance toward commensal microbiota [23–25]. The expression of Foxp3 and the formation of pTregs is most effective when TCR signaling is coupled with reduced co-stimulator signaling, such as CD28 [26]. Additionally, the presence of low levels of co-stimulatory molecules (e.g., B7-1/B7-2) and the secretion of anti-inflammatory cytokines by tolerogenic dendritic cells (DCs) promote the differentiation of pTregs [27]. In addition to suboptimal TCR and co-stimulator signaling, the presence of TGF-β and IL-2 is necessary for pTreg generation [28]. Inducible T regulatory type 1 (Tr1) cells are a subgroup of pTregs distinguished by their capacity to produce the anti-inflammatory cytokine IL-10. Upon stimulation, these cells can temporarily increase their levels of Foxp3 expression and have demonstrated their ability to sustain tolerance in peripheral tissues, regulate the responses of effector T cells in autoimmune conditions, and hinder the rejection of transplanted organs. The ability to cultivate antigen-specific Tr1 cells *in vitro* has encouraged their practical application in clinical settings related to transplant medicine and autoimmune diseases [29].

Both tTregs and pTregs are found in various peripheral organs, yet the most prevalent generation of Tregs occurs within the gut [30]. Gut Tregs encompass both tTreg and pTreg populations. tTregs typically originate in the thymus and predominantly recognize self-antigens. Once they leave the thymus, tTregs become active in secondary lymphoid organs and further differentiate into effector Tregs within the gut. This transformation is triggered by signals such as IL-2, IL-33, TGF-β, and signals from microbiota, and their metabolites [30]. Recently, an alternative pathway for tTreg development has emerged in research. These specialized tTregs, originating from CX3CR1+ DCs in the intestine, possess the capability to migrate to the thymus and present antigens from commensal microbiota to thymocytes, inducing their differentiation into tTregs. These specialized tTregs carry TCRs capable of recognizing foreign antigens and relocate to the gut, contributing significantly to establishing peripheral tolerance [31]. Conversely, pTregs originate from Tconvs stimulated by foreign antigens presented by RORγt + type-3 innate lymphoid cells (ILC3s) and/or RORγt + DCs in the gut-draining lymph nodes [32,33]. Once activated within the gut, pTregs acquire the suppressive characteristics of effector Tregs. The significance of TGF-β in pTregs/iTregs development has been well established since it has the capacity to stimulate Foxp3 expression in Tconvs. In mice lacking TGF-β, tTreg development in the thymus proceeds normally, but there is a noticeable decrease in pTregs [34,35]. The TGF-β signaling pathway involves the activation of Smad transcription factors 2 and 3 (Smad2 and Smad3), forming a complex with Smad4. Similar to TGF-β knockouts, mice lacking Smad2 and Smad3 exhibit normal tTregs in the thymus, yet they display reduced Treg counts in the periphery and diminished Foxp3 induction [36,37]. Fig. 1 illustrates Treg development, differentiation, and the key factors involved in various tissue Tregs.

The differentiation of Tconvs into tTregs and pTregs/iTregs is tightly regulated by the Foxp3 locus. This gene locus contains four intronic enhancers known as conserved noncoding sequences (CNSs) that play crucial roles in this process [38]. Upon signaling through TCR/CD28, TGF-β, and IL-2, downstream transcription factors bind to these CNSs, resulting in Foxp3 expression and the subsequent development of tTregs and pTregs/iTregs [39,40]. Notably, CNS1 has two consecutive Smad-binding sites that aid in generating iTregs/pTregs [36–38]. Downstream transcription factors associated with TCR/CD28 and IL-2, such as NFAT, AP-1, *c*-Rel, and JAK/STAT5, bind to Foxp3 promoter, CNS2, and CNS3, leading to tTregs development and stability in pTregs/iTregs [39]. Recently, Satb1, a chromatin organizer, was identified as binding to the CNS0 region and activating Treg cell-specific super enhancer before the Foxp3 promoter. Satb1 also influences other genes linked to Tregs, such as CTLA-4 and IL2-Rα, during the initial stages of tTreg cell differentiation. However, the specific induction of Satb1 and its exclusive binding to Treg cell-specific super enhancer remain unclear [41]. A depiction of the CNSs regions and related transcription factors can be found in Fig. 2.

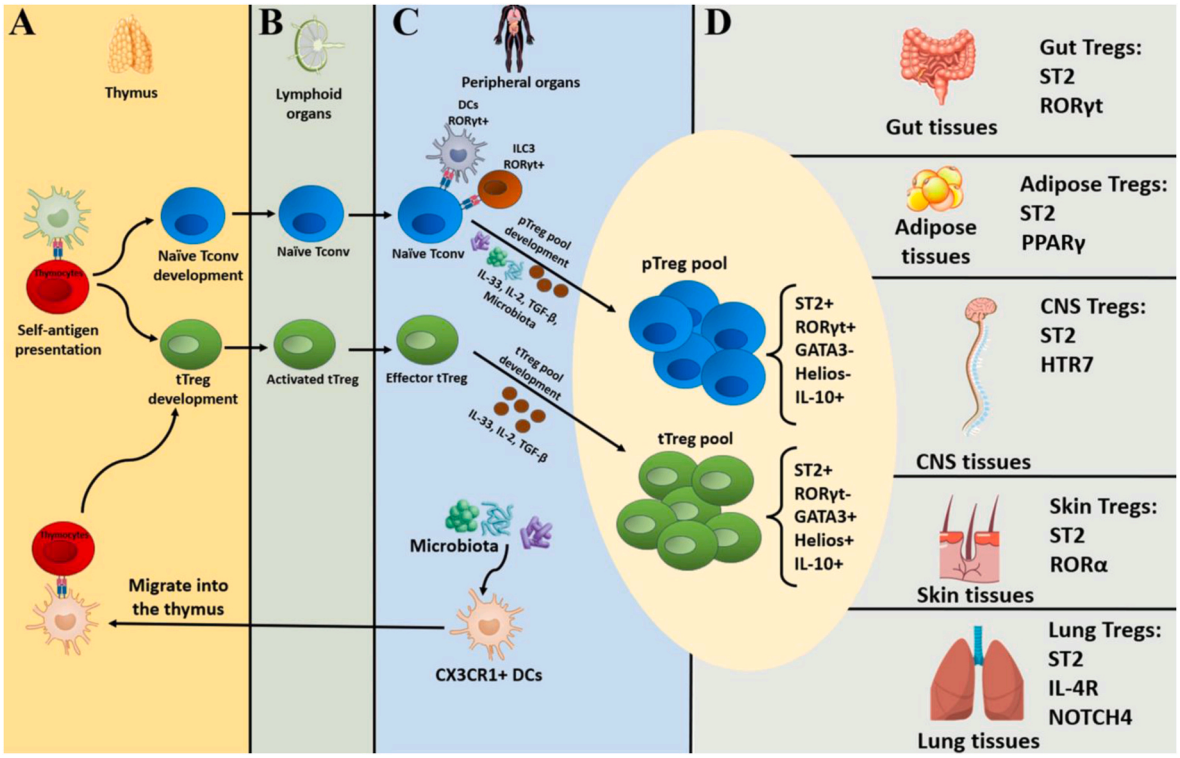

Fig. 1. Treg development, differentiation, and the key factors: (A) Recognition of self-antigens in the thymus leads to the development of both tTregs and Tconv cells, which then migrate to lymphoid organs **(B)**. **(C)** In peripheral organs, both tTregs and Tconv cells receive Treg-specific cytokines like IL-33, IL-2, and TGF-β, and encounter microbiota and their byproducts, leading to the formation of tTregs and pTregs pools in peripheral tissues. For example, pTregs originate from Tconv cells stimulated by RORγt + ILC3s and/or RORγt + DCs, along with Treg-specific cytokines in the gut. Additionally, CX3CR1+ DCs in the intestine can migrate to the thymus and present antigens from commensal microbiota to thymocytes, prompting their differentiation into tTregs. pTregs in peripheral tissues exhibit ST2+/RORγt+/GATA3-/Helios-//IL-10+, while tTregs in peripheral tissues are mostly ST2+/RORγt-/GATA3+/Helios+//IL-10+. **(D)** However, effector Tregs expressing ST2 migrate to peripheral organs, and their equilibrium is maintained via the IL-33/ST2 pathway. Different tissue-specific Tregs exhibit distinct key factors: gut Tregs demonstrate higher RORγt levels, adipose Tregs show elevated PPARγ, CNS Tregs express more Htr7, skin Tregs display higher RORα levels, and lung Tregs have increased IL4R and Notch4 expression. These characteristics are primarily observed in studies conducted on mice. **Abbreviations arranged in alphabetical order: CX3CR1:** CX3C motif chemokine receptor 1, **DCs:** Dendritic cells, **GATA3:** GATA Binding Protein 3, **Helios:** Transcription factor Ikzf2, **HTR7:** Serotonin receptor 7, **IL-10:** Interleukin-10, **IL-2:** Interleukin-2, **IL-33:** Interleukin-33, **IL4R:** IL-4 receptor, **ILC3:** Type-3 innate lymphoid cell, **NOTCH4**: Neurogenic locus notch homolog 4, **PPARγ:** Peroxisome proliferator-activated receptor gamma, **pTregs:** Peripheral-Derived Tregs, **RORα:** RAR-related orphan receptor alpha, **RORγt:** RAR-related orphan receptor gamma, **ST2:** IL-33 receptor, **TGF-β:** Transforming Growth Factor Beta, **tTregs:** Thymic-Derived Natural Tregs.

Another regulatory mechanism for Treg development involves the methylation status of the Treg-specific demethylated region (TSDR) within the CNS2 enhancer of the Foxp3 locus. Demethylation of the CNS2 region maintains Foxp3 expression by enabling stable binding of transcription factors like the ATF/CREB or RunX1/CBFβ complex to the demethylated CNS2 [38]. This demethylation is mediated by ten-eleven-translocation (Tet) enzymes and 5-hydroxymethylcytosine (5 hmC). Unlike tTregs, iTregs exhibit instability, which poses a challenge in using *ex vivo*-expanded iTregs for adoptive immune therapy. Stability in Foxp3 expression in tTregs is maintained through the demethylation of CpG islands in the CNS2 region of the Foxp3 locus [42–44]. Hence, identifying the factor(s) responsible for inducing demethylation *ex vivo* could be key in establishing methods to generate stable iTregs for clinical applications.

External factors can also regulate CNSs regions, influencing iTregs/pTregs induction *in vitro*, *ex vivo*, and *in vivo*. For instance, retinoic acid induces the binding of retinoid X receptor (RXR) and retinoic acid receptor (RAR) to CNS1, increasing histone acetylation in Smad3 at CNS1, enhancing Smad3 binding, and promoting iTregs/pTregs induction [45]. Vitamin D3 interacts with the vitamin D receptor (VDR), forming a heterodimer with RXR, which binds to the vitamin D response element on the Foxp3 enhancer CNS1 in naïve T cells. This interaction prompts Foxp3 expression and contributes to iTregs/pTregs generation [46]. Additionally, butyrate, as a histone deacetylase (HDAC) inhibitor, enhances acetylation in the Foxp3 promoter and CNSs, fostering iTregs generation [47]. Vitamin C has been shown to enhance Tet activity, facilitating the demethylation of the CNS2 region and increasing Foxp3 stability in TGF-β-induced iTregs [48].

The primary function of Tregs is to control or regulate self-reactive T cells and provide self-tolerance through direct and indirect mechanisms. Tregs directly influence target cells by secreting immunosuppressive cytokines like IL-10, IL-35, and TGF-β1, the immunomodulatory molecule kynurenine, and cytotoxic products (e.g., perforin and granzyme). Indirect mechanisms involve generating extracellular adenosine and AMP as immunosuppressive molecules via CD39/CD73 expressed on their surface, expressing CTLA-4 which suppresses effector T cells by endocytosing CD80/CD86 molecules expressed on DCs, and trapping IL-2 via CD25 expression on their surface and effectively "starving" surrounding cells of this cytokine. The choice of suppression mechanism depends on the specific target cell and microenvironment, with Tregs utilizing different mechanisms based on the context [49,50].

In addition to conventional $CD4^+$ Tregs, $CD8^+$ Tregs represent a compelling subset crucial for maintaining immune balance and preventing autoimmunity, although their biology is still unfolding amidst the well-established understanding of $CD4^+$ Foxp3+ Tregs [51]. A noteworthy aspect of $CD8^+$ Tregs is their heterogeneity and subsets, with various $CD8^+$ T cell subsets demonstrating regulatory properties, referred to as "CD8+ Tregs." Examples include CD8+/Foxp3+ Tregs observed in tissue transplantation and alloantigen-induced immune responses, as well as CD8+/CD103+ Tregs induced from naïve $CD8^+$ T

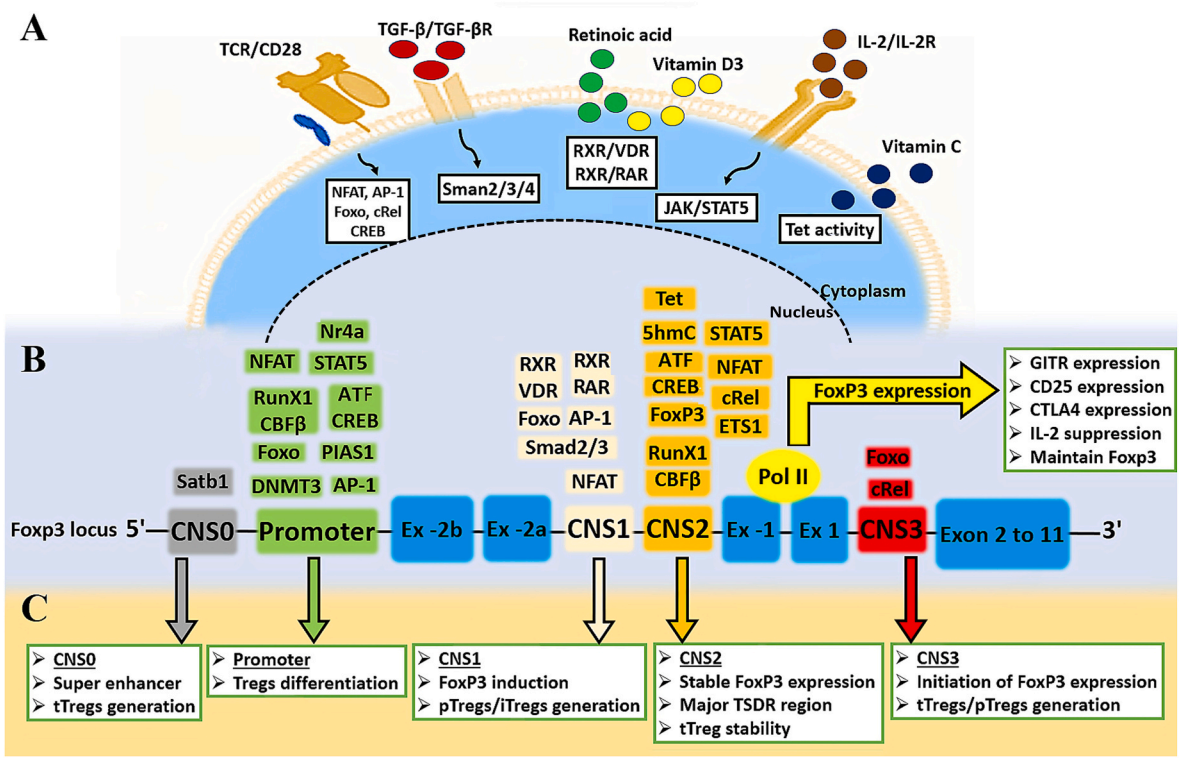

Fig. 2. CNSs regions and related transcription factors: (A) T cell outer membrane: T cells express various transcription factors in response to different external stimulants like TGF-β, IL-2, vitamins D and C, retinoic acid, and TCR/CD28 stimulation, alongside internal regulators such as Tet enzymes. These factors bind to the FoxP3 promoter and enhancers, known as CNSs within the FoxP3 locus. **(B) T cell nucleus**: The FoxP3 locus comprises four CNS regions and 14 exons. These CNS regions enhance and regulate Foxp3 expression in response to downstream transcription factors (Shown with same colors) and the methylation status of the TSDR regions. The expressed Foxp3 protein acts as a master regulator transcription factor, promoting the expression of Treg-specific genes like GITR, CD25, CTLA-4, IL-2 suppression, and the maintenance of Foxp3 expression. **(C) CNSs outcomes:** Each CNS region responds to specific transcription factors and methylation statuses, thereby contributing to the generation of different Treg subsets such as tTregs, pTregs, and iTregs. **Abbreviations arranged in alphabetical order: 5 hm C**: 5-hydroxymethylcytosine, **AP-1:** Activator protein 1, **ATF**: Activating transcription factor, **CBFβ**: Core Binding Factor Beta, **CNS**: Conserved Noncoding Sequences, **CREB**: cAMP Response Element-Binding Protein, **cRel**: *c*-Rel homology Protein, **CTLA4**: Cytotoxic T-lymphocyte associated protein 4, **DNMT3**: DNA Methyltransferase 3, **ETS1**: Protein *C*-ets-1, **Ex**: Exon, **Foxo**: Forkhead box O, **Foxp3**: forkhead box P3, **GITR**: Glucocorticoid-induced TNFR-related protein, **IL-2:** Intelukin-2, **IL-2R**: Intelukin-2 receptor, **iTregs**: Induced Tregs, **NFAT**: Nuclear Factor of Activated T Cells, **Nr4a**: Nuclear Receptor Subfamily 4 A, **PIAS1**: Protein Inhibitor of Activated STAT1, **Pol II**: RNA polymerase II, **pTregs**: Peripheral-Derived Tregs, **RAR**: retinoic acid receptor, **RunX1**: Runt-related transcription factor 1, **RXR**: retinoid X receptor, **Satb1**: Special AT-rich sequence binding protein 1, **Smad2/3/4**: Smad transcription factors 2, 3, and 4, **STAT5**: Signal transducer and activator of transcription 5, **TCR**: T cell receptor, **Tet**: ten-eleven-translocation enzymes, **TGF-β**: Transforming Growth Factor Beta, **TGF-βR**: Transforming Growth Factor Beta Receptor, **Tregs**: Regulatory T cells, **TSDR**: Treg-specific demethylated region, **tTregs**: Thymic-Derived Natural Tregs, **VDR**: Vitamin D Receptor.

cells *in vitro*. Additionally, CD122high/Ly49+/CD8+ Tregs, found in naïve mice, rely on the transcription factor Helios and IL-15, playing essential roles as non-redundant regulators of germinal center reactions. However, despite progress, questions persist regarding their precise differentiation, function, and true identity, with comparisons to $CD4^+$ follicular Tregs remaining relevant [52–54]. This ongoing exploration highlights the potential of $CD8^+$ Tregs in immune-related diseases, offering promise for future therapeutic interventions.

By continuously monitoring and regulating self- and allo-reactive T cells, Tregs play a vital role in reinstating tolerance in autoimmune conditions and transplanted organs. In transplantation medicine, long-term graft acceptance remains a challenge despite improvements in short-term outcomes through better donor-recipient matching and immunosuppressive regimens [55]. Likewise, existing therapies for autoimmune disorders frequently depend on the use of immunosuppressive medications. While these regimens initially control immune responses, immunocompromised patients are vulnerable to infections, cancers, and organ failures, which can result in patient death or graft rejection [56]. Based on the strong potential of Tregs to preserve immune tolerance, immunotherapy targeting Tregs has been proposed as a potentially effective tool for restoring immune balance. This has led to the establishment of numerous clinical trials in the field of immunotherapy worldwide, with a focus on harnessing Tregs for the treatment of transplantation and autoimmune diseases (as reviewed in Ref. [57]).

Adoptive Treg therapies demonstrate considerable potential in addressing various diseases, yet they encounter significant limitations that merit thorough consideration [58–60]. Firstly, the lack of antigen specificity poses a notable hurdle. While Treg cells possess inherent immunosuppressive capabilities, their broad specificity may lead to generalized immunosuppression. To enhance efficacy and minimize unintended immune suppression, there is a pressing need to target antigen-specific Treg cells, which have shown increased potency in preclinical models of autoimmune diseases and transplantation. Secondly, the heterogeneity within the Treg population presents a complex scenario. Treg cells exhibit functional diversity, with some subsets displaying greater effectiveness in suppressing immune responses than others. Strategies aimed at enriching for highly suppressive Treg subsets are vital to optimize therapeutic outcomes. Additionally, addressing challenges such as poor persistence, functional impairment in disease states, and *in vivo* plasticity are essential for the successful translation of Treg cell therapies from preclinical research to clinical applications [61–63].

Despite these challenges, ongoing clinical trials are shedding light on successful manufacturing processes and favorable safety profiles for Treg cell therapy. Advances in Treg cell engineering and antigen-specific approaches show potential in overcoming these limitations, thereby

expanding the therapeutic possibilities of Treg-based treatments. Efforts to tackle these obstacles are crucial for fully realizing the therapeutic benefits of adoptive Treg cell therapies in clinical settings. To maximize the efficacy of Treg immunotherapy, it is imperative for these cells to migrate to specific target tissues, maintain stability within local organs, enhance their suppressive capabilities, and ensure the longevity of their intended function. Pursuing these objectives, the utilization of biomaterials emerges as an appealing supportive strategy for Treg immunotherapy, addressing these challenges effectively. Consequently, the prospect of employing biomaterial-enhanced Treg immunotherapy holds immense promise as a treatment option for organ transplant recipients and individuals grappling with autoimmune diseases in the near future.

1.2. Biomaterial-based strategies to provoke regulatory T cells

Various biomaterials have been designed to interact with biological systems for medical purposes, and they are utilized in a wide range of applications, including nanomedicine, drug delivery, tissue engineering, implants, diagnostics, and surgical sutures [64–68]. These materials can be derived from natural resources or synthesized in the laboratory through various chemical approaches. Naturally-derived biomaterials are categorized into three main types: **I)** polysaccharide-based (e.g., cellulose, glucose, and chitin/chitosan); **II)** protein-based (e.g., fibrin, silk, and gelatin); **III)** acellular tissue-based (e.g., liver, heart and blood vessels). Synthetic biomaterials are also classified into three categories: **I)** polymeric-based (e.g., poly (lactic-*co*-glycolic acid) (PLGA), polyethylene glycol (PEG) and citrate-based polymers); **II)** ceramic-based (e.g., calcium phosphate, bioactive glass, hydroxyapatite); **III)** metal-based (e.g., gold, platinum, and iron). Beyond the nature of biomaterials, they can be used to fabricate various engineering platforms, such as bio-links, scaffolds, hydrogels, and nanoparticles. When designing biomaterials, certain properties need to be considered to ensure their effectiveness and compatibility with the body. These properties include biodegradability; biocompatibility; mechanical strength; low toxicity; half-life; and non-immunogenicity. Some of these compounds have been approved by the U.S. Food and Drug Administration (FDA) for diagnostic and therapeutic uses due to their advantageous properties [69].

The application of various biomaterials to manipulate the immune system has been extensively documented. In this regard, biomedical engineers have developed various approaches to strengthen and/or attenuate the immune system using biomaterials *in vitro* or *in vivo* [7,8]. These approaches include delivering antigens to DCs [70], macrophage polarization [71], provoking T lymphocyte cell responses [72], inducing tolerogenic DCs [73] and Tregs [74], controlled-release of cytokines [75], immune-regulating camouflage [76], adoptive immune cell therapies [77], and tracking various immune cells [78]. Several studies have been conducted to improve the efficacy of immunotherapies for cancer and infections using biomaterials. According to the two-signal theory of T cell activation, signal-1 involves the engagement of the TCR by the peptide-major histocompatibility (peptide-MHC) complex, while signal-2 arises from co-stimulatory molecules on antigen-presenting cells (APCs). However, cancer and infections disrupt these pathways through various mechanisms, evading the immune system response. To address this, biomaterial-based artificial APCs (aAPCs) could provide two signals for the full activation and proliferation of antigen-specific T lymphocytes [79].

On the other hand, there has been extensive research on the use of biomaterials to provoke Tregs in the treatment of organ transplant medicine and autoimmune diseases. Tregs are uniquely suited to alleviate uncontrolled hyperimmune responses that cause severe damage to transplanted or healthy tissues, and biomaterial-based approaches could play a crucial role in improving and facilitating the functionality of Treg therapies [80,81]. The ultimate goal of biomaterial-stimulated Tregs is to increase therapeutic efficacy based on Tregs. In this particular case, a population of efficient effector Tregs can be recruited to target tissues, persist in pathological tissue environments, and actively provoke favorable therapeutic effects. To these aims, biomaterials can control the release of Tregs-stimulated cytokines (e.g., TGF-β1, and/or IL-2), and act as a slow stimulating source of Tregs over time [82]. They can also co-deliver modulatory signals and self- or allo-antigens to Tregs [83], induce antigen-specific Tregs [84], act as aAPCs and expand effector/-memory Tregs [85]. To enhance the efficiency of signal delivery using biomaterials, these approaches can be optimized by using targeted biomaterials to deliver immunomodulating agents to Tregs. Figs. 3–8 depict several strategies through which biomaterials can enhance Treg therapy. These approaches involve mechanisms like expanding pools of antigen-specific or poly-clonal Tregs, increasing Tregs populations from a low baseline, improving Tregs functionalities, and tracking Tregs, among others. These strategies are discussed in detail below. They have the potential to enhance the effectiveness and strength of immunotherapies by promoting Tregs against various complications and diseases, both *in vivo* (pTregs) and *in vitro* (iTregs) settings.

2. Direct Functions of biomaterials on Treg Activation and expansion

One of the promising approaches to expanding Tregs is by directly manipulating them using biomaterials. These biomaterials have been ingeniously designed to deliver specific signals, acting as controlled platforms to regulate Treg behavior and function. By mimicking the natural microenvironment of Tregs, these biomaterial-based release systems offer precise control over immunomodulation. In this section, we will explore various biomaterial-based strategies that directly contribute to Treg activation and expansion.

2.1. Biomaterial-controlled release of treg-specific signals

Treg-specific signals are critical for the proper functioning of Tregs and maintaining immune balance in the body. In the field of biomaterials, a significant breakthrough has been the development of platforms that can release these specific signals in a controlled manner. This can be achieved by encapsulating cytokines or other immunemodulatory molecules within biomaterial matrices, enabling localized delivery. The precise control of signal release allows for targeted and sustained effects on Tregs, finely tuning the immune response for potential therapeutic applications (Table 1 and Figs. 3 and 4) [80].

The survival and function of Treg subsets heavily depend on cytokine signaling such as IL-2 and TGF-β1. Although low-dose recombinant cytokines have shown promise in certain conditions, their short half-lives require frequent doses for effectiveness while minimizing off-target signaling. Additionally, the administration of soluble cytokines is hindered by their pleiotropic toxic effects. To overcome these challenges, Li et al. designed a TGF-β1-releasing PLGA microparticle, an FDA-approved degradable polymer, for localized cytokine delivery. The microparticles were carefully designed to have a microscale size (54 ± 51 μm) to prevent phagocytosis and enhance retention at the transplanted beta-islets site. This innovative approach effectively generated Foxp3+/CD3+ Tregs *in vitro* and *in vivo* (Fig. 3A–B) [80].

Similarly, a PLGA scaffold was designed to seed allogeneic beta-islets and incorporate IL-33, a cytokine of interest for inducing Tregs, resulting in the generation of Foxp3+/CD4+ and ST2+ (IL-33 receptor) Tregs (Fig. 3C) [87]. To achieve robust Treg expansion, McHugh et al. co-encapsulated TGF-β1 and IL-2 into spherical PLGA nanoparticles, inducing naïve T cells to differentiate into Foxp3+/CD4+ Tregs that secreted IL-10, IL-2, and TGF-β1 *in vitro* after 5 days. To avoid the pleiotropic toxic effects of cytokines, they designed anti-CD4 functionalized PLGA nanoparticles to deliver these cytokines to naïve T cells with controlled-release capacity up to 14 days. The encapsulation of IL-2 and TGF-β1 in PLGA demonstrated enhanced bioactivity compared to soluble cytokines. This enhancement could be attributed to increased local concentration gradients that allow for their controlled release over

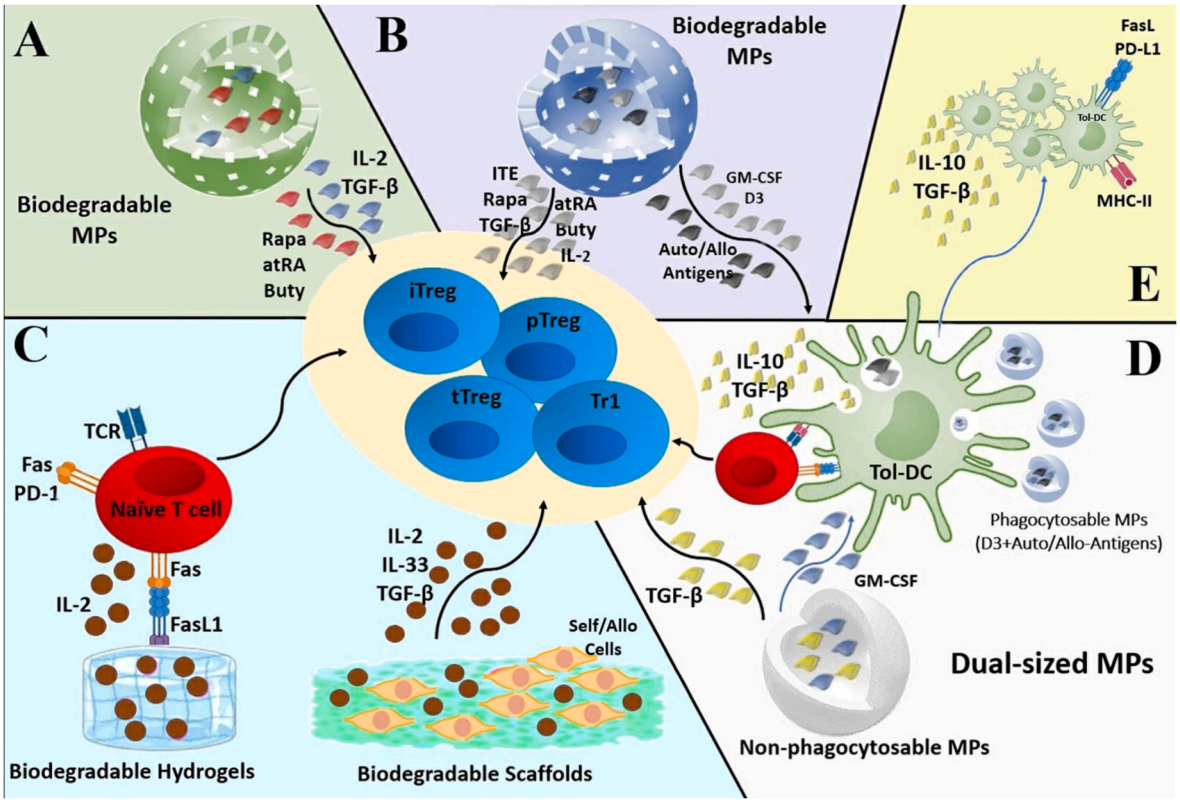

Fig. 3. Controlled Release of Treg-Specific Signals: (A) Biomaterial-based platforms can control the release of specific-Treg signals (e.g., immunomodulators like Rapa, atRA, ITE, and Buty; cytokines such as IL-2 and TGF-β) over time, and they can also deliver auto- or allo-antigens alongside these signals **(B)**, leading to the expansion of Tregs. **(C)** Biomaterial-based hydrogels and scaffolds, when functionalized with Treg modulators (e.g., FasL1, IL-2, IL-33, and TGF-β), can trigger tissue-specific Tregs in conjunction with self or allo cells. **(D)** In the dual-sized MP system, phagocytosable MPs are employed to deliver modulators (e.g., vitamin D3) and auto- or allo-antigens to DCs, while non-phagocytosable MPs are used for the controlled release of Treg modulators (e.g., TGF-β) and DC differentiation cytokines (e.g., GM-CSF), resulting in the expansion of Tol-DCs **(E)** and Tregs. **Abbreviations arranged in alphabetical order: atRA**: All-trans Retinoic Acid, **Buty**: Butyrate, **D3**: Vitamin D3, **FasL**: Fas Ligand, **GM-CSF**: Granulocyte-Macrophage Colony-Stimulating Factor, **IL-10**: Interleukin-10, **IL-2**: Interleukin-2, **IL-33**: Interleukin-33, **ITE**: 2-(1′Hindole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester, **iTregs**: Induced Tregs, **MHC**: Major Histocompatibility Complex, **MPs**: Microparticles, **PD-1**: Programmed cell death protein 1, **PD-L1**: Programmed death-ligand 1, **pTregs**: Peripheral-Derived Tregs, **Rapa**: Rapamycin, **TCR**: T Cell Receptor, **TGF-β**: Transforming Growth Factor Beta, **Tol-DC**: Tolerogenic Dendritic Cells, **Tr1**: Inducible T Regulatory Type 1, **Treg**: Regulatory T cells, **tTregs**: Thymic-Derived Natural Tregs.

specific time frames [82].

Beyond the role of biomaterials in the controlled release of different cytokines, biomaterial-based combination therapy with cytokines, drugs, and modulators has been reported to induce Tregs. Researchers achieved the release of rapamycin (Rapa), IL-2, and TGF-β1 by separately encapsulating them in PLGA nanoparticles, allowing for controlled release over specific time frames. This reduced the PLGA size to the micrometer scale and proved as effective as soluble factors in inducing Tregs. The combination therapy resulted in the induction of a Treg phenotype (Foxp3+/FR4+/CD25+/GITR+) *in vitro* and effectively suppressed naïve T cell proliferation. The reported microparticles had no adverse effects on Tregs and were equally effective at inducing human Tregs [75]. In a similar study, McBride et al. encapsulated Rapa into mono-(6-amino-6-deoxy)-β-cyclodextrin (Rapa-βCD-NH2), a cyclic oligosaccharide, resulting in increased aqueous solubility of Rapa and its functionality on Tregs, maintaining Rapa bio-activity for one month. Combining Rapa-βCD-NH2 and soluble TGF-β1 synergistically increased Rapa functions and promoted the expansion of CD25+/CD4+/Foxp3+ Tregs (Fig. 3A–B) [86].

As part of biomaterial-based combination therapies, an innovative dual-sized microparticle system has been developed to enable the precise delivery of Treg signals, thereby promoting immune tolerance. In this strategy, microparticles are engineered with different sizes to encapsulate specific molecules. Keselowsky et al. conducted three studies using this approach. They utilized small, phagocytosable PLGA microparticles (less than 3 μm) to facilitate the phagocytosis of entrapped autoantigens and modulatory agents such as vitamin D3, Rapa, and all-trans retinoic acid (atRA), leading to tolerogenic antigen presentation. Conversely, larger, non-phagocytosable microparticles (more than 30 μm in diameter) were designed to provide sustained local release of cytokines like TGF-β1, IL-10, and GM-CSF. This dual-sized microparticle approach not only directly activates Foxp3+ Tregs but also creates a tolerogenic microenvironment, promoting Treg expansion (Fig. 3D–E) [88–90].

Another innovative biomaterial-based strategy aims to expand Tregs by directly acting as a trap for Treg-specific signals. In this approach, Zamecnik et al. fabricated polycaprolactone (PCL) nanowires conjugated to *anti*-IL-2, with size of in 37 mm length and 200 nm pore diameter. This materials-based strategy focuses on locally suppressing tissue-resident T cells that promote inflammation and activating Tregs to alleviate the disease. This injectable nanowire was well tolerated for several weeks, resisted phagocytosis, induced minimal foreign body response, and activated tissue-resident Tregs in a mice model of skin autoimmunity. Despite their ability to expand Tregs, the nanowires selectively neutralize specific T cell subsets by removing IL-2 from naïve T cells, affecting the local immune repertoire. This strategy shows promising results in a murine model of autoimmune disease [91].

Hydrogels can trap drugs or biomolecules within their three-dimensional structure. The rate of drug release can be adjusted by modifying the crosslinking density of the hydrogel. PEG hydrogels, specifically, are widely used in tissue engineering to create scaffolds that mimic the extracellular matrix, supporting cell adhesion, proliferation,

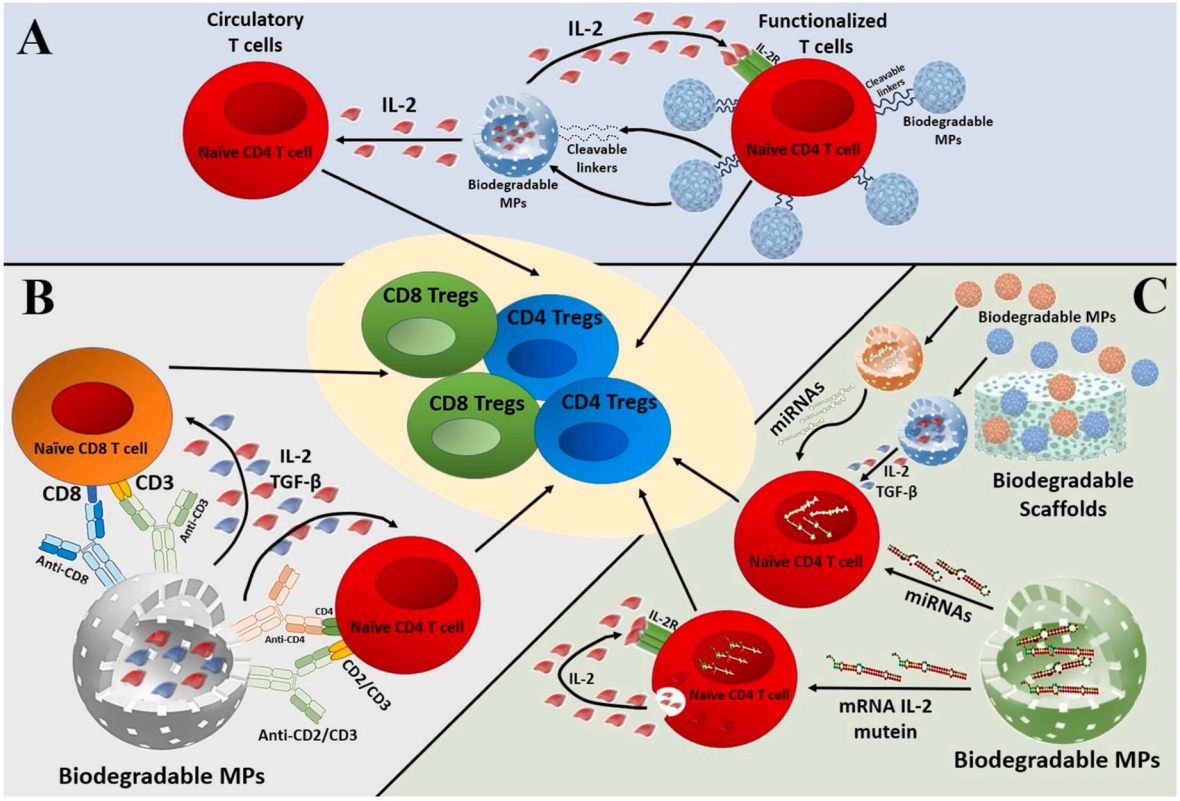

Fig. 4. Direct Functions of Biomaterials on Tregs Activation and Expansion: (A) The "backpack strategy" results in the activation of functionalized Tregs and, through a bystander mechanism, induces the conversion of circulatory T cells into Tregs. **(B)** Functionalized biomaterials equipped with anti-CD2/CD3/CD4/CD8 molecules deliver specific Treg signals (e.g., IL-2 and TGF-β) to T cells, thereby activating specific Treg subsets. **(C)** Biomaterial-based gene manipulation is employed to increase Treg gene expression of growth factors (e.g., IL-2 mRNA) or essential genes via miRNAs. This approach can be combined with Tregs' cytokines (e.g., IL-2 and TGF-β) to enhance the efficiency of Treg expansion. **Abbreviations arranged in alphabetical order: IL-2**: Interleukin-2, **IL-2R**: Interleukin-2 Receptor, **MPs**: Microparticles, **TGF-β**: Transforming Growth Factor Beta, **Treg**: Regulatory T cells.

and differentiation. They are also utilized in drug delivery, offering localized and sustained release of hydrophilic drugs or proteins. In this context, Medina et al. developed a PEG-hydrogel platform to co-deliver an analogue of IL-2, a Treg stimulator, and Fas Ligand 1 (FasL1), a Treg presenting ligand, to establish localized immune tolerance for transplanted beta-islets. The PEG-hydrogel efficiently prolonged the presence of IL-2 analogue at the graft site, while FasL1-presenting microgel mimicked the Treg extracellular matrix, optimizing tolerance signaling. The hydrogel platform had no negative impact on the effectiveness of the IL-2 analogue and FasL1, making it suitable for pancreatic islets in cell replacement therapies, and significantly increased Tregs. Although indefinite allograft function was not achieved, the study successfully demonstrates a robust biomaterial-based method for delivering bioactive immunomodulatory proteins, extending their presence to a specific site (Fig. 3C) [74]. This approach holds promise for treating autoimmune diseases and facilitating organ transplantation in localized areas.

In the context of modulating Tregs, butyrate (Buty), atRA, and Rapa play significant roles in regulating immune responses. These compounds enhance Treg function, promote the expansion of iTregs, and contribute to immune tolerance. To effectively deliver these signals to Tregs, Carey et al. developed a delivery system using PLGA microparticles. These microparticles were designed to carry type 1 diabetes (T1D)-relevant antigens along with one of the modulators (Rapa, atRA, or Buty) to induce the proliferation of antigen-specific Tregs. The designed PLGA microparticles, loaded with different modulators, were carefully crafted to be of phagocytosable size (2–6 μm in diameter) and successfully expanded antigen-specific Tregs. Interestingly, when the antigen was co-loaded with Rapa and atRA, it induced a higher level of Treg differentiation, suggesting that Tregs have varying responses to encapsulated-modulators (Fig. 3A–B) [83].

Biomaterials offer exciting possibilities for immune modulation through the controlled release of Treg-specific signals, including both cytokines and modulators. Encapsulating cytokines and drugs within biomaterial matrices shows promise in inducing Tregs while avoiding adverse effects. Nonetheless, these innovative strategies hold the key to transformative breakthroughs in personalized treatments for immune-related diseases (Table 1 and Fig. 3). Further research and optimization are crucial to fully harness their benefits.

2.2. *Nano-formulations for induction of antigen-specific tregs*

Desensitization therapy involves training the immune system to be less reactive to specific antigens, aiming to induce tolerance and produce antigen-specific Tregs. Traditional desensitization techniques like oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and subcutaneous immunotherapy (SCIT) are commonly used to manage allergies and immune-related conditions. However, these methods have their limitations, including unknown mechanisms, limited efficacy for all antigens, time-consuming processes, potential for adverse reactions, and varying levels of effectiveness. To address these limitations and induce tolerance against a wide range of antigens, researchers have focused on biomaterial-based strategies. Nano-formulations, which involve the use of biomaterial-based nanoparticles, have emerged as a promising approach. These nanoparticles release antigens slowly, enabling a more controlled and sustained delivery of auto- and allo-antigens. By presenting antigens in a tolerogenic context, these nano-formulations leverage the unique abilities of Tregs to recognize and suppress specific immune responses (Fig. 3B). As a result, they offer great potential

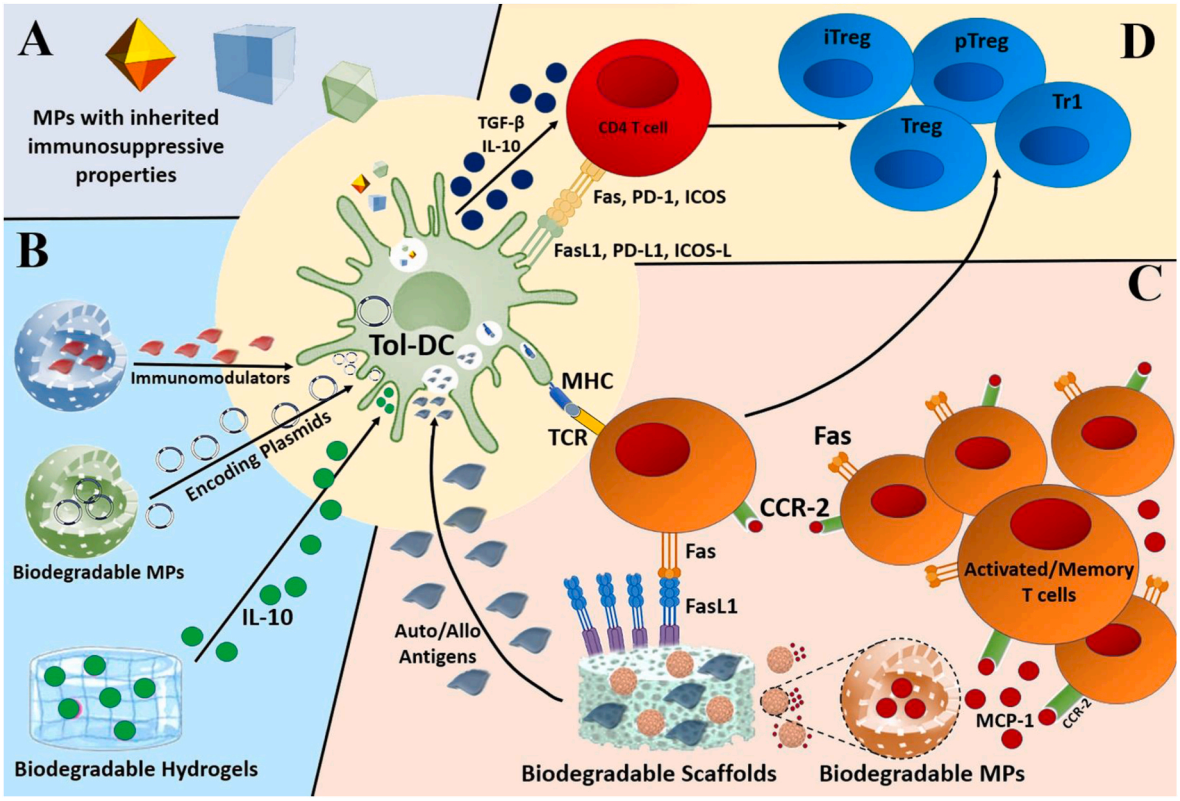

Fig. 5. Biomaterial-Induced Tol-Mic for Tregs: (A) Certain biomaterials possess inherent tolerogenic properties due to their unique physicochemical characteristics. When taken up by DCs, this leads to the differentiation of Tol-DCs. **(B)** Biomaterial-based delivery systems are employed to transport essential immunomodulators (e.g., vitamin D3 and IL-10) to Tol-DCs. These systems can also involve gene manipulation (e.g., encoding IL-10) to expand the population of Tol-DCs. **(C)** Biomaterial-based scaffolds are utilized to recruit T cells through chemoattractant cytokines like MCP-1 to the scaffold matrix. Subsequently, the differentiation of Tregs occurs through the interaction of modulatory molecules, such as Fas-FasL1, between Tregs and the scaffold. Simultaneously, the delivery of released auto- or allo-antigens to DCs results in the formation of a Tol-Mic and the expansion of antigen-specific Tregs. **(D)** The expanded Tol-DCs secrete anti-inflammatory cytokines like IL-10 and TGF-β, and they express immunomodulatory molecules such as FasL, PD-L1, and ICOS-L. These molecules, in turn, promote the expansion of Tregs. **Abbreviations arranged in alphabetical order: CCR-1**: CC chemokine receptor 1, **FasL**: Fas Ligand, **ICOS**: Inducible T-cell CO-Stimulator, **ICOS-L**: Inducible T-cell CO-Stimulator Ligand, **IL-10**: Interleukin-10, **iTregs**: Induced Tregs, **MCP-1**: Monocyte Chemoattractant Protein-1, **MHC**: Major Histocompatibility Complex, **MPs**: Microparticles, **PD-1**: Programmed cell death protein 1, **PD-L1**: Programmed death-ligand 1, **pTregs**: Peripheral-Derived Tregs, **TCR**: T Cell Receptor, **TGF-β1**: Transforming Growth Factor Beta 1, **Tol-DC**: Tolerogenic Dendritic Cells, **Tol-Mic**: Biomaterial-induced tolerogenic microenvironment, **Tr1**: Inducible T Regulatory Type 1, **Tregs**: Regulatory T cells, **tTregs**: Thymic-Derived Natural Tregs.

for preventing graft rejection and treating autoimmune disorders.

To harness the beneficial characteristics of biomaterial delivery systems, Quintana et al. employed gold nanoparticles (AuNPs) as carriers to deliver a T cell epitope derived from MOG (myelin oligodendrocyte glycoprotein) – a potential antigen implicated in neuro autoimmune diseases – to DCs in an EAE (experimental autoimmune encephalomyelitis) model. To enhance the generation of antigen-specific Tregs, they attached MOG-coated AuNP to ITE (2-(1′Hindole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester), which serves as a Tregs modulator. ITE functions as an AhR (aryl hydrocarbon receptor) ligand, regulating the differentiation of IL-10+/Foxp3+ Tregs in both mice and humans. To stabilize the AuNP-MOG-ITE construct and enable controlled release, they added a layer of thiol-PEG, yielding particles of approximately 60 nm in diameter. This innovative approach showcased four main strengths: I) Utilizing AuNPs as a delivery system due to their immunomodulatory properties and efficient cellular uptake; II) Simultaneously delivering the antigen and ITE as a Tregs inducer; III) Employing AuNP-covered PEG as a stabilizer, facilitating the controlled release of the antigen and ITE; IV) Using a specific form of MOG (MOG35–55) to facilitate the generation of Tregs by DCs. The AuNP-MOG-ITE construct effectively suppressed EAE by promoting the generation of tolerogenic DCs, leading to the expansion of Foxp3+ Tregs [92]. This research group also applied the same strategy to ameliorate T1D in a mouse model. They used the beta-islet cell antigen proinsulin (Ins) and constructed AuNP-Ins-ITE nanoparticles, which successfully suppressed autoimmune diabetes by inducing tolerogenic DCs and expanding Foxp3+ Tregs [81]. Similarly, the research group utilized nanoliposome particles (NLPs) to encapsulate MOG and ITE, with average size 100 nm in diameter. Nanoliposomes are FDA-approved nanoparticles that are more biocompatible and biodegradable compared to AuNPs. The NLP-MOG-ITE construct suppressed EAE by expanding IL-10-producing Tr1 and MOG-specific Foxp3+ Tregs [100]. The researchers demonstrated that nanoparticles-ITE induced a tolerogenic state in DCs by activating AhR, resulting in the induction of suppressor of cytokine signaling 2 (SOCS2) (Fig. 3B). As a result, the activation of NF-κB (nuclear factor kappa B) and the generation of pro-inflammatory cytokines were suppressed [81].

Nanoliposome-based biomaterials have found wide application as carriers for antigens, providing protection against degradation and burst release, enhancing solubility, and enabling targeted delivery to specific locations in the body. These NLPs are biodegradable and biocompatible, making them suitable for use in the human body. Moreover, their unique structure allows them to carry both hydrophilic and hydrophobic antigens. Leveraging the distinct properties of NLPs, the Broere et al. designed an anionic DSPG (1,2-distearoyl-*sn*-glycero-3-phosphoglycerol) NLPs with inherent tolerogenic characteristics. Within this NLP platform, they incorporated a human-proteoglycan-derived peptide (a candidate antigen for rheumatoid arthritis [RA]) with atRA (a Treg

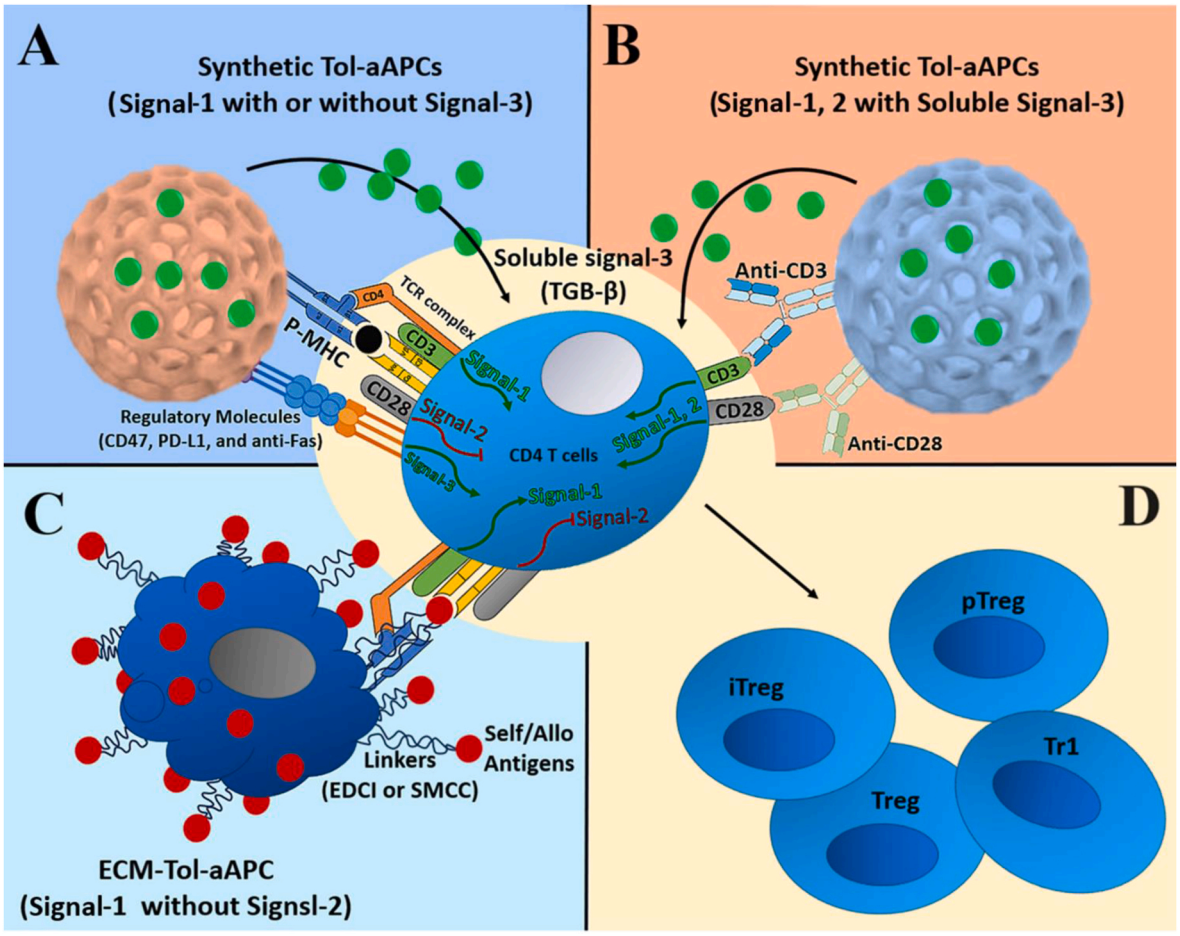

Fig. 6. Biomaterials as Tol-aAPCs: (A) Biomaterials initiate signal-1 through *p*-MHC with or without signal-3, utilizing regulatory molecules like PD-L1, *anti*-Fas/FasL1, and CD47, or soluble modulators such as TGF-β. In the absence of signal-2 from co-stimulators like CD28, CD4 T cells either differentiate into Tregs or become anergic cells. **(B)** When biomaterials transduce signals-1 and 2 along with soluble signal-3 (e.g., TGF-β), this results in the differentiation of Tregs or induction of T cell anergy. **(C)** By crosslinking self/allo antigens with EDCI or SMCC onto syngeneic splenocytes or erythrocytes, antigens are presented to T cells with a disruption of MHC signals, ultimately leading to Treg differentiation **(D)**. **Abbreviations arranged in alphabetical order: aAPCs**: Artificial APCs, **ECDI**: 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide, **ECM-Tol-aAPC**: Engineered Cell Membrane-Derived Tol-aAPC, **iTregs**: Induced Tregs, ***p*-MHC**: Peptide-Major Histocompatibility Complex, **PD-L1**: Programmed death-ligand 1, **pTregs**: Peripheral-Derived Tregs, **SMCC**: Sulfosuccinimidyl-4-(*N*-maleimidomethyl) Cyclohexane-1-carboxylate, **TCR**: T Cell Receptor, **TGF-β**: Transforming Growth Factor Beta, **Tol-aAPCs**: Tolerogenic artificial Antigen-Presenting Cells, **Tr1**: Inducible T Regulatory Type 1, **Tregs**: Regulatory T cells.

modulator). This combination resulted in the induction and expansion of antigen-specific Tregs in a mouse model of treated arthritis [104]. Similarly, Thomas et al. adopted a similar approach by encapsulating a high-affinity $CD4^+$ mimic-epitope derived from the beta-islet autoantigen chromogranin A (as the candidate antigen) with vitamin D3 (another Treg modulator) within NLPs. Their findings demonstrated the expansion of chromogranin A-specific Tregs in the peripheral system, along with the inhibition of IFN-γ+ antigen-specific $CD8^+$ T cells [98]. These studies, along with others [99], have described nano-formulation platforms containing antigens and Treg modulators for the stimulation of antigen-specific Tregs in autoimmune disorders (Fig. 3B).

One of the most effective approaches for inducing tolerance involves administering antigens coupled with ECDI (1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide) to apoptotic cells, such as splenocytes. This regimen relies on macrophages increasing their production of IL-10, the generation of Tregs, and the co-inhibition of T cells via the PD-1 and CTLA-4 pathways. However, this approach is limited by the availability of donor cells, the costs associated with cell procurement, and the regulatory complexities related to cell-based therapies. To address these challenges, it is crucial to identify a particle carrier that can target similar regions in the body without triggering immune activation pathways. Biomaterial nanoparticles have been developed that not only control the release of antigens but also enhance their uptake by phagocytes, leading to the induction of antigen-specific Tregs. For instance, the Miller et al. designed polystyrene beads and biodegradable ECDI-PLGA microparticles linked with myelin proteolipid protein epitope (PLP139-151). These microparticles successfully increased Tregs and IL-10 levels, preventing and treating the EAE mouse model. The mechanism behind this effect involved the uptake of microparticles by macrophages expressing the “MARCO” scavenger receptor in the marginal zone, which facilitated the functioning of Tregs [94]. In addition, their research team loaded a hybrid peptide construct (consisting of a peptide from chromogranin-A fused to an insulin *C*-peptide fragment) onto ECDI-PLGA microparticles. This led to an increase in the ratio of Foxp3+/CD4+ Tregs to IFN-γ+ T cells and provided protection against T1D in mice [95]. Similar approaches were also utilized for diabetogenic peptides conjugated on ECDI-PLGA microparticles, yielding similar results [96,97].

In a similar vein, the Luo et al. developed PLGA particles delivering peptides containing donor antigens to improve skin allograft survival. They used both peptide-encapsulated and ECDI-conjugated PLGA, with the latter resulting in a significant expansion of graft-infiltrating Tregs compared to peptide-encapsulated PLGA [106]. Furthermore, in an allogeneic beta-islet transplant model, they coupled donor splenocytes

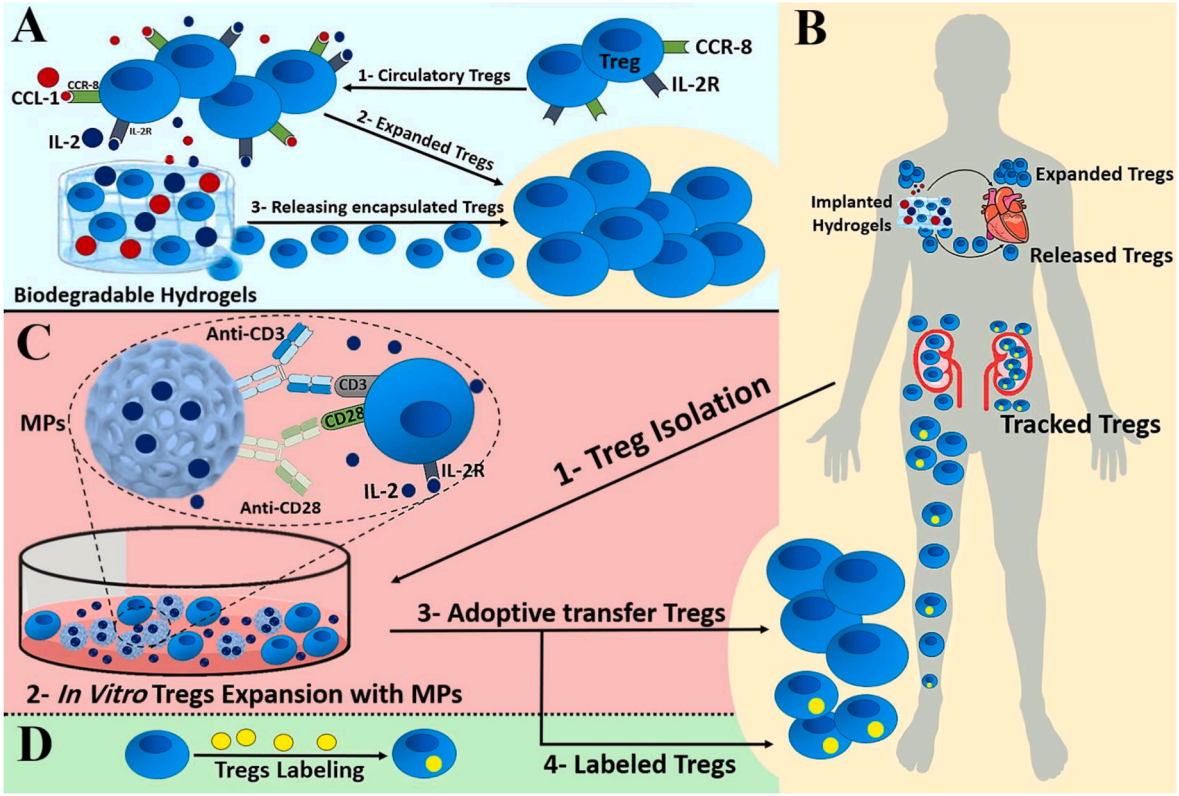

Fig. 7. Biomaterials to Facilitate Functional Tregs: (A) Gradual release of Tregs over time from biomaterial-based scaffolds, which are recruited to inflamed sites like transplanted organs, contributes to the alleviation of inflammation. Incorporating chemoattractant cytokines such as CCL-1 and IL-2 into Treg scaffolds enhances the effectiveness of implanted scaffolds **(B)**. **(C)** Isolated Tregs with a low frequency can be expanded *in vitro* using MPs beads, leading to an increase in the efficiency of adoptive transfer Treg therapy **(B)**. **(D)** By labeling expanded Tregs, it becomes possible to track the transferred cells over time and determine the fate of Tregs after adoptive transfer **(B)**. **Abbreviations arranged in alphabetical order: CCL-1**: Chemokine ligand 1, **CCR-8**: CC chemokine receptor 8, **IL-2:** Interleukin-2, **IL-2R**: Interleukin-2 Receptor, **MPs**: Microparticles, **Tregs**: Regulatory T cells.

lysate antigens to ECDI-PLGA particles, leading to prolonged graft survival and the induction of antigen-specific Tregs [105]. These studies illustrate promising strategies for using particle-based carriers to enhance immune regulation and promote transplant tolerance, potentially offering new avenues for effective therapies in various conditions.

As mentioned earlier, the dual-sized microparticle system can also be utilized to deliver antigens to induce specific-antigen Tregs. This system employs two different sizes of microparticles: firstly, phagocytosable microparticles are used to deliver modulators and antigens to APCs. Secondly, non-phagocytosable microparticles are utilized for the controlled release of substances. In this context, Keselowsky et al. conducted three separate studies demonstrating that encapsulating the MOG antigen and vitamin D3 into the phagocytosable MPs, along with the Treg-inducing factor (TGF-β1) and DC growth factor (GM-CSF) into non-phagocytosable PLGA MPs, resulted in the alleviation of EAE symptoms in mice. This effect was achieved through reduced allogeneic TCR signaling and proliferation, increased frequency of Tregs producing IL-10, and enhanced expression of PD-1 (Fig. 3D) [84,88,90]. These findings offer a promising and innovative approach to restoring immune balance in the EAE mouse model. The dual-sized MP system demonstrates robust and long-lasting antigen-specific autoimmune protection, surpassing the effectiveness of soluble substances or irrelevant antigen formulations. Furthermore, the versatility of this platform is evident, as it allows for personalized, disease-specific responses by substituting antigens and/or factors, potentially inducing either tolerogenic or immunogenic effects based on the specific requirements.

Biomaterials can act as scaffolds for auto- and allo-antigens, facilitating the infiltration of specific immune cells into the material. This approach offers various advantages, including improved control over the localization of antigens, limiting their dissemination to areas affected by inflammation and disease. Moreover, when these scaffolds are administered subcutaneously, they are easily accessible and can be monitored through minimally invasive biopsies. In one study conducted by Mooney et al., they utilized alginate hydrogel conjugated with the beta-cell directed peptide (BDC) for antigen-specific tolerogenic immune modulation. Their findings revealed that antigen-specific T cells accumulated within the hydrogel, and notably, around 60% of the antigen-specific $CD4^+$ T cells within the hydrogel were Tregs [107]. In another investigation by Graham et al., beta-islet cells were loaded onto microporous PLGA scaffolds and transplanted into the abdominal fat of a nonobese diabetic (NOD) mouse model. Co-transplantation of Tregs around the scaffold induced antigen-specific Tregs, resulting in systemic tolerance in the NOD mice [108].

In a study by Kim et al., loading MOG antigen into the mesoporous silica nanoparticles (MSNPs) induced the differentiation of antigen-specific Foxp3+ Tregs in the spleen, leading to systemic immune tolerance in the EAE mouse model. This approach also resulted in a reduction of CNS-infiltrating autoreactive $CD4^+$ T cells and APCs [101]. In a study conducted by Herkel et al., two nanoparticles were used, including CdSe/CdS/ZnS double shell quantum dots and iron oxide nanocrystals. These nanoparticles were encapsulated into EDCI-poly (maleic anhydride-*alt*-1-octadecene) and coupled with MOG peptide. This coating allowed for the specific delivery of autoantigens to LSECs (liver sinusoidal endothelial cells), which act as both inducers and preservers of Tregs in the liver. The results of their study revealed a key concept: using nanoparticles to target autoantigens to LSECs can activate antigen-specific Tregs, successfully treating autoimmune disorders [102]. Therefore, biomaterial-based strategies, including nano-formulations and particle carriers, show promising potential in inducing antigen-specific Tregs for immune tolerance in autoimmune

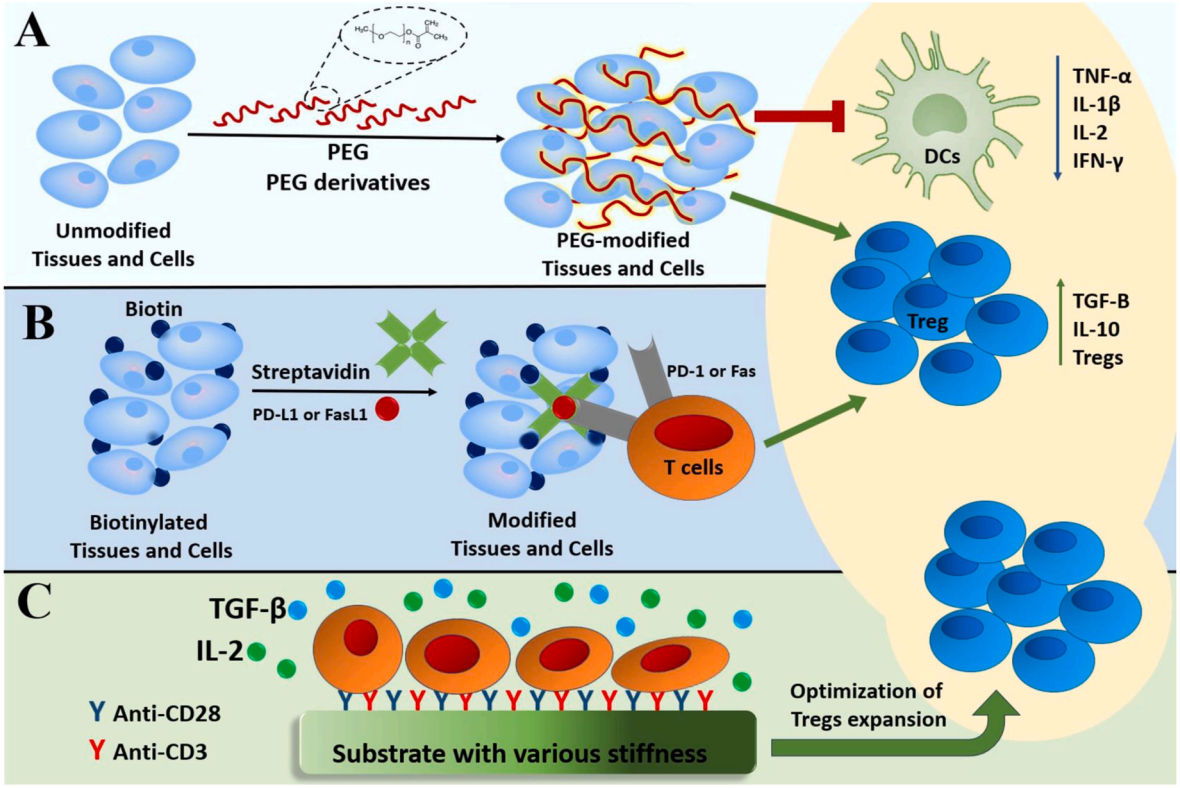

Fig. 8. Immune Camouflage and Controlled Substrate Rigidity by Biomaterials: (A) PEG and its derivatives can effectively minimize or prevent host immune responses through PEG-based charge and steric camouflage, resulting in the inhibition of DCs, reduced levels of inflammatory cytokines (e.g., IL-1β, IL-2, TNF-α, and IFN-γ), expansion of Tregs, and an increase in anti-inflammatory cytokines (e.g., IL-10 and TGF-β). **(B)** Tissues/cells biotinylated for functionalization with streptavidin-modulators (e.g., PD-L1 and FasL1) can induce Tregs. **(C)** Biomaterials with varying stiffness are employed to optimize the expansion of Tregs, thereby increasing the efficiency of adoptive transfer Treg therapy. **Abbreviations arranged in alphabetical order: DCs:** Dendritic Cells, **FasL**: Fas Ligand, **IFN-γ**: Interferon Gamma, **IL-10**: Interleukin-10, **IL-1β**: Interleukin-1 beta, **IL-2**: Interleukin-2, **PD-1**: Programmed cell death protein 1, **PD-L1**: Programmed death-ligand 1, **PEG**: Polyethylene Glycol, **TGF-β**: Transforming Growth Factor Beta, **TNF-α**: Tumor Necrosis Factor Alpha, **Tregs**: Regulatory T cells.

disorders and transplantation (Table 1 and Fig. 3).

2.3. Biomaterial-formulated "backpacks" for tregs

The controlled release of Treg-specific signals, such as the IL-2 cytokine, using biomaterials has shown promise as a potential strategy to enhance Treg function and promote immune tolerance. However, it is essential to carefully consider the potential side effects that could arise from this approach. For instance, the prolonged release of IL-2 from biomaterials may inadvertently stimulate the activities of natural killer (NK) cells and effector T cells, leading to unintended immune responses and inflammation. Similarly, the use of nano-formulations for inducing antigen-specific Tregs and decorated biomaterials for targeting Tregs also presents some challenges. While these approaches offer innovative ways to manipulate the Tregs for therapeutic purposes, there is a risk of non-specific accumulation of the nanoparticles in organs other than the intended targets. This can result in the unintended release of antigens, triggering uncontrolled immune responses and potentially exacerbating the very condition that the therapy seeks to treat. Moreover, in immune-related disorders such as autoimmune diseases or situations where immune function is compromised, such as in organ transplantation, the population of naturally occurring Tregs may be inadequate or dysfunctional. This poses a significant obstacle in harnessing the full potential of Tregs for therapeutic purposes. Efforts to augment Treg populations or enhance their activity in these contexts are challenging and require careful consideration to ensure safety and efficacy.

To overcome these challenges, innovative biomaterial strategies have made significant advancements, resulting in the development of what is now known as "Treg backpacks." These biomaterial-formulated backpacks serve as specialized carriers, delivering specific immunomodulatory cargos directly to Tregs. The concept behind Treg backpacks involves modifying small populations of Tregs with immunomodulatory cargos within the biomaterial carriers (Table 2 and Fig. 4A). This modification allows for a controlled and sustained release of the cargos, enhancing the suppressive capabilities of Tregs. By continuously delivering immunomodulatory agents directly to Tregs, these cells are maintained in a primed and activated state, ready to effectively regulate immune responses as needed.

In this context, Eskandari et al. developed protein nanogels containing IL-2-Fc fusion proteins with cleavable NHS-SS-NHS (bis-*N*-hydroxy succinimide) crosslinkers. These nanogels that release IL-2 in response to reducing conditions, such as those present at the surface of T cells when they receive stimulation via the TCR. When Tregs were surface-conjugated with IL-2 nanogels, they exhibited more allograft-protective effects compared to stimulated Tregs with IL-2 or unmodified Tregs. They demonstrated that Tregs-modified nanogels carrying an IL-2 cargo were more effective in suppressing allo-immunity in murine skin transplant models and humanized mouse allo-transplantation models than conventional Tregs [109]. This cleverly designed approach offered several key advantages: I) By utilizing IL-2-Fc, the half-life of IL-2 in the body was prolonged, thanks to the Fc part of the fusion protein. II) The nanogels were constructed to respond to high redox potential. As the redox potential of circulating T cells increased in correlation with the concentration of donor antigens, particularly in inflamed areas, it triggered the controlled release of IL-2 in response to this heightened redox potential, allowing for a precise and localized release of the stimulator. III) The nanogels were stabilized on the surface of Tregs by employing an anti-CD45 monoclonal antibody, acting as a

Table 1
Direct functions of biomaterials on Tregs activation and expansion (Part 1).

Control of Treg-Specific Signal Release				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
- Controlled release of cytokines and/or drugs over specific time frames - Usability in autoimmunity and transplantation	Spherical PLGA microparticles (15–26 μm)	- Encapsulation and controlled-release of cytokines (IL-2 and TGF-β1) and drug - Combination therapy with encapsulated cytokines and Rapa	- Induction of Treg phenotype (Foxp3+/FR4+/CD25+/GITR+) - Effective suppression of naïve T cell proliferation by Tregs	[75]
	βCD-NH2 particles	- Encapsulation of Rapa along with soluble TGF-β1	- Increase in the bioavailability and function of Rapa - Promotion of CD4+/CD25+/Foxp3+ Tregs expansion - Decrease in the production of IFN-γ	[86]
- Beta-islet transplantation - T1D	Spherical PLGA microparticles (54 ± 51 μm)	- Co-transplantation of PLGA microparticles releasing TGF-β1 and mouse islets	- Generation of Foxp3+/CD3+ Tregs *in vitro* and *in vivo* - Induction of polyclonal and antigen-specific Tregs *in vitro* - Reduction of intra-islet $CD3^+$ cells invasion - Elevation of CD3+/Foxp3+ Tregs at the peri-transplantation site - long-term functioning grafts	[80]
	Spherical PLGA microparticles (less than 6 μm)	- T1D-relevant antigens co-loaded with Treg modulators (Rapa, atRA, or buty)	- Treg differentiation - Expansion of antigen-specific Tregs - Decrease in the activation of APCs	[83]
	PLGA layered scaffold	- Seeding allogeneic beta-islets on PLGA layered scaffold incorporated with IL-33	- Induction ofFoxp3+/ST3+ Tregs - Upregulation of Th2 response - Prolongation of graft survival	[87]
	PEG-hydrogel	- Analogue of IL-2, and FasL1-presenting microgels - FasL1-presenting microgel that mimicked the Treg extracellular matrix - Establishment of localized immune tolerance for transplanted beta-islets	- Prolongation of the presence of IL-2 analogue and FasL1 at graft site - Upregulation of Tregs	[74]
- T1D - EAE	Dual-sized PLGA microparticles (<3, >30 μm)	- Encapsulation of the MOG autoantigens and vitamin D3 into the phagocytosable PLGA - Encapsulation of Treg-inducing factor (TGF-β1) and DCs growth factor (GM-CSF) into non-phagocytosable PLGA	- Activation of Foxp3+ Tregs resulting in IL-10 production - Increase in PD-1 expression - Reduction in allogeneic TCR signaling - Establishment of a tolerogenic microenvironment - Amelioration of T1D and EAE	[73,84, 88–90]
- Skin autoimmunity	PCL nanowires (37 mm length with 200 nm pore diameter)	- Conjugation of PCL nanowires with *anti*-IL-2 leading to capturing endogenous IL-2	- Activation of Tregs - Recruitment of Tregs to skin - Suppression of tissue-resident T cells - Improvement of skin autoimmune disease	[91]
Induction of antigen-specific Tregs Formation				
- T1D - EAE	AuNP (60 nm)	- AuNP decorated with autoantigens and ITE (an AhR agonist) - MOG autoantigen for EAE - Ins autoantigen for T1D	- Suppression of autoimmune diseases - Generation of tolerogenic DCs - Expansion of Foxp3+ Tregs - Induction of SOCS2 and inhibition of NF-κB signaling	[81,92]
	Biodegradable PLGA (464–522 nm) or ECDI-PLGA	- Delivery of autoantigens to tolerogenic APCs - Biodegradable ECDI-PLGA conjugated with autoantigens - Biodegradable PLGA loaded with diabetogenic autoantigens - Encephalomyelitis autoantigens: PLP, MOG - Diabetogenic autoantigens: chromogranin A, IGRP, and insulin *C*-peptide fragment	- Induction of T cell anergy - Decrease in effector T cells release pro-inflammatory cytokines - Increase in Foxp3+ Tregs - Increase in IL-10 levels - Protection against T1D and EAE	[93–97]
	NLPs	- NLPs co-carried diabetogenic autoantigen, chromogranin A, and vitamin D3	- Expansion of chromogranin A-specific Tregs - Expansion of Foxp3−/PD-1+/CD73+/ICOS+/IL-10+ peripheral Tregs - Inhibition of IFN-γ+ autoantigen-specific $CD8^+$ T cells - Suppression of the development diabetes	[98]
-EAE	PLGA particles	- PLGA particles co-carrying encephalomyelogenic autoantigens and Rapa	- Expansion of endogenous autoantigen-specific Tregs - Therapeutic efficacy against EAE	[99]
	NLPs (average size 100 nm)	- Encapsulation of autoantigens in NLPs with ITE (an AhR agonis)	- Suppression of EAE - Generation of tolerogenic DCs	[100]

(*continued on next page*)

Table 1 (*continued*)

Control of Treg-Specific Signal Release				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
		- MOG autoantigen for EAE	- Expansion of specific Foxp3+ Tregs and IL-10-producing Tr1 - Reduction of infiltrating effector T cells in the CNS	
	Cerium oxide-functionalized MSNPs (10–30 nm size)	- Delivery of autoantigens to tolerogenic APCs - Loading MOG antigen into the MSNPs - Functionalization of MSNPs with cerium oxide (As a scavenging reactive oxygen species)	- Induction of antigen-specific Foxp3+ Tregs in the spleen - Reduction of CNS-infiltrating APCs and autoreactive $CD4^+$ T cells	[101]
	Superparamagnetic iron oxide and CdSe/CdS/ZnS double shell quantum dots nanoparticles	- Delivery of autoantigen peptides to LSECs - Encapsulation of nanoparticles into EDCI-polymer (maleic anhydride-*alt*-1-octadecene) and coupling with MOG peptide	- Activation of antigen-specific Tregs - Control and prevention of the onset of EAE	[102]
-EAE -Hypersensitivity -Hemophilia	PLGA biodegradable nanoparticles	- PLGA loading with autoantigens and Rapa	- Increase in Tregs - Enhancement and sustenance of B cell tolerance - Inhibition of $CD8^+$ and $CD4^+$ T cell activation - Reduction of relapses in EAE - Suppression of hypersensitivity reactions - Inhibition of production of anti-coagulation factor VIII antibodies	[103]
-Rheumatoid arthritis	DSPG-liposome nanoparticles (183.7 ± 4.9 nm)	- Incorporation of hPG-derived peptide with atRA into NLPs	- Induction and expansion of autoantigen-specific Tregs - Prevention of RA	[104]
-Skin transplant -Beta-islet transplantation -T1D	Biodegradable PLGA (458.8 ± 14.8 nm) or ECDI-PLGA (486.5 ± 26.4 nm)	- Delivery of allo- and auto- antigens to induce tolerance - Biodegradable PLGA with skin alloantigens - Biodegradable ECDI-PLGA linked with splenocytes lysate autoantigens	- Expansion of graft-infiltrating Tregs - Induction of antigen-specific Tregs - Prolonged graft survival - Promotion of tolerance for beta-islet	[105, 106]
	Alginate hydrogel loaded PLGA microparticles	- Antigen-specific tolerogenic immune modulation - Hydrogel delivering GM-CSF with BDC-loaded PLGA	- Accumulation of antigen-specific Tregs within the hydrogel - Improvement of pancreatic islets transplantation	[107]
	Microporous PLGA scaffolds	- Beta-islet cells loaded onto microporous PLGA scaffolds and transplanted into the abdominal fat - Co-transplantation of Tregs around the scaffold	- Infiltration of Tregs around islet transplants - Establishment of systemic tolerance against transplanted beta-islet cells in the NOD mice	[108]

Abbreviations arranged in alphabetical order: AhR: Aryl Hydrocarbon Receptor, **APCs**: Antigen-Presenting Cells, **AuNP**: Gold Nanoparticles, **BDC**: Beta-Cell Directed Peptide, **Buty**: Butyrate, **CNS**: Central Nervous System, **DCs**: Dendritic Cells, **DSPG**: 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol, **EAE**: Experimental Autoimmune Encephalomyelitis, **ECDI**: 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide, **FasL1**: Fas Ligand 1, **Foxp3**: Forkhead Box P3, **hPG**: Human Proteoglycan, **IFN-γ**: Interferon Gamma, **IGRP**: Glucose-6-Phosphatase Catalytic Subunit–Related Protein, **IL-2**: Interleukin-2, **IL-10**: Interleukin-10, **Ins**: Proinsulin, **ITE**: 2-(1′Hindole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester, **LSECs**: Liver Sinusoidal Endothelial Cells, **MOG**: Myelin Oligodendrocyte Glycoprotein, **MSNPs**: Mesoporous Silica Nanoparticles, **NF-κB**: Factor Kappa B, **NLPs**: Nanoliposome Particles, **NOD**: Nonobese Diabetic, **PCL**: Polycaprolactone, **PD-L1**: Programmed death-ligand 1, **PLGA**: Poly (lactic-*co*-glycolic acid), **PLP**: Myelin Proteolipid Protein Epitope, **RA**: Rheumatoid Arthritis, **Rapa**: Rapamycin, **SOCS2**: Suppressor of cytokine signaling 2, **T1D**: Type 1 Diabetes, **TGF-β1**: Transforming Growth Factor Beta 1, **Tr1**: Inducible T Regulatory Type 1, **Tregs**: Regulatory T cells, **Vitamin A or atRA**: All-trans Retinoic Acid, **βCD-NH2**: Mono-(6-amino-6-deoxy)-β-cyclodextrin. *The particle sizes in the table are for diameters.

non-internalizing surface linker specifically expressed on leukocytes. Importantly, CD45 ligation itself did not impact T cell proliferation. Similarly, the Brusko et al. achieved easy co-encapsulation of IL-2 and harmine, a mitogenic drug promoting beta-islet replication, in 472 nm PLGA nanoparticles. The hydrophobic nature of both IL-2 and harmine facilitated their encapsulation in these nanoparticles. The nanoparticles were then efficiently conjugated on Tregs using Poly-L-lysine (PLL) as a method for backpack conjugation. This design ensured the viability of Tregs while having no adverse effects on cell migration. The administration of functionalized Tregs successfully prevented the onset of T1D in pre-diabetic NOD mice [7]. These backpack platforms represent successful approaches to target low-population Tregs, preventing autoimmune conditions, and facilitating successful organ transplantation.

2.4. Decorated biomaterials for targeting tregs

Over the years, biomaterials have evolved to incorporate ligands that specifically target Treg surface markers, like CD25. This section explores the concept of decorated biomaterials, which selectively bind to Tregs, enhancing their enrichment and expansion. This strategy opens up exciting possibilities for Treg-targeted therapies, as it allows localized immunomodulation, reduces off-target effects, and minimizes activation of other immune cells. The utilization of decorated biomaterials represents a sophisticated approach for Treg-targeted therapies, enabling precise immunomodulation while minimizing potential side effects (Table 2 and Fig. 4B). In this regard, the Fahmy et al. designed biodegradable PLGA nanoparticles loaded with IL-2 and TGF-β1, specifically targeted to $CD4^+$ T cells by anti-CD4 antibodies. These particles, with an average size of 168 nm in diameter, effectively induced and expanded stable Foxp3+ $CD4^+$ Tregs with immunosuppressive activity both *in vitro* and *in vivo* after 4–5 days. The introduction of targeted cytokine-loaded PLGA nanoparticles improved the stability of iTregs, ensuring the maintenance of their suppressive characteristics, even in the presence of pro-inflammatory cytokines. This outcome highlights the importance of using nanocarriers-based methods to stabilize and expand iTregs, which are essential for Tregs immunotherapy in the context of autoimmune diseases and inflammation [82].

Targeted biomaterials can also lead to the induction of other subsets of Tregs. In contrast to conventional Tregs, characterized by Foxp3+/CD4+/CD25+, there exists a unique subset known as $CD8^+$ Tregs, which

Table 2
Direct functions of biomaterials on Treg activation and expansion (Part 2).

Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
Treg Backpacking				
- Skin transplant	Nanogels containing cleavable NHS-SS-NHS	- Nanogels loaded with IL-2 and harmine conjugated on Tregs - Release of IL-2 in response to reducing conditions such as TCR interactions	- Enhanced effectiveness of Tregs carrying IL-2-loaded nanogels - Suppression of allo-immunity in murine skin transplant - Protection against allograft rejection	[109]
- T1D	PLGA nanoparticles (472 nm)	- PLGA loaded with IL-2 and harmine - PLGA conjugated on Tregs using PLL	- Enhanced effectiveness of Tregs conjugated with IL-2 - Inhibition of diabetes	[7]
Targeting Tregs				
- Controlled Release of cytokines over specific time frames - Murine lupus - GvHD	Spherical PLGA nanoparticles (100–300 nm)	- Co-encapsulation and controlled-release of IL-2 and TGF-β1 - Specific delivery of IL-2 and TGF-β1 to naïve T cells - Anti-CD2/CD4 or anti-CD3/CD4/CD8 functionalized nanoparticle	- Induction of CD4+/CD25hi/Foxp3+/CD127- and CD8+/Foxp3+ Tregs - Suppression of *anti*-DNA antibody production - Suppression of murine lupus - Prevent GvHD	[82, 110, 111]
Gene manipulation of Tregs				
- SLE	Modified spherical PLGA with mPEG and PLL (158.2 ± 1.48 nm)	- Delivery platform for miR-125a into splenic T cells	- Effective delivery of miR-125a into splenic T cells - Enhancement in the differentiation and suppressive potency of Tregs - Reversal of the imbalance of regulatory/effector T cells - Reduction in lupus nephritis and alleviation of SLE progression	[115]
- Effective miRNA-based therapies in Treg-mediated immune therapy such as periodontal bone loss	PEGylated PLLA nanofibrous spongy microspheres, PEGylated PLLA co-functionalized with MSNPs, and PLGA microspheres integrated into one delivery vehicle	- Injectable scaffold to accommodate Tregs - MSNPs facilitating the release of IL-2/TGF-β1 - PLGA component for controlled-release miR-10a	- Increase in the number of Tregs and promotion of their expansion	[116]
- GvHD - EAE - Usability in autoimmunity and transplantation	NLPs	- NLPs containing mRNAs to encode a modified human IL-2 mutein with a prolonged half-life and enhanced affinity for IL-2Rα - NLPs containing Foxp3 mRNA	- Expansion of Tregs without directly alter genes in Tregs - Decrease in GvHD and EAE severity - Effective delivery of Foxp3 mRNA to primary human $CD4^+$ T cells - Increase in TGF-β and IL-10 secretion - Suppression of primary effector CD4 and CD8 T cell proliferation *ex vivo*	[117, 118]

Abbreviations arranged in alphabetical order: **EAE**: Experimental Autoimmune Encephalomyelitis, **GvHD**: Graft-Versus-Host Disease, **IL-2**: Interleukin-2, **IL-2Rα**: Interleukin-2 Receptor Alpha, **mPEG**: Monomethoxypolyethylene Glycol, **MSNPs**: Mesoporous Silica Nanoparticles, **NHS-SS-NHS**: bis-*N*-hydroxy succinimide, **NLPs**: Nanoliposome Particles, **PLGA**: Poly (lactic-*co*-glycolic acid), **PLL**: Poly-L-lysine, **PLLA**: Poly (L-lactic acid), **SLE**: Systemic Lupus Erythematosus, **T1D**: Type 1 Diabetes, **TCR**: T Cell Receptor, **TGF-β1**: Transforming Growth Factor Beta 1, **Tregs**: Regulatory T cells. *The particle sizes in the table are for diameters.

possess immunosuppressive properties and express Foxp3/CD8/CD25. Unlike conventional $CD8^+$ T cells that eliminate infected or cancerous cells, $CD8^+$ Tregs modulate the immune responses by suppressing the activation of other immune cells through the direct cell-to-cell interactions and production of anti-inflammatory cytokines. Their role in maintaining immune tolerance is crucial, and studying their function holds potential for innovative immunotherapies to treat autoimmune diseases and successful organ transplantation [51]. For the suppression of murine lupus (an autoimmune condition) and graft versus host disease (GvHD) a transplant complication), Antonio et al. developed PLGA nanoparticles containing TGF-β1 and IL-2, targeting different subsets of Tregs [110,111]. In the first study, PLGA nanoparticles coated with anti-CD2/CD4 antibodies were administered to mice model of lupus. *In vitro*, these PLGA nanoparticles induced the generation of $CD8^+$ and $CD4^+$ Foxp3+ Tregs. When administered in a lupus mouse model, these nanoparticles resulted in the expansion of $CD4^+$ and $CD8^+$ Tregs, significant inhibition of *anti*-DNA antibody production, and ameliorated renal manifestation [111]. In the next study, PLGA nanoparticles were decorated with anti-CD3/CD4/CD8 antibodies. These engineered nanoparticles induced CD4+/CD25hi/Foxp3+/CD127-and CD8+/Foxp3+ Tregs, which were functional in a humanized mice model [110]. Decorated biomaterials offer promising avenues for Treg-targeted therapies, enabling localized immunomodulation and potential treatments for inflammation, autoimmune disorders, and transplant complications. The use of decorated biomaterials and the exploration of $CD8^+$ Tregs show encouraging results for future immunotherapy development (Table 2 and Fig. 4B). Moreover, $CD8^+$ Tregs have been demonstrated to effectively regulate memory responses to a greater extent than $CD4^+$ Tregs. Conversely, $CD4^+$ Tregs exhibit higher efficiency in controlling naive immune responses compared to $CD8^+$ Tregs. This suggests the likelihood of a synergistic interaction between $CD4^+$ and $CD8^+$ Tregs. Therefore, there exists significant potential to

refine and augment the functionality of both $CD4^+$ and $CD8^+$ Tregs for innovative cell therapies [112–114].

However, there are various delivery systems designed to deliver TGF-β and IL-2 to Tregs. When considering the challenges and critical parameters associated with these delivery systems targeting Tregs, it becomes evident that achieving effective immunomodulation presents several obstacles. Ensuring adequate bioavailability, targeting specificity, stability, and safety of the delivery system are paramount challenges. Additionally, scalability and manufacturing considerations are crucial for clinical translation. Key parameters influencing efficacy include controlled release kinetics, biocompatibility, tissue penetration, optimization of dosing regimen, and long-term stability. Addressing these challenges and optimizing parameters will undoubtedly advance the development of immunomodulatory therapies, improving therapeutic outcomes, and facilitating clinical application in immune-mediated disorders.

2.5. Biomaterial for treg gene manipulation

This section delves into the cutting-edge application of gene-editing tools for manipulating Treg genes, a promising strategy to enhance their regulatory capabilities (Table 2 and Fig. 4C). Currently, viral-vector-based transduction methods used for this purpose come with drawbacks such as high costs, time-consuming vector production, and safety concerns. Therefore, there was a need for more cost-effective and efficient methods to genetically modify $CD4^+$ Tregs. Biomaterials emerged as ideal candidates for delivering gene-editing tools and nucleic acids, like microRNAs, into Tregs, allowing targeted modifications and genetic enhancements. This innovative approach holds significant potential for personalized immunotherapies, leveraging genetically engineered Tregs to achieve improved therapeutic outcomes in immune-related disorders. In this context, Gamrad et al. developed an effective nucleic acid delivery approach using AuNPs for Tregs. They used AuNPs as a carrier platform (diameter: 5 nm), functionalizing them with an oligonucleotide (siRNA) and a nuclear localization signal peptide (NLS-peptide) to ensure efficient translocation of the transporter species. Upon treating splenocytes with functionalized AuNPs, CD11c + DCs and CD11b + macrophages demonstrated the highest uptake capacity for AuNPs. Furthermore, approximately 4% of $CD8^+$ T cells and $CD19^+$ B cells exhibited uptake of the particles, while within the $CD4^+$ T cell population, around 9% of $CD25^+$ Tregs displayed the ability to bind to and take up the AuNPs. The researchers successfully achieved down-regulation of GFP expression in GFP + Tregs through the administration of GFP silencer siRNA-conjugated AuNPs [119].

MiRNA dysregulation is known to contribute to the development of autoimmunity, and their ability to regulate several disease-related genes makes them attractive targets for treatments. Previous research has indicated a decrease in miR-125a expression levels in circulating T cells of systemic lupus erythematosus (SLE) patients [115]. Additional investigations conducted on miR-125a deficient mice unveiled compromised immunoregulatory capabilities and Treg maintenance. It appears that miR-125a plays a notable role in governing the decision-making process within Tregs by directly inhibiting crucial factors like STAT3, IL-13, and IFN-γ. These factors are pivotal for effector lineage T cells and can hinder the differentiation of Tregs. As a result, miR-125a functions somewhat similarly to Foxp3, contributing to the improved stability of Treg-mediated self-tolerance [120]. However, miRNA faces challenges in therapeutic applications due to its susceptibility to nuclease degradation and its difficulty in being internalized by cells, largely given its molecular weight and polyanionic charge. Although chemical modification of miRNAs has shown therapeutic potential *in vivo*, concerns about safety and the need for high dosages have limited their widespread use.

To address these challenges, Jiali Zhang et al. developed a PLGA nanoparticle delivery platform for targeting miR-125a in a mouse model of SLE. They modified PLGA with monomethoxy-PEG and PLL to enhance stability and miR-125a encapsulation efficiency, respectively. The resulting PLGA nanoparticles, with a diameter of 158.2 ± 1.48 nm, were preferentially enriched in the spleen and effectively delivered miR-125a into splenic T cells without any negative effects on splenocytes. This led to an enhancement in the differentiation and suppressive potency of Tregs, which reversed the imbalance of regulatory/effector T cells, subsequently reduced lupus nephritis and alleviated SLE progression in the mouse model. Furthermore, the researchers demonstrated that PLGA-encapsulated miR-125a, when combined with TGF-β1, significantly increased Tregs expansion [115]. In line with the findings, Peter et al. pioneered the development of nanofibrous spongy microsphere scaffolds using PEGylated Poly (L-lactic acid) (PLLA) as a niche for recruiting Tregs. These scaffolds were multi-functionalized with PLGA to incorporate miR-10a, serving as an inducer for naïve T cells to differentiate into Tregs, and MSNs to incorporate Treg growth factors (TGF-β1/IL-2). The resulting injectable biomolecule-delivering system effectively increased the Treg population and promoted their expansion [116]. These innovative delivery approaches show great potential for effective miRNA-based therapies in Treg-mediated immune therapy.

As mentioned earlier, IL-2 plays a crucial role in regulating the function and balance of Tregs. When administered at low levels, it can effectively suppress immune disorders through promoting the expansion of Tregs expressing IL-2Rα. However, even at low doses, IL-2 signaling through the IL-2Rβ/γ complex may activate pro-inflammatory non-Treg T cells. Therefore, achieving greater specificity towards Tregs could be advantageous for therapeutic purposes. In pursuit of this goal, Huang et al. developed lipid nanoparticles containing mRNAs (messenger RNAs) to encode a modified IL-2 with a prolonged half-life. This mRNA produced a fusion protein of human albumin and IL-2, designed to enhance IL-2's stability, and included specific mutations that increased its affinity for IL-2Rα. When these lipid-encapsulated mRNA nanoparticles were administered subcutaneously in mouse models of GvHD and EAE, they selectively activated and expanded Tregs, leading to a reduction in disease severity in the mice. Although these mRNA-nanoparticles did not directly alter genes in Tregs, their products had a direct impact on the Tregs population [117]. Additionally, Thatte et al. developed an NLPs platform for delivering Foxp3 mRNA to $CD4^+$ T cells. Their results indicated that this delivery system effectively delivers Foxp3 mRNA to primary human $CD4^+$ T cells, resulting in an increase in TGF-B and IL-10 secretion, along with the suppression of primary effector CD4 and CD8 T cell proliferation *ex vivo*. This platform holds promise for engineering immunosuppressive Tregs, with potential applications in transplant medicine and autoimmune therapies [118].

3. Indirect functions of biomaterials on Treg Activation and expansion

Developing a specialized microenvironment to enhance the function of Tregs using biomaterials has demonstrated success. The innovative concept of biomaterial-induced tolerogenic microenvironment (Tol-Mic) signifies an advanced immunotherapy strategy with the aim of bolstering the effectiveness of Tregs. Diverse techniques involving biomaterials are employed to establish a Tol-Mic (Table 3 and Fig. 5). These methods encompass the induction of tolerogenic DCs, characterized by their low expression of co-stimulatory molecules (e.g., B7-1/B7-2) and MHC molecules, along with the presence of inhibitory molecules such as PD-L1 or FasL1 on their surface. Moreover, these DCs secrete anti-inflammatory cytokines like TGF-β1 and IL-10, which encourage Treg responses and quell pro-inflammatory immune reactions in transplantation and autoimmune diseases—all facilitated through biomaterial utilization (Reviewed by Ref. [121]). Additionally, certain biomaterials inherently possess tolerogenic attributes due to their unique physicochemical properties (Fig. 5A) (Reviewed by Refs. [121, 122]).

Table 3
Indirect functions of biomaterials on Treg activation and expansion.

Biomaterial-induced tolerogenic microenvironment (Tol-Mic) for Tregs				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
- Generation of Tregs - Useable in autoimmunity and transplantation	PEG-4MAL hydrogels	- IL-10-functionalized hydrogels - Extended duration and protection of DCs	- Generation of CD25+/Foxp3+ Tregs *in vitro* settings - Enhancement of the function of immunosuppressive DCs	[123]
	PLGA particles modified with O10H6 (297 ± 14.1 nm)	- PLGA encapsulated plasmids encoding IL-10 - Promotion of inherent expansion of DCs	- Enhanced uptake of PLGA by DC - Enhancement of the function of immunosuppressive DCs - Enhancement of the growth of Foxp3+ Tregs *in vitro* conditions	[124]
	Oval-shaped GQDs (Average lateral sizes of 23.6 ± 7.0 and 65.6 ± 8.7 nm)	- Engineered nanoparticles with inherited immunosuppressive properties - Phagocytosis by DCs	- Hindrance of the production of ROS in DCs - Inhibition of NF-kB and mTOR signaling pathways in DCs - Development of suppressive CD4+/CD25high/Foxp3+ Tregs	[125]
	NLPs (184–205 nm)	- Liposomes loaded with vitamin D3	- Promotion of the development of a Foxp3+/CD127low/TIGIT+/CD4+ Tregs	[126]
- Allergic airway inflammation	PS50G nanoparticles	- Engineered nanoparticles with inherited immunosuppressive properties	- Induced recruitment and activation of monocytes and DCs in the lung - Expansive growth of TNFR2+/Foxp3+ Tregs - Resistance to allergic airway inflammation	[127]
- Colitis - T1D - EAE	PLGA polymer, co-functionalized with MSNPs and PEG	- Encapsulation of MCP-1 within the MSNPs - Immobilization of FasL1 on the microsphere's surface - Encapsulation of well-known autoantigens relevant to colitis, nonobese diabetic conditions, and encephalomyelitis within the PLGA	- Autoantigen-specific T cell apoptosis - Increased production of TGF-β1 by macrophages - Restoration of Tregs - Reduction in disease severity across all mouse models	[128]
- Osteoarthritis	Lipid nanoparticles (215.4 ± 53.8 nm)	- Encapsulation of type II collagen along with Rapa	- Promotion of the generation of antigen-specific Tregs - Amelioration of osteoarthritis	[129]

Abbreviations arranged in alphabetical order: DCs: Dendritic Cells, **EAE**: Experimental Autoimmune Encephalomyelitis, **FasL1**: Fas Ligand 1, **Foxp3**: Forkhead Box P3, **GQDs**: Graphene Quantum Dots, **IL-10**: Interleukin-10, **MCP-1**: Monocyte Chemoattractant Protein-1, **MSNPs**: Mesoporous Silica Nanoparticles, **mTOR**: mammalian Target of Rapamycin, **NF-κB**: Factor Kappa B, **NLPs**: Nanoliposome Particles, **PEG**: Polyethylene Glycol, **PEG-4MAL**: 4-arm poly (ethylene glycol)-maleimide, **PLGA**: Poly (lactic-*co*-glycolic acid), **PLGA-O10H6**: Cationic Peptide O10H6, **PS50G**: Synthetic Glycine-Coated 50 nm Polystyrene Nanoparticles, **ROS**: Reactive Oxygen Species, **T1D**: Type 1 Diabetes, **TGF-β1**: Transforming Growth Factor Beta 1, **Tregs**: Regulatory T cells. *The particle sizes in the table are for diameters.

3.1. Biomaterial-induced tolerogenic microenvironment (Tol-Mic) for tregs

Interleukin-10 (IL-10) plays a crucial role as a cytokine that significantly influences immune responses, particularly by contributing to the expansion of tolerogenic DCs. This expansion contributes to the establishment of a Tol-Mic, which in turn supports the expansion of Tregs. Biomaterials also play a facilitating role in this process. For instance, a study conducted by Beskid et al. developed IL-10-functionalized hydrogels with the aim of enhancing the function of immunosuppressive DCs. By carefully releasing IL-10, they utilized a hydrogel based on PEG-4MAL (4-arm poly (ethylene glycol)-maleimide) to effectively prolong and protect the viability of DCs. Consequently, this led to the generation of CD4+/CD25+/Foxp3+ Tregs *in vitro* settings [123]. Another investigation led by Meng et al. focused on shaping the Tol-Mic by promoting the inherent expansion of DCs capable of producing IL-10. Their approach involved using PLGA-encapsulated plasmids encoding IL-10. To further improve the efficiency of this method, they modified the surface of these particles using the cationic peptide O10H6 (PLGA-O10H6). This modification significantly enhanced the uptake of PLGA by DCs. Notably, these modified DCs efficiently assimilated the IL-10-encoding plasmids, subsequently driving the growth of Foxp3+ Tregs *in vitro* conditions [124]. These adaptable biomaterial strategies offer extensive potential for diverse applications in molding immune tolerance (Fig. 5B).

Engineered nanoparticles can act as inducers of tolerogenic DCs and contribute to the formation of a Tol-Mic, fostering the expansion of Tregs, based on their physicochemical properties including shape, size, dosage, surface charge, and duration of exposure to DCs (Fig. 5A). In pursuit of this goal, Tomic et al. designed nanoparticles using graphene quantum dots (GQDs), which possess numerous advantages such as being phagocytosed by DCs, biocompatibility, and biodegradability within the body. They demonstrated that these nanosized GQDs were engulfed by DCs, subsequently hindering the production of ROS (reactive oxygen species), as well as inhibiting NF-kB and mTOR signaling pathways in DCs. DCs treated with GQDs showed an enhanced tendency for Th2 polarization and instigated the development of suppressive CD4+/CD25high/Foxp3+ Tregs [125]. Mohamud et al. fabricated PS50G (synthetic glycine-coated 50 nm polystyrene nanoparticles) with the potential to induce the production of chemokines, initiating the recruitment and/or maturation of DCs and monocytes. Their findings highlighted that PS50G nanoparticles induced activation of DCs in the lung, correlating with the expansive growth of CD4+/Foxp3+/TNFR2+ Tregs and conferring resistance to in mice model of allergic airway inflammation [127]. Furthermore, AuNPs possess unique properties, including flexibility in terms of size, surface modification, shape, and being easily phagocytosed by DCs. These attributes position AuNPs as favorable nanoparticles for inducing tolerogenic DCs both *in vitro* and *in vivo*, as outlined in a review by Ref. [122].

The expansion of antigen-specific Tregs within the biomaterial-induced Tol-Mic can also be achieved through the utilization of polymeric or liposomal nanoparticles loaded with immunomodulatory agents, particularly Rapa [75,83,86,103,129], and vitamin D3 [73,88,90,126]. This approach can be combined with the encapsulation of both

auto- and allo-antigens, along with immunomodulatory agents, within a single nanoparticle, as previously detailed in this review article. Both strategies result in the uptake of nanoparticles by DCs, transforming them into a tolerogenic state.

In addition to serving as a delivery system to target DCs, biomaterials also contribute to enhancing the stability of immunomodulatory agents in the body. For example, Rapa, being poorly soluble in water, benefits from encapsulation in βCD-NH2, leading to improved aqueous solubility and increased effectiveness on Tregs [86]. Liposome nanoparticles can fuse with the cell membrane of DCs, enveloping fat-soluble agents like vitamin D3 and facilitating their delivery to the DCs. In this context, Nagy et al. demonstrated that liposomes loaded with vitamin D3 can prime DCs, promoting the development of a Foxp3+/CD127low/TIGIT+/CD4+ Tregs phenotype within the skin [126]. Similarly, another study by Sohn et al. employed lipid nanoparticles encapsulating type II collagen, an autoantigen in osteoarthritis, along with Rapa to induce Tregs in a mouse model of osteoarthritis. Their findings indicated that these nanoparticles effectively prompted the generation of antigen-specific Tregs, which were also transportable into the osteoarthritis mice, subsequently ameliorating the disease (Fig. 5B) [129].

Microparticles can also be deliberately designed to shape a Tol-Mic for the reestablishment of Tregs without impacting DCs. In this context, Jin et al. fabricated microparticles to treat various mouse models of autoimmune conditions, including colitis, nonobese diabetic conditions, and encephalomyelitis. They engineered microspheres using PLGA polymer, co-functionalized with MSNPs and PEG. In the initial phase, they encapsulated MCP-1 (monocyte chemoattractant protein-1) within the MSNPs for controlled release, and also immobilized FasL1 on the microsphere's surface. The underlying idea was that MCP-1 would attract activated T cells in the context of autoimmunity, and as these activated T cells increasingly exhibited Fas molecules on their surface, the immobilized FasL1 would trigger apoptosis in these activated T cells. This, in turn, would lead to the reestablishment of Tregs at the site of inflammation. They demonstrated that the apoptotic T cells were engulfed by specialized macrophages, resulting in the production of substantial amounts of TGF-β1, thus, promoting further differentiation of Tregs. In the subsequent stage, by encapsulating well-known autoantigens relevant to colitis, nonobese diabetic conditions, and encephalomyelitis within the microspheres, they observed a reduction in disease severity across all mouse models. This reduction was achieved through the controlled release of autoantigens subsequent to the

Table 4
Biomaterials as tolerogenic artificial antigen-presenting cells (Tol-aAPC).

Synthetic Tol-aAPC				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
- Generation of Tregs - Useable in autoimmunity and transplantation	NLPs	- Antigen presentation to T cells without signal-2 - Liposomes carrying peptide-MHC complexes (Signal-1)	- Induction of antigen-specific Tregs - Induction of Tregs both *in vitro* and *in vivo* with suppressive activities	[130]
	Spherical PLGA-PBAE microparticles (3 μm)	- PLGA functionalized with anti-CD3 (Signal-1) and anti-CD28 (Signal-2) - PLGA loaded with TGF-β1 (Signal-3)		[131]
- T1D - Rheumatoid Arthritis - EAE	Magnetic (Iron oxide) bead nanoparticles	- Autoantigen presentation to T cells without signal-2 - Iron oxide nanoparticles coated with autoimmune relevant peptides-MHC complexes (Signal-1) - T1D relevant peptides: GAD65, PPI, and IGRP - EAE relevant peptides: MOG - Arthritis relevant peptides: collagen II	- Transformation of autoreactive T cells into Tregs - Expansion of autoregulatory T cells - Effective prevention of the development of T1D, EAE, and arthritis	[132,133]
	Spherical PLGA-PBAE nanoparticles (700 ± 200 nm)	- PLGA functionalized with anti-CD3 (Signal-1) and anti-CD28 (Signal-2) - PLGA loaded with TGF-β1 (Signal-3)	- Expansion of Foxp3+ Tregs - Improved engraftment of beta-islets	[85]
	Spherical PLGA nanoparticles (180–240 nm)	- Antigen presentation to T cells without signal-2 - PLGA carrying MOG autoantigen-MHC complexes (Signal-1) - PLGA loaded with TGF-β1 (Signal-3) - PLGA functionalized with regulatory molecules (e.g., *anti*-Fas, PD-L1, and CD47)	- Increase in Tregs - Decrease in MOG-reactive Th17 and Th1 cells - Improvement of demyelination and reduced neuroinflammation	[134]
Engineered cell membrane-derived Tol-aAPC (ECM-Tol-aAPC)				
- Generation of Tregs - Useable in autoimmunity and transplantation - T1D	ECDI-fixed splenocytes	- Antigen presentation to T cells without signal-2 - Allo- or auto- antigens linked to syngeneic splenocytes - linker used: ECDI	- Expansion of Foxp3+/CD4+ Tregs - Expansion of antigen- specific Tregs - Long-term survival of beta-islet transplants - Induction of peripheral tolerance through CTLA-4 and PD-L1, and the release of anti-inflammatory cytokines like IL-10	[135–138]
	SMCC-fixed erythrocyte	- Antigen presentation to T cells without signal-2 - Allo- or auto- antigens linked to erythrocytes - linker used: SMCC	- Antigen-specific T cell deletion - Induction of T cell anergy - Expression of Tregs - Induction of peripheral tolerance through PD-1/PD-L1 interactions - Establishment of memory of tolerance	[139]

Abbreviations arranged in alphabetical order: CTLA-4: Cytotoxic T-lymphocyte-Associated Protein 4, **EAE**: Experimental Autoimmune Encephalomyelitis, **ECDI**: 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide, **Foxp3**: Forkhead Box P3, **GAD65**: Glutamic Acid Decarboxylase, **IGRP**: Glucose-6-Phosphatase Catalytic Subunit–Related Protein, **IL-10**: Interleukin-10, **MHC**: Major Histocompatibility Complex, **MOG**: Myelin Oligodendrocyte Glycoprotein, **NLPs**: Nanoliposome Particles, **PBAE**: Poly (beta-amino ester), **PD-1**: Programmed death 1, **PD-L1**: Programmed death-ligand 1, **PLGA**: Poly (lactic-*co*-glycolic acid), **PPI**: Pre-proinsulinI, **SMCC**: Sulfosuccinimidyl-4-(*N*-maleimidomethyl) Cyclohexane-1-carboxylate, **T1D**: Type 1 Diabetes, **TGF-β1**: Transforming Growth Factor Beta 1, **Tregs**: Regulatory T cells.
*The particle sizes in the table are for diameters.

induction of T cell apoptosis and the secretion of TGF-β1 by macrophages. This multifaceted approach facilitated the establishment of a Tol-Mic, effectively contributing to the reestablishment of Tregs (Fig. 5C–D) [128].

3.2. Biomaterials as tolerogenic artificial antigen-presenting cells (Tol-aAPCs)

T cells require two signals to activate and expand, and these signals are provided by APCs. The first signal involves the TCR on T cells binding to the peptide-MHC complex presented by APCs. This interaction is facilitated by the CD3 and CD4/CD8 co-receptors on T cells. The second signal comes from co-stimulatory molecules on APCs (e.g., B7-1/B7-2), which interact with receptors on T cells, like CD28. Without this second signal, T cells become anergic or unresponsive to antigens, potentially leading to the expansion of antigen-specific Tregs. On the other hand, when both signals 1 and 2 are transduced in a regulatory microenvironment (for example, in the presence of TGF-β1 as a soluble-released signal-3), it can lead to the expansion of Tregs. Biomaterials can play a role as tolerogenic artificial antigen-presenting cells (Tol-aAPCs) in mediating the expansion of Tregs. This section discusses two approaches to fabricating Tol-aAPCs: one involves using synthetic materials, and the other involves engineered cell membrane biomimetics (Table 4 and Fig. 6).

3.2.1. Synthetic Tol-aAPC

Liposome-based biomaterials can imitate cell membrane properties, making them suitable for functionalizing with peptide-MHC complexes. By providing signal-1 to T cells in the absence of signal-2, they can induce specific-antigen Tregs. One of the pioneering attempts to induce Tregs using liposome-functionalized peptide-MHC was conducted by Jan Klein's research team. They demonstrated that liposomes carrying peptide-MHC complexes can induce Tregs by presenting ovalbumin to T cells [130]. Furthermore, other nanoparticles can also be utilized for peptide-MHC presentation to T cells. For instance, Clemente-Casares et al. designed magnetic bead nanoparticles coated with T1D-disease-relevant peptides (e.g., IGRP [glucose-6-phosphatase catalytic subunit–related protein], PPI [pre-proinsulin], and GAD65 [glutamic acid decarboxylase]), bound to MHC-class II (MHC-II). These nanoparticles primed autoreactive T cells into Treg cells, leading to the alleviation of T1D in mice [133]. In a similar study, IGRP peptide-coupled MHC on iron bead nanoparticles in a mouse model of T1D resulted in the expansion of cognate low-avidity autoreactive CD8 T cells with regulatory functions. These Treg subsets successfully restored normoglycemia in mice [132]. Overall, peptide-MHC-based nanoparticles offer a promising category of medications for the treatment of various autoimmune disorders through a targeted approach that focuses on the underlying diseases (Fig. 6A).

According to the MHC-restriction theory, creating a generalized nanoparticle-functionalized peptide-MHC suitable for all patients is very complicated. Moreover, designing a peptide antigen-fitted MHC with the right properties is time-consuming and limited because we don't know all the best candidate auto- and allo-antigens. To address these challenges, Jordan et al. developed spherical PLGA microparticles coated with poly (beta-amino ester) (PBAE). These microparticles were loaded with TGF-β1 (as a soluble-released signal-3) and functionalized with anti-CD3 (to facilitate signal-1 transduction) and anti-CD28 (to induce signal-2). The results of their study demonstrated that this synthetic Tol-aAPC induced Tregs both *in vitro* and *in vivo*, exhibiting suppressive activities [131]. Similarly, in another study conducted by Sarah et al., PLGA/PBAE particles encapsulating TGF-β1 and coated with anti-CD3 and anti-CD28 were fabricated. These spherical particles, with a size of 700 ± 200 nm, strongly induced Tregs, leading to an improvement in beta-islet engrafts in mice with T1D [85]. Synthetic Tol-aAPCs can be externally designed as biomimetic biomaterials to induce tolerance against auto- and allo-antigens (Table 4 and Fig. 6B).

3.2.2. Engineered Cell Membrane-Derived Tol-aAPC (ECM-tol-aapc)

The use of synthetic Tol-aAPC-based nanoparticles is limited due to concerns about organ toxicity. As an alternative approach, engineering cell membranes of APCs with biomaterials provides a way to induce T cell anergy or unresponsiveness by lacking signal-2 on their surface, leading to the expansion of Tregs. Syngeneic splenocytes-functionalized with ECDI have been shown to induce T cell clonal anergy both *in vivo* and *in vitro* [140,141]. Stephen D. Miller et al. conducted four separate studies that demonstrated the effectiveness of administering peptide-coupled, ECDI-fixed splenocytes in inducing T cell unresponsiveness [135,138]. This approach has led to the induction of peripheral tolerance in autoimmune complications [137] and donor-specific tolerance in beta-islet transplants [136]. The tolerance is mediated through the expansion of Foxp3+/CD4+ Tregs, the presence of inhibitory molecules (e.g., PD-L1 and CTLA-4), and the release of anti-inflammatory cytokines (e.g., IL-10).

The use of syngeneic splenocytes may not apply to all patients. Therefore, an alternative approach is to utilize erythrocytes as a source of aAPCs. The concept behind erythrocyte-binding antigens is that as erythrocytes age, circulate, and eventually get cleared from the body, the attached antigen payload on the erythrocyte surface will also be cleared in a tolerogenic manner along with the aging erythrocytes. In a study by Grimm et al., the authors conjugated ovalbumin to erythrocytes using SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) linker. This erythrocyte-binding antigen approach led to antigen-specific T cell deletion, induction of T cell anergy, and the expression of Treg markers through PD-1/PD-L1 interactions, resulting in the establishment of long-term immune tolerance [139]. So, engineering APC cell membranes with biomaterials offers a promising alternative to induce T cell anergy and expand Tregs (Table 4 and Fig. 6C–D).

4. Biomaterials to facilitate enhancement in treg functions

In this section, we delve into an exploration of several innovative biomaterial strategies that hold promise for optimizing Treg functionality. These strategies include scaffolds designed to act as Treg niches, techniques for Treg isolation, tracking Tregs after adoptive transfer, and cryopreservation methods for Tregs. By exploring these approaches, we aim to gain insights into enhancing the effectiveness and therapeutic potential of Tregs in immune regulation and tolerance induction (Table 5 and Fig. 7).

4.1. Scaffold-based biomaterials for delivering tregs

Treg-therapy shows promise in establishing immune tolerance in transplant recipients and individuals with autoimmune diseases. Current clinical trials often involve the systemic adoptive transfer of *ex vivo* expanded Tregs, yielding promising results. However, the low population of Tregs in peripheral blood presents challenges in generating a sufficient number of cells through *ex vivo* expansion, making the process time-consuming and costly. To address these challenges, researchers have explored alternative strategies, such as utilizing scaffolds as Treg niches. These scaffolds create a supportive microenvironment that enhances Treg's survival, expansion, and functionality. A specific targeted approach, developed by Kim et al. involves encapsulating human natural and induced Tregs into alginate-gelatin methacryloyl (GelMA) hydrogel for localized immunosuppression, particularly applicable in beta-islet transplantation. The GelMA hydrogel effectively retains Treg viability, stable phenotype, and function within its structure. Furthermore, supplementing the GelMA with specific bioactive factors like CCL-1 (chemokine ligand 1) and IL-2 enhances Treg viability and suppressive activities, recruited CCR-8+ (CC chemokine receptor 8+) T cells, as a Tregs subpopulations, to the GelMA [77].

In a similar study, Bushman et al. utilized Tregs encapsulated within PEG-norbornene (PEGNB) biodegradable hydrogel around allografted

Table 5
Biomaterials to facilitate enhancement in treg functions (part 1).

Scaffold-based biomaterials				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
- Beta-islet transplantation - T1D	GelMA hydrogel	- Functionalization of hydrogel with IL-2 and CCL-1 - Encapsulation of natural and induced Tregs into functionalized hydrogel	- Retention of Treg viability, stable phenotype, and function within hydrogel - Inclusion of Tregs and attraction of CCR-8+ Tregs within hydrogel	[77]
- Nerve allografts	PEGNB biodegradable hydrogel	- Encapsulation of Tregs into hydrogel - Transplantation of hydrogel containing Tregs around nerves' allografts	- Gradual release of Tregs from the hydrogel into the graft - Suppression of host immune response - Promotion of nerve regeneration in recipients	[142]
Isolation of Tregs				
- Facilitate *ex vivo* isolation, activation, and expansion of Foxp3+/CD4+/CD25+ Tregs - Study the behaviors of Tregs	Superparamagnetic particles (Dynabeads™ Treg CD3/CD28)	- Optimization of Dynabeads™ Treg for isolation, activation and expansion of Foxp3+/CD4+/CD25+ Tregs	- Several hundred-fold expansions of Treg cell populations - Isolation of Tregs with high expression of Foxp3 and suppressive activity	[143]
	Soluble polymer nanomatrix	- Polymer conjugation with anti-CD3 and anti-CD28 - Inclusion of a high amount of exogenous IL-2	- Isolation of CD4+/CD127-/CD25+/CD45RA+ and CD4+/CD127-/CD25hi Treg subsets - Enhancement of Treg functions during *in vitro* expansion	[144]
	Microfluidic platform	- Microfluidic Platform for Manipulation and Isolation of Tregs	- High yield and speed for Tregs isolation - Assessment of Treg behaviors in interplay between TCR/CD3 and LFA-1 adhesion and localization of PKC-θ	[145, 146]
Tregs Tracking				
- Tracking of polyclonal expanded Tregs	Radiolabeled Tregs with ^{89}Zr-oxine	- *In vivo* tracking using SPET/CT	- Viability and activities of Tregs unaffected by radiolabeling - Successful tracking and monitoring of polyclonally expanded human Tregsin immunodeficient mice for 5 days	[147]
	Paramagnetic metal ion, gadolinium-III	- Labeling of Tregs with metal ion, gadolinium-III - *In vivo* tracking employing LA-ICP-MS technique	- Successful tracking and monitoring of labeled Tregs in immunodeficient mice for 10 days - Detection of Tregs at the single-cell level	[148]
- Tracking of expanded Tregs in skin transplanted mice	Radiolabeled Tregs with Tc-99 m	- *In vivo* tracking via SPET/CT	- Viability and activities of Tregs unaffected by radiolabeling - Successful tracking and monitoring of Tregs in skin transplanted mice for 1 day	[149]
	Dynabeads™ Treg	- Tracking and monitoring of Tregs expressing NIS-GFP fusion protein	- Viability and activities of Tregs unaffected by the transduced gene - Successful tracking and monitoring of Tregs in skin transplanted mice for 40 days	[78]
- Tracking of expanded Tregs in of heart and lung transplantation	PEGylated nano-sized iron-oxide particles	- Particles serving as MRI contrast agent	- labeling efficiency of over 90% in T cells - High intracellular iron concentration and transverse relaxivity	[150]

Abbreviations arranged in alphabetical order: **Tc-99m**: Technetium-99 m pertechnetate, **CCL-1**: Chemokine ligand 1, **CCR-8**: CC chemokine receptor 8, **GelMA**: Alginate-gelatin methacryloyl, **GFP**: Green Fluorescent Protein, **IL-2**: Interleukin-2, **LA-ICP-MS**: Laser Ablation Inductively Coupled Plasma Mass Spectrometry, **LFA-1:** Lymphocyte function-associated antigen 1, **MRI**: Magnetic resonance imaging, **NIS**: Human sodium Iodide Symporter, **PEG**: Polyethylene Glycol, **PEGNB**: PEG-norbornene, **PKC-θ**: Protein kinase C theta, **SPET/CT**: Single-photon emission computed tomography, **T1D**: Type 1 Diabetes, **TCR**: T Cell Receptor, **Tregs**: Regulatory T cells. *The particle sizes in the table are for diameters.

nerves. The findings of their research revealed that, for 14 days, Tregs were gradually released from the hydrogel, migrated into the graft, and assumed a dual role. They effectively suppressed the host immune response while also promoting the nerves regeneration of recipient rats, comparable to the positive control of autografts. By employing these advanced techniques, researchers aim to optimize Treg-therapy and develop more effective and targeted treatments for immune-related conditions. These innovative approaches show significant promise in enhancing immune tolerance and improving patient outcomes in transplantation and autoimmune diseases (Fig. 7A–B) [142].

Designing scaffolds for delivering Tregs presents multiple challenges crucial for effective therapy. Retaining Tregs within target tissues is paramount, alongside maintaining their suppressive function post-delivery to adequately regulate immune responses. Optimizing Treg dosage and understanding their longevity *in vivo* are essential for sustained therapeutic effects and to prevent adverse outcomes. Combining Treg therapy with adjunctive approaches, such as low-dose IL-2 or other immunomodulatory agents, can augment efficacy. Tailoring Treg therapy based on the microenvironment's role in autoimmune diseases and transplantation allows for personalized treatment strategies. Moreover, advancements in autoantigen discovery and cutting-edge technologies like T cell receptor sequencing and chimeric antigen receptor (CAR) technology offer promising avenues for personalized CAR-Treg therapies. Successfully addressing these challenges could establish Treg immunotherapy as a personalized medicine option for effectively managing autoimmune diseases and transplantation [151–153]. However, ongoing research and clinical trials are indispensable to refine these approaches and enhance patient outcomes.

4.2. Biomaterials for isolating tregs

The frequency of Tregs in bloodstream is relatively low, comprising only about 5–10 percent of $CD4^+$ T cells. This underscores the necessity of expanding Tregs *ex vivo* before their adoptive transfer in most clinical settings. In Treg-therapy applications, having an adequate number of functional Tregs is crucial to achieve therapeutic benefits. However, due

to their limited presence in the bloodstream, it becomes essential to enhance and enrich Tregs through *ex vivo* expansion techniques before reintroducing them to the patient.

Researchers have developed a type of superparamagnetic spherical polymer particle called Dynabeads™ CD3/CD28 CTS™, which serve the purpose of *ex vivo* isolation, activation, and expansion of T cells. These particles are particularly useful for adoptive T cell-based therapy. Similarly, Aarvak et al. have introduced a product called Dynabeads™ Treg CD3/CD28, specifically designed to facilitate *ex vivo* isolation, activation, and expansion of CD4+/CD25+/Foxp3+ Tregs. Over a 14-day culture period, they observed a remarkable high expansion of Tregs, all while maintaining high expression of Foxp3 with suppressive activities. The Dynabeads Treg CD3/CD28 product is tailored to optimize the activation and expansion of CD4+/CD25+/Foxp3+ Tregs, demonstrating its potential in enhancing Treg-based therapies (Fig. 7B–C) [143].

As mentioned earlier, Tol-aAPCs can also serve as effective tools for *in vitro* expansion of Tregs. In a study conducted by Janssens et al., researchers developed a soluble polymer nanomatrix that was conjugated with anti-CD3 and anti-CD28, along with the addition of exogenous IL-2, to facilitate Treg expansion. The results of their study demonstrated a significant increase in functional Tregs during *in vitro* expansion, with a remarkable 185-fold and over 70-fold increase in two Treg populations, namely CD4+/CD25high/CD127-and CD4+/CD25+/CD127-/CD45RA+, respectively. This approach shows great promise in enhancing rare Treg populations [144].

Microfluidic and fluidic switching technologies play a significant role in the biomedical field, promising to improve cell manipulation. Microfluidics deals with the behavior, control, and manipulation of cells within microscale channels and chambers, making it a crucial area in cell therapy. In one study by Lee et al., they developed a microfluidic platform to study the behaviors of Tregs. The research showed that these rare CD4+/CD25+ Tregs responded differently to micro-scale features of anti-CD28 and anti-CD3 antibodies compared to CD4+/CD25+ conventional T cells. The response of Tregs was dependent on the balance between LFA-1 adhesion and TCR/CD3, the localization of protein-kinase-*C*-theta (PKC-θ) in Tregs, and the interplay between TCR/CD3 and LFA-1-based adhesion [145]. Furthermore, microfluidic platforms can also be utilized for Treg isolation. Berglund et al. developed a microfluidic-based system with high yield and speed for Tregs isolation. They successfully obtained 150,000 to 1.7 million cells from an initial pool of 450 million freshly isolated peripheral blood mononuclear cells in under 4 h. These isolated cells had an average Foxp3+ expression of 85% and a viability of 97%. Such advancements hold great promise for advancing cell therapies and research in the biomedical field [146].

4.3. Biomaterials for tracking tregs

Despite the increasing number of clinical trials involving Treg therapies, there are still crucial questions about the behavior and monitoring of Tregs within the body. Therefore, it is essential to monitor their distribution, *in vivo* fate, functional activity, and interactions with other cells after infusion. This monitoring is crucial to evaluate, predict, and enhance the effectiveness of adoptive cell therapies (Fig. 7B–D). In a study by Jacob et al., polyclonal expanded human Tregs were successfully tracked and monitored using radiolabeled ^{89}Zr-oxine in immunodeficient mice for three days. The results showed that this radiolabeling did not affect the viability of Tregs or their suppressive activities [147]. Another study involved radiolabeling of $CD4^+$ T cells with Tc-99 m (Technetium-99 m pertechnetate), enabling Treg biodistribution assessment within a day [149,154]. However, the short half-life of the radioisotope Tc-99 m (6 h) limited the ability to track Tregs over the long-term [154]. The use of longer half-life radioisotopes, such as ^{89}Zr-oxine, could extend tracking times, but it comes with higher radioactive doses, which may limit the duration of the study.

For long-term cell tracking, indirect cell labeling using genetic engineering to express reporter genes has proven effective with radionuclide imaging. In another study by Jacob et al., Tregs were tracked and monitored in humanized mice with human skin transplants for up to 40 days. These traceable Tregs were expanded using anti-CD3/CD28 beads, IL-2, and Rapa and transduced with lentiviral particles carrying the NIS-GFP fusion gene. Human sodium iodide symporter (NIS) and green fluorescent protein (GFP) were shown to be promising reporter genes for cell tracking, being well tolerated in Tregs and detectable within 24 h after administration. Through this approach, the researchers identified the role of innate immune cells in Treg trafficking to skin grafts [78]. Other strategies for Treg tracking include deuterium labeling (up to 180 days) [155], carboxy fluorescein succinimidyl ester (CSFE, up to 10–100 days) [59,60,156–159], the luciferase gene-reporter system (up to 21–294 days) [160–162], and Treg-specific monoclonal-Ab staining (up to 98 days) [163].

Traditional cell tracking techniques, such as gene reporter systems, radiolabeling, and small-molecule fluorescent probes, have inherent limitations in efficiently and continuously detecting cells in deep tissues. These limitations are primarily due to their restricted signal penetration and rapid degradation. Additionally, there is a risk of cytotoxicity from using these labeling materials, which can impact the functionality of the labeled immune cells. To address these challenges, scientists have developed non-invasive cell labeling using biomaterials. These agents facilitate real-time *in vivo* cell tracking and possess outstanding emission capabilities, photostability, and minimal cytotoxicity [164]. In a study conducted by Managh et al., a paramagnetic metal ion, gadolinium-III (Gd-III), was utilized as a contrast agent for Treg monitoring both *in vitro* and *in vivo*, using a sensitive technique named LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry). The findings demonstrated that the labeled cells remained detectable for up to 10 days after labeling, at the single-cell level, both *in vitro* and *in vivo* [148].

AuNPs are widely used as nanoimaging agents in CT (computed tomography)-mediated immunoimaging. Their adjustable parameters, such as functionalization, size, and shape, make them suitable for various immune cell labeling, leading to improved intracellular stability and imaging intensity. In this regard, Meir et al. developed 20-nm glucose-coated AuNPs for *in vivo* monitoring of tumor-specific T cells. By modifying the AuNPs with glucose, their stability and internalization into T cells were enhanced. This strategy maintained the biological activity of tumor-specific T cells and effectively showed the recrutation of them in tumor tissues for up to three days after adoptive cell transfer [165]. Gadolinium nanoparticles (GdNPs) can also be used for T cell tracking in MRI (Magnetic Resonance Imaging). In a study conducted by the Yao et al., core-shell sodium gadolinium fluoride (NaGdF4) nanoparticles functionalized by DSPE-PEG-NH2 and HIV-1 *trans*-activator peptides were fabricated for quantitative MRI tracking of transferred autologous T cells. Systemic adoptive transfer of T cells labeled with these designed GdNPs in a glioma mouse model showed significant T cell accumulation at the glioma site 24 h after intravenous infusion [166]. In another study, Chien et al. developed an MRI contrast agent using PEGylated nano-sized iron-oxide particles (IOPC–NH2) for the *in vivo* tracking of T cells, specifically Tregs. They achieved a labeling efficiency around 90 percent in T cells, resulting in a high intracellular iron concentration and transverse relaxivity. Importantly, they accomplished this without resorting to conventional methods (e.g., electroporation, transfection reagents, or transactivator peptides). The process of labeling T cells involved incubating them with these particles in a culture medium. They successfully detected IOPC-NH2 labeled Tregs in a mouse model of lung and heart transplantation [150].

Biomaterial tracking can be used for *in vivo* monitoring of chimeric antigen receptor (CAR) T cells after adoptive transfer. For example, Harmsen et al. developed a CAR T cell imaging platform that employed NIRF/PET (near infrared fluorescence and positron emission tomography). They labeled CAR T cells with NIRF silica nanoparticles and ^{89}Zr. These labeled cells were then administrated into ovarian peritoneal carcinomatosis mice model, and NIRF/PET imaging was utilized to track

the distribution of CAR T cells *in vivo*, aiding in the assessment of the therapeutic efficacy of CAR T-therapy for tumors [167]. In another study, PEGylated functionalized AuNPs labeled with ^{64}Cu(II) using tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, a macrocyclic chelator) were introduced into transgenic CD19-targeted CAR T cells using a synthetic DNA transposon/transposase system. The labeled CAR T cells were then injected into mice, and their distribution in the body was detectable using PET imaging [168]. Additionally, Xie et al. synthesized ultra-small superparamagnetic iron oxide nanoparticles (USPIOs) covered with amino alcohol derivatives of glucose to monitor the dynamic infiltration and persistence of CAR T cells in glioblastoma. The CAR T cells could be traced for 3–14 days following the injection of USPIO-labeled CAR T cells [169]. Similar approaches could also be used for specific organ tracking of Tregs in organ transplantation, autoimmune diseases, and after Treg adoptive transfer.

4.4. Biomaterials for cryopreserving tregs

Human Treg therapy poses distinctive challenges due to the limited presence of these cells in the bloodstream. To prevent graft rejection or treatment of autoimmune disorders, a substantial amount of Tregs is required. While the precise critical Treg number is uncertain, animal studies suggest that maintaining a Treg to conventional T cell ratio between 1:1 and 1:2 is crucial. Achieving this ratio would necessitate infusing billions of Tregs to effectively suppress the immune response [170]. Recent progress in isolating and expanding Tregs through biomaterial-based *ex vivo* expansion has tackled the scarcity issue, enabling the acquisition of sufficient fresh Tregs for clinical purposes [85,131,133]. Nevertheless, improving the feasibility of Treg-based therapies entails the ability to sort, isolate, and expand Tregs over an extended period, as needed. This strategy would provide greater flexibility in treatment scheduling, infusion timing, and the potential for subsequent Treg administrations.

However, our current understanding of how cryopreservation might impact Tregs remains limited. Based on the available literature, cryopreservation could potentially influence the production of cytokines and the expression of surface markers that play a crucial role in Treg function (as reviewed by Ref. [171]). There are two cell banking approaches for Treg-based therapies: I) Cryopreservation of *ex vivo* expanded Tregs. II) Cryopreservation of CD4$^+$ T cells for next isolation and expansion of Treg. Both of these approaches hold promise as solutions to counteract the negative effects of cryopreservation on Tregs [172]. In this regard, a study conducted by Ulbar et al. demonstrated that Tregs obtained from patients with end-stage kidney and liver disease can be isolated and expanded using paramagnetic beads. The cryopreserved Tregs were successfully expanded and remained fully functional after thawing. These Tregs were utilized in therapy for solid organ transplantation [173]. Similarly, Levings et al. employed the Dynabeads Tregs method to cryopreserve expanded Tregs. Their study also showed successful expansion of thawed Tregs after cryopreservation [174]. These cryopreservation techniques utilizing biomaterials have proven effective in preserving a substantial number of functional Tregs.

Biomaterials not only aid in the isolation and expansion of Tregs before and after cryopreservation but can also serve as cryoprotectants to mitigate the adverse effects of cryopreservation and preserve Treg function after thawing. Traditionally, 10% concentration of DMSO (dimethyl sulfoxide) is widely used as a cryoprotective agent in clinical adoptive cell therapies. However, using it for long-term storage could potentially alter the functions of preserved cells and lead to significant toxicity when administered to patients [175]. Kaiser et al. addressed this concern by incorporating PEG into the freezing medium, which reduced the DMSO concentration in Treg cryopreservation to as low as 5%. Their research showed that the PEG-DMSO composition enhanced Treg functionality, viability, and post-cryopreservation recovery rates both in *ex vivo* and *in vivo* settings [176]. Beyond PEG, various other cryoprotectant agents such as synthetic proteins, polymers, sugar alcohols, and sugars are extensively employed for DMSO-free cryopreservation of immune cells in adoptive cell-based immunotherapy (as reviewed by Ref. [177]).

Despite the existence of intracellular biocompatible cryoprotectants like trehalose, the challenge lies in effectively delivering these protective agents into cells. This challenge has hindered their broader usage in preserving immune cells, especially lymphocytes that are difficult to transfect [178]. To overcome this obstacle, Yao et al. designed a nanocarrier utilizing tripolyphosphate (TPP) and chitosan nanoparticles. This nanocarrier encapsulated trehalose and facilitated its transport into NK cells cytoplasm. When compared to both free trehalose and DMSO, this nanocarrier-based trehalose delivery method effectively maintained the robust viabilities and antitumor capabilities of thawed NK cells following a standard freeze-thaw cycle [179]. This cryopreservation strategy involving biomaterials holds potential for application to other immune cells, especially Tregs.

5. Immune-regulating camouflage biomaterials

The concept of using biomaterials-based immune camouflage has arisen as a promising strategy for altering immunogenic antigens. These biomaterials are chemically bound to proteins on the cell surface, allowing them to evade immune detection or minimize immune responses. This is achieved through various mechanisms, such as hiding antigens, the formation of a steric barrier between cells or receptors and ligands, and the transmission of immunomodulation signals to T cells and the formation of Tregs via inhibitory molecules like PD-L1 and FasL1. By shielding the antigenic epitopes, this strategy can reduce or prevent autoimmunity and graft loss (Table 6 and Fig. 8A–B).

Polyethylene glycol (PEG) and its derivatives which have been approved by the FDA are non-toxic polymer which can minimize or prevent host immune responses through PEG-based charge and steric camouflage. In this context, Hamada et al. administered mPEG (monomethoxypolyethylene glycol)-covered CM (cardiac myosin) to EAM (experimental autoimmune myocarditis) mice model prior to inducing active or passive EAM, effectively inhibiting its induction. They theorized that this suppression was brought about by an expansion of antigen-specific CD4$^+$ and CD8$^+$ Treg subsets [180]. In a similar study, Wang et al. employed another PEG derivative known as mPEG-SPA (methoxy polyethylene glycol succinimidyl propionate) to coat corneal alloantigens, resulting in reduced corneal graft rejection in an animal model. Although they did not analyze Tregs in this model, they demonstrated decreasing IFN-γ and IL-2 levels, along with raising IL-10 in animals treated with mPEG-SPA [181]. In an effort to evaluate the impact of PEG-modified antigens on Tregs, Lu et al. transplanted PEG-modified acellular adipose matrix into a xenotransplantation mouse model. Their findings indicated that adipogenesis was enhanced through an increase in Tregs within the transplanted matrix (Fig. 8A) [76].

Using biomaterial-engineered ligands represents another strategy for immune camouflage aimed at inducing immune tolerance by dampening effector T cell responses to auto- and allo-antigens, and converting conventional T cells into CD4+/Foxp3+ Tregs. In pursuit of this objective, Shirwan et al. conducted three separate studies utilizing FasL1 in combination with streptavidin (SA-FasL1) for beta-islet transplantation in a T1D mouse model. In the first study, they transplanted beta-islets displaying SA-FasL1 into a T1D mouse model, demonstrating that tolerance was initiated and sustained by CD4+/CD25+/Foxp3+ Tregs [185]. In the second study, they engineered a SA-FasL1-functionalized PEG microgel for localized immunomodulation and acceptance of allogeneic beta-islet grafts. Their findings revealed the inhibition of alloantigen-specific T cells and an increase in CD4+/CD25+/Foxp3+ Tregs, resulting in the acceptance and functional persistence of allografts for over 200 days [183]. In their third study, allogeneic beta-islets were seeded onto SA-FasL1-functionalized PEG particles measuring 860 ± 40 nm and transplanted into the

Table 6
Biomaterials to facilitate functional tregs (part 2).

Camouflage for immune regulation				
Application(s)	Biomaterial(s) used	Strategy (es)	Result(s)	Ref.
- Autoimmune myocarditis - Graft of corneal tissue - xenotransplantation of adipose	mPEG	- Minimization or prevention of host immune responses through PEG-based charge and steric camouflage	- Expansion of cardiac myosin-specific $CD4^+$ and $CD8^+$ Treg subsets - Inhibition of EAM induction	[180]
	mPEG-SPA	- Concealment of the autoantigenic epitopes - CM antigens modified with mPE - Corneal antigen coated with mPEG-SPA	- Reduction inIL-2 and IFN-γ levels - Increase inIL-10 levels - Reduction in corneal graft rejection	[181]
	PEG	- Acellular adipose matrix modified with PEG	- Augmentation of Tregs - Enhanced augmentation of Tregs within the transplanted matrix - Improved adipogenesis	[76]
- Allogeneic transplant survival - Transplantation of Beta-islet - T1D	Biotinylated PLGA scaffolds (860 ± 40 nm)	- Functionalization of Hydrogels and scaffolds with FasL1 - Seeding of allogeneic beta-islets on hydrogels and scaffolds	- Increase inCD4+/CD25+/Foxp3+ Tregs - Inhibition of alloantigen-specific T cells - Induction of localized immune tolerance	[182]
	Biotinylated PEG hydrogels		- Acceptance and function of beta-islet allografts	[183]
	Biotinylated beta-islets	- Biotinylated beta-islets linked to PD-L1 or FasL1	- Development of CD4+/CD25+/Foxp3+ Tregs - Increase in TGF-β1 and IL-10 levels - Decrease inIL-1β, TNF-α, and IFN-γ levels - Establishment of systemic tolerance and long-term graft acceptance - Prolonged survival and functionality of grafts	[184–186]
- Allografts of cardiac tissue	Biotinylated splenocytes	- Biotin-fixed donor splenocytes displayed FasL1	- Induction and expansion of CD4+/CD25+/Foxp3+ Tregs - Long-term acceptance of graft in recipient rats	[187]
Regulation of substrate rigidity				
- Optimization of *ex vivo* expansion to induce Tregs	PDMS	- Development of an adjustable substrate rigidity platform to support anti-CD3 and anti-CD28 signals along with TGF-β1 and IL-2	- Significant increase in Treg induction at lower substrate rigidities compared to higher levels of rigidity	[188]
	PA-gels		- Optimization of inducing Tregs expansion *ex vivo* or *in vivo* - Mechanosensitivity of Treg metabolism - Utilization of OXPHOS process for Tregs expansion	[189]

Abbreviations arranged in alphabetical order: **CM**: Cardiac Myosin, **EAM**: Experimental Autoimmune Myocarditis, **FasL1**: Fas Ligand 1, **Foxp3**: Forkhead Box P3, **IFN-γ**: Interferon Gamma, **IL-10**: Interleukin-10, **IL-1β**: Interleukin-1 beta, **IL-2**: Interleukin-2, **mPEG**: Monomethoxypolyethylene Glycol, **mPEG-SPA**: Methoxy Polyethylene Glycol Succinimidyl Propionate, **OXPHOS**: Oxidative Phosphorylation, **PA-gels**: Polyacrylamide Gels, **PD-1**: Programmed cell death protein 1, **PD-L1**: Programmed death-ligand 1, **PDMS**: Poly(dimethylsiloxane), **PEG**: Polyethylene Glycol, **PLGA**: Poly (lactic-*co*-glycolic acid), **T1D**: Type 1 Diabetes, **TNF-α**: Tumor Necrosis Factor Alpha, **Tregs**: Regulatory T cells. *The particle sizes in the table are for diameters.

peritoneal fat of T1D mouse models, resulting in sustained engraftment acceptance. They hypothesized that this acceptance was facilitated by the induction and expansion of CD4+/CD25+/Foxp3+ Tregs [182].

Similarly, Yolcu et al. pursued a comparable approach by designing biotinylated beta-islets and subsequently functionalizing them with streptavidin PD-L1 (SA-PDL1) to achieve localized immunomodulation in T1D mice model. Their findings revealed that the successful surface display of SA-PDL1 protein on the biotinylated beta-islets without negatively impacting islet insulin secretion or viabilities. The transplantation of SA-PDL1-modified beta-islets led to the sustained survival and functionality of grafts for over 100 days in more than 90% of recipient mice. This prolonged survival was linked to the expansion of Foxp3+ Tregs, an increase in anti-inflammatory cytokines such as IL-10 and TGF-β1, as well as the inhibition of T-bet expressing T cells and a decrease in pro-inflammatory cytokines like IFN-γ, TNF-α, and IL-1β [186]. They also employed beta-islets engineered with SA-FasL1 to investigate the underlying mechanisms responsible for the enduring protection of allografts in a mouse model of T1D. Their research revealed that this immune protection unfolds in two phases: 1) Induction phase characterized by decrease in the population of proliferating T cells that target the transplanted tissue, and 2) Maintenance phase sustained by the presence of CD4+/CD25+/Foxp3+ Tregs [184]. Hence, the manipulation of cell and tissue surfaces using biomaterials offers a practical, effective, and secure avenue for precise immunomodulation. This discovery holds significant implications for fields such as autoimmunity and transplantation (Table 6 and Fig. 8B).

6. Treg induction by biomaterials with controlled substrate rigidity/stiffness

While re-stimulating, activating, and expanding Tregs using biomaterial strategies like aAPC, scaffolds, and bead particles (such as Dynabeads) is widely practiced for manipulating Tregs *ex vivo* and *in vivo* before their infusion or cryopreservation, the effectiveness of these methods heavily relies on the mechanical properties of the Treg substrates. These properties can influence their activation, proliferation, and differentiation outcomes. This is due to the fact that immune cells encounter diverse microenvironments with varying degrees of stiffness and fluid forces within the body. As a result, they adapt both their physical structure and their functions to accommodate these unique biomechanical cues [190,191].

For instance, robust T cell activation occurs when peptide-MHC complexes and anti-CD3 agonists (like the OKT3 antibody) are immobilized on solid supports [191]. Milone et al. has devised a straightforward approach for transmitting signal-1 (via the TCR/CD3 engagement) and signal-2 (through the CD28 molecules) to T cells. They accomplished this using a biocompatible silicone elastomer known as poly (dimethylsiloxane) (PDMS), which comes with varying levels of substrate rigidity. Their work demonstrates that substrates with lower stiffness result in a remarkable four-fold increase in production of IL-2 and *ex vivo* expansion among both $CD8^+$ and $CD4^+$ T cells in compare stiffer substrates. This rigidity factor significantly influences T cell activation, proliferation, and the differentiation of T helper subsets and

cytotoxic T cells. Therefore, when crafting T cell culture systems and interpreting outcomes based on the solid-phase immobilization of CD28 ligands and TCR/CD3, it is essential to consider the rigidity of the substrate [192,193].

Hence, biomaterials can be fine-tuned to closely mimic the microenvironments within the body and replicate its conditions, thereby boosting the effectiveness of Treg manipulation. In this context, Nataraj et al. employed PDMS as a controllable substrate with varying rigidity to support anti-CD28 and anti-CD3 signals in the presence of IL-2 and TGF-β1. Their study highlighted that inducing Tregs from conventional $CD4^+$ T cells *ex vivo* is influenced by the stiffness of the substrate. The findings revealed a significant rise induction of Treg at lower substrate stiffness compared to higher levels of rigidity [188]. Similarly, Shi et al. undertook a comparable investigation, crafting an adjustable substrate rigidity platform utilizing polyacrylamide gels (PA-gels) to support anti-CD3 and anti-CD28 signals, along with TGF-β1 and IL-2, for the induction of Tregs from PBMCs-isolated T cells. Their results pointed towards the mechanosensitivity of Treg metabolism and induction, which relied on increased utilization of the oxidative phosphorylation (OXPHOS) process [189]. Consequently, to efficiently induce Tregs *ex vivo* or *in vivo*, it is imperative to optimize both mechanical cues (such as the duration of TCR stimulation) and chemical regulators (like TGF-β1 and IL-2). Utilizing biomaterials represents one of the most effective strategies to achieve this objective (Table 6 and Fig. 8C).

7. Applications of biomaterial-boosted tregs

Tregs play a crucial role in preserving tolerance within the body. Consequently, Treg immunotherapy shows significant promise as a treatment option for organ transplants and autoimmune disorders. Currently, individuals who receive organ transplants must undergo lifelong immunosuppression to prevent rejection, and there is no definitive cure for autoimmune disorders. In the past decade, there has been substantial progress in our understanding of both antigen-specific and polyclonal Tregs biology. Furthermore, clinical trials involving Tregs manufactured under strict good manufacturing practices (GMP) have revealed that Treg-based therapies are safe and exhibits early effectiveness for both organ transplants and autoimmune diseases [194,195].

Numerous clinical trials are currently underway or have been completed, examining polyclonal Treg immunotherapies and low-dose IL-2 therapies in the context of transplant medicine and autoimmune conditions [58]. These trials involve the use of GMP-sorted Tregs, specifically CD4+/CD127low/CD25high or CD4+/CD25high subsets, which are then expanded through TCR stimulation or administered via subcutaneous low-dose IL-2. To maximize the effectiveness of Treg immunotherapy, it is imperative that these cells successfully migrate to specific target tissues, maintain stability within local organs, enhance their suppressive capabilities, and ensure their continued survival while fulfilling their intended functions [194,195]. In pursuit of these objectives, the use of biomaterials emerges as a compelling and supportive strategy for augmenting Treg immunotherapy and addressing these formidable challenges [73,74,76,78,81,83,84,91,96,108]. As a result, the prospect of employing biomaterial-enhanced Treg immunotherapy holds tremendous promise as a treatment option for patients undergoing organ transplants and those grappling with autoimmune diseases in the near future. In this section, we will delve into the utilization of biomaterial-boosted Tregs to the enhance survival of transplanted organs and treatment of autoimmune diseases (Tables 1–6).

7.1. Treating autoimmune diseases

The onset of autoimmune disorders is influenced by a combination of environmental factors, genetic predispositions, and the composition of gut microbiota. These conditions can impact a broad spectrum of organs and tissues within the body, exhibiting either organ-specific manifestations—such as autoimmune hepatitis affecting the liver, T1D mellitus targeting the pancreas, Grave's disease affecting the thyroid, Guillain-Barré syndrome impacting the nervous system, and Crohn's disease affecting the gastrointestinal tract—or manifesting as systemic disorders like SLE, RA, systemic sclerosis, primary antiphospholipid syndrome (APS), and systemic vasculitis. Both systemic and organ-specific autoimmune disorders are characterized by immune-mediated processes of unknown origin, stemming from disruptions in immune homeostasis. This disruption involves a lack of control over self-reactive T effector cells by Tregs, and reinstating tolerance may alleviate disease activity in affected individuals [194,196].

Treatments for autoimmune diseases often aim to dampen pro-inflammatory immune responses, either by inhibiting activation of immune cell within the affected organs or by decreasing the population of organ-specific T cells. Given that Treg cells possess immune-homeostatic properties crucial for averting autoimmunity, there has been considerable interest in Treg-based immunotherapies over the past decade. Although most existing strategies rely on polyclonal Tregs because of their effectiveness in animal models, they come with limitations like the potential for widespread immunosuppression and an increased risk of infection for patients [194–196]. Researchers are now concentrating on selectively suppressing autoreactive T cells in an autoantigen-specific manner. Studies involving TCR transgenic mice have shown that antigen-specific Tregs exhibit enhanced potency and effectiveness, particularly in the context of autoimmune diabetes [197]. Nevertheless, challenges persist in the isolation and expansion of antigen-specific Tregs, understanding the intricacies of their suppression mechanisms, survival, and plasticity [194–196]. To address these challenges, biomaterials have emerged as valuable tools for enhancing antigen-specific Treg therapies in the context of autoimmune disorders.

The presentation of self-antigens through tolerogenic APCs plays a vital role in inducing self-tolerance and preventing autoimmune disorders [198]. Among the organs where this process is particularly significant, the liver and spleen stand out due to their substantial populations of both parenchymal and non-parenchymal sinusoidal tolerogenic APCs [199,200]. These organs also readily take up biomaterial-based micro- and nano-particles [201,202]. Recognizing this, Nguyen et al. designed MSNPs functionalized with cerium oxide (known for scavenging reactive oxygen species) and loaded with MOG peptide to prevent EAE in mice, which serves as a model for human multiple sclerosis (MS). They demonstrated that tolerogenic macrophages, DCs, and B cells were the primary cell types taking up these MSNPs in the spleen. Treating EAE-afflicted mice with these engineered MSNPs led to an expansion of Tregs in the spleen and promoted systemic immune tolerance. This treatment also reduced autoreactive $CD4^+$ T cells in the CNS [101].

In a similar study, Miller et al. developed polystyrene beads and biodegradable PLGA microparticles linked to encephalitogenic peptides, including MOG and PLP, to prevent and treat the EAE mouse model. They observed that these microparticles were engulfed by macrophages expressing the scavenger receptor called "MARCO" in the liver's marginal zone. This uptake resulted in increased Tregs and elevated levels of IL-10, ultimately alleviating EAE in mice [94]. In another investigation conducted by Herkel et al., they fabricated CdSe/CdS/ZnS double shell quantum dots or iron oxide nanocrystals which were encapsulated into EDCI-poly (maleic anhydride-*alt*-1-octadecene) coupled with MOG peptide. These engineered nanoparticles delivered MOG peptides to LSECs, efficiently inducing CD4+/Foxp3+ Tregs and effectively controlling and preventing the onset of EAE in mice [102].

Immunomodulatory components, such as atRA, vitamin D3 [203,204], and mTOR inhibitors (e.g., Rapa) [205] have the potential to increase the populations of circulating Tregs and modulating their phenotypes in autoimmune disorders, thus improving disease activity. The controlled release of these components, along with autoantigens, to tolerogenic APCs, can be facilitated by biomaterials, leading to the expansion of autoantigen-specific Tregs. In this context, Keselowsky et al. developed phagocytosable PLGA microparticles containing

autoantigens and vitamin D3, along with non-phagocytosable microparticles loaded with Treg growth factors (e.g., GM-CSF and TGF-β1). This approach effectively blocked the progression of advanced EAE [84,90] and inhibited T1D-specific autoreactive T cell responses in mice [73,88]. Similarly, Thomas et al. encapsulated the chromogranin A, a diabetogenic autoantigen, along with vitamin D3, into liposomes. Their findings indicated an expansion of autoantigen-specific Foxp3-/IL-10+/CD73+/PD-1+/ICOS+ and Foxp3+ peripheral Tregs, suppression of the development of $CD4^+$ T cells in diabetic NOD-SCID mice, and the inhibition of autoantigen-specific $CD8^+$ T cells, along with reduced IFN-γ production [98].

In another study led by Broere et al., atRA and a peptide derived from human proteoglycan (hPG), which acts as an autoantigen in RA, were incorporated into liposome nanoparticles for the prevention of RA in an arthritis mouse model. Their findings demonstrated that co-delivering atRA and the autoantigen within liposomes stimulated the induction of autoantigen-specific Tregs in the RA model [104]. Similarly, Carey et al. developed PLGA nanoparticles that co-encapsulated autoantigens relevant to T1D along with molecules known to induce Tregs, such as Rapa and atRA. This approach resulted in an expansion of Treg differentiation, a decrease in the activation of APCs, and a reduction in inflammatory cytokines in pancreatic-reactive T cells [83]. Furthermore, LaMothe et al. designed PLGA particles that co-carried encephalomyelogenic autoantigens and Rapa for the treatment of a mouse model of EAE. Their results indicated that PLGA particles triggered the expansion of endogenous autoantigen-specific Tregs, leading to therapeutic efficacy against EAE [99]. Kishimotoa et al. described PLGA biodegradable nanoparticles loaded with autoantigens and Rapa. These tolerogenic nanoparticles were employed in treating mouse models of EAE, hypersensitivity, and hemophilia. The results of this treatment showed an increase in Tregs, inhibition of $CD4^+$ and $CD8^+$ T cell activation, enhanced and sustained B cell tolerance. These outcomes led to a reduction in relapses in EAE, the suppression of hypersensitivity reactions, and the production of anti-coagulation factor-VIII antibodies in hemophilia A mice [103].

Biomaterials can also establish an immune-balanced environment in inflamed tissues to induce and restore Tregs. Jin et al. developed immune-homeostatic microspheres for the treatment of autoimmune conditions in mouse models, including EAE, T1D, and colitis. They constructed these microspheres using a PLGA polymer and co-functionalized them with MSNPs and PEG. In the initial phase, they incorporated MCP-1 within the MSNPs for controlled release and anchored FasL1 on the microsphere's surface. The concept behind this design was that MCP-1 would attract activated T cells in autoimmune contexts. As these activated T cells displayed Fas molecules on their surface, the immobilized FasL1 would induce apoptosis in these T cells, ultimately leading to the restoration of Tregs at the site of inflammation. Specialized macrophages then engulfed the apoptotic T cells, resulting in the production of substantial amounts of TGF-β1, further promoting Treg differentiation. In the subsequent phase, by encapsulating well-known autoantigens relevant to various autoimmune conditions within the microspheres, they observed a reduction in disease severity in all mouse models. This reduction occurred through the controlled release of autoantigens following T cell apoptosis and the secretion of TGF-β1 by macrophages. This comprehensive approach facilitated the development of a tolerogenic microparticle, effectively assisting in the restoration of Tregs [128]. Rohimah et al. designed synthetic PS50G nanoparticles with the potential to stimulate the secretion of chemokines responsible for attracting and maturing monocytes and DCs at sites of inflammation. Their results revealed that these nanoparticles triggered the activation of tolerogenic DCs in the lungs, resulting in resistance to allergic airway inflammation in a mouse model. This effect was accompanied by the induction of TNFR2+/Foxp3+ Tregs [127].

The AhR is a ligand-activated transcription factor that plays a crucial role in regulating the immune response [206]. AhR signaling can influence the expansion of T cell subsets [207] and the functioning of APCs, particularly DCs [208] and macrophages [209]. Numerous studies have shown that AhR mediates the maturation of APCs and their abilities to activate and differentiate naïve T cells into Tregs [81,92,210]. Consequently, these findings suggest the potential therapeutic applications of AhR. Activation of AhR using synthetic ligands or natural compounds has been shown to improve autoimmune disorders such as inflammatory bowel disease (IBD) [211], T1D [212], and MS [208]. The delivery of AhR ligands via nanoparticles represents a promising approach to therapeutically restore antigen-specific tolerance in autoimmune diseases. In this regard, Quintana et al. decorated gold nanoparticles (AuNPs) with diabetogenic or encephalomyelogenic autoantigens and ITE, an AhR agonist, to alleviate T1D [81] and EAE [92] in a mouse model. Their results indicated that AuNPs carrying autoantigens and ITE expanded Foxp3+ Tregs and suppressed the development of EAE and autoimmune diabetes by inducing SOCS2 followed by NF-κB inhibition [81]. In a similar study, nanoliposomes loaded with ITE and MOG autoantigen induced tolerogenic DCs and suppressed the development of EAE by expanding MOG-specific Foxp3+ Tregs and Tr1, while reducing infiltrating effector T cells in the CNS [100].

The key regulatory cytokines and growth factors for Tregs are TGF-β1 and IL-2. These cytokines are pivotal for initiating, expanding, and enabling the function of Tregs in autoimmune complications [213]. There has been a proposal to manipulate these cytokines to potentially address deficiencies in Tregs. For example, low-dose IL-2 therapies have been employed to rectify Treg defects and improve symptoms in autoimmune conditions [214]. Utilizing biomaterial-based strategies to control the release or delivery of these cytokines presents a promising strategy to restore the functionality of deficient Tregs. In pursuit of this goal, the Cava et al. designed PLGA nanoparticles loaded with TGF-β1 and IL-2, which were functionalized with anti-CD4 and anti-CD8 to specifically deliver these cytokines to CD4 and CD8 T cells. Their study revealed that these nanoparticles induced both $CD8^+$ and $CD4^+$ T cells to differentiate into Foxp3+ Tregs, leading to the suppression of lupus in murine models [110,111].

Similarly, Keselowsky et al. introduced a strategy known as the "backpack" using PLGA nanoparticles containing IL-2, which were conjugated onto the surface of Tregs through PLL. The adoptive transfer of IL-2 nanoparticles conjugated on Tregs significantly inhibited diabetes in a mouse model of T1D [7]. Desai et al. also developed injectable PCL nanowires conjugated with *anti*-IL-2 to ameliorate skin autoimmune disease in a mouse model. These designed nanowires activated tissue-resident Tregs in the skin autoimmune model, selectively neutralized specific T cell subsets by removing IL-2 from naïve T cells and resulted in the prevention of complicated skin autoimmune conditions in the mouse model [91]. Huang et al. created lipid nanoparticles encapsulating mRNA to encode human IL-2, aiming for the selective activation and expansion of Tregs. When these lipid nanoparticles were administered subcutaneously in GvHD mice models and EAE, they revealed a significant reduction in disease severity in the mice [117].

Research has shown that miRNA dysregulation contributes to autoimmune diseases, making them attractive therapeutic targets. Specifically, reduced levels of miR-125a have been observed in the T cells of SLE patients, leading to impaired Treg function [115]. Increasing miR-125a levels enhances Treg-mediated self-tolerance [120]. However, the therapeutic use of miRNA faces challenges such as susceptibility to degradation and difficulties in cell uptake. To overcome these challenges, Zhang et al. developed a delivery platform using PLGA nanoparticles to target miR-125a in a mouse model of SLE. These modified nanoparticles efficiently delivered miR-125a to splenic T cells without harming other splenic cells. This enhanced Treg differentiation and function, rebalancing the immune response, reducing lupus nephritis, and alleviating SLE progression in the mouse model. Additionally, combining PLGA-encapsulated miR-125a with TGF-β1 significantly increased Treg expansion [115].

As previously mentioned, T cells require two signals for activation

and expansion, which are provided by APCs. When antigen presentation occurs without the second signal, T cells can become unresponsive to antigens and lead to the development of antigen-specific Tregs. Biomaterials can play a role as Tol-aAPCs in promoting Treg expansion in autoimmune conditions. In this context, Santamaria et al. fabricated iron oxide nanoparticles carrying autoimmune-relevant peptides bound with MHC-II in the context of T1D, EAE, and arthritis. These functionalized nanoparticles, bearing peptide-MHC complexes, effectively prevented the development of T1D [132,133], EAE [133], and arthritis [133] in mouse models by promoting the transformation of autoreactive T cells into Tregs and expanding autoregulatory T cells. Similarly, Shen et al. designed PLGA nanoparticles co-coupled with the MOG autoantigen-MHC complex, along with multiple regulatory molecules (e.g., CD47, PD-L1, and *anti*-Fas), and loaded them with TGF-β1. When administered intravenously in EAE mice, these nanoparticles significantly improved demyelination and reduced neuroinflammation by increasing Tregs and decreasing MOG-reactive Th17 and Th1 cells [134].

In addition to presenting autoantigen-MHC complexes through biomaterials, biomaterials can also deliver autoantigens in a controlled and tolerogenic manner, even in the absence of co-stimulatory molecules. This approach has the potential to reverse the immune response against autoantigens in autoimmune conditions and promote the expansion of autoantigen-specific $CD4^+$ Tregs. In this context, Miller et al. loaded diabetogenic autoantigens (such as chromogranin A, IGRP, and insulin *C*-peptide fragment) and encephalomyelitis autoantigens (e.g., MOG) into biodegradable PLGA nanoparticles. Treating mice with EAE and T1D using these tolerogenic nanoparticles resulted in a decrease in the ability of effector T cells to produce pro-inflammatory cytokines, leading to anergy. This approach also increased the population of Foxp3+ Tregs, ultimately preventing diabetes [95–97] and relapsing EAE [93,96] in the mouse models.

Chemically concealing autoantigens with biomaterials like PEG and its derivatives enables them to evade detection by the immune system or minimizes immune responses—a phenomenon referred to as “immune camouflage," as previously discussed. One particularly effective and non-toxic polymer for immune camouflage is m-PEG. In a study by Hamada et al., they injected m-PEG-modified CM to EAM mice model before inducing active or passive EAM. This modification of CM autoantigens proved effective in inhibiting EAM by expanding CM-specific $CD4^+$ and $CD8^+$ Treg subsets in mice. Therefore, by concealing the antigenic epitopes, this approach holds promise for reducing or preventing autoimmunity [180].

7.2. Enhancing survival of transplanted organs

Transplantation has become a widely adopted clinical treatment option for numerous patients, offering the best chance of survival in cases of cancer or organ failure. Organs like kidneys, livers, beta-islets, hearts, and bone marrow are routinely transplanted in many countries. In the initial stages following transplantation, both the direct and indirect pathways of antigen presentation come into play, involving APCs, such as DCs, from both the donor and recipient. These processes can contribute to graft rejection [215]. To prevent the loss of transplanted organs, recipients are required to undergo long-term immunosuppressive therapy. However, immunosuppression can lead to a range of side effects, including sepsis, diabetes, hypertension, renal dysfunction, and the potential for long-term complications like post-transplant lymphoproliferative disorder and malignancies [216].

Graft rejection primarily occurs due to an imbalance in adaptive immune cells, particularly a reduction in the frequency of Tregs or a malfunction in their regulatory function, which is responsible for controlling alloantigen-specific T cells. To address this issue and prevent graft rejection, a promising therapeutic approach involves rebalancing the immune system in favor of the regulatory arm through Treg therapies. Recipient Tregs can be generated either in a non-specific, polyclonal manner by expanding recipient Tregs using CD28, CD3 beads, IL-2, and Rapa, or in an antigen-specific manner by expanding recipient Tregs using donor APCs primed with the donor antigens. Consequently, polyclonal, autologous, or antigen-specific Treg therapies can be administered after organ transplantation [58,194]. To enhance the efficacy of Treg immunotherapy, biomaterial-based strategies offer the potential to enhance Treg immunotherapies through various mechanisms as previously mentioned, ultimately leading to improved survival of transplanted organs.

Biomaterial-based scaffolds designed for the localized delivery of Treg growth factors and alloantigens are widely employed to induce polyclonal and/or alloantigen-specific Tregs, thereby facilitating local immunomodulation in organ transplant settings. In this context, Mooney et al. developed an alginate hydrogel-based scaffold for delivering a beta-cell directed peptide encapsulated within PLGA microparticles. Their findings indicated that approximately 60 percent of the recruited antigen-specific $CD4^+$ T cells within the hydrogel were Tregs, which were also enriched in the transplanted pancreatic islets [107].

Graham et al. loaded beta-islet cells onto microporous PLGA scaffolds and transplanted them into the abdominal fat of a NOD mouse model. Co-transplanting Tregs around the scaffold resulted in the expansion of alloantigen-specific Tregs, leading to systemic tolerance in the NOD mice. Furthermore, systemic tolerance against transplanted beta-islet cells was achieved through the infiltration of Tregs around second islet transplants [108]. Similarly, Liu and Shea designed a PLGA scaffold seeded with allogeneic beta-islets and incorporated IL-33. PLGA scaffolds implanted into the epididymal fat revealed that the delivery of IL-33 led to a localized increase in graft-protective T cells. Notably, approximately 80 percent of the local $CD4^+$ T cell population expressed CD4+/Foxp3+ and ST2+ (IL-33 receptor), indicating their regulatory functions. This resulted in the prolonged survival of the transplanted graft [87].

The adoptive transfer of Tregs represents a promising immunotherapy approach aimed at improving the survival of organ transplants and is currently undergoing clinical trials. However, this strategy comes with certain drawbacks, primarily related to the time-consuming and costly protocols necessary for generating a sufficient number of Tregs to achieve meaningful clinical outcomes [58]. Since Tregs are present at low frequencies in human peripheral blood, it would be beneficial to explore more targeted approaches that require fewer Tregs. One such approach involves the use of biomaterial-based scaffolds for encapsulating Tregs and providing localized immunosuppression. In this context, Kim et al. developed a GelMA hydrogel that contained Tregs and their growth factors like CCL-2 and IL-2. This hydrogel effectively preserved the viability and suppressive phenotype of Tregs while also protecting pancreatic islets [77]. Similarly, in a study conducted by Bushman et al., Tregs were encapsulated within a biodegradable hydrogel made of PEGNB and transplanted around allografts of peripheral nerves. The encapsulated Tregs were gradually released from the hydrogel and migrated into the graft site, resulting in the suppression of the host immune response and promoting regeneration of nerve in recipient animal [142].

Immunosuppressive drugs like Tacrolimus, Sirolimus, and Cyclosporine, widely used in transplant medicine, interfere with T cell signaling pathways involving TCR, CD28, mTOR, NFAT, and IL-2 signaling. This interference leads to the general inhibition of various T cell subsets, including Tregs, impacting their survival and proliferation during graft implantation [217,218]. Biomaterial-based strategies can be designed to specifically target and enhance the function of this small population of Tregs during graft implantation. In this context, Eskandari et al. developed a novel approach where they conjugated Tregs with nanogels containing IL-2 that could be released under reducing conditions, such as when Tregs receive stimulation through the TCR upon encountering alloantigens at the transplant site. This innovative strategy, known as the “backpack", involved Tregs carrying IL-2-loaded nanogels, which proved to be more effective in suppressing

allo-immunity in skin transplantation and humanized allo-transplantation mouse models compared to systemic IL-2 stimulated Tregs or unmodified Tregs [109]. Huang et al. also made significant progress by developing lipid nanoparticles loaded with modified human IL-2 mRNAs, which resulted in increased mRNA stability and enhanced affinity for IL-2Rα. When these nanoparticles were subcutaneously injected into mouse models of GvHD, they selectively activated and expanded Tregs, ultimately leading to a reduction in disease severity in the mice models [117].

Alloantigen presentation via Tol-aAPCs represents an additional approach to inducing tolerance against alloantigens in the field of transplantation medicine. In this context, Luo et al. developed two types of Tol-aAPCs utilizing donor alloantigens from both skin and beta-islets. These alloantigens were conjugated onto PLGA nanoparticles using EDCI. The engineered Tol-aAPCs successfully promoted tolerance in a mouse model of allogeneic skin [106] and beta-islet [105] transplantation, ultimately enhancing graft survival. This effect was achieved through the expansion of graft-infiltrating Tregs. Similarly, Neshat et al. designed a Tol-aAPC by incorporating TGF-β1 into PLGA particles while also incorporating anti-CD3 and anti-CD28 molecules to stimulate T cells. Their results demonstrated that the activation of T cells with anti-CD28 and anti-CD3, along with TGF-β1, led to the expansion of Foxp3+ Tregs and improved engraftment of beta-islets in mice model [85].

Administering intravenous therapy involving the use of EDCI as a crosslinking agent, fixed onto syngeneic splenocytes or erythrocytes, with or without alloantigens, represents a potent approach for inducing *in vitro* anergy and promoting peripheral T cell tolerance *in vivo*, particularly in the context of transplantation [135]. This therapy is based on the concept that MHC molecules on donor lymphocytes are believed to play a role in graft rejection, and ECDI treatment is thought to disrupt these MHC signals [219,220]. On the other hand, when EDCI-coupled alloantigens are presented on the surface of donor splenocytes or erythrocytes to T cells in the absence of signal-2, it results in the induction of T cell anergy and the expansion of Tregs [140,141]. In a study by Miller et al., ECDI-treated donor splenic cells were intravenously injected into allogeneic beta-islet recipient mice, leading to long-term survival of beta-islet transplants through the expansion of CD4+/CD25+/Foxp3+ Tregs [136]. Similarly, Hubbell et al. induced CD4+/CD25+/Foxp3+ Tregs and established memory of tolerance by utilizing antigens fixed onto erythrocytes, which could have practical applications in transplant medicine [139]. Both studies revealed that the PD-1/PD-L1 pathway and Tregs are crucial contributors to tolerance induction [136,139]. In another approach, Shirwan et al. developed a strategy involving biotin-fixed donor splenocytes that displayed FasL1 protein on their surface to induce tolerance to cardiac allografts. This innovative strategy resulted in long-term graft acceptance in recipient rats, primarily mediated by the induction and expansion of CD4+/CD25+/Foxp3+ Tregs [187].

Biomaterial strategies based on immune camouflage are extensively employed to induce immunomodulation and ensure the survival of allogeneic transplants. In their work, Shirwan et al. biotinylated beta-islets and then linked them separately to PD-L1 [186] and FasL1 [184,185]. Pancreatic islets engineered with PD-L1 and FasL1 promote the development of CD4+/CD25+/Foxp3+ Tregs, leading to systemic tolerance and long-term graft acceptance. In other studies, they created PEG hydrogels [183] and PLGA scaffolds [182] that were functionalized with FasL1, and allogeneic beta-islets were seeded on their surfaces. Both FasL1-functionalized biomaterials induced localized immune tolerance, fostering the acceptance and function of beta-islet allografts, mediated by CD4+/CD25+/Foxp3+ Tregs. Wang et al. utilized methoxy mPEG-SPA, a derivative of PEG, to coat corneal antigens. The mPEG-SPA-coated corneas resulted in decreased levels of IL-2 and IFN-γ, an increase in IL-10, and reduced corneal graft rejection in an animal model [181]. Additionally, Feng Lu et al. transplanted PEG-modified acellular adipose matrix into a xenotransplantation mouse model, which led to enhanced adipogenesis mediated by an increase in Tregs [76].

8. Challenges and future perspectives in biomaterial-based treg induction

The remarkable clinical advancements achieved through Treg adoptive transfer therapy have ignited a compelling exploration of this treatment approach in a wider range of autoimmune disorders and transplantation medicine. Nonetheless, numerous challenges have surfaced in both preclinical investigations and clinical trials. Notably, these challenges encompass suboptimal yields in *ex vivo* Treg expansion, inefficient homing of Tregs to target tissues, and limited longevity of transferred Tregs—all of which hinder their effective translation into clinical practice. To tackle these hurdles, significant efforts have been directed towards the development of state-of-the-art biomaterial-based strategies. The integration of biomaterials into Treg immunotherapy holds tremendous promise within the realm of medical treatments. These advancements are particularly encouraging for patients undergoing organ transplantation and individuals dealing with autoimmune diseases. This review delineates six pivotal roles of biomaterials in adoptive Treg cell therapy, encompassing the facilitation of robust *in vitro* and *in vivo* Treg activation and expansion, the enablement of functional engineering of these cells, the provision of protective delivery mechanisms for Tregs, the facilitation of precise tracking of the transferred cells, the capacity for immune-regulating camouflage through biomaterials, and the optimization of Treg induction by biomaterials with controlled substrate rigidity/stiffness.

The incorporation of biomaterials into Treg immunotherapy permits precise control over the release of crucial cytokines, thereby ultimately amplifying the therapeutic efficacy of Tregs. Furthermore, biomaterials can concurrently deliver modulatory signals, induce antigen-specific Tregs, act as artificial antigen-presenting cells, and promote the expansion of effector and memory Tregs. These multifaceted strategies underscore the potential of biomaterials in reshaping the immune landscape for therapeutic advantage. However, the presence of challenges, such as inadvertent immune responses and nonspecific nanoparticle accumulation in non-targeted organs, emphasizes the need for ongoing refinement in this field. While biomaterial-based approaches in Treg immunotherapy have been meticulously devised to surmount obstacles associated with Treg expansion, functional engineering, *in situ* delivery, and precise tracking, additional considerations are essential to ensure safety, scalability, and reproducibility. Safety concerns regarding potential immune responses and non-targeted accumulation of biomaterials in organs necessitate thorough evaluation through rigorous preclinical studies and clinical trials. Comprehensive immunotoxicity assessments, including evaluation of inflammatory responses, cytotoxicity, and immunogenicity, are imperative to mitigate potential adverse effects associated with biomaterials.

Scalability remains a critical challenge, as the transition from laboratory-scale production to large-scale manufacturing for clinical applications requires optimization of manufacturing processes and quality control measures to ensure consistent product quality and safety. Consideration of factors such as scalability of production methods, reproducibility of biomaterial synthesis, and cost-effectiveness of manufacturing processes is essential for the successful translation of biomaterial-based Treg induction methods from bench to bedside. Moreover, reproducibility is paramount for the success of biomaterial-based Treg induction methods. Standardization of protocols, rigorous quality control measures, and validation studies are indispensable to ensure reproducible results across different research settings and clinical applications. Implementation of good manufacturing practices (GMP) and adherence to regulatory guidelines for biomaterial production and characterization are essential to guarantee the reliability and consistency of biomaterial-based Treg induction methods.

Additionally, optimization of delivery efficiency for proteins, genes,

and small molecules to Tregs through biomaterials necessitates meticulous attention to factors such as particle rigidity, cellular uptake mechanisms, and combination strategies for multiple tracking techniques. Advanced techniques such as microfluidics-based platforms, 3D bioprinting, and personalized medicine approaches hold promise for enhancing the scalability and reproducibility of biomaterial-based Treg induction methods, thereby facilitating their widespread clinical application. In the context of personalized medicine, biomaterial design must evolve to interact with individual patient conditions and the specific targets of diseases, fostering synergistic therapeutic effects. The integration of biomaterials to enhance Treg immunotherapy, potentially in conjunction with other therapeutic modalities, represents a promising direction for the future of medical treatments. In conclusion, the ongoing refinement of biomaterial-based platforms holds the potential to broaden the use of immune cell-based therapies and accelerate their adoption in clinical settings. This holds the promise of improved outcomes and renewed hope for patients facing various medical conditions.

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CRediT authorship contribution statement

Kazem Mashayekhi: Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Conceptualization. **Khashayarsha Khazaie:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **William A. Faubion:** Supervision, Funding acquisition, Conceptualization, Writing – original draft, Writing – review & editing. **Gloria B. Kim:** Conceptualization, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interests

No declaration for G.B.K.

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