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Strategies to direct the enrichment, expansion, and recruitment of regulatory cells for the treatment of disease

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Abstract

Disease and injury perturb the balance of processes associated with inflammation and tissue remodeling, resulting in positive feedback loops, exacerbation of disease and compromised tissue repair. Conversely, under homeostatic healthy conditions, these processes are tightly regulated through the expansion and/or recruitment of specific cell populations, promoting a balanced steady-state. Better understanding of these regulatory processes and recent advances in biomaterials and biotechnology have prompted strategies to utilize cells for the treatment and prevention of disease through regulation of inflammation and promotion of tissue repair. Herein, we describe how cells that regulate these processes can be increased in prevalence at a site of disease or injury. We review several relevant cell therapy approaches as well as new strategies for directing endogenous regulatory cells capable of promoting environmental homeostasis and even the establishment of a pro-regenerative micro-environment. Collectively, these examples may provide a blueprint for next-generation “medicine” that spurs the body’s own cells to action and replaces conventional drugs.

Keywords

regulatory T cells; mesenchymal stem cells; biomaterials; homeostasis; regeneration

Homeostasis and Disease

Biological homeostasis is most commonly referred to as a balance or ‘steady state’ between two competing processes such as inflammation and immune regulation and anabolism and catabolism in tissue remodeling. In actuality, biological homeostasis is more aptly described as a dynamic equilibrium (as opposed to a true “steady state”) involving the sensing of perturbations, and in turn, processes that regulate these perturbations. Specifically, a perturbation produces a change in outcome that is then detected, inducing a corresponding regulatory activity that leads to negative feedback to restore balance. A simple analogy to this mechanism is a thermostat that measures the temperature with a sensor and, in response to the sensor’s reading, regulates heating/cooling to stabilize the environmental temperature. Notably, in a disease state or following injury, perturbations in biological process are not always properly regulated, resulting in an instability that can lead to improper healing and even tissue destruction and functional impairment.

Excessive inflammation is often a key characteristic of a tissue that has lost homeostasis, which is evident, for instance, in unbalanced mucosal inflammation of the gut culminating in inflammatory bowel diseases (Crohn’s disease, ulcerative colitis)¹. Even in sites conventionally thought of as immune privileged such as the eye, aberrant, unbalanced inflammation can lead to uveitis, dry eye disease and glaucoma². Moreover, traumatic injury, which starts with tissue damage, can induce abnormal signaling pathways³ and an inflammatory response that becomes a major perturbator of homeostasis⁴, leading to tissue degradation⁵, dysregulation³ and failure to repair. Finally, tissue homeostasis and regulated inflammation are key to the survival of transplanted tissues and organs⁶.

Different therapeutic approaches have emerged over the past several decades that attempt to address excessive inflammation and homeostatic imbalance. Anti-inflammatory drugs, such as COX-1/2 inhibitors, resolvins, glucocorticoids, and more recently small molecule anti-proliferative agents such as methotrexate, cyclosporine, tacrolimus and rapamycin have been used in attempt to suppress inflammation during disease or tissue repair⁷. However, the negative side effects of such treatments that have limited specificity can potentially outweigh any benefits (e.g. steroid induced osteoporosis, impaired wound healing, thrombosis and cardiovascular disease)⁷.

Recently, anti-inflammatory biologics, such as TNF blockers⁷, have emerged as more effective and specific therapies for treating aberrant inflammation. However, biologics are still primarily administered systemically in relatively large quantities, leading to complications such as increased susceptibility to infection and cancer development⁷. Furthermore, the clinical efficacy of targeting specific cytokines has not yielded the robust results that were expected based on animal models, such as in Crohn’s disease where one-third the patients do not respond to TNF antagonists, likely because inflammatory perturbations leading to disease are complex processes governed by many cytokine and signaling networks (for example, in Crohn’s disease the IL-12, and IL-23 cytokine pathways have a major role in the disease in addition to TNF)^{8,9}.

In recent years, several approaches have emerged to deliver or boost the expression of anti-inflammatory cytokines and molecules, as opposed to blocking inflammatory cytokines. For example recombinant IL-10 and IL-11 have been used in phase II clinical trials for inflammatory Crohn's disease (reviewed⁸), however such treatments had disappointing efficacy compared to steroid treated controls possibly due to the overwhelming abundance of inflammatory mediators present in disease sites^{8, 10}. In contrast, the mechanisms that cells use to regulate these processes *in situ* are sophisticated and dynamic, which is difficult to reproduce using current technology. One viable approach, therefore, is to somehow boost the number of cells capable of performing these complex regulatory functions at the site of a disease or injury. Presumably, cells would be more effective at naturally avoiding a disease state or restoring a local environment to a healthy state, than administration of a single cytokine or factor. Two cell types that hold great promise in regards to treatments of disease and the restoration of homeostasis to promote tissue regeneration are mesenchymal stem cells (MSCs) and CD4+CD25+Foxp3+ regulatory T cells (Tregs). Both cell types are capable of regulating their environment to direct the restoration of tissue homeostasis through cell-cell interactions and extracellular secretion of multiple factors (reviewed^{11–14}). Accordingly, MSCs and Tregs have been extensively explored for cell therapies (involving the isolation, *ex vivo* expansion, and re-administration of the cells) for a large number of applications through clinical trials^{11, 14, 15}.

Mesenchymal Stem Cells as Mediators of Homeostasis

MSCs are commonly used in tissue engineering and regenerative medicine applications because of their well-known capacity to differentiate into multiple cell types. Their key feature of interest here, though, is their ability to migrate to a damaged tissue (homing) in response to the locally released inflammatory chemokines and, once there, to inhibit the inflammation by modulating innate and adaptive immune cells via soluble factors or cell-cell interaction^{16, 17}. The most straightforward way to increase the number of MSCs in a biological environment would be to administer the desired cell type after *ex vivo* expansion and in some instances differentiation (defined as a cell therapy). For instance, several clinical trials are ongoing to use MSCs injected in the central nervous system as a treatment for ischemic stroke and traumatic brain injury. In this instance, MSCs are used as promoters of tissue regeneration, mediated by the MSCs produced factors¹⁸. Interestingly, infusion of MSCs into the body has been reported in numerous animal studies and clinical trials to treat a variety of additional conditions such as graft versus host disease (GvHD), myocardial infarction, osteogenesis imperfecta, Type I diabetes, and Crohn's disease^{19, 20}, but, although functional improvements of tissues were reported, only few engrafted MSCs were detected *in vivo*^{21, 22}. These findings support the concept of MSCs achieving tissue repair not only through their *in situ* differentiation and engraftment, but also by their regulatory function, promoting regeneration from other cell types and suppressing the immune response¹⁹. Moreover, it is reported that secretion of heme oxygenase-1 (HO-1) by MSCs can promote the formation of another regulatory cell type, Tregs²³, which in turn suppress the pro-inflammation reaction of other immune cells as described in detail later. Thus, it is likely that these two key regulators of the body, MSCs and Tregs, are, in fact, connected to one another and perhaps even act in a cooperative fashion.

In addition to immunomodulatory cytokines, MSCs also secrete other factors to support cell survival (trophic factors), and promote regeneration. These factors include, G-CSF, GM-CSF, SCF, LIF, CSF, IL-11, IL-6, VEGF, HGF, angiopoietin-1, EGF, KGF, and bFGF, as extensively described elsewhere²⁴, and can enhance cell growth, proliferation, differentiation, extracellular matrix production, and favor cell recruitment and vascularization, all processes crucial in tissue repair and regeneration¹⁴. In wound healing, both preclinical and clinical studies have shown that MSCs can accelerate wound closure by modulating the inflammatory response, inhibiting apoptosis, and promoting effective vascularization and the migration of keratinocytes¹¹. Moreover, the antifibrotic, angiogenic and regulatory potential of MSCs is being explored to treat or prevent ongoing alloreactivity, so much that the first clinical trials in transplant recipients are underway^{25, 26}.

Regulatory T-cells as Mediators of Homeostasis

Regulatory T-cells (or Tregs) are a heterogeneous population of CD4⁺CD25⁺FoxP3⁺ T helper cells composed of thymic-derived natural Tregs (nTregs) or peripherally induced Tregs (iTregs, Th3, and FoxP3⁺ Tr1)²⁷, and are characterized by high levels of secretion of the anti-inflammatory cytokine IL-10, and of TGFβ, which mediates part of their suppressor functions¹². Given their potent regulatory phenotype, the local enrichment of autologous or *ex vivo*-expanded adoptively transferred Tregs is a very desirable therapeutic approach for a multitude of immune-mediated destructive pathologies^{12, 13, 28}. *Ex vivo* produced Treg cells have been already explored in several clinical trials. For example, the isolation and *ex vivo* expansion of Tregs from umbilical cord blood sources was shown to reduce the incidence of acute GvHD in phase I trials for bone marrow transplantation²⁹. The success of this trial appears to be related to the Tregs ability to suppress CD4⁺ and CD8⁺ effector T cells also present in the hematopoietic cell transfers³⁰. Similarly, it has been reported that the combined cell therapy of Tregs and T effector cells infused in phase I clinical trial patients receiving HLA-mismatched bone marrow helped reconstitute the patients immune system, while preventing GvHD³¹. These Treg cell therapies take the place of strict systemic immunosuppression protocols that are normally followed for patients receiving mismatched donor bone marrow transplants, thus greatly alleviating the risk for infection or other illnesses³². Treg cell therapies also have the potential to complement MSC therapies. In a mouse critical size cranial defect model, the adoptive transfer of both MSCs and Tregs yielded robust wound healing and bone regeneration, whereas MSC therapy alone only resulted in modest tissue regeneration³³. In this case, it was shown that the addition of Tregs greatly diminished the levels of inflammatory cytokines IFNγ and TNF that would otherwise inhibit bone regeneration, likely through the expression of effector immune cells³³. This finding corroborates the notion that Tregs and MSCs do indeed function synergistically to regulate the local microenvironment and promote tissue healing. While several MSC- and Treg-based cell therapies have proven successful in the clinic, their utilization still faces a number of significant obstacles.

Limitations of Cell Therapies

Harnessing the full functional repertoire of cells via cell therapy has led to exciting advances in treatment of disease and injury, however there are many challenges yet to overcome. The

hurdles of cell therapies are well known and have been thoroughly reviewed elsewhere^{11, 14, 15, 34}. These can be briefly described as: 1) difficulty in establishment of cell sources capable of generating sufficient numbers of cells^{35, 36}, 2) inability to specifically expand and separate target therapeutic cells^{15, 37}, 3) difficulty in maintaining functional capabilities of re-introduced cells due to T cell plasticity³⁸, and possible tumorigenicity³⁹. Furthermore, when taking into account clinical therapy needs, the ability to adhere to good manufacturing practice (GMP) at every step of the process is non-trivial and poses significant limitations to the widespread use of many cell therapies. A very promising therapeutic alternative to cell therapies would be the direct, selective recruitment of a patient's own endogenous MSCs or Tregs to the desired body compartment, where they could modulate the host response to the introduction of a foreign material (bio implantation), of a transplanted organ, or establish a regenerative *milieu* after tissue damage.

Harnessing the Potential of the Body's Own Endogenous Regulators

Recent advances in the understanding of the biological processes that govern inflammation and regulation of inflammation have led to development of synthetic strategies aimed at recruiting or expanding the body's own endogenous regulators in order to restore homeostasis and promote regeneration, while avoiding the complications associated with *ex vivo* cell expansion and re-administration. A large number of both synthetic and natural biomaterials have been investigated for many different applications, reviewed elsewhere^{40–44}, including strategies to recruit cells of the immune system (for example, pro-regenerative M2 macrophages) to promote tissue regeneration^{45, 46}. However, several recent approaches have specifically harnessed the endogenous immune regulators in attempt to restore homeostasis and promote regeneration.

One strategy to increase the prevalence of regulators at a local site is the specific recruitment of a patient's own natural, endogenous Tregs using a recombinant chemokine, CCL22. Although the chemokine receptor for CCL22 (CCR4) is expressed on a variety of different lymphocytes, it appears that it is overexpressed on FoxP3⁺ Treg⁴⁷ and CCL22 seems to be a primary chemokine that directs natural Treg homing *in vivo*⁴⁸. Accordingly, controlled release of recombinant CCL22 using degradable poly(lactide-co-glycolide) (PLGA) microspheres is an effective strategy for direct site-specific recruitment of natural Tregs *in vivo*^{49, 50}. These recruited endogenous Tregs were shown to also effectively reduce the severity of inflammatory periodontal disease in both mouse and dog models⁴⁹. More so, Tregs have also been recruited in order to protect pancreatic islets and prevent autoimmune diabetes in mice, however, CCL22 production was induced by adenovirus gene delivery (encoding for CCL22) to the islets⁵¹. Recently, others have developed targeted nanoparticles for the delivery of Treg-inducing factors, specifically, using anti-CD4 tagged PLGA nanoparticles releasing leukemia inhibitory factor (LIF) capable of inducing the expansion of non-human primate Tregs *in vitro*⁵². Furthermore, leukemia inhibitory factor released from PLGA nanoparticles *in vivo* was shown to enhance β -islet transplantation to restore insulin production in a mouse model for diabetes (presumably through the induction of Tregs)⁵³. Another strategy to harness endogenous Tregs for murine diabetes combined the delivery of antisense oligonucleotides directed against CD40, CD80 and CD86 from polymeric microspheres⁵⁴. These combination particles encapsulating antisense

oligonucleotides were capable of inducing the expansion of endogenous Tregs (potentially mediated by tolerogenic dendritic cells) and preventing type 1 diabetes in mice. Furthermore, isolated Tregs from treated mice were capable of preventing T-cell mediated β -islet destruction in secondary mouse recipients, suggesting that the particles led to the induction of stable Treg phenotypes⁵⁴.

An alternative strategy to bolster endogenous regulatory T cells is to differentiate a more prevalent population of cells (such as naïve CD4⁺ T cells) into regulatory T-cells. To this end, it was found that the combination of TGF β , rapamycin and IL-2 robustly converts both mouse and human naïve CD4⁺ lymphocytes to FoxP3⁺ regulatory T cells *in vitro*⁵⁵. This strategy of inducing peripheral Tregs *in situ* is particularly attractive as it may address problems that arise if patients have insufficient numbers of natural regulatory T cells as one of the factors attributing to disease or lack of regeneration. Indeed, this multi-factor strategy was capable of inducing the specific proliferation and expansion of CD4⁺ FoxP3⁺ Tregs with 80% pure Treg population, as opposed to only 2% pure Treg population proliferation after delivery of IL-2 along⁵⁵. The application of this technique *in vivo* could represent a promising approach for generating far more induced Treg at a local site than is possible by recruiting naturally occurring Treg.

Regulatory T cells as regulators of regeneration

Although it is well known that the strategic modulation of endogenous immune regulators, such as Tregs, can effectively mediate destructive inflammation, it is not clear how these cells also appear to promote regeneration⁴⁹. One way in which endogenous regulators may promote tissue healing and regeneration is through the release of pleiotropic cytokines such as IL-10 and TGF β . For example, IL-10 is known to play an important role in bone metabolism *in vivo*, accordingly IL-10-deficient mice display both decreased osteoblast generation and bone formation⁵⁶. Similarly, TGF β has been shown to play a role in promoting cell growth, differentiation and extracellular matrix production, and therefore is considered an anabolic cytokine^{57–59}. Indeed, TGF β is known to recruit osteoblast precursors, induce their differentiation and up-regulate the expression of collagen type I, even in disease conditions^{60, 61}. The controlled release of CCL22 and subsequent recruitment of endogenous Tregs (as shown by local increases in FoxP3) also appears to upregulate both IL-10 and TGF β expression in periodontal tissue⁴⁹. In addition, a host of both hard and soft tissue pro-regenerative factors such as BMP4, BMP7, RUNX2, ALP, DMP1, COL1A1⁴⁹ were upregulated as well concurrently with the downregulation of factors known to be involved with both hard and soft tissue destruction⁴⁹. Indeed, the addition of Tregs has been an emerging trend in regenerative therapies, where Tregs have been utilized for complete repair of critical size defects in mouse skulls³³, and ischemic kidney repair⁶².

Engineering techniques for harnessing endogenous mesenchymal stem cells

An analogous approach to what is discussed above could be to synthetically reproduce the natural mechanisms of migration of endogenous mesenchymal stem cells. For instance,

MSCs migrate to the injured or inflamed site by sensing chemokines gradients, and locally regulate immune reactions and promote tissue regeneration by cell-cell contact or secretion of cytokines. A synthetically driven homing of MSCs would then theoretically promote these same functions, and benefit broad area of diseases including inflammatory and degenerative diseases.

The most obvious strategy to recruit endogenous MSCs would rely on the creation of a local gradient of a chemoattractant molecule of choice to induce cells chemotaxis⁶³. Several chemokines have been implicated to induce MSC homing⁶⁴; one of the most widely used chemoattractant is stromal cell-derived growth factor-1 (SDF-1/CXCL12), although it is not specific to MSCs as it also recruits, for instance, certain lymphocytes⁶⁵. Another MSC chemoattractant is platelet-derived growth factor (PDGF)⁶⁶ which is also non-specific for MSCs, but PDGF receptors are highly expressed on MSCs⁶⁷. It is worth noting that besides chemokine gradients, the surrounding extracellular matrix (ECM) and the environmental mechanical forces can recruit MSCs as well⁶⁸.

The chemoattractant molecule is generally released from a point source via a carrier that can be in the form of micro/nanospheres⁵⁰, of scaffolds^{66, 69}, especially when aimed at tissue engineering applications⁷⁰, or, in fact, of any other implantable structure (stents, subcutaneous patches, sutures, etc.)^{69, 71, 72}. Other strategies that are utilized in tissue engineering could also be helpful, including the patterning of scaffolds to induce and direct cell invasion⁷³.

Current strategies to recruit endogenous MSCs typically rely on several different techniques to delivery the chemoattractant or MSC boosting factor (reviewed^{74, 75}). For example, release of CXCL12 from a poly(lactide ethylene oxide fumarate) (PLEOF) hydrogels or from chitosan/poly(γ -glutamic acid) polyelectrolyte complexes have been utilized to recruit MSCs *in vitro*^{76, 77}. Furthermore, CXCL12 has also been released from poly(lactide-co-glycolide) (PLGA) microspheres to promote MSCs recruitment *in vitro*⁷⁸. Alternatively, PDGF has been released to enhance both cell migration and vasculogenesis in a rat dorsal-wound healing model⁷⁹. Notably, modern degradable carriers can now offer a high degree of tunability of the release behavior for any given chemoattractant^{80, 81}. Since a controlled release carrier may also be loaded with practically any “instructional” molecules, (e.g. growth factor inducing stem cells differentiation⁸²), combining multiple carriers (for instance, different microparticles formulation containing different molecules or a scaffold/microparticles combinatorial structure⁸³) allows a more sophisticated cellular control. For instance, this technique could be used for the sequential recruitment of different cells as well as the recruitment of a given cell precursor followed by the release of instructional molecules to direct cell differentiation or tissue production. For instance the sequential delivery of PDGF/simvastatin, vascular endothelial growth factor (VEGF)/sphingosine 1-phosphate (S1P), or basic fibroblast growth factor (bFGF)/PDGF for sustained neovascularization in subcutaneous rat and mice implants^{84–86} or for dentoalveolar bone regeneration in rat molar sockets after tooth removal⁸⁷.

As opposed to delivery systems that are “programmed” to deliver according to a predetermined schedule, systems capable of responsive release (where release is induced by

endogenous cells) are one of the exciting new frontiers of nanomedicine as they hold promise for instructional-based biomimetic drug delivery. Generally the polymeric components of the carrier incorporate specific sequences that can be cleaved only under the desired environmental conditions (e.g., low pH) or by specific molecules (e.g. inflammatory cytokines, MMPs, etc.)^{88, 89}. Such carriers would allow dynamic release of intended molecules in response to cellular activity.

Concluding remarks and future vision

The role of endogenous regulators, such as Tregs or MSCs, for the promotion of homeostasis is a promising strategy to treat a number of diseases where current treatments are lacking. Cell therapies utilizing MSCs and Tregs have shown great promise even in the clinic, and have revealed the power of cells to promote immunological and regenerative homeostasis for the treatment of disease. Yet newer, fully synthetic approaches to localized recruitment, expansion and activation of a patient's own endogenous cells bypasses many of the hurdles of cell therapies. Indeed, these synthetic approaches seem to (at least in some capacity) mimic the body's natural mechanisms to cause cell homing or expansion when necessary. Each of these approaches represent potential future medical treatments that harness the body's innate regulatory capacity in a way that is dramatically more sophisticated and complex than what is possible in the clinic today.

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Figure 1.
How MSCs migrate to sites of disease or injury and potential modes of regulating immune cells, reviewed²⁴.

Figure 2. Synthetic engineering strategies to expand and chemoattract endogenous MSCs and Tregs.

Figure 3. Synthetic strategies to recruit and enhance Tregs for the amelioration of disease, restoration of homeostasis, and promotion of tissue regeneration.