Harnessing and Modulating Inflammation in Strategies for Bone Regeneration

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Inflammation is an immediate response that plays a critical role in healing after fracture or injury to bone. However, in certain clinical contexts, such as in inflammatory diseases or in response to the implantation of a biomedical device, the inflammatory response may become chronic and result in destructive catabolic effects on the bone tissue. Since our previous review 3 years ago, which identified inflammatory signals critical for bone regeneration and described the inhibitory effects of anti-inflammatory agents on bone healing, a multitude of studies have been published exploring various aspects of this emerging field. In this review, we distinguish between regenerative and damaging inflammatory processes in bone, update our discussion of the effects of anti-inflammatory agents on bone healing, summarize recent *in vitro* and *in vivo* studies demonstrating how inflammation can be modulated to stimulate bone regeneration, and identify key future directions in the field.

Introduction

INFLAMMATION IS A highly regulated biological response that plays an immediate and crucial role in promoting regeneration after bone fracture or injury.^{1,2} However, in certain clinical contexts, such as in inflammatory diseases or severe foreign body reactions to implanted materials, the normal inflammatory response fails to resolve, resulting in chronic inflammation that has destructive effects on bone tissue. A tremendous amount of effort has been invested in developing pharmacologic agents to disable this uncontrolled inflammatory signaling and thus prevent bone damage.^{3,4} In the process, a growing body of evidence has emerged that indicates that a complex balance exists between bone tissue and the immune system, which is responsible for the inflammatory response, and that obliterating inflammation also has damaging effects on bone.^{1,3,5} We previously published a review¹ that introduced this information to the bone tissue engineering community by identifying inflammatory signals critical for bone regeneration and describing the inhibitory effects of anti-inflammatory agents on bone healing. Since our previous review, a multitude of studies have been published exploring new strategies to harness and modulate inflammation to facilitate bone regeneration. The objectives of this review are to (1) distinguish between regenerative and damaging inflammatory processes in bone; (2) identify anti-inflammatory agents that inhibit bone healing; (3) summarize recent *in vitro* and *in vivo* studies to

demonstrate how inflammation can be modulated to stimulate bone regeneration; and (4) combine this information to identify key future directions in the field.

Regenerative Versus Damaging Inflammatory Processes in Bone

Inflammatory signaling during bone regeneration

Inflammation is an immediate response to bone injury, and a growing body of evidence indicates that the signaling cascades initiated during the week-long acute inflammatory response play a critical role in priming bone regeneration.^{1,2,5} Bone fracture stimulates expression of several dozen inflammatory cytokines, including interleukin-1 α (IL-1 α), IL-1 β , IL-6, IL-18, and tumor necrosis factor alpha (TNF- α).^{6–8} In murine models, TNF- α , IL-1 α , IL-1 β , and IL-6 signaling peaks 24 h after bone injury and returns to baseline levels after a few days.^{6,7} Together with a variety of growth factors, including proteins from the transforming growth factor beta (TGF- β) and bone morphogenetic protein (BMP) families, fracture-induced inflammatory mediators recruit inflammatory cells, promote angiogenesis, and guide mesenchymal stem cell (MSC) differentiation.^{6–8} Several cytokines, including TNF- α , IL-6, and stromal cell-derived factor-1 (SDF-1), have been shown to directly impact the *in vivo* migration of MSCs.^{9–12} Levels of most inflammatory mediators return to baseline after the week-long acute inflammatory phase.^{6–8}

Fracture healing involves overlapping phases of inflammation, renewal, and remodeling. During the renewal phase, the fracture site is stabilized by a cartilaginous callus, which then calcifies and undergoes endochondral bone formation. At this point, the remodeling phase begins. Levels of TNF- α and several other cytokines rise for a second time as the mineralized fracture callus is remodeled into lamellar bone by the renewing and resorptive actions of osteoblasts and osteoclasts, respectively.^{1,6,7,13} *In vivo* studies have identified cytokines from both the inflammatory and remodeling phases that are critical for bone regeneration. For instance, mice lacking either IL-6 or TNF- α have severely impaired fracture healing, with bone formation being delayed by several weeks.^{14,15} Emerging strategies to harness this signaling and use it to stimulate bone regeneration are discussed in a separate section of this review.

Role of inflammatory cells in bone regeneration

Although inflammatory cells, mainly neutrophils and macrophages, are immediately recruited to the injury site and release a cascade of cytokines and growth factors, *in vivo* studies have not yet identified a crucial inflammatory cell type for fracture healing. Inflammatory cells are primarily present at the fracture site during the initial week-long inflammatory phase of bone healing.^{2,16} Within 3–7 days of injury, and continuing during the subsequent renewal and remodeling phases of healing, native bone cells in the regenerating fracture callus, mainly osteoblasts and chondrocytes, are the primary source of inflammatory cytokines such as TNF- α .⁶ This overlapping inflammatory signaling by native bone cells may explain the normal bone healing seen in the absence of inflammatory cells. For instance, absence of neutrophils and macrophages does not adversely affect *in vivo* tissue repair.¹⁷ Absence of lymphocytes significantly accelerates *in vivo* fracture healing and remodeling, resulting in a mineralized fracture callus with superior biomechanical properties.^{18,19} In a unique chimeric animal model, transplantation of inflammatory cells from aged mice with impaired bone regeneration into otherwise healthy juvenile mice did not affect *in vivo* fracture healing. However, in the inverse case, consisting of a middle-aged mouse with inflammatory cells derived from a juvenile mouse, fracture callus healing and remodeling were accelerated.²⁰ It is possible that the beneficial effect of acute inflammation after bone injury may not be attributable to a particular cell type, but the relative proportions of the cells recruited to the injury site. A recent study in a sheep model indicated that the composition of the injury-induced inflammatory infiltrate is tissue-specific.¹⁶ For instance, 1 h after injury, a bone fracture hematoma contained fewer neutrophils than a hematoma taken from a muscle injury site. However, by 4 h after injury, this difference was no longer apparent, and the relative proportions of neutrophils, macrophages, and lymphocytes at the fracture site were identical to those seen in circulating blood.¹⁶ Any variation in the number and type of inflammatory cells, even for just a few hours, directly affects the balance between bone tissue and the immune system by altering the local concentration of growth factors and inflammatory cytokines that regulate bone healing.

Aberrant inflammation results in bone damage

The normal inflammatory response can be disrupted by many factors, including massive trauma and inflammatory diseases.^{3,4} Aberrant inflammatory signaling has been implicated as a significant factor in bone injuries that fail to heal. Osteoblasts taken from human patients with fracture nonunion had anomalous expression of 281 genes, including sets of genes that regulate growth factor activity, osteogenesis, angiogenesis, cytokine activity, and the inflammatory response.²¹ Similar results were seen in an animal model of delayed osseous union; at several timepoints after bone injury, the tissue from the nonunion site had reduced expression of several growth factors, including BMP-2, BMP-4, BMP-7, and TGF- β 1, as well as the cytokine TNF- α .²² These findings underscore the importance of regulated acute inflammatory signaling in priming bone regeneration.

The impact of inflammatory diseases on bone is not a direct effect of inflammatory cells. In a model of rheumatoid arthritis, mice with reduced osteoclast numbers were protected from bone damage, despite the infiltration of high numbers of inflammatory cells into the bone.²³ However, osteoclast-deficient mice were not protected from other destructive effects of inflammatory arthritis, like cartilage damage.²³

When acute inflammation fails to resolve, for example, due to the presence of an underlying inflammatory disease like rheumatoid arthritis, the resulting prolonged, unregulated inflammatory signaling has damaging effects on bone. For example, although TNF- α and IL-6 have been shown to be critical for *in vivo* bone regeneration in murine models,^{14,15} chronic exposure to high levels of these cytokines has damaging effects on bone. In murine models, prolonged, systemic exposure to high levels of TNF- α triggers tissue damage and symptoms resembling those of rheumatoid arthritis, including chronic inflammation, decreased bone volume, and reduced bone mechanical strength.²⁴ In a recent study of a mouse model of type 1 (i.e., autoimmune) diabetes, impaired fracture healing was attributed to excessive callus remodeling triggered by elevated expression of several inflammatory mediators, including TNF- α .²⁵ Systemic treatment with a TNF- α inhibitor starting 10 days after bone fracture significantly improved fracture healing in the diabetic mice.²⁵ Abnormally elevated levels of IL-6 also have damaging effects on bone. In human patients, abnormally high serum IL-6 levels in the days and even months after bone fracture correlate with decreased load-bearing capability of the injured bone.^{26,27} In a recent study, serum from human patients with polyarticular juvenile idiopathic arthritis, an autoimmune disorder that causes chronic joint inflammation, significantly suppressed *in vitro* osteogenesis of human osteoblasts, as determined by comparing their alkaline phosphatase (ALP) expression, osteocalcin protein level, and calcified matrix deposition to that of osteoblasts cultured in serum from healthy controls.²⁸ Analysis of the arthritic serum indicated that IL-6 was the only cytokine that was abnormally elevated.²⁸ This highlights the difference between the highly regulated acute inflammation that occurs during bone fracture healing, compared to the unregulated chronic inflammation seen in arthritis and autoimmune disease.

Effects of Anti-Inflammatory Drugs on Bone Healing

A tremendous amount of effort has been invested in developing pharmacologic agents to disable uncontrolled inflammatory signaling and thus prevent bone damage in patients with inflammatory diseases.^{3,4} A wide variety of anti-inflammatory medications are available to treat ailments ranging from musculoskeletal pain to chronic inflammation associated with autoimmune disease. However, therapeutic strategies have been complicated by the complex balance that exists between bone and the immune system, which is responsible for the inflammatory response; a growing number of studies indicate that anti-inflammatory medications have predominantly negative effects on bone healing.^{1,29} In most cases, instead of recommending the discontinuation of anti-inflammatory medications, current clinical guidelines instruct physicians to assess the risk/benefit ratio for each individual patient.³⁰ For researchers, considering the effects of the anti-inflammatory medications discussed in this section will aid in the design of more effective bone regeneration strategies.

Corticosteroids

Although corticosteroids are commonly used *in vitro* to induce osteogenic differentiation of MSCs,³¹ they are known to have negative effects on *in vivo* bone homeostasis and regeneration. In human patients taking corticosteroids, for example, to treat the chronic inflammation associated with rheumatoid arthritis, monitoring for adverse effects on bone, including reduced bone density and bone necrosis, is part of standard clinical practice.^{32,33} Numerous studies have shown that corticosteroids suppress *in vivo* fracture healing in rodents and rabbits by reducing osteogenesis, angiogenesis, and mechanical strength at the fracture site.^{34–38} Studies of genetically altered mice have demonstrated that baseline native corticosteroid signaling in osteoblasts is necessary for bone morphogenesis, but not for bone regeneration.³⁹ Mice lacking corticosteroid signaling in osteoblasts had normal bone healing after tibial fracture.³⁹ In recognition of their negative effects on bone, corticosteroids are not prescribed to human patients with bone injuries.²⁹ In fact, corticosteroids are a common therapeutic agent for human patients with fibrodysplasia ossificans progressiva, a disease associated with excessive bone formation.⁴⁰

Although long-term treatment with corticosteroids inhibits bone regeneration, the effects of short-term *in vivo* exposure remain unclear. Recent tissue engineering studies have reported a wide variety of effects of short-term corticosteroid exposure, ranging from no effect to substantial, beneficial effects on bone regeneration. Two recent studies have reported that 3D poly(DL-lactic-co-glycolic acid) (PLGA)-based scaffolds delivering dexamethasone stimulated *in vivo* bone regeneration in critical-sized defects after 4–8 weeks, compared to empty defects.^{41,42} However, neither study demonstrated a clear benefit of dexamethasone delivery, since equivalent bone regeneration was seen with dexamethasone-free scaffolds in each case.^{41,42} The effect on *in vivo* osteogenesis may depend on the method and temporal pattern of corticosteroid delivery. In a study of rat MSC-seeded titanium fiber meshes implanted in critical-sized rat cranial defects for 4 weeks, MSC preculture in media supplemented with dexamethasone for 4 days re-

sulted in superior bone regeneration compared to MSCs cultured without dexamethasone. However, exposure to dexamethasone for 16 days resulted in much less bone formation than seen with untreated MSCs.⁴³ In another study, MSCs precultured with dexamethasone for 1 week and seeded onto hydroxyapatite scaffolds resulted in increased ectopic bone formation in a rat subcutaneous implantation model; greater bone formation occurred when dexamethasone was supplied through a nanoparticle-based delivery system added to the preculture medium rather than as a free drug in solution.⁴⁴ In a rabbit model, systemic injection of the corticosteroid methylprednisolone increased ectopic bone formation in subcutaneously implanted chondrocyte-seeded PLGA scaffolds compared to rabbits not treated with corticosteroids.⁴⁵

In contrast to the diverse effects of corticosteroids in these animal models, a recent case study reported that implanting a dexamethasone-loaded gelatin scaffold in human patients with osseous nonunion stimulated bone regeneration.⁴⁶ Autologous bone marrow and bone fragments were collected from the noninjured femur of each patient and incubated *in vitro* in a high concentration (10^{-5} M) of dexamethasone for 1–2 h. The liquid portion of the mixture was then absorbed into a gelatin scaffold, which was packed into the site of osseous nonunion, and then surrounded by the dexamethasone-treated bone fragments. After 6–8 months, only 1/13 patients had persistent nonunion, and 6/13 had achieved complete union.⁴⁶ Additional research is necessary to establish the role of corticosteroids in the various phases of bone regeneration and better understand the mechanism of the diverse effects seen in humans and animals with bone injuries.

Cytokine-specific antagonists

To avoid the negative effects of nonspecific agents like corticosteroids, the chronic inflammation seen in autoimmune diseases is increasingly being treated with medications that specifically target a portion of the immune system. For instance, rheumatoid arthritis is treated using monoclonal antibodies and other synthetic biologic agents that target TNF- α , IL-1, and IL-6. These cytokine-specific inhibitors are a new class of medications, having only been approved for use in humans within the past 10 years.^{47,48} Seven agents that antagonize specific cytokines are currently approved for use in human patients: infliximab, adalimumab, etanercept, golimumab, and certolizumab-pegol inhibit TNF- α , whereas anakinra and tocilizumab antagonize IL-1 and IL-6, respectively.⁴⁹ Only a small number of studies have reported the effects of these agents on bone healing, and the results have been inconclusive. Most studies have focused on the oldest cytokine-specific antagonists, the TNF- α blockers infliximab, adalimumab, and etanercept. Several studies of human patients undergoing orthopedic surgery have shown no significant impact of TNF- α antagonists on the incidence of adverse events or wound healing complications.^{50,51} In contrast, other studies have shown that patients treated with TNF- α antagonists had a statistically significant increased risk of orthopedic surgical site infection and other complications.^{52,53} Since absence of TNF- α has been shown to be detrimental to bone regeneration in an animal model,¹⁴ the inconsistent inhibition of bone healing seen in human

patients is believed to stem from the very low doses of these medications that are prescribed, since higher doses suppress the immune system and increase the risk of severe infections like tuberculosis.^{47,49} However, the specific mechanism of each agent may also play a role. A recent *in vitro* study of human osteoblasts exposed to therapeutic doses of two TNF- α antagonists indicated that infliximab had a negative effect on osteoblast proliferation, whereas etanercept had no effect.⁵⁴

Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs), a large group of medications ranging from over-the-counter pain relievers like ibuprofen to prescription-only anti-inflammatories like celecoxib (Celebrex[®]), are the most widely studied anti-inflammatory medications in bone biology. The detrimental effects of various NSAIDs on bone regeneration have been documented in both human patients and animal models for several decades (recently reviewed by Refs.^{55,56}).

NSAIDs target the cyclooxygenase (COX) enzymes, which catalyze the rate-limiting step of prostaglandin synthesis. There are two forms of COX; COX-1 is constitutively expressed in bone and results in low levels of prostaglandin synthesis, whereas COX-2 is triggered by bone injury and results in high levels of prostaglandin production.²⁹ Recent studies have identified COX-2 as a critical enzyme in bone regeneration. In mouse models, absence of COX-1 due to genetic mutation did not affect bone regeneration, whereas absence of COX-2 impaired fracture healing and increased the risk of osseous nonunion.^{57,58} Consistent with these results, studies in rodent and rabbit models have shown that selective COX-2 inhibitors cause greater reductions in bone mineral density and mechanical properties at the injury site than nonselective NSAIDs, which inhibit both COX-1 and COX-2.^{59–61}

The detrimental effects of COX-2 inhibition are most profound in the initial inflammatory phase of fracture healing. In rat models, administration of COX-2 inhibitors for 5–7 days immediately after bone injury resulted in measurable effects 4–8 weeks later. COX-2 inhibition significantly reduced fracture callus mechanical strength and bone mineral density, and also increased the incidence of osseous nonunion.^{59,62,63} However, treating the rats with a COX-2 inhibitor either before bone fracture or starting 14 days after fracture had no effect on bone healing.⁶³ Systemic COX-2 has been shown to be critical for *in vivo* bone regeneration; local delivery of COX-2 within a bone graft in mice lacking COX-2 could not compensate for the detrimental effects on bone healing.⁶⁴

Studies of NSAID effects in human patients with bone injuries support the results of these animal studies. NSAIDs have been shown to significantly increase the risk of delayed union or nonunion after long bone injury.^{65,66} As with corticosteroids, NSAIDs are consequently used to prevent and treat ectopic or excessive bone formation in human patients.^{40,67,68} For instance, NSAIDs are used to prevent heterotopic bone formation after hip surgery, with as little as 5–7 days of postoperative NSAID administration being sufficient to inhibit ectopic bone formation.⁶⁸ However, in hip surgery patients who also have long bone fractures, NSAID

use increases the risk of nonunion in the long bone by fourfold.⁶⁹ This underscores the fact that excessively suppressing inflammation can disrupt normal bone healing. In light of these findings, current clinical guidelines recommend that NSAIDs be avoided for the 2 weeks after a bone fracture, particularly in patients with a higher risk of osseous nonunion.^{30,55}

Harnessing Inflammation to Stimulate Bone Regeneration

Stimulation of bone inflammation has not been extensively studied *in vivo* due to the risk of systemic inflammation and subsequent tissue damage. However, increasing awareness of the importance of certain inflammatory signals in priming bone regeneration has led to several recent *in vitro* and even some *in vivo* studies.

Prostaglandin E2 receptor agonists

Among the various prostaglandin types, prostaglandin E2 has been shown to have the most profound effects on bone homeostasis. Injury-induced increased prostaglandin E2 production is critical for bone regeneration.^{70,71} The specific bone cell types that respond to prostaglandin E2 remain unclear, although both osteoclast progenitors and mature osteoblasts have been implicated based on *in vitro* studies.^{61,72} *In vivo*, signaling via the prostaglandin E2 type 1 (EP1) receptor has been shown to negatively regulate bone regeneration, whereas signaling via the EP2 and EP4 receptors stimulates bone formation.⁷² Synthetic molecules selectively targeting these latter receptors have consequently been used in bone regeneration strategies. Local injection of selective EP2 agonists improved *in vivo* fracture healing in rodent models.^{73,74} In addition, implantation of a PLGA matrix encapsulating an EP2 agonist into a critical-sized canine ulnar defect stimulated osseous union after 24 weeks.⁷⁴ Delivery of selective EP4 receptor agonists has also shown promising results. In rodent models, both local and systemic delivery of EP4 selective agonists stimulated *in vivo* osteogenesis and angiogenesis, resulting in accelerated fracture healing.^{73,75} Dual delivery of BMP-2 and an EP4 agonist from a poly(ethylene glycol)-based hydrogel stimulated bone formation in a critical-sized murine cranial defect model; bone regeneration was greater with dual delivery than with delivery of either agent alone.⁷⁶

Leukotriene antagonists

Modulation of leukotriene signaling is a very new bone regeneration strategy.⁷⁷ Leukotrienes are a group of inflammatory signaling molecules that have the same metabolic precursor, arachidonic acid, as prostaglandins. However, unlike COX-2, which is the rate-limiting enzyme in prostaglandin synthesis and has been shown to play a critical role in bone regeneration, the key enzyme in leukotriene synthesis, 5-lipoxygenase, is detrimental to fracture healing.⁷⁷ One proposed mechanism for the impaired fracture healing seen with COX-2 inhibition is increased arachidonic acid availability to 5-lipoxygenase,⁷⁷ which results in abnormally elevated leukotriene levels at the fracture site.⁷⁸ In a rat model, administration of a 5-lipoxygenase inhibitor for 3 weeks after femoral fracture resulted in faster fracture

bridging and superior mechanical properties at the fracture site compared to untreated rats.⁷⁹ Similar accelerated fracture healing was reported in mice genetically altered to lack 5-lipoxygenase.^{78,79} Two clinically available leukotriene antagonists, montelukast sodium (Singulair®), a cysteinyl leukotriene type-1 receptor antagonist, and zileuton (Zyflo®), a 5-lipoxygenase inhibitor, also have beneficial effects on femoral fracture healing in a mouse model.⁸⁰ Both agents are currently approved by the U.S. Food and Drug Administration (FDA) for the treatment of inflammatory airway diseases such as asthma. Systemic administration of either leukotriene antagonist significantly accelerated bone regeneration compared to fracture healing in untreated controls.⁸⁰ Further mechanistic analysis indicated that the leukotriene antagonists enhanced fracture healing by directly affecting chondrocyte activity within the fracture callus.⁸⁰ These recent results highlight the promise of modulating leukotriene synthesis as a new strategy to stimulate bone regeneration.

Modulating cytokine activity

Direct modulation of cytokine activity to promote bone regeneration is a new strategy that remains largely at the cell culture testing stage, though a few *in vivo* studies have been recently published. The most commonly studied cytokines are TNF- α , IL-1, and IL-6.

TNF- α . Since the mechanism of TNF- α in bone regeneration remains unknown, studies to date have been mainly conducted *in vitro*. These studies have revealed weaknesses in the typical *in vitro* culture conditions used to study MSC osteogenic differentiation and have necessitated changes to standard cell culture protocols. TNF- α does not affect *in vivo* embryonic skeletal development,¹⁴ but plays a critical role in bone regeneration. Mice lacking TNF- α have severely impaired fracture healing, with bone formation being delayed by several weeks.^{14,81} Exposure to TNF- α significantly affects the *in vitro* protein expression, proliferation, and migration of undifferentiated human MSCs, but has not been reported to induce differentiation.^{11,82} Thus, to study the effects of TNF- α on osteogenically differentiating MSCs, supplements must be added to induce MSC osteogenic differentiation. Studies of the effects of TNF- α on osteogenic differentiation have been complicated by the fact that two supplements typically used to stimulate *in vitro* MSC osteogenic differentiation, dexamethasone and ascorbic acid, antagonize TNF- α signaling.^{83–85} While commonly used in *in vitro* MSC cultures, dexamethasone, an anti-inflammatory corticosteroid, is not present in the *in vivo* bone fracture environment.¹ Additionally, as discussed above, corticosteroids have been implicated in impaired bone fracture healing. One strategy to overcome this limitation and create a more realistic *in vitro* model of the fracture healing environment has been to preculture MSCs in osteogenic medium to trigger differentiation and then use medium without dexamethasone to study the effects of TNF- α .⁸³ In the absence of dexamethasone, treatment of osteoprogenitors with TNF- α resulted in dose-dependent increases in ALP activity and mineralized matrix deposition, which are, respectively, early and late markers of osteogenesis.^{12,83,86–88} In contrast, when added to dexamethasone-containing osteogenic medium, TNF- α had the opposite effect on MSCs.^{89,90} The pro-regenerative effects of TNF- α

seen in dexamethasone-free *in vitro* MSC cultures were recently confirmed *in vivo* through repeated injections of TNF- α into a fracture healing model. Daily local injections of TNF- α during the first 2 days after injury resulted in significantly increased fracture callus mineralization 4 weeks later.¹² These findings for TNF- α emphasize the importance of developing clinically realistic *in vitro* culture models to improve the predictions of the *in vivo* effects of various cytokines.

IL-1. Unlike TNF- α , which does not affect skeletal development but is required for normal bone regeneration, absence of IL-1 does not affect *in vivo* fracture healing.⁹¹ However, mice lacking IL-1 α and/or IL-1 β , the two forms of IL-1, have significantly higher bone mineral density and femoral bone mass than normal mice, which has been attributed to impaired osteoclast development.⁹² *In vitro* studies of the effects of IL-1 α and IL-1 β on osteogenically differentiating MSCs have shown that these cytokines have similar effects as TNF- α .^{87,89,93} MSCs precultured to induce osteogenic differentiation and subsequently exposed to IL-1 β (in dexamethasone-free medium) had significant dose-dependent increases in early and late markers of osteogenic differentiation,⁸⁷ whereas simultaneous exposure to IL-1 β and dexamethasone had the opposite effect.⁸⁹ Simultaneous delivery of IL-1 β and TNF- α had synergistic effects on *in vitro* MSC deposition of mineralized matrix.⁸⁷ A recent study reported that daily local IL-1 β injections during the first 3 days after bone fracture resulted in slightly accelerated *in vivo* bone regeneration.⁹¹ When compared to the results of *in vitro* studies, this finding once again emphasizes the need to deliver cytokines to MSCs under culture conditions simulating the *in vivo* fracture healing environment (e.g., no dexamethasone) to effectively predict *in vivo* results.

IL-6. IL-6 is required for both normal skeletal development and bone regeneration. Mice lacking IL-6 have reduced bone mineral density and also have impaired fracture healing.¹⁵ *In vitro* studies of osteogenically differentiating cells have indicated that exposure to IL-6 for 3–6 days increases gene expression of osteogenic markers RUNX2 and osteocalcin.⁹⁴ In an *in vivo* fracture healing model, repeated local injections of parathyroid hormone fragments and IL-6 in the first 2 weeks after injury significantly increased fracture callus mechanical strength and accelerated osseous union.⁹⁵ However, this treatment reduced the mechanical strength of noninjured bones, which was attributed to a systemic effect of the injected agents.⁹⁵ This underscores the need for controlled release systems to provide targeted cytokine delivery to the site of bone injury.

IL-4. Although abnormally elevated IL-4 levels have long been known to inhibit *in vivo* bone remodeling and result in extremely weak, osteoporotic bones,⁹⁶ absence of this cytokine due to genetic mutation was recently shown to have no effect on *in vivo* fracture healing.⁹⁷ However, in a study of *in vivo* ectopic bone formation stimulated by implantation of xenogeneic demineralized bone matrix, absence of IL-4 and IL-13 reduced angiogenesis,⁹⁷ a finding that may have implications for the development of bone tissue engineering strategies.

Interferon- γ . Many studies of the effects of interferon (IFN)- γ on bone regeneration have focused on signal transducer and

activator of transcription 1 (STAT1), an intracellular signal that is a component of the IFN- γ pathway. Absence of IFN- γ signaling, due to absence of either STAT1 or the IFN- γ receptor, increases the number of osteoclasts in bone *in vivo*, but also increases bone mass.^{98,99} Absence of STAT1 also accelerates *in vivo* fracture healing and subsequent bone remodeling.⁹⁹ Gelatin scaffolds loaded with fludarabine, a STAT1 inhibitor, stimulated *in vivo* ectopic bone formation when implanted subcutaneously in a mouse model. Dual delivery of BMP-2 and fludarabine revealed an additive effect of the two agents on *in vivo* ectopic bone formation.⁹⁹

Stromal cell-derived factor-1. SDF-1 is a new target in bone regeneration strategies. In animal models with reduced SDF-1 signaling, new bone formation 2 weeks after injury was 60%–80% less than normal.⁹ In another study, porous PLGA scaffolds loaded with SDF-1 triggered MSC chemotaxis both *in vitro* and *in vivo*, resulting in MSC migration toward, and subsequent attachment to, the scaffold.¹⁰ SDF-1 delivery from subcutaneously implanted PLGA scaffolds significantly reduced *in vivo* fibrotic encapsulation and inflammatory cell accumulation near the scaffold,¹⁰ demonstrating the potential of this cytokine as a component of future immunomodulatory strategies to prevent foreign body reactions to biomedical implants.

TP508. TP508 (rusalotide acetate; Chrysalin[®]) is a synthetic peptide that stimulates the same signaling pathways activated by cytokines like TNF- α and IL-1.¹⁰⁰ This peptide has been shown to be a very potent stimulator of *in vivo* bone regeneration, with a single injection into a rat femoral fracture increasing the strength of the healed bone by over 30%.¹⁰¹ In rabbit models, TP508 has been shown to stimulate bone regeneration of critical-sized defects when incorporated into a PLGA microparticle-based controlled release system.^{102,103} However, in a recent Phase III clinical trial of this agent, a single injection of TP508 into the site of a radius fracture failed to significantly improve (vs. placebo) the primary measure of success, which was the total time that the fracture required immobilization.¹⁰⁴ However, further analysis revealed that in a subset of the clinical trial participants, that is, female patients with osteopenia, TP508 was successful; it significantly reduced the amount of time that the fractures required immobilization, and also accelerated fracture healing.¹⁰⁴ Further studies are needed to clarify the efficacy of TP508 in patients with impaired bone regeneration capacity.

Modulating inflammatory cell activity

Another emerging strategy is to directly target and modulate inflammatory cell function in bone. Small molecule inhibitors of cathepsin K, an enzyme specific to osteoclasts and other bone cells of the monocyte lineage, effectively reduced *in vivo* bone resorption and uncontrolled joint inflammation in a rat model of rheumatoid arthritis.¹⁰⁵ Although the inherent immunomodulatory properties of MSCs have been recognized for approximately a decade, clinical strategies utilizing these cells have focused on systemic injection of culture-expanded MSCs to treat immune diseases.¹⁰⁶ A recent study indicates a strategy through

which native MSC function can be modulated via local delivery of cytokines. MSCs exposed to IFN- γ and either TNF- α , IL-1 α , or IL- β have been shown to secrete factors that suppress T lymphocyte function *in vitro*.¹⁰⁷ The MSCs also recruited additional T lymphocytes to the area, which were then also inactivated.¹⁰⁷

Concluding Points

Harnessing and modulating inflammatory signaling is a promising new strategy for bone regeneration. Since our previous review¹ 3 years ago, which introduced this topic to the bone tissue engineering community by identifying key inflammatory cytokines that prime bone regeneration and describing the inhibitory effects of anti-inflammatory agents on bone healing, a multitude of *in vitro* and *in vivo* studies have focused on this topic. These studies have provided crucial information, as well as identified important questions to be answered in future work.

The initial week-long inflammatory phase of fracture healing is characterized by the influx of inflammatory cells, that is, neutrophils, lymphocytes, and macrophages, and the release of a variety of cytokines and growth factors. However, the mechanism by which these complex signaling cascades trigger scar-less bone regeneration remains unknown. Several cytokines, including TNF- α and IL-6, have been shown to play a critical role in bone healing and have even been successfully harnessed to trigger regeneration in animal models of massive bone trauma. Although they are the primary source of inflammatory cytokines immediately after bone injury, no particular inflammatory cell type has been identified as critical for bone regeneration. In fact, lymphocytes have been shown to impair bone healing, suggesting that the native bone cells are the most promising targets for rational control of bone inflammation.

Engineering strategies to rationally control inflammatory signaling are necessary because of the complex balance that exists between bone tissue and the immune system, which is responsible for the inflammatory response. Precise spatial and temporal control is necessary when delivering cytokines because both prolonging and obliterating this signaling impair bone healing. For instance, complete absence of TNF- α delays regeneration, whereas prolonged exposure to high levels of this cytokine stimulates bone destruction.

A tremendous amount of effort has been invested in developing pharmacologic agents to treat uncontrolled, chronic inflammation and prevent bone damage. In the case of NSAIDs, the negative effects on bone regeneration are clear. However, leukotriene antagonists, which are currently FDA-approved to treat chronic airway inflammation, have shown immense potential as agents to stimulate bone healing. The role of corticosteroids in this field remains unclear. *In vivo*, they have been shown to have a wide range of effects depending on factors including the method and temporal pattern of administration. This is a particularly important lesson for the development of future tissue engineering strategies, as the composition of the implanted material can profoundly affect the immune and, therefore, inflammatory response.

Another very important lesson is the need for clinically realistic *in vitro* models of bone regeneration. Historically, bone tissue engineers have commonly used dexamethasone, an anti-inflammatory corticosteroid, to stimulate *in vitro* os-

teogenic differentiation of MSCs and thus generate models of bone healing. However, comparison of *in vitro* results with the available *in vivo* studies of cytokine delivery has indicated that this osteogenic supplement cannot be used when studying cytokine delivery. Concurrent delivery of corticosteroids and cytokines such as TNF- α to MSC cultures has resulted in findings that are completely opposite to those seen *in vivo*. Pretreatment of MSCs with corticosteroids to stimulate *in vitro* osteogenic differentiation, followed by cytokine delivery alone, has yielded more realistic results. Important future directions for *in vitro* studies in this field include studies to elucidate the mechanisms underlying inflammatory signaling, additional optimization of cell culture models, as well as studies of the interplay between various cytokines and growth factors found at the fracture site. Learning to rationally control inflammation will provide a wealth of new directions in the development of bone tissue engineering strategies, and will ultimately improve the quality and specificity of therapies available to stimulate bone regeneration in patients with impaired healing or severe bone injuries.

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