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Review article

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IMMUNOMODULATORY AND POTENTIAL THERAPEUTIC ROLE OF MESENCHYMAL STEM CELLS IN PERIODONTITIS

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Periodontitis is a chronic inflammatory disease leading to alveolar bone destruction, and eventually tooth loss. In genetically or environmentally predisposed individuals periodontopathogenic bacteria trigger an inflammatory immune response where activated macrophages secrete inflammatory cytokines and T helper 17 cells produce interleukin-17, receptor activator of nuclear factor kappa B ligand (RANKL) and tumor necrosis factor-α. Inflammation and the production of RANKL, the key cytokine responsible for osteoclast activation, cause excessive activation of osteoclasts. This results in a decoupling between bone formation and resorption, leading to bone loss. As conventional treatment does not target the inflammatory response and osteoclast activation, its effectiveness is limited. Novel treatments are thus required if we are to cure this disease. Mesenchymal stem cells (MSCs), including those of dental origin, are potent immunomodulators and are known to be suitable for tissue regeneration. MSCs can inhibit the immune response by suppressing T cells, inducing regulatory T cells and converting dendritic cells and macrophages into a regulatory phenotype. Additionally, genetic modulation may enhance the therapeutic potential of MSCs. In the present review the authors describe the potential use of MSCs, either unmodified or engineered for therapeutic purposes in periodontitis, with special emphasis on MSCs from dental pulp and periodontal ligament. The paper envisions that multiple targeting of this inflammatory disease by modulating the immune response, promoting bone regeneration and inhibiting bone resorption might yield significantly improved treatment outcomes when combined with conventional treatment modalities.

Key words: *mesenchymal stem cells, periodontitis, regeneration, immunomodulation, regulatory T cells, dental, periodontal, immunosuppression, osteoprotegerin, BMP-2, gene therapy*

INTRODUCTION

The oral cavity is inhabited by diverse microbiota that plays an important role in maintaining oral and systemic health (1). The presence of oral commensals inhibits colonization by pathogens by occupying the binding sites on oral surfaces (2). Salivary glycoproteins selectively absorbed on teeth form a pellicle. Salivary pellicle provides an attachment surface to primary colonizer oral bacteria (3, 4). Other bacteria will then co-aggregate with the primary colonizers *via* coaggregation interactions (5). The result is a dental plaque, but its formation can be kept to a minimum by regular tooth cleaning. When oral hygiene is poor, the proportion of Gram-negatives and anaerobic species increases in the maturing plaque, and the presence of endotoxin and other bacterial products leads to periodontal disease (1). Periodontal diseases are highly prevalent and can affect up to 90 percent of the human population worldwide (6). Their accurate diagnosis can still be a challenge (7). In essence, periodontal diseases are the pathological manifestation of the host response against bacterial challenge from dental biofilm at the tooth/gingival interface (8). The mildest and fully reversible form of periodontal disease is gingivitis, which does not affect tooth supporting tissues (9). Untreated gingivitis may advance to periodontitis over time. Dental plaque formation spreads apically and the junctional epithelium at the bottom of the gingival sulcus is destroyed by accumulating bacterial complexes. The resulting epithelial downgrowth and pocket formation allows plaque bacteria to colonize subgingival root surfaces. Toxins from Gram-negative bacteria such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (and a number of other ones) in the pocket induce an inflammatory host reaction. The subsequent tissue destruction is driven by mediators of the host immune-inflammatory response to the infection (9) resulting in increased pocket depths. At the same time, gingival connective tissue, Sharpey fibers and alveolar bone are destroyed. Unfortunately, as the destructive process mostly presents only with very mild symptoms, the disease remains untreated until teeth lose their ligamentous connection to the alveolar bone, become mobile and are eventually lost (10, 11).

Alveolar bone loss and chronic inflammatory process are important clinical hallmarks of chronic periodontal disease. Therapeutic intervention should ideally aim at dampening the inflammatory immune response regenerating the lost

anantomical structure and function of the periodontal region as well as possibly restoring the normal commensal microbiota. Conventional treatment options include the removal of bacterial deposits and surgical tissue regeneration procedures in periodontitis (12). Such treatments also include oral hygiene instructions and scaling/root planing, aiming to prevent the disease, slowing down or stopping its progress and maintain the therapeutic goals achieved. However, such interventions are usually insufficient to promote the regeneration of damaged structures (13). Thus, novel treatment modalities such as using anti-inflammatory substances (14, 15) are required for successful treatment. One innovative approach, the use of mesenchymal stem cells (MSCs), offers the possibility to simultaneously target the inflammatory response and to promote the regeneration of the periodontal structure. MSCs have been isolated from a number of adult tissues such as bone marrow, adipose tissue, gingiva and dental tissues. Adult MSCs are known to be capable of differentiating into various cell types, thereby facilitating tissue regeneration after damage due to injury, inflammation or other pathologies (16, 17). MSCs also have potent immunomodulatory capabilities *via* secretion of regulatory cytokines and other modulatory factors or *via* direct cell-cell contact (18, 19). MSCs of dental origin, including dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs), are of particular interest. Despite their small size, teeth are an abundant source of DPSCs and PDLSCs (20, 21). Importantly, MSCs are normally obtained from teeth removed during routine dental care and they can be cultured under standard conditions (22). Therefore, the process involves no additional burden on the patient beside from that of routine tooth extraction. The use of various biomolecules may also help to reduce the pathological bone loss (23) as well as promote and support the regenerative processes (24). Several growth factors such as BMP-2 (25, 26) or bFGF (27) have already been proposed and tested in animal models for this purpose. The pharmaceutical targeting of the RANKL/OPG balance to reduce bone loss in periodontitis has also been introduced and reported in animal experiments (28).

The aim of this review is to discuss the immunopathomechanism of periodontitis and its relation to bone loss, to address the potential use of mesenchymal stem cells, especially those of dental origin, either unmodified or engineered to express biomolecules for therapeutic purposes, to treat this complex disease.

PATHOLOGICAL BONE LOSS AND IMMUNOREGULATION IN PERIODONTITIS

A persistent host inflammatory response and the resulting chronic inflammation is one of the key factors in the destruction of soft and mineralized periodontal tissues in periodontitis (29). It is characterized by the destructive activity of multiple elements of the immune system along with the overactivation of osteoclasts and diminished osteoblast activity, resulting in an imbalance in bone formation and breakdown. The process is not fully understood, but what is known already offers several potential targets for the treatment of periodontal disease.

A shift in the balance between osteoclast and osteoblast function in periodontitis

Physiological and pathological bone resorption is carried out by a specialized cell type called the osteoclasts. Osteoclasts are hematopoietic lineage derived cells originating from monocytes. Monocytes are released from the bone marrow into the blood, where they home into different tissues and differentiate into tissue-resident macrophages (30). Osteoclasts are formed in response to appropriate stimuli by the fusion of mononuclear precursors within the monocyte fraction of peripheral blood. Macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) are the two key factors that drive osteoclast formation and activity (*Fig. 1*) (31, 32). M-CSF, produced by osteoblasts or bone marrow stromal cells, stimulates the expression of RANK (the receptor for RANKL) on osteoclast precursors, allowing RANKL to drive mature osteoclast formation (33). Under normal circumstances, RANKL is expressed on the surface of osteoblasts and osteocytes (34). Osteoprotegerin (OPG), a soluble decoy receptor for RANKL, inhibits RANKL-induced osteoclast formation and activation (*Fig. 1*) (35, 36). OPG is produced by human periodontal ligament cells, gingival fibroblasts and epithelial cells (37, 38). Thus, osteoclast activity depends on the balance between RANKL and OPG.

Osteoblasts are bone forming cells, which also produce bone morphogenetic proteins, including BMP-2, which plays a major role in the differentiation of bone cell lineages, bone development and bone repair (39). BMP-2 also promotes RANKL secretion by osteoblasts, and is crucial for osteoclasts by offering a suitable microenvironment for their differentiation (40) (*Fig. 1*). Furthermore, under physiological conditions, they are the main sources of RANKL and M-CSF for the development of osteoclasts (40). Bone formation and bone resorption are thus normally coupled (41, 42). A shift in this balance toward bone resorption occurs in a number of chronic inflammatory diseases, which results in bone loss.

As discussed above, osteoclast activity depends on the RANKL/OPG ratio (23). The main sources of RANKL under normal circumstances are osteoblasts and osteocytes (34). In periodontal lesions the excess amounts of RANKL, produced mostly by activated T and B cells (43), increase the RANKL/OPG ratio which favors osteoclast activation and bone resorption. Importantly, RANKL inhibition by OPG blocks bone loss in experimental periodontitis (28) (*Fig. 1*). As a prototype of novel therapeutic approaches, denosumab, a fully human monoclonal antibody that targets RANKL, *i.e.* mimics the effect of OPG, is already in use in humans to prevent bone loss in a number of diseases including osteoporosis and periodontitis (44).

In summary, bone resorption is mediated by osteoclasts, whose activity is determined by the RANK/RANKL/OPG axis. In periodontitis, the development and maintenance of a pathogenic inflammatory milieu leads to inappropriately high RANKL levels, excessive osteoclast activation and bone loss. Therapeutic methods are being developed to inhibit inflammatory processes and osteoclast function.

The role of dendritic cells

T cell response, a major component of the adaptive immunity, is induced and controlled by antigen presenting cells (45). Dendritic cells (DCs) and macrophages are the most important professional antigen presenting cells. Dendritic cells may be subdivided into classical DCs (cDCs) and plasmacytoid DCs (pDCs) (46). cDCs specialize in antigen presentation to T cells and thereby initiating and controlling immunity (30). They are characterized by high phagocytic activity as immature cells and high cytokine producing capacity as mature cells. cDCs are generally short-lived (30, 47), whereas plasmocytoid DCs (pDCs) are relatively long-lived (30, 48). pDCs are found both in bone marrow and in peripheral organs. Besides from controlling T cell responses, they produce large amounts of type I interferons in response to viral infection (49). There are conflicting reports on the presence of DCs in periodontitis lesions and on their role in the pathogenesis of the disease (50). A recent study by Allam

and coworkers showed a sharp decrease in the number of CD1a+ DCs in chronic periodontitis lesions compared with healthy oral mucosa (51).

The role of macrophages

Macrophages eliminate dead cells that underwent apoptosis or necrosis, produce growth factors and cytokines and act as a first line of defense against invading bacterial pathogens. They differentiate from circulating peripheral blood mononuclear cells that migrate into tissues in steady state or during inflammation (52). In addition, by presenting antigens, they have a key role in T cell activation and in the polarization of the adaptive immune response. Three populations of activated macrophages have been identified: classically activated, wound healing and regulatory macrophages (*Fig. 1*) (53). Classically activated macrophages function in the defense against intracellular pathogens and secrete high levels of pro-inflammatory cytokines. However, the cytokines and mediators they produce, can be harmful to the host: tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-23 produced by classically activated macrophages promote the development and expansion of Th17 cells (53) (*Fig. 1*). Wound healing macrophages arise in response to IL-4 or IL-13 and mostly contribute to the synthesis of extracellular matrix (54). Regulatory macrophages produce high levels of the immunosuppressive cytokine IL-10 and downregulate the production of IL-12. Since IL-10 inhibits the production and counteracts the effects of several pro-inflammatory cytokines, regulatory macrophages are strong inhibitors of inflammation (53).

Macrophages were detected in considerable numbers in inflammatory infiltrates from periodontitis subjects (51, 55). They play key roles in regulating the immune response in periodontitis not only by presenting antigens to T cells but also by secreting a variety of inflammatory cytokines such as IL-1, IL-6 and TNF-α (56-58), which directly or indirectly influence the differentiation and function of osteoclasts, the key mediators of bone resorption. For example, TNF-α secreted by activated macrophages stimulates osteoclast differentiation both directly (56, 59) and indirectly *via* stimulating RANKL production by osteoblasts (60) (*Fig. 1*). Furthermore, osteoclasts are derived from the monocyte/macrophage lineage, and therefore macrophages themselves can differentiate into new osteoclasts (61). Therefore, the relationship between monocytes/macrophages and the biology of osteoclasts is well established.

The role of T cell subsets

T cell differentiation, a key factor in the immune response, is regulated by antigen presenting cells *via* the production of cytokines (62, 63). Naive CD4+ T helper cells may differentiate

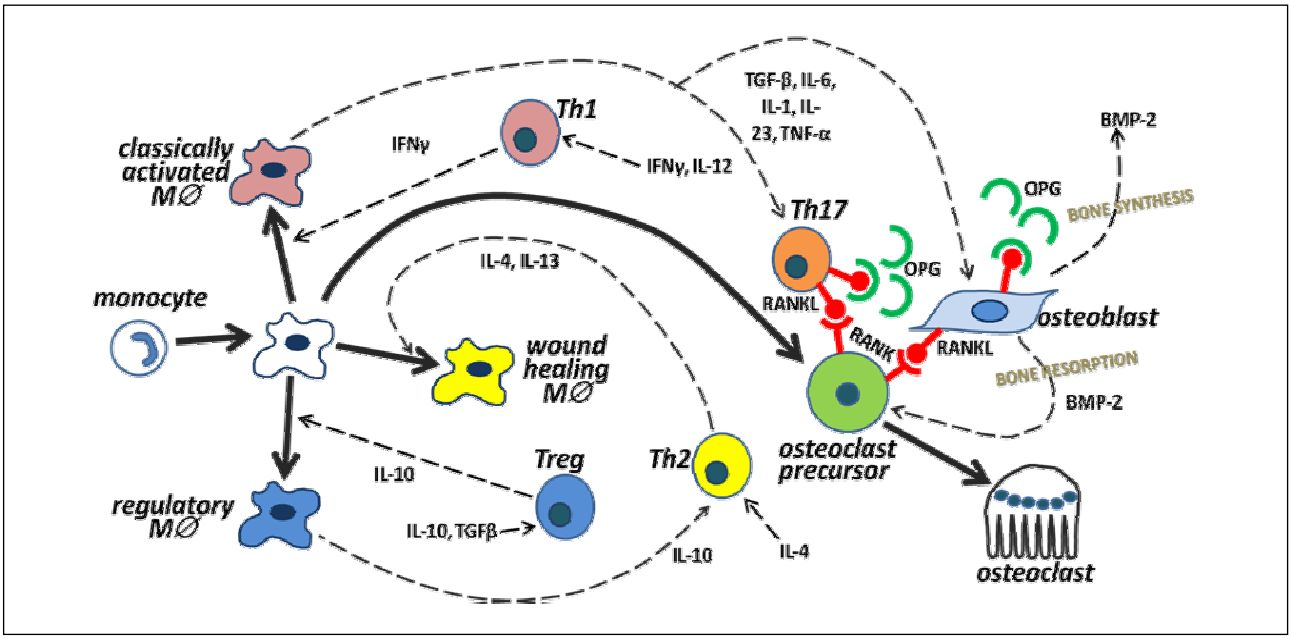

Fig. 1. Immune regulation of osteoclast function in periodontal inflammation. This figure depicts a very complex mechanism that is presently not fully understood. Osteoclasts are derived from the monocyte/macrophage lineage. Circulating monocytes home to tissues to become macrophages (MØ) and, depending on environmental cues, can assume classically activated, wound healing and regulatory phenotypes. These innate immune cells phagocytose and kill invaders and interact with the components of the adaptive immune system by presenting antigens and producing cytokines. Key components of the adaptive immune response in periodontitis are CD4+ helper T cells (Th). Th1, Th2, Th17 and regulatory T cells (Treg) differentiate in response to certain cytokines, some of which are shown in the figure. Also shown are certain cytokines by which they regulate the differentiation of MØ subsets and of osteoclasts. Osteoclast differentiation and function is regulated by the receptor RANK on the surface of osteoclasts and osteoclast precursors. Its activating ligand is RANKL, most of which is produced by Th17 cells in periodontitis. The effect of RANKL is blocked by its decoy receptor OPG, produced by bone marrow stromal cells and osteoblasts, the cell type that produces bone and secretes bone morphogenetic proteins (BMPs), including BMP-2, which is a key morphogen for bone formation, but an osteoclast activator as well. Osteoclast activity is determined by the ratio of the amounts of RANKL and OPG. In addition to BMP-2 and OPG, osteoblasts also produce RANKL. Under physiologic conditions, this ensures that the synthesis and the resorption of bone are coupled.
Bold arrows, cell development pathways; dashed arrows, cytokines with a certain effect produced by a cell type; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; BMP, bone morphogenetic protein; RANK, receptor activator of NFκB; RANKL, RANK Ligand; OPG, osteoprotegerin; IL, interleukin; Th, helper T cell; Treg, regulatory T cell; MØ, macrophage.

into T helper 1 (Th1), Th2 (64), Th17 (65) and regulatory T (Treg) (66) cells.

Th1 differentiation is induced by IFN-γ and IL-12 (67, 68) (*Fig. 1*). Th1 cells are critical in cell-mediated immunity to clear intracellular pathogens. Th2 cells are induced by IL-4 (*Fig. 1*) (67). The Th2 effector cytokines are IL-4, IL-5 and IL-13 (69, 70). A Th2 immune response is more effective against extracellular pathogens.

Th17 cells are a new pathogenic Th cell subpopulation (65, 71) (*Fig. 1*). These cells play a role in the pathogenesis of several autoimmune/inflammatory diseases (72-75). In mice, the key cytokines for Th17 cell differentiation are TGF-β and IL-6/IL-21 (76, 77). IL-23 is required for Th17 cell maintenance and expansion (78, 79). IL-1β and TNF-α also amplify Th17 development (79, 80). In humans, TGF-β, IL-1β, IL-6, IL-21 and IL-23 are required for Th17 differentiation (81, 82). IL-23 is secreted by innate immune cells such as DCs and macrophages in response to stimulation of certain pattern recognition receptors or other signals (83). IL-23 receptor is expressed on T cells, natural killer cells, DCs and macrophages (84). The effector cytokines secreted by Th17 cells are IL-17, IL-17F, IL-21, IL-22 and IL-9 (85, 86). The amount of IL-17 correlates with the presence and degree of periodontitis (87). IL-17 and IL-17F induce the production of proinflammatory cytokines such as TNF-α, IL-1β, GM-CSF, G-CSF, IL-6 and certain chemokines (85), whose proinflammatory effects are exerted on a variety of cell types including fibroblasts, monocytes, macrophages, epithelial cells and osteoblasts.

Th17 cells have been shown to be involved in periodontitis (88). IL-17 produced by Th17 cells induces RANKL expression by osteoblasts, leading to osteoclast stimulation (89, 90) (*Fig. 1*). IL-17 also elicits the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α by fibroblasts, endothelial cells, macrophages and epithelial cells (91, 92), thus producing and maintaining an inflammatory milieu. Th17 cells also themselves express RANKL (89), and TNF-α (93) which induces osteoclastogenesis on osteoclast precursors in synergy with RANKL. These characteristics clearly link Th17 cells to sustained inflammation and pathologic bone loss in periodontal disease.

Regulatory T cells (Treg) modulate the immune response and inhibit inflammation (94, 95) (*Fig. 1*). They develop in the thymus and play an essential role in preventing the inappropriate activation of the immune system (96). To date, the most specific marker of Tregs is the transcription factor Foxp3 (97-99). Secretion of IL-10 and TGF-β is an important aspect of immune regulation by Treg cells (100).

Tregs are also involved in the regulation of immune response in periodontitis (*Fig. 1*). Their presence has been demonstrated in the periodontal environment (101). In humans P. gingivalis infection has been shown to be associated with Tregs dysregulation leading to diminished Treg function (102). In a mouse model of periodontitis, periodontopathogen infection increased the number of Treg cells in periodontal tissue (103). Inhibition of Treg function decreased the production of Treg signaling molecules IL-10, CTLA-4 and TGF-β, stimulated the production of the inflammatory cytokines IFN-γ, TNF-α and RANKL, and increased the number of leukocytes and bone loss in periodontal tissues. This clearly demonstrates that Tregs attenuate periodontitis (103). This is also supported by a more recent study that has also demonstrated the induction of IL-10 producing Treg cells in periodontitis (104). Very recent data indicate that the modulation of the Th17/Treg imbalance by retinoic acid administration provides protection against periodontitis by enhancing Treg cell activation and inhibiting Th17 cell activation, suggesting the potential for clinical prevention of periodontitis (105).

MESENCHYMAL STEM CELL THERAPY FOR PERIODONTITIS

Mesenchymal stem cells (MSCs) are a class of multipotent cells originally identified in bone marrow (106, 107). Their high

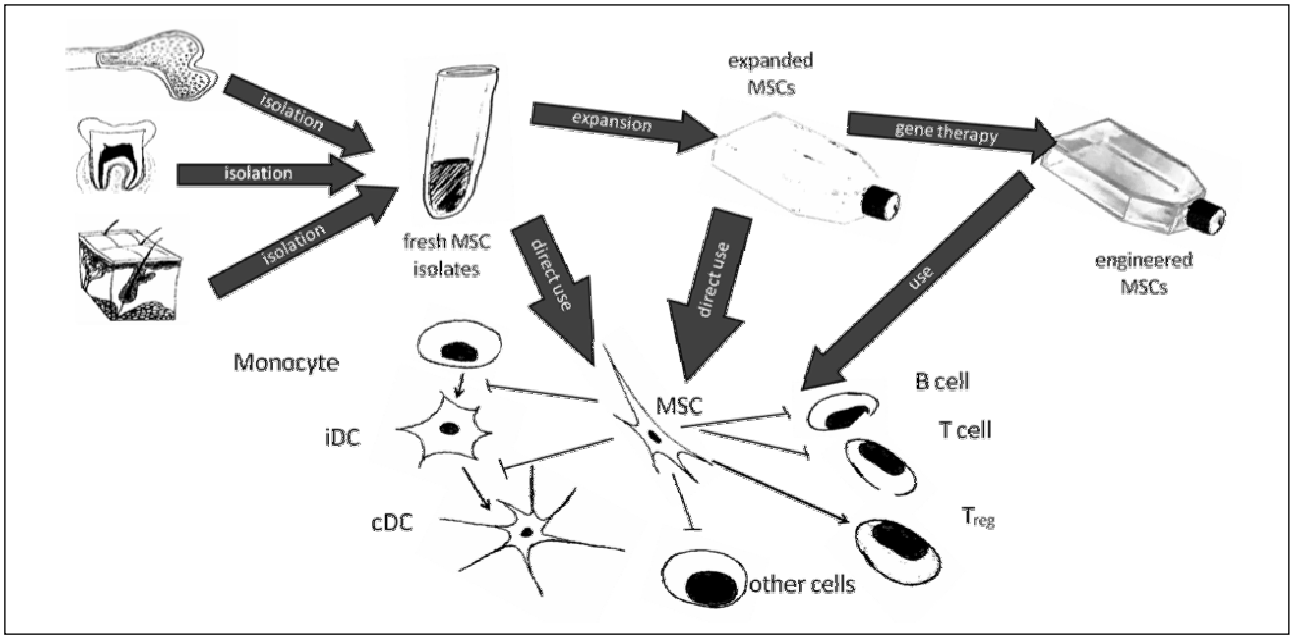

Fig. 2. Multiple ways of immunoregulation by mesenchymal stem. MSCs, collected from various sources such as bone marrow, teeth or skin, can be used directly or after expansion, with or without *ex vivo* gene transfer. MSCs stimulate regulatory T cell (T_{reg}) function and inhibit all other cell types of the immune system, such as B and T cells, monocytes, immature dendritic cells (iDCs) and classical dendritic cells (cDCs). Gene transfer of an immunomodulatory or other factor may further enhance the efficacy of MSC therapy.

plasticity and their presence in adult humans make them a very attractive candidate for clinical use. In addition to bone marrow, they have been discovered in a number of other tissues such as brain, skin, skeletal muscle and the gastrointestinal tract (108-110). They were also found in oral tissues such as dental pulp and periodontal ligament (111-115). MSCs can be used for the regeneration of bone, liver, neuronal tissue, blood and skeletal muscle (111, 112, 116-120). MSCs have the potential to directly differentiate to multiple lineages of mesenchymal origin, including bone, cartilage, fat, connective tissue, smooth muscle and hematopoietic supportive stroma (121).

MSCs of dental origin, including dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs), are of particular interest. Despite their small size, teeth are an abundant source of DPSCs and PDLSCs (111, 114, 115). Importantly, MSCs are normally obtained from teeth removed during routine dental care. At present, a major focus in our laboratory is to study the differentiation, regeneration and immunomodulatory potential of MSCs of dental origin. Since stem cell cultures of dental origin exhibit mesenchymal stem cell characteristics (111, 114, 115), one of the most plausible directions for differentiation and potential utilization of these cells is the osteogenic one. Indeed, in response to appropriate pharmacological induction MSCs of human dental origin can be induced *in vitro* to differentiate into an osteogenic/odontogenic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules (111-116, 122). In this respect it is important to note that oral MSCs derive from neural crest cells, whereas bone marrow derived MSCs (BMSCs) originate from the mesoderm. Therefore, some differences in their behaviour are predictable (123, 124) as they may be more suitable for craniofacial regeneration, such as periodontal area reconstruction, than bone marrow originated MSCs.

IMMUNOMODULATION BY MESENCHYMAL STEM CELLS

Importantly, MSCs can escape immune recognition and inhibit immune response (125) (*Fig. 2*). Therefore, MSCs are excellent candidates as immunosuppressive agents during solid-organ transplantation and to treat graft-vs-host disease, inflammatory diseases, and other autoimmune diseases (126-128). MSCs exert their immunomodulatory function *via* several mechanisms and acting on various cell types of the immune system.

T-cell suppression by mesenchymal stem cells

One of the major mechanisms of immunosuppression by MSCs is the effect on T-cells. BMSCs inhibit the proliferation of T-cells in mixed lymphocyte cultures *in vitro* (20, 129-131). The inhibitory effect is independent of antigen presenting cells, major histocompatibility complex molecules and Treg cells (130). BMSCs suppress T-cells primarily *via* the secretion of soluble factors (132, 133) such as TGFβ (129, 134), hepatocyte growth factor (129), interleukin-10 (135) and prostaglandin E_2 (136). In addition, BMSCs inhibit T-cell proliferation by the secretion of indoleamine 2,3-dioxygenase (IDO), which is induced by IFN-γ (137, 138). The production of nitric oxide (NO) is also a potential mechanism by which BMSCs inhibit T-cell proliferation (139).

Induction of regulatory T cells by mesenchymal stem cells

MSCs also modulate immune responses through the induction of Treg cells. In mitogen-stimulated cultures of peripheral blood mononuclear cells, MSCs can induce the generation of CD4+ CD25+ T cells displaying a regulatory phenotype (136, 140-142). According to a recent study, the induction of CD4+ CD25+ FoxP3+ Treg cells by MSCs resulted in increased allograft tolerance in kidney transplant patients (143). Furthermore, MSCs prevent autoimmune B cell destruction and subsequent diabetes in NOD mice *via* the induction of IL-10 producing Treg cells (144) and induce Treg cells in a cell contact by a PGE_2 and TGF-β1 dependent manner (145). Additional studies suggest that the effects of MSCs in the treatment of autoimmune diseases and diabetes may rely on the induction of *de novo* generation of Treg cells (146-148), which then promote regerenation over inflammation.

B cell and natural killer (NK) cell regulation by mesenchymal stem cells

MSCs can block pathogenic B-cell response *in vivo* (149). When B cells and MSCs were cocultured *in vitro*, humoral factor(s) released by MSCs suppressed B-cell terminal differentiation (150) and proliferation (151). However, other investigators found that MSCs stimulate IgG secretion by B cells and support their proliferation (152, 153). Therefore, their effect remains controversial. Natural killer (NK) cells have been shown to contribute to inflammatory conditions such as inflammatory bowel disease (154) and periodontitis (155). BMSCs were reported to suppress proliferation, cytokine secretion and cytotoxicity of NK cells (136, 142, 156).

Modulation of antigen presenting cells by mesenchymal stem cells

BMSCs were shown to inhibit the differentiation of CD14+ human monocytes into dendritic cells and to revert mature DC into an immature state with decreased expression of antigen presentation molecules and costimulatory molecules and reduced IL-12 secretion *via* both cell-cell contact and the secretion of soluble factors (157) (158). BMSCs were reported to block monocyte differentiation into DCs by preventing their entry into the G1 phase of the cell cycle (159). The soluble factor secreted by BMSCs to suppress DC differentiation and maturation was shown to be PGE_2 (160). Furthermore, PGE_2 secreted by exogenously administered BMSCs was reported to reprogram macrophages to produce IL-10 in an experimental cecal ligation and puncture sepsis model (161). BMSCs were not only shown to inhibit DC differentiation and maturation, but also to alter the cytokine secretion profile of DCs and convert them into a regulatory phenotype (135, 136). In addition, macrophages co-cultured with MSCs acquired an anti-inflammatory and wound-healing phenotype and accelerated wound healing *in vivo* (162).

In summary, mesenchymal stem cells exhibit their immunomodulatory effect *via* several mechanisms: they suppress the proliferation and function of T cells, stimulate Treg cells, inhibit the differentiation of dendritic cells and convert DCs and macrophages into anti-inflammatory phenotypes (*Fig. 2*).

IMMUNOMODULATION BY MESENCHYMAL STEM CELLS OF DENTAL ORIGIN

MSCs of dental origin have also been shown to possess potent immunomodulatory functions comparable to those of BMSCs, which make them promising alternative cell sources for MSC-based therapies (163). Considerable evidence supports that mesenchymal stem cells of oral origin may be useful therapeutic agents for immunosuppression not only in oral inflammation but also in various other chronic inflammatory conditions (18).

T cell suppression by mesenchymal stem cells of dental origin

DPSCs are able to induce the apoptosis of already activated T cells *in vitro* (164). DPSCs were also shown to inhibit the proliferation of stimulated T cells, and this inhibitory effect was actually stronger than that of bone marrow derived MSCs (130, 165). The immunosuppressive activity of DPSCs is mediated most probably at least in part by soluble factors such as TGF-β (166). Toll-like receptors, which are widely distributed on immune cells, are able to trigger immunosuppression by DPSCs through the increased expression of TGF-β and IL-6 (167).

PDLSCs were also shown to suppress T cells (166, 168). In an animal experimental periodontitis model, PDLSCs cured the inflammation in part by suppressing the activation of T cells in a prostaglandin E_2-dependent manner (169). Another recent study has shown that PDLSCs from inflamed periodontal ligament can inhibit T cell proliferation, Th17 differentiation and IL-17 production (170). Th17 cell activity is also inhibited by stem cells from human exfoliated deciduous teeth (SHED), even more efficiently than by BMSCs, an effect that is sufficient to reverse systemic lupus erithematosus-associated disorders in a mouse model (171).

MSCs derived from human gingiva (GMSCs) also inhibit T cell proliferation. This effect is mediated by IFN-γ induced stimulation of IDO, IL-10, COX-2, and inducible nitric oxide synthase (iNOS) expression (172).

Treg induction by mesenchymal stem cells of dental origin

A recent study has shown induction of CD4+ CD25+ Foxp3+ regulatory T cells and suppression of Th17 differentiation by normal PDLSCs, and this capacity was present, even though to a lesser extent, in inflamed PDLSCs (170). Furthermore, MSCs derived from human gingiva (GMSCs) have been shown to ameliorate inflammation in experimental colitis by inducing a wide panel of anti-inflammatory factors and by increased infiltration of Treg cells to sites of inflammation (172).

In summary, MSCs of dental origin have been reported to exert immunomodulatory effects by a similar mechanism as MSCs of other sources (*Fig. 2*).

ENGINEERED MESENCHYMAL STEM CELLS IN THE TREATMENT OF PERIODONTITIS

Genetic modification enhances the therapeutic potential of MSCs (173). MSCs engineered to express IL-10 reduce the expression of inflammatory cytokines, prevent acute allograft rejection and promote survival after experimental orthotopic liver transplantation (174), exert local anti-inflammatory effects in an ear inflammation model (175), and prevent ischemia-reperfusion injury after experimental lung transplantation (176).

Since increased osteoclast activity is a key feature of periodontitis (23), the beneficial effects of MSCs on bone loss, exerted through their immunmodulatory properties, can be enhanced by directly influencing the OPG/RANKL balance *via* expressing osteoprotegerin in PDLSCs or DPSCs. To this end, using adenoviral transduction we expressed mouse osteoprotegerin (mOPG) in human periodontal ligament stem cells prepared as described before (112). Our data showed that PDLSCs could be engineered to express transgenic proteins by transduction with a recombinant serotype 5 adenovirus encoding either LacZ (AdLacZ) or mOPG (AdmOPG). We found that AdmOPG transduction of PDLSCs resulted in dose and time dependent expression of mOPG in cell culture supernatants (*Fig. 3B*). When we delivered PDLSCs transduced with AdmOPG systemically into rats by retro-orbital injection, we found that injection of AdmOPG-transduced but not of AdLacZ-transduced PDLSCs resulted in mOPG expression in rat sera above background levels (*Fig. 3C*). Thus, injection of PDLSCs engineered by adenoviral transduction can be used for systemic delivery of therapeutic proteins *in vivo*. These proof-of-concept experiments clearly show that human mesenchymal stem cells of periodontal ligament origin, after transduction by an adenoviral construct, are able to efficiently deliver therapeutic osteoprotegerin into experimental animals.

Taken together, mesenchymal stem cells from dental sources such as the dental pulp, the periodontal ligament and the gingiva, represent a high, but at present not fully explored potential for immunomodulation and tissue regeneration not only in periodontital diseases but also in other inflammatory disorders. Although this potential is well understood in some aspects, it is completely unknown in others (18, 19), indicating the need for more research to speed the path to clinical applications. Also, besides the immunomodulatory effects, this potential can be further advanced in the future by possible gene therapy interventions, either to target destructive osteoclast activity or to enhance regenerative osteoblast function.

MESENCHYMAL STEM CELLS OF DENTAL ORIGIN AS BUILDING BLOCKS FOR PERIODONTAL TISSUE REGENERATION

Mesenchymal stem cells from a number of sources have been suggested to have high potential for periodontal tissue regeneration (177). BMSCs were shown to induce periodontal regeneration of class III furcation defects in dogs (17, 178). GFP tracing revealed that transplanted BMSCs differentiated into cementoblasts, osteoblasts, osteocytes, and fibroblasts of the regenerated tissue. BMSCs induced periodontal regeneration in human subjects as well (179).

Adipose-derived mesenchymal stem cells

(ASCs) were also observed to be capable of osteogenic (180), as well as condrogenic and myogenic (181, 182) differentiation. ASCs mixed with platelet-rich plasma facilitated alveolar bone regeneration and the formation of a periodontal ligament-like structure in rat periodontal tissue defects (183).

Among the different cellular sources PDLSCs have been reported to be useful for the treatment of periodontal inflammation and bone loss due to their regenerative effects in an experimental porcine model of periodontitis (184). Periodontal lesions were created by the surgical removal of bone mesially to the first molars in miniature pigs (184). Autologous stem cells were isolated from the periodontal ligament of cuspids, expanded *ex vivo*, loaded on HA/TCP scaffolds, and transplanted into the bone defects. Twelve weeks later, bone regeneration was significantly improved and inflammatory cell infiltration was reduced in PDLSC-HA/TCP treated pigs compared to the control and HA/TCP only treated groups (184). By tracing transplanted PDLSCs using a retroviral vector encoding green fluorescent protein, it was shown that GFP-labeled cells were present in newly formed periodontal bones and differentiated into osteoblasts (184). Feng and coworkers also demonstrated the utility of transplanted autologous PDL progenitors, a cell population highly similar to PDLSCs, for periodontal regeneration in human patients (185). Similar results were obtained with human PDLSCs by another group in rats (186).

Most previous studies aimed to prepare stem cells of periodontal origin from normal human tissues (112, 114-116,

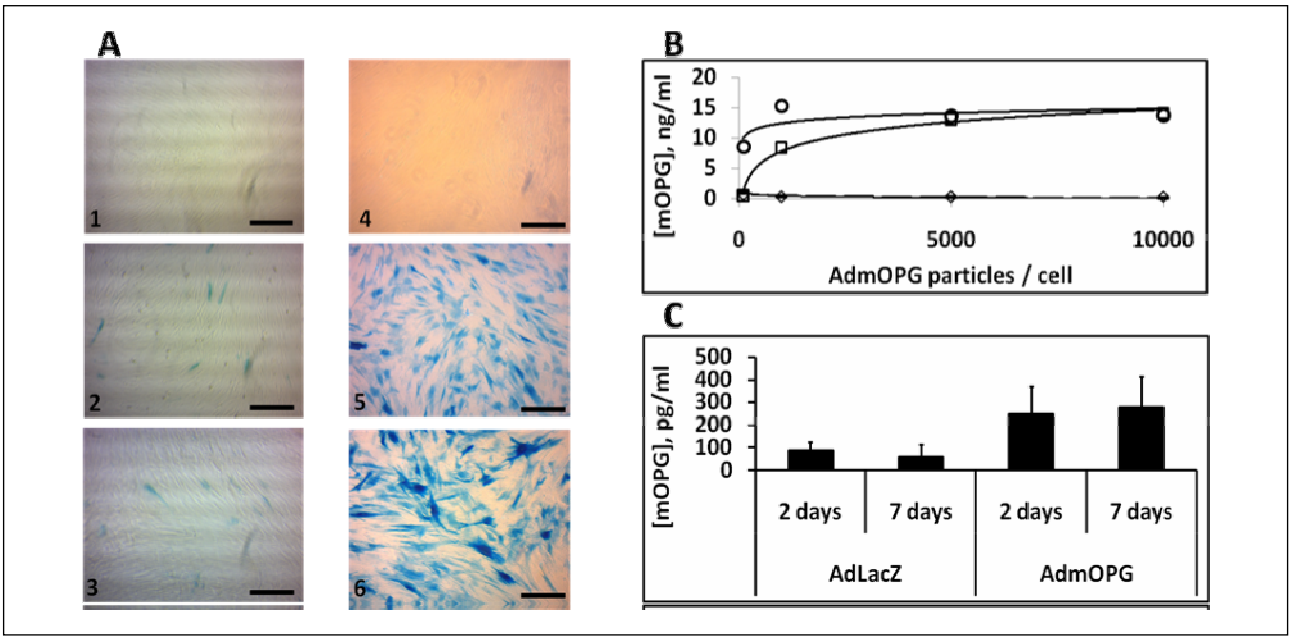

Fig. 3. Osteoprotegerin is effectively expressed in mesenchymal stem cells of dental origin,, the *in vivo* delivery of transduced cells elevates plasma osteoprotegerin level in rats. *Ex vivo* gene transfer of periodontal ligament stem cells (PDLSCs) using adenoviral vectors. (*A*) Demonstration of AdLacZ transduction of PDLSCs. Cells were transduced with AdLacZ at different viral particles (vp) per cell ratios (1–3: 100 vp/cell; 4–6: 5000 vp/cell) for various durations (1, 4: 0 day; 2, 5: 1 day; 3, 6: 4 days). Cells were stained for β-galactosidase. (*B*) mOPG production by PDLSCs after *in vitro* transduction. PDLSCs were transduced with either AdLacZ (symbols rhombus, and line, –) or AdmOPG (symbols square, and circle,) at the indicated vp/cell ratios and for the durations of 1 day (symbols rhombus, and square,) or 4 days (symbols line, – and circle,). mOPG concentrations determined by ELISA kit from supernatants. (*C*) PDLSC-mediated adenoviral gene therapy for systemic production of mOPG in rats *in vivo*. PDLSCs transduced *ex vivo* with either AdLacZ or AdmOPG at 5000 vp/cell, were injected retro-orbitally to rats (4.3×10^6 cells/animal). Sera were collected after 2 days and 7 days as indicated and mOPG concentrations were measured by ELISA. AdLacZ was purchased from the Vector Development Laboratory at the Baylor College of Medicine. AdmOPG was prepared at our laboratory.

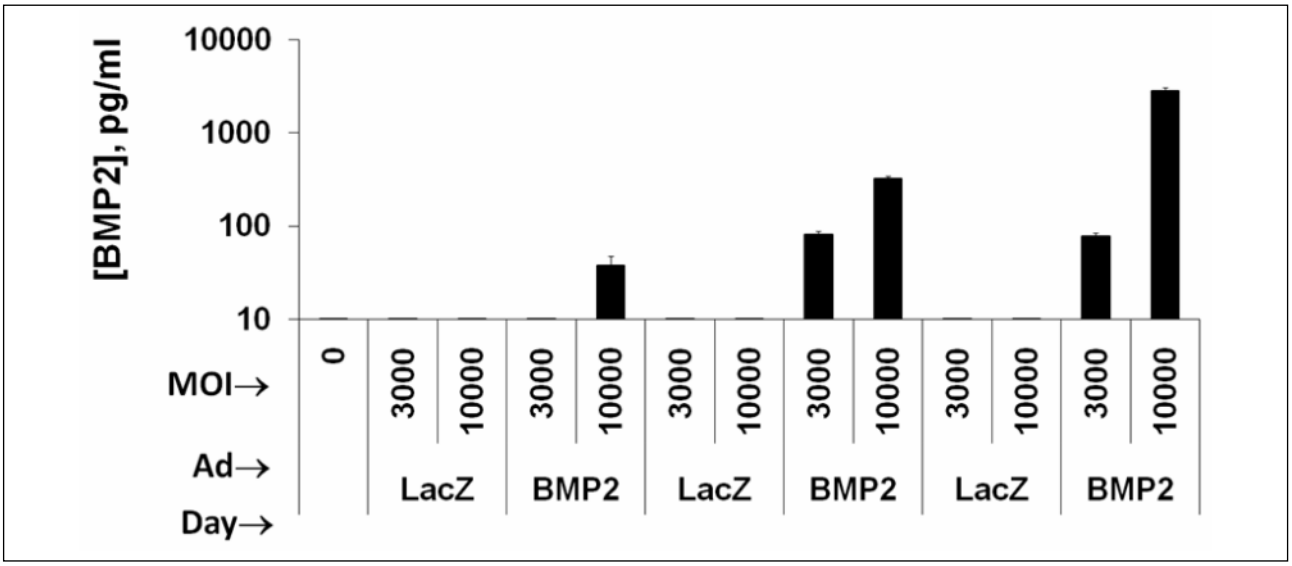

Fig. 4. Adenoviral infection of mesenchymal stem cells of dental origin is an efficient way to transduce BMP-2. The graph shows production of BMP-2 by dental pulp stem cells (DPSCs) after adenoviral transduction. DPSCs were transduced by either AdLacZ or AdBMP-2, at different vp/cell ratios (MOI) and for different durations as indicated, supernatant media were collected, and BMP-2 concentrations determined by ELISA kit. AdBMP-2 was a generous gift from Dr. Elizabeth A. Davis, Baylor College of Medicine.

122). However, a recent work clearly showed that PDLSCs may also be isolated from patients suffering from chronic periodontitis with a severe degree of inflammation. This work demonstrated that stem cells can be easily isolated from granulation tissue being extracted during minimally-invasive periodontal surgery (187). Their full capacity to differentiate into both osteogenic and neuronal lineages, just like PDLSCs isolated from uninflamed periodontium, was also shown (187).

When these PDLSC cells obtained from inflamed human periodontium were layered onto collagen sponges and implanted into periodontal defects on the right buccal cortex of the mandible of immunodeficient nude rats, accelerated reformation of periodontal ligament-like tissue, collagen fibers and elements of bone were found indicating partial but not full regeneration (188). These results are very similar to those obtained by using PDLSCs isolated from non-inflamed human periodontal tissue, then implanted into the experimentally created periodontal defects in rats (189) and in minipigs (184). Collectively, these data support the notion that periodontal progenitors isolated either from normal or from inflamed periodontium are able to promote periodontal wound healing. This is extremely important since in the future it might become possible to isolate periodontal stem cells from the inflamed area which needs treatment anyway, in order to cultivate, enrich and possibly genetically modify the cells for subsequent reimplantation into the inflamed area to achieve tissue regeneration.

In the context of tissue regeneration, it is of note that MSCs were first identified as a cell population in the bone marrow that can make bone and reconstitute a hematopoietic microenvironment (190) and they can give rise to, among other cell types, osteoblasts (121). Osteoblasts are the cells that produce new bone. They are important sources of RANKL and M-CSF for the development of osteoclasts (40). They also produce bone morphogenetic proteins, including BMP-2, which plays a major role in the differentiation of bone cell lineages, bone development and bone repair (39), and is approved for human clinical use in orthopedic surgery (16). Therefore, genetic engineering of MSCs to express BMP-2 should enhance their therapeutic effect in bone regeneration. Indeed, MSCs expressing transgenic BMP2 (or VEGF) accelerate bone healing when co-implanted into critical-sized bone defects (191). Furthermore, PDLSCs transduced with AdBMP-2 had better osteogenic potential than PDLSCs alone or treated with recombinant BMP-2 (192). In another study, MSCs transduced with AdBMP-2 mediated regeneration of alveolar bone defects better than MSCs alone (193). These results show that genetic engineering of MSCs, including those of dental origin, to express BMP-2 enhances their osteoblastogenic potential and thereby promotes bone regeneration.

Our own results also show that human MSCs of dental origin, namely DPSCs, can be engineered by adenoviral transduction to express BMP-2. We transduced DPSCs with either AdLacZ or AdBMP2 at 3000 or 10000 particles/cell and measured BMP-2 concentration in media supernatants after 1, 3 and 7 days. We found that AdBMP-2 but not AdLacZ transduction lead to BMP-2 production by human DPSCs in a dose and time dependent manner (*Fig. 4*). Importantly, transduction with either AdLacZ or AdBMP-2 had no substantial effect on the viability of DPSCs at either dose or time point. These proof-of-concept results are in line with the available literature data described above. These data further confirm that, in addition to unmodified mesenchymal stem cells utilized in the future as building blocks for periodontal regeneration, genetically engineered MSCs of dental or other origin may also be used. This will exploit the fact that they produce therapeutic proteins that will increase their efficacy of tissue reconstructing capacity.

CONCLUDING REMARKS

Conventional treatments including the removal of bacterial deposit, surgical tissue regeneration procedures and enhanced oral hygiene may not be satisfactory to prevent, to slow down or to stop periodontitis. Such interventions are usually insufficient to promote the regeneration of damaged structures. The use of mesenchymal stem cells to treat inflammatory diseases such as periodontitis is an emerging area of interest. Importantly, MSCs can be obtained from a variety of sources such as bone marrow, skin or teeth, and they can be expanded *ex vivo* to generate a sufficient number of cells. The therapeutic effects of allogeneic or autologous MSCs can be primarily attributed to their immunomodulatory effects. As it appears, their immunoregulatory function can be augmented by genetic modification. Genetically engineered mesenchymal stem cells offer multiple ways to target periodontal disease: in addition to the potent immunomodulatory effect, they may inhibit bone loss or promote bone regeneration, respectively. Thus, engineered mesenchymal stem cells from sources such as the dental pulp and the periodontal ligament represent a high, but at present not fully explored potential for immunomodulation and bone regeneration in periodontotal diseases.

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