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INFLAMMATORY MACROPHAGES ENHANCE THE REGENERATIVE PERFORMANCE OF MINERAL BONE SUBSTITUTE THROUGH SYNERGY WITH A BLOOD CLOT IN ECTOPIC OSTEOGENESIS MODEL

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Early inflammatory response plays a key role in initiating tissue repair, but its contribution to bone regeneration depends on the local microenvironment. The aim of this study was to evaluate the effect of inflammatory macrophages, alone or combined with a blood clot, on the regenerative performance of mineralized bone substitute (MBS) in a mouse subcutaneous implantation model of ectopic osteogenesis. Thioglycollate-induced peritoneal macrophages (TEPM) were included in MBS implants with either blood clot (MTB) or saline (MT), while MBS alone served as a control (M). Implants were histologically analyzed two and eight weeks after implantation. At two weeks, MTB implants showed increased cellular infiltration, improved vascularization, early connective tissue infiltration into MBS granules, and active phagocyte-mediated material remodeling compared with MT and M groups. These features were associated with the presence of osteoblast-like cells along the material surface. At eight weeks, MTB implants showed the most advanced tissue organization, with well-developed collagen, sustained vascularization, progressive granule degradation, and noticeable regenerative activity. In contrast, MT implants showed moderate regenerative activity, while M implants were predominantly characterized by fibrous tissue formation and limited osteogenic characteristics. The results indicate that inflammatory

macrophages drive a pro-regenerative response only when supported by a blood clot that provides a temporary matrix and endogenous signaling factors. This collaborative action of macrophages and the blood clot create a microenvironment that enhances angiogenesis, tissue maturation, and early osteogenesis. These findings support the concept that mimicking the early microenvironment after injury can enhance the regenerative performance of bone substitute materials, which can be applied to advance tissue engineering and lead to superior clinical outcomes.

Key words: macrophages, blood clot, bone substitute, ectopic osteogenesis, tissue regeneration

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INFLAMATORNI MAKROFAGI POBOLJŠAVAJU REGENERATIVNE PERFORMANSE MINERALNOG KOŠTANOG SUPSTITUENTA KROZ SINERGIJU SA KRVNIM UGRUŠKOM U MODELU EKTOPIČNe OSTEOGENEZE

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Rani inflamatorni odgovor igra ključnu ulogu u pokretanju reparacije tkiva, ali njegov doprinos regeneraciji kostiju zavisi od lokalnog mikrookruženja. Cilj ove studije je bio da se proceni efekat inflamatornih makrofaga, samih ili u kombinaciji sa krvnim ugruškom, na regenerativne performanse mineralizovanog koštanog substituenta (MBS) na mišjem modelu ektopične potkožne implantacije. Tioglikolatom-indukovani peritonealni makrofagi (TEPM) bili su uključeni u MBS implante sa krvnim ugruškom (MTB) ili fiziološkim rastvorom (MT), dok je sam MBS služio kao kontrola (M). Implanti su histološki analizirani dve i osam nedelja nakon implantacije. Nakon dve nedelje, u MTB implantima je uočena povećana ćelijska infiltracija, poboljšana vaskularizacija, rana infiltracija vezivnog tkiva u MBS granule i aktivno remodeliranje biomaterijala posredovano fagocitima, u poređenju sa MT i M grupama. Ove karakteristike su bile povezane sa prisustvom ćelija sličnih osteoblastima duž površine materijala. Nakon osam nedelja, MTB implantati su pokazali odličnu tkivnu organizaciju, sa dobro razvijenim kolagenom, razvijenom vaskularnom mrežom, progresivnom degradacijom granula i primetnom regenerativnom aktivnošću. Nasuprot tome, MT implantati su pokazali umerenu regenerativnu aktivnost, dok su M implantati pretežno

okarakterisani formiranjem fibroznog tkiva i ograničenim osteogenim potencijalom. Rezultati ukazuju da inflamatorni makrofagi pokreću proregenerativni odgovor samo kada su podržani krvnim ugruškom koji obezbeđuje privremenu matricu i endogene signalne faktore. Ovo zajedničko dejstvo makrofaga i ugruška stvara mikrookruženje koje poboljšava angiogenezu, sazrevanje tkiva i ranu osteogenezu. Naši rezultati podržavaju koncept da oponašanje ranog mikrookruženja nakon povrede može poboljšati regenerativne performanse koštanih substituenata, što se može primeniti za unapređenje inženjerstva tkiva i dovesti do pogodnih kliničkih ishoda.

Ključne reči: makrofagi, krvni ugrušak, koštani zamenik, ektopična osteogeneza, regeneracija tkiva

INTRODUCTION

The process of bone repair following trauma involves the coordinated activity of multiple cell types, cytokines, and growth factors. This complex and highly regulated process goes through three overlapping phases: inflammatory, reparative and remodeling phase (1-3). Among the early key orchestrators in tissue repair are macrophages, which play a central role during inflammatory phase and typically exhibit a proinflammatory phenotype at this stage. Their primary functions include the elimination of pathogens and damaged cells, as well as the initiation of the repair process through secretion of growth factors, chemokines, and matrix-remodeling enzymes that influence the behavior of surrounding cells. Through interaction with fibroblasts, endothelial cells, and stem/progenitor cells, macrophages regulate cell proliferation, angiogenesis, and the formation of new tissue. These activities create the conditions necessary for effective tissue repair and the subsequent transition toward healing and regeneration. A timely switch from an inflammatory to a reparative phenotype is therefore essential for successful tissue restoration (3,4). Importantly, these processes reflect general principles of wound healing and tissue regeneration, which in most tissues involve inflammation, provisional matrix formation, angiogenesis, and extracellular matrix remodeling (5).

Bleeding and subsequent formation of a blood clot (BC) represent an inevitable consequence of tissue injury, including bone damage. This creates a favorable microenvironment for the activation of repair-related cells, as the clot is rich in numerous cytokines, growth factors, and various cell populations, including monocytes, the precursors of macrophages (6).

Macrophage phenotype and function are strongly influenced by the surrounding microenvironment into which they are introduced (7). In this regard, the blood clot, an unavoidable component of bone injury, plays a crucial role in modulation of macrophage activity and establishing a proregenerative ambient. The clot serves as a provisional extracellular matrix rich in fibrin, platelets, cytokines, and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF). Together, these components promote macrophage recruitment, polarization, and survival, and also stimulate angiogenesis, a critical step for successful tissue repair (8-11).

Biomaterials are widely used in orthopedic surgery not only for reconstruction of large bone defects but also as osteoconductive scaffolds which serve as carriers for cells and bioactive

substances capable to accelerate the bone repair process (12-14). For biomaterials, structural and compositional similarity to the mineral component of native bone is a key requirement (15). The incorpotation of cells and bioactive molecules into biomaterial scaffolds creates a microenvironment that is crucial for efficient osteogenic process in bone tissue engineering (10,16-19).

Although the role of macrophages in bone healing has been widely investigated, the regenerative potential of inflammatory macrophages in combination with a blood clot and bone substitute materials as tissue engineering construct remains insufficiently understood. The aim of this study was to evaluate the effect of inflammatory macrophages on induction of ectopic osteogenesis in mineral bone substitute implants and to determine how the presence of a blood clot modulates their regenerative potential, which is important for tissue healing process.

MATERIALS AND METHODS

Mineral Bone Substitute (MBS)

The bone substitute material used in this study was Bio-Oss® (Geistlich Pharma, Wolhusen, Switzerland), a biocompatible, deproteinized bovine mineral matrix whose architecture closely resembles the hydroxyapatite component of human bone (20).

Experimental Animals

Male BALB/c mice (weighing 22–24 g; 10–12 weeks of age) obtained from the Military Medical Academy (Belgrade, Serbia), and kept in the Vivarium of the Faculty of Medicine, University of Niš, where experiments were performed. They were housed under standard laboratory conditions with free access to food and water. Animal procedures were performed in accordance with the Animal Welfare Act of the Republic of Serbia and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123).

Experimental procedures on animals were approved by Ethical Committee of the Faculty of Medicine, University of Niš (Approval No. 01-5518-6) and also following the favorable Opinion of the Ethics Committee for the Protection of the Welfare of Experimental Animals of the Faculty

of Medicine, University of Niš, approval was issued by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (decision number 323–07-00073/2017–05/3 from 06.01.2017).

Thioglycollate-elicited Peritoneal Macrophages

Thioglycollate-elicited peritoneal macrophages (TEPMs) were harvested by lavage of the mouse peritoneal cavity with RPMI-1640 medium (PAA Laboratories, Pasching, Austria) supplemented with heparin (Galenika, Belgrade, Serbia), four days after intraperitoneal injection of 3% thioglycollate (Fluka, Munich, Germany), as previously described (21). After centrifugation (1.200 rpm for 10 min at 4°C), the cells were resuspended in heparin-free RPMI-1640 medium (PAA Laboratories, Pasching, Austria).

Blood Collection

Blood was collected from the retro-orbital plexus of mice and immediately diluted either with TEPM suspension or with saline solution at a ratio of 1:4, as previously described (21).

Implant Preparation

Three types of implants were prepared using mineral bone substitute (MBS): MBS alone, MBS combined with thioglycollate-elicited peritoneal macrophages (TEPMs) suspension, and MBS combined with both TEPMs and blood clot (BC), as described below.

1) Group MTB: 10 mg MBS + 30 µL TEPMs suspension with blood

2) Group MT: 10 mg MBS + 30 µL TEPMs suspension with saline

3) Group M: 10 mg MBS + 30 µL saline

For the MTB implants, blood was mixed with a suspension of TEPMs at a ratio of 1:4 forming a blood clot (BC) through a clump of MBS granules. For the MT implants, the TEPMs suspension was mixed with saline solution using the same ratio. Implants with macrophages (MTB and MT) contained 2.5×10^5 TEPMs per implants.

Implantation procedure

For the implantation procedure, 24 BALB/c mice were randomly assigned to three experimental groups, each comprising six animals. Implantations were carried out following the induction of general anesthesia via intraperitoneal administration of ketamine, in accordance with guidelines for mouse anesthesia. The implants were placed into subcutaneous interscapular pockets using a biopsy needle. Each animal received four implants of the same type. Explants were harvested two and eight weeks after implantation.

Histology

After routine fixation, histological processing, and sectioning, explanted specimens were stained by Azan trichrome (AT) staining. Microscopic evaluation and image acquisition were performed on a Leica DMLS light microscope (Leica Microsystems, Germany) equipped with AxioCam 105 color digital camera and Zen 2 Blue software (Carl Zeiss, Oberkochen, Germany).

RESULTS

Histological observation two weeks after implantion

Two weeks after implantation, MTB implants (Figs. 1a, 1b) showed advanced granule degradation. Multinucleated phagocytes were frequently observed on the MBS granule surfaces, together with numerous mononucleated cells. Intergranular tissue was highly cellular and vascularized, containing developing collagen fibers. In several areas, connective tissue penetrated into the granule structure. Early osteogenic activity was indicated by the presence of osteoblast-like cells lining the MBS surfaces.

MT implants (Figs. 1c, 1d) exhibited lower MBS granule degradation and less pronounced phagocytic activity. The connective tissue surrounding MBS granules contained fewer cells, weakly developed collagen fibers, and only occasional blood vessels compared with the MTB implants. Osteoblast-like cells were present along the material surface, but less frequently compared to MTB implants.

In M implants (Figs. 1e, 1f) intergranular connective tissue was relatively rich in cells, comparable to that observed in the MTB implants. Collagen fibers were present but poorly organized. Multinucleated phagocytes and blood vessels were observed more frequently than in MT implants. Flattened osteoblast-like cells were occasionely seen along the surfaces of MBS particles.

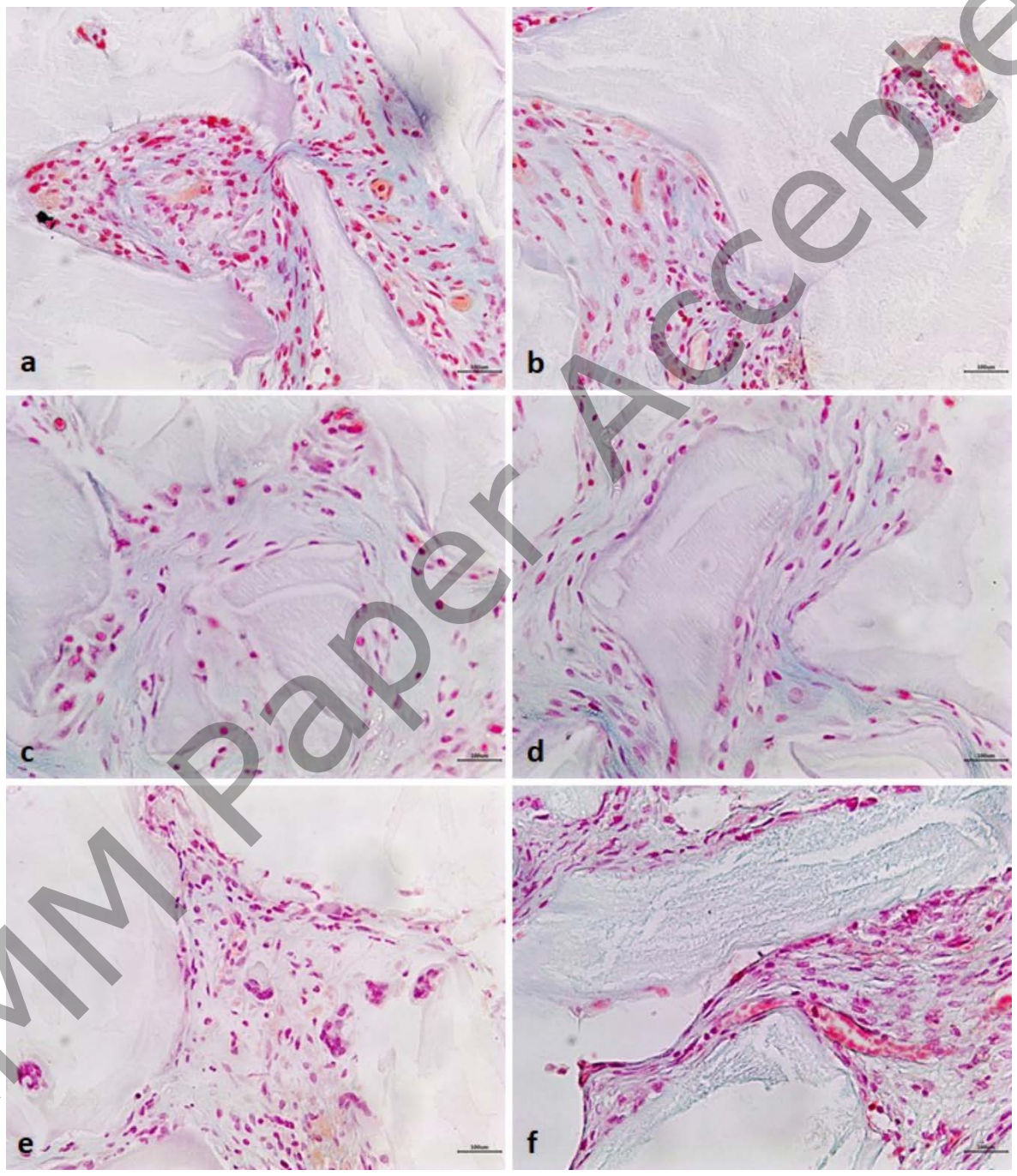

Figure 1. *Histological evaluation two weeks after implantation. MTB (a, b), MT (c, d), M (e, f). Azan trichrome staining, brightfield, objective magnification×20*

Histological observation eight weeks after implantion

Eight weeks after implantation, MTB implants (Figs. 2a, 2b) demonstrated the most pronounced fragmentation of MBS granules, with ongoing but reduced phagocytic activity. The intergranular tissue was well organized, showing increased collagen deposition, moderate cellularity, and the presence of large blood vessels.

In MT implants (Figs. 2c, 2d), granule fragmentation was more pronounced compared to the previous time point, but intergranular tissue remained poorly cellular, with weak collagen organization. The number of blood vessels was higher than at the two-week time point, although the overall tissue organization was less advanced compared to MTB implants.

M implants (Figs. 2e, 2f) were characterized by dense collagen-rich connective tissue with reduced cellularity. Phagocytic activity was stil evident, and vascularization appeared to be intermediate between MTB and MT implants. However, osteogenic features were limited, and the tissue organization suggested a predominantly fibrous healing response.

Overall, the MTB group exhibited the most favorable tissue response, with increased cellularity, vascularization, progressive material degradation, and early osteogenic process development. The MT group showed moderate regenerative activity, whereas the M group was characterized predominantly by fibrous tissue formation and limited osteogenic features.

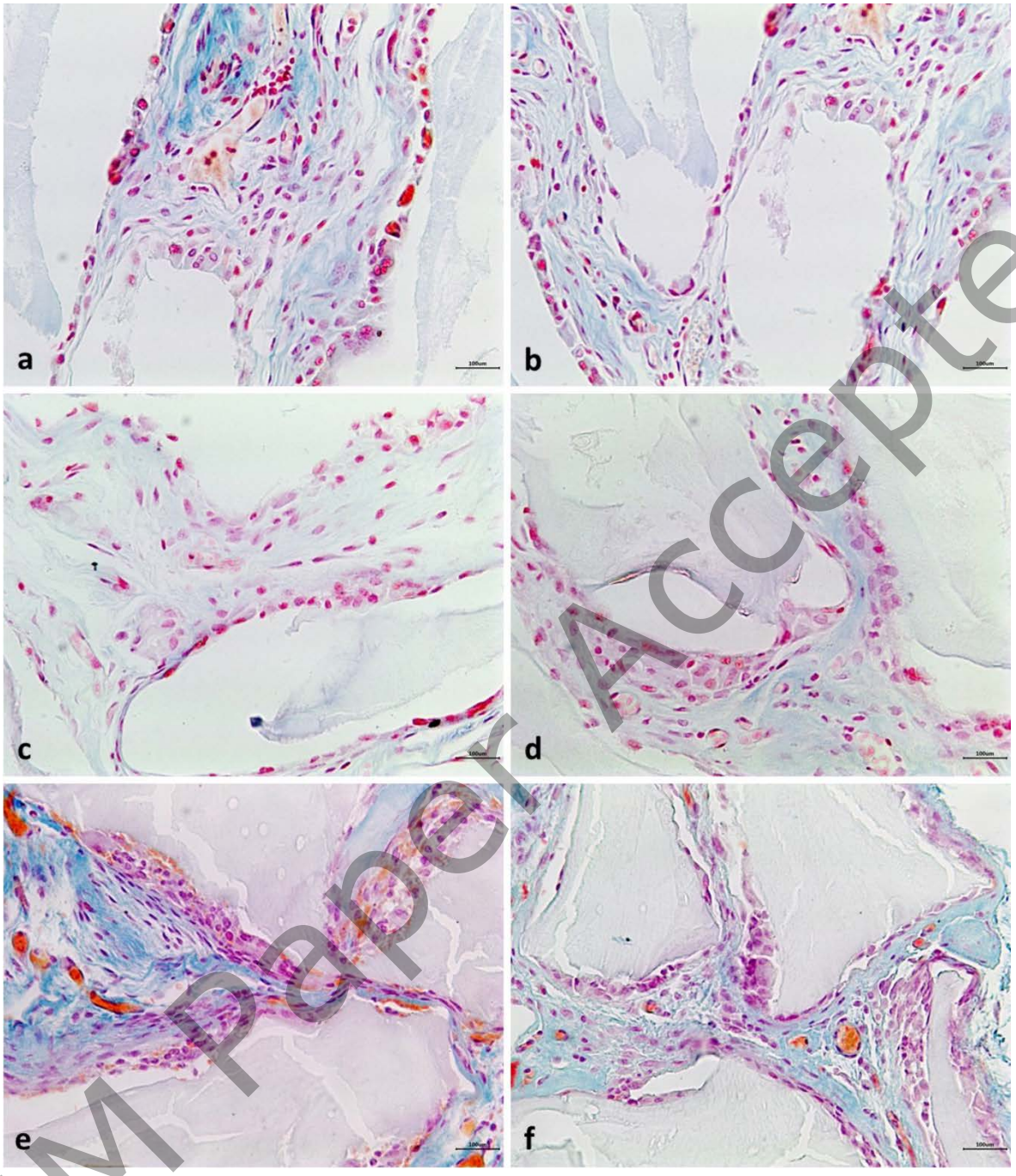

Figure 2. *Histological evaluation eight weeks after implantation. MTB (a, b), MT (c, d), M (e, f). Azan trichrome staining, brieghtfield, objective magnification ×20.*

DISCUSSION

Inflammatory responses, often associated with pathology and tissue demage, also play a crucial role in tissue healing. Inflammation is now recognized as an active and tightly regulated process driven by immune cell-derived mediators that support repair and regeneration. Among immune cells, macrophages represent key regulators of this process. Through their dynamic functional states, macrophages coordinate the transition from inflammation to tissue repair, whereas their dysregulation contributes to chronic inflammatory conditions (22).

Following bone injury, blood clot formation represents a critical early event that limits blood loss and provides a provisional extracellular matrix rich in cells and signaling factors that orchestrate subsequent repair and remodeling (23). Platelet-derived mediators create an initially proinflammatory environment that recruit neutrophils and monocytes, enabling debris and pathogens clearance, while simultaneously establishing conditions favorable for bone regeneration (24). Based on these principles, the present experimental design was intended to mimic the early post-injury bone environment. Mineral bone substitute (MBS) simulated the bone tissue, while thioglycollate-elicited peritoneal macrophages (TEPMs) represented inflammatory macrophages (21). The addition of a blood clot (BC) simulated the early cellular and inflammatory milieu present after tissue injury. Ectopic bone formation was evaluated in these engineered implants using a mouse subcutaneous implantation model, comparing them with implants lacking the BC and those composed only of MBS.

Our results demonstrated that MTB implants containing mineral bone substitue (MBS), macrophages, and a blood clot (BC), exhibited the most favorable regenerative response compared to implants without a clot (MT) or composed of biomaterial alone (M) at both time points. This was reflected by increased cellularity of the intergranular tissue, enhanced vascularization, progressive but controlled granule degradation mediated by multinucleated phagocytes, and the presence of well-organized collagen-rich connective tissue (21, 25). Likewise, two weeks after implantation, cell-rich connective tissue penetrated MBS granules in MTB implants, while mononuclear cells arranged on granules' surfaces, resembling intramembranous bone formation (26).

The pronounced phagocytic activity observed in MTB implants, especially two weeks after implantation, can be attributed to the added macrophages, but also to monocytes from the BC

that can gradually differentiate into macrophages, as well as other phagocytic cells depending on the microenvironmental conditions (27, 28). In contrast, implants containing macrophages without blood clot (MT implants) showed weaker phagocytic activity, not only in comparison to MTB implants, but also M ones which contained only MBS. This finding suggests that the presence of inflammatory macrophages in the absence of a supportive inflammatory milieu may limit further immune cell recruitment and promote premature inflammatory resolution. In M implants, stronger phagocytosis could be a consequence of the organism's classic defense response to a foreign body gradually developing over the time (29). Likewise, because MBS particles alone do not have sufficient adhesive capacity to form a stable mass, their separation may trigger a local inflammatory response (30). The presence of already differentiated inflammatory macrophages in MT implants but without a supportive inflammatory milieu, limits additional phagocyte recruitment and has inflammatory resolution as consequence (31). These differences suggest that blood clot provides definitive support to the inflammatory process (32), whose properly regulated duration is of critical importance for optimal osteoreparative healing.

Prominent phagocytosis in MTB implants may be partially responsible for pronounced but regulated angiogenesis, because degradation of the blood clot creates a prolonged and spatially organized angiogenic signaling. Namely, blood clot is a natural proangiogenic matrix composed of a fibrin network, which is a scaffold for endothelial cells, and growth factors such as PDGF, TGF-β, VEGF and fibroblast growth factor (FGF) (8) could be released by clot breakdown. On the other hand, macrophages by themselves have the ability to produce proangiogenic signals, as well as enzymes essential for the formation of spaces that will be inhabited by endothelial cells (21,25,33). Adequate vascularization is a key prerequisite for successful osteogenesis, because cells, cytokines, growth factors, and nutrients reach the implant through blood vessels (10, 11). A more pronounced process of angiogenesis in M implants could be a consequence of a strong acute inflammatory response (34, 35), but such vascularization is often characterized by instability (36). Weaker angiogenesis in MT implants could be explained as an outcome of the fact that the inflammatory response is already partially taken care of, without the appropriate maintenance support that exists in MTB implants.

The presence of well-developed collagen in combination with high cellularity of intergranular tissue is an important indicator of a favorable osteogenic process in MTB implants, because, among other things, it can indirectly indicate active tissue maturation and progression

toward osteogenic differentiation (21, 25). On the other hand, weaker collagen organization and reduced cellularity in MT implants may indicate a delay in the osteogenic process. In contrast, dense collagen deposition with low cellularity in M implants may be attributed to an intense inflammatory response, likely reflecting a predominantly fibrotic rather than osteogenic outcome (37).

Taken together, our results indicate that inflammatory macrophages, especially when combined with a blood clot, enhance the regenerative response to mineral bone substitutes by promoting a controlled inflammatory environment associated with angiogenesis, material remodeling, and early osteogenesis. These findings support the concept of macrophage-mediated osteoimmunomodulation as a potential strategy for improving bone regeneration.

Importantly, the mechanisms observed in the present study reflect general principles of wound healing and tissue regeneration. In most tissues, successful repair depends on a precisely regulated early inflammatory response, provisional matrix formation, angiogenesis, and subsequent tissue remodeling. The macrophage–clot interaction identified here represents a biological microenvironment that mimics these early post-injury events and may therefore be relevant not only for bone regeneration but also for broader applications in regenerative medicine.

Conclusion

Our results indicate that ectopic osteogenesis is influenced by the interplay between inflammatory macrophages, the biomaterial, and the presence of a blood clot. The most pronounced regenerative response was observed when inflammatory macrophages and a blood clot were combined in imlants. This combination created conditions that supported cell infiltration, blood vessel formation, collagen deposition, and gradual biomaterial degradation, all of which are essential for new bone formation. In contrast, the presence of inflammatory macrophages alone was insufficient to fully support ectopic osteogenesis.

The blood clot appears to play a critical role as a provisional biological matrix that provides structural support and represents a source of endogenous signaling molecules. Such an environment likely contributes to the maintenance of macrophage activity and facilitates the recruitment of additional cells involved in tissue healing. When this supportive environment was missing, bone formation was delayed and less organized.

These findings further emphasize successful bone repair and regeneration depends on a properly regulated inflammatory response. Rather than being detrimental, early inflammation, when spatially and temporally controlled, can promote angiogenesis, matrix remodeling, and osteogenic differentiation. Mimicking the natural post-injury environment by combining biomaterials, immune cells, and physiological signaling components, such as blood clot, is a promising osteoimmunomodulatory strategy. This approach enhances the regenerative performance of bone substitutes, advances tissue engineering, and leads to superior clinical outcomes.

Also, these findings reflect general principles of wound healing, where controlled early inflammation, provisional matrix formation, and angiogenesis are essential for successful tissue regeneration.

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