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Interdependencies and interfaces in bone regeneration - the immune system at its core

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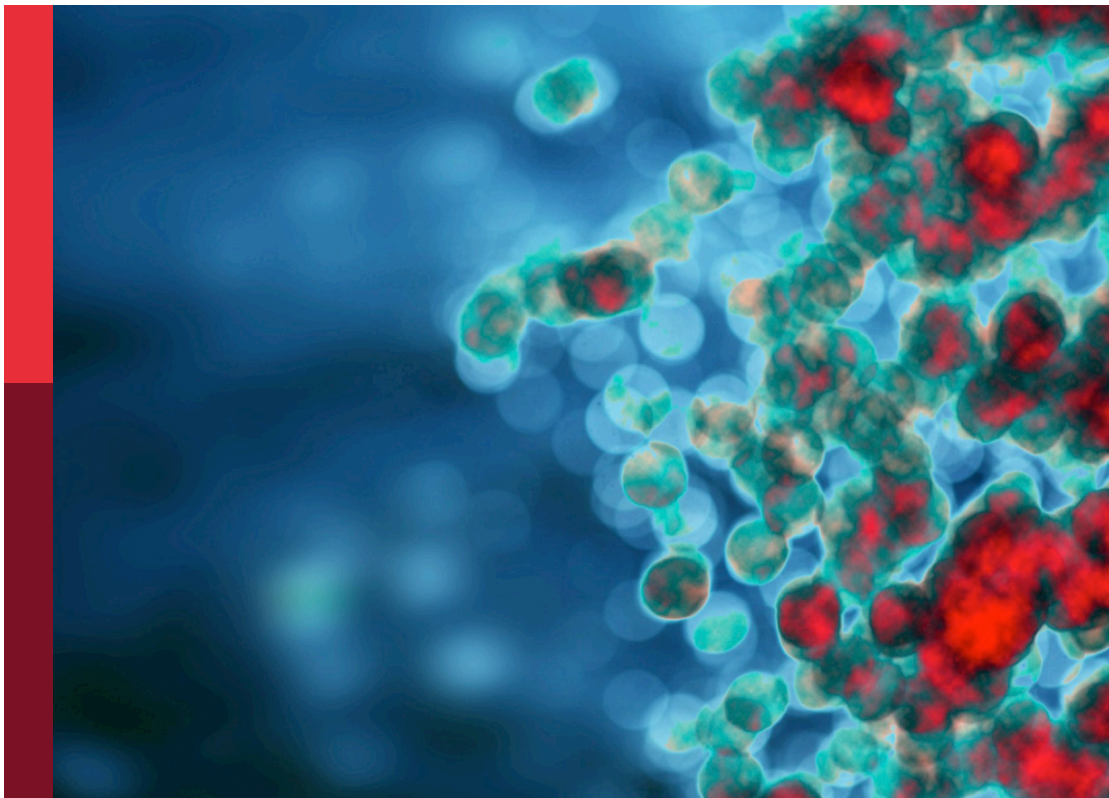

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Interdependencies and interfaces in bone regeneration - the immune system at its core

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Editorial: Interdependencies and interfaces in bone regeneration – the immune status at its core

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bone regeneration, immune reaction, inflammatory phase, bone healing, immune cells, interfaces

Editorial on the Research Topic
Interdependencies and interfaces in bone regeneration - the immune system at its core

Incentive

Regeneration stands as the optimal outcome following an injury. However, leveraging endogenous regenerative mechanisms for therapeutic purposes, necessitates a profound understanding of the underlying processes. Researchers utilize bone as a model for regeneration, aiming to elucidate the interdependencies and interfaces within the regenerative process. Bone healing is a highly complex process that is tightly orchestrated and the immune reaction evolves as a crucial control system herein (1, 2). Distinct phases, each with unique characteristics, succeed one another, overlapping and dependent on each other, resulting in complete form and functional restoration upon successful accomplishment. However, the complexity of this intricate process exposes it to potential derailments, leading to unsatisfactory outcomes. Bone healing processes can be affected by underlying genetic, metabolic, traumatic and neoplastic conditions, all of which are interdependent with immune cell functions.

Recent findings highlight the pivotal role played by the interaction between the inflammatory response and its surrounding mechanical environment (3), metabolism (4), and revascularization (5) in the facilitating successful regenerative processes. This Research Topic provided an opportunity to compile papers exploring the regenerative process, including the healing environment beyond the scope of bone cells. Nine papers were selected from 24 submitted, comprising four original papers, four review articles, and one perspective paper. The contributions of 55 authors from around the world, including Belgium, Chile, China, Germany, New Zealand, Sweden, the United Kingdom, and the United States, are included in this Research Topic.

Research

The articles in this Research Topic focus on a current research trend: the utilization of computational capabilities. To aid histological analysis, in silico models are being created. E. Borgiani et al. introduced COMBINI, an in silico method that allows for simulation of the early inflammatory reaction during bone healing at tissue, cell and molecular levels. The model's output has been verified against experimental ex vivo immunofluorescent images. This innovative tool holds significant potential for exploring the mechano-biological interdependencies in the process of regenerative bone healing. Haffner-Luntzer et al. focused on altered metabolism and neuro-endocrine regulation during bone formation, emphasizing the importance of the early inflammatory response for a successful healing. They examined the impact of a concurrent brain and bone injury, particularly investigating mast cells and their involvement in osteoclastogenesis. Yang et al. utilised a bibliometric analytical approach to provide an overview of the research field concerning the interdependence of macrophages and osteoarthritis over the past 30 years. Meanwhile, Wang et al. emphasised the altered inflammatory pathways during the aging process. While inflammatory pathways are still active with progressive aging (and indeed may be overactive), signals that promote bone formation decrease. Wang et al. undertake an expression analysis harnessing several online tools and were able to thus identify a total of nine potential drugs to prevent age-related bone loss.

The original research articles emphasize the significance of the initial inflammatory healing stage, highlighting the availability of new analytical tools, due to recent advancements in computing technology. Additionally, the articles shed light on the interdependence between inflammation and biomechanics, inflammation and metabolic and endocrine signalling, inflammation and age-related bone loss and inflammation and degenerative diseases such as osteoarthritis.

Review

Reviews in this Research Topic underscore the interdependence of the immune response in musculoskeletal conditions, further highlighting the close link between the immune system and bone homeostasis, along with the pivotal role of the immune system in pathological musculoskeletal conditions. Capobianco et al., provide an overview of approaches studying inflammatory cells in fracture healing, thereby summarizing the current knowledge of the immune-stromal crosstalk including identifying gaps that still need investigating. Zheng et al. reviewed osteoimmunology focusing on chronic inflammation and detail the pathophysiological mechanism of osteonecrosis. Altered osteoimmune functions, e.g. due to glucocorticoids or alcohol, affect bone metabolic homeostasis causing osteonecrosis. The authors propose new treatment ideas based on this literature review. Albrektsson et al. investigated the impact of osteoimmunomodulation by endosseous implants. In this context, the implant triggers a foreign body response that affects osseointegration, which can either enable or derail ingrowth, leading to peri-implant bone loss.

In the fourth review, Ren et al. introduced myeloid-derived suppressor cells (MDSCs), immature cells derived from myeloid that exhibit immunosuppressive functions. In chronic inflammation, these cells aim to counterbalance the overactive immune system. Displaying the versatility of the immune system, these cells can also differentiate into osteoclasts, further affecting bone metabolism. These reviews place the immune response at the centre of bone homeostasis in heathy and chronic inflammatory environments, proposing new therapeutic approaches to prevent bone loss in specific patient situations.

Perspective

In the context of the research theme, a perspective article proposed a speculative hypothesis, suggesting that cell-free DNA and its activation of the innate immune response might substantially contribute to postoperative bone loss following alveolar bone grafting (Huang et al.). While cell-free DNA has been studied in the context of periodontitis, the authors speculate on its broader role in bone loss by activating the innate immune response, triggering NF-kB activation, and increased TNFa (tumor necrosis factor alpha) expression. TNFa serves as a marker cytokine for pro-inflammatory processes. Cell-free DNA includes endogenous nuclear and mitochondrial DNA, along with exogenous bacterial or viral DNA, representing a DAMP (danger-associated molecular pattern) that would be highly present in an injury situation.

Conclusion

This Research Topic highlights the significance of the inflammatory response, particularly the initial reaction, in relation to bone formation. Furthermore, it emphasizes the interdependence and interaction of factors such as mechanics, endocrine signalling, degenerative co-morbidities, chronic inflammation, ageing, and osteoimmunology. The CRC 1444 "Directed Cellular Self-Organisation to Advance Bone Regeneration" clarifies interdependencies and expands on the research that has been initiated within this Research Topic.

Author contributions

TK: Writing – original draft, Writing – review & editing. KH: Writing – original draft, Writing – review & editing. BW: Writing – original draft, Writing – review & editing. KS-B: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bibliometric and visualization analysis of macrophages associated with osteoarthritis from 1991 to 2021

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Background: Macrophages significantly contributes to symptomology and structural progression of osteoarthritis (OA) and raise increasing attention in the relative research field. Recent studies have shown that tremendous progress has been made in the research of macrophages associated with osteoarthritis. However, a comprehensive bibliometric analysis is lacking in this research field. This study aimed to introduce the research status as well as hotspots and explore the field of macrophages research in OA from a bibliometric perspective.

Methods: This study collected 1481 records of macrophages associated with osteoarthritis from 1991 to 2021 in the web of science core collection (WoSCC) database. CiteSpace, VOSviewer, and R package "bibliometrix" software were used to analyze regions, institutions, journals, authors, and keywords to predict the latest trends in macrophages associated with osteoarthritis research.

Results: The number of publications related to macrophages associated with osteoarthritis is increasing annually. China and the USA, contributing more than 44% of publications, were the main drivers for research in this field. League of European Research Universities was the most active institution and contributed the most publications. *Arthritis and Rheumatism* is the most popular journal in this field with the largest publications, while *Osteoarthritis and Cartilage* is the most co-cited journal. Koch AE was the most prolific writer, while Bondeson J was the most commonly co-cited author. "Rheumatology", "Orthopedics", and "Immunology" were the most widely well-represented research areas of OA associated macrophages. "Rheumatoid arthritis research", "clinical symptoms", "regeneration research", "mechanism research", "pathological features", and "surgery research" are the primary keywords clusters in this field.

Conclusion: This is the first bibliometric study comprehensively mapped out the knowledge structure and development trends in the research field of

macrophages associated with osteoarthritis in recent 30 years. The results comprehensively summarize and identify the research frontiers which will provide a reference for scholars studying macrophages associated with osteoarthritis.

KEYWORDS

osteoarthritis, macrophages, bibliometric, CiteSpace, VOSviewer

Introduction

Osteoarthritis (OA) remains the most common form of arthritic disease which affects the whole joint. By 2030, there would be 35% of people in the general population suffering from OA, and it is predicted to be the single greatest cause of disability (1). In the USA, over 27 million OA patients are estimated to suffer from this disease, and caused tremendous social and economic burdens (2). It is now accepted that some risk factors such as genetic predisposition, obesity, aging, and joint trauma plays a major role in OA development (3). Despite improved pain alleviation through the development of treatment therapies, the joint function restoration and damaged cartilage repair for OA patients is still lacking promising advances (4). Recently, OA has been defined as a low-degrade inflammatory disease that involving cartilage loss, synovitis, subchondral bone remodeling, osteophyte formation and meniscus and ligament changes (5). Therefore, it is urgent to elucidate the pathophysiological basis of inflammation and tissue damage repair processes of OA to benefit the advances of prognosis and therapeutics of OA diseases.

In recent years, the role of macrophage-mediated inflammation in the pathogenesis of OA has gained wide attention. Currently, the role of synovial inflammation in the OA progression still remains to be determined. It has been demonstrated that multiple factors act as danger-associated molecular patterns (DAMPs) that result in macrophage activation can initiate synovial inflammation during OA. One possible theory is that, exogenous pathogen-associated molecular patterns (PAMPs) and endogenous DAMPs selectively activate surface pattern recognition receptors (PRRs) on macrophages, subsequently induce inflammatory cytokines and chemokines secretion (6). Another primary activation way refers to inflammasome mediated pathways, such as the NLR pyrin domain containing 3 (NLRP3) inflammasome. NLRP3, belongs to a member of NLR family, was proved to recognize different DAMPs to form NLRP3 inflammasome in the cytosol and initiate inflammations (7). As such, macrophages could serve as a possible treatment target in OA. For example, the clearance of macrophages by anti-CD14-conjugated magnetic beads successfully reduce production of IL-1 and TNF-α (8). Moreover, as a kind of plastic cells, macrophages are classified as classically activated M1 and alternatively activated M2 macrophages (9). The macrophage subtypes can be generated *in vitro*, as interferon (IFN)-γ/lipopolysaccharide (LPS) can induce M1 subtype formation while M2 macrophages can be generated by exposing M0 macrophages to interleukin (IL)-4/IL-13 (10, 11). Compared to pro-inflammatory M1 macrophages, M2 macrophages are known as immunomodulatory macrophages and contribute to tissue repair and regeneration (12, 13). This information indicates the significance of regulating macrophage polarization in alleviating OA progression. For instance, a canine OA model treated with intra-articular injections of recombinant human IL-1ra which refer to M2 marker presented an reduction of osteophytes formation and cartilage loss (14). However, the imbalance between M1 and M2 macrophages requires further investigations and new advances of macrophage reprogramming may yield significance for prevent OA. Despite the increasing interests on the topic of OA associated macrophages, comprehensive and meaningful analysis of publication trends of this research area remains highly insufficient and requires to be summarized urgently.

Recently, bibliometric analysis has been widely adopted to analyze massive scientific research data and identify developing trends (15). Importantly, it can summarize publication evolution, predict research hotspots, and further evaluate frontiers in specific fields though a citation network (16–18). As far as we know, although related academic researchers have published bibliometric studies of stem cells in OA (19), no similar analysis about macrophage in OA have as yet been reported. Notably, several bibliometric tools such as CiteSpace, VOSviewer, R package "bibliometrix" have been applied to visualize the specific medical literature analysis fields (20–22). Therefore, in the present study, we used bibliometric statistics to fill this knowledge gap. This paper comprehensively analyzed the literatures related to OA associated macrophages and performed visualization analysis over the last three decades (from 1991 to 2021) to identify its significant features and predict future research directions.

Materials and methods

Data source and search strategy

Web of science core collection (WoSCC) database originating from Clarivate Analytics was considered one of the most authoritative and comprehensive database platforms which contains more than 12000 international academic journals (23). Therefore, we selected it to obtain global academic information for bibliometric analysis according to previous studies (24–26). All the published literatures were extracted from WOS and the date of the search were from 1 January 1991 to 31 December 2021. In present study, the search terms were as follows: theme = osteoarthritis or degenerative arthritis *AND theme = macrophage or macrophages or histocyte or histocytes AND publishing year = (1991–2021) AND Document types = (ARTICLE OR REVIEW) AND Language = (English). The detailed information of certain countries of regions in the WoSCC was refined by indexing country/region when search. Additionally, all valid data of literatures, including publishing year, title, author names, nationalities, affiliations, abstract, keywords, and name of journals were saved in the format of download.txt files from WoSCC database and subsequently imported into Excel 2021. Coauthors (YZ and LJJ) independently searched and extracted all data from these literatures. Any disagreement was resolved by consulting with experts to reach the final consensus. Finally, all the coauthors separately cleaned and analyzed the data with Origin 2021 and GraphPad Prism 8.

Bibliometric analysis and visualization

As we know, the intrinsic function of WoSCC was to explore the basic features of eligible literatures. Therefore, the number of literatures and corresponding citations were reflected. The relative research interest (RRI) was deemed as the number of publications in a certain field by all field literatures per year. The world map was acquired by R software including python + numpy + scipy + matplotlib. The time curve of publications was drawn according to previous article (19). The H-index, which refers to a scholar who has published H papers and they have been cited at least H times, was defined to measure the impact of scientific research (27). We chose the VOSviewer (Leiden University, Leiden, The Netherlands) software to construct and visualize bibliometric networks of the publications in our present study. And the VOSviewer was performed for analyzing the bibliographic coupling, co-citation, and co-occurrence analyses in detail. In addition, we choose R package "bibliometrix" software to visualize publications production among countries, map the international collaboration between countries, and visualize a three-field plot analysis. Moreover, CiteSpace (6.1. R2) which was developed by Professor Chen C, was used to construct dual-map overlay for journals, cluster analysis of co-cited keywords, and detection of references and keywords with intense citation bursts.

Results

Overall performance of global literatures

According to the search criteria, a total of 1556 literatures were collected from the year of 1991 to 2021. Subsequently, 1489 of literatures were identified by excluding the meeting abstract (20), proceedings papers (3), correction book chapter (3), and retracted publication (1). Finally, 1481 literatures were identified by excluding 8 non-English literatures (Figure 1). As shown in Figure 2A, the trend of global literatures was increasing steadily year by year. The number of literatures increased from 10 (1991) to 161 (2021). The most research was published in 2021 (161, 11.14%) (Figure 2A). In addition, the relative interest in this field has also increased over the past few years (Figure 2A).

In total, 65 countries/regions have made contributions in literatures in this field. As shown in Figures 2B, C, the USA published the most papers (394, 29.266%), followed by China (247, 17.093%), Japan (166, 11.488%), Germany (129, 8.927%) and England (115, 7.958%). It is shown in Figure 2D that the annual number of publications of top 10 countries/regions rose from 10 (0.705%) in 1991 to 166 (11.707%) in 2021. Before 2019, the annual number of publications of the USA and Japan increased faster than that of China. For predicting the future global literatures trend, a logistic regression model was performed to create a time curve of the number of literatures. Figure 2E illustrates the fitting curve of the annual publication trend and the correction coefficient R2 is 0.9434. The predicted number of publications will be was estimated to 1000 in the year of 2031. Overall, these results indicating that the research on macrophages associated with osteoarthritis has attracted increasing researchers' focus and reached a staged of rapid development.

Analysis of countries

As we can see from Figure 3A, publications from the USA had the highest total citation frequencies (22978). Netherlands ranked second in total citation frequencies (8340), followed by Japan (7760), England (7744) and Germany (5291). Regarding the global collaboration network analysis, the Figure 3B showed that the USA exhibited the highest output volume and worked closely with Netherland, South Korea, and France. From the Figure 3E, we can figure out that the network diagram of cooperation mainly exists in North America, West Europe,

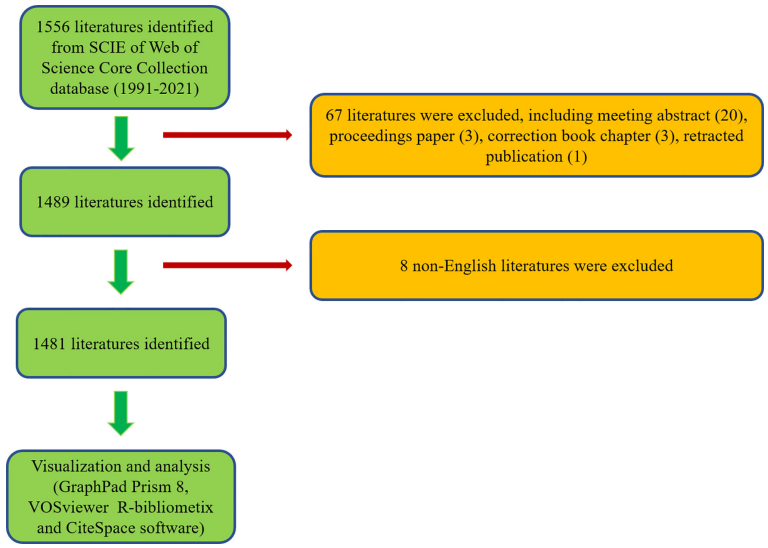

FIGURE 1
Flowchart of the screening process.

and East Asia. In terms of every citation frequency, publications from Scotland had the highest average citation frequencies (124.58). Wales ranked second in average citation frequency (99), prior to the Netherlands (73.16), England (67.34) and Switzerland (61.76) (Figure 3C). Additionally, the USA (80) dominated in this field in the relative publications of H-index, followed by Netherlands (51), Japan (47), England (42) and Germany (42) (Figure 3D).

Analysis of institutions and authors

Regarding publication ranking, the top 25 contributive institutions were listed in Figure 4A. The first was League of European Research Universities (127 publications), followed by Northwestern University (39 publications), and Radboud University Nijmegen ranked third (36 publications). Figure 4B exhibits the network diagram of collaboration between institutions, which shows that that there is strong cooperation relationship between institutions such as Shanghai Jiao Tong University, Zhejiang University, and Nanjing Medical University in China and Duke University, Stanford University, and Harvard University in the USA.

The top 10 authors contributed a total of 217 publications, which accounted for approximately 15% of all publications in this field. Koch AE published the most studies, with 29 publications, followed by Haines GK with 26 publications and Tak PP with 23 publications (Table 1). CiteSpace visualizes the network between authors, as shown in Figure 4C. Authors from the same country collaborate more frequently with strong connection. However, the connections between authors from different countries are still inadequate. The co-citation analysis considered the relatedness of the items based on the numbers they were co-cited. A total of 871 authors with a minimum of 10 documents were analyzed using VOSviewer (Figure 4D). The top 5 authors with largest total link strength were as follows: Bondeson J (total link strength =5889 times), Blom AB (total link strength = 5513 times), Goldring MB (total link strength = 4692 times), Scanzello CR (total link strength = 4543 times), and Koch AE (total link strength = 4359 times).

Analysis of journals and research areas

Table 2 lists the top 10 productive journals involved in this study. The journal *Arthritis and Rheumatism* (impact factor = 8.955, 2021) published the most with 98 publications. There were 92 publications in *Osteoarthritis and Cartilage* (IF = 7.507, 2021), 77 publications in *Arthritis Research Therapy* (IF = 5.606, 2021), 47 publications in *Journal of Rheumatology* (IF = 5.346, 2021) and 45 articles in *Annals of the Rheumatic Diseases* (IF = 27.973, 2021). The names of journals of co-citation analysis were performed using VOSviewer, and the journal with a minimum number of citations over 10 was defined. As plotted in Figure 5A, 824 journals were shown in the total link strength. The top 5 journals with best total link strength were as follows: *Osteoarthritis and Cartilage* (total link strength = 184826 times), *Arthritis and Rheumatism* (total link strength =152813 times), *Annals of the Rheumatic Diseases* (total link strength = 135410 times), *Journal of Immunology* (total link strength = 105307 times), and *Arthritis Research Therapy* (total link strength = 93494 times).

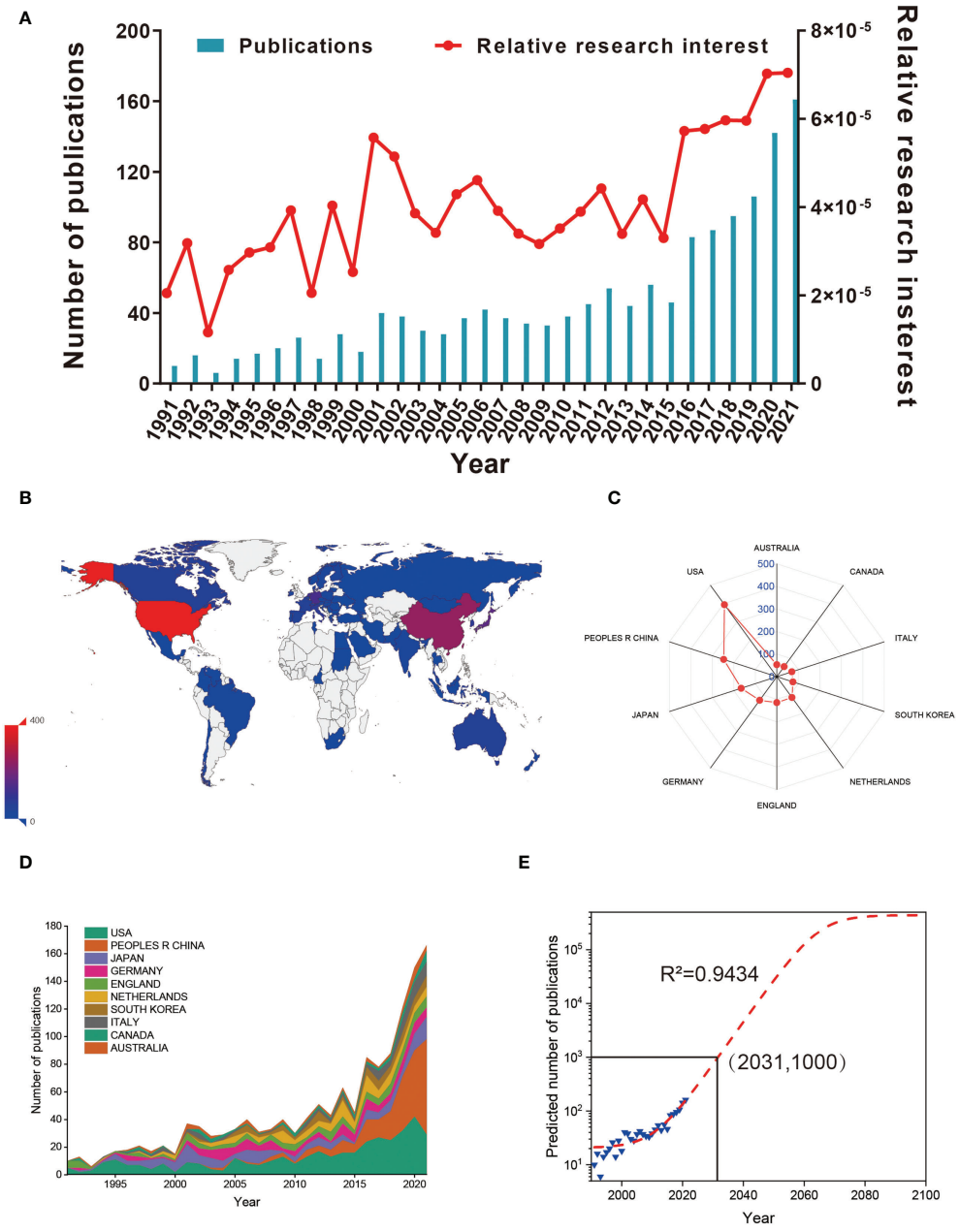

FIGURE 2
(A) The global number (blue bars) and relative research interests (red curve) of publications related to macrophages associated with osteoarthritis. **(B)** Distribution of macrophages associated with osteoarthritis research in world map. **(C)** The sum of publications related to macrophages associated with osteoarthritis from the top 10 countries and regions. **(D)** The annual number of publications in the top 10 most productive countries from 1991 to 2021. **(E)** Model fitting curves of global trends in publications related to macrophages associated with osteoarthritis per year (R^2 = 0.9434, (2031,1000) indicates that the total publications will up to 1000 in year of 2031).

We performed a visual analysis of the research orientations using VOSviewer (Figure 5B), which is also summarized in Table 3. In details, the most prevalent research fields were rheumatology, orthopedics, immunology, cell biology, and biochemistry molecular biology. The spline wave from left to right describes the citation association, which is represented by the colored path. The Figure 5C depicted three primary citation paths marked in orange and green. The two primary paths showed that documents published in molecular/biology/genetics were primarily cited by researchers published in molecular/biology/immunology and medicine/medical/clinical journals, while the third path showed that documents published in

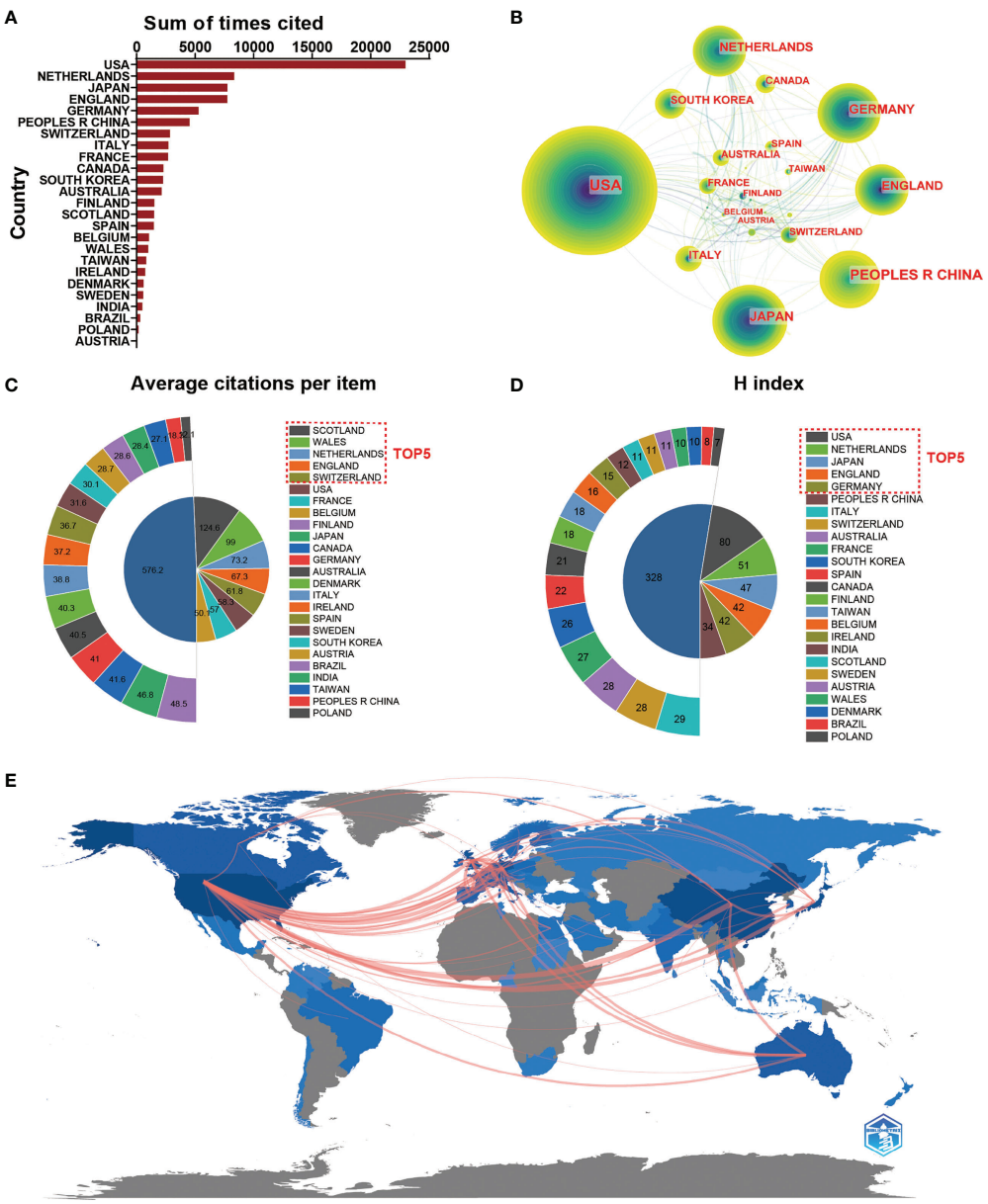

FIGURE 3
(A) The top 25 countries/regions of total citations related to macrophages associated with osteoarthritis. **(B)** Country/regional collaboration analysis. **(C)** The top 25 countries/regions of the average citations per publication related to macrophages associated with osteoarthritis. **(D)** The top 25 countries/regions of the publication H-index related to macrophages associated with osteoarthritis. **(E)** The geographical network map of macrophages associated with osteoarthritis.

sports/rehabilitation/sport was primarily cited by researchers published in molecular/biology/immunology.

Citation and co-citation analysis

A total of 674 articles in this field have more than 25 citations (Figure 6A). The top 10 most cited documents are shown in Table 4. There were 878 citations for "Discovery and development of folic-acid-based receptor targeting for Imaging and therapy of cancer and inflammatory diseases", followed by "The role of cytokines in osteoarthritis pathophysiology", with 784 citations. The third-ranked article with the largest number of citations was "Increased Concentrations of Nitrite in Synovial-Fluid and Serum Samples Suggest Increased Nitric-Oxide Synthesis in Rheumatic Diseases", with 624 citations.

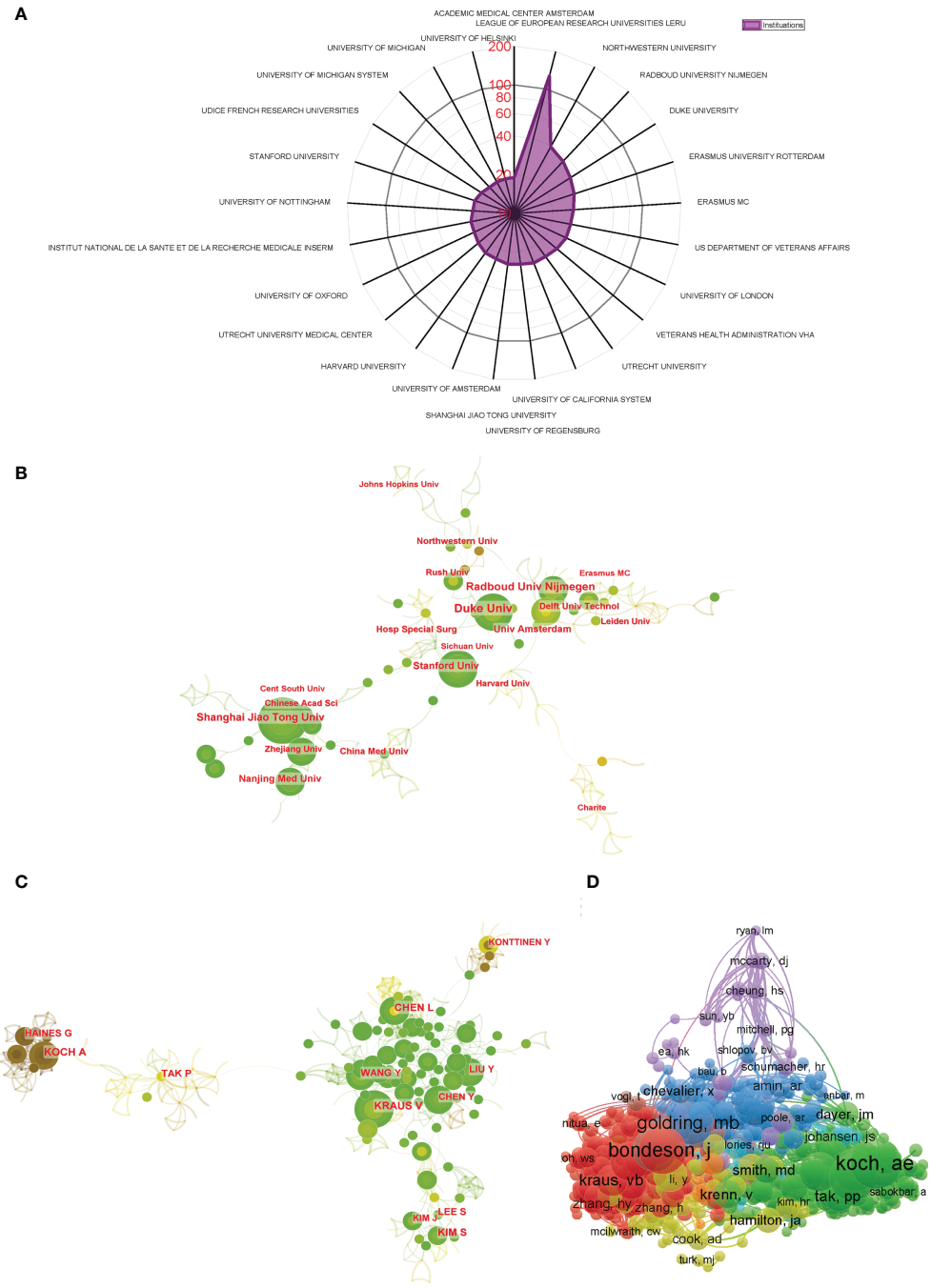

FIGURE 4
(A) The top 25 institutions with most publications related to macrophages associated with osteoarthritis. **(B)** Institutional collaboration analysis. **(C)** Author collaboration analysis. **(D)** Network visualization diagram of the co-cited authors of the Publications.

Moreover, co-cited references were analyzed by VOSviewer (Figure 6B) to show the most influential literature. In addition, citation burst is a valuable indicator that reflects the references of interest to researchers in a particular domain in a period (28). In our study, the top 25 references with the strongest citation bursts were identified by CiteSpace and presented in Figure 6C, among which the citation burst for duration of references. The article titled "Synovial macrophage M1 polarisation exacerbates experimental osteoarthritis partially through R-spondin-2", published in 2018, ranked first (strength = 16.3). Meanwhile, the citation bursts of articles published by Daghestani H lasted from 2016 to 2021.

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TABLE 1 The top 10 authors with the most publications on macrophages associated with osteoarthritis.

Rank	High Published Authors	Country	Article counts	Percentage %
1	Koch AE	USA	29	2.007
2	Haines GK	USA	26	1.799
3	Tak PP	Netherlands	23	1.592
4	Van Den Berg WB	Netherlands	22	1.522
5	Kraus VB	USA	21	1.453
6	Pope RM	USA	20	1.384
7	Van Der Kraan PM	Netherlands	20	1.384
8	Straub RH	Germany	19	1.315
9	Van Lent PLEM	Netherlands	19	1.315
10	Van Osch GJVM	Netherlands	18	1.246

Analysis of keywords and hotspots

CiteSpace's algorithm was also used to detect the burst of keywords based on burst detection. The top 25 keywords with the highest burst strength are shown in Figure 7A. We found that the keyword with highest citation outbreaks was interleukin 1 (strength = 13.5), followed by messenger RNA (13.17) and necrosis factor alpha (13.09). The keyword with the longest burst time was human monocyte, which lasted 18 years from 1991 to 2008. More meaningfully, the keyword "mice" had outbreak citations most recently (2009-2018), which implied that the research on the linkage between macrophages associated with osteoarthritis and animal models researches might be research hotspots in the future. We also built a network map to visualize keyword clusters (Figure 7B), and we found that "osteoarthritis" (Cluster0), "necrosis factor alpha" (Cluster1), "infrapatellar fat pad" (Cluster2), "t cell" (Cluster3), "collagen induced arthritis" (Cluster5), "nitric oxide" (Cluster7), and "synovial fluid" (Cluster11) were the hotspots of research since 1991.

Figure 7C represents a three-field graph in which authors, keywords, and journals were associated. It was possible to observe the links between the main elements through this three-field graph and their relationship was exhibited directly by the strength of the connection links (29). The keywords most frequently used were "expression", "rheumatoid-arthritis" "inflammation" and "osteoarthritis", which coincide with the keywords presented in Figure 7B. The author's Koch AE, Haines GK and Pope RM are strongly connected with the keyword "expression" and "rheumatoid-arthritis" establishing the relatively strongest links. In turn, it can be found that the heaviest links were related to the *Osteoarthritis and Cartilage*. Moreover, it can be seen that the *Arthritis and Rheumatism* covered most of the papers related to the keyword "expression", "rheumatoid-arthritis", and "inflammation". Therefore, this visualization suggested that rheumatoid arthritis as a kind of arthritis was relative referential for osteoarthritis research.

For bibliometrics, the keywords co-occurrence analysis is a prevalent way to identify hot research topics and areas, and it also plays a vital role in monitoring the developments in scientific research. In a co-occurrence analysis, the keyword was defined as the words used more than 5 times in titles or abstracts in all papers, which were chosen and analyzed *via* VOSviewer. As shown in Figure 8A, the 527 identified keywords were mainly classified into six clusters as follows: cluster 1: rheumatoid arthritis research (red), cluster 2: clinical symptoms (green), cluster 3: regeneration research (yellow), cluster 4:

TABLE 2 The top 10 productive journals related to macrophages associated with osteoarthritis.

Rank	Journal	Article counts	Percentage%	IF
1	Arthritis and Rheumatism	98	6.773	8.955
2	Osteoarthritis and Cartilage	92	6.358	7.507
3	Arthritis Research Therapy	77	5.321	5.606
4	Journal of Rheumatology	47	3.248	5.346
5	Annals of the Rheumatic Diseases	45	3.110	27.973
6	Journal of Orthopedic Research	33	2.281	2.728
7	Arthritis Rheumatology	27	1.866	15.483
8	Plos One	25	1.728	3.752
7	Scientific Reports	25	1.728	4.996
10	Journal of Immunology	22	1.520	5.426

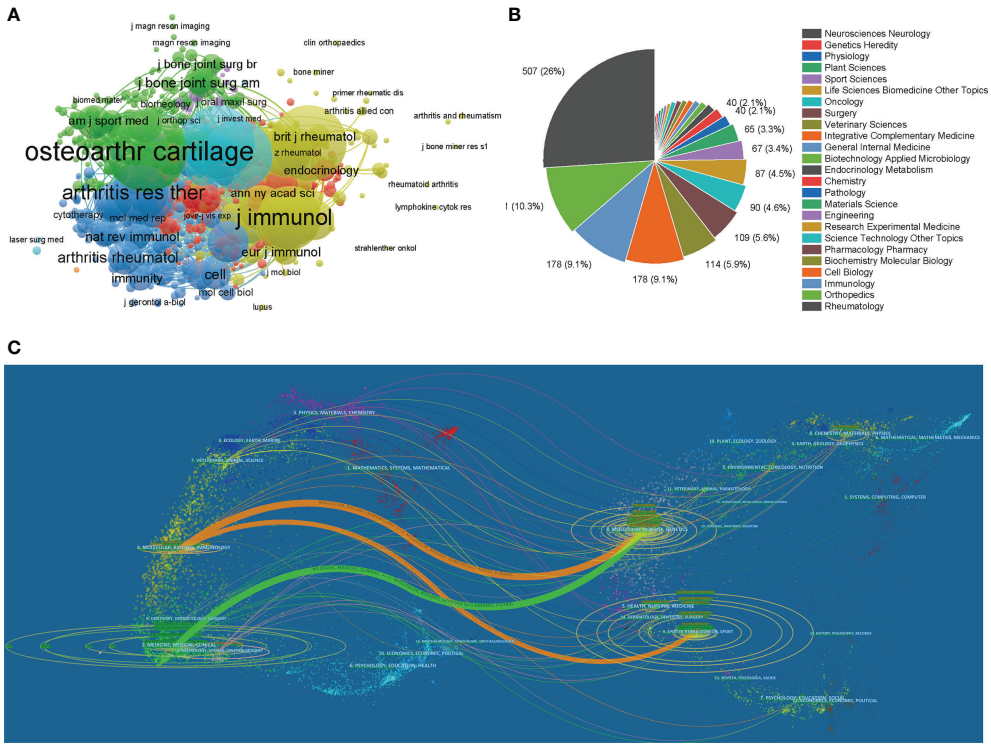
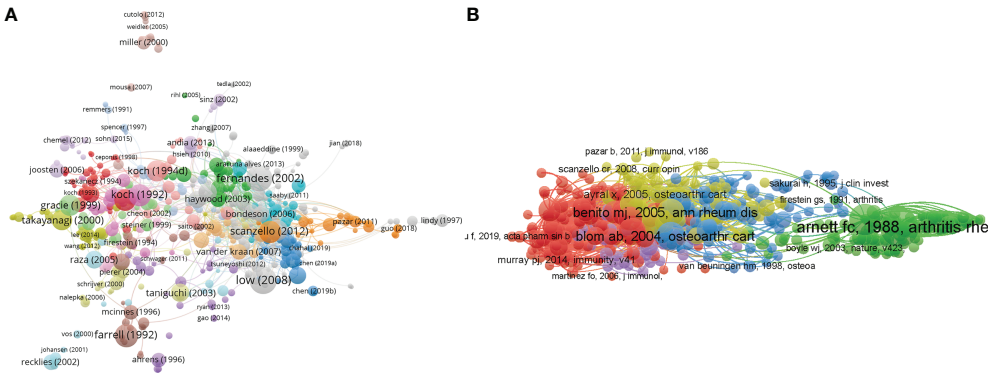


FIGURE 5
(A) Network map of journals that were co-cited in more than 20 citations. **(B)** Mapping of the top 25 research directions related to macrophages associated with osteoarthritis. **(C)** The dual-map overlay of journals related to macrophages associated with osteoarthritis.

mechanism research (dark blue), cluster 5: pathological features (orange), and cluster 6: surgery research (light blue). These results exhibited the most prominent research topics in macrophages associated with osteoarthritis so far. In the "rheumatoid arthritis research" cluster, the primary keywords were: T cells, interleukin-1, and classification. For the "clinical symptoms" cluster, the frequently used keywords were: pain, synovitis, and adipose tissue. As for the "regeneration research" cluster, the main used keywords were: inflammation, polarization, and repair. For the "mechanism research" cluster, the dominantly used keywords were: activation, apoptosis, and nitric oxide. When talking about the "pathological features" cluster, the frequently used keywords were: inhibition, osteoporosis, and mineralization. And cluster "surgery research" consist of the frequently used keywords as follows: replacement, bone-resorption, and joint-destruction. These

TABLE 3 The top 10 well-represented research areas related to macrophages associated with osteoarthritis.

Rank	Research Areas	Records	Percentage%
1	Rheumatology	508	35.107
2	Orthopedics	201	13.891
3	Immunology	178	12.301
4	Cell Biology	155	10.712
5	Biochemistry Molecular Biology	107	7.395
6	Pharmacology Pharmacy	102	7.049
7	Medicine Research Experimental	87	6.012
8	Multidisciplinary Sciences	73	5.045
9	Engineering Biomedical	63	4.354
10	Materials Science Biomaterials	56	3.870



C

Top 25 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	1991 - 2021
Arnett F, 1988, ARTHRITIS RHEUM, V31, P315, DOI 10.1002/art.1780310302, DOI	1988	7.76	1991	1993	
Koch A, 1991, LAB INVEST, V64, P313	1991	8.16	1993	1996	
Koch A, 1991, J IMMUNOL, V147, P2187	1991	7	1994	1996	
Blom A, 2004, OSTEOARTHR CARTILAGE, V12, P627, DOI 10.1016/j.joca.2004.03.003, DOI	2004	8.47	2005	2008	
Kapoor M, 2011, NAT REV RHEUMATOL, V7, P33, DOI 10.1038/nrrheum.2010.196, DOI	2011	12.46	2012	2016	
Bondeson J, 2010, ARTHRITIS RHEUM-US, V62, P647, DOI 10.1002/art.27290, DOI	2010	10	2012	2015	
Sellam J, 2010, NAT REV RHEUMATOL, V6, P625, DOI 10.1038/nrrheum.2010.159, DOI	2010	8.88	2012	2015	
Scanzello C, 2012, BONE, V51, P249, DOI 10.1016/j.bone.2012.02.012, DOI	2012	12.1	2013	2017	
De L, 2012, OSTEOARTHR CARTILAGE, V20, P1484, DOI 10.1016/j.joca.2012.08.027, DOI	2012	7.8	2014	2017	
Berenbaum F, 2013, OSTEOARTHR CARTILAGE, V21, P16, DOI 10.1016/j.joca.2012.11.012, DOI	2013	11.78	2015	2018	
Fahy N, 2014, OSTEOARTHR CARTILAGE, V22, P1167, DOI 10.1016/j.joca.2014.05.021, DOI	2014	8.8	2015	2019	
Daghestani H, 2015, ARTHRITIS RHEUMATOL, V67, P956, DOI 10.1002/art.39006, DOI	2015	11.81	2016	2021	
Sokolove J, 2013, THER ADV MUSCULOSKEL, V5, P77, DOI 10.1177/1759720X12467868, DOI	2013	7.49	2016	2018	
Kraus V, 2016, OSTEOARTHR CARTILAGE, V24, P1613, DOI 10.1016/j.joca.2016.04.010, DOI	2016	15.89	2017	2021	
Robinson W, 2016, NAT REV RHEUMATOL, V12, P580, DOI 10.1038/nrrheum.2016.136, DOI	2016	11.45	2017	2021	
Wojdasiewicz P, 2014, MEDIAT INFLAMM, V2014, P0, DOI 10.1155/2014/561459, DOI	2014	7.8	2017	2019	
Rahmati M, 2016, BONE, V85, P81, DOI 10.1016/j.bone.2016.01.019, DOI	2016	7.8	2017	2019	
Glyn-jones S, 2015, LANCET, V386, P376, DOI 10.1016/S0140-6736(14)60802-3, DOI	2015	7.66	2017	2021	
Raghu H, 2017, ANN RHEUM DIS, V76, P0, DOI 10.1136/annrheumdis-2016-210426, DOI	2017	7.05	2017	2021	
Mathiessen A, 2017, ARTHRITIS RES THER, V19, P0, DOI 10.1186/s13075-017-1229-9, DOI	2017	11.84	2018	2021	
Utomo L, 2016, OSTEOARTHR CARTILAGE, V24, P2162, DOI 10.1016/j.joca.2016.07.018, DOI	2016	9.18	2018	2021	
Wu C, 2017, ARTHRITIS RHEUMATOL, V69, P1772, DOI 10.1002/art.40161, DOI	2017	8.52	2018	2021	
Zhang H, 2018, ANN RHEUM DIS, V77, P1524, DOI 10.1136/annrheumdis-2018-213450, DOI	2018	16.3	2019	2021	
Liu B, 2018, EXP THER MED, V16, P5009, DOI 10.3892/etm.2018.6852, DOI	2018	9.04	2019	2021	
Manferdini C, 2016, ARTHRITIS RES THER, V18, P0, DOI 10.1186/s13075-016-0983-4, DOI	2016	7.52	2019	2021	

FIGURE 6
(A) Network map of citation analysis of documents with more than 25 citations. **(B)** Network map of co-citation analysis of references. **(C)** Top 25 references with strongest citation bursts of publications related to macrophages associated with osteoarthritis.

results exhibited that the most prominent fields of macrophages associated with osteoarthritis research included the abovementioned five directions.

According to Figure 8B, the VOSviewer colored all keywords based on the average times they appeared among the published papers. Specifically, the color blue indicates that the keywords appeared relatively early, while the color yellow indicates a more recent appearance. As shown in Figure 8B, the research trends of most studies in the six clusters were changed from rheumatoid arthritis research (cluster1), pathological features (cluster 5), and surgery research (cluster 6) to clinical symptoms (cluster 2), regeneration research (cluster 3), mechanism research (cluster 4), suggesting that future research hotspots might lie in the research of clinical symptoms, regeneration and mechanism exploration.

Discussion

In the past few decades, researchers have put enormous efforts into macrophages associated with osteoarthritis research, and considerable progress has been achieved in diagnosing and treating osteoarthritis (30). The critical role of macrophages in inflammatory and destructive responses in OA pathogenesis is currently widely recognized. It should be noticed that increased macrophages in OA patients' synovium and subchondral bone tissue were identified with multiple cell surface markers such as CD163, CD68, CD14, MHC class II genes and F4/80, and the increase of CD14 and CD163 is associated with OA severity (8, 31). Therefore, a significant obstacle within macrophages associated with osteoarthritis research is the development of basic studies and effective treatments.

TABLE 4 The top 10 documents with the most citations in the field of macrophages associated with osteoarthritis.

Rank	Title	Corresponding Author	Journal	IF	Publication year	Total citations
1	Discovery and development of folic-acid-based receptor targeting for Imaging and therapy of cancer and inflammatory diseases	Doorneweerd, DD	Accounts of Chemical Research	24.466	2008	878
2	The role of cytokines in osteoarthritis pathophysiology	Pelletier, JP	Biorheology	1.615	2002	784
3	Increased Concentrations of Nitrite in Synovial-Fluid and Serum Samples Suggest Increased Nitric-Oxide Synthesis in Rheumatic Diseases	Moncada, S	Annals of The Rheumatic Diseases	27.973	1992	624
4	The role of synovitis in osteoarthritis pathogenesis	Goldring, SR	Bone	4.626	2012	595
5	Enhanced Production of Monocyte Chemoattractant Protein-1 In Rheumatoid-Arthritis	Strieter, RM	Journal of Clinical Investigation	19.456	1992	579
6	Localization of Tumor-Necrosis-Factor-Alpha in Synovial Tissues and At the Cartilage Pannus Junction in Patients with Rheumatoid-Arthritis	Maini, RN	Arthritis and Rheumatism	8.955	1991	537
7	A proinflammatory role for IL-18 in rheumatoid arthritis	McInnes, IB	Journal of Clinical Investigation	19.456	1999	531
8	A clinical perspective of IL-1 beta as the gatekeeper of inflammation	Dinarello, CA	European Journal of Immunology	6.688	2011	520
9	Vascular Endothelial Growth-Factor - A Cytokine Modulating Endothelial Function in Rheumatoid-Arthritis	Ferrara, N	Journal of Immunology	5.426	1994	518
10	Involvement of receptor activator of nuclear factor kappa B ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis	Tanaka, S	Arthritis and Rheumatism	8.955	2000	483

The trend overview of development of macrophages associated with osteoarthritis

As shown in this study, a significant increase in the number of publications per year has been found from 1 January 1991 to 31 December 2021. Moreover, the RRI has also increased slightly over the past few years, suggesting the popularity of this area is also increasing. In terms of national contributions, in our study, approximately 65 countries have published papers on the macrophages associated with osteoarthritis field. Particularly, The USA contributed the largest papers (394, 29.266%) than China (247, 17.093%), Japan (166, 11.488%), Germany (129, 8.927%), and England (115, 7.958%). Recently, the number of total citations, per citations, and H-index are critical parameters in the bibliometric study and can also show the quality and academic impact of different countries. As shown in Figure 2 and Figure 3, the USA contributed the most publications, more extensive total citations, and the largest H-index, suggesting that the USA was a highly productive and leading country in this field. The USA possesses the most elite researchers and institutions worldwide, suggesting the USA's leading position in the field of macrophages associated with osteoarthritis research. Interestingly, Scotland ranked first in terms of average citations (124.6), followed by Wales (99) and the Netherlands (73.2). Regarding the top countries or regions, it can be seen that the Netherlands, ranking sixth in the number of publications, is still making a significant progression in this field of total citation, and H-index for it ranked second and fourth, respectively. Although China ranked the second largest number of total publications, it showed weaker performance in total citations, average citations, and H-index, suggesting that China might not catch up with the USA in the following decades. The contradiction between the quantity and quality of publications in China also requires more in-depth studies. Among the scientific institutions, League of European Research Universities ranked second (127 publications), Northwestern University (39 publications), and Radboud University Nijmegen (36 publications) actively contributed to the research front. Notably, the leading top 5 institutes have contributed significantly to the research regarding with macrophages associated with osteoarthritis, which is consistent with the global publications produced by the top 5 countries. It is noted that approximately the top 25 institutes come from the top 5 countries, indicating the leading role of first-class institutes in improving one country's academic research ranking. Therefore, this evidence collectively infers that further in-depth studies with cooperation could play a vital role in macrophages associated with osteoarthritis research, guiding researchers to publish high-quality papers in the future.

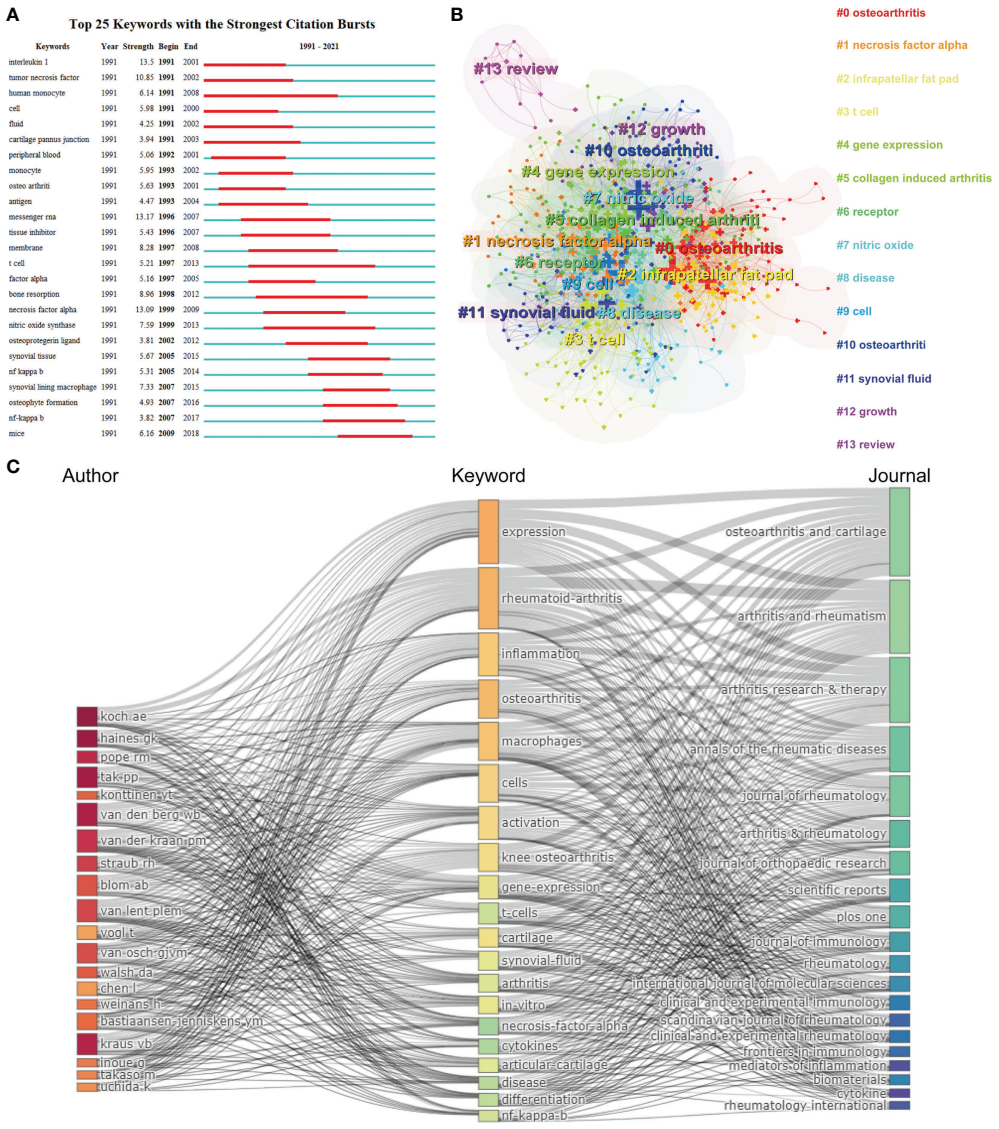


FIGURE 7
(A) Top 25 keywords with the strongest citation bursts based on CiteSpace. **(B)** Clustering analysis of the keyworks network based on CiteSpace. **(C)** Three-field plot of the Keywords Plus analysis on macrophages associated with osteoarthritis Notes: three-field plot of the keywords analysis: (middle field: keywords; left field: authors; right field: journals).

Status and quality of authors, journals, and studies

Regarding authors, the top-ranked authors with the most publications are Americans, together with the largest funds provided by the USA National Institutes of Health (NIH), which means that the USA has played the most crucial role in the field of macrophages associated with osteoarthritis research. The top-ranked authors listed in Table 1 with the most publications were relative earlier entrants and might have been given prior attention to obtaining the new advancements in macrophages associated with osteoarthritis research. Additionally, the collaboration analysis in Figure 4C showed that the research relationship among authors in different countries is relatively scattered, indicating a lack of academic connection and communication among authors. Therefore, authors in different countries and institutions should strengthen their cooperation to improve macrophages' research on osteoarthritis jointly. As shown in Figure 4D, Bondeson J, Blom AB, and Goldring MB might be the top authors with the highest citation frequency, which represents the international attention and recognition of these researchers in this field.

Besides the authors' analysis, the journals associated with publications were further explored, and the results are shown in Table 2. The journal *Arthritis and Rheumatism*, *Osteoarthritis and Cartilage*, and *Arthritis Research Therapy* published most papers. Recently, the impact factors were generally high. Interestingly, the top 5 journals published more than 40 papers in total, and, predictably, the listed top 10 journals might be the possible choices for researchers to publish high-quality research in the future. Furthermore, the co-citation analysis based on journals was conducted to investigate the impacts of publications by analyzing the total citation number. Figure 5A showed that *Osteoarthritis and Cartilage* had made the most outstanding contributions in this field. Among the top 10 research orientations, two are specialized in the clinical study and five are in basic research. More specifically, the dual-map analysis reflected the concentration of research in genetics, immunology, and rehabilitation studies.

The impact of published literature was evaluated in citation analysis of documents (Figure 6A) and co-citation network

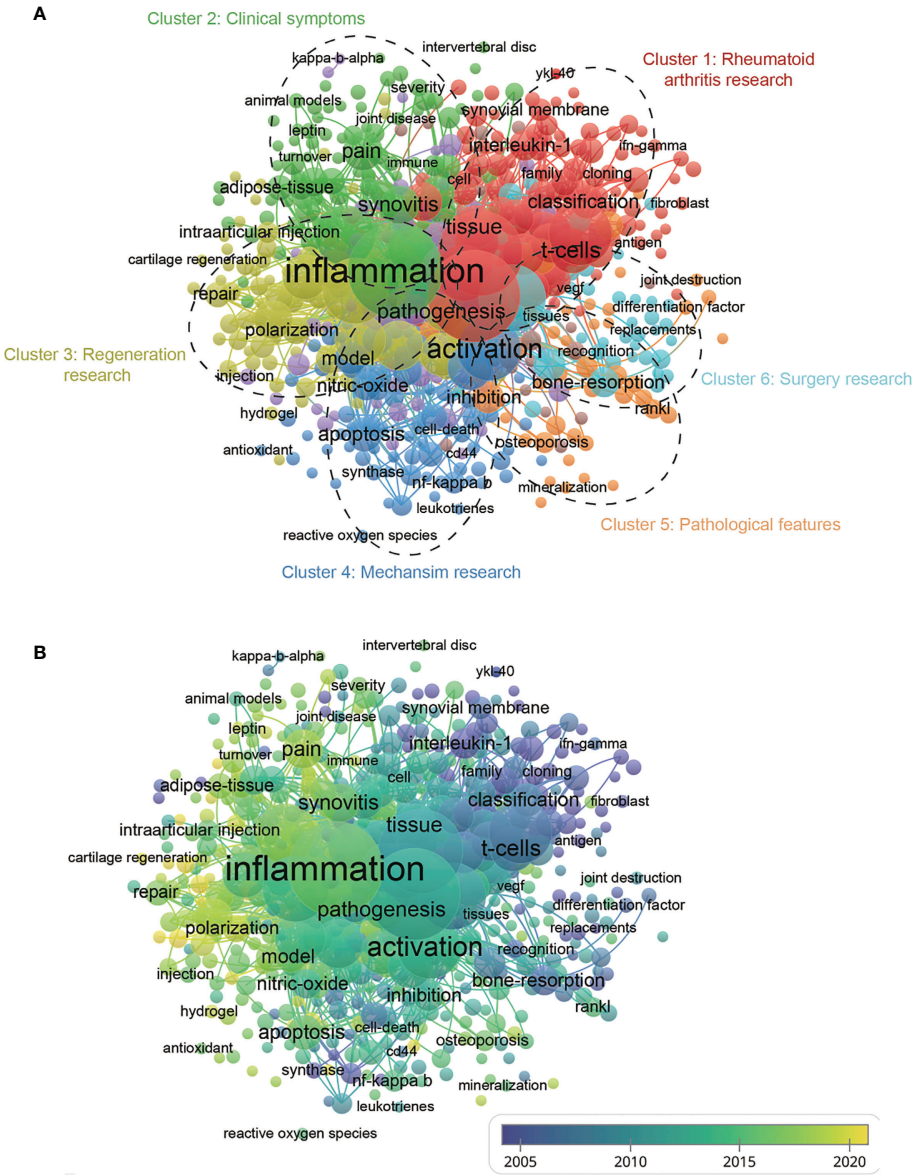


FIGURE 8
(A) Mapping of keywords in the research related to macrophages associated with osteoarthritis; the frequency is represented by point size and the keywords of research fields are divided into six clusters: rheumatoid arthritis research (red), clinical symptoms (green), regeneration research (yellow), mechanism (dark blue), pathological features (dark brown), and surgery research (baby blue). **(B)** Distribution of keywords according to the mean frequency of appearance; keywords in yellow appeared later than those in blue.

analysis (Figure 6B). Table 4 showed that the most cited article was the exploitation of the well-characterized up-regulation of folate receptors on activated macrophages, which may be a target for rheumatoid arthritis and inflammatory osteoarthritis treatment (32). Another study focused on the role of cytokines in OA pathophysiology was written by Pelletier JP et al. (33). Among the ten most cited articles, most types of literature are of the basic research type, focusing on the pathology, pathogenesis, diagnosis, and treatment of OA and other kinds of arthritis.

Interestingly, co-citation analysis of references can figure out which publications have made the most outstanding contributions in this field. As shown in Figure 6B, "Differential role for interleukin-1 in induced instability osteoarthritis and spontaneously occurring osteoarthritis in mice" authored by Blom AB et al. might be the top reference with the highest citation frequency. In Figure 6C, most of the top 25 cited articles with the strongest citation bursts were related to OA pathophysiology, diagnosis, and therapy, indicating that these directions are hot topics in macrophages associated with osteoarthritis research field.

Research hotspots and frontiers

The co-occurrence analysis of keywords and bursts reflected the developing trends and hotspots in macrophages associated with osteoarthritis research. As shown in Figure 7A, "interleukin 1" is the keyword with the highest citation outbreaks, which represents the initial status of this keyword in OA research. For example, as early as the 1990s, Arend WP et al. proposed the IL-1 receptor antagonists (IL-1Ra) intervention in the treatment of OA and confirmed a reduction of cartilage destruction associated with this therapy (34, 35). As shown in Figures 7B, C, it is shown that the primary research clusters mainly refer to "osteoarthritis", "necrosis factor alpha", "t cell", "gene expression", and "synovia fluid", indicating that molecular biology exploration in OA disease is another hotspot.

In our study, the keywords' co-occurrence network was depicted based on the determination of keywords in the titles/abstracts of all included publications. Figure 8A showed 6 main research trends, which could be divided into 6 clusters: rheumatoid arthritis research (red), clinical symptoms (green), regeneration research (yellow), mechanism research (dark blue), pathological features (orange) and surgery research (light blue). These results could not only comply with hopeful hotspots in this field of macrophages associated with osteoarthritis research but also forecast the directions of future studies, as follows.

(I). Rheumatoid arthritis research: Co-occurrence analysis of keywords identified "T cells", "interleukin-1", and "classification" as important research hotspots which deserve further attention. Rheumatoid arthritis (RA) has been considered an autoimmune disease because it presents with a chronic systemic inflammatory disorder (36). T lymphocytes (T cells), mainly categorized into helper T cells (Th cells) and cytotoxic T cells (Tc cells), secrete cytokines to modulate the behavior of cells involved in immunologic response (37). In RA, T-lymphocytes stimulate macrophages to overproduce inflammatory cytokines. Notably, the role of T cells in OA disease progression is also an emerging topic of investigation. For example, OA patients present with enhanced T helper cells in synovial tissue and synovial fluid. Furthermore, multiple T cells such Th1, Th9, and Th17 cells are located in OA synovial fluid, while Th1, Th17, and cytotoxic T cells mainly existed in OA synovial tissue, all of these cells secrete various catabolic cytokines, including IL-2, IFN-γ, and TNF-α (38). Notably, the classification of osteoarthritis subtypes according to the distinct molecular signatures was performed recently. A study conducted by Yuan, Chunhui, et al. divided OA patients into four subtypes based on the symptoms: glycosaminoglycan metabolic disorder subtype, collagen metabolic disorder subtype, activated sensory neuron subtype, and inflammation subtype (39). This study provided distinct molecular subtypes in knee OA, which may shed light on the precise diagnosis and treatment of this disease.

(II). Clinical symptoms: One primary topic of OA is studying the mechanism of pain in symptomatic OA. Generally, pain is a complex process including sensory, affective, and cognitive experiences, while some kinds of tissue (infrapatellar fat pad (IFP) and the synovial membrane) have been investigated as a potential source of pain in OA (40). Regarding the role of synovitis in OA pain, Baker et al. proved the strong connection between contrast-enhanced MRI-detected synovitis and Knee OA severity (41). Another potential therapeutic target refers to adipose tissue in IFP. Hypointense IFP signal and greater volume of IFP were demonstrated to be highly correlated with OA pain (42). Specifically, the molecular mechanisms involved in OA pain refer to the IFP-Synovial membrane can be divided into neuropeptides and peptide hormones, growth factors, and cytokines (40). Interestingly, IL-1β-producing macrophages regulate calcitonin receptor-like receptor (CLR) expression in synovial cells and are reported to be involved in pain transmission and neurogenic inflammation (43). In addition, the high level of Neuropeptide Y (NPY) detected in OA patients synovial fluid was also correlated with OA severity and pain (44). Both synovial fluid CD14 and CD163 were positively associated with osteophyte progression (45). Importantly, previous studies discovered that several subsets of macrophages might contribute to OA pain through nerve growth factor (NGF) and calcitonin gene-related peptide (CGRP) expression (46–48). Takano et al. discovered that

CD14-positive macrophages could regulate NGF by inflammatory cytokines (IL-1β and TNF-α) production (49). In addition, Shotaro et al. reported that elevated CGRP by CD14-positive macrophages may contribute to increased OA pain (48). In addition, researchers reported that CD163+CD14^{low} macrophages expressing TNF-α might be a vital contributor to the OA pain (50). These molecular factors contribute to the pain of OA and as a potential therapeutic target in OA pain treatment and should be further explored in the future.

(III). Regeneration research: Promising regeneration strategies for OA are urgently needed since the OA involves articular cartilage destruction, synovitis, subchondral bone remodeling, osteophyte formation, and meniscus and ligament changes (5). Several specific mediators (PAMPs, DAMPs, and inflammasome) act as microenvironment stimuli that induce synovial macrophage activation and polarization (51). Since macrophage polarization plays a fundamental role in OA progression and regeneration, many efforts have been made to explore novel specific targets to inhibit or slow the progression of OA. For instance, M2 macrophage membrane-coated nanoparticles (Au-M2 NPs), a unique drug platform, could be applied as a highly anti-inflammatory and specific polarize macrophages to M2 type and eventually alleviate OA inflammation as well as matrix degradation (52). On the other hand, investigating the underlying molecular pathology of OA is also a pivotal research direction for differential treatment. For example, Yin, Jianbin, et al. performed an RNA sequencing of OA M1-polarized macrophages and successfully identified that pentraxin 3 (PTX3) is highly expressed in OA patients. Moreover, PTX3 was upregulated when miR-224-5p was insufficient, which activated the p65/NF-κB pathway to induce M1 macrophage polarization by targeting CD32 (53). Therefore, blockade of this pathway and PTX3 may alleviate the OA development.

(IV). Mechanism research: Although multiple proinflammatory factors (including IL−1, IL−6, IL−17, and TNF−α) released by chondrocytes and proliferating synoviocytes affects the mobilization, polarization and apoptosis of macrophages, the underlying mechanisms are not completely understood (54). Therefore, exploring the advanced therapeutic targets for macrophage polarization which involves OA progression, is urgently needed. Notably, nitric oxide (NO), a small bioactive molecule, can significantly inhibit the inflammatory response by activating the AMP-activated protein kinase (AMPK) signal pathway (55–57). However, the role of NO in the OA disease process remains to be elucidated; some studies suggested that NO was responsible for inducing apoptosis and proinflammatory cytokines secretion, while other studies indicated that NO and its redox derivatives might also protect chondrocytes to a certain extent (58). A study by Chen, Xu, et al. proved that A photothermal-triggered nitric oxide nanogenerator combined with siRNA attenuates macrophage-mediated inflammation, showing promising effects for OA treatment (59).

(V). Pathological features: For OA pathological progression, pathological calcification or mineralization in the affected joint is an important feature. The most common site of pathological calcification was cartilage, while other soft tissues, including the meniscus, synovium, and tendons, were also commonly affected (60). In detail, the two most common forms of pathological articular minerals refer to Basic calcium phosphate (BCP) and calcium pyrophosphate dehydrate (CPPD) (61). Several pathological processes were involved in abnormal mineralization as follows: pathological rejuvenation of chondrocytes, changes in ECM structure and composition, changes of extracellular calcium level, disordered pyrophosphate (PPi) and phosphate (Pi) metabolism, mitochondria-mediated calcification, and imbalance between inhibitors and promoters in non-collagenous proteins (NCP) (60). The relationship between osteoporosis and OA requires further investigation. In addition to commonly observed subchondral sclerosis in OA, some patients may suffer from pain and disability, thus encountering osteoporosis with increased fracture risk (62). Regarding the current situation of OA study, we suggest future research should focus on conducting more systematic prospective studies to comprehensively understand the OA pathological features.

(VI). Surgery research: The surgical indication is pivotal for OA patients because surgery is always a relative indication. Multiple indications include symptoms, OA stage, and individual patient factors (age, physical activity, and patient's comorbidities) that should be taken into consideration in surgical interventions (63). The surgical treatment for OA main refers to arthroscopic lavage and debridement, cartilage repair techniques, osteotomies around the knee, and joint arthroplasty (63). For joint arthroplasty, it is vital to determine appropriate OA progression time points for joint replacement. Biomarkers in plasma or other body fluids could be an ideal indicator for diagnosis and determination of OA progression. For example, the CRTAC1 protein in plasma was found to be associated with joint pain and hand OA severity, and it is not associated with other inflammatory joint diseases such as rheumatoid arthritis (64). In addition, after joint replacement surgery, the protein profile in plasma also changed, indicating that these biomarkers can be used to predict prosthesis survival time or early prosthesis failure.

Future research trends

According to the analysis above, it is significant to predict the future trends and possible future impact on search of macrophages associated with osteoarthritis. As depicted in Figure 7, the primary research clusters mainly refer to "osteoarthritis", "necrosis factor alpha", "T cell", "gene expression", and "synovia fluid", indicating that molecular biology exploration in OA disease is another hotspot and future direction. In addition, as shown in Figure 8, the research directions have changed from rheumatoid arthritis research, pathological features, and surgery research to clinical symptoms, regeneration research, mechanism research, which could significantly influence future researchers. In terms of clinical symptoms research, many key molecules associated with OA have been identified and the relationship between subsets of macrophages and OA clinical symptoms has also been discussed, which could assist clinicians to better manage patients' symptoms. As for the regeneration research, many researchers dedicated to explore specific targets to slow down or inhibit the progression of OA by targeting M1 or M2 macrophages. In addition, the mechanism research of macrophages has also drawn many researchers' attention. For example, NO was found to induce apoptosis and proinflammatory cytokines secretion, while others reported that it could protect chondrocytes and attenuates macrophage-mediated inflammation (49–53). Therefore, exploring the mechanisms underlying on the macrophage and OA progression. Based on these findings, the development of basic research of molecular biology and mechanism exploration could benefit the relief of clinical symptoms.

Limitation

There are still some limitations to be discussed: (1) Due to the limitation of our bibliometric software, all of the studies collected from WoSCC, PubMed, Cochrane, Scopus and Embase library databases have not been included, which may lead to publication bias. Therefore, more data sources and powerful software are recommended in the future research. (2) We only extracted research and review articles in English, and the articles published in non-English language or non-research/review articles were not included in this study, which may result in some omissions. (3) We did not visualize the keywords with a timeline, which may result in hotspot prediction bias due to neglection of temporal data. (4) Since the new studies are updated daily, we might neglect some influential newly published studies. (5) As the data selection is done by two authors, encountered problems were resolved by consulting with experts to reach the final consensus.

Conclusion

In conclusion, this study is the first bibliometric analysis to scientifically and comprehensively analyze the global macrophages associated with osteoarthritis research trends over the past 30 years. This study systematically summarized the global publication trends and helped scholars identify the essential authors, institutions, and journals in this field. Moreover, the keyword and co-citation clustering analysis also guide researchers to choose new research directions mainly in five directions as follows "rheumatoid arthritis research", "clinical symptoms", "regeneration research", "mechanism research", "pathological features", and "surgery research". We can expect that further cooperation among authors, institutions, and countries in the future would accelerate the development of macrophages associated with osteoarthritis research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

DX, JHL contributed to conception and design of the study. ZY, ZH, HaL organized the database. ZY, ZH, HaL organized the database. ZY, JJL, LL and HuL performed the statistical analysis. YZ, HaL wrote the first draft of the manuscript. JJL, DX, and JHL wrote sections of the manuscript. JJL and HuL contributed to data acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of immune cells in modulating chronic inflammation and osteonecrosis

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Osteonecrosis occurs when, under continuous stimulation by adverse factors such as glucocorticoids or alcohol, the death of local bone and marrow cells leads to abnormal osteoimmune function. This creates a chronic inflammatory microenvironment, which interferes with bone regeneration and repair. In a variety of bone tissue diseases, innate immune cells and adaptive immune cells interact with bone cells, and their effects on bone metabolic homeostasis have attracted more and more attention, thus developing into a new discipline - osteoimmunology. Immune cells are the most important regulator of inflammation, and osteoimmune disorder may be an important cause of osteonecrosis. Elucidating the chronic inflammatory microenvironment regulated by abnormal osteoimmune may help develop potential treatments for osteonecrosis. This review summarizes the inflammatory regulation of bone immunity in osteonecrosis, explains the pathophysiological mechanism of osteonecrosis from the perspective of osteoimmunology, and provides new ideas for the treatment of osteonecrosis.

KEYWORDS

osteonecrosis, inflammation, immune cells, osteoimmunology, cytokines, bone regeneration

1 Introduction

Osteonecrosis is the death of bone and marrow cells as a result of chronic inflammation. Continuous stimulation by various adverse factors induces an immune response that, if unchecked, creates a chronically inflamed microenvironment that inhibits bone regeneration and repair. Osteonecrosis can be triggered by drugs, alcoholism, presence of sickle cell disease, or treatment with radiotherapy or chemotherapy (1–4). Osteonecrosis can occur in many parts of the body, especially around the joints, causing the collapse of mechanically encumbered subchondral bone and secondary osteoarthritis, which in turn causes pain and dysfunction that seriously affect the patient's quality of life

and eventually require surgery (1, 5–9). Each year, 20,000-30,000 new cases of osteonecrosis of the femoral head (ONFH) are diagnosed in the United States (10, 11) and about 150,000 cases of osteonecrosis in China (10, 12). Among cancer patients who received zoledronic acid for three years, the incidence of bisphosphonate-related osteonecrosis of the jaw is approximately 1.3% to 3.2% (13). As osteonecrosis can be a slow, progressive disease, its cumulative, long-lasting consequences place a significant burden on society, especially as populations around the world live longer.

The original intention of inflammation is to remove harmful stimuli or pathogens and promote tissue repair. The inflammatory response helps recruit factors that remove necrotic bone and intramedullary tissue. Indeed, bone injury causes an inflammatory response in bone tissue that is necessary for repair. Pro-inflammatory chemokines are secreted from injured tissues to recruit macrophages, neutrophils and other immune cells to remove harmful stimuli and regulate the resolution of inflammation. Bone marrow mesenchymal stem cells are also recruited to initiate bone repair (14, 15). Under normal conditions, the inflammatory response needs to dissipate in order to give way to regenerative processes. Otherwise, inflammation can become prolonged and thus impair tissue regeneration. In osteonecrosis, the persistence of harmful factors stimulates local immune cells to continuously secrete inflammatory factors, prolonging inflammation until it becomes chronic and impairing bone repair (16–18).

Osteoimmunology is an academic discipline that studies the interactions between bone cells (e.g., osteoblasts, osteoclasts, bone marrow mesenchymal stem cells) and immune cells (e.g., macrophages, T cells, B cells, neutrophils, dendritic cells) in the same microenvironment (19–22). These interactions are mediated by cytokines and signal transduction pathways. In the past, osteonecrosis was considered to result from the death of osteoblasts and osteocytes as well as abnormal activation of osteoclasts. However, studies have found a close link between abnormal immune responses and immune cell infiltration in osteonecrotic tissues, which show signs of uncontrolled inflammation (23–28). How various immune cells regulate inflammation in osteonecrosis has not been fully elucidated. This review summarizes current knowledge about the regulation of inflammation in osteonecrosis, and how immune cells perpetuate or abrogate osteonecrosis. In this way, the review elaborates the pathophysiological mechanism of osteonecrosis from an immunological perspective.

2 Uncontrolled inflammation leads to the failure of bone repair in osteonecrosis

The healing process after bone injury can be divided into three general stages: inflammation, callus formation, and remodeling (18). Bone injury results in death of bone cells and bone marrow cells, release of platelet-derived factors and complement fragments, and damage to the extracellular matrix. The net effect is that endogenous molecules act as damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PRRs) on local cells, which in turn activates inflammatory cascades (14, 18). Stimulated cells release cytokines and chemokines that induce immune cells to release even more pro-inflammatory factors, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, C-C motif chemokine ligand 2 (CCL2) and stromal cell-derived factor 1 (SDF1) (14). This inflammatory response is a critical first step for eradicating harmful stimuli and removing cellular debris in order to help initiate the reconstruction of normal bone tissue. Inflammatory factors recruit neutrophils, macrophages, and osteoclasts to phagocytose and remove bone fragments and cell debris, while also activating mesenchymal stem cells to initiate osteogenic and angiogenic activities (14, 29–31). (Figure 1) The initial inflammatory response to bone injury usually dissipates within one week after the stimulus is removed. In the callus formation stage, bone marrow mesenchymal stem cells and osteoprogenitor cells participate in bone formation, which usually takes 1-3 months. The final remodeling stage takes months to years, during which new bone tissue is formed and shaped (14, 18).

Bone tissue repair depends on successful removal of harmful stimuli and suitable regulation of inflammation. An uncontrolled inflammatory response, either excessive or insufficient, is deleterious to bone repair. In the case of excessive inflammation, an overabundance of reactive oxygen species is produced, and proteases that damage the surrounding normal tissue are activated (32). Persistently high levels of inflammation inhibit the normal osteogenic response (16, 33). In the early stage of bone injury, transient signaling by TNF-α and IL-6 recruit the progenitors of osteoblasts required for bone regeneration, but persistently high levels of TNF-α and IL-6 inhibit osteogenesis and further damage bone tissue (14, 34). Excessive inflammation also stimulates osteoclast differentiation and activation, resulting in inflammatory osteolysis. Conversely, when the inflammatory response to bone injury is insufficient, local dead cell debris and bone debris are not completely removed, allowing DAMPs to persist in the microenvironment (14, 16). In either case, an excessive or insufficient inflammatory reaction eventually translates to chronic inflammation, which is the bridge between bone injury and osteonecrosis. Chronic inflammation hinders bone repair and regeneration following bone injury, which finally leads to osteonecrosis (10, 14, 16, 35–40). (Figure 2)

3 Immune cells and osteonecrosis

Chronic inflammation, the most prominent feature of osteonecrosis, occurs when inflammation prolongs resulting

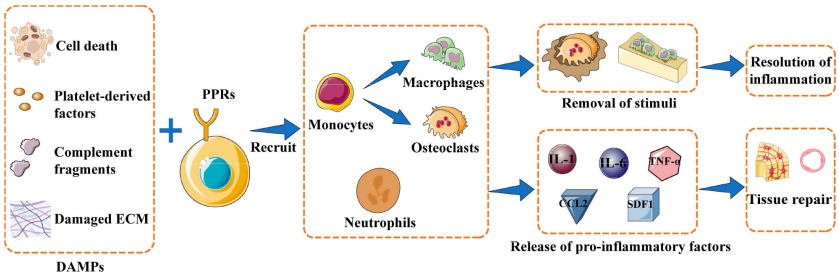

FIGURE 1
Inflammation initiates bone repair. When bone injury occurs, damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs) expressed on the surface of local cells. These cells are activated to release inflammatory factors that recruit immune cells, which can phagocytose bone fragments and cell debris or produce pro-inflammatory factors to recruit mesenchymal stem cells and initiate osteogenesis and angiogenesis. The overall result is resolution of inflammation and new bone tissue. Abbreviations: CCL2, C-C motif chemokine ligand 2; ECM, extracellular matrix; IL-1, interleukin 1; IL-6, interleukin 6; PMN, polymorphonuclear leukocytes; SDF1, stromal cell-derived factor 1; TNF-α, tumor necrosis factor–alpha.

from the impaired resolution program (41–47). Persistent production of pro-inflammatory cytokines, progressive tissue injury and aberrant tissue remodeling are vital characteristics of this process (46, 48). In necrotic bone tissue, inflammatory cytokines/chemokines continuously recruit innate immune cells (macrophages, neutrophils, dendritic cells) and adaptive immune cells (T cells and B cells), which further release inflammatory factors in a positive feedback loop in order to amplify the overall inflammatory response (19, 20, 49). Furthermore, chronic inflammation excessively activates bone resorption and inhibits bone formation, driving osteonecrosis. In this way, disruption of the normal coordination between pro-inflammatory activation and anti-inflammatory silencing during bone repair may be the pathophysiological basis of osteonecrosis. Given that immune cells are the most important "modulators" of inflammation, elucidating how innate and adaptive immune cells regulate inflammation associated with osteonecrosis could provide insights into its pathogenesis and treatment.

3.1 Innate immune cells in osteonecrosis

3.1.1 Macrophages

Macrophages are sentinels of the immune system. They identify and remove pathogens, kill target cells, present antigens, and regulate immune functions (50, 51). Macrophages differentiate mainly from monocytes and can be

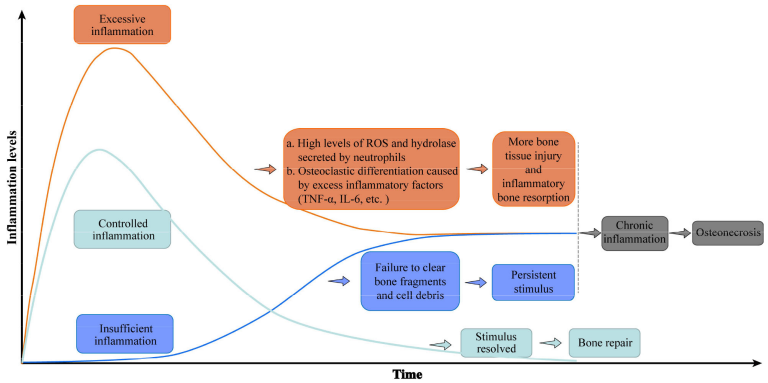

FIGURE 2
Uncontrolled inflammation promotes osteonecrosis. A controlled inflammatory response to bone injury activates immune cells to remove damaged tissue, then returns to baseline levels conducive to bone regeneration. Excessive inflammation maintains high levels of inflammatory factors that further destroy bone, while an insufficient inflammatory response fails to clear immune-activating factors. Either inflammatory disorder eventually leads to chronic inflammation and osteonecrosis. The green curve represents the change in the inflammatory level of controlled inflammation over time, while the orange and blue curves represent the inflammation level of excessive inflammation and insufficient inflammation, respectively. Abbreviations: IL-6, interleukin 6; ROS, reactive oxygen species; TNF-a, tumor necrosis factor–alpha.

divided into classically activated macrophages (M1 phenotype) or alternatively activated macrophages (M2 phenotype) (50, 51). After bone injury, DAMPs released by bone and marrow cells recruit macrophages to the injured area and polarize them to the M1 phenotype, leading them to secrete pro-inflammatory factors such as TNF-α, IL-1β, and IL-6, which initiate and maintain inflammation (52–54). Four to seven days after bone tissue injury, secretion of anti-inflammatory factors such as tumor growth factor (TGF)-β and IL-10 into the microenvironment polarize M1 macrophages to the M2 phenotype. This shift in phenotype helps resolve inflammation, promotes secretion of mineralized matrix by bone marrow mesenchymal stem cells, and induces expression of the osteogenic factors alkaline phosphatase and osteocalcin, which enhance the osteogenic activity of osteoblasts. At the same time, anti-inflammatory factors inhibit osteoclast-mediated bone resorption, further supporting bone tissue repair (14, 55, 56). The regeneration and repair of bone tissue after injury depend on the precise order of macrophage polarization from M1 to M2.

In osteonecrosis, macrophages become locked in the M1 phenotype and continue to release inflammatory factors that exacerbate the initial tissue injury. Animal models of osteonecrosis showed high numbers of macrophage infiltration in areas with osteonecrosis, high ratio of M1 to M2 macrophages, and significant upregulation of pro-inflammatory factors TGF-β, IL-1β and IL-6 (57–59). Interestingly, a recent study of specimens from patients with non-traumatic ONFH also found that the main macrophage subset in the osteonecrosis area had the M1 phenotype, the local microenvironment was enriched with IL-1β and IL-6, and the ratio of M1 to M2 macrophages was significantly increased as osteonecrosis progressed (35). Inhibiting M1 macrophage polarization and reducing the M1/M2 ratio in femoral head and jaw reduced the secretion of local pro-inflammatory factors and the apoptosis of bone cells caused by inflammation, relieving steroid-induced osteonecrosis of the femoral head (SONFH) and bisphosphonate-related osteonecrosis of the jaw to some extent (60, 61). In addition, specifically regulating macrophage polarization from M1 to M2 to reduce the M1/M2 ratio downregulated the expression of pro-inflammatory factors in the osteonecrotic area, promoted the secretion of anti-inflammatory factors such as TGF-β and IL-10, reduced osteocyte apoptosis and promoted bone formation, allowing the regeneration and repair of necrotic bone tissue to a certain extent (62, 63). These findings suggest that M1 macrophage enrichment is an important osteoimmune feature of osteonecrosis and that targeting M1 macrophages is a promising therapeutic approach to treating osteonecrosis.

Strategies employed so far have targeted the upstream signaling pathways responsible for M1 polarization. Extracellular DAMPs released from injured bone bind to pattern recognition receptor toll-like receptor 4 (TLR4) on cell membranes and thereby activate the TLR4/MyD88/NF-κB signaling pathway, which promotes macrophage recruitment and M1 polarization (57, 64–66). Inhibition of TLR4/MyD88/NF-κB signaling *in vivo* by calycosin or TLR-4 inhibitor TAK-242 reduced the expression of various pro-inflammatory factors and promoted bone formation, effectively alleviating osteonecrosis in animals with SONFH and in bisphosphonate-related osteonecrosis of the jaw (33, 61, 66). On the other hand, some extracellular pro-inflammatory factors could activate the JAK/STAT1 pathway, which is another important pathway to promote M1 macrophage polarization (61, 67). Inhibition of the JAK/STAT1 pathway by using IL-17 inhibitor or curcumin inhibited the polarization of M1 macrophages in mice, significantly reduced the ratio of M1 to M2 macrophages, and prevented inflammatory-mediated apoptosis of osteocytes (60, 61). Therefore, methods to inhibit M1 polarization need to be further explored in order to develop potential therapeutic strategies for osteonecrosis. (Figure 3)

3.1.2 Neutrophils

Neutrophils are derived from hematopoietic stem cells and mainly circulate in the peripheral blood. They have strong chemotactic and phagocytic properties (68). Once recruited to sites of bone injury, neutrophils secrete inflammatory and chemotactic mediators, such as IL-6 and MCP-1, which further recruit monocytes and macrophages (14).

The ability of neutrophils to promote inflammation in necrotic bone tissue is one of the important causes of osteonecrosis. Strong neutrophil infiltration occurs within one week of injury in ischemic osteonecrosis, but then neutrophil numbers taper off over time, although a low number persists in the microenvironment. These remaining neutrophils foster the occurrence and development of osteonecrosis through immune regulation of acute and chronic inflammation (49). The percentage of neutrophils in blood has been associated with the severity of SONFH, which may be because neutrophils promote osteoclast formation to accelerate bone resorption (23, 68). At the same time, neutrophils activated by necrotic tissue secrete neutrophil extracellular traps (NETs), which directly or indirectly induce the secretion of inflammatory factors (69–71). In ONFH patients, neutrophils are enriched in femoral head microvessels and the corresponding NETs interfere with blood flow, resulting in ischemic necrosis (69). Further studies in rats found that intravenous administration of neutrophils capable of forming NETs promoted the development of SONFH (69). Given the deleterious role of neutrophils in osteonecrosis, the removal of neutrophils may be a treatment for osteonecrosis. (Figure 3)

3.1.3 Dendritic cells

In innate immunity, the main functions of dendritic cells (DCs) are phagocytosis and antigen presentation. DCs express a large number of PRRs, such as TLRs, C-type lectin receptors and NOD-like receptors, which recognize various DAMPs and

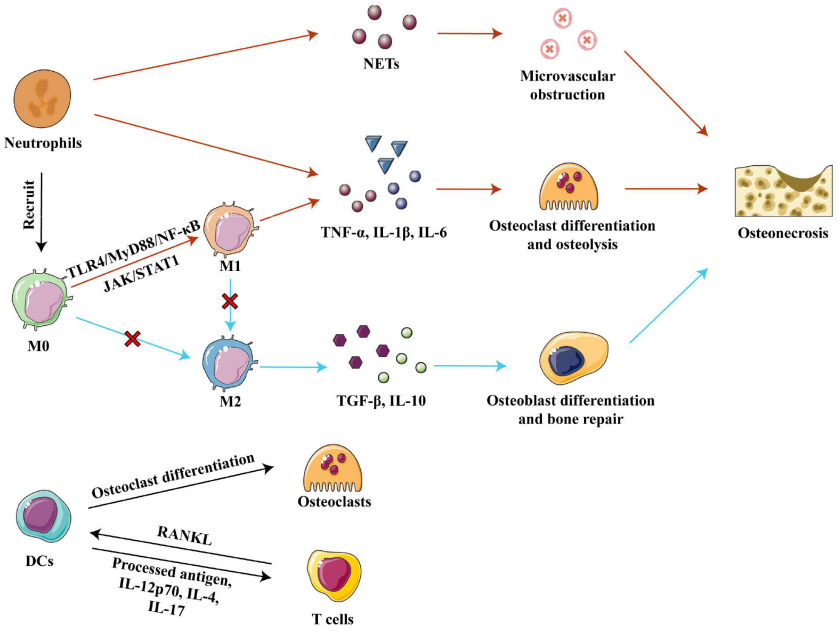

FIGURE 3
Innate immune cells in osteonecrosis. Neutrophils cause microvascular blockage and osteolysis by secreting, respectively, NETs and pro-inflammatory factors, resulting in osteonecrosis of the femoral head. Activation of the TLR4/MyD88/NF- κB and JAK/STAT1 pathways polarizes macrophages to the M1 phenotype, and they secrete inflammatory factors TNF-α, IL-1β and IL-6 to promote osteoclast differentiation and osteolysis. In osteonecrosis, macrophage polarization to the M2 phenotype is blocked, further impairing bone repair. DCs can differentiate into osteoclasts and participate in bone remodeling under the stimulation of RANKL secreted by T cells. DCs present processed antigens and secrete inflammatory factors that affect T cell differentiation. Abbreviations: DCs, dendritic cells; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-12 p70, interleukin 12 p70; IL-17, interleukin 17; M0, Macrophages; M1, classically activated macrophages; M2, alternatively activated macrophages; NETs, neutrophil extracellular traps; RANKL, receptor activator of nuclear factor kappa-B ligand; TGF-β, tumor growth factor beta; TNF-α, tumor necrosis factor–alpha.

pathogen-associated molecular patterns and quickly amplify local immune responses (72, 73). The contribution of DCs in osteoimmunology is two-fold: (1) DCs can differentiate into osteoclasts when stimulated by receptor activator of nuclear factor kappa-B ligand (RANKL) released from T cells, and the new osteoclasts participate in local bone remodeling; and (2) DCs can heavily influence the type of T cell responses by presenting processed antigen *via* major histocompatibility complex (MHC) class I and class II molecules, or by secreting pro- or anti-inflammatory cytokines such as IL-12 p70, IL-4, and IL-17 (45, 73, 74). (Figure 3) Which T-cell subtypes become involved and whether their net effect is to exacerbate or mitigate osteonecrosis will be discussed later in this review.

DCs serve as an important link between innate and adaptive immune responses by maintaining osteoimmune homeostasis. In contrast to the other innate immune cells, DCs may actually ameliorate osteonecrosis. In a mouse model, bisphosphonates impaired DC differentiation, maturation, migration and antigen presentation, ultimately inhibiting T cell activation and local immune responses, which translated to a higher risk of osteonecrosis of the jaw (75, 76). Two bioinformatic analyses showed decreased infiltration of activated DCs in ONFH (23, 27). These observations suggest that osteonecrosis may be due in part to DCs deficiency that impairs osteoimmune functions.

3.2 Adaptive immune cells in osteonecrosis

3.2.1 T cells

T cells or T lymphocytes are an important component of cell-mediated adaptive immunity, and antigen-specific receptors on their surface can recognize antigens that antigen-presenting cells display on MHC complexes (77, 78). T cells can be divided into several subgroups based on their functions, and these subgroups can influence bone homeostasis. Various T cell subtypes work together to maintain the balance between osteogenic and osteoclastic metabolism by secreting osteoprotegerin (OPG) and RANKL or regulating the local inflammatory microenvironment, which in turn affects bone metabolism (77–79).

Interestingly, T helper (Th) cells and cytotoxic T lymphocytes (CTLs) contribute to the progression of

osteonecrosis, while regulatory T cells (Tregs) alleviate it. Th17 cells are enriched and activated in local tissues of ONFH and osteonecrosis of the jaw, and Th17 cells secrete IL-17 to maintain a chronic inflammatory microenvironment (80). IL-9 secreted by Th2, Th9 and Th17 cells upregulates inflammatory factors and enzymes related to cartilage degradation, promoting ONFH progression (42, 81). High numbers of CTLs infiltrate areas of osteonecrosis and contribute to it (24). They promote interactions between T cells and osteoclasts and enhance the activity of osteoclasts by secreting cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (79). Conversely, Tregs may play a positive role in osteonecrosis, unlike Th and CTLs. The number of Tregs was found reduced in areas of osteonecrosis in mice (82). Further research found that Tregs secrete anti-inflammatory factors such as IL-4, IL-10 and TGF-β in non-traumatic ONFH in order to promote the resolution of inflammation while inhibiting osteoclast activity and osteolysis (79). Therefore, regulating the differentiation of T cells may be a strategy to treat osteonecrosis. (Figure 4)

3.2.2 B cells

B cells or B lymphocytes secrete antibody molecules to initiate adaptive humoral immune responses and present antigens to activate specific T cell immunity (83, 84). B cells help maintain a normal bone microenvironment, and abnormal numbers of some B cell subtypes may be associated with osteonecrosis. Compared to healthy people, ONFH patients show significantly higher numbers of CD5+CD19+ B1 cells, CD86+CD19+ and CD95+CD19+ activated B cells, and CD27+CD95+CD19+ memory B cells in the blood (79, 85). Conversely, osteonecrotic tissue shows local decreases in the number of memory B cells and the total number of B cells (86). These observations emphasize the importance of B cells in maintaining the normal bone microenvironment and the ability of different B cell subtypes to influence the progression of osteonecrosis.

Different subtypes of B cells regulate bone metabolism by exerting different regulatory effects on osteogenic and osteoclast metabolism. Regulatory B cells (Breg) are a newly discovered

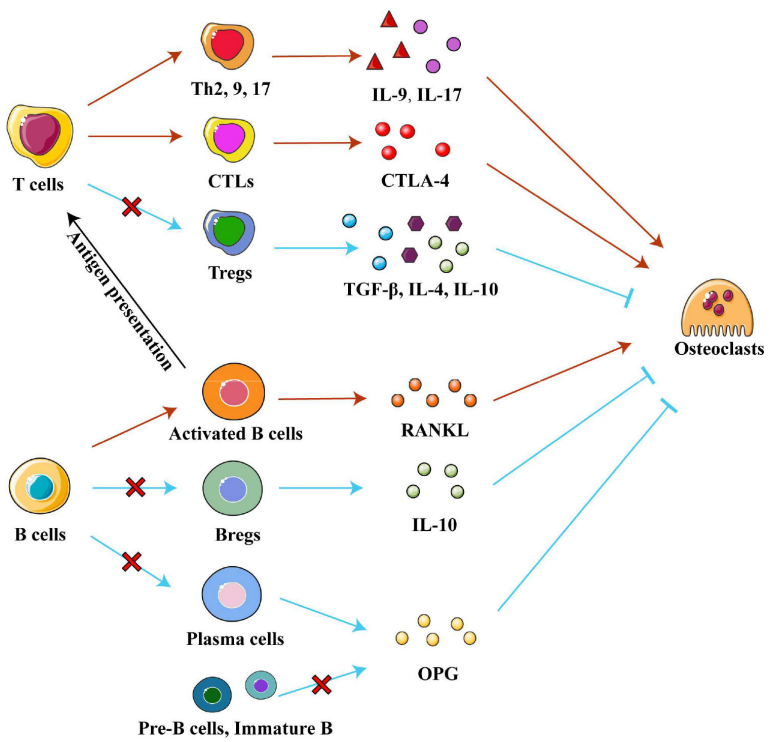

FIGURE 4
Adaptive immune cells in osteonecrosis. T cells can differentiate into the T helper cells (Th), cytotoxic T lymphocytes (CTLs) and regulatory T cell (Tregs) subtypes, which secrete various cytokines to influence chronic inflammation and osteoclast differentiation in different ways. Pre-B-cells and immature B cells are found only in bone marrow, while Bregs, plasma cells and activated B cells are recruited into osteonecrosis tissue. Activated B cells affect differentiation of T cell subtypes by presenting processed antigens and secrete RANKL to promote osteoclast differentiation. Bregs, plasma cells, Pre-B-cells and immature B cells secrete IL-10 and OPG respectively to inhibit osteoclast differentiation. Abbreviations: Bregs, regulatory B cells; CTLA-4, cytotoxic T lymphocyte-associated protein 4; IL-4, interleukin 4; IL-9, interleukin 9; IL-10, interleukin 10; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TGF-β, tumor growth factor beta.

subpopulation of B cells, which can secrete the anti-inflammatory factor IL-10 and inhibit osteoclast differentiation (83, 87, 88). An *in vivo* study found that low levels of Bregs led to low levels of IL-10 and activation of osteoclastic metabolism (88). In an *in vitro* study, regulating Breg differentiation reduced the levels of IL-6, IL-17 and TNF-α as well as promoted Treg differentiation (87, 88). In addition, OPG/RANKL system is another pathway through which B cells affect bone metabolism. Pre-B cells, immature B cells, and antibody-secreting B cells (plasma cells) inhibit osteoclast differentiation by producing copious amounts of OPG to block the RANK/RANKL system. (Indeed, this OPG production accounts for 40-60% of total OPG in the bone marrow.) On the contrary, activated B cells secrete RANKL under pro-inflammatory conditions to activate osteoclast formation (89–91). Boosting beneficial B cell subtypes over detrimental subtypes may be a treatment for osteonecrosis, which future studies should explore. (Figure 4)

4 Conclusion

During the development of osteonecrosis, necrotic bone damages local immune function, which leads to uncontrolled inflammation that creates a chronic inflammatory microenvironment, hindering bone regeneration and repair. This review summarizes the importance of immune cells and the regulation of their inflammatory responses in the pathogenesis of osteonecrosis on the basis of several original theories of osteonecrosis. It explains the pathophysiological mechanism of osteonecrosis from an immunological perspective according to the literature.

The immune system clearly exerts complex, pleiotropic effects on the development and severity of osteonecrosis. Abnormal infiltration of injured bone by M1 macrophages, neutrophils, and certain T cell subsets worsens disease by creating an abundance of pro-inflammatory factors, while DCs, Bregs and Tregs dampen immune responses by secreting anti-inflammatory and osteoclast-inhibiting factors. Despite these insights, we still do not understand the role of most immune cells in the progression of osteonecrosis. This will require making sense of how specific environmental cues influence the differentiation of immune cell subtypes and sub-lineages, and how these various subpopulations communicate with one another. The cellular heterogeneity in bone will make this work particularly challenging. Nevertheless, such research is quite important for the development of potential treatments for osteonecrosis.

Author contributions

ZT conceived the manuscript. ZT and JZ drafted the manuscript. JZ designed the figures. ZY provided valuable comments. LX and DW revised the manuscript critically for important intellectual content. ZT performed manuscript review and final version approval. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The potential role of cfDNA-related innate immune responses in postoperative bone loss after alveolar bone grafting

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The purpose of treating alveolar bone cleft is to restore a normal maxilla structure. Multiple factors have been identified that can affect the success of alveolar bone grafting. However, with consistent treatment modifications, the surgical outcomes have been improved, but alveolar bone loss still exists. Thus, a new aspect should be found to solve this problem. As alveolar bone belongs to the periodontal tissues, the mechanism of the alveolar bone loss after bone grafting in patients with alveolar bone cleft may be similar to the development of alveolar bone loss in periodontitis. Cell-free DNA (cfDNA) has been demonstrated as a key promoter of alveolar bone loss during periodontal inflammation. We hypothesized that cfDNA-related innate immune responses could be a major inducement for postoperative bone loss after alveolar bone grafting. In this perspective, we preliminarily proved the potential association between cfDNA, TLR9 pathway, and alveolar bone grafting operation, and it might verify that surgical trauma could accumulate cfDNA, which can further activate cellular TLR9 signaling.

KEYWORDS

alveolar bone cleft, alveolar bone grafting, innate immune response, cell free DNA, TLR9, proinflammation

Introduction

Patients with alveolar bone cleft need alveolar bone grafting to restore a normal maxilla structure, and the grafting of autogenous bone like iliac bone is still the most common choice (1, 2). However, bone loss after the surgery happens a lot (3). Clinical studies demonstrated that the operation age (1, 4), the structure of the alveolar cleft (5–7),

and the pre- and post-operative maneuvers, especially poor management of oral hygiene (3), can affect the final outcomes of bone grafting. With consistent modifications of the treatment, the surgical outcomes have been improved, but the alveolar bone loss still exists (8, 9).

As alveolar bone belongs to the periodontal tissues, we hypothesize that the mechanism of the alveolar bone loss after bone grafting surgery might be similar to the development of alveolar bone loss in periodontitis. In periodontitis, tartar (mineralized plaque, soft scale, and food residue around the gingival sulcus) is the pathogenic factor that initiates the periodontal innate immune response and leads to inflammatory alveolar bone loss (10). For tartar, oral hygiene helps remove plaque and keep the tartar away (11), which will inhibit the innate immune response and stop the progress of inflammatory bone loss (12). Periodontal inflammation and related tissue destruction are more severer in patients with alveolar bone cleft than in those without alveolar bone cleft (13, 14), and the structure of alveolar defect can affect oral hygiene, then adversely exacerbate the periodontal status (15). Therefore, the local environment of the cleft is risky for enhancing bone loss. Oral hygiene, which can eliminate the local stimulus for periodontal inflammation, helps avoid bone grafting failure (16), which preliminarily supports our hypothesis that inhibition of local inflammation and innate immune responses could benefit bone grafting treatment.

Cell-free DNA (cfDNA)-related innate immune response is a key promoter to the progress of alveolar bone loss when periodontal inflammation happens (17, 18). Cell-free DNA (cfDNA) includes endogenous nuclear and mitochondrial DNA, and exogenous bacterial or viral DNA (19, 20). cfDNA plays the role of the ligands to DNA-sensing pathways, such as Toll-like Receptor 9 (TLR9), which can initiate the innate immune response, activate NF-κB signaling that leads to the secretion of proinflammatory cytokines like TNF-α, and cause inflammatory alveolar bone loss (17). In patients with periodontitis, cfDNA level in the gingival crevicular fluid is correlated with the degree of periodontitis (21–23). We recently confirmed that clearance of cfDNA can help alleviate alveolar bone loss by inhibiting TLR9 activation (17). As we have demonstrated the possible similarity between bone loss after bone grafting and the development of alveolar bone loss in periodontitis, herein, we hypothesize that cfDNA- and TLR9-related innate immune responses can also take part in the postoperative bone loss after alveolar bone grafting.

Postoperative bone loss after bone grafting possibly happens as the following: (1) surgery leads to sterile Inflammation, which increases the levels of damage-associated molecular patterns (DAMPs) (24, 25); (2) It is impossible to be a totally sterile environment in oral and maxillofacial surgery (26), which can lead to the increasing levels of pathogen-associated molecular patterns (PAMPs); (3) cfDNA levels will be increased because of the accumulation of DAMPs and PAMPs, and will consequently activate the TLR9/NF-κB pathway (19, 20) and may lead to the bone loss after alveolar bone grafting. In this perspective, we try to preliminarily demonstrate that cfDNA- and TLR9-related innate immune responses could happen after alveolar bone grafting in patients with alveolar bone cleft, which possibly is associated with postoperative bone loss, by showing pilot analyses of the pre- and post-operative levels of cfDNA in the gingival crevicular fluid (GCF) and serum of patients and use *in vitro* study to confirm that the cfDNA- and TLR9-related innate immune response can be activated after bone grafting surgery.

cfDNA-related innate immune responses after alveolar bone grafting

Increasing cfDNA levels after alveolar bone grafting in GCF and serum of patients

To determine whether cfDNA levels were increased in the body fluids of patients after alveolar bone grafting, 16 patients with alveolar bone cleft and without obvious periodontal inflammation were enrolled in this study. Patients were asked to have oral hygiene one month before the surgery, and the periodontal health of all patients with intact periodontium had no probing attachment loss, probing pocket depth ≤ 3mm, bleeding on probing < 10%, and no radiological bone loss (27). All the patients finished their cleft lip repair and palatoplasty before 2 years old. The surgery for the alveolar bone grafting was performed by the same surgeon, and the bone for grafting was collected from the iliac bone. Patient sample collection was performed with the approval of the Ethics Committee of West China Hospital of Stomatology, Sichuan University (WCHSIRB-CT-2020-272). All participants in this study signed an informed consent form before sample collection.

GCF and serum sampling were conducted before (preoperative) and 2 days after alveolar bone grafting (postoperative) (17, 18, 28). GCF sampling was performed on the teeth nearest to the cleft and surgical sites, which indicated the local inflammatory environment change at the surgical sites. The serum might demonstrate possible inflammatory environment change of the whole body because of the surgery, as surgery can cause damage to the tissue and lead to sterile inflammation. Extraction of cfDNA from GCF and serum was performed with a DNeasy Blood & Tissue Kit (QIAGEN, Germany). Concentrations of cfDNA in GCF and serum were measured with a Quant-iT PicoGreen double-stranded DNA Assay Kit. The statistical analyses were accomplished by Prism 8

(GraphPad). Paired t-test was used to compare the mean value between the two groups.

We found that 2 days after alveolar bone grafting, which involved the trauma to the periodontal tissues near the cleft and the trauma to the iliac bone, cfDNA levels in the GCF and serum increased significantly, while the increase was more significant in GCF (Figures 1A, B). Herein we confirmed that cfDNA could be associated with surgery, and the changing of cfDNA levels might be imputed to surgical damage to the local tissues and the potential infection in the oral environment. Based on the results, with cfDNA increasing in local sites of GCF, the following cfDNA-induced inflammation can happen, so then we carried out the *in vitro* study for preliminary exploration.

Cellular TLR9 signaling activated by the body fluids of the patients after alveolar bone grafting

Next, we evaluated whether the GCF and serum could lead to higher activation of the cellular TLR9 signaling. Stable hTLR9-overexpressing HEK-Blue cells were purchased from *In vivo*Gen (San Diego, CA, U.S.A.) and were initially propagated in DMEM with 10% (v/v) FBS and maintained in growth medium supplemented with selective antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin, 2 mM L-glutamine). Before treatment, certain numbers of HEK-Blue hTLR9 cells (8×10^4 cells/well hTLR9 cells) were seeded and cultured in

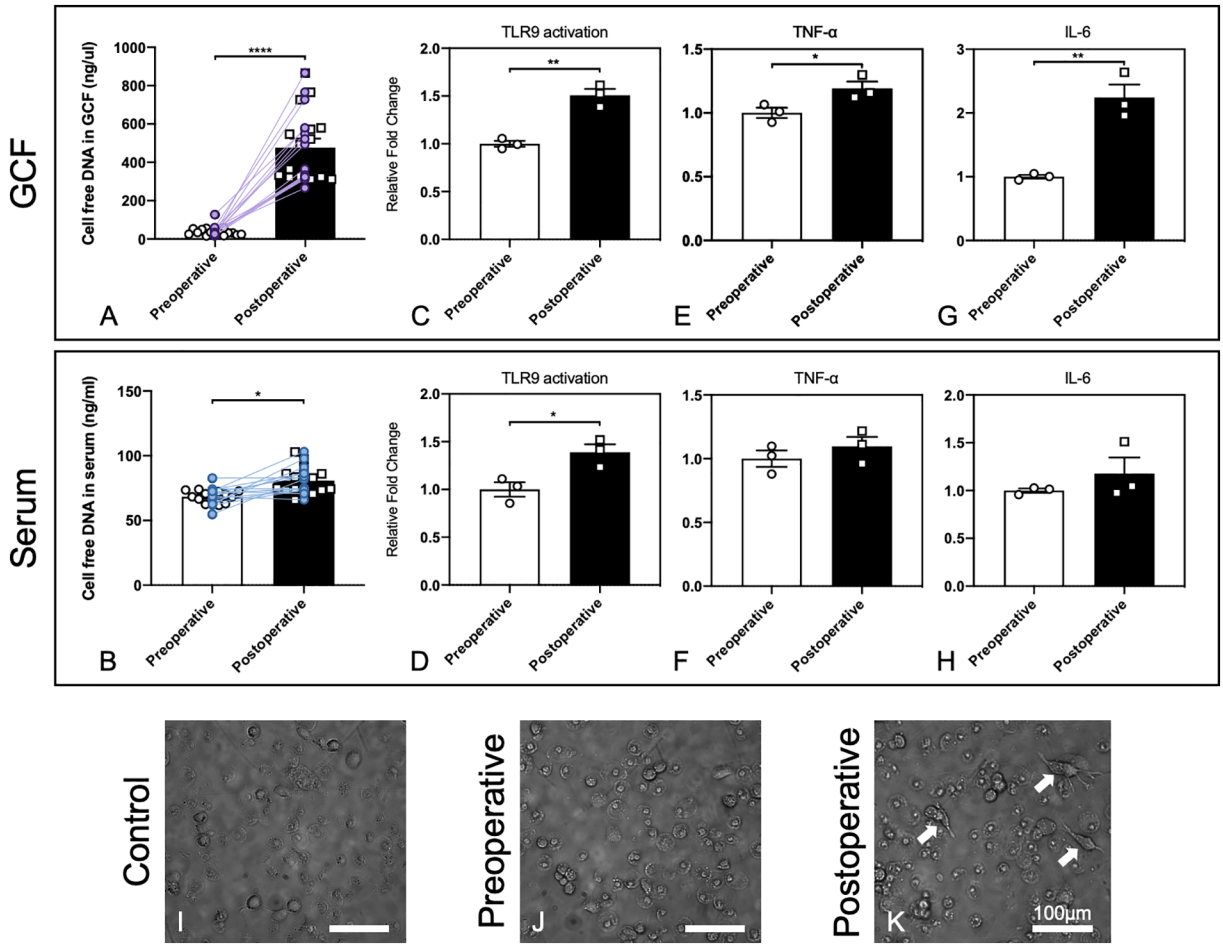

FIGURE 1
GCF and serum in patients after alveolar bone grafting induced stronger cfDNA-related innate immune responses. **(A, B)** Increasing cfDNA levels after alveolar bone grafting in GCF and serum of patients with alveolar bone cleft. Data are means ± SEM; $*P < 0.05$, $****P < 0.0001$ assessed by paired t-test (n = 16). **(C, D)** Activation of HEK-TLR9 reporter cells by GCF and serum from patients with alveolar bone cleft before and after alveolar bone grafting. Data are means ± SEM; $*P < 0.05$, $**P < 0.01$ by Student's *t*-test ($n = 3$). **(E, F)** TNF-α expression activated by GCF and serum from patients with alveolar bone cleft before and after alveolar bone grafting. Data are means ± SEM; $*P < 0.05$ by Student's *t*-test ($n = 3$). **(G, H)** IL-6 expression activated by GCF and serum from patients with alveolar bone cleft before and after alveolar bone grafting. Data are means ± SEM; $**P < 0.01$ by Student's *t*-test ($n = 3$). **(I–K)** Morphological changes of macrophages by patients' GCF after alveolar bone grafting. Arrows show significant morphological changes in cells. Scale bar, 100 µm.

basal DMEM overnight in 96-well plates, then stimulated with one microliter of human GCF and 20 μL of human serum from the patient who had alveolar bone grafting pre- and 2 days postoperatively, respectively. After 24 h, the activation of reporter cells was determined with the QUANTI-Blue medium with testing the secreted embryonic alkaline phosphatase (SEAP) activity. Student's t-test was used to compare the mean value between the two groups. Different compositions and levels of cfDNA can lead to different levels of TLR9 response, so the higher cellular activation of HEK-Blue TLR9 cells means the cfDNA in the GCF and serum can stimulate higher TLR9 activation (17). Our results demonstrated that 2-days-postoperative GCF and serum induced significantly higher TLR9 activation in HEK-Blue TLR9 cells than the GCF and serum from the preoperative (Figure 1C, D), which verified that cfDNA from 2-days-postoperative GCF and serum could possibly cause a more significant proinflammatory response by inducing TLR9 pathway.

We then tested whether 2-days-postoperative GCF and serum caused a prominent increase in TNF-α and IL-6 levels in RAW 264.7 macrophages than the GCF and serum from the preoperative. RAW 264.7 cells were seeded and cultured in basal DMEM overnight at 2×10^4 cells per well in a 96-well plate. One microliter of human GCF and 20 μL of human serum were then added into the well. After incubation for 24 h, the supernatants were collected and TNF-α and IL-6 levels were measured using ELISA kits purchased from Thermo Scientific (Waltham, Massachusetts, U.S.A). Paired t-test was used to compare the mean value between the two groups. The outcomes demonstrated that both TNF-α and IL-6 levels were increased, while the increase was significant by stimuli of GCF (Figure 1E–H). Together, these results suggested that cellular TLR9 signaling can be activated by the body fluids of the patients after alveolar bone grafting.

Morphological changes of macrophages by the patients' GCF after alveolar bone grafting

Macrophage polarization could be affected by the microenvironment in the periodontal tissues, and the phenotypes of macrophages could determine the final osteogenesis of the alveolar bone (29). Thus, we carried out a preliminary experiment by observing the morphological changes of macrophages by the stimuli of preoperative and postoperative GCF following the published protocol (17). Human monocyte THP-1 cells were purchased from ATCC (Manassas, VA, U.S.A.) and applied. THP-1 cells were cultured in RPMI-1640 media

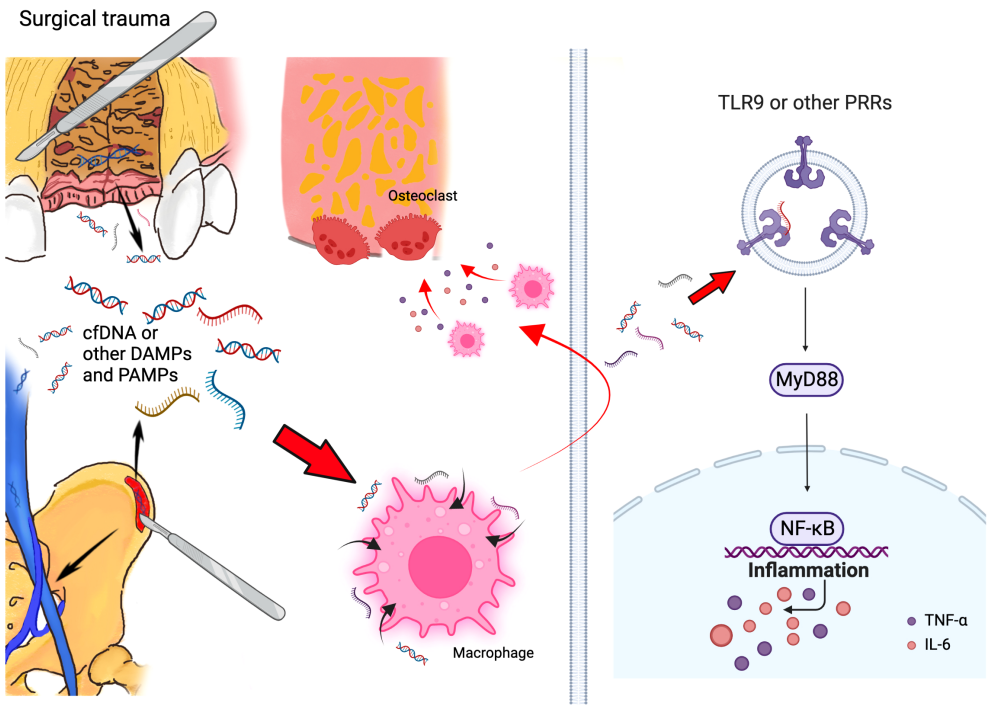

FIGURE 2
Schematic of mechanism that cfDNA and other DAMPs and PAMPs can promote bone loss initiated by surgical trauma during alveolar bone grafting. Surgery can accumulated cfDNA and other DAMPs and PAMPs, and these molecules can consistently activate the TLR9 and other PRR pathways, which activate the innate immune response and lead to bone loss after alveolar bone grafting. (Created with BioRender.com).

supplemented with 10% FBS and selective antibiotics. 8×10^4 cells were plated in 96-well plates in 200 μL RPMI media plus 25 ng/mL phorbol myristate acetate (PMA) for 48 h, and one microliter of pre- and post-operative GCF was added during the final 18 h of treatment. After incubation, the morphology of cells was observed, which was altered by treatment with postoperative GCF, and dendrite-like change could be found (Figures 1I–K). This finding demonstrated postoperative GCF might have more stimulus that could alter the phenotypes of macrophages, but needed further investigation to confirm this hypothesis.

Discussion and perspectives

Alveolar bone cleft is one of the most common craniofacial birth defects, often companied by cleft lip and palate (30). Alveolar bone cleft can influence the development of tooth and dental germ, including the quantity, morphology and position of tooth (31–33). Alveolar bone grafting is the standard treatment of clinics for alveolar bone cleft at present (34). A successful alveolar bone grafting has several purposes, including the bony continuity in the maxillary arch (5, 6), the stabilization of maxillary dental arch (7), the preservation for periodontal health of adjacent teeth (35, 36), the induction of permanent tooth eruption (1, 4) and implant placement (37). For getting successful operation outcomes, it's indispensable to comprehend how multiple factors influence the surgical outcome.

Significant controversy for influence factors to a successful operation exists, in which the operation age (38), the cleft width (1, 39) and the cleft volume (40), presence of the lateral incisor, and the eruption and root development of the cleft-adjacent canine (41) are in a heated discussion. However, even though the aforementioned factors have been taken care of, the improvement in surgical outcomes was not significant yet, and postoperative alveolar bone loss still exists. Recently, poor oral hygiene became another hotspot in the success of alveolar cleft reconstruction surgery, which was similar to periodontitis (3, 16, 42). Thus, a new aspect based on this concept could be the potential for solving this problem.

As alveolar bone belongs to the periodontal tissues, we hypothesize that the mechanism of the alveolar bone loss in patients with alveolar bone cleft after bone grafting surgery may be similar to the development of alveolar bone loss in periodontitis. Another inflammation, peri-implantitis, which also happens in periodontal tissue, should also be mentioned in terms of our concept. It was found that a more pronounced inflammatory response was expressed in peri-implantitis than in periodontitis, which caused alveolar bone loss and failure of implant treatments (43, 44).

From our perspective, the progress of alveolar bone loss in surgical treatment for alveolar bone cleft can be similar to periodontitis and peri-implantitis. In periodontitis and peri-implantitis, during the innate immune response, the levels of PAMPs increase with dying bacteria (45); meanwhile, local inflammation causes cell death and accumulates DAMPs (46). Thus, in the inflammatory microenvironment of periodontitis and peri-implantitis, a collection of both PAMPs and DAMPs could continuously activate the immune systems and promote alveolar bone loss. In the situation of alveolar bone grafting in patients with alveolar bone cleft, surgeries in both the alveolar region and iliac bone region might contribute to the increase of DAMPs, accompanied by PAMPs generated in the oral cavity, which triggered the local immune response together and led to the postoperative alveolar bone loss (Figure 2).

Pattern-recognition receptors (PRRs), such as TLRs, which detect DAMPs and PAMPs, can initiate innate immune response (47, 48). Inappropriate activation of TLR9 happened in patients with periodontitis (49), as increased TLR9 levels can be found in their periodontal tissue (50). Meanwhile, TLR9-deficient mice are resistant to periodontitis (51, 52). We have confirmed that cfDNA can be a major source that enhances periodontal tissue destruction by activating TLR9 pathway, and targeting cfDNA and TLR9 pathway can help ameliorate periodontitis (17). Thus, based on the possible similarity between postoperative alveolar bone loss and periodontitis, we assumed that cfDNA- and TLR9-related innate immune responses could be a major inducement for postoperative bone loss after alveolar bone grafting. According to our outcomes, we preliminarily proved the potential association between cfDNA, TLR9 pathway, and alveolar bone grafting operation: Surgical trauma could accumulate cfDNA, and activate cellular TLR9 signaling *in vitro*.

Macrophages in the mononuclear phagocyte system are important in periodontal inflammation, as M1/M2 phenotypes can switch dynamically with the progression of periodontitis (53–55). We also observed morphological changes in macrophages with the stimuli of postoperative GCF, which is similar to the situation of periodontitis (17), which could be related to the M1/M2 phenotypes alteration. However, further study should be carried out to confirm this concept.

In this perspective, we hypothesize the potential enhancement by DNA sensing and TLR9-related innate immune responses to postoperative bone loss, and further experiments are necessary to elucidate the association between cfDNA, TLR9 pathway, and alveolar bone grafting operation associated with surgical trauma. Meanwhile, other PRR-related pathways should also be investigated in further study. For example, the TLR2 pathway has been confirmed both in the pathogenesis of periodontitis and peri-implantitis (56, 57); LPS and TLR4 pathway has been widely studied for the regulation in periodontitis and peri-implantitis (43, 56); and also other PRRs pathways should be considered (47, 48). Similarly, multiple cells, such as natural killer cells, mast cells, and neutrophils, should also be studied in the future as they are involved in innate immune responses (58). For the polarization of macrophages, further study should be concentrated on detecting phenotype markers by histology and flow cytometry.

In summary, inflammation can be a potential source and target for managing postoperative bone loss after alveolar bone grafting.

Concluding remarks

From this perspective, we propose that cfDNA can be the major source that enhances postoperative bone loss after alveolar bone grafting in patients with alveolar bone cleft, and targeting cfDNA and related pathways could be the potential therapeutic strategy to improve the treatment for patients with alveolar bone cleft.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of West China Hospital of Stomatology, Sichuan University (WCHSIRB-CT-2020-272). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

HH and RY contributed to the collection of data, analyses of the data, and writing and revising of the paper. HH and BS supervised the research. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Osteoimmune regulation underlies oral implant osseointegration and its perturbation

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In the field of biomaterials, an endosseous implant is now recognized as an osteoimmunomodulatory but not bioinert biomaterial. Scientific advances in bone cell biology and in immunology have revealed a close relationship between the bone and immune systems resulting in a field of science called osteoimmunology. These discoveries have allowed for a novel interpretation of osseointegration as representing an osteoimmune reaction rather than a classic bone healing response, in which the activation state of macrophages ((M1−M2 polarization) appears to play a critical role. Through this viewpoint, the immune system is responsible for isolating the implant biomaterial foreign body by forming bone around the oral implant effectively shielding off the implant from the host bone system, i.e. osseointegration becomes a continuous and dynamic host defense reaction. At the same time, this has led to the proposal of a new model of osseointegration, the foreign body equilibrium (FBE). In addition, as an oral wound, the soft tissues are involved with all their innate immune characteristics. When implant integration is viewed as an osteoimmune reaction, this has implications for how marginal bone is regulated. For example, while bacteria are constitutive components of the soft tissue sulcus, if the inflammatory front and immune reaction is at some distance from the marginal bone, an equilibrium is established. If however, this inflammation approaches the marginal bone, an immune osteoclastic reaction occurs and marginal bone is removed. A number of clinical scenarios can be envisioned whereby the osteoimmune equilibrium is disturbed and marginal bone loss occurs, such as complications of aseptic nature and the synergistic activation of pro-inflammatory pathways (implant/wear debris, DAMPs, and PAMPs). Understanding that an implant is a foreign

body and that the host reacts osteoimmunologically to shield off the implant allows for a distinction to be drawn between osteoimmunological conditions and peri-implant bone loss. This review will examine dental implant placement as an osteoimmune reaction and its implications for marginal bone loss.

KEYWORDS

bone healing, bone regeneration, osteoimmunology, immune reaction, osteomechanobiology, osteometabolics, osteoneurology, revascularization

1 Introduction

Osseointegration is needed for oral implant function. Provided that properly trained individuals place clinically controlled oral implant systems, the general outcome is most positive with 10 year failure rates varying between 0-4% (1), and osseointegrated oral implants have in case studies been shown to function over 50 years in the body (2). However, the original view of osseointegration as just a simple bone repair process after osteotomy does not appear to be valid. As demonstrated originally by Donath and co-workers (3), an implant is recognized as a non-self material by the immune system of the body, i.e, in successfully osseointegrated cases. This was recently demonstrated through a quantitative polymerase chain reaction (qPCR)- and histological animal model study where the host established a clear and regulated inflammatory response which thereafter shielded-off the implanted biomaterial in bone (4). Therefore, what is seen when implants are placed in the hard tissues is an *Osteoimmune reaction*, a term that would better describe actual tissue reactions than the original term osseointegration. A recently published suggested definition reads "Osseointegration is a foreign body reaction where interfacial bone is formed as a defense reaction to shield off the implant from the tissues" (5).

In the vast majority of cases the immunological/inflammatory response mounted by the host will lead to implant integration rather than its rejection. Due to the immunologically and mechanically stimulated bone shield-off reaction and the osteoimmune/immunological equilibrium that is established in the case of oral implants, clinicians may load the implants that will then survive for many years in function. However, the immune and healing responses are not only transient one-time reactions, but instead represent a temporal continuum of dynamic hard and soft tissues changes (6). Therefore, today the focus is on modulation of the osteoimmune microenvironment at the bone-implant interface (7–9), understanding that if the host-biomaterial equilibrium becomes perturbed, the result can be marginal bone loss (MBL) or peripheral bone loss around the implant. If the temporal shift in equilibrium at the marginal bone is limited, MBL may be small and does not necessarily challenge the implant's long term survival i.e a new host-biomaterial equilibrium is established (6). However, if continuous and of substantial magnitude, the provocation may result in a shift in the immune/re-balancing response from shielding off the implant to rejection of it (Figure 1). Taken together, these observations confirm differences between the teeth of an individual and implants – rules that apply to the former are generally irrelevant for the latter and vice versa. MBL around implants, in this context, should be considered a condition rather than a disease (10, 11). This paper aims to present an overview of osteoimmunology of relevance for osseointegration and threats to this condition, and to furthermore, analyze the situation from a bone cell/tissue point of view. We start with an overview of osteoimmunology and oral microbiology and discuss then perturbation of osteoimmune responses and marginal bone loss from different perspectives. The importance of the implant passivation layer is presented as well as potential sequale of primary and secondary corrosion phenomena. The paper ends with concluding remarks centered on the paradigm shift that is the result of a greater understanding of osteoimmunology, a core area of knowledge for interpreting implant outcome.

2 Basics of osteoimmunology

Traditionally, three types of bone cells have been described in bone tissue; osteoblasts, osteoclasts and osteocytes. Osteoblasts are responsible for bone growth and osteoclasts favor bone resorption. Activities of both depend on signaling cues (cytokines) and cell-cell interactions. Especially prominent is the receptor activator of nuclear factor κ B (RANK)-receptor activator of nuclear factor-kappa B ligand (RANKL) interaction. Osteocytes, which act in response to mechanical stimuli largely control the osteoclastic/osteoblastic activity through both net bone growth (via e.g. parathyroid hormone PTH, osteocalcin, mechanical stimuli and Wnt ligands) and bone resorption (via e.g. mechanical unloading, sclerostin and dickkopf signals (12)).

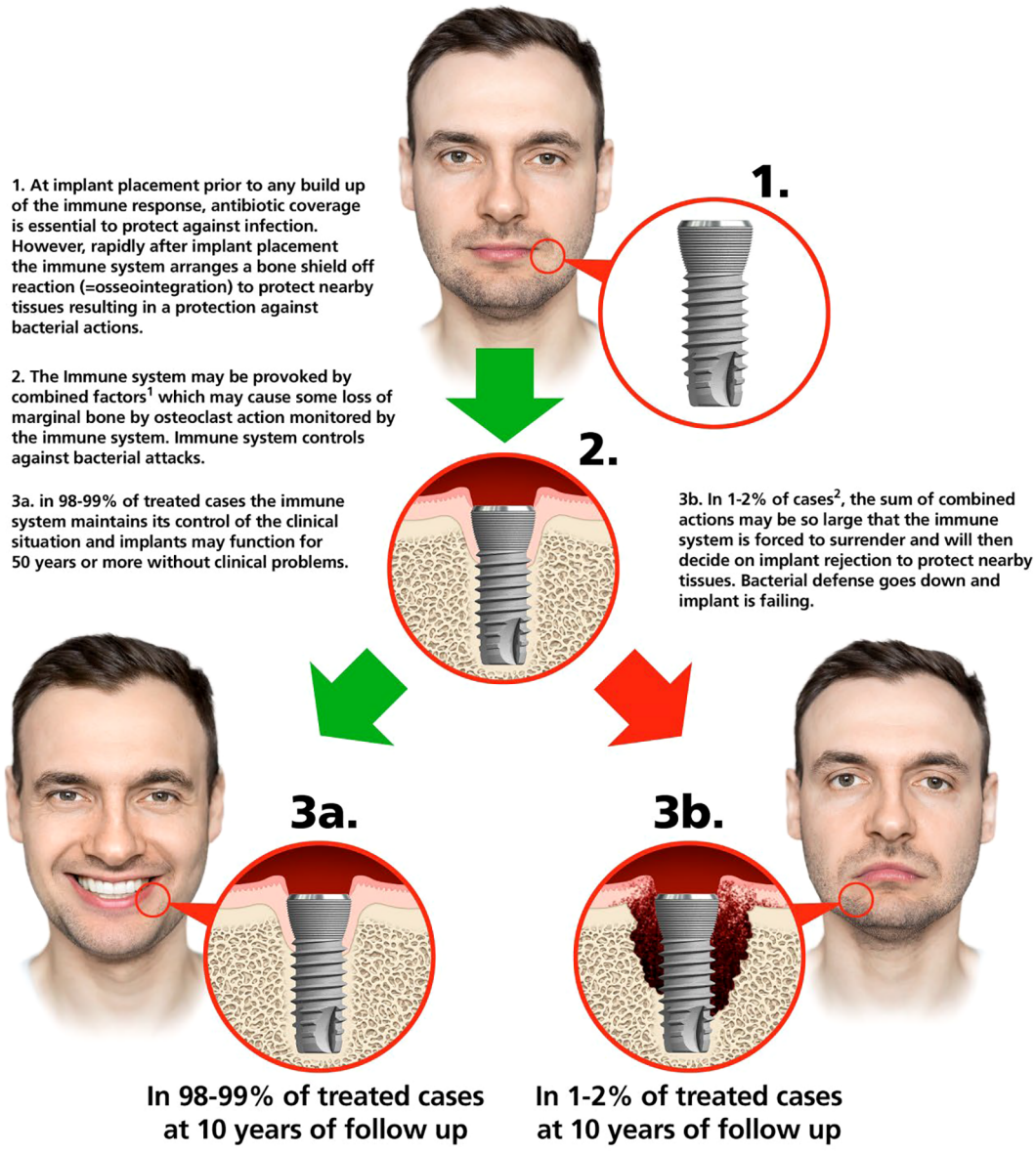

FIGURE 1
A general overview of immune system actions in relation to oral implants. Computerized image of human face.

The always ongoing bone remodeling process has thus traditionally been described as the carefully coordinated interaction between osteoblastic, osteocytic and osteoclastic activities, a process that is carried out by active basic multicellular units (BMUs) (13). Further, it has been described that the activity of RANKL and consequently osteoclastogenesis, is controlled *via* production of osteoprotegerin (OPG) by osteoblasts and other stromal cells. Hence, the OPG/RANKL

balance has been proposed as the determining factor to maintain bone density (14).

In addition, a number of molecular and cellular mechanisms constitute a permanent interaction between bone tissue and the immune/inflammatory system. In this sense, the cells of both systems share common origins, since osteoclasts originate from stem cells of the monocyte-macrophage hematopoietic stem cell lineage and osteoblasts from the mesenchymal stem cell lineage (15). Furthermore, lymphocytic, dendritic cell and macrophage cytokines are all known to act as local bone remodeling regulatory factors (16). The molecular basis of the underlying mechanisms was identified only 20 years ago with the discovery of the essential role of the RANK/RANKL axis in bone and immune cell physiopathology. From that moment, the term "osteoimmunology" was coined to define a new discipline covering the interplay between bone and the immune systems (17).

A rapid evolution in our knowledge of immunology has taken place during the past decades. The adaptive immune response (mainly *via* T and B cells) was long thought to drive innate immunity. However, immunology had it backwards, as now macrophages and the innate immunity are increasingly in the focus of attention, not least in oral implantology. Indeed, because of the discovered macrophage polar-opposite kill and repair activities, the independence of these responses from T cells, and that these types of responses stimulate Th1- or Th2-type responses, macrophages were renamed M1 and M2 to highlight the importance of innate immunity (18). In recent years, it is also understood that bone formation and remodeling are influenced by the inflammatory state of the local microenvironment. In this regard, the eventual phenotypic switch of M1 to M2 macrophage seems to play a crucial role in modulating osteogenesis (19). Moreover, it has been proposed that an efficient and timely switch from M1 to M2 macrophage phenotype facilitates an osteogenic cytokine release and with it the formation of new bone tissue around implanted biomaterials. This is the basis for the concept of an osteoimmunomodulatory material (20). This was confirmed for titanium implants e.g. by Trindade´s works since 2018 (significantly up-regulated ARG1 gene expression around titanium at 10 days) (4, 21, 22). In relation to this, it has been postulated that mainly bone macrophages (osteomacs) would be responsible for the recruitment of osteoprogenitor cells to build new peri-implant bone, since the surface of the titanium implant would directly induce differentiation towards a pro-regenerative M2 macrophage (23). In addition, it is known that once macrophages acquire a functional polarization, they still retain the ability to continue changing in response to new environmental stimulation (24). This was shown in a recently detailed mapping of the mouse mandibular alveolar bone where a unique immune microenvironment was demonstrated under active bone remodeling and immunomodulation (25, 26).

All these findings indicate that oral osseointegration is maintained in a dynamic and likely immunologically dynamic environment. With this in mind, a new dynamic model of osseointegration has been proposed to represent an interplay between the complex osteoimmune/inflammatory events and oral implants, coined the Foreign Body Equilibrium (FBE). This model has in turn allowed a view of marginal bone loss (MBL) around oral implants to be a result of FBE susceptibility to peri-implant environmental conditions (27), (Figure 2). Therefore, MBL can be viewed as a biological, and maybe transient, imbalance in the local immune/inflammatory state (28) adjacent to artificial devices instead of as a disease (10).

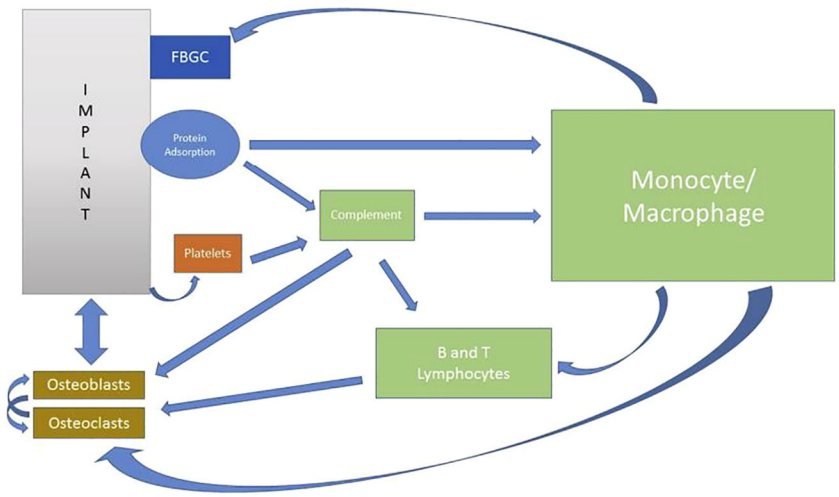

FIGURE 2
Hypothetical model for osseointegration dynamics. FBGC: foreign body giant cells. From Trindade, et al. Ref. (27).

3 Osseointegration and oral microbiology

The first study in which a direct bone anchorage to titanium was suggested as a clinical possibility was published in 1969 (29), and the term "osseointegration" was first coined in 1977 (30). After that and based on classical bone physiology and fracture healing studies, oral implants were considered bio-inert (31) and were considered similar to teeth by some investigators. Since teeth may suffer from periodontitis, it has been assumed that oral implants are also subjected to a hereditary inflammatory disease with relation to bacteria. Hence, the term peri-implantitis was introduced and seen as a bacterially related disease of oral implants (32). Over the last two decades this opinion about MBL has been accepted at several meetings arranged for the purpose of consensus (33). After these conferences, the discussion has continued "mirroring" the progression of gingivitis to periodontitis where peri-implant mucositis is assumed to precede peri-implantitis. However, features or conditions characterizing the conversion from peri-implant mucositis to peri-implantitis have not been identified, despite the scientific advances of the last decades (34). However, during the latest years large progress has been made in oral microbiology, with significance also to implants. For example, oral bacteria have the capability to produce mucosa and bone degrading peptides (35, 36), but are largely balanced by the presence and activities of B-cells, neutrophils, and different T-cells and their molecular products. In addition, the inherent immunomodulating role of the biomaterial and its interplay with the host's innate immunity has been ignored. In fact, the fate of a bone implant appears to be largely determined by its effects on the host immune response. In general, persisting inflammation impedes tissue repair and favors bacterial overgrowth. Therefore, a balanced inflammatory environment around a biomaterial is critical, since both downregulated and excessive inflammatory responses lead to suboptimal bone regeneration clinically (37).

4 Perturbation of osteoimmune reactions

There appears to be two principal reasons for perturbation of the osteoimmune equilibrium in the area of the marginal bone around osseointegrated implants; septic and aseptic reactions:

4.1 Septic reactions

Currently, MBL is considered mostly to be due to septic reactions as evidence has emerged that bacteria can be present also in bone tissue itself. Apparently healed alveolar bone in the dental implant bed displayed bacterial species that further were found locally in the bone even in som cases of tooth agenesis (38, 39). The assumed mechanism of septic causes for MBL is bacterial recuitment of inflammatory bone resorbing cells (40) that may result in implant failure if the infection is maintained. The plethora of bacteria everywhere in the oral cavity may be interpreted as a substantial threat for implant survival. However, in reality oral implants fare quite well despite all bacteria. Analyzing situations where bacteria are known to cause clinical problems with implants include the case of oral implants placed without simultaneous antibiotic coverage, with a consequent increase in implant failure rates (41). In addition, bacteria can secondarily cause MBL (40) in the case of oral implants where a failing process has already been initiated for other reasons. It is of particular interest that these two situations with known possibilities for infection occur either prior to completed osseointegration or once the process of osseointegration failure has already begun. Considering the very high implant survival rates over long periods of time (42), such observations indicate the presence of very strong bacterial defense mechanisms as an inherent capacity of the body, and hence favor osseointegration. This bacterial defense was initially regarded synonymous with the establishment of hemi-desmosome formations (30). More recently, cellular mechanisms have been regarded as the reason for the defense such as a combination of inflammatory and immune cell types or keratinocytes (28, 43). Other potential mechanisms coupled to the defense may be associated with the immune reaction per se, a reaction inevitable in the case of oral implant placement. Another septic reaction close to implants may be seen originating from bacterial leakage between implant parts. However, this type of septic reaction is local and is not known to, on its own, generalize to attacks on the osseointegration process (26). In other words, presence of bacteria is inevitable in the oral cavity, but particular defense mechanisms may guard against bacterial actions in form of marginal bone resorption.

4.2 Aseptic reactions

Immune homeostasis of alveolar bone can be directly affected by microorganisms as noted above, However, new evidence shows that mechanical stimulation could promote the conversion of myeloid-derived monocytes into an activated state, suggesting that occlusal force could drive the immune microenvironment difference between alveolar and long bone. In fact, within the complex immune sensing microenvironment of the alveolar bone (44), alveolar macrophages are critical during the early stages of osseointegration (45). Therefore, more recent research has pointed attention to a largely aseptic reason for MBL. For example, high levels of oxidants are produced during chronic hypoxia and inflammation leading to bone loss. This leads to tissues or bone becoming hypoxic by losing their

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vasculature when exposed to overpressure. Conversely, when insufficient pressure is exerted on bone due to lack of mechanical activity, oxidant production also increases (46). As described above, bone cells such as osteoblasts and osteoclasts have been identified as not only bone building and bone degrading cells but also as a functioning part of the immune system (47, 48). The skeletal system and immune- and inflammatory systems seem independent of one another but, in fact, are inseparable and closely related (49). The aseptic mechanism of MBL may simply be viewed as the immune system stimulating macrophage and osteoclastic function more than osteoblastic activity which inevitably will lead to bone resorption. Osteoblasts and osteoclasts have long been known to be functionally coupled to one another (50). More recently, the data is overwhelming that both these cells act as part of the broader immune system (51). Other factors known to cause MBL such as unsuitable oral implant designs (25), clinical handling (activities of individual surgeons/restorative dentists (Figures 3A, B) (52) or, pharmaceutical treatments (53) are in all probably aseptic in nature. Other aseptic causes of MBL may be disuse atrophy and, possibly, resorption due to old age of the implant host. Most certainly, there are many cases when it is uncertain whether the origin of MBL is septic or aseptic or their combination.

4.3 Ligature model in question

A great number of "ligature studies" have been published, allegedly serving as the experimental approach to prove the bacterial origin of MBL (54). However, when ligatures were placed around implants in tibial sites, not known to harbor any bacteria, some interesting findings were reported. Firstly, there was a clearly enhanced immunological response to implants with ligatures compared to control implants without ligatures. Secondly, despite the apparent absence of bacteria, MBL was observed anyhow around implants with ligatures, but not around controls without ligatures (55). These findings from long bones of animals indicate a general relevance with respect to the noticed increase of immune reactions to ligatures, a new observation that in all probability would be present as a primary reaction also in maxillofacial bone. However, in the latter site there are numerous bacteria too and, particularly if the immune system is repeatedly provoked by the placement of new ligatures at two week intervals (54) as is commonly done, a rejection phenomenon will occur with due lowering of the bacterial defense leading to implant failure. Researchers in these cases, may not have known about nor appreciated the immune reactions to implants and ligatures/ligature placement in the past, hence they have generally not been concerned with this strong provocation of the immune system. In light of our new knowledge however, ligature studies appear to be excellent at provoking an immune dis-equilibrium and initial aseptic bone resorption. When the ligature-provoked immune system switches over from a shield-off reaction to rejection, the contribution of the ligature trauma and ligature accumulated bacteria to the observed bone resorption is unknown but appears to be similar to what is observed around failing clinical implants.

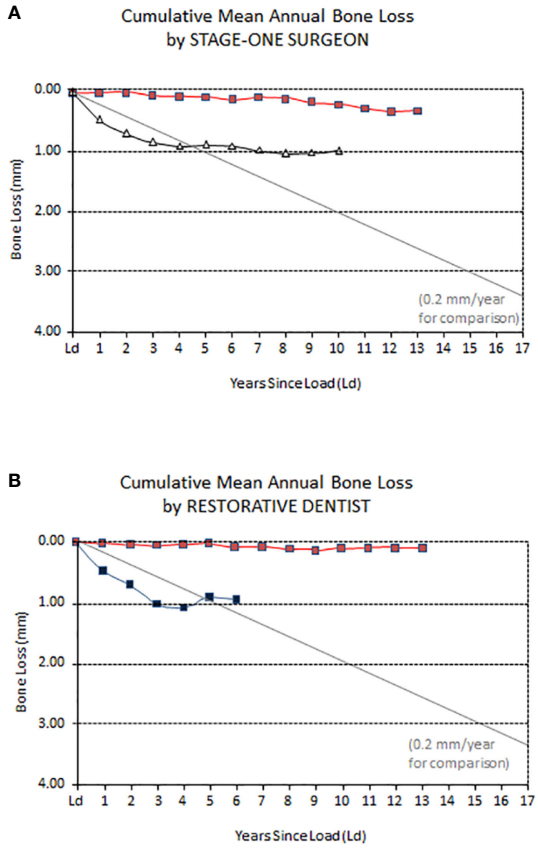

FIGURE 3
(A) This figure depicts the performance of one individual surgeon with respect to the cumulative, average, annual loss in marginal bone that was associated to this clinician (squares) whereas the triangles depict the average annual performance of another oral surgeon who saw much greater accumulated bone loss than his peer, despite them using the same implant type in similar patients. Both these surgeons were active at the University of Toronto, Canada. **(B)** The squares in this figure represent the cumulative, annual loss in marginal bone associated to two restorative dentists active at the University of Toronto, Canada some 20 years ago. The bone loss curves were constructed so that they started from zero levels by the investigator S Ross Bryant. Figures **(A, B)** indicate that if a given patient had the poor luck to be treated by the least good surgeon and followed up by the least good prosthodontist, this meant an average accumulated loss of marginal bone of 2 millimeters at about 3-4 years after implant treatment. These curves support the notion that marginal bone loss around oral implants need not always have a septic background. Created using data from S R Bryant, Ph D thesis, University of Toronto Canada.

5 Stages of osseointegration failure

During the last years, the concept of osteoimmunology has been highlighted, and osseointegration seems to be a foreign body reaction (FBR) equilibrium whose mechanism depends on a complex cellular heterogeneity and dynamic changes within the implant-mediated osteoimmune microenvironment. This was demonstrated in a recent study that mapped the general osteoimmune microenvironment around the bone implant through single cell RNA sequencing, scRNA-seq (56). Under this biological context, it has been suggested that primary (early) failure, MBL, and periimplantitis (late loss/failure) are clinical terms that, respectively, describe a picture of early, transitory or late breakdown of osseointegration (57). In recent years, thanks to the better knowledge of immunologically caused tissue responses, it is understood that these so-called "biological complications" could be related, and it is possible that they represent different manifestations of the same condition, that is, a local peri-implant imbalance of the innate immune system, either site specific (MBL) or involving the circumference of the shield-off bone (10). Therefore, a possible mechanism may be that a balanced plasticity in peri-implant macrophages could be related to a long-term FBE. On the contrary, an increase in the M1/M2 ratio (imbalance) could be behind peri-implant bone loss, likely a clinical manifestation of an incipient or ongoing FBR (Figure 4).

5.1 Primary or early failure

Primary failure is the clinical scenario where osseointegration is never achieved. The frequency of such failures is low, in the range of 0–2% in most clinical reports (57). Clinically, this corresponds to oral implants that are found to be mobile at the abutment connection, and already before the placement of the definitive prosthesis and in the absence of other pathological signs. The major histologic findings show that such implants are surrounded by a connective tissue capsule. Also, in some cases, an epithelial down growth is observed with epithelial cells attached to the implant surface *via* hemidesmosomes (58).

In the field of bone biomaterials, it is known that a prolonged M1 polarization phase leads to increased fibrosis-enhancing cytokine release pattern by M2 macrophages, resulting in the formation of a fibrocapsule (20). In fact, in an animal model of osseointegration, a prolonged M1 polarization phase with high M2 phenotypic activity was demonstrated around copper when compared to titanium, and the formation of a fibrocapsule around copper was observed (36). It is known that when M2 macrophages take an important pro-fibrotic role it is because the lesion is persistent in that environment. M2 cell populations are known to be able to secrete large amounts of pro-fibrotic factors such as TGF-β and Galactin-3 (59). Interestingly, M2 macrophages can also induce the epithelial-to-mesenchymal transition (EMT) through TGF-β (60). EMT, in turn would play a role in the development of fibrosis, as the matrix-producing myofibroblasts arise from cells of the epithelial lineage in response to injury (60). In this sense, a link has been proposed between EMT, fibrosis and foreign body response (61). In addition, M1/M2 imbalance on copper, could be related to a non-enzymatic oxidation catalyzed by Cu2+ and the generation of host-derived oxidation-specific epitopes, which represent danger associated molecular patterns, DAMPs, whose major mechanism of recognition is *via* pattern recognition receptors (PRRs) primarily expressed on macrophages (62). Therefore, a similar mechanism could hypothetically be related to primary failures.

Indeed, several DAMPs and their accompanying PRRs have been associated with the activation of inflammatory responses, wound healing and biomaterial implantation, especially in non-infectious environments. Recently it was demonstrated that the inhibition of HMGB1 (prototypic DAMP) or receptor RAGE impair osseointegration, resulting in a foreign body reaction with persistence of M1 macrophages, necrotic bone, and the presence of MNGCs (63). In turn, a prolonged M1 polarization phase may be dependent on cytosolic multiprotein oligomers of the innate immune system responsible for the activation of inflammatory (inflammasome) activation, creating a pro-inflammatory environment susceptible to bone resorption (64). Specifically, the NLRP3 inflammasome senses a variety of signals referred to as DAMPs, including those triggered by degradation products of the extracellular matrix. Thus, the bone DAMP/NLRP3 inflammasome axis has been proposed as a novel mechanism that sustains bone resorption, mainly at conditions of low-grade inflammation (65). In addition, low-grade inflammation decreases access to oxygen and nutrients in affected tissues. Hypoxia could then lead to tissue necrosis, thereby increasing the local immunogenicity *via* the generation of DAMPs (66). On the other hand, the epithelial downgrowth observed on implant failures may therefore be related to the role of M1/M2 macrophage balance in EMT/MET (mesenchymal epithelial transition) plasticity (67).

5.2 Late implant failure

Late losses (after prosthesis placement) can sometimes be attributed to overload and/or secondary corrosion, or to a combination of these. In advanced failure cases, there is an excessive loss of marginal bone, implant mobility and interestingly, the presence of a stratified connective tissue (capsule). Further epithelial downgrowth migration is observed (58, 68). Recently, it has been shown that this could possibly be due to the repolarization of both M1 to M2 and vice versa, and that the macrophage phenotypes are defined by the current

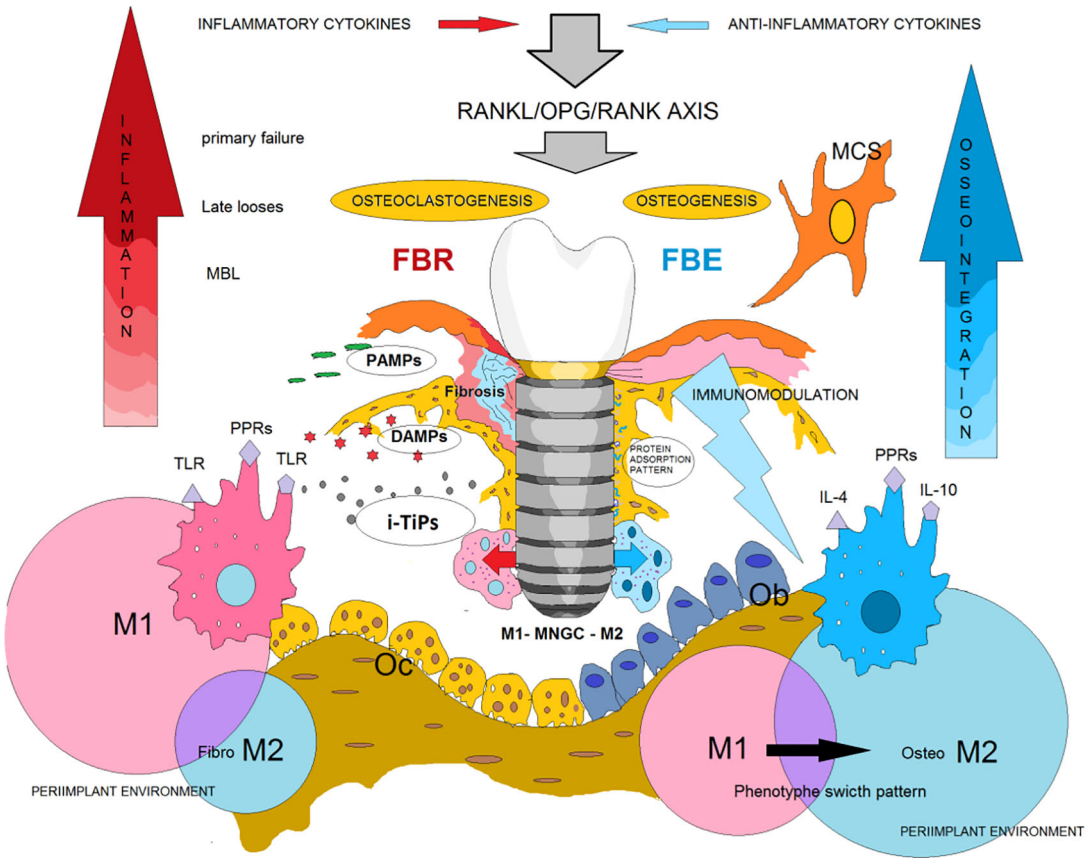

FIGURE 4
Implant-Osteoimmune interaction. Osseointegration is a condition of continuous and dynamic implant-osteoimmune interaction. If the implant surface evokes an initial and long-term immunomodulation, interfacial bone is formed to shield off the implant from the tissues (FBE). In addition, the M2 anti-inflammatory environment would induce adequate defense reactions to handle transient septic and aseptic threats (PAMPs, DAMPs, Implant-derived Titanium particles (i-TiPs)), which is clinically reflected with 10 year failure rates varying between 0-4%. However, if it is continuous and of considerable size, the provocation and the consequent M1 inflammatory environment can generate Inflammatory cytokines that alters the expression of RANK/RANKL axis, counteracting the ability of implant surface osteoimmunomodulation, then a partial, progressive or total FBR can occur. Modified from Zetao Cheng, et al (ref.20).

cellular microenvironment (24). Moreover, MNGCs present at implant interfaces have also the potential to shift between pro-inflammatory M1-MNGCs (often previously referred to as FBGCs) and wound-healing M2-MNGCs polarization states, whose precursor cells are thought to be derived from osteomacs (69, 70). It is important to note that M1-MNGCs may express a different repertoire or concentration of inflammatory factors (cytokines and chemokines), which are also time-dependent if M1-MNGCs switch towards an anti-inflammatory phenotype. Therefore, the FBR could differ between different biomaterials (71). In fact, the results of FBR, such as chronic inflammation, excessive granulation, collagen fiber deposition, and fibrous tissue formation, are related to the persistence of a microenvironment with upregulation of genes related to inflammation (IL- 1) and the ability of the biomaterial to continue serving as an immunomodulator (72). These are critical findings, because macrophages and other cells of the innate immune system respond to a myriad of signals emanating from their local environments, including signals resulting from the interaction between prosthetic byproducts and periprosthetic cells (66).

DAMPs can be products of necrotic or stressed cells as a result of long-term ischemia and/or toxic effects of prosthetic debris. For this reason, several studies have examined the role of DAMPs in periprosthetic osteolysis (PPOL) (66), as there are several potential sources of ions and particles in implant dentistry (73). Moreover, presence of organic and inorganic contaminants onto some surfaces (74) and the potential exposure of less stable elements such as vanadium and aluminum after surface modification procedures, can also trigger an inflammatory response (75). Regarding Ti ions and particles, it is known that both can coexist in the peri-implant environment. A recent study showed that metal particles embedded in an experimental rat mandible defect triggered chronic inflammation with a foreign body granulomatous reaction characterized by the presence of histiocytes and

MNGCs, i.e, Ti metal particles induced a chronic inflammatory cell infiltrate associated with a foreign body reaction (76). Interestingly, new evidence suggests a spatiotemporal distribution of macrophages in the FBR, therefore, a microenvironment may exist or be created within and around the biomaterial and that different macrophage phenotypes are associated with these different spaces (77).

Human macrophages develop a specific response to Ti particles. Upon contact, M1 exhibits increased production of pro-inflammatory cytokines, chemokines and growth factors, but a decreased phagocytic activity, while M2 macrophages have been suggested to mediate particle uptake (78). This could be related to the absence of MNGC or frustrated phagocytosis in the vicinity of titanium particles in granulation tissue harvested from peri-implantitis cases, as shown in a recent article, even though there was a significantly higher expression of CD68 (79). For example, it has been shown that proinflammatory M1 macrophages predominate in soft tissue biopsies from peri-implantitis sites over M2 macrophages (80, 81). As indicated in a recent paper (4), qPCR-techniques were used to verify such immune responses. However, measurable foreign body reactions are a shortlived phenomena and M1-MNGCs may not be possible to study in chronic specimens as done in a recent paper (79). In normal foreign body reactions, M1-MNGCs and associated granulomatous tissue are formed at approximately 4 days after implantation, increase up to about 14 days, but subsequently gradually disappear (82) to be replaced with other immune derived reactions such as machrophage responses. The M1 polarization observed in peri-implantitis lesions also suggests a robust response by the immune system against local factors; and thus, more tissue destruction (81). We should keep in mind that reactive oxygen species (ROS) always dissolve some Ti-oxide during an inflammatory phase. One plausible interpretation is therefore that later dissolved material is “shielded off” due to local immune activation, very similar to the later shield off of macroscopic implants. Inflamed tissues maintain a persistent low level of inflammation and thereby enhance over time the dissolved material that precipitates to particles and necessitate a response, a “shield off” process, or alternatively, a low response due to immunecompromized tissues in the vicinity of implants.

6 Marginal bone loss from different perspectives

At present, it is thought that an increase or decrease in bone response is related to implant mechanical stability and the initial response modulated by the immune system (40). In fact, macrophage ablation impairs woven bone formation around oral implants (45), and the impact on the immune response by Vitamin D deficiency has been related to low early implant healing (83). Furthermore, it is known that some intraoral sites support osseointegration better than others. In this sense, studies revealed a strong positive correlation between bone remodeling rate, mitotic activity, and osteotomy site healing and high endogenous Wnt signaling (84). Also, findings suggest a role for an autocrine Wnt signaling in macrophages during the immune response to implanted biomaterials (85).

Histologically, osseointegrated oral implants show a heterogeneous interface with variable degrees of mineralized bone-implant contact (BIC) (86). Therefore, in some cases, there could be a mechanically weak bone-to-implant interface (87). This is clinically relevant since functional loading and mechanical strain are the main causes for bone remodeling. Osteocytes are known to translate signals related to mechanical strain into biochemical signals and largely regulate the osteoblast–osteoclast axis. As a result, bone remodeling may change the peri-implant crestal bone contours (87). In turn, the macrophage-osteoclast axis is involved in regulating the balance of bone remodeling and resorption that is essential for the maintenance of normal bone morphology (88). On the other hand, the rate of new bone formation depends also on proteins secreted by macrophages that regulate undifferentiated mesenchymal cells to transform to bone-forming osteoblasts (89).

The activation of inflammatory processes is followed by physiological bone repair mechanisms. However, there could be typical individual mediator-related signaling patterns of inflammatory cytokines. In this sense, a unique bone remodeling situation appears to occur when fatty degenerative tissue is present in the medullary cavity of the jawbone, which could be related to a dysregulated programming in stem cell expansion (90). Recent findings demonstrate that alveolar bone monocytes/macrophages tend to express a high level of oncostatin M (Osm), which promotes osteogenic differentiation and inhibits adipogenic differentiation of MSCs (44). Therefore, if there is a weak bone-to-implant interface, associated personalized signal patterns, continuous stress signals and immunogenicity of the elements present, there is a risk that initially transitory and site specific peri-implant bone loss may progress to a more damaging and vicious stage (91). Such a mechanism may be especially evident at the marginal bone area.

6.1 Macrophage polarization and the osteoimmunological mechanisms behind marginal bone loss as a condition but not as a disease

Macrophages are highly plastic cells that rapidly respond to their microenvironment by adopting different phenotypes with important roles in regulating the healing response to biomaterials.

The prolonged presence of inflammatory M1 macrophages can exacerbate tissue damage and prevent biomaterial integration. In contrast, the immune response favorable to healing by M2 macrophages precedes osteoinduction. In recent years, an increasing number of studies have investigated the response of M2 macrophages to biomaterials. In fact, the interaction between M1 and M2 dominated microenvironments and the temporal modulation of the M1 to M2 transition provide an interesting line of investigation to search for new therapeutic approaches focused on the immune system to improve osseointegration. Such studies include modification of implant surface properties, ionic-treated implant surfaces with LiCl or Mg, use of polarizing cytokines such IL-4 and mechanical stimuli to promote the innate immunomodulatory capacities of BMMSCs (91).

Peri-implant tissues may thus be considered as an immunologically active microenvironment with immunological sentinels present such as macrophages modulated by neutrophils, dendritic cells, T-cells, B-cells and MNGCs being able to activate and direct an immune-mediated and controlled inflammatory response (91). Furthermore, it is known that prolonged inflammation plays a critical role in bone resorption, because pro-inflammatory cytokines (such as IL-17A) (92) alter negatively the RANK/RANKL axis balance (93). In this sense, proinflammatory M1 macrophage polarization can be induced by implant/wear debris, damage associated molecular patterns (DAMPs), and pathogen associated molecular patterns (PAMPs), resulting in the production of high levels of pro-inflammatory cytokines (e.g. TNF-a, IL-1b, IL-6) through NF-kB activation. In addition to secreting cytokines, M1 macrophages show potential to differentiate into osteoclasts, and may serve as an osteoclast reservoir. Conversely, M2 activation is often characterized by the expression of anti-inflammatory cytokines (e.g. IL-4, TGF-b and IL-10) and antigen presentation ability, suppress osteoclastic activity and promoted osteogenesis through the inhibition of NF-κB signaling pathway (94, 95). Although the mechanism underlying the observed plasticity in macrophages is not well understood, It is thought that macrophage polarization represents a “fluid state”. In this regard, polarization reversibility is a target of therapeutic interest, especially when the M1/M2 imbalance may compromise the immune response (96). In a recent study, researchers analyzed the subpopulations of M1 (CD68 and iNOS) and M2 (CD68 and CD206) macrophage polarization through Immunofluorescence staining, noting a statistically significant increase in population of macrophage M1 phenotype from peri-implantitis samples compared to periodontal disease samples. In the same line, an immunohistochemical analysis showed a significantly higher expression of M1 (CD80) inflammatory phenotype at advanced peri-implantitis sites (80, 81). These studies correlate the increase of the M1/M2 ratio with a high response of the immune system against local signals in the cases of peri-implant lesions, which could possibly play a critical role in the underlying pathogenesis of peri-implant bone loss (80, 81).

6.2 Implant-abutment site and marginal bone loss

The connections of various implant components to the top of the implant and their emergence from the body’s hard and soft tissues have implications for tissue attachment and turnover. Generally, components are placed, removed and replaced on multiple occasions including closure screws, healing caps, temporary abutments, final abutments and temporary and final restorations. These component placement and removal procedures not only prevent stable soft tissue attachment onto the implant component but also provide an avenue for fretting and galvanic corrosion, and bacterial access to interfaces including the interface at the top of the implant. Many studies have documented bacterial contamination of these interfaces regardless of whether the connections are internal or external to the implant (97). These contaminated interfaces therefore provide an ecological niche for bacterial colonization and their products such that the host response is unable to eliminate or mitigate the bacterial challenge. As such, the host must provide an immunological response adjacent to the interface. Clinicians generally place the top of a bone level or “submerged” implant at or slightly below the crest of the bone meaning that a bacterially contaminated interface, and consequently, a host inflammatory reaction is located directly at the marginal bone level.

Broggini et al. (98) documented that a peak of inflammatory cells was located approximately 0.50 mm coronal to the interface in tissues adjacent to the implant. This inflammation consisted primarily of neutrophilic polymorphonuclear leukocytes indicative of a persistent acute inflammatory reaction at the marginal bone level. Mononuclear cells were evenly distributed along the implant surface, and this inflammation was associated with bone loss. Interestingly, the absence of an interface at the bone level (using a tissue level or “non-submerged” implant) resulted in only sparse cells and no peak of inflammation at the marginal bone level and minimal bone loss (98). The peri-implant cellular infiltrate immediately coronal to the implant-abutment interface decreased gradually and progressively in the soft tissues toward either bone or gingival epithelium. This study provided histomorphometric data that a unique pattern of inflammatory infiltrate develops adjacent to implant interfaces with associated bone loss. The differential pattern of peri-implant neutrophil accumulation suggests that the bacterial accumulation at the interface results in a chemotactic stimulus that both initiates and sustains the recruitment of inflammatory cells. Such activation of the host defense system (such as cytokines, complement, and antibodies) can result in a gradient of inflammatory cells perpetuating an acute inflammatory process which is exacerbated by an inability to access the interface for oral hygiene (98). This study, in addition to documenting the intense inflammatory process, also demonstrated significantly greater bone loss around implants

with an interface at the marginal bone level compared to implants without such an interface (98). It was hypothesized that the interface at the marginal bone level leads to microbial leakage, colonization and a persistent bacterial presence. The chemotactic signaling promotes a sustained neutrophil accumulation and, in parallel, mononuclear cells are recruited to the surface. The combined and sustained activation of inflammatory cells can then promote osteoclast formation and activation resulting in marginal bone loss.

Another study compared the distribution and density of inflammatory cells surrounding implants with an implant-abutment interface placed supracrestally, at the crest or, subcrestally and correlated that with bone loss (99). This study revealed that, in spite of location, all implant interfaces had a similar pattern of peri-implant inflammation. That pattern consisted of polymorphonuclear leukocytes concentrated at or immediately coronal to the interface. Interestingly, peri-implant neutrophil accumulation increased progressively as the interface depth increased and marginal bone loss was significantly correlated with inflammatory cell accumulation, i.e. the deeper the interface, the greater the magnitude of peri-implant inflammation (99). In contrast, mononuclear cells were relatively uniformly located along the entire surface of the implants. Furthermore, there was significantly greater bone loss associated with subcrestal implants compared to implants placed at the crest or supracrestally. These findings reveal that the implant-abutment interface defines the degree of inflammatory cell accumulation and its location in the tissues and, suggests that the inflammatory cells contribute directly or indirectly to the extent of marginal bone loss (99).

The study above identified a highly significant relationship between the degree of peri-implant inflammation and the magnitude of marginal bone loss. A number of previous studies have also demonstrated a spatial relationship between inflammation and bone loss supporting the observed association between contaminated implant-abutment interfaces, inflammatory cell infiltrate accumulation and marginal bone loss (100, 101). In the late 1970's, Waerhaug (100) described in periodontal disease an "extended arm" of inflammation while Garant (101) described an "effective radius off action" of inflammation to bone loss. More recently, Graves and Cochran (102) described such a relationship as an "inflammatory front" where an increase in the host inflammatory response resulted in an increase in bone loss. This cause-and-effect relationship was demonstrated with inhibitors to the pro-inflammatory molecules IL-1 and TNF-alfa (103). This spatial relationship between inflammation and the immune system and bone has resulted in an area of science referred to as "osteoimmunology" as noted above and involves the science related to osteoclast development (104, 105). Taken together, these studies demonstrate that the location of an implant-abutment interface can be an important determinant of marginal bone loss as has been noted when evaluating marginal bone loss for implant success (106) where up to a mean of 1.5 mm of marginal bone loss was allowed for in the first year after implant placement.

In summary, bacterial-induced inflammation and corrosion may together with other factors contribute to MBL by jointly affecting peri-implant bone rather than as isolated factors. Secondary corrosion is a late implant response that may, in clinical cases which have previously resulted in some MBL, facilitate a transitional shift in the immune system from being a sentinel of implant shield off, to implant rejection, even if this is not an inevitable outcome of secondary corrosion (107) that will be discussed in greater detail under next heading.

7 Peri-implant phenomena involved in osteoimmune regulation

7.1 Implant passivation layer

The coronal portion of the implant exists in a spatially singular situation where it interacts directly and simultaneously with the oral microenvironment (Figure 5), the peri-implant soft tissue barrier. As discussed previously in this article, no biomaterial is fully bioinert. However, select non-toxic biomaterials such as titanium can achieve a homeostatic state within the peri-implant tissues enabling a long-term functional stability (108). This state is dynamic and contingent upon the biomaterial's capacity to reach an electrochemical equilibrium, while present in biological fluids. For titanium biomedical implants, the success of primary osseointegration is dependent upon the establishment of a surface "passivation" layer (109, 110). The chemical composition of this layer is distinct from that of the underlying metal, being mainly (>98%) composed of titanium dioxide, TiO_2. The passivation layer is formed rapidly but not instantly on titanium surfaces under atmospheric conditions and protects from further passive oxidation of the implant. Therefore, it contributes to the long-term stability of the implant within the tissues without further corrosion. The establishment and development of the passivation layer is also dynamic and the electrochemical changes that occur due to insertion of the implant in an osteotomy within the bone result in electrochemical changes that move hand in hand with the process of osseointegration. During successful osseointegration the passivation layer thickness maximizes, while a direct bone-to-implant contact is established and maintained (110). Importantly, osseointegration is achieved between the titanium passivation layer and host bone cells, and not between the underlying metal and host tissues (111). In fact, no published data has ever shown cellular attachment on titanium surfaces without protective passivation layers.

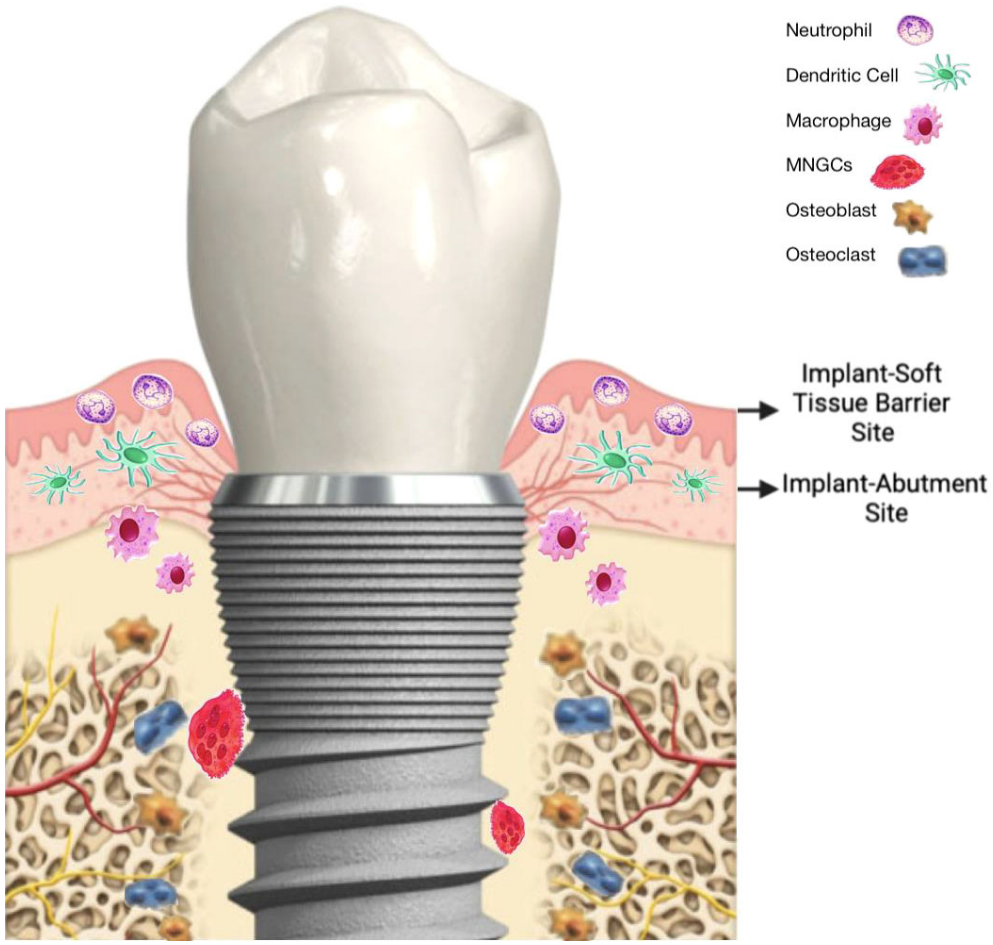

FIGURE 5
Two critical sites involved in marginal bone loss exist at the coronal aspect of the implant where it emerges through the bone and soft tissues.

In essence these electrochemical changes that occur at the titanium surface represent a controlled primary corrosion of the metal under the definition of passivation as the "conversion of a refined metal into a more chemically stable form, such as the spontaneous formation of an ultrathin film of corrosion products, known as a passive film, on the metal's surface that act as a barrier to further oxidation" (112). As mentioned previously (see Figure 2) immune and bone cell populations respond to these early electrochemical events that occur during implant osseointegration with a specific role being played by alveolar macrophages during the early stages of osseointegration (4, 45). In addition to the direct signaling of the RANKL-OPG pathway that occurs in response to the surgical trauma induced to osteocytes during implant placement, at least two independent *in vivo* animal models have demonstrated that the first two- (rat model) (45) to four-weeks (rabbit model) (4) of the osseointegration phase are dominated by CD68+ macrophages expressing both M1 and M2-related genes, suggestive of a inflammation-driven remodeling. Depletion of macrophages in the rat model led to compromised osteogenesis during early osseointegration, highlighting the central effect that immunity has in regulating the biomaterial-bone interface (45). The important role of implant surface passivation in ensuring an optimal tissue response to the implanted metal is evidenced by the fact that when the implant passivation layer thickens, as in the case of Mg-oxidized implants (113, 114), that increased thickness of the passivation layer provides improved bone anchorage.

7.2 Implant-soft tissue barrier with focus on inflammation and primary corrosion

When discussing host immune/inflammatory responses to biomaterials it is important to destigmatize the term "inflammation" because it has traditionally been linked to the

host defense process against harmful microorganisms. However, it is now well established that inflammatory responses are part of host physiology and are necessary processes to regulate tissue and organ function, wound healing and cell death. Inflammation is therefore critical to eubiosis (115) and not necessarily results in tissue destruction (116). Inflammatory responses only become implicated in the pathophysiology of diseases when they become deregulated, non-resolving and as a result become chronic. In the context of implant biomaterial-host equilibrium, successful osseointegration is characterized by a controlled immune/inflammatory response that is critical to peri-implant wound healing and, in most cases, resolves timely to allow chronic immune surveillance to aid in maintaining tissues homeostasis. Nonetheless, if the tissue environment is not conducive to the electrochemical stability of the titanium passivation layer, destructive corrosion can occur leading to titanium dissolution from the implant surfaces (107, 108). Wennerberg et al. (117) addressed the extent of primary corrosion during the osseointegration of titanium implants with various surface modifications by artificial material aging in solution for 1-month at atmospheric conditions. None of the implant surfaces exhibited dissolution of titanium from the surface during the experiment in buffered saline suggesting that an electrochemical equilibrium is rapidly established and sustained under favorable conditions, which resemble healthy tissue, i.e. oxygen availability, neutral pH=7.3 (117). However, when the same surfaces were placed in strongly acidic lactate solution (pH=2.3) and aged for 1 month up to 250ng of dissolved titanium were identified in solution (117). Therefore, aggressive electrochemical conditions, such as a strongly acidic environment or chemically reductive conditions, may lead to electrochemical instability of the passivation layer and titanium release *in vitro* even in the absence of bacterial and frictional challenges (107). Vascular interruption as a result of surgical trauma in the case of implant placement is another example of a micro environmental factor that may contribute to electrochemical instability. In corroboration, a separate study (118) showed that the corrosion resistance of titanium is diminished under inflammatory conditions that included oxidative attack by reactive oxygen species (119), acidic environment (pH~3) and reduced oxygen availability (anaerobic conditions in peri-implant pockets) (118). Among these environmental factors, lack of oxygen achieved by de-aeration was the strongest determinant of diminished electrochemical impedance (118). Although these environmental challenges have been described from a biomaterials viewpoint, it is clear that they are bidirectional and affect the host tissues as well. When the electrochemical equilibrium on the titanium passivated surface is displaced, more titanium ions are generated and dissolved in tissue fluids. It has been suggested that these titanium ions rapidly aggregate in protein-rich fluids forming highly biologically active titanium microparticles (119, 120).

7.3 Implant passivation layer and secondary corrosion

When the chronic electrochemical oxidation of titanium leads to gradual destruction of the passivation layer, the effects of corrosion are not limited to the biomaterial but also affect osteoimmune regulation of osseointegration. This has been evidenced by two recent studies (108, 121) from independent research groups showing that abrasive dental treatments, such as ultrasonic instrumentation with steel instruments used to clean the implants surface, leads to destructive corrosion. This can be regarded as secondary corrosion when compared to the primary oxidation, i.e. corrosion, which occurs during healing of implants and has a protective effect in most cases *via* the formation of the passivation layer. In the case of secondary corrosion, the resulting damage to the passivation layer results in accelerated titanium release from the implant surface to the tissues with detrimental effects locally and deregulation of the osteoimmune axis (107, 108). It was long thought that the scratch exposed metal would, however, be re-oxidized in water/air within tens of milliseconds to seconds (122) as the re-passivation of titanium in water or air is an undoubtable scientific fact. Nonetheless, it is not translational to the dental implant clinical reality. Earlier studies were conducted in atmospheric conditions or in water but neither of these conditions represent the microenvironment of the peri-implant pocket. As a result, the fallacy that clinicians can damage the implant surfaces to "clean" them from bacterial biofilm was developed under the assumption that the titanium passivation layer will re-passivate after abrasion within milliseconds (108). Conversely, Berbel et al. (108) showed that when replicating anaerobic inflammatory conditions that exist in the peri-implant pocket to repeat these experiments, scratching of the passivation layer for cleaning resulted in long-term reduction in corrosion resistance. These changes led to secondary corrosion appearing as microgranular corrosion on the titanium surfaces (108, 118). In a subsequent paper it was further shown that these abrasions of the passivation layer led to vastly accelerated titanium release to the environment in simulated body fluid during titanium aging. As such, it is imperative to highlight that the notion that titanium will rapidly re-passivate does not stand true under clinical conditions.

These findings have important clinical ramifications to avoid initiation or perpetuation of peri-implantitis due to iatrogenic reasons, such as preventive abrasion of implants with steel instruments to remove bacteria. Importantly, the released implant-derived Titanium Particles (i-TiPs) cause fibroblast cell death and activate macrophages towards an M1 phenotype (108, 121). Importantly, the persistent effect of i-TiPs activates inflammasomes in immune cells that lead to IL-1β release through activation of the complement system (4, 123, 124). As discussed above, IL-1β is a major osteoclast activating factor and provides a means of communication from immune and tissue resident cells to the local bone eliciting osteoclastic differentiation with destructive downstream effects. Therefore,

the biological plausibility exists for regarding the electrochemical instability of the titanium surface occurring either through tribocorrosion (i.e. surface transformations resulting from the interaction of mechanical loading and chemical/electrochemical reactions), local chemical attack (ROS or Fluorides) or damage by dental implant instruments as a potential cause of marginal bone loss within the implant-soft tissue barrier Interface.

8 Synergistic activation of pro-inflammatory pathways

Macrophages and other cells of the innate immune system respond to a large number of signals emanating from their local environment; therefore, the inflammatory potential can be multiplied due to the synergistic activation of pro-inflammatory pathways. As described above, proinflammatory M1 macrophage polarization can be induced by implant/wear debris, DAMPs, and PAMPs (95).

It appears that titanium particles do not tend to be encapsulated in the tissues around dental implants, but instead migrate through peri-implant tissues causing immune reactions, with smaller particles tending to produce greater toxicity and enhanced pro-inflammatory response (125). In relation to this, it is known that particles of a diameter smaller than 1 μm, or nanoparticles, generate the most biological toxicity and can induce cellular mutations. In a recent study, it was shown for the first time that Titanium nanoparticles (TiNPs) affect the transcriptional program in human macrophages (GDF-15 over-production and strong suppression of stabilin-1), which could interfere with the long-term integration of the implant through the imbalance between inflammation and healing processes (126).

While the molecular mechanism of DNA damage induced by TiO_2 NPs is unknown, it is suggested that exposure to TiO_2 NPs causes aberrant DNA methylation levels that can lead to unusual gene expression, altering epigenetic integrity (127).

It is observed that the macrophage reactivity upon activation by wear particles is driven by cell membrane contacts through surface receptors, such as CD14 and TLRs (128), or through the phagocytosis of wear debris and the stimulation of the NALP3 inflammasome(NLRP3, Cryopyrin) (129). In bone and its surrounding tissues this results in an influx of immune cells, osteoclasts and other cells. The resulting pro-inflammatory environment leads to increased bone destruction and suppressed bone formation (130).

It is not known in detail how these molecular and cellular interactions translate into a specific biologic response of either inflammation or tolerance in a particular patient (66). However, the osteo-immune response could be conditioned not only by local and systemic oxidative stress but also by the local innervation state (Figure 6). In support of the latter, recent *in vivo* experiments using Ti-implants in rat femur indicated strongly that neural regulation of bone directly modulates its formation and, as a consequence, osseointegration (131). The significance of this finding is not currently understood, but almost certainly there exist tight connections to the immune/inflammatory system. It is well known that both the inflammatory reaction and the wound healing process are intimately connected to changes in the redox balance, and even though at low concentrations, oxidative stress exhibits various physiological roles. Upregulation of Reactive Oxygen Species (ROS) production and persistence over a long period of time can then prove to be harmful to the host (132). In fact, recent discoveries, have demonstrated a link between oxidative stress and an aberrant innate immune system response in sterile inflammatory diseases (133).

The general presumption that biomaterial implantation allows opportunistic bacteria to flourish by providing a surface for biofilm formation likely is biased. The dysregulated host response opens the opportunity for bacteria to invade immune compromised tissues and hence contribute to the susceptibility of implants to infection (37). In this sense, the beginning of understanding bone loss as a condition is a great paradigm shift that allows osseointegration to be considered from a different point of view. Reincorporating oral implantology to the field of biotechnology where the emergence of omic sciences such as implantogenomics (134), epigenetic effects of nanoparticles (135) and advanced immunomodulation (136) acquire enormous relevance when maintaining implant health in our patients.

9 Concluding remarks

Since periodontitis may cause loss of teeth, peri-implantitis was assumed to cause loss of oral implants with increasing time of follow up. Accelerating loss of marginal bone around implants was, therefore, regarded as a disease that logically, as it seemed, would best be treated by a similar type of surgery as periodontitis. One cannot blame the doctor for interpreting the numerous bacteria present in the end stage of bone resorption to be what caused the problem in the beginning, since there was no alternative explanation for this development that was known at the time.

However, today we have identified alternative explanations behind implant threatening bone loss; adverse immune reactions that can be demonstrated to be behind failure of oral as well as orthopedic implants (11, 137). The science of osteoimmunology is relatively new and has been established first in our new millennium and mainly after the initial attempts to couple all marginal bone loss to a bacterial disease. Furthermore, we recognize today that teeth are natural parts of our human bodies whereas implants represent foreign bodies with clearly measurable immune reactions (4). It is to

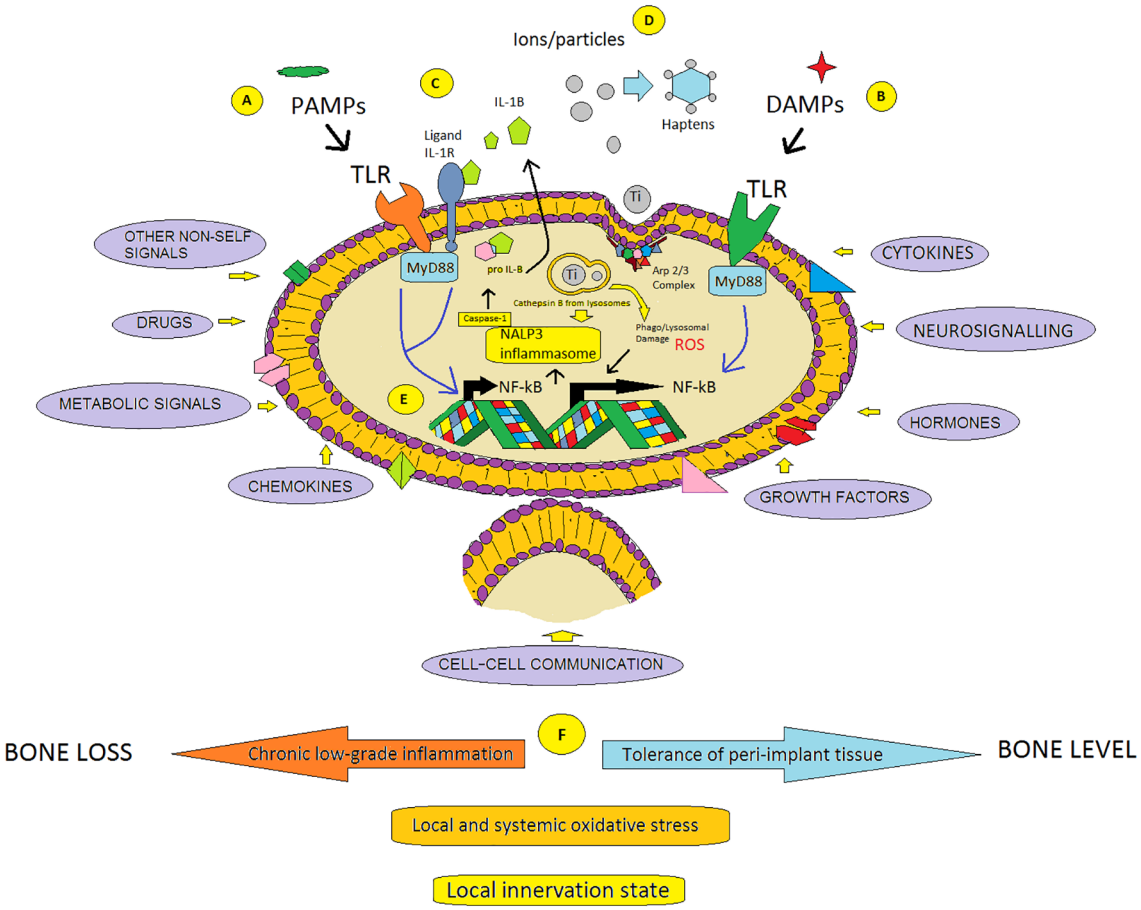

FIGURE 6
Common molecular pathways and environmental signals. **(A, B)** Toll-like receptors (TLRs) and other types of pattern recognition receptors recognize PAMPs and DAMPs and trigger inflammation via the activation of the transcription factor NF-Kb. Signaling pathway that requires the adaptor molecule MyD88. **(C)** In addition, inflammation in response to necrotic cells is mostly mediated by IL-1 receptor (IL-1R), which leads to NF-kB activation. **(D)** On the other hand, titanium particles can induce acute inflammation due to activation of the NALP3 inflammasome, which leads to increased IL-1 secretion and IL-1-associated signaling. Process mediated by protein complexes such as the Arp 2/3 complex. Also, titanium ions can bind to proteins, such as albumin or transferrin, creating a bioavailable metalloprotein that could serve as an antigen in immunological reactions. **(E)** Activation of NF-κB , the master inflammatory transcription factor. **(F)** Macrophages and other cells of the innate immune system respond to a large number of signals emanating from their local environment, therefore, the inflammatory potential can be multiplied due to the synergistic activation of pro-inflammatory pathways. In this sense, it is known that the crosstalk between the skeletal system and the immune system can lead to osteoclastogenesis, for example, through IL-1. A specific biologic response of either inflammation or tolerance in a particular patient could be related to local and systemic oxidative stress, and other basal states, such as the state of local innervation. All these possible cellular and molecular mechanisms would be constantly counteracted/balanced by both the long-term immunomodulatory capacity of the implant and the dynamic osteo immune environment. (Modified from Goodman SB, et al. ref. 66).

no great surprise that investigators have demonstrated clear differences between periodontitis and peri-implantitis (107, 138). One study compared teeth and implants in the same jaw of patients and found that when teeth lost bone, implant bone level was stable and, conversely, when implants lost bone, teeth bone was stable. In only 3% of cases was there simultaneous bone loss around teeth and implants reported (139). Surgery for what has been seen as threatening marginal bone loss around oral implants have, at best, presented questionable clinical results with a clear tendency of causing more patient problems than non-surgical approaches (43, 140). In addition, implants with a diagnosed state of alleged disease at a mean of 12.5 years after placement (141) were re-investigated 9 years later when it was demonstrated that 91.4% of the allegedly sick implants had seen no further bone loss and 95.3% of the previously as sick declared implants still functioned in the jaw of the patients (142). In another study, a decreased risk for oral implant losses with increasing time was reported (143). Increasing plaque index was found associated with lower levels of MBL (144) and Menini et al. (52) was unable to find any MBL associated with increasing plaque index in an up to 14 year followed up clinical

study. There are indeed several reasons for MBL which are most difficult to explain with a primary infection etiology. These situations include MBL associated with the responsible surgeon or prosthodontist (53), MBL associated with intake of pharmaceutical products (145) and at least initial MBL due to accidental presence of cement in the soft tissues. However, the latter example is of dual nature; MBL due to (nano-micron sized) cement particles will immediately stop if the cement is removed, indicative of this bone loss being immune driven since bacterial actions would not disappear instantly. However, if cement is not removed in time, then the immune system may start a rejection phenomenon whereby a secondary infection will ensue. Taken together, the evidence for functioning, osseointegrated implants suffering from an infectious disease is insufficient. The paradigm shift is that we today know that implants are not bio-inert as previously believed (146); instead an immune system activation follows the placement of an oral implant (4). The immune system has two ways of responding to an implant; either to embed it in bone to protect other tissues (bone shield off; osseointegration) or rejection of the foreign body (3). In the great majority of cases there will be an immune system caused shield-off of the implant. Some marginal bone loss can be monitored by the immune system control of the osteoblast/osteoclast combined action (10). A more dangerous development would be if the immune system is overwhelmed by implant threatening attacks; it may then shift over to rejection of the oral implant.

This view does not exclude the role of infection in particular cases. When the implants have a maintained immune-caused shield-off, there appears to be bacterial protection. However, there may be situations when this protection may not be active and then a direct infection with subsequent MBL is a possibility that can be exemplified by broken implant components where parts of the implants are not stable. Further, we cannot exclude situations when the immune system is overwhelmed by bacteria that then may act as a regulator of the osteoimmune system, e.g. if the immune system is compromised in some way and the normal bacterial flora becomes pathogenic. Bacterial presence may be controlled by the immune system, but the bacteria will always be present and do not disappear. Therefore, in the age of osteoimmunology, one must always remember that, under the right circumstances, it would be sufficient with only a few surface located and slime protected bacteria to cause infection and severe tissue problems, e.g. as described *via* the "race for the surface" mechanisms (147).

10 Conclusions

1. Osseointegration is needed for oral implant function.
2. Recent advances in osteoimmunology suggest that osseointegration is an osteoimmune defence reaction, more than a simple bone repair process.
3. The bone-anchored implant integration process should in the future be termed"the immunoinflammatory process" instead of only the "inflammatory process". In this process the innervation development adjacent to implants is also important.
4. Osteoimmunological mechanisms underlie marginal bone loss (MBL) as a condition, not a disease.
5. The immune system is capable of causing MBL through its control over the osteoblast/osteoclast coupled function.
6. As far as is known today, bacteria may affect oral implants secondarily once a rejection reaction by the immune system has been initiated. Local bacterial reactions, not affecting implant stability, may occur adjacent to leakage from the abutment implant connection.
7. Patient related factors such as smoking, consumption of certain pharmaceuticals and genetic disorders as well as surgical and prosthodontic techniques, local microbes, foreign bodies such as small cement particles, primary corrosion and implant fractures can cause MBL monitored by the immune system. Secondary corrosion may later add to these oral implant survival challenges that, taken together, may, lead to a shift in the immune reactions from bone shield-off to rejection of the implant.

Author contributions

AT; concept/design, author contribution, data analyses/interpretation. TP; critical revision of article, drafting article. AL; author contribution, critical revision of article. CP; author contribution, concept/design, data analysis/interpretation, drafting article. KG; author contribution, data analysis/interpretation, drafting article. CD; author contribution, data analysis/interpretation, drafting article. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

BIC	bone-implant contact
BMMSC	bone marrow mesenchymal stem cell
BMU	basic multicellular unit
CD68	cluster of differentiation 68
DAMP	danger associated molecular pattern
EMT	epithelial-to-mesenchymal transition
FBE	foreign body equilibrium
FBGC	foreign body giant cell
FBR	foreign body response
GDF-15	growth/differentiation factor 15, a member of transforming growth factor beta family
HMGB1	High mobility group box 1 protein
IL-4	interleukin 4
iNOS	inducible nitric oxide synthase
i-TiP	implant-derived titanium particles
M1	macrophage phenotype 1, pro-inflammatory
M2	macrophage phenotype 2, pro-regenerative
MBL	marginal bone loss
MET	mesenchymal epithelial transition
MNGC	multinucleated giant cell
MSC	mesenchymal stem cell
NF-κB	nuclear factor-κB (NF-κB), a transcription factor
NLRP3	NLR family pyrin domain containing 3
NP	nanoparticle
OPG	osteoprotegerin
Osm	oncostatin M
PAMP	pathogen associated molecular pattern
PPOL	periprosthetic osteolysis
PRR	pattern recognition receptors
PTH	parathyroid hormone
RAGE	receptor for advanced glycation end products, a pattern recognition receptor
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
ROS	reactive oxygen species
scRNA-seq	single cell RNA sequencing technology
TGF-β	transforming growth factor
TLR	toll like receptor
TNF-a	tumor necrosis factor alfa
Wnt	evolutionarily conserved paracrine or autocrine signaling pathways

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Altered early immune response after fracture and traumatic brain injury

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Introduction: Clinical and preclinical data suggest accelerated bone fracture healing in subjects with an additional traumatic brain injury (TBI). Mechanistically, altered metabolism and neuro-endocrine regulations have been shown to influence bone formation after combined fracture and TBI, thereby increasing the bone content in the fracture callus. However, the early inflammatory response towards fracture and TBI has not been investigated in detail so far. This is of great importance, since the early inflammatory phase of fracture healing is known to be essential for the initiation of downstream regenerative processes for adequate fracture repair.

Methods: Therefore, we analyzed systemic and local inflammatory mediators and immune cells in mice which were exposed to fracture only or fracture + TBI 6h and 24h after injury.

Results: We found a dysregulated systemic immune response and significantly fewer neutrophils and mast cells locally in the fracture hematoma. Further, local CXCL10 expression was significantly decreased in the animals with combined trauma, which correlated significantly with the reduced mast cell numbers.

Discussion: Since mast cells and mast cell-derived CXCL10 have been shown to increase osteoclastogenesis, the reduced mast cell numbers might contribute to higher bone content in the fracture callus of fracture + TBI mice due to decreased callus remodeling.

KEYWORDS

fracture healing, traumatic brain injury, inflammation, mast cells, polytrauma

1 Introduction

Despite the remarkably high regeneration capacity of the skeletal system as well as ongoing improvement in fracture treatment during recent decades, orthopaedic complications such as delayed fracture healing or non-unions are still challenging (1). The healing process of bone is strongly dependent on age, trauma severity, fracture fixation, existing comorbidities and other biomechanical and biological factors (2, 3). It has been shown that severe trauma might be a risk factor for orthopaedic complications, especially an additional thoracic trauma or hemorrhagic shock was demonstrated in preclinical models to delay bone regeneration (4–7). On the other hand, both clinical and pre-clinical data suggest that an additional traumatic brain injury (TBI) might lead to accelerated fracture (Fx) healing (8–10), although clinical data are not consistent (11). Bigger fracture calli with higher bone content were found in patients and animals with combined Fx and TBI. Furthermore, TBI patients are more prone to heterotopic ossification (12). Preclinical studies investigating the molecular mechanisms behind this phenomenon linked the additional traumatic brain injury to alterations in metabolism and neuro-endocrine regulations (13, 14). Further, inflammatory mediators were altered in the intermediate phase of fracture healing (14). However, the very early systemic and local inflammatory response towards Fx+TBI has not been investigated in detail so far. This is of great importance in this context, since the early inflammatory phase of fracture healing is known to be essential for the initiation of downstream processes for adequate fracture and tissue repair (15–17). Disturbances in this highly complex process consequently result in delayed or impaired healing, as for example demonstrated by the surgical removal of the fracture hematoma (18, 19). In contrast, an overwhelming local inflammation, induced by immune cell activating agents or systemic immune responses in polytrauma patients also disturbs bone regeneration (19, 20). Among the immune cells present in the hematoma, mast cells and polymorphonuclear neutrophils dominate early after fracture with their non-specific defense mechanisms (21–23). MC-mediated neutrophil recruitment has been shown during fracture healing (24–26) and is also reported in chronic inflammatory diseases (27–29). Therein, MCs regulate vascular leakage and attract neutrophils *via* IL-1β, TNF, KC, and MIP-2 (30–33). Downstream, neutrophils recruit macrophages to the fracture site, which have been shown to be of utmost importance for bone regeneration (34). Besides innate immune cells, also cell populations of the adaptive immune system were found to be involved in fracture healing (35) (36, 37). Especially mast cells (MCs) were shown to be master regulators during the early inflammatory phase of bone regeneration, as they appear during the whole time course of fracture healing, interacting with both innate and adaptive immune cells (24, 25, 38–40). Effector T cells are attracted by MC-derived RANTES and antigen presentation of MCs to cytotoxic T cells was shown (41). Various MC-derived chemokines and leukotrienes additionally contribute to T cell recruitment in distinct inflammatory scenarios (39, 41, 42). Therefore, the aim of this study was to analyze the presence of inflammatory mediators and various immune cells in the circulation and locally in the fracture hematoma early after Fx or combined trauma (Fx+TBI). These data should give additional insights into molecular mechanisms which might be responsible for accelerated fracture healing in case of additional head trauma.

2 Methods

2.1 Experimental design

24 male C57BL/6J mice (provided by Jackson Laboratories) were included in the present study at the age of 10-12 weeks and a body weight of 25-30 g. All experiments were approved by the local animal welfare committee (IACUC UCSF AN143402-03B) and were performed in compliance with international regulations for laboratory animal welfare and handling (ARRIVE guidelines for animal experiments). Half of the mice received a unilateral tibia fracture, and the other half received an unilateral tibia fracture and an ipsilateral traumatic brain injury. 6 mice per group were euthanized at 6h after injury and 24h after injury, respectively. Blood was collected and tibiae were embedded into paraffin for further analysis.

2.2 Tibia fracture

Mice anaesthesized with 2% isoflurane were placed in a pronated position under a fracture apparatus. The apparatus consists of a blunt two-pronged base to frame the tibia and a 2 mm-thick blunt punch connected to a guided 500 g weight. The right tibia was centered in the frame under the punch before the weight was lifted to 5 cm above the tibia. When dropping the weight, a closed fracture was created *via* three-point bending. The fracture was not stabilized, and the animals were allowed to move freely after the surgery. The animals received pain medication by buprenorphine injections (sustained-release buprenorphine HCl 1.2 mg/kg) every 6 h. Fracture location and full fracture were confirmed intraoperatively by radiological examination with a Fluoroscan device.

2.3 Traumatic brain injury (TBI)

Ipsilateral traumatic brain injury was conducted as described previously (14). Briefly, controlled open cortical contusions were applied on the left side of the brain by compressing the cortex 1.7 mm at a rate of 4.5 m/s for 150 ms using a 3 mm wide convex probe. After contusion, the cortex was covered with saline-soaked gelfoam and the wound was closed in separate anatomical layers using sterile sutures. Animals were closely monitored after the injury. Mice received a peri-operative dose of sustained-release buprenorphine HCl (1.2 mg/kg) as an analgesic.

2.4 Sample collection

Mice were euthanized using carbon dioxide. Blood was taken by cardiac puncture. Plasma was collected after centrifugation for 5 min (800 x g, 4°C) and a second centrifugation step for 2 min (13000 x g, 4°C). The samples were stored at -80°C until further analysis.

Fractured tibiae were removed, fixed in 4% formalin for 48h, decalcified for 14 days by EDTA and embedded into paraffin.

2.5 Multiplex analysis

To analyze systemic inflammatory mediators, plasma from mice was analyzed by using the ProcartaPlex Immunoassay (ThermoFisher, Waltham, MA, USA) for granulocyte-colony stimulating factor (G-CSF), interleukin (IL)-6, keratinocyte chemoattractant (KC), IL-10, tumor necrosis factor (TNF), CXCL10 and monocyte chemoattractant protein-1 (MCP-1). All procedures were performed according to the manufacturer's instructions. Some plasma parameters have been published as control samples in a previous study regarding cardiac inflammation after trauma (43).

2.6 Histology, immunohistochemistry, immunofluorescence

Paraffin-embedded tibiae were cut for histological analysis, immunohistochemistry and RNA analysis from formalin-fixed, paraffin-embedded (FFPE) sections. First, tibiae were cut to 7 µm thick longitudinal sections for histological and immunohistochemical analysis. Toluidin blue staining was conducted to analyze mast cell numbers, as granula of mast cells appear as dark violet in this staining. Afterwards, two 15 µm thick RNase-free sections from each block were cut serially and stored in RNase-free tubes at -20°C until further processing. Before cutting, the blade of the microtome and all other used materials were treated with RNaseZap to avoid RNase contaminations. As described below, RNA can be isolated from FFPE sections by using a specific RNA isolation kit. This technique allowed us to use all mice simultaneously for histological analysis, immunohistochemical staining and qPCR analysis. The fractured bones were cut until the bone marrow was visible on both sides of the fracture making sure that the middle part of the fracture hematoma was displayed on the slices. With this technique, we made sure that always the same area was analyzed.

Staining for Ly6G, F4/80, CD8 and CXCL10 was performed using the following primary antibodies incubated overnight at 4°C: rat anti-mouse Ly6G (1:200; 127632, BioLegend, San Diego, CA, USA) and rat anti-mouse F4/80 (1:500; #MCA497GA, Biorad, Hercules, CA, USA), goat anti-mouse CXCL10 (1:50; #AF-466-NA, R&D systems), rabbit anti-mouse CD8 (1:500, Bioss #bs-0648R). As secondary antibodies, goat-anti rabbit IgG-biotin (1:200; #B2770, Life Technologies, Carlsbad, CA, USA) and goat anti-rat IgG-biotin (1:100 and 1:200 respectively for Ly6G and F4/80 staining; A10517, Invitrogen, Carlsbad, CA, USA) were used and incubated at room temperature (RT) for 30 min or 1 h, respectively. For signal detection, horseradish peroxidase (HRP)-conjugated streptavidin (#PK-6100, VECTASTAIN® Elite ABC-HRP Kit, Peroxidase, Vector Laboratories, Burlingame, UK) was applied according to the manufacturer`s protocols. NovaRED (#SK-4800, Vector® NovaRED® Substrate Kit, Peroxidase (HRP), Vector laboratories) was used as chromogen and the sections were counterstained with hematoxylin (1:2000; #2C-306, Waldeck, Münster, Germany).

Immunofluorescence double staining for CXCL10 and Avidin was performed using the following antibodies: goat anti-mouse CXCL10 (1:50; #AF-466-NA, R&D systems) and Avidin Texas Red (1:150 A820, ThermoFisher) incubated at RT for 1 h. Rabbit anti-goat IgG (H+L) FITC (#A16143, Life Technologies) was used in a concentration of 1:50 for CXCL10 staining as the secondary antibody. Species-specific non-targeting immunoglobulins were used as isotype controls. We have demonstrated previously that Avidin is a very good tool to stain mast cells in tissue sections in various animal models (24, 25, 44, 45).

2.7 RNA isolation and qPCR

Total RNA isolation was performed using the FFPE RNEasy kit from Qiagen and RT-PCR was performed as described previously (46). Quantitative RT-PCR was performed using the SensiFAST SYBR Hi-ROX One-Step Kit (Bioline, Memphis, TN, USA). *B2m* was used as the housekeeping gene (F: 5′-ccc gcc tca cat tga aat cc-3′, R: 5′-tgc tta act ctg cag gcg tat-3′). Relative gene expression of TNFa (5'- GGC CAC CAC GCT CTT CTG TCT ACT -3', 5'- TGA TCT GAG TGT GAG GGT CTG GGC -3'), IL1beta (5'-aca agg aga acc aag caa cg-3', 5'-ggg tgt gcc gtc ttt cat ta-3'), IL-6 (5'-tcc ttc cta ccc caa ttt cc-3', 5'-gcc act cct tct gtg act cc-3'), IL-10 (5'-GGC AGA GAA GCA TGG CCC AGA AAT C-3', 5'-ACT CTT CAC CTG CTC CAC TGC CT-3') and CXCL10 (5'-GGATCCCTCTCGCAAGGA-3', 5'-ATCGTGGCAATGATCTCAACA-3') was calculated using the delta-delta CT method (relative to B2m and the Fx group).

2.8 Statistical analysis

Group size was n=6 for each treatment and time point. Data from Fx and Fx + TBI groups were compared by using the unpaired Student's t-test. P-values of less than 0.05 were considered as statistically significant. Correlation analysis was done by matching CXCL10 protein expression scores from each mouse to the cell counts from the same mouse at both 6h and 24h. Data were analyzed by simple linear regression. Statistical analysis and graphs were done by GraphPad Prism 9. Data are displayed as mean + standard deviation with individual values indicated as black dots for the Fx group and black boxes for the Fx+TBI group.

3 Results

3.1 Systemic inflammation after fracture and TBI

To analyze systemic inflammation after fracture and combined trauma, several pro- and anti-inflammatory mediators known to be involved in fracture healing were determined in plasma samples at 6h and 24h after injury (Table 1). G-CSF, IL-6 and IL-10 levels did not differ between Fx and Fx+TBI mice at all time points. KC was significantly increased in the combined trauma group at 6h, but not at 24h after injury. MCP1 was significantly increased in the combined trauma group at 24h, but not at 6h after injury. CXCL10 was significantly reduced in the Fx+TBI mice at both time points. These data indicate a dysregulated

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TABLE 1 Inflammatory mediator levels in the plasma.

	6h		24h	
	Fx	Fx + TBI	Fx	Fx + TBI
Plasma				
G-CSF	40.7 ± 12.3	154.6 ± 172.4	84.4 ± 93.8	94.1 ± 12.2
KC	253.7 ± 131.1	483.8 ± 129.9*	107.5 ± 102.5	91.5 ± 7.9
IL-6	155.6 ± 56.8	291.7 ± 281.4	207.7 ± 260.3	120.6 ± 68.0
IL-10	6.2 ± 6.9	13.6 ± 4.1	16.9 ± 14.2	29.6 ± 12.1
CXCL10	87.2 ± 35.5	40.5 ± 29.7*	129.1 ± 48.1	66.7 ± 12.1*
MCP1	30.2 ± 7.9	50.41 ± 20.5	49.9 ± 13.8	68.9 ± 12.0*

*Significantly different ($p<0.05$) compared to the Fx group, Student's t-test, Fx, isolated fracture; Fx + TBI, fracture and additional traumatic brain injury; IL, Interleukin; CXCL, C-X-C motif chemokine ligand.

systemic inflammatory response after combined trauma. In general, variations of cytokine levels between the individual mice of one group were especially high in the Fx+TBI group 6h after trauma, which might be due to the combination of two traumata and could influence conclusions draws from that data.

3.2 Local expression of pro- and anti-inflammatory mediators in the fracture hematoma

To analyze local immune reaction after fracture or combined trauma, several pro- and anti-inflammatory mediators known to be present in the early fracture hematoma, were determined by qPCR analysis after 6h and 24h (Figure 1). Interleukin-6 gene expression was significantly reduced in the hematoma of Fx+TBI mice compared to FX mice 6h, but not 24h after injury (Figures 1A, B). CXCL10 gene expression was significantly reduced at both time points (Figures 1C, D). Gene expression levels of IL-1beta, TNFalpha and IL-10 did not differ locally in the fracture hematoma at all time points (Figures 1E–K). To further verify the reduced expression of CXCL10 also on protein levels, immunohistochemical staining was performed (Figure 2). Indeed, CXCL10 was less expressed in the early fracture hematoma of Fx+TBI mice compared to Fx only mice. Expression was found in bone marrow/hematoma areas around the fracture site.

3.3 Immune cell infiltration into the fracture hematoma

Immune cell infiltration into the fracture hematoma was characterized by immunohistochemical staining for Ly6G

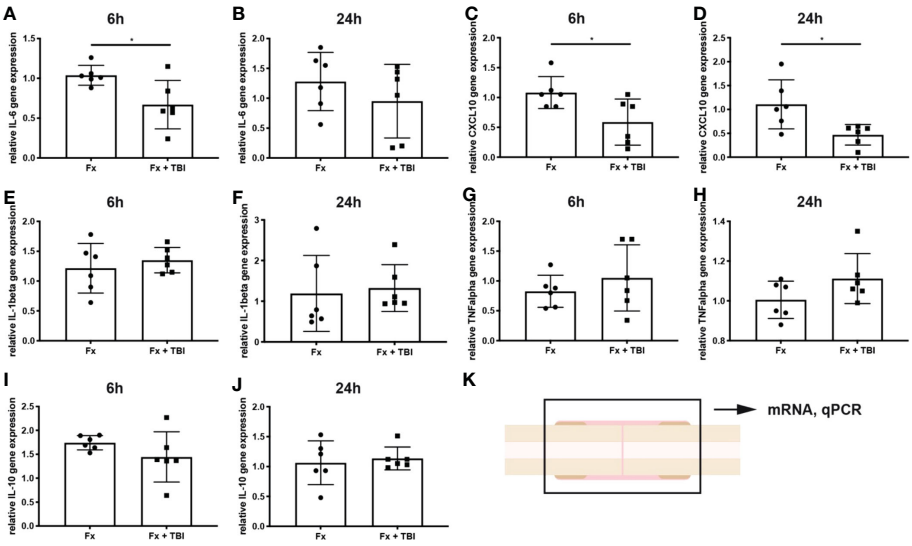

FIGURE 1
Local gene expression of inflammatory mediators in the hematoma. RNase-free sections of the fractured bones were cut, RNA was isolated and gene expression analyzed by qPCR. Data are normalized to the housekeeping gene B2M and the Fx group, respectively. **(A)** Relative IL-6 gene expression at 6h and **(B)** 24h after injury. **(C)** Relative CXCL10 gene expression at 6h and **(D)** 24h after injury. **(E)** Relative IL-1beta gene expression at 6h and **(F)** 24h after injury. **(G)** Relative TNFalpha gene expression at 6h and **(H)** 24h after injury. **(I)** Relative IL-10 gene expression at 6h and **(J)** 24h after injury. **(K)** Experimental design. * indicates p-value below 0.05 for comparison between Fx and Fx+TBI.

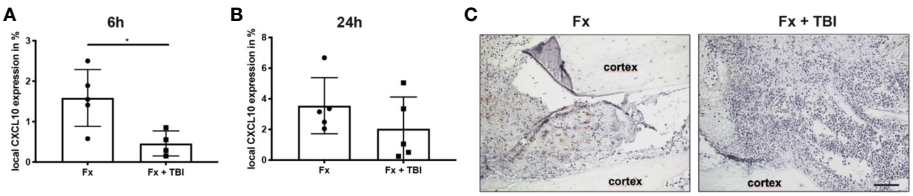

FIGURE 2
Local protein expression of CXCL10 in the hematoma. Longitudinal sections of the fracture bones were cut and stained for CXCL10. Staining was quantified by positive pixel amount relative to the total pixel. **(A)** Local CXCL10 protein expression at 6h and **(B)** 24h after fracture. **(C)** Representative images from the fracture area at 6h after fracture. Scale bar = 50 µm. * indicates p-value below 0.05 for comparison between Fx and Fx+TBI.

(neutrophils), F4/80 (macrophages), and CD8 (cytotoxic T-lymphocytes). Mast cells were counted based on their violet-appearing granules in Toluidin blue staining. Significantly fewer neutrophils were found in the hematoma of Fx + TBI mice at both 6h and 24h after injury (Figures 3A, B). Macrophage and $CD8^+$ T-cell numbers did not differ between the groups (Figures 3C–F). Further, significantly fewer mast cells were found in the hematoma of Fx + TBI mice at both 6h and 24h after injury (Figures 3G–I). These data indicate a dampened neutrophil and mast cell infiltration into the fracture hematoma after combined trauma. To further analyze if the reduced protein expression of CXCL10 might be due to reduced neutrophil and/or mast cell numbers, we performed a correlation analysis between the parameter's neutrophil numbers, mast cell numbers, and local CXCL10 protein expression in all samples (Figures 4A–C). We detected no significant correlations between neutrophils and CXCL10 protein expression and between neutrophil and mast cell numbers, however, mast cell numbers and local CXCL10 protein expression correlated significantly with $R^2 = 0.5417$. We further established an immunofluorescence double staining method for mast cells and CXCL10 and confirmed increased CXCL10 staining in areas with many mast cells (Figures 4D–F). However, not all mast cells were positive for CXCL10 also non-mast cells were detected to express CXCL10, therefore we assume that mast cells are not the only cell population secreting this protein in the fracture hematoma, but might be one of the most important sources.

4 Discussion

Pre-clinical data strongly indicates an accelerated fracture healing after a combined traumatic brain injury (10). Clinical observations also suggested an increased callus and bone formation after combined injury (47), however strong evidence is still lacking due to the challenging monitoring of polytrauma patients (11). In preclinical models, the observation of a stronger fracture callus has been linked to altered metabolism and neuro-endocrine regulations (13, 14). In more detail, it has been shown that blood-brain barrier leakage after TBI leads to increased release of osteogenic factors from peripheral nerves (12). Further, the spenic pro- and anti-inflammatory response towards fracture was altered in TBI mice (13, 14). Other studies have linked increased bone content in the fracture callus after TBI with factors like SDF-1 (48), prolactin (49) and leptin (50). The latter was shown to influence metabolic parameters like insulin and posttraumatic osteocalcin secretion (13) and thereby altering osteoblast differentiation. Further, serum samples from patients

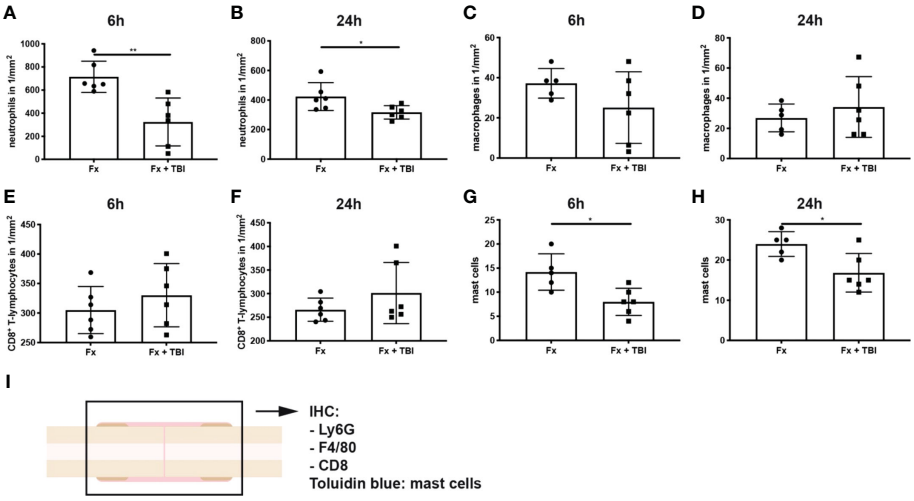

FIGURE 3
Immune cell populations in the fracture hematoma. Longitudinal sections of the fracture bones were cut and stained for immune cell markers. **(A)** $Ly6G^+$ neutrophil numbers at 6h and **(B)** 24h after injury. **(C)** $F4/80^+$ macrophage numbers at 6h and **(D)** 24h after injury. **(E)** $CD8^+$ T-lymphocyte numbers at 6h and **(F)** 24h after injury. **(G)** Mast cell numbers were determined in Toluidin blue staining at 6h and **(H)** 24h after injury. **(I)** Experimental design. * indicates p-value below 0.05 for comparison between Fx and Fx+TBI; ** indicates p-value below 0.01 for comparison between Fx and Fx+TBI.

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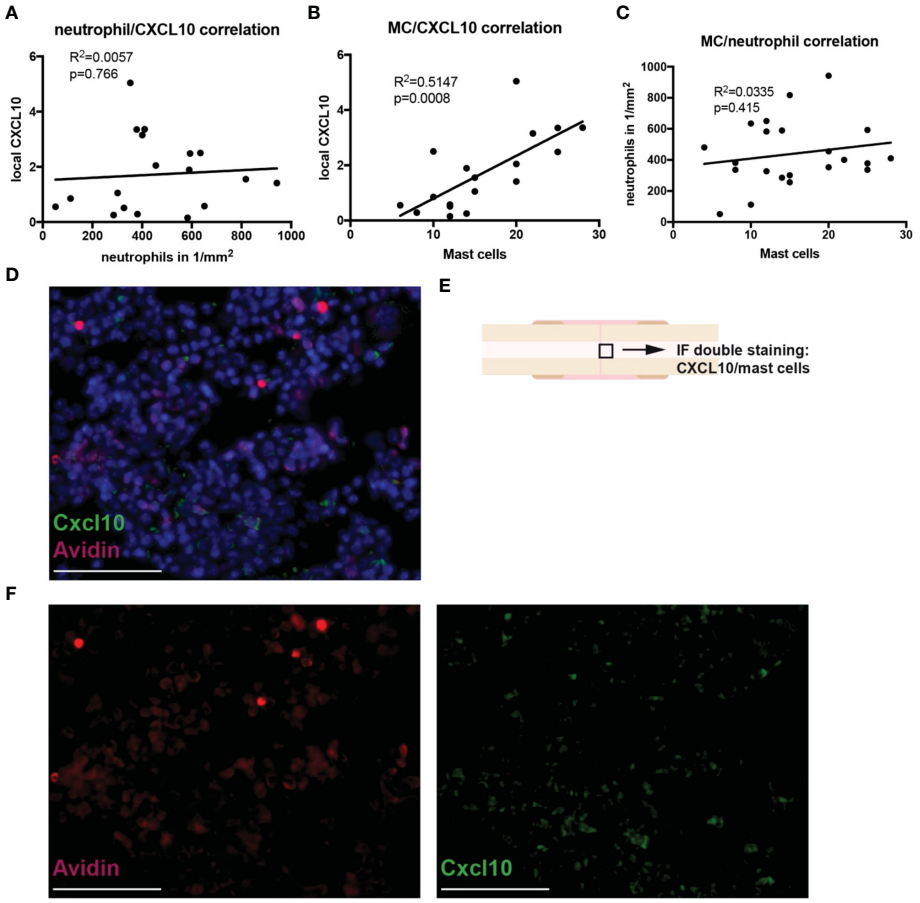

FIGURE 4
Correlations between neutrophil/mast cell numbers and CXCL10 expression in the fracture hematoma during the early inflammatory phase. Correlation analysis by simple linear regression was performed between the parameters neutrophil numbers, mast cell numbers and local CXCL10 protein expression in all samples. **(A)** Neutrophil number/CXCL10 correlation. **(B)** Mast cell number/CXCL10 correlation. **(C)** Mast cell number/neutrophil number correlation. **(D)** Immunofluoresence double staining for mast cells (Avidin staining, red) and CXCL10 (green). DNA was counterstained with Hoechst (blue). Scale bar = 50 µm. E) Black box marked the area which is shown in **(E). (F)** Single fluorescent channels for Avidin (red) and CXCL10 (green). Scale bar = 50 µm.

with TBI were shown to accelerate osteogenic differentiation thereby indicating that systemic humoral factors might be involved (51, 52). However, the very early inflammatory phase of fracture healing after combined trauma has not been investigated in detail so far. This would be important since early inflammation has clearly been linked to fracture healing outcome. Therefore, the aim of this study was to analyze the early inflammatory reaction to fracture and fracture+TBI in a mouse model of ipsilateral polytrauma.

We found a dysregulated systemic inflammatory reaction in the combined trauma group with the pro-inflammatory cytokines KC and MCP1 being significantly increased. In contrast, the cytokine CXCL10 was significantly reduced in Fx+TBI mice both systemically and locally in the hematoma. This correlated significantly with reduced mast cell numbers in the fracture hematoma, while neutrophil numbers were also significantly decreased. KC, also known as CXCL1, has been shown to be highly expressed after a fracture event in mice (53) and is produced by a lot of different inflammatory cell types. KC is important for the recruitment of neutrophils to sites of injury. MCP1, also known as CCL2, is a major regulator of monocyte recruitment and is secreted by a variety of different cell types upon inflammatory stimulus (54). CXCL10, also known as IP-10, is a pro-inflammatory cytokine which is produced by mast cells, but also by some other cell types like fibroblasts and endothelial cells. Interestingly, it has been shown in the context of fracture healing, that mast cell-derived CXCL10 contributes to increased osteoclastogenesis in the fracture callus in osteoporotic mice after femur fracture (25). Mast cell-deficient mice displayed less osteoclasts in the fracture callus, a reduced callus remodelling (24) and were protected from delayed fracture healing after ovariectomy (25) and additional thoracic trauma (44). This indicates a critical role of mast cells during fracture healing and might suggest that reduced mast cell numbers could have positive effects on the healing process regarding callus bone mass, although of course callus remodeling during later healing phases is also important for fracture healing outcome in patients. We suggest that reduced mast cell numbers in the fracture hematoma and decreased local CXCL10 expression in fracture+TBI mice might lead to reduced osteoclast numbers in the later fracture callus and thereby contributing to the increase bone mass seen frequently in fracture+TBI mice in previous studies (13, 14). Clinical data supporting the hypothesis of reduced callus remodeling after TBI is available from Andermahr et al., showing reduced markers of collagen degradation in polytrauma patients with TBI (55). Further, mast cells can also influence osteoblast differentiation by secreting factors like IL-6 or Midkine (56). However, to really prove the involvement of mast cells, it would be necessary to investigate fracture healing after TBI in mast cell-deficient mice and to

analyze later healing stages. This would be an interesting perspective for future studies to link mast cell appearance with osteoclastogenesis and osteoblastogenesis in the fracture callus after additional TBI.

It was also shown previously that mast cells regulate the recruitment of neutrophils to the fracture hematoma (24, 25). Therefore, it was not surprising to us that we found both reduced mast cell and neutrophil numbers in the fracture hematoma. However, we did not detect a direct correlation between mast cell and neutrophil numbers in the fracture hematoma of all animals, indicating that mast cells are not the only important regulator in neutrophil recruitment. It was also demonstrated that mast cells might be involved into the recruitment of T lymphocytes during inflammatory conditions (41). Various MC-derived chemokines and leukotrienes contribute to T cell recruitment in distinct inflammatory scenarios (39, 41, 42). Since we did not detect a difference in $CD8^+$ T cells in the fracture hematoma, we suggest that during fracture healing, other stimuli are more important to recruit cells of the adaptive immune system to the fracture hematoma. This is also in line with previous data showing no differences in T cells numbers between mast cell-competent and mast cell deficient mice after fracture (24). Interestingly, previous studies have demonstrated reduced macrophage and monocyte numbers in the fracture callus of mast cell-deficient mice (24). Since we did not detect such differences in the present study, mast cells might not be critical for macrophage recruitment in the context of an additional TBI.

Limitations of our study are, as mentioned above, that we did not investigate later time points of fracture healing in this study. However, as we have demonstrated previously in that model, mice with a tibia fracture and an additional TBI displayed increased bone area in the fracture callus, while total callus area, cartilage and vascular tissue area were not altered (14). This indicates accelerated fracture healing in those mice. Another limitation is that we used a non-stabilized tibia fracture model and therefore the interfragmentary strains might differ between different animals. And since it was shown that local strains and stresses do also influence inflammation (57–59), this undefined mechanical situation might lead to higher standard deviations in the inflammatory parameters as seen in some of our datasets. Therefore, although we did a power analysis previous to our study, it would be recommended to increase sample size for future studies. Another limitation is that we did not investigate molecular mechanisms leading to the altered inflammatory status in the mice with fracture + TBI in detail. We hypothesize that traumatic brain injury leads to a recruitment of inflammatory cells to the brain rather than to the fracture location and therefore the additional injury dampens the inflammation in the fracture hematoma. There is evidence from the literature that TBI leads to influx of immune cells due to disruption of the blood-brain barrier and that this neuroinflammation can also cause long-lasting brain disfunctions (60, 61). Mast cells in the brain seems to also play a role during that process (62–64). Therefore, in our next study using the present model of fracture + TBI, we will carefully investigate also the brain tissue to further analyze neuroinflammation and bone-brain trauma crosstalk in more detail.

In conclusion, we found a dysregulated systemic inflammatory response towards fracture in mice with an additional TBI with some inflammatory cytokines being increased and some being decreased. Further, we could link decreased local expression of CXCL10 to reduced mast cell numbers in the fracture hematoma of combined trauma mice. This might contribute to accelerated fracture healing frequently seen in mice with fracture and an additional TBI as increased mast cell numbers has been linked to delayed fracture healing. Investigating the molecular mechanisms in more detail might give further insights into the molecular and cellular regulation of successful bone regeneration.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by IACUC UCSF AN143402-03B.

Author contributions

Design of experiments: MH-L, BW, MK, KM, RM, and TM. Funding of experiments: MH-L, BW, MK, RM, AI, and TM. Conduction of experiments: MH-L, BW, KM, IL, VF, and CB. Drafting the manuscript: MH-L, RM, and TM. Editing the manuscript: MH-L, BW, KM, IL, VF, CB, AI, MK, RM, and TM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Inflammation produced by senescent osteocytes mediates age-related bone loss

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Purpose: The molecular mechanisms of age-related bone loss are unclear and without valid drugs yet. The aims of this study were to explore the molecular changes that occur in bone tissue during age-related bone loss, to further clarify the changes in function, and to predict potential therapeutic drugs.

Methods: We collected bone tissues from children, middle-aged individuals, and elderly people for protein sequencing and compared the three groups of proteins pairwise, and the differentially expressed proteins (DEPs) in each group were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). K-means cluster analysis was then used to screen out proteins that continuously increased/decreased with age. Canonical signaling pathways that were activated or inhibited in bone tissue along with increasing age were identified by Ingenuity Pathway Analysis (IPA). Prediction of potential drugs was performed using the Connectivity Map (CMap). Finally, DEPs from sequencing were verified by Western blot, and the drug treatment effect was verified by quantitative real-time PCR.

Results: The GO and KEGG analyses show that the DEPs were associated with inflammation and bone formation with aging, and the IPA analysis shows that pathways such as IL-8 signaling and acute-phase response signaling were activated, while glycolysis I and EIF2 signaling were inhibited. A total of nine potential drugs were predicted, with rapamycin ranking the highest. In cellular experiments, rapamycin reduced the senescence phenotype produced by the H_2O_2-stimulated osteocyte-like cell MLO-Y4.

Conclusion: With age, inflammatory pathways are activated in bone tissue, and signals that promote bone formation are inhibited. This study contributes to the understanding of the molecular changes that occur in bone tissue during age-related bone loss and provides evidence that rapamycin is a drug of potential clinical value for this disease. The therapeutic effects of the drug are to be further studied in animals.

KEYWORDS

age-related bone loss, senescence, inflammation, proteomics, osteocyte, rapamycin

Introduction

Bone is a dynamic organ in which bone formation mediated by osteoblasts balances against bone resorption mediated by osteoclasts to maintain bone homeostasis (1). With age, this balance gradually tilts toward bone resorption, leading to bone loss and osteoporosis (2, 3). The most important complication of osteoporosis is fracture (4), which leads to increased mortality and makes a significant impact on the health and quality of life of patients (5). As the population ages, the incidence of fractures due to osteoporosis is also increasing, which is a major health problem (6).

Bone senescence is a highly complicated process, which results from the interplaying of systemic and local factors with a variety of bone-related cells, including osteocytes, osteoblasts, osteoclasts, bone-marrow-derived mesenchymal stem cells (BMSCs), and bone-marrow-derived macrophages (BMDMs) in response to various intracellular and extracellular stimuli, such as oxidative stress, genetic damage, and the altered responses of bone cells to various biological signals and to mechanical loading (7). During bone aging, senescent osteocytes and myeloid cells are the main sources of senescence-associated secretory phenotype (SASP) in the bone microenvironment, and the expression levels of SASP components including p53, p21, and p27 were significantly elevated (8). SASP is the most important feature of senescent cells and is a conserved cellular response that manifests as a low-grade chronic inflammatory state that emerges with age (9). The pro-inflammatory phenotype of SASP is mediated by NF-κB cascade amplification signals (10).

A hallmark of the aging process is a progressive increase of chronic inflammation, which was originally called "inflamm-aging" (11). Although restricted inflammation is beneficial for bone repair, systemic chronic inflammation yielding excessive proinflammatory cytokines such as IL-1, IL-6, and TNF is detrimental to bone formation and fracture healing (12). Macrophages were considered as the primary player in mediating the inflammatory responses (13). However, several studies indicated that aged macrophages are less responsive to IFNγ or LPS by secreting the lower levels of inflammatory cytokines (14, 15). Osteocytes, accounting for over 90% of the bone cells, can transmit signals to each other by forming a network of tubules through axons (16). Current studies have shown that bone tissue expression of pro-inflammatory factors is elevated in mice with osteoporosis, such as TNF-α (17), IL-6 (18), and IL-1 (19). Nevertheless, the cells mainly mediating aging-associated inflammatory responses are unclear.

Proteins are the most important functional executor in a living organism. Proteomics based on label-free liquid chromatography-mass spectrometry (LC-MS/MS) routinely quantifies thousands of proteins across multiple samples in a single run, the following annotation providing an important path for the study of disease pathology and the discovery of therapeutic targets. Several groups have performed a proteomics approach to explore the pathology of bone-related diseases, including osteoporosis (20, 21), osteosarcoma (22, 23), osteoarthritis (24), and bone fracture (25). Most of the proteomics studies used cultured cell samples, including BMSCs (26–29), osteoblasts (30, 31), and osteoclasts (32–34). However, the proteomic alteration of cultured cells in response to a certain stimulus cannot simulate the actual situation of bone tissues *in vivo*. Moreover, previous proteomics studies on human bone tissues are scarce, and the overall research in bone primarily focused on genomics and transcriptomics (35). It might result from the lack of access to obtain in clinics and the costs. Also, postmenopausal osteoporosis cannot be equated with age-related bone loss. In addition to all the above restraints, proteomics analysis about bone aging was limited so far.

In the present study, the bone specimens from children, middle-aged patients, and older individuals were subjected to proteomics analysis by LC-MS/MS. The differentially expressed proteins (DEPs) from the pairwise comparison or from three groups continuously up- or downregulated with age were annotated. We also compared and investigated the possibility of osteocytes as the main cells producing the inflammatory-associated DEPs or signaling pathways during bone aging. In addition, rapamycin was predicted as an inhibitor of bone aging. Finally, we confirmed the reliability of our proteomics results and the effect of rapamycin on the expression of the inflammatory or SASP marker genes. Our study will advance a better understanding of the molecular mechanisms of bone aging.

Methods

Collection of human samples

The project was approved by the Ethics Committee of the Union Hospital of Tongji Medical College, Huazhong University of Science and Technology (Ethics No. 2020-S001). The procedure was according to approved guidelines. Human bone samples were collected from patients undergoing surgical treatment in the orthopedic surgery department at Union Hospital. These bone samples would usually have been discarded as part of joint replacement surgery or associated surgery. The study included 33 subjects, with 11 samples from 2 to 12 years old, 11 samples from 41 to 54 years old, and 11 samples from 69 to 88 years old. Subjects who had tumors or systemic diseases, were immunologic, were treated with steroids or hormones, or had other factors that might affect bone metabolism were excluded. In order to avoid the influence of bone-related diseases on the local bone microenvironment, we sampled the site as far away from the lesion as possible. When the tissue is collected, it is washed using saline to remove blood from the surface, then stored in liquid nitrogen. The collected bone tissue does not contain bone marrow or cartilage tissue, its cellular component is mainly osteocytes, and other components include mineral salts and various proteins (collagen and non-collagen). The basic information of the 33 individuals and the anatomical sites from which the samples were collected are included in Supplementary Table 1.

Label-free quantitative proteomics analysis

The bone tissue was fully ground to a powder by adding liquid nitrogen, and each sample was lysed by adding 4 times the volume of powder lysis solution (1% SDS, 1% protease inhibitor), sonicated at 4°C, and centrifuged at 12,000*g* for 10 min. The supernatant was transferred to a new centrifuge tube for protein concentration determination using a BCA kit. Trypsin was added and enzymatically cleaved into peptide fragments. The peptides were dissolved with liquid chromatography mobile phase A and separated using EASY-nLC 1200 UHPLC system and then injected into an NSI ion source for ionization and then into a mass spectrometer (Q Exactive™ HF-X) for analysis. The data acquisition mode was performed using a data-dependent scanning (DDA) program.

Functional enrichment analysis

Proteins in the three groups were compared with each other, and proteins with p-value <0.05 and fold change >1.5 or <1/1.5 determined by Student's t-test were defined as differentially expressed proteins (DEPs). Pearson's correlation coefficient was used to detect correlations between groups of samples, visualized by TBtools (36). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) functional enrichment analyses were performed on the DAVID database (https://david.ncifcrf.gov/), and the parameter settings are all default values. The results of the GO analysis were plotted using GraphPad Prism 8.0, and the results of KEGG analysis were visualized using an online platform (http://www.bioinformatics.com.cn). To analyze protein temporal changes with age, the DEPs were analyzed by the k-means clustering algorithm and then visualized by an online platform (http://www.bioinformatics.com.cn).

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) was used to predict the activation or inhibition state of the canonical pathway (37), and it was analyzed based on the reported literature. The lists of DEPs were uploaded to the IPA software (QIAGEN). The "core analysis" of DEPs was first performed in the software, and the results can be obtained for the canonical signaling pathways and upstream regulatory molecules. In addition, a "comparative analysis" can be performed for the pairwise comparison groups. Utilizing the software, predictions are scored by z-score: when the z-score is greater than or equal to 2, predictions are activated, and when the z-score is less than or equal to −2, predictions are suppressed.

Connectivity map analysis

To explore potential drugs by Connectivity Map (CMap) analysis (https://clue.io/query), the dataset allows for drug prediction based on gene changes. So, we predicted potential therapeutic drugs by targeting proteins that change when age-related bone loss occurs. The database scores all predicted drugs from −100 to 100. All drugs predicted were selected for the generation of a heatmap according to the scores. A score of 100 means that the drug produces exactly the same perturbation as the change in the input gene, while −100 means that the drug produces a perturbation exactly opposite to the change in the input gene. When screening for therapeutic drugs, drugs with changes opposite to the DEPs and scores less than −90 are considered meaningful.

GSEA

Gene set enrichment analysis (GSEA) was performed using the pre-ranked method in GSEA Java (http://software.broadinstitute.org/gsea/msigdb), and genes from GSE141595 were used for the analysis (8). For our study, we used all the C5 collection and interesting signaling pathways related to inflammation for GSEA. The minimum and maximum numbers for the selection of gene sets from the collection were 10 and 500 genes, respectively.

Animals

All experimental procedures involving animals were approved by the Animal Care and Use Committee of Wuhan Union Hospital (Ethic No.3047). Three of each of the 6-week-old (young) and 18-month-old (old) C57BL/6J mice were bought from Beijing Vital River Laboratory Animal Technology (Beijing, China). Mice were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and subsequently executed by cervical dislocation followed by immersion in 75% alcohol for 5 min. The mouse skin and muscle were scissored to separate the mouse tibia and femur. The bone marrow cavity of the mice was opened in a sterile operating table and then flushed with PBS to remove the bone marrow, leaving the bony part. Bones from each mouse were mixed and placed in liquid nitrogen and then ground with a mortar and pestle. Bone pieces were lysed in 1*RIPA buffer (Beyotime, China) with proteinase inhibitor cocktail (Beyotime, China) for 15 min at 4°C. Bone debris was removed after centrifugation at 3,000 rpm for 5 min at 4°C. Bone samples were stored at −80°C for the subsequent experiments.

Cell culture

MLO-Y4 cells were utilized as osteocytes in our research which were bought from iCell Bioscience (China). They were cultured in 12-well plates in α-MEM supplemented with 10% FBS and 1% PS. Mild concentrations of H_2O_2 at 400 μM for 12 h were utilized to construct an induced senescent phenotype (38, 39), and then the phenotype was treated with different concentrations of rapamycin for 24 h.

Quantitative real-time PCR

The total RNA of MLO-Y4 cells was extracted by TRIzol (Biosharp), and cDNA was reverse-transcribed using HiScript 1st Strand cDNA Synthesis Kit (Vazyme) and real-time PCR using SYBR qPCR Mix (Vazyme). The primer sequences were as follows: β-actin (mouse): 5′-CATTGCTGACAGGATGCAGAAGG-3′ (forward) and 5′-TGCTGGAAGGTGGACAGTGAGG-3′ (reverse); IL-6 (mouse): 5′-TACCACTTCACAAGTCGGAGGC-3′ (forward) and 5′-CTGCAAGTGCATCATCGTTGTTC-3′ (reverse); P53 (mouse): 5′-CCTCAGCATCTTATCCGAGTGG-3′ (forward) and 5′-TGGATGGTGGTACAGTCAGAGC-3′ (reverse); P21 (mouse): 5′-TCGCTGTCTTGCACTCTGGTGT-3′ (forward) and 5′-CCAATCTGCGCTTGGAGTGATAG-3′ (reverse); P27 (mouse): 5′-AGCAGTGTCCAGGGATGAGGAA-3′ (forward) and 5′-TTCTTGGGCGTCTGCTCCACAG-3′ (reverse); and Opg (mouse): 5′-CGGAAACAGAGAAGCCACGCAA-3′ (forward) and 5′-CTGTCCACCAAAACACTCAGCC-3′ (reverse).

Western blot analysis

The human and mouse bone protein lysates were loaded into 10% SDS-PAGE gels, and the gels were cut into two parts. They were transferred into a 0.45-μm polyvinylidene difluoride membrane (Millipore) and separated. The large molecule protein CSPG4 (A3592, ABclonal) was processed at 300 mA for 3 h at 4°C with 10% methanol, and ITGA2B (A5680, ABclonal), tubulin (GB11017,

Servicebio), and β-actin (GB11001, Servicebio) were processed at 300 mA for 1.5 h at 4°C with 20% methanol. The intensity of the protein was analyzed with ImageJ software.

Statistical analysis

Student's *t*-test was the statistical method used to compare protein sequencing results. GraphPad Prism 8.0 was used to perform one-way ANOVA with Bonferroni correction for comparisons among more than two groups in the cellular experiments. Significance was determined at $p < 0.05$. All experiments were performed at least in triplicate and in three independent experiments.

Results

Characterization of proteomics of human bone tissues at different ages

To identify the key proteins/pathways and candidate biomarkers during bone aging, we performed label-free LC-MS/MS proteomic sequencing on bone tissues from the three cohorts: children (group A), middle-aged individuals (group B), and older individuals (group C). As shown in Figure 1, the DEPs ($p < 0.05$, fold change > 1.5 or fold change < 0.667) were subjected to further bioinformatic analysis, including GO analysis, KEGG analysis, and IPA analysis. The potential drugs to treat bone aging were also predicted based on the DEPs, and we also verified the expression of several key DEPs and the effect of the predicted drugs on bone cell senescence (Figure 1).

As shown by Pearson's correlation analysis (Figure 2A) and principal component analysis (PCA, Figure 2B), the within-group variation is relatively low in the children group, whereas the variations are high in both the middle-aged group and the older group, implying large individual differences after bone maturation. Moreover, the children group was significantly different from the other two groups. Accordingly, there is a great difference in protein profiling between the children group and the other two groups which had some overlapped individuals (Figure 2B). The heatmap of DEPs also shows more DEPs between the children group and the other two groups (Figure 2C). As shown in Figure 2D, the total number of DEPs when comparing the middle-aged and children groups (B–A) is 622, of which 365 were downregulated and 257 were upregulated. There are 513 DEPs with 278 downregulated and 235 upregulated in the bone tissues from the older group compared with the children group (C–A). Only a small number of DEPs (112) were found between the older group and the middle-aged group (C–B). All data indicated that the proteins in bone tissues were differentially expressed with aging.

Analysis of the DEPs from the pairwise comparison

The DEPs from the pairwise comparison between middle-aged individuals and children (B–A), older individuals and children (C–A), or older and middle-aged individuals (C–B), respectively, were annotated to GO and KEGG analyses. Figure 3A shows the results of GO analysis for the three paired comparison groups, and the top 15 molecular functions, the top 5 cellular components, and the top 15 biological processes were listed. The complete GO analysis data are listed in Supplementary Table 2. Notably, in the B–A groups, biological processes were enriched in aging, blood coagulation, positive regulation of I-κB kinase/NF-κB signaling, and innate immune response (Figure 3A, left). In the C–A groups, biological processes were enriched in skeletal system development, collagen fibril organization, osteoblast differentiation, and innate immune response (Figure 3A, middle). In the C–B groups, biological processes were enriched in the intrinsic apoptotic signaling pathway in response to oxidative stress and acute-phase response (Figure 3A, right). These suggest that DEPs are associated with inflammation and bone formation.

KEGG analysis showed that the DEPs in the B–A groups mainly mediated ribosome, phagosome, glycolysis/gluconeogenesis, complement and coagulation cascades, HIF-1 signaling pathway, etc. (Figure 3B, left). Likely, the DEPs in the C–A groups mediated phagosome, glycolysis/gluconeogenesis, pyruvate metabolism, and HIF-1 signaling pathway (Figure 3B, middle). The DEPs of the C–B groups mostly participated in endocytosis, ribosome, Huntington disease, prion disease, and Parkinson disease (Figure 3B, right).

Then, we used IPA to determine whether the signaling pathways were activated or inhibited with age. The results show that the neuroinflammation signaling pathway, coagulation system, IL-8 signaling, acute-phase response signaling, and CXCR4 signaling are significantly activated in the middle-aged and older groups, suggesting that inflammatory signaling pathways are significantly activated in bone tissue with age (Figure 3C). In contrast, EIF2 signaling and glycolysis I, which facilitate bone formation (40–43), are significantly inhibited in the middle-aged and older groups (Figure 3C). The complete canonical signaling pathway prediction data are listed in Supplementary Table 3.

Inflammation might be generated from osteocytes

The upstream regulator analysis (performed by IPA) allowed us to predict transcription factors, small RNAs, and drugs causing the observed protein alterations. The heatmap according to *z*-score shows the top 5 activated and inhibited transcription factors in the three paired comparison groups (Figure 4A). The complete upstream regulator prediction data are listed in Supplementary Table 4. We identified RELA proto-oncogene, NF-κB subunit (RELA, also known as P65) as the top predicted activated transcription factors of the DEPs between the B–A groups and the C–A groups (Figure 4A). As a key subunit of the NF-κB complex, RELA plays an important role in multiple biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis (44). RELA was activated in the bone tissues from middle-aged and older individuals, implying an inflammatory response of bone cells to the aging microenvironment. Upregulation of RELA promotes the expression of CYBB, HMOX1, and ICAM1 which are associated with the neuroinflammation signaling pathway and IL-8 signaling (Figure 4B). C-C motif chemokine receptor 2 (CCR2) was the top inhibited transcription factor of the

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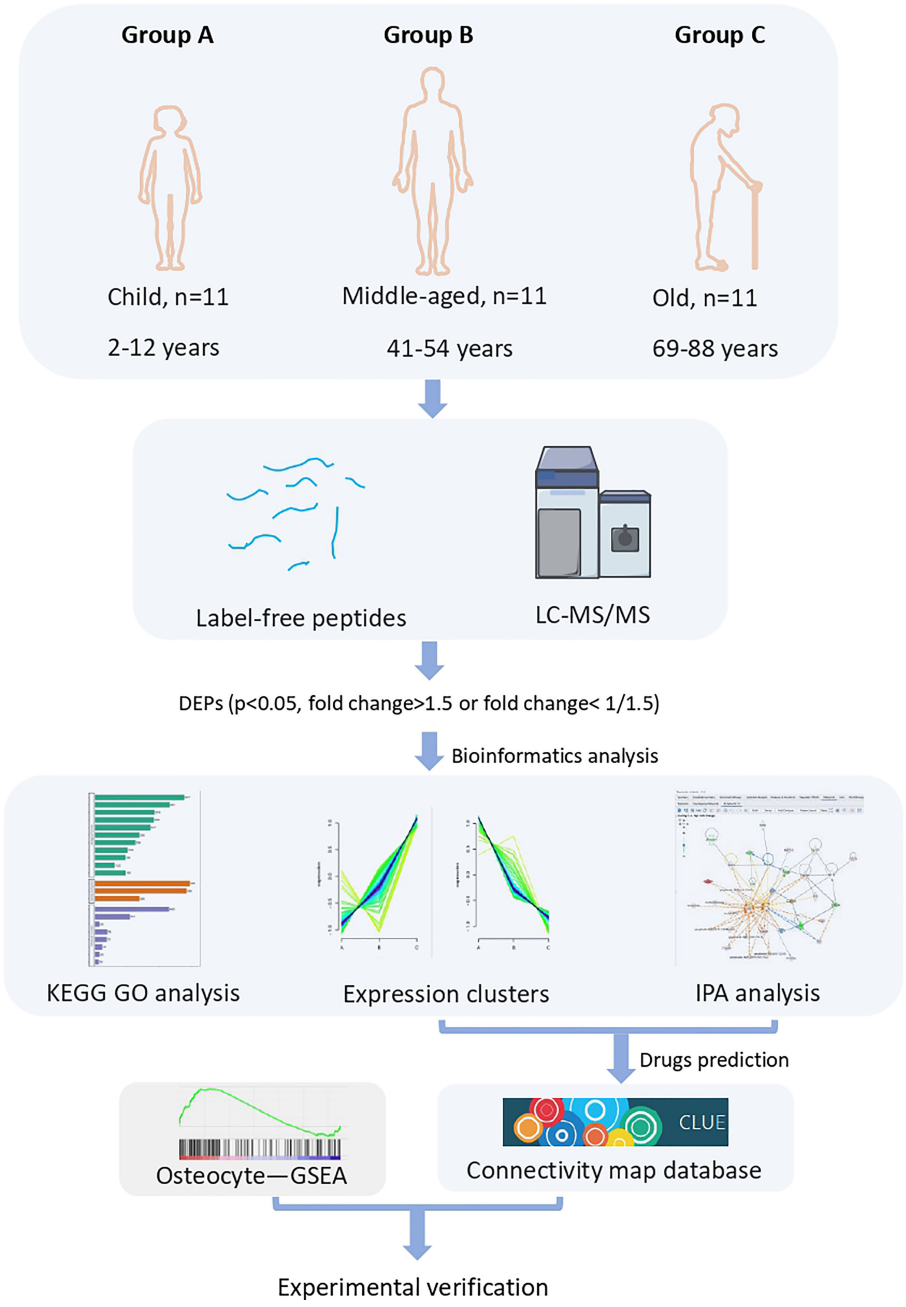

FIGURE 1
Flow diagram of label-free LC-MS/MS proteomics.

DEPs between the B–A groups and the C–A groups (Figure 4A). Downregulation of CCR2 inhibited the expression of bone matrix proteins, such as collagens, BGN, and VCAN (Figure 4C), all of which are crucial factors involved in cell adhesion, angiogenesis, and inflammation. In addition, the top 5 activated transcription factors included APP, MAPK14, FKBP10, and EIF4E (Figure 4A), of which MAPK14 is an important molecule in the MAPK signaling pathway. The top 5 inhibited transcription factors include IL10RA, SRF, IGF2BP1, and TGFB1 (Figure 4A), of which IL10RA is an anti-inflammatory factor (45), while SRF, IGF2BP1, and TGFB1 are all reported to be important molecules in promoting bone formation (46–48).

As described above, the inflammatory response of bone cells was activated along with aging. Since bone cells, including osteoblasts, osteocytes, and osteoclasts, coordinated with each other to maintain bone homeostasis, osteocytes make up over 90% of the cellular content of bone. As the bone samples in which we performed protein sequencing had mainly osteocytes, cellular composition A sequencing data (GSE141595) have shown that osteocytes may be the primary mediator of bone senescence (8). We next explored whether the inflammatory pathways predicted and activated in our study were associated with osteocytes. Previous data showed that RELA and MAPK14 (Figure 4A) were predicted to be significantly activated upstream transcription factors, so we focused on whether their corresponding NF-κB signaling pathway and MAPK signaling pathway were activated, which are related to inflammation (44, 49). In addition, the neuroinflammation signaling pathway and CXCR4 signaling were predicted to be significantly

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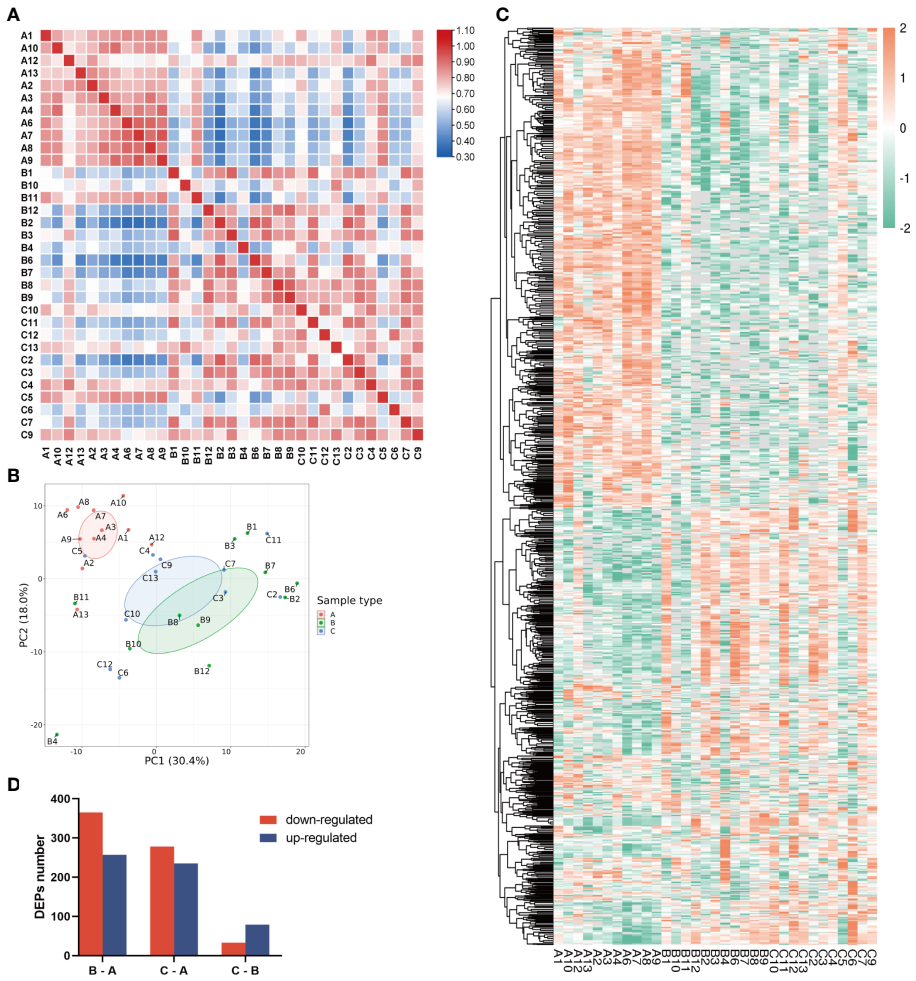

FIGURE 2
Characterization of proteomic of human bone tissue at different ages. **(A)** Pearson's correlation matrix of 33 samples. The color of the square represents the magnitude of the correlation: blue represents a small correlation coefficient, while red represents a large correlation coefficient as the color bar shows. **(B)** PCA plot of the three groups. Group A was distinct from groups B and C. **(C)** Heatmap of all protein expression in the three groups. **(D)** The numbers of differentially expressed proteins (DEPs) in the three pairwise-compared groups. The red bar indicates the downregulated proteins, and the blue bar indicates the upregulated proteins. In the chart, group A refers to the children, group B refers to the middle-aged individuals, and group C refers to the older individuals.

activated inflammatory pathways (Figure 3C), so we focused on whether the above four signaling pathways were activated. We performed GSEA analysis of published data on osteocyte-enriched tissues (8) (Figures 4D–G), and osteocytes in the aged group were enriched in the NF-κB signaling pathway [normalized enrichment score (NES) = 1.5, *p*-value = 0.007], MAPK signaling pathway (NES = 1.49, *p*-value=0.001), neuroinflammatory response (NES = 1.31, *p*-value = 0.11), and CXCR4 pathway (NES = 1.16, *p*-value = 0.23), suggesting that the inflammatory-associated signaling pathways during bone aging were likely to be generated from osteocytes.

Analysis of the DEPs continuously up- or downregulated with age

Chronological expression analysis was applied to better explore protein temporal changes with age. As the C–A groups had the largest age gap, the 513 DEPs (Figure 2D) were targeted, and the expression values of these proteins in children, middle-aged individuals, and older individuals were analyzed. K-means clustering analysis was performed on the 513 DEPs, and they were classified into six types based on expression patterns (Figure 5A). The number of proteins in cluster 1 to cluster 6 is 93, 96, 70, 65, 72, and 117, respectively. The expression values of the 513 DEPs and proteins of the six clusters are listed in Supplementary Table 5. Among the six clusters, DEPs of cluster 3 and cluster 4 were of primary interest to us due to the DEPs upregulated or downregulated continuously with age. The continuously increased or decreased DEPs were subjected to GO-BP enrichment analysis (Figure 5B). The continuously upregulated DEPs (cluster 3) were largely involved in signal transduction, cytoskeleton organization, regulation of cell shape, and response to endoplasmic reticulum stress, whereas the continuously downregulated DEPs (cluster 4) were enriched in cell adhesion, skeletal system development, and collagen fibril organization; actually, the overall pathways enriched by continuously downregulated DEPs were closely related with osteogenesis, ossification, and bone mineralization, reflecting that decreased bone formation was a key feature of bone aging (Figure 5B).

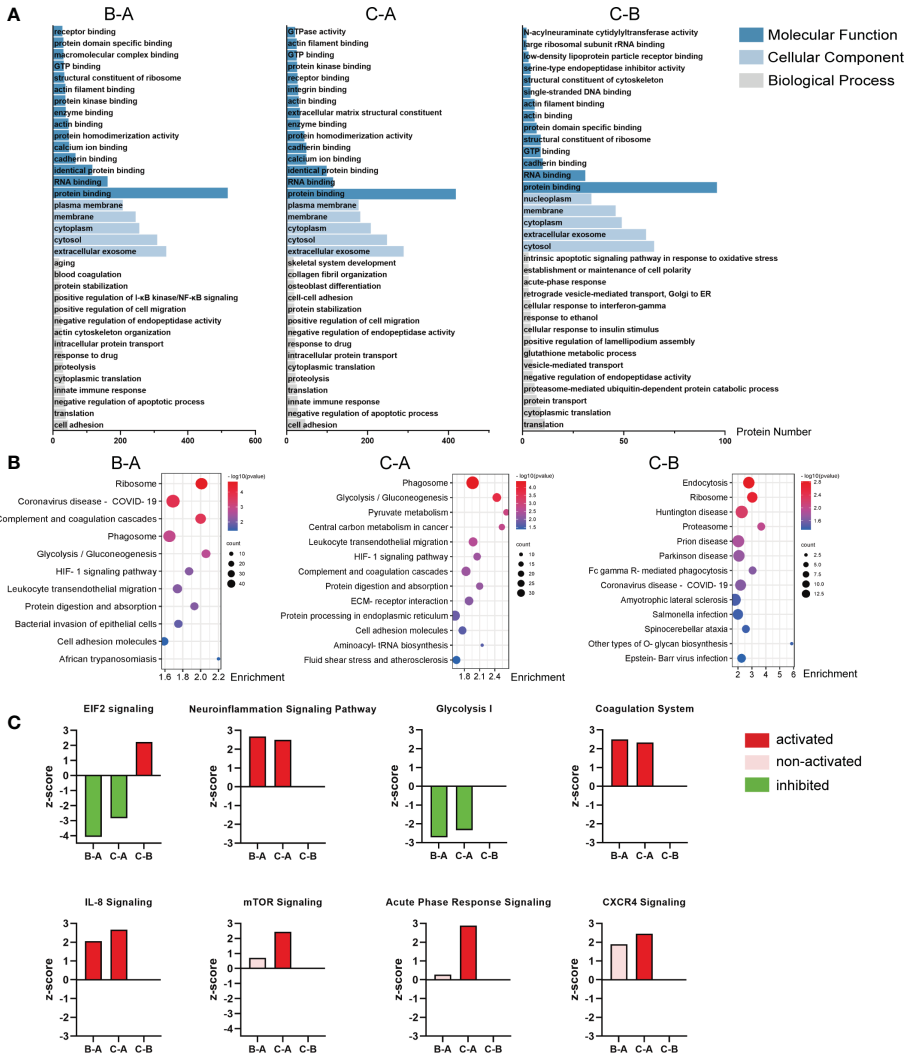

FIGURE 3
Analysis of the DEPs from the pairwise comparison. **(A)** Representative GO enrichment in the three pairwise comparison groups; the horizontal axis indicates the number of enriched genes. **(B)** Bubble plot of KEGG enrichment analysis of the three comparison groups; the color of the bubble represents the enriched *p*-value, and the size of the bubble represents the number of enriched genes. **(C)** Activation or inhibition of several canonical signaling pathway in the three comparison groups. *Z*-score >2 means the pathway is activated, indicated in red, while *z*-score <−2 means the pathway is inhibited, indicated in green; the pink bar means non-activated pathway; and the missing values in the C−B groups mean no valid prediction. In the chart, B−A refers to middle-aged individuals compared with children, C−A refers to older patients compared with the middle-aged individuals, and C−B refers to older patients compared with middle-aged individuals.

Then, the IPA program was used to predict the activation/inhibition of the signaling pathways of the continuously up- or downregulated DEPs. We identified three pathways that were significantly activated in continuously upregulated DEPs, namely, NRF2-mediated oxidative stress response, Fcγ receptor-mediated phagocytosis, and ferroptosis signaling pathway (Figure 5C), whereas three pathways were significantly inhibited in the continuously downregulated DEPs, namely, GP6 signaling pathway, wound healing signaling pathway, and osteoarthritis pathway (Figure 5D).

Moreover, the core molecules in the clusters of continuously up- or downregulated DEPs were selected by the IPA program. Integrin Subunit Alpha 2b (ITGA2B), which increased more than 10-fold (Supplementary Table 5) in the older group compared with the children group, was the core molecule among the continuously upregulated DEPs (Figure 5E). Collagen Type I Alpha 1 Chain (COL1A1), as the most important bone matrix protein, was the core molecule of continuously downregulated DEPs (Figure 5F). COL1A1 was decreased by more than 50% (Supplementary Table 5) in the older group compared with the children group, indicating that the reduction of COL1A1 might be primarily responsible for bone aging or aging-related bone loss.

Potential drug prediction

To find the potential small molecule drugs against age-related bone loss, we employed the CMap approach to analyze the continuously upregulated (cluster 3) and downregulated (cluster 4) DEPs among the three groups. A total of nine drugs were predicted to be potentially effective (score <−90) (Figure 6A). The top predicted drug was sirolimus (also known as rapamycin), and rapamycin forms a complex with FKBP12 and then specifically binds to mTORC1 and

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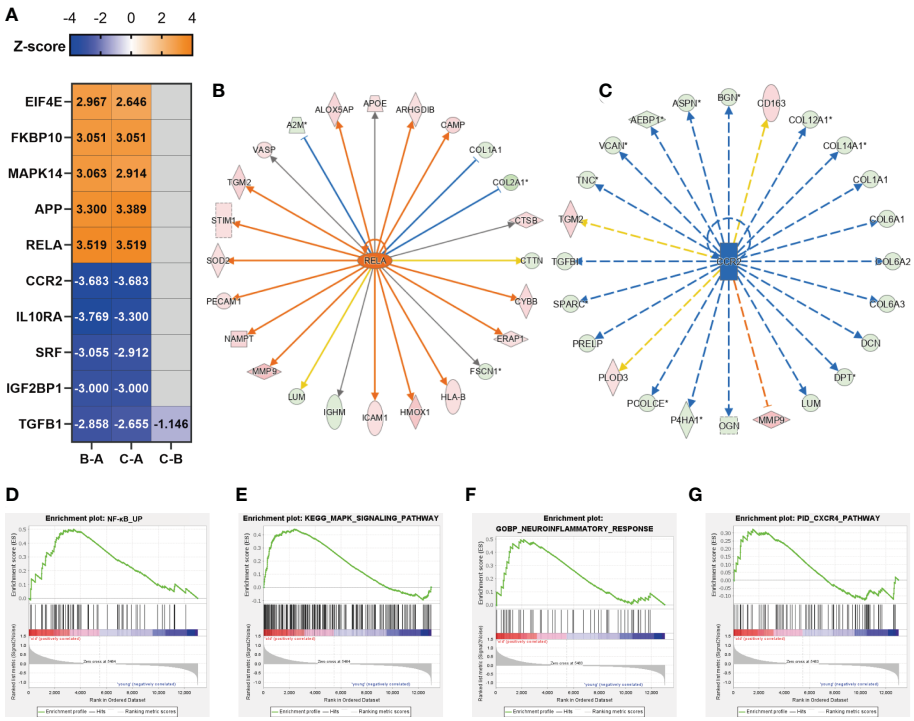

FIGURE 4
Inflammation might be generated from osteocytes. **(A)** Representative predicted upstream transcription factors in the three comparison groups; the number indicates *z*-score. *Z*-score >2 means the molecular is activated, indicated in orange, while *z*-score <−2 means the molecular is inhibited, indicated in dark blue; the negative prediction was indicated in gray and the non-activated pathway was indicated in light blue. **(B)** The significantly activated molecule RELA and its regulatory map in the C−A groups. **(C)** The significantly inhibited molecule CCR2 and its regulatory map in the C−A groups. **(D–G)** GSEA plots of mRNA sets of several inflammatory-associated signaling pathways.

inhibits its kinase activity (50). Our prediction suggested a beneficial role of rapamycin against bone cell aging, which was consistent with the current reports characterizing rapamycin as a star drug against cellular aging (50–52). We further analyzed the interaction between rapamycin and the DEPs by using the Search Tool for Interactions of Chemicals (STITCH) database (53). The results showed that rapamycin could interact with HMOX1 (upregulated with age), RPS6KA3 (upregulated with age), and TF (downregulated with age) (Figure 6B). Rapamycin also can ameliorate inflammation induced by various stimuli (54–56), which was proper for aged bone in which the inflammatory response was activated in our study.

Validation of our bioinformatic predictions by *in-vivo* and *in-vitro* experiments

We first validated the expression pattern of the key DEPs from the proteomics sequencing results. The core molecules of the continuously upregulated or downregulated DEPs were ITGA2B and COL1A1, respectively. We observed a severe overexposure of COL1A1 in Western blotting, which may be due to its extremely high abundance in the bone matrix; thus, a cell surface proteoglycan, chondroitin sulfate proteoglycan 4 (CSPG4), another representative downregulated protein, was chosen for further validation. The Western blot assay indicated an increase of ITGA2B and a decrease of CSPG4 in human bone tissues from older individuals than those from children (Figures 7A, B), which was consistent with our proteomics sequencing results (Supplementary Table 5). The levels of ITGA2B and CSPG4 were also determined in the bone tissues from 6-week-old mice and 18-month-old mice, respectively. In agreement with that of human specimens, ITGA2B was increased significantly, whereas CSPG4 was reduced remarkably in 18-month-old mice (Figures 7C, D).

Our bioinformatics analysis showed that the inflammatory-associated DEPs or signaling pathways during bone aging were likely to be generated from osteocytes. As the top-predicted drug against bone aging, rapamycin has been reported to attenuate inflammatory responses. Thus, we explored whether rapamycin reduced the phenotype of cell senescence or senescence-associated inflammation in osteocytes. The mouse osteocyte cell line MLO-Y4 was exposed to hydrogen peroxide (H_2O_2) to mimic the senescence microenvironment. The results showed that H_2O_2 exposure indeed induced a significant increase of the aging-associated inflammatory cytokine *IL-6* and the senescence markers, including *p53*, *p21*, and *p27*, but there was an obvious decrease of osteoprotegerin (*Opg*), a molecule that inhibits bone resorption; however, rapamycin effectively relieved H_2O_2-induced cell damage, indicated by the lower expression of *IL-6*, *p53*, *p21*, and *p27* and the higher level of *Opg* when compared with the H_2O_2-treated group (Figure 7E). Collectively, we experimentally confirmed the reliability of our proteomics sequencing results and validated the potential of rapamycin against bone aging.

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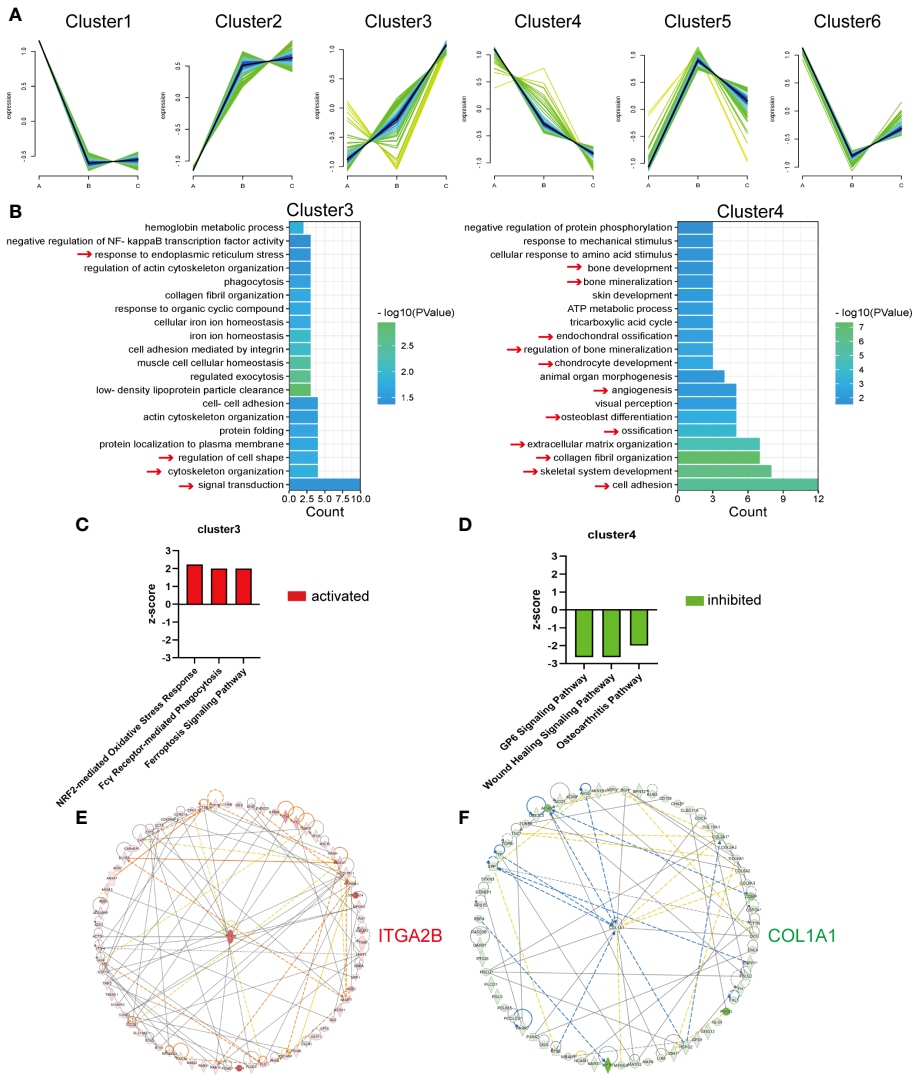

FIGURE 5
Analysis of the DEPs continuously up- or downregulated with age. **(A)** Six clusters of the 513 DEPs in the three groups. A refers to the children, B refers to the middle-aged individuals, and C refers to the older individuals. **(B)** Representative biological process analysis of cluster 3 (left) and cluster 4 (right). **(C, D)** Activation or inhibition of several canonical signaling pathways in cluster 3 and cluster 4. **(E, F)** The core molecular of cluster 3 and cluster 4.

Discussion

Age-related bone loss remains understudied, and we examined protein changes in the bone tissue of three age groups by proteomics for the first time. In this study, we first characterized the traits of DEPs from pairwise comparison, including DEP numbers and types and GO and KEGG enrichments, respectively. The data indicated that children were markedly different from middle-aged and old individuals with a great number of DEPs and those DEPs were enriched in inflammation and bone formation processes. On this basis, we next analyzed proteins continuously upregulated and downregulated along with age from 513 DEPs screened by comparing old individuals with children. In addition, we predicted drugs that may treat age-related bone loss, with rapamycin as a potential therapeutic agent. In cellular experiments, rapamycin treatment reversed the aging-associated phenotype of MLO-Y4.

Pearson's correlation analysis of the samples shows that there is a lower intragroup variability in children's bone tissues, while there is a higher intragroup variability in middle-aged and older individuals' bone tissues. Although the site of bone tissue collection varied more in children, the sites in middle-aged and elderly people were derived from the hip joint. We speculate that this phenomenon may be due to a combination of factors such as nutritional status, exercise habits, and dietary habits in middle-aged and older adults. Bone tissue samples were obtained from men and women of different ages, and gender was not excluded from the analysis, leading to an overall result that may better describe age-related bone loss rather than postmenopausal osteoporosis. Although we lack direct evidence of bone loss in the elderly samples, the majority of elderly cases were from patients with femoral neck fractures, which could serve as a suggestive basis for bone loss (57–59). It should be pointed out that the reasons for surgery are different in different age groups, and we have tried our best to exclude the influence of systemic factors on bone tissue. However, the influence of bone-related diseases on the local microenvironment cannot be completely excluded. Although the sampling site is far from the lesion, it may still have some influence on the sequencing results.

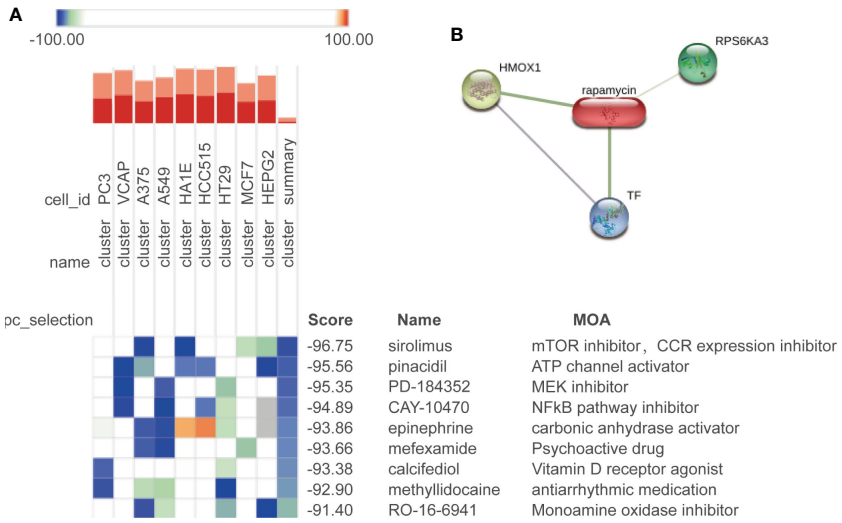

FIGURE 6
Potential drug prediction. **(A)** Drug candidates predicted by CMap in nine cell types; colors of the heatmap represent the prediction scores: blue means negatively correlated to input genes, and orange means positively correlated to input genes. We show the drugs with a composite score of less than −90. **(B)** Correlation between the predicted drug rapamycin and its target.

GO enrichment results suggest that with age, DEPs can be enriched in biological processes associated with inflammation, such as blood coagulation, positive regulation of I-κB kinase/NF-κB signaling, innate immune response, and acute-phase response. We further determined whether inflammatory pathways are indeed activated with age by IPA. The results showed significant activation of various inflammatory signaling pathways, such as neuroinflammation signaling pathway, coagulation system, IL-8 signaling, acute-phase response signaling, and CXCR4 signaling. If the major cells that produce inflammation can be identified, targeting them for intervention may be a way to treat age-related bone loss. We attempted to analyze this by combining single-cell sequencing data, which is currently scarce for bone tissue of different ages, with one study that performed single-cell RNA sequencing of primary human femoral head tissue cells (60). However, their sample size was only four cases, with the younger group being 45 and 31 years old (older than our children group) and already diagnosed with osteoarthritis and osteopenia, obviously not applicable to our study. Considering that the main cell type in the sampling site is the osteocyte, we then selected data from GSE141595, with a tissue source of osteocyte-enriched samples from young and old women, and performed RNA-seq (8), which is closer to our sequencing sample source. The GSEA enrichment analysis reveals that the elderly group is enriched in NF-κB signaling, MAPK signaling, neuroinflammatory response, and CXCR4 signaling. However, IL-8 signaling, acute-phage response signaling, and coagulation system, which were significantly activated in the IPA, were not enriched in the elderly group. It is probably due to that transcriptomics and proteomics are not an exact match, or the difference is caused by the source of the samples which is all women. Although most of the cells in our bone tissue samples are osteocytes, the effects of osteoprogenitors, osteoblasts, and osteoclasts could not be completely excluded.

The current drugs for the treatment of osteoporosis include bisphosphonates, teriparatide, and estrogen, but they are limited by side effects, and research on more effective drugs is necessary. New drugs have been discovered, such as parathyroid hormone-related peptide analogs, sclerostin inhibitors, cathepsin K inhibitors, and senolytics. We selected proteins that consistently increased and decreased with age based on the k-means clustering algorithm and used this to predict potential drugs for age-related bone loss, with rapamycin being the highest-scoring drug. Rapamycin forms a complex with FKBP12 and then specifically binds to mTORC1 and inhibits its kinase activity (50). It has been shown to be an anti-aging drug (51) and has additionally been widely reported as an anti-inflammatory and immunosuppressive agent, but studies of its effects on bone are controversial. Rapamycin alleviated age-related bone trabecular loss in mice (61) and reduced the level of oral inflammation in aged mice (62). Conversely, it has also been reported that rapamycin has a negative effect on bone quality in young mice and rabbit bone tissue (63–65). These results seem to suggest that the effect of rapamycin on bone is dependent on age status. A recent study showed that mTORC1 has age-specific effects on bone (66), which may explain why rapamycin has a two-way effect on bone.

In our study, rapamycin was suggested to attenuate the osteocyte senescence phenotype. We simulated osteocyte senescence by stimulating the mouse osteoid cells MLO-Y4 with H_2O_2 *in vitro*. MLO-Y4 produced a significant senescence-related secretory phenotype after H_2O_2 stimulation, with significantly elevated mRNA levels of *IL-6*, *P53*, *P21*, and *P27* along with decreased *Opg* levels, while its senescence marker expression decreased and *Opg* levels increased after treatment with rapamycin. Although H_2O_2 stimulation is one of the reported methods to induce osteocyte senescence (38, 39), different chemical stimuli or physical radiation does not fully mimic the effects of natural senescence. Although MLO-Y4 is widely used to study osteoblasts *in vitro* (67–70), there are still differences between MLO-Y4 and primary osteocytes; for example, the expression of Sclerostin (*Sost*) is difficult to detect in MLO-Y4 cells (71), which is expressed in primary osteocytes (72). Therefore, it needs to be further validated by primary cells from senescent mice or by animal experiments.

Several other drugs predicted in the CMap database may also be potential drugs for age-related bone loss. The second-ranked drug

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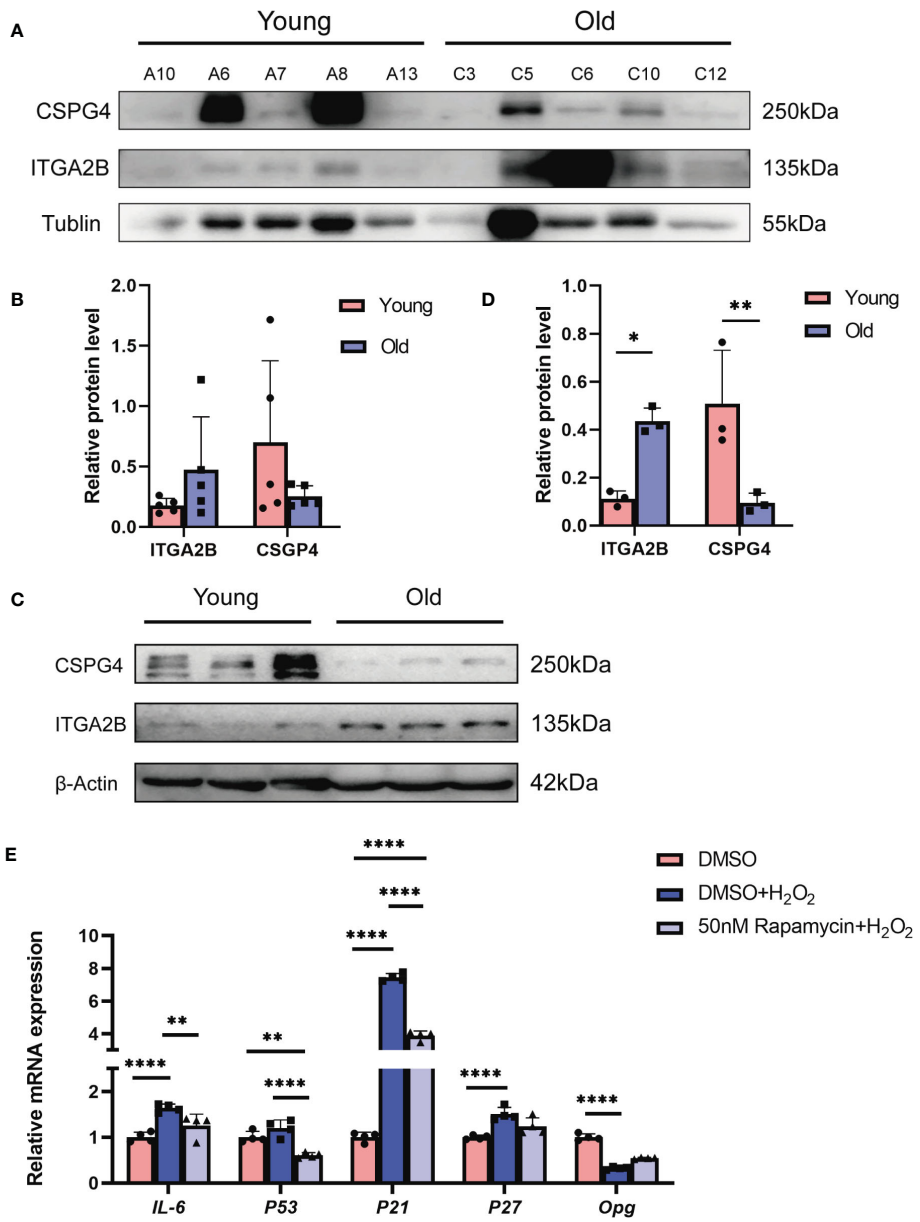

FIGURE 7
The validation of our bioinformatic predictions by *in-vivo* and *in-vitro* experiments. **(A)** Validation of representative proteins from sequencing. β-Tubulin was used as the control. **(B)** The quantitative results of Western blotting from **(A)**. **(C)** Validation of proteins in young (6 weeks) and old (18 months) mice bone. **(D)** The quantitative results of Western blotting from **(C)**. **(E)** Representative qRT-PCR quantitation for the marker of inflammatory and SASP. All data were presented as the mean ± SD; *p < 0.05; **p < 0.01, **** p < 0.0001.

pinacidil is an oral antihypertensive drug that relaxes vascular smooth muscle and is a K^+ channel opener (73). Several studies have shown that it prevents damage to osteoblast function from reactive oxygen species and may have a positive effect on bone (74, 75). The third-ranked PD-184352 is a MEK inhibitor, and the MEK/ERK pathway enhances the production of several pro-inflammatory cytokines (76, 77). MAPK14 was predicted to be an upregulated transcription factor in middle-aged and older individuals in our results, and additionally, the GSEA analysis shows that RNA from osteocyte-enriched samples in older women could be significantly enriched in the MAPK pathway, suggesting that targeting the MAPK signaling pathway may be a direction of treatment. It has been shown that PD-184352 inhibits osteoclast differentiation (78), but its effect on osteogenic differentiation is mostly negative (79, 80). In addition, PD-184352 alleviates the phenotype of human rheumatoid arthritis (81), and its study on age-related bone loss was not reported, and further studies are needed in the future. In addition, this study did not target a specific molecule, and the transcription factors predicted by IPA are also the subject of our future research, perhaps to clarify the functions of these transcription factors which might contribute to the discovery of new drugs for age-related bone loss.

In summary, we have utilized proteomics for the first time to characterize age-related bone tissue changes, and based on the proteomics results, we have predicted and experimentally validated potential therapeutic agents, providing a basis for the potential molecular characterization of age-related bone loss.

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the PRIDE (82) partner repository with dataset identifier PXD039538.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Union Hospital of Tongji Medical College, Huazhong University of Science and Technology. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Animal Care and Use Committee of Wuhan Union Hospital.

Author contributions

ZW, XZ, HW, and JZ contributed to the conception and design of the research. ZW and XZ contributed to the writing and drafting of the manuscript. ZW contributed to the drawing of the figures and tables and analysis of the data. ZW and TR performed the animal experiments. XC, WX, and JL collected the human samples. All authors critically reviewed and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1114006/full#supplementary-material

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The role of myeloid derived suppressor cells in musculoskeletal disorders

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The immune system is closely linked to bone homeostasis and plays a pivotal role in several pathological and inflammatory conditions. Through various pathways it modulates various bone cells and subsequently sustains the physiological bone metabolism. Myeloid-derived suppressor cells (MDSCs) are a group of heterogeneous immature myeloid-derived cells that can exert an immunosuppressive function through a direct cell-to-cell contact, secretion of anti-inflammatory cytokines or specific exosomes. These cells mediate the innate immune response to chronic stress on the skeletal system. In chronic inflammation, MDSCs act as an inner offset to rebalance overactivation of the immune system. Moreover, they have been found to be involved in processes responsible for bone remodeling in different musculoskeletal disorders, autoimmune diseases, infection, and cancer. These cells can not only cause bone erosion by differentiating into osteoclasts, but also alleviate the immune reaction, subsequently leading to long-lastingly impacted bone remodeling. In this review, we discuss the impact of MDSCs on the bone metabolism under several pathological conditions, the involved modulatory pathways as well as potential therapeutic targets in MDSCs to improve bone health.

KEYWORDS

myeloid derived suppressor cell (MDSC), bone metabolism, osteoclast, osteoblast, immune cells, inflammation, osteoimmunology

1 Introduction

Bone is a versatile organ that is an essential component for the ambulatory ability and is host to essential cell lineages such as hematopoietic stem cells, as well as bone cells and immune cells. The solid bone matrix is constantly being remodeled in response to changes in physical stress (1). This self-regulated biological remodeling process is mainly driven by bone resorption and formation. While osteoclasts (OCs) eliminate damaged or aged bone tissue, osteoblasts (OBs) are responsible for secretion of new bone matrix and mediation of matrix calcification (2). Both cell types are vital for responding to biomechanical or

metabolic changes, remodeling the microstructure of the bone accordingly, and maintaining bone homeostasis.

This equilibrium is governed by several cells and mediating cytokines (3). In particular, the immune system interacts tightly with the bone metabolism (4–6). However, in various pathologies such as tumor metastasis or local inflammation, this delicate equilibrium is distorted (7, 8). Besides focusing on the causative disease, recent research has also focused on identifying key regulatory players to influence bone homeostasis (9, 10). Myeloid derived suppressor cells (MDSCs), a group of immature cells of the myeloid lineage, represent a cell type with immune regulatory function through interaction with effector or regulatory lymphocytes. These cells are activated and proliferate in diseases, including chronic bacterial infection, autoimmune diseases, and cancer (11–15).

Recent studies have described the role of MDSCs in bone-related disease. Bone lesions ranging from systemic bone loss (osteoporosis, autoimmune diseases) to local destruction (osteomyelitis, implant related infection, bone fracture and bone metastasis of tumor) can create a long-lasting inflammatory environment (4, 6, 16, 17). These signals play a key role in myeloid lineage cell activation and differentiation to MDSCs, which in turn impact disease progression and the regenerative capabilities of bone. MDSCs can interact with nearby lymphocytes in the bone, indirectly influencing the bone metabolism through stimulation of the immune system. Additionally, MDSCs were found to impact bone directly, i.e., by differentiating into osteoclasts, or secreting cytokines. In this review, we aim to illustrate how MDSCs can affect bone health and their role in musculoskeletal morbidities.

2 Bone remodeling and its interaction with the immune system

Bone serves as one of the most important immune organs as the origin of several immune cells is the bone cavity and its metabolic activity is closely linked to the immune system. The recently coined term “osteoimmunology” connects the metabolic activity of the bone with the immune system (18). The bone forms a relatively closed space that supplies a suitable cradle for the reciprocal interactions of immune cells and bone cells.

Mediators secreted by bone cells can either stimulate or obstruct processes of immune development. Bone cells contribute to the maturation and expansion of various immune cells derived from hematopoietic stem cells (HSCs). Mesenchymal stromal cells expressing the C-X-C motif chemokine-12 (CXCL-12) are required for HSC maintenance (19). Additionally, OBs are essential in maintaining common lymphoid progenitors (CLPs) through expression of IL-7 and CXCL-12 (20). Ablation of OBs results in severely decreased hematopoiesis in the bone marrow, in particular the generation of B cells (21). Osteocytes also support the lymphocyte development and show positive impact on B cell generation (22, 23). Moreover, OCs are fundamental to create bone marrow cavities sufficient in size for HSCs to sustain their physiological capabilities and indirectly support HSCs by recruiting osteoblasts (24). They are also engaged in establishing a livable milieu in the bone to induce HSC homing and niche formation (25).

At the same time the immune system has significant impact on bone homeostasis (26). Over- or under-regulation of the immune system results in abnormal bone mineralization through different mechanisms. Different T cell populations including CD8+, CD4+ T helper cells (Th), and regulatory T cells (Treg) impact the bone metabolism through secretion of various cytokines. CD8+ T cells and Th17 favor osteoclastogenesis by secretion of tumor necrosis factor-α (TNF-α) and IL-17 (17, 27). B cells, as supportive regulators of osteoclasts, limit bone remodeling (28, 29). Macrophages are characterized into two phenotypes, proinflammatory M1 and anti-inflammatory M2, which support and hinder bone regeneration, respectively. Besides their phagocytic function, these cells also differentiate into osteoclasts and secrete TNF-α and various ILs balancing bone formation and resorption (30, 31).

In this regard, MDSCs, a type of immature myeloid cells, have recently started to attract attention due to their impact on the bone metabolism and their immunosuppressive capacities. First described as a key modulator in tumor microenvironment, the role of MDSCs is becoming undeniably important during disease progression due to their potential to regulate immune balance and crosstalk with the bone system.

3 MDSCs are induced in a chronic inflammatory setting

MDSCs were first discovered in a tumor mouse model. Aggregation of these cells around the tumor site lead to suppression of T-cell induced immunity and boosted cancer metastasis (32). While MDSC has become a comprehensive term to describe a specific origin, phenotype, and immunosuppressive capacities, it covers a heterogeneous group of distinct subphenotypes (33). Since several years, interest in MDSC-related immune regulation has been soaring in different disease settings, including chronic inflammatory diseases, infection and obesity (13). Deepening the understanding of the stimulating factors affecting differentiation of MDSCs may offer novel therapeutic targets.

Together with neutrophils and macrophages, MDSCs derive from the myeloid lineage but gain distinguished immunosuppressive functions during differentiation (34–36). Circulating MDSCs have been found in tumor, autoimmune, and septic patients but not or in very limited quantities in healthy individuals (37). In these chronic inflammatory environments, continuous low-grade stimulation of IMCs skews differentiation to increased generation of MDSCs (13). MDSCs generated under these conditions are poorly phagocytic and display potent immune-suppressive potential. Key factors involved in the differentiation of IMCs are granulocyte-macrophage colony stimulating factor (GM-CSF), G-CSF, and M-CSF (38–41), as well as inflammatory cytokines TNF-α, IL-1β, and IL-6 (41–43). These effectors from the microenvironment stimulate and regulate several

intracellular pathways involving various key nodes that are crucial for the survival and immunosuppressive function of MDSCs (44–46).

MDSCs are commonly classified as granulocytic (G-MDSC, also known as polymorphonuclear MDSC, PMN-MDSC), monocytic (M-MDSC), and other subgroups such as early-stage MDSC (e-MDSC) and fibrocytic MDSC (F-MDSC) (47, 48). In humans, MDSCs expresses CD11b and CD33–markers related to immunosuppressive functions, while in mice, CD11b, Ly6C and Ly6G were defined as phenotypic markers (34, 49). Additionally, expression of CD84 has been recently identified on MDSCs in tumor settings (36). However, these markers alone cannot sufficiently phenotype all MDSC subpopulations (34). Besides their shared suppressive capabilities against adaptive immunity, their immunosuppressive capability differs in various nuances. In patients with head and neck cancer, PMN-MDSCs displayed the most prominent immunosuppressive features and have been associated with poor clinical outcome (50), while in a tumor mouse model, MDSCs with monocytic features showed heightened suppressive capability and blocked the T cell responses (51, 52).

4 Potential interactions of MDSCs with osteoclasts

Osteolysis occurs in several disease including osteoporosis, autoimmune arthritis, bone infection, and bone metastasis, where osteoclasts surpass the speed of regeneration of osteoblasts (7, 53). Related to the destruction of the cancellous bone microstructure, the trabeculae become thinner and more fragile with larger trabecular separation, subsequently manifesting in reduced bone volume (54, 55). MDSCs are osteoclast progenitors that can break the dynamic balance of bone remodeling in disease.

In inflammation, overactivated osteoclastogenesis can be observed, where monocytes and macrophages are functionally calibrated by various cytokines leading to activation of the receptor activator of nuclear factor kappa-B ligand (RANKL) pathway and receptor osteoprotegerin (OPG). T cells bind to RANK, the receptor of RANKL expressed on osteoclast progenitor cells, while OPG competitively binds to RANKL to hinder the stimulating effect of RANK (18). Other inflammatory components including TNF-α, IL-1, and IL-6 also disrupt the bone metabolism by triggering RANKL expression of osteoblasts, cell fusion, multinucleation, and functional activation of osteoclasts (56–58). The inflammatory cytokines stimulate osteoclasts to eliminate defective bone tissue. At the same time, bone regeneration is inhibited by interfering cells supporting the bone metabolism, particularly osteoblasts, osteocytes, and bone marrow mesenchymal stromal cells (BMSCs). Elevated levels of TNF-α, IL-1α, and IL-7 usually found in chronic inflammatory settings lead to osteoblast apoptosis, negatively affecting the osteogenic capacity of osteoblasts and differentiation of BMSCs (7, 59). Additionally, osteoblasts and osteocytes not only sustain the normal bone mineralization process, but also regulate osteoclast differentiation through secreting soluble proteins, inflammatory cytokines, and through direct cell-cell interactions (2, 60).

MDSCs mainly generate where myelopoiesis takes place including the bone marrow, spleen, and other lymphatic organs, but they can be also reprogrammed from mature myeloid cells in the periphery (37). Besides their immune modulatory ability, MDSCs can differentiate into mature and functional osteoclasts (61–65). An *in vitro* experiment using murine Gr1+CD11b+ MDSCs showed that a combination of RANKL and M-CSF can initiate differentiation into osteoclasts. Additionally, in a fluorescent mice model osteoclast generation was increased after MDSC injection, indicating MDSCs as an origin of these bone-resorbing cells (61). Likewise, allogenic transfusion can increase osteoclast differentiation in inflammation (62). Recently, obesity was also suggested to promote expansion of M-MDSCs and subsequent differentiation to osteoclasts (64, 65). MDSC-induced osteolysis is linked to chronic pathological diseases (36, 38). However, MDSCs are a heterogenous group consisting of several subgroups with different immune functions and capacity to differentiate to osteoclasts.

MDSCs and osteoclasts derive from the myeloid lineage, as do monocytes, macrophages, and dendritic cells. Both, MDSCs and osteoclasts share some common intracellular signaling pathways related to differentiation, proliferation, and osteoclastic cell functions. The osteoclastogenic capability of both cell types are repressed after treatment with bisphosphonates, suggesting a shared pathway in MDSCs and osteoclasts (63). Osteoclast differentiation of MDSCs is initiated by activation of the RANKL and NF-κB pathway (62). RANKL also activates the immune regulatory functions of MDSCs and promotes the expansion of M-MDSCs (66). The role of other pathways that have interactions with RANKL/RANK in osteoclast differentiation is of ongoing investigation (67). Additionally, MDSCs and OCs share similar immunosuppressive functions through secretion of the immunosuppressive cytokines IL-10 and transforming growth factor (TGF-β) (3). Both cell types are also capable of inhibiting the T cell mediated immune response. However, they also share immune regulatory features with mature myeloid cells that support the inflammatory environment. They have been shown to be able to sustain a proinflammatory environment under pathological conditions by presentation of antigens, secretion of proinflammatory cytokines, and inducing proliferation of T effector cells (3, 68).

5 MDSCs are a link between the immune and skeletal system

MDSCs also regulate other immune cell types which directly affect the musculoskeletal system. They modulate macrophage polarization from M1 to M2. Anti-inflammatory M2 macrophages stimulate the osteogenic capacity of BMSCs (69, 70). Interaction between MDSCs and regulatory B cells (Bregs) positively impact the bone metabolism (71, 72). Additionally, MDSCs stimulate the proliferation of Tregs that act as key helpers in prolonging osteoblast survival (73). This indicates a complicated interaction triangle among MDSCs, the bone, and

components of immune system. Figure 1 summarizes an overview of the interaction among MDSCs, immune cells and skeletal system.

5.1 Soluble factors from MDSCs

A broad range of secreted factors are related to the function of MDSCs, some of which were described immunosuppressive that can prolong the chronicity. However, they also play a versatile role in osteogenesis. TGF-β and IL-10 are two of the most important factors supporting proliferation of Tregs (51, 74), and also play a key role in the generation of osteoblasts (75). Additionally, adenosine which is generated by CD39 and CD73 on the surface of MDSCs can lead to activation of the A2A receptor subsequently increasing production of Tregs (73, 76). Adenosine also has a direct proliferative effect on BMSCs and osteoblasts by activation of the A2B receptor, and therefore contributes to bone regeneration (77, 78). Moreover, other molecules secreted by MDSCSs such as S100A8/A9 and NO have also been shown to positively impact osteoblast differentiation (79, 80).

5.2 Immunosuppressive surface markers on MDSCs

Cell-cell contact through immunosuppressive ligands and receptors plays a key in immune dysregulation. Previous studies have suggested a variety of immunoregulatory surface functional molecules to be found on MDSCs (81). These membrane proteins can directly interact with T effector cells, promote the expansion of Tregs and Bregs, and thus regulate systemic immunity in viral infections, autoimmune diseases, and cancer. There are few studies on the direct contact of MDSCs to osteoblasts, but several studies discussed how these surface markers can affect their fate. In particular, the PD-1/PD-L1 axis might have regulatory effect on bone remodeling by limiting osteoclastogenesis (82). Additionally, Galectin-9 is widely expressed in various tissues that were reported to induce osteoblast differentiation (83). CD155, an important receptor mediating cell adhesion, was reported to be expressed on osteoclast precursors and regulate differentiation processes (84). CD276 is membrane-bound but can be also released from the surface as a soluble molecule. Deficiency of CD276 results in lower osteoblastic activity and reduced mineralization (85). Research on ADAM17 demonstrated its role in stimulating osteoclastogenesis by degrading interferon (IFN)-γ (86) and inhibiting osteoblast differentiation through interaction with RUNX2 (87).

5.3 MDSC-derived exosome and immune response

Exosomes are a group of lipid bilayer vesicles with nanoscale size (usually 30-100nm), shed by various types of cells during the

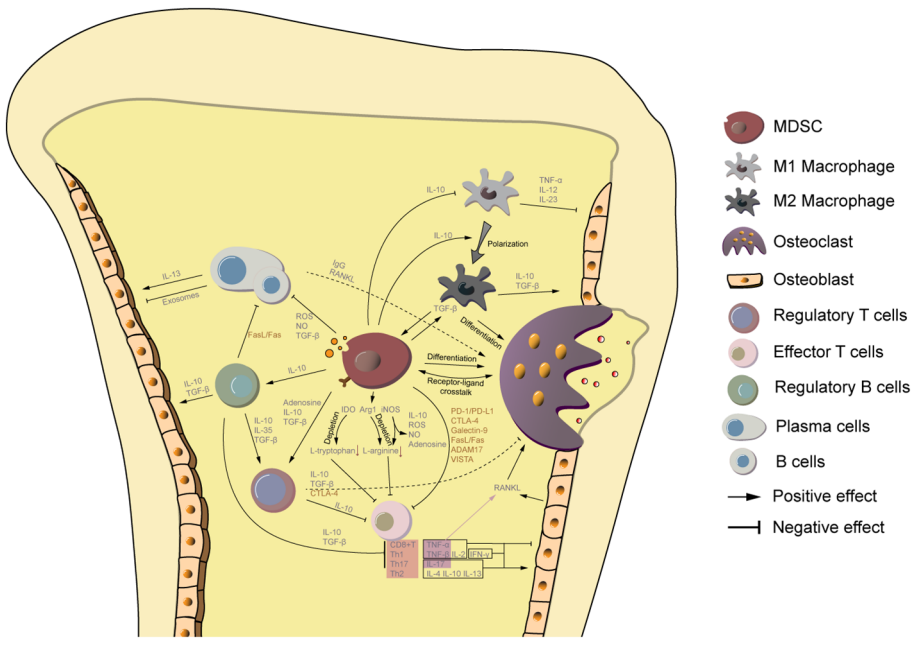

FIGURE 1
MDSCs are a key link between the bone metabolism and immune system. MDSCs are immature cells of the myeloid lineage that can differentiate to osteoclasts. Additionally, they secret IL-10 to promote macrophage polarization from M1 to M2, of which the latter one is also capable to differentiate to osteoclasts. MDSCs are also involved in the regulation of other counterparts of the immune system. Small molecules from MDSCs, including TGF-β, IL-10, adenosine, and ROS/NO hamper the immune reaction directly or indirectly by supporting proliferation of regulatory T cells, regulatory B cells, M2 macrophages, and inhibiting the activity of effector T cells, B cells, plasma cells, and M1 macrophages. Among them, regulatory T/B cells and M2 macrophage support osteogenic processes. Cytokines from M1 macrophage, CD8+ T cells, and Th1 cells limit osteoblast function, while Th2 and Th17 promote osteogenesis. The effect of plasma cells and B cells on osteoblast activity is controversial and depends on different biological settings. Moreover, immunosuppressive ligands and surface receptors on MDSCs interact with lymphocytes and osteoblasts to regulate their function.

intercellular communication and regulation. Compared to bone marrow from healthy individuals, exosomes of MDSCs in a tumor environment are excreted in larger numbers and contain more cytokines related to tumor invasion, angiogenesis, and myeloid cell activation or function (88), as well as mRNAs, microRNAs, and other protein molecules involved in immune modulation (89–91). Through proteomic analysis, several typical surface markers on exosomes were found to be representative of their parental MDSCs and beneficial for MDSC migration (92). MDSCs secret exosomes to interfere with their neighborhood in response to changing immune circumstances. CD8+ T cells treated with these small vesicles display a trend towards anergy, while Tregs increase their regulatory activity (88). G-MDSCs were reported to attenuate immune responses of Th1 and Th17 cells and thus reduce the severity of autoimmune arthritis by releasing exosomes (93). Additionally, TGF-β and IL-10 have been found in MDSC-exosomes – two molecules involved in inhibition of autoimmunity and stimulation of osteoblastic growth (94).

6 Role of MDSCs in skeletal diseases

MDSCs are activated by inflammation to limit the immune response and to protect against tissue damage. However, in a tumor or chronic bacterial infection environment the immunosuppressive function of MDSCs contribute to disease progression and prolongation. In the skeletal system, MDSCs can not only dampen immune activity, but also cause bone erosion by differentiating to OCs. Despite their importance for bone health, knowledge on their involvement in various different skeletal diseases remains limited.

6.1 Ageing and osteoporosis

Osteoporosis is a chronic disease featuring low bone mineral density, pronounced bone loss, bone fragility, and subsequently increased risk for fracture with or without external force. Aging, female gender, genomics, lack of nutrients and other comorbidities are important pathogenic factors impairing bone health and causal to the development of osteoporosis.

Osteoporosis is characterized by gradual degradation of bone tissue with aging. Besides impaired osteoblast function and increasing number of osteoclasts, immune dysfunction has been shown to play a significant role in osteoporosis (6). The aging process of the immune system that is accompanied by progressive immune dysfunction affecting both lymphogenesis and myelogenesis is called “immunosenescence” (95). Specifically, with increasing age there is a gradual decline of T- and B- cells, increased generation of cells from the myeloid lineage, and upregulation of proinflammatory cytokines including IL-6 and TNF-α from senescent cells. The phenomenon of these inflammatory changes within an aging body is called “inflammageing” (96). The resulting chronic proinflammatory environment forms a suitable milieu for proliferation and expansion of MDSCs in bone of the elderly (97–99). Additionally, MDSCs are stimulated towards osteoclast differentiation in inflammageing. Aged individuals show increased MDSC-dependent osteoclast differentiation (99, 100). These changes are driven by increased production of reactive oxygen species (ROS) and nitric oxide (NO). ROS are a set of oxygen-containing molecules aggravating oxidative stress and aging process (101, 102), while NO is synthesized from precursor L-arginine. These molecules damage biologically active molecules, such as DNA, RNA, and enzymes relevant for repairing DNA and cell mitosis (103). In aged individuals, ROS and NO are a potential pathomechanism for enhanced osteoclastogenesis (99, 100). Studies in a murine model of osteoporosis suggest that the resulting bone loss can be alleviated by treatment against these products of oxidative stress (104, 105). Besides being inducers of osteoclastogenesis, ROS and NO function as immune modulators produced by G-MDSCs and M-MDSCs, that suppress T cell generation and function.

Proinflammatory IL-1β, IL-6, and TNF-α, as well as growth factor M-CSF are key regulators in age-related osteoporosis (96, 100). Long-term stimulation by these cytokines leads to increased osteoclastogenesis of MDSCs by upregulation of RANKL – an important regulator of expansion and survival of MDSCs. With increasing age, MDSCs gain more sensitivity to RANKL and are subsequently more stimulated and activated (100). Inhibition of RANKL significantly lowers the proportion of MDSCs vice versa (106). Additionally, chronic NF-κB pathway activation in aged individuals contributes to differentiation of MDSCs (97). The severity of bone loss in osteoporosis is closely related to the activity of the NF-κB pathway (107).

Commonly, bisphosphonates are used to treat age-related osteoporosis. These molecules can dose-dependently abrogate expansion of MDSCs and limit their osteoclastic ability by inhibition of protein prenylation (63), suggesting MDSCs play an essential role in this pathology. Given the impact of MDSCs on the bone metabolism, targeting this cell population is a potential novel therapeutic target against osteoporosis (108).

6.2 Autoimmune arthritis and bone destruction

Autoimmune diseases are a range of morbidities characterized by abnormal generation of self-reactive antibodies (4). In contrast to autoinflammatory diseases caused by the innate immune system, adaptive immune cells are responsible for the development of autoimmune diseases. However, both morbidities share inflammation as a common feature. This proinflammatory environment increases osteoclast differentiation and subsequently causes bone erosion as a discernable sign of autoimmune diseases compared to degenerative arthritis.

In autoimmune diseases, MDSCs have been pointed out to be deleterious to bone formation. Charles et al. first described a group of M-MDSC-like myeloid cells with $CD11b^{-/low}Ly6C^{hi}$ phenotype with high differentiation potential and myeloid suppressor function in a rheumatoid arthritis (RA) mice model (109). Zhang et al. later identified that co-stimulation of MDSCs with M-CSF and RANKL

contributes to bone erosion in a collagen induced arthritis (CIA) model (62). Similar, in another murine autoimmunity model (MFG-E8 knockout mice), bone mass was compromised by enhanced inflammation due to increased osteoclast differentiation of MDSCs (110). In humans, Chen et al. found a strong correlation of M-MDSCs and Th17 cells with osteolysis. Th17 cells can switch to a pro-osteoclastogenic phenotype with high expression of RANKL and reciprocally induce M-MDSCs differentiating into OCs (111). Of note, M-MDSCs were found to secret Arg-1 instead of NO to regulate RANKL expression on Th17 cells (111), which contrasts previous findings that M-MDSCs usually secret NO to modulate the immune responses (13).

Besides their impact on the bone, MDSCs can actively regulate the activity of autoimmune diseases by interacting with T and B effector cells. The immunosuppressive ability of MDSCs has been described in various diseases prone to arthritic lesions, including RA, systemic lupus erythematosus (SLE), and ankylosing spondylitis and the adoptive transfer of allogenic MDSCs has been shown to be a novel treatment approach in affected patients (93, 112). In an autoimmune arthritis model, adoptive transfer of MDSCs skewed the T cell population toward Treg generation, reduced the Th1 and Th17 cell population, and decreased the expression of inflammatory cytokines (113). Similar, transfusion of PD-L1 expressing MDSCs resulted in expansion of regulatory T and B cells and subsequent down-regulation of overactive autoimmunity in a murine SLE model (114).

In contrast to these findings, MDSCs have been reported to prolong or even exaggerate inflammation and thus enhance disease activity. In several reports on the adoptive MDSC transfer in SLE, MDSCs increased disease severity by secreting Arg-1 stimulating Th17 cell differentiation (14, 74). Similar, some reports found higher expression of TNF-α and IL-1β and subsequently increased diseases progression in autoimmune arthritis after MDSC transfer (115, 116). This effect may be caused by selecting MDSCs using Gr-1 and CD11b which can also be found on potentially proinflammatory mature myeloid cells. Another potential mechanism responsible for increased inflammation may be MDSCs potential to differentiate to macrophages or neutrophils depending on the local complex inflammatory environment (117). In addition to an adverse immune response, MDSCs are potential osteoclast precursors when transferred into an autoimmune condition and may deteriorate affected bony structures further.

6.3 Orthopedic implant-related infection

Despite increased use of antibiotics and improved aseptic surgical techniques, orthopedic implant-associated infections still remain one of the most challenging complications in orthopedics for patients, physicians, and the health care system alike (118, 119). Chronic inflammation at the bone-implant interface can impact healing and subsequently lead to septic loosening. Once osteolysis sets on, the bone quality decreases over time and the risk for fracture or implant failure significantly increases (118).

In chronic implant-related infection, low virulent bacteria form a layer of biofilm to protect themselves against the immune system and antibiotics (120). Inside the biofilm, bacteria form communities with a reduced metabolic rate, described as a "dormant state" (121, 122). This biofilm gradually elicits the immunosuppressive function of local reactive leukocytes, and therefore prolongs bacteria survival, further complicating successful treatment (16). Additionally, the proinflammatory environment attracts MDSCs to accumulate in the bone niche and attenuate the antibacterial function of polymorphonuclear cells (123).

MDSCs were recently revealed to be involved in the pathogenesis of periprosthetic joint infections. Besides elevated local cell prevalence, their presence in the peripheral blood persists over a long period of time, suggesting a systemic process potentially affecting other organs. However, despite their assumed role in disease progression, knowledge on the impact of MDSCs in implant-associated infections remains severely limited. Their immunosuppressive function has been shown to prolong infection by inhibiting the immune responses mediated by T cells, B cells, and natural killer cells (16, 124, 125). Compared to other myeloid derived cells or lymphocytes, prevalence of MDSCs was particularly high and increased over time in chronic infections (124, 126). Additionally, there has been large numbers of MDSCs observed infiltrating the biofilm, accounting for nearly half of the detectable MDSC population (16). G-MDSCs have been shown to be particularly relevant for heightened bacterial resistance (11). They produce IL-10 leading to increased bacterial persistence (11, 127) and susceptibility to infections (128). After antibody depletion of the G-MDSC population by targeting Ly6G, Ly6C+ monocytes and macrophages expand and regain proinflammatory function essential for clearing bacterial infection (124). Besides G-MDSC, M-MDSC are found around the biofilm albeit in much smaller numbers (16). At the biofilm, M-MDSCs differentiate to anti-inflammatory M2 macrophages that hinder T-cell mediated immunity and thus also contribute to infection persistence (129). Employment of anti-bacterial additions to implants can significantly reduce the number of MDSCs, limit their anti-inflammatory function, and increase efficiency of antibiotics (130, 131). Additionally, successful treatment can positively impact the bone metabolism, as MDSCs differentiate to OCs in infection (132). After surgical addressing of the biofilm, the septic bone destruction recovers significantly (131).

The relationship of the pathogenesis of orthopedic infection and MDSCs is reciprocal. Increased prevalence of MDSCs is linked to heightened risk of infection. Of note, in one *in vivo* human study, the number of G-MDSCs was elevated after aseptic orthopedic surgeries while relative occurrence of total leukocytes and MDSCs remained the same (128). These results suggest during and immediately after surgery risk for bacterial infection may be highest and targeting MDSCs may be a viable prophylactic treatment.

The PD-1/PD-L1 signaling axis has been suggested as a potential target. MDSCs down-regulate T-cell induced pathogen elimination through PD-1/PD-L1 signaling (133, 134). Additionally, *in vivo* experiments suggest a crucial role of PD-1 in differentiation of MDSCs to OCs. PD-1 knockout in osteoporotic mice halved the number of OCs and led to a 2-fold increase in bone volume (82). Inhibition of PD-1 using immune checkpoint inhibitors interrupts OC precursor cell differentiation in areas

with bone lesions involving downregulation of CC-chemokine ligand 2/CC-chemokine receptor 2 (CCL2/CCR2) pathway, whereas it exerts no effect on physiological bone structures (135). Conversely, targeting the PD-1/PD-L1 axis may improve clinical outcome, yet can also aggravate inflammation and disrupt the bone metabolism (136). Similar, bisphosphonate can dampen the osteolytic effects of OCs and inhibit MDSC differentiation, however, they have been associated with higher bacterial burden and increased risk for infection (53, 137). Promising novel strategies such as using bisphosphonate as carrier for antibiotics still have to prove effective in a clinical setting (138).

6.4 Bone fracture

A traumatic fracture is described as partially or completely disrupted continuity of the bone potentially leading to persisting pain, immobility, and even death due to blood loss (139). However, the bone tissue possesses the potential to fully recover from if treated appropriately. Despite adequate conservative or surgical treatment around 5-10% of affected patients develop mal- or non-union fractures and need additional intervention (140).

Fracture union encompasses consecutive and overlapping phases, from formation of hematoma, soft callus, fibrous tissue to hard callus, and finally remodeled bone (9). The metabolic phases during bone healing interact with the innate and adaptive immune system. The processes involved promote angiogenesis and osteoblast differentiation from BMSCs (9). Dysregulation of the immune response can retard the fracture healing process and is a significant risk factor for mal- or non-union fracture healing. Thus, restoring the physiological immune environment in general and targeting MDSCs in particular is a promising novel therapeutic approach in affected individuals (17, 31).

Currently, there exist conflicting evidence on the role of MDSCs in the bone healing process. Traumatic injury leads to increased cytokine production of IL-1β, IL-6, and G-CSF prompting accumulation of MDSCs (141). Cheng et al. described a long-term dysregulated immune pattern in delayed bone healing (142). By computational analysis, they found a negative correlation of circulating MDSCs and bone healing. MDSCs indirectly suppress the regenerative capability of BMSCs by inhibition of B cell differentiation and elevated IL-10 expression (72). Conversely, MDSCs show a protective effect on injured bone tissue and can even support tissue remodeling (143, 144). After arthroplasty, there is a high concentration of MDSCs that support development of new blood vessel at the polymethyl methacrylate induced periosteal membrane. Local transplantation of MDSCs enhances the formation of these capillaries around the membrane (145). In traumatic fracture healing, significantly elevated number of MDSCs were observed in the transitional area, facilitating the recovery of the bone injury by suppressing local inflammation to stimulate osteoblast differentiation and function (146). However, while MDSCs promote bone regeneration by improving angiogenesis and limiting the inflammatory response, continuous presence of MDSCs pose a risk for infection due to their immunosuppressive capabilities (142).

6.5 Bone malignancy and metastasis

Cancer growth depends on both the vigorousness of the tumor itself and a compromised anti-tumor ability of the immune system. MDSCs can facilitate tumor growth through their immunosuppressive capabilities. Research on MDSCs and their involvement in tumor progression has been a main focus and inspires hope for novel therapeutic approaches.

Osteosarcoma (OS) is one of the most prevalent primary bone malignancies in children and teenagers. Both surgical intervention and chemotherapy are employed to enhance quality of life and overall survival. A better understanding of the role MDSCs in supporting growth of OS may open up new treatment options. In the tumor microenvironment, MDSCs, most of them PMN-MDSCs, accumulate and inhibit the T-cell mediated immune responses induced by high expression of IL-18 and CXCL12 (147, 148). Blocking these inducive factors has been shown to sharpen the anti-PD-1 treatment efficacy in mice indicating the importance of the PD-1/PD-L1 axis in MDSCs during the growth of OS (147–149). Activation of the PI3K/Akt pathway was also found to be pivotal in OS tumor growth (148, 149). Additionally, the STAT3 pathway has been related to immunosuppression in this tumor pathology. Inhibition of STAT3 and PI3K/Akt signaling can reverse the suppressive effects on local immunity and reduce tumor size (148–150).

Besides primary bone tumors, the skeletal system is much more commonly affected by metastasis of several types of cancer. In cases of bone metastasis, a variety of growth factors and chemokines produced by the bone and immune regulating cells facilitate the proliferation and expansion of MDSCs (151, 152). At tumor site, malignant cells can precondition the immunosuppressive behavior of BMSCs. These cells subsequently promote the expansion of MDSCs and can attract cancer cells to migrate from the blood into the bone (153). Additionally, MDSCs contribute to epithelial-mesenchymal transition (EMT), thus enhancing mobility, invasion, and resistance to apoptotic stimuli of cancer cells. $CXCR2^{+}$PMN-MDSCs were found to be a major regulator and initiator of EMT through releasing IL-6 during breast cancer progression (154). M-MDSCs can also modulate EMT by secretion of nitric oxide synthase modulate (155). Moreover, MDSCs are involved in the formation of the pre-metastatic niche (PMN). They aggregate at the PMN where they support the construction of the nutritious “soil” for tumor metastases to “plant in” by promoting neovascularization (12, 156) and increasing the activity of neutrophil extracellular traps that can catch circulating tumor cells to colonize (157, 158). Lastly, MDSCs enhance direct differentiation to M2 macrophages (159, 160) and facilitate the differentiation of M1 to tumor-supportive M2 macrophages (161).

The cancer-driven accumulation of MDSCs also has impact on the bone metabolism by differentiating to OCs. This hinders bone regeneration both at the site of osteolytic bone metastases and by dissemination to the bone site *via* blood stream (61). Of note, osteoclast differentiation is MDSC-dependent in bone metastasis, signifying the essential crosstalk between tumor cells and myeloid progenitors in the bone microenvironment (162). Once tumor cells spread to the bone and meet the primed MDSCs they start a continuous stimulate each other reciprocally challenging the bone health. In multiple myeloma,

the impact on the bone is even more severe as this malignancy originates from the bone marrow (63). Additionally, the generated OCs enhance tumor immune evasion of multiple myeloma cells from T cell surveillance *via* PD-L1, galectin-9, and CD200 (163, 164). Treatment with immune checkpoint blockers targets this mechanism to revert the MDSC-driven anti-tumor immunosuppression (147).

7 Conclusion

The delicate balance of bone resorption and regeneration interacts with and is influenced by the regulatory immune system both physiologically and in disease. In this review, we discuss the impact of MDSCs on the bone metabolism under several pathological conditions, the involved modulatory pathways as well as potential therapeutic targets in MDSCs to improve bone health. MDSCs have a regulatory function on the immune system and can significantly and lastingly impact the process of bone remodeling through differentiation into osteoclasts. In chronic inflammatory conditions, generation of MDSCs is induced. MDSCs have previously been identified in several diseases affecting the bone including tumor, autoimmune diseases, fractures, and infection. They are part of a complex network in which they interact with and regulate other immune cells by releasing soluble proteins, exosomes, and through surface protein-receptor interactions. However, there remains paucity on several of the involved pathways linking MDSCs to osteoclast differentiation and function as well as osteoblast activity and behavior. Emerging evidence suggests a key role of MDSCs in these diseases making them a promising target for novel therapeutic approaches in several diseases.

Author contributions

Conceptualization, YR. Project administration, MM. Resources, MM. Supervision, AK and MM. Visualization, YR. Writing – original draft, YR, HB, and AK. Writing – review & editing, YR, HB, AK, and MM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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COMMBINI: an experimentally-informed COmputational Model of Macrophage dynamics in the Bone INjury Immunoresponse

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Bone fracture healing is a well-orchestrated but complex process that involves numerous regulations at different scales. This complexity becomes particularly evident during the inflammatory stage, as immune cells invade the healing region and trigger a cascade of signals to promote a favorable regenerative environment. Thus, the emergence of criticalities during this stage might hinder the rest of the process. Therefore, the investigation of the many interactions that regulate the inflammation has a primary importance on the exploration of the overall healing progression. In this context, an *in silico* model named COMMBINI (COmputational Model of Macrophage dynamics in the Bone INjury Immunoresponse) has been developed to investigate the mechano-biological interactions during the early inflammatory stage at the tissue, cellular and molecular levels. An agent-based model is employed to simulate the behavior of immune cells, inflammatory cytokines and fracture debris as well as their reciprocal multiscale biological interactions during the development of the early inflammation (up to 5 days post-injury). The strength of the computational approach is the capacity of the *in silico* model to simulate the overall healing process by taking into account the numerous hidden events that contribute to its success. To calibrate the model, we present an *in silico* immunofluorescence method that enables a direct comparison at the cellular level between the model output and experimental immunofluorescent images. The combination of sensitivity analysis and a Genetic Algorithm allows dynamic cooperation between these techniques, enabling faster identification of the most accurate parameter values, reducing the disparity between computer simulation and histological data. The sensitivity analysis showed a higher sensibility of the computer model to the macrophage recruitment ratio during the early inflammation and to proliferation in the late stage. Furthermore, the Genetic Algorithm highlighted an underestimation of macrophage proliferation by *in vitro* experiments. Further experiments were conducted using another externally fixated murine model, providing an independent validation dataset. The

validated COMMBINI platform serves as a novel tool to deepen the understanding of the intricacies of the early bone regeneration phases. COMMBINI aims to contribute to designing novel treatment strategies in both the biological and mechanical domains.

KEYWORDS

bone fracture healing, inflammatory phase, macrophages, *in silico* model, multiscale model, sensitivity analysis, genetic algorithm, immunofluorescence

1 Introduction

Fracture healing in long bones is a complex process where numerous biological factors cooperate for the complete restoration of the original bone structure and functionality. What makes this process fascinating is the innate capacity of the bone to autonomously initiate its own healing following an injury [Bigham-Sadegh and Oryan (1)]. Immediately after the injury, biological and mechanical factors within the healing region guide the progression of fracture repair [AI-Aql et al. (2); Hankenson et al. (3); Bahney et al. (4)]. The haematoma that forms within the bone fracture has a strong osteoinductive potential [Tsunoda et al. (5); Kolar et al. (6)], generating the environment for successful initiation of the healing process. The early stage of bone fracture healing is characterized by a cascade of events that involves numerous cells, molecules and chemicals recruited from disrupted blood vessels, bone marrow and periosteum niches.

The inflammatory stage is the initial step of bone fracture healing [Schmidt-Bleek et al. (7)]. It starts immediately after the injury as a first response and clears the fracture region of debris, apoptotic cells and necrotic tissue [Niu et al. (8)]. When an open fracture occurs, the inflammatory response prevents the unhindered invasion of external pathogens, thereby reducing the risk of diseases or infection [Loi et al. (9)]. The inflammatory environment is formed promptly after the injury through the invasion and recruitment of specialized cells [Baht et al. (10)], namely innate immune cells. The haematoma region, where the initial phases of healing take place, is formed by a blood clot as a result of disrupted vessels [Kolar et al. (6); Schell et al. (11)] This clot, which contains bone debris and other dead cells forms a region where the inflammatory response is promoted (pro-inflammatory) [Kolar et al. (6)]. The recruitment of innate immune cells such as neutrophils and macrophages will guarantee the cleansing of the healing area from debris and dead cells, which are phagocytized and degraded [Wu et al. (12); Loi et al. (9); Maruyama et al. (13); Gierlikowska et al. (14)]. During the initial inflammation by innate immune cells, a specialized adaptive immune response is triggered with the recruitment and activation of T and B cells, natural killer cells and dendritic cells [Baht et al. (10)]. Especially T cells of the adaptive immune system have been found to regulate the tissue formation beyond the hematoma phase [Reinke et al. (15); Schlundt et al. (16); Bucher et al. (17)]. The innate immune response is initiating the healing cascade whereas the adaptive immune response is dynamically regulating the ongoing inflammatory process. The current version of the COMMBINI model focuses on this inevitable inflammatory stage initiated primarily by macrophages after bone injury.

The physiological development of the inflammatory stage is paramount for the successful repair of the injury [Mountziaris and Mikos (18); Wu et al. (12); Loi et al. (9); Gu et al. (19); Hoff et al. (20); Duda et al. (21)]. However, due to the many factors involved, disruption to the healing cascade is not rare. While some disturbances may have minimal impact, there is a possibility for the occurrence of compromising events, leading to healing delay or non-unions [Bishop et al. (22); Wildemann et al. (23)]. Scenarios where a depleted quantity of macrophages is induced show compromised repair [Alexander et al. (24); Vi et al. (25); Schlundt et al. (26)]. Additionally, prolonged inflammation can have detrimental effects on the healing process, leading to chronic inflammation [Maruyama et al. (13)]. Therefore, it is crucial to regulate and buffer the inflammation (anti-inflammatory response) after a certain number of days [Newman et al. (27)]. Accordingly, a well-coordinated sequence of events is required to generate a suitable environment for the repair and remodeling stages, which will complete the healing process in the following weeks [Baht et al. (10)]. Due to its "dance-opener" role, the successful development of the inflammatory stage is essential to guarantee a productive healing progression. Consequently, many recent studies on bone fracture healing have shifted their focus to this initial stage [Maruyama et al. (13); Newman et al. (27); Baratchart et al. (28)]. Therapeutics and treatments that support the correct initiation of bone fracture healing hold clinical significance in the new generation of biological and mechanical instruments aimed at reducing the risk of failure to heal.

Most of the available literature utilizes *in vitro* models to investigate the immune events that characterize the inflammatory stage of bone healing [Ying et al. (29); Lin et al. (30); Nathan et al. (31)]. However, evaluating the role of dynamics and interactions in the complete scenario remains experimentally challenging. Computer modeling is gaining more and more interest in the academic field for the investigation of mechano-biological processes occurring at multiple levels [Giorgi et al. (32); Vavourakis et al. (33); Lafuente-Gracia et al. (34)]. The possibility to simulate cellular and molecular dynamics and interactions is a valuable asset for the detailed study of bone fracture healing [Borgiani et al. (35); García-Aznar et al. (36)]. Despite their

potential, existing computer models of bone fracture healing are mostly limited to the study of the mechano-biological process during repair phases, neglecting the role of the inflammatory stage [Lafuente-Gracia et al. (34)]. To date, only few computer models explored this stage of bone healing by using continuous domains to investigate the dynamics of inflammatory cell and cytokine concentrations [Kojouharov et al. (37); Trejo et al. (38); Baratchart et al. (28)]. However, while those models only evaluate the temporal evolution of the inflammatory cells and cytokines dynamics, the multiscale *in silico* model that we propose employs the computational potentialities to extend the investigation to the spatial dimensions.

In this manuscript, we present a novel *in silico* framework to investigate the mechano-biological interactions in the early inflammatory stage of bone fracture healing at tissue, cellular and molecular levels. A multiscale model is proposed to investigate the interactions between different levels of biological components (*e.g.* cells, cytokines). The agent-based modeling approach provides a new perspective on the role of immune cell populations during the inflammatory stage and their intrinsic capacity to regulate - and be regulated - by the pro- and anti-inflammatory cytokines at the molecular level. The model combines multiple algorithms, to simulate the complete spectrum of multiscale interactions and regulations that happen during the inflammation phase in bone healing. Model calibration was performed using a combination of *in vitro* and *in vivo* results reported in the literature, in part analyzed using a newly developed *in silico* immunofluorescence pipeline. Model validation was executed using an in-house *in vivo* experiment. With this study, we deliver a computational tool that supports the investigation of novel therapeutics and treatments to enhance bone fracture healing with dedicated attention to the multiscale events that interlace during the inflammatory stage.

2 Materials and methods

2.1 The agent-based model to investigate the cellular level

To investigate the inflammatory stage of bone fracture healing, a multiscale *in silico* model has been developed. The model, named COMMBINI (COmputational Model of Macrophages dynamics in the Bone INjury innate Immunoresponse), aims to simulate the biological and mechanical environment during the progression of the healing of a long bone fracture. To date, only the cellular and molecular modules of COMMBINI have been developed with the support of PhySiCell [Ghaffarizadeh et al. (39)], an open-source software that simulates the cells as single entities within an agent-based model. These virtual cells perform phenotype-specific activities (*e.g.* migration, proliferation) and regulate the molecular level (*e.g.* consumption and production of cytokines).

During the inflammatory stage, the cellular level plays a major role, as the innate immune cells actively contribute to initiating the healing response. To simulate this cellular level, an agent-based model has been developed. With this approach, each cell was simulated independently and not as a passive component of a cell population, thereby providing stochasticity to the investigation and guaranteeing the spatio-temporal variability that characterizes biological systems [Wehrens et al. (40); Allen et al. (41)]. The simulation was performed within a geometrical domain that represents the shape of a murine tibia fracture over a virtual period of 3 days, encompassing the early inflammatory stage. For the current study, a fracture opening in the center of the bone was simulated. The size of the fracture gap depends on the specific case study under investigation (cfr. § 2.3, 2.7). The model geometry was created by assuming a hollow cylinder as a simplified shape for the bone and a spheroid shape for the callus domain (healing region), following the same assumptions as previous studies [Wang and Yang (42); Borgiani et al. (43); Perier-Metz et al. (44)]. The healing region is the spatial domain where cell activities and molecular dynamics are simulated. Boundary conditions are imposed on the surfaces of the healing region. Bone marrow is simulated as a reservoir of non-polarized macrophages: they are recruited from the bone marrow compartment to invade the healing region. Furthermore, once the inflammation is over, the macrophages leave the region and emigrate back to the marrow compartment. The same conditions are imposed on the curved surface of the healing region, which simulates the periosteal boundaries. A zero-flux condition is imposed on the surface of the bone cortex as it is assumed that cells cannot migrate and cytokines cannot diffuse through it. The 2D model is generated by an intersecting plane along the middle axis (Figure 1A).

The iterative nature of the model allowed the investigation of the cellular environment evolution with a time resolution of Δt_{cell} = 1 min. In each iteration, virtual cells within the Region of Interest (ROI) perform specific actions based on phenotype-specific ratios, and the cellular environment is updated accordingly. Four different cell phenotypes are described in this computer model: non-polarized macrophages (M0), pro-inflammatory macrophages (M1), anti-inflammatory macrophages (M2) and polymorphonuclear neutrophils (PMN) (Figure 1B). The PMNs are the only cell type simulated within the healing region at the initial time-point. They are uniformly distributed within the region with an initial concentration $[\mathbf{PMN}]_0$. Macrophages start to appear from the first iterations of the simulation onward. At the molecular level, an initial concentration of fracture debris ($\mathbf{Db_0}$) is homogeneously distributed within the healing region. This initial condition is crucial as the presence of debris chemotactically promotes the invasion of the healing region by the immune cells. No inflammatory cytokines are simulated within the region at the initial time-point but they start being secreted from the first iteration onwards. The M0 recruitment from the marrow cavity and tissues surrounding the healing region is stimulated by the presence of debris. The PMNs and macrophages phagocytose the debris, leading to a decrease in its concentration and recruitment capacity as healing progresses. To simulate this behavior in the computational model, the M0 recruitment ratio follows a dynamic pattern that decreases along with the physiological reduction of debris concentration within the healing region [Trejo et al. (38)]:

$$\frac{\Delta M0}{\Delta t} = k_{R(M0)}\left(1 - \frac{[M\Phi]}{[M\Phi]_{max}}\right)[\text{Debris}] \quad (1)$$

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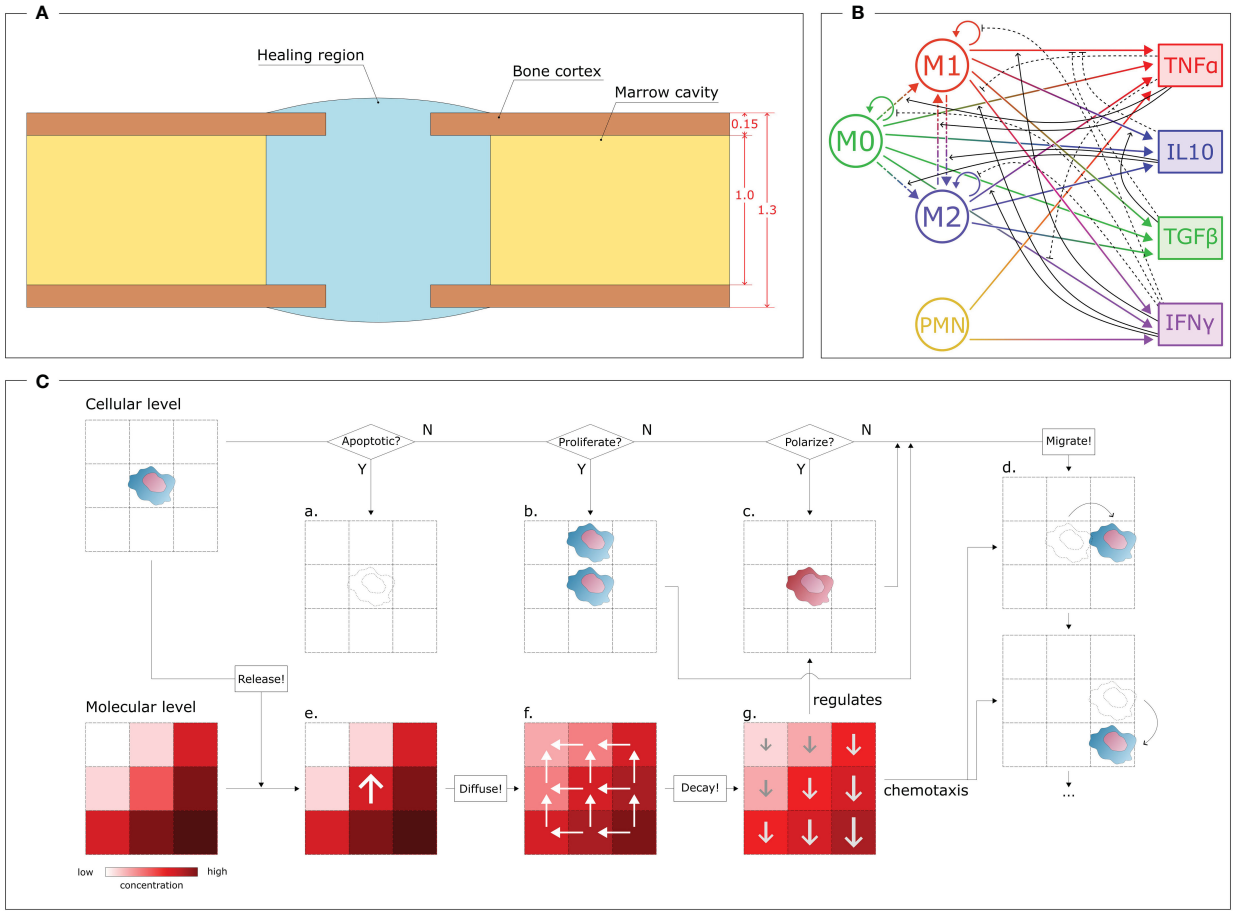

FIGURE 1
Overview of the COMMBINI components. **(A)** Simulation domain (blue), based on the callus geometry for bone fracture healing, wherein the cellular and molecular levels are simulated. Dimensions reported in mm. **(B)** Multiscale interactions between the cellular level (left) and the molecular level (right). Circular arrows: proliferation/population doubling; dash-dotted arrows: macrophage polarization/interpolarization; gradient arrows: cytokine secretion; black arrows: cellular activity regulations (solid: promotion, dashed: inhibition). M0: non-polarized macrophages, M1: pro-inflammatory macrophages, M2: anti-inflammatory macrophages, PMN: polymorphonuclear neutrophils, TNFα: Tumor Necrosis Factor alpha, IL10: Interleukin 10, TGFβ: Transforming Growth Factor beta, IFNγ: Interferon gamma. **(C)** Schematic representation of the rules that regulate the two levels. At the cellular level (top), for each cell it is checked if the cell is in an apoptotic, proliferative or polarized state, according to dynamics reported in the literature and translated into computer model algorithms. If apoptotic **(a)**, the cell is removed by the model; if proliferative **(b)**, a daughter cell is created in one of the surrounding positions; if polarized **(c)**, the phenotype changes. The cell can migrate **(d)** by performing a sequence of jumps. Each cell releases specific molecules at the molecular level (bottom) by increasing their concentration in the specific position **(e)**. Then, the molecules diffuse **(f)** from regions of high concentrations to low; and degrade **(g)** following exponential dynamics, therefore having a faster decay in more concentrated regions. The molecular environment regulates the polarization algorithm **(c)** and drives cell migration **(d)** through chemotaxis.

Equation (1) halts macrophage recruitment when no more debris needs to be removed or if the maximal concentration of macrophages is reached within the healing region. The dynamic is regulated by two parameters, whose variation can lead to faster or slower recruitment of macrophages: $\mathbf{k_{R(M0)}}$ is the maximum non-polarized macrophage recruitment ratio and $[\mathbf{M\Phi}]_{\mathbf{max}}$ is the maximal macrophage concentration allowed within the healing region. To create a realistic evolution of the cellular environment during the inflammatory stage of bone healing, the macrophages and PMNs perform additional activities, *i.e.* they migrate, proliferate, polarize and are subject to apoptosis (Figure 1C).

In COMMBINI, cellular migration is stochastically simulated as a sequence of "jumps" in random directions to create a movement pathway in the 2D space [Allen et al. (41)]. The span of each jump is defined by the migration speed ($\mathbf{k_v}$) associated with each cell phenotype. Cellular proliferation is simulated by generating a daughter cell with identical characteristics to its mother cell in one of the neighboring positions. The proliferation ratio ($\mathbf{k_p}$) associated with each cell phenotype determines the frequency of cell division within each iteration. Apoptosis is simulated as the removal of cells by programmed cell death. The apoptosis ratio ($\mathbf{k_a}$) of a cell increases with the accumulation of phagocyted debris [Bratton and Henson (45)] and the number of other cells in its vicinity, mimicking the consumption of essential nutrients for survival. Furthermore, macrophages have the ability to change their phenotype in response to the surrounding inflammatory environment as perceived at the molecular environment (more details in § 2.2). The M0 macrophages can, under specific molecular conditions, polarize into either an M1 or M2 phenotype (Figure 1B) [Yunna et al. (46)]. In our model, this process is simulated as the

change of the phenotype flag associated with the macrophage. Following the phenotype switch, the virtual macrophage adjusts its behavior by modifying its parameter values and algorithm dynamics according to the characteristics assigned to the new phenotype. Moreover, although infrequent, interpolarization can occur between M1 and M2 phenotypes, depending on whether pro-inflammatory macrophages reside in an anti-inflammatory environment, and vice versa (Figure 1B)[Yunna et al. (46)]. Inter-polarization into pro-inflammatory macrophages is rare compared to the interpolarization into the anti-inflammatory phenotype due to the natural progression of the bone healing process. To avoid unnecessary complexity, this model does not include further subdivisions within the M2 subtypes. However, the model can be readily expanded to include such subdivisions if the scope is extended beyond the inflammatory stage to incorporate the repair phase.

In the discrete agent-based model, the apoptosis, proliferation and polarization conditions are reported as probability values for the respective event to occur within the iteration period Δt_{cell}. Therefore, during each iteration, a random floating point value between 0 and 1 (precision 10^{-6}) is assigned to each cell for each event. If the value exceeds the probability value, the event does not get triggered (N paths in Figure 1C). Conversely, if the value is lower than the probability value, the cell is removed, generates a daughter cell or changes its phenotype (Y paths in Figure 1C). For cell proliferation, the position of the daughter cell is randomly selected from the four adjacent positions that are not occupied by other cells. Migration is performed at every iteration by allowing the cell to jump multiple times to adjacent positions based on their migration speed and the spatial and temporal resolution of the model. In this model, assuming a spatial resolution of 1 μm (cellular model spatial resolution) and an iteration period of Δt_{cell} = 1 min (temporal resolution), a PMN (k_v = 5.00 μm min^{-1}) will perform five jumps during each iteration. The direction of each jump is randomly chosen among the four surrounding positions that are not occupied by other cells, when chemotaxis is not involved. However, a large part of the phagocytic cells included in this work is driven by the fracture debris gradient. Chemotaxis is incorporated into the model by directing cell movement according to the gradient of the chemotactic agent concentration (Figure 1C).

While macrophages are recruited, PMNs promote the onset of the inflammatory response. In the first version of COMMBINI, PMNs are the only non-macrophage population considered at the cellular level. At the start of the simulation, PMNs are uniformly distributed within the healing region with an initial concentration $[PMN]_0$. Through the course of the inflammation, PMNs are recruited from the surrounding tissues by following a dynamic analogous to (1). PMNs are short-lived cells that tend to disappear from the healing region after triggering the initial inflammatory signal and its amplification [Summers et al. (47)]. Therefore, the proliferation of PMNs is not included in the model (Figure 1B). To simulate the natural behavior of neutrophils, PMNs simulated in COMMBINI release pro-inflammatory cytokines and clear debris from their surroundings to generate a pro-inflammatory environment [Kovtun et al. (48, 49)].

2.2 Differential equations to describe the molecular level dynamics

The cellular level has a mutual regulatory relationship with the molecular level. Consequently, we simulated the molecular model within the same agent-based model that simulates the cellular environment. The dynamics of cytokine concentration at the molecular level are simulated using partial differential equations (PDE) with function descriptions obtained from the literature (Supplementary Table 2). The equations were solved using the BioFVM solver [Ghaffarizadeh et al. (50)] on a 2000 μm x 2000 μm square 2D grid within the healing region, with a resolution of 10 μm. The concentration of each inflammatory cytokine is evaluated in each grid element. This setup enables multiscale interactions, as each element in the molecular model shares its position with one or more cells in the cellular environment, according to the common coordinate system. The activities of the cells within the same element are regulated by the cytokine concentration within it (Figure 1C). Conversely, the presence of cells within each element regulates the intrinsic variation of cytokine concentration, reproducing phenotype-specific dynamics (Figure 1C). Macrophage polarization is regulated by the molecular level as the macrophages simulated at the cellular level polarize according to the cytokine concentration predicted in the same spatial location of the healing region (Figure 1C). Tumor Necrosis Factor alpha (TNFα) and Interleukin 10 (IL10) have been chosen for this model to respectively represent pro- and anti-inflammatory cytokines at the molecular level. Therefore, we described the macrophage polarization rules as probability functions, which are regulated by the concentration of those cytokines [Trejo et al. (38)]:

$$P(M0 \rightarrow M1) = k_{01} \frac{[TNF\alpha]}{a_{01} + [TNF\alpha]} \quad (2)$$

$$P(M0 \rightarrow M2) = k_{02} \frac{[IL10]}{a_{02} + [IL10]} \quad (3)$$

$$P(M1 \rightarrow M2) = k_{12} \frac{[IL10]}{a_{12} + [IL10]} \quad (4)$$

$$P(M2 \rightarrow M1) = k_{21} \frac{[TNF\alpha]}{a_{21} + [TNF\alpha]}. \quad (5)$$

In equations (2 - 5), the parameters **k** represent the macrophage polarization ratios and the parameters **a** represent the cytokine half-saturation for macrophage polarization.

The molecular environment is, in turn, regulated by the immune cells (Figure 1B). These release pro- and anti-inflammatory cytokines, according to the dynamics included in the model. In addition to TNFα and IL10, the model includes Transforming Growth Factor beta (TGFβ) and Interferon gamma (IFNγ), as they regulate cell activity in the healing region: *e.g.* TGFβ lowers secretion of pro-inflammatory cytokines by M1 macrophages [Nagaraja et al. (51)], and IFNγ downregulates macrophage proliferation (Figure 1B). All the cell-specific cytokine secretion dynamics simulated in this model are reported in Table 1.

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TABLE 1 Cell-specific cytokine secretion dynamics for each cytokine included in the *in silico* model.

M0	TNFα	$k_{TNF}\left(1+\frac{k_{TNI}}{1+e^{(a_{TNI}-[IFN\gamma])}}\right)$
	IL10	k_{IL10}
	TGFβ	k_{TGF}
	IFNγ	$k_{IFN}e^{-a_{ITN}[TNF\alpha]}$
M1	TNFα	$k_{TNF}(k_{TNIL}e^{-a_{TNIL}[IL10]}+b_{TNIL})(k_{TNTG}e^{-a_{TNTG}[TGF\beta]}+b_{TNTG})\left(1+\frac{k_{TNI}}{1+e^{(a_{TNI}-[IFN\gamma])}}\right)$
	TGFβ	k_{TGF}
	IFNγ	$k_{IFN}e^{-a_{ITN}[TNF\alpha]}$
M2	IL10	k_{IL10}
	TGFβ	k_{TGF}
PMN	TNFα	k_{TNF}
	IFNγ	k_{IFN}

M0: non-polarized macrophages, M1: pro-inflammatory macrophages, M2: anti-inflammatory macrophages, PMN: polymorphonuclear neutrophils, TNFα: Tumor Necrosis Factor alpha, IL10: Interleukin 10, TGFβ: Transforming Growth Factor beta, IFNγ: Interferon gamma.

The cytokines diffuse through the molecular level by following Fick's law of diffusion, with a specific diffusion coefficient **(D)** associated with each cytokine. Neumann boundary conditions (no-flux) have been assigned to the borders of the healing region and bone cortex. Additionally, decay rates (**d**) have been set for each cytokine to simulate their enzymatic degradation, leading to a decrease in concentration. A temporal resolution of Δt_{mol} = 1 s was assigned to iteratively simulate the dynamics within the molecular level. To coordinate the temporal dynamics between the two levels, which are characterized by different temporal resolutions, the cellular environment updates every 60 iterations of the molecular level.

Additionally, the molecular level simulates the dynamical spatio-temporal variation of the concentrations of debris within the healing region. In this study, the term debris is used to define the agglomerate of dead cell bodies and necrotic tissue pieces resulting from the bone fracture. The presence of debris elicits the release of Damage Associated Molecule Pattern (DAMP) inflammatory stimuli. The distribution of debris concentration is included at the molecular level as a biological variable capable of influencing the inflammatory stage development [Chow et al. (52)]. In COMMBINI, the macrophages follow the debris concentration gradient at the molecular level to orient their migration towards the zones of the healing region characterized by a higher concentration of debris. Phagocytosis has been implemented in the model as the capacity of macrophages and PMNs to remove debris in their spatial surroundings, hence clearing the healing region. An engulfment ratio $\mathbf{k_e}$ was defined to quantify the debris phagocyted by those cells within the iteration period.

2.3 Dedicated *in vivo* experiments for model calibration

The model parameters at both cellular and molecular levels were obtained from previously published *in vitro* works that investigated the biological characteristics of macrophages and cytokines (Supplementary Tables 1, 2). Afterward, a parameter calibration was performed to minimize the differences between the simulation outcomes and the experimental results from dedicated *in vivo* studies through the use of immunofluorescent imaging of macrophage populations. The *in vivo* experiments have received approval from the Ethical Committee for Animal Experimentation of the KU Leuven (approval number 020/2022). Tibial osteotomies (1 mm) were created in male C57BL/6 mice, fixated with an external Ilizarov fixator as previously described [van Gastel et al. (53)]. Three samples were obtained from the animals at 3 days post-fracture and prepared for immunohistology. The samples were fixated in formalin overnight at 4°C and decalcified with an edetic acid (EDTA) solution. The decalcified fracture samples were embedded in paraffin and 5 µm thick sections were mounted on glass slides. One slide from the center of each sample has been selected for immunofluorescence staining, obtaining $n = 3$ *ex vivo* images to use for calibration. The slides were deparaffinized with Histo-Clear (National Diagnostics, cat. no. HS-202) and dehydrated, followed by enzymatic antigen retrieval using 1 mg mL^{-1} Pepsin in 0.02M HCl. The samples were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.1% Tween20 (Merck, cat. no. P1379) and 0.01% Tergitol (AppliChem, cat. no. A9780) for 45 minutes at room temperature. The samples were stained with immunofluorescent markers for macrophages and their specific subtypes. DAPI identifies all the nuclei and the Cluster of Differentiation 68 (CD68) is a general marker for macrophages [Schlundt et al. (26)]. Co-expression of CD68 and CD80 is specific for pro-inflammatory macrophages, while co-expression of CD68 and CD206 identifies anti-inflammatory macrophages [Schlundt et al. (26)]. The samples have been incubated overnight at 4°C with a 1:500 dilution of anti-CD68 antibody (ThermoFisher, cat. no. 14-0681-82) and a 1:100 dilution of anti-CD80 (ThermoFisher, cat. no. PA5-85913) or anti-CD206 antibody (ThermoFisher, cat. no. PA5-101657) in

blocking buffer. On the second day, the samples were incubated for 4 hours at room temperature with Goat anti-rat IgG, Alexa Fluor Green 488 antibody with a 1:500 dilution (ThermoFisher, cat. no. A-11006) and Donkey anti-Rabbit IgG, Alexa Fluor Red 594 with a 1:1000 dilution (ThermoFisher, cat. no. A32754) in blocking buffer. Since bone is autofluorescent, the Vector TrueVIEW autofluorescence quenching kit (Vector, cat. no. SP-8400-15) was used. Finally, a counterstaining was performed with 5 μg mL^{-1} DAPI for 10 minutes. The samples were dried and mounted in VECTASHIELD Vibrance Antifade (Vector, cat. no. H-1700). Samples were imaged using the Olympus IX83 inverted microscope within 48 hours. The sections were conserved at -20°C for additional image acquisitions.

2.4 Deep-learning cell quantification and *in silico* immunofluorescence

A custom Python script was developed to analyze the immunofluorescent images and extract quantitative information at the cellular level. The outcome of the pipeline generates a fully segmented image with spatial information about macrophage distribution. Whole-cell segmentation was performed by Mesmer (DeepCell), a deep-learning tool trained on an extensive database of tissue image data and validated by experts [Greenwald et al. (54)]. Dimension filtering is applied to the images and the elements with a surface area below 80 μm^2 or larger than 200 μm^2 are not classified as cells [Cannon and Swanson (55)]. An ROI is chosen on the immunofluorescent image by selecting the fracture region, avoiding the bone cortex and staining artifacts. All cells within the ROI are labeled according to phenotype and quantified. For each macrophage phenotype, concentrations are calculated by dividing the number of cells by the ROI area. This data is compared with the macrophage concentrations simulated by the cellular level in the agent-based model. To perform a more direct qualitative comparison, *in silico* immunofluorescence was generated as output of the computational model by assigning the same color-coded pattern to the virtual cells as the immunofluorescent images. For example, the bright green fluorescence assigned to the CD68 channel was used to paint the cytoplasm of all the virtual macrophages, as they are supposed to express that marker (Figure 2). Co-marking is represented by the chromatic combination of the two markers: *i.e.* M1 cells that are co-marked by CD68 (green) and CD80 (red) are represented *in silico* with a yellow color (Figure 2). Additionally, this novel computational technique delivers a dataframe that contains information about all the cells identified within the ROI of the immunofluorescence image. Each cell is categorized in detail according to its size, the 2D position of its centroid and marker positivity.

2.5 Design of experiments to reduce the calibration complexity

The parameter calibration of the computer model was performed by following an optimization pathway to reduce the difference between quantified experimental and simulation outcomes. The calibration process can be time-exhaustive when many model parameters are included. Therefore, a sensitivity analysis was performed to determine the model parameters that most strongly influence the quantitative outcome of COMMBINI. The model was run multiple times with different combinations of parameter values. Reduction of the number of simulation runs was possible by cutting non-necessary repetitions with the support of Taguchi's orthogonal arrays [Kacker et al. (56)]. This strategy is convenient when many parameters have to be analyzed: the model is regulated by 36 parameters and a 2-level sensitivity analysis would have required 2^{36} simulation repetitions to analyze all the parameter combinations (full factorial). With Taguchi's orthogonal array, we reduced this number to 72, drastically dropping the estimated runtime of the analysis. An analysis of variance (ANOVA) was performed on the model outputs to evaluate the percentage of the total sum of squares (%TSS) for each parameter [Isaksson et al. (57)]. The absolute value of this percentage represents how sensitive the output is to variation of the

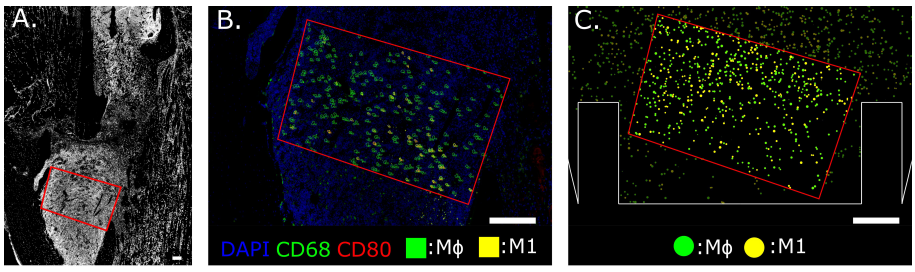

FIGURE 2
Region of Interest selection in the distal bone fracture **(A)** and cellular level comparison between *ex vivo* **(B)** and *in silico* **(C)** immunofluorescence at day 3 post-fracture. The staining utilized for *ex vivo* immunofluorescence marked nuclei in blue (DAPI), generic macrophages (MΦ) in green (CD68) and pro-inflammatory macrophages (M1) in yellow (co-expression of CD68 and CD80, green + red = yellow). *In silico* immunofluorescence used the same color-code associated with the specific macrophage markers used in the experiments to facilitate a direct qualitative comparison between experimental data and the simulation results. For quantitative comparison, macrophage concentration within the same area (red outline) is compared, located at the callus site indicated in **(A)**. Scalebar = 200 μm.

parameter value: higher %TSS expresses a more significant influence on the output. The sign associated with the %TSS indicates the influence on the output variation: if positive, an increase in the parameter value results in an increase in the output value and vice versa. For each output, the four most influential parameters were selected according to the highest %TSS absolute value.

2.6 Genetic Algorithm to perform the model parameter calibration

Once the most significant parameters were identified by the sensitivity analysis, we calibrated them with the support of a Genetic Algorithm (GA) [McCall (58)]. A fitness function was generated employing data from experimental images, with the aim of reducing the quantitative differences between the *in silico* model and *ex vivo* immunofluorescence images. Numerical differences between experimental data (*e.g.* concentration of macrophages) and the corresponding quantitative output from the agent-based model of the cellular level were employed as the fitness function. The GA follows an evolutionary approach based on subsequent generations, aiming to minimize the fitness function. If a combination of parameter values did not reduce the function, it was removed by the algorithm in the following generation, allowing it to keep only the most promising ones. The selection of the most promising values and their cross-combination with the other components of the population minimized, generation after generation, the fitness function until a predetermined threshold was met. A more detailed explanation of the GA methodology employed to calibrate this model is reported in Supplementary Materials.

2.7 Model validation with an independent experimental dataset

Validation of the results was performed on a different dataset of experimental immunofluorescent images (n = 2), previously reported by Schlundt et al. (26). Differently from the dataset that was used for calibration, the model of the validation set is characterized by a smaller fracture gap size (0.7 mm), in a different bone (femur) from female mice. The mouse strain (C57BL/6) was analogous to our in-house experiment and the same immunofluorescent staining markers were used to investigate the macrophage distribution in *ex vivo* images. The model domain was adapted to match the dimensions of the validation experiment's bone and fracture gap. The biological parameter values obtained from the GA calibration process were validated by quantitatively comparing the macrophage populations concentrations simulated on this new domain and the ones measured from *ex vivo* immunofluorescent images. The success of the validation process supports the claim that confirms the assertion that the additional calibration step using data from *in vivo* experiments is important and leads to a more accurate representation of the inflammatory phase of fracture healing in murine long bones than when using parameter values derived from *in vitro* experiments reported in the literature.

2.8 Statistical analysis of the *in silico* results

Due to the involvement of the discrete agent-based framework, the multiscale model has a stochastic nature. The variability is shown by the mean and standard deviation of multiple repetitions (n = 5) of the simulation under the same investigative conditions and different initial random seeds. One-tailed student's T-test was performed to investigate the differences between the calibrated and non-calibrated models.

3 Results

3.1 *In silico* immunofluorescence with literature values

When *in vitro* experiments reported in the literature are used to parametrize the model, the simulation results show a concentration of macrophages within the healing region of 346.4 ± 9.3 mm^{-2} after 1 day, followed by an average increase of 12.7% between day 1 and day 3. Specifically, at day 1 the M0 concentration is 207.5 ± 8.1 mm^{-2}, the M1 concentration is 99.2 ± 7.4 mm^{-2} and the M2 concentration is 39.7 ± 7.1 mm^{-2}. As the inflammation progresses, the concentrations vary between day 1 and day 3: M0 decreases by 84.4 ± 4.1%, M1 and M2 increase 2.2-fold (± 0.3) and 3.2-fold (± 0.8) respectively (Figure 3A). At the molecular level, the cellular engulfment leads to a reduction in fracture debris over time, resulting in the complete clearance of debris from the healing region within 3 days (Figure 3B). Pro- and anti-inflammatory cytokines secreted by immune cells exhibit analogous dynamics throughout the onset of bone healing, though pro-inflammatory cytokine secretion is more intense during the early stage of healing (Figure 3C), followed by a delayed anti-inflammatory wave (Figures 3C, D).

3.2 Sensitivity analysis to evaluate the most influential parameters for *in silico* outputs

When considering the total macrophage concentration output, the ANOVA test revealed that the *in silico* model exhibited the highest sensitivity to the macrophage recruitment ratio ($k_{R(M0)}$) during the early stage of inflammation (%TSS = 46.9% at day 1, reduced to %TSS = 3.3% at day 3). In the later stage, it was observed that the initial concentration of PMNs ($[PMN]_0$) had the largest impact, although with a negative trend (%TSS = -37.5% at day 3). Additionally, the non-polarized macrophage proliferation ratio ($k_{p(M0)}$) influenced the results at day 1 (%TSS = 12.9%), while the pro-inflammatory macrophage proliferation ratio ($k_{p(M1)}$) had a greater effect on the output at day 3 (%TSS = 15.9%). Furthermore, the debris engulfment ratio associated with PMNs ($k_{e(PMN)}$) exhibited an influence on the predicted macrophage concentration at day 3, with a negative trend (%TSS = -13.4%). The complete list of %TSS

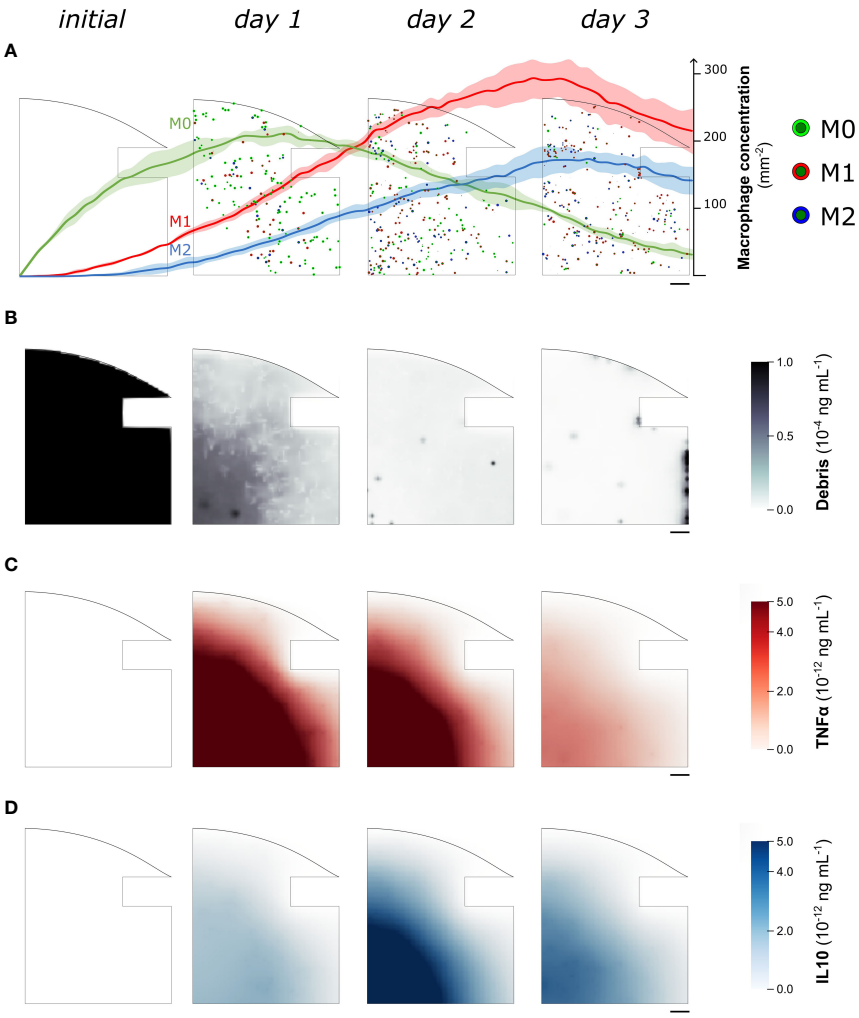

FIGURE 3
Representative images of the temporal evolution of the cellular level **(A)** and the molecular level **(B–D)** during the fracture healing progression. Model results were collected from one quarter of the healing callus every 24 hours since the fracture induction (initial). In **(A)** we superimposed the quantitative variation of macrophage concentration (mean ± standard deviation, n = 5) over the course of the healing process. Neutrophil population is not shown to improve readability. M0: non-polarized macrophages, M1: pro-inflammatory macrophages, M2: anti-inflammatory macrophages, TNFα: Tumor Necrosis Factor alpha, IL10: Interleukin 10. Scalebar = 100 μm.

associated with each parameter at day 1 and 3 is reported in Supplementary Table 3.

3.3 Genetic Algorithm to identify optimal parameter set

By minimizing the fitness function, defined as the difference in the macrophage concentration within the healing region between values obtained from computer simulations and experiments on day 3 postfracture, the GA identified the optimal combination of values for the most influential parameters at that time-point ($[PMN]_0$, $k_{R(M0)}$, $k_{p(M1)}$, $k_{e(PMN)}$). The algorithm converged after nine generations for the parameters (Figure 4), and it resulted in a clear tendency for higher macrophage proliferation rates ($k_{p(M1)}$) to better capture the experimental data (1.07 10^{-3} min^{-1}, +28.5% compared to literature value). Calibrated values for macrophage recruitment and neutrophil engulfment ratios showed smaller yet still considerable divergence from literature-based values ($k_{R(M0)}$ = 2.33 10^{-2} h^{-1}, +10.9%; $k_{e(PMN)}$ = 2.71 10^{-3} min^{-1}, -18.6%) and the initial PMN population tended to maintain the concentration value found in the literature ($[PMN]_0$ = 984.38 μm^{-3}, -1.6%). Throughout the iteration of the GA, the average difference between *in silico* output and *ex vivo* immunofluorescent image quantification decreased from 240.9 mm^{-2} to 107.1 mm^{-2}, resulting in a 56.5% reduction of the fitness function (Figure 4).

When the model was run with the optimized parameters, the M0 concentration peaked around day 1 (213.1 ± 17.4 mm^{-2}) and decreased with the progression of the inflammation (35.7 ± 6.5 mm^{-2} on day 3). Pro- and anti-inflammatory macrophage concentrations increased from day 1 (M1: 122.1 ± 14.6 mm^{-2}, M2: 43.2 ± 8.3 mm^{-2}) to day 3 (M1: 281.9 ± 26.6 mm^{-2}, M2: 140.0 ± 25.4 mm^{-2}) (Figure 5A). The M1 concentration showed a significant influence of the calibration on day 1 (p = 0.016) and

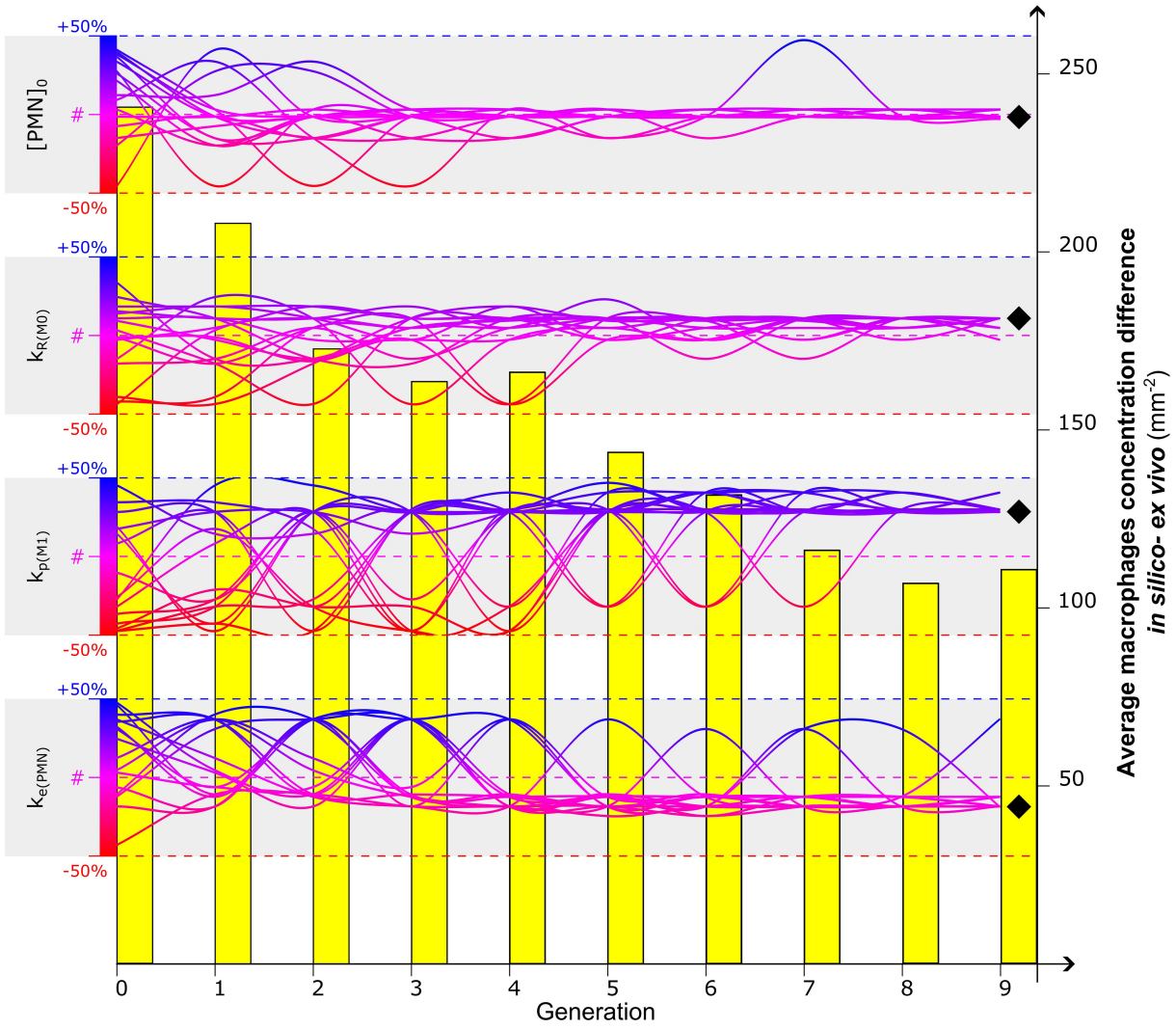

FIGURE 4
Calibration of the four most influential parameters ($[PMN]_0$, $k_{R(M0)}$, $k_{p(M1)}$, $k_{e(PMN)}$) to optimize the *in silico* predicted macrophage concentration at day 3. Evolution of a 16-sample population is represented by the gradient-colored lines: each sample represents a combination of values associated with the four parameters. The Genetic Algorithm is initialized at generation 0 by randomly associating to each parameter a value within the range of +/-50% of the values found in the literature (identified by #) (Supplementary Tables 1, 2). The dynamic evolution of the algorithm led the combination of the parameter to converge to values that better calibrate the model. Diverging bumps observed in the evolution of the lines are associated with mutations, singularities of the Genetic Algorithm to increase the investigative variability. After a full run of Genetic Algorithm (9 generations in this case), a value is identified for each parameter to calibrate the model (black diamond). The capacity of the Genetic Algorithm to minimize the fitness function is observed in the evolutionary reduction of the difference of macrophage concentration between *in silico* and *ex vivo* data (yellow bars). For additional details about the Genetic Algorithm, the reader is addressed to Supplementary Materials.

day 3 (p = 0.008), in contrast to both M0 and M2 concentrations where no significant influence was observed ($p > 0.05$).

When comparing the results of the model using literature-based values (Figure 3A) with those obtained with the calibrated model, we observed that the qualitative dynamics of macrophage concentration during the inflammation processes remained unaltered for all the subtypes. However, there was an increase in the number of cells within the healing region. The pro-inflammatory macrophage concentration in particular increased (+31.0%) due to the GA-driven increment of the proliferation ratio. This observation aligns with the fitness objective of the calibration to reduce the difference in macrophage concentration on day 3 between *ex vivo* immunofluorescence (518.8 ± 8.3 mm^{-2}, identified as CD68+ cells) and computer model results (non-calibrated: 389.3 ± 36.5 mm^{-2}; GA optimized: 457.6 ± 51.5 mm^{-2}, p = 0.033) (Figure 5B).

3.4 Validation of the calibration results with an alternative dataset

In the case of the 0.7 mm fracture dataset, we observed a decrease in macrophage concentration within the healing region. The *ex vivo* immunofluorescence data at day 3 showed a

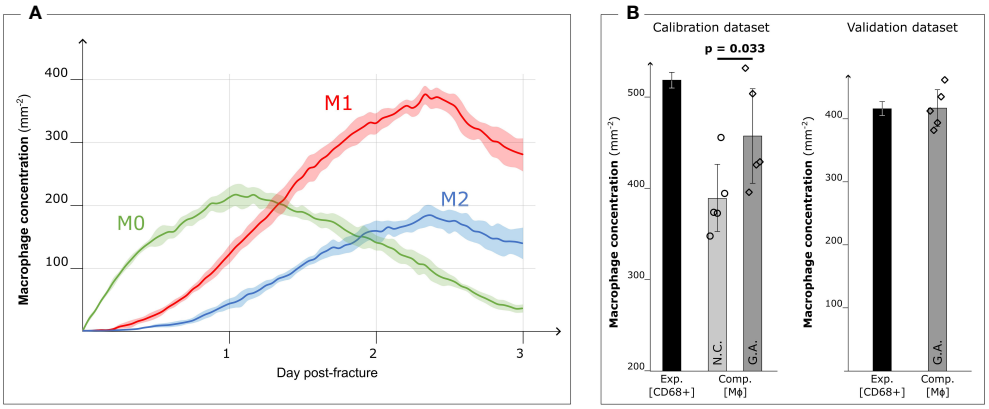

FIGURE 5
Results after Genetic Algorithm calibration and comparison with experimental data. **(A)** Dynamic variation of the concentration of the different macrophage types (M0: non-polarized macrophages, M1: pro-inflammatory macrophages, M2: anti-inflammatory macrophages, mean ± standard deviation, n = 5) over the course of the inflammation progression, when Genetic Algorithm calibrated parameters are used. **(B)** Comparison between the experimental immunofluorescence concentration of macrophages (Exp. [CD68+]) (black bars, mean ± standard deviation, n = 3 for calibration dataset, n = 2 for validation dataset) and computational predicted concentration of macrophages (Comp. [MΦ]) (gray bars, mean ± standard deviation, n = 5) with parameter based on literature data (Non-Calibrated, N.C.) or calibrated with Genetic Algorithm (G.A.). Scatter plots of the 5 results from the computer model are added to show the model stochasticity.

concentration of 414.0 ± 22.6 mm^{-2} (CD68+). This reduction in macrophage concentration is also predicted by the GA-calibrated *in silico* model for the 0.7 mm gap. Specifically, the *in silico* model predicted a concentration of 415.2 ± 28.8 mm^{-2} at day 3 post-fracture (Figure 5B).

4 Discussion

This manuscript presents an integrated *in silico-in vivo* pipeline for the development and calibration of a computational model capturing the early phase of fracture healing, called COMMBINI. By employing agent-based modeling, each biological cell is represented as a single discrete entity and not as an element of a dynamic continuous concentration, providing a novel perspective on the investigation of the early phase of the bone healing process. The agent-based model is designed with stochastic algorithms to faithfully reproduce the biological behavior of cells [Andrews et al. (59); Wehrens et al. (40); Allen et al. (41)]. However, COMMBINI also includes deterministic rules to investigate the processes that drive healing progression, such as chemotaxis. These deterministic rules are essential for introducing spatial information and preventing the agent-based model from generating a homogeneous environment. Chemotactic attraction is one of the deterministic factors promoting the directional migration of the immune cells within the healing region [Kolar et al. (6)]. Specifically, debris chemotaxis was observed to be essential to simulate the recruitment of the first macrophages from the bone marrow and surrounding tissues to the center of the fracture gap. The implementation of a spatio-regulated debris clearance rule to reproduce the natural behavior of macrophages [Gordon and Plüddemann (60); Westman et al. (61)] was necessary to complete the callus invasion, reducing the recruitment of further macrophages.

The molecular level has been simulated by solving diffusion-decay differential equations within a region that shares the coordinate system with the cellular level, following the approach in Borgiani et al. (43). The domain size has been chosen to fully include the healing domain and its spatial resolution has been adapted to create a sufficiently fine grid on which to solve the equations, avoiding to increase the computational costs. With the proposed resolution (10 μm), the molecular level is capable of adequately reproducing the cytokine dynamics without increasing the simulation time. Also, temporal resolution differs between the cellular and molecular levels. By following the in-code values proposed by BioFVM, the time resolution has been kept to the order of seconds to guarantee an accurate and smooth simulation of the molecular dynamics, with no detriment to computational performances. These settings have been based on previous benchmarks of the solver, where adequate accuracy has been obtained in diffusion-decay systems under the same temporal resolution utilized in this work [Ghaffarizadeh et al. (50)]. Furthermore, the overall timespan of the inflammatory stage is limited to few days and there is no necessity to use hour- or day-scale resolution to reduce the number of iterations, as in simulations of later stages of bone healing, which progresses through months [Borgiani et al. (43); Nasello et al. (62)]. In light of an eventual upscaling of the model to 3 dimensions, the spatial and temporal resolutions used in this study might be adapted after performing convergence analyses.

At the molecular level, the *in silico* model accurately simulates the transition from a pro-inflammatory to an anti-inflammatory environment, replicating the dynamic changes in the concentration of specialized inflammatory cytokines that occur during the initial phases of bone fracture healing [Maruyama et al. (13)]. Within the healing region, it is possible to observe a first pro-inflammatory wave of TNFα, with peak concentrations in the marginal regions during the first hours. This is followed by a progressive invasion of

the defect site as the inflammatory response progresses (Figure 3C). The IL10 concentration was more prominent in the healing region around 2 days post-operation (Figure 3D), generating an anti-inflammatory environment to extinguish the inflammatory response and progress to the following repair stage. Modulation of the duration of the pro- and anti-inflammatory phases is critical to avoid unnecessary extended inflammation, which may lead to chronicity [Loi et al. (9)]. Therefore, the multiscale computer model might be used to investigate the two-way interactions between the cellular and molecular levels to predict how regulations at the smaller scale can have spatial-related implications on larger scales. Exogenous provision of treatments can be implemented at the molecular level by simulating a user-defined concentration spike in the healing region within a defined spatio-temporal frame. Molecular therapeutics targeting the inflammatory response, such as non-steroidal anti-inflammatory drugs [Lisowska et al. (63)], could be preliminarily tested with COMMBINI to investigate their effect on enhancing bone healing at the cellular level.

The computer model parametrized with literature data predicted a lower macrophage concentration within the callus region when compared to experimental data. To improve the model predictions, we performed a sensitivity analysis on the model outputs, followed by a sensitivity analysis on the model outputs followed by optimization of the most influential parameters using a GA and experimental results from a dedicated *in vivo* experiment. The sensitivity analysis showed that the model was particularly sensitive to changes in the macrophage recruitment ratio during the initial stage of healing and to the macrophage proliferation constant in the later inflammation. This result follows the expected monotonic relationship between the recruitment and proliferation ratio values and the macrophage concentration within the healing region. The GA calibration with experimental results on day 3 post-operation confirmed that an increasing value of the macrophage proliferation ratio was necessary to reduce the difference in macrophage concentration between the *in silico* and experimental results. The literature data (Supplementary Tables 1, 2), which we used to originally parametrize the model, underestimates the capacity of macrophages to proliferate within the healing region. Specifically, the value assigned to macrophage proliferation ratio has been obtained from *in vitro* cellular assays of isolated mature macrophages [Chitu et al. (64)]. However, while performing *in vitro* experiments on macrophages is less challenging than *in vivo*, only these last provide more exhaustive information on the behavior of those cells [Luque-Martin et al. (65)]. A valid compromise might be the use of advanced *in vitro* models, as organ-on-chip, to generate the investigative environment that more closely resembles the inflammatory scenario [Wikswo (66); Zhang et al. (67)]. Additionally, increasing the range of the GA (beyond the current upper bound of 50% variation) and including additional targets beyond the general macrophage concentration (*e.g.* macrophage subtypes) could further enhance the calibration.

To ensure accurate alignment between the simulated and experimental results, we developed an *in silico* immunofluorescence pipeline. In the simulation results, each macrophage subtype is visualized with a specific color, corresponding to the fluorescent staining used for the corresponding macrophages observed in the immunofluorescent images of the experimental outcomes. The calibration of the model was performed by quantitatively comparing the macrophage concentration inside a user-defined ROI on both *in silico* and experimental immunofluorescent images. The same procedure was employed to validate the model results with a second set of immunofluorescence images obtained from an independent experiment performed in murine femurs with a 0.7 mm osteotomy, collected at day 3 post-operation. The *in silico* fracture geometry was adapted to the validation dataset by reducing the dimension within the callus domain, while the model itself remained unaltered. Similar to the trend observed from experimental images, the *in silico* model predicted a reduced macrophage concentration for the smaller fracture gap. The validation data set was smaller than the calibration data set ($n = 2$) but we deemed it sufficient for the purpose of this proof of concept study where the focus is on the model development, calibration and the use of *in silico* immunofluorescence. In follow-up studies, when additional features will be added to the model (*e.g.* third spatial dimension, influence of mechanical loading), dedicated validation experiments will be run with sufficient power, including additional time points and spatial information to validate all aspects of the cellular and intracellular dynamics. Additionally, while the original parameter set used to calibrate the model was obtained from a male mouse population, the validation was performed in female animals. Macrophage characteristics in mice have been observed to be diverse between males and females [Chen et al. (68); Varghese et al. (69)]. Nevertheless, no obvious sex-specific influences were detected between the calibration and validation phase, though this might be due also to other potentially influencing factors such as age and strain. In this study, we have developed the model to capture normal healing in healthy adult mice. Its behavior when simulating other (patho)physiological states (ageing, disease-associated alterations or genetic modification), will be the subject of follow-up studies.

The model presented in this work aims to fill a wide gap in the *in silico* skeletal modeling field. While most of the state-of-the-art models limit their analysis to the later stages of bone fracture healing (repair and remodeling) [Ghiasi et al. (70); Borgiani et al. (35)], COMMBINI provides a new perspective on the role of the immune response in supporting and guiding bone healing during the first hours and days post-injury. The project's overall aim is to build a mechano-biological environment that can simulate how changes at the molecular level (*e.g.* administration of exogenous pro-/anti-inflammatory cytokine) and the cellular level (*e.g.* specialized macrophage colonies seeded on a scaffold) might affect bone tissue regeneration. To date, COMMBINI includes only the biological regulators of the inflammatory phase. Future work will include the role of mechanical loading (*e.g.* from gait) on the regulation of the biological processes as it is well known that macrophages are mechanosensitive cells [Li et al. (71)]. The inclusion of the mechanical loading will add another source of (spatial) variation in the model, which might allow to capture the spatially non-uniform distribution in macrophage subtype observed

experimentally [Stefanowski et al. (72)]. Additional limitations that will be included in future iterations of COMMBINI are the inclusion of cytokine chemotaxis [Edderkaoui (73)] and further refinement of the multiscale regulations of the macrophage population dynamics related to the development of the natural pro- and anti-inflammatory environment [Schlundt et al. (26); McCauley et al. (74); Frade et al. (75)]. Moreover, to limit the computational complexity of the current model, COMMBINI excludes the investigation of adaptive immune cells. Adaptive response plays a role in the late inflammatory stage and, therefore, its regulation is relevant for the subsequent regeneration stages [Baht et al. (10); Bucher et al. (76)]. The inclusion of additional macrophage subsets (*e.g.* M2 subsets: M2a, M2b, M2c, M2d) and lymphocytes could increase granularity at the cellular level and it is a possible route to cover also the subsequent repair and remodeling stages with this model [Bucher et al. (17); Gharavi et al. (77); Nikovics et al. (78)].

The model will be extended to include the transition into the early repair stage of bone healing, characterized by skeletal tissue formation. The addition of specialized cells (*e.g.* skeletal progenitor cells, osteoblasts, endothelial cells) will simulate the progression from the inflammatory to the repair stage and the revascularization within the healing region. Finally, the current simulation version of the model has been executed in 2D which is a choice made in relation to compute costs and the calibration/validation data available. In order to validate the 3-dimensional version of the model, 3D imaging techniques or reconstruction of stacked 2D slices will be required.

With the presented model, we developed a calibrated tool to investigate bone fracture healing progression starting from the initial inflammatory stage. To date, COMMBINI can simulate the natural innate immune response progression but will integrate the role of external interferences in the future. We believe that the *in silico* approach could favor a novel predictive strategy to plan adequate therapeutical strategies before surgical intervention when disruptive mechano-biological conditions occur (*e.g.* wide segmental defect, chronic inflammation). Furthermore, due to its multiscale nature, the model will be able to include alteration of the tissue, cell or molecular environment related to skeletal diseases. Osteomyelitis is a bacterial infection of the bone that might occur in case of open fracture [Slyamova et al. (79)]. The computational model can be integrated with the bacterial population and antibiotic treatment provision to investigate the role of the treatment on the infection and its influence on the natural development of the inflammatory response. The possibility of predicting the quantitative and qualitative outcome of the treatment strategy before its practical application will assist the operator in choosing the optimal path to follow, especially in case of challenging scenarios. For example, the impact of scaffolding the fracture with smart biomaterials, which sense environmental stimuli and respond accordingly, can be evaluated *in silico* with this model. The COMMBINI project fits well in the new trend of *in silico* trials [Pappalardo et al. (80); Viceconti et al. (81)] where validated computer models are employed to better inform or augment traditional *in vitro* and *in vivo* (animal and human) studies during the development of new therapeutic strategies.

5 Conclusions

With COMMBINI we developed a multiscale integrated *in silico* model for the study of the early inflammatory stage of bone fracture healing. An original approach with *in silico* immunofluorescence was presented and employed to calibrate the model with data from *in vivo* experiments. The calibration with a GA showed that *in vitro* models could not fully capture the macrophage proliferation process during bone healing inflammation. The validation with data from an independent experiment demonstrated the capacity of COMMBINI to capture the essential biological elements at play during the inflammatory phase of bone healing.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

Author contributions

EB and LGe conceptualized and designed the study; EB and GN developed the computer model and the *in silico* immunofluorescence; GN, LO and TH designed the *in vivo* study that was used to calibrate the model; GN, LO, TH, and LGr performed the surgeries on the animals and collected the bone samples; EB and LO performed immunofluorescence on bone samples; EB developed the code to run the sensitivity analysis and Genetic Algorithm calibration; CHB and KS-B provided the experimental data for the validation set. All the authors helped with the analysis of the experimental results. EB wrote the first draft of the manuscript and all the other authors contributed to the article. All authors approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1231329/full#supplementary-material

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Temporal dynamics of immune-stromal cell interactions in fracture healing

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Bone fracture repair is a complex, multi-step process that involves communication between immune and stromal cells to coordinate the repair and regeneration of damaged tissue. In the US, 10% of all bone fractures do not heal properly without intervention, resulting in non-union. Complications from non-union fractures are physically and financially debilitating. We now appreciate the important role that immune cells play in tissue repair, and the necessity of the inflammatory response in initiating healing after skeletal trauma. The temporal dynamics of immune and stromal cell populations have been well characterized across the stages of fracture healing. Recent studies have begun to untangle the intricate mechanisms driving the immune response during normal or atypical, delayed healing. Various *in vivo* models of fracture healing, including genetic knockouts, as well as *in vitro* models of the fracture callus, have been implemented to enable experimental manipulation of the heterogeneous cellular environment. The goals of this review are to (1): summarize our current understanding of immune cell involvement in fracture healing (2); describe state-of-the art approaches to study inflammatory cells in fracture healing, including computational and *in vitro* models; and (3) identify gaps in our knowledge concerning immune-stromal crosstalk during bone healing.

KEYWORDS

fracture healing, osteoimmunology, inflammation, bone, crosstalk

Introduction

Unlike most tissues in the body, bone has the unique ability to regenerate - this process is dependent on carefully orchestrated crosstalk between immune and stromal cells. Although the term 'osteoimmunology' was coined over twenty years ago to describe the role of immune cells in normal and pathological bone remodeling, there is much that remains unknown about mechanisms guiding immune-stromal cell interactions during the process of bone repair (1). With 600,000 yearly cases of malunion or non-union fractures in the US, there is a

critical need to understand both restorative and detrimental properties of immune-stromal crosstalk during the fracture healing response (2).

Overview of fracture healing

Fracture repair involves recruitment of immune cells in a temporal and spatial manner that influences the proliferation and differentiation of stromal cells. During the initial stages of long bone callus formation, a fracture hematoma forms, followed by inflammation and stromal progenitor cell recruitment as illustrated in Figure 1. Bone formation occurs next via direct, osteoblast-mediated mechanisms (intramembranous ossification) and via indirect, chondrocyte-mediated mechanisms (endochondral ossification) (3). The majority of pre-clinical fracture studies occur in rodents due to feasibility, reproducibility, and similarities in dynamics of fracture healing to that of humans (4).

Hematoma formation and inflammatory phase

This phase occurs over the first 1-5 days post fracture in humans (5, 6).

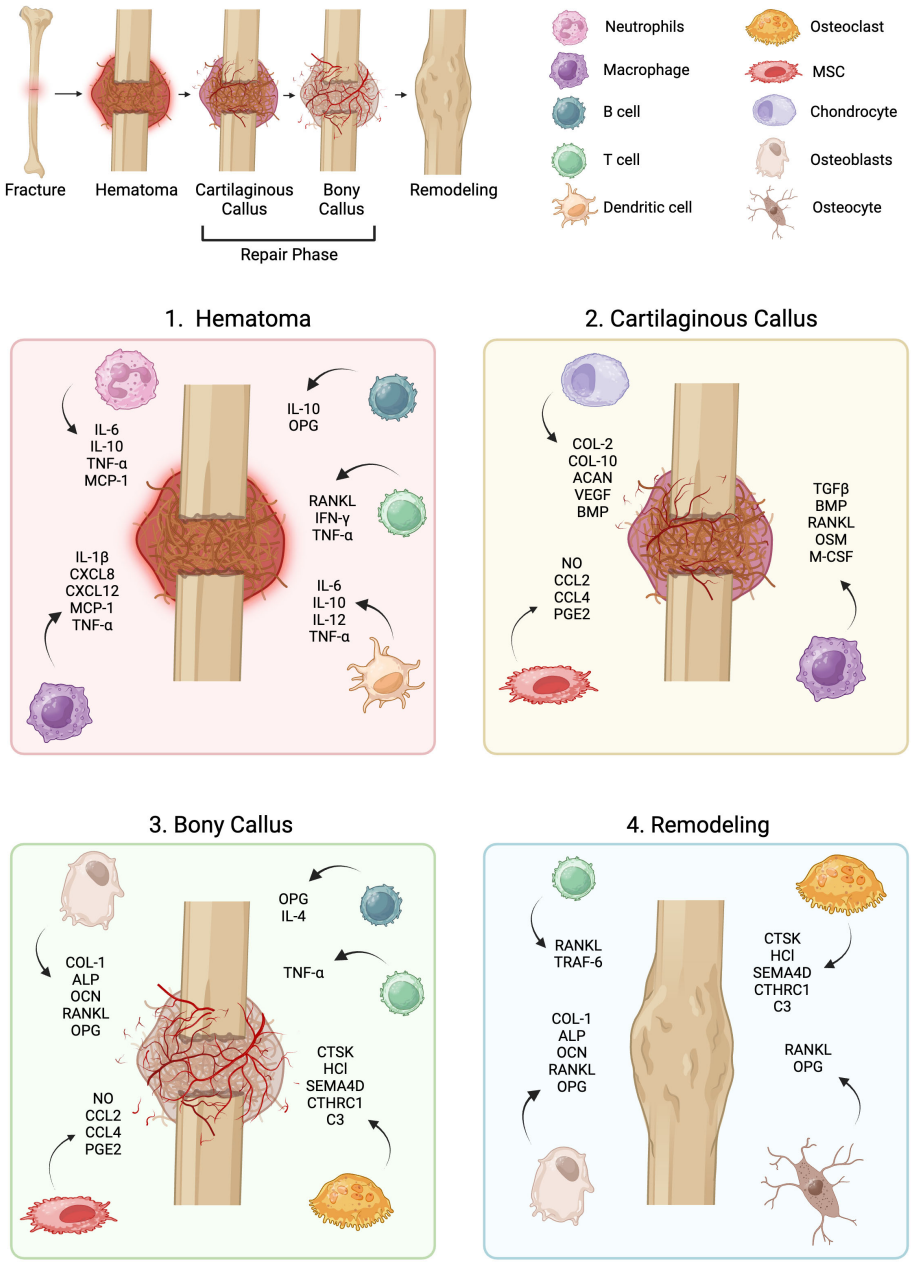

FIGURE 1
Overview of fracture repair. Fracture repair occurs across distinct phases, each of which involves dynamic stromal-immune cell interactions: the hematoma phase, repair phase, and remodeling phase. MSC, mesenchymal stromal cell.

Neutrophils

In the first 24 hours of fracture healing, a hematoma forms and is infiltrated by granulocytic cells (predominantly neutrophils) that act as 'first responders' (7, 8). These cells recruit monocytes via secretion of cytokines like interleukins (IL-) 1, 6, and 10; tumor necrosis factor alpha (TNF-α); and monocyte chemoattractant protein 1 (MCP-1) (9–14). Neutrophils have also been implicated in contributing to the initial fibrin-rich clot. Within 48 hours of fracture, neutrophils make up the vast majority of cells present at the injury site and synthesize a fibronectin-containing extracellular matrix (ECM) (15). Fibronectin binds fibrin and provides binding sites for other ECM proteins, cells, and growth factors (16). Neutrophil depletion by anti-Ly6G antibody treatment impairs fracture healing, highlighting the essential role of neutrophils in the early inflammatory response (17). While neutrophil infiltration is key to the formation of the hematoma, sustained neutrophil activation leads to diminished osteogenic activity, reduced callus mineralization, and impaired/delayed healing (18, 19).

Monocytes/macrophages/dendritic cells

Upon recruitment, systemically-derived monocytes differentiate into macrophages and dendritic cells. Dendritic cells are present during the early phases of fracture healing, and express inflammatory cytokines (IL-6, IL-12, TNF-a, IL-10) (20–24). Furthermore, CD8+ dendritic cells are known to stimulate CD8+ T cells (20). Early on, macrophages remove cellular debris and secrete inflammatory cytokines including IL-1, TNF-α, IL-6, chemokine (C-X-C motif) ligand (CXCL) 8, CXCL12, and MCP-1 (25–27). Macrophage polarization occurs along a spectrum but is often simplified into 3 subclasses: a naïve, pro-inflammatory, or pro-regenerative phenotype. While macrophages are present throughout the healing process, macrophage depletion studies have identified that their presence is most critical in the immediate aftermath of injury during the pro-inflammatory phase (28–31). Polarized macrophages have been shown to exhibit plasticity in their ability to revert back to a naïve resting state *in vitro* (32) and through predictive modeling (33). Inflammatory macrophages demonstrate reduced inducible nitric oxide synthase (iNOS) signaling as time progresses after pro-inflammatory stimulation, eventually returning to a naïve state. While this observation may hold for inflammatory macrophages in tissue repair, it has yet to be described in the context of fracture healing. Macrophage-derived cytokines IL-1β and TNF-α also stimulate fibroblast proliferation within the fracture callus (34). Some studies posit that cytokines, such as TNF-α, secreted by pro-inflammatory macrophages, induce bone morphogenetic protein (BMP) 2, the transcription factor RUNX2, and expression of alkaline phosphatase in mesenchymal stromal cells (MSC) (35, 36). However, other studies suggest that later pro-regenerative macrophages secrete BMP2 and oncostatin M (OSM) to promote ECM mineralization, underscoring the importance of temporal dynamics in fracture healing (37, 38). It has also been demonstrated that during this initial phase pro-inflammatory macrophages secrete vascular endothelial growth factor (VEGF) to stimulate neovascularization. As the pro-inflammatory to pro-regenerative shift occurs, pro-regenerative macrophages secrete platelet-derived growth factor (PDGF) (39). Importantly, although the acute inflammatory phase following hematoma formation is critical for fracture healing, chronic inflammation and persistence of pro-inflammatory macrophages impairs fracture healing (40, 41).

Natural killer cells

Little is known about the function of natural killer (NK) cells during fracture repair; however, it is hypothesized that they likely assist in debridement of the fracture callus and recruit macrophages to the injury site (9). Early work suggested that NK cell activity was suppressed in fracture patients; whereas recent studies indicate an important role for NK cells in MSC recruitment to the fracture site through neutrophil activating peptide 2 secretion, and in regulation of osteoclastogenesis (42–44). Different classes of NK cells regulate progenitor cell survival during digit tip regeneration that may be comparable to events during fracture healing (45). NK cells also show interdependency with MSC, where MSC secretion of IL-10, transforming growth factor beta (TGF-β), and prostaglandin E2 (PGE2), has been linked to suppression of NK cells (46–48).

Lymphocytes

Lymphocytes arrive as the initial inflammatory phase wanes. T cells express the pro-osteoclastogenic cytokine, receptor activator of nuclear factor κB ligand (RANKL), whereas B cells express osteoprotegerin (OPG), which blocks RANKL activity, inhibiting osteoclastogenesis (49). Spatio-temporal studies of T and B cells in fracture healing have established increased T cells in the bone marrow immediately after injury, with a significant increase in CD4+ T cells compared to CD8+ T cells. Following this initial spike in T and B cells, they retreat from the injury site, reappearing later during bone formation and remodeling (49). Notably, Reinke et al. determined that CD8+ T cells release interferon ɣ (IFN-ɣ) and TNF-α, and that their persistence throughout the fracture repair process greatly impairs osteoblast differentiation and healing (50). To prevent this, IgM+ CD27+ regulatory B cells release IL-10, suppressing IFN-ɣ, TNF-α, and IL-2 signals from CD8+ T cells to promote resolution of the inflammatory response (51).

Repair phase

The repair phase occurs between 5 and 21 days in humans and consists of the formation of a cartilaginous soft callus that then converts to a hard bony callus (5). During the repair phase, bone will heal by endochondral ossification, where it goes through a cartilaginous intermediate, or direct intramembranous ossification where MSC differentiate into osteoblasts and deposit a mineralized

ECM (31, 52). Both processes are necessary for fracture repair, however the amount that each contributes to healing depends on fracture stabilization and mechanical forces (53). During the soft and hard callus phases, MSC, chondrocytes, osteoblasts, macrophages, osteoclasts, T cells, and B cells are the dominant cell populations (5, 9).

Macrophages/osteoclasts

Bone-resident macrophages regulate bone formation and play a key role in MSC differentiation. Activated macrophages release the cytokines TGFβ, BMP, and OSM to induce MSC differentiation (54). Chang et al. coined the term 'osteomacs' to define a discrete F4/80^{pos} Mac-2$^{neg/lo}$ TRACP^{neg} macrophage population found on the periosteum and endosteum lining the bone (55, 56). Osteomacs promote intramembranous ossification and have been shown to exert control over osteoblast maintenance. Within calvarial cultures, the removal of osteomacs results in decreased mineralization, reduced osteocalcin (OCN) induction, and a limited TNF-α response to LPS, demonstrating an integral role in bone homeostasis and osteoblast function (55, 57). Studies have further demonstrated the importance of the osteomac population in a murine tibia fracture model, where depletion resulted in decreased bone formation (56). During the latter part of the repair phase, inflammatory macrophages, described as F4/80^{pos} Mac-2^{pos} TRACP^{neg} differentiate into osteoclasts through macrophage colony-stimulating factor (M-CSF) and RANKL signaling (56). Osteoclasts can induce osteoblast differentiation through secretion of soluble factors like including collagen triple helix repeat-containing protein 1 (CTHRC1) and complement component C (C3) (58, 59). In contrast to osteomac depletion, depletion of osteoclasts, which resorb cartilaginous ECM through catabolic activity, did not impair bone formation (56). Notably an MSC-derived population of septoclasts have also been recently implicated in cartilage resorption during fracture healing as well as developmental ossification, potentially augmenting this activity. However, septoclast importance in bone remodeling post-fracture is still under investigation (60).

Lymphocytes

During fracture repair, T and B cells infiltrate the fracture site and assist in osteoblast maturation and retention. In this 'second-wave' lymphocytes are absent from the cartilaginous regions of the fracture callus, however they are present near the regions of woven bone (49). Konnecke et al. reported that B cells maintain bone homeostasis through the production of OPG to reduce osteoclastogenesis, and physically interact with osteoblasts to influence their differentiation and function (49). Numerous studies have likewise described T cells as critical for fracture repair (61–65). T cells secrete TNF-α to induce osteogenesis and are necessary for normal deposition of collagen I by osteoblasts during fracture healing (61). T cell depletion further exhibited similar premature mineral deposition as seen in Rag1-deficient mice (which lack mature lymphocytes), pointing toward a T cell-osteoblast interaction pathway (61).

MSC/chondrocytes/osteoblasts

MSC derive from various sources including the periosteum and bone marrow (66). In the healing callus they begin to differentiate into chondrocytes and osteoblasts. MSC modulate the immune environment by secreting regulatory molecules including nitric oxide (NO) (67), chemokine ligand (CCL) 2 and 4, and PGE2, to recruit macrophages which trigger MSC chondrogenic and osteogenic differentiation (54, 68, 69). Current literature suggests that skeletal MSC derive from multiple sources including the periosteum, endosteum, bone marrow, and vasculature (66). Periosteal-derived MSC at the callus edges have increased osteoblastogenic potential and undergo intramembranous ossification, secreting collagen 1 (COL-1), OCN and alkaline phosphatase (ALP) (70). On the other hand, bone marrow-derived MSC at the fracture site are more predisposed toward endochondral ossification, depositing collagens 2 (COL-2) and 10 (COL-10) as well as sulfated glycosaminoglycans such as aggrecan (ACAN) (10, 71, 72). Under injury conditions, periosteal-derived MSC have also been shown to contribute to endochondral ossification (71). During this process, the cartilaginous callus begins to stimulate vascular infiltration as hypertrophic chondrocytes secrete angiogenic factors VEGF (73), PDGF (74), and placental growth factor (PGF) (75). Vascular infiltration has been demonstrated to be crucial for the replacement of the cartilaginous callus by bone (76). Although immune-derived cues may direct MSC differentiation pathways, recognized contributors to this spatial phenomenon of MSC becoming either osteoblasts or chondrocytes are mechanical cues and hypoxia (40, 77, 78).

Remodeling phase

This phase typically takes around 18 weeks but can last for up to 1 year under typical fracture healing conditions in humans (5, 79). During fracture remodeling, the initial fracture callus is replaced with mature mineralized tissue and normal bone structure is restored. This coordinated response to injury is the last stage of fracture repair and is the longest, and the least well-studied (80). During the remodeling phase, inflammatory cells (other than osteoclasts) are dramatically reduced, and remodeling is driven by continuous local and systemic cell signaling (81). Bone remodeling occurs as a function of the stresses that bone receives due to forces acting upon it, including muscle actions (82, 83). The ability of bone to remodel post-fracture declines with age in humans. Indeed, children are more likely than adults to experience overgrowth of mineralized tissue, resulting in ectopic bone formation (84). Studies in mice have corroborated the age-related decline in fracture healing potential in humans, showing significant delays in bone remodeling and decreased bone recovery in elderly mice post-fracture (85, 86).

Osteoclasts

Although osteoclast activity is present early on in fracture repair, it is most prominent in the remodeling phase (87). Osteoclasts work in a balance with osteoblasts and osteocytes to first degrade immature woven bone which is then replaced with more mature bone. Osteoclasts create a reversal zone where the bone surface is eroded, leaving a canopy where osteoprogenitors are found. The basic multicellular unit -an assembly of osteoblasts, osteoclasts, and capillaries- is a prominent hallmark of bone remodeling (81, 88). Osteoclast differentiation is positively regulated by RANKL signaling and negatively regulated by OPG (89). Osteoclasts dissolve bone through secretion of cathepsin K (CTSK) and hydrochloric acid, and degrade ECM via secreted matrix metalloproteinases (90, 91).

Osteoprogenitors/osteoblasts

MSC differentiate into osteoblasts, which deposit mineral in equilibrium with osteoclast activity (21). Osteoprogenitors and osteoblasts constitute the canopy around blood vessels, serving as the main source of cells contributing to bone formation. A bone remodeling compartment forms near capillaries and sinusoids, providing access to osteoprogenitors including bone lining cells and pericytes (88). Pericytes encircle capillaries, however evidence suggests that these pericytes can migrate to the bone surface and differentiate into mature osteoblasts (92, 93). Osteoblasts secrete RANKL and OPG to modulate osteoclastogenesis (94).

Lymphocytes

T cells regulate osteoblast-osteoclast equilibrium by secretion of RANKL (95). Although T cell expression of RANKL may drive osteoclastogenesis during bone remodeling, T cells also drive degradation of TNF receptor associated factor 6 (TRAF6), acting as a negative feedback mechanism for osteoclast activity (96).

Osteocytes

Osteocytes make up 90% of healthy adult bone and function in response to changes in their microenvironment, such as mechanical deformation, to initiate remodeling responses via RANKL and OPG production (97).

Fracture modeling approaches

The mechanisms by which immune and stromal cells orchestrate fracture repair are not fully understood. To interrogate these complex biological interactions, various models of fracture healing have been developed. Herein follows an overview of models of *in vivo* fracture healing, *in vitro* fracture models, and computational models, to replicate both typical and impaired fracture healing.

In vivo murine fracture model

Animal models most faithfully recapitulate the physiological environment and allow for manipulation of cell responses through genetic knockouts and pharmacological or environmental intervention. Selective ablation of immune cell types in mice has contributed heavily to our understanding of the immune system in fracture healing. Fracture models of comorbidities illustrating immune disruption in fracture healing has been thoroughly reviewed (98–100). Numerous studies have utilized transgenic cre drivers such as LysM-Cre, Mrp8-Cre, and Lck-Cre, as well as Macrophage-Fas Induced Apoptosis (MAFIA) mice to generate immune cell-type specific targeting (101–111). Closed long bone fractures in rodent models are often employed to study fracture healing (112). Factors such as age, ischemia, osteoporosis, and immune deficiency are then incorporated to examine causes of impaired healing (57, 98, 113–117). Fracture in aged populations exhibit increased pro-inflammatory macrophage recruitment as well as increased apoptotic markers in human (118) and mouse (119) systems. Lopez et al. demonstrated that anti-inflammatory modulation of the aged fracture rescues callus formation and healing in aged mice (119). The ischemic fracture model exhibits distinctly smaller callus formation and increased fibrosis (114). Ovariectomy produces postmenopausal osteoporosis in mice, leading to chronic inflammation and increased catabolic activity within bone. Fracture following ovariectomy demonstrates delayed callus mineralization, and remodeling (120, 121). Macrophage populations also exhibit increased IFN-ɣ, nitric oxide, and IL-6 expression (57, 122). Interestingly, MSC isolated from osteoporotic patients do not have impaired potential to regenerate bone, emphasizing the critical role of the immune environment in vivo (123). Multiple studies have revealed that fracture healing is greatly impaired in immunodeficient mice, underscoring the necessity of the immune response in fracture repair (101, 124). While the importance of the innate immune system is indisputable, studies have contested the importance of the adaptive response; Toben et al. demonstrated that eradication of the adaptive immune response using *RAG1*$^{-/-}$ mice accelerated fracture healing and improved bone quality (125). However others have stressed the immunoregulatory importance of adaptive immune cells (particularly T cells) in guiding the repair response and enabling osteoblast activity (63, 126). This emphasizes the complexity of the immune response in fracture repair and the necessity for diverse models to better dissect these pathways.

In vitro fracture callus

While the gold standard of preclinical studies is animal models, these models may have limited transferability due to differences in timeline, physiologic structure, pharmacologic response, and variation in specific gene pathways across species, supporting the

need for *in vitro* models using human cells and tissues to complement animal work (4, 127, 128). *In vitro* models have been developed over the past decade to create a more physiologically-relevant system for studying human fracture. Along with reducing the number of animals necessary to carry out fracture research, the use of human cells carries additional translational transferability. Pfeiffenberger et al. extensively developed a human-based fracture gap model to interrogate immune-stromal crosstalk *in vitro* (128, 129). While other models, in particular co-culture models (130), focus on later stages of regeneration, this approach uses coagulation of human peripheral blood and MSC to model hematoma development and its progression through fracture repair (129). The hematoma is combined with scaffold-free bone-like constructs made from mesenchymal condensation and allows for manipulation of molecular and environmental cues such as oxygen availability. Hoff et al. developed a human hematoma model using tissue from total hip arthroplasties to monitor and characterize the immune response under bioenergetically-controlled conditions. Cells were exposed to hypoxia with limited nutrients, generating an inflammatory response representative of that seen in fracture after the first 24 hours (131). Increased vascular endothelial growth factor and IL-8 secretion under hypoxia in this model resulted in a decreased granulocytes and increased lymphocytes, as seen *in vivo* (131). Sridharan et al. investigated the interaction of MSC and macrophages in different collagen scaffolds functionalized with hydroxyapatite particles of varying shapes and sizes (132). This emphasized the ability of microenvironmental stimuli to modulate the immune system and presents a unique opportunity to study these interactions in a cell-specific manner. The hydroxyapatite scaffold polarized macrophages toward a pro- or anti-inflammatory phenotype depending upon changes in scaffold particle size and shape, and the authors also demonstrated that macrophage presence increased osteogenesis. Importantly, these studies demonstrate comparable results from an *in vitro* human hematoma model with that shown *in vivo*. *In vitro* models present a powerful tool to understand discrete mechanisms of fracture healing selective to specific cell populations.

In silico fracture modeling

Only recently has computational modeling of fracture healing incorporated intrinsic and extrinsic effects of the immune system, to ascertain their influence on mechanical and biological properties of the callus (133, 134). Computational models are a powerful complementary tool for guiding hypotheses when integrated with *in vivo* and *in vitro* experiments. State-of-the-art *in silico* models encompass continuous, discrete, or hybrid models to interrogate the complex spatiotemporal aspects of fracture healing. No model can holistically capture these processes; however, a corpus of literature is available that aims to help researchers build their own *in silico* models to study the spatiotemporal effects of the immune response on fracture repair (133). Continuous models function at the tissue and cellular level; these models use partial differential equations to create a continuous overview of a given scenario to study inflammation, bone mechanics, and bone repair. Discrete models study specific individual behaviors at the subcellular level, using agent-based approaches or cellular automata models to understand mechanistic processes in response to their environments (133).

Hybrid models aim to bridge the gap from subcellular mechanisms to the tissue. According to Lafuente-Gracia et al., to address the physiologic processes of the inflammatory response, a compartment model is required, where each compartment is assigned its own equation and set of agents (molecules or cells) and transitions (biological processes like phagocytosis or differentiation) between compartments (133).

Kojouharov et al. developed a mathematical model of the early inflammatory response in fracture healing using nonlinear ordinary differential equations (135). It was then elaborated on further in subsequent papers to consider unactivated (M0), classically activated (M1) and alternatively activated (M2) macrophages as separate variables (136) as well as migration due to molecular factors (137). This study was one of the first to incorporate both the primary hematoma formation and the inflammatory response, by identifying the primary entities involved in the early fracture - bone debris, pro- and anti-inflammatory cytokines, macrophages, MSC and osteoblasts. Informing the computational model with the known progression from fracture hematoma to cartilaginous fracture callus to repair, the authors developed a model for differentiation and cytokine production that includes known events such as initial MSC density, debridement rate, proliferation rate, and synthesis of cartilage and bone (135, 136). The model also maintains assumptions such as the inability of M1 and M2 to dedifferentiate back to M0 (136). This model provides an instrument for studying normal and impaired fracture repair, and for extrapolating mechanistic pathways that may otherwise be overlooked and could be adapted clinically to infer the effects of pharmacologics on fracture repair. This group has most recently extended their model to study the direct effects of phagocytes and inflammatory cytokines on macrophage and MSC cell migration during the initial inflammatory and repair phases (137).

Ghiasi et al. also developed a computational model of human fracture with a specific emphasis on the initial inflammatory stage of fracture healing, however they approached it from a mechanobiology perspective (138). This model employs a finite element-based approach that simulates the processes of fracture healing, and the entities present, such as MSC and debris. Both the Kojouharov and Ghiasi models incorporate initial fracture size and cellular density; however, Kojouharov et al. placed emphasis on the cytokines released, while Ghiasi et al. emphasized the Young's modulus of the granulation tissue along with stresses and mechanical responses that shape hematoma formation and influence callus formation (135, 136, 138).

Most recently, Borgiani et al. developed the COMMBINI model, an agent-based computational model to understand macrophage dynamics that occur during the early inflammatory phase (up to 5 days post-injury) (139). This model utilizes deep-learning algorithms on immunofluorescent stained slides to generate spatial information about different macrophage populations. It uniquely addresses phenotype-specific cell activities (eg. cell proliferation, migration, phagocytosis, apoptosis) and incorporates polarization and cytokine signaling. While the

COMMBINI includes neutrophils, the focus of the model is on macrophages, subdivided into categories M0, M1, and M2. To understand the inflammatory phase of fracture repair in guiding healing, the model focuses on expression of key pro-inflammatory and pro-regenerative cytokines like TNFα, IL10, TGFβ, and IFNγ (139). While valuable and an important primary step, the field recognizes that macrophages exist on a spectrum of functionality, more nuanced than discrete M0, M1, or M2 states.

Discussion

Fracture repair is a complex process orchestrated by immune and stromal cells to regenerate bone tissue. Inducing ischemia results in aberrant repair and regeneration of tissues, underscoring the importance of systemic immune cells to guide healing (140, 141). Studies involving the effect of limb ischemia on fracture healing date back to the 1960s and while the importance of the immune response during fracture repair is well acknowledged, immune alterations in fracture healing under ischemic conditions remain unclear (142). This is true for other impaired healing conditions as well, for instance in aged models or diabetic models where there is increased systemic inflammation. Numerous methods for inducing and modulating fracture repair have been developed to study tissue healing and remodeling *in vivo* – including the integral roles of immune cells. *In vitro* systems allow for the study of mechanisms in discrete phases and specific cell interactions, with the advantage of utilizing human cells. Computational models enhance our study of fracture healing by expanding upon our understanding of networks underlying the fracture microenvironment and simulating the healing response. Importantly, they serve as a tool to study pharmacologic intervention in fracture repair, in conjunction with *in vivo* and *in vitro* models. Used together, these models provide a powerful and holistic approach for interrogating immune dynamics and mechanisms in normal and impaired fracture healing, and will continue to evolve and incorporate more complex variables.

Author contributions

CC: Conceptualization, Writing – original draft, Writing – review & editing. KH: Conceptualization, Writing – review & editing. AK: Conceptualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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