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Macrophage Depletion Reduces Bone Loss and Alters Inflammatory Responses: A Mouse Peri-Implantitis Model

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ABSTRACT

Aim: To evaluate the impact of macrophage depletion on bone loss and inflammatory responses in a mouse model of peri-implantitis, assessing macrophage depletion potential as a therapeutic strategy.

Materials and Methods: Using 6-week-old male C57BL/6 mice, peri-implantitis was induced by placing a silk ligature around osteointegrated dental implants. Mice were divided into three groups: Healthy control (Healthy group); peri-implantitis with liposomal phosphate-buffered saline (PBS group); and peri-implantitis with liposomal clodronate for macrophage depletion (CLOD group). Two weeks after ligature placement, micro-CT, histological and real-time PCR analyses were performed to assess bone density, leukocyte infiltration and cytokine levels.

Results: The CLOD group showed a significantly higher bone-to-implant contact (74%) and bone volume relative to total volume (79%) compared to the PBS group (53% and 54%, respectively) and Healthy group (64% and 66%, respectively). Histological analysis revealed significantly reduced leukocyte and macrophage counts in the CLOD group. Additionally, TNF-α and IL-10 levels were significantly decreased in the CLOD group compared with the PBS group.

Conclusion: Macrophage depletion effectively reduces bone loss and inflammation in peri-implantitis. This study highlights targeting macrophages as a promising approach for managing peri-implantitis, although further research is needed to optimize therapeutic strategies.

1 | Introduction

Peri-implantitis poses a significant challenge in contemporary implant dentistry, characterized by inflammation and progressive loss of supporting bone tissue surrounding dental implants (Berglundh et al. 2018). This condition compromises both the longevity and success of dental implants, while also imposing a considerable burden on patient morbidity and healthcare costs (Berglundh et al. 2024; Karlsson et al. 2022). The prevalence of peri-implantitis has been reported to range between 20% and 22%, underscoring its widespread impact and the urgent need for effective management strategies (Berglundh et al. 2024).

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Peri-implantitis is fundamentally a bacterial infection; however, the resulting tissue destruction is notably influenced by the host's immune response (Berglundh et al. 2018, 2024). Previous studies on the immune cell composition in peri-implantitis have identified a high prevalence of plasma cells and polymorphonuclear cells, with T cells and B cells being also present but in lower quantities (Carcuac and Berglundh 2014; Ghighi et al. 2018; Ginesin et al. 2023; C.-W. Wang et al. 2021). Previous studies have shown that macrophages constitute approximately 6%–15% of the cells in peri-implantitis lesions (Carcuac and Berglundh 2014; Galarraga-Vinueza et al. 2021; Ginesin et al. 2023; Villalobos et al. 2024).

Monocytes, originating from monoblasts in the bone marrow, exit the bloodstream and migrate to tissues where they differentiate into macrophages. Macrophages are crucial components of the innate immune response, with the ability to recognize and engulf foreign entities. They function as antigen-presenting cells and can create either pro-inflammatory (M1) or anti-inflammatory (M2) environments (Das et al. 2015; Y. Li et al. 2024b). Analysis of human biopsies has shown that macrophages in peri-implantitis exhibit distinct features compared to those in healthy peri-implant tissue and periodontitis, with a higher prevalence of M1 macrophages in peri-implantitis (Fretwurst et al. 2020; Galarraga-Vinueza et al. 2021; J. Li et al. 2024a). Additionally, monocytes serve as precursors to osteoclasts under the influence of M-CSF, underscoring their dual role in modulating immune responses and bone resorption in peri-implantitis.

Current treatments for peri-implantitis primarily focus on bacterial removal, which is undoubtedly crucial. However, additional modulation of the immune response could synergistically mitigate peri-implantitis progression. Previous research has proposed various immunomodulation therapies, including anti-tumour necrosis factor (TNF)-α treatments, drugs that influence macrophage polarization such as PPARγ agonists and other agents such as zoledronic acid and statins (Di Paola et al. 2006; Fujita et al. 2010; Hassumi et al. 2009; Mayer, Balbir-Gurman, and Machtei 2009; Pers et al. 2008; Stienstra et al. 2008; Viniegra et al. 2018). However, none of these approaches has achieved significant success or led to effective treatments yet.

Macrophage depletion is an experimental tool used in an animal model to study the role of macrophages in the pathogenesis of inflammatory conditions (Du et al. 2020; Hotchkiss, Clark, and Olivares-Navarrete 2018). Previous studies have examined the role of macrophages during the osteointegration phase of implants and have shown alterations in immune response and delayed bone healing (Hotchkiss, Clark, and Olivares-Navarrete 2018; X. Wang et al. 2020). Both studies utilized clodronate-loaded liposomes for macrophage depletion. This process occurs when macrophages phagocytose clodronate liposomes and, after liposome degradation, free clodronate accumulates within the cells, leading to apoptosis of macrophages (N. Van Rooijen and Sanders 1996). These liposomes are commonly used to investigate macrophage functions in various inflammatory conditions in mice (Kozutsumi et al. 2023; Turner et al. 2018; Nico van Rooijen and van Kesteren-Hendrikx 2002). The current study aims to explore the effects of non-specific immune response modulation on the initiation of peri-implantitis: specifically, evaluating how macrophage depletion influences the initiation of peri-implantitis using a mouse preclinical model of dental implant. We hypothesize that macrophage depletion reduces inflammatory infiltrate in a ligature-induced peri-implantitis mouse model and decreases peri-implant bone loss.

2 | Materials and Methods

2.1 | Animal Care

All procedures followed protocols approved by the Technion Committee on Animal Research (approval number: IL-092-05-2023). All experiments were performed in accordance with ARRIVE guidelines and regulations. Six-week-old male C57BL/6 mice (22–24 g; ENVIGO, Ness Ziona, Israel) were used. The animals were housed in a temperature-controlled environment with food and water provided ad libitum.

Mice were sedated using an intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (16 mg/kg) and local infiltration of lidocaine 2% with 1:100,000 epinephrine. During each surgical procedure, long-lasting buprenorphine (0.3 mg/kg body weight) was administered subcutaneously. All surgeries were performed under a surgical microscope (Zeiss).

2.2 | Peri-Implantitis Induction

The study timeline is described in Figure SS1. The upper left maxillary molar was extracted. Three weeks post extraction, a full-thickness mucoperiosteal flap was raised, and an implant osteotomy was performed using a low-speed dental drill (900 rpm). A titanium implant (0.6 mm diameter, titanium-6 aluminium-4 vanadium alloy, 'Retopins', NTI Kahla GmbH, Germany) was screwed into the implant bed for primary stability (Mouraret et al. 2014). The implants were placed supracrestally to allow space for future ligature placement, which resulted in light occlusal contact, resulting in a final length of approximately 2.2 mm. The flap was repositioned, with two threads of the implant exposed to the oral cavity. All implants were positioned in the same anatomical location to minimize heterogeneity between subjects. The left side was selected based on the surgeon's personal preference.

Three weeks post implant insertion, mice were divided into three groups: healthy implant (Healthy group), peri-Implantitis with phosphate-buffered saline PBS (PBS group) and peri-implantitis with clodronate (CLOD group). In the PBS and CLOD groups, peri-implantitis was induced by securing a 5-0 silk ligature around the head of the implant (Hiyari et al. 2018; Nguyen Vo et al. 2017; Reinedahl et al. 2024).

For macrophage depletion, the CLOD group received 200 μL of liposomal clodronate (Liposoma BV, Amsterdam, Netherlands) (Moreno 2018; Nico van Rooijen and van Kesteren-Hendrikx 2002; X. Wang et al. 2019) via intraperitoneal injection starting 3 days before ligature placement, with subsequent injections every 7 days (total of three injections per mouse). The PBS group received the same volume of liposomal

PBS (Liposoma BV, Amsterdam, Netherlands) at the same time points. The Healthy group did not receive further treatment after implant placement.

Two weeks after ligature placement, mice were anaesthetized with isoflurane and sacrificed by cervical dislocation. For micro-CT and histological analyses, the maxillae were carefully dissected, removing the skin and superficial muscle, and then fixed in 4% paraformaldehyde for 48 h to preserve tissue integrity. For real-time qPCR, an incision of 1 mm circumference of gingiva around the implant was made, and the soft tissue was immediately snap-frozen in liquid nitrogen and stored at −80°C. The spleens were also collected, fixed in 4% paraformaldehyde for 48 h and processed accordingly.

2.3 | Micro-CT Analysis

Micro-CT scans (PBS: $N = 6$; CLOD: $N = 6$; Healthy: $N = 7$) (SkyScan 1276; Bruker MicroCT, Aartselaar, Belgium) were conducted with the following parameters: source voltage 70 kV, source current 60 μA, image pixel size 11 μm and a rotation step of 0.3°. Volumetric data were reconstructed using NRecon version 1.7.5.4 (Skyscan, Kontich, Belgium). The micro-CT analysis was a modification of established protocols (Carli et al. 2012; Cuijpers et al. 2014; Haugen et al. 2013). Briefly, the DataViewer software (version 1.5.6.2; Skyscan, Kontich, Belgium) was used to position each implant perpendicular to the horizontal plane in the *x*- and *y*-axes. The volume of interest (VOI) was defined as follows: the bone crest was identified based on the position of the adjacent tooth. Within the region extending from the bone crest to two threads apically (Figure 1A), the VOI was specified as a circular area with a radius of 20 pixels, positioned 2 pixels away from the implant surface (Bruker MicroCT, Aartselaar, Belgium). The VOI was analysed using the CTan software (version 1.18; SkyScan, Aartselaar, Belgium), with thresholds of 90 and 60 selected for the implant and bone, respectively.

2.4 | Histological Preparation

Processing of the tissue and histological preparations followed a modification of a well-established protocol (Mouraret et al. 2014; Tian et al. 2022; X. Wang et al. 2020). Briefly, following fixation, bone tissue samples were rinsed in PBS and then decalcified gently using Mol-DECALCIFIER (EUH210, MI, USA) at 37°C for 5 days. After decalcification, tissue samples were dehydrated by washing them in an ascending series of ethanol concentrations. At this stage, both soft and hard tissues reached comparable stiffness, with minimal tissue shrinkage, allowing for the smooth and careful removal of the implants without disturbing the surrounding peri-implant bone or soft tissues. The maxilla was then trimmed buccally and embedded in paraffin. Paraffin blocks were sectioned sagittally at 5 μm and stained with haematoxylin and eosin (H&E).

2.5 | Immunohistochemistry Analysis

Antigen retrieval was performed at the Department of Pathology, Rambam Health Care Campus, using the BenchMark Ultra slide staining system (Roche, Basel, Switzerland). Pretreatment conditions were set automatically. Histological sections were blocked with Block Buster (Background Buster, Innovex Biosciences, California, USA) for 30 min, rinsed twice with PBS and then immunolabelled with antibodies: anti-CD45 antibody (1:1000, PBS: $N = 4$; CLOD: $N = 4$, 20103-1-AP, Proteintech, IL, USA) anti-F4/80 (1:100 dilution, PBS: $N = 4$; CLOD: $N = 4$, #70076, CST, Massachusetts, USA) for 1 h at room temperature. This was followed by immunolabelling with horse radish peroxidase (HRP) (ZytoChem Plus HRP Polymer anti-Rabbit, Zytomed, Berlin, Germany) for 30 min, staining with 2,4-diaminobenzidine (DAB) (SuperPicture Polymer Detection Kit, Thermo Fisher Scientific, Massachusetts, USA) for 15 min and additional staining with haematoxylin (10% haematoxylin, 90% distilled water) for 1 min. To demonstrate the specificity of the immunoreactions, samples were labelled only with the

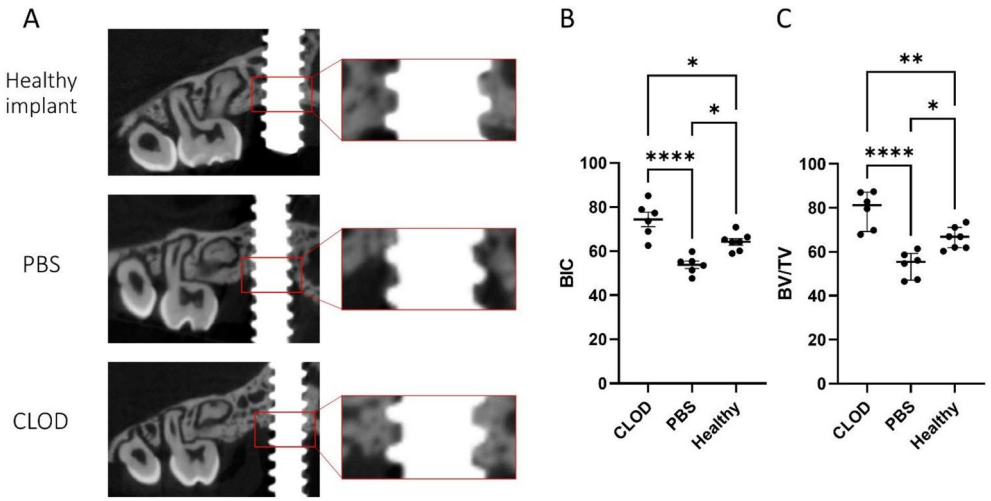

FIGURE 1 | Micro-CT evaluation of peri-implant bone volume changes. (A) Representative micro-CT image of each group—healthy, peri-implantitis and liposomal PBS (PBS) and peri-implantitis and liposomal clodronate (CLOD). Red squares indicate higher magnification of the volume of interest (VOI) representing the higher bone loss in PBS group. (B) Bone-to-implant contact (BIC) for each group after treatments. (C) Bone volume relative to total volume (BV/TV) for each group after treatments. $N = 6$ for PBS and CLOD groups and $N = 7$ for healthy group analysed using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s., no significant difference.

secondary antibody, excluding the primary antibody. This approach served as a negative control. Histological slides were scanned with an automatic Olympus VS200 Slide Scanner (Evident, Tokyo, Japan) and analysed using the OlyVIA version 3.4.1 software (Evident, Tokyo, Japan). To calculate the proportion of the stained area, an ROI was defined for each slide, specifically locating the soft tissue above the first implant thread embedded in intact bone (Figure 2A). The proportion of the stained area relative to the total area was then automatically calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

2.6 | Real-Time PCR

For RT-qPCR analysis, three mice from the CLOD group, three mice from the PBS group and two from the healthy group were used in two independent experiments. Tissue was dissected into fragments of <1 mm in size and homogenized manually, and total RNA was extracted using the HP total RNA kit (Omega BIO-TEK, Norcross, GA, USA). Fifty micrograms of RNA was used for the reverse transcription reaction. cDNA was generated using the high-capacity cDNA reverse transcription kit (QuantaBio, Beverly, MA, USA). Quantitative real-time PCR

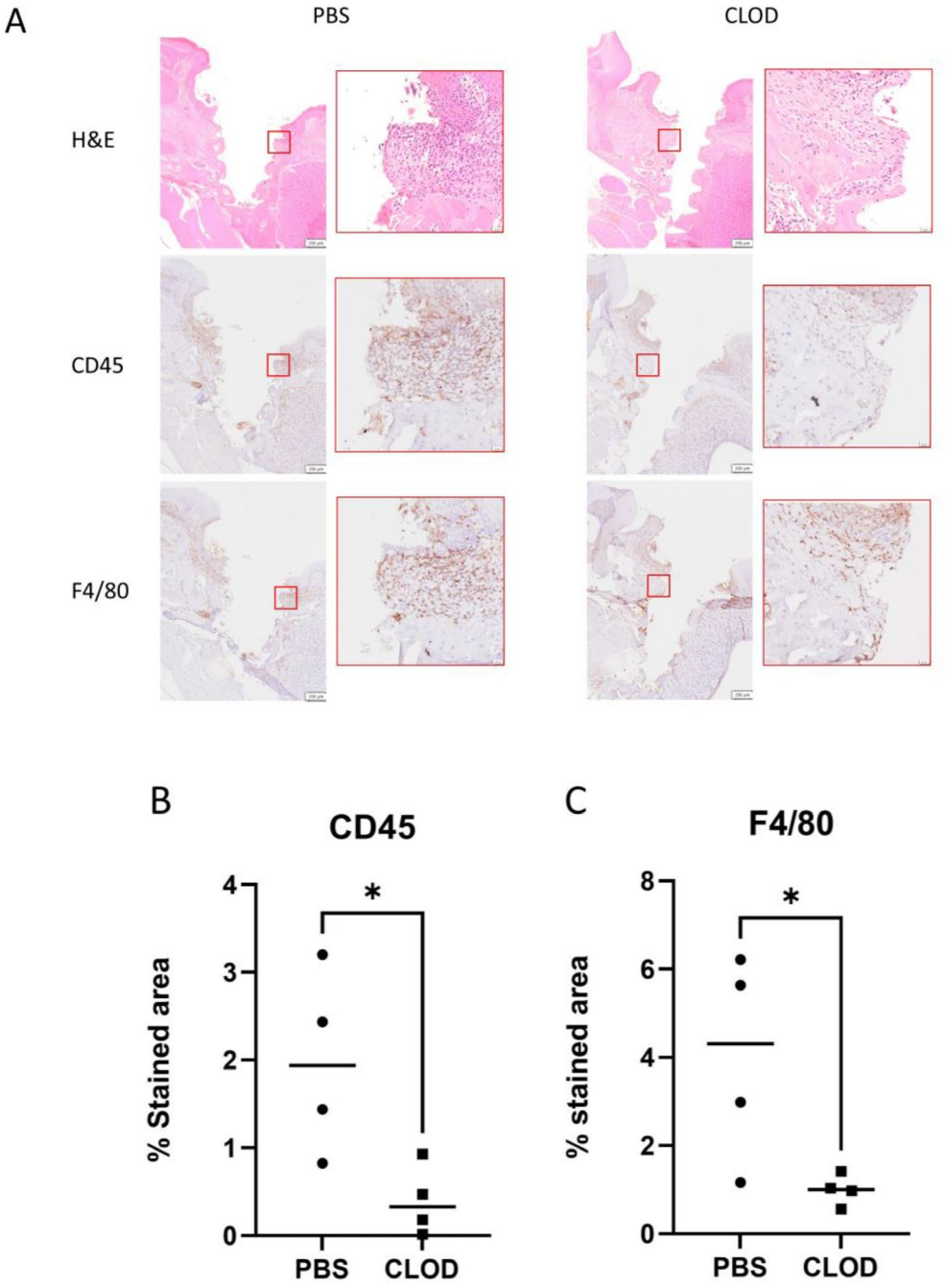

FIGURE 2 | Histological evaluation of liposomal clodronate treatment for peri-implantitits. (A) Hematoxilin and eosin staining of mice treated with liposomal PBS (PBS) and liposomal clodronate (CLOD) with immunohistochemistry of CD45 and F4/80. Red square shows that the first thread above bone level contains the area of interest and quantified in (B and C), respectively. Scale bar: 200 μm in lower magnification images and 20 μm in higher magnification images. $N=4$ for PBS and CLOD groups. Analysed using an unpaired t-test. $^*p<0.05$.

TABLE 1 | List of primers.

Gene	Forward	Reverse
GAPDH	AGAACATCATCCCTGCATCCAC	TCAGATCCACGACGGACACA
IL-10	GAGAAGCATGGCCCAGAAATC	GAGAAATCGATGACAGCGCC
TNF-α	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC

analysis was performed using SYBR Green (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA, USA). The primer sequences are listed in Table 1. The results were analysed using QuantStudio software and the ΔΔCt method. Data were normalized, and fold change for each gene was calculated using the ΔΔCt method.

2.7 | Statistical Analysis

Sample size calculation was performed using $\alpha = 0.05$ and $\beta = 0.2$, based on data from a previous study (Deng et al. 2020). For the micro-CT evaluation of peri-implant bone loss, Deng et al. reported a bone resorption of 0.25 mm^3 (SD = 0.09) in the peri-implantitis group after 2 weeks compared to 0.1 mm^3 in the Healthy group (Deng et al. 2020). Based on these values, a minimum of six animals per group was required for this outcome. A similar approach was applied to determine the sample size for secondary outcomes. A total of 36 mice were required for the assays (estimated sample size). To account for potential animal loss due to the surgical procedures (10%), we included 40 mice in the study (actual sample size).

Statistical parameters including means, medians, ranges, SDs and standard errors (SEs) were calculated. Comparisons between groups were performed using one-way analysis of variance (ANOVA) and Student's *t*-test. A significance level of 5% was set. Statistical analysis was conducted with GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA).

3 | Results

3.1 | Macrophage Depletion Reduces Peri-Implant Bone Loss

Two weeks after ligature placement, including three injections of liposomal clodronate, a 52% reduction in spleen macrophages was observed compared to the PBS control group. Mice with peri-implantitis that had received liposomal clodronate treatment (CLOD) had a bone-to-implant contact (BIC) of approximately 74% (SD = 7.92), which was significantly higher than (64%; SD 3.97) that in the Healthy group (p-value < 0.05) and 53% (SD = 4.12) in the PBS group (p-value < 0.0001). The difference between the PBS and Healthy groups was also statistically significant (p-value < 0.05) (Figure 1B). The bone volume relative to total volume (BV/TV) showed similar trends, with the CLOD group having the highest BV/TV at 79% (SD = 8.46), followed by the Healthy group at 66% (SD = 4.99, $p < 0.01$), and the lowest BV/TV in the PBS group at 54% (SD = 6.01, $p < 0.0001$). A significant difference was also observed between the PBS and Healthy groups ($p < 0.05$) (Figure 1C).

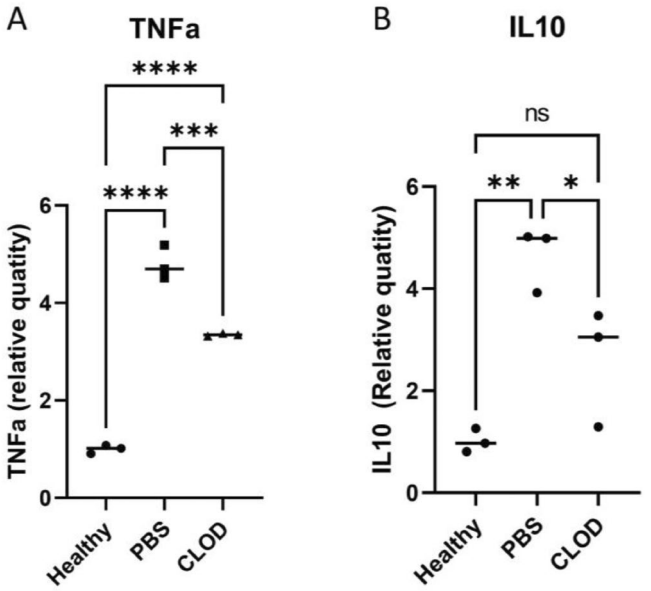

FIGURE 3 | Macrophage depletion using clodronate reduced the expression of TNF-α and Il-10 in the soft tissue around implants: (A) TNF-α, (B) IL-10. Representative data from one assay is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n.s., no significant difference.

3.2 | Macrophages Depletion Alters Leukocyte Composition in Peri-Implantitis Tissue

Following maxilla harvesting and decalcification, implants were removed and the tissue was subjected to immunohistochemical analysis. Sequential sections were stained with H&E, and immunostained for CD45 (leukocyte marker) and F4/80 (macrophage marker) (Figure 2A). The connective tissue coronal to the bone in the PBS group showed a higher cell density compared to the CLOD group (Figure 2). The area of CD45-positive cells was 5 times greater in the PBS group than in the CLOD group (average 1.97, SD = 1.03 for PBS; 0.39, SD = 0.39 for CLOD; p-value < 0.05, Figure 2B). Similarly, the area of F4/80-positive cells was four times larger in the PBS group compared to the CLOD group (Average 4, SD 2.35 for PBS; 0.99, SD 0.34 for CLOD; $p < 0.05$, Figure 2C).

3.3 | Macrophages Depletion Affects Pro-Inflammatory Mediators in Peri-Implantitis Tissue

Two weeks post ligature placement, the Healthy group showed significantly lower levels of the examined cytokines (TNF-α and IL-10) compared to both the PBS and CLOD groups (Figure 3).

Notably, TNF-α levels were significantly reduced in the CLOD group compared to the PBS group ($p < 0.05$), while IL-10 levels were elevated in the PBS group relative to the CLOD group ($p < 0.05$).

4 | Discussion

Peri-implantitis represents a significant challenge in dental implantology. Despite extensive research, effective treatment options remain elusive. While peri-implantitis is primarily a bacterial infection, the associated tissue damage is largely driven by the host's immune response (Berglundh et al. 2018, 2024). We explored the role of macrophages, the key players in the non-specific immune response, in the initiation of peri-implantitis.

Clodronate, a bisphosphonate primarily used for osteoporosis, has not yet been adopted for treating inflammatory diseases. Clodronate induces apoptosis in monocyte–macrophage lineage cells both in vitro and in vivo (Volpi et al. 2007). However, its effect on osteoclasts is not cytotoxic, as clodronate inhibits the proton pump in osteoclasts (Volpi et al. 2007). A 52% reduction in spleen macrophages was observed in the CLOD group. This level of depletion is consistent with previous studies, which used a comparable dosage and injection frequency (Davison et al. 2014; X. Wang et al. 2020). Notably, macrophage depletion resulted in a significant reduction in bone loss compared to the PBS group, and also led to unexpectedly higher BIC and BV/TV compared to the Healthy group. This indicates the achievement of a greater bone density, suggesting that macrophage depletion may also contribute to a reduction in bone turnover.

Macrophages also play a critical role in recruiting and activating specific immune cells to the site of inflammation (Y. Li et al. 2024a; Sima and Glogauer 2013; Tsukasaki and Takayanagi 2019). In our study, histological analysis showed that macrophage depletion led to a significant reduction in both leukocytes and macrophages at the site of inflammation, alongside attenuation of bone loss. These observations suggest a reduced induction of peri-implantitis. Similar findings were reported in study on ligature-induced periodontitis, where macrophage depletion mitigated disease induction, decreased the number of macrophages and reduced the expression of pro-inflammatory cytokines (Clark et al. 2021). The significant presence of macrophages in the peri-implant tissue, coupled with increased levels of TNF-α, may account for the greater peri-implant bone loss observed in the PBS group. These results may suggest that macrophages are pivotal in modulating the extent and severity of the inflammatory response.

TNF-α is a pro-inflammatory cytokine known to stimulate osteoclastogenesis through RANK signalling (Souza and Lerner 2013). Human studies have identified macrophages and T cells as major sources of TNF-α in inflamed gingiva, with TNF-α colocalizing with macrophages in the connective tissue of peri-implantitis lesions (Matsuki, Yamamoto, and Hara 1992; Villalobos et al. 2024). High levels of TNF-α were found in chronic osteolytic sites, such as periodontally compromised areas, and targeting TNF-α therapeutically improved clinical outcomes and reduced bone resorption (Andrukhov et al. 2011; Górska et al. 2003; Mayer, Balbir-Gurman, and Machtei 2009; Stashenko et al. 1991). In our study, elevated TNF-α levels were found in the PBS group compared to the Healthy group, supporting previous findings (Nguyen Vo et al. 2017). Notably, the CLOD group exhibited lower TNF-α levels compared to the PBS group, consistent with observations from a periodontitis mouse model where macrophage depletion was applied (Clark et al. 2021).

IL-10, an anti-inflammatory cytokine, is known to inhibit osteoclast differentiation and is released by various immune cells, including macrophages and T cells (Souza and Lerner 2013). While studies on a peri-implantitis mouse model have generally reported reduced IL-10 levels compared to healthy implants (Tzach-Nahman et al. 2017), other human and in vivo studies indicate elevated IL-10 levels in periodontal disease (Bertoldo et al. 2024; Deng et al. 2020; Escalona, Mastromatteo-Alberga, and Correnti 2016). In our study, elevated IL-10 levels were observed in the PBS group compared to the Healthy and CLOD groups. This discrepancy may be attributed to heightened leukocyte activity in the PBS group, which could lead to increased secretion of IL-10.

The current study has several limitations. First, although male mice were chosen to minimize biological variability, this restricts the generalizability of the findings to both sexes. Further studies including female mice are necessary to determine the translational value of these results. Additionally, while liposomal clodronate effectively depletes macrophages, its effects are partial and diminish over time (Nico van Rooijen and van Kesteren-Hendrikx 2002), and it may also influence osteoclastogenesis and tissue repair, potentially confounding the results (Davison et al. 2014; Michalski et al. 2019; Schlundt et al. 2021).

Additionally, the relatively high BIC observed in the peri-implantitis and PBS groups may be due to protocol differences, such as peri-implantitis induction without bacteria or LPS, induction timing, sacrifice timing, implant surface and structure variations and implants' light occlusal contact.

Regarding tissue processing, although implant removal was performed according to a well-established protocol (Mouraret et al. 2014; Tian et al. 2022; X. Wang et al. 2020), it might have still resulted in damage to the tissue–implant interface, potentially affecting the accuracy of subsequent immunohistochemical analysis. However, despite this limitation, the outline of the implant threads remained detectable, allowing reliable assessment of the interface.

Lastly, natural variation among individuals may explain the heterogeneity in staining among PBS-treated mice, with some showing severe peri-implantitis and others only the mild form. In contrast, the other treatment groups displayed striking uniformity, indicating that the interventions had a significant effect, beyond the intrinsic natural variability.

5 | Conclusion

This study emphasizes the critical function of macrophages in the onset of peri-implantitis. The reduction of macrophages leads to a decrease in bone loss and a lower recruitment of leukocytes

at the interface between bone and the implant. These results suggest that targeting macrophages could be a promising avenue for therapeutic interventions in peri-implantitis; however, additional studies are necessary to overcome existing limitations and enhance strategies for the modulation of macrophages. Future studies should also investigate the impact of macrophage depletion on other immune cell populations and their subpopulations, particularly the M1/M2 macrophages, which play a dual role in both tissue destruction and the resolution of inflammation. Future research should also focus on developing local therapies to modulate macrophage activity, translating these findings into clinical practice.

Author Contributions

All authors contributed substantially to the interpretation of the data for the work and to drafting and critically revising the manuscript. They gave their final approval of the version to be published and agreed to be accountable for all aspects of the work. Additionally, Hadar Zigdon-Giladi, Yotam Bar-On, Benjamin R. Coyac and Ofir Ginesin contributed to the concept and design of the study. Hadar Zigdon-Giladi, Ofir Ginesin Ofri Doppelt-Flikshtain and Tal Berg for collecting the data. Hadar Zigdon-Giladi, Ofir Ginesin, Benjamin R. Coyac and Yotam Bar-On for securing funding and Hadar Zigdon-Giladi for managing the group.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.