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INSIGHTS

The stunning clodronate

Elvira Mass[1]

Not only macrophages, but also neutrophils, are a main target of clodronate. In this issue of *JEM*, Culemann et al. (2023. *J. Exp. Med.* https://doi.org/10.1084/jem.20220525) demonstrate that anti-inflammatory effects of clodronate liposomes are driven via stunning of polymorphonuclear neutrophils and not solely through depletion of macrophages.

Clodronate liposomes have been broadly used to study the function of macrophages in autoimmunity, infections, transplantation, cancer, neuroinflammation, and other immune cell–driven pathophysiologies (Ponzoni et al., 2018; Ravichandran et al., 2022; Rosowski, 2020). Due to its ability to induce apoptosis of macrophages (Van Rooijen and Sanders, 1994), clodronate liposomes have been the method of choice to deplete macrophages in a temporally controlled manner in many labs worldwide. The administration procedure is straightforward in comparison with complex transgenic mouse models: depending on the targeted macrophage population, clodronate liposomes can be injected intravenously to target macrophages systemically, e.g., in the liver, the spleen, the gut, and the bone marrow, or applied locally, e.g., into the lung or the brain to target only tissue-specific populations. Although it is a well-known fact that clodronate also induces apoptosis of monocytes and dendritic cells and that the simultaneous death of millions of immune cells leads to inflammation (Mass, 2018), most studies detecting a clodronate-dependent phenotype have been connected to macrophages, indicating that targeting macrophages alone may be a potential therapeutic intervention.

To delineate the impact of clodronate liposome treatment on mononuclear phagocytes, including macrophages, monocytes, and neutrophils, Culemann et al. (2023) used the K/BxN serum transfer arthritis (STA) mouse model in which the immunological mechanisms occurring in rheumatoid arthritis (RA) can be studied. They first validated the previous finding that clodronate liposome treatment in the STA model leads to an almost complete inhibition of arthritis. Due to the controversial literature regarding monocytes/macrophages being detrimental or beneficial in RA (Knab et al., 2022), they continued with transgenic mouse models targeting single mononuclear phagocyte populations. First, using *Nr4a1* knockout mice, they showed that a lack of Ly6^{low} monocytes had no impact on the regular onset of STA. Similar results were observed in the *Cx3cr1Cre*; *iDTR* model, in which continuous injection of diphtheria toxin leads to the depletion of synovial macrophages and monocytes. Changing the treatment protocol in the *Cx3cr1Cre*; *iDTR* model, allowing a repopulation of blood monocytes while synovial macrophages remained absent, even increased joint inflammation during STA. The same experiments in a mouse model of gouty arthritis recapitulated these findings, suggesting that the amelioration of arthritis upon clodronate liposome administration is likely not due to the manipulation of the monocyte/macrophage compartment. To confirm this hypothesis, clodronate liposomes were injected into *Cx3cr1Cre*; *iDTR* mice treated continuously with diphtheria toxin, which phenocopied the anti-inflammatory effects of clodronate even in the absence of monocytes and macrophages.

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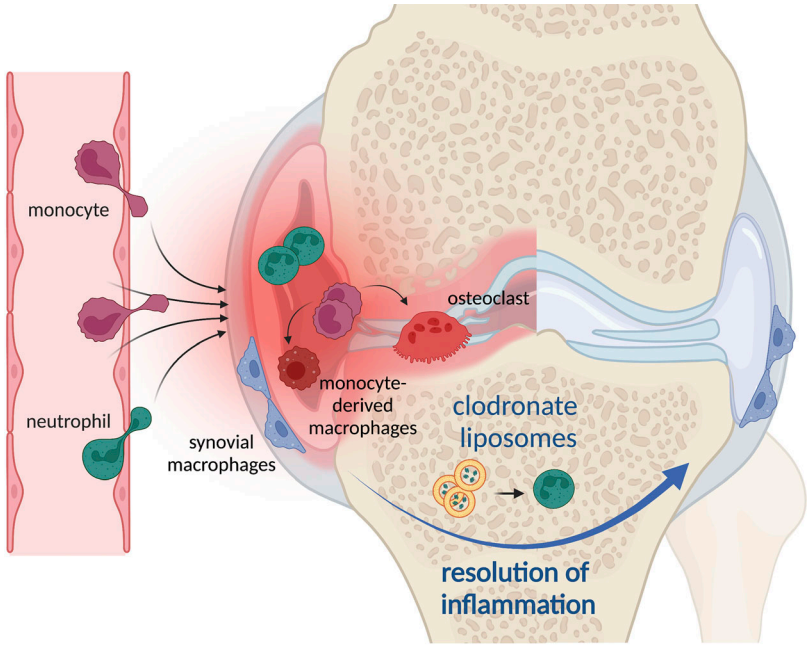
Culemann et al. (2023) continued their search for an alternative cell type taking up clodronate liposomes and serving as an effector cell during arthritis. Intriguingly, they observed that 1–2 d after injection, ∼50% of neutrophils contained fluorescently labeled clodronate liposomes, although their numbers were unaltered in comparison to controls. Next, they depleted neutrophils via injection of a Ly6G-specific antibody or deleted neutrophils using a *Mrp8Cre*; *iDTR* mouse model. In both cases, the rescue of the STA phenotype corresponded to the phenotype observed in

[1]Developmental Biology of the Immune System, Life & Medical Sciences Institute, University of Bonn, Bonn, Germany.

Elvira Mass: elvira.mass@uni-bonn.de.

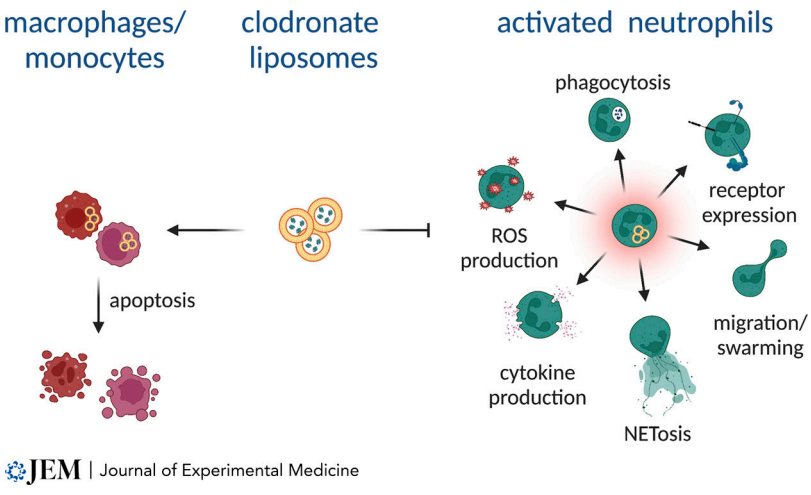


Clodronate liposomes inhibit inflammation in the joint in arthritis mouse models. During arthritis, immune cells, including monocytes and neutrophils, are recruited to the site of inflammation. Monocytes can undergo differentiation towards monocyte-derived macrophages and osteoclasts. Upon clodronate liposome injection, monocytes and monocyte-derived macrophages are depleted while synovial macrophages survive. Nevertheless, the inflammation-resolving mechanism of clodronate is mediated via stunning of pro-inflammatory neutrophils. Created with BioRender.com.

response to clodronate liposomes alone. To pinpoint neutrophils as a major pro-inflammatory driver of STA, mice injected with clodronate liposomes were injected with neutrophil-enriched bone marrow cells, which indeed led to an acerbated inflammatory response in comparison to an adoptive transfer of control cells. Finally, the authors show that clodronate-treated neutrophils show a reduction in the production of ROS and cytokines and less formation of neutrophil extracellular traps. Furthermore, they lose their ability to phagocytose and migrate—a change of neutrophil core functions previously defined as "stunning." In summary, the anti-inflammatory effects of clodronate liposomes in arthritis models are not primarily linked to the depletion of pro-inflammatory monocytes/macrophages but can be attributed to the stunning of detrimental neutrophil effector functions.

The study by Culemann et al. (2023) has broad implications that go beyond the specific inflammatory model of arthritis. First, "simple" causal relationships between disease onset/progression and macrophages after clodronate administration should be interpreted with caution. Second, many macrophage-related studies should be revisited, for reasons beyond the modes of action of clodronate, which may also explain the previous controversial literature about the role of macrophages in arthritis models: macrophages are no longer the one simple immune cell population that can be stratified into pro-inflammatory M1 and anti-inflammatory M2 macrophages. Instead, every tissue harbors fetal-derived and monocyte-derived macrophages with different life spans and distinct effector functions during health and disease (Schultze et al., 2019; Mass et al., 2023). Thus, targeting all macrophage subsets via clodronate simultaneously may even worsen disease outcome and makes interpretation of data with respect to the contribution of individual macrophage subsets impossible without combining disease models with more sophisticated transgenic/conditional mouse models, as demonstrated by the current study of Culemann et al. (2023).

Of note, clodronate is a widely used drug that has been effective in treating various osteometabolic disorders in humans since the 1960s. It is known for its ability to inhibit excessive bone resorption in several conditions such as hypercalcemia malignancy and osteolytic bone metastases and has been particularly used for the prevention and treatment of postmenopausal osteoporosis (Markell et al., 2020). In recent years, clodronate has also shown promise in preventing bone loss and providing anti-inflammatory and analgesic benefits for a range of conditions like complex regional pain syndrome and bone marrow edema. There is also evidence to suggest that clodronate may be even effective in treating



Modes of action of clodronate liposomes. Uptake of clodronate liposomes leads to apoptosis of macrophages and monocytes, but not neutrophils. Instead, clodronate causes stunning of neutrophils, thereby preventing neutrophil-induced inflammation via inhibition of phagocytosis, migration and swarming behavior, ROS production, cytokine production, formation of neutrophil extracellular traps (NETosis), and expression of receptors. Created with BioRender.com.

rheumatoid and osteoarthritis (Frediani and Bertoldi, 2015; Moretti et al., 2021). However, the mode of action of clodronate in humans seems distinct from the macrophage-depleting mechanism in animal models. Here, the main target cells are osteoclasts, specialized bone-resident macrophage-like cells, that are inhibited in their activity or undergo apoptosis upon clodronate uptake (Frediani and Bertoldi, 2015). On the one hand, it would be of interest to investigate in patients with osteometabolic disorders whether the anti-inflammatory properties of clodronate are a result of stunned neutrophils instead of or in addition to the osteoclast-targeting activity. On the other hand, similar drugs belonging to the bisphosphonate group and frequently used to treat these disorders should be tested for their neutrophil-stunning activity. Recent studies highlight neutrophils as a possible contributing factor for RA and other autoimmune diseases (Thieblemont et al., 2016; Wright et al., 2021). Thus, the novel neutrophil-stunning mechanism of clodronate in mice may open new therapeutic concepts to target human pathophysiologies driven by chronic neutrophil activation with clodronate or other types of bisphosphonates.

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