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The Effect of Inhalant Organic Dust on Bone Health

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Abstract

Purpose of Review: Agriculture remains a major economic sector globally, and workers experience high rates of chronic inflammatory lung and musculoskeletal diseases. Whereas obstructive pulmonary diseases are known risk factors for bone loss, the underlying relationship between lung inflammation and bone health is not well-known.

Recent Findings: An agriculture organic dust extract inhalation animal model has recently linked lung injury-induced inflammation to systemic bone loss. This process is dependent upon lipopolysaccharide and the Toll-like receptor 4 (TLR4) signaling pathway. Downstream systemic interleukin-6 is a key mediator that subsequently activates osteoclastogenesis. Age is a host factor that impacted bone disease with younger mice demonstrating increased susceptibility to bone loss following inhalant exposures as compared to older mice. Supplemental dietary vitamin D was shown to prevent organic dust-induced bone loss, but not lung disease, in animals.

Summary: Recent animal studies provide new mechanistic insight into the lung-bone inflammatory axis. Host factors, diet, and lipopolysaccharide/TLR4 signaling pathways play a significant role in explaining how inhalant organic dust exposures impact bone health. These investigations might lead to specific targeted therapeutic approaches.

Keywords

Organic Dust; Bone; endotoxin; COPD; interleukin (IL)-6; Toll-like receptor 4/TLR4

Introduction

Agriculture work is associated with adverse health effects, with the most prevalent issues of chronic inflammatory lung and musculoskeletal diseases. Development of chronic obstructive pulmonary disease (COPD), chronic bronchitis, asthma, and hypersensitivity pneumonitis have all been associated with agricultural exposure (1,2,3,4). Exposed workers also have an increased life-time risk (90%) of developing musculoskeletal diseases (5). There is a high prevalence of osteoarthritis of upper and lower extremities, low back pain, and rheumatoid arthritis (RA) with subsequent increase in morbidity and mortality from hip fractures. (5,6,7). There is a similar association of bone loss and osteoporosis in persons with chronic inflammatory respiratory diseases like COPD, asthma, cystic fibrosis, and post-

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lung transplantation (8,9,10,11). Furthermore, it has been shown that having COPD is an independent risk factor for developing bone mineral density (BMD) loss, irrespective of body-mass index (BMI), sex, age, steroid use, smoking, or severity of COPD (12). Recognizing these similarities, it is reasonable to suggest that exposure to agriculture-related occupational inhalant insults triggers an airway inflammatory process that could impact systemic bone health.

More than two decades ago, associations between occupational exposure and bone disease were described for Ewing's sarcoma (13). Analysis of medical and occupational family history of children with this bone disease revealed an increased risk (RR: 8.8, CI: 1.8–42.7) with fathers who were agricultural workers (13). Expounding on this initial observation, a later case-controlled study of 196 children from 64 U.S. institutions demonstrated that mothers or fathers who had perinatal or postnatal exposure to occupational organic dusts lead to an increased odds ratio (OR: 2.1, CI: 1.0–4.2) of their offspring developing Ewing's sarcoma (14). The odds ratio was further increased (OR: 3.5, CI: 0.4–33.0) if exposure was primarily from the mother's occupational exposure (14). Ewing's sarcoma is a rare form of bone malignancy that is primarily found in adolescents (15). The primary mechanism associated with Ewing's sarcoma is through overexpression of receptor activator of nuclear factor kappa-β ligand (RANKL), which leads to enhanced osteoclastogenesis (16).

Balance of osteoclast and osteoblast activity is central to the maintenance of bone homeostasis (17). Osteoclasts are bone-specific, bone-resorbing macrophages derived from bone marrow osteoclast precursor cells that express membrane bound RANK. Osteoclast maturation and activation occurs in the presence of macrophage colony-stimulating factor (M-CSF) and RANKL (17). Following osteoclast activation and bone resorption, bone morphogenetic proteins, fibroblast growth factors, and transforming growth factor β are released from the bone matrix (18). These factors stimulate osteoblasts, which in turn produce new bone matrix and promote mineralization. Several factors stimulate lining cells to balance RANK/RANKL expression and bone homeostasis. These include mechanical stress, tumor necrosis factor (TNF)-α, insulin growth factor-I, parathyroid hormone, and interleukins (18). The inflammatory cytokines, interleukin (IL)-17, IL-1β, IL-6, and TNF-α, have all been shown to play a role in RANKL expression and osteoclastogenesis (18). Understanding these mechanisms regulating bone homeostasis could provide explanation linking airway inflammatory injury and bone disease. An overview schematic of known mechanisms involved in the lung-bone inflammatory axis are depicted in Figure 1.

Agriculture organic dust exposure and airway inflammatory disease

Agricultural workers have an increased risk of COPD and asthma-like disease (19). Following exposure to organic dust, human airways develop an inflammatory response marked by Th1/Th17 activation and neutrophil airway influx (20,21,22,23). Genetic polymorphisms in Toll-like receptors and neutrophil adhesion-molecule expression have been associated with agriculture respiratory disease. (25,26,27). Toll-like receptor activation is highly dependent on the adaptor protein, myeloid differentiation factor 88 (MyD88) (28). Repetitive exposure leads to an attenuated response in lung inflammation (29) but a sustained systemic effect (30). Animal models resembling human disease including

approaches of intranasal instillations and nebulizations of agriculture organic dusts and extracts or direct facility exposure in mice and rats have all demonstrated similar patterns of inflammatory markers (21,23,28,31,32). Repetitive organic dust inhalation induces neutrophil influx, release of inflammatory cytokine/chemokine (i.e. TNF-α, IL-6, neutrophil chemoattractants), Th1/Th17 microenvironment, activation of macrophages, and lung pathology marked by lymphoid aggregates and peribronchial and perivascular inflammation (21,22,23,31,32,). It was recently reported that there is also a role for B cells in ODE-induced airway inflammation with further evidence for an autoreactive response marked by increased levels of IgG anti-citrullinated protein antibody and anti-malondialdehyde-acetaldehyde autoantibodies (24). Homeostatic regulators of the ODE-induced airway inflammatory process include protein kinase C epsilon (PKCε) (33), nucleotide-binding oligomerization domain 2 (NOD2) (34,35), and scavenger receptor class A (SRA) (36). Organic dusts obtained from swine confinements are commonly utilized due to their strong inflammatory consequences, and these dusts consists of a complex mixture of particulates containing gram negative and gram positive bacterial components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) (37,38).

Animal model of inhalant organic dust-induced bone loss

To investigate the lung-bone inflammatory axis following organic dust exposures, a murine model has been developed and characterized (39). Namely, C57BL/6 mice were intranasally instilled with swine confinement organic dust extract (ODE), LPS, PGN, or saline control daily for 3 weeks whereupon hind limbs were collected 5 hours following final exposure. Bone images as well as bone quantity and quality analysis was conducted by micro-computed tomography (CT) imaging technology and software analysis with comparisons to bone histology. Initial studies focused on the distal calcaneus bone and found that as compared to saline inhalation, inhalant ODE resulted in significant loss (−24%) in bone mineral density and a −25% decrease in the trabecular bone volume fraction, which are markers of bone demineralization. ODE exposure also increased bone surface area (marker of bone resorption) by +20%, and resulted in deterioration in morphological bone structure as noted by decreased trabecular thickness (−12%), decreased trabecular number (−15%), increased trabecular separation (+18%), and disconnected trabecular pattern factor (+325%). ODE exposure induced a loss of torsional resistance (i.e. mean polar moment of inertia) by −43%. Similarly, bone histology findings demonstrated reduction in collagen and proteoglycan. Intranasal inhalation of LPS and PGN daily for 3 weeks also resulted in increased bone deterioration as compared to control. LPS demonstrated the most significant bone loss and deterioration as compared to ODE and PGN. This observation was in contrast to lung inflammation because PGN and ODE exposures induced enhanced lung tissue pathology as compared to LPS, which the authors interpreted as a compartmental response to inhalant ODE exposure (19).

Organic dust mediated lung-bone inflammatory axis is dependent on TLR4

To further understand the role of specific components driving the bone loss to inhalation of this complex organic dust, investigators employed Toll-like receptor 2 (TLR2) and TLR4 knockout mice. TLR2 is a key signaling pathway in cellular response to gram positive

bacterial components, while TLR4 recognizes and responds to LPS from gram negative bacteria. TLR2 and TLR4 are also expressed on bone osteoclasts and play roles in osteoclastogenesis (40). Moreover, both TLR2 and TLR4 signaling pathways have been implicated in mediating airway inflammatory disease in agriculture workers, particularly swine confinement workers (25,26). Animals deficient in TLR2 or TLR4 demonstrate an approximate 50% reduction in airway inflammatory makers following organic dust challenge (28,41,42).

Using same exposure model and micro-CT analysis as already described (39), Staab, et al. (2016) investigated the inhalant ODE-induced bone consequences of the proximal tibia in TLR2 and TLR4 knockout mice to wild type animals (43). As compared to WT animals, ODE exposed TLR4 knockout mice were protected from adverse changes in bone mineral density, trabecular separation, bone surface area, trabecular pattern factor, and polar moment of inertia. However, the bone quantity and quality measurements with the TLR2 knockout mice were no different than the wild type mice exposed to ODE. Both wild type and TLR2 knockout animals instilled with ODE had significant changes in bone mineral density (−16%), polar moment of inertia (−41%), trabecular separation (+12%), bone surface area (+13%), and trabecular pattern factor (+13%) when compared to saline instilled animals. These studies demonstrated that TLR4 is necessary for mediating the bone loss consequences following respiratory injury with ODE.

In this same study (43), ODE-induced airway neutrophil influx was reduced by approximately 50% in both TLR2 and TLR4 knock out mice compared to wild type animals. Similar reductions were demonstrated with ODE-induced cytokines/chemokine in the lavage fluid. Moreover, inhalant ODE-induced systemic levels of were reduced by 51% in TLR2 KO and 64% in TLR4 KO mice as compared to wild type animals. These studies would suggest that the reduction in lung inflammation alone is not the explanation for the bone protective effect observed in TLR4 KO mice, but suggest the involvement of TLR4 signaling pathway in bone pathology.

Interleukin 6 Involvement in Osteoclastogenesis

IL-6 has been shown to play important roles in the pro-inflammatory state of COPD (44), heart disease (45), rheumatoid arthritis (46), and osteoclastogenesis (18). This suggests that systemic IL-6 may be important for explaining the lung-bone inflammatory axis. Ant-IL-6 therapies, such as tocilizumab, have been successful in the treatment of rheumatoid arthritis (47,48). IL-6 has also been shown to impact RANKL expression and influence osteoclastogenesis (18). In addition, IL-6 is a key cytokine that is consistently associated with organic dust induced airway injury (31,49,50,51), and murine serum levels of IL-6 have been detected following inhalant ODE exposure (30,52). Therefore, it was hypothesized that systemic IL-6 could be responsible for mediating inhalant-ODE induced bone loss.

In a study of 3 week inhalant exposure to ODE, airway inflammatory and bone outcomes in wild type mice were compared to IL-6 knockout animals (30). Unexpectedly, there was no difference in inflammatory cell influx, cytokine/chemokine release or lung pathology between ODE-treated wild type and IL-6 knockout animals, suggesting that the airway

inflammatory response to ODE is not dependent on IL-6 (30). However, micro-CT analysis of the distal tibia demonstrated that IL-6 knockout mice were protected from inhalant ODE-induced bone loss. Specifically, there was no difference in bone mineral density, polar moment of inertia, trabecular separation, bone surface area, trabecular pattern factor, or bone volume ratio between saline and ODE-treated IL-6 knockout animals as there was in the ODE-treated wild type mice (30).

Bone marrow cells collected from these same saline and ODE-treated wild type and IL-6 knockout mice were investigated for osteoclast precursor cell (OCP) populations. Whereas wild type animals expressed increased OCP populations following inhalant ODE exposure compared to saline controls, there was no significant increase in OCP populations in the IL-6 knockout mice. These studies highlight the important role systemic IL-6 plays in the lung-bone inflammatory axis.

Organic dust-induced osteoclastogenesis

Osteoclasts and osteoclast precursor (OCP) cell count and activity correlate well with arthritis severity and bone loss (53,54). Osteoclasts are myeloid-derived cells, and osteoclast precursor cells express CD115, CD117, and CD27. CD115 and CD117 are receptors for M-CSF and stem-cell factor respectively; expression of both markers is necessary for osteoclast differentiation and proliferation (53). CD27 expression has also been shown to identify OCPs with high proliferation potential (55). CD70 is the ligand responsible for activation of CD27 and in chronic inflammatory states such as rheumatoid arthritis; CD70 is overexpressed and promotes arthritic affects (55). Using flow cytometry, OCPs can be isolated from lineage negative (CD3-/CD45R-/CD11b-) cells with expression of CD115/CD117/CD27. In the context of ODE inhalation, it has been demonstrated that osteoclast frequency as well as bone marrow derived osteoclast precursor cell populations are increased (30,43,56).

Tartrate-resistant acid phosphatase (TRACP 5b) is a marker of osteoclast number and bone resorption activity (57). Serum levels of TRACP 5b has been measured in animal studies. Serum TRACP 5b levels have been shown to be increased in wild type mice treated with ODE as compared to saline control animals (43). However, in TLR4 knockout animals, there is no difference in serum TRACP 5b levels with ODE exposure as compared to saline control, which further implicates the TLR4 signaling pathway in mediating ODE-induced osteoclastogenesis.

Toll-like receptors are activated by either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (58). TLRs 2, 3, 4, and 9 have been identified on osteoclast precursor cells and are key to stimulating maturation, while mature osteoclasts express TLR 2 and 4 (58,59). All ten functional human TLRs, except for TLR3, use the MyD88-dependant signaling pathway once activated (58). Each TLR has specific agonist that it responds to, such as PGN (TLR2), viral ds-RNA (TLR3), LPS (TLR4), and bacterial DNA (TLR9) (58,59). These TLRs are present on osteoclasts and act synergistically (59). When osteoclast precursor cells are committed to become mature osteoclasts by binding RANK-ligand with membrane bound RANK, TLR 2, 3, 4, and 9 all

promote osteoclast maturation (59). Furthermore, TLR 4 and 9 stimulate an increase in IL-6 and TNF-α which feedback to bone marrow stromal cells to produce more RANK-ligand (59). However, if an osteoclast precursor cell is not yet committed with RANK/RANKL, TLR 9 activation will inhibit osteoclastogenesis by decreasing expression of membrane bound RANK and M-CSF receptor (59).

In vitro osteoclastogenesis assays using RANKL and M-CSF (43) pretreated bone marrow cells demonstrated that further stimulation with ODE, LPS, or peptidoglycan promoted osteoclast maturation as assessed by cell surface mRANKL expression (43). In comparison to WT, cells derived from TLR4 knockout mice demonstrated reduced expression of mRANKL following ODE treatment whereas TLR2 knockout cells demonstrated a normative response (43). Consistent with these in vitro studies, in vivo studies of TLR4 knockout mice treated with ODE inhalation, OCP populations were not increased. However, in both wild type and TLR2 knockout animals treated with ODE, these OCP populations were significantly increased compared to saline controls (43). Collectively, these studies suggest that TLR4 is sufficient to promote osteoclastogenesis following ODE exposure.

Host Factors

Host factors influence disease development and severity. In COPD, morbidity and mortality worsens with age (60,61). COPD is more common with male gender, history of frequent respiratory infections, smoking history, and alpha-1-anti-trypsin genotypes (62). Bone disease is also dependent upon several host factors. Gender, age, and body mass index (BMI) are associated with lower vitamin D levels and increase systemic TNF-α (63). Lower estrogen levels increase bone resorption and osteoporosis (64). Increased BMI and increased estrogen levels have been shown to improve bone regeneration following maxillary bone regeneration procedures (65). Host factors likely effect the lung-bone inflammatory axis.

American farmers are an aging population, with approximately 30% being older than 65 years of age (66). The impact of age in ODE inhalation-induced bone loss was investigated in a study of mice that were 7 to 9 weeks old (young mice) as compared to 12 to 14 month old mice (older mice) (52). This age difference approximately correlates to human ages of 20’s and 50’s, respectively. Older mice were noted to have less lung neutrophil influx and cytokine release following acute (one-time) ODE exposure and an increased lymphocyte and macrophage influx following repetitive (3 week) ODE exposures as compared to younger animals. Histological examination of the lungs also revealed an increase in alveolar inflammatory infiltrates in the older mice after ODE repetitive exposure, but no difference in the bronchiolar infiltrates. Older mice also had an increase in lung CD8+ T lymphocytes compared to the younger animals. As compared to the older animals, the younger mice demonstrated an increase in peripheral blood neutrophils and serum IL-6 following acute ODE inhalant exposure. Interestingly, the serum IL-6 response lessened with repetitive ODE exposures in the younger mice, but remained elevated in the older mice. Although the older animals had a blunted airway inflammatory response to ODE, they had higher inflammatory markers at baseline and a sustained systemic response with repetitive exposure.

Given the sustained systemic IL-6 response in the older animals, it was postulated that these older mice would have worse bone deterioration following repetitive ODE exposure. However, the younger, but not older, animals were susceptible to ODE-induced bone deterioration as assessed by micro-CT analysis of the distal tibia. Not surprisingly, in comparing saline control young and older mice, the older mice had considerably more bone loss and features of bone deterioration than the younger animals. This suggests that older age alone is a risk factor for bone deterioration, but that inhalant ODE exposure could have a greater impact on the young.

Current and Future Therapies

A mainstay treatment of osteoporosis has included bisphosphonates, which incorporate into bone structure to prevent resorption and cause general osteoclast apoptosis (67). With improved understanding of bone homeostasis, newer therapies have emerged that target specific pathways. Hormone based therapies include the selective estrogen receptor modulators (SERMs), such as Raloxifene, that are estrogen agonists which promote bone homeostasis (68). Teriparatide is a recombinant DNA which mimics parathyroid hormone and promotes osteoblast activity (68). Both of these therapies target specific bone-hormone interactions. Although this has improved treatment, these medications still carries potential side effects from altered hormone homeostasis such as thromboembolism, headaches, muscle cramping, nausea, or diarrhea (68). Targeting osteoclastogenesis directly could render potentially more effective treatment options with fewer side effects. One such pathway is the RANK/RANKL osteoclast interaction that stimulates osteoclastogenesis. Denosumab is a human monoclonal antibody that targets RANKL and inhibits osteoclastogenesis (69). Osteoprotegerin is a receptor protein that binds RANKL and inhibits osteoclastogenesis (70). Although these both have promise in osteoporosis treatment, denosumab use has an association with increased risk of infections (71) and osteoprotegerin use has an association with increased heart disease (72). Tocilizumab is an antibody that binds soluble and membrane bound IL-6 and has been approved for treatment of rheumatoid arthritis (73), but it might also increase the risk of upper airway infections (74). The IL-6 signaling pathway is comprised of two pathways called the classic and the *trans* pathways, which has been generally considered anti-inflammatory and pro-inflammatory, respectively (75). The *trans* IL-6-signaling pathway is dependent on a transmembrane protein named gp130, and current research is investigating the benefit of trans-signaling blockade via anti-gp130 recombinant protein use in several diseases (76,77,78).

Vitamin D is a low cost and low side-effect profile hormone that has been shown to directly inhibit osteoclastogenesis without compromising other systems (56). Vitamin D is an active hormone that is synthesized in the skin via sunlight exposure or can be absorbed from dietary intake. In a murine study, animals were fed either a low vitamin D (1 IU/g) or a high vitamin D (10 IU/g) diet for 5 weeks, followed by 3 weeks of daily intranasal inhalation of either saline, ODE, or LPS (56). The high vitamin D treatment group demonstrated protection against ODE and LPS-induced bone mineral density loss, bone volume loss, and bone micro-architecture deterioration (56). Furthermore, $TRAP^{+}$ osteoclasts were frequently present in the low vitamin D mice but were nearly absent in the high vitamin D treatment

group exposed to either ODE or LPS (56). There was no difference in lung pathology, airway neutrophil influx, lavage fluid levels of TNF-α, IL-6, IL-1β, IL-17A, and IFN-γ, or serum IL-6 levels between the ODE exposed low and high vitamin D treatment groups (56). The only detectable difference was a slight increase in serum IL-10 levels in the high vitamin D treatment group (56). This suggested that vitamin D plays a selective role in preventing osteoclast associated bone reabsorption without altering the airway inflammatory response. Other studies have confirmed that active vitamin D can indirectly inhibit osteoclastogenesis via decreased expression of RANKL from bone marrow stromal cells (79) or increase expression of osteoprotegerin (80). Vitamin D has also been shown to suppress c-fos (81) and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) (82), which are both transcription factors required for osteoclast maturation.

Conclusion

Occupational agricultural work exposure and the development of musculoskeletal diseases have been well-recognized for more than two decades. With studies linking bone mineral density loss with COPD and asthma, recent studies have emerged investigating bone loss and mechanisms mediating bone loss following agriculture-related organic dust-induced airway injury in animal models. TLR4- and IL-6 signaling pathway have been shown to be important in explaining bone consequences following organic dust extract induced bone deterioration. As osteoclastogenesis is recognized as a primary mechanism of inflammatory disease related bone loss, it has been demonstrated that agriculture organic dusts induce osteoclasts/osteoclastogenesis. These events are also dependent upon TLR4 and IL-6 signaling pathways. The agricultural workforce is an aging population and whereas older age alone is an independent risk factor for bone loss, studies have shown that younger animals exposed to organic dusts appear to have increased susceptibility to bone loss following airway injury. Thus, attention to younger workers' risk of potentially developing bone disease from occupational exposures is warranted. Vitamin D has been shown to prevent bone loss induced by inhalant organic dust exposure, which was associated with inhibition of osteoclastogenesis. Vitamin D supplementation is an inexpensive intervention that could be promoted among the agricultural workforce for bone loss/fracture risk protection.

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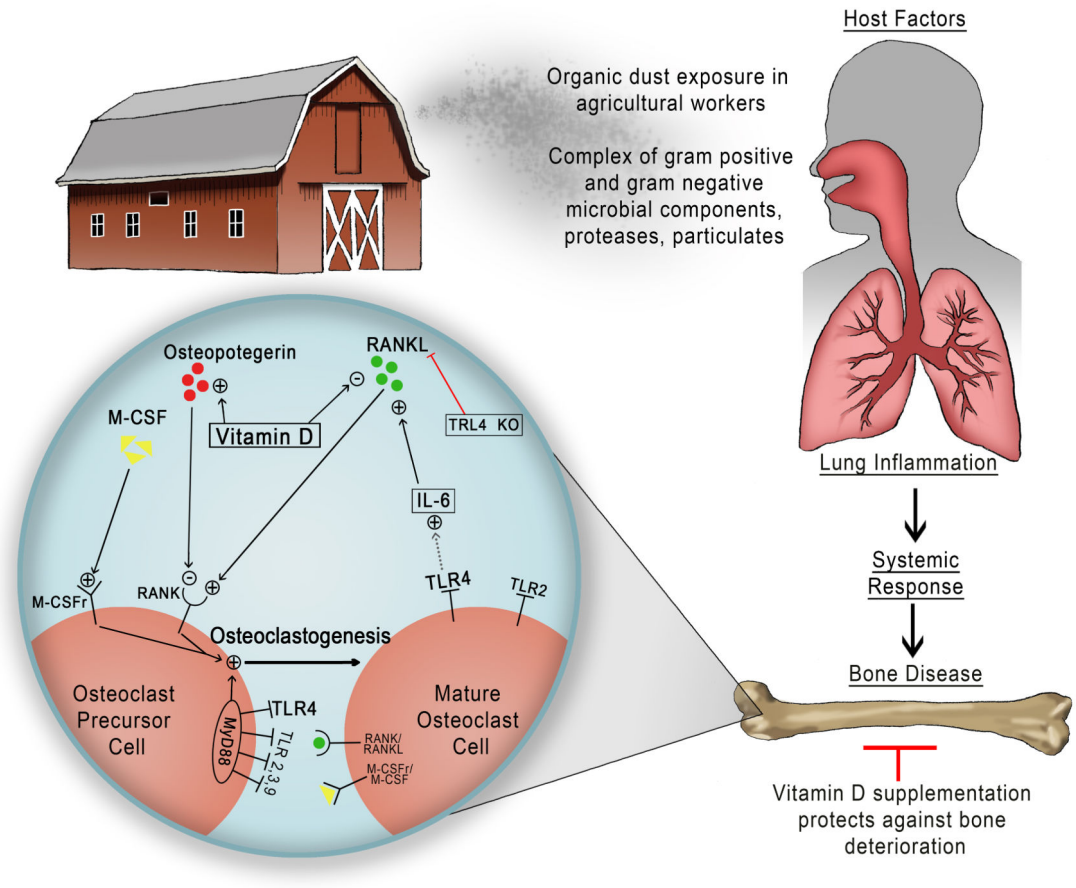

Host Factors: Age.

Lung Inflammation: TLR2/TLR4 – MyD88 dependent; Scavenger Receptor A, nucleotide oligomerization domain (NOD)2, and protein kinase C pathway involvement; TNF-α, IL-6, CXCL1, CXCL1 release; Neutrophil influx; Th1/Th17 and B lymphocytes; Activated macrophages.

Systemic Response: Increased serum IL-6; Neutrophilia; Bone marrow osteoclast precursor cells; Increased TRACP 5b.

Bone Disease: Bone mineral density loss; Bone deterioration; increased osteoclasts; TLR4 dependent; IL-6 dependent.

Figure 1. Overview schematic of the proposed relationship of inhalant organic dust exposures induction of bone loss.

Inhalation of complex agriculture organic dust induces an airway inflammatory response that leads to a systemic response and bone deterioration findings. The "+" sign = positive activator of the pathway. The "-" sign = negative influence or blockade of the pathway. M-CSF = macrophage colony-stimulating factor. M-CSFr = macrophage colony-stimulating factor receptor. RANK = receptor activator of nuclear factor kappa-β. RANKL = receptor activator of nuclear factor kappa-β ligand. TLR = Toll-like receptor. MyD88 = myeloid differentiation factor 88. IL-6 = Interleukin-6.