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Aging in an osteoimmunological context; the contribution of the macrophage.

by
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DISSERTATION

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Abstract

Aging in an osteoimmunological context; the contribution of the macrophage.

Daniel Clark

Aging is characterized by physiologic changes leading to a predisposition to a myriad of age-related diseases. Accompanying these age-related diseases is a process of systemic inflammatory dysregulation known as inflamm-aging. This body of work focuses on fracture healing and periodontal disease, as both involve dysregulation of inflammation within bone and both demonstrate increased complications or prevalence with age. We chose to investigate the macrophage and its contribution to age-related pathologies affecting bone. Macrophages are important regulators of inflammation during fracture healing and periodontal disease. An improved understanding of the age-related changes to macrophages will advance our understanding of the biology of aging and lead to enhanced healthcare for the aging population.

Mouse models of fracture healing and periodontal disease using young and old mice were employed in this work. Macrophages from young and old mice were characterized via RNA-seq. Macrophage were depleted pharmacologically during disease or fracture healing to elucidate the contribution of macrophages within the given models.

The results demonstrated that macrophages from old mice present an aged-macrophage phenotype with increased pro-inflammatory and M1 gene expression. By eliminating the influence of the aged macrophage phenotype via macrophage depletion, periodontal disease severity was significantly reduced and fracture healing was significantly improved. Further, we have demonstrated that TREM2 expression on macrophages is decreased with age which drove increased inflammatory cytokine expression and poorer fracture healing outcomes. Taken together, the aged-related changes that occur to the macrophage are likely involved in numerous disease pathologies, and this work presents potential therapeutic targets to address the macrophage-driven inflammatory dysregulation in the elderly population.

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Chapter 1. The effects of aging on fracture repair

1.1 Introduction

The elderly population in the United States has been steadily increasing, and those aged 65 years old and older are expected to comprise 17% of the population by 2030 (1,2). This growing population presents their own unique health needs, and in order to meet these needs a better understanding of the physiologic changes that occur with aging is necessary. The skeletal system exhibits physiologic changes that occur with increasing age. Conditions such as osteoporosis and osteoarthritis increase with age. Additionally, many reports demonstrate a higher rate of bone fracture, and these are associated with increased morbidity and mortality (3,4,5). A decline in healing potential is observed in the elderly, and this contributes to increased rates of delayed healing or non-unions (6). Delayed healing, and the resulting incapacitation, can have more severe and systemic consequences in the elderly, which poses unique challenges for the treating clinician (3,7). While increased age has been generally associated with a wide range of physiological changes, the mechanisms that result in decreased capacity for fracture healing are not fully understood.

An understanding of the age-related effects to fracture healing is complicated by a lack of a complete understanding of fracture healing in healthy and in young individuals. However, by analyzing the individual facets of fracture healing that we do understand we can compare differences in fracture healing between the young and old humans and animals. This review will highlight the phases of fracture healing and the respective physiologic changes that occur with age. Cellular, molecular and genetic differences between young and old humans and animal models will be characterized to illustrate the current understanding of the effect of age on bone fracture healing. Figure 1.1 summarizes the effect of age on critical cells that contribute to fracture healing.

1.2 Physiology of bone fracture healing

Fracture healing proceeds through multiple phases characterized by anabolic and catabolic processes (8). The early healing stage is characterized by a robust inflammatory response that is responsible for debriding the fracture site and contributing to the signaling milieu that will propagate the successive stages of healing, including recruitment and differentiation of skeletal tissue progenitor cells (9). The anabolic phase follows the initial inflammatory response. Progenitor cells give rise to a soft callus with a central cartilaginous region and new bone formation at the periphery (9,10). The soft callus is characterized by avascular cartilage tissue that induces vascularization (11). A hard callus begins to develop through endochondral ossification with increased mineralization and replacement of chondrocytes with osteoblast, in part, through transdifferentiation (12,13). Finally, remodeling of the callus occurs through catabolic processes. The callus is reduced in size and osteoblastic and osteoclastic processes alternate to reestablish the normal hematopoietic and trabecular structure, restoring bone to its pre-injured state (14).

Histological and molecular changes within the callus have been described during the stages of fracture healing as mentioned above and provide healing comparisons between old and young animals. Numerous studies have reported delayed fracture healing in elderly animals and have shown decreased cartilage and bone formation, delayed cartilage resorption, and slower mineralization within the callus (15,16,17). Delayed bone healing may be associated with age-related changes in the osteochondral stem cells. In general, there is an age-related decrease of stem cell quantity compounded by a decrease in proliferation and differentiation potential as demonstrated in humans and animal models (18,19,20). Lopas et al. demonstrated a decrease in osteochondral stem cell proliferation associated with a significant decrease in bone and cartilage content within the fracture callus of aged mice compared to young (15).

Chondrocytes and osteoblasts arise from stem cells predominantly located in the periosteum during fracture healing (9,21,22). Senescence and greater oxidative damage was associated with

periosteal derived progenitor cells from old humans compared to young (23). Additionally, a decreased number of periosteal cells were able to be derived from the periosteum of old humans compared to young (21). The chondrogenic potential of stem cells in the periosteum is decreased in elderly mice compared to young mice, and chondrogenic differentiation from periosteal cells is delayed in old versus young mice (22,24). Older animals have delayed expression of Type 2 collagen(CollII) and delayed cartilage matrix deposition at early time points of fracture healing (24). Similarly, osteoblast differentiation and osteocalcin expression is delayed from periosteal cells at the fracture site in elderly mice compared to young mice (24).

Stem cells contributing to fracture healing may also arise from other tissue sources that could be negatively affected by age related changes. Cells located in muscle appear to contribute to bone fracture healing. While muscle stem cells, known as satellite cells, may contribute only a small number of cells that comprise the skeletal tissues, they appear to regulate fracture healing possibly through signaling interactions (25). With aging, satellite cell quantity and function decline which may negatively affect their ability to support fracture healing (26). However, the role of other cell types that reside in muscle is not known and worthy of investigation. The bone marrow is also a potential source of osteochondral stem cells (27,28). Similar to satellite cells, aging results in decreased function and quantity of bone marrow stem cells that could have a negative effect on fracture healing (29,30).

Age also affects later stages of healing during endochondral ossification. The characteristic histological findings that describe endochondral ossification, hypertrophic chondrocytes, expression of type 10 collagen (ColX), and vascular invasion were all delayed in elderly mice compared to young mice (24). Completion of endochondral ossification, characterized by complete conversion of cartilage to bone within the callus, was also delayed in elderly mice versus young mice (24).

The age-related alterations to the cellular processes that are evident during fracture healing are accompanied by changes in the regulation of critical genes involved in bone fracture healing. By

using whole genome expression analysis of fracture calluses in rats, significant differential regulation of 144 genes were found in young compared to elderly rats (31). Functional analysis of these genes suggested they were largely involved in cell migration (31). More specifically, bone morphogenic protein (*Bmp-2*) and Indian Hedgehog (*Ihh*) expression in the fracture callus of elderly rats was decreased compared to young rats (32). Other studies have shown comparable levels of gene expression in the early time points of fracture healing; however, in elderly rats expression of *Ihh*, *Bmp*, and *Tgf-β* decreased at the same rate as the young rats despite requiring almost twice as long for complete healing to be detected radiographically (33). Thus, molecular changes occurring in cells comprising the fracture callus are evident in animals of advanced age, and these changes may contribute to the alterations in healing that are observed in older animals. However, systemic changes as a result of the normal process of aging also occur and could contribute to the delays in healing.

1.3 Inflammation and fracture healing

The initial phase of fracture healing is characterized by a robust inflammatory response. Secretion of pro-inflammatory cytokines at this time are necessary to initiate the healing process and achieve adequate healing (34). Temporal control of the inflammatory response is crucial for proper bone fracture healing. After the initial pro-inflammatory phase, inflammation must be resolved in order to allow the anabolic processes to begin and continue the subsequent healing phases. However, changes in the inflammatory system occur with age, and chronic inflammation and/or a decreased ability to resolve inflammatory processes could negatively affect bone fracture healing.

1.4 Inflamm-aging and immunosenescence

An excessive or prolonged inflammatory phase can have detrimental effects on fracture healing (35,36). In animal models, prolonged inflammation results in delayed chondrogenesis and smaller callus size (37). Recently, it was shown that elevated systemic levels of a pro-inflammatory cytokine, TNF α , in induced diabetic mice negatively affected angiogenesis during fracture healing

(38). In humans, an elevated inflammatory status is related to certain systemic conditions, including diabetes, smoking, and increased age. These conditions are all associated with poorer fracture healing outcomes, but the mechanisms remain incompletely understood. The term “inflamm-aging” has been used to describe a chronic increased pro-inflammatory status in the elderly (39). Elderly people are found to have higher levels of circulating pro-inflammatory cytokines, even in healthy individuals. It appears the increased pro-inflammatory status in the elderly predisposes them to range of systemic diseases including osteoporosis, Alzheimer’s disease, Type II diabetes, atherosclerosis, and Parkinson’s disease (40,41,42). Currently, it is unclear what drives this increased inflammation. Inflamm-aging has been suggested to be the result of a defect in the proper resolution of the normal inflammatory response, or the result of an unknown chronic mechanism that signals and prolongs the inflammatory response (43,44). As the inflammatory response is a critical step in proper fracture healing, any disruption of the inflammatory response could negatively affect fracture healing.

Inflamm-aging may also be a result of age-related changes to the immune response. Aging of the adaptive immune response has been described as immunosenescence (45). Immunosenescence describes a loss of immune function that is associated with a predisposition to infection and disease in the elderly (45). Increased age is associated with changes in T and B cell production and maturation. T cell production and maturation is negatively affected by age related changes to the bone marrow hematopoietic compartment and to the thymus (46,47). With increasing age the source of T cell progenitors, the hematopoietic compartment, decreases in size and is associated with a decrease in T cell progenitor quantity and proliferation potential (47). The T cell progenitors migrate to the thymus for further differentiation and maturation. A decrease in thymus function and T cell output is seen with increasing age as involution and atrophic changes are present (46). The lack of a sufficient quantity of T cells produced by the thymus negatively affects the ability to mount an effective immune response. Additionally, the lack of T cells limits the availability of regulatory T cells that are present to resolve the mounted immune

and inflammatory response (46,48). Immune cell trafficking has also been shown to be of importance in fracture healing studies showing impaired fracture healing in CCR2 deficient mice (49). CCR2 is a chemokine receptor for the ligands Ccl2, Ccl7, and Ccl12 that is expressed on monocytes, myeloid derived cells, a subset of T cells and mesenchymal stem cells. Recent studies in a muscle regeneration model have suggested that CCR2 deficiency in young mice results in an inflamm-aging environment similar to changes seen with aging (50). In this way, it has been proposed that the elevated pro-inflammatory status of inflamm-aging may be a response to age-related defects of the immune response. Figure 1.2 demonstrates a conceptual model of the effects of inflammatory response dysregulation on the subsequent stages of fracture healing. The age-related effects on inflammation during fracture healing can be investigated independently of other age-related changes to the organism by using a chimeric animal model. Xing et al. used such a chimeric model of lethal irradiation followed by bone marrow transplant (51). By irradiating aged mice and transplanting the bone marrow of young mice to those animals, investigators found that the osteochondral stem cells were derived from the aged host and the inflammatory cells were derived from the young donor in a fracture model. In this manner, the older mice receiving young bone marrow had larger calluses and more bone in the early healing time points, more rapid callus remodeling at later stages of healing, and a rejuvenation of the inflammatory response compared to older mice receiving age-matched bone marrow transplants (51). A similar study by Baht et al. utilized a parabiosis and a bone marrow transplant model of old and young mice in a fracture healing study (52). Fractures in old mice healed significantly better with shared circulation from a young mouse or with bone marrow transplants from young mice compared to age-matched old mouse donors. No cells from the young donor circulation or bone marrow differentiated into skeletal cells in the callus suggesting a component of the circulation or bone marrow could revert osteochondral stem cells of old mice to a more youthful phenotype. They further found that β -catenin was differentially expressed in old and young mice during fracture healing. Shared circulation with young mice resulted in decreased levels of β -catenin and was

associated with improved fracture healing. Decreasing β -catenin activity in old mice resulted in similar improvements in healing as sharing circulation with young mice. The two studies described above suggest a crucial component regulating fracture healing of the hematopoietic environment that is affected by age-related changes.

1.4 Cellular regulation of inflammation

An understanding of the intrinsic age-related changes to cells involved in the inflammatory response may explain, in part, the poorer healing potential in the elderly. Cells of the innate and adaptive immune system local to the fracture site assist in regulating the inflammatory response. Macrophages are powerful regulators of inflammation. In the early inflammatory stage of healing, macrophages are classically activated and have an M1 phenotype (53,54). M1 macrophages are pro-inflammatory and release cytokines IL-1, IL-6, TNF- α , iNOS to elicit and propagate the inflammatory response (53,54). As inflammation is down-regulated in the later phases of fracture healing the macrophages acquire an alternatively activated M2 state. M2 macrophages express anti-inflammatory cytokines, such as IL-10 and promote healing through secretion of growth factors TGF β , VEGF, and PDGF (53,55). Temporal control of the polarization of M1 and M2 macrophages is important to regulate inflammation during the healing process.

Intrinsic age-related changes to macrophages may perturb the inflammatory response in the elderly and may have negative consequences for fracture healing. Aged macrophages were found to be less responsive to granulocyte macrophage colony-stimulating factor (GM-CSF) that resulted in decreased proliferation compared to young macrophages (56). Additionally, elevated serum levels of chitotriosidase, a marker for chronically activated macrophages, was found in elderly humans compared to young controls (57). The negative effects of age-related changes to macrophages are further supported in animal models of healing. Cutaneous wounds heal slower in aged mice and can be rescued with transplants of macrophages from young mice (58). Further, aged macrophages appear to be detrimental to healing. In blocking macrophage recruitment to the fracture site, elderly mice appear to exhibit better fracture healing compared to elderly mice

that had normal macrophage activity (59). Conversely, in the same experiment, blocking macrophage recruitment to the fracture site in young mice had a negative effect on fracture healing (59).

Macrophages are known to contribute to bone healing. A tissue resident population of macrophages, termed “osteomacs,” has recently been described (60). Osteomacs regulate osteoblast function and promote fracture healing; blocking their activity has deleterious effects on bone healing (60,61). While it appears clear that circulating macrophages that are recruited to the site of bone injury have age-related changes that hinder fracture healing, whether age-related changes occur in osteomacs is not known.

Cells of the adaptive immune system also contribute to fracture healing (62). T and B cells are present within the early callus during the inflammatory phase (63). Regulatory B cells negatively regulate inflammation through expression of IL-10 and downregulating pro-inflammatory cytokine expression in T cells within the callus (64). B cells from patients with poor fracture healing outcomes had decreased expression of IL-10 compared to patients that healed normally (64). As healing continues, cartilage formation within the callus is associated with a local increase in Treg cells (65). At later time points of healing, T and B cells are readily present at sites of mineralization and in direct contact with osteoblasts and osteoclasts (63). Models of fracture healing using mice with genetic modifications have further elucidated the role of T and B cells. Deficient T and B cell function in Rag1^{-/-} mice demonstrated impaired fracture healing which was associated with decreased IL-17F expression (66). IL-17F was shown to promote osteoblast maturation in vivo (66). Additionally, dysregulated T cell recruitment and activation within the bone in a mouse model of lupus was associated with increased bone turnover and decreased bone fraction within the fracture callus (65). As discussed above, T and B cell quantity and function are negatively affected by age. Due to the involvement of T and B cells in fracture healing, such age-related changes could have a negative effect on fracture healing.

Mesenchymal stem cells are also involved in the inflammatory response and act as powerful immunomodulators at the site of injury to control excessive inflammation and promote repair (67,68,69). Suppression of inflammation mediated by MSCs occurs, in part, through signaling and interactions with local inflammatory cells. MSCs have been shown to interact with T-cells, B-cells, NK cells, and dendritic cells through signaling that limits proliferation of inflammatory cells or promotes secretion of anti-inflammatory molecules (70). Additionally, research has suggested MSC and macrophages interact to promote alternatively activated macrophages in the downregulation of inflammation and promotion of healing (71). The immunomodulatory properties of MSCs have been utilized clinically and have been reported beneficial in the treatment of graft versus host disease, Crohn's disease, renal failure, and heart failure (72-75).

Intrinsic age-related changes to MSCs could explain the impaired healing in older humans and animals as a result of dysregulated inflammatory response. The quantity of MSCs isolated from bone marrow is decreased with age (30). Additionally, there are increased markers for oxidative damage of MSCs from elderly human samples (58). These age-related changes to MSCs corroborate findings in experimental wound healing studies that show a benefit of therapeutic administration of MSCs from young animals but no benefit in healing with aged MSCs (58).

1.5 Vasculature and fracture healing

Successful bone fracture healing requires adequate vascularization of the tissue (76). The contribution of the vasculature to fracture healing includes providing a blood supply for delivery of nutrients and cells, providing the endothelial cells that express angiogenic and osteogenic signaling molecules locally, and providing the source of oxygen to the healing callus (9,77,78). However, the complete contribution of the vasculature to fracture healing is not fully understood. With increasing age, perturbations in bone fracture healing are associated with age-related dysfunction to the bone vascular system and its ability to regenerate in healing. Generally, the vascular perfusion of the skeleton decreases with age (79). Elderly rats display significantly higher ossifications and decreased patency of bone marrow blood vessels compared to younger

rats (80). The decrease in vascularization at the time of fracture may delay angiogenesis during the fracture healing. The fracture callus at early healing time points in young mice have a higher surface density of blood vessels compared to the elderly mice (77). The increase in vascular density was associated with an early detection of vascular endothelial growth factor (VEGF) and Hypoxia inducible factor 1 α (HIF-1 α) in young mice but not elderly (77). Additionally, increased Mmp9 and Mmp13 transcripts were detected throughout early and late stage healing in young compared to old mice (77). Differential expression of angiogenic factors, including VEGF, HIF-1 α , and Mmps, have been well demonstrated in young animals versus old during bone fracture healing (81,82,83).

1.6 Conclusions

Increasing age has been shown to negatively affect the cellular and molecular processes throughout the different stages of bone fracture healing. Inflammatory regulation, cellular differentiation and signaling cascades are all affected, in part, by age-related changes. Our current understanding of these age-related changes explains, only partially, the decreased healing potential and increased complications observed during fracture healing in elderly patients. A more complete understanding is necessary to allow for therapeutics to target the specific age-related deficiencies and provide better care for the increasing geriatric population.

Figures: Chapter 1

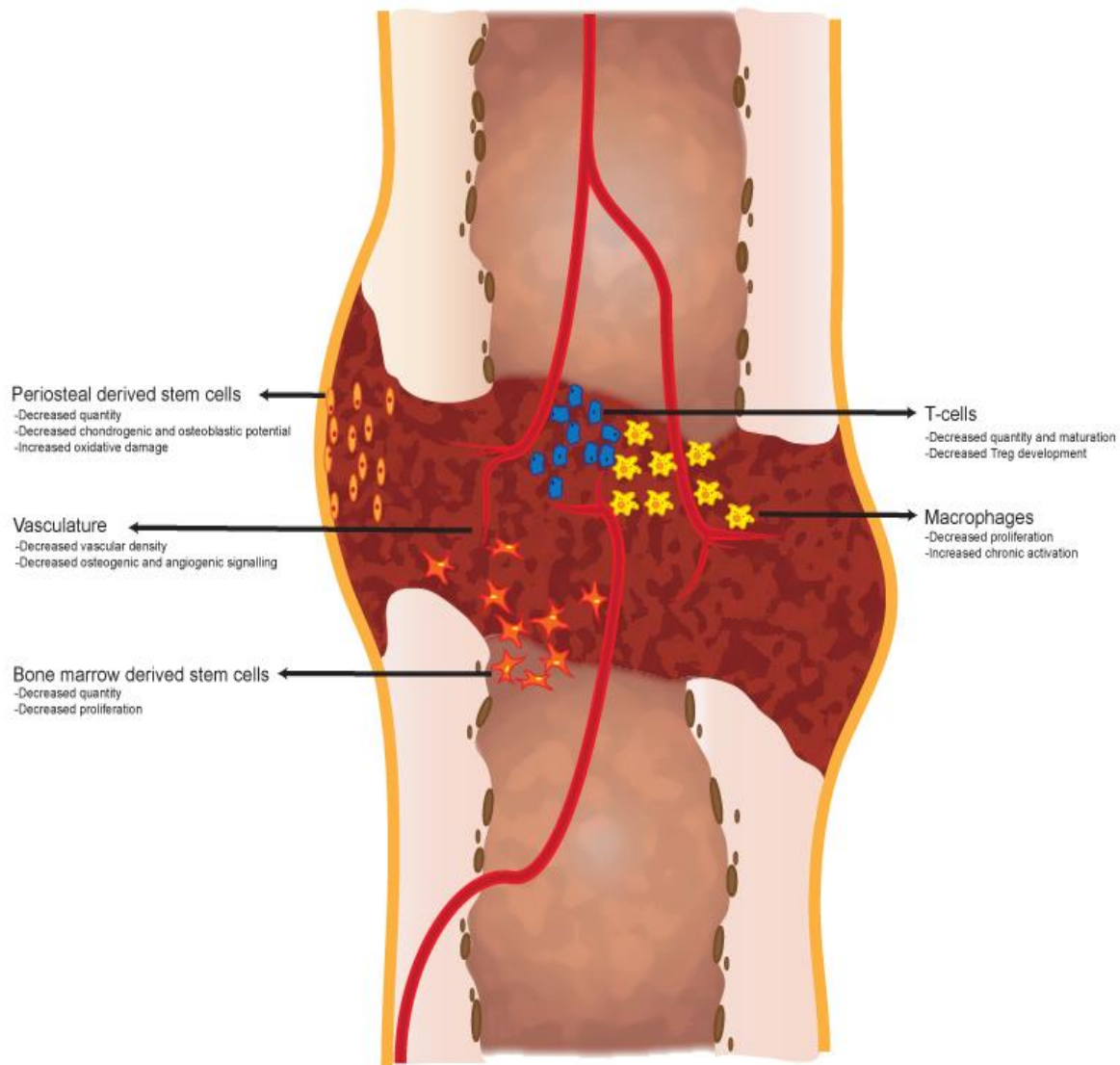


Figure 1.1 The effect of age on the cellular contribution to fracture healing. Stem cells and immune cells involved in fracture healing demonstrate age-related changes that may negatively affect fracture healing. Osteochondral stem cells arise from the periosteum and bone marrow and demonstrate decreased quantity, increased oxidative damage, and decreased osteoblastic and chondrogenic differentiation potential with age. T-cells contribute to fracture healing and production and maturation of T-cells is negatively affected by age related changes to the bone marrow hematopoietic compartment and to the thymus. Macrophages are important regulators of inflammation during fracture healing. Aged macrophages demonstrate decreased proliferation and increased activation that may contribute to the poorer healing outcomes associated with aged macrophages compared to young. Finally, adequate vascularization is required for successful fracture healing. Aged animals demonstrate decreased vascular density within the callus which is associated with decreased levels of key angiogenic factors required for healing.

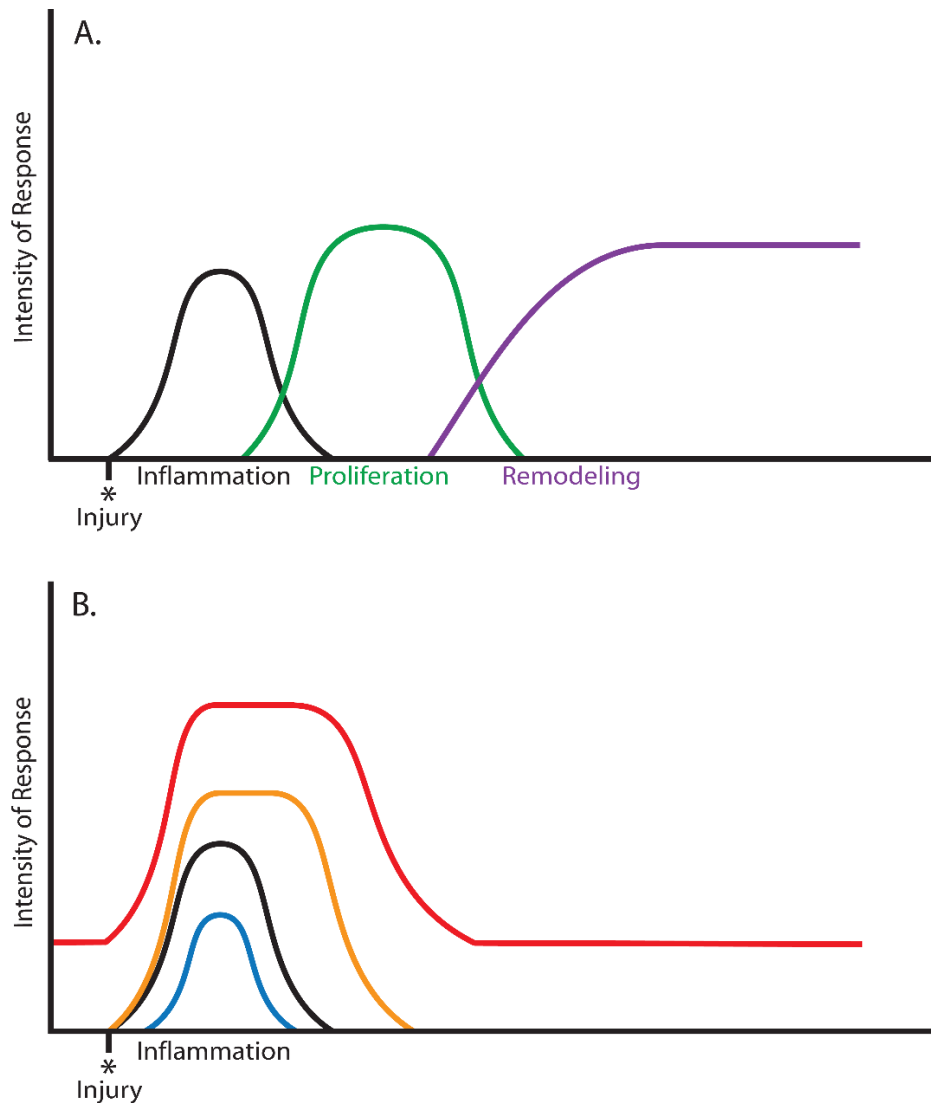


Figure 1.2. The effects of inflammatory response perturbation on the stages of fracture healing. (A) Fracture healing follows three general stages of inflammation, proliferation and remodeling. The initial inflammatory response is tightly regulated and crucial in initiating the subsequent stages of healing. (B) Systemic conditions, including increased age, have an effect on inflammation and may result in differential inflammatory responses during fracture healing. Senescence of the inflammatory response results in a decreased and limited inflammatory response (blue curve) that may result in inadequate activation of the proceeding healing stages. An exaggerated and sustained response (orange and red curves) can result from inadequate resolution of the response and may negatively affect the proceeding stages. An increased basal level of inflammation (red curve) is proposed to occur with inflamm-aging and would have possible negative effects throughout the healing process.

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Chapter 2: The effects of aging on periodontal disease

2.1 Introduction

Periodontal disease affects 48% of adults in the United States (1). Disease is initiated by a host inflammatory response to the local presence of bacterial plaque (2). Inadequate clearance or sustained presence of the bacterial plaque will prolong the host inflammatory response and eventually lead to tissue damage to the host (2). The sustained host response can eventually lead to the induction of periodontal disease with the associated loss of tooth-supporting soft and hard tissue. Through the progression from health to periodontal disease, complex changes occur to the microbial composition as well as the cellular and molecular immune response that all contribute to the pathogenesis of periodontal disease (3).

The prevalence and severity of periodontal disease increases with age, affecting over 60% of those over the age of 65 (1,4). As periodontal disease progresses, the loss of tooth support results in difficulty eating, speaking, unacceptable esthetics, and ultimately to the loss of teeth (5). Periodontal disease in elderly individuals can be detrimental to their systemic health. A lack of proper function when eating can restrict their diet and result in malnourishment (6). Similarly, the systemic inflammatory burden as a result of periodontal disease can contribute to or worsen the myriad of diseases and conditions that have higher prevalence with age, including diabetes, kidney disease, Alzheimer disease, and cardiovascular disease (7). Therefore, it is important to understand why the prevalence of periodontal disease increases with age to allow for more directed treatment in a susceptible population.

2.2 Periodontal disease pathogenesis

During periodontal disease, tissue destruction is ultimately caused by the host inflammatory response. Therefore, it is important to understand how the inflammatory response changes as a function of age. Periodontal disease is one of many diseases that become more prevalent with increased age. As described in the previous section, diseases such as Alzheimer's disease, Type

II diabetes, atherosclerosis, and Parkinson's disease all become more prevalent with increased age and all share an underlying dysregulated inflammatory component that predisposes or contributes to such diseases. The term inflamm-aging has been used to describe the dysregulated pro-inflammatory status in the elderly (8). It is unknown if inflamm-aging results from a chronic signal that maintains the inflammatory response or from a defect in the resolution of inflammation (9). As periodontal disease arises from a dysregulated host response, it can be appreciated how the dysregulation that occurs with inflamm-aging would impact the pathogenesis of periodontal disease in elderly individuals. Disruption of systemic inflammation has been shown to be associated with periodontal disease. Systemic disease such as type II diabetes and obesity are characterized by elevated systemic inflammation and both diseases demonstrate increased prevalence of periodontal disease. In obese patients, higher levels of circulating TNF α was associated with the higher prevalence of periodontal disease in those patients (10). In diabetic patients, higher blood glucose levels were associated with increased alveolar bone loss (11). It also should be noted that there is a likely a bidirectional relationship, where there is an increased systemic inflammatory burden as a result of periodontal disease that can predispose or worsen type II diabetes and obesity in an individual (12). The association of periodontal disease with obesity and type II diabetes again points to a shared underlying inflammatory component.

The cause of inflamm-aging remains unknown and is likely multifactorial. Investigating age-related changes in immune cells may lead to a better understanding of the biology of aging and could lead to therapeutic targets to mitigate the effects of inflamm-aging in periodontal disease. Cells of the innate and adaptive immune response are involved in the pathogenesis of periodontal disease. Macrophages and T cells are both important regulators of inflammation and have demonstrated age-related changes. Their role in periodontal disease and possible contribution to inflamm-aging is expanded upon below.

2.3 Macrophages and the innate immune response

Age-related changes to cells of the innate immune response have largely been implicated in inflamm-aging. The innate immune system is responsible for the initial host response to bacterial plaque (13). Neutrophils, macrophages, natural killer cells, and dendritic cells are involved in the innate response, with each cell type presenting different aging phenotypes (8,30). The macrophage is a key modulator of the innate immune system and can display both a pro-inflammatory (M1) and anti-inflammatory (M2) phenotype to regulate the disease process (14,15). The M1 phenotype is involved with bacterial killing and, thus, is present in response to bacterial plaque during periodontal disease (13). After adequate removal of the bacterial plaque, a timely phenotypic switch away from M1 is required to prevent tissue damage as a result of the pro-inflammatory phenotype, and the M2 phenotype is then present to aid in the resolution of inflammation and promote healing of damaged tissues. The presence of M1 and M2 type macrophages has been shown to be related to increased and decreased severity of periodontal disease respectively (16,17). The higher prevalence of M1 macrophages was associated with increased inflammatory cytokines within the gingiva of humans with periodontal disease compared to healthy controls (18).

The plasticity of the macrophage makes it an interesting cell to study, as control over the differing phenotypes is an attractive therapeutic target. Systemic inflammatory diseases, such as metabolic syndrome, cardiovascular disease, and rheumatoid arthritis, have shown the involvement of macrophages in their pathogenesis, and treatment directed towards macrophages in these conditions has shown promise (19,20,21,22). Additionally, chronic inflammatory conditions affecting the bone, such as arthritis, have shown improvements in animal models by depleting the macrophages (23), further suggesting that macrophages are a viable therapeutic target in osteolytic diseases like periodontitis

As described in the previous chapter, macrophages have demonstrated age-related changes that affect cytokine and growth factor production as well as being implicated in poor wound healing outcomes. The systemic disease mentioned above (metabolic syndrome, cardiovascular disease, rheumatoid arthritis) that have benefited from macrophage-targeting therapies are also disease that are more prevalent with increased age. This observation further suggests that age-related changes to macrophages may contribute to inflamm-aging and the associated age-related diseases such as periodontal disease.

In general, the age-related changes to macrophages would be predicted to contribute to the pathogenesis of periodontal disease by promoting a local increased pro-inflammatory status. Circulating macrophages from elderly individuals demonstrated an increased pro-inflammatory status (24,25). Defects in phagocytosis in old macrophages have also been demonstrated (26,27). Inefficient clearance of microbes or necrotic and apoptotic debris within the periodontium would result in the maintenance of a pro-inflammatory state. However, age-related changes demonstrated in circulating macrophages or using in vitro models are not necessarily replicated within the periodontium (28,29). To date, a full understanding of the age-related changes to macrophages and its affect on periodontal disease is lacking. However, our understanding of macrophage activity in other diseases suggests that age-related changes to macrophages are a likely contributing factor to the pathogenesis of periodontal disease and could serve as a therapeutic target in the management of disease in both young and elderly patients.

2.4 T Cells and the adaptive immune response

While age-related changes to the innate immune system has been linked to inflamm-aging, age-related changes to the adaptive immune response have generally been associated with immunodeficiency. Activation of the adaptive immune response is characterized by the activity of T cells and B cells in the production of antibodies and cellular immunity (31). Age-related changes to T and B cells affect their activation and proliferation centrally as well as their

recruitment and function local to the site of inflammation (32). The immunodeficiency observed in elderly individual has been associated with immune senescence, specifically a decrease in T cell and B cell function. With increased age there is an involution of the thymus, the site of naïve T cell production, resulting in an overall decline in naïve T cell numbers (33). Similarly, decreased generation of progenitor B cells from the bone marrow is observed with increased age (32). Overall, these age-related declines in naïve B and T cell quantity results in a decline of antigen-specific immunity and the resulting immunodeficiency with increased susceptibility to infection in the elderly (31). The immunodeficient status associated with increased age would be expected to result in a susceptibility to subgingival microbial infection during periodontal disease. However, it is unclear if the increased prevalence of periodontal disease is a result of the immune senescence or from the inflamm-aging phenotype described earlier.

As T cells interact with antigen presenting cells, they further differentiate into sub-populations with distinct functions. CD4+ T helper cells are generally characterized as Th1 and Th2 based upon their cytokine secretory profile and resulting ability to activate pro-inflammatory cells (Th1) or involvement in the resolution of inflammation (Th2) (34). One subset of Th1 cells that has been implicated in periodontal disease is Th17 cells, named after their characteristic production of IL-17 (35). Th17 cell expansion and IL-17 expression are pathogenic mediators in periodontal disease (36,37). There are multiple cellular sources of IL-17 within the gingiva; however, during periodontal disease Th17 cells represent 80% of the IL-17+ cells (36). Inhibition of Th17 cell differentiation was shown to decrease disease severity (36). Interestingly, Th17 cells demonstrate increased homeostatic expansion with increased age (38); however, it is unknown what drives this age-related expansion. Homeostatic Th17 cell expansion requires IL-6 (38), pathogenic expansion of Th17 cells requires IL-6 and IL-23 signaling (35). IL-6 expression from the epithelium has been reported to promote homeostatic expansion of Th17 cells (38); however, other drivers of homeostatic and pathogenic Th17 expansion are unknown. Given the pro-

inflammatory nature of IL-6 and IL-23, cells of the innate immune response known to express those cytokines could be involved in Th17 expansion. The effect of inflamm-aging on those innate cells could result in further production of the cytokines and increased Th17 expansion with age.

2.5 Conclusion

The inflamm-aging phenomenon contributes to the myriad of age-related disease and more recent work has similarly shown inflamm-aging to have a detrimental effect on periodontal disease. While this review focused on the immune system, a host of other age-related changes may also contribute to the increased prevalence of periodontal disease in the elderly. Known age-related changes to the microbiota may have a detrimental effect on periodontal disease (39). Additionally, age-related changes to the periodontal tissue structure and integrity may result in a susceptibility to disease (40). Finally, decreased progenitor capacity of mesenchymal stem cells in older humans may result in perturbed tissue repair after an inflammatory insult during periodontal disease (41,42). These topics, and more, may further contribute to the increased prevalence of periodontal disease in elderly populations and warrant further investigation and discussion.

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Chapter 3: Age-related changes to macrophages and the impact on fracture healing.

3.1 Introduction

Perturbation of the inflammatory phase of fracture repair can have detrimental effects on the healing outcome (1,4). This is evident in patients with chronic inflammatory conditions such as diabetes, rheumatoid arthritis, and increased age (2). The disturbance of inflammation in these conditions is associated with poorer fracture healing outcomes (5-8). Similarly, in experimental animal models, induced local and systemic inflammation has negative effects on fracture healing outcomes (9-11).

As described in Chapter 1, the elderly population, including those in good health, are found to have higher levels of circulating pro-inflammatory cytokines, which is associated with a predisposition to a range of systemic disease including osteoporosis, Alzheimer's disease, Type II diabetes, atherosclerosis, and Parkinson's disease (19-21). The chronic, increased pro-inflammatory status associated with aging is described as "inflamm-aging" (22,23). Currently, the mechanisms responsible for inflamm-aging are unclear. Therefore, understanding how dysregulation of the inflammatory process in elderly populations affects fracture healing represents a critical area for investigation.

To this effort our group has been investigating age-related changes to the macrophage as a possible contributor to the inflammatory dysregulation observed in elderly populations. The macrophage is an important inflammatory cell involved in fracture healing (25,26). Throughout the course of healing, macrophages polarize among various states of inflammation in response to their environment. During early phases of healing macrophages are classically activated and exhibit pro-inflammatory activities. These have been generally considered M1 macrophages (27,28). As healing progresses macrophages switch to anti-inflammatory states and are generally considered M2 macrophages that are responsible for downregulating inflammation and promoting healing (28,29). However, these are very broad categories and not strict definitions of cell types, and in actuality these categories are likely comprised of multiple subsets of macrophages

comprising these populations. Nonetheless, enhancement of M2-like macrophages at the fracture site has been shown to improve fracture repair (30). In addition, tissue-resident macrophages, osteomacs, have been observed in close proximity to osteoblasts on the bone surface, and contribute to osteoblast regulatory functions (31). Macrophages promote osteoblast differentiation during fracture healing (32), and we have also shown the importance of macrophages in fracture healing. Fracture healing in mice that lack *C-C Motif Chemokine Receptor 2 (Ccr2)* exhibits disruption of macrophage trafficking to the fracture callus and delayed fracture healing (33), and others have observed similar results after depletion of macrophages in mouse models (25,30,32). Finally, age-related changes to macrophage activity have been previously demonstrated. Dysregulated chemokine and cytokine expression has been observed in aged macrophages compared to young (34). Additionally, decreased growth factor production is associated with aged macrophages (35). Hence, these changes could significantly impact fracture healing in aged animals.

In this work, our goal was to better understand the contribution of the inflammatory response in aged animals, and specifically the macrophage, during fracture healing. We assessed influx of inflammatory cells to the fracture site in young and elderly mice and used next-generation RNA sequencing to assess age-related changes in the transcriptome of macrophages derived from the fracture callus. Finally, we manipulated macrophages in elderly mice to assess the extent to which they can be targeted for therapy. Our results add to the increasing body of evidence supporting a role for the inflammatory system in bone fracture healing.

3.2. Materials and methods

3.2.1. Animals

All procedures were approved by the UCSF Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines for humane animal care. All mice (C57B6/J) were obtained from the National Institute on Aging's Aged Rodent Colony.

Specific pathogen-free mice were bred and raised in barriers and utilized for experiments at 24 months (old adult mice) or 3 months (young adult mice) of age.

3.2.2. *Tibia fractures*

Mice were anesthetized and subjected to closed, non-stable fractures of the right tibia created by three-point bending, as previously described (36). Analgesics were administered post-surgery and mice were permitted to ambulate freely. To inhibit macrophage recruitment during fracture healing in old mice, treatment groups received the compound PLX3397 (275mg/kg) (Plexikon Inc. Berkeley, CA) *ad libitum* in their chow. Control groups received the same chow without PLX3397. PLX3397 is a small molecule kinase inhibitor of Colony stimulating factor 1 receptor (M-CSF1R) (37). The compound significantly reduces the quantity of macrophages in the fracture callus (38). Treatment with PLX3397 was started 24 hours before fracture and continued for 3 or 10 days after fracture until the animal was euthanized and the tissues harvested for analysis.

3.2.3. *Tibia processing and stereology*

Mice were sacrificed at day 10-post fracture for stereological analysis of the fracture callus. Fractured tibiae were collected and fixed for 24 hours in 4% paraformaldehyde. The tibiae were decalcified in 19% EDTA for 14 days and dehydrated in graded ethanol prior to paraffin embedding. Serial sagittal sections (10 μ m) were cut through the entire tibia using a microtome (Lecia, Bannockburn, IL) and mounted on slides. Sections were stained using Hall Brunt Quadruple Stain (HBQ) to visualize bone and cartilage. An Olympus CAST system (Center Valley, PA) and software by Visiopharm (Hørsholm, Denmark) was used to quantify tissue volumes according to stereological methods developed by Howard and Reed (39). A 2x magnification setting was utilized to outline the boundary of the fracture callus. Then, bone and cartilage were identified and labeled at 20x magnification. The Cavalieri formula was used to estimate the absolute volume of the total callus, bone, and cartilage tissue as previously described (17,24,33).

3.2.4. Flow cytometry

Old and young mice were sacrificed at days 1, 3, 10, and 14 post fracture. Fractured tibiae were collected and the calluses were dissected, weighed, disassociated manually through a 100 μ m nylon cell strainer, and digested with Collagenase type I (0.2mg/mL; Worthington, Lakewood, NJ) for 1 hour at 37 degrees. Cells were rinsed, collected by centrifugation, and resuspended in incubation buffer (0.5% BSA in PBS). Isolated cells were blocked for 10 minutes at room temperature in 10% rat serum and then stained with directly conjugated fluorescent antibodies: CD45 (clone 30-F11), CD3 (145-2C11), B220 (RA3682), Gr-1 (RB6-8C5), NK1.1 (PK136), MHC Class II (M5/114.15.2) Ly6 C (HK1.4) Ly6G (clone 1A8), F4/80 (clone BM8), and CD11b (clone M1/70) (Biolegend, San Diego, CA). Staining with Fixable Red Dead (Thermo Fisher, Waltham, MA) was used for the detection of dead cells. Isotype controls and fluorescence minus one controls were used to gate for background staining. Cells were sorted on a FACSAria (BD Biosciences, San Jose, CA), and FlowJo Software 9.6 (Treestar, Ashland, OR) was used for analysis.

3.2.5. RNA-seq Analysis

Macrophages were isolated from the fracture callus of old (n=10), old treated with PLX3397 (n=6) and young (n=11) mice at day 3 post-fracture. The callus was dissected and cells were collected as described above. For the detection and isolation of macrophages, cells that stained with the following directly conjugated fluorescent antibodies CD3 (145-2C11), B220 (RA3682), NK1.1 (PK136), Ly6G (clone 1A8) were excluded, and macrophages were collected by staining with CD45 (clone 30-F11), F4/80 (clone BM8), and CD11b (clone M1/70) (Biolegend, San Diego, CA). Callus macrophages were sorted to 99.8% purity on FACSAria (BD Biosciences, San Jose, CA). RNA was extracted using Invitrogen RNA aqueous Micro Kit (AM1931). The library was prepared using Illumina Truseq Stranded mRNA Library Prep Kit and Single-end 50 bp RNAseq was performed on Illumina HiSeq 4000. An average read depth of 60.7 million reads per sample was

generated. Reads were aligned using STAR_2.4.2a to the mouse genome (Ensemble Mouse GRCm38.78). Differential gene expression was assessed using DEseq2. Gene ontology and KEGG pathway analysis was performed using DAVID (<http://david.abcc.ncifcrf.gov>) and the mouse genome was used as background.

3.2.6. Statistics

GraphPad Prism v.7 software was used for analysis. Comparisons between groups was made by first using a 2-way ANOVA multiple comparisons test followed by a 2-tailed Student's *t* test. $P < 0.05$ was considered statistically significant. Differential gene expression was considered significant at $FDR < 0.1$. For term enrichment in gene ontology and KEGG pathway analysis the level of significance was set using a modified Fisher Exact P-value of $p < 0.05$.

3.3 Results

3.3.1. Old mice demonstrate delayed fracture healing compared to young mice

Previously, we have shown that the rate of fracture healing is directly related to the age of mice. Eighteen month-old mice healed slower than 1 and 6 month old mice (17). Therefore, to begin this work we compared fracture healing in 24 month-old mice, because these are considered elderly (40), to young adult mice (3 months old). Fracture healing was assessed using stereology to quantify the volume of bone and cartilage within the fracture callus. At 10 days post-fracture, old mice had smaller calluses with significantly less bone and more cartilage ($p < 0.05$) compared to young adult mice (Fig. 3.1). Thus, these data are in agreement with our earlier work.

3.3.2. Immune cell infiltration into the fracture callus is similar in old and young mice

Our objective was to examine the effect of age on inflammation during fracture healing. First, we assessed the inflammatory response during fracture healing in old and young mice by quantifying lymphocyte infiltration into the fracture callus at 1, 3, 10, and 14 days post fracture via flow cytometry. The quantity of T cells, natural killer T cells, and natural killer cells isolated from the callus was similar in young and old mice at all time points examined ($n=5$ mice/ group) (Fig. 3.2A).

In contrast, B cells were significantly increased in young mice at day 10 ($p < 0.05$). Macrophages were the most prevalent immune cell analyzed among cells derived from the fracture callus. The quantity of macrophages peaked 3 days after fracture, and the macrophages were reduced dramatically by day 14, but, no significant differences were noted in the quantity of F4/80+ macrophages in old and young fracture calluses at any time point analyzed (Fig. 3.2B). However, in examining subpopulations of macrophages, the F4/80+, Ly6C- population, indicative of a “restorative” macrophage phenotype (41) was increased within the fracture callus of young mice compared to old mice at day 1, but this difference had resolved by day 3 (Fig. 3.2C). Our data suggest that there may be slight differences in the cellular inflammatory response in young and old mice, but the differences are subtle.

3.3.3 Callus macrophages from old mice are transcriptionally distinct from callus macrophages from young mice.

Since the quantity of immune cells infiltrating the fracture callus was similar in young and old mice, functional, rather than quantitative, changes in these cells may contribute to inflammatory dysregulation upon aging. The macrophage was selected for further analysis, because macrophages were the most abundant immune cell type analyzed, and we observed differences in a subpopulation of macrophages, F4/80+, Ly6C-, between young and old mice. To evaluate intrinsic age-related changes in macrophages, RNA-seq analysis was performed on macrophages isolated from the fracture callus of old and young mice at 3 days post fracture. In total, 1222 genes were significantly differentially expressed in old macrophages compared to young; 364 genes were upregulated and 200 genes were down-regulated more than 2-fold (Fig. 3.3A). Gene ontology enrichment analysis was performed to begin exploring the implications of these differentially expressed genes in macrophages (Fig. 3.3B). A number of significantly enriched disease processes are identified that are related to aging and the immune response. These enriched terms include rheumatoid arthritis, graft versus host disease, inflammatory bowel

disease. Additionally, molecular and cellular processes important in macrophage function were significantly enriched, including antigen processing and presentation, response to wounding, and cytokine activity. These suggest that fundamental differences in function of young and old macrophages may contribute to age-related differences in inflammatory response to fracture.

The 1222 significantly differentially expressed genes in the macrophages from old and young fracture calluses is compared with a heat map (Fig. 3.3C). Hierarchical clustering based on Euclidean distances demonstrates old and young animals sort largely based upon differential gene expression patterns (Fig. 3.3C). The heatmap further characterizes old mice as more heterogeneous in their transcriptomes than young animals. To further assess differences between old and young macrophages, samples were hierarchically sorted based on their differential expression of 14 genes associated with characteristic macrophage cytokines and markers of M1 and M2 macrophages. This analysis demonstrated that mice sort by age and that old mice have increased expression of pro-inflammatory cytokines and markers of M1 macrophages (Fig. 3.3D). Principal component analysis further demonstrates distinct clustering of the young mice from old (Fig. 3.3E). The heterogeneity of the old macrophage transcriptome is further seen in the principal component analysis with young mice demonstrating closer clustering compared to old mice that span PC1 and PC2 (Fig. 3.3E). Median PC scores of old and young mice across the individual principal components additionally demonstrate the difference by age and the increased variability in the old mice (Supplementary Fig. 3.1). PC1 appears to largely separate the old from young mice. Although, three old mice are seen clustering with the young mice in all analyses performed, further supporting the idea that cells from old mice are more variable in their gene expression profiles than young mice. Genes with the highest and lowest eigenvalues on PC1 are presented in Supplementary Table 3.1.

3.3.4. Inhibition of macrophage recruitment improves fracture healing in old mice

Macrophages from the fracture calluses of old mice were transcriptionally distinct and displayed a more pro-inflammatory phenotype compared to young macrophages. Thus, we sought to inhibit macrophage recruitment during fracture healing to assess if healing outcomes could be improved in old mice. We administered a CSF-1R inhibitor, PLX3397, that inhibits recruitment of macrophages from the bone marrow. Administering PLX3397 for 10 days after fracture healing improved fracture healing outcomes (Fig. 3.4). Stereological analysis demonstrated a larger fracture callus with significantly increased bone volume in treated old mice compared to control old mice (Fig. 3.4B). Flow cytometry demonstrated significant reduction of macrophages within the callus of PLX3397 treated mice (Fig. 3.5A).

3.3.5. Improved fracture healing is associated with transcriptionally “younger” macrophages

To understand how the transcriptional profile of callus macrophages changes with PLX3397 treatment, we collected macrophages from the fracture callus of mice treated with PLX3397 at 3 days post-fracture. RNA-seq analysis demonstrated that only 64 genes were significantly differentially expressed in old macrophages from mice treated with PLX3397 compared to young macrophages; 27 genes were up-regulated and 28 genes were down-regulated more than 2-fold (Fig. 3.5B). Hierarchical clustering by Euclidean distance demonstrates that macrophages from old mice treated with PLX3397 cluster between macrophages from young and old mice without treatment (Fig. 3.5C). Principal component analysis further demonstrates that the macrophages from old mice treated with PLX3397 cluster closely with the young mice compared to the old with less transcriptomic heterogeneity (Fig. 3.5D). This suggests that the inflammatory macrophages that are recruited to the bone fracture are substantially different between old and young mice, but the remaining tissue resident macrophages demonstrate less age-related changes.

3.4 Discussion

The results from this study demonstrate that an aged macrophage phenotype is detrimental to fracture healing. Using an unbiased next generation sequencing approach, we demonstrate differences in the gene expression signatures of macrophages that infiltrated the fracture site in young and old mice. Macrophages from old mice have a more M1, pro-inflammatory gene signature than macrophages from young animals. Further, we demonstrated that a pharmacologic (PLX3397) leading to a decrease in macrophages recruitment to the fracture site of old mice improves fracture healing outcomes. In older mice treated with PLX3397, macrophages that are present within the callus appear transcriptionally “younger”, suggesting that the detrimental age-related changes may occur in infiltrating macrophages.

The delayed fracture healing we observed in the elderly mice compared to young adults here (Fig. 3.1) is similar to our previous findings (17). Other groups have shown delayed healing with decreased callus size and decreased bone volume at multiple time points post fracture in old mice compared to young (18,42). Substantial alterations in inflammation may affect fracture healing in aged animals. For example, inflammation induced with Lipopolysaccharide led to decreased callus strength in young animals (10), and delayed healing in aged animals has been directly associated with inflammatory dysregulation within the callus in aged mouse models of fracture healing (24,43). Other studies have demonstrated an association of systemic inflammatory dysregulation, as a result of increased age or disease, with poor fracture healing outcomes in humans and animal experiments (4-6,44). In fact, we have shown that transplantation of juvenile bone marrow into lethally irradiated middle-aged animals stimulates bone fracture healing, and this is associated with decreased inflammation (24). This work was subsequently confirmed (45). However, the inflammatory cells responsible for the stimulatory effect remain largely unknown.

Recent work has suggested that macrophages may underlie the dysregulation of inflammation seen during fracture healing in aged animals. Our preliminary research demonstrated that

reducing the influx of inflammatory macrophages into the callus of aged animals stimulated healing (38). Other work has advanced this observation and supports the idea that aging macrophages are deleterious to healing (45,24). Interestingly, depletion of macrophages in healthy young mice has also been shown to be deleterious to fracture healing (30,32,33). Collectively, this work supports the important role for macrophages in fracture healing and the deleterious effect of age-related changes to macrophages on fracture healing outcomes.

The age-related changes in fracture healing does not appear to be a function of significant differential inflammatory cell recruitment. We observed similar numbers of immune cells infiltrating the fracture callus in young and old mice (Fig. 3.2). T cells may contribute to fracture healing largely through the recruitment and activation of osteoclasts (46). The aging immune system is characterized by decreased naive T cell quantity and weaker activation in elderly populations compared to young (47). However, the quantity of T cells within the fracture callus did not differ by age (Fig. 3.2A). Similarly, the quantity of F4/80+ macrophages did not differ by age; however, a sub-population of macrophages, identified as F480+ Ly6C-, was increased at day 1 post fracture in young animals but this difference was not apparent by day 3 (Fig. 3.2C). Macrophages that are F4/80 positive and Ly6C negative have been suggested to be anti-inflammatory or M2-like macrophages (41,48,49), suggesting that the M2-like macrophages were present in the fracture callus of young mice to a greater extent at earlier time points than in old mice. The significance of this subtle alteration is not known.

At day 10 we observed increased B-cells in the fracture callus of young mice compared to old mice (Fig. 3.2A). During fracture healing B cells regulate osteoclast activity through the expression of osteoprotegerin (46), and interactions between B cells and macrophages have been demonstrated to regulate inflammation during infection (50,51). While these are intriguing observations the specific role of B-cells in mediating effects of age on fracture healing are

unexplored, but the novelty of these cells in an inflammatory response to injury is potentially interesting.

The importance of macrophages to fracture healing and bone regeneration is beginning to emerge (33,45,52). Different macrophage sub-types have been proposed to mediate separate functions during healing. Pro-inflammatory, or M1-type, macrophages appear early in fracture healing and produce pro-inflammatory cytokines (IL-1, IL-6, TNF α , iNOS) that further propagate the inflammatory response (53). These cells likely phagocytize remnants of dead tissues to debride the fracture site. Later, a switch to anti-inflammatory, or M2-type, macrophages occurs within the fracture callus. M2 macrophages initiate down regulation of the inflammatory response with production of IL-10 and TGF β (53,54). M2 macrophages also promote tissue repair through the production of growth factors (TGF β , PDGF, VEGF) (55). However, *in vivo* phenotyping of macrophages is complex, and given the plasticity of macrophages, the M1/M2 distinctions are likely not dichotomous and probably represent poles on a large spectrum of macrophage phenotypes. Here, RNA-seq analysis allowed an unbiased analysis of the transcriptomic differences of macrophages within the fracture callus of young and old mice. Macrophages from the fracture callus of old mice were transcriptionally distinct from macrophages of young mice (Fig. 3.3). Further, a M1/M2 gene expression signature, comprised of 14 selected genes for cytokines and cell markers associated with traditional M1 and M2 phenotypes, was distinct between age groups and able to differentiate macrophages from old mice versus young. Here, macrophages from old mice demonstrated expression of more pro-inflammatory or M1 genes suggesting that old macrophages contribute to the pro-inflammatory phenotype evident in elderly populations.

With the demonstrated pro-inflammatory, and potentially deleterious, phenotype of aged macrophages, we wanted to understand the effect of limiting macrophage recruitment into the fracture callus. PLX3397 treatment prevented macrophage recruitment and resulted in improved

fracture healing in old mice (Fig. 3.4). The magnitude of change appears small in the PLX3397 treated old mice when compared to total callus volume at 10 days post fracture. However, the change represents a 35% increase in bone volume in the treated group compared to age-matched controls, which is consistent with other bone fracture research in mice that shows improvements in bone volume within a fracture callus of 25-50% using other experimental agents to improve fracture healing (56-58).

RNA-seq analysis demonstrated that 1222 genes were significantly differently expressed in macrophages in old mice compared to young (Fig. 3.3A), and this quantity is important considering the breadth of biological and disease processes that these genes are associated with (Fig. 3.3B). In old mice, when recruitment of macrophages was inhibited with PLX3397 the number of significantly differentially expressed genes between old and young macrophages were reduced by 95% (Fig. 3.5B), and the treated old mice clustered between the young and old controls (Fig. 3.5C). These findings suggest the presence of a more youthful macrophage population after PLX3397 treatment in old mice.

One potential “youthful” macrophage population is osteomacs. Osteomacs are resident tissue macrophages in bone and have been shown to co-localize with osteoblasts and contribute to osteogenesis (52,59). Specific depletion of osteomac populations in vivo was shown to be deleterious during both intramembranous and endochondral fracture repair processes (60). After inhibition of M-CSF with PLX3397, the infiltrating macrophages were decreased and this is accompanied by a decrease in inflammation mediated by macrophages (38,61). The pharmacological effect of PLX3397 works largely on infiltrating inflammatory macrophages by antagonizing CSF1R and preventing the monocyte to macrophage differentiation. Therefore, we suspect that the resident osteomacs are less affected by PLX3397. The improved fracture healing in old mice treated with PLX3397 could be a result of decreased inflammatory macrophages and/or an expansion or activation of a more youthful and beneficial osteomac population. Further

work is needed to understand the age-related changes to osteomacs and their contribution to fracture healing.

A potentially important observation that we made using RNA-seq analysis is that macrophages from old animals were much more heterogenous than those from young animals (Fig. 3.3C). The heatmaps demonstrated more homogenous gene expression levels between the individual young mice than old animals. The extent of heterogeneity in old mice is present despite all mice being from the same genetic background, sourced from the same laboratory and colony, housed in similar environments, and samples prepared on the same day. Complex disease processes and biological traits, including aging, are often defined by a heterogenous phenotypic presentation (62,63,64). Heterogenous changes are present across many aspects of the biology of aging with an impact that is not fully understood. Age-related genetic heterogeneity could result from cumulative effects from the environment or an unknown mechanism (65). How to properly analyze the heterogeneity present in large genetic datasets is not clearly defined. Largely, the heterogeneity is accepted as normal and sample size is increased so that differences may be detected. However, further research would be better aimed at understanding the biological cause and significance of such variation, as the increased variance in old animals may be a substantial contributor to phenotypic outcomes. Further, this may aid in identifying at risk individuals who would benefit from individualized treatment plans.

3.5 Conclusion

In summary, this study characterizes the cellular immune response during bone fracture healing and demonstrates age-related changes at the cellular level that are reflective of the altered physiology present in elderly populations. Robust transcriptional differences differentiated macrophages infiltrating the callus of old mice compared to young. The old macrophages demonstrated a pro-inflammatory M1 macrophage phenotype. The aged macrophage phenotype was detrimental to fracture healing outcomes as fracture healing was improved in old mice when

aged macrophages were inhibited from accumulating in the callus during fracture healing. The therapeutic targeting of macrophages during fracture healing may be an effective therapy to improve fracture healing outcomes in elderly populations. Finally, understanding the variance and its underlying mechanism(s) may contribute significantly to directed treatments of the patient with musculoskeletal injuries.

Figures: Chapter 3

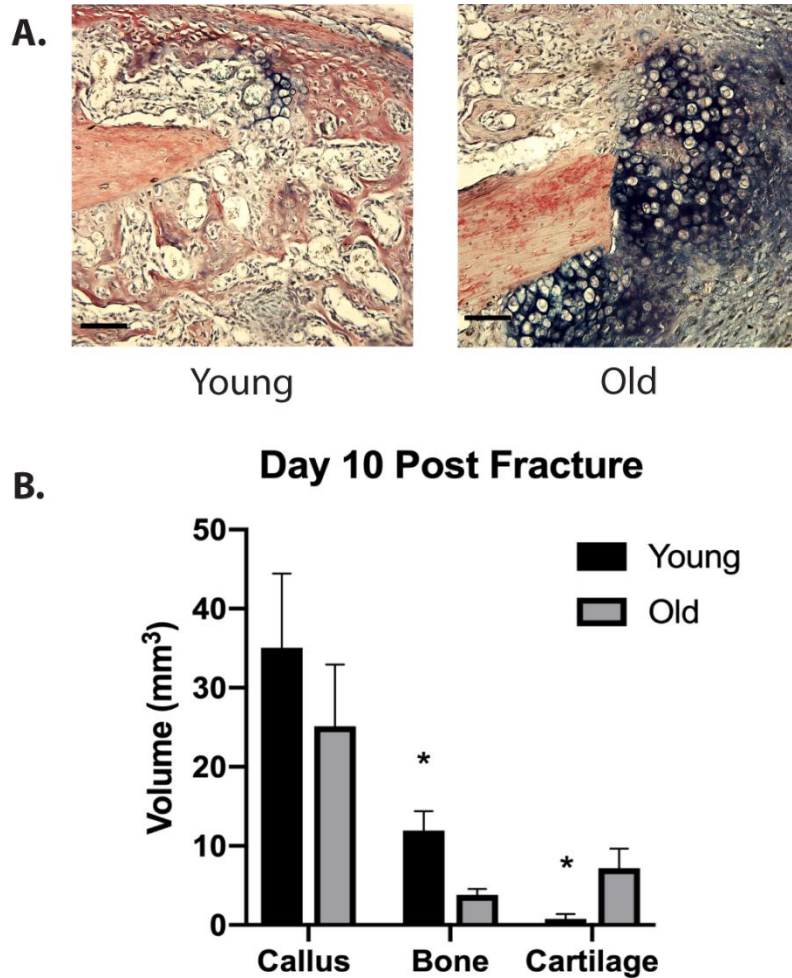


Figure 3.1: Old mice demonstrate delayed fracture healing compared to young. (A) Representative histological images with Hall Brunt quadruple stain (HBQ) (mineralized tissue stains red and cartilage stains blue) of fracture calluses in old (24 months) and young (3 months) mice (scale bar= 200um). Stereological analysis was performed and the volume of the total callus and the volume of bone and cartilage tissue within the callus was calculated at 10 days after closed tibial fracture (n=5/group). (B) Old mice demonstrate delayed healing with smaller callus size and significantly less bone and more cartilage (* p<0.05).

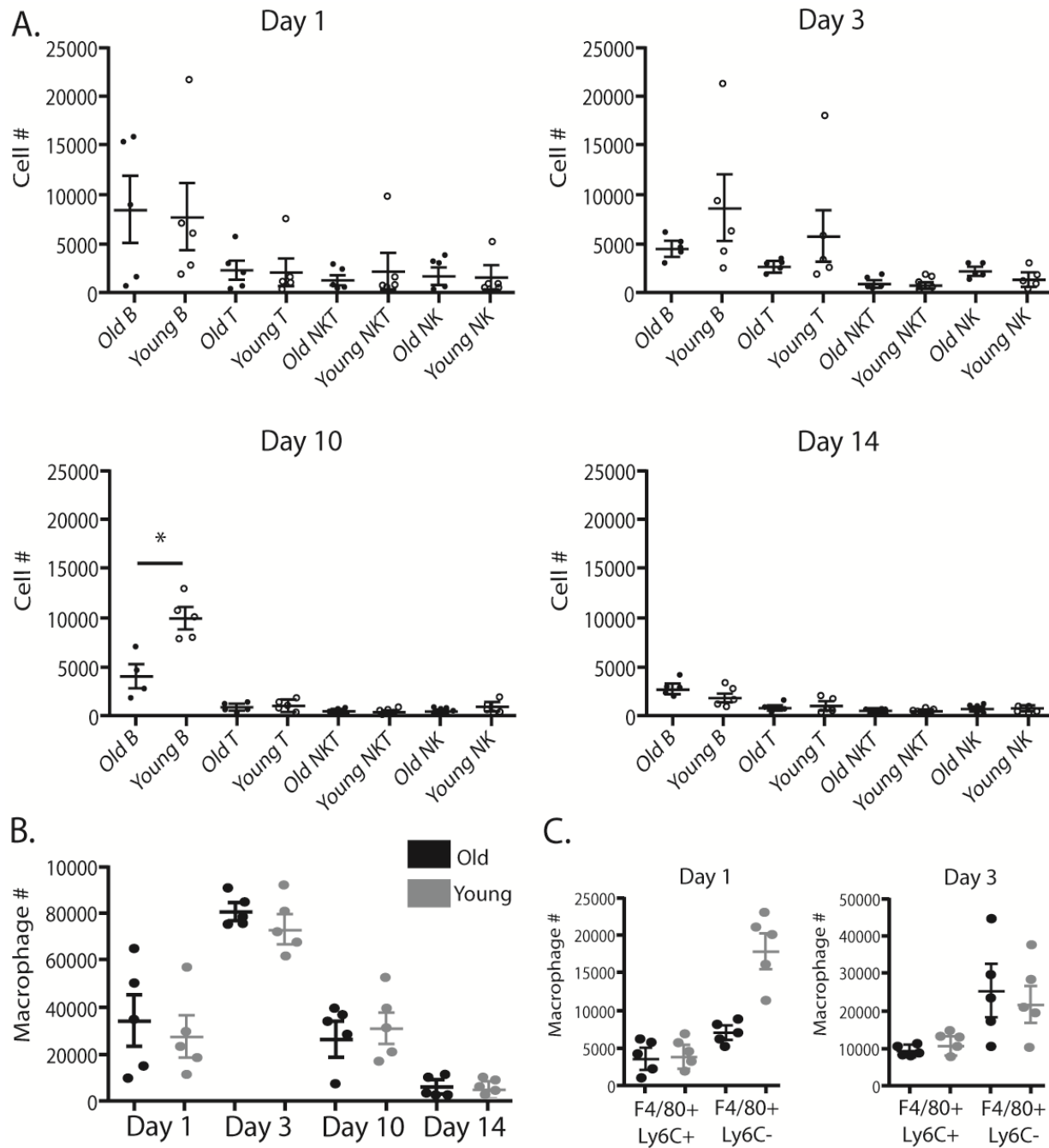


Figure 3.2: Immune cell infiltration into the fracture callus is similar in old and young mice. (A) The quantity of B cells, T cells, NKT cells, and NK cells were similar within the fracture callus at days 1, 3, 10 and 14 post fracture measured via flow cytometry in old (n=5) and young (n=5) mice. B cell quantity at day 10 was the only significant differences between age groups. (B) Macrophages (F4/80+) were the most abundant immune cell analyzed within the fracture callus and demonstrated no significant difference in quantity between young (gray) and old (black) mice at any of the time points analyzed. (C) A sub-population of macrophages (F4/80+, Ly6C-) was increased in young mice at day 1 compared to old mice. (* $p < 0.005$).

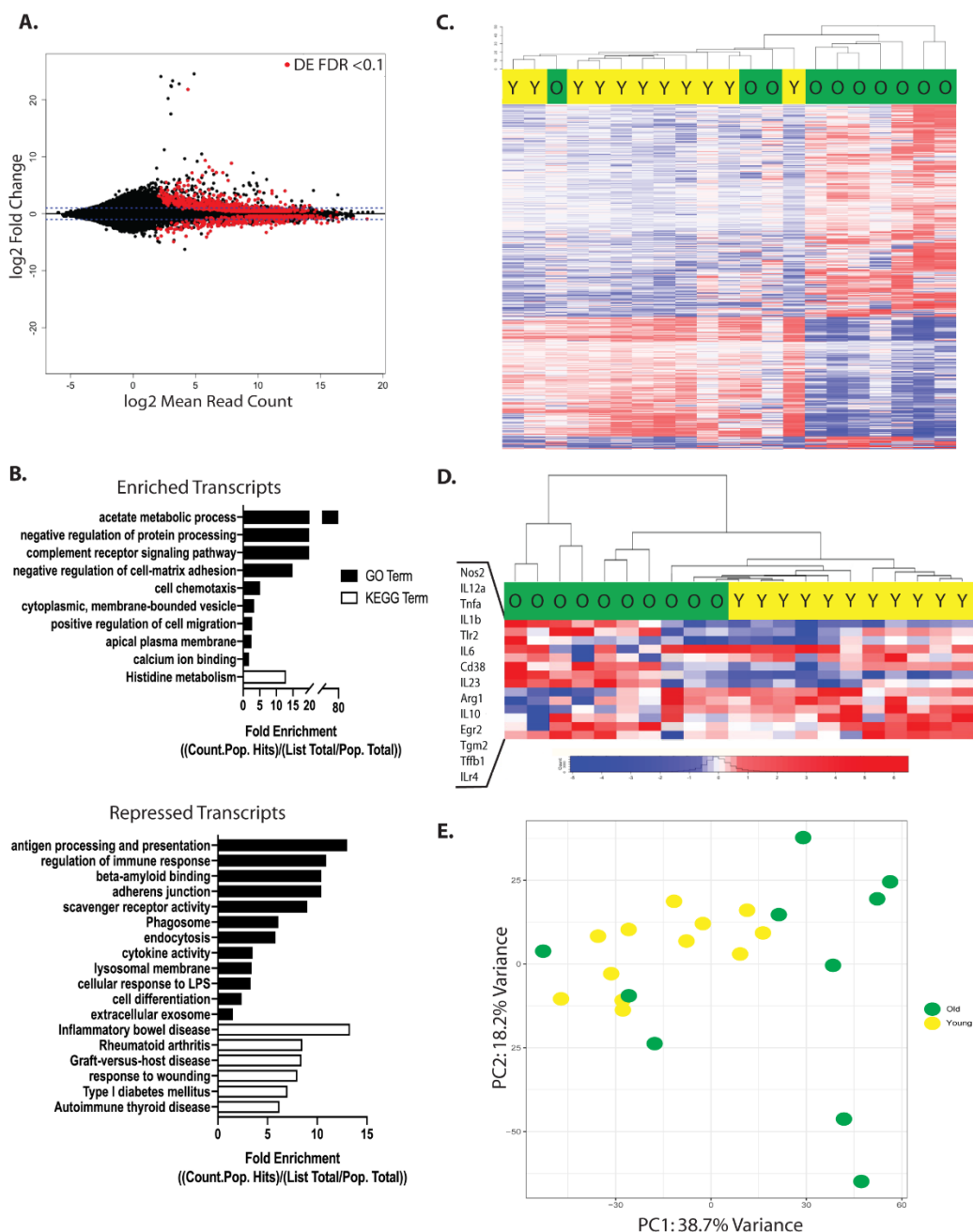


Figure 3.3: Macrophages from the fracture callus of old mice are transcriptionally distinct from young mice. RNA-seq analysis of callus macrophages in old ($n=10$) and young ($n=11$) mice collected at 3 days post fracture. (A) 1222 genes were significantly differentially expressed in old macrophages compared to young (FDR < 0.1) (red dots). (B) Enriched gene ontology terms associated with the significantly up and down regulated genes. (C) Heat map demonstrates hierarchical clustering of young (yellow) and old (green) mice based on the differential expression of the 1222 genes. (D) Hierarchical clustering of young (yellow) and old (green) mice, based on the differential expression of a M1/M2 gene signature, demonstrates a more pro-inflammatory/M1 gene expression signature in macrophages from old mice compared to young. (E) Principal component analysis of differential gene expression in macrophages from old mice and young demonstrates clustering of young (yellow) mice and a heterogeneous spread of old (green) mice across PC1 and PC2.

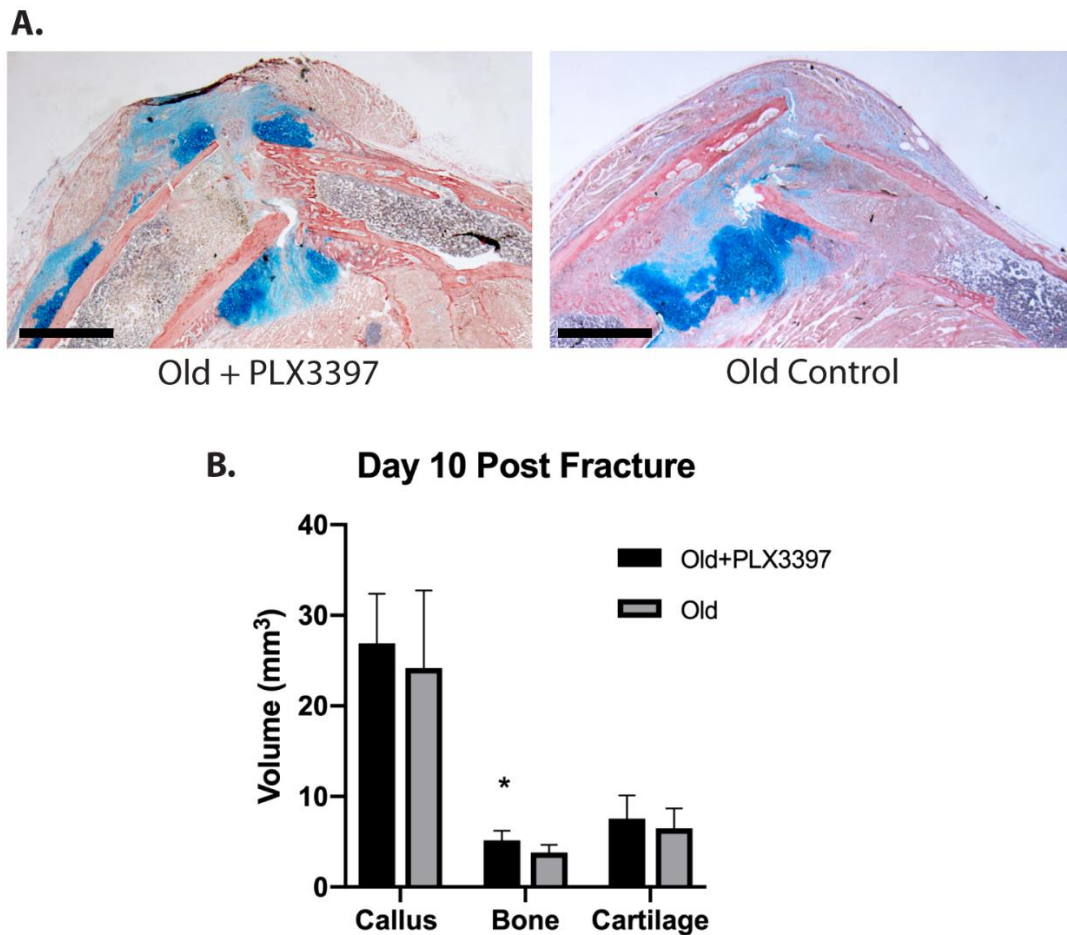


Figure 3.4: Inhibition of macrophage recruitment improves fracture healing in old mice. (A) Representative histological images (HBQ stain) of fracture calluses in old mice treated with PLX3397 (CSF-1R inhibitor) and aged matched old controls 10 days after closed tibial fracture (n=11/group) (scale bar= 200um). Stereological analysis was performed and the volume of the total callus and the volume of bone and cartilage tissue within the callus was calculated. (B) Old mice treated with PLX3397 demonstrate significantly more bone volume compared to old non-treated mice (* $p < 0.05$).

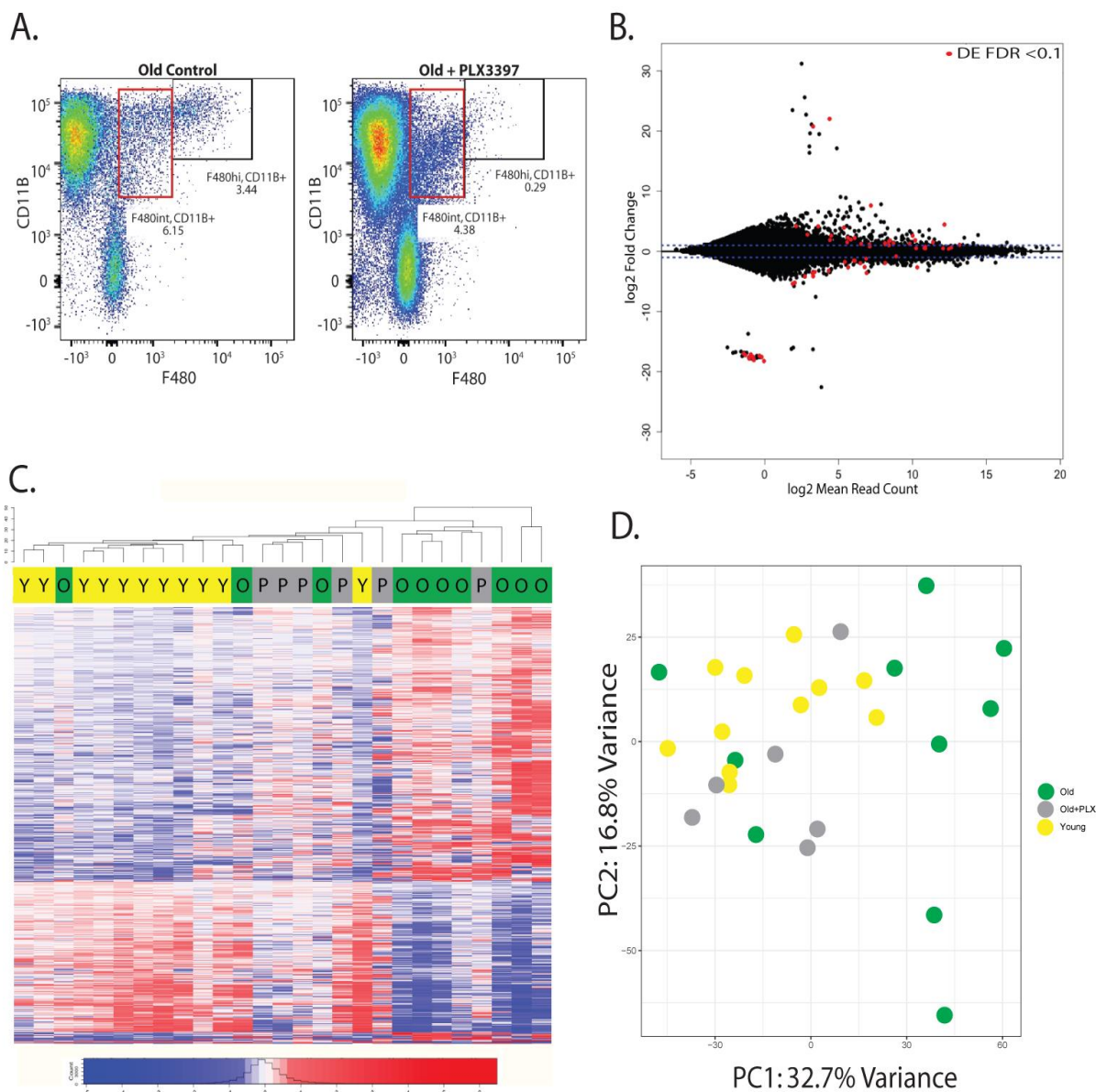
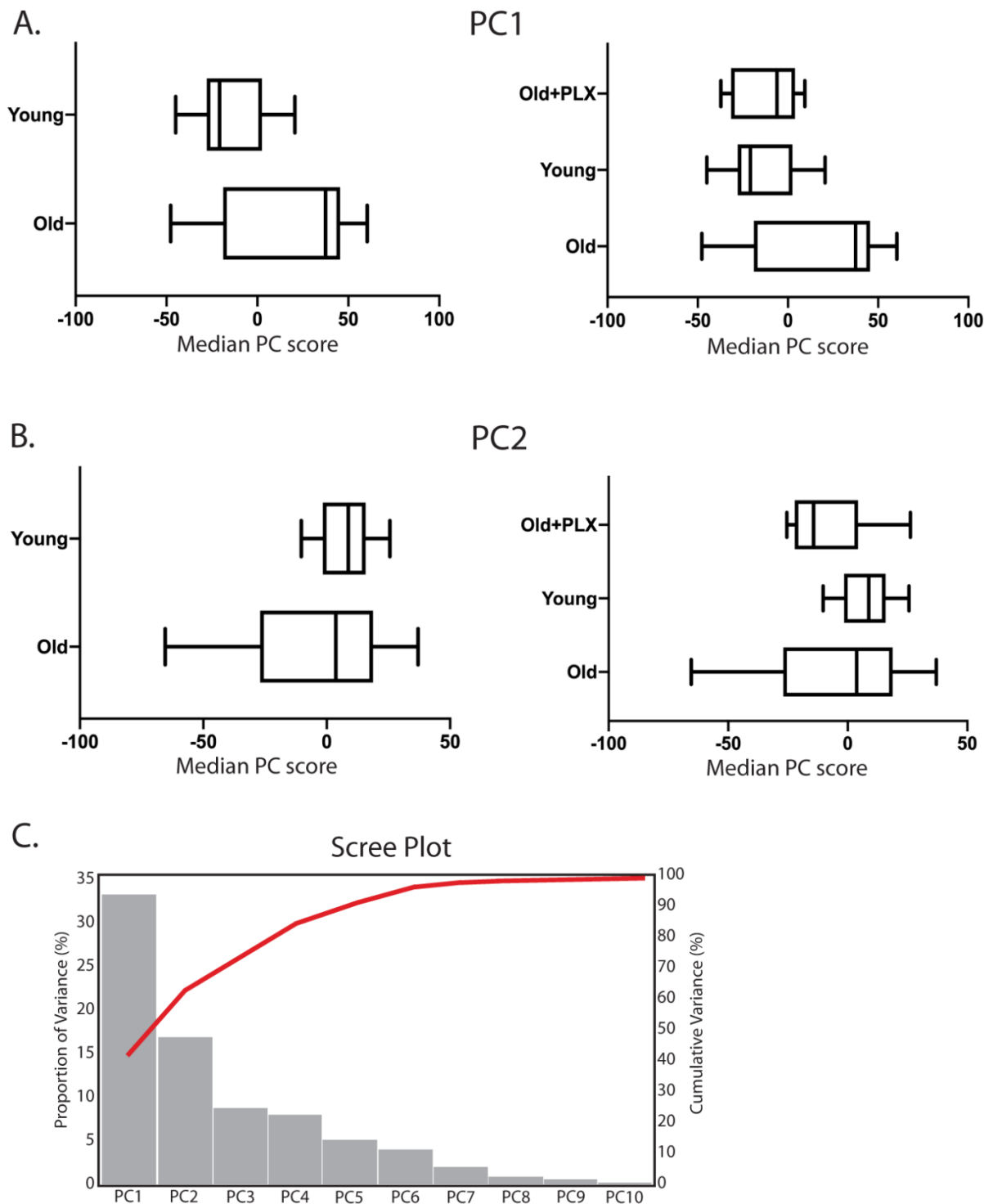


Figure 3.5: Improved fracture healing in treated old mice is associated with transcriptionally “younger” macrophages. (A) PLX3397 treatment during fracture healing resulted in significant decrease in macrophage quantity within the fracture callus, as analyzed through flow cytometry. (B) RNA-seq analysis demonstrates 64 genes were significantly differentially expressed in old macrophages treated with PLX3397 compared to young (red dots) (FDR < 0.1). (C) Hierarchical clustering demonstrates old macrophages treated with PLX3397 (gray) cluster between young (yellow) and old (green) control macrophages. (D) Principal component analysis of differential gene expression in macrophages demonstrates close clustering of young (yellow) mice with old mice treated with PLX3397 (gray).



Supplemental Figure 3.1: Plot of the individual PC scores along PC1 (A) and PC2 (B) of young, old, and old mice treated with PLX3397. PC1 separates old from young (A). The variance of PC scores is greater across both PC1 and PC2 in old mice compared to young. (C) Scree plot demonstrates that the majority of the variance (56.9%) is accounted for in PC1 and PC2.

Supplemental Table 3.1: Gene list of the upper and lower 2% of eigenvalues along PC1

Upper 2%		Lower 2%	
Gene	Eigenvalue	Gene	Eigenvalue
Igkv15-103	0.0584	Gpnmb	-0.0425
Ighg2c	0.0556	Selenop	-0.0426
Ighv7-1	0.0547	Slc6a8	-0.0428
Igkc	0.0537	P2ry6	-0.0429
Ighg2b	0.0531	Pdlim4	-0.0432
Igkj5	0.0439	Tppp3	-0.0436
Ighv6-3	0.0435	Lpl	-0.0437
Igkv5-39	0.0430	Fcgr1	-0.0440
Igkv1-117	0.0426	Cav1	-0.0443
BC100530	0.0426	Igf1	-0.0445
Stfa2l1	0.0425	Pid1	-0.0446
Igkv1-88	0.0418	Tmem37	-0.0447
Igkv6-32	0.0388	H2-Ab1	-0.0448
Asprv1	0.0385	Cx3cr1	-0.0449
Stfa2	0.0383	Gdf15	-0.0449
Igkj2	0.0368	Il10	-0.0449
Ighv14-2	0.0356	Mmp14	-0.0450
Aspa	0.0351	Ednrb	-0.0451
Stfa1	0.0343	H2-Aa	-0.0452
Ighv11-2	0.0343	Ifi27l2a	-0.0453
Gm5483	0.0335	C3ar1	-0.0458
Pou2af1	0.0334	H2-Eb1	-0.0461
Iglc2	0.0325	Ccr5	-0.0465
Igha	0.0323	Stab1	-0.0468
Ighj4	0.0322	Olfml3	-0.0469
Ighv5-16	0.0316	Aif1	-0.0477
Hoxa9	0.0309	Emp1	-0.0477
Ctla2a	0.0309	Amz1	-0.0482
Cacna1h	0.0307	Abcc3	-0.0483
Gm27252	0.0305	Pmp22	-0.0487
Ighm	0.0301	Apoe	-0.0489
Gimap6	0.0301	Pltp	-0.0489
D630045J12Rik	0.0298	Cd36	-0.0494
Stfa3	0.0297	Spp1	-0.0495
Il5ra	0.0296	Ccl12	-0.0497
Hoxa7	0.0288	Dab2	-0.0497
Fam110c	0.0287	Gas6	-0.0497
Saa3	0.0287	Ifi205	-0.0498
Pdgfrb	0.0286	Nxpe5	-0.0500
Dmwd	0.0284	Lgmn	-0.0507

Supplemental Table 3.1 (cont.): Gene list of the upper and lower 2% of eigenvalues along PC1

Upper 2%		Lower 2%	
Gene	Eigenvalue	Gene	Eigenvalue
Spns2	0.0284	Ccl8	-0.0519
Mpl	0.0279	Pf4	-0.0520
Igkj4	0.0279	Flrt3	-0.0524
Cxcr2	0.0278	C1qc	-0.0526
Ighj2	0.0277	Folr2	-0.0527
Iglv1	0.0277	Siglec1	-0.0539
Retnlg	0.0274	Ms4a14	-0.0539
Igkv5-43	0.0273	Cxcl1	-0.0545
Il17rb	0.0273	Pbbp	-0.0548
Ighv9-3	0.0270	Sdc3	-0.0551
Scrg1	0.0269	C1qb	-0.0558
Ighj1	0.0268	Cbr2	-0.0570
Ighv1-15	0.0266	Pdpn	-0.0582
Myct1	0.0265	Arg1	-0.0589
Crispld2	0.0265	C1qa	-0.0596
Muc13	0.0265	Ccl7	-0.0598
Cd55	0.0265	Cd209a	-0.0598
Epx	0.0264	Ms4a7	-0.0610
Itga2b	0.0263	Mrc1	-0.0656
Serpina3g	0.0263	Fcrls	-0.0737

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Chapter 4: Age-related changes to macrophages contribute to the pathogenesis of periodontal disease

4.1 Introduction

Periodontal disease results from a dysbiosis between the bacterial plaque and the host inflammatory response (1). In disease, the host inflammatory response results in the osteolytic destruction of bone supporting the teeth, which is the clinical hallmark of periodontal disease (1,2). Management of periodontal disease involves, in part, controlling the presence of bacterial plaque. However, adequate management of the host inflammatory response remains elusive. Susceptibility to periodontal disease is typically associated with systemic inflammatory dysregulation (3). Obesity, type II diabetes, rheumatoid arthritis, and Alzheimer's disease are associated with a higher incidence of periodontal disease, and each disease presents with an underlying perturbation of inflammation that predisposes or directly contributes to the pathogenesis of the disease (3,4). The elderly population is an additional group that demonstrate systemic inflammatory dysregulation. Inflamm-aging, as described in previous chapters, describes the elevated chronic inflammatory status of the elderly (4). This dysregulated inflammatory status in the elderly appears to contribute to the pathogenesis of periodontal disease, as increased prevalence of periodontal disease is associated with increased age (5,6).

The focus of this work was to investigate the macrophage due to its shared contribution to inflamm-aging as well as to the pathogenesis of periodontal disease. As reviewed in Chapter 2, the macrophage contributes to periodontal disease via the M1/pro-inflammatory macrophage phenotype that presents within the periodontium in response to the presence bacterial plaque. Persistence of the M1 phenotype or lack of the M2/anti-inflammatory phenotype contributes to the pathogenesis of periodontal disease (7,8). Our work in Chapter 3 demonstrates distinct transcriptional differences of macrophages from old mice compared to young and characterizes an age macrophage phenotype as being more M1 and pro-inflammatory. From this data, we

hypothesized that the aged macrophage phenotype would be detrimental in the proper regulation of inflammation and contribute to the pathogenesis of periodontal disease.

4.2 Materials and Methods

4.2.1 Periodontal disease induction

All animal procedures were approved by the UCSF Institutional Animal Care and Use Committee. Old (24-month) and young (3-month) male C57BL/6 mice were obtained from the NIA Aging Rodent Colony. Periodontal disease induction was accomplished by first anesthetizing the mice with a 1:1 solution of dexmedetomidine and ketamine delivered IP. Then a 6.0 silk suture inoculated with *Porphyromonas gingivalis* (ATCC 33277) was tied in a subgingival position around the second maxillary molars bilaterally. Control mice in both age groups received a sham surgery where mice were anesthetized, sterile sutures were placed subgingivally and then immediately removed. The sutured remained in place for 7 days to adequately induce periodontal disease. At the end of the 7-day induction period mice were euthanized, or in a second arm of the study, the sutured was removed and the animal was allowed to recover for an additional 7 days. At the end of disease induction or disease recovery period, animals were euthanized, and maxillae were collected for analysis.

4.2.2 Macrophage depletion

During disease induction or disease recovery, half the mice in each age group received a pharmacologic, PLX3397 (Plexxikon, Berkeley, CA), to deplete macrophages within the periodontium. PLX3397 was delivered throughout the 7-day period via mouse chow *ad libitum* at an average dose of 40mg/kg/day. PLX3397 is a small molecule inhibitor of the macrophage colony stimulating factor 1 (M-CSF-1) receptor. Antagonizing the M-CSF-1 receptor prevents differentiation of monocytes into macrophages and subsequent recruitment to the site of inflammation. Non-treated groups received the control chow provided by the drug manufacturer.

4.2.3 Micro-CT analysis

Primary outcomes of disease severity were determined by bone volume/total volume and bone mineral density as measured via micro-CT. Dissected maxillae were separated into the left and right alveolar processes. One hemimaxilla had the gingiva removed (used for qRT-PCR analysis) and the alveolar process containing the 3 molar teeth was isolated, defleshed, and soaked in 30% hydrogen peroxide overnight to allow for complete removal of the soft tissue. The alveolar processes were fixed in 10% buffered formalin. Samples were scanned using Scano Medical μ CT set at 55kVp and 109 μ A with a slice thickness of 5 μ m. Analysis of the scanned images was performed using Scanco Medical μ CT Evaluation Program v6.5. The region of interest was delineated along transverse slices isolating alveolar bone supporting teeth and excluding tooth structure. Mesial and distal boundaries were made at the buccal furcation of the first molar and the buccal furcation of the third molar.

4.2.4 qRT-PCR

Inflammatory cytokine expression within the gingiva was compared across treatments and age groups via qRT-PCR. Gingiva was isolated from one half of the dissected maxilla and prepared for qRT-PCR analysis. Briefly, gingiva was homogenized in Trizol and mRNA was isolated. cDNA was reverse transcribed using Superscript III (Invitrogen). qRT-PCR was performed with SYBR Green and the following primers; GAPDH (F: 5'-AGCCTCGTCCCGTAGACAAAAT-3'; R: 5'-CCGTGAGTGGAGTCATACTGGA-3'), IL-1 β (F: 5'-TGTAATGAAAGACGGCACACC-3'; R: 5'-TCTTCTTTGGGTATTGCTTGG-3'), TNF α (F: 5'-TGCCTATGTCTCAGCCTCTTC-3'; R: 5'-GAGGCCATTTGGGAAGTCT-3'), IL-6 (F: 5'-TCCAATGCTCTCCTAACAGATAAG-3'; R: 5'-CAAGATGAATTGGATGGTCTTG-3'), IL-17 (F: 5'-GCTCCAGAAGGCCCTCAGA-3'; R: 5'-CTTCCCTCCGCATTGACA-3'), IL-23 (F: 5'-TGTGCCCGTATCCAGTGT-3'; R: 5'-CGGATCCTTTGCAAGCAGAA-3'), iNOS (F: 5'-GAAGGGGACGAACTCAGTGG -3'; R: 5'-GTGGCTCCCATGTTGCATTG -3'), TREM2 (F: 5'-AAAGTACTGGTGGAGGTGCTG-3'; R: 5'-

AGGCTAGAGGTGACCCACAG-3'). Gene expression was normalized to the house keeping gene GAPDH and presented as relative gene expression ($\Delta\Delta C_T$) or fold change ($2^{-\Delta\Delta C_T}$).

4.2.5 Immunohistochemistry

Quantification of macrophages within the periodontium was performed via immunohistochemical staining for F4/80+ macrophages. One half of the maxilla was isolated and fixed in 4% paraformaldehyde for 24 hours. The tissue was then decalcified with 19% ethylenediaminetetraacetic acid (pH 8) for 28 days with the solution changed every other day. After complete decalcification, the samples were dehydrated and embedded in paraffin. Paraffin sections were cut with a microtome at 8 micrometers thick and prepared for staining. Sections were first blocked with 3% H₂O₂ (45 minutes), followed by antigen retrieval with Trypsin 0.1% in dH₂O for 30 minutes. Sections were then blocked with 5% goat serum albumin for 1 hour. The primary antibody, rat anti-mouse F4/80 (BD Biosciences), was incubated with the section at a 1:200 dilution (in 5% goat serum) overnight at 4°C. The secondary anti-body, HRP conjugated goat anti-mouse IgG, was applied at 1:500 dilutions and incubated for 1 hour. Secondary antibody signal was amplified using the VectaStain ABC Kit (Vector) and finally stained with 3,3'-diaminobenzidine (DAB) substrate. Photographs were taken of one representative slide at 200x, and 3 regions of interest (ROI) per slide were analyzed at 400x. The ROIs were adjacent to the tooth and included the epithelium, connective tissue or bone in the coronal 1/3 of the root. Each ROI had a field of view (FOV) of 0.23mm². F480+ macrophages were quantified using Image J imaging software. The 3 ROIs were averaged per sample for each mouse.

4.2.6 Statistical Analysis

The BV/TV, BMD, and macrophage quantification was calculated per sample and presented as mean \pm SD. Groups were first analyzed via ANOVA for significant differences, and between group differences were analyzed using a two tailed T-test. qRT-PCR utilized technical triplicates and the mean QT value was calculated. Relative gene expression ($\Delta\Delta C_T$) was calculated and presented

as mean \pm SEM and analyzed using an ANOVA followed by between group comparisons using a two-tailed T-Test. Significance for all analysis was determined at $p < 0.05$. All statistical analysis was performed using GraphPad Prism v.7 software.

4.3 Results

4.3.1. *Old mice demonstrate an aged periodontal phenotype*

Alveolar bone height was compared in old (24 month) and young (3 month) healthy control mice (n=5/group). Old mice demonstrated significantly increased bone loss (0.19mm \pm 0.007) compared to young (0.09mm \pm 0.015) ($p < 0.01$) (Fig. 4.1). Increased bone loss in control old mice was associated with inflammatory dysregulation within the gingiva. IL-1 β , TNF α , IL-6, IL-17 demonstrated significant increased expression in the gingiva of old control mice compared to young ($p < 0.05$) (Fig. 4.1).

4.3.2. *Macrophage quantity within the periodontium is similar in old and young mice.*

The ligature model of periodontal disease resulted in significant bone loss in old and young mice after an induction period of 7 days (Fig. 4.2). Macrophage quantification was analyzed in old and young mice (n=5/group) at three time points; baseline, after 7 days of disease induction, and after 7 days of recovery. At all time points, the macrophage quantity within the periodontium was similar in old and young mice ($p > 0.05$) (Fig. 4.2).

4.3.3. *Macrophage depletion attenuates bone loss and pro-inflammatory cytokine expression in old and young mice*

Periodontal disease was induced in old and young mice (n=5/group). Half the mice in each group received PLX3397. Administration of PLX3397 throughout the induction period resulted in a significant decrease in macrophage quantity within the periodontium of old and young mice ($p < 0.05$) (Fig. 4.3). In old mice, treatment with PLX3397 resulted in significantly increased bone volume/total volume compared to non-treated controls (61 \pm 3% vs 53 \pm 3%) ($p < 0.001$) and significantly increased bone mineral density compared to non-treated controls (822 \pm 51mg

HA/mm³ vs 707±58 mg HA/mm³) (p<0.001) (Fig. 4.3). Improved bone measurements with PLX3397 treatment was associated with decreased inflammatory cytokine expression. Treated old mice demonstrated significant decreases in relative mRNA expression of IL-1β, TNFα, and IL-6 (p<0.05) (Fig. 4.3). In young mice, a similar trend was also demonstrated. During disease induction in young mice, treatment with PLX3397 resulted in significantly increased bone volume/total volume compared to non-treated controls (59±0.4% vs 46±1.0%) (p<0.001) and significantly increased bone mineral density compared to non-treated controls (776±7mg HA/mm³ vs 598±20 mg HA/mm³) (p<0.001) (Fig. 4.3). Relative mRNA expression of iNOS was significantly reduced in young mice with treatment compared to non-treated controls (p<0.05) (Fig. 4.3).

4.3.4. During disease recovery, macrophage inhibition differentially affects bone loss and pro-inflammatory cytokine expression in old compared to young mice.

PLX3397 administration in old mice during disease recovery demonstrated similar significant improvements as was seen during disease induction. Treatment in old mice resulted in significantly increased bone volume/total volume compared to non-treated controls (62±2% vs 56±2%) (p<0.05) and significantly increased bone mineral density compared to non-treated controls (815±37mg HA/mm³ vs 750±33 mg HA/mm³) (p<0.001) (Fig. 4.4). Relative mRNA expression of IL-1β was also significantly decreased within the gingiva of treated mice compared to non-treated controls (p<0.05) Fig. 4.4). However, in young mice, no benefit of PLX3397 administrated was demonstrated during disease recovery. Compared to non-treated controls, treated young mice demonstrated no significant differences in bone volume/total volume (54±1% vs 55±1%) (p>0.05) or bone mineral density (690±26mg HA/mm³ vs 709±28 mg HA/mm³) (p>0.05). There were also no significant differences in the relative mRNA expression of the inflammatory genes analyzed in young treated mice compared to non-treated controls during disease recovery (p>0.05) (Fig. 4.4).

4.4 Discussion

The results from this work support the role of macrophages in periodontal disease pathogenesis and further demonstrate age-related changes to the macrophage as a contributing factor to the increased prevalence of periodontal disease in elderly populations.

We demonstrated an aged periodontal phenotype in old mice with increased bone loss and associated increased pro-inflammatory cytokine expression. This periodontal phenotype is represented in humans that demonstrate increased prevalence of periodontal disease with age and an associated systemic inflammatory dysregulation (4,5). The systemic inflammatory dysregulation is known as inflamm-aging and has been associated with the myriad of age-related disease (9). Other groups have similarly shown that periodontal disease in mice increases with age (10). However, specific mechanisms responsible for the age-related increased in disease prevalence are unknown.

Our group decided to investigate the macrophage in the context of aging and periodontal disease after understanding the age-related changes that occur to macrophages involved in fracture healing, as presented in Chapter 3. Macrophages were demonstrated to be transcriptionally distinct in the fracture callus of an old mouse compared to young as measured via RNA-seq. Both periodontal disease and fracture healing can be considered osteoimmunological models to study macrophages. In both models, local sites of inflammation affecting bone result in the recruitment and infiltration of macrophages, and the necessary resolution of inflammation depends, in part, on a change in macrophage activity or phenotype (11). In the periodontal disease model, the stimulus is bacterial in nature, while the fracture model exhibits “sterile” inflammation without a bacterial or infectious component. It would be assumed that the macrophage activity would differ in some aspects with and without the presence of bacteria. However, we contend that the extensive transcriptional differences between macrophages in old versus young fracture calluses can be compared across osteoimmunological models. The increased pro-inflammatory and M1

gene expression by macrophages within the fracture callus of old mice would be similarly pathogenic in periodontal disease and could contribute to the increased prevalence and severity of periodontal disease associated with increased age.

We have demonstrated the quantity of infiltrating macrophages into the periodontium did not differ by age group. The quantity of macrophages at baseline, after 7 days of disease induction, and after 7 days of disease recovery were similar in the periodontium of both old and young mice. These findings demonstrate that age related changes do not result in differential recruitment or substantivity of the macrophage at the site of inflammation. Certainly, a prolonged or stronger signal resulting in increased recruitment of inflammatory cells could result in more severe disease presentation. However, the increased prevalence of disease in old mice does not appear to be a result of increased macrophages present within the periodontium. Our previous findings demonstrated similar macrophage numbers in the fracture callus of old and young mice (Chapter 3). Taken together, these findings suggest the demonstrated transcriptional differences in old and young macrophages are an important contributing factor to the pathogenesis of periodontal disease in old mice.

The depletion of macrophages during periodontal disease induction resulted in decreased disease severity in both young and old mice. The decreased disease severity was associated with decreased inflammatory cytokine expression locally within the gingiva. Other groups have shown similar improvements in periodontal disease severity with the administration of compounds that targeted the inflammatory response. Alveolar bone loss was significantly reduced in a mouse model of periodontal disease by local delivery of a cytokine (CL22) that increased the local recruitment of T regulatory cells (12). Similarly, promoting the resolution of inflammation in a mouse model of periodontitis with the administration of resolvins resulted in decreased bone loss (13).

It was interesting to see a benefit with macrophage depletion during disease recovery in old mice but not in young. We hypothesize this differential effect by age was due to the transcriptional difference demonstrated in old mice compared to young. Macrophages from old mice demonstrated more M1 and pro-inflammatory cytokine gene expression (Chapter 3). Therefore, depleting the old macrophages reduced a source of cytokines that could propagate the inflammatory response. However, when young macrophages were depleted, there was not the same reduction of pro-inflammatory cytokines. We hypothesize that the young macrophage had already made the phenotypic switch to an M2-like macrophage during the recovery phase. Thus, less of a benefit was demonstrated by depleting M2-like macrophages in young mice compared to depleting M1-like macrophages in old mice. From the analyses here, it is unknown if there was a deleterious effect from depleting M2-like macrophages in young mice. The M2 phenotype is involved in inflammatory resolution as well as producing growth factors to aid in tissue healing (11). Therefore, it could be reasoned that the depletion of M2 macrophages may be detrimental during recovery from periodontal disease. Our micro-CT data demonstrate a minimal reduction in bone volume/total volume and bone mineral density in the treated young mice compared to the non-treated controls during disease recovery. These findings were far from statistically significant; however, additional quantitative methods or macrophage depletion over a longer period of time may demonstrate the potential detrimental effect of depletion of M2-like macrophages.

We demonstrated the administration of PLX3397 significantly reduced the number of macrophages within the periodontium. Multiple macrophage depletion strategies exist that could be applied in a similar manner to a periodontal disease mouse model. Clodronate liposomes can be administered to deplete macrophages. The liposomes are phagocytized by the macrophages and the clodronate is delivered intracellularly and results in apoptosis of the cell (14). This method has similarly shown effective depletion rates of macrophages within bone (15), but it first requires

the presence of a differentiated and active mature macrophage. Thus, by first allowing the differentiation of the macrophage at the site of inflammation, cytokine secretion activity could already have an effect on the disease process before the macrophage is depleted. Another macrophage depletion strategy utilizes the transgenic MaFIA mouse (Mac Fas-induced apoptosis). The MaFIA mouse used a Cre-loxP system to target the *Csfr1r* promoter that allows for inducible apoptosis upon administration of diphtheria toxin (16). The MaFIA mouse system is similarly effective in depleting macrophages but lacks specificity and targets other CD11b cells that may result in confounding effects in periodontal disease (17). Similarly, PLX3397 may have an effect on cells other than macrophages. By antagonizing the m-CSF receptor, the drug blocks monocyte differentiation of other cell types that require M-CSF activation. These other cell types importantly include osteoclasts which would be involved in the pathogenesis of periodontal disease. In examining the activity of osteoclasts during periodontal disease, we found TRAP+ cells present in the periodontium of PLX3397 treated mice similar to what was observed in non-treated mice (Supplementary Figure 4.1). The difference in quantity of osteoclasts between treated and non-treated mice could not be specifically associated as a direct effect of the drug, as PLX3397 administration also reduced expression of inflammatory cytokines that are upstream of osteoclast activation (18).

4.5 Conclusion

The work presented here demonstrates that the aged macrophage phenotype, as characterized in Chapter 3 via RNA-seq, contributes to the pathogenesis of periodontal disease. Similar beneficial outcomes were demonstrated from macrophage depletion in aged models of fracture healing and periodontal disease, suggesting the age-related changes to macrophages strongly contribute to the inflamm-aging phenotype in an orthopaedic context. Interestingly, the beneficial outcome demonstrated from macrophage depletion during disease recovery could serve as a

model for a more directed therapeutic strategy for the management of periodontal disease in the elderly population.

Figures: Chapter 4

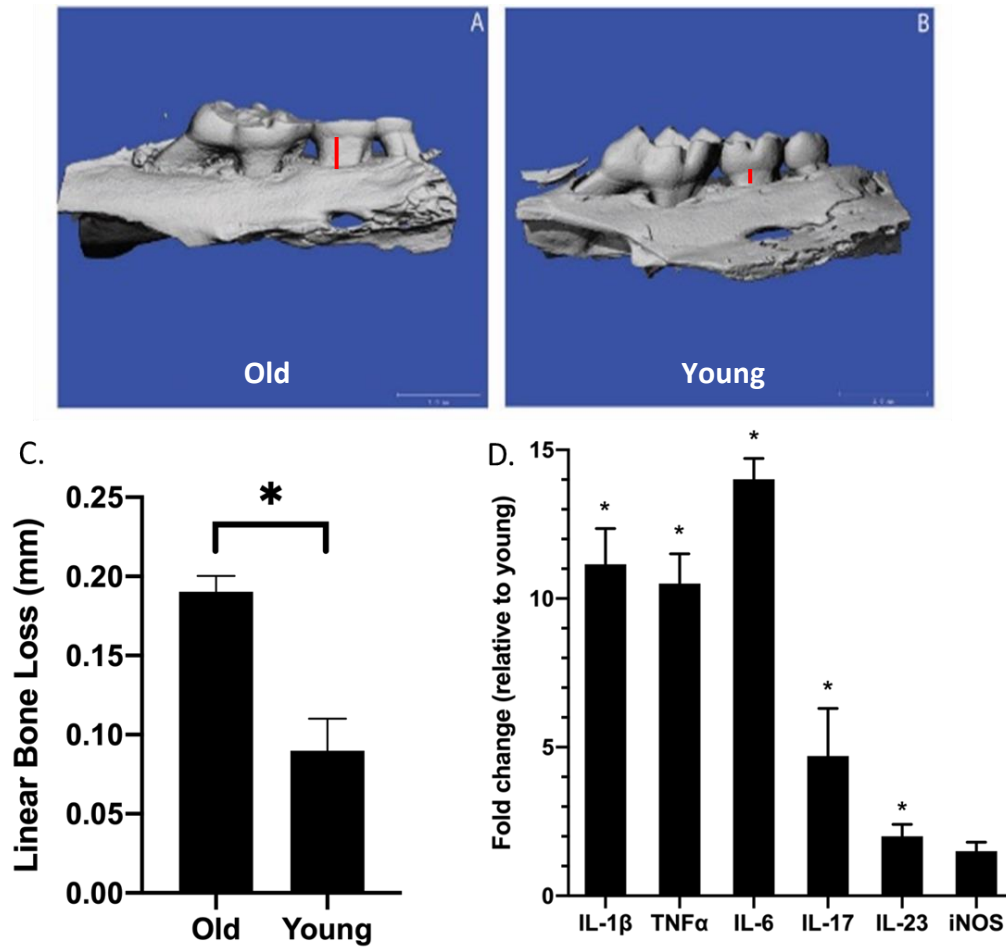


Figure 4.1: Old mice present an aged periodontal phenotype. Bone loss in healthy control old mice is associated with increased pro-inflammatory gene expression. Linear bone loss (measured from CEJ to alveolar bone crest-red line) in old mice (24 month) (A) was compared to young mice (3 month) (B), and quantified (C). Fold change of gene expression in gingiva of old relative to young (D). (* $p < 0.05$).

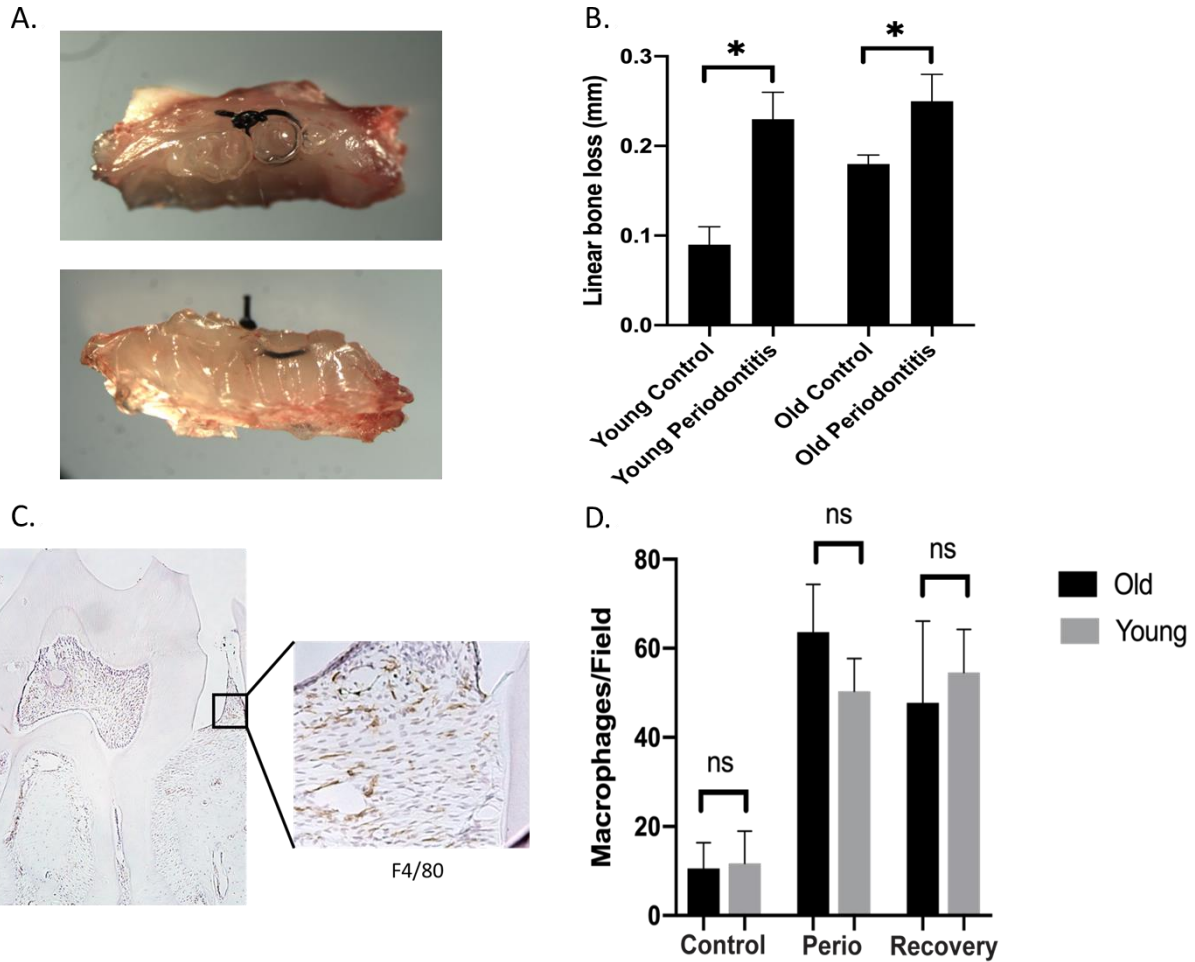


Figure 4.2: Macrophage quantity within the periodontium is similar in old and young mice. Disease induction via the ligature model (A) resulted in significant bone loss compared to control mice in young (3 month) and old (24 month) mice (B). Macrophage quantity within the periodontium was measured via immunohistochemistry for F4/80+ macrophages (brown) (C). The quantity of macrophages within the periodontium at baseline, after 7 days of disease induction, and after 7 days of disease recovery was not significantly different in young and old mice (D).

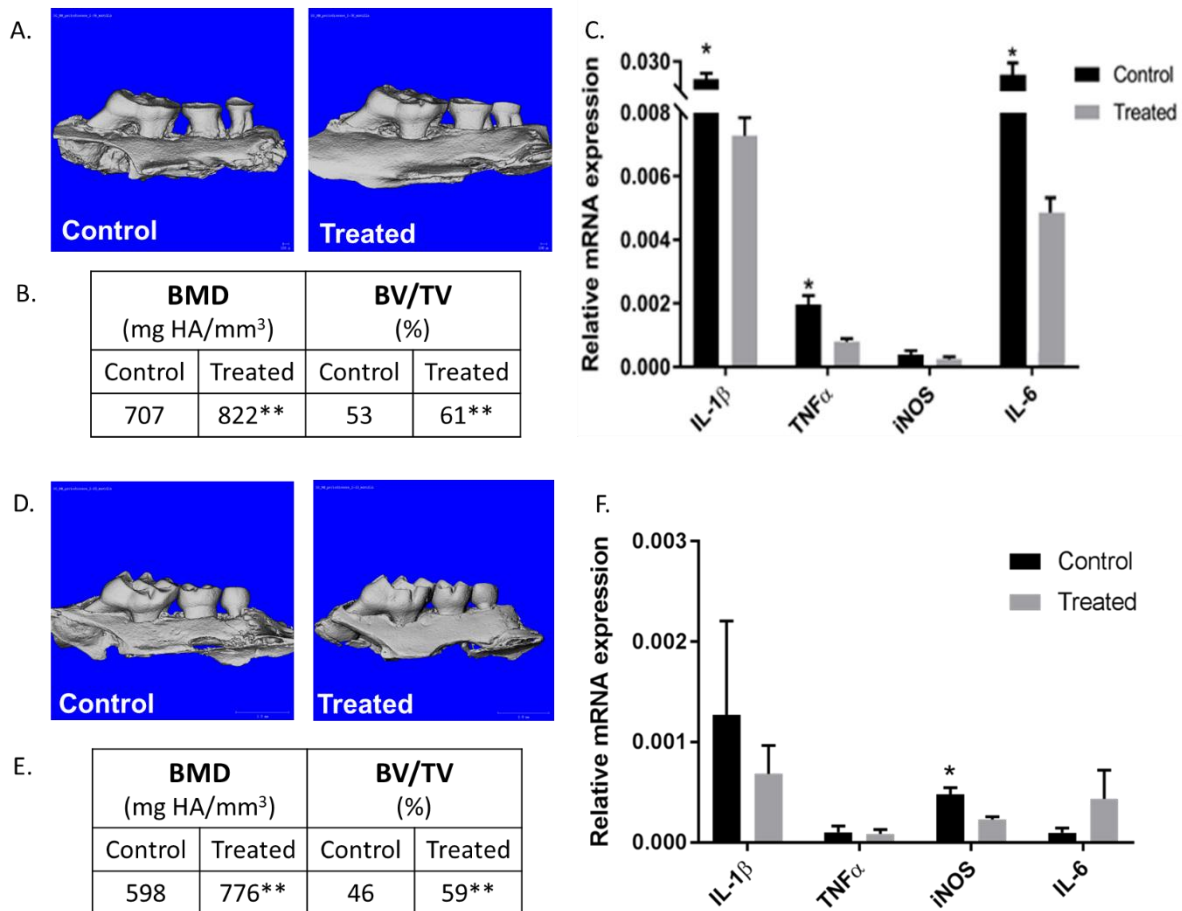


Figure 4.3: Macrophage depletion attenuates bone loss and pro-inflammatory cytokine expression in old and young mice. After 7 days of disease induction dissected hemi-maxillae were analyzed via micro-CT from old (A) and young (D) mice. Treated groups received PLX3397 throughout the induction period to deplete macrophages. Control groups received a control chow. As a measure of disease severity bone mineral density (BMD) and bone volume/total volume (BV/TV) were analyzed via micro-CT in old (B) and young mice (E) as a function of macrophage depletion treatment. Gingiva was removed from the hemi-maxillae and analyzed via qRT-PCR for expression of pro-inflammatory cytokines. Relative mRNA expression ($\Delta\Delta C_T$) was compared between treated and control group in old (C) and young (F) mice. (* $p < 0.05$, ** $p < 0.001$).

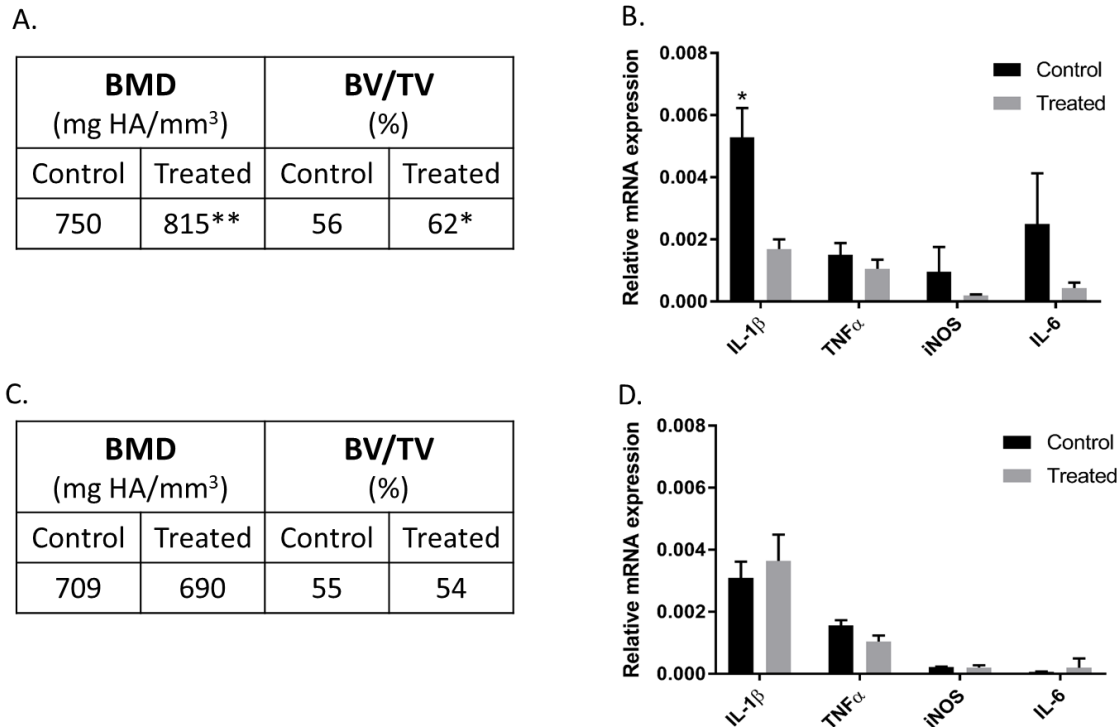
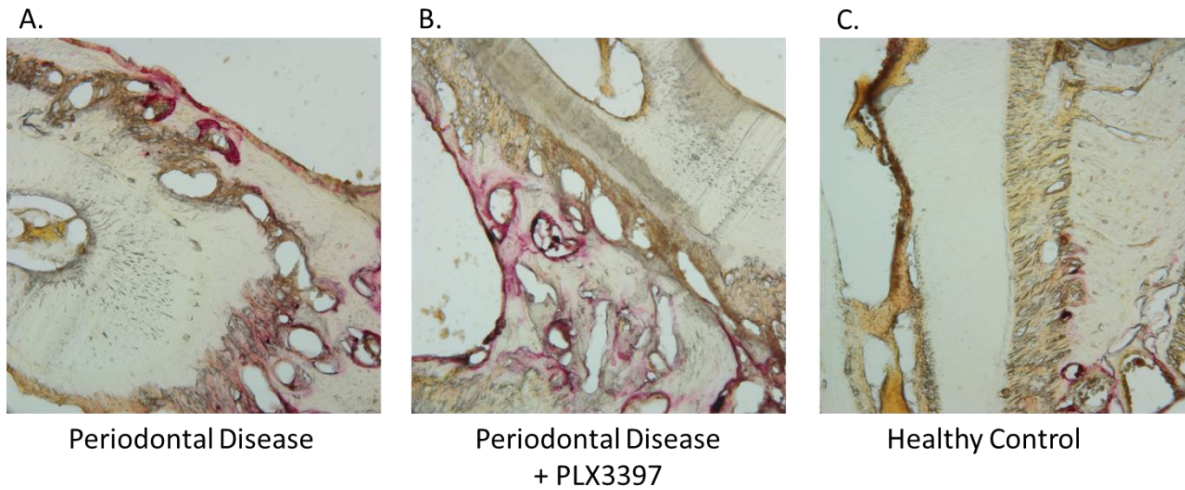


Figure 4.4: During disease recovery, macrophage inhibition differentially affects bone loss and pro-inflammatory cytokine expression in old compared to young mice. After 7 days of disease recovery dissected hemi-maxillae were analyzed via micro-CT from old and young mice. Treated groups received PLX3397 throughout the recovery period to deplete macrophages. Control groups received a control chow. As a measure of disease severity bone mineral density (BMD) and bone volume/total volume (BV/TV) were analyzed via micro-CT in old (A) and young mice (C) as a function of macrophage depletion treatment. Gingiva was removed from the hemi-maxillae and analyzed via qRT-PCR for expression of pro-inflammatory cytokines. Relative mRNA expression ($\Delta\Delta C_T$) was compared between treated and control group in old (B) and young (D) mice. (* $p < 0.05$, ** $p < 0.001$).



Supplemental Figure 4.1: Osteoclastogenesis is present after PLX3397 treatment. TRAP staining of histological slides of the periodontium in mice after 7 days of periodontal disease induction (A), after 7 days of periodontal disease induction with administration of PLX3397 throughout the induction period (B) and in healthy controls (C). (20x magnification)

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Chapter 5: Mechanisms driving inflammatory dysregulation in aged macrophages

5.1 Introduction

The previous work has robustly demonstrated the age-related changes that occur to the macrophage at the transcriptional level and has characterized an aged macrophage phenotype with increased M1/pro-inflammatory gene expression. Further, it has shown how depletion of the aged macrophages has beneficial effects in two distinct osteoimmunological models, fracture healing and periodontal disease. The goal of the proceeding work was to further understand mechanisms driving the age-related changes in macrophages and to understand the effect of the aged macrophage phenotype on other inflammatory cells during fracture healing and periodontal disease. Therefore, we have investigated age-related changes to the expression of triggering receptor expressed on myeloid cells (*TREM2*) that may contribute to the differential inflammatory gene expression in aged macrophages. Further, we investigated how the aged macrophage phenotype may promote other downstream cellular regulators of inflammation.

Regulation of macrophages activity occurs, in part, through the activity of *TREM2*. Activation of *TREM2* on macrophages results in downregulation of inflammation (1). *TREM2* signals through its transmembrane coreceptor DNAX-activation protein 12 (DAP12) to inhibit TLR stimulation of pro-inflammatory cytokines production (2) (Fig. 5.1). Studies have demonstrated that inhibition of *TREM2* results in increased pro-inflammatory cytokine production and increased inflammatory-related damage to tissue (3,4). Despite being an attractive target to regulate inflammation, no natural ligand of *TREM2* has been discovered (1). Polymorphisms of *TREM2* gene can result in a condition known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), which presents with bone cysts, osteoporotic features, and neurodegeneration possible due to a systemic wide disturbance in inflammatory regulation (5).

The role of *TREM2* in an osteoimmunological context has not been previously investigated. Our genetic understanding of PLOSL and the associated bone cysts suggest *TREM2* may be involved

in inflammatory regulation within the skeletal system. Previous work has shown that knockout of *Dap12* has been shown to be detrimental in fracture healing in mice (6). Understanding the mechanisms of how TREM2 regulates inflammation within bone and the effect of age on TREM2 is important for the improved management of disease and conditions within elderly populations.

The effects of the aged macrophage phenotype on other immune cells are unknown. Given the increased pro-inflammatory cytokine gene expression demonstrated by aged-macrophages, it is reasonable to assume other immune cells in the inflammatory cascade may be differentially affected by aged macrophages compared to young. One potential pathogenic cellular interaction in periodontal disease is between the macrophage and Th17 cells. Th17 cell expansion and IL-17 expression are pathogenic mediators in periodontal disease (7,8). There are multiple cellular sources of IL-17 within the gingiva; however, during periodontal disease Th17 cells represent 80% of the IL-17+ cells (7). Inhibition of Th17 cell differentiation was shown to decrease disease severity (7). Drivers of pathogenic Th17 cell expansion in periodontal disease are beginning to be elucidated but are not fully understood. Pathogenic expansion of Th17 cells requires IL-6 and IL-23 signaling (7,8). With increased age, Th17 cells demonstrate increased homeostatic expansion (9); however, it is unknown the extent to which pathogenic Th17 cell expansion is differentially regulated by age. Understanding how the aged macrophage phenotype may drive pathogenic Th17 expansion may lead to potential therapeutic targets.

5.2 Materials and Methods

The methodology is described in brief below with full descriptions included in the previous chapters.

5.2.1. Animals

All procedures were approved by the UCSF Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines for humane animal care. Mice (C57B6/J) were obtained from the National Institute on Aging's Aged Rodent Colony.

Specific pathogen-free mice were bred and raised in barriers and utilized for experiments at 24 months (old adult mice) or 3 months (young adult mice) of age. *Trem2* knockout mice were generated, authenticated, and provided by our collaborator Mary Nakamura.

5.2.2. *Tibia fractures*

Mice were anesthetized and subjected to closed, non-stable fractures of the right tibia created by three-point bending, as previously described (10). To inhibit macrophage recruitment during fracture healing in old mice, treatment groups received the compound PLX3397 (275mg/kg) (Plexxikon Inc. Berkeley, CA) *ad libitum* in their chow. Control groups received the same chow without PLX3397. Treatment with PLX3397 was started 24 hours before fracture and continued for 3 or 10 days after fracture until the animal was euthanized and the tissues harvested for analysis.

5.2.3. *Tibia processing and stereology*

Mice were sacrificed at day 10-post fracture for stereological analysis of the fracture callus. Fractured tibiae were collected, fixed, and decalcified. Serial sagittal sections (10 μ m) were mounted on slides and stained using Hall Brunt Quadruple Stain (HBQ) to visualize bone and cartilage. Total callus, bone and cartilage volume was quantified according to stereological methods developed by Howard and Reed (11).

5.2.4. *Flow cytometry*

Old and young mice were sacrificed at day 3 post fracture. Fractured tibiae were collected and the calluses were dissected and digested with Collagenase type I (0.2mg/mL; Worthington, Lakewood, NJ) for 1 hour at 37 degrees. Cells were isolated and resuspended in incubation buffer (0.5% BSA in PBS). Isolated cells were blocked and then stained with directly conjugated fluorescent antibodies: CD45 (clone 30-F11), CD3 (145-2C11), B220 (RA3682), Gr-1 (RB6-8C5), NK1.1 (PK136), MHC Class II (M5/114.15.2) Ly6 C (HK1.4) Ly6G (clone 1A8), F4/80 (clone BM8), and CD11b (clone M1/70) (Biolegend, San Diego, CA). Cells were sorted on a FACSAria (BD

Biosciences, San Jose, CA), and FlowJo Software 9.6 (Treestar, Ashland, OR) was used for analysis.

5.2.5. RNA-seq Analysis

Macrophages were isolated from the fracture callus of old (n=10) and young (n=11) mice at day 3 post-fracture. The callus was dissected and cells were collected and stained as described above. Macrophages were collected by staining with CD45 (clone 30-F11), F4/80 (clone BM8), and CD11b (clone M1/70) (Biolegend, San Diego, CA). RNA was extracted using Invitrogen RNA aqueous Micro Kit (AM1931). The library was prepared and Single-end 50 bp RNAseq was performed on Illumina HiSeq 4000. Reads were aligned using STAR_2.4.2a to the mouse genome (Ensemble Mouse GRCm38.78). Differential gene expression was assessed using DEseq2. Gene ontology and KEGG pathway analysis was performed using DAVID (<http://david.abcc.ncifcrf.gov>) and the mouse genome was used as background.

5.2.6 qRT-PCR

Five days post fracture, the animal was sacrificed and the whole callus was dissected. Similarly, 7 days after periodontal disease induction (see chapter 3 for disease induction methodology) or in healthy controls, gingival tissue was dissected bilaterally from the maxillary alveolar process. Dissected tissue was placed in Trizol and homogenized. RNA extraction was then performed with isopropanol. cDNA was synthesized and quantitative real time PCR was performed on biological and technical triplicates. Relative gene expression of IL-1 β , IL-6, TNF- α , iNOS, IL-23 was quantified. GAPDH was used as the house keeping gene.

5.2.7. Statistics

GraphPad Prism v.7 software was used for analysis. Comparisons between groups was made by first using a 2-way ANOVA multiple comparisons test followed by a 2-tailed Student's *t* test. P<0.05 was considered statistically significant. Differential gene expression was considered

significant at $FDR < 0.1$. For term enrichment in gene ontology and KEGG pathway analysis the level of significance was set using a modified Fisher Exact P-value of $p < 0.05$.

5.3 Results

5.3.1 *TREM2 is upregulated during fracture repair*

Three days after tibia fracture in old and young mice, the fracture callus was dissected, and macrophages were isolated. Isolated macrophages were Cd45+, and CD11b+, and further analyzed for expression of F4/80+. Additionally, macrophages were isolated from non-fractured bone marrow of old and young mice as controls for comparison. Figure 5.2 demonstrates that TREM2 expression is nearly absent in macrophages from the non-fractured bone marrow of old and young mice, suggesting in the absence of an inflammatory process, TREM2 is not active. However, the lower panels demonstrate an increase in TREM2 expression on macrophages isolated from the fracture callus. There also appears to be a trend towards increased TREM2 expression in the macrophages from young mice compared to old.

5.3.2 *TREM2 is downregulated in old mice compared to young*

To understand how TREM2 expression on macrophages is affected by age, macrophages were collected from the fracture callus of old and young mice 3 days after fracture and analyzed via bulk RNA-seq. Figure 5.3 demonstrates the list of genes involved in the TREM2 signaling pathway. Of all the genes involved, only TREM2 expression was significantly differentially affected by age ($FDR < 0.1$), with decreased expression in the old macrophages compared to young. Further, when periodontal disease was induced in old and young mice, TREM2 expression was significantly lower within the gingiva of old mice compared to young (Fig. 5.3).

5.3.3 *TREM2 deficient mice demonstrate increased pro-inflammatory cytokines expression in the fracture callus and in the gingiva*

To understand the impact of TREM2 in regulating inflammation, we obtained mice with genetic deletion of TREM2. Gingival tissue was dissected from TREM2^{-/-} mice and aged matched wild

type controls. Pro-inflammatory cytokine expression was increased in $TREM2^{-/-}$ mice compared to controls (Fig. 5.3). Similarly, fractures were created in $TREM2^{-/-}$ and aged-matched wild type controls and the calluses were isolated five days post fracture. Analysis of the fracture callus via qRT-PCR similarly demonstrated increased pro-inflammatory cytokine expression in the fracture callus of $TREM2^{-/-}$ mice compared to controls (Fig. 5.4)

5.3.4 TREM2 deficient mice demonstrate delayed fracture healing

As $TREM2$ deficiency drove inflammatory dysregulation in the fracture callus, we wanted to understand the impact of $TREM2$ deficiency on fracture healing outcomes. Tibia fractures were created in $Trem2^{-/-}$ mice and wild type controls. Animals were sacrificed and the healing callus analyzed after 7, 10, and 14 days post fracture. Calluses were analyzed via stereology to quantify the volume of total callus, bone and cartilage tissue. $Trem2^{-/-}$ mice demonstrated significant delayed healing by day 10 with significantly decreased callus volume and bone volume compared to wild type controls (Fig. 5.4).

5.3.5 An aged macrophage phenotype may drive Th17 cell expansion to further promote inflammatory dysregulation in periodontal disease.

The pathogenesis of periodontal disease involves, in part, activation and expansion of Th17 cells locally within the gingiva. Th17 cell expansion is driven by activation by IL-6 and IL-23. The specific tissue and cellular sources of IL-6 and IL-23 that drive Th17 cell expansion are unknown. We hypothesized that the aged macrophage phenotype may drive the TH17 expansion more so than a young macrophage through the dysregulated expression of pro-inflammatory cytokines. To test this hypothesis, gingiva from old and young mice was dissected and processed for qRT-PCR. Expression of IL-6 and IL-23 was significantly increased in the gingiva of old mice compared to young (Fig. 5.5). Further, periodontal disease was induced in old mice, and half the groups were given PLX3397, a compound that depleted macrophages. Depletion of macrophages during

periodontal disease resulted in significant decreases in IL-6 and IL-23 expression within the gingiva during periodontal disease (Fig. 5.5).

5.4 Discussion

The results presented in this chapter demonstrate that inflammatory dysregulation in macrophages may be derived from dysregulated expression of TREM2. The aged macrophage phenotype was further shown to have the potential to promote the expansion of Th17 cells in the pathogenesis of periodontal disease. These results are of interest as they provide a mechanism, TREM2 dysregulation, for inflammatory dysregulation seen in macrophages from old mice, which may be a target for future translational therapeutic approaches. In addition, the relationship of the aged macrophage phenotype to Th17 cells, a potent promoter of periodontal disease, suggests a stronger link between the aged macrophage phenotype and the pathogenesis of periodontal disease.

TREM2 has recently become of interest to investigators studying macrophage regulation of inflammation, specifically within the central nervous system (12). Much of the focus has been placed on TREM2 dysregulation associated with neurodegenerative diseases including dementia and Alzheimer's disease (12). The focus of TREM2 in the central nervous system was initiated after understanding the polymorphism to *TREM2* that was involved in PLOSL and the associated early onset dementia (5). Since then, recent work has demonstrated the importance of TREM2 in regulating inflammation within the central nervous system, specially its role in proper phagocytosis and clearance of cellular debris (13,14). Additional polymorphisms of *TREM2* have since been strongly associated with Alzheimer's disease and autism (15,21). As stated previously, PLOSL additionally present with cystic bone lesions and osteoporotic features, suggesting TREM2 may also be involved in the regulation of inflammation within bone. However, there is a lack of research investigating the role of TREM2 in inflammatory regulation within the skeletal system.

Our work has demonstrated that *Trem2*^{-/-} mice have poorer healing outcomes compared to their age-matched controls with significantly smaller callus and less bone at day 10 post fracture. At later time points of healing, histological analysis demonstrated an absence of cartilage within the callus of *Trem2*^{-/-} mice (data not shown), suggesting the fracture healed to completion. This data suggests TREM2^{-/-} mice are still capable of healing but demonstrate a delayed healing response. We have also shown delayed fracture healing in old animals compared to young in Chapter 3, which was associated with decreased TREM2 expression in macrophages from old mice. The delayed fracture healing in old mice, and the associated decreased TREM2 expression suggest TREM2 is important for proper fracture healing.

Our work demonstrated an increased expression of TREM2 on macrophages at 3 days post fracture. However, the expression levels of TREM2 remained low, at less than 5% of all macrophages isolated. While targeting the activation of the TREM2 receptor could be of therapeutic interest, it is also important to understand how the expression of TREM2 at the surface of macrophages is controlled. Studies have shown that the administration of LPS into macrophage cell cultures resulted in a significant decrease in TREM2 expression, suggesting during periods of infection the anti-inflammatory effects of TREM2 are down regulated (3). In other studies, TREM2 was over expressed on macrophages which resulted in decreased inflammatory cytokine expression (16). Further work on TREM2 expression regulation during fracture healing may look at multiple time points throughout the healing phase to understand when TREM2 expression is highest and what cytokine expression may be associated with its regulation.

Mechanisms regulating the phenotypic presentation of macrophages along the pro-inflammatory (M1) and anti-inflammatory (M2) spectrum and are unknown and are likely multifactorial considering the complex signaling involved in inflammatory regulation. However, TREM2 may serve as a promising target to regulate the macrophage phenotype, as activation of TREM2 results in anti-inflammatory activity by the macrophage similar to a M2 phenotype. The presence

of M1 and M2 type macrophages has been shown to be related to increased and decreased severity of periodontal disease respectively (17). Thus, promotion of an M2-like polarization state in macrophages may demonstrate a therapeutic benefit (18). Delivery of IL-10 was shown to downregulate the M1 phenotype and was associated with decreased severity of muscular dystrophy (19). In addition, promotion of M2 macrophages via delivery of IL-4/IL-13 resulted in improved fracture healing (20). Targeting TREM2 activation has demonstrated heterogeneous outcomes. Reported ligands for TREM2 include mammalian cells such as macrophages and other immune cells, phospholipids, and whole bacteria or bacterial components; however, there is no consensus on a natural ligand for TREM2 (1). Activation of TREM2 has resulted in general downregulation of inflammatory cytokine expression or increase in phagocytic activity depending on the stimulus (1). However, more work is needed to understand how TREM2 activation may control macrophage phenotype or directly impact wound healing.

Finally, we wanted to understand how the aged macrophage phenotype interacted with other inflammatory cells. While there are likely many effects of the increased pro-inflammatory cytokine expression by aged macrophages, we chose to focus on Th17 cells due to its pathogenic role in periodontal disease (7). This work showed that the known cytokines that drive pathogenic Th17 cell expansion (IL-6, IL-23) are significantly increased within the gingiva of old mice compared to young. Expansion of Th17 cells is also increased in old mice compared to young (9). Further, depleting macrophages during periodontal disease induction resulted in significantly decreased IL-6 and IL-23 expression. During periodontal disease we only examined the cytokine expression within the gingival tissue; however, isolated macrophages from the fracture callus of old mice also demonstrated increased expression of IL-6 and IL-23 compared to young (Chapter 3, Fig. 3). This data suggests an interaction of the aged macrophage phenotype and Th17 cells that contribute to the increased prevalence of periodontal disease in elderly populations.

5.5 Conclusion

Work in the previous chapters have shown that macrophages from old mice present an aged-macrophage phenotype with increased pro-inflammatory and M1 gene expression. By eliminating the influence of the aged macrophage phenotype via macrophage depletion, periodontal disease severity was significantly reduced and fracture healing was significantly improved. Further, we tried to understand intrinsic changes within the macrophage that may be responsible for the pro-inflammatory characteristics of the aged macrophage phenotype. To this effort, we have shown that TREM2 expression is decreased with age which drove increased inflammatory cytokine expression and poorer fracture healing outcomes. Finally, we have shown a mechanism in which the aged macrophage phenotype can further promote inflammatory dysregulation and disease severity by driving Th17 cell expansion in periodontal disease. Taken together, the aged-related changes that occur to the macrophage are likely involved in numerous disease pathologies, including periodontal disease and fracture healing complications (Fig. 5.6), and this work presents potential therapeutic targets to address the macrophage-driven inflammatory dysregulation in the elderly population.

Figures Chapter 5

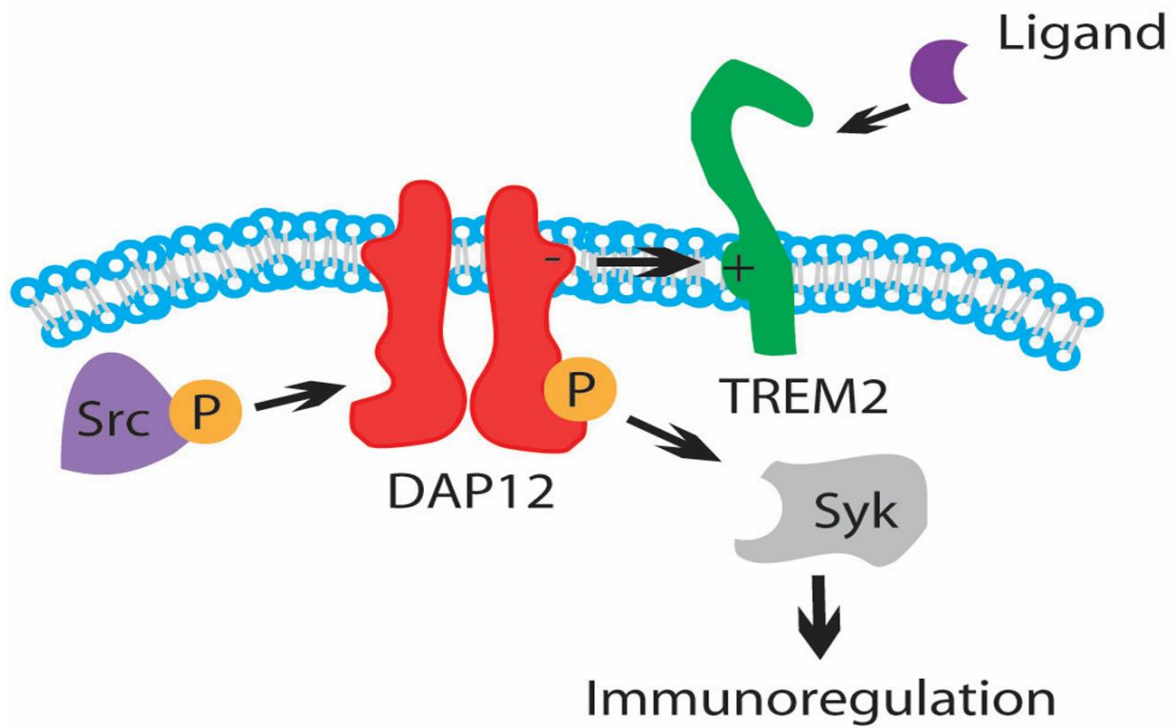


Figure 5.1: Schematic of the triggering receptor expressed on myeloid cells 2 (TREM2) signaling pathway. Activation of TREM2 results in activation of immunoregulatory functions of macrophages to downregulate inflammation. Signaling through TREM2 requires the intramembranous co-receptor DNAX activation protein of 12kDa (DAP12).

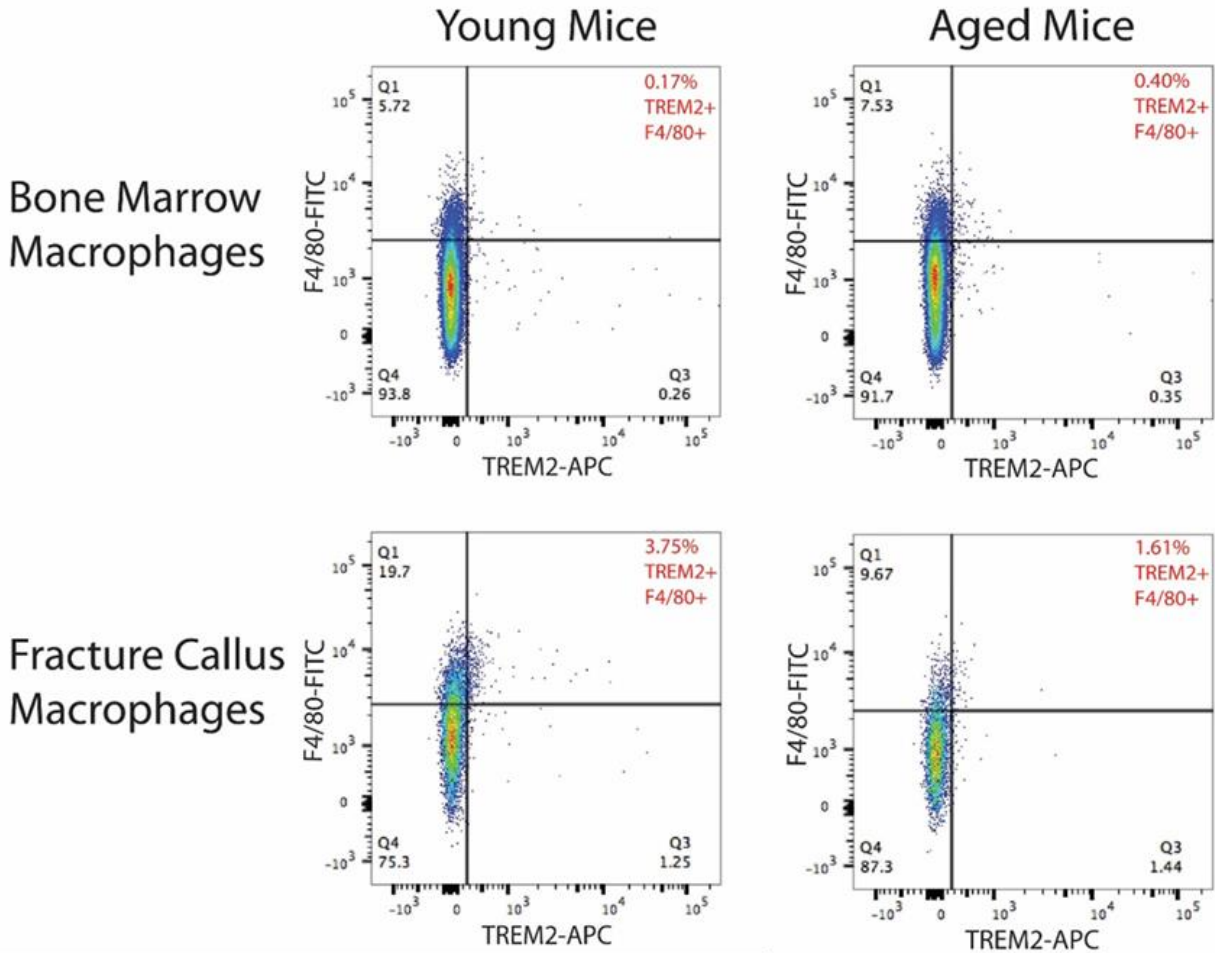


Figure 5.2: TREM2 expression on macrophages increases during fracture repair. Bone marrow stromal cells were isolated from the intact femur or from within the fracture callus. Macrophages were isolated via flowcytometry as CD11b+, F4/80+, and additionally analyzed for TREM2+ expression. TREM2 expression is low on macrophages from non-fractured bone marrow in young and old mice (Top Panels). Increased TREM2 expression is demonstrated on macrophages isolated from the fracture callus at Day 3 post fracture in young and old mice (Bottom Panels).

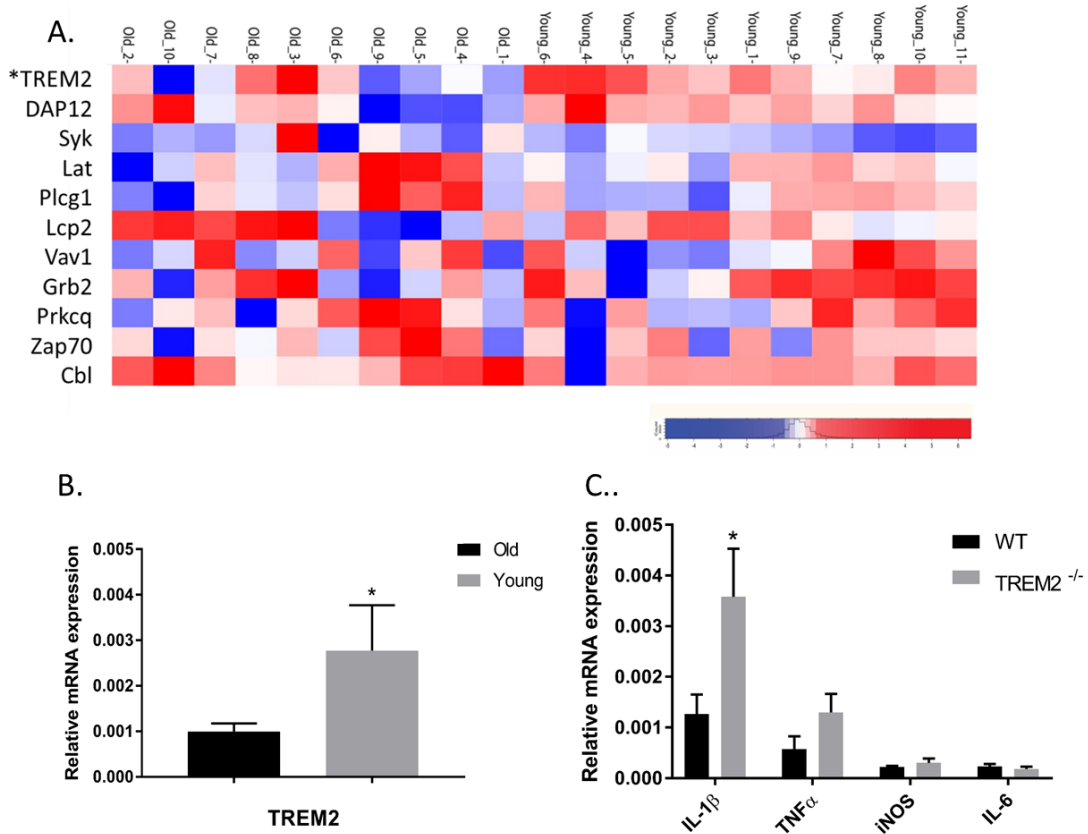


Figure 5.3: TREM2 expression is downregulated in old mice. Macrophages were isolated from the callus of old and young mice 3 days post fracture and analyzed via RNA-seq for the expression of the genes involved in the TREM2 signaling cascade (A). Gingiva was isolated from young, and old mice after disease induction and analyzed for expression of TREM2 via qRT-PCR (B). Gingiva in TREM2^{-/-} mice demonstrated increased inflammatory cytokine expression compared to age matched wild type (WT) controls (C). ($p < 0.05$).

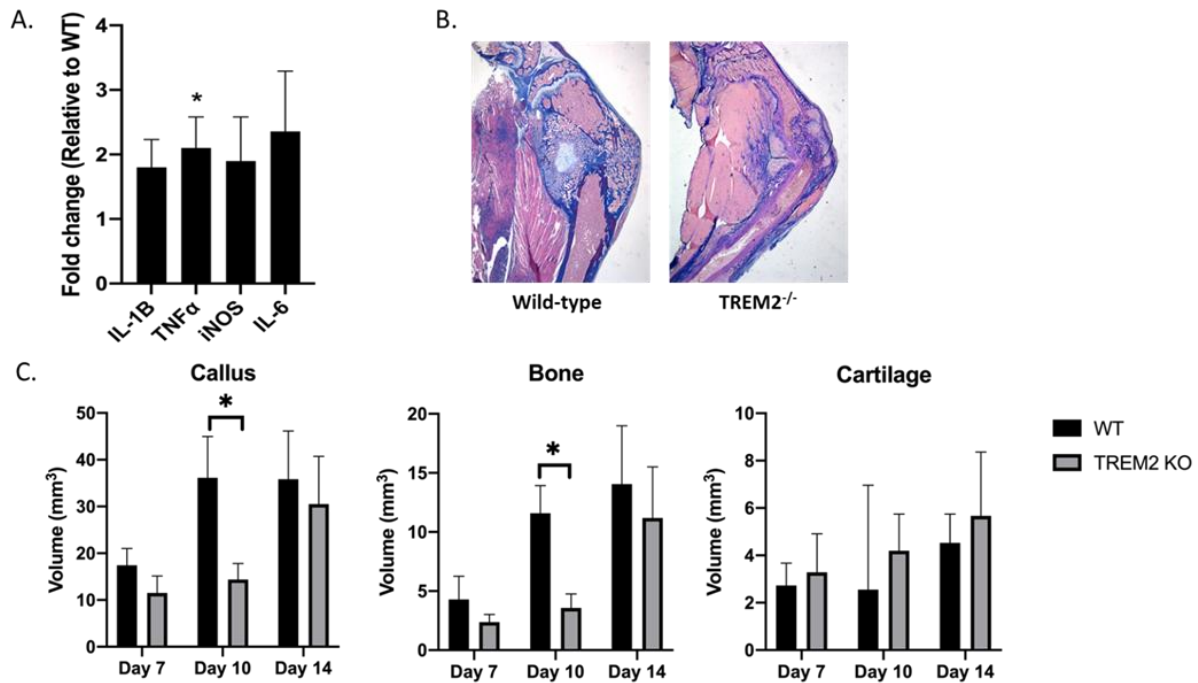


Figure 5.4: TREM2 deficient mice demonstrate delayed fracture healing. Tibia fractures were created in TREM2^{-/-} and age matched wild type (WT) controls. The fracture callus was isolated 5 days post fracture and analyzed via qRT-PCR for expression of inflammatory cytokine genes (A). Fractures were isolated 7, 10, and 14 days post fracture and histological slides were prepared and stained with HBQ (B). Slides were analyzed via stereology for quantification of callus, bone, and cartilage volume (C). ($p < 0.05$).

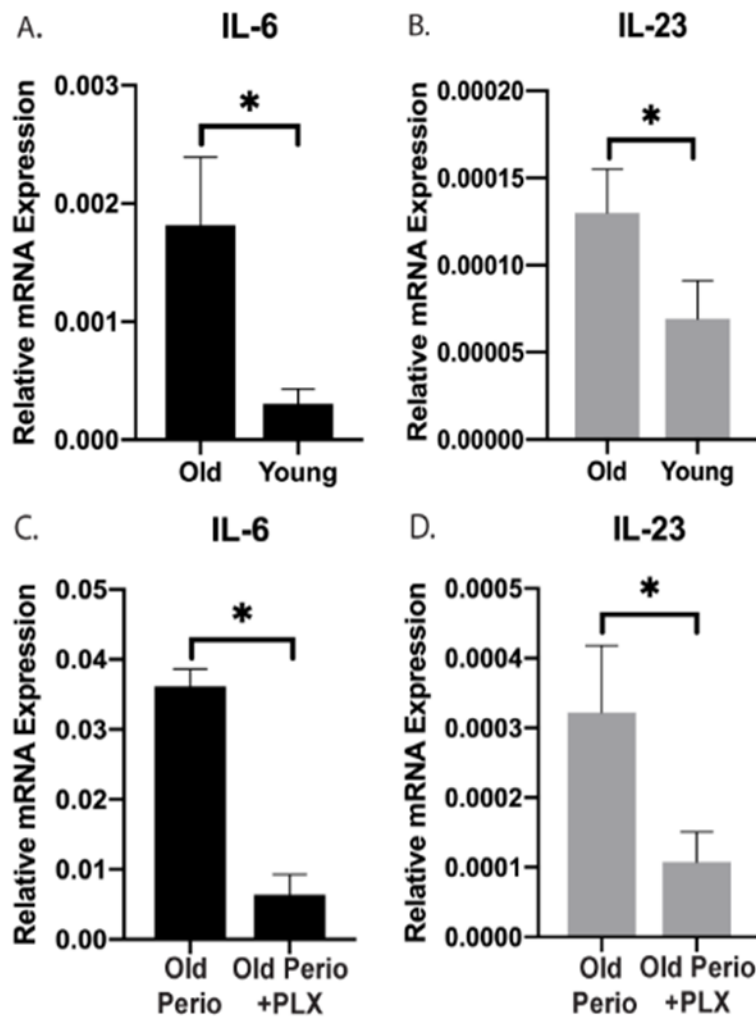


Figure 5.5: IL-6 and IL-23 expression is increased in the gingiva of old mice and can be reduced by macrophage depletion. Gingiva was isolated from healthy old and young mice and analyzed via qRT-PCR for relative expression of IL-6 (A) and IL-23 (B). Periodontal disease was induced in old mice and half the mice were given PLX3397 to deplete macrophages throughout the induction period. After 7 days of disease induction the gingiva was and analyzed via qRT-PCR for relative expression of IL-6 (C) and IL-23 (BD. ($p < 0.05$).

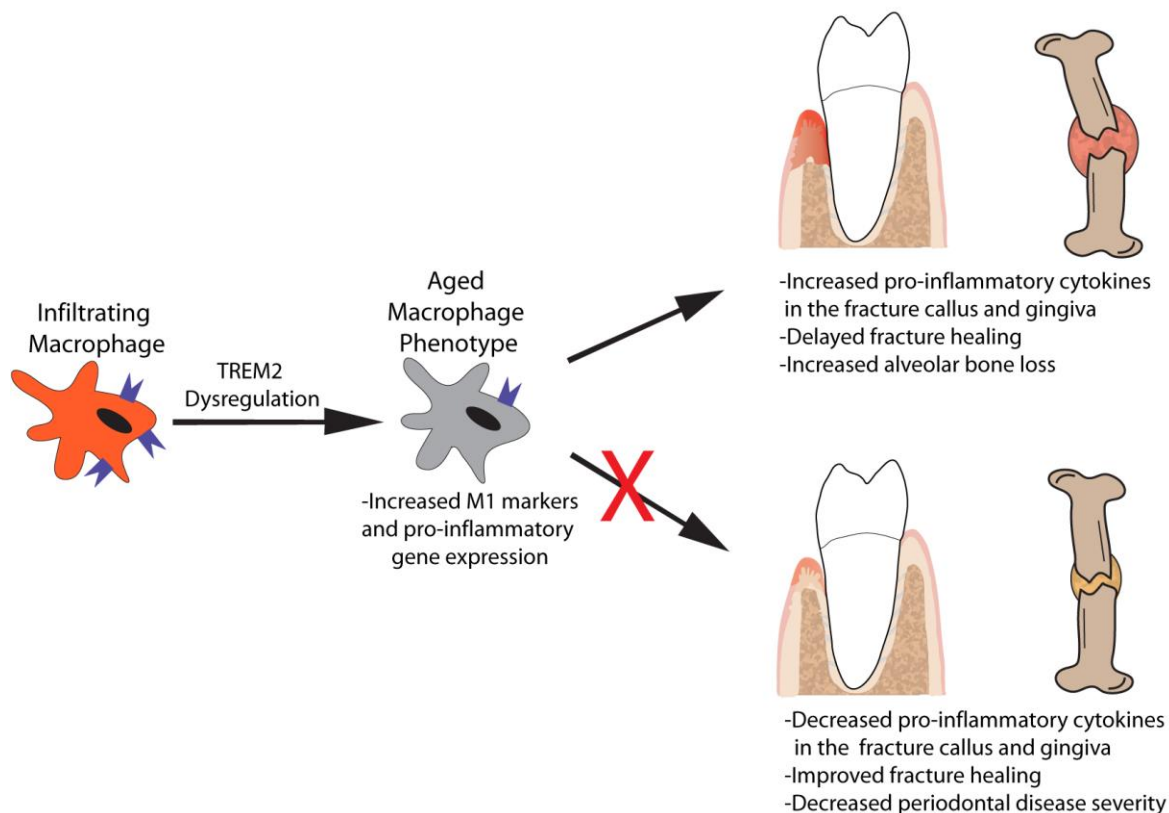


Figure 5.6: Graphical summary of findings. Infiltrating macrophages at the site of inflammation are transcriptionally distinct in old mice compared to young. Infiltrating macrophages from old mice demonstrate increased M1 markers and pro-inflammatory gene expression compared to young. TREM2 expression was decreased in macrophages from old mice which is hypothesized to be a driver of the inflammatory dysregulation in old macrophages. Old mice demonstrated increased periodontal disease severity and delayed fracture healing. Disease severity and fracture healing were improved with the depletion of macrophages in old mice.

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