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Los Angeles

**Role of Osteoclasts in the potential pathogenic
mechanism of Osteonecrosis of Jaw**

A thesis submitted in partial satisfaction of the requirements for the
Master of Science in Oral Biology

by

Kawaljit Kaur

2015

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ABSTRACTS OF THE THESIS

Role of Osteoclasts in the potential pathogenic mechanism of Osteonecrosis of the Jaw

By

Kawaljit Kaur

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Dr. Anahid Jewett, Chair

Osteonecrosis of jaw, is a bone disorder affecting the jaw bone. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a condition found in patients who have received intravenous and oral forms of bisphosphonate therapy for various bone-related conditions. Bisphosphonates (BPs) are a class of drugs used to treat osteoporosis and malignant bone metastasis. Osteoclast's are the bone cells capable of resorbing bone, are the potential target of BP therapy. The objective of this study is to demonstrate the role of osteoclasts in ONJ, effect of BP on the differentiation

and function of osteoclasts, inflammatory mediators secreted by osteoclasts involved in ONJ model, interaction of osteoclasts with other immune cells especially NK cells, as well as role of IL-10 in osteoclasts and BP interaction. This study demonstrate that, *i.v* nitrogen-containing bisphosphonates Zolendronic Acid and oral nitrogen-containing bisphosphonates Alendronate and oral non-nitrogen containing Etidronate modulate the pro-inflammatory functions of osteoclasts. Nitrogen containing Bisphosphonates mediated significant dose dependent release of pro-inflammatory cytokines IL-6, TNF- α , IL-1b and they inhibited anti-inflammatory IL-10 secretion by osteoclasts. Zolendronic Acid, Alendronate and Etidronate each had a different effect on the secretion of cytokines, with Zolendronic Acid having the highest and Etidronate the lowest ability to up-regulate the cytokine secretion. Zolendronic Acid, Alendronate and Etidronate each had a different effect on cell viability, Zolendronic Acid caused highest reduction in osteoclasts numbers, followed by Alendronate and Etidronate almost cause no cell death. The surface expression of CD14, CD33, CD54, CD44, CD11b, MHC class I and II and B7H1 were significantly increased when osteoclasts were treated with Zolendronic Acid, effect was higher at 500nM conc. and down-modulated at higher concentration of Zolendronic Acid, but still higher than untreated OC. All three bisphosphonates were capable of decreasing pit formation by osteoclasts. Osteoclasts act as key immune effectors capable of modulating the function of Natural Killer (NK) cells. Treatment of osteoclasts with Zolendronic Acid and much less with Alendronate was capable of inhibiting NK cell cytotoxicity whereas it induced significant secretion of cytokines, IFN- γ , IL-6, IL-10 and IL-18 and chemokines in the cultures of NK cells with osteoclasts. Resistance to NK cell cytotoxicity was higher at low concentration

of Zolendronic Acid, comparative to higher concentration. NK cells were able to lyse osteoclasts much more than their precursor cell monocytes and this correlated with the decreased expression of MHC class I and CD54 expression on osteoclasts. These results suggest that Zolendronic Acid treated osteoclasts may remain viable in the microenvironment for a prolonged period of time during interaction with NK cells providing continuous secretion of pro-inflammatory cytokines and chemokines in the absence of anti-inflammatory cytokine IL-10 resulting in the chronicity of inflammation.

Zolendronic Acid injections could similarly increase IFN-g and IL-6 secretion and Cytotoxicity against the tumor cells, in the bone marrows but decrease the secretion of IFN-g in gingival, pancreatic and adipose tissues. These observations are of significant value since they may provide potential mechanisms for the pathogenesis seen in Osteonecrosis of the Jaw (ONJ). It is possible that an increase in activation of Bone Marrow derived cells by Zolendronic Acid result in the eventual loss or inhibition of IFN-g secreting cells when they reach to the tissues. Since gingival derived immune cells are of activated phenotype when compared to bone marrow derived immune cells in healthy subjects, prior activation of bone marrow derived cells when mobilized to the gingival tissues and are exposed to additional activation signals from antigens in gingiva may undergo activation induced cell death, or simply become exhausted, and result in the lower secretion of IFN-g by the immune cells as seen in our in vivo model system. These possibilities are under investigation in our laboratory. The phenotype and nature of immune cells, including NK cells, one of the main contributors of IFN-g secretion in bone marrow and tissues are currently being investigated in our laboratory.

The thesis of Kawaljit Kaur is approved

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2015

This Thesis is dedicated, my mentor, Dr. Anahid Jewett, for believing in me, and encouraging me to learn and press on boundaries of knowledge, to my husband for his love, endless support and encouragement and my parents, Sr. Major Singh and Smt. Pyar Kaur, who always taught me to trust in God and hard work, and for earning in honest living for us.

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CHAPTER 1

INTRODUCTION

Osteonecrosis of jaw (ONJ)

Osteonecrosis of the jaw (ONJ) is a severe bone disease that affects the jaw bones, the maxilla and the mandible. Both local and systemic factors have been implicated in ONJ pathogenesis, dental and bacterial infection can increase the risk of inflammation [1-3]. Osteonecrosis of jaw, is the potential complication of bisphosphonates therapy, more common among cancer patients with metastatic bone disease treated with bisphosphonates, and is known as bisphosphonate-related osteonecrosis of jaw (BRONJ), or MRONJ, medication-related osteonecrosis of jaw. The term BRONJ was first described in Marx RE et al. in 2001 [4]. Although, the true incidence of BRONJ is unknown, an overwhelming proportion (97%) of the reported cases are related to high-dose intravenous BP used in cancer patients, frequency is higher in multiple myeloma (55.9%), followed by breast cancer (33.4%) [5-7]. A plausible explanation for increased risk in cancer patients is that the severity of cancer, chemotherapy and corticosteroids may modulate immunological status, increasing the risk of infection and impairing wound healing. Also, cancer patients receive up to 12-50 times higher doses of BP than osteoporosis patients. Factors such as trauma from dental surgery, advanced cancer, chemotherapy, corticosteroids, dental infection, diabetes, tobacco and alcohol use seem to increase the risk of BRONJ development [5, 6, 8-11].

The American Association of Oral and Maxillofacial Surgeons (AAOMS) defines BRONJ as “exposed bone in the maxillofacial area occurring in the absence of head and neck irradiation and showing no evidence of healing for at least 8 weeks after identification in patients treated with BP therapy”, [8] later modified the term BRONJ to medication-related osteonecrosis of the jaw (MRONJ). The risk of osteonecrosis associated with phosphorous compounds was first described in the 19th century [12]. An occupational disease termed “phossy jaw” or

“phosphorus necrosis of the jaw” was noted among workers in the matchmaking industry who worked with white phosphorus [13]. Workers presented with pain, infection, and exposure of the jaw bone. The incidence of “phossy jaw” started decreasing over the years with reduced use of white phosphorous in industry and improved working conditions. However, in 1999 reports of ulceration of the oral mucosa as a complication of oral BP therapy started emerging [14]. It was thought the ulceration were a result of direct mucosal injury, similar to esophageal ulceration that is another recognized side effect of alendronate therapy [15]. The clinical manifestations of ONJ vary significantly from asymptomatic small fistulation to painful swelling with extensive bone exposure leading to pathological bone fracture [9, 11, 16]. BP accumulate more and induce more profound effect in the jaws due to the fact that alveolar model remodels at the ten times the rate of tibia and at about five time the rate of basilar bone [17].

Osteoclasts

Osteoclasts are multinuclear cells, have the unique ability to degrade bone to initiate normal bone remodeling and mediate bone loss in pathologic conditions by increasing their resorptive activity. Osteoclasts are derived from hematopoietic stem cells, precursors in the myeloid/ monocyte lineage that circulate in the blood after their formation in the bone marrow, and are tartrate-resistant acid phosphatase (TRAP)-positive cells [18-20], and their differentiation is controlled by interactions between osteoblasts and/or stromal cells and pre-osteoclasts [21], M-CSF and RANKL are the essential factors expressed by osteoblasts, stromal cells and lymphocytes required for osteoclasts formation. M-CSF is a cytokine released from osteoblasts as a result of endocrine stimulation from parathyroid hormone [22]. It binds to receptors on

osteoclast precursor cells (OPC) and induces differentiation into OCs. M-CSF is required for both the proliferative and differentiation phase of osteoclast development [23, 24]. RANKL is critical for osteoclastogenesis and bone resorption [25-27]. RANKL interacts with its receptor RANK (receptor activator of NF- κ B), a transmembrane receptor that is a member of the tumor necrosis factor (TNF) receptor superfamily and is expressed on the surface of pre-osteoclasts and mature osteoclasts [28]. Osteoprotegerin (OPG) is a soluble decoy receptor which is produced by osteoblasts and can block osteoclast formation in vitro and bone resorption in vivo by binding to RANKL and reducing its ability to bind to RANK [29] [30, 31]. When osteoclasts are activated for resorption, a tight attachment to the bone surface is made via a membrane domain called the sealing zone (SZ). The formation of this region involves the rearrangement of the cytoskeleton and the formation of an F-actin ring. When resorption begins, the area of membrane within the actin ring forms the ruffled border (RB). This is a highly convoluted membrane domain which provides a large surface area for the release of protons and proteolytic enzymes that dissolve the bone matrix [32].

Bone resorption is necessary for many skeletal processes. It is an obligatory event during bone growth, tooth eruption and fracture healing, and is also necessary for the maintenance of an appropriate level of blood calcium. Bone resorption is tightly coupled to bone formation in the healthy skeleton, however several diseases manifest as a result of an imbalance between resorption and formation. Osteopetrosis is a disease caused by a lack of osteoclast activity, leading to an increase in bone mass, whereas osteoporosis is a disease caused by osteoclast over activity, therefore leading to reduced bone mass and an increased risk of fracture.

Bisphosphonates

Bisphosphonates, earlier known as diphosphonates, later renamed bisphosphonates, appeared in 1969. It was not until the 1990s that their biochemical actions were elucidated. BPs show high binding capacity to the bone matrix, especially in sites of active bone metabolism [33]. It has been noted that patients treated with intravenous BP, have increased risk of developing ONJ [34-36]. Bisphosphonates have become treatment of choice for a variety of bone diseases in which excessive osteoclast activity is an important pathological feature, including Paget's disease of bone, metastatic and osteolytic bone disease, and hypercalcaemia of malignancy, as well as osteoporosis, BP binds to osteoclasts and inhibit their ability to resorb bone, resulting decreased bone turnover and thus decreasing loss of structural integrity of the bone [37-39]. In addition, BP are reported to have direct antiproliferative and proapoptotic effects on cancer cells which can further reduce the incidence of bone metastases [40-45]. Etidronate was the first bisphosphonate to be used in humans, was used in a child with myositis ossificans progressiva, now called fibrodysplasia ossificans progressiva. In United states, etidronate was the first approved BP in 1977 followed by pamidronate in 1991, alendronate in 1995, risedronate in 1998, zoledronate in 2001, and ibandronate in 2005. Currently there are at least eleven bisphosphonates (etidronate, clodronate, tiludronate, pamidronate, alendronate, ibandronate, risedronate, and zoledronate, olpadronate, neridronate and minodronate) have been registered for various clinical applications in various countries.

Based on molecular Mode of Action, BP are classified as, Non-nitrogen containing bisphosphonates (can be metabolically incorporated into non-hydrolysable analogues of ATP, which interfere with ATP-dependent intracellular pathways) eg: etidronate and clodronate, and Nitrogen-containing bisphosphonates, they are more potent (inhibit key enzymes of the

mevalonate/cholesterol biosynthetic pathway. The major enzyme target for bisphosphonates is farnesyl pyrophosphate synthase (FPPS) [46] eg: pamidronate, alendronate, risedronate, ibandronate, and zoledronate. Inhibition of FPPS prevents the biosynthesis of isoprenoid compounds (notably farnesol and geranylgeraniol) that are required for the post-translational prenylation of small GTP-binding proteins (which are also GTPases) such as rab, rho and rac, which are essential for intracellular signaling events within osteoclasts [47-52]. Based on the mode of administration, BP are further classified into oral (etidronate, tiludronate alendronate, risedronate, ibandronate) and intravenous BP (pamidronate, zoledronate). For oral administration, patients are required to fast several hours before drug ingestion and to remain upright and avoid eating food for at least 30 minutes after drug ingestion. Under ideal conditions less than 1% of the orally administered dose is absorbed. About fifty percent of the absorbed dose binds to the bone surface, mostly at sites of active bone remodeling. The skeletal uptake varies with age, sex and with the dose and nature of the compound. Although bioavailability of oral BP is very low, adequate levels to inhibit bone resorption can be reached within a few weeks [53]. However, the amount absorbed is inadequate to inhibit a strong pro-resorptive stimulus that occurs in metastatic bone disease and multiple myeloma, and are mainly used to treat osteoporosis and Paget's disease [54, 55]. Therefore, for metastatic bone disease and multiple myeloma, intravenous infusions of nitrogen-containing BP such as pamidronate and zoledronate are preferred [56, 57]. Intravenous infusions of BP produce rapid anti-resorptive action within 24-48 hours of infusion.

Bisphosphonate preparations currently available in the US

	Primary indication	Nitrogen containing	Dose	Route	Relative potency†
Etidronate (Didronel)	Paget's disease	No	300–750 mg day ⁻¹ for 6 months	Oral	1
Tiludronate (Skelid)	Paget's disease	No	400 mg day ⁻¹ for 3 months	Oral	50
Alendronate (Fosamax)	Osteoporosis	Yes	10 mg day ⁻¹	Oral	1 000
			70 mg week ⁻¹		
Risedronate (Actonel)	Osteoporosis	Yes	5 mg day ⁻¹	Oral	1 000
			35 mg week ⁻¹		
Ibandronate (Boniva)	Osteoporosis	Yes	2.5 mg day ⁻¹	Oral	1 000
			150 mg month ⁻¹		
Pamidronate (Aredia)	Bone metastases	Yes	3 mg every 3 months	IV	
			90 mg every 3 weeks	IV	1 000–5 000
Zoledronate (Zometa)	Bone metastases	Yes	4 mg every 3 weeks	IV	10 000+
(Reclast)	Osteoporosis		5 mg year ⁻¹	IV	

†Relative to etidronate. IV, intravenous.

S. L. Ruggiero *et al.* 2009

Osteoclasts and Bisphosphonates interaction

Many studies have shown that bisphosphonates can affect osteoclast-mediated bone resorption in a variety of ways that include effects on osteoclast differentiation and their recruitment to the bone surface [58] inhibition of osteoclast adhesion [59], and inhibition of osteoclast activity on the bone surface [60, 61], shortening of the osteoclast life span by promoting its apoptosis [62–67]. It is widely accepted that BPs exert their major effect on mature osteoclasts.

All bisphosphonates share a common P-C-P backbone. The P-C-P backbone of bisphosphonates allows them to bind divalent metal ions such as calcium (Ca²⁺) [38], and due to their high affinity for hydroxyapatite crystals, BP are directed to areas of bone turnover and are especially concentrated at sites of undergoing active bone resorption [68, 69], minimal amounts of BP are released into the circulation [70]. Once BP bind to bone, their half-life is approximately for 11–30 days [71]. BP action on mature osteoclasts causes subtle changes in osteoclast structure

that affect the osteoclast ability to resorb bone. A characteristic feature is the loss of osteoclast ruffled border [72-74]. Also, BP can disrupt the osteoclast cytoskeleton and cause loss of actin rings [62, 69, 72, 75], which are required for polarization and the formation of a sealing zone at the bone surface. Furthermore, inhibition of cellular metabolism could indirectly affect processes required for resorption such as release of lysosomal enzymes[63] or acidification brought about by the activity of the ATP-dependent proton pump in the ruffled border [76]. They inhibit osteoclasts which in turn reduces bone resorption, substantially reducing fracture risk by 40%-70% in osteoporosis patients, and improve quality of life in cancer patients by preventing skeletal complications. However, prolonged BP use is associated with a significant dental complication termed “Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)”. To date, the true incidence, etiology, and risk factors that contribute to BRONJ pathogenesis are unknown.

Kimachi *et al* (2011) [77], suggested that Nitrogen-containing bisphosphonates not only inhibit mature osteoclasts but also prevent osteoclast precursors from differentiating and migrating towards inflammatory osteolytic lesions. Previous studies also, suggested that some BP may also inhibit osteoclast precursors, thereby preventing osteoclast formation [58, 78]. BP can have direct effects on osteoclasts or its precursors, or indirectly exert action on cells that modulate the osteoclasts. However since mature, multinucleated osteoclasts are formed by the fusion of mononuclear precursors of hematopoietic origin, bisphosphonates might also inhibit bone resorption by preventing osteoclast formation, in addition to affecting mature osteoclasts [79]. It was shown that some bisphosphonates could inhibit the formation of osteoclast-like cells in long-term cultures of human bone marrow *in vitro*, in a dose-dependent manner [80].

Furthermore, BP alter angiogenesis [81, 82] and signal transduction between osteoclasts and osteoblasts [83]. The effects of Zoledronate are truly remarkable, not only in reducing the excessive destructive activity taking place within the bone, but in producing often very long lasting effects, data demonstrate that the antiresorptive effects of a single 5-mg dose of Zoledronate are sustained for 3 years [84] . Many patients will not need further treatment after just one infusion of zoledronic acid (Reclast, 5 mg infusion) [84, 85]. Zoledronic acid IV is used to treat the cancer that has spread to the bone from solid tumors, lesions in the bone of the disease multiple myeloma, increased calcium in the blood from cancer, Paget's disease of bone, osteoporosis, decreased bone mass following menopause, glucocorticoid-induced osteoporosis prevention, osteoporosis caused by anti-androgen drugs.

Alendronate (AL) is commonly used for prevention and treatment of osteoporotic fractures. AL may inhibit human osteoclastogenesis by affecting the key regulatory genes in marrow cells. A number of clinical trials showed that AL decreases biomarkers of osteoclastic bone resorption, increases bone density, and reduces the incidence of fractures in osteoporotic patients [54, 86, 87]. Alendronate can inhibit bone resorption at 10^{-4} and 10^{-8} M of bone graft [88]. ALN inhibit bone resorption primarily by a reduction in osteoclast activity without marked effect on osteoclast number at 10^{-7} M, but at higher concentration (10^{-5} M), both osteoclast number and resorption profoundly decreases [65]. Etidronate is used to treat the paget's disease of bone and osteoporosis, it works by slowing the breakdown of old bone.

Interaction of Osteoclasts with other immune cells

Bone and immune system are functionally interconnected. Immune and bone cells derive from same progenitors in the bone marrow, they share a common microenvironment and are being influenced by similar mediators, different immune cells such as macrophages, B lymphocytes, mast cells, natural killer cells (NK), etc. have been shown to influence bone cells as well [89]. Immune cells and their products (cytokines) play an important role in the regulation of skeletal development and function, particularly of the osteoclast, implies that immune cell dysfunction may be involved in the pathogenesis of certain skeletal disorders [90]. IFN- γ , produced by both NK cells and Th1 lymphocytes, has been shown to inhibit osteoclastogenesis *in vitro* [91]. However, the *in vivo* effects of IFN- γ on bone tissue are less clear since often provide a contrasting effect when compared to *in vitro* studies [92, 93]. Reduced functioning of osteoclast and NK cell function coexist in osteopetrotic mutant rat [90]. OC progenitor activity is positively regulated by TNF- α and negatively regulated by IFN- γ [94]. IFN- γ binds to its receptor on osteoclasts, degrades RANKL signaling and thus inhibits the activation of osteoclasts and protects our bones from being resorbed. This cytokine is produced predominantly by NK and natural killer T (NKT) cells involved in the innate immune response, and by CD4⁺ Th1 and CD8⁺ cytotoxic T lymphocyte (CTL) effector T cells, once antigen-specific immunity develops [95]. ITIM-bearing NK receptor, positively regulates osteoclasts differentiation, immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for osteoclast differentiation [96, 97]. Cross-talk between the skeletal system and T cells, is termed as osteoimmunology. RANKL expressed by CD4⁺ and CD8⁺ T-cells can induce osteoclastogenesis, providing a link between immune and skeletal system. Osteoclasts produce chemokines that recruit CD8 positive T cells. Osteoclasts induced the secretion of IL-2, IL-6,

IFN- γ and induced the proliferation of CD8 positive T cells. CD8 positive T cells activated by osteoclasts expressed FoxP3, CTLA4, and receptor activator of NF- κ B ligand [98]. Anti-CD3/CD28-stimulated $\gamma\delta$ T cells or CD4⁺ T cells inhibit human osteoclast formation and resorptive activity in vitro. Cytokine production by CD3/CD28-stimulated $\gamma\delta$ T cells and observed a lack of IL-17 production, with activated $\gamma\delta$ T cells producing abundant interferon (IFN)- γ . Neutralization of IFN- γ markedly restored the formation of osteoclasts from precursor cells and the resorptive activity of mature osteoclasts, suggesting that IFN- γ is the major factor responsible for the inhibitory role of activated $\gamma\delta$ T cells on osteoclastogenesis and resorptive activity of mature osteoclasts [98].

NK cells

Natural Killer (NK) cells are granular lymphocytes that function at the interference of innate and adaptive immunity [99]. Discovered in the early 1970's by accident when investigators were studying specific cytotoxic effects of lymphocytes, it was not until the 1980's that they became generally accepted despite the accumulated evidence [100]. NK cells are a subset of 8 cytotoxic lymphocytes able to recognize and lyse tumor cells and virus infected cells without prior sensitization [101]. Traditionally they have been classified as effectors of innate immunity due to the lack of antigen specific cell surface receptors [102]. NK cells are known to mediate direct and antibody dependent cellular cytotoxicity (ADCC) against tumors as well as to regulate the function of other cells through the secretion of cytokines and chemokines [103]. NK cells derive from CD34⁺ hematopoietic stem cells (HSC's) found in the bone marrow. They can be found throughout the body in the spleen, liver, placenta, and peripheral blood [104]. Human NK cells are defined phenotypically by the surface expression of CD56 and CD16, and by their

lack of CD3 surface expression [105]. CD56 is a human neural-cell adhesion molecule, but its function on human NK cells is yet to be understood. Although the function of CD56 is unknown, its expression correlates with the expression of other surface markers that confer important functional properties to NK cells [99]. Two subsets of NK cells have been identified based on surface expression of CD56 and CD16. The major subset of NK cells, about 90% of human NK cells, is defined by low expression of CD56 (CD56dim) and high expression of CD16 (CD16 bright). The minor subset makes up approximately 10% of human NK cells and is defined by high expression of CD56 (CD56 bright) and low or lack of CD16 (CD16 dim) expression [99, 105]. The CD56dim CD16 bright cells were found to be the more cytotoxic subset of human NK cells. On the other hand, CD56bright CD16dim/- NK cells were found to secrete more cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), TNF- β , granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-10 (IL-10), and IL13 after being stimulated with pro-inflammatory cytokines [99, 105, 106].

NK cells develop in the bone marrow and constitute about 5-10% of total lymphocytes in the peripheral circulation and secondary lymphoid organs [107]. Effector function of NK cells include direct natural cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), as well as secretion of inflammatory cytokines and chemokines that indirectly regulate the functions of other immune cells [108, 109]. NK cells mediate cytotoxicity against transformed tumor cells, as well as healthy cells, by releasing pre-formed granules of proteins, known as perforin and granzyme B, which can induce apoptosis, or programmed cell death in target cell [110-112]. NK cells have also been identified within inflamed synovial fluid and express RANKL and M-CSF which during their interaction with monocytes can trigger the formation of osteoclasts in a

process that is RANKL and M-CSF dependent [113]. Zolendronic acid can induce IFN-gamma secretion in NK cells, and can activate NK cells [114].

PURPOSE OF STUDY

To investigate the underlying mechanism involved in BP induced osteoclasts activation and its consequences on the activation of NK cells.

AIMS OF THE THESIS

This study has three specific aims and several sub aims in terms of phenotypic and functional characterization of BP treated osteoclasts, and osteoclasts interaction with NK cells.

Specific Aim 1: To investigate the phenotypic, morphological and functional characterization of Osteoclasts with/without BP treatment.

Our studies demonstrated that nitrogen-containing bisphosphonates Zolendronic acid and Alendronate and non-nitrogen containing Etidronate results the morphological variation of osteoclasts, cause the cell death, and modulation of cytokine secretion by human osteoclasts. Nitrogen containing Bisphosphonates mediate significant dose dependent release of pro-inflammatory cytokines IL-6, TNF- α , and IL-1 β whereas they inhibit the anti-inflammatory IL-10 secretion by osteoclasts. The surface expression of CD14, CD33, CD54, CD44, CD11b, MHC class I and II and B7H1 were modulated when monocytes differentiated to osteoclasts, macrophages and dendritic cells, and these surface expressions were significantly increased when osteoclasts were treated with Bisphosphonates. All three bisphosphonates decreased pit formation by osteoclasts.

Specific Aim 2: To investigate the role of immunosuppressive cytokine IL-10, in BP-treated Osteoclasts, mediated stimulation of NK cells.

Osteoclasts acts as a key immune effectors capable of modulating the function of Natural Killer (NK) cells and nitrogen-containing bisphosphonates Zolendronic acid and Alendronate and non-nitrogen containing Etidronate maintains a pro-inflammatory microenvironment during interaction with NK cells. Bisphosphonates, particularly those of Zolendronic acid and Alendronate trigger significant levels of pro-inflammatory cytokines and chemokines from osteoclasts, and the levels synergistically rises when cultured with activated NK cells. Treatment with IL-10 down-regulate the pro-inflammatory cytokines increased as a result of Zolendronic acid treatment on osteoclasts, but when we treated the cells with IL-10 and anti-IL-10 inhibition of cytokines was reversed, providing evidence that absence of anti-inflammatory cytokine IL-10 as a result of BP treatment, resulting in the chronicity of inflammation.

Treatment of osteoclasts with Zolendronic acid and much less with Alendronate was capable of inhibiting NK cell cytotoxicity whereas it induced significant secretion of cytokines and chemokines in the cultures of NK cells with osteoclasts. NK cells were able to lyse osteoclasts much more than their precursor cell monocytes and this correlated with the decreased expression of MHC class I and CD54 expression on osteoclasts.

Specific Aim 3: To investigate the effect of Zolendronic acid in different tissue compartments in Zolendronic acid injected and NACL injected B6 WT mice.

Our *in-vivo* data showed, Zolendronic acid injections could similarly increase IFN-g secretion and Cytotoxicity against the tumor cells, in the bone marrows but decrease the secretion of IFN-g in gingival, pancreatic and adipose tissues. These observations are of significant value since they may provide potential mechanisms for the pathogenesis seen in Osteonecrosis of the Jaw (ONJ). It is possible that an increase in activation of Bone Marrow derived cells by Zolendronic acid result in the eventual loss or inhibition of IFN-g secreting cells when they reach to the tissues. Since gingival derived immune cells are of activated phenotype when compared to bone marrow derived immune cells in healthy subjects, prior activation of bone marrow derived cells when mobilized to the gingival tissues and are exposed to additional activation signals from antigens in gingiva may undergo activation induced cell death, or simply become exhausted, and result in the lower secretion of IFN-g by the immune cells as seen in our *in vivo* model system. These possibilities are under investigation in our laboratory. The phenotype and nature of immune cells, including NK cells, one of the main contributors of IFN-g secretion in bone marrow and tissues are currently being investigated in our laboratory.

CHAPTER 2

To investigate the phenotypic, morphological and
Characterization of Osteoclasts with/without BP treatment.

ABSTRACT

Osteoclasts, macrophages and dendritic cells are derived from blood monocytes. Osteoclasts are identified based on morphological identification through histochemical and biochemical studies through the tartrate resistance acid phosphatase stain (TRAP). To demarcate the difference between the osteoclasts, macrophages, dendritic cells and monocytes, we carried out cytokine, chemokine and surface markers analysis. Osteoclasts are found to be TRAP positive. The analysis of cytokines, chemokines and growth factors using multiplex cytokine array demonstrated a gradual increase in the secretion of IL1RA, IL2R, IL12, IL-15, IFN- α cytokines and MIP-1a, MIP-1b and Rantes chemokines whereas a decrease in IL-6 cytokine secretion can be observed from day 2 to day 21 of differentiation of monocytes to osteoclasts (Table 1).. The levels of MCP-1 and IL-8 remained significantly high at all-time points tested. No significant secretion of IL-1b, IL-2, IL-4, IL-5, IL-7, IL-13, IL-17 and Eotaxin at the time points and concentration tested (Table 1). There was an inverse modulation of IL-6 and IL-10 during differentiation of osteoclasts from monocyte precursors. The amounts of cytokines were largely similar between freshly isolated monocytes, M2 macrophages and Osteoclasts with Osteoclasts having the lowest secretion (Table 2). When cell surface receptor expression was compared between Osteoclasts and freshly isolated autologous monocytes, a significant down-modulation of all the cell surface receptors were observed, and osteoclasts found to lower surface receptor expressions when compared with macrophages and dendritic cells. We then analyzed the effect of BP on osteoclasts, there was a dose dependent increase in cell death when nitrogen containing Zolendronic acid and Alendronate were added to Osteoclasts, with Zolendronic acid having higher toxicity than Alendronate. The non-nitrogen containing Etidronate did not mediate

cell death at any concentration. Both nitrogen-containing Zolendronic acid and Alendronate but not Etidronate were able to induce secretion of IL-6, TNF- α , IL-1 β in dose-dependent and time dependent manner. In contrast to elevated secretion of pro-inflammatory cytokines by nitrogen containing bisphosphonates, the levels of anti-inflammatory cytokine IL-10 was severely suppressed at the concentrations of 1-50 μ M. Treatment of Osteoclasts with Zolendronic acid increased all the cell surface receptors at lower concentration of Zolendronic acid which correlated with the increased cytokine induction. There was dose dependent decrease in the resorptive activity of osteoclasts by the treatment with the three bisphosphonates, however Zolendronic acid exerted the most severe inhibition. Both the numbers of the pits and the size of the pits were affected by the treatment with the three bisphosphonates.

BACKGROUND AND INTRODUCTION

Osteoclasts, the multinucleated cells that resorb bone, develop from hematopoietic cells of monocyte/macrophage lineage [115]. Osteoclasts are the giant cells, size varies from 20 to several hundred micrometers in diameter with 3-20 nuclei. The nuclei in one cell vary, and appear euchromatic or heterochromatic and can be round or irregular. Osteoclasts consist of foamy cytoplasm having abundant vacuoles, golgi complexes around the pleotropic mitochondria and vesicles filled with lysosomes. Important feature of osteoclasts is the ruffled border which secrete enzymes, to resorb bone after providing contact with calcified bone surface. This ruffled borders are deep-in fold of plasma membrane, surrounded by sealing zone, a sphere of contractile proteins.

Osteoclasts are derived from Monocytes. Monocytes develop from myelo-monocytic stem cells in the bone marrow, and then enter to bloodstream account for 1 to 10% of the circulating leukocytes, circulate there in order to enter to tissues such as spleen, liver, lung and bone marrow, where they mature to macrophages, the main scavengers of immune system, they also mature to osteoclasts. Blood monocytes are also heterogenous and are of mainly two subsets, classical CD14⁺⁺ monocytes and the nonclassical CD14⁺CD16⁺⁺ monocytes. Monocytes play major role in the immune defense, inflammation and tissue remodeling through phagocytosis, antigen processing and presentation and by cytokine production. Inflammation is a protective response to infection by the immune system that requires communication between different classes of immune cells to coordinate their actions. Acute inflammation is an important part of the immune response, but chronic inflammation can lead to destruction of tissues in autoimmune disorders.

Identifying and characterizing the surface expression markers on different subtypes on the differentiated counterparts of osteoclast precursor cells (monocytes), broaden the understanding of immune functioning of the osteoclasts. Monocytes express CD45 for first four weeks of culture. After 14 days, during culture monocytes express CD68, which further increase during 28 days of culture [116]. Monocytes express CD14, CD11b, and CD58 on their surface. IFN-gamma and GM-CSF treatment up-regulate MHC-II and CD11b expressions and down-regulate the CD14 and CD16, but M-CSF treatment up-regulate CD14 and CD16 expression of Monocytes [117]. CD11b expression increase during differentiation of Monocytes to Macrophages, while CD14 expression reduced while differentiation of Monocytes to Macrophages. Dendritic cells express MHC-I, MHC-II, CD1, Fc gamma RII, CD40, B7, CD44, ICAM-1, CD8, CD11c, and lack CD14 [118-120]. CD44 is found to be expressed on the

basolateral plasma membrane of the osteoclasts and is required for osteoclasts motility and bone resorption [121]. OC express MHC molecules and the expression of these molecules is up-regulated by LPS and IFN- γ [122]. OC secrete IL-10, TNF- α , IL-6, TGF- β , and IL-1 β [122].

Bisphosphonates treatment result modulation of cytokine secretion by osteoclast. Secreted cytokine proteins provide signals between immune cells to coordinate the inflammatory response. Cytokines that play a major role in the innate immune response include, TNF-alpha, IL-1, IL-10, IL-12, type I interferon, IFN-gamma, and chemokines. Cytokines especially IL-1, IL-6, TNF-alpha act to broadly provoke the inflammatory response, they play particular importance in bone physiology [123]. Interleukin (IL)-6 is produced at the site of inflammation and plays a key role in the acute phase response as defined by a variety of clinical and biological features such as the production of acute phase proteins. The main sources of IL-6 in bone are osteoblastic cells and stromal cells, and IL-6 has effect on osteoclastogenesis and bone resorption [124-126]. TNF- α is one of the most potent osteoclastogenic cytokines produced in inflammation, TNF- α mediates RANKL stimulation of osteoclast differentiation through an autocrine mechanism [127]. TNF- α stimulate the osteoclastic bone resorption in vivo as well as in vitro [128, 129]. IL-1 also involved along with TNF- α in the bone resorption during inflammatory diseases [130]. IL-10, an anti-inflammatory cytokine, secreted by variety of cells, including T cells, B cells, and monocytes/macrophages [131-133]. IL-10, is known to suppress a broad range of inflammatory response and is known to be important factor in maintaining homeostasis of overall immune responses [134, 135]. IL-10, as an anti-inflammatory mediator, play a significant role in counterbalancing the proinflammatory response in various infectious diseases [136-138].

Characteristic morphological feature of bisphosphonate-treated osteoclasts is the lack of a ruffled border, the region of invaginated plasma membrane facing the resorption cavity. Bisphosphonates were also shown to disrupt the cytoskeleton of the osteoclast [72]. There are alterations in interleukins in patients with BRONJ, value of IL-1 β was found higher in saliva of patients with bis-phosphonates-related osteonecrosis of the jaws [139]. IL-6 production is evaluated during bisphosphonate treatment, in order to monitor the jaw osteonecrosis onset, IL-6 was significantly elevated in mucosa from patients with ONJ versus without jaw necrosis [140]. Zoledronic acid induces transient TNF-alpha and IL-6 increases and that these increases are higher in patients who have developed fever, suggesting that these cytokines could be responsible for fever pathogenesis [141]. Zoledronic acid (ZA) induces an acute phase response in association with elevation of serum cytokines, ZA induces short term changes in thyroid hormones, characteristic of nonthyroidal illness syndrome (NTIS), in association with an increase in TNF- α and IL-6 [142]. Bisphosphonates, and in particular the aminobisphosphonates (nBPs), are known to have a number of side-effects including a rise in body temperature and accompanying flu-like symptoms that resemble a typical acute phase response. The mechanism for this response has been partially elucidated and appears to be associated with the release of tumour necrosis factor (TNF)-alpha and interleukin (IL-6), by peripheral blood gamma-delta T cells induced by aminobisphosphonates [143]. Specific cytokine production, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF alpha)] following a single dose of pamidronate in patients with increased bone turnover. In vivo, there was a statistically significant ($P < 0.001$) increase in median values of TNF alpha in all post-baseline measurements. Median values for IL-6 also showed a significant ($P < 0.001$) increase at 24 hours after dosing. There were no statistically significant changes in median IL-1 values. In vitro, an increase in TNF

alpha and a mild increase in IL-6 was seen with all bisphosphonates, with the greatest effects seen with the highest concentration of both pamidronate and zoledronate. No changes were observed in IL-1 with any agent. Significant changes in both TNF alpha and IL-6 were observed within 3 days of a single dose of pamidronate in patients treated for the first time confirming previous findings. The results of the *in vitro* study are consistent with the *in vivo* findings [144]. Zolendronic acid inhibited secretion of IL-10 from mature Dendritic cells. The amount of secreted IL-10 in medium treated with ZOL at 10 μ M was about three times less than the control group [145].

BPs, bind strongly to hydroxyapatite crystals, they suppress osteoclast-mediated bone resorption, they are retained in long time in the bone structure, and BP are excreted unmetabolized in urine [146]. The presence of the bis-phosphonates (Alendronate) in the hydroxyapatite nanocomposites inhibits osteoclasts differentiation, and promote their apoptosis [147, 148]. Zolendronate-hydroxyapatite, prevents osteoclast formation and promote apoptosis, and Zolendronate-hydroxyapatite exerts more influence than Alendronate-hydroxyapatite on osteoclast apoptosis [149]. The structural and *in vitro* results put into evidence that zoledronate not only displays a greater affinity than alendronate for HA structure, but exerts an even greater influence on osteoclast apoptosis [149]. Hydroxyapatite (HA), an inorganic calcium phosphate material, is one of the most widely used bone regenerative biomaterials, as its composition mimics the inorganic extracellular matrix of bone tissue [150, 151].

Our studies showed that there is down-modulation of surface receptor when monocytes differentiated to osteoclast, and these surface expression varies from other monocytes lineage such as macrophages and dendritic cells. We analyzed CD14, CD54, CD44, MHC-I, MHC-II, CD11b, CD33, B7H1, CD124 and CD15. CD14 is a myelomonocytic differentiation antigen

expressed by monocytes, macrophages, and activated granulocytes, which after combining with the Ag, activate the inflammatory response [152]. CD54, intercellular adhesion molecule-1 is associated with increased adhesion of monocytes characterizing the chronic inflammation [153]. CD54 expression was also higher on alveolar macrophages than on blood monocytes, making them more important immune cells [154]. MHC molecules are membrane-bound proteins, MHC I molecules are found on almost all tissues of the body, while MHC II molecules are found only on antigen-presenting cells especially macrophages, dendritic cells and B cells. MHC molecules possess a deep groove that is capable of holding a short peptide. MHC I molecules process proteins present inside the cell and present them on their surface. MHC II molecules present antigens taken from the phagosome digestion, most often foreign cells, and present them to the immune system. The immune system monitors the proteins present on MHC I molecules and activates when a foreign protein, from an intracellular parasite, is detected. This normally results in the destruction of the cell. CD44 is a glycoprotein cell surface receptor for the extracellular matrix molecules hyaluronan, fibronectin, collagen, and fibrin. It is widely expressed in a number of tissues, including leucocytes, keratinocytes, chondrocytes, many epithelial cell types, and some endothelial and neural cells [155]. A number of different functions have been ascribed to CD44, including cellular adhesion and migration, lymphocyte activation and proliferation, and tumour cell metastasis [156]. Phagocytosis of apoptotic neutrophils, but not apoptotic lymphocytes, by human monocyte-derived macrophages is augmented rapidly following ligation of CD44 by bivalent Abs in vitro, suggesting that CD44 has a role in the clearance of apoptotic neutrophils by monocyte derived macrophages [157]. B7-H1 (PD-L1) is a novel member of the B7 family proteins which exert costimulatory and immune regulatory functions. B7-H1 is constitutively expressed on monocytes and differentially matured DC, but

not on B cells. Neutralization of B7-H1 on monocytes or differentially matured monocyte-derived DC markedly increases the secretion of the pro-inflammatory cytokines, IFN-gamma [158]. Stimulation of monocytes with IL-10 alone could significantly increase B7-H1 expression [159, 160]. Human CD33 is a myeloid-restricted transmembrane protein of the sialic acid-binding Ig-like lectin (Siglec) family. Amongst the 11 human siglecs, there are eight proteins highly related to CD33 which have biochemical features of inhibitory receptors, containing two conserved tyrosine-based inhibitory motifs. Five of these (CD33/siglec-3, -5, -7, -9 and -10) are expressed on circulating monocytes. Monocyte-derived dendritic cells down-modulated siglec-7 and -9 following maturation with LPS. Plasmacytoid dendritic cells in human blood expressed siglec-5 only. Siglecs are differentially expressed on mononuclear phagocytes and dendritic cells and that some can be modulated by stimuli that promote maturation and differentiation [161]. sialic acid (CD33 ligand) removal from the monocyte surface by neuraminidase resulted in IL-1 beta up-regulation, while the addition of red blood cells or sialyllactosamine (but not lactosamine) reversed the effect of neuraminidase treatment, thus demonstrating the importance of ligand recognition by CD33 for repression of monocyte activation, indicating that by controlling monocyte activation, CD33 is a key molecule in the inflammatory response, depending on the sialic acid microenvironment for its repressor activity [162].

Based on previous findings osteoclasts express CD13, CD14, CD31, CD44, CD51, CD53, CD54, CD61, CD63, CD68, CD115, CD130, CD254, CD265, CD280. In our study. We showed that osteoclasts express CD14, CD44, CD54, B7H1, CD33, CD11b, CD124, CD15, MHC-1, MHC-II, although B7H1, CD15, CD33 and CD124 surface expression were almost same as on monocyte surface. We have six sub aims in which we demonstrate characteristic difference between monocytes, macrophages, dendritic cells, and osteoclasts, followed by effect

of bisphosphonate on osteoclasts and the functional characteristic of bisphosphonates treated osteoclasts.

Sub Aim 1: Phenotypic characterization of osteoclasts cultured from human monocytes.

Sub Aim 2: Differential induction of cytokines, chemokines and growth factors by monocytes, M1 and M2 macrophages, Dendritic cells and Osteoclasts.

Sub Aim 3: Comparison of cell surface receptor expression between Osteoclasts, monocytes, Dendritic cells and macrophages.

Sub Aim 4: Uptake, morphological and the pro-inflammatory effect of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate on Osteoclasts.

Sub Aim 5: Zolendronic acid modulated surface receptor expression on Osteoclasts.

Sub Aim 6: Decreased pit numbers and size by Bisphosphonate-treated Osteoclasts.

MATERIAL AND METHODS

A. Cell culture and reagents

Monocytes, Dendritic cells (DCs), Macrophages (M1 and M2) and Osteoclasts (OCs)

Monocytes purification kit was purchased from stem cell Technologies. RPMI 1640 was supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% non-essential amino acids (Invitrogen by Life Technologies, CA) was used to cultured monocytes, M1 macrophages and dendritic cells. Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts.

B. Antibodies

Monocyte purification kit was purchased from stem cell Technologies (Vancouver, Canada) Human recombinant IFN- γ , LPS, human recombinant IL-4 and human GM-CSF were dissolved in PBS with 1%BSA and stored at -20°C. Human recombinant IFN- γ (50ng/ml), LPS (15ng/ml) was used for M1 macrophages culture. Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -20°C, and used for osteoclasts culture. ELISA kits were purchased from Biogends, CA. Zolendronic acid, Alendronate and Etidronate were purchased from UCLA Ronald Reagan Pharmacy. Fluorescent Zolendronic acid analogs were synthesized via a linker strategy (Hokugo et al. 2013). PE conjugated IgG1 and 2b, PE-CD14, PE-CD11b, PE-CD124, PE-B7H1, PE-CD15, PE-CD33, PE-CD44, PE-CD54, PE-MHC-I and PE-MHC-II were all purchased from Biolegend, CA.

C. Human Peripheral Blood Monocytes purification

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for 1 hour after which the adherent subpopulation of PBMCs was detached from the tissue culture plates and the monocytes were purified using isolation kits obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved for each subset based on flow cytometric analysis of CD14. To confirm the low contamination of NK cells, anti-CD16 antibody staining was analyzed, which remained low, at 3 ± 1 % similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures.

D. Monocytes cultures for Osteoclasts, Macrophages and Dendritic cells generation

For macrophages, monocytes were cultured in RPMI 1640 containing 15ng/ml LPS and 50ng/ml rh-IFN-gamma for 8 days. For dendritic cells, monocytes were cultured in RPMI 1640, containing 150ng/ml GM-CSF and 50ng/ml rh-IL-4. For osteoclasts, monocytes were cultured in alpha-MEM medium containing 25ng/mL M-CSF, day 3 after culture media was refreshed with addition of M-CSF (25ng/ml), and day 6 after culture media was refreshed with addition of M-CSF (25ng/ml) and Rank Ligand (25ng/mL). Medium was refreshed every 3 days with alpha-MEM containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days.

E. TRAP staining

Osteoclasts were detached from tissue culture plate and seeded in 96-well plate at 3×10^4 cells/well for 18-24 hours. Afterwards the cells were rinsed twice with 1X PBS and fixed with 10% formaldehyde for 5 minutes at room temperature. The cells were then rinsed three times with 1X PBS and incubated with Chromogenic Substrate solution (Primary Cell, Co., Japan) for 30 minutes or until stained TRAP is clearly seen. Finally, cells were then rinsed with deionized water to neutralize the reaction and images were taken with Leica DMI 6000B inverted microscope.

F. Enzyme-Linked Immunosorbent Assays (ELISAs) and Multiplex Cytokine Arrays

Enzyme-linked Immunoassorbent Assays (ELISAs), for IFN- γ , IL-6, IL-10, TNF- α , IL-1 β (Biolegends, CA) and IL-18 (R&D Systems, Minneapolis, MN), were performed on the harvested supernatant after cultures and bisphosphonates treatments on osteoclasts to measure the concentration levels of cytokines and growth factors secreted by monocytes, macrophages, dendritic cells, and bisphosphonate non-treated/treated osteoclasts, as described by manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines. To perform single ELISA plates were coated with recommended amount of capture antibodies and incubated overnight. After overnight incubation, plates were washed three or four times, and antibodies were blocked with ELISA PBS containing 1% BSA for 1 hour. Thereafter plates were washed three or four times, and standards and samples were added in respective wells. For IL-6, IL-10, IL-1 β , and IL-18 plates were incubated for 2 hours, and for TNF- α plates were incubated overnight. After incubation plates were washed four times, detection antibodies were added and plates were incubated for 1 or 2 hours. Plates were washed, followed for the addition of Avidin-HRP and continued incubation for next 30 mins or 1 hour. Finally the plates were washed, and incubated with alkaline phosphate substrate (Sigma

Chemical Co. 104), reaction was stopped with stop solution, 2N H₂SO₄. Readings of plated were done with ELISA reader using 405nm or 450nm filter. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer.

G. Surface Staining

Staining was performed by coating the cells with the antibodies. The cells, monocytes, dendritic cells, macrophages and osteoclasts were detached from the tissue culture plates and washed twice with ice-cold PBS containing 1% BSA. Predetermined optimal concentrations of specific human monoclonal antibodies were added to 1×10^4 cells in 50 μ l of cold-BSA and cells were incubated on ice for 30 min. Thereafter cells were washed in cold PBS-BSA and brought upto 500 μ l with PBS-BSA. An Epics C (Coulter) flow cytometry was used for analysis.

H. Pit resorption assay

Purified Human osteoclasts were generated from healthy donor's monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Afterwards, osteoclasts were rinsed, detached from tissue culture plates and seeded at 1×10^4 cells/well in 24 well plate pre-coated with synthetic carbonate apatite (Cosmo Bio Co, Japan) for 7 days. After the incubation period, culture medium was removed and cells were rinsed with 5% sodium hypochlorite for 5 minutes. The cells were then washed with water and photographed using Leica DMI 6000B inverted microscope.

I. Statistical Analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS

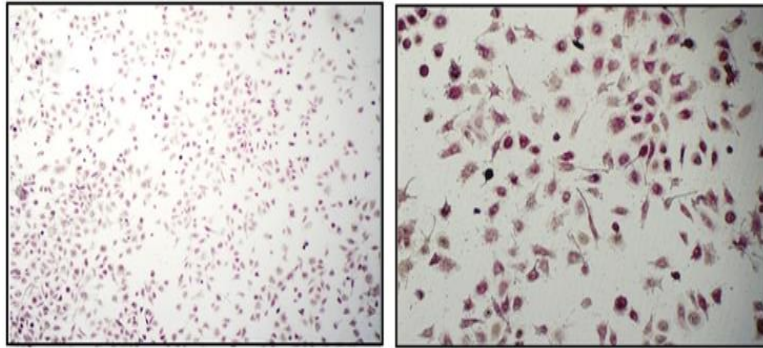
1. Phenotypic characterization of osteoclasts purified from human monocytes.

Human osteoclasts were generated using purified monocytes treated with Rank Ligand and M-CSF as described in the Materials and Methods section. Osteoclasts cultured from monocytes at day 21 were histochemical analyzed with TRAP staining. Osteoclasts were positive for TRAP staining (Fig. 1). The analysis of cytokines, chemokines and growth factors using multiplex cytokine array demonstrated a gradual increase in the secretion of IL1RA, IL2R, IL12 cytokines and MIP-1a, MIP-1b and Rantes chemokines whereas a decrease in IL-6 cytokine secretion can be observed from day 2 to day 21 of differentiation of monocytes to osteoclasts (Table 1). Increased detection of IL-15 and IFN- α but not IFN- γ was also observed (Table 1). The levels of MCP-1 and IL-8 remained significantly high at all-time points tested. No significant secretion of IL-1b, IL-2, IL-4, IL-5, IL-7, IL-13, IL-17 and Eotaxin at the time points and concentration tested (Table 1). Therefore, osteoclasts have the ability to secrete inflammatory cytokines and chemokines which can play important roles in differentiation and tissue remodeling.

Since there was a gradual decrease in IL-6 secretion from day 2 to 21 we compared the levels of IL-6 decrease to the release of anti-inflammatory cytokine IL-10 (Fig. 2). In contrast to gradual decrease in IL-6 secretion from day 3 to day 16 of culture, IL-10 secretion in osteoclast precursors exhibited a gradual increase from day 3 to day 16th (Fig. 2). Therefore, there was an inverse modulation of IL-6 and IL-10 during differentiation of osteoclasts from monocyte precursors.

Figure 1:

A:



B:

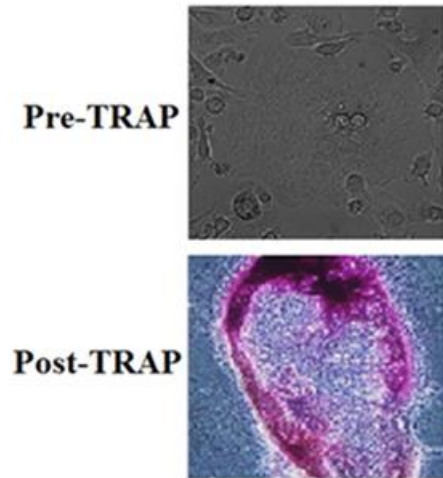


Fig. 1. Osteoclasts generated from monocytes *in vitro* are TRAP positive.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, cells were rinsed with 1X PBS, detached from tissue culture plate and seeded in 96-well plate at 3×10^4 cells/well for 24 hours. Afterwards, the cells were fixed with 10% formaldehyde and stained with Chromogenic Substrate. Images were taken with Leica DMI 6000B inverted microscope. A) Full well picture. B) Single cells focused before and after TRAP stain.

Table 1:

	Day 2	Day 6	Day 10	Day 14	Day 21
IL-1RA	680.21	673.11	3168.01	4897.2	4342.09
IL-2R	30.7	30.7	65.33	156.18	212.19
IL-12	19.8	7.8	17.25	23.56	32.06
IL-15	58.53	71.99	162.6	78.49	51.51
IFN-alpha	90.08	107.54	125.92	105.93	68.78
IFN-gamma	0	0	0	0	0
IL-6	108.12	14.35	58.17	33.92	15.58
MIP-1 alpha	39.71	43.28	639.82	1931.8	5070.89
MIP-1 beta	116.41	109.21	1914.16	3907.9	5168.19
RANTES	271.49	54.85	63.02	238.27	432.49
MCP-1	>6100	>6100	>6100	>6100	3627.89
IL-8	20001.6	24988.3	22072.3	0	22267
MIG	0	2.15	27.67	12.39	14.25
IP-10	15.87	23.73	55.62	19.48	12.82
Eotaxin	0	0	0	0	0

Table 1. Production of cytokines, chemokines and growth factors by Osteoclasts.

Human monocytes purified from healthy donor's PBMCs were differentiated into osteoclasts in culture with medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Culture medium was harvested on days 2, 6, 10, 14 and 21 and the levels of cytokine and chemokine production were measured using multiplex cytokine array kit.

Figure 2:

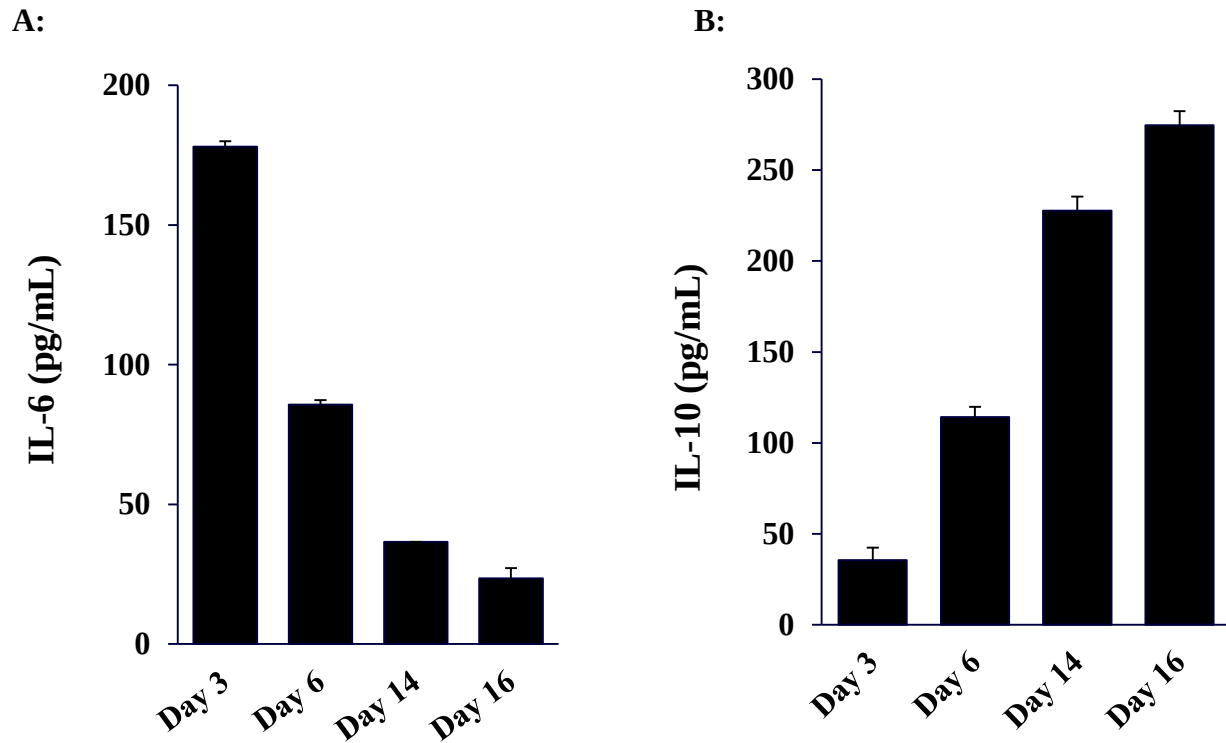


Fig. 2. Gradual decrease in IL-6 and increase in IL-10 production during osteoclast differentiation from monocytes.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 16 days. Culture medium was collected at various time points and the levels of IL-6 (**A**) and IL-10 (**B**) produced by osteoclasts were measured using specific ELISAs.

2. Differential induction of cytokines, chemokines and growth factors by monocytes, M1 and M2 macrophages, Dendritic cells and Osteoclasts.

To investigate the cytokine and chemokine profiles of Monocytes, Osteoclasts, Dendritic cells, M1 macrophages, and M2 macrophages were generated with different activation and time duration as shown in Fig. 3. The profile and amounts of cytokines and chemokine secretion in osteoclasts resembled those of the freshly purified monocytes and cultured M2 macrophages, and were greatly distinct from M1 macrophages and Dendritic cells (Table 2). M1 macrophages demonstrated the highest secretion of cytokines followed by the dendritic cells which had lower overall secretion for the majority of cytokines tested, although there were some exceptions such as IL-1Ra which was higher from Dendritic cells (Table 2). The amounts of cytokines were largely similar between freshly isolated monocytes, M2 macrophages and Osteoclasts with Osteoclasts having the lowest secretion (Table 2). Interestingly, the levels of chemokines were high in all the subsets, M1 macrophages having the highest for the MIP-1a and MIP-1b and the lowest for MCP-1 (Table 2). IL-8 secretion was the highest from M1 macrophages and M2 macrophages, and monocytes had the next highest secretion, whereas dendritic cells and Osteoclasts secreted lower amounts (Table 2). Secretion of Rantes was the lowest for the M1 macrophages and higher in the other subsets (Table 2). Monocytes, M2 macrophages and Osteoclasts secreted highest levels of MCP-1 and IP-10 when compared to DCs or M1 macrophages (Table 2). Osteoclasts had the lowest amounts of growth factor secretion whereas M1 macrophages had the highest with the exception of GM-CSF where they secreted the least (Table 2). Overall, these results indicated that the profiles of cytokine, chemokine and growth factor secretion of Osteoclasts resemble to the monocytes and M2 macrophages.

Figure 3:

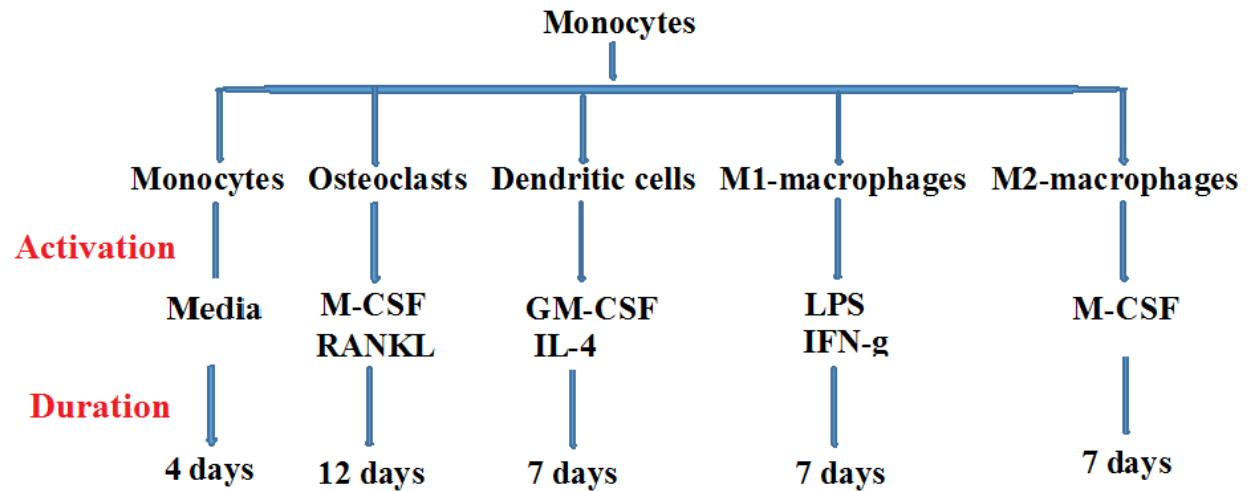


Fig. 3: Generation of dendritic cells, M1 macrophages, M2 macrophages and osteoclasts.

Highly purified Human monocytes were left untreated or treated with different stimuli and time periods, as shown in figure above. For Monocytes, culture Monocytes with media for 4 days, for Osteoclasts culture with 25ng/ml of M-CSF and 25ng/ml RANKL for 12 days, for Dendritic cells culture with 150ng/ml of GM-CSF and 50ng/ml of IL-4 for 7 days, for M1 macrophages culture with 15ng/ml of LPS and 50ng/ml of IFN-g for 7 days, for M2 macrophages culture with 25ng/ml of M-CSF for 7 days.

Table2:

	IL-1b	IL-1Ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-9	IL-10	IL-12p70	IL-13	IL-15	IL-17	IFN- γ	TNF- α
Monocytes	8.09	159	12.84	2.75	0	293.4	2.51	11.73	21	23.96	0	16.6	42	0	26
DCs	10.25	9397	21.91	NA	4	686.8	3.78	11.3	86.9	6	4.7	18	72	0	47
M1 macrophages	>1952	4434	35.23	6.42	0	32200	13.5	14.63	1081	73.15	12.3	0	70	NA	710
M2 macrophages	14.76	434	20.06	3.7	4	395.1	8.25	18.21	32.9	33.21	3.75	20.1	76	0	44
Osteoclasts	0	91.3	11.06	2.12	0	46.38	0	9.53	13.6	10.42	0	21.5	32	0	24

	MCP-1	MIP-1 α	MIP-1 β	RANTES	Eotaxin	IL-8	IP-10
Monocytes	1152	17.5	205.3	>1890.95	0	7210	619
DCs	110.8	21.4	146.5	>1890.95	0	3110	175
M1 macrophages	93.68	207	2100	673	34	22262	481
M2 macrophages	3002	19.8	297.8	>1890.95	0	27886	1878
Osteoclasts	2133	16.6	186.8	1560	0	3874	1285

	Basic FGF	VEGF	PDGF-BB	G-CSF	GM-CSF
Monocytes	0	151	281.2	66.3	85
DCs	0	19.4	324.8	86	NA
M1 macrophages	0	996	337.5	9349	53
M2 macrophages	0	251	424.7	81.3	62
Osteoclasts	0	76.1	117.1	31.3	90

Table 2: Production of cytokines, chemokines and growth factors by monocytes, Dendritic cells, M1 and M2 macrophages and Osteoclasts.

Highly purified Human monocytes were left untreated or treated with different stimuli as described in figure 3. Supernatants were harvested from each sample on the indicated days and the levels of cytokines and chemokines were measured using multiplex cytokine array kit.

3. Comparison of cell surface receptor expression between Osteoclasts, monocytes, Dendritic cells and macrophages

When cell surface receptor expression was compared between Osteoclasts and freshly isolated autologous monocytes, a significant down-modulation of all the cell surface receptors were observed (Fig. 4). Profound decreases in CD14, CD11b, CD44, MHC-class I and II and CD54 were observed on the surface of osteoclasts as compared to freshly isolated autologous monocytes (Fig. 4). However, when monocytes were cultured in media in the absence of Rank ligand and M-CSF and the levels compared to Osteoclasts, the differences between monocytes and osteoclasts substantially decreased (Fig. 5). The expression levels of CD14, CD11b B7H1 and CD54 increased and CD44 decreased, whereas no differences for MHC class I and II, CD33, CD15, CD124 could be seen (Fig. 5). In contrast, the expression of all the cell surface receptors was significantly increased on the surface of macrophages as compared to either monocytes or osteoclasts (Fig. 5). DCs expressed higher levels of CD11b, CD54, MHC class II, CD33, B7H1 and CD44 and lower levels of CD14 and MHC class I when compared to monocytes and osteoclasts (Fig. 5). When the surface expression of day 8 differentiated Osteoclasts were compared with day 21, a slight increase in CD14, CD11b, CD33, and B7H1 and a decrease in MHC class II were noted. It is of note that even though mean channel fluorescence intensity of CD14 and CD44 were increased, the percentages of the cells expressing these surface receptors were decreased (Fig. 5). Addition of combination of IFN- γ and TNF- α decreased CD44 and increased CD14, CD54, MHC class II, CD33, CD124 and B7H1 whereas no change in CD11b, MHC class I, CD15 were noted (Fig. 5).

Figure 4:

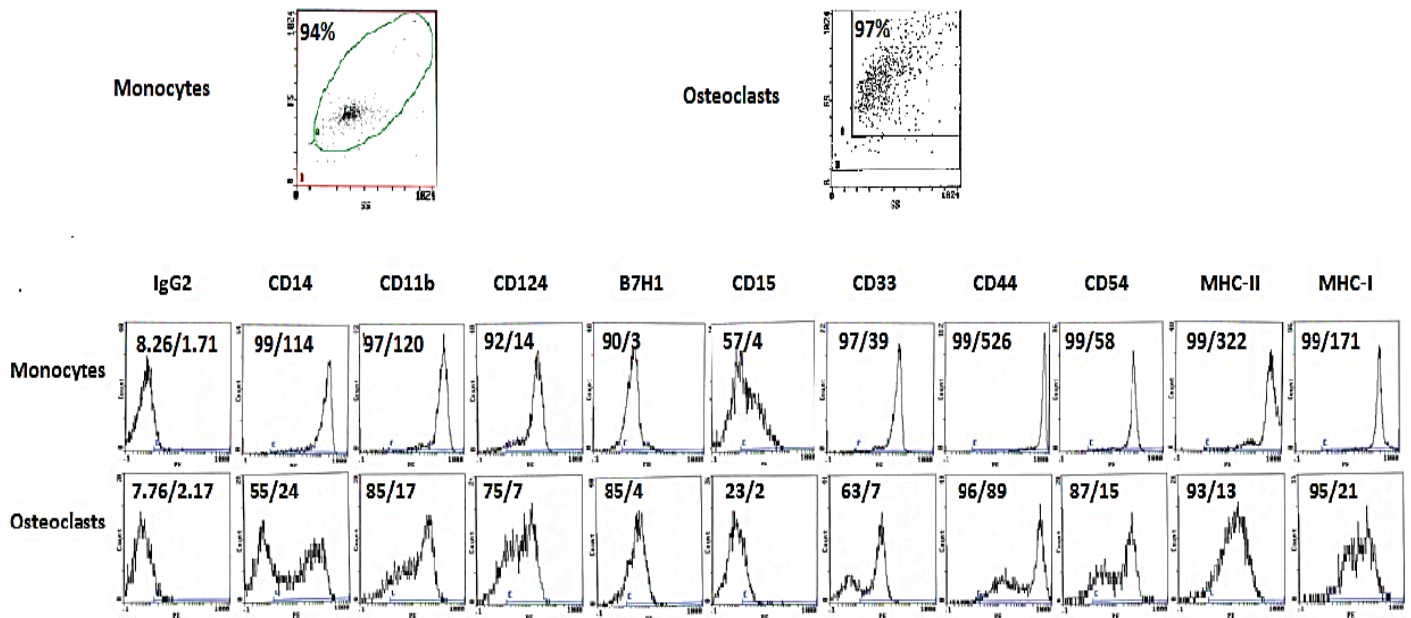


Fig 4: Significant levels of decrease in CD14, CD11b, CD44, MHC-class I and II and CD54 expression were observed on the surface of osteoclasts as compared to freshly isolated autologous monocytes.

Surface expression of Monocytes and Osteoclasts was analyzed by flow cytometry. Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. 1×10^4 Monocytes and osteoclasts were used to stain and analyze for the surface expression of CD14, CD11b, CD124, B7H1, CD15, CD33, CD44, CD54, MHC-I, and MHC-II.

Figure 5:

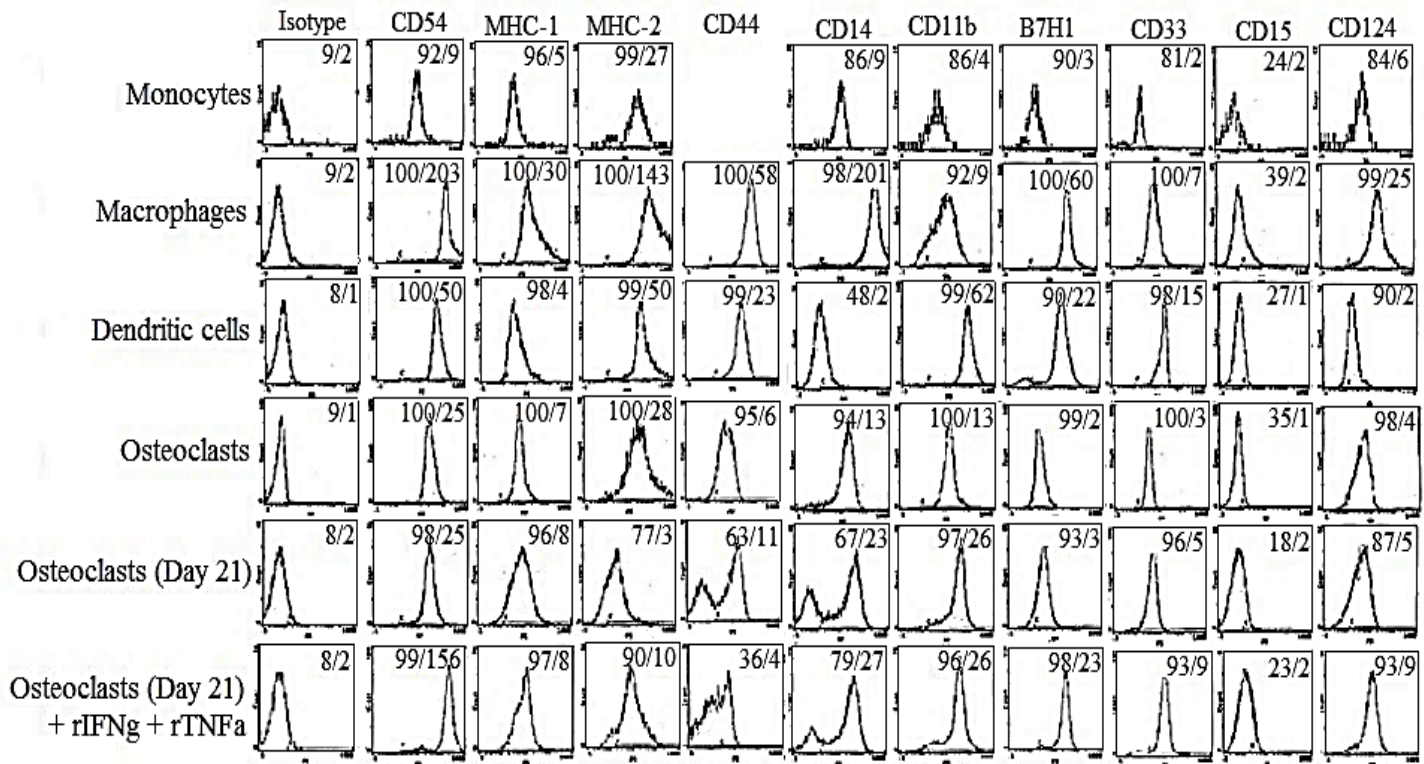


Fig 5: Comparison of surface expression of Monocytes, Macrophages, Dendritic cells, and Osteoclast.

Monocytes were cultured with RPMI for 8 days. Macrophages were generated from monocytes cultured in medium containing LPS (15ng/ml) and rh-IFN- γ (50 ng/ml) for 8 days. Dendritic cells were generated from monocytes, cultured in medium containing GM-CSF (150 ng/ml) and IL-4 (50 ng/ml) for 8 days. Osteoclasts were generated from monocytes cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 8 days and 21 days. Osteoclasts at day 20 of differentiation were treated with rh-IFN- γ (10ng/ml) and rh-TNF- α (20ng/ml) overnight. 1×10^4 cells were used to analyze the surface expression of, CD14, MHC-I, MHC-II, CD11b, CD44, CD54, and CD33.

4. Uptake, morphological and the pro-inflammatory effect of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate on Osteoclasts.

To determine the effect of bisphosphonates on Osteoclasts, first we determined the specific uptake of fluorescently labeled Zolendronic acid by Osteoclasts (Fig. 6). As shown in the figure 6 Osteoclasts which were taken up Zolendronic acid appeared red under the microscope since Zolendronic acid was labeled with red florescence. We analyzed the structural difference after BP treatment, took pre-TRAP and post-TRAP pictures using inverse microscope at 20 X magnification (Fig. 7). With BP treatment Osteoclasts lost their sealing zones. We then determined the effect of all three bisphosphonates on cell viability. As shown in Fig. 8 there was a dose dependent increase in cell death when nitrogen containing Zolendronic acid and Alendronate were added to Osteoclasts, with Zolendronic acid having higher toxicity than Alendronate (Fig. 8). The non-nitrogen containing Etidronate did not mediate cell death at any concentration (Fig. 8). Both nitrogen-containing Zolendronic acid and Alendronate but not Etidronate were able to induce secretion of IL-6 at the level of 1 μ M and the levels significantly decreased with the 25-100 μ M concentration of Zolendronic acid. Alendronate at the levels of 100 μ M exhibited significant decrease in IL-6 secretion when compared to 1-25 μ M levels (Fig. 9). Etidronate at all different concentrations were not able to induce secretion of IL-6. In contrast to elevated secretion of IL-6 by nitrogen containing bisphosphonates, the levels of anti-inflammatory cytokine IL-10 was severely suppressed at the concentrations of 1-50 μ M (Fig. 10). Etidronate at all concentrations had the ability to increase or retain the secretion of IL-10 by osteoclasts (Fig. 10).

Since the highest increase in IL-6 secretion was observed at 1 μ M, we then determined the ability of lower concentrations of bisphosphonates to induce IL-6 secretion at the range of 10 nM to 1 μ M (Fig. 9). The results demonstrated a dose and time dependent increase in IL-6 secretion

by Zolendronic acid and Alendronate, with Zolendronic acid having higher ability to induce IL-6 (Fig. 9). Etidronate had no or minimal effect on the secretion of IL-6 at all different concentrations (Fig. 9). Both Zolendronic acid and Alendronate inhibited IL-10 secretion at the concentrations of 1-50 μ M (Fig. 10). The inhibitory effect of Zolendronic acid and Alendronate were dose and donor dependent.

In addition, to IL-6 and IL-10 secretion, the levels of TNF- α (Fig. 11) and IL-1 β (Fig. 11) were also determined after bisphosphonate treatment. Dose dependent increase in TNF- α (Fig. 11) and IL-1 β (Fig. 11) secretion was also observed by Zolendronic acid followed by Alendronate and no secretion by Etidronate. Overall, these data demonstrated the ability of both Zolendronic acid and Alendronate but not Etidronate to induce pro-inflammatory cytokines, whereas they had inhibitory effect on the release of anti-inflammatory IL-10 since the release of this cytokine was greatly affected by the decreased viability of the cells.

Figure 6:

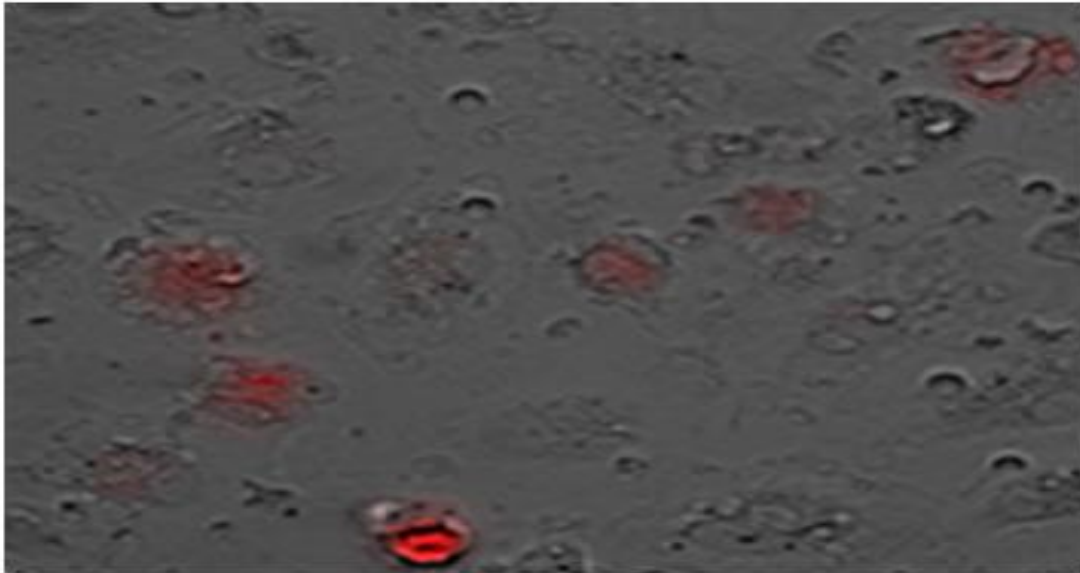


Fig 6. Uptake of fluorescently labeled Zolendronic acid by Osteoclasts.

Purified Human osteoclasts were generated from healthy donor's monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, osteoclasts were rinsed with 1X PBS, detached from the tissue culture plate and seeded at a density of 0.3×10^5 cells/well in 24 well plate. After an overnight incubation, the cells were treated with Zolendronic acid conjugated with 5 - Carboxy - X - rhodamine (5uM) for 24 hours and the image was taken with Leica DMI 6000B microscope

Figure 7:

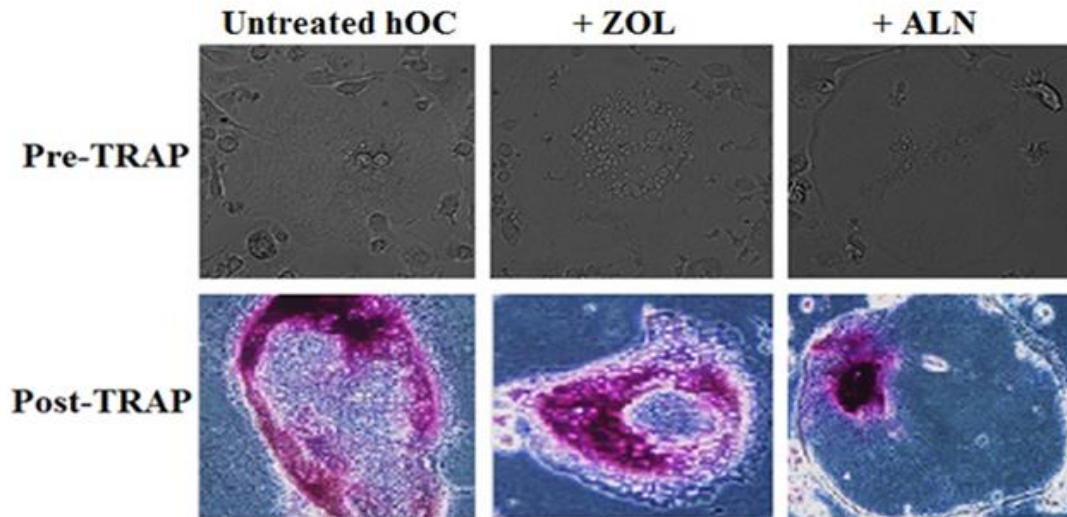


Fig. 7: Morphological variations of Osteoclasts on BP treatment.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 3×10^5 ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid and Alendronate. After 72 hours of treatment, cells were fixed with 37 % formaldehyde. Pictures were taken using Leica microscopy at 20X magnification. Cells were TRAP stained using Sigma Kit, and Pictures were taken using Leica microscopy at 20X magnification.

Figure 8:

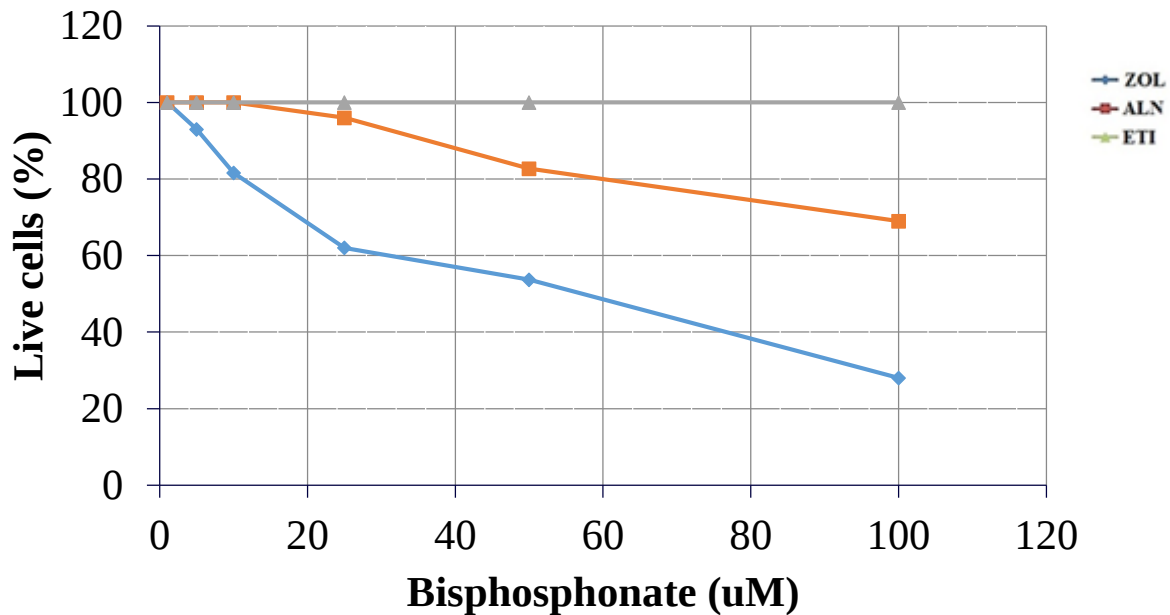
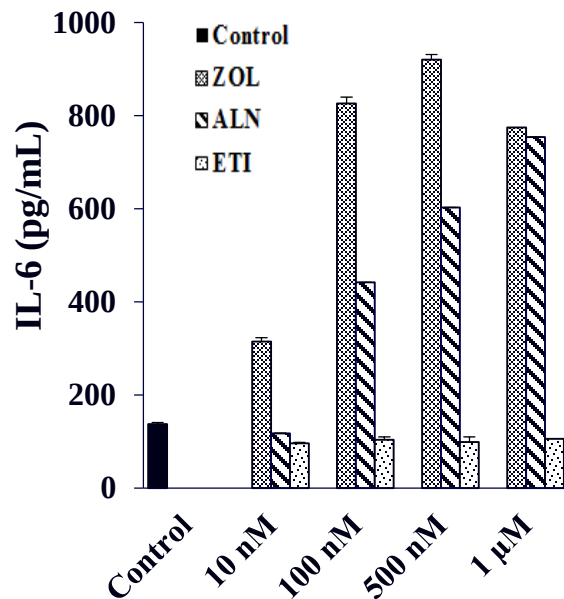


Fig 8. Nitrogen-containing Zolendronic acid and Alendronate, but not Etidronate, were able to induce death in Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. After the differentiation period, osteoclasts were rinsed with 1X PBS, detached from tissue culture plate and seeded at 1.5×10^4 cells/well in 24 well plate for 24 hours. Cells were then treated with Zolendronic acid, Alendronate or Etidronate at 1, 5, 10, 25, 50 and 100uM for 6 days. The cells were then rinsed with 1X PBS, stained with propidium iodide and analyzed by flow cytometry.

Figure 9:

A.



B.

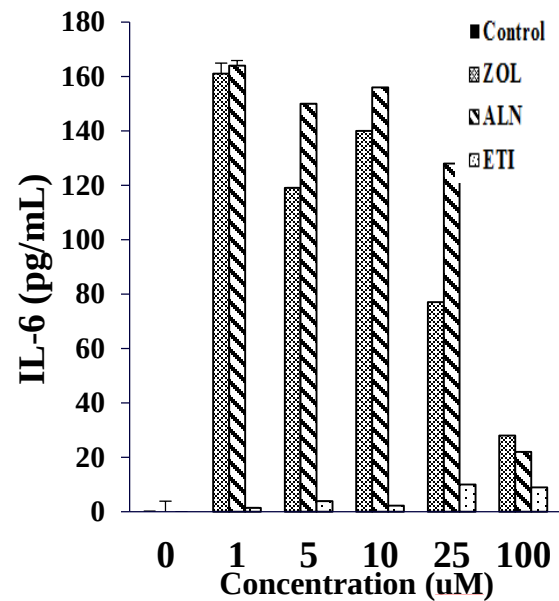
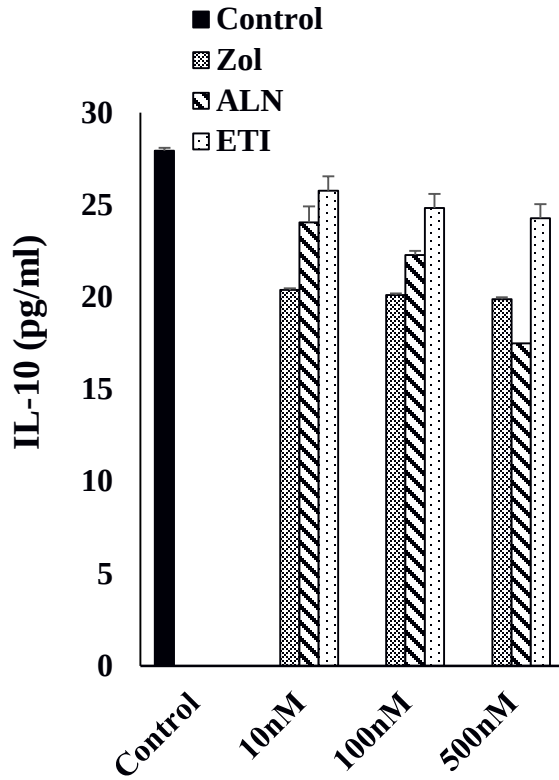


Fig 9: Increased IL-6 secretion by Zolendronic acid and Alendronate but not Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2 \times 10^5/\text{ml}$ were treated with variable concentrations of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 6 days, after which the supernatants were harvested and subjected to a specific ELISA for IL-6. At concentrations 10nM-1uM, Zolendronic acid showed gradual increase of IL-6 secretion, Alendronate showed IL-6 increased secretion at 100nM-1uM, but Etidronate showed no increased secretion (Fig.A). At concentration 1uM-100uM both Zolendronic acid and Alendronate showed decrease of IL-6 secretion, Etidronate again showed no effect on IL-6 secretion (Fig.B)

Figure 10:

A.



B.

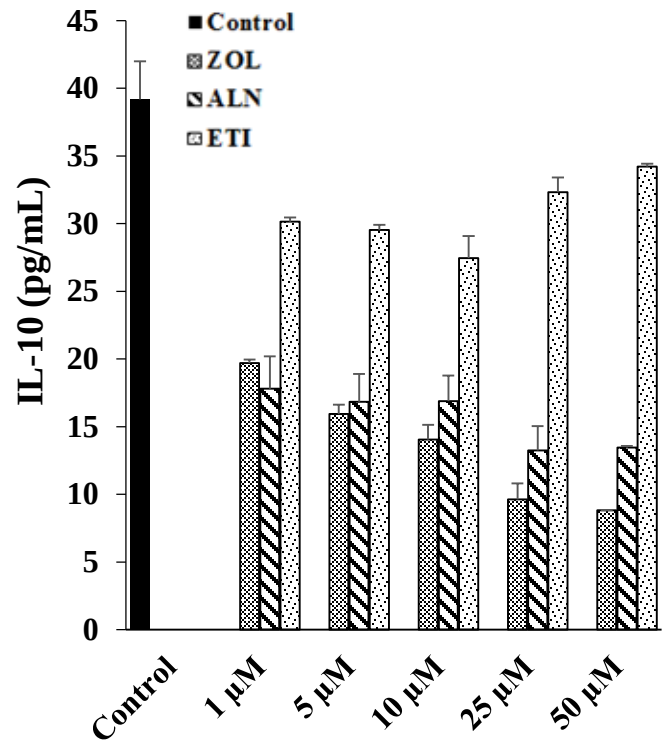
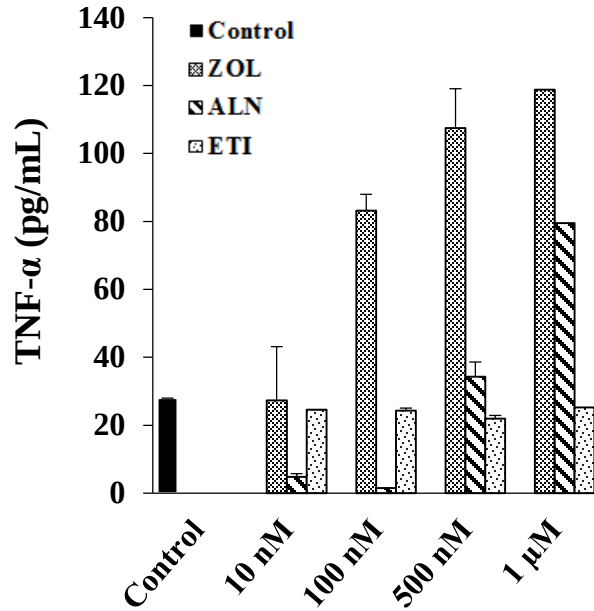


Fig 10: IL-10 secretion by Zolendronic acid and Alendronate and Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2 \times 10^5/\text{ml}$ were treated with 10nM-500nM (Fig. A) and 1uM-50uM (Fig. B) concentrations of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 6 days, after which the supernatants were harvested and subjected to a specific ELISA for IL-10.

Figure 11:

A:



B:

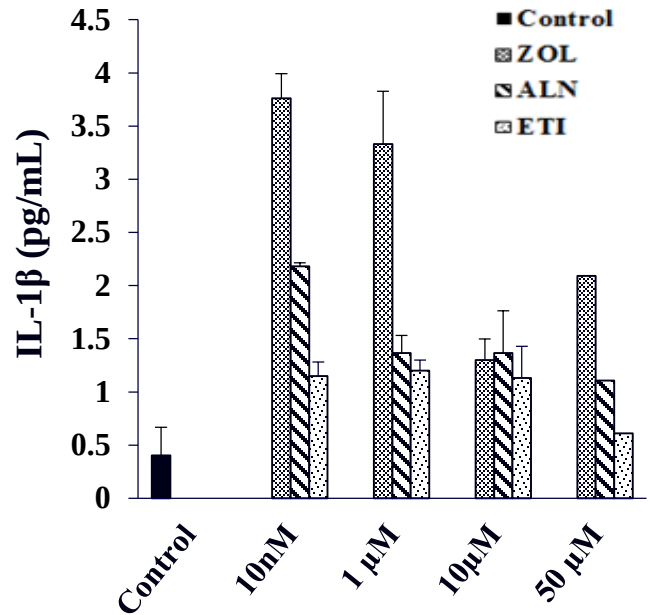


Fig 11: Increased TNF- α secretion by Zolendronic acid and Alendronate but not Etidronate treated Osteoclasts, Increased IL-1 β secretion by BP.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of 2×10^5 /ml were treated with concentrations 10nM-1 μ M (Fig.A) and 10nM-50 μ M (Fig.B) of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate as indicated in the figure for the duration of 6 days, after which the supernatants were harvested and subjected to a specific ELISA for TNF- α and IL-1 β .

5. Zolendronic acid modulated surface receptor expression on Osteoclasts

Treatment of Osteoclasts with Zolendronic acid increased all the cell surface receptors at lower concentration of Zolendronic acid which correlated with the increased cytokine induction (Fig. 12). At higher concentration of Zolendronic acid there was less increase in the cell surface receptors (Fig. 12). Comparison between Zolendronic acid treated Osteoclasts and those treated with the supernatants prepared from the activated NK cells demonstrated higher induction of cell surface receptors by Zolendronic acid with the exception of CD54 where supernatant treated Osteoclasts had higher induction (Fig. 13). Of note both CD14 and CD44 expression were significantly down-modulated by NK supernatant treated Osteoclasts (Fig. 13).

Figure 12:

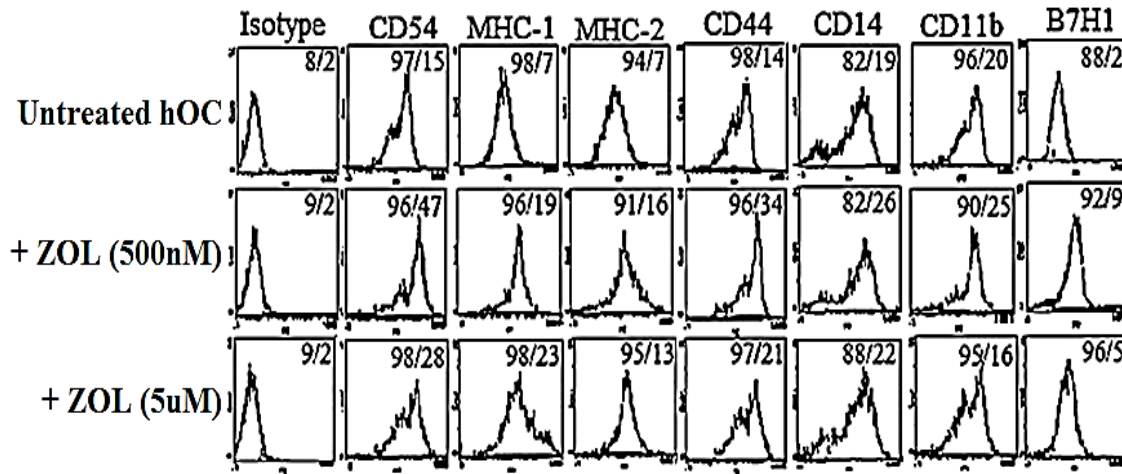


Fig 12: Zolendronic acid modulated surface receptor expression on Osteoclasts.

Surface expression of Zolendronic acid treated osteoclasts were analyzed by flow cytometry. Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts were then treated with Zolendronic acid at 500 nM and 5 μ M. After 4 days of incubation, surface expression of CD14, CD44, CD54, B7H1, MHC-I, MHC-II, and CD11b were determined by flow cytometric analysis.

Figure 13:

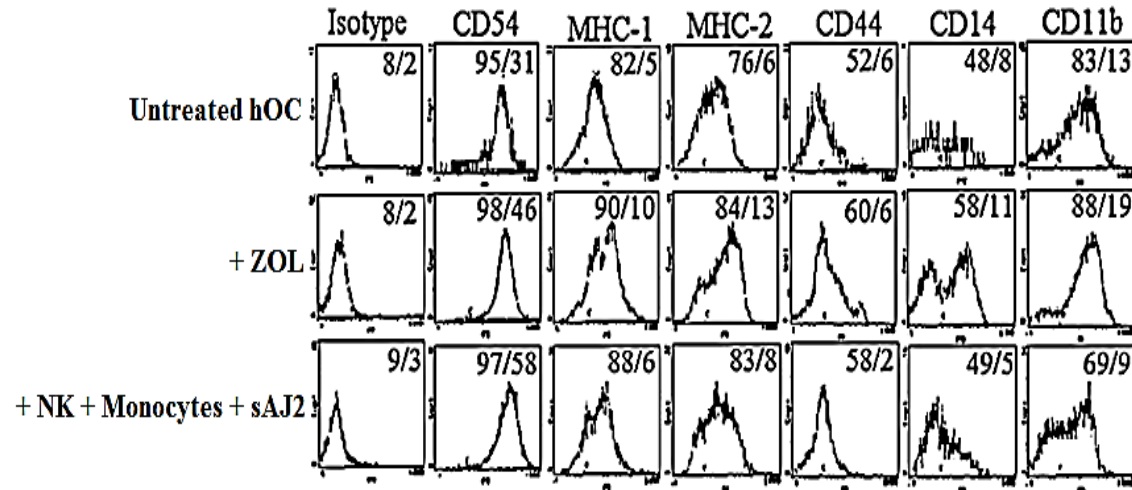


Fig 13. Osteoclasts treated with supernatants from activated NK cells or Zolendronic acid demonstrated higher induction of cell surface receptors.

Surface expression of Zolendronic acid treated osteoclasts were compared to NK supernatant treated osteoclasts. Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts were treated with Zolendronic acid for 5 days and supernatant from NK cells and monocyte co-culture for 2 days. CD54, MHC-I, MHC-II, CD44, CD14 and CD11b expression were determined by flow cytometric analysis.

6. Decreased pit numbers and size by Bisphosphonate-treated Osteoclasts.

The resorptive activity of the osteoclasts was determined after treatment with Zolendronic acid, Alendronate and Etidronate. Although dose dependent decrease in the resorptive activity of osteoclasts could be seen by the treatment with the three bisphosphonates, Zolendronic acid exerted the most severe inhibition (Fig. 14). Both the numbers of the pits and the size of the pits were affected by the treatment with the three bisphosphonates (Fig. 14).

Figure 14:

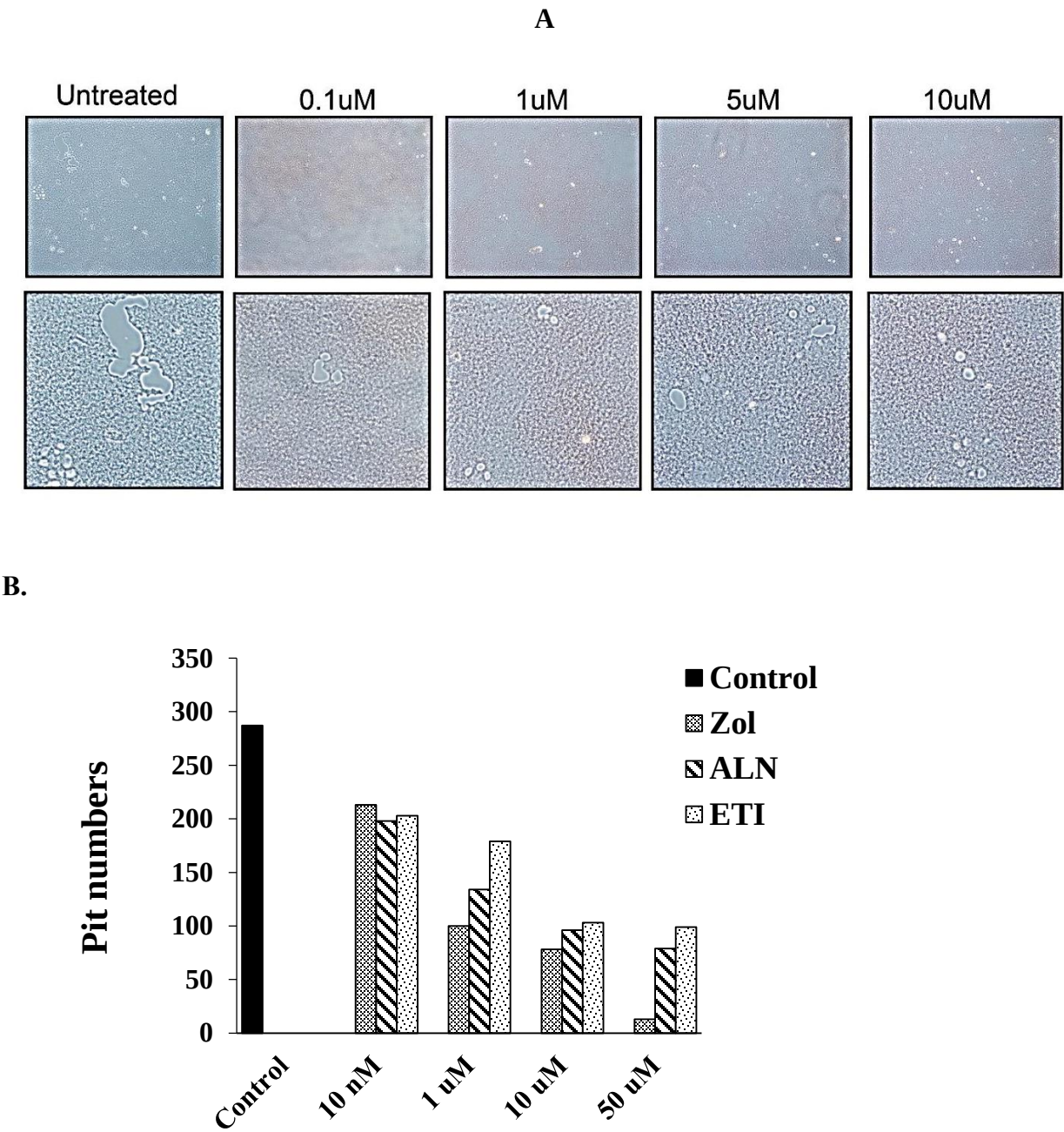


Fig 14: Decreased size and the numbers of pits by Zolendronic acid, Alendronate, and Etidronate treated Osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 12 days. Osteoclasts at a concentration of $2.5 \times 10^5/\text{ml}$ were treated with different concentrations of nitrogen-containing Zolendronic acid and Alendronate and non-nitrogen containing Etidronate in carbonate-apatite coated plate as described in figure 2, after which, osteoclasts were washed using 5% bleach, and both the pictures **(A)** the number of pits counted **(B)** using microscopy at 5X magnification.

DISCUSSION

Phenotypic, functional and morphological characteristics of osteoclasts treated with and without two nitrogen containing Zolendronic acid and Alendronate and one non-nitrogen containing bisphosphonate Etidronate were determined in this aim. Osteoclasts were generated from their precursor cells monocytes and differentiated with RANKL and M-CSF. Initial characterization indicated that during differentiation with RANKL and M-CSF osteoclasts gradually increased the secretion of a number of chemokines and cytokines from day 2 to day 21 of culture, and the profiles of secretion was similar to those of M2 macrophages and monocytes than DCs or M1 macrophages. In comparison to all other subsets, osteoclasts, in general, secreted lower amounts of cytokines, however, they secreted substantial amounts of chemokines (Table 2). Interestingly, secretion of IL-6 by osteoclasts decreased whereas the IL-10 secretion gradually rose from day3 of differentiation to day16. Zolendronic acid and Alendronate but not Etidronate treated osteoclasts triggered dose dependent secretion of IL-6, TNF- α , IL1 β whereas they inhibited the secretion of IL-10. Indeed, our in vivo experiments with Zolendronic acid injected mice during and after tooth extraction demonstrated significantly higher secretion of IL-6 by osteoclasts when determined in in situ immunohistochemical analysis of oral gingival mucosa indicating clear agreement between our in vitro and in vivo experiments (Nishimura *et al*). The dose dependent increase in IL-6 secretion by Zolendronic acid was evident when Osteoclasts were treated with 10nM-1 μ M, and at higher concentration of Zolendronic acid from 1 μ M-100 μ M a dose dependent decrease in the secretion of IL-6 could be observed which related to the ability of Zolendronic acid to induce functional suppression and/or cell death in osteoclasts. Zolendronic acid induced increase of IL-6 was higher when compared to Alendronate whereas Etidronate demonstrated no ability to induce IL-6 secretion. In contrast, IL-10 secretion was not changed from doses 10nM-1 μ M, and

at higher concentration of Zolendronic acid and Alendronate but not Etidronate a dose dependent suppression of IL-10 secretion could be observed, however, this could be due to the ability of Zolendronic acid and Alendronate to induce cell death at high concentrations. Zolendronic acid was also able to induce TNF- α and IL-1 β from osteoclasts. The increase in inflammatory cytokines induced by Zolendronic acid and to a lesser extent by Alendronate correlated with the inability of Zolendronic acid and Alendronate treated osteoclasts to retain their resorptive activity since both the number and size of the pits formed on the resorptive plates were decreased. Interestingly, even though Etidronate did not induce inflammatory cytokines it was able to decrease the ability of osteoclasts to resorb hydroxyapatite significantly.

To determine whether Zolendronic acid had the ability to modulate surface receptors on osteoclasts we first analyzed a number of key cell surface receptors on osteoclasts and compared it to monocytes, macrophages and DCs. As can be seen in Fig. 4 Osteoclasts had significantly down-modulated many of the cell surface receptors, notably, MHC class I and II, CD14, CD11b and CD54. There was 4-25 fold decrease in the expression of surface receptors and the most decrease was seen for MHC class I (8 fold) and II (25 fold) expression on osteoclasts. Considering the size of the osteoclasts such a decrease in surface receptors is quite substantial and it may have significant physiological consequences for the activation of immune inflammatory cells.

Since osteoclasts were compared to freshly isolated monocytes, we next determined the surface receptors when both osteoclasts and monocytes were cultured for 8 days and compared the expression to monocytes treated with IFN- γ and LPS and Dendritic cells generated from monocytes treated with GM-CSF and IL-4. Monocytes cultured for 8 days also down-modulated their surface receptors and the levels of expression remained lower when compared to either 8 day or 21 day cultured osteoclasts for CD14, CD11b and CD54. The levels of MHC class I and II did

not change substantially, however, lower amounts of MHC class II were seen on 21 day osteoclast culture. The surface expressions on osteoclasts were quite different from either macrophages or DCs. Activation of osteoclasts with IFN- γ and TNF- α up-regulated the majority of surface receptors, however, the increase never reached to the levels obtained on the surface of macrophages. These experiments suggested that monocytes in the periphery may be less activating for innate immune cells such as NK cells since they retain higher levels of key surface expression such as MHC class I, whereas once they move to the tissues and down-modulate their surface receptors they may become more activating. Indeed, this may be one reason why NK cells in peripheral blood remain relatively quiescent, even in the presence of competent NK cytotoxic machinery.

Treatment of osteoclasts with Zolendronic acid up-regulated surface receptors significantly and this increase was comparable or even higher when osteoclasts were treated with culture supernatants from NK cells and monocytes treated with sonicates of gram positive bacteria (Fig. 13).

CONCLUSION

The results from this study indicated, Bisphosphonates are causing decrease in number of osteoclasts, and increasing the pro-inflammatory cytokines on interaction with osteoclasts, at the same time this interaction is inhibiting the anti-inflammatory chemokines. Osteoclasts differ from monocytes and other monocyte lineages based on their surface receptors, there was down-modulation of surface receptors when monocytes differentiate to osteoclasts, but when osteoclasts were treated with low concentration of Zolendronic acid, they start up-regulating all those receptors. All three BP used in the study reduced the pit formation by the osteoclasts.

CHAPTER 3

To investigate the role of immunosuppressive cytokine IL-10, in BP-treated Osteoclasts, mediated stimulation of NK cells.

ABSTRACT

Treatment of osteoclasts with Zolendronic acid and much less with Alendronate was capable of inhibiting NK cell mediated cytotoxicity whereas it induced significant secretion of cytokines and chemokines in the cultures of NK cells with osteoclasts. NK cells were able to lyse osteoclasts much more than their precursor cell monocytes and this correlated with the decreased expression of MHC class I expression on osteoclasts. These results suggest that Zolendronic acid treated osteoclasts may remain viable in the microenvironment for a prolonged period of time during interaction with NK cells and trigger continuous secretion of pro-inflammatory cytokines and chemokines in the absence of anti-inflammatory cytokine IL-10 resulting in the chronicity of inflammation. Treatment of IL-10 reduce inflammatory cytokines by Zolendronic acid treated osteoclasts, that inhibition was blocked by anti-IL-10.

BACKGROUND AND INTRODUCTION

NK cells are the large granular lymphocytes, identified by the lack of T cell receptor, surface expression of CD16 and CD56. NK cells exhibit cytotoxic activity against the cancer and provide crucial cytokines to activate adaptive immunity.

Interleukin-10 (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. IL-10 generated by a variety of cells, including T cells and macrophages, functions in a negative feedback loop that suppresses the generation of inflammatory cytokines and dampens the acute inflammatory response. Interleukin 10 (IL-10) indirectly prevents antigen-specific T-cell activation, which is associated with down-regulation of the antigen presentation and accessory cell functions of monocytes, macrophages, Langerhans

cells and dendritic cells [163]. IL-10 suppresses osteoclast formation, suggesting that it plays a role in modulating bone loss in inflammatory disorders [164, 165]. IL-10 inhibited RANKL-induced osteoclastogenesis [166]. IL-10 acts directly on mononuclear precursors to inhibit NFATc1 expression and nuclear translocation, disrupting Ca^{2+} mobilization, indicating that one of the ways in which IL-10 directly inhibits osteoclastogenesis is by suppressing NFATc1 activity [167]. IL-10 may down-regulate osteoclastogenesis mainly through inhibition of the expression of NFATc1, c-Fos and c-Jun, providing evidence of inhibitory action of IL-10 on RANKL-mediated osteoclastogenesis [168]. IL-10 treatment abolished OC differentiation in a dose-dependent manner. Cy Luo *et al.* (2011), showed that, as the ratio of *Treg* cells to BMC was up-regulated, there was inhibition of OC differentiation and bone resorption, by increased secretion of IL-10 and TGF- β 1, and this mechanism was reversed by anti-IL-10 and anti-TGF- β 1 antibodies [169]. Inhibition of major histocompatibility complex (MHC) class II expression by macrophages is the primary mechanism by which interleukin-10 (IL-10) exerts immune suppression. Human rIL-10 were able to downregulate proinflammatory cytokine production, suggesting that rIL-10 could be tried as an anti-inflammatory agent in neonates with a high risk of chronic lung disease [170]. IL-10 may be an important inherent component of the cytokine network of congestive heart failure [171]. IL-10 inhibits the antigen-presenting capacity of synovial fluid macrophages, which further emphasizes the anti-inflammatory potential of IL-10 in inflammation. Importantly, IL-10 is able to downregulate the APC function of synovial fluid macrophages even when they are efficiently activated [172]. IL-10 has important regulatory effects on immunological and inflammatory responses because of its capacity to downregulate class II MHC expression and to inhibit the production of proinflammatory

cytokines by monocytes [173]. IL-10 down-regulate the expression of TNF- α , TNF- α is the required factor for osteoclast differentiation.

Accumulating evidence indicated that the skeletal and immune systems are closely related through cellular and molecular interactions, the immune and skeletal systems share cytokines, signaling molecules, transcription factors and membrane receptors [174, 175]. Original are differentiated from same precursors as macrophages and dendritic cells, which are professional antigen presenting cells, studies showed that osteoclasts can also function as APCs and activate both CD4⁺ and CD8⁺ T cells [122]. IFN- γ would have different effect on early-stage and late-stage osteoclast precursors. Osteoclasts precursors exposed to RANKL for 1-2 days can be rendered resistant to maximal osteoclast-inhibitory doses of IFN- γ . IFN- γ resistant pre-osteoclasts produced low level of NO, and were resistant to IFN- γ induced, mac-1-induced and RANKL induced surface expression [176]. T-cell produced cytokines play a pivotal role in the bone loss caused by inflammation, infection, estrogen deficiency. IFN- γ is a major product of activated T helper cells that function as a pro- or anti-resorptive cytokine. IFN- γ blunts osteoclasts formation through direct targeting of osteoclast precursors, but indirectly stimulates osteoclasts formation and promotes bone resorption by stimulating antigen-dependent T- cell activation and T cell secretion of the osteoclastogenic factors RANKL and TNF- α [177]. Suppressive effect of IFN- γ , is reduced when osteoclast precursors are pre-exposed to the receptor activator of NF- κ B (RANKL). IFN- γ markedly suppress the RANKL-induced expression of nuclear factor of activated T-cells c1 (NFATc1) in normal, but not RANKL-pretreated bone marrow macrophages (BMM). Also, IFN- γ impairs the activation of nuclear factor- κ B (NF- κ B) and c-Jun N-terminal kinase (JNK) pathway in normal, but not RANKL pretreated BMM. These findings indicate that IFN- γ inhibits osteoclastogenesis partially by

suppressing the expression of NFATc1 and the activation of NF- κ B and JNK pathways [178]. IFN- γ inhibits the RANKL-induced expression of osteoclast genes, but RANKL pretreatment reprograms osteoclast genes into a state in which they can no longer be suppressed by IFN- γ , indicating that IFN- γ inhibits osteoclastogenesis by blocking the expression of osteoclast genes. IVVY motif in the cytoplasmic domain of RANKL is responsible for rendering BMMs refractory to inhibitory effect of IFN- γ . All these findings provides the important mechanistic insights into the biphasic effects of IFN- γ on osteoclastogenesis [178]. IFN- γ mediates the fusion of pOCs into multinucleated osteoclasts by inducing DC-STAMP expression which, in turn, is regulated by NFATc1 expression via the induction of c-Fos [179]. Zolendronic acid treated monocytes when co-cultured with NK cells, IL-15, MIP-1 β , and IFN- γ secretions were higher in Zolendronic acid treated monocytes compared with the untreated monocytes [180]. Zolendronic acid treated DC stimulated NK cells to produce higher levels of IFN- γ [114]. Studies showed that postmenopausal women with low bone mass showed elevated serum levels of IFN- γ . Characteristic morphological feature of bisphosphonate-treated osteoclasts is the lack of a ruffled border, the region of invaginated plasma membrane facing the resorption cavity. Bisphosphonates were also shown to disrupt the cytoskeleton of the osteoclast [72]. Many studies have shown that bisphosphonates can affect osteoclast-mediated bone resorption in a variety of ways that include effects on osteoclast recruitment, differentiation, and resorptive activity, and some may induce apoptosis [60, 65-67]. It is widely accepted that BPs exert their major effect on mature osteoclasts.

NK cells express both RANKL and M-CSF and when co-cultured with Monocytes trigger the differentiation of osteoclasts, a process dependent on RANKL and M-CSF [181]. NK cells have the potential to contribute to the pathogenesis of inflammatory disorders both through the

inflammatory mediators that they produce and their interactions with other immune (Fig. 19) [182]. In our study, we found that Zolendronic acid treatment results in up-regulation of the cytokines, chemokines and growth factors during osteoclasts and NK cells interaction. Treatment with rh-IL-10 was able to reduce the inflammatory cytokines. This study consist of several sub-aims.

Sub Aim 1: Osteoclasts are targets of NK cells and induce significant cytokine and chemokine secretion by the NK cells.

Sub Aim 2: NK cells secrete significant levels of inflammatory cytokines and chemokines in culture with Bisphosphonate treated Osteoclasts and Monocytes.

Sub Aim 3: Osteoclasts form clumps by NK cells and BP combination.

Sub Aim 4: Zolendronic acid treated Osteoclasts and oral tumor cells are resistant to NK cell mediated cytotoxicity.

Sub Aim 5: BP inhibit IL-10 secretion by Osteoclasts, addition of IL-10 reduce inflammatory cytokines, that inhibition was blocked by anti-IL-10.

Figure 15:

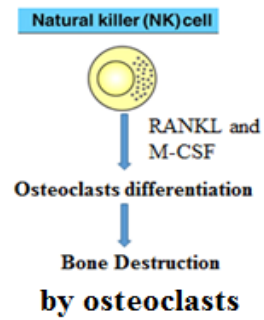


Figure 15: How Natural Killer cells interact with osteoclasts. NK cells are capable of interacting with osteoclasts through cytokine production, M-CSF and RANKL.

MATERIAL AND METHODS

A. Cell culture and reagents

Monocytes, Dendritic cells (DCs), Macrophages (M1 and M2) and Osteoclasts (OCs)

Monocytes purification kit was purchased from stem cell Technologies. RPMI 1640 was supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% non-essential amino acids (Invitrogen by Life Technologies, CA) was used to cultured monocytes, M1 macrophages and dendritic cells. Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts.

B. Antibodies

Monocyte purification kit was purchased from stem cell Technologies (Vancouver, Canada) Human recombinant IFN- γ , LPS, human recombinant IL-4 and human GM-CSF were dissolved in PBS with 1%BSA and stored at -20°C. Human recombinant IFN- γ (50ng/ml), LPS (15ng/ml) was used for M1 macrophages culture. Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -20°C, and used for osteoclasts culture. ELISA kits were purchased from Biogends, CA. Zolendronic acid, Alendronate and Etidronate were purchased from UCLA Ronald Reagan Pharmacy. Fluorescent Zolendronic acid analogs were synthesized via a linker strategy (Hokugo et al. 2013). PE conjugated IgG1 and 2b, PE-CD14, PE-CD11b, PE-CD124, PE-B7H1, PE-CD15, PE-CD33, PE-CD44, PE-CD54, PE-MHC-I and PE-MHC-II were all purchased from Biolegend, CA.

C. Human Peripheral Blood Monocytes purification

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for 1 hour after which the adherent subpopulation of PBMCs was detached from the tissue culture plates and the monocytes were purified using isolation kits obtained from Stem Cell Technologies (Vancouver, Canada). Greater than 95% purity was achieved for each subset based on flow cytometric analysis of CD14. To confirm the low contamination of NK cells, anti-CD16 antibody staining was analyzed, which remained low, at 3 ± 1 % similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures.

D. Monocytes cultures for Osteoclasts, Macrophages and Dendritic cells generation

For M1 macrophages, monocytes were cultures in RPMI 1640 containing 15ng/ml LPS and 50ng/ml rh-IFN-gamma for 8 days. For dendritic cells, monocytes were cultured in RPMI 1640, containing 150ng/ml GM-CSF and 50ng/ml rh-IL-4. For osteoclasts, monocytes were cultured in alpha-MEM medium containing 25ng/mL M-CSF, day 3 after culture media was refreshed with addition of M-CSF (25ng/ml), and day 6 after culture media was refreshed with addition of M-CSF (25ng/ml) and Rank Ligand (25ng/mL). Medium was refreshed every 3 days with alpha-MEM containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days.

E. TRAP staining

Osteoclasts were detached from tissue culture plate and seeded in 96-well plate at 3×10^4 cells/well for 18-24 hours. Afterwards the cells were rinsed twice with 1X PBS and fixed with 10% formaldehyde for 5 minutes at room temperature. The cells were then rinsed three times with 1X PBS and incubated with Chromogenic Substrate solution (Primary Cell, Co., Japan) for 30 minutes or until stained TRAP is clearly seen. Finally, cells were then rinsed with deionized water to neutralize the reaction and images were taken with Leica DMI 6000B inverted microscope.

F. Enzyme-Linked Immunosorbent Assays (ELISAs) and Multiplex Cytokine Arrays

Enzyme-linked Immunoassorbent Assays (ELISAs) especially for IFN- γ , IL-6, IL-10, TNF- α , IL-1 β (Biolegends, CA) and IL-18 (R&D Systems, Minneapolis, MN), were performed on the harvested supernatant after cultures and bisphosphonates treatments on osteoclasts to measure the concentration levels of cytokines and growth factors secreted by monocytes, macrophages, dendritic cells, and bisphosphonate non-treated/treated osteoclasts, as described by manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines. To perform single ELISA plates were coated with recommended amount of capture antibodies and incubated overnight. After overnight incubation, plates were washed three or four times, and antibodies were blocked with ELISA PBS containing 1% BSA for 1 hour. Thereafter plates were washed three or four times, and standards and samples were added in respective wells. For IL-6, IL-10, IL-1 β , and IL-18 plates were incubated for 2 hours, and for TNF- α plates were incubated overnight. After incubation plates were washed four times, detection antibodies were added and plates were incubated for 1 or 2 hours. Plates were washed, followed for the addition of Avidin-HRP and

continued incubation for next 30 mins or 1 hour. Finally the plates were washed, and incubated with alkaline phosphate substrate (Sigma Chemical Co. 104), reaction was stopped with stop solution, 2N H₂SO₄. Readings of plated were done with ELISA reader using 405nm or 450nm filter. Fluorokine MAP cytokine multiplex kits were purchased from R&D Systems (Minneapolis, MN) and the procedures were conducted as suggested by the manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines provided by the manufacturer.

G. Surface Staining

Staining was performed by coating the cells with the antibodies. The cells, monocytes, dendritic cells, macrophages and osteoclasts were detached from the tissue culture plates and washed twice with ice-cold PBS containing 1% BSA. Predetermined optimal concentrations of specific human monoclonal antibodies were added to 1 X 10⁴ cells in 50 µl of cold-BSA and cells were incubated on ice for 30 min. Thereafter cells were washed in cold PBS-BSA and brought upto 500 µl with PBS-BSA. An Epics C (Coulter) flow cytometry was used for analysis.

H. Pit resorption assay

Purified Human osteoclasts were generated from healthy donor's monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Afterwards, osteoclasts were rinsed, detached from tissue culture plates and seeded at 1X10⁴ cells/well in 24 well plate pre-coated with synthetic carbonate apatite (Cosmo Bio Co, Japan) for 7 days. After the incubation period, culture medium was removed and cells were rinsed with 5% sodium

hypochlorite for 5 minutes. The cells were then washed with water and photographed using Leica DMI 6000B inverted microscope.

I. ^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [183]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr -labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

J. Statistical Analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS

1. Osteoclasts are targets of NK cells and induce significant cytokine and chemokine secretion by the NK cells

Since osteoclasts express lower levels of MHC class I on the surface they may be targets of NK cell lysis. Human osteoclasts were generated using purified monocytes treated with Rank Ligand and M-CSF as described in the Materials and Methods section. Highly purified autologous NK cells were cultured with or without IL-2 and/or anti-CD16mAb for 24 hours before they were added to ⁵¹Chromium labeled osteoclasts. (Fig. 16A). Both untreated and IL-2 treated NK cells were able to lyse osteoclasts although the levels of IL-2 treated NK cells were significantly higher than the untreated NK cells. Addition of anti-CD16 mAb triggering antibody with IL-2 inhibited IL-2 induced NK cell cytotoxicity (Fig. 16A). Osteoclasts were able to induce significant secretion of IFN- γ , by the IL-2 treated NK cells, although IFN- γ secretion was highly induced by IL-2 in combination with anti-CD16mAb (Fig. 16B). To determine whether osteoclasts are better or not than monocytes as the targets of NK cells, NK cell cytotoxicity against osteoclasts and monocytes was analyzed using untreated and IL-2 treated NK cells. As shown in Fig. 17A NK cells lysed osteoclasts much more than monocytes. In addition, both monocytes and osteoclasts were able to induce significant secretion of IFN- γ (Fig. 17B), by the IL-2 treated NK cells, albeit the levels were higher when NK cells were cultured with osteoclasts than monocytes, as expected, based on surface expression. We next analyzed IL-6, IL-10, IL-18 using single ELISA. In case of osteoclasts, IL-6 and IL-10 secretion did not changed much with untreated NK cells, but was induced with IL-2 treated NK cells, although was highly induced by IL-2 in combination with anti-CD16mAb NK cells treated NK cells (Fig. 18A and 18B). On the other hand monocytes induced IL-6 secretion with IL-2 treated NK cells and by IL-2 in combination with anti-CD16mAb NK

cells, but induction was about 1/5 to 1/7 of that of osteoclasts (Fig 18A). In case of monocytes IL-10 secretion was reduced with untreated, IL-2 treated NK cells and also with combination of IL-2 and anti-CD16mAb treated NK cells, although it was slightly higher in case of IL-2 and anti-CD16mAb treated NK cells, comparatively to untreated and IL-2 treated NK cells, but not significantly. We also analyzed IL-18 secretion (Fig. 19), although it did not give us proper correlation, but highest induction was in case of osteoclasts with IL-2 in combination with anti-CD16mAb treated NK cells. All these findings suggested that osteoclasts induced higher cytokines and chemokines as compared to monocytes when interact with NK cells, are more susceptible to NK cells mediated cytotoxicity.

We then compared the levels of IFN- γ secretion by IL-2 and IL-2+anti-CD16mAb treated NK cells cultured either with osteoclasts, DCs or monocytes from after day 5 of cultures (Fig. 20). As shown in Fig. 20B, the levels of IFN- γ secreted in the cultures of NK cells with osteoclasts was much higher than those induced in the cultures of NK cells either with monocytes or DCs. We then determined whether osteoclasts can support expansion of NK cells. Both osteoclasts and DCs, and much less monocytes, are able to support the expansion of NK cells (data not shown). NK cells expanded by osteoclasts, DCs and monocytes were counted and equal numbers of NK cells from each expanded subset were used in cytotoxicity assay against K562. As shown in Fig. 20A, IL-2 treated NK cells expanded by osteoclasts had the highest cytotoxicity, followed by those expanded by dendritic cells and the least cytotoxicity was seen by IL-2 treated NK cells which were expanded by monocytes. Addition of anti-CD16mAb antibody with IL-2 abrogated NK cell mediated cytotoxicity in all expanded NK cells (Fig.20A). When we supplemented the IL-2 treated NK cells and IL-2 in combination with anti-CD16mAb treated NK cells, expanded by osteoclasts, DCs and monocytes, there was up-regulation of cytotoxic function in all the cases, keeping higher level in

case of osteoclasts expanded NK cells (Fig 21). These results indicated that osteoclasts are potent inducers of NK cell function and expansion.

Figure 16:

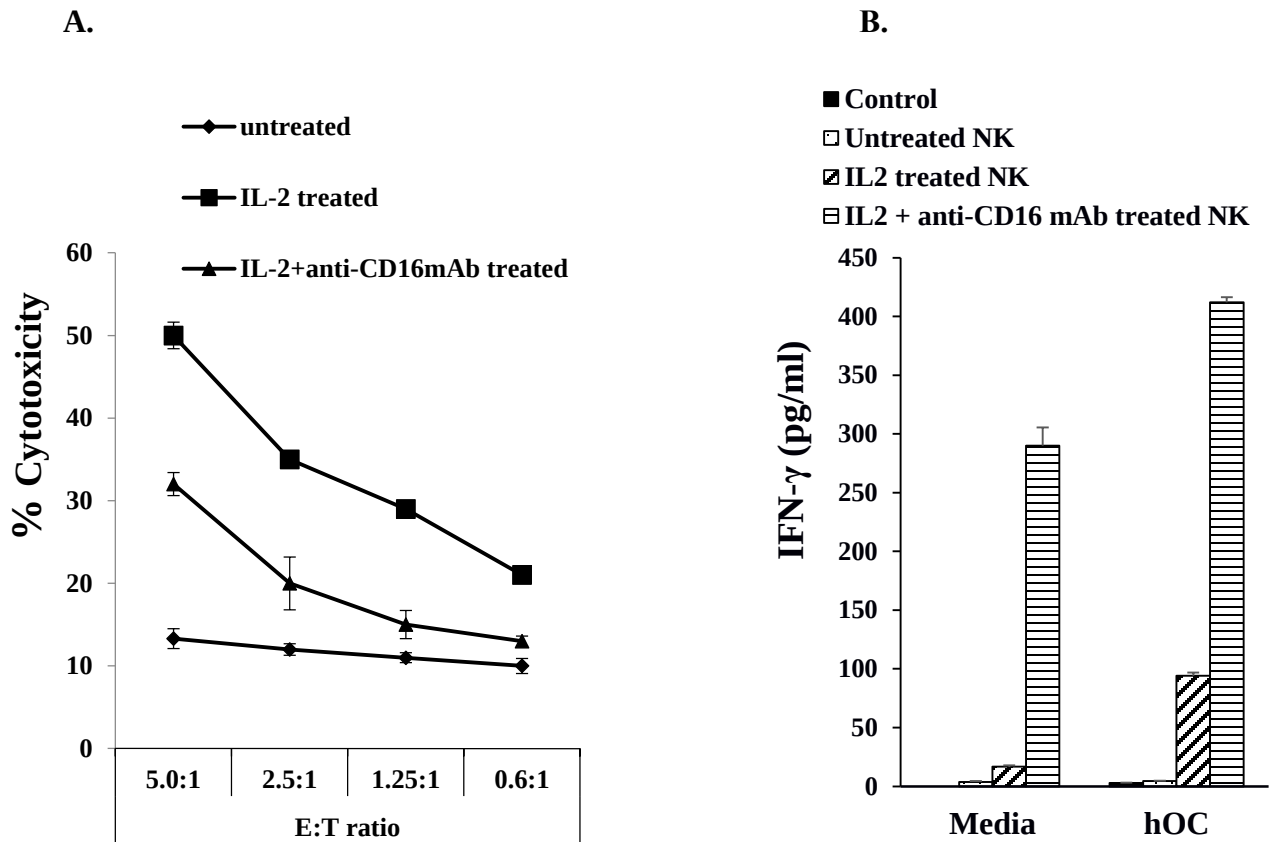


Figure 16: Osteoclasts are susceptible to NK cells mediated cytotoxicity and induces significant IFN- γ secretion by the NK cells.

Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before they were added to ^{51}Cr labeled autologous osteoclasts at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay (A). Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before the cells were added to autologous osteoclasts at an effector to target ratio of 0.3 :1 NK cells to osteoclasts. After an overnight incubation, the supernatants were removed and the level of secreted IFN- γ is measured using specific ELISA (B).

Figure 17:

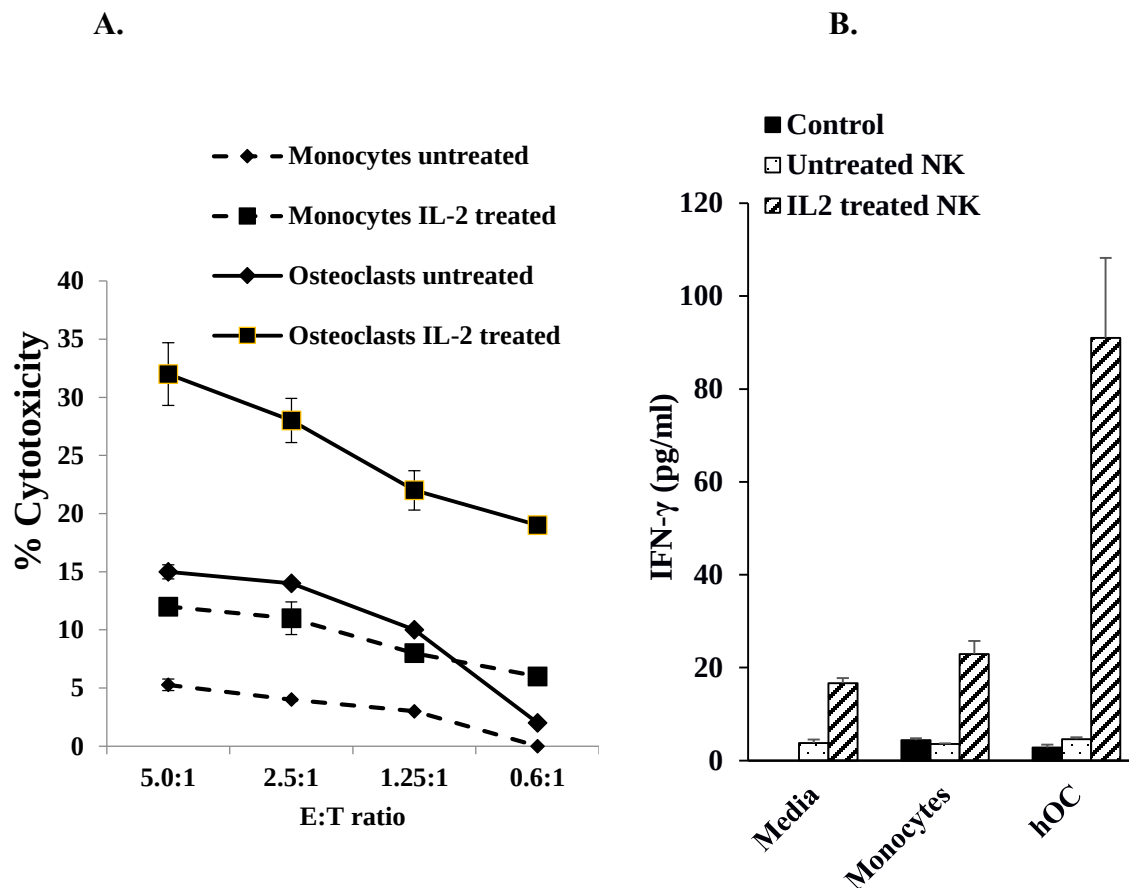
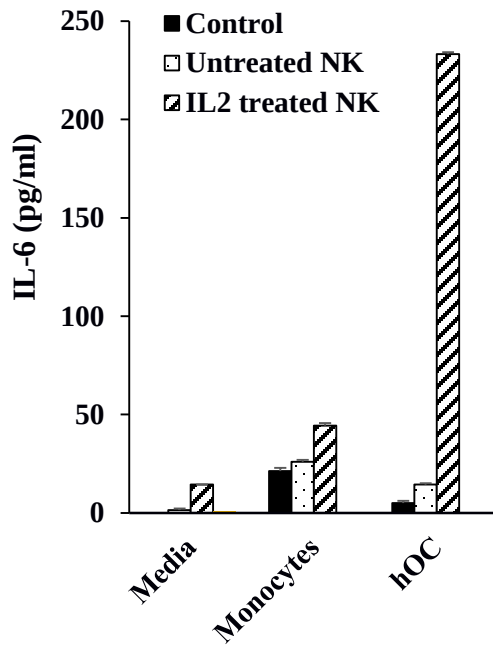


Figure 17: Osteoclasts are more susceptible to NK cells mediated cytotoxicity and induce more IFN- γ secretion than monocytes by the NK cells.

Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml) for 18 hours before they were added to ^{51}Cr labeled autologous osteoclasts and monocytes at various effector to target ratios. NK cell mediated cytotoxicity were determined using a standard 4 hour ^{51}Cr release assay (A). Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous osteoclasts and monocytes at an effector to target ratio of 0.3 :1 NK cells to osteoclasts/monocytes. After an overnight incubation, the supernatants were removed and the level of secreted IFN- γ is measured using specific ELISA (B).

Figure 18:

A.



B.

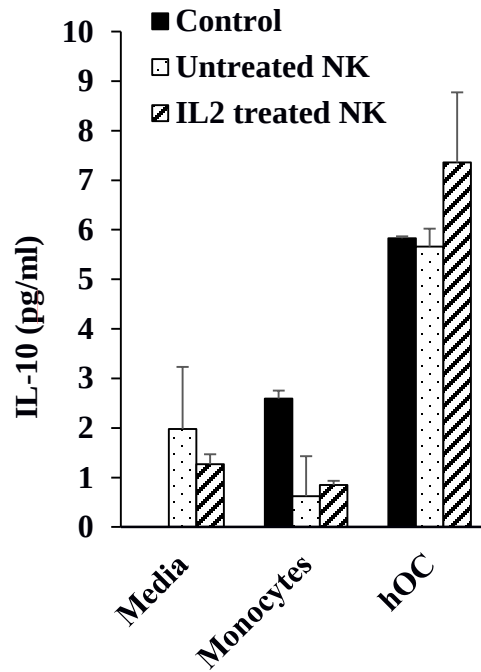


Figure 18: Osteoclasts induced more IL-6 and IL-10 secretion than monocytes by the NK cells.

Highly purified autologous NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous osteoclasts and monocytes at an effector to target ratio of 0.3 :1 NK cells to osteoclasts/monocytes. After an overnight incubation, the supernatants were removed and the level of secreted IL-6 (A) and IL-10 (B) is measured using specific ELISA.

Figure 19:

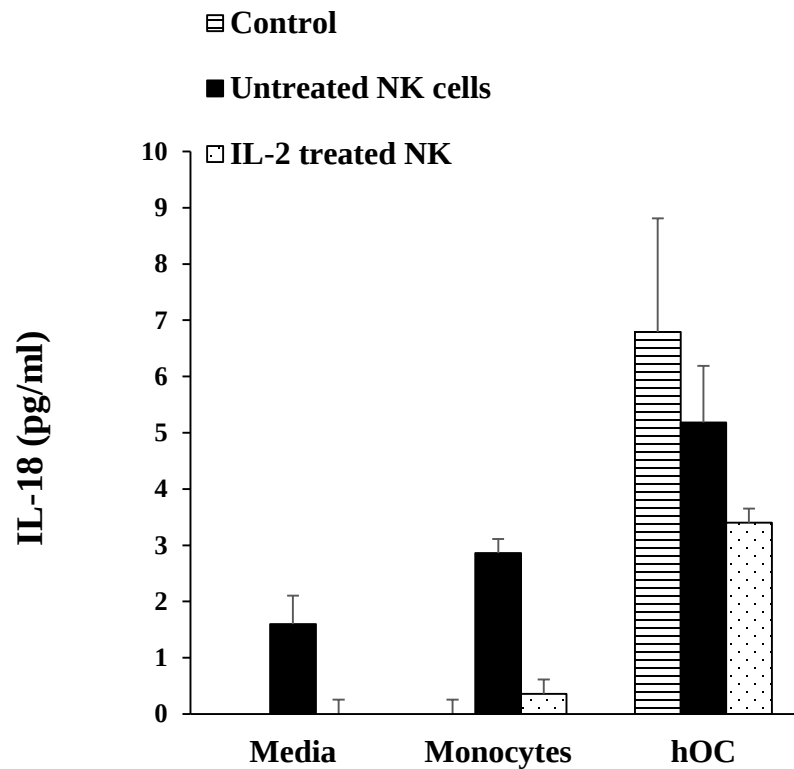
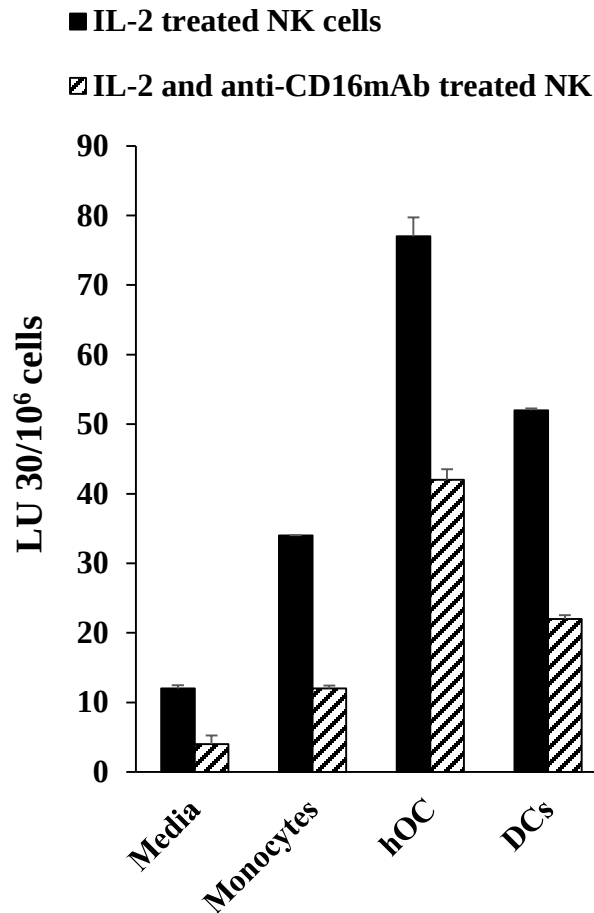


Figure 19: Osteoclasts induced more IL-18 secretion than monocytes by the NK cells.

Highly purified autologous NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous osteoclasts and monocytes at an effector to target ratio of 0.3 :1 NK cells to osteoclasts/monocytes. After an overnight incubation, the supernatants were removed and the level of secreted IL-18) is measured using specific ELISA.

Figure 20.

A.



B.

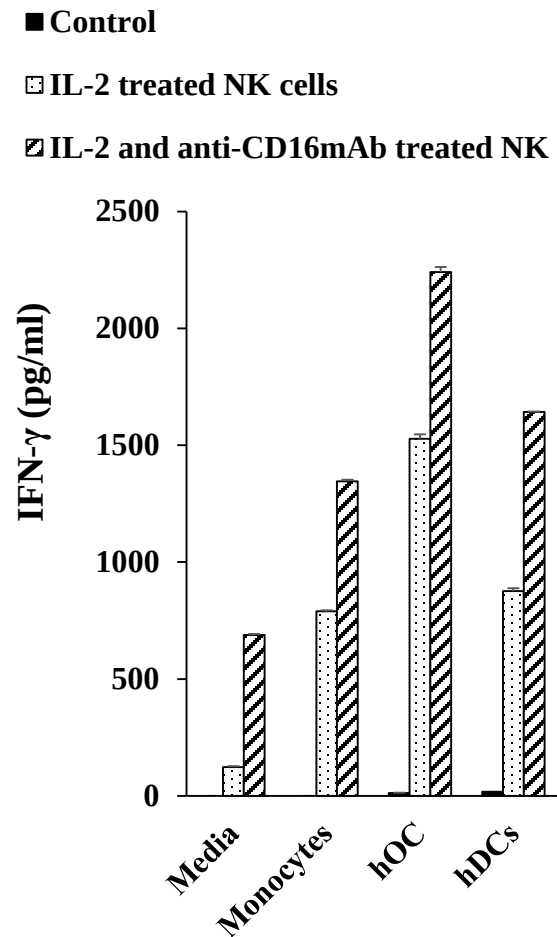


Figure 20: Osteoclasts keeps the NK cells functional and induce significant level of IFN- γ secretion by the NK cells.

Dendritic cells (DCs), osteoclasts (hOC) and monocytes were prepared as described in figure 3. Highly purified NK cells (1×10^6 cells/ml) were treated with IL-2 (1000 units/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with autologous cells at 1:1 titrations. The supernatants from each culture condition were harvested day 5, and NK cells were used against K562. NK cell mediated cytotoxicities were determined using a standard 4 hour ⁵¹Cr release assay (A). The level of IFN- γ produced by NK cells was measured using a specific ELISA (B).

Figure 21:

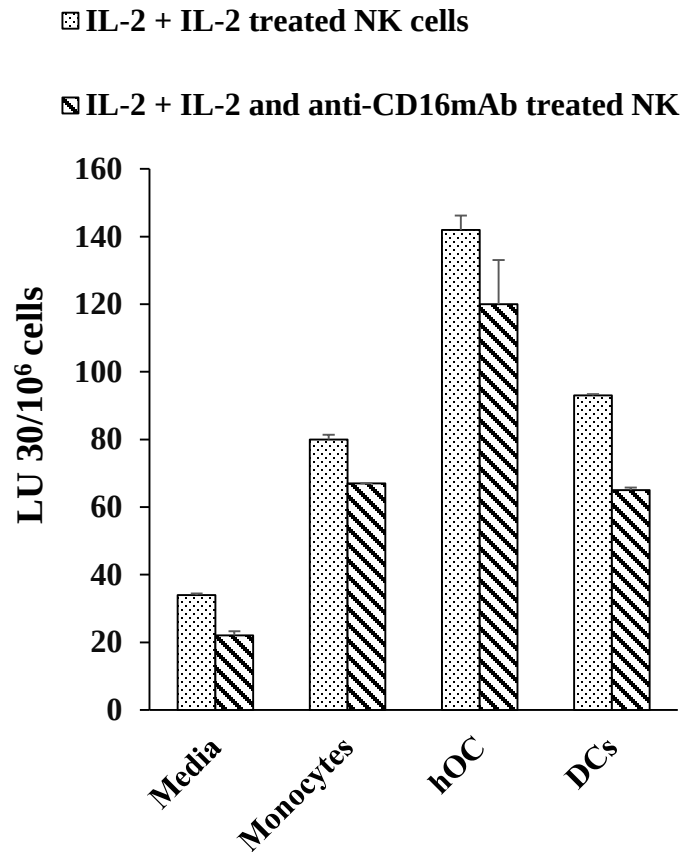


Figure 21: Osteoclasts keeps the NK cells functional.

Dendritic cells (DCs), osteoclasts (hOC) and monocytes were prepared as described in figure 3. Highly purified NK cells (1×10^6 cells/ml) were treated with IL-2 (1000 U/ml) or the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with autologous cells at 1:1 titrations. Day 5 after co-culture, IL-2 or IL-2 in combination with anti-CD16mAb treated NK cells were supplemented with fresh IL-2 (1000U/ml). 12 hours after IL-2 addition, NK cells were used against K562. NK cell mediated cytotoxicities were determined using a standard 4 hour ⁵¹Cr release assay.

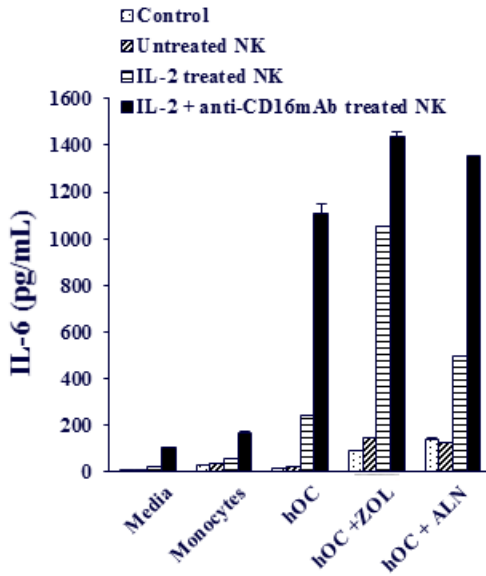
2. NK cells secrete significant levels of inflammatory cytokines and chemokines in culture with Bisphosphonate treated Osteoclasts and Monocytes.

We next determined the effect of bisphosphonates when NK cells were cultured with either Zolendronic acid or Alendronate treated osteoclasts. As shown in Table 3 Zolendronic acid treated osteoclasts triggered significantly higher induction of cytokines and chemokines in the co-cultures with NK cells, and the effect was higher when compared to Alendronate treated Osteoclasts (Fig. 22, Fig.23, Fig.25, Fig.26), whereas Etidronate had no enhancing effect. As expected, based on surface expression, osteoclasts triggered cytokine and chemokine secretion significantly more than monocytes (Fig. 22-24 and Table 3). IL-2 treated NK cells triggered significantly higher release of IL-6, IFN- γ , IL-10, IL-8, MCP-1, MIP-1a and MIP-1b in the cultures with osteoclasts than monocytes (Table 3). Zolendronic acid treated osteoclasts upregulated secretion of cytokines and chemokines 2-6 fold higher for IL-6, IFN- γ , MIP1a and MIP-1b by IL-2 and IL-2+anti-CD16mAb treated NK cells when compared to untreated osteoclasts (Table 3 and Fig. 22-Fig. 26). The levels of IL-8 and MCP-1 secretion were very high and plateaued in the cultures of NK cells with osteoclasts (Table 3). Both Zolendronic acid and Alendronate treated osteoclasts demonstrated decreased secretion of IL-10 in the cultures with NK cells when compared to untreated osteoclasts (Fig. 23A). Zolendronic acid treated osteoclasts secreted higher levels of IL-18 when compared to monocytes, the levels significantly increased when cultured with NK cells (Fig. 23B). When monocytes were treated with Zolendronic acid, there was induction of IFN- γ on Zolendronic acid treated monocytes by NK cells, and the level of IFN- γ reached to the induction level of untreated osteoclast with NK cells (Fig. 24). Now, we wanted to analyze the cytokine secretion by variable concentration of nitrogen containing bisphosphonate Zolendronic acid and Alendronate and no-nitrogen containing bisphosphonate, we treated osteoclasts with three concentration

100nM, 250nM, and 500nM of three BP. Zolendronic acid at all concentration showed induction of IFN- γ , but highest level at particular concentration is donor dependent. Alendronate showed induction at concentration 250nM and higher, but induction level is lower than Zolendronic acid, Etidronate showed very low or no induction of IFN- γ (Fig. 26).

Figure 22:

A.



B.

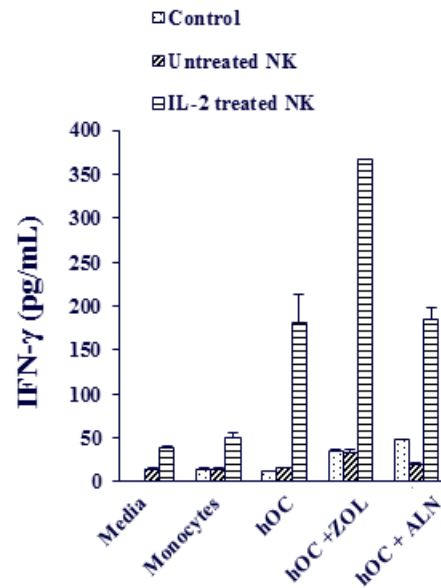


Figure 22: Zolendronic acid treated hOC secrete higher IL-6 and IFN- γ , when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Monocytes were purified from autologous donor. Osteoclasts at a concentration of 3×10^5 ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid and Alendronate. After 72 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:3 of NK cells to monocytes or osteoclasts. After an overnight incubation, the supernatants were harvested and subjected to a specific ELISA for IL-6 (A), IFN- γ (B).

Figure 23:

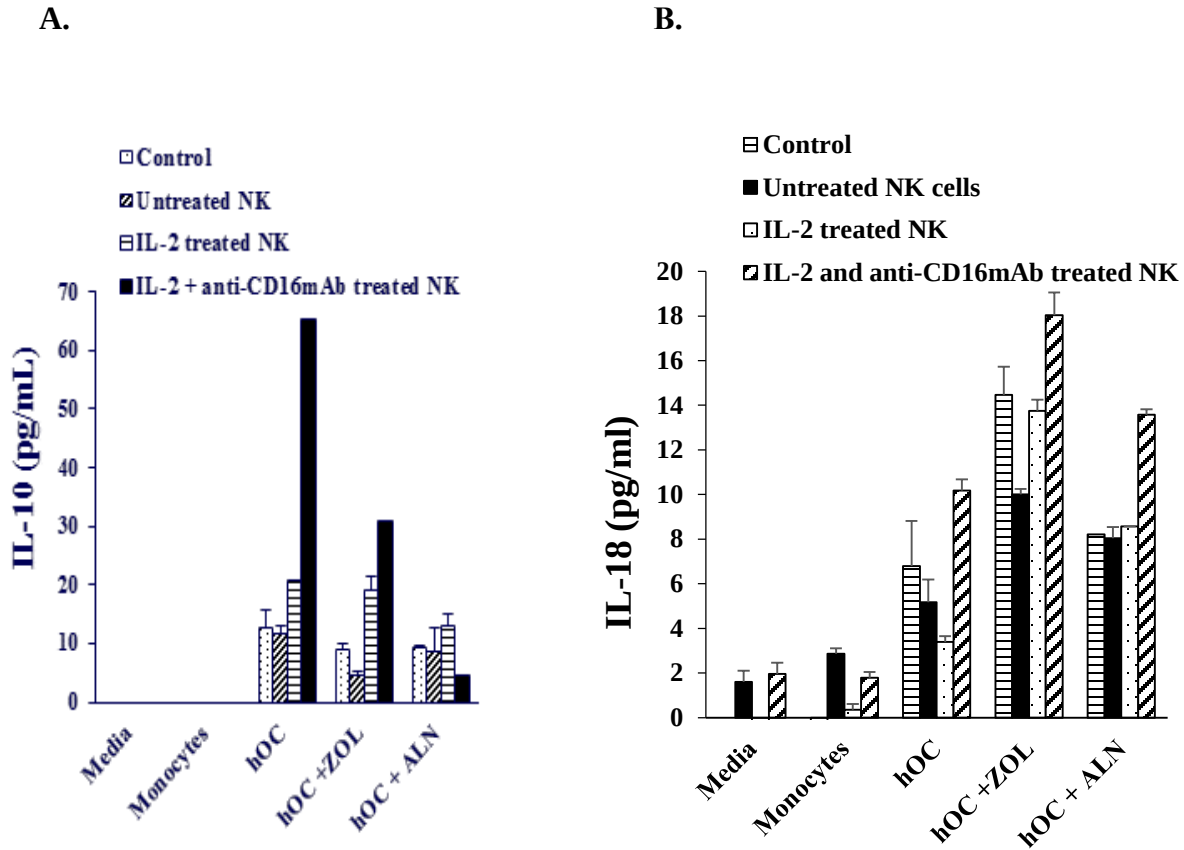


Figure 23: Zolendronic acid treated hOC secrete higher IL-10 and IL-18, when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Monocytes were purified from autologous donor. Osteoclasts at a concentration of 3×10^5 ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid and Alendronate. After 72 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:3 of NK cells to monocytes or osteoclasts. After an overnight incubation, the supernatants were harvested and subjected to a specific ELISA for IL-10 (A) and IL-18 (B).

Table 3.

		IL-6	IFN- γ	IL-10	MCP-1	IL-8	MIP-1 α	MIP-1 β
Media	Media alone	7	1	0	56	7	1039	0
	Untreated NK alone	24	1	0	999	1514	942	375
	IL-2 treated NK alone	54	4	0	1252	3638	1039	586
	IL-2 + anti-CD16mAb treated NK alone	252	25	0	3818	16969	5803	20368
Monocytes	Untreated	106	4	0	239	11217	1377	375
	+ Untreated NK	99	2	0	264	10426	1135	163
	+ IL-2 treated NK	136	4	10	1232	11125	1232	374
	+ IL-2 + anti-CD16mAb treated NK	424	33	10	28195	21638	1619	4229
Osteoclasts	Untreated	41	4	14	31691	16607	1377	2129
	+ Untreated NK	63	4	23	29258	16544	1715	2185
	+ IL-2 treated NK	535	4	24	38655	19349	1908	5680
	+ IL-2 + anti-CD16mAb treated NK	3866	57	82	38916	23766	6114	61555
Osteoclasts + ZOL	Untreated	196	4	14	37820	18589	1425	2157
	+ Untreated NK	312	6	14	38159	20986	1715	2538
	+ IL-2 treated NK	3267	12	20	39332	22631	4294	11587
	+ IL-2 + anti-CD16mAb treated NK	11605	80	31	38748	24420	6105	38411

Table 3: Production of cytokines, chemokines and growth factors in cultures of NK cells with monocytes and Osteoclasts.

Human monocytes purified from healthy donor's PBMCs were differentiated into osteoclasts in culture with medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. After 72 hours of treatment of monocytes and cultured osteoclasts with bisphosphonates at 1uM concentration, treated monocytes and osteoclasts were washed extensively with fresh media and cultured with untreated, IL-2 treated and IL-2 + anti-CD16mAb treated NK cells. Supernatants from the co-cultures of monocytes or osteoclasts with NK cells were harvested after 24 hours of incubation and the levels of cytokine and chemokine production were measured using multiplex cytokine array kit.

Figure 24:

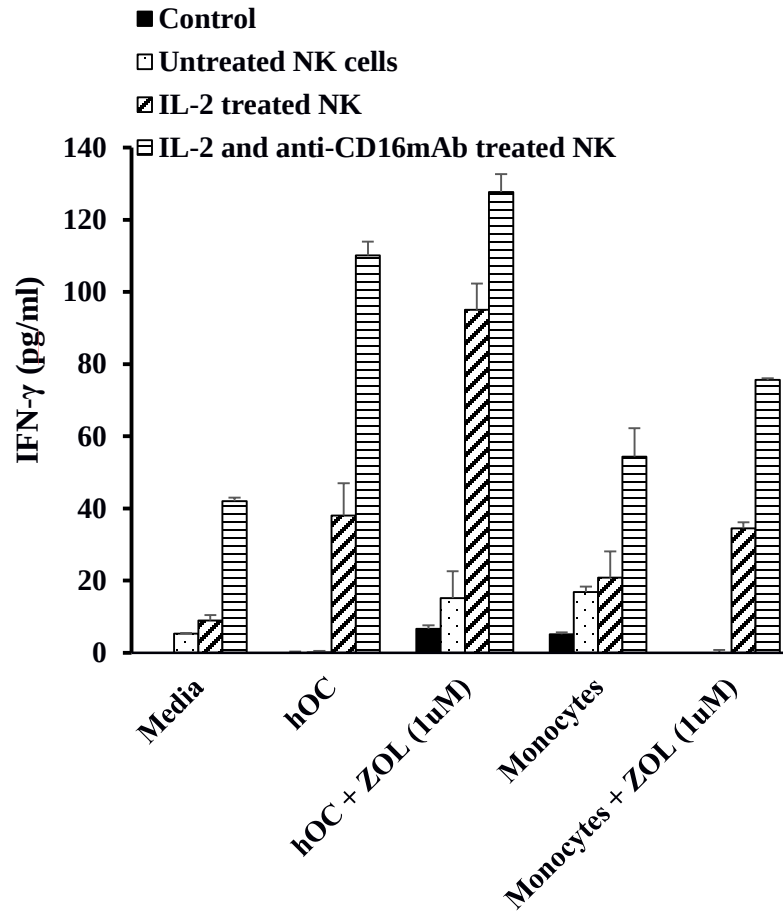


Figure 24: Zolendronic acid treated hOC and monocytes secrete higher IFN- γ , when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Monocytes were purified from autologous donor. Osteoclasts and monocytes at a concentration of 1×10^5 /ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid. After 72 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:3 of NK cells to monocytes or osteoclasts. After an overnight incubation (A), and day 4 after co-culture (B), the supernatants were harvested and subjected to a specific ELISA for IFN γ .

Figure 25:

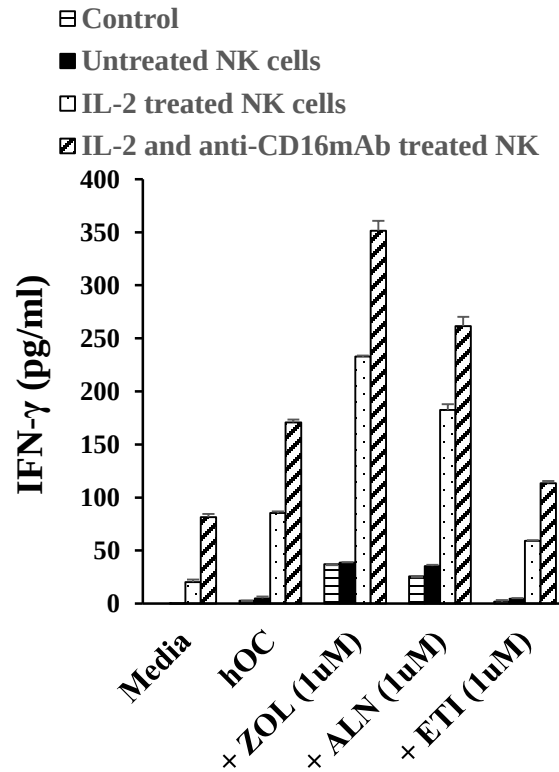


Figure 25: Zolendronic acid treated hOC secrete higher IFN- γ , when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Osteoclasts at a concentration of 1×10^5 /ml were treated with 1μM of nitrogen-containing BP, Zolendronic acid and Alendronate and non-nitrogen containing Etidronate. After 12 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:2 of NK cells to osteoclasts. After an overnight incubation after co-culture the supernatants were harvested and subjected to a specific ELISA for IFN- γ .

Figure 26:

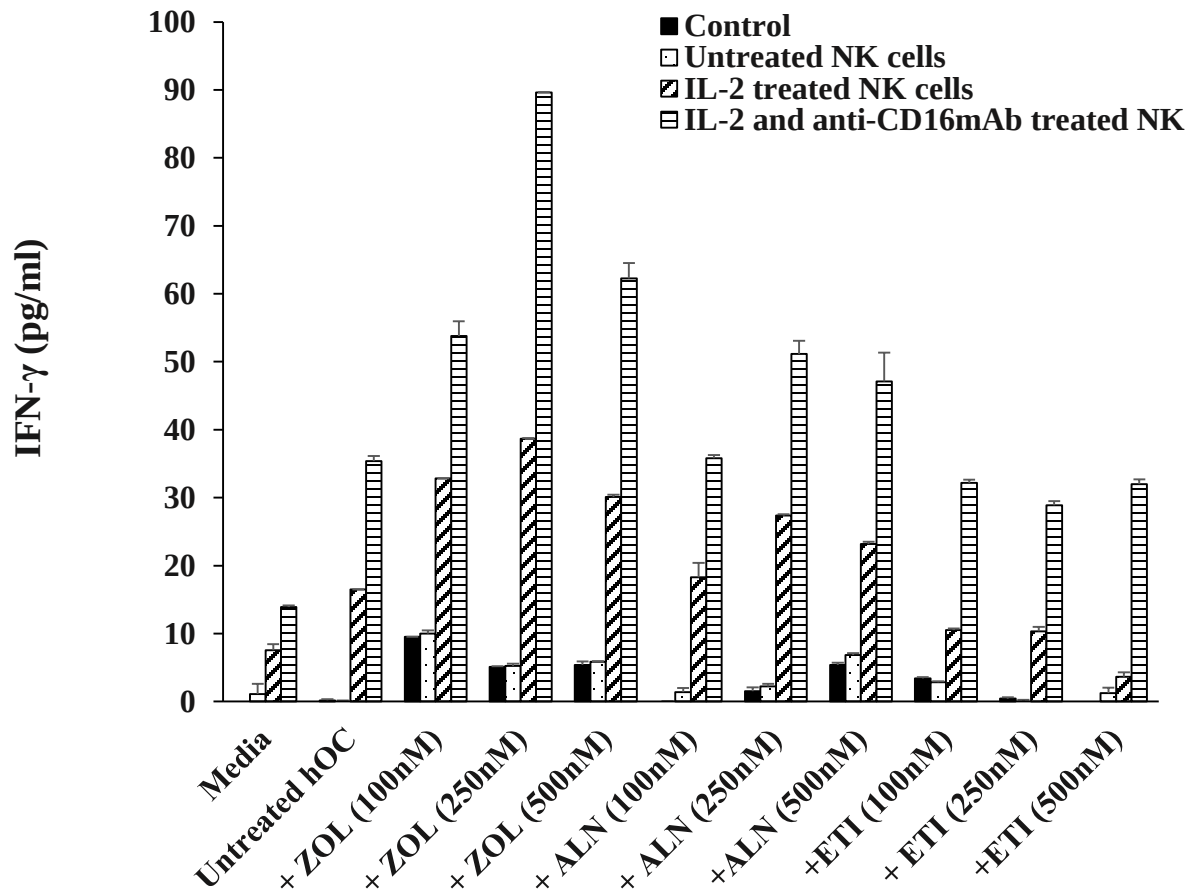


Figure 26: Zolendronic acid treated hOC secrete higher IFN- γ , when co-cultured with IL-2 treated and combination of IL-2 and anti-CD16mAb treated NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 21 days. Osteoclasts at a concentration of 1×10^5 /ml were treated with 100-500nM of nitrogen-containing BP, Zolendronic acid and Alendronate and non-nitrogen containing Etidronate. After 12 hours of treatment, BP were washed off, and fresh media was added. Purified NK cells were left untreated, treated with IL-2 (1000 units/ml) and a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18 hours before the cells were added to autologous monocytes or osteoclasts at an effector to target ratio of 1:2 of NK cells to osteoclasts. After an overnight incubation after co-culture the supernatants were harvested and subjected to a specific ELISA for IFN- γ .

3. Osteoclasts form clumps by NK cells and BP combination.

As we found that osteoclasts cultured with untreated NK, IL-2 treated and IL-2 in combination with anti-CD16 treated NK cells were resulting in significant up-regulation of cytokines and chemokines, and this effect was higher when osteoclasts were pretreated with BP, we were interested to see what happen to osteoclasts structure with this combinations. To determine this, we pre-treated osteoclasts for 3 days, and co-cultured with autologous NK cells, followed by fixing the cells and taking pictures with inverse microscope at 20 X magnification. We found that, without any addition, osteoclasts had proper membrane and ruffled borders, on the other had when we added NK cells and BP or combination of both, osteoclasts lose their membrane and ruffled borders, and this effect was worst in the co-culture with IL-2 and anti-CD16mAb combination treated NK cells, this co-relate with higher cytokine and chemokine in this condition, providing evidence of higher damage to cells or inflammation with this combination (Fig 27A and 27B). These ruffled borders are responsible for the resorptive function of osteoclasts, by losing these osteoclasts are no more functioning properly. With Zolendronic acid and NK cells combination, we found that osteoclast start fusing, followed by distortion of shape and forming clumps, and this was the combination where we saw higher secretion of IFN-gamma, suggesting us that it is possible that Zolendronic acid with NK cells combination is moving osteoclasts to differentiate more in fast speed and ultimately losing their resorptive function.

Figure 27A:

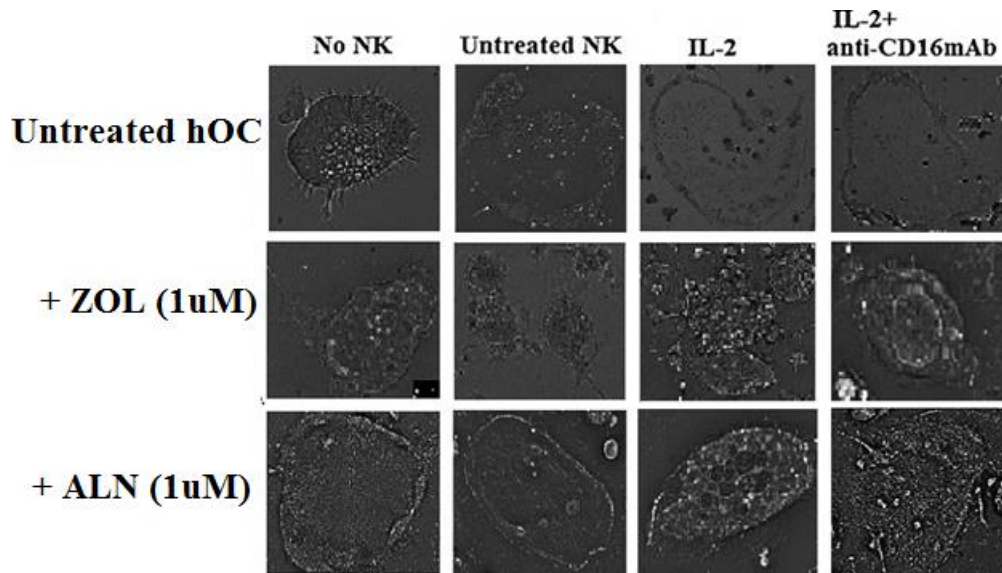


Figure 27A: Morphological feature of Untreated and BP treated osteoclasts with NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 1.5×10^5 /1ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid and Alendronate. After 72 hours of treatment, media was removed, cells were washed. Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to osteoclasts culture. After 12 hours of incubation, all detached cells were washed off and attached cells were fixed with 37 % formaldehyde. Pictures were taken using Leica microscopy at 20X magnification.

Figure 27 B:

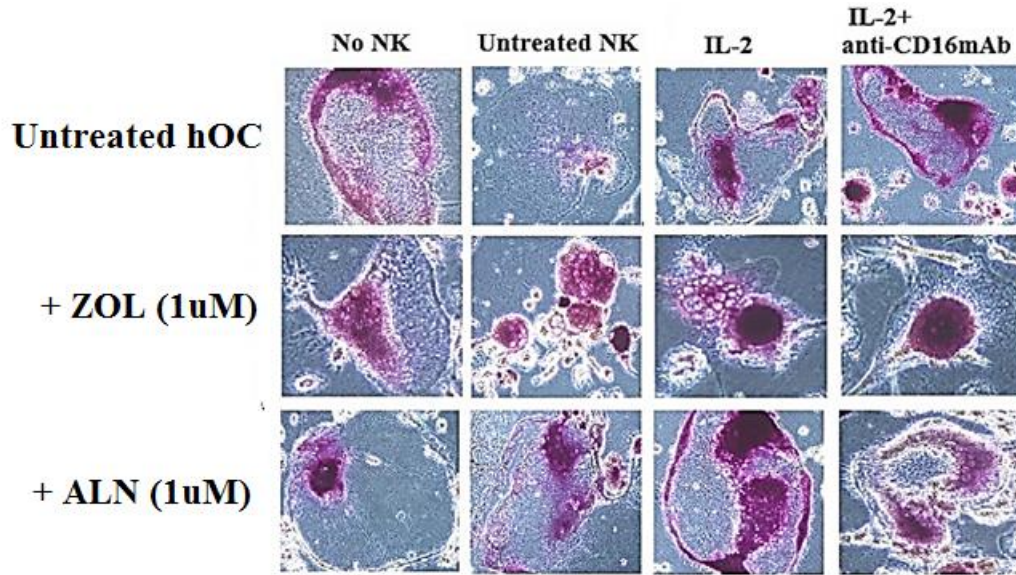


Figure 27 B: Morphological feature of Untreated and BP treated osteoclasts with NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 1.5×10^5 /ml were treated with 1 μ M of nitrogen-containing BP, Zolendronic acid and Alendronate. After 72 hours of treatment, media was removed, cells were washed. Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to osteoclasts culture. After 12 hours of incubation, all detached cells were washed off and attached cells were fixed with 37 % formaldehyde. Pictures were taken using Leica microscopy at 20X magnification. Cells were TRAP stained using Sigma Kit, and Pictures were taken using Leica microscopy at 20X magnification.

4. Zolendronic acid treated Osteoclasts and oral tumor cells are resistant to NK cell mediated cytotoxicity

We then determined the cytotoxic activity of NK cells against bisphosphonate treated osteoclasts and OSCSCs. IL-2 treated NK cells lysed untreated osteoclasts and the treatment with Zolendronic acid induced resistance in osteoclasts against NK cell cytotoxicity. Zolendronic acid at 500 nM caused more resistance to NK cell mediated cytotoxicity than at 1 μ M (Fig. 26B). The resistance to cytotoxicity is also seen with Alendronate but at much lower levels (Fig. 26A). To determine whether the ability to induce resistance in NK cells is specific for osteoclasts, we treated OSCSCs for 15-30 min and used in cytotoxicity assay against NK cells. Treatment of OSCSCs with Zolendronic acid also induced resistance against NK cytotoxicity, as seen in Fig 27A and 27B. The effect of Zolendronic acid treated osteoclasts on NK cells is similar to that induced by IL-2+anti-CD16mAb treated NK cells in which NK cytotoxicity is suppressed whereas there is significant induction of cytokine secretion by the NK cells (split anergy), although in experiments with osteoclasts we never saw lytic units comparable to OSCSCs, lytic units of cytotoxicity against osteoclasts were 1/3 -1/2 depending on donors. To further explore the effect Zolendronic acid on NK cells mediated cytotoxicity we treated the osteoclasts with 100nM-1 μ M of Zolendronic acid, we found that Zolendronic acid at 100nM start inhibiting NK cells cytotoxicity and this effect become more effective upto 250nM (Fig. 28), or in some donors upto 500nM (data not shown), and afterwards it start increasing the NK cells mediated cytotoxicity, but still it never approach to untreated osteoclasts. BPs treatment on NK cells also reduced their cytotoxic function against the osteoclasts (Fig. 29C). When NK cells were treated with IL-2 there was up-regulation of their cytotoxic function, ZA and ALN treatment alone do not have any effect on NK cells cytotoxic function, but when NK cells were treated in combination with IL-2 and ZA there was reduction in

cytotoxic function of NK cells against the osteoclasts, although the fold decrease was not much significant. ALN in combination with IL-2 cause not much effect on cytotoxic function of NK cells (Fig. 29C). To summarize, we evaluated that Zolendronic acid is suppressing the NK cells mediated cytotoxicity against the OSCSCs and osteoclasts, Alendronate suppress NK cells mediated cytotoxicity to some extent at low concentration, but Etidronate has almost no effect on NK cells mediated cytotoxicity against the osteoclasts.

Figure 28:

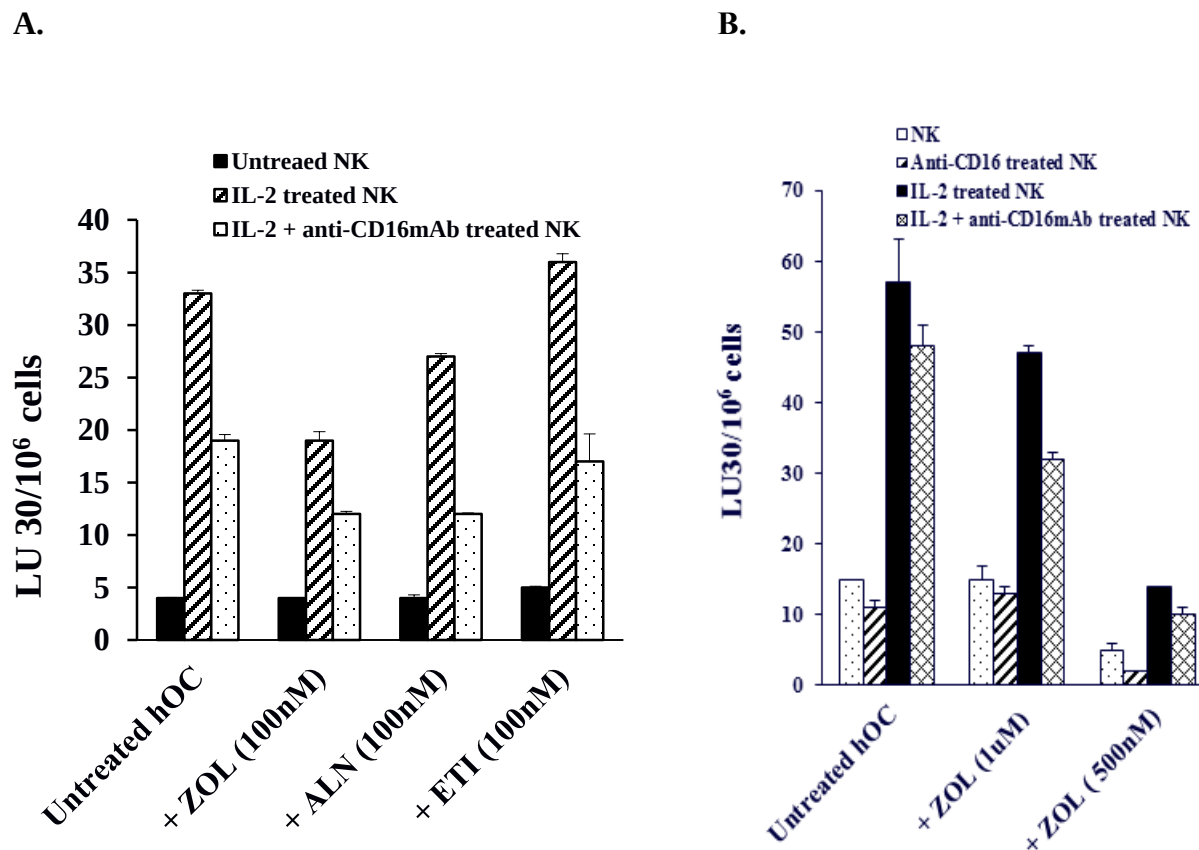
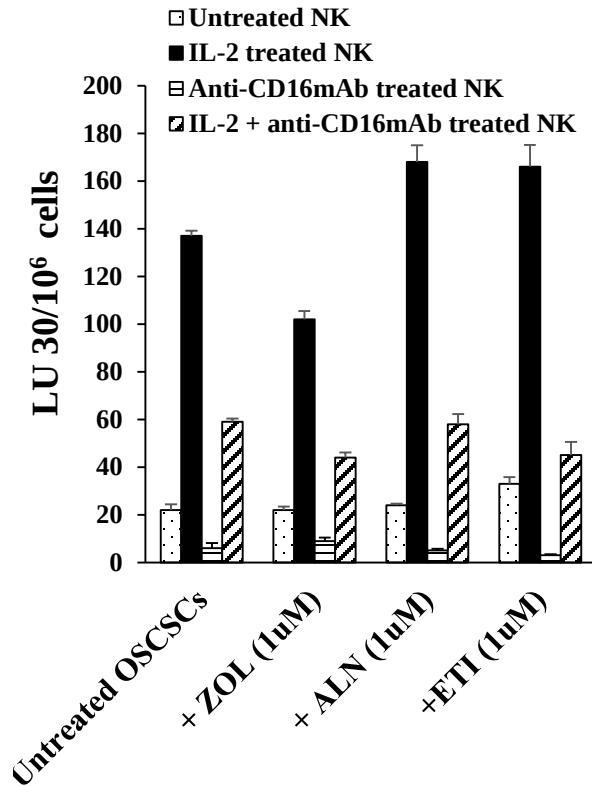


Fig. 28: Zolendronic acid treated osteoclasts are more resistant to NK cells mediated cytotoxicity.

Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml), treated with anti-CD16mAb (3 ug/ml), and with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours before they were added to ^{51}Cr labeled osteoclasts at various effector to target ratios. Osteoclasts were pre-treated with nitrogen-containing BP, Zolendronic acid and Alendronate for 3 days. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. Zolendronic acid treated osteoclasts were more resistant to NK cell mediated cytotoxicity (A), and more resistant at lower concentration of Zolendronic acid (B).

Figure 29:

A.



B.

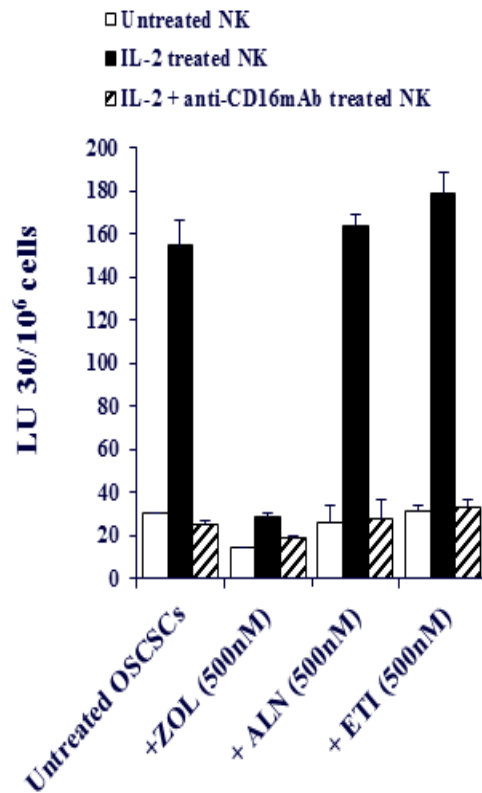


Fig. 29: Zolendronic acid treated OSCSCs are more resistant to NK cells mediated cytotoxicity.

Highly purified NK cells (1×10^6 cells/ml) were left untreated, treated with IL-2 (1000 units/ml), treated with anti-CD16mAb (3 μ g/ml), and with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 μ g/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs at various effector to target ratios. OSCSCs were pre-treated with nitrogen-containing BP, Zolendronic acid and Alendronate and non-nitrogen containing BP, Etidronate for 30 mins at $1\mu\text{M}$ (A) and 500nM (B) concentration. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. Zolendronic acid treated OSCSCs were more resistant to NK cell mediated cytotoxicity.

Figure 29C:

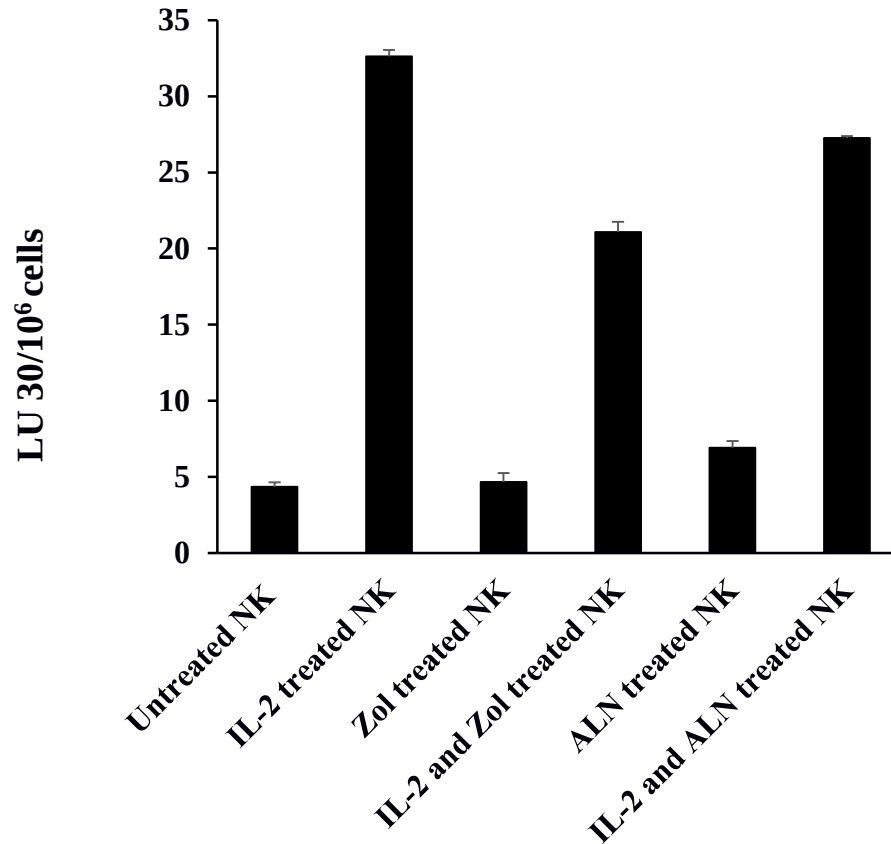


Figure 29C: BP treated NK cells exhibit lower cytotoxicity against tumors as compared with IL treated NK cells.

Highly purified NK cells (1×10^6 cells/ml) were left untreated or treated with IL-2 (1000 units/ml) alone or a combination of IL-2 (1000 units/ml) and ZA (500nM) or ALN (500nM) for 18 hours before they were added to ^{51}Cr labeled osteoclasts (hOC) at various effector to target ratios. NK cell mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of the osteoclasts X100.

5. BPs inhibit IL-10 secretion by Osteoclasts, addition of IL-10 reduce inflammatory cytokines, that inhibition was blocked by anti-IL-10.

Zolendronic acid has the inhibitory effect on IL-10 secretion of osteoclasts, and Zolendronic acid treatment induced the secretion of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, IFN- γ , TNF- α and IL-1 β . Recombinant IL-10, has shown to have inhibitory effect on the cytokines secretion, as we showed in our background material. To determine the cytokine inhibitory effect of rh-IL-10 on our cells we treated osteoclasts with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10. Untreated osteoclasts were also treated with rh-IL-10. Purified NK cells were left untreated and rh-IL-10 treated, treated with IL-2 with/without rh-IL-10 and a combination of IL-2 and anti-CD16mAb with/without rh-IL-10. We used 2 ng/ml concentration of rh-IL-10, and found the inhibition of cytokines induced by Zolendronic acid treatment or by NK cells on the osteoclasts. This cytokine inhibitory of rh-IL-10 become more dominating after the day 5 of the treatment, there was very low or no inhibition 24 hours after treatment. There was almost 50-80% reduction of IFN- γ secretion on Zolendronic acid treated osteoclasts (Fig. 30), and 30-50% reduction of IFN- γ on untreated osteoclasts (Fig. 30). We also analyzed IL-8 (Fig. 31) and IL-6 (Fig. 32) on Zolendronic acid and rh-IL-10 treated, and found that rh-IL-10 inhibited the secretion of both IL-8 and IL-6, although extent of IL-8 inhibition was less comparatively to IFN- γ and IL-6 inhibition on Zolendronic acid treated osteoclasts. To further confirm that this inhibition of cytokines was due to rh-IL-10, we treated rh-IL-10 treated cells with anti-IL-10, as expected the inhibitory effect of rh-IL-10 was reversed, although it did not reached upto the level of cytokines induced by anti-IL-10 alone (Fig 33-34).

Figure 30:

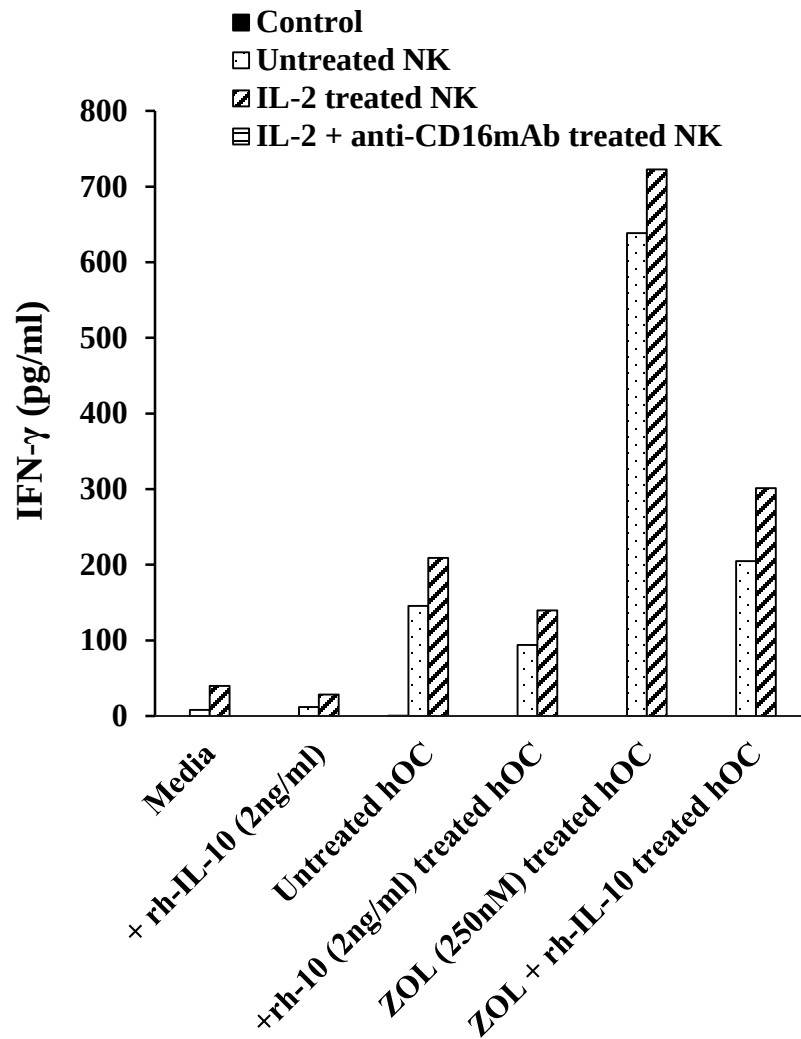


Figure 30: rh-IL-10 reduce the cytokine secretion induced by Zolendronic acid treated osteoclasts by NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 2×10^5 /1ml were treated with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10 (2ng/ml). Highly purified NK cells (1×10^6 cells/ml) were left untreated without rh-IL-10 (2ng/ml) treated, treated with IL-2 (1000 units/ml) with/without rh-IL-10 (2ng/ml), treated with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) with/without rh-IL-10 (2ng/ml) for 18 hours, before they were added to osteoclasts culture. Supernatant was harvested on day 5 after culture and subjected to specific ELISA for IFN- γ .

Figure 31:

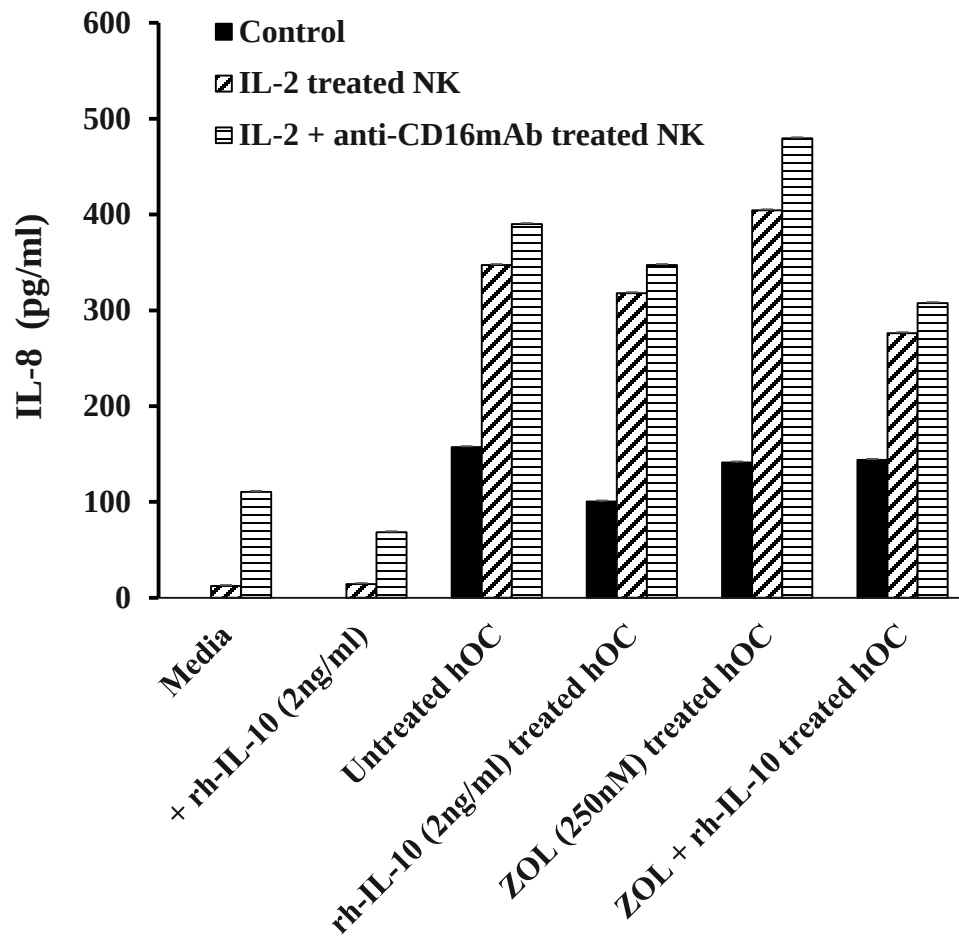


Figure 31: rh-IL-10 reduce the cytokine secretion induced by Zolendronic acid treated osteoclasts by NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 2×10^5 /1ml were treated with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10 (2ng/ml). Highly purified NK cells (1×10^6 cells/ml) were left untreated without rh-IL-10 (2ng/ml) treated, treated with IL-2 (1000 units/ml) with/without rh-IL-10 (2ng/ml), treated with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) with/without rh-IL-10 (2ng/ml) for 18 hours, before they were added to osteoclasts culture. Supernatant was harvested on day 5 after culture and subjected to specific ELISA for IL-8

Figure 32:

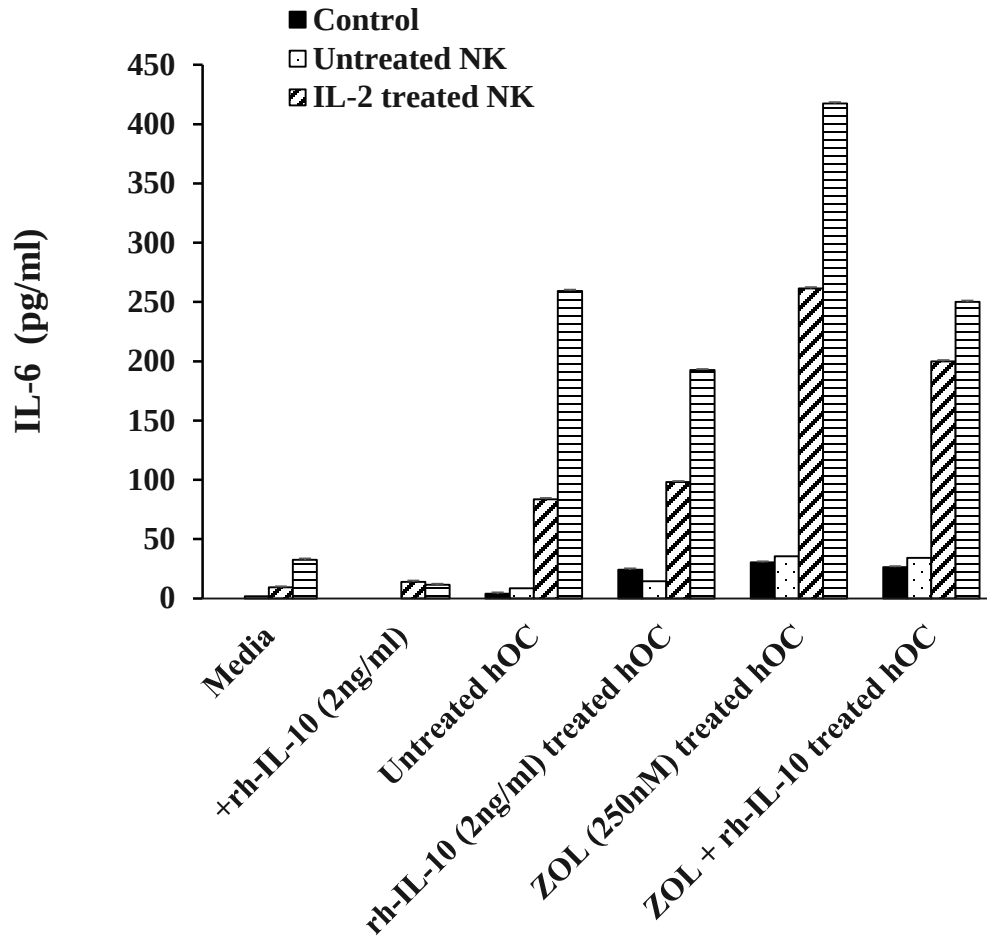


Figure 32: rh-IL-10 reduce the cytokine secretion induced by Zolendronic acid treated osteoclasts by NK cells.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 2×10^5 /ml were treated with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10 (2ng/ml). Highly purified NK cells (1×10^6 cells/ml) were left untreated without rh-IL-10 (2ng/ml) treated, treated with IL-2 (1000 units/ml) with/without rh-IL-10 (2ng/ml), treated with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) with/without rh-IL-10 (2ng/ml) for 18 hours, before they were added to osteoclasts culture. Supernatant was harvested on day 5 after culture and subjected to specific ELISA for IL-6.

Figure 33:

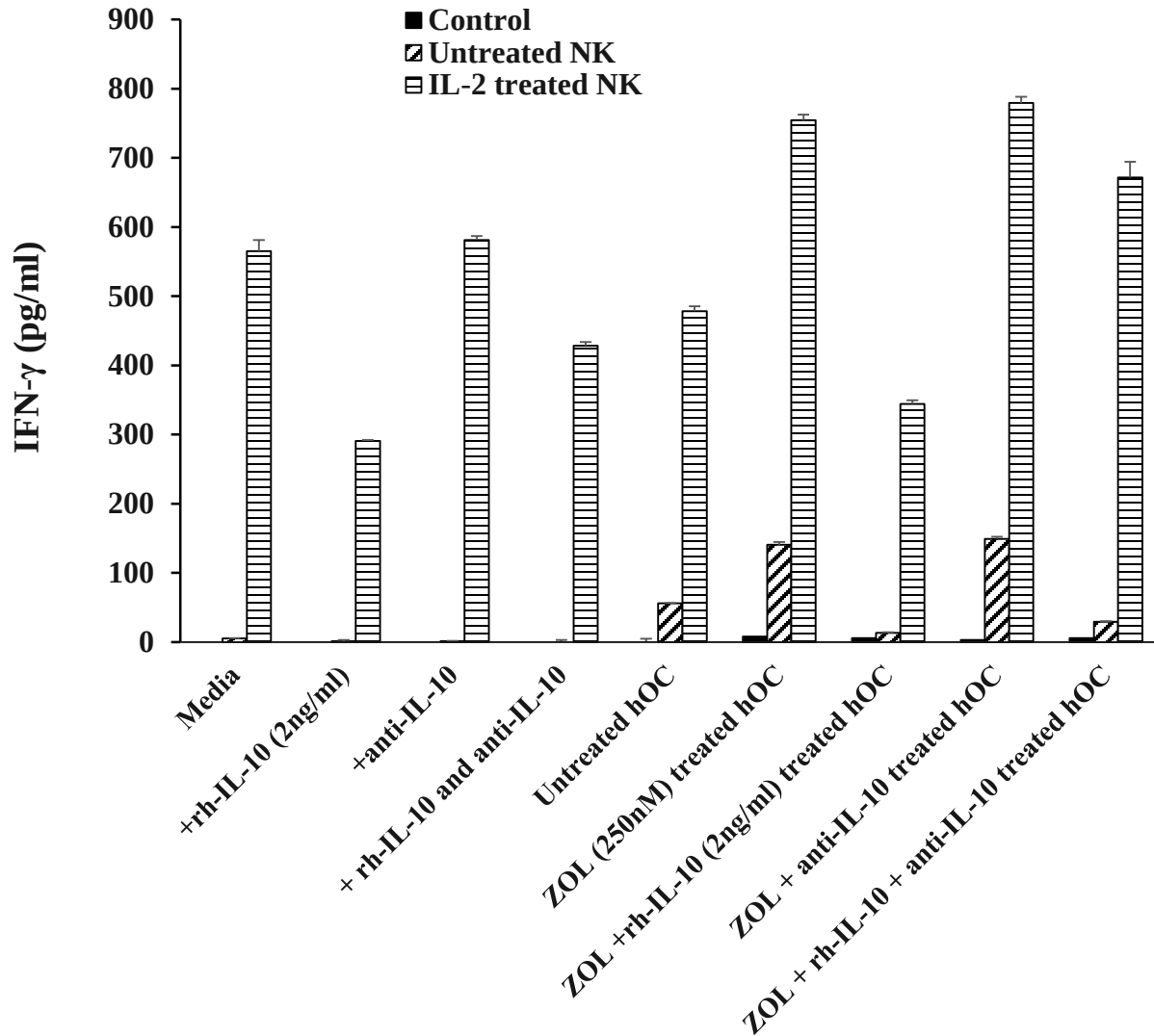


Figure 33: anti-IL-10 reversed the cytokine inhibitory effect of rh-IL-10 on osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 2×10^5 /ml were treated with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10 (2ng/ml) and also with/without anti-IL-10. Highly purified NK cells (1×10^6 cells/ml) were left untreated with/without rh-IL-10 (2ng/ml) treated and with/without anti-IL-10, treated with IL-2 (1000 units/ml) with/without rh-IL-10 (2ng/ml) and with/without anti-IL-10, treated with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) with/without rh-IL-10 (2ng/ml) and with/without anti-IL-10, for 18 hours, before they were added to osteoclasts culture. Day 5, after culture, supernatant was harvested and subjected to specific ELISA for IFN- γ .

Figure 34:

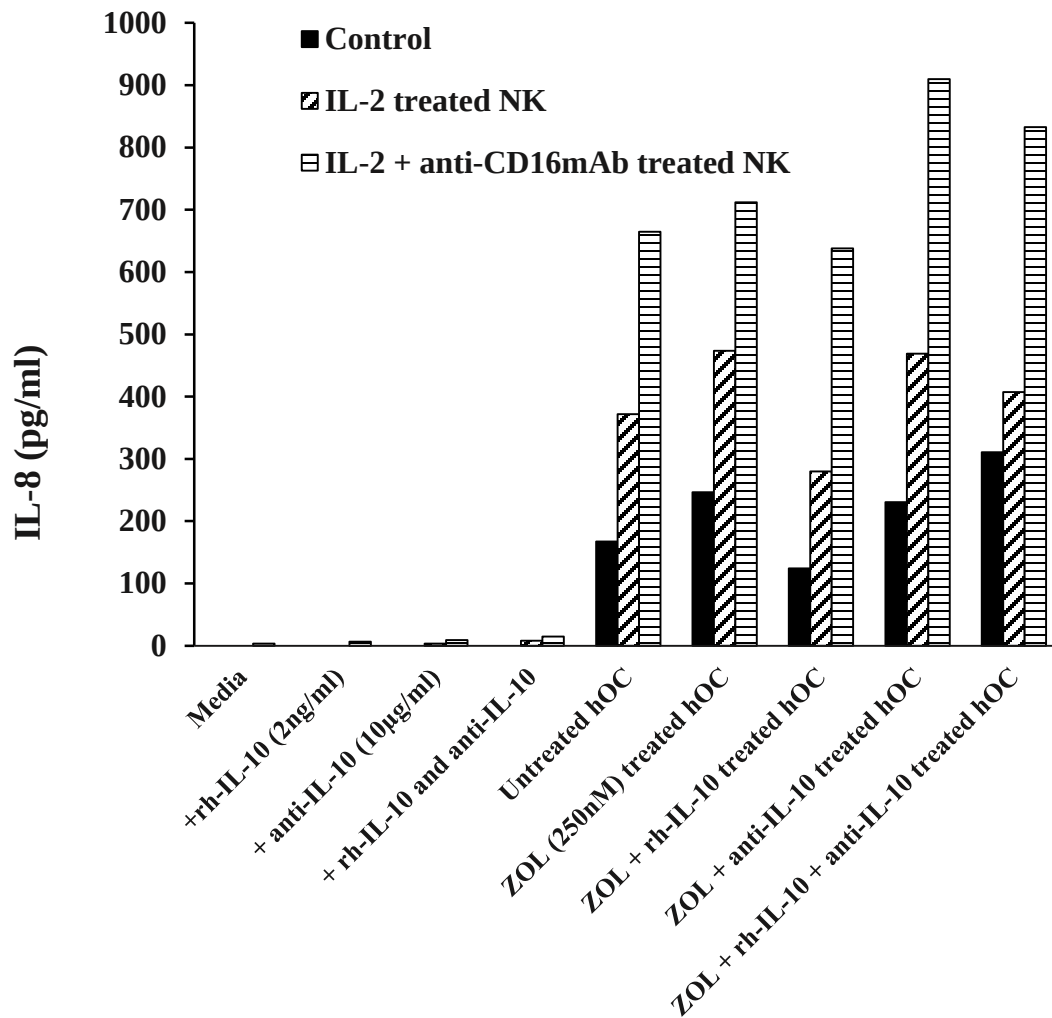


Figure 34: Anti-IL-10 reversed the cytokine inhibitory effect of rh-IL-10 on osteoclasts.

Osteoclasts were generated from monocytes and cultured in medium containing M-CSF (25ng/mL) and RANKL (25ng/mL) for 17 days. Osteoclasts at a concentration of 2×10^5 /1ml were treated with 250nM of nitrogen-containing BP, Zolendronic acid with/without rh-IL-10 (2ng/ml) and also with/without anti-IL-10. Highly purified NK cells (1×10^6 cells/ml) were treated with IL-2 (1000 units/ml) with/without rh-IL-10 (2ng/ml) and with/without anti-IL-10, treated with combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) with/without rh-IL-10 (2ng/ml) and with/without anti-IL-10, for 18 hours, before they were added to osteoclasts culture. Day 5, after culture, supernatant was harvested and subjected to specific ELISA for IL-8.

DISCUSSION

Previous studies in our lab, determined that the stage of maturation and differentiation of healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have shown that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [112]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [112]. Based on these results, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact (Tseng et al, “in press”). In addition, we have shown previously that CD14+HLADR- monocytes can condition NK cells to lose cytotoxicity and gain the ability to secrete inflammatory cytokines [103, 184-187]. The signals received from the stem cells or monocytes alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function is found to ultimately aid in driving differentiation of surviving, healthy, as well as transformed stem cells [103, 184-187]. Differentiation of stem cells and their resistance to NK cell mediated cytotoxicity correlated with significant increase in the expression of MHC class I, CD54, B7H1 surface expression in a number of healthy and tumor stem cell models, and it was blocked by the addition of the combination of anti-TNF- α and anti-IFN- γ which restored NK cell cytotoxicity and blocked the increased expression of above-mentioned surface markers in addition to inhibition of cytokine and chemokine secretion (manuscript submitted). Since Zolendronic acid increased MHC class I, CD54 and B7H1 on osteoclasts we reasoned that it may behave as a differentiation agent capable

of decreasing NK cell mediated cytotoxicity. Indeed, treatment of osteoclasts with Zolendronic acid and much less with Alendronate was able to inhibit NK cell cytotoxicity significantly. In addition, NK cells were able to lyse osteoclasts much more than freshly isolated monocytes and this correlated with the decreased expression of MHC class I and CD54 expression on osteoclasts.

In contrast to the decrease in cytotoxicity, Zolendronic acid mediated dose dependent increase in cytokine secretion such as TNF- α and IFN- γ in the co-cultures of NK cells with osteoclasts. As mentioned above, TNF- α and IFN- γ secreted by the NK cells synergistically increase differentiation of stem cells resulting in increase in MHC class I, CD54 and B7H1 and their resistance to NK cell mediated cytotoxicity and decrease in cytokine and chemokine secretion by the NK cells cultured with differentiated OSCSCs (Tseng et al, “in press”). It is interesting to note that Osteoclasts express lower levels of MHC class I and II and resist increase in MHC class I surface expression when either treated with the combination of TNF- α and IFN- γ or with activated NK supernatants known to increase maximally MHC class I and II. This may be one reason why Osteoclasts are found to be the best targets for the expansion of fully functional NK cells under the optimized conditions of NK cell stimulation, as shown in this paper. Indeed, such observation is of outmost importance since this strategy may be used to expand NK cells for delivery to cancer patients. Osteoclast expanded NK cells not only exhibited high cytotoxic activity but also they mediated significant secretion of IFN- γ when compared to DC and monocyte stimulated NK cells. Osteoclast expanded NK cells responded to IL-2 activation and substantially increased IFN- γ secretion per cell basis when compared to DCs or monocytes. Interestingly, the addition of Zolendronic acid was found to increase MHC class I on Osteoclasts, and the mechanism underlying such increase by Zolendronic acid is presently unknown. However, decrease in NK cell cytotoxicity by Zolendronic acid is observed after 30 minutes treatment of Osteoclasts or tumor

cells with Zolendronic acid which is very fast and may not relate to the function of MHC class I. Therefore, Zolendronic acid may have a direct effect on inducing resistance of cells to NK cell mediated cytotoxicity. Thus, these results suggest that Zolendronic acid treated osteoclasts may remain viable in the microenvironment for a prolonged period of time and continuously trigger high levels of cytokines and chemokines resulting in the chronicity of inflammation. Indeed, under the conditions where supernatants from the NK cells induce differentiation in stem cells such as DPSCs or OSCSCs, there is a significant inhibition of both cytotoxicity and cytokine and chemokine secretion, however, in the presence of Zolendronic acid, even though NK cell cytotoxicity is inhibited, cytokine secretion continues at a high level which may be the reason for the adverse effects mediated by Zolendronic acid. In addition, Zolendronic acid treated osteoclasts may survive longer and provide continuous NK cell stimulation by the increased production and synergistic functions of NK activating cytokines such as IL-18, IL-15, IL-12 and IFN- γ which we have shown to be secreted by the osteoclasts. This possibility is under investigation in our laboratory and is the subject of a future report. Both Zolendronic acid and Alendronate but not Etidronate are able to increase cytokine and chemokine secretion by the NK cells in the co-cultures of NK cells with osteoclasts.

CONCLUSION

NK cells were able to lyse osteoclasts, and osteoclasts can be better immune cells as compared to the monocytes and Dendritic cells, as they were able to secrete more pro-inflammatory cytokines. Osteoclasts induced higher cytokines and chemokines as compared to monocytes when interact with NK cells, are more susceptible to NK cells media cytotoxicity. Zolendronic acid treated osteoclasts triggered significantly higher induction of cytokines and chemokines in the co-cultures with NK cells, and the effect was higher when compared to Alendronate treated Osteoclasts, whereas Etidronate had no enhancing effect. Osteoclasts triggered cytokine and chemokine, like IL-6, IFN-g, IL-10, IL-8, MCP-1, MIP-1a and MIP-1b secretion significantly more than monocytes. When monocytes were treated with Zolendronic acid, there was induction of IFN-gamma on Zolendronic acid treated monocytes by NK cells, and the level of IFN- γ reached to the induction level of untreated osteoclast with NK cells. Zolendronic acid induced resistance in osteoclasts and OSCSCs, against NK cell cytotoxicity. The resistance to cytotoxicity is also seen with Alendronate but at much lower levels. Bisphosphonates especially Zolendronic acid has the inhibitory effect on IL-10 secretion of osteoclasts, and there was reduction of IFN- γ on Zolendronic acid treated osteoclasts by rh-IL-10, inhibitory effect of rh-IL-10 was reversed by anti-IL-10.

CHAPTER 4

To investigate the effect of Zolendronic acid in different tissue compartments in Zolendronic acid injected and NACL injected B6 WT mice.

ABSTRACT

Our *in-vivo* data showed, Zolendronic acid injections could increase IFN-g and IL-6 secretion and Cytotoxicity against the tumor cells, in the bone marrows but decrease the secretion of IFN-g in gingival, pancreatic and adipose tissues and IL-6 in gingival tissues. BM derived NK cells have more cytotoxic function and cytokines secretion after Zolendronic acid injections. Surface expressions of NK cells, T cells, B cells etc. did not changed within the Zolendronic acid and NACL group on the examined tissues. It is possible that an increase in activation of Bone Marrow derived cells by Zolendronic acid result in the eventual loss or inhibition of IFN-g secreting cells when they reach to the tissues. Since gingival derived immune cells are of activated phenotype when compared to bone marrow derived immune cells in healthy subjects, prior activation of bone marrow derived cells when mobilized to the gingival tissues and are exposed to additional activation signals from antigens in gingiva may undergo activation induced cell death, or simply become exhausted, and result in the lower secretion of IFN-g by the immune cells as seen in our *in vivo* model system.

BACKGROUND AND INTRODUCTION

Once we examined the *in-vitro* effect of BP, we were more interested to see the effect of Zolendronic acid *in-vivo*. To explore this, we injected the B6 WT with ZA and NACL. Osteonecrosis of jaw after ZA injection was determined by histological studies. Osteonecrosis of the jaw (ONJ) is an emerging condition in patients undergoing long-term administration of bisphosphonates (BP) for the treatment of osteoporosis and hypercalcaemia associated with malignancy, multiple myeloma, and metastatic breast and prostate cancers [188]. Bisphosphonates are the potent inhibitors of osteoclasts and are beneficial for the treatment of bone metastasis. BP are widely and successfully used in treatment of skeleton diseases such as Paget disease, postmenopausal osteoporosis and tumor bone diseases [189]. Low concentration of Zolendronic acid rapidly and directly affected the oral mucosa tissue through the induction of gene regulated apoptotic process, supporting the potential for soft tissue injury as an initiating/potentiating event for osteonecrosis [190]. Lung metastases were absent in all Zol-treated mice that survived during the study period [191].

ZOL induces growth inhibition and apoptosis on pancreatic cancer cell lines and interferes with growth and survival pathways downstream to p21(ras), suggesting that BPs can be used cancer treatment [192]. Zoledronic acid has a cytotoxic potential even at pharmacologic dosage. Zoledronic acid does not only induce apoptosis by inhibiting the Ras-pathway but has also an anti-metastatic effect as shown in migration assays and by down-regulation of urokinase plasminogen activator. Freshly prepared gammadelta T cells consisting mainly of Vdelta2 cells showed increased cytotoxicity against bisphosphonate-treated pancreatic carcinoma cells [193]. Pancreatic cancer, PC cells are highly sensitive to ZOL-induced growth perturbation and induction of apoptosis, which is caspase-9- caspase-6- and PARP-dependent. Inhibition of p21^{ras}/Raf-

1/MEK1/ERK signalling as well as PK-B/Akt inhibition might be relevant for the antitumour effects of ZOL [192]. ZOL and gemcitabine GEM, when used in combination, have significant antitumor, anti-metastatic and anti-angiogenic effects on pancreatic cancer cells [194].

ZOL interfered with myeloma Bone Marrow Stromal Cells (BMSCs) by reducing proliferation, increasing apoptosis, and modifying the pattern of expression of adhesion molecules, especially those involved in plasma cell binding. These effects on BMSCs might explain the antitumor activity of ZOL [195]. Bisphosphonates (Bps) interfere as well with bone microenvironment inhibiting the survival of stromal cells and hampering the contact between plasma and stromal cells [196].

There is increased risk of ONJ with obesity [197]. Obesity has been linked to significant increases in systemic markers of inflammation such as c-reactive protein and circulating inflammatory cytokines such as leptin, IL-6, and TNF- α [198]. It is possible that amino-bisphosphonates could contribute to the pathogenesis of ONJ by regulating macrophage responses to cytokines such as leptin and IL-6. Inhibition of SOCS3 by ZA resulted in significant increases in IL-6 production [199]. Suppressor of Cytokine Signaling-3 (SOCS3), a member of the SOCS family of proteins, has multiple domain-specific functions that include inhibition of Janus kinase (Jak) activity, competition with signal transducer and activator of transcription (Stat) proteins, regulation of protein degradation, and suppression of cytokine signaling [200].

Exposure of prostate cancer, PC3 cells to various concentrations of ZA resulted in induction of apoptosis and autophagy. The expression of inflammatory biomarkers including interleukin 6 (IL-6), cyclooxygenase-2 (COX-2), and NF- κ B was remarkably upregulated in response to ZA treatment in a dose- and time-dependent manner. The anti-apoptotic protein Bcl2

was increased with parallel increased level of IL-6, suggesting that treatment with low concentrations of ZA enhances the inflammatory profile and may serve as a prosurvival signaling pathway in PC3 cells [201].

Our studies showed that there was increased cytokine secretion and cytotoxic function in BM and BM derived NK cells after ZA injection, but decreased cytokine secretion in other tissue compartments examined. We also analyzed the surface markers of NK cells, T cells, gamma-delta T cells, and B cells in various tissue compartments, but there was not much difference among ZA and NACL group. We have three sub-aims for this study.

Sub-Aim 1: Injection of Zolendronic acid has not much effect on the surface markers of BM derived cells and gingival cells.

Sub-Aim 2: Injection of Zolendronic acid in animals increased the amounts of IFN-g and IL-6 secretion by Bone Marrow cells, but inhibited those from gingival, pancreas and adipose cells.

Sub-Aim 3: Zolendronic acid injections increased the BM derived NK mediated cytotoxicity against the tumor cells, whereas no differences could be seen in other tissues examined.

MATERIAL AND METHODS

A. In vivo injection of Zolendronic acid or NACL in mice

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days as described previously. All mice were euthanized 2 weeks after tooth extraction. After euthanasia, gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals and they were digested using collagenase II (gingival tissue and peri-pancreatic fat) and collagenase IV (pancreas) (Invitrogen, CA) and DNase (Sigma-Aldrich, CA). Femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted and cultured in the presence and absence of IL-2 treatment using RPMI 1640 media (Life technologies, CA). Single cells released from the gingival tissues, peri-pancreatic fat and pancreas, were suspended in media and cultured in the presence of IL-2.

B. Cell culture and reagents

Bone Marrow, Gingiva, Pancreas, peri-pancreatic fat and BM derived NK cells.

NK purification kit was purchased from stem cell Technologies. NK cells from BM were purified with negative selection. More than 91% purity was analyzed within the lymphocyte gate using PE-CD49b subset. RPMI 1640 was supplemented with 10% FBS (Gemini Bio-Products, CA), 5 % antibiotic antimycotic, 1% sodium pyruvate, and 1% non-essential amino acids (Invitrogen by Life Technologies, CA) was used to cultured BM, gingiva, pancreas, peri-pancreatic fat and BM derived NK cells. Rh-IL-2 (10000 U/ml) was used to activate the cells.

C. Antibodies

ELISA kits were purchased from Biogends, CA. ZA was purchased from UCLA Ronald Reagan Pharmacy. PE conjugated IgG1 and 2b, PE-CD3, PE-CD19, PE-CD49b, PE-f4/80 anti-mouse antibodies were purchased from Biolegend, CA.

D. Enzyme-Linked Immunosorbent Assays (ELISAs) and Multiplex Cytokine Arrays

Enzyme-linked Immunoassorbent Assays (ELISAs) especially for IFN- γ and IL-6 (Biolegends, CA) were performed on the harvested supernatant after to measure the concentration levels of cytokines, as described by manufacturer. To analyze and obtain the cytokine concentration, a standard curve was generated by either two or three fold dilution of recombinant cytokines. To perform single ELISA plates were coated with recommended amount of capture antibodies and incubated overnight. After overnight incubation, plates were washed three or four times, and antibodies were blocked with ELISA PBS containing 1% BSA for 1 hour. Thereafter plates were washed three or four times, and standards and samples were added in respective wells. Plates were incubated for 2 hours, were washed and added detection antibody. 1 hour after another incubation, added Avidin-HRP and continued incubation for next 30 mins or 1 hour. Finally the plates were washed, and incubated with alkaline phosphate substrate (Sigma Chemical Co. 104), reaction was stopped with stop solution, 2N H₂SO₄. Readings of plated were done with ELISA reader using 405nm or 450nm filter.

E. Surface Staining

Staining was performed by coating the cells with the antibodies. The cells were washed twice with ice-cold PBS containing 1% BSA. Predetermined optimal concentrations of specific human monoclonal antibodies were added to 1×10^4 cells in 50 μ l of cold-BSA and cells were incubated on ice for 30 min. Thereafter cells were washed in cold PBS-BSA and brought upto 500 μ l with PBS-BSA. An Epics C (Coulter) flow cytometry was used for analysis.

F. ^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [183]. Briefly, different numbers of purified NK cells were incubated with ^{51}Cr -labeled tumor target cells. After a 4 hour incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

G. Statistical Analysis

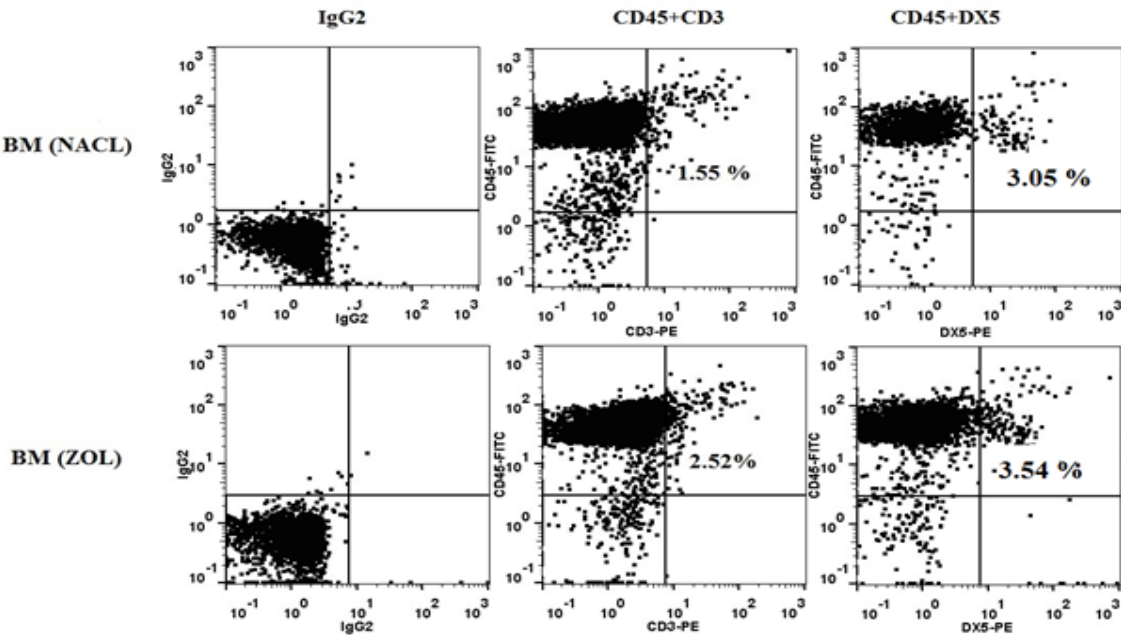
An unpaired, two-tailed student t- test was performed for the statistical analysis. One way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS

1. Zolendronic acid injections has not much significant effect on the surface markers of the BM and Gingival tissues.

When cell surface receptor expression was compared between ZA and NACL injected animals in Bone Marrow derived cells and gingival cells for NK cells, T cells, B cells and monocytes no significant difference was observed, but minor increase CD3T cells surface marker was observed Bone Marrow derived cells (Fig 35 A), but effect was opposite in case on gingival tissue. NACL injected gingival tissue has higher percentage of CD3T positive cells (Fig. 35B).

Figure 35: A.



B.

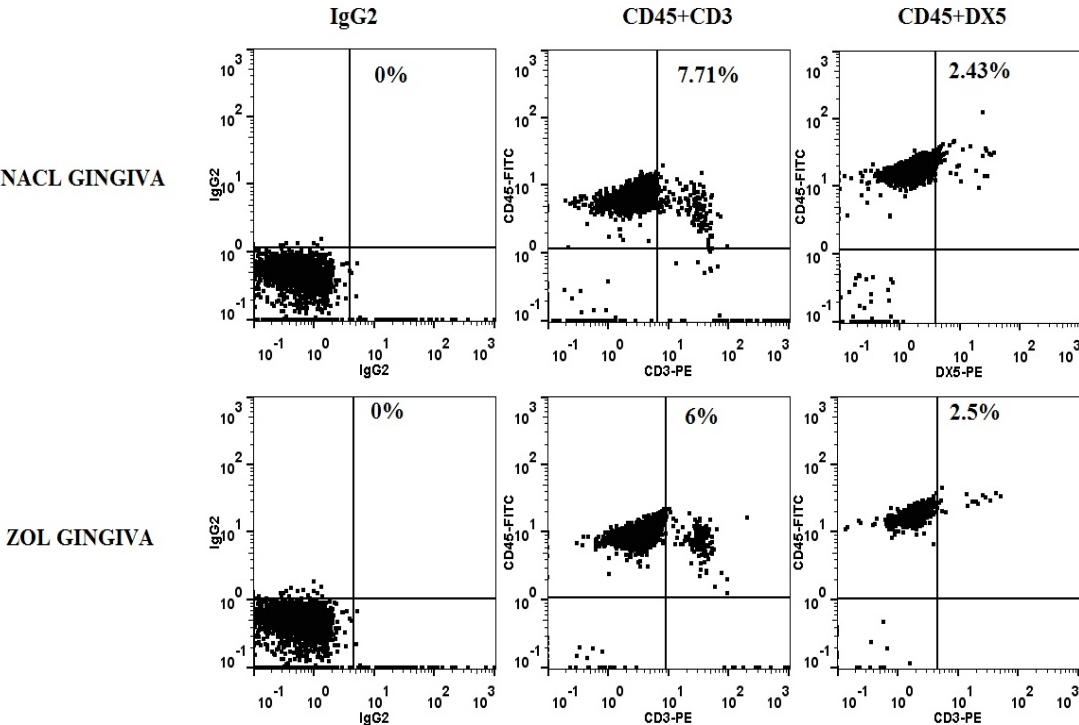


Figure 35: Surface Expression of Gingiva after Zolendronic acid and NACL injections

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, gingival tissues were obtained from Zolendronic acid and NACL injected animals and they were digested as described in materials and method. Femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted. Afterwards, the surface expression of CD45-FITC, CD45-FITC+CD3-PE, CD45-FITC+DX5-PE, were assessed with flow cytometric analysis after staining with the respective PE and FITC-conjugated antibodies. Isotype control antibodies were used as control. Percentage of CD3 and DX5 within the CD45 population was calculated for BM (A) and Gingiva (B).

2. Zolendronic acid stimulated significant IFN-g and IL-6 secretion by bone marrow derived cells but inhibited secretion by gingival cells.

Bone Marrow cells and cells obtained from gingival tissues after injection of Zolendronic acid in vivo were left untreated or treated with IL-2 and cultured for 5 days before the levels of IFN-g and IL-6 secretion were assessed. As shown in Fig. 36 and Fig. 37, injection of Zolendronic acid in animals increased the amounts of IFN-g and IL-6 secretion by Bone Marrow cells (Fig. 36B and Fig. 37A) but inhibited those from gingival cells (Fig. 36A and Fig. 37B). Conversely, NACL injected animals had higher levels of IFN-g and IL-6 secretion in gingival associated cells treated with IL-2 but lower induction with IL-2 treated Bone Marrow cells when compared to Zolendronic acid treated animals (Fig. 36A and Fig. 37B). Similarly, IL-2 treated dissociated pancreatic cells and adipose tissue from Zolendronic acid injected animals had lower secretion of IFN-g when compared to NACL injected animals (Fig. 36A), but lower secretion of IL-6 secretion (Fig. 37C). Thus, Zolendronic acid induced IFN-g and IL-6 secretion in different tissue compartments are distinct depending on the location and type of cells.

Figure 36:

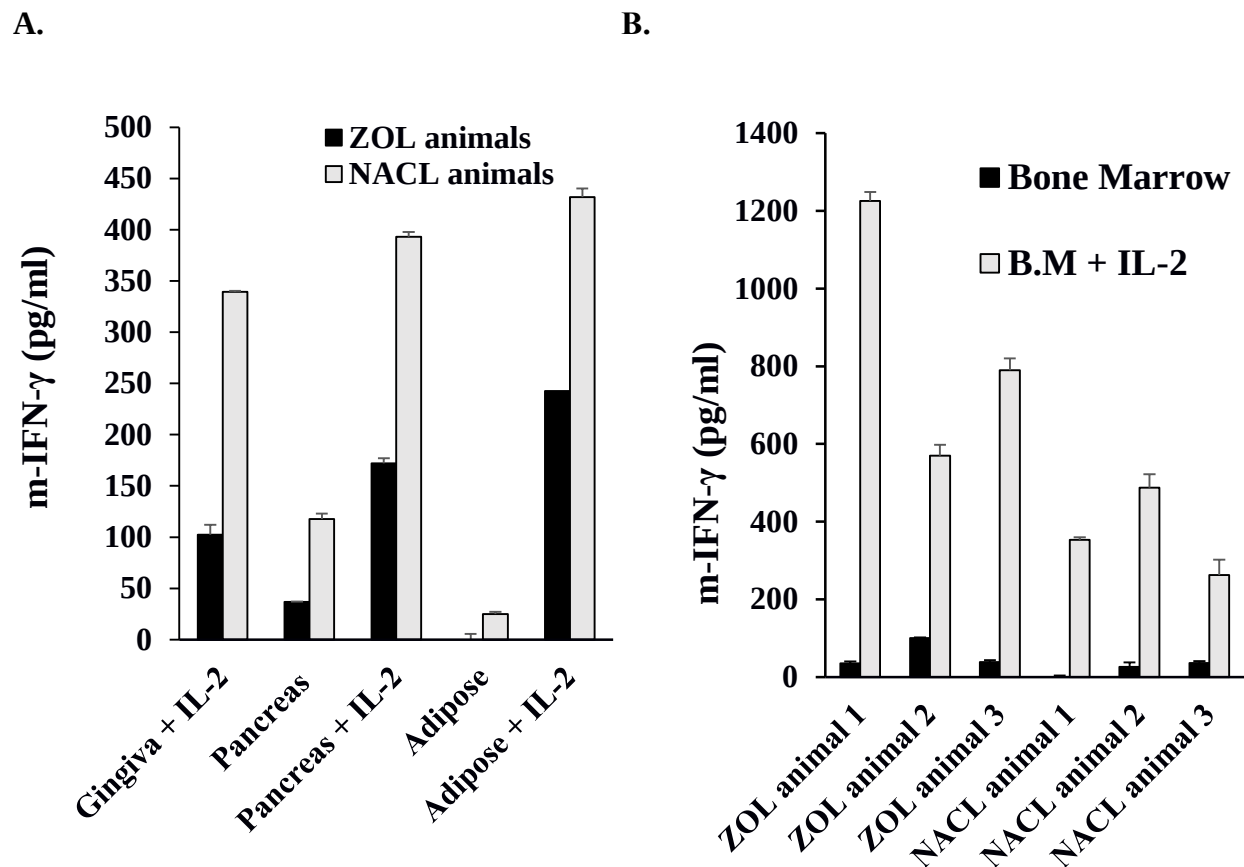


Figure 36: Zolendronic acid stimulated significant IFN-g secretion by bone marrow derived cells but inhibited secretion by gingival cells.

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals and they were digested as described in materials and method. Femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted and cultured (1×10^6 /ml) in the presence and absence of IL-2 (10,000 U/ml) treatment in 12 well culture plate. Single cell released from gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals were cultured in the presence and absence of IL-2 (10,000 U/ml) in 24 well culture plate. Supernatants were harvested on Day 5 after culture of Bone Marrow (Fig. B), gingival tissue, adipose and pancreas (Fig. A) and the levels of IFN-γ were measured with specific ELISA.

Figure 37:

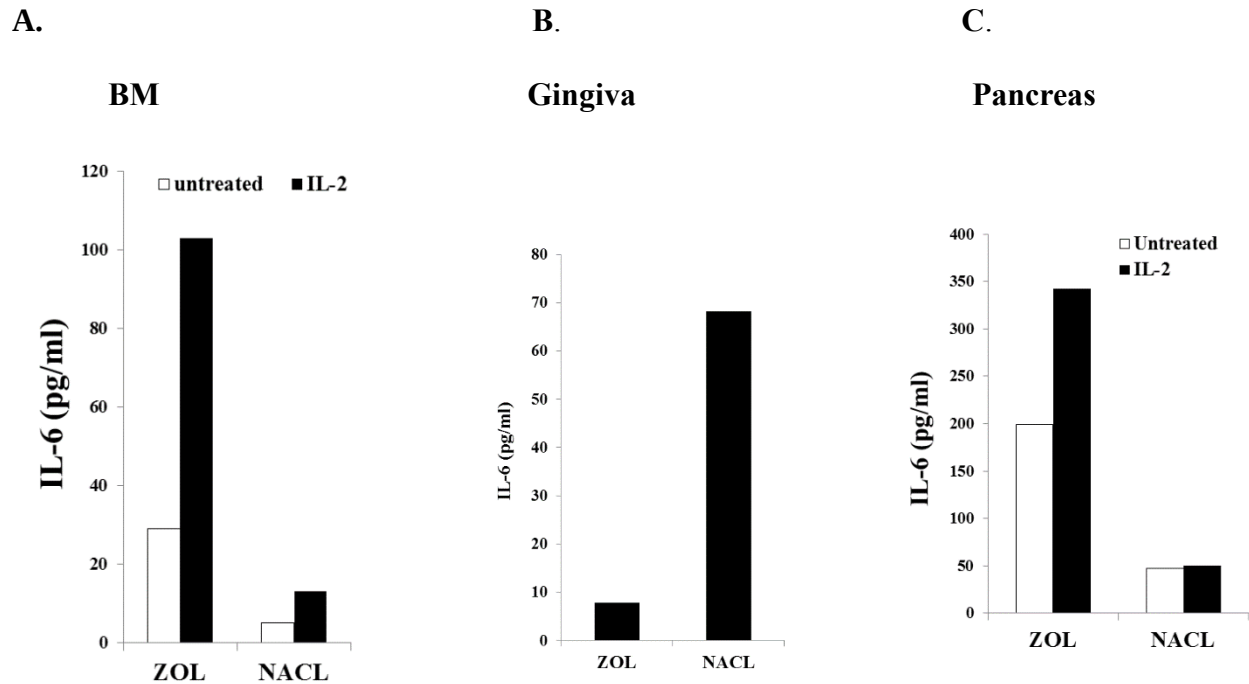


Figure 37: Zolendronic acid stimulated significant IL-6 secretion by bone marrow derived cells but inhibited secretion by gingival cells.

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals and they were digested as described in materials and method. Femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted and cultured (1×10^6 /ml) in the presence and absence of IL-2 (10,000 U/ml) treatment in 12 well culture plate. Single cell released from gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals were cultured in the presence and absence of IL-2 (10,000 U/ml) in 24 well culture plate. Supernatants were harvested on Day 5 after culture of Bone Marrow (Fig. A), gingival tissue (Fig. B) and Pancreas (Fig. C). The levels of IFN- γ were measured with specific ELISA.

3. Zolendronic acid up-regulate the Cytokine secretion and cytotoxic function of BM derived cells and BM derived NK cells.

Bone Marrow cells and cells obtained from gingival tissues after injection of Zolendronic acid in vivo were left untreated or treated with IL-2 and cultured for 5 days before they were for cytotoxicity against the tumor cells. As shown in Fig. 38 and Fig. 39 injection of Zolendronic acid in animals increased the cytotoxic function of BM derived cells and BM derived NK cells, and increase the amounts of IFN-g and IL-6 secretion by Bone Marrow derived NK cells (Fig. 40A and Fig 40B). Conversely, NACL injected animals had lower levels of IFN-g and IL-6 secretion in BM derived NK cells and less cytotoxic function against the tumor cells. IL-2 treated dissociated pancreatic cells, gingival tissue and adipose tissue from Zolendronic acid and NACL injected animals didn't show much difference based of cytotoxic function against the tumor cells (Fig. 38B).

Figure 38:

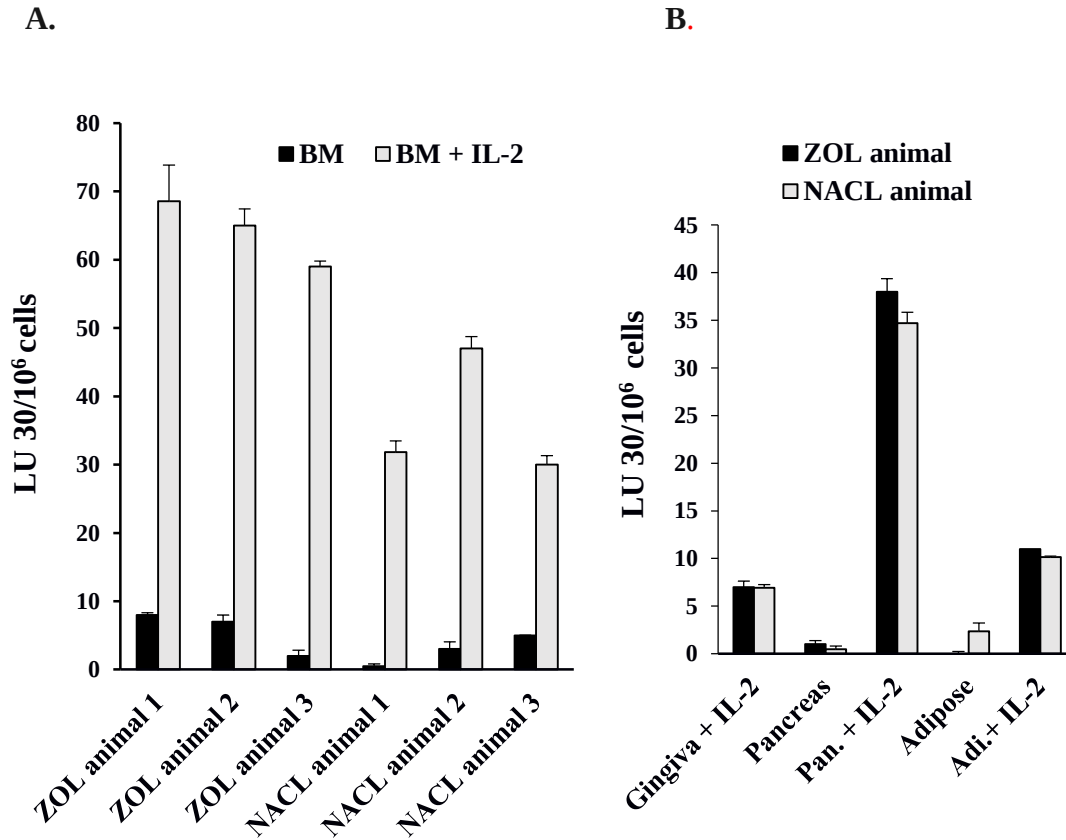


Figure 38: Zolendronic acid results in increased cytotoxic function in BM.

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals and they were digested as described in materials and method. Femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted and cultured (1×10^6 /ml) in the presence and absence of IL-2 (10,000 U/ml) treatment in 12 well culture plate. Single cell released from gingival tissues, pancreas and peri-pancreatic fat, were obtained from Zolendronic acid and NACL injected animals were cultured in the presence and absence of IL-2 (10,000 U/ml) in 24 well culture plate. BM derived cells (Fig. A), gingival tissue, pancreatic and adipose tissue (Fig. B) mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of the St63 X100.

Figure 39:

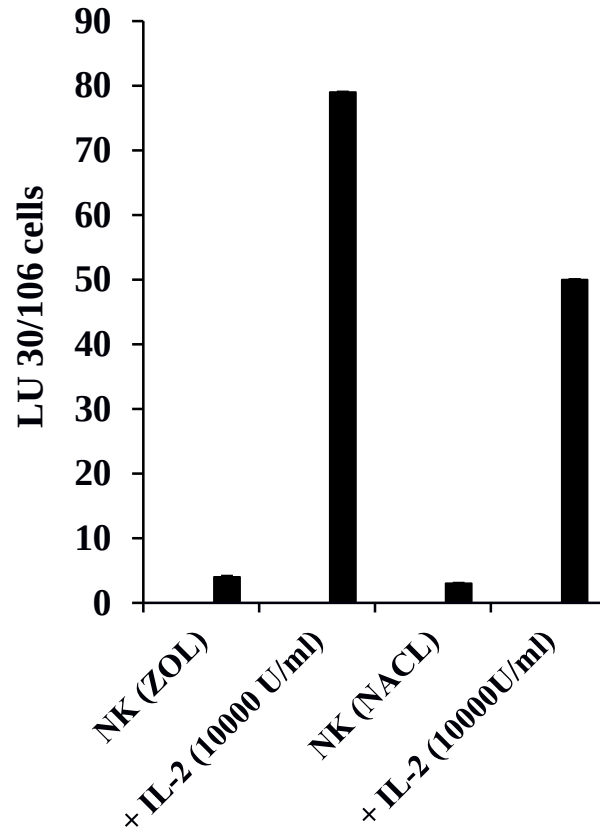


Figure 39: BM derived NK cells exhibit more cytotoxic functions after Zolendronic acid Injection.

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, femurs from Zolendronic acid and NaCl injected animals were harvested and bone marrow cells were extracted. NK cells were purified using negative selection kit and cultured (1×10^6 /ml) in the presence and absence of IL-2 (10,000 U/ml) treatment in 12 well culture plate. BM derived NK mediated cytotoxicities were determined using a standard 4 hour ^{51}Cr release assay. The lytic units 30/ 10^6 cells were determined using inverse number of NK cells required to lyse 30% of the St63 X100.

Figure 40:

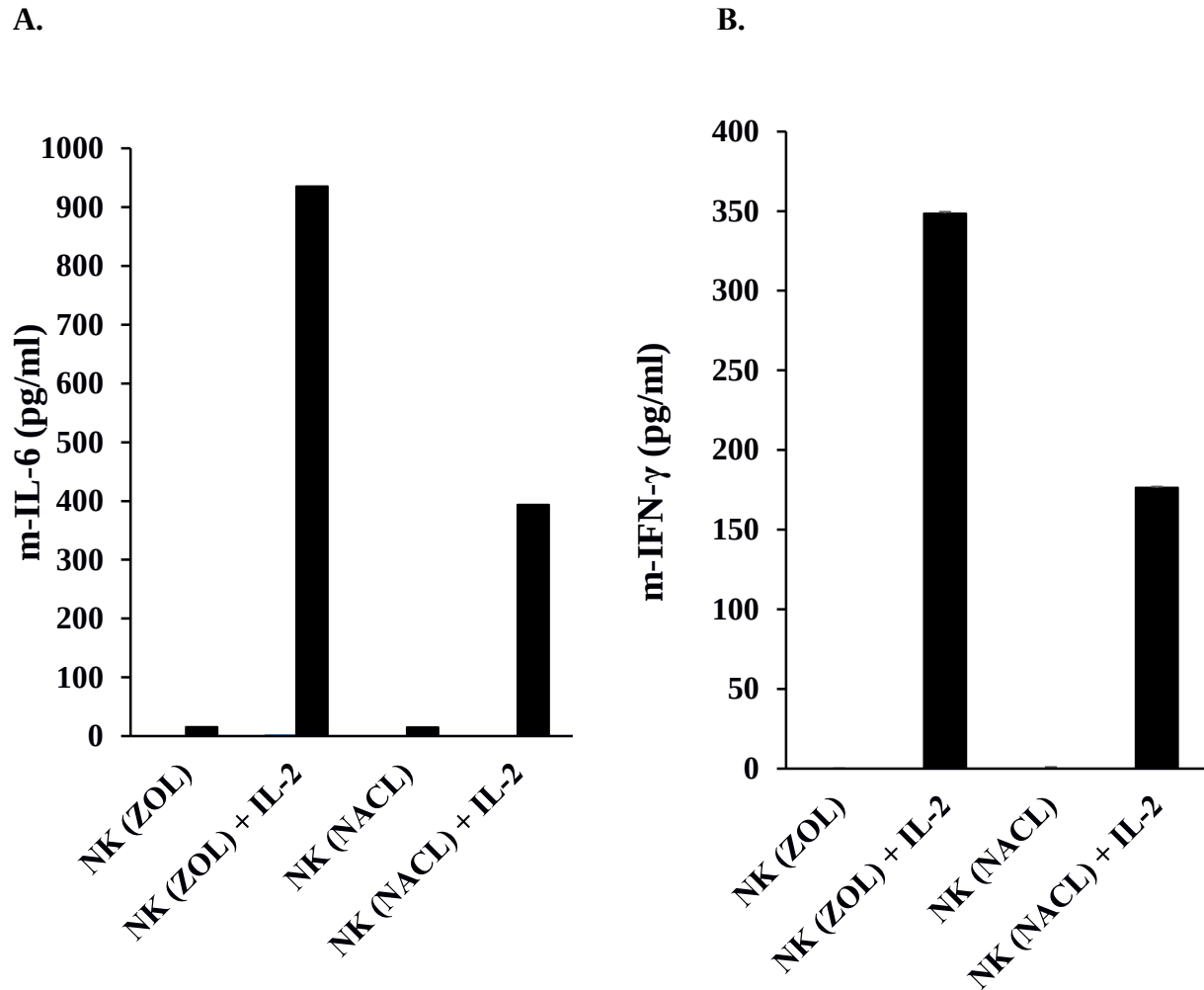


Figure 40: BM derived NK cells secrete more IFN-g and IL-6 after Zolendronic acid Injection.

Female WT B6 mice received IV injection of 500 µg/Kg ZOL or 0.9% NaCl vehicle solution followed by maxillary first molar extraction after 6 days. 2 weeks after tooth extractions, animals were sacrificed. After euthanasia, femurs from Zolendronic acid and NACL injected animals were harvested and bone marrow cells were extracted. NK cells were purified using negative selection kit and cultured (1×10^6 /ml) in the presence and absence of IL-2 (10,000 U/ml) treatment in 12 well culture plate. Supernatant was harvested day 5 after culture and level of IL-6 (Fig. A) and IFN-g (Fig. B) by specific ELISA.

DISCUSSION

Zolendronic acid could similarly increase IFN-g and IL-6 secretion in bone marrow when injected to the animals. Reciprocal increase in the secretion of IFN-g in Bone Marrow vs. those of gingival, pancreatic and adipose tissues were observed. IL-6 secretion was higher in bone marrow and pancreas, but lower in gingiva after ZA injection. These observations are of significant value since they may provide potential mechanisms for the pathogenesis seen in Osteonecrosis of the Jaw (ONJ). It is possible that an increase in activation of Bone Marrow derived cells by Zolendronic acid result in the eventual loss or inhibition of IFN-g secreting cells when they reach to the tissues. Since gingival derived immune cells are of activated phenotype when compared to bone marrow derived immune cells in healthy subjects, prior activation of bone marrow derived cells when mobilized to the gingival tissues and are exposed to additional activation signals from antigens in gingiva may undergo activation induced cell death, or simply become exhausted, and result in the lower secretion of IFN-g and IL-6 by the immune cells as seen in our *in vivo* model system. The phenotype characteristics of bone marrow and gingival tissue based on surface markers were very similar to each other but just minor difference based on CD3 positive surface expressions. Zolendronic acid injected BM has higher surface expression whereas NACL injected gingival tissues has higher surface expression of CD3 positive. Just like bone marrow derived cells, bone marrow derived NK cells exhibit higher cytotoxic function against the tumor cells and increased secretion of IFN-g and IL-6, suggesting that this might be the one of mechanism of reducing the bone metastasis in cancer patients after the ZA treatment.

CONCLUSION

Zolendronic acid induced IFN-g and IL-6 secretion in different tissue compartments were distinct depending on the location and type of cells. Zolendronic acid injections increased the BM derived cells and BM derived NK cells mediated cytotoxicity against the tumor cells, whereas no differences could be seen in other tissues examined. Zolendronic acid increased the cytotoxic function of BM derived NK cells against the tumors.

FINAL CONCLUSION

The results from this study indicated, Osteoclasts differ from monocytes and other monocyte lineages based on their surface receptors and secretion of pro-inflammatory cytokine. Osteoclasts can be better immune cells as compared to the monocytes and Dendritic cells, as they were able to secrete more pro-inflammatory cytokines while their interaction with NK cells. Osteoclasts have low surface receptors as compared to monocytes, macrophages and dendritic cells, but when osteoclasts were treated with low concentration of Zometa, they start up-regulating all those receptors. Zometa reduced the number of osteoclasts, inhibited the IL-10 secretion and induced the secretion of pro-inflammatory cytokines. Zometa, Alendronate and Etidronate reduced the pit formation by the osteoclasts. NK cells were able to lyse osteoclasts. Osteoclasts secreted higher cytokines and chemokines when compared to monocytes after their interaction with NK cells and they were more susceptible to NK cells mediated cytotoxicity. Zometa treated osteoclasts triggered significantly higher induction of cytokines and chemokines in the co-cultures with NK cells and the effect was higher when compared to Alendronate treated Osteoclasts, whereas Etidronate had no enhancing effect. When monocytes were treated with Zometa, there was induction of IFN- γ on Zometa treated monocytes by NK cells and the level of IFN- γ reached almost to the induction level of untreated osteoclast with NK cells. Zometa induced resistance in osteoclasts and OSCSCs against NK cell cytotoxicity. Zometa has the inhibitory effect on IL-10 secretion of osteoclasts and treatment with rh-IL-10 significantly reduced the IFN- γ secretion by Zometa treated OCs and that inhibitory effect of rh-IL-10 was blocked by the addition anti-IL-10 monoclonal Ab. Our *in-vivo* data showed that Zometa induced IFN-g secretion in different tissue compartments were distinct depending on the location and type of cells. Zometa could increase

IFN-g secretion in bone marrow when injected to the animals. Reciprocal increase in the secretion of IFN-g in Bone Marrow vs. those of gingival, pancreatic and adipose tissues were observed. Zometa injections increased the BM derived NK mediated cytotoxicity against the tumor cells, whereas no differences could be seen in other tissues examined.

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