UNIVERSITY OF CALIFORNIA

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Bone analysis of pancreatic tumor-bearing hu-BLT mice

A thesis submitted in partial satisfaction of the

requirements of the degree Master of Science in Oral Biology

by

Patricia E. Reese

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ABSTRACT OF THE THESIS

Bone analysis of pancreatic tumor-bearing hu-BLT mice

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Patricia E. Reese

Master of Science in Oral Biology

University of California, Los Angeles, 2020

Professor Anahid Jewett, Chair

This is a novel report of the correlation and potential impact of the immune system, specifically NK cells and Interferon Gamma, in bone quality alteration related to pancreatic cancer in hu-BLT mice. For this purpose, we studied how MP2 tumors injected in hu-BLT mice affected the bone structure. Also, we analyzed how MP2 tumor-bearing mice injected with NK cells and fed with and without AJ2 influenced the bone morphology.

We demonstrated the suppression of NK cell cytotoxicity and decreased secretion of IFN-γ in tumor-bearing mice within all tissue compartments and restoration by super-charged NK Cells.

There was a remarkable correlation between the micro-CT analysis results, the induction and secretion of IFN-γ, and bone morphology. The MP2 tumor-bearing mice injected with NK cells' cultures and fed with AJ2 presented increased bone formation with statistically significant higher trabecular bone volume compared to MP2 tumor-bearing mice group and MP2 tumor-bearing mice fed with AJ2 group, respectively. On the contrary, MP2 tumor-bearing mice showed decreased IFN-γ and decreased bone formation.

Consistent with the micro-CT findings, histological analysis of the AJ2 treatment group exhibited increased bone formation when compared to the Control and MP2 tumor groups. TRAP staining suggested more osteoclast activity and bone resorption in the MP2 tumor sample compared to the rest of the samples.

Therefore, we can hypothesize that IFN-γ induce secretion by NK cells, can inhibit tumor growth, and decreases skeletal complications of malignancy by directly acting on host cells to inhibit osteoclast formation and function.

In our study, the enhanced osteolytic lesion formation in BLT tumor-bearing mice and NK cells' ability to secrete IFN-γ to significantly reduce bone loss in tumor-bearing mice strongly supports a direct anti-osteoclastogenic role for IFN-γ in the setting of cancer-induced bone disease. Furthermore, this report suggests that IFN-γ can directly promote bone formation.

Overall, our data demonstrated that the injection of NK cells into tumor-bearing mice increased IFN-γ secretion in hu-BLT mice. It also suggests that IFN-γ has direct anti-tumor effects and potentially may suppress tumor-induced bone loss by directly targeting host osteoclasts to inhibit osteolysis.

A better understanding of the bone marrow microenvironment and processes that influence tumor cell maintenance and bone growth should present opportunities to understand bone remodeling and osteolysis.

To the best of our knowledge, the present work is a novel report of bone quality alteration related to pancreatic cancer in hu-BTL mice and the role of IFN-γ secreted by NK cells in the suppression of tumor-induced bone loss and induction of bone formation.

The thesis of Patricia E. Reese is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2020

DEDICATION

I dedicate this thesis to my mother, Elizabeth, to my father Alfredo's memory, who I miss dearly, and my husband, Shawn.

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Anahid Jewett. I have been blessed to have a mentor who is intelligent, patient, with high virtue and integrity. Thank you for your leadership and support during my journey to become a better professional.

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Finally, heartfelt thanks go out to my beloved husband for his immense sacrifice. Thank you for always supporting my goals and dreams.

INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer death worldwide, with an incidence rate nearly comparable to its mortality rate [1]. The 5-year survival rate has remained less than 6% for the past three decades [1, 2], demonstrating the aggressiveness and lethal nature of this disease. Clinical manifestations are usually non-specific, and patients commonly complain of abdominal pain, poor appetite, jaundice, and wasting syndrome.

The usual sites of metastases in pancreatic cancer are the liver and peritoneal cavity. Other less common sites are the lung, bone, and brain. Skeletal metastases from pancreatic cancer have thus far been considered an infrequent occurrence. The first case of pancreatic cancer with skeletal metastases was described in Russian literature in 1963 [3]. Besides, pancreatic cancer patients usually suffer from severe nutrition deficiency, muscle wasting, and bone mass loss [4].

In pancreatic cancer, the current research focus has mainly been devoted to clarifying the carcinogenesis and developing potential therapeutics to inhibit tumor growth. In contrast, rare studies have been conducted on bone metastasis and the progression of bone changes. What is important; in clinical practice, pancreatic cancer patients at advanced stages frequently develop cachexia-induced bone loss, leading to a high incidence of bone fracture. While muscle wasting has been under extensive investigation, bone metastasis, bone loss, and related molecular mechanisms in pancreatic cancer are still poorly understood.

We have previously established that the stage of differentiation of pancreatic tumors has a profound effect on the function of NK cells and that stem-like/poorly differentiated tumors were preferentially targeted by the NK cells. Also, our studies indicated that an intact immune system

is required for the elimination of tumors. However, tumors have been shown to cause immune suppression, in particular, NK suppression, and this defect occurs in NK cells at many levels. NK cells from cancer patients and humanized mice implanted with tumors lose their ability to kill and differentiate tumors. NK cells' inability to curtail tumor growth through increased lysis and differentiation of tumors is a profound deficiency that could be improved by administering supercharged NK cells, as we have seen in hu-BLT mice implanted with poorly differentiated pancreatic tumors. In this study, we investigated the potential impact of the immune system, specifically supercharged NK cells and Interferon Gamma, in mediating tumor dissemination and bone quality alteration related to pancreatic cancer hu-BLT mice.

To the best of our knowledge, the present work is a novel report of bone quality alteration related to pancreatic cancer in hu-BTL mice and the role of IFN-γ secreted by NK cells in the suppression of tumor-induced bone loss and induction of bone formation.

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 [3] [4] [5], and their differentiation is controlled by interactions between osteoblasts and/or stromal cells and pre-osteoclasts [6], 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 [7]. 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 [8] [9]. RANKL is critical for osteoclastogenesis and bone resorption [10] [11]. RANKL interacts with its receptor RANK (receptor activator of NF-kB), 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 [12]. 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 [13] [14] [15]. 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 [16]. 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 [17].

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.

IFN-γ interaction with Osteoclasts and other immune cells

IFN-γ is a multifunctional cytokine produced mainly by NK cells and activated T cells that plays a critical role in host immune responses against pathogens and cancer [18]

Previously, it has been reported that IFN-γ can inhibit the critical osteoclast regulator, receptor activator of NFkB ligand (RANKL), by activating ubiquitin-mediated degradation of its signaling pathway adaptor protein TRAF-6 (7,8).

Further studies showed that IFN-γ could also be used to inhibit experimental tooth movement and tumor-induce bone erosion by decreasing osteoclastic activity [37,38]. It has been shown that IFN-γ participates in the regulation of RANKL signaling and bone destruction [39].

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, T and B lymphocytes, mast cells, natural killer cells (NK), etc. have been shown to influence bone cells as well [19]. (**Fig. 1**).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 [20, 21]. IFN-γ, produced by both NK cells and Th1 lymphocytes, has been shown to inhibit osteoclastogenesis in vitro [21]. 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 [22] [23]. Reduced functioning of osteoclast and NK cell function coexist in osteopetrotic mutant rat [20]. OC progenitor activity is positively regulated by TNF-α and negatively regulated by IFN-γ [24]. 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 [25]. ITIM-bearing NK receptor, positively regulates osteoclasts differentiation, immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for osteoclast differentiation [26] [18]. Crosstalk between the skeletal system and T cells, is termed as osteoimmunology. RANKL expressed by CD4+ and CD8+ T-cells can induce osteoclast genesis, 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-kB ligand. Anti- CD3/CD28-stimulated γδ T cells or CD4+ T cells inhibit human osteoclast formation and resorptive activity in vitro. Cytokine production by CD3/CD28-stimulated γδ T cells and observed a lack of IL-17 production, with activated γδ 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 γδ T cells on osteoclastogenesis and resorptive activity of mature osteoclasts.

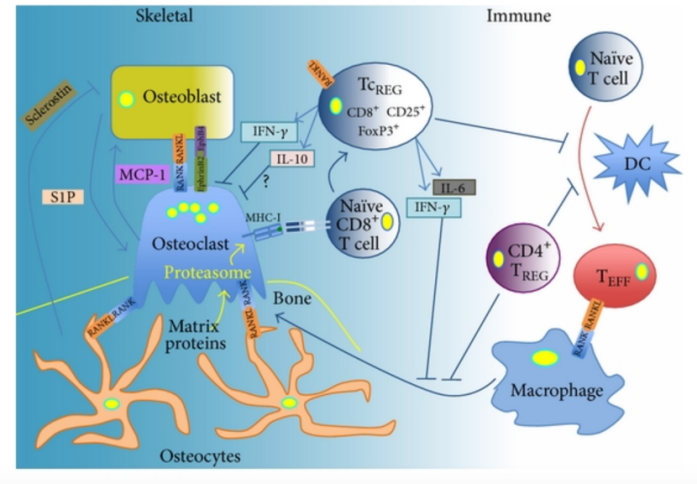


Fig. 1: Regulatory interactions that maintain homeostasis of the skeletal systems.

Based on the observation that bone destruction in rheumatoid arthritis is always caused by an excessive activation of the immune system, researchers identified a close relationship between immune system and osteoclasts, which is termed osteoimmunology. In fact, RANKL is secreted by activated T-cells, and dysregulation of RANKL leads to defective formation of lymph nodes and lymphocyte differentiation as well as impaired osteoclastic bone resorption [27]. A number of molecules known to be involved in the regulation of immune system, including TNF alpha, IL-1, IL-7, IL-17, IL-6, IFN-γ, IFN- alpha also play critical roles in osteoclastogenesis [28].

IFN-γ, also known as immune interferon, is the only type II IFN and was discovered in 1965. It is secreted predominantly by T-cells, natural killer cells, and some other cells such as macrophages, dendritic cells and B cells. IFN-γ signal transduction is mediated by binding with IFNGR1 and IFNGR2 resulting in activation of intracellular molecular signaling networks such as JAK-STAT pathway and STAT-independent pathways such as the MAP kinease, NF- _B, and PI3K pathways [35]. Briefly, following binding with IFN-GRs, JAK1 and JAK2 facilitate trans-phosphorylation

of the JAKs and the receptor subunits are activated. Subsequently, STAT1 is recruited to the receptor and becomes phosphorylated, resulting in active form of the STAT known as IFN-γ activated factor (GAF).GAF then binds to the IFN-γ activation site (GAS) on the promoter of IFN-inducible genes leading to target gene expression [29]. Although they share considerably overlapping functions with type I IFNs, IFN-γ uniquely regulates a variety of autoinflammatory and autoimmune diseases, such as systemic lupus erythematosus [9],hemophagocytic lymphohistiocytosis (HLH), and macrophage activation syndrome (MAS) [30]. IFN-γ has long been used as an immunosuppressive. Further studies showed that IFN-γ could also be used to inhibit experimental tooth movement and tumor-induce bone erosion by decreasing osteoclastic activity [31] [32]. It has been shown that IFN-γ participates in the regulation of RANKL signaling and bone destruction [33].

IFN-γ has also recently been found to participate in a number of signaling pathways in osteoclastogenesis. Li et al. revealed that IFN-γ mediates a previously unknown feedback loop that exits between osteoclasts and activated T-cells by inducing indoleamine 2,3-dioxygenase (IDO) expression in osteoclasts [34]. Ji et al. identified another mechanism through which IFN-γ regulates osteoclastogenesis. In contrast to previous studies, they found that IFN-γ alone did not affect TRAF6 expression in human osteoclast precursors, whereas, IFN-γ cooperated with TLRs to suppress RANK expression by inhibiting colony stimulating factor 1 receptor (c-Fms), a potent stimulator of RANK expression [35]. In addition to directly inhibiting osteoclast formation.

IFN-γ was recently reported to promote osteoblastogenesis and bone formation both in vitro [36] and in vivo [37]. The anabolic effect of IFN-γ is mediated by increasing both osteoblastogenesis and osteoclastogenesis [38] with a predominant stimulatory effect on the osteoblast lineage, thus increasing bone mass and rescuing oophorectomized (OVX) mice from osteoporosis [37] [9]. In

addition, IFN-γ significantly increases the expression of osteogenic markers in differentiating mesenchymal stem cell (MSC) into osteoblasts in vitro, including runt-related transcription factor 2 and osteopontin [36].

Overall, the effects of IFN-γ on bone remain complex [39] with some investigators reporting contrasting findings about its effect on osteoclastogenesis [38] and bone resorption [40]. High doses of IFN-γ have been used as treatment in patients with osteopetrosis [41] to induce bone resorption and reduce bone mass. Other studies report that IFN-γ directly inhibits osteoclast differentiation [37] [42] and induce osteoclast apoptosis [42]. In addition, the effect of IFN-γ on osteoclast differentiation and function could be affected, among others, by estrogen deficiency, inflammation, and bacterial toxins [43], [44].

NK cells

Natural Killer (NK) cells are granular lymphocytes that function at the interference of innate and adaptive immunity [45]. 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 [46]. NK cells are a subset of 8 cytotoxic lymphocytes able to recognize and lyse tumor cells and virus infected cells without prior sensitization [47]. Traditionally they have been classified as effectors of innate immunity due to the lack of antigen specific cell surface receptors [48]. 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 [49]. 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 [50]. Human NK cells are defined phenotypically by the surface expression of CD56 and CD16, and by their lack of CD3 surface

expression [51]. 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 [45]. 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 [45] [51]. 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 [45] [51] [52].

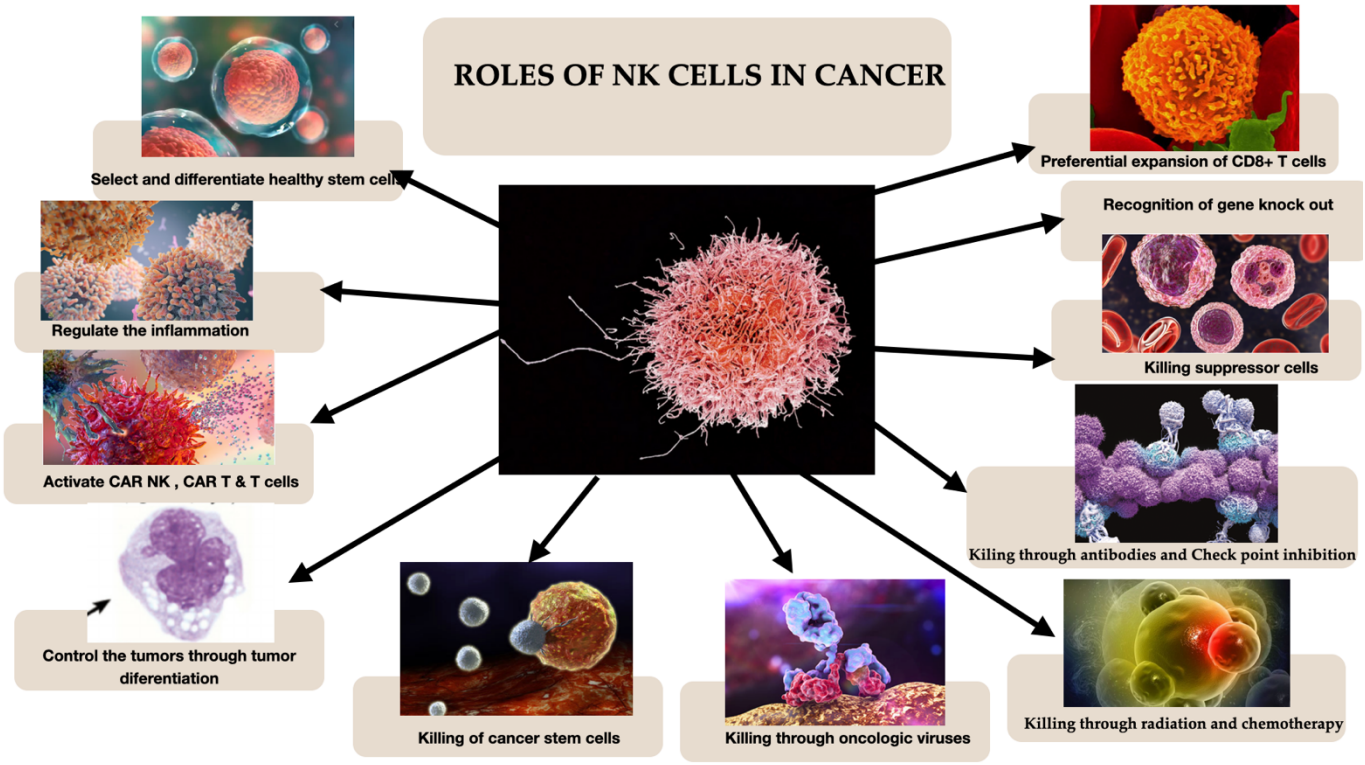
NK cells develop in the bone marrow and constitute about 5-10% of total lymphocytes in the peripheral circulation and secondary lymphoid organs [53]. Effector function of NK cells include direct natural cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), as well as secretion of inflammatory cytokines and chemokines that indirectly regulate the functions of other immune cells [54] [55]. 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 [56] [57] [58]. 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 [59].

NK cell cytotoxic activity in cancer patients is severely reduced, correlating with the decreased expression of NK cell activating receptors even at the early stages of disease, and are further diminished in advanced cancers [60-62]

Patients' NK cells are significantly defective in their function, and the defect is seen at both preneoplastic and neoplastic stages of pancreatic cancer [63-65] Patients' NK cells do not recover their full functional potential even when the best activating conditions are provided for their expansion and function. Moreover, patients' NK cells under the expansion conditions give rise to T cell expansion at a much faster rate, with a subsequent decrease in the percentages of NK cells when compared to those from healthy individuals [63]. Therefore, the use of allogeneic NK cells from healthy individuals is preferable than autologous NK cells for therapeutic purposes. The use of allogeneic NK cells in hematologic malignancies following HLA-haploidentical HSCT in clinical trials has previously been reported [16],[66, 67]. In addition, allogeneic NK cells exert less collateral damage due to graft versus host disease (GVHD) when used therapeutically in solid tumors [67-70]. In search of a more potent therapeutic dose of NK cells, we have recently established a novel strategy to expand highly functional NK cells, coined as super-charged NK cells, by employing osteoclasts as feeder cells in the presence of a combination of sonicated probiotic bacteria (sAJ2) [71]. The potency and effectiveness of super-charged NK cells are significantly superior to those established by other methodologies or when compared to primary activated NK cells. The term super-charged was used to describe the magnitude and superiority of their functional potential in lysing and differentiating CSCs/poorly differentiated tumors [63, 72]. At our lab, we were studying the biology of NK cells for a long time, and we establish that NK cells have the following roles: 1) Selection and differentiation of healthy stem cells. 2) Regulate inflammation, 3) activate CAR NK, CAR T, and T cells, 4) control the tumors through tumor

differentiation, 5) kill cancer stem cells, 6) kill through oncologic viruses. 7) act synergistically with radiation and chemotherapy, 8) kill through antibodies and checkpoint inhibition, 9) kill suppressor cells. 10)exert preferential expansion of CD8+ T cells 11) gene knockout recognition. (Figure 2).

Figure 2: **Roles of Natural Killer Cells in Cancer**



AJ2 probiotic Bacteria [73]

AJ2 is a combination of eight strains of gram-positive probiotic bacteria with the ability to induce synergistic production of IFN-γ when added to IL-2-treated or IL-2 + anti-CD16 monoclonal antibody-treated NK cells (anti-CD16mAb). The combination of strains was used to provide bacterial diversity in addition to synergistic induction of a balanced pro and anti-inflammatory cytokine and growth factor release in NK cells. Moreover, the quantity of each bacteria within the combination of strains was adjusted to yield a closer ratio of IFN-γ to IL-10 to that obtained when NK cells are activated with IL-2 + anti-CD16mAb in the absence of bacteria. The rationale behind such selection was to obtain a ratio similar to that obtained with NK cells activated with IL-2 + anti-CD16mAb in the absence of bacteria since such treatment provided significant differentiation of the cells [72, 74].

NK cells Immunotherapy

NK cells have very diverse biological functions including significant roles in defense against tumor cells. Based on knowledge of NK cell function and evidence that they become nonfunctional in cancer patients, several approaches have been proposed for the use of NK cells in immunotherapy:

1) Cytokines

Many cytokines such as IL-2, IL-21, IL-12, IL-15, and IFN- γ have been known to activate and boost NK cells function [75]. Cytokines can be used to boost NK cells for immunotherapeutic means, both in vitro and in vivo. Cytokine administration for cancer treatments has been implemented clinically but has never been considered as a success due to severe side-effects and cytotoxicity [76].

2) Antibodies

Antibodies can be applied to NK cell immunotherapy based on different approaches. NK cells can target tumor cells coated with IgG antibodies through the ADCC. There are several monoclonal antibody treatments available specific to different tumor antigens such as the use of anti-CD20 for the treatment of B cell lymphoma or anti-Her2 for the treatment of Her2-overexpressing invasive breast cancer [77]. Antibodies can be also being used to block NK cells' checkpoint inhibitors. Studies have shown that NK cells express checkpoint molecules such as PD-1, CTLA-4, TIM-3 and TIGIT [78]. The role of these checkpoint inhibitor drugs in NK cells have not been well studied.

3) Adoptive transfer of NK cells

Transferring functionally competent NK cells as an immunotherapy approach has been established for many years. NK cells can be harvested from different sources, and their functional competencies may vary depending on the strategies used to separate, activate or expand them.

Sources of natural killer cells for adoptive transfer:

a) NK cells isolated from Peripheral Blood Mononuclear Cells (PBMCs)

NK cells can be isolated from autologous and allogeneic PBMCs. The strategies to expand and activate NK cells from PBMCs are different. Investigators have tried to Expand NK cells directly from PBMCs without isolating the NK cells population, or by depleting CD3+ cells, or selecting CD16+, or CD16+ and CD56+ cells. Different cells have been used as feeder cells to improve NK cells expansion. Irradiated K562 and OK432 are two of the most popular feeder cells for NK expansions. Different Cytokines and other activators also have been applied to expand NK cells in vitro. IL-2, IL-21, IL-15 and IL-18 are some of the cytokines used for this means [79] [80], [81]. Our laboratory has a novel strategy to expand NK cells up to 21,000- to 132,000-fold in 20 days.

In this technique NK cells are treated with IL-2 and CD16 antibody and probiotic bacteria and Osteoclasts are used as feeder cells. The Expanded NK cells, called "Super Charged NK cell" have high cytotoxicity and IFN- γ secretion [63].

Autologous NK cells as a source of therapy has not been very effective. We showed that NK cells from cancer patients are less functional both in terms of cytotoxicity and of IFN- γ secretion in vitro[63]. In an in vivo study adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression [82]. In another study autologous NK cells have also demonstrated a limited effect on tumor suppression in malignant glioma [83]. Allogenic NK cells can be a better resource for NK cell therapy since NK cells from healthy donors have better functionality than NK cells in cancer patient [43], besides the KIR receptor from the donor mismatch the MHC class I of the recipient letting NK cells to skip some of the inhibitory processes [16].

b) Stem cell- derived NK cells

Due to the pluripotency of stem cells, using them as a precursor of NK cells has become one of the interesting sources of NK cells. Generation of NK cells from ESCs and iPSCs generally requires two steps. First, CD34+ hematopoietic precursors must be generated. The CD34+ cells are then sorted and differentiated into NK cells with cytokines and feeder cells (usually murine stromal cells). When NK cells were generated from hESCs, they were mostly CD56+CD45+ NK cells, which also expressed inhibitory and activating receptors typically found on adult NK cells. These NK cells were also able to mediate cytotoxicity against of leukemic cells, K562 (erythroleukemia), and several solid tumors, including breast cancer (MCF7), testicular embryonal carcinoma (NTERA2), prostate cancer (PC3), and glioma (U87) cell lines [84]. A studied showed that when efficacy of Induced pluripotent stem (IPS) cell-derived natural killer cells with NK cells

isolated from peripheral blood that had been activated and expanded in long-term culture, and overnight activated Peripheral blood isolated NK cells, were compared, NK cells derived from IPS mediate anti-ovarian cancer killing in NSG mouse at least as well as NK cells isolated from blood [85, 86]. The Kaufman research group established a feeder-free, sorting-free approach to generate NK cells from human ESCs. These authors used a spin-embryoid body [70] system with BMP4 and VEGF to derive hematopoietic progenitors. After 11 days of culture, the spin-EBs were transferred to the NK cell culture containing the cytokines IL-3, IL-15, IL-7, SCF and Flt3L for 28 days. This feeder-free system can generate NK cells that have no difference from those derived from the murine embryonic liver cell line EL08-1D2 as feeder cell. Meanwhile, this group established a clinical-scale derivation of NK cells from ESCs and iPSCs without cell sorting and in the absence of feeder cells [86].

c) NK cells isolated from Umbilical Cord blood.

Umbilical cord blood (UCB) has become a known source for NK cells. The NK cells from UCB and peripheral blood (PB) have some differences. UCB NK cells express similar levels of CD56, NKp46, NK30 and NKG2D as PB NK cells but lower levels of CD16 CD2, CD11a, CD18, CD62L), KIRs, DNAM-1, NKG2C, IL-2R, and CD57 and CD8 [86]. UCB has a higher percentage of NK cells but these cells have lower cytotoxicity in comparison to NK cells from PB which could be due to lower levels of Granzyme B and perforin in CB NK cells. Studies have shown that with proper signaling NK cells from UCB can be expanded to create many cells with proper function [87-89].

d) Genetically modified NK cells

The genetic modification can be used to promote the efficacy of NK cells by different means. NK cells can be genetically modified to secrete cytokines in favor of their survival and activation.

Engineering Chimeric antigen receptor (CAR) NK cells has currently become the topic of interest. Currently, several tumor antigen-binding domains have been designed as CAR extracellular domains and tested [90-92]. One of the problems associated with genetic modification of NK cells is that these cells are very hard to virally infect and approaches to improve their transfection rate should be introduced.

Although various approaches are being used to use NK cells for cancer therapy, the long-term efficacy of these protocols is not very promising. NK cells have diverse biological functions and to benefit patients the ideal NK cells therapy should focus on boosting all functions of NK cells.

PURPOSE OF THE STUDY

This study's objective was to investigate the potential impact of the immune system, specifically NK cells and Interferon Gamma, in mediating tumor dissemination and bone quality alteration related to pancreatic cancer in hu-BLT mice. For this purpose, we determine the impact of MP2 tumors, super-charged NK cells, and AJ2 on the secretion of IFN Gamma and its association with bone loss. Also, we analyzed how MP2 tumors injected in hu-BLT mice affected the bone structure. Furthermore, we determined how MP2 tumor-bearing mice injected with NK cells and fed with and without AJ2 influenced the bone structure.

THESIS OUTLINE

Specific aim 1: Determine the impact of MP2 tumors, super-charged NK cells and AJ2 on the secretion of IFN Gamma and its association with bone loss.

Specific aim 2: Investigate how MP2 tumor-bearing mice injected with NK cells and fed with and without AJ2 influence the bone structure.

- Sub-Aim 1*: Determine how stem-like/undifferentiated tumors implanted in hu-BLT mice alter the bone microenvironment structure*
- Sub-Aim 2*: Demonstrate how mice fed with AJ2 influence the bone structure.*
- Sub-Aim 3*: Establish how MP2 tumor-bearing mice fed with AJ2 affects the bone structure*
- Sub-Aim 4*: Determine how MP2 tumor-bearing mice injected with cultures of NK cells and fed with AJ2 influence the bone structure*
- Sub-Aim 5*: Investigate the correlation and potential impact of Interferon Gamma in bone quality alteration related to pancreatic cancer in hu-BLT mice.*
- Sub-Aim 6*: Study the presence of osteoclasts in hu-BLT mice after implantation with MP2 tumors in the presence and absence of supercharge NK cells and feeding with AJ2.*

Methods and Materials

Generation of hu-BLT mice

When human $CD34^+$ progenitor cells are provided with an appropriate thymic microenvironment, they can mature into naive single-positive human T cells[9,10]. We therefore asked whether $CD34^+$ cells introduced by bone marrow transplantation could systemically repopulate the mouse and sustain thymopoiesis in the implanted human thymic tissue. We used NOD/SCID mice because they support significantly higher percentages of reconstitution following transplantation with human $CD34^+$ cells. In essence, we first implanted NOD/SCID mice with human fetal liver and thymic tissues and allowed them to recover from surgery. We then preconditioned the implanted mice with a sublethal dose of gamma radiation and transplanted them with autologous $CD34^+$cells obtained from fetal liver. Thy-liv–implanted mice that received a bone marrow transplant with autologous $CD34^+$ cells had readily detectable numbers of human cells in the peripheral blood (49% ± 22% (mean ± s.d.), range 14–82%, n = 20), consisting of B cells, monocytes and macrophages, DCs and, specifically, T cells (Fig. 3).

BLT mice demonstrated a high proportion of human $CD45^+$ cells in bone marrow (48% ± 14% (s.d.), range 23–75%, n = 24), spleen (42% ± 21% (s.d.), range 5–84%, n = 24) and lymph nodes (71% ± 22% (s.d.), range 6–95%, n = 14). Human T cells, B cells, monocytes and macrophages, and DCs were present in all tissues. The mean percentage of $CD4^+$ T cells was 71% (± 11% (s.d.), range 47–91%, n = 73) and the mean percentage of $CD8^+$ T cells was 21% (± 11% (s.d.), range 4–48%, n = 73) for all tissues examined. We also determined the differentiation state of the human T cells in the peripheral blood of BLT mice. BLT mice kept

under sterile conditions had a higher percentage of naive T cells ($CD45RA^{+}CD27^{+}$) than did healthy human controls.

Liver and lung from BLT mice contained substantial numbers of human T cells, B cells, monocytes and macrophages, and both $CD11c^{+}$ and $CD123^{+}$ DCs. We also observed multilineage reconstitution in the gut of BLT mice analyzed by immunohistology. These data show that BLT mice can generate an extensive state of sustained systemic multilineage reconstitution with human hematopoietic cells (Fig. 4).

Figure 3

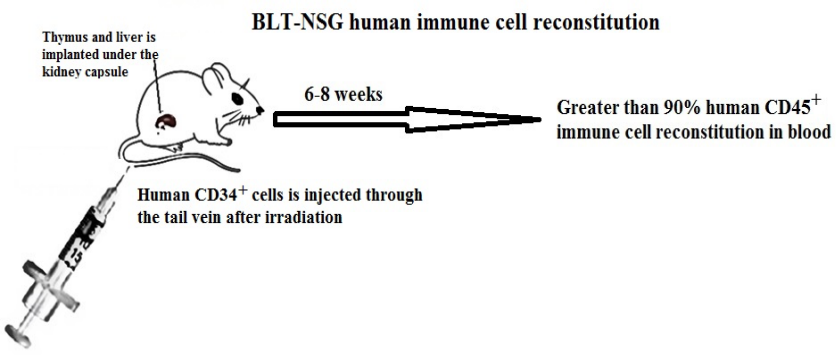


Figure 3: BLT-NSG human immune cell reconstitution.

Figure 4

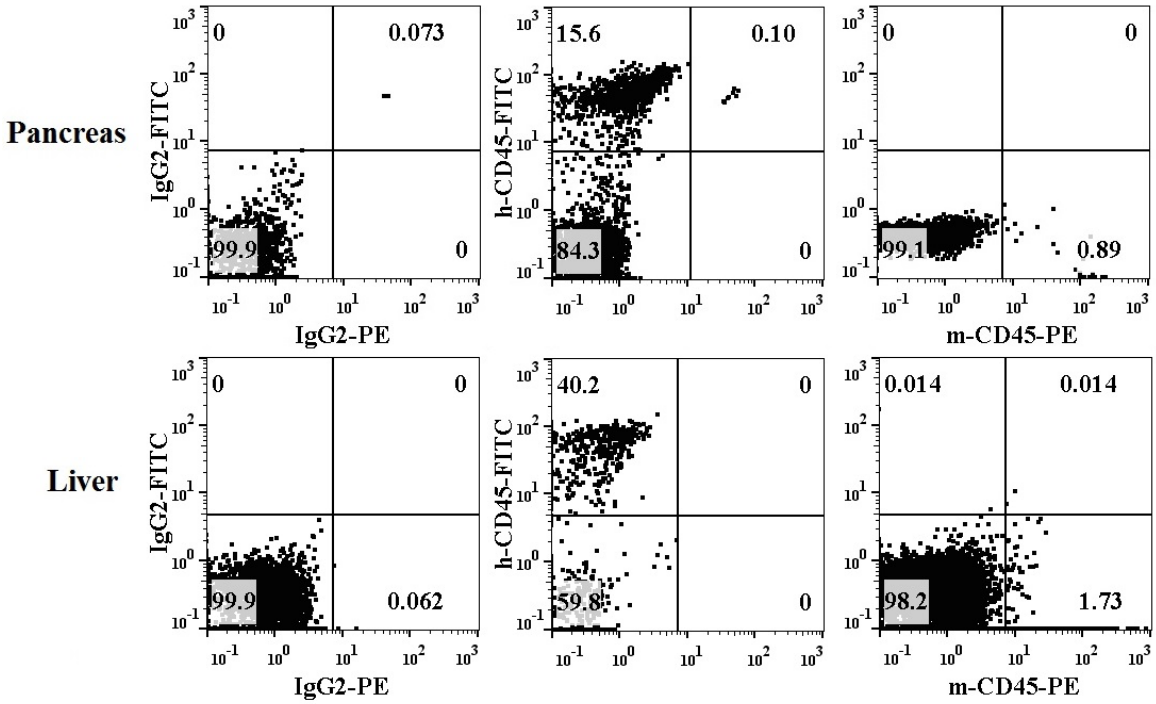


Figure 4: Greater than 90% of tissue infiltrated immune cells in BLT-NSG mice are of human immune cells: In pancreas of hu-BLT mice: IgG shows 99.9 % of Human immune cells, m-CD45 99 %. In liver of hu-BLT mice: Ig G presents 99.9% of human immune cells and m-CD45 of 98 %.

Cell lines, Reagents, and Antibodies

Recombinant human IL-2 was obtained from NIH-BRB. Human TNF-α and IFN-γ was obtained from Biolegend (San Diego, CA, USA). Antibody to CD16 was purchased from Biolegend (San Diego, CA, USA). Fluorochrome-conjugated human and mouse antibodies for flow cytometry were obtained from Biolegend (San Diego, CA, USA). Monoclonal antibodies to TNF-α and IFN-γ were prepared in our laboratory and used at 1:100 dilutions to block rhTNF-α and rhIFN-γ

functions. The human NK cell and monocyte purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Ethics Approval and Consent to Participate

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained and all procedures were approved by the UCLA-IRB (IRB#11-000781). Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC) (protocol # 2012-101-13).

Purification of Human NK Cells and Monocytes

NK cells and monocytes were negatively selected from PBMCs using isolation kits from Stem Cell Technologies (Vancouver, BC, Canada). Greater than 96% purity was obtained both for purified NK cells and monocytes based on flow cytometric analysis.

Cell Dissociation and Cell Culture of Tissues from hu-BLT Mice

Pancreatic tumors were harvested from hu-BLT mice and cut into 1 mm 3 pieces and placed into a digestion buffer containing 1 mg/mL collagenase IV, 10 U/mL DNAse I, and 1% bovine serum albumin (BSA) in DMEM media for 20 min at 37 °C. The samples were then filtered through a 40 mm cell strainer and centrifuged at 1500 rpm for 10 min at 4 °C. To obtain single-cell suspensions from BM, femurs were flushed using media, and filtered through a 40 μm cell strainer. Spleens were removed and single cell suspensions were prepared and filtered through a 40 μm cell strainer and centrifuged at 1500 rpm for 5 min at 4 °C. The pellets were re-suspended in ACK buffer to remove the red blood cells. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-hypaque centrifugation.

Isolations of NK Cells, T Cells and Monocytes from hu-BLT Mice

NK cells and T cells from hu-BLT splenocytes were obtained as described previously by using

the human CD56+ and CD3+ selection kits respectively (Stem Cells Technologies, Vancouver, BC, Canada). Monocytes from hu-BLT bone marrow were isolated using human CD14 isolation kit (eBioscience, San Diego, CA, US

Generation of Osteoclasts and Expansion of Human and hu-BLT NK Cells

Monocytes were purified form human peripheral blood or hu-BLT BM and cultured using alpha-MEM medium containing M-CSF (25 ng/mL) and RANKL (25 ng/mL) for 21 days (medium was refreshed every 3 days). NK cells were activated with rh-IL-2 (1000 U/mL) and anti-CD16 mAb (3μg/mL) for 18–20 h before they were cultured with osteoclasts and sonicated-AJ2 to generate supercharged NK cells. The medium was refreshed every 3 days with RMPI containing rh-IL-2 (1000 U/mL).

Enzyme-Linked Immunosorbent Assays (ELISAs) and Multiplex Cytokine Assay

Human ELISA kits for IFN-γ and IL-6 were purchased from Biolegend (San Diego, CA, USA). The assays were conducted as recommended by the manufacturer. For certain experiments multiplex arrays were used to determine the levels of secreted cytokines and chemokines. Analysis was performed using MAGPIX (Millipore, Danvers, MA, USA) and data was analyzed using xPONENT 4.2 (Luminex, Austin, TX, USA).

Surface Staining and Cell Death Assays

Staining was performed by staining the cells with antibodies as described previously, briefly, antibodies were added to 1 × 104 cells in 50 μL of cold-PBS+ 1% BSA and cells were incubated on ice for 30 min. Thereafter cells were washed in cold PBS+ 1% BSA and flow cytometric analysis was performed using Beckman Coulter Epics XL cytometer (Brea, CA, USA) and results were analyzed in FlowJo vX software (Flowjo, Ashland, OR, USA).

51Cr Release Cytotoxicity Assay

The 51Cr release assay was performed as described previously [60]. Patient-derived OSCSCs were used as a specific and sensitive NK target to assess NK cell-mediated cytotoxicity [49]. Briefly, different numbers of effector cells were incubated with 51Cr–labeled OSCSCs. After 4 h incubation the supernatants were harvested from each sample and counted on a gamma counter. The percentage specific cytotoxicity was calculated using the following formula:

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

Lytic unit 30/106 is calculated by using the inverse of the number of effector cells needed to lyse 30% of tumor target cells × 100.

Sonicating AJ2

AJ2 was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10 mg/mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 seconds, at 6 to 8 amplitudes. Sonicated samples were then incubated for 30 seconds on ice. After every five pulses, a sample was taken to observe under the microscope until at least 80 percent of cell walls were lysed. It was determined that approximated 20 rounds of sonication/incubation on ice, were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ2) were aliquoted and stored in a -80 degrees Celsius freezer.].

Analysis of Human Pancreatic Cancer Cell Growth in Humanized-BLT Mice

Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were generated as previously described [55,56]. In vivo growth of pancreatic tumors was performed by orthotopic tumor implantation in the pancreas hu-BLT mice. To establish orthotopic tumors, mice were anesthetized using isoflurane, and tumors in a mixture with Matrigel (10 μL) (Corning, NY, USA) were injected in the pancreas using insulin syringe. Mice received 1.5 × 106 super-charged NK cells via tail vein injection 7 to 10 days after the tumor implantation. They were also fed AJ2 (5 billion/dose) orally. The first dose of AJ2 was given one or two weeks before tumor implantation, and feeding was continued throughout the experiment at an interval of every 48 h. Mice were euthanized when signs of morbidity were evident. Lumbar vertebrae, pancreas, pancreatic tumors, bone marrow, spleen, and peripheral blood were harvested, and single cell suspensions were prepared from each tissue as described previously and below.

Bone Analysis

The assessment of bone architecture was made by micro computed tomography (micro-CT). Samples were harvested, formalin-fixed and imaged using high-resolution microCT (Skyscan 1275, Skyscan, Belgium) at an image resolution of 10 u pixels and analyzed using Data Viewer, Recon, CTAn and CTVol software provided by the manufacturer.

Specimen preparation for scanning

At the final time point, all mice will be euthanized in a CO2 chamber with the appropriate CO2 concentrations and exposure times. Lumbar vertebrae (L3) were dissected and fixed in 70% ethanol. In this study, vertebras were scanned with Skyscan 1275 (Bruker microCT N.V., Belgium), equipped with a 5-μm focal spot micro-focus x-ray tube at the resolution of 10μm (60 kVp, 166 mA, and 1mm Al Filter). Specimens were aligned with the vertical axis of the scanner,

and low-density foam (a non-attenuating material) was used to stabilize the specimens firmly into a 0.25-diameter-tube. Phantom calibration was performed to relate the micro-CT values to a mineral-equivalent value (g/cm3) of calcium hydroxyapatite.

Reconstruction

To process images, scanned images were reconstructed with NRecon (Bruker microCT N.V., Belgium) for attenuation correction, ring artifact reduction, and beam hardening.

After data acquisition, images were aligned in 3D view for vertical orientation with Data Viewer software for accuracy.

Segmentation of Volume of Interest

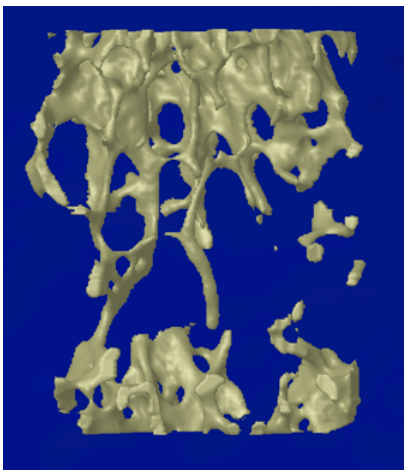
Segmentation of the images will be completed manually by comparing the binarized image with the unsegmented image, and a single global threshold of 60 will be applied. An irregular ROI selection will be manually drawn parallel and close to the endocortical surface.

Ex vivo assessment of bone architecture by micro-CT analysis

Length of the ROI was adjusted in proportion to the total vertebral height. For third lumbar vertebrae, transverse micro-CT slices were acquired for the entire vertebral body, and trabecular bone was evaluated within the region of 0.5 mm away from the growth plate. To ensure accuracy, each ROI were drawn manually in a sequential manner for each trans-axial micro-CT slice. Morphometric parameters were computed from the binarized images using a direct three-dimensional approach that does not rely on any prior assumptions about the underlying structure. For trabecular morphology, assessment of bone volume fraction (BV/TV %), trabecular thickness (Tb. Th, mm), trabecular number (Tb. N, mm), and trabecular separation (Tb.Sp, mm) were used. All analyses were performed with CTAn software (Bruker microCT N.V., Belgium).

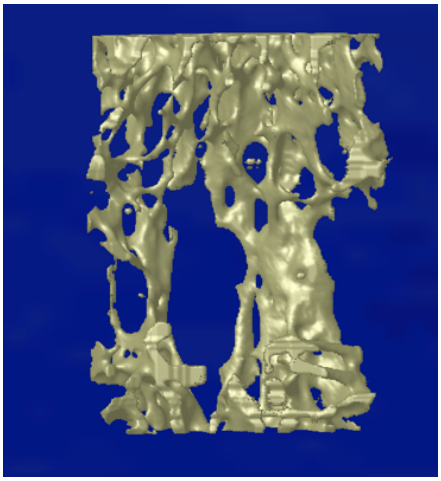
A 3D rendered model of lumbar vertebrae was constructed by CTVol software (Bruker microCT N.V., Belgium). One representative sample was taken from each group. Comparison of the 3D rendered volume was performed to show differences in trabecular structure of the treated group and the control group. Figures 5 and 6.

Figure 5



MP2

Figure 6



MP2+NK+AJ2

Figures 5 and 6: Representative images of MP2 and MP2+NK+AJ2

Histology and quantitative histomorphometry

Static histomorphometry was carried out on hu-BLT mice. Third lumbar vertebras (L3) were dissected, fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Frontal sections, 5 µm thick and stained with 0.1% toluidine blue, pH 6.4.

Static parameters of bone formation (OB) and resorption (OC) were measured in a defined area between 0.25mm from both growth plates and endochondral bone surfaces. Additional

histochemical stain tartrate-resistant acid phosphatase (TRAP) was performed to identify osteoclasts.

Statistical Analysis

For the micro-CT results we used Linear Mixed Effects Models to determine if there were any differences between the comparisons (as difference in means between the groups) in the table. We choose a linear model because the outcome is on an interval scale (as opposed to a categorical scale) and we used a mixed effects model to account for the correlation between the different outcomes within each mouse.

For the rest of the procedures, we performed an unpaired, two-tailed Student t-test for the statistical analysis. One-way ANOVA using Prism-7 software (Graphpad Prism, San Diego, CA, USA) was used to compare different groups. [1] denotes the number of mice used for each condition in the experiment. The following symbols represent the levels of statistical significance within each analysis, *** (p-value <0.001), ** (p-value 0.001–0.01), * (p-value 0.01–0.05).

Chapter 1: **Determine the impact of MP2 tumors, super-charged NK cells and AJ2 on the secretion of IFN Gamma and its association with bone loss.**

Background

We have previously demonstrated that the stage of differentiation of tumors has a profound effect on NK cells' function and that stemlike / poorly differentiated tumors were preferentially targeted by the NK cells. Also, we determined the role of super-charged NK cells in immune mobilization, lysis, and differentiation of stemlike/ undifferentiated tumors implanted in the pancreas of humanized-BLT (hu-BLT) mice fed with or without AJ2 probiotics.

Stem-like/undifferentiated pancreatic tumors grew rapidly and formed large tumors, and exhibited lower expression of above-mentioned differentiation antigens in the pancreas of hu-BLT mice. Unlike stemlike/undifferentiated tumors, NK differentiated MP2 (MiaPaCa-2) tumors or patient-derived differentiated tumors could not grow or grew smaller tumors and were unable to metastasize in hu-BLT mice, and they were susceptible to chemotherapeutic drugs. Stem-like/undifferentiated pancreatic tumors implanted in the pancreas of hu-BLT mice and injected with super-charged NK cells formed much smaller tumors, proliferated less, and exhibited differentiated phenotype. When the differentiation of stemlike tumors by the NK cells was prevented by the addition of antibodies to IFN-γ and TNF-α, tumors grew rapidly and metastasized, and they remained resistant to chemotherapeutic drugs.

More significant numbers of immune cells infiltrated the tumors of NK-injected and AJ2-probiotic bacteria fed mice. Moreover, increased IFN-γ secretion in the presence of decreased IL-6 was seen in tumors resected and cultured from NK-injected and AJ2 fed mice. Tumor-induced decreases in NK cytotoxicity and IFN-γ secretion were restored/increased within PBMCs, spleen, and bone

marrow when mice received NK cells and were fed with AJ2. Therefore, we concluded that NK cells prevent the growth of pancreatic tumors through lysis and differentiation, thereby curtailing the growth and metastatic potential of stem-like/undifferentiated-tumors [75]

Aim 1 Results

Hu-BLT NK cells cultured with osteoclasts, expanded greatly and increased secretion of IFN-γ

Hu-BLT NK cells purified from the spleen of mice responded to the activation signals provided by the IL-2 and anti-CD16 mAb treatment and expanded greatly and demonstrated increased secretion of IFN-γ when cultured with both autologous and allogeneic osteoclasts in the presence of sAJ2 treatment (Figure 7 A-B), indicating close similarity between hu-BLT and human donor derived NK cell expansion and function by osteoclasts.

AJ2 and super-charged NK cells increased the levels of IFN-γ in the serum of the hu-BLT mice

Sera from the peripheral blood of either NK injected or NK injected/AJ2 fed tumor-bearing mice exhibited 2.73 and 4.8-fold more IFN-γ, respectively, when compared to tumor-bearing mice (Figure 8 and 9).

Mice with implantation of the tumor in the absence of any treatment had the least amount of IFN-γ in the sera (Figure 8 and 9).

Feeding AJ2 alone, or injecting super-charged NK cells in the absence of tumor implantation, or feeding AJ2 with implantation of tumors, or injecting super-charged NK cells and feeding AJ2 all increased the levels of IFN-γ in the serum of the hu-BLT mice moderately when compared to control mice in the absence of any treatments. These mice had much less IFN-γ in the sera when compared to those which were implanted with the tumor and fed with AJ2 and injected with super-charged NK cells (Figure 9).

On average, a decrease in IFN-γ secretion from the pancreatic cell cultures could be observed in

mice implanted with MP2 tumors, when compared to control mice with no tumors.

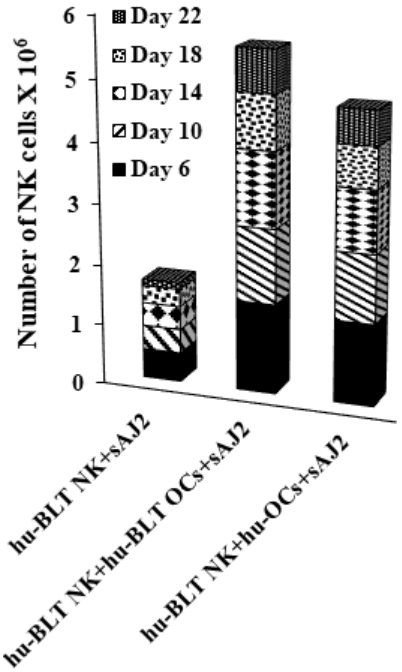
Injection of NK cells into tumor-bearing mice restored IFN-γ secretion in pancreatic cell cultures and the levels exceeded those seen in the control mice with no tumors.

Although slight differences could be seen between NK alone injected or NK-injected and AJ2 fed mice in terms of tumor weight/tumor growth in pancreas, there was, on average, higher secretion of IFN-γ by NK injected and AJ2 fed pancreatic cell cultures (Figure 10).

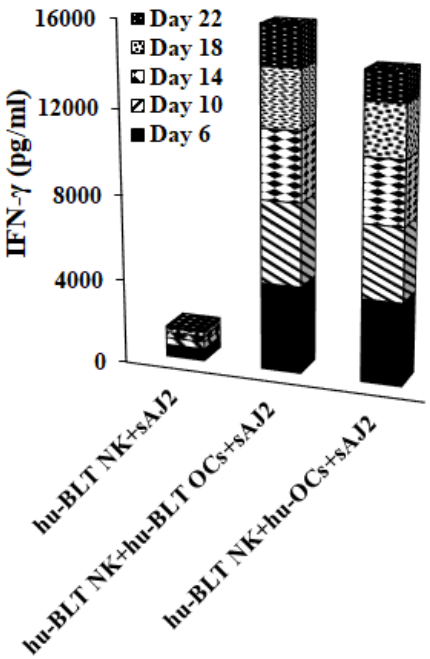
Blocking MP2 differentiation with anti-IFN-γ and anti-TNF-α antibodies resulted in the inhibition of tumor differentiation and generation of tumors with higher tumor weights. (Fig 11).

Figures 7 A-B

A



B



Figures 7 A-B: Phenotypic characteristics of bone marrow, spleen, peripheral blood, pancreas in hu-BLT mice. Lack of tumor growth, metastasis and long-term survival of NSG mice after orthotopic implantation of NK-supernatant differentiated MP2 tumors in pancreas. MP2 tumors were differentiated by the NK-supernatants. Patient-derived differentiated PL12 (2 × 106) (n = 3), NK-differentiated MP2 tumors (diff-MP2) (5 × 105) (n = 3), and MP2 tumors (3 × 105) (n = 3) were implanted into the pancreas of NSG mice.

OCs were generated from hu-BLT bone marrow monocytes and human peripheral blood monocytes. **NK cells purified from hu-BLT splenocytes** were pre-treated with IL-2 (1000 U/mL) and anti-CD16mAb (3 μg/mL) for 18 hours and then either cultured alone or with hu-BLT-OCs or human OCs in the presence of sAJ2 (NK: OCs: sAJ2; 2:1:4) and the numbers of expanding NK cells were counted on days 6, 10, 14, 18 and 22. At each day of culture equal numbers of NK cells from each group were cultured and cell growth determined (A). The supernatants from the NK cells and OCs cultures in the presence of sAJ2, were harvested on days 6, 10, 14, 18 and 22, the levels of IFN-γ were determined using single ELISA [1].

Figure 8: Single injection of super-charged NK-cells with/without feeding with AJ2 inhibited tumor growth due to differentiation of tumors in hu-BLT mice.

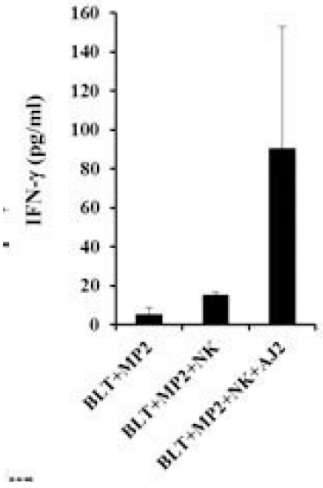


Figure 8: Implantation of tumor cells in the pancreas and tail vein injection of super-charged NK cells were carried out, and at the time of sacrifice mice were bled and the levels of IFN-γ in the serum were determined using multiplex array (n = 3)

Figure 9

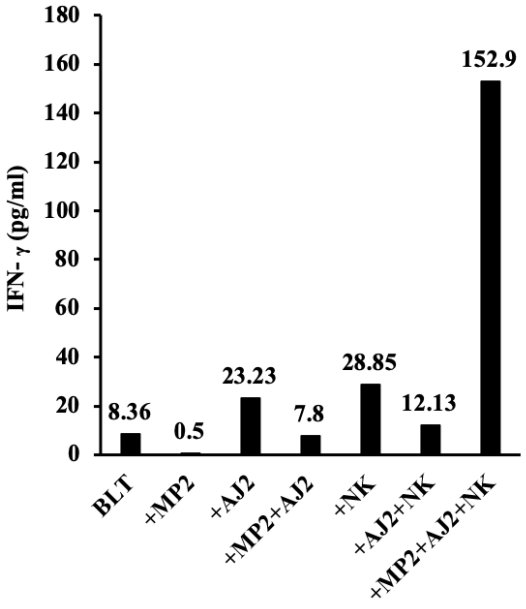


Figure 9: Single injection of super-charged NK-cells with/without feeding with AJ2 inhibited tumor growth due to differentiation of tumors in hu-BLT mice. Hu-BLT mice were implanted with 1 × 106 tumor cells in the pancreas, and after 1–2 weeks mice received 1.5 × 106 super-charged NK cells via tail vein injection, and disease progression was monitored for another 3–5 weeks. Mice were also fed AJ2 (5 billion/dose) starting 1–2 weeks before tumor implantation, and thereafter every 48 h throughout the experiment. Implantation of tumor cells in the pancreas and tail vein injection of super-charged NK cells were carried out, and at the time of sacrifice mice were bled and the levels of IFN-γ in the serum were determined using multiplex array (n = 3)

Figure 10

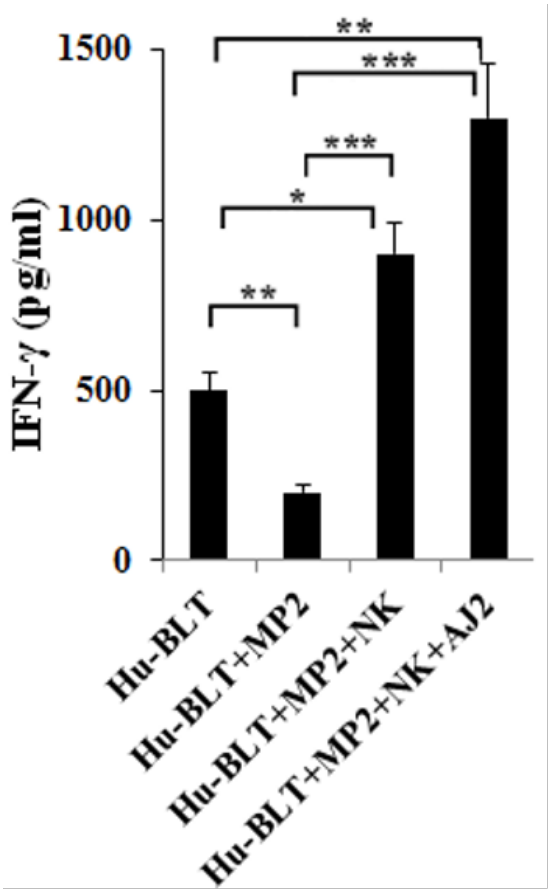


Figure 10: Hu-BLT mice were implanted with MP2 tumors and injected with NK cells and fed with AJ2. Upon sacrifice, tumors were resected, and single **cell cultures** were prepared, and equal numbers of tumors were cultured on day 7 or 11 and the levels of IFN-γ were determined in culture supernatants (n = 2/each experimental condition).

Figure 11

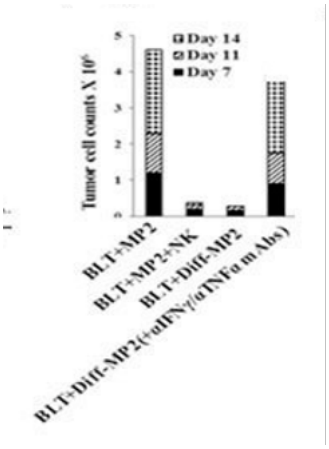


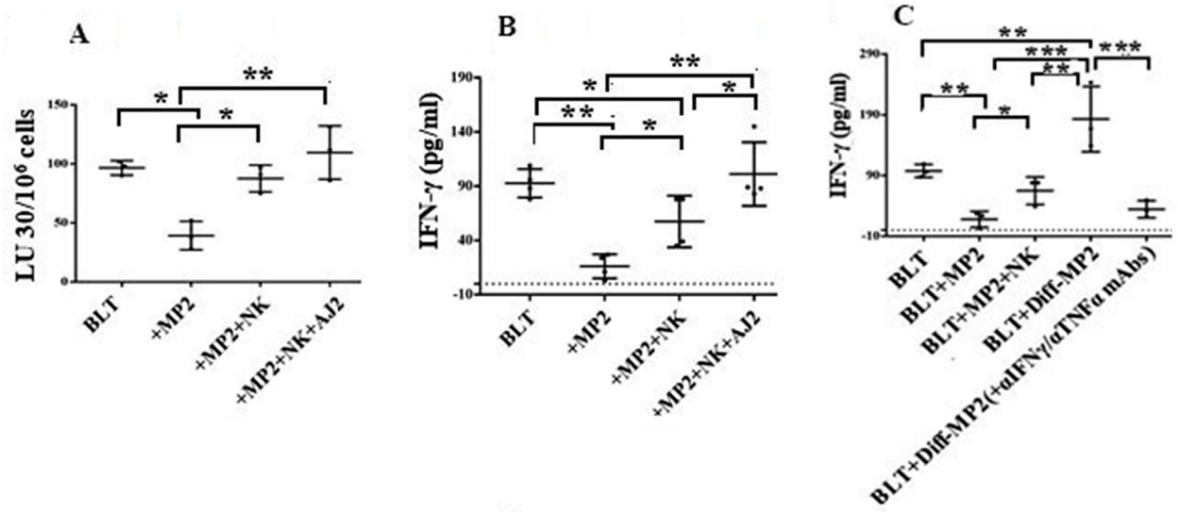
Figure 11: Hu-BLT mice were implanted with MP2 tumors and injected with NK cells or implanted with NK-differentiated tumors. At the end of the experiment pancreatic tumors were harvested and tumor growth was assessed on days 7, 11 and 14, and on day 7 attached tumors from each well were counted and equal numbers of tumors from each group were re-cultured and tumor growth in each well was determined every 3 days (n = 12/each experimental condition, one representative experiment is shown in the figure).

Suppression of NK Cell Cytotoxicity and Decreased Secretion of IFN-γ in Tumor-Bearing Mice within All Tissue Compartments; Restoration by Super-Charged NK Cells

PBMCs from tumor-bearing mice which were similar to PBMCs and NK cells from pancreatic cancer patients, had significantly lower NK cell-mediated cytotoxicity and exhibited decreased IFN-γ secretion, when compared to those from healthy mice or humans, respectively. (Figure 12 A-C)

Tumor-bearing mice had much lower cytotoxicity and/or secretion of IFN-γ in cells obtained from all tissue compartments, in comparison to those obtained from control mice without tumor, or tumor-bearing mice injected with NK cells, or those implanted with NK-differentiated tumors (Figure 13).

Figure 12 A-C



Figures 12 A-C: NK cell cytotoxicity and ability to secrete IFN-γ is severely decreased in pancreatic cancer patients

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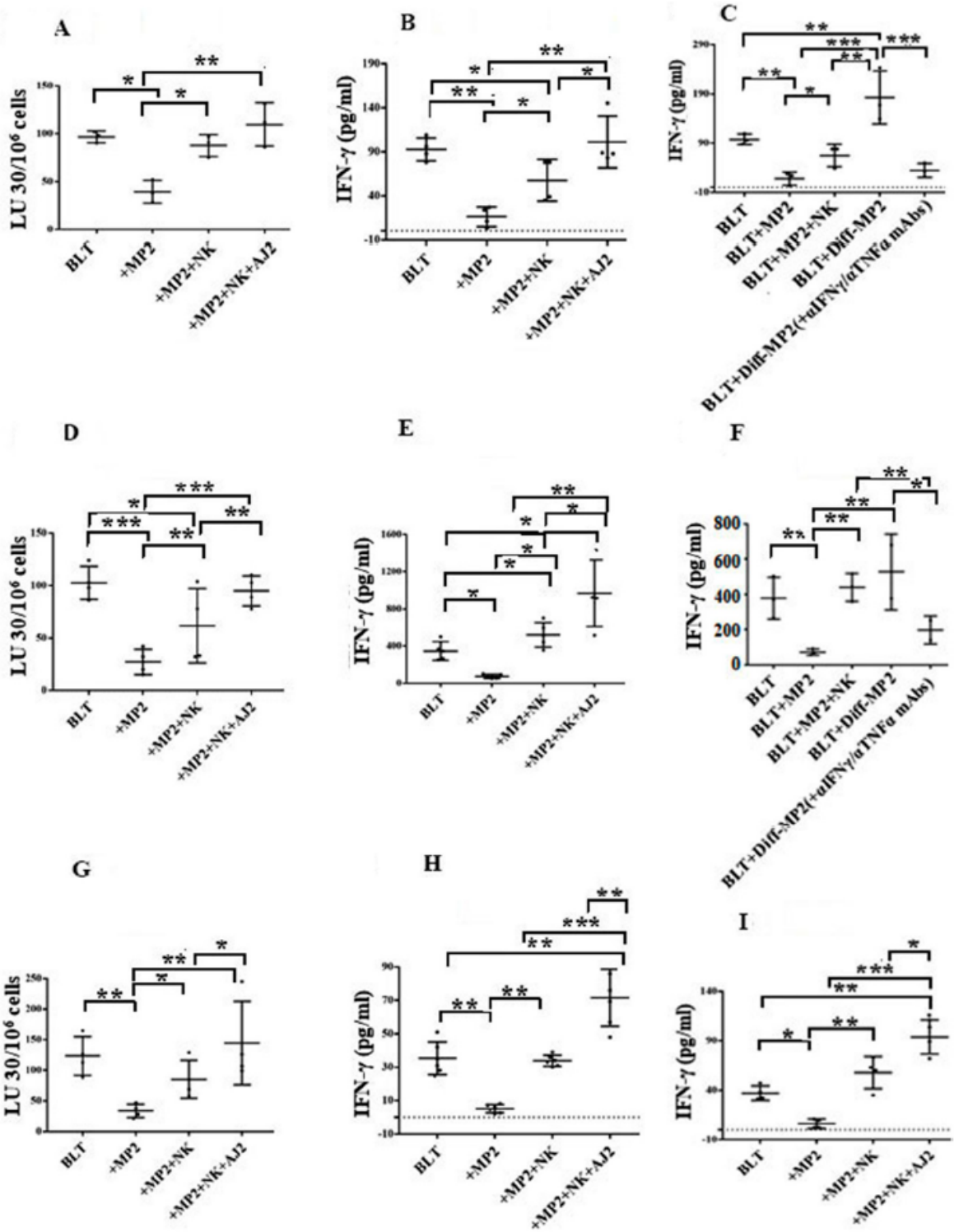
Figure 13 A-L


A
LU 30/10⁶ cells
BLT
+MP2
+MP2+NK
+MP2+NK+AJ2
B
IFN-γ (pg/ml)
C
IFN-γ (pg/ml)
BLT+Diff-MP2(+αIFNγ/αTNFα mAbs)
D
E
F
G
H
I

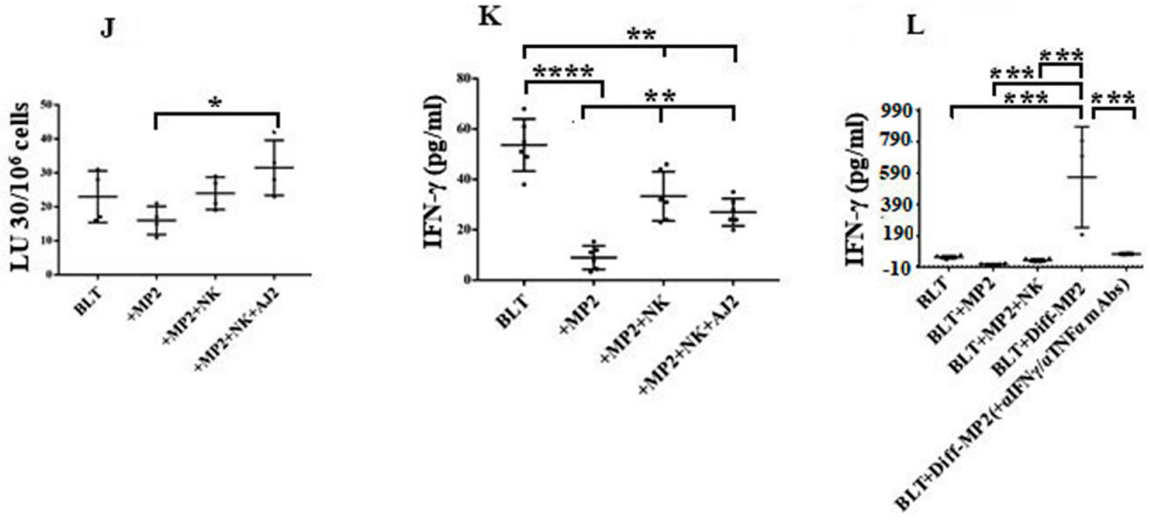

J
LU 30/10⁶ cells
*
BLT
+MP2
+MP2+NK
+MP2+NK+AJ2
K
IFN-γ (pg/ml)
**
****
**
BLT
+MP2
+MP2+NK
+MP2+NK+AJ2
L
IFN-γ (pg/ml)
***
***
***
***
990
790
590
390
190
-10
BLT
BLT+MP2
BLT+MP2+NK
BLT+Diff-MP2
BLT+Diff-MP2(+αIFNγ/αTNFα mAbs)

Figure 13 A-L. Injection of super-charged NK-cells with/without feeding with AJ2 restored and increased IFN-γ secretion and/or cytotoxic function of NK cells from different tissues of tumor-bearing hu-BLT mice. Upon sacrifice, PBMCs were isolated from blood and treated with IL-2 (1000 U/mL) before they were used in cytotoxicity assay against OSCSCs using 4 h 51Cr release assay. Lytic units 30/106 cells were determined using inverse number of NK cells required to lyse 30% of the target cells × 100. Procedures were carried out before the PBMCs were isolated and treated with (1000 U/mL) and the supernatants were harvested and IFN-γ secretion was determined using ELISA (n = 4/each experimental condition) (B,C). Procedures were carried out before spleens were harvested, and single-cell suspensions were prepared. Splenocytes were treated with IL-2 (1000 U/mL) before they were used for cytotoxicity against OSCSCs using 4 h 51Cr release assay. Lytic units 30/106 cells were determined using inverse number of NK cells required to lyse 30% of the target cells × 100 (n = 4/each experimental condition) [1]. Procedures were carried out as previously described before the supernatants were harvested from day 3 or 7 cultures of splenocytes, and IFN-γ secretion was determined using ELISA (n = 5/each experimental condition). NK-enriched cells were isolated from splenocytes and were cultured with IL-2 (1000 U/mL) before they were used for cytotoxicity against OSCSCs using 4 h 51Cr release assay.

Supernatants were harvested from day 3 or 7 NK-enriched cultures and IFN-γ secretion was determined using ELISA (n = 6/each experimental condition) [1]. The CD3+ T-cells were isolated from splenocytes and were cultured with IL-2 (100 U/mL), and on day 3 or day 7 the supernatants were harvested and IFN-γ secretion was determined using ELISA (n = 4/each experimental condition) [2]. BM cells were harvested and treated with IL-2 (1000 U/mL) for 7 days before they were used for cytotoxicity against OSCSCs using 4 h 51Cr release assay. Supernatants were harvested on day 3 or day 7 of BM cultures and IFN-γ secretion was determined using ELISA (n = 6/each experimental condition) (K,L). The following symbols represent the levels of statistical significance within each analysis, *** (p-value < 0.001), **

Aim 1 Conclusions

In previous studies, we have shown that NK cells prevent pancreatic tumors' growth through lysis and differentiation, thereby curtailing the growth and metastatic potential of stem-like/undifferentiated-tumors. Also, we demonstrated that NK cells induced differentiation of MP2 tumors through the functions of IFN-γ and TNF-α.

In this study, supercharged NK cells in the presence and absence of feeding AJ2 increased IFN-γ secretion in different compartments of hu-BLT mice; like the serum, cell cultures from pancreatic tumors, NK cells purified from hu-BLT splenocytes, PBMCs, splenocytes cell cultures, and bone marrow. On the contrary, MP2 tumor-bearing hu-BLT mice showed decreased IFN-γ in the same compartments.

Chapter 2: **To investigate how MP2 tumor-bearing mice injected with cultures of NK cells and fed with and without AJ2 affect the bone structure**.

Mice fed with AJ2 presented increased bone formation when compared to the CTRL group.

The AJ2 group presented increased bone formation with increased bone volume and trabecular number when compared to the CTRL group. However, there was no statistical difference between AJ2 vs. Control in any of the variables. (Figure 14, A-B).

MP2 tumor-bearing mice injected with cultures of NK cells and fed with AJ2 presented increased bone formation with statistically significant higher trabecular bone volume when compared to MP2 tumor and MP2+AJ2 group, respectively.

MP2 tumor-bearing mice injected with NK cells and fed with AJ2 group showed a statistically significant increase in bone volume fraction (BV/TV), augmented trabecular thickness (Tb.Th),

higher trabecular number (Tb.n), and decreased trabecular spacing (Tb.Sp) when compared to the MP2 tumor group. (Figure 15, A-B).

Similarly, MP2 tumor-bearing mice injected with NK cells and fed with AJ2 group showed a statistically significant increase in bone volume fraction (BV/TV), augmented trabecular thickness (Tb.Th), higher trabecular number (Tb.n), and decreased trabecular spacing (Tb.Sp) when compared to the MP2+ AJ2 group. (Figure 15 A-B)

Stem-like/undifferentiated tumors implanted in hu-BLT mice injected with cultures of NK cells and fed with or without AJ2 presented similar bone formation when compared to the control group.

After six weeks of tumor transplantation, lumbar vertebrae were harvested and scanned with high-resolution micro-CT. Stem-like/undifferentiated tumors implanted in hu-BLT mice injected with NK cells' cultures and fed with or without AJ2 presented similar bone formation compared to the control group. (Figure 16, A-B).

Figure 14 A-B: CTRL vs AJ2

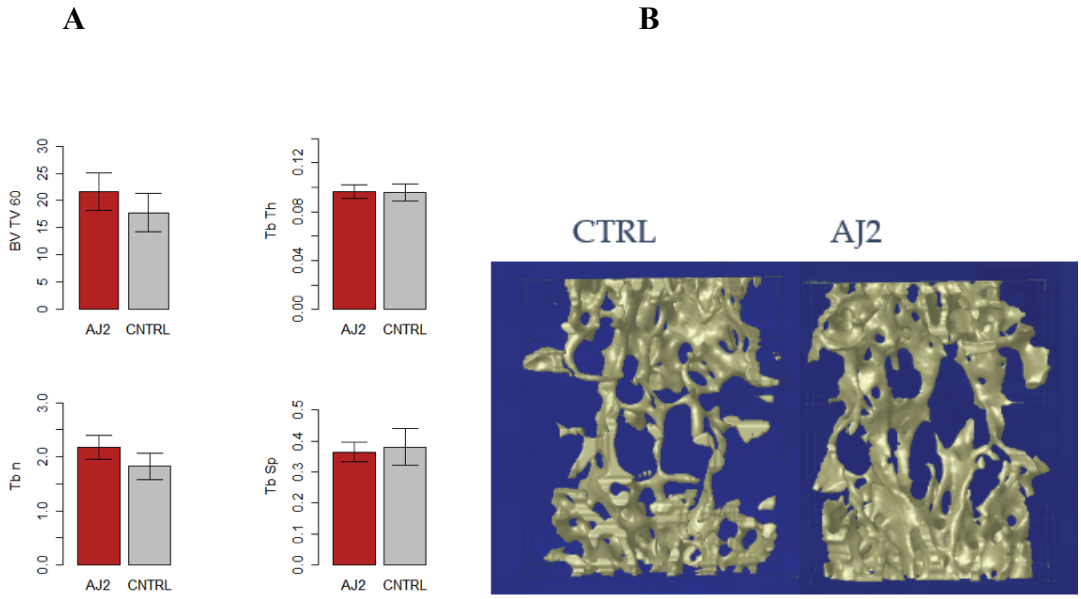


Figure 14 A-B: Mice fed with AJ2 presented increased trabecular bone formation when compared to the CTRL group

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Figure 15 A-B: MP2 vs **MP2+AJ2** vs **MP2+NK+AJ2**

A **B**

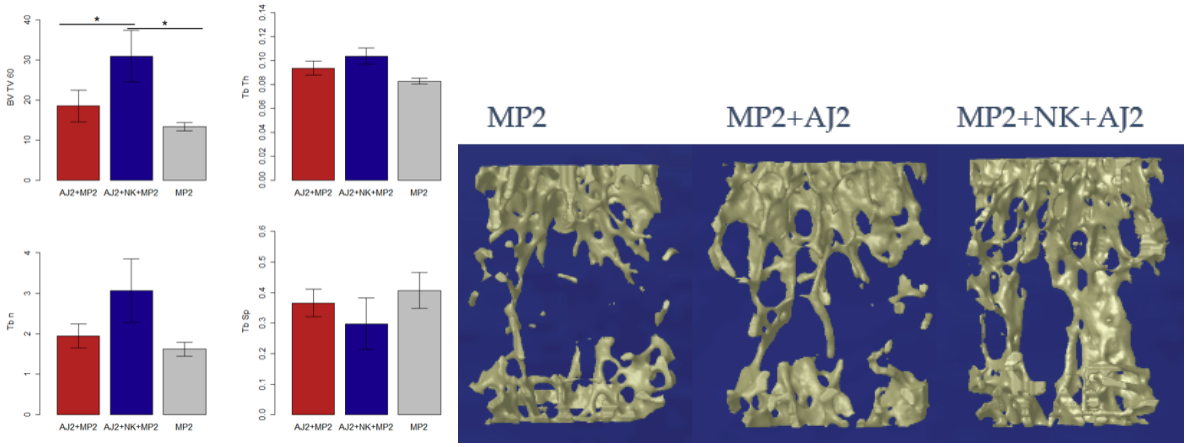


Figure 15 A-B: MP2 tumor-bearing mice injected with NK cells and fed with AJ2, presented statistically significant higher trabecular bone volume when compared to MP2 tumor and MP2+AJ2 group, respectively.

Figure 16 A-B

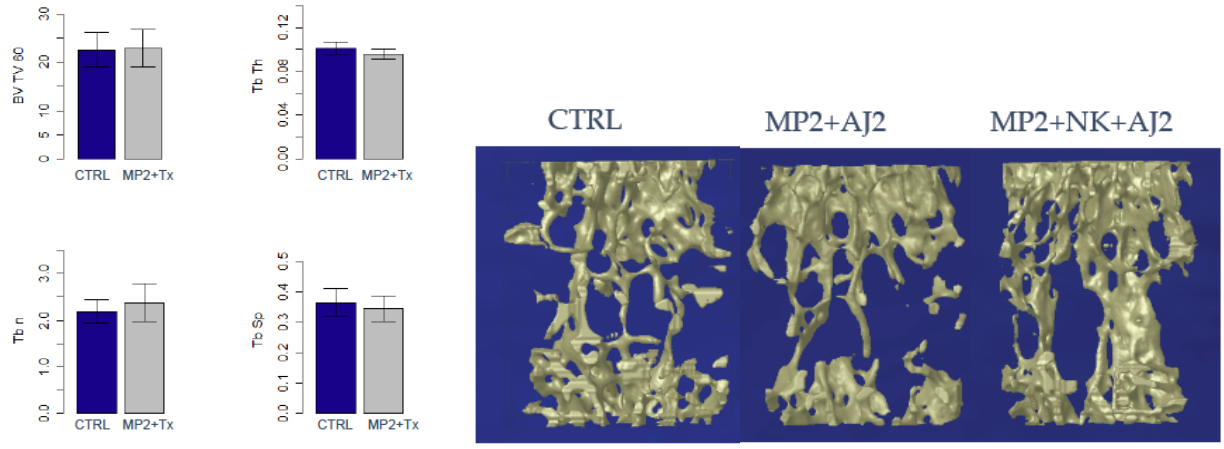
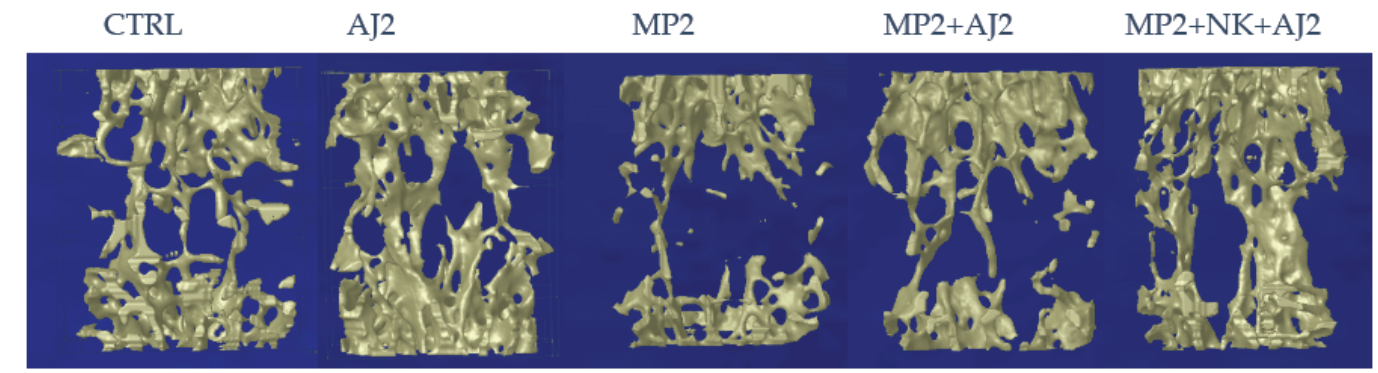


Figure 16 A-B: Stem-like/undifferentiated tumors implanted in hu-BLT mice injected with cultures of NK cells and fed with or without AJ2 presented similar bone formation when compared to the control group.

Figure 17



	CTRL	AJ2	MP2	MP2+AJ2	MP2+NK+AJ2
Serum IFN -g (pg/ml)	8.36	23.23	0.5	7.8	152.9
IL-6 in tumor	11±6.6		716±487		23.7±9.3
IFN-g in tumor	495±68		179.5±19		1390±130
PBMCs	93.5±18		18±8.6		98±29
Splenocytes	354±126		136.6±28.6		944.9±346
NK cells	38±10		5.4±1.8		65.6±15
Bone marrow	55±12		10±5.8		31±6.56

Figure 17: There was a remarkable correlation between induction and secretion of IFN-γ and the bone morphology. In the MP2 tumor-bearing mice injected with NK cells and fed with AJ2 group there was increased IFN-γ in serum, cell cultures from pancreatic tumors, NK cells purified from splenocytes, PBMCs, splenocytes cell cultures, and bone marrow cells. On the contrary, MP2 tumor-bearing hu-BLT mice showed decreased IFN-γ in the same compartments.

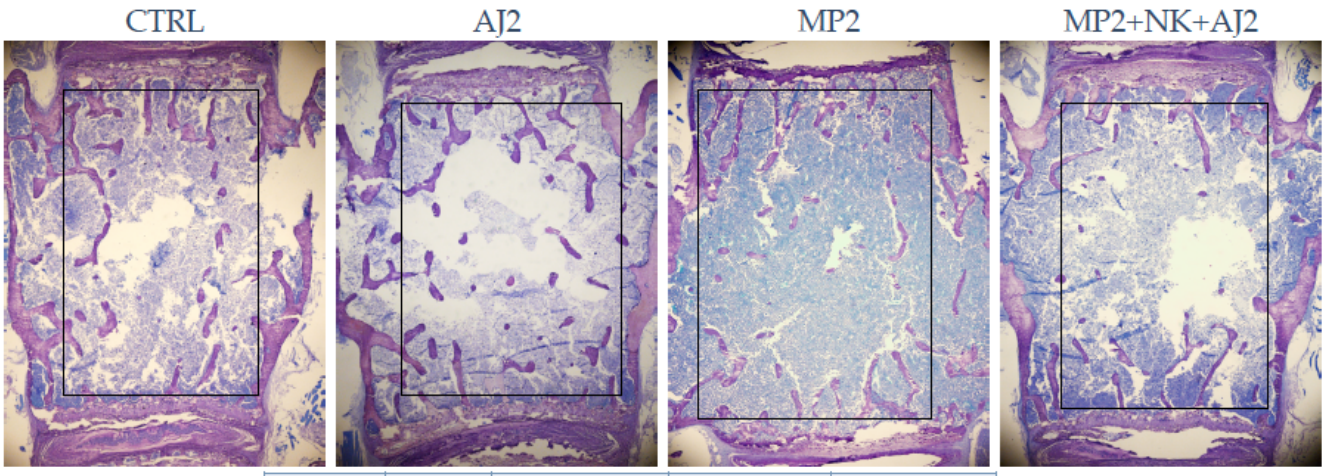
Histological, immunohistochemical analysis, and static indices assessments at the third lumbar vertebra exhibited an increase of trabecular bone formation in the AJ2 treated group when compared with the Control and MP2 groups.

To assess the bone remodeling process's underlying cellular mechanisms, histological analysis and static indices assessment at the third lumbar vertebra were performed.

Consistent with the micro-CT findings, histological analysis of the AJ2 treatment group exhibited an increase in bone volume fraction (BV/TV), augmented trabecular thickness (Tb.Th), higher trabecular number (Tb.n), and decreased trabecular spacing (Tb.Sp) when compared to the Control and MP2 tumor groups. The histomorphometric values for the MP2+NK +AJ2 sample could not be confirmed due to staining failure. (Figure 18)

For the static parameters of bone resorption (OC), the MP2 tumor sample was positive for TRAP staining. On the contrary, the control, AJ2, and MP2+NK+AJ2 samples were negative for TRAP staining. These results are in accordance with the micro-CT results, suggesting more osteoclast activity and bone resorption in the MP2 tumor sample when compared to the control, AJ2, and MP2+NK+ AJ2 samples. (Figure 19).

Figure 18



	BV/TV	Tb.Th	Tb.Sp	Tb.N
MP2	8.2565	31.0183	344.667	2.6618
CTRL	11.1283	35.841	286.2289	3.1049
AJ2	12.394	33.9481	239.9596	3.6509

Figure 18: Histological analysis of the AJ2 sample exhibited increased bone formation when compared to the Control and MP2 samples. The histomorphometric values for the MP2+NK +AJ2 sample could not be confirm due to staining failure.

Figure 19

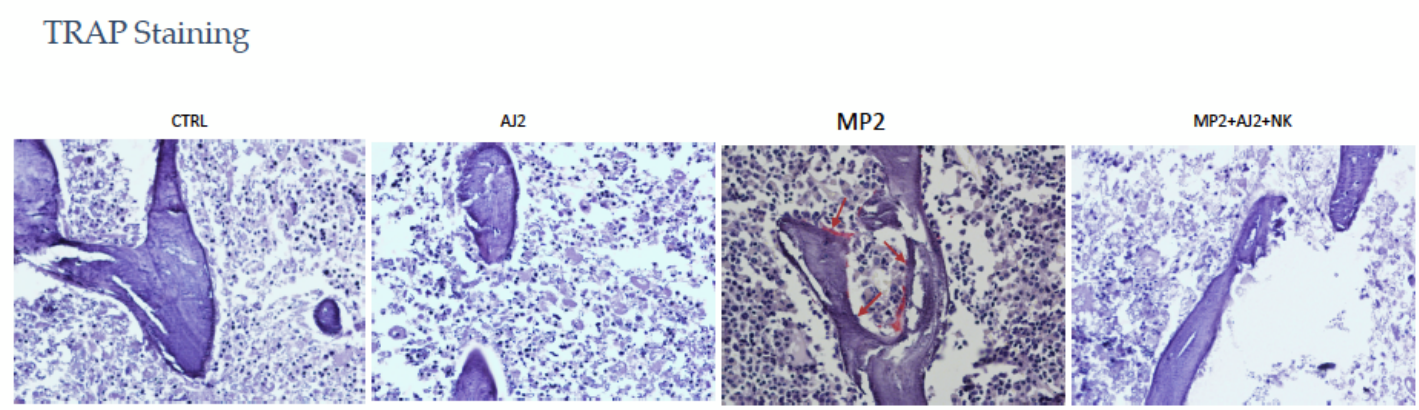


Figure 19: The MP2 tumor sample exhibits positive TRAP staining.

Aim 2 Conclusions

We found a remarkable correlation between the 3D images generated from the micro-CT results, the induction and secretion of IFN-γ, and bone morphology. In the tumor-bearing mice injected with NK and fed with AJ2, there was increased IFN-γ secretion in different compartments like the serum, cell cultures from pancreatic tumors, NK cells purified from splenocytes, PBMCs, splenocytes cell cultures, and bone marrow cells. Consistently, the MP2 tumor-bearing mice injected with NK cells' cultures and fed with AJ2 presented increased bone formation with

statistically significant higher trabecular bone volume compared to MP2 tumor-bearing mice group and MP2 tumor-bearing mice fed with AJ2 group, respectively.

On the contrary, MP2 tumor-bearing mice showed reduced bone formation and decreased IFN-γ secretion in the same compartments. (Figure 17). Accordingly, MP2 tumor-bearing mice group presented statistically significant reduced trabecular bone volume compared to the treatment groups.

Consistent with the micro-CT findings, histological analysis of the AJ2 treatment group exhibited increased bone formation when compared to the Control and MP2 tumor groups. TRAP staining suggested more osteoclast activity and bone resorption in the MP2 tumor sample compared to the rest of the samples.

Discussion

The discovery of the association between immune system and skeletal system has led to profound progress in our understanding of osteoclastogenesis. Accumulating evidence suggests that IFNs play critical roles in regulating osteoclast formation and bone resorption.

-Interferons play essential roles in regulating osteoclast differentiation and bone resorption. Over the last decade, we have seen tremendous developments in our understanding of the mechanisms by which interferons regulate osteoclastogenesis (, #103).

IFN-γ is a cytokine produced by innate and adaptive cells in the immune system [42] as well as by MSC [36]. IFN-γ interacts with its receptors expressed constitutively to promote cell signaling and transcription through Jak1/STAT1 pathway [93]. Its production is regulated by cytokines (positively, IL-12 and IL-18; negatively, IL-4, IL-10, transforming growth factor-b, and

glucocorticoids) to exert antiviral activity (principally) and both directly and indirectly affecting a number of other biological pathways [93]. In terms of osteoimmunology, IFN-γ plays an important role in the regulation of both bone formation and bone resorption in a dose-dependent manner [94] [95]. Depending on the dose and the experimental model, IFN-γ could regulate osteoclastogenesis and osteoclastic activity either positively [21] [44] [96], or negatively [43]. In addition, IFN-γ is required in early phases of osteoblastogenesis [37] while treatment with low doses of exogenous IFN-γ induces osteoblastogenesis both in vitro [37] and in vivo [37].

Vidal et al, reported that the anabolic effect of low doses of IFN-γ on bone is quite consistent [36, 37], thus suggesting that IFN-γ could become a useful therapeutic approach to bone loss in the near future. However, to understand the potential therapeutic effect of IFN-γ on osteoporosis and bone loss, it is essential to assess its effect on other cellular components of the bone marrow milieu and specifically on marrow fat.

IFN-γ has been shown to regulate bone cell differentiation and function with complex effects on skeletal health. However, the role of IFN-γ in pathological bone disease is controversial. Previously, it has been reported that IFN-γ can inhibit the critical osteoclast regulator, receptor activator of NFkB ligand (RANKL), by activating ubiquitin-mediated degradation of its signaling pathway adaptor protein TRAF-6 (7,8). Mice deficient for IFN-γ or its receptor develop enhanced bone loss associated with collagen induced arthritis (CIA) [97] [98].

Also, it was reported that IFN-γ can stimulate superoxide production in osteoclasts, leading to enhanced osteoclast apoptosis [99].

In contrast, Gao et al. found that IFN-γ indirectly stimulates osteoclast formation and bone loss after ovariectomy via antigen-driven T cell activation, resulting in the production of osteoclast activating factors [100]. Interestingly, IFN-γ has been used to treat infantile osteopetrosis in which

patients suffered from high bone mass secondary to osteoclast dysfunction or osteoblast hyperactivity, but the mechanism of action may be through modulation of the host immune system immunity rather than direct effects on bone cells [101] [102] [103].

Kohara et al. demonstrated that IFN-γ suppressed TNF-alpha induced osteoclastogenesis by decreasing nuclear entry of NFATc1, and stimulating Fas/FasL apoptosis signaling in vivo and in vitro [104].

Taken together, these studies suggest that IFN-γ plays a critical role in osteoclastogenesis under conditions of inflammation or over-activated T-cells. IFN-γ can either inhibit osteoclast formation or enhance osteoclastogenesis according to the conditions to which the osteoclast precursors are exposed. (, #103)

Other reports suggest that mice lacking an intact IFN-γ signaling pathway are more susceptible to collagen-induced arthritis [105] [106]and associated bone loss.

Xu et al, indicates that IFN-γ has two modes of action in experimental models of bone metastases. First, IFN-γ can inhibit tumor growth through direct effects on tumor cells. Secondly, IFN-γ decreases skeletal complications of malignancy by directly acting on host cells to modulate osteoclast function. [107]

More recently, other studies have shown the pro-osteogenic effects of IFN-γ in vitro and in vivo [36] [37].

The mechanisms accounting for the various cell-type specific responses to IFN-γ are not well understood. For example, cell-type specific expression of downstream target genes may dictate IFN-γ responses. Additionally, mutations in many components of the IFN-γ signaling pathway have been identified in tumor cells, many having consequences on IFN-γ responsiveness. [108, 109] [110].

Xu et al, confirmed that IFN-γ directly inhibits osteoclastogenesis in vitro and that this inhibitory effect is mediated by IFN-γ induced increased degradation of TRAF-6 protein in RANKL-stimulated bone marrow-derived macrophages [21] [111]. In vivo, however, the role of IFN-γ in osteoclast biology and pathological bone diseases remains controversial. Mice lacking an intact IFN-γ signaling pathway are more susceptible to collagen-induced arthritis [112] [113] [114] and associated bone loss. This could be mediated indirectly through the host immune system rather than by direct targeting of OCs. This study also reported that unlike patients with autoimmune diseases such as rheumatoid arthritis and T cell-mediated bone loss in postmenopausal osteoporosis [115] [116] [117], many cancer patients, particularly those with advanced disease are in a immunosuppressive state [118] [119] [120]. Therefore, we hypothesize that the diminished skeletal health and increased tumor associated bone loss observed in advanced stage cancer patients could be due to a lack of IFN-γ mediated inhibition of both osteoclasts and tumor cells.

Previous studies found that IFN-γ negatively regulates M-CSF-mediated proliferation and survival of osteoclast progenitors. This is consistent with observations by Xaus et al [121].

The phenomenon of bone resorption is very complex and has been coordinated by cellular and hormonal factors at in vivo conditions. IFN-γ inhibits bone resorption and has an inhibitory effect on osteoclasts at the level of differentiation [98] [100-102] [103]. In addition, IFN-γ is an important control mechanism in osteoclastogenesis. [100] [122] [123] [124, 125].

Duque et al, indicated that there is indirect evidence linking IFN-γ with osteoblastogenesis. Disruption of nitric oxide (NO) signaling in mice results in defective bone formation. [126] [127]. Because IFN-γ, together with interleukin 1 (IL-1), stimulates NO production to high levels in bone, [128] [129] these earlier studies support a role for IFN-g in bone formation. Furthermore, IFN-γ induces expression of Best5, a gene expressed during bone formation in rats. [101]. This data

strongly supports a physiologic role of IFN-γ for maintenance of skeletal integrity and suggest that modulation of its signaling pathway may be used advantageously to improve bone strength.

Mermut et al, revealed that IFN-γ inhibits bone resorption stimulated by interleukin (IL)-1 and blocks collagenase production caused by parathyroid hormone, prostaglandin E2, 1.25(OH)2 vitamin D3, IL-1, tumor necrosis factor-alpha, and epidermal growth factor [31].

The pro-osteogenic effect of IFN-γ has been previously reported both in vitro and in vivo [36] [37].

In our study, there was a remarkable correlation between induction and secretion of IFN-γ and increase bone formation. In the tumor-bearing mice injected with NK and fed with AJ2, there was increased IFN-γ in different compartments and augmented bone formation. On the contrary, MP2 tumor-bearing mice showed decreased IFN-γ and decreased bone formation in the same compartments. Therefore, we can hypothesize that IFN-γ induce secretion by NK cells, can inhibit tumor growth, and decreases skeletal complications of malignancy by directly acting on host cells to inhibits osteoclast formation and function.

Conclusions

This is a novel report of the role of NK cells and AJ2 in the treatment and prevention of tumor growth and their role in increasing the levels of IFN-γ in the hu-BLT mice suppressing tumor-induced bone loss.

We previously demonstrated that NK cells prevented the growth of pancreatic tumors through lysis and differentiation, thereby curtailing the growth and metastatic potential of stem-like/undifferentiated-tumors through the functions of IFN-γ and TNF-α. Increased IFN-γ secretion

in the presence of decreased IL-6 was seen in tumors resected and cultured from NK-injected, and AJ2 fed mice.

IFN-γ is a multifunctional cytokine produced mainly by NK cells and activated T cells that play a critical role in host immune responses against pathogens and cancer.

Hu-BLT NK cells cultured with osteoclasts expanded greatly and increased secretion of IFN-γ. Hu-BLT NK cells were purified from the spleen of mice, responded to the IL-2 and anti-CD16 mAb treatment's activation signals, expanded greatly and demonstrated increased secretion IFN-γ when cultured with both autologous and allogeneic osteoclasts in the presence of sAJ2 treatment. AJ2 and super-charged NK cells increased the levels of IFN-γ in the serum of the hu-BLT mice. On average, an increase in IFN-γ secretion from the pancreatic cell cultures could be observed in mice implanted with MP2 tumors, injected with NK and fed with AJ2 when compared to mice implanted with MP2 tumors and control mice.

We demonstrated the suppression of NK cell cytotoxicity and decreased secretion of IFN-γ in tumor-bearing mice within all tissue compartments and restoration by super-charged NK Cells. We found a remarkable correlation between the 3D images originated from the micro-CT analysis results, the induction and secretion of IFN-γ, and bone morphology. In the tumor bearing mice, injected with NK and fed with AJ2 we found increased IFN-γ in different compartments like the serum, cell cultures from pancreatic tumors, NK cells purified from splenocytes, PBMCs, splenocytes cell cultures, and bone marrow cells. On the contrary, MP2 tumor-bearing mice showed decreased IFN-γ in the same compartments. Besides, there was a decreased bone formation and more production of IL-6 in the MP2 group.

In this study, the overall effect of decreased IFN-γ secretion significantly reduced bone volume, indicating that deficiency in IFN-γ results in a depletion in bone formation. Assessment of bone

architecture with 3D micro-CT analysis, histology, and histomorphometry demonstrated that MP2 tumor mice with decreased IFN-g secretion had a significant deficit in trabecular bone.

Our results suggest that decreased IFN-γ secretion results in lower osteoblast differentiation of MSCs, a significant reduction in bone volume, indicating that deficiency in IFN-γ results in a depletion in bone formation. This study outcome also suggests that IFN-γ has direct anti-tumor effects and suppresses tumor-induced bone loss by directly targeting host OCs to inhibit osteolysis. Also, there was correspondence between increased IFN-γ induction and secretion, and increased bone formation presented in the 3-D images generated from the micro-CT analysis results. Consistent with the micro-CT findings, histological analysis of the AJ2 treatment group exhibited increased bone formation when compared to the Control and MP2 tumor groups. TRAP staining suggested more osteoclast activity and bone resorption in the MP2 tumor sample compared to the rest of the samples.

In our study, the enhanced osteolytic lesion formation in BLT tumor-bearing mice and NK cells' ability to secrete IFN-γ to significantly reduce bone loss in tumor-bearing mice strongly supports a direct anti-osteoclastogenic role for IFN-γ in the setting of cancer-induced bone disease.

Our study corroborates the close interplay between the immune and skeletal systems. Among the cytokines that have been found to regulate osteoclastogenesis, IFN-γ seems to be a critical regulator of bone resorption. In this report, IFN-γ decreased tumor growth and prevented tumor-associated bone loss by inhibiting tumor cell growth and osteolysis.

Our data strongly support the physiologic role of IFN-γ for skeletal integrity maintenance and suggest that modulation of its signaling pathway may be used advantageously to improve bone strength. Besides, our results indicate that IFN-γ suppresses osteoclastogenesis.

Our findings demonstrate the significance of super-charged NK cells and AJ2 in the treatment and prevention of tumor growth and their role in increasing the levels of IFN-γ in hu-BLT mice suppressing tumor-induced bone loss.

This report proposes a protective role for NK cells and IFN-γ that warrants additional research into novel therapeutic treatments for patients with osteolytic malignancies.

A better understanding of the bone marrow microenvironment and processes that influence tumor cell maintenance and growth in the bone should present opportunities for targeting hematopoietic cell populations as part of anticancer therapy.

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