UNIVERSITY OF EASTERN PIEDMONT AMEDEO AVOGADRO

PhD in Molecular Medicine:

Role of the ICOS/B7H system in the bone Microenvironment

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Background: Osteoblasts and osteoclasts are the two major bone cells that are involved in the bone remodelling. Osteoblasts arise from mesenchymal stem cells and are responsible for bone formation; whereas osteoclastic cells have haematopoietic origin and are involved in bone resorption. These two processes of bone formation and bone resorption are crucial to maintain the bone homeostasis. An imbalance leads to bone disorders such as osteopetrosis and osteoporosis respectively. Also auto-immune disease such as Rheumatoid Arthritis (RA) of the bone is due to an imbalance in the pro and anti-inflammatory cytokines which results in chronic inflammation and joint damage. T cells are critical regulators of osteocell differentiation and function in the bone, but whether in turn they can regulate T cell homing and response to stimuli in unclear. A possible way of interaction could be via costimulatory molecules such as ICOS (Inducible Costimulator of T cell). ICOS is a co-stimulatory molecule involved in T-cell activation and proliferation. B7h is the ligand and it is expressed by both haematopoietic and non-haematopoietic cells. The interaction of ICOS: B7h controls T cells function at sites of inflammation.

Aims: During my work, I would be looking into the expression and functional role of B7H on normal and neoplastic osteoclasts and osteoblasts, evaluating the effect of interaction between T-cells and osteo-cells. In particular, I will evaluate the effect of triggering of B7h in proliferation, cytokine secretion, and migratory activity of these cells. Moreover, osteoblast activity will be evaluated by assessing their alkaline phosphatase activity, osteocalcin expression, and mineralization activity. A positive result may lead to the better explanation of the role of the ICOS/B7h system in bone related disease such as osteoporosis and rheumatoid arthritis and open the way to use of drugs interfering with this system in the therapy of these diseases.

INTRODUCTION:

Aron and Choi have very aptly introduced the term Osteoimmunology, wherein they talk about the cells of the immune system and that of bone regulating the functions of each other. Bone is the repository and main source of calcium in human's body; two major cells maintaining the bone homeostasis are osteoblasts (including osteocytes and bone lining cells) and osteoclasts where the formers are involved in the secretion of bone matrix and the latters resorb the bone[1]. The extracellular matrix (ECM) of the bone is subdivided into an inorganic and organic part. The organic matrix is mainly constituted of type I collagen (approximately 95%), as well as other types of collagens, noncollagenous proteins and proteoglycans, whereas the inorganic matrix predominantly contains calcium and phosphorus, appearing as hydroxyapatite crystals ([3Ca 3 (PO 4) 2] (OH) 2) deposited into

the collagenous matrix[2]. An imbalance in these cells leads to bone disorders as in the case of autoimmune inflammatory diseases such as rheumatoid arthritis (RA) where in the rate of resorption exceeds the rate of mineral deposition leading to bone mass loss in the joints[3]. In the mean time, bone marrow is the principle site of immune cell generation and the interaction between the bone cells and the immune cells that co-exist in the same micro-environment is unclear[1].

Osteoblasts originate from the mesenchymal stem cell (MSC) [2, 4]. Core binding factor 1 (Cbfa1, also termed runt related transcription factor 2, runx2) and the downstream factor osterix are crucial transcription factors for lineage commitment and osteoblast differentiation[5, 6]. Osteoblasts function by synthesizing the collagen-rich organic matrix and also provide optimal conditions for matrix mineralization by secreting numerous bone matrix proteins and matrix metalloproteinases (MMP) [7]. Mature osteoblasts express alkaline phosphatase, bone sialoprotein as well as osteocalcin and osteopontin as their cell markers [8, 2]. The importance of osteoblasts has been well demonstrated by Taichman and Emerson, who showed their involved in establishment of hematopoietic stem cell niches, as well as in the engraftment and maintenance of hematopoietic stem cells (HSC) in the bone marrow by an intimate cell-to-cell contact via integrins [9]. Macrophages are one of the important cells involved in the genesis of Osteoblasts by secreting IL-18 [2].

Osteoclasts precursors are generated in the bone marrow and circulate in the monocyte fraction and are of haematopoietic origin. MCSF is a crucial cytokine involved in proliferation and survival of osteoclast precursor cells by signalling through its receptor c-fms [10, 11]. Osteoclasts are tissue-specific giant polykaryons derived from the monocyte/macrophage hematopoietic lineage and are the only cells capable of breaking down mineralized bone, dentine and calcified cartilage[2, 12, 13]. Differentiation of osteoclast is regulated by macrophage colony-stimulating factor (M-CSF) and RANKL [14, 15]. The osteoclast precursors must undergo transendothelial migration on their way to the bone and their homing into the bone possibly involves $\alpha_v\beta_3$ integrin (Vitronectin) recognizing laminins present in the vascular endothelial basement membrane [16, 17]. Mature osteoclasts express cell markers such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor (CTR) and integrin receptors that are quite unique for them [18-23]. Osteoclasts start resorbing bone by attaching tightly to the matrix, which creates an isolated lacuna, inside which is an acidic environment necessary for matrix dissolution. Ruffled borders contain ATPases that transport protons and play an important role by releasing acid into the resorption lacuna and initiating rapid dissolution of the hydroxyapatite crystals [24]. The enzymes involved in the bone dissolution are tartrate-resistant acid phosphatase (TRAP), cathepsin K and matrix metalloproteinase 9 (MMP-9) which aid in the degradation of the organic matrix. Some of the factors which are shown promote osteoclast genesis are IL-1, IL-6 and TNF-α [2]. IFNβ

functions as a negative-feedback regulator or an inhibitor that inhibits the differentiation of osteoclasts by interfering with the RANKL signalling [25, 26].

RANKL is one of the most important molecules that explicitly link the bone and immune systems [25]. The extensive distribution of RANKL throughout the body indicates its multiple functions, whereas the most important one is dedicated to the induction of osteoclastogenesis. RANKL is a type II transmembrane protein and member of the TNF superfamily and is mainly expressed in preosteoblasts/stromal cells as well as on activated T cells. TNFR and TNF superfamily members are involved in cellular responses such as proliferation, differentiation, inflammation, and cell survival and death [16, 27]**.** Induction of RANKL is dependent on protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and calcineurin-mediated signaling pathways [28]. Its production is been reported to be upregulated in the presence of PTH and TNF-α in pathologic conditions [2, 29]. RANKL/RANK/OPG is the major signalling pathway that governs the osteoclastogenesis and controls the bone resorbing osteoclasts. RANKL is normally expressed on osteoblasts and binds to RANK on the osteoclasts where it transmits activation signals through 'adapter proteins' (AP-1). TRAF6 is one such adaptor proteins and allows RANKL to activate several downstream signalling pathways (including the transcription factor NF-kB and the protein kinases JNK and c-Src) which control the osteoclast specific genes. Osteoprotegrin (OPG) is a decoy receptor of RANKL that prevents RANKL from binding to RANK and activating osteoclasts [1]. Nuclear factor of activated T cells c1 (NFATc1) has been termed as the master regulator of osteoclastogenesis as many downstream effectors of RANK such as NF-κβ and AP-1 ultimately contribute to the activation of NFATc1 [2]**.** Activation of NFAT is mediated by a specific phosphatase, calcineurin, which is activated by calcium–calmodulin signalling. NFAT binding sites and NFATc1 specifically autoregulates its own promoter AP1 (containing cFOS) and continuous activation of calcium signalling is crucial for this auto-amplification [25, 30, 31].

Activation of spleen tyrosine kinase (SYK) and phospholipase Cγ (PLCγ) by the phosphorylation of ITAM is crucial for induction of NFATc1 as these molecules are involved in the calcium signalling pathway [25, 32]. Immunoreceptor tyrosine-based activation motifs (ITAMs), containing adaptor molecules such as DAP (DNAX-activating protein) 12 (DAP-12) and Fc common receptor γchain (FcRγ) act as co-stimulators for osteoclastogenesis and helps in mediating Ca^{2+} signalling induced by RANKL. RANKL serves as both a chemotactic and survival factor for osteoclasts [14]

T-Cells have been implicated in a series of pathological conditions including bone and joint diseases such as rheumatoid arthritis. Local inflammation within the bone, due to metastasis or fractures, or joint inflammation in arthritis, attracts T cells which appear to actively participate in bone remodelling through production of RANKL.[28]. Naive T-cells are of no effect on the process of osteoclastogenesis until they get activated. It is speculated that the proinflammatory cytokines, which are produced by macrophages upon stimulation by T

cells, act on synovial fibroblasts/osteoblasts which are the main source of RANKL responsible for osteoclast differentiation, although RANKL has also been shown to be produced by T cells. Kotake et al have also detected RANKL expression on CD3+ and CD4+ T cells in synovial tissue from RA patients [33]. The surface expression of RANKL on marrow stromal cells, B cells, and T cells has been documented which might suggest that upregulation of RANKL on stromal cells and lymphocytes in the bone marrow could mediate increased bone resorption [2]. RANKL expressing T cells have been found to be involved in dendritic cell (DC) activation and survival, implicating the role of RANKL-RANK in Tcell mediated DC regulation and lymph node organogenesis. As T cell-triggered osteoclasts display a typical osteoclast phenotype (TRAP+, CTR+, cathepsin K+, b3-integrin+, RANK+), it suggests that activated T cells can directly trigger osteoclastogenesis through membrane-bound and soluble RANKL implying that activated T cells can directly cause bone loss in vivo [28]. The long sought after TH cell subset that was involved in osteoclastogenesis was found to be IL-17-producing TH-cell subset (TH17 cells). These TH cell subset were classified under the pro-osteoclast genesis because these cells expressed low amounts of INF-γ and were also found to be involved in the production of inflammatory cytokines such as TNF-α which have direct implication on osteoclast genesis by promoting expression of RANKL on osteoblasts and lastly these cell subset by themselves express RANKL and can accelerate osteoclast genesis.[25]**.** The mode of action is as follows, IL-17 first acts on osteoblastic cells, stimulates cyclooxgenase-2 (COX-2)-dependent prostaglandin E2 (PGE2) synthesis, then induces RANKL gene expression, which, in turn, induces differentiation of osteoclast progenitors into mature osteoclasts [34, 35]**.**

Antigen presenting cells (APCs) play a pivotal role in cellular immunity by processing and presenting antigens to specific T cells [37]. For the T cells to be activated it takes three signals of which the first is provided by antigen peptide-MHC I and peptide-MHC II complexes on the surface of APCs that provide the antigen-specific signal to T-cell receptor; the second is called costimulatory signal and is provided by B7 family and TNF family CD80 (B7-1) and CD86 (B7-2) through binding to CD28; and the third is mediated by cytokines secreted by APCs such as IL-12, IL-10, TGF-β , IL-6,and IL-23, which direct the differentiation of activated lymphocytes into different effector T-cell subsets. Haiyan Li and et al have reported antigen presenting capacity of osteoclasts similar to dendritic cell during T-cell activation. They have also showed the presence of MHC and CD80, CD86 on osteoclasts which thereby functions as an APC and activate both CD4+ and CD8+ T cells. They have also showed that these co-stimulatory molecules expressions can be upregulated by LPS and IFN-γ stimulation [36].

The ICOS ligand, B7 homologous protein (B7h) is constitutively expressed on B cells and is inducible on monocytes and dendritic cells at low levels [39, 40]. ICOS is expressed only on the activated T-cells in contrast to B7h which is constitutively expressed on B-cells without any stimulation. Binding of ICOS to its ligand B7h leads to production of multiple cytokines such

as IL-4, IL-5, IFN-γ, and IL-10 [39, 41, 42]. Ruth et al have shown that CD14+ monocytes isolated from the RA synovial fluid expressed ICOS, B7RP.1, B7RP.2, B7H1, and B7H2 [38]. B7RP-1 is a member of the B7 family of costimulatory molecules that specifically interacts with inducible costimulator (ICOS) expressed on activated T cells. Iwai et al have observed the involvement of the ICOS-B7h costimulatory pathway in the pathogenesis of an experimental murine autoimmune arthritis and also have proved this by administration of anti-B7h mAb which significantly ameliorated the RA. They have also moved forward in describing the presence ICOS and B7h in the inflamed synovial tissue as well as in the draining lymph nodes (LNs). Their study indicated that blockade of B7h during RA leads to the inhibition of both Th1 and Th2 mediated immune responses and suggests that the ICOS-B7h interaction plays an important role in the pathogenesis of RA [43]. In addition to this, Nurieva et al., showed that ICOS knockout mice was resistant to RA. However, analysis of B7h expression on other types of cells in the synovium, such as osteoclast, has never been done and would be important for better understanding of the possible involvement of ICOS-B7h interactions in osteogenesis and bone diseases. Intervention of the ICOS costimulatory pathway may also be a novel strategy for the treatment of human rheumatoid arthritis and possibly other chronic inflammatory diseases [43].

Activation of the immune system is essential for host defence against pathogens, but aberrant and prolonged activation under pathological conditions results in tissue damage owing to the persistent over activation of effector cells. In RA, a long-standing question is how abnormal T-cells get activated in the inflamed joints and mechanistically induces bone damage [14]. In this study, we would try to understand the function and the role of ICOS-B7h pathway in the activation of T-cells in the case of RA and would make an attempt to address the expression of B7h on the osteoclast found in inflamed tissues and link it to the activation of T-cells during the disease progression, as ICOS-B7H interaction has been shown to regulate the expression of IL-17 in the joint and mediate inflammatory responses [44].

Materials and Method:

Materials

Tissue culture media, Duldecco's Modified Eagles Medium (DMEM), RPMI1640 were supplemented with 10% hest-decomplemented Fetal bovine serum (FBS) Penicillin, streptomycin and Gentamycin. Recombinant hM-CSF and Recombinant hRANKL were from R&D systems (Minneapolis, MN, USA). Monoclonal antobodies for CD antigens were from e-biosciences and MACS (magnetic cell sorting) magnetic separation column were purchased from Miltenyo Biotec (Bergisch Gladbach, Germany). Acid Phosphatase, Leukocyte (TRAP Staining) (Sigma diagnostics, St. Louis, MO, USA).

Preparation of total PBMC and Isolation of CD14+ cells

Blood of healthy donors were provided anonymously. To isolate the PBMC the blood samples were diluted 1:1 (v/v) in PBS, layered over Lympholyte-H solution and centrifuged (1800rpm, 25min). The PBMC layer was collected and washed with PBS followed by 2%FBS in PBS, isolated by centrifugation (1700rpm, 10 min) and resuspended in media containing 10% serum. Prior to CD14+ enrichment, the PBMC was washed twice with PBS and collected (1200 rpm, 10min, $4^{o}C$). The PBMS was then washed with purification buffer and the supernatant was discarded. 1X 10^{8} cells were resuspended in 300ul of purification buffer before adding 100ul of FCR blocking reagent and 100ul of biotin-antibody cocktail following incubation at $4^{o}C$ for 10 min. After incubation, we added 300ul of purification buffer followed by 200ul of Anti-biotin microbeads, mixing and incubating at $4^{o}C$ for 15 min. Then, cells were washed with 1-2ml of purification buffer and centrifuged at 1200rpm for 10min. The supernatant was aspirated and the cells were resuspended in purification buffer at a concentration of 10^{8} cells/500ul. The cell suspension was then added to LS negative selection column mounted on a magnetic separator. The CD14+ cell population was collected as effluent fraction with unlabeled cells representing enriched monocytes. CD14+ sample was later treated with anti-CD14 conjugated to FITCH and TCR conjugated to PE and analysed by fluorescence-activated cytometry using FACS calibur (Becton Dickson).

Differentiation of Osteocalsts from CD14+ monocytes isolated from PBMC

CD14+ cells were seeded onto to a tissue culture plate at a density of $3*10^{6}$ cells in 2ml of DMEM medium containing 10% FBS, penicillin, streptomycin and gentamycin. Cell culture was performed in these conditions (base medium) with the presence of RANKL (30 ng/ml) and M-CSF (25ng/ml). Cultures were washed after the seventh day in PBS and subjected to trypsin at $37^{o}C$ for approximately 20 minutes to remove non-adherent cells, and were maintained for 21 days at $37^{o}C$ in a 5% CO_2 humidified atmosphere. Culture medium and cytokines were replaced twice weekly. After 21 days of culture multinucleated cells (MNC) appeared. Cells were gently washed with PBS to remove medium containing cytokines. Cells

were removed by scrapping off and were reseeded on sterile glass cover slips with equal volume of medium containing the cells. Cells were cultured for one more day with the conditioned medium. Cultures were characterized at day 21 as described.

TRAP Staining

Cover slips were washed with PBS and fixed in fixative solution (citrate solution, acetone, 37% formaldehyde) for 30 seconds, at day 21. Then the cover slips were washed thoroughly with deionized water and stained for TRAP using an acid phosphotase, leucocyte (TRAP) kit, according to manufacturer's instruction. Briefly, cells were incubated in naphtol AS-BI in presence of tartrate and Fast garnet GBC at 37°C for 1 hour in dark. Then, the cells were washed with water and digital micrographs of multinucleate cells were taken using a 20X objective.

Treatment with ICOS

The cells that were reseeded on coverslips were treated with 1ug/ml of ICOS H/H and were incubated at 37°C, 5% CO_2 humidified atmosphere for 24 hours before subjecting to TRAP staining as described before.

RESULTS:

Expression of B7h on Primary osteoblasts

The expression of B7h on the primary osteoblasts was examined using flow cytometric analysis. The primary osteoblast cell lines showed no expression of B7h (**Fig1)**. So, we tried to stimulate these cells using various cytokines/stimuli such as TNF-α (**Fig-2**), combinations of Ascorbic acid, β-glucan and dextramethasone (**Fig-3**) and also a combination of dextramethasone, VitD_3 and β-corticoids (**Fig-4**). The cells showed no expression of B7h. We also tried activating the cells using Pro and anti-inflammatory cytokines of RA such as IL4, IL6 and GM-CSF and their respective combinations but the cells showed no expression of our target B7h (Data not shown).

Characterization of OCs

OCs were differentiated *in vitro* from monocytes of healthy donors in cell culture with the addition of M-CSF and RANKL. To detect the activity of TRAP in OCs, cultured cells were washed and stained with TRAP kit. Mature osteoclasts (**Fig-5**) were transferred onto glass slides for TRAP staining. As shown in **Figure 6**, OCs showed strong TRAP activity, which appeared as purplish to dark red granules in the cytoplasm. As OCs was formed

through the merge of precursors, OCs were much larger than monocytes, and multiple cell nuclei could be seen in OCs.

Expression of B7h on Osteoclasts

To determine the expression of B7h on the osteoclast, flowcytometry was performed using specific mAb against B7h and CD14. The flow cytometric analysis did not yield any positive results and the cells did not express any B7h (**Fig-6)**. CD14 was used as a negative marker as the depletion in the count was observed during the process of osteoclast differentiation from monocytes. There were a very few population of cells that were double positive (**Fig-7**). PTH was also used as a differentiating agent in the initial experiments as it was shown to promote the process of osteoclast genesis thereby facilitating the differentiation, activation and survival of osteoclasts. However, there was no difference between the experiments run without PTH stimulation (data not shown).

Expression of B7h on Osteosarcoma cell lines

To check the expression of B7h on the human osteosarcoma cell lines namely MG63 and U2OS, flowcytometry was performed using specific mAb against B7h. The flowcytometric analysis showed clear presence of B7h on the osteosarcoma cell lines (**Fig 9)**. This expression of B7h by osteosarcoma cells provides hint about the involvement of these cells in abnormal T cell activation during bone cancer.

Treatment of differentiated osteoclasts with ICOS

The osteoclasts that were differentiated from monocyte precursors for 21 days were treated with 1µg of ICOS. The cells when stained for TRAP showed changed cell morphology, the number of cells in the observed microscopic field was low when compared to control and there was cytoplamic eruptions leading to the leak to cytoplasmic contents into the external environment (**Fig-8**). This indicates the cells had suffered due to the treatment with ICOS.

DISCUSSION:

Bone system and the immune system share an abundance of molecules and regulatory mechanisms, and this, together with the importance of the interactions between the two systems might has resulted in the emergence of the field of osteoimmunology[25]**.** We stared differentiating osteoclasts beginning from CD14+ monocytes as it was shown by Danks et al that CD14-synovial cells from the RA inflamed synovial tissues were the sole cells that promoted osteoclast formation in the inflamed joints[10]. During our initial differentiation PTH was used as it was said to upregulate the production of interleukin-6 and RANKL by osteoblasts, thereby facilitating the differentiation, activation and survival of osteoclasts. However, we did not see any significant changes between the cells differentiated in presence and in the absence of PTH. This indicates that this stimuli works well in the

presence of osteoblast cells by upregulating the cytokine production needed for the osteoclastogenesis. In our preliminary screening, we have not yet as such establish the presence of the B7h on the osteoclast and we are trying to setup experiment to visualize the B7h marker on the cell surface of osteoclasts. It seems that during the differentiation and maturation stages from the monocytes the expression of B7h by the cells may be downregulated, so checking the expression of B7h during the differentiation of osteoclasts may provide an insight into the expression of B7h during the different stages of the cell differentiation. This may also act as a cell stage marker wherein losing the expression of B7h by the monocytes may indicate the maturity of the cell during the osteoclast genesis cell cycle. On the contrary the osteosarcoma cell lines MG63 and U2OS that was examined showed the presence of B7h, hinting the role of ICOS-B7h pathway in the bone tumour. This study takes us a step closer to understand the mechanism by which bone cells regulate the immune response in autoimmune disease such as RA and in cases of bone tumour. As we have preliminarily seen the effect of ICOS on the mature osteoclasts, wherein the cells started to suffer after treatment hints us of the overlap of the two systems which may become a realistic targeted therapeutic option to reduce the resorptive capacity of osteoclasts and opens a possibility of cytokine therapy for bone tumours.

Figures:

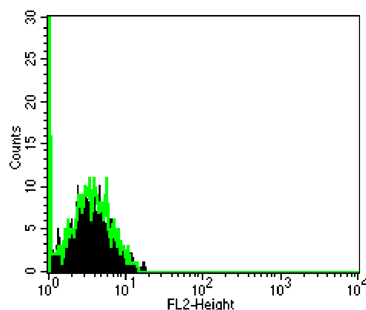


Fig1: Expression of B7H on primary osteoblast: Primary osteoblasts were cultured and stained with antibodies and analyzed by flow cytometry. Histogram with unstained control (Dark/black) overlayed with B7h stained sample (Green line).

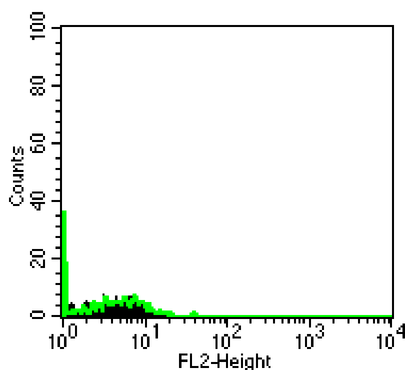


Fig2: Expression of B7H on primary osteoblast treated with TNF-α: Primary osteoblasts were cultured in presence of TNF-α and stained with antibodies and analyzed by flow cytometry. Histogram with unstained control (Dark/black) overlayed with B7h stained sample (Green line).

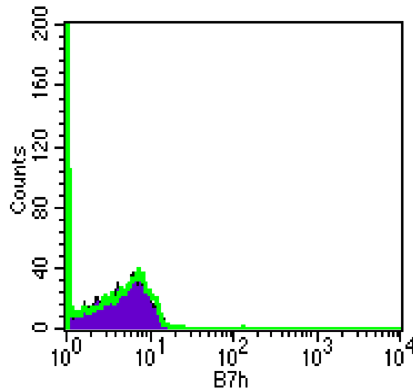


Fig3: Expression of B7H on primary osteoblast treated with Ascorbic acid, β-Glucan and Dextramethasone: Primary osteoblasts were cultured in presence of Ascorbic acid, β-Glucan and Dextramethasone and stained with antibodies and analyzed by flow cytometry. Histogram with unstained control (Dark/black) overlayed with B7h stained sample (Green line).

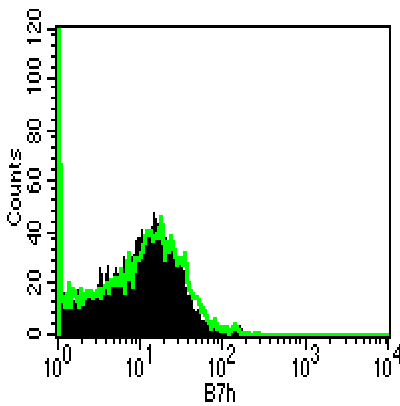


Fig4: Expression of B7H on primary osteoblast treated with dextramethasone, $VitD_3$ and β-corticoids: Primary osteoblasts were cultured in presence of dextramethasone, $VitD_3$ and β-corticoids and stained with antibodies and analyzed by flow cytometry. Histogram with unstained control (Dark/black) overlayed with B7h stained sample (Green line).

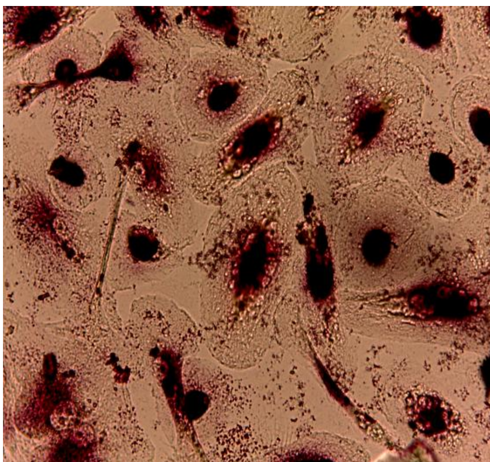


Fig 6: Osteoclasts were generated from monocytes with M-CSF and RANKL for 21 days. TRAP staining to detect TRAP activity in osteoclasts. TRAP activity was observed under light microscope. Numerous TRAP+ MNS's are shown (dark red colour)

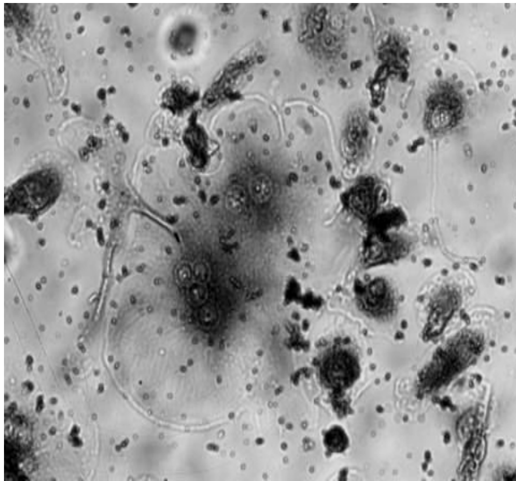


Fig 5: Phase contrast microscopy of osteoclasts cultured in presence of M-CSF and RANKL for 21 days.

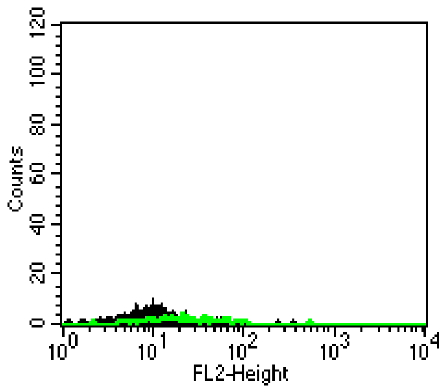
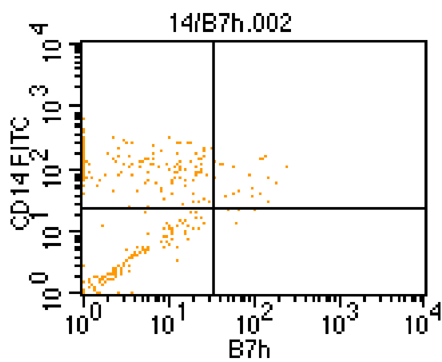
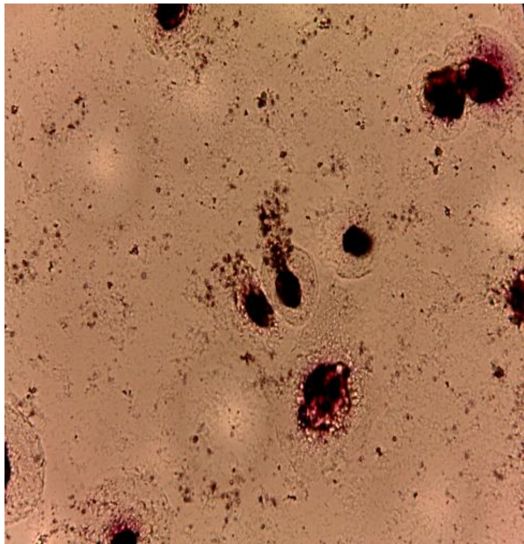


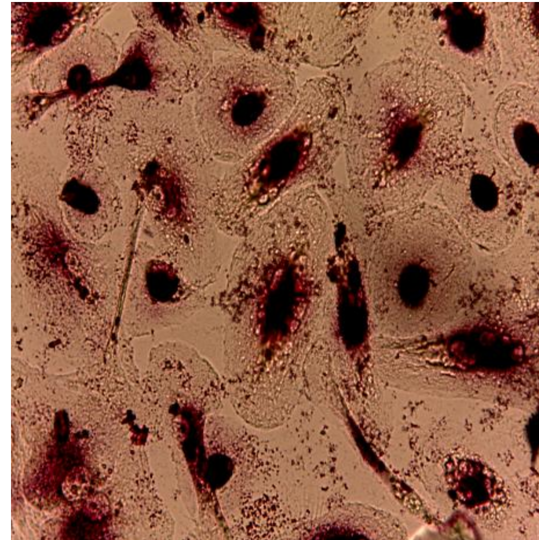
Fig- 6: Expression of B7h on the differentiated osteoclasts cultured in the presence of M-CSF and RANKL for 21 days and stained with antibodies and analyzed by flow cytometry. The dark/black background is the control and the green is the sample.



Fif-7: The quadrant showing very few cells double positive to B7H and CD14

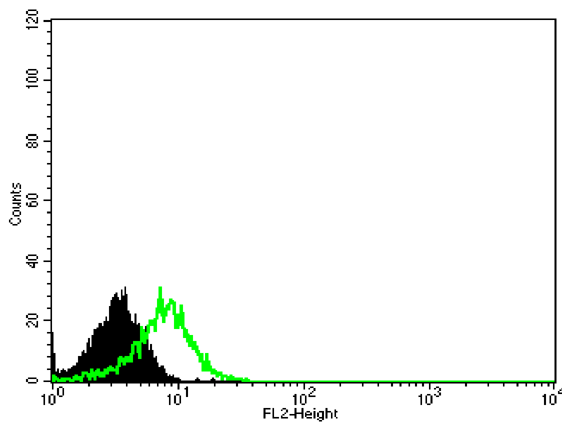
Quad	% Gated	% Total
UL	67.22	28.10
UR	5.02	2.10
LL	27.51	11.50
LR	0.24	0.10

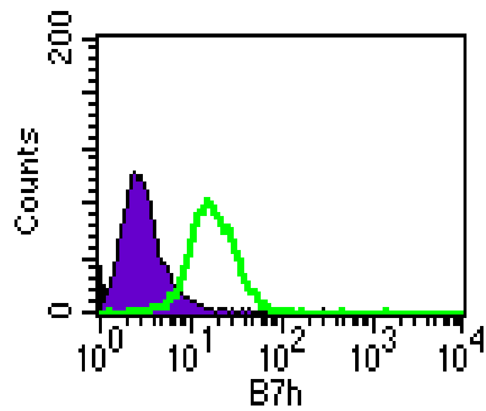




A **B**

Fig-8: Osteoclasts were obtained by culturing the CD14+ monocyte fraction in presence of M-CSF and RANKL for 21 days. A: shows the TRAP stained cells that was treated with 1 µg of ICOS; B: The untreated osteoclast.





A: MG63 **B: U2OS**

Fig 9: Expression of B7h on the differentiated osteosarcoma stained with antibodies and analyzed by flow cytometry. The dark/black background is the control and the green is the sample. A: MG 63 B: U2OS

Reference

1. Joseph R. Arron and Yongwon Choi: Bone versus immune system. Nature,vol 408, 30 November 2000, 535-536

2. Martina Rauner, Wolfgang Sipos, Peter Pietschmann: Osteoimmunology. Int Arch Allergy Immunol 2007;143:31–48

3. Gideon A. Rodan and T. John Martin: Therapeutic Approaches to Bone Diseases; Science 289, 1508 (2000); Science 289, 1508-1514, 2000

4. Aubin J: Regulation of osteoblast formation and function. Rev Endocrinol Metab Disord 2001; 2: 81–94.

5.Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G: Osf2/cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997; 89: 747–754.

6.Nakashima K, et al: The novel zinc fingercontaining transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002; 108: 17–29.

7. Fujisaki K, Tanabe N, Suzuki N, Mitsui N, Oka H, Ito K, Maeno M: The effect of IL-1 on the expression of matrix metalloproteinases, plasminogen activators, and their inhibitors in osteoblastic ROS 17/2.8 cells. Life Sci 2006; 78: 1975–1982.

8. Marom R, Shur I, Solomon R, Benayahu D: Characterization of adhesion and differentiation markers of osteogenic marrow stromal cells. J Cell Physiol 2005; 202: 41–48.

9. Taichman RS, Emerson SG: The role of osteoblasts in the hematopoietic microenvironment. Stem Cells 1998; 16: 7–15.

10. L Danks, A Sabokbar, R Gundle, N A Athanasou: Synovial macrophage-osteoclast differentiation in inflammatory arthritis. Ann Rheum Dis 2002;61:916–921

11. Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA: The human osteoclast precursor circulates in the monocyte fraction. Endocrinology 1996;137:4058–60

12. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T: Origin of osteoclasts: Mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow- derived stromal cells. Proc Natl Acad Sci USA 1990; 87: 7260–7264.

13. Kurihara N, Chenu C, Miller M, Civin C, Roodman GD: Identification of committed mononuclear precursors for osteoclast-like cells formed in long term human marrow cultures. Endocrinology 1990; 126: 2733–2741.

14. Tomoki Nakashima & Hiroshi Takayanagi: Osteoimmunology: Crosstalk Between the Immune and Bone Systems. J Clin Immunol (2009) 29:555–567

15. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ: Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev. 1999;20:345–357.

16. Charles Kaplan , Alison Finnegan: Osteoclasts, pro-inflammatory cytokines, rank-l and bone remodeling in rheumatoid arthritis. Frontiers in Bioscience 8, d1018-1029, May 1, 2003

17. Horton M. A., J. H. Spragg, S. C. Bodary, and M. H. Helfrich: Recognition of cryptic sites in human and mouse laminins by rat osteoclasts is mediated by beta 3 and beta 1 integrins. Bone 15, 639 (1994)
18. Kodama H, Nose M, Niida S, Yamasaki A:Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. J Exp Med 1991; 173: 1291–1294.
19. Jacquin C, Gran DE, Lee SK, Lorenzo JA, Aguila HL: Identification of multiple osteoclast precursor populations in murine bone marrow. J Bone Miner Res 2006; 21: 67–77.
20. Luchin A, Purdom G, Murphy K, Clark MY,Angel N, Cassady AI, Hume DA, Ostrowski MC: The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. J Bone Miner Res 2000; 15: 451–460.
21. Hattersley G, Chambers TJ: Calcitonin receptors as markers for osteoclastic differentiation: correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures. Endocrinology 1989; 125: 1606–1612.
22. Hansen T, Otto M, Gaumann A, Eckardt A, Petrow PK, Delank KS, Kirkpatrick CJ, Kriegsmann J: Cathepsin K in aseptic hip prosthesis loosening: expression in osteoclasts without polyethylene wear particles. J Rheumatol 2001; 28: 1615–1619.
23 Teti A, Grano M, Carano A, Colucci S, Zambonin Zallone A: Immunolocalization of beta 3 subunit of integrins in osteoclast membrane. Boll Soc Ital Biol Sper 1989; 65: 1031–1037.
24. Teti A, Blair HC, Schlesinger P, Grano M, Zambonin-Zallone A, Kahn AJ, Teitelbaum SL, Hruska KA: Extracellular protons acidify osteoclasts, reduce cytosolic calcium, and promote expression of cell-matrix attachment structures. J Clin Invest 1989; 84: 773– 780.
25. Hiroshi Takayanagi: Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems .292, april 2007 , volume 7
26. Takayanagi, H. et al.: RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-β. Nature 416, 744–749 (2002).
27. Baker S. J., and E. P. Reddy: Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene 12, 1 (1996)
28. Young-Yun Kong, Ulrich Feige, Iidiko Sarosi, Brad Bolon, Anna Tafuri, Sean Morony, Casey Capparelli, Ji Lik, Robin Elliottk, Susan McCabek, Thomas Wong¶, Giuseppe Campagnuolo, Erika Moran, Earl R. Bogoch, Gwyneth Van, Linh T. NguyenI, Pamela S. OhashiI, David L. Lacey, Eleanor Fish¶, William J. Boylek & Josef M. Penninger: Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature.402,304–309;1999.
29. Dai J, He P, Chen X, Greenfield E: TNFa and PTH utilize distinct mechanisms to induce IL-6 and RANKL expression with markedly different kinetics. Bone 2006; 38: 509–520.
30.Takayanagi, H. et al:. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling for terminal differentiation of osteoclasts. Dev. Cell 3, 889–901 (2002).

31. Matsuo, K. et al:. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. J. Biol. Chem. 279, 26475–26480 (2004).
32. Mao, D., Epple, H., Uthgenannt, B., Novack, D. V. & Faccio, R.: PLCγ2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. J. Clin. Invest. 116, 2869–2879 (2006).
33. Shigeru Kotake, Nobuyuki Udagawa, Masayuki Hakoda, Makio Mogi, Kazuki Yano, Eisuke Tsuda, Ken Takahashi, Takefumi Furuya, Shigeru Ishiyama, Kang-Jung Kim, Seiji Saito, Toshio Nishikawa, Naoyuki Takahashi, Akifumi Togari, Taisuke Tomatsu, Tatsuo Suda,2 and Naoyuki Kamatani : Activated Human T Cells Directly Induce Osteoclastogenesis From Human Monocytes : Possible Role of T Cells in Bone Destruction in Rheumatoid Arthritis Patients Arthritis & Rheumatism Vol. 44, No. 5, May 2001, pp 1003–1012
34. Nobuyuki Udagawa: The mechanism of osteoclast differentiation from macrophages: possible roles of T lymphocytes in osteoclastogenesis J Bone Miner Metab (2003) 21:337–343
35. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T: IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest (1999) 103:1345–1352
36. Haiyan Li, Sungyoul Hong, Jianfei Qian, Yuhuan Zheng, Jing Yang, and Qing Yi : Cross talk between the bone and immune systems: osteoclasts function as antigen-presenting cells and activate CD4+ and CD8+ T cells
37. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975-1028.
38. Jeffrey H. Ruth, James B. Rottman, Gillian A. Kingsbury, Anthony J. Coyle, G. Kenneth Haines , Richard M. Pope, and Alisa E. Koch: ICOS and B7 Costimulatory Molecule Expression Identifies Activated Cellular Subsets in Rheumatoid Arthritis. Cytometry Part A 71A:317–326 (2007)
39. Ruth JH, Shahrara S, Park CC, Morel JC, Kumar P, Qin S, Koch AE.: Role of macrophage inflammatory protein-3a and its ligand CCR6 in rheumatoid arthritis. Lab Invest 2003;83:579–588.
40. Katschke KJ Jr, Rottman JB, Ruth JH, Qin S, Wu L, LaRosa G, Ponath P, Park CC, Pope RM, Koch AE:. Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. Arthritis Rheum 2001;44:1022–1032.
41. Rottman JB, Smith T, Tonra JR, Ganley K, Bloom T, Silva R, Pierce B, Gutierrez-Ramos JC, Ozkaynak E, Coyle AJ.: The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. Nat Immunol 2001;2:605–611.
42. Verwilghen J, Lovis R, De Boer M, Linsley PS, Haines GK, Koch AE, Pope RM:. Expression of functional B7 and CTLA4 on rheumatoid synovial T cells. J Immunol 1994;153:1378–1385.
43. Hideyuki Iwai, Yuko Kozono, Sachiko Hirose, Hisaya Akiba, Hideo Yagita, Ko Okumura, Hitoshi Kohsaka, Nobuyuki Miyasaka, and Miyuki Azuma : Amelioration of Collagen-Induced

Arthritis by Blockade of Inducible Costimulator-B7 Homologous Protein Costimulation1. J Immunol 2002; 169:4332-4339.
44. I. Nurieva, Piper Treuting, Julie Duong, Richard A. Flavell, and Chen Dong: Inducible costimulator is essential for collagen-induced arthritis Roza J. Clin. Invest. 111:701–706 (2003)

Attended seminars

Speaker	Title
"Neuronal Death: Recruitment of programmed necrosis and autophagy"	**Philip BEART** Florey Neuroscience Institutes, University of Melbourne, Parkville, Australia
"Next generation sequencing in T-ALL"	**Dr. Kim De Keersmaecker** VIB Center for the Biology of Disease, Center for Human Genetics KU Leuven (Belgium)
"Recent Advances in Hematopoietic Stem Cell Gene Therapy: from microRNA Regulation to Targeted Gene Transfer"	**Prof. Luigi Naldini** San Raffaele, Telethon Institute for Gene Therapy (HSR-TIGET)
"Molecular control of human fetal globin expression: towards a potential cure for B-thalassemia and sickle cell anemia"	**Prof. Sjaak Philipsen** Erasmus MC-Cell Biology, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
"**High-throughput Biochemical Target Investigation Unveils a Novel Function of miR-21 as a Negative Modulator of Signal Transduction in T-lymphocytes**"	**Prof. J. N. Bouwes BAVINCK** Department of Dermatology, Leiden University, Olanda
"Linfomi cutanei primitivi"	**Prof. Pino Macino** Dipartimento di Biotecnologie Cellulari ed Ematologia Università degli Studi di Roma "La Sapienza"
"Numerical simulations as virtual microscope at the nanoscale: some examples with dendritic molecules"	**Prof. Andrea Danani** Chief of the Laboratory of Applied Mathematics and Physics-LAMFI Department of Innovative Technologies-DTI, University of Applied Science of Southern Switzerland-SUPSI, Lugano, CH
"Microparticles as novel effectors in Inflammation"	**Prof. Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London

	London, UK
"Resolvins and Omega-3 in Inflammation"	**Prof. Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs"	**Prof.ssa Isabel MERIDA** Centro Nacional de Biotecnologia, Madrid
"Signalling pathways controlling integrin trafficking during invasion"	**Dott.ssa Elena RAINERO** Beatson Insitute for Cancer Research, Glasgow, UK
"Exosomes Shuttle RNA"	**Prof. Lötvall J.**, Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg
"Next-generation DNA sequencing and target arrays in the clinics"	**Dr. Paolo Fortina** Department of Cancer Biology, Jefferson Genomics Laboratory, Kimmel Cancer Center, Thomas Jefferson University Jefferson Medical College, Philadelphia, PA, USA
"Alpha-MSH and the melanocortin system in inflammation"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"Galectins-carbohydrate binding protein: sweet or sour?"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"The resolution of inflammation: players and targets"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK

“New trends in allergy and immunology”	**Prof. Joseph A.Bellanti**, Professor of Pediatrics and Microbiology-Immunology Director, International Center for Interdisciplinary Studies of Immunology Georgetown University School of Medicine

Participation at courses

Course title and location	**Organizer(s) and affiliation(s)**	**Number of hours**	
Genetics and Molecular Medicine, Universita' del Piemonte Orientale "A. Avogadro", (NO)	Prof. Steve Ellis (Louisville University), COREP, Universita' del Piemonte Orientale "A. Avogadro"	20 hrs/ 10 days	21 May- 1 June 2012

Lab Meeting -
Sub: Annual Progress report
Date: 27/06/2010