Μεταπτυχιακό Πρόγραμμα Σπουδών στην
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The role of Tpl2 and Nalp3 proteins in the process of Osteoclastogenesis

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Μελέτη του ρόλου της Tpl2 και της Nalp3 πρωτεΐνης στην διαδικασία της Οστεοκλαστογένεσης

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ΠΕΡΙΛΗΨΗ

Οι οστεοκλάστες διασπούν την οστική μάζα, χάρις στα ιδιαίτερα χαρακτηριστικά τους που τους παρέχουν την δυνατότητα να απορροφούν οργανική και ανόργανη οστική μάζα. Σημαντικό στοιχείο της δράσης του οστεοκλάστη είναι το γεγονός ότι η διαφοροποίηση και η δράση του επηρεάζεται στενά από το μικρο-περιβάλλον του, και κυρίως από τις φλεγμονώδεις κυτταροκίνες, όπως η ιντερλευκίνη-1 και ο TNF-α. Συνεπώς, η δημιουργία και η δράση των οστεοκλαστών είναι αυξημένη σε φλεγμονώδεις καταστάσεις, όπως η ρευματοειδής αρθρίτιδα, με αποτέλεσμα την δημιουργία οστεοπόρωσης ή οστεοπενίας. Η μελέτη μορίων που εμπλέκονται στην διαφοροποίηση ή την λειτουργία των οστεοκλαστών μπορεί να εισαγάγει νέα μόρια-στόχους στην θεραπεία της απώλειας οστικής μάζας στα πλαίσια φλεγμονωδών καταστάσεων.

Σε αυτά τα πλαίσια, οι πρωτεΐνες Tpl2 και Nalp3 συμμετέχουν, με διαφορετικό τρόπο, στην διαδιακασία της φλεγμονής και της ανοσίας. Η Tpl2 είναι μια κινάση σερίνης-θρεονίνης, της οικογένειας των κινασών MAP3K, που ενεργοποιείται καθοδικά διαφόρων υποδοχέων, όπως οι TLRs και ο TNFR, που δρουν σημαντικά στα πλαίσια της ανοσιακής απάντησης και της φλεγμονής. Η ERK κινάση, που ενεργοποιείται καθοδικά της Tpl2, εμπλέκεται σε τρία καθοριστικά σηματοδοτικά μονοπάτια κατά την διαφοροποίηση των οστεοκλαστών: την αλληλεπίδραση RANK/RANKL, την πρόσδεση c-fms/MCSF και το μονοπάτι των ιντεγκρινών α_v. Η μελέτη του ρόλου της Tpl2 σε διαφορετικά είδη κυττάρων, όπως οι οστεοκλάστες, μπορεί να αποκαλύψει νέους ρόλους της πρωτεΐνης και θεραπευτικούς στόχους στη φλεγμονή. Η Nalp3 πρωτεΐνη εμπλέκεται στην ανοσιακή απάντηση, μέσω της συμμετοχής της στον σχηματισμό του πολυπρωτεϊνικού φλεγμονοσώματος NALP3 inflammasome, το οποίο μεσολαβεί κυρίως την παραγωγή των κυτταροκινών ιντερλευκίνης 1β και 18. Οι οστεοκλάστες επηρεάζονται σε μεγάλο βαθμό από το φλεγμονώδες μικρο-περιβάλλον, και ειδικά από την ιντερλευκίνη-1β και τον TNFα, με αποτέλεσμα να αυξάνεται η διαφοροποίηση και η δράση τους. Επομένως, ίσως υπάρχει αλληλεπίδραση μεταξύ του φλεγμονοσώματος NALP3 και της λειτουργίας των οστεοκλαστών, σε συνθήκες φλεγμονής.

Συνολικά, τα δεδομένα από την βιβλιογραφία που παρουσιάστηκαν, μας οδήγησαν στην υπόθεση ότι οι πρωτεΐνες Tpl2 και Nalp3 μπορεί να υπεισέρχονται στην διαδικασία της οστεοκλαστογένεσης. Για τον σκοπό αυτό, στην μελέτη αυτή, διαφοροποιήθηκαν οστεοκλάστες από κύτταρα του μυελού των οστών από ποντίκια $Tpl2^{-/-}$ και $Nalp3^{-/-}$ και ελέγχθηκε η διαδικασία της οστεοκλαστογένεσης σε σχέση με ποντίκια αγρίου τύπου (WT), με πειράματα βασισμένα στην χρώση TRAP, με ανάλυση κυτταρομετρίας ροής

(FACS), με τον καθορισμό των επιπέδων φωσφορυλίωσης της ERK κινάσης κατά Western blot και με την διερεύνηση των επιπέδων έκφρασης γονιδίων ειδικών για τους οστεοκλάστες. Με τα αποτελέσματα που προέκυψαν από την μελέτη αυτή, αποδείχθηκε ότι δεν υφίστανται σημαντικές διαφορές στην οστεοκλαστογένεση στα δύο KO (Tpl2 και Nalp3) ποντίκια σε σχέση με το WT, όπως πολύ καθαρά δείχθηκε από τα αποτελέσματα της real-time PCR για τα ειδικά για τους οστεοκλάστες γονίδια (TRAP, NFATc1, MMP9 και cathepsin K). Συμπερασματικά, αποδείχθηκε ότι οι πρωτεΐνες Tpl2 και Nalp3 δεν συμμετέχουν στην διαδικασία διαφοροποίησης των οστεοκλαστών, δεδομένου ότι ο σχηματισμός των οστεοκλαστών παραμένει ανέπαφος και στα δύο KO μοντέλα ποντικών.

SUMMARY

The osteoclast is characterized as the ‘sole’ bone-resorbing cell, and this is due to its unique characteristics, that provide osteoclast with the ability to dissolve organic and mineral bone. An important feature of the osteoclast is the fact that osteoclasts’ development and function are largely controlled by the microenvironment, especially by pro-inflammatory cytokines such as IL-1 and TNFa. Therefore, osteoclast formation and function are enhanced in inflammatory situations, such as rheumatoid arthritis, leading to the clinical feature of osteopenia and osteoporosis, a condition of systemic reduced bone mass. Finally, studying molecules associated with the differentiation and function of osteoclasts can reveal the identification of new targets in the therapy of osteoclast-associated bone loss, a common feature of inflammation related diseases.

In this context, Tpl2 and Nalp3 protein are involved, in different ways, in inflammatory conditions. Tpl2 as a serine/threonine kinase of the MAP3Ks family, that is activated downstream of TLRs (TLR2,3,4,7,9) and TNFR (TNFR1 and CD40), that are important for the immune response and inflammation. ERK kinase, that is phosphorylated downstream of Tpl2, is implicated in three major steps of osteoclast differentiation: RANK/RANKL interaction, c-fms/M-CSF binding and integrins signaling pathway. To this end, it is important to delineate the role of Tpl2 in different cell types, such as osteoclasts, that are closely linked to the immune response. Nalp3 protein is involved in the immune response, through its participation in the formation of the multi-protein complex of inflammasome, that mainly mediates the production of IL-1b and IL-18. Osteoclasts are highly influenced, and positively affected in terms of differentiation and activation, by pro-inflammatory cytokines, and most importantly IL-1 and TNFa. Thus, the manipulation of Nalp3 could possibly affect the differentiation or function of osteoclasts.

Collectively, the data from the literature presented above led us to formulathe the hypothesis that Tpl2 and Nalp3 proteins could be implicated in the differentiation process towards osteoclasts. To this end, osteoclasts were differentiated from bone marrow cells isolated from $Tpl2^{-/-}$, $Nalp3^{-/-}$ and wild type mice and the differentiation process was examined based on the osteoclast-specific TRAP staining, with the assesement of ERK phosphorylation on $Tpl2^{-/-}$ osteoclasts and mainly with the analysis of mRNA levels of osteoclast-specific genes in all three mouse strains. From the results obtained, it was apparent that not significant differences in osteoclast formation appeared in the two knock-out mouse strains. Phosphorylation of ERK was not different in $Tpl2^{-/-}$ mice as

compared to WT, upon the activation of osteoclasts with LPS or RANKL. Next, and most importantly, from the real-time PCR data, it was clearly shown that the levels of expression of osteoclast-specific genes, were comparable between all three mouse strains and for all genes studied (TRAP, NFATc1, MMP9 and cathepsin K). Subsequently, it was concluded that neither Tpl2 nor Nalp3 proteins are involved in the differentiation process of osteoclasts, since osteoclasts formation was not impaired in both $Tpl2^{-/-}$ and $Nalp3^{-/-}$ knock-out mice, and this was examined in different stages of the differentiation process.

1. INTRODUCTION

1.1 OSTEOCLAST BIOLOGY

Bone, unlike other rigid structures, such as teeth, tendons and cartilage, is continuously renewed by the process of bone remodeling. The equilibrium between bone synthesis and resorption, exerted by osteoblasts and osteoclasts respectively, determines both phases of physiological bone turn-over: bone modeling, during development, and bone remodeling, a lifelong process intended for the renewal of tissue. The mature osteoclast is the sole bone resorbing cell, revealing its importance in the regulation of bone metabolism, but is also interacting with the immune system, provided that its activity is affected in inflammatory conditions, such as rheumatoid arthritis. On this notion, a new interdisciplinary field called "osteoimmunology" has emerged, to study the close relationship between the immune and the skeletal system.

Osteoclasts are terminally differentiated, multinucleated, non-dividing cells of relatively short life-span, that derive from hematopoietic progenitors in the bone marrow, which also give rise to monocytes of peripheral blood, and to the various types of tissue macrophages (1). The osteoclast is a tissue-specific macrophage polykaryon, created by the cytoplasmic fusion of its mononuclear precursor cells. The hematopoietic origin of the osteoclast was firstly demonstrated after the restoration of osteopetrosis after bone marrow transplantation (figure 1) (2). A hematopoietic stem cell (HSC), common to lymphocytes, red blood cells, platelets, granulocytes and mononuclear phagocytes, progresses through the colony-forming unit for granulocytes and macrophages (CFU-GM) and the CFU for macrophages (CFU-M), into the pre-osteoclast and multinucleated cell. Finally, the mature active resorbing osteoclast is formed (3).

The activation of osteoclast function and osteoclastic bone resorption consists of multiple steps, starting with the proliferation of osteoclast progenitor cells, which then differentiate into mononuclear prefusion osteoclasts (pOCs); these fuse into multinuclear osteoclasts (up to eight nuclei can be found in a normal mammalian osteoclast) and osteoclasts migrate to the resorption sites. This activation occurs at or near the bone surface, since cell to cell contact between stromal and bone marrow cells, as well as osteoclasts and osteoblasts, is necessary (4). The adherence of the osteoclast to the bone matrix, through the $\alpha_v\beta_3$ integrins, is important for the initiation of the osteoclastic function (5). Integrins are transmembrane proteins that combine α and β subunits and

realize cell to cell and cell to matrix interactions, necessary for osteoclast activation. The integrin $\alpha_v\beta_3$ strongly is expressed by osteoclasts, and its binding to bone matrix is pivotal to the resorptive process (6). In fact, mice lacking β_3 integrins are osteosclerotic, due to dysfunctional osteoclasts (7).

The unique characteristics of the osteoclast are adopted to achieve mineral and organic bone resorption. The size of the cell (300μm as compared to 10-20μm of a macrophage) facilitates the osteoclast to cover the large matrix area. In order to resorb bone, it is essential for the osteoclast to attach the bone and assume a polarized morphology. The actin is reorganized and forms an 'actin ring' and the 'sealing zone' (a tight junction between the cell and the bone), which in turn encircles the 'ruffled border', that serves as the actual resorbing site (8). This compartment, the site of resorption, isolated from the general extracellular space, is acidified to a pH of approximately 4.5, by the fusion of acidic vesicles with the ruffled border, and by an electrogenic proton pump (H^+-ATPase) coupled to a Cl^- channel. The sealing zone separates the ruffled border and its acidic environment from the rest of the cell membrane, so the release of proton and chloride ions is directional only to the resorption pit (Howship's lacunae) (9). HCL dissolves the hydroxyapatite, to Ca^{2+}, HPO_4^{2-} and H_2O. To enable continuous release of HCl into the resorption area, protons are continuously produced, by the activity of carbonic anhydrase II, an enzyme that is highly expressed in osteoclasts and facilitates the hydration of CO_2, resulting in production of protons and HCO_3^-. HCO_3^- is exchanged to chloride by the chloride-bicarbonate exchanger located in the basolateral membrane. The osteoclast utilizes more than one enzyme to degrade organic matrix, and cathepsin K is the main collagen-degrading enzyme (10). This enzyme is the predominant proteinase in human osteoclasts, but other proteinases (cathepsin D, B, and L) are also present in these cells (11). The resorptiom products are endocytosed and secreted into the circulation, thus contributing to calcium and phosphate homeostasis. Then, the osteoclast is detached from the bone surface, depolarizes and continues to a new resorption site or undergoes apoptosis.

Remodeling begins with degradation of matrix by osteoclasts, a mixture of insoluble proteins, where type I collagen is predominant (>90%) and hydroxyapatite. Osteoblasts are then recruited to the site, where they secrete and mineralize new matrix. Until age 30–35, bone production exceeds or equals removal, thus increasing or maintaining bone mass. Thereafter, bone mass decreases, reflecting the predominance of osteoclast activity. Most adult skeletal diseases are due to increased osteoclast activity, leading to a

disequilibrium in bone remodeling, resulting to bone resorption and osteopenic situations (12,13).

Osteoporosis is a family of disorders where systemic bone mass is reduced, creating the risk of a spontaneous fracture, and all forms of osteoporosis reflect the increased osteoclast function compared to that of the osteoblast. This increase can involve either increase of activity or increase of the overall number of osteoclasts. Paget bone disease (PBD) is characterized by localized osteopenic lesions, also found in familial expansile osteolysis. In PBD, osteoclasts contain more nuclei than normal (up to 100 nuclei per cell). The process begins by an increased osteoclast-mediated bone resorption, originated by a gain of function of osteoclasts (14), which is compensated by an increase in bone formation, resulting to a disorganized mosaic of woven and lamellar bone (15). Osteopetrosis is characterized by an increase in bone mass, accompanied with the loss of distinction between the bone marrow space and the cortex. The 'malignant' form of osteopetrosis is inherited in an autosomal recessive way and reflects a decrease in osteoclast function as well as recruitment, while the 'benign' form of osteopetrosis is autosomal dominant (and occasionally recessive) and is characterized by dysfunctional osteoclasts. Osteopetrosis caused by arrested osteoclastogenesis can be further divided into osteoclast autonomous and non-autonomous osteopetrosis, where the cell responsible for the molecular defect is the osteoclast or its precursor in the first case and cells supporting osteoclast differentiation in the latter (16).

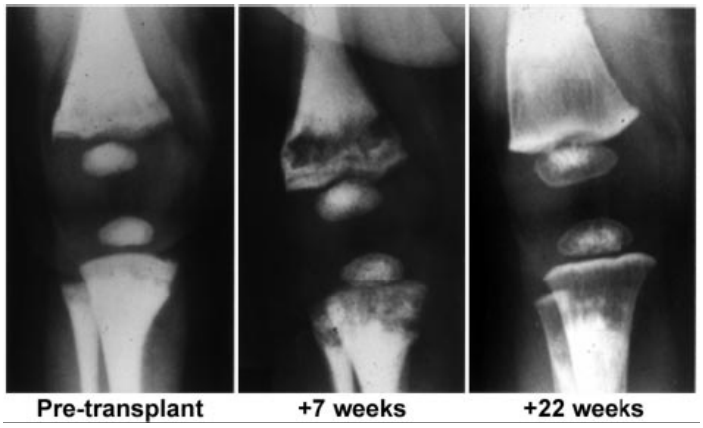

Figure 1: First cure of patient with malignant osteopetrosis. The patient, a 3-month-old female, received a marrow transplant from her HLA/MLC compatible brother. Dramatic resolution of the sclerotic bone was evident within 7 weeks. The patient is well 27 years later. The presence of Y-chromosomes in her osteoclasts after transplant established the cell's hematopoietic ontogeny in man

The differentiation and activation of the osteoclast is mainly regulated by a, recently identified, TNF family receptor molecule, RANK (Receptor Activator of Nuclear factor NF-κB) and its ligand RANKL (17). RANK is a type I homotrimeric transmembrane

protein, widely expressed. Mutations of RANK have not been described in humans, although there is a line of transgenic mice with spontaneously occurred deletion mutation, presented with osteopetrotic phenotype (18). RANKL is a type II homotrimeric protein, expressed as membrane bound and as a secreted protein, after the proteolytic cleavage generated from matrix metalloproteases on the ADAM domain (disintegrin and metalloprotease domain) (19). RANKL is expressed in lymph nodes, thymus, lung, and to a lower level, in tissues like bone marrow and spleen. RANKL expression is stimulated in osteoblast/stromal cells, by hormones and factors stimulating bone resorption, and binds RANK, expressed on osteoclast progenitor cells, thus activating the signalling pathway downstream RANK, which includes the recruitment of the adaptor protein TRAF6 (tumor necrosis factor receptor associated factor-6), leading to NF-κB activation and translocation to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 (nuclear factor of activated T cells-1) to trigger the transcription of osteoclastogenic genes (20). OPG (osteoprotegerin) acts as a decoy receptor of RANKL, and inhibits RANKL binding to its cellular receptor (21). OPG is expressed on osteoblasts in response to anabolic agents (as estrogens and TGF-β related bone morphogenic proteins) and protects the skeleton from excessive bone resorption. Therefore, the RANKL/OPG ratio is a determinant factor for bone mass and skeletal integrity. RANKL regulates osteoclast differentiation, but always in the presence of MCSF. MCSF (macrophage colony stimulating factor) is expressed by osteoblasts and their precursors, as a membrane bound and soluble protein, and in combination with RANKL are together sufficient to promote osteoclastogenesis (22). Mice lacking MCSF (op/op mice) develop osteoclast deficient osteopetrosis (23). MCSF stimulates osteoclast differentiation from its precursor cells, directly inducing RANK expression, but also promotes its functions as a mature cell, including spreading, motility and cytoskeletal organization (24, 25). The only MCSF receptor is c-fms, a tyrosine kinase, that autophosphorylates when occupated, activating ERK1/2 and PI3K/AKT signaling pathways, thus promoting proliferation and survival (26, 27). The osteoclast-associated receptor (OSCAR) is an activating-type immunoglobulin-like receptor induced in RANKL-stimulated BMMs and expressed on osteoclasts (28). OSCAR is involved in cell to cell interaction between osteoblasts and osteoclasts. FcRγ chain, a signal transducing adaptor molecule for Fc receptors, is associated with OSCAR and is involved in the cell surface expression of OSCAR. Furthermore, FcRγ is required for signal transduction by OSCAR, suggesting that the FcRγ-mediated signal transduction by OSCAR is involved

in osteoclast differentiation (29). OSCAR is expressed in all human myeloid cells, where it promotes dendritic cells survival. Stimulation of hOSCAR acts in conjunction with the Toll-like receptor (TLR) ligands, (LPS, polyI:C), to increase the expression of dendritic cell maturation markers, and to modulate cytokine release (30). hOSCAR is a functional receptor also on monocytes and neutrophils, involved in the induction of the primary proinflammatory cascade and the initiation of downstream immune responses (31). Kim et al showed that NFATc1 expression precedes that of OSCAR during RANKL-mediated osteoclastogenesis, since inhibition of NFATc1 by cyclosporin A (an inhibitor of calcineurin, which is a phosphatase that activates NFAT proteins) abolishes RANKL-induced OSCAR expression and subsequent osteoclast differentiation. The 1.0-kb promoter region of the OSCAR gene contains three potential NFATc1-binding sites. Furthermore, NFATc1 synergistically activates OSCAR, together with Mitf and PU.1, transcription factors previously shown to be critical for osteoclast differentiation. Elucidation of NFATc1 as a transcription factor for OSCAR expression implies the presence of a positive feedback circuit of RANKL-induced activation of NFATc1, involving NFATc1-mediated OSCAR expression and its subsequent activation of NFATc1, necessary for efficient differentiation of osteoclasts (32).

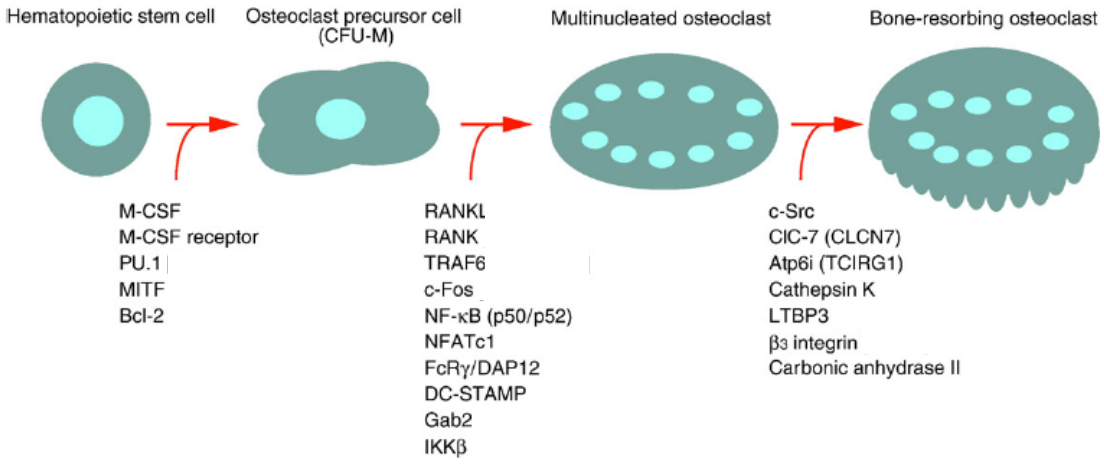

Figure 2: Osteoclast differentiation program, main regulatory molecules

1.2 OSTEOCLAST DIFFERENTIATION PROGRAM

As mentioned above, osteoclasts are of hematopoietic origin; a hematopoietic stem cell (HSM) progresses through the colony-forming unit for granulocytes and macrophages (CFU-GM) and after that, through the CFU for macrophages, in order to become a pre-osteoclast, only along with the presence of MCSF for this latter step. Then, the pre-osteoclasts form the multinucleated osteoclasts, which will finally generate mature and bone-resorbing osteoclasts (figure 2).

1.2.1 Factors regulating Osteoclasts Differentiation

In the first stage of osteoclastogenesis, four key molecules are involved, in order to generate common progenitors for macrophages and osteoclasts. The molecule that mediates the earliest event in osteoclastogenesis is PU.1, in interplay with Mitf (microphtalmia-associated transcription factor), and knockouts of both generate osteopetrosis. Mitf targets Bcl2, an antiapoptotic protein. MCSF and its only receptor, c-Fms, that dimerizes and autophosphorylates several tyrosine residues when occupated, provide signals for survival and proliferation of early osteoclast precursors. MCSF upregulates RANKL expression, that is prerequisite for the next step of differentiation. The transcription factor PU.1 binds to the promoter region of c-fms gene and activates its transcription. Moreover, the transcription factor Mitf binds to the promoter of Bcl2 gene and upregulates the expression of Bcl2, an anti-apoptotic protein, involved also in the osteoclast lineage. Tyrosines 559 and 807, in the cytoplasmic domain of c-Fms, are essential, while tyrosines 697, 706 and 721 are dispensable for osteoclast proliferation and differentiation (33).

M-CSF collaborates with $a_v\beta_3$ integrins, which mediate cell to cell and cell to matrix interactions, and actually both share a common pathway. For example, growth factors like MCSF, activate intracellular pathways, like ERK-c-Fos pathway, which is indispensable for proliferation and differentiation, but prolongation of such pathways, requires cell adhesion. Faccio et al studied $\beta_3^{-/-}$ preosteoclasts for components of MCSF-receptor that are crucial for osteoclastogenesis. They actually ascertained that high doses of MCSF can rescue the defected osteoclastogenesis (but not resorptive capacity) in $\beta_3^{-/-}$ precursors, by restoring the defective ERK signaling, suggesting that both the receptor and the integrin utilize ERK signaling pathway to promote cell proliferation, survival and differentiation, through enhanced c-fos expression. In fact, tyrosine Y697 is required for

sustained ERK phosphorylation and rescue of defective osteoclastogenesis in $\beta_3^{-/-}$ cells (34).

Mitf, a basic helix-loop-helix zipper protein, plays a key regulatory role in several cell types such as mast cells, melanocytes, and osteoclasts. Mitf regulates the expression of various genes, including mast cell protease 6, OSCAR, and cathepsin K. The mice with mutations at the *mi* locus develop severe osteopetrosis due to defective osteoclasts (25, 26), suggesting a key role for Mitf in osteoclastogenesis. Mitf interacts with c-fos and PU.1 in the cytoplasm and controls their subcellular localization (35). Recently, PIAS3 has been shown to suppress Mitf transcriptional activity by blocking its DNA binding domain, leading to the intriguing possibility that PIAS3 may play a key role in the regulation of bone remodeling (36).

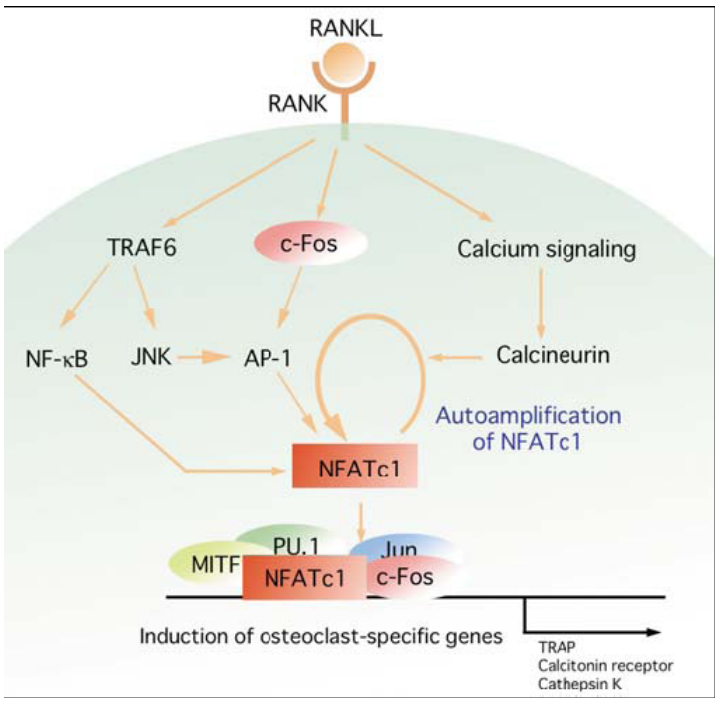

Figure 3: Integration of RANKL signaling by NFATc1, the master switch for osteoclastogenesis. RANKL signaling events are reconstituted in the context of NFATc1 induction and activation. RANKL induces the NFATc1 gene via TRAF6 and c-Fos signaling pathways, both of which are essential for osteoclastogenesis. Furthermore, RANKL evokes calcium oscillation, leading to the calcineurin mediated activation of NFATc1. RANKL triggers the sustained NFATc1-dependent transcriptional program, through autoamplification of the NFATc1 gene expression, during osteoclast differentiation. At the final stage of differentiation NFATc1 cooperates with AP-1 composed of c-Fos and Jun family members to induce osteoclast-specific genes such as TRAP, calcitonin receptor, and cathepsin K. MITF and PU.1 may also participate in the osteoclastogenic NFATc1: AP-1 complex in some promoters (27)

In the second step of differentiation, where multinucleated pre-osteoclasts are formed, the most important molecules participating are those that transduce the signal after the binding of RANKL to its receptor (4). The binding of RANKL, as a membrane bound protein of osteoblasts, to its receptor RANK, expressed on BMMs-osteoclast precursors, activates their differentiation into osteoclasts. RANK lacks intrinsic enzymatic activity in its intracellular domain, so it transduces signaling by recruiting adaptor molecules. RANK-RANKL interaction results to the recruitment and trimerization of TRAF6 (TNF-

receptor associated factor), and in parallel, the induction of expression of c-Fos. TRAF6 activates three downstream pathways: NF-κB, that translocates to the nucleus, Akt and MAPKs, including JNK and p38, and c-Fos activates the activator protein AP-1, a complex of transcription factors, composed of Fos, Jun and ATF. RANKL itself also activates NFATc1 indirectly, through calcium signaling and calcineurin, a phosphatase that activates NFAT family of factors when cytoplasmic calcium levels increase. All these together activate NFATc1, which plays the role of the master regulator of osteoclastogenesis, NFATc1 activation induces its autoamplification and then NFATc1, acting as a transcription factor, activates the transcription of osteoclastogenic genes (37-39).

In the final step of osteoclasts differentiation, mature multinucleated osteoclasts are generated. Bone degradation and terminal osteoclastogenesis require physical contact between the osteoclast and its precursor, respectively, and bone matrix. So, the molecules that osteoclasts can recognize and attach to the bone matrix are essential for resorption. It is now clear that the integrin $\alpha_v\beta_3$ is central to osteoclast/bone recognition, because mice with the $\alpha_v\beta_3$ gene knocked out have dysfunctional osteoclasts and become progressively osteopetrotic $\alpha_v\beta_3$ recognizes proteins in the bone matrix that contain the Arg-Gly-Asp (RGD) amino-acid motif. Osteoclasts that lack $\alpha_v\beta_3$ still attach to bone but fail to organize their actin cytoskeleton into a sealing zone and have abnormal ruffled membranes (40). In the cascade of events following integrins signalling, Pyk2 is phosphorylated, thus activating c-Src, which inhibits c-Cbl and matrix adhesion. c-Cbl, an E3 ubiquitin ligase that is also involved in osteoclast recruitment, polyubiquitylates c-Src, which probably leads to degradation of the Pyk2/c-Src/c-Cbl complex in the proteasome. Despite in vitro documentation of the importance of $\alpha_v\beta_3$, c-Src, Pyk2 and c-Cbl in osteoclast function, only mice that lack the integrin or c-Src have been shown to have a substantial bone phenotype. Therefore, if Pyk2 and c-Cbl are important to the osteoclast in vivo, there are compensatory mechanisms that function in their absence.

The mature bone-resorbing osteoclast expresses genes whose activity is critical for its function. Cathepsin K, which degrades collagen, carbonic anhydrase, TRAP and integrins $\alpha_v\beta_3$ are the most important molecules for the resorbing capacity of osteoclasts. Cathepsin K (CTSK) is a secreted protease and its levels increase with osteoclast differentiation and activation, regulated by RANKL, via TRAF6 and AP1 complex. RANKL stimulates cathepsin K expression and promoter activity in a dose- and time-dependent manner and

this activation is inhibited by either dominant negative (DN) TRAF6 or DN-c-fos (41). Carbonic anhydrase II (CA II), an enzyme necessary for osteoclast activity, is transcriptionally upregulated by c-Fos/AP-1. A functional AP-1 binding site is present in the CA II promoter and is necessary for this regulation. Furthermore, AP-1 binding activity is induced by treatment of bone marrow cultures with the osteoclastogenic hormone 1,25 dihydroxyvitamin D3. Thus, cFos/AP-1 can directly regulate the expression of the osteoclast marker CA-II and that AP-1 activity is upregulated in osteoclast progenitors in response to osteoclastogenic signals (42).

1.2.2 The osteoclast as a member of the osteoimmune system

Osteoclastogenesis is highly dependent on the cellular microenvironment, that apart its basic regulators MCSF and RANKL, provides further signals, such as proinflammatory cytokines, prostaglandins, the crosstalk with T cells, all induced in inflammatory conditions, known for the implication of bone loss, such as rheumatoid arthritis, periodontal disease, inflammatory bowel disease etc. A hallmark of RA is the rapid erosion of periarticular bone, which is often followed by general secondary osteoporosis (or osteopenia) and osteoclasts are found to be prevalent at the site of focal erosion.

In this context, the differentiation towards osteoclasts is affected by activated T cells, both directly and indirectly. Activated T cells produce themselves RANKL that promotes osteoclastogenesis, but also pro-inflammatory cytokines, such as IL-1, TNFα, IL-6, IL-11 and IL-15 that exert a stimulatory effect on osteoclasts differentiation, by inducing RANKL expression on osteoblasts and marrow stromal cells (43). Another factor that links osteoclasts with the immune system is the expression of TLRs on osteoclasts. In more details, TLRs activation on osteoclast precursors, mature osteoclasts and osteoblasts results in increased osteoclastogenesis, while activation of TLRs on early osteoclast precursors exerts an anti-osteoclastogenic effect (44). Most importantly, the process of osteoclast differentiation is highly influenced by the presence of pro-inflammatory cytokines, such as TNFα and IL-1.

TNF-α is among the most potent of the inflammatory cytokines involved in osteoclastogenesis. It induces catabolic processes, implicated in the pathogenesis of osteopenic conditions, such us rheumatoid arthritis and chronic inflammatory osteolysis. Actually, TNF-α induces LPS-stimulated osteoclastogenesis, via its p55 receptor, through the activation of NF-κB (45). It is shown that TNF-α alone, at any concentration, cannot induce the differentiation of murine osteoclast precursors, but can enhance dramatically

the differentiation of RANKL-primed cells, even if RANKL is at very low, even insufficient, levels of inducing osteoclastogenesis. Also, TNFα acts synergistically with RANKL in activating SAPK/JNK and nuclear translocation of NF-κB. Finally, TNF-α potentiates osteoclastogenesis by directly targeting osteoclast precursors, exposed to RANKL, since only TNF-R efficient cells respond to the osteoclastogenic signal of TNF and upregulate RANKL expression (46). Furthermore, TNF-α induces in vivo the expression of the stromal cell product M-CSF, that maintains survival and longevity of osteoclast precursors (47).

IL-1, like TNF-α, is another osteoclastogenic cytokine that promotes RANKL expression in marrow stromal cells and osteoblasts. In fact, IL-1 mediates TNF dependent RANKL expression, via enhanced expression of IL-1R, as it was confirmed by Wei et al, using a IL-1R antagonist and then measuring the amounts of RANKL mRNA produced. This action is mediated by phosphorylation and activation of p38 MAPK. Finally, IL-4, an abundant anti-inflammatory cytokine, produced mainly from activated T cells, suppresses TNF- and IL-1 mediated RANKL expression by inhibiting p38 activation. Actually a model has been proposed: TNFα, signaling through p38 MAPK, induces stromal cell expression of IL-1, which in turn upregulates its own receptor. Occupancy of now abundant IL-1R similarly activates p38, which promotes RANKL production (48).

1.3.1 TPL2 KINASE – TPL2 KNOCKOUT MICE

Tpl2 protein (Cot protein in humans) was first identified as an oncoprotein, overexpressed or structurally modified in rodent tumors (49). Tpl2 is expressed in the spleen, thymus, lungs and mostly in macrophages, B and T cells. The Tpl2 gene (Tumor progression locus 2) encodes for a serine/threonine kinase of the MAP3Ks family, that regulate the activation of MAPKs signaling pathways (ERK, JNK, P38). Tpl2 overexpression leads to the activation of these kinases and also ERK5, NFkB and NFAT (50-53). $Tpl2^{-/-}$ macrophages activated by LPS, CD40L or TNFa elicit a decreased phosphorylation of ERK and NFkB, but not JNK or p38, thus suggesting a more limited physiological role of the kinase (54, 55). Also, $Tpl2^{-/-}$ B cell activation leads to a decreased ERK phosphorylation (56). Tpl2 is activated downstream of several receptors, including TLRs (TLR2,3,4,7,9) and TNFR (TNFR1 and CD40), that are important for the immune response and inflammation, by signals as LPS (for TLR4), produced by gram-bacteria, and TNFa, abundant in situations of inflammation (57, 58).

In non-activated cells, Tpl2 forms a complex of strong avidity with the cytoplasmic factor p105 NFkB1 (inhibitor of NFkB) and with ABIN-2 inhibitor (NFkB2). The interaction of NFkB1, Tpl2 and ABIN-2 stabilizes and inactivates the Tpl2 kinase activity (59, 60). In this context, LPS and TNF-a when binding to their receptors, cause the release of Tpl2 from p105; then the active Tpl2, though unstable, is being degraded by the proteosome (61). IKK is activated, that phosporylates NFkB1 in a serine residue and NFkB1 is degraded also by the proteasome (62). The catalytic subunit of IKK, IKKb, is required for the signaling of NFkB and Tpl2/ERK activation, especially since IKKb is necessary for the phosphorylation of threonine 290 of Tpl2, that regulates Tpl2-NFkB1 binding (63,64). However, several more molecules are expexted to be required for Tpl2 activation and regulation. Recently several proteins with tyrosine kinase activity have been implicated in the activation of Tpl2 after LPS stimulation (65); Syk kinase is already shown that participates in Tpl2 activation upon TNFa stimulation of macrophages (66). In addition, the serine/threonine kinase Akt interacts with Tpl2, in order to regulate the function of Tpl2 in the NFkB signaling pathway in T cells (67).

The participation of Tpl2 in the pathways of TLRs and TNFRs exerts its importance in the immune response. It is expected that the lack of Tpl2 will result in decreased phosphorylation of MEK/ERK upon LPS or TNFa stimulation, and therefore decreased levels of pro-inflammatory cytokines produced. Actually, Tpl2 knockout mice ($Tpl2^{-/-}$) produce low levels of TNFa after LPS stimulation (54) and are resistant to LPS septic shock, as well as to TNFa-induced inflammatory bowel disease (57) and arthritis. However, these two syndromes are initiated by different cell types. To this end, it is important to delineate the role of Tpl2 in different cell types.

1.3.2 Tpl2 and Osteoclastogenesis

Accordingly, little is known about the participation of Tpl2 in the differentiation program towards osteoclast formation. However, several lines of evidence support the participation of ERK kinase in three major steps of osteoclast differentiation: RANK/RANKL interaction, c-fms/M-CSF binding and integrins signaling pathway. Therefore, RANK-RANKL interaction is known to result to the recruitment of TRAF6, that activates three downstream pathways: NF-κB, Akt and MAPKs (ERK, JNK and p38). TRAF6 itself interacts directly with Tpl2, upstream of MEK, that is required for ERK activation following anti-CD40 stimulation (68). Moreover, inhibition of ERK potentiates RANKL-induced osteoclast differentiation, suggesting that ERK is involved

in negative regulation of osteoclastogenesis (69). Recent data have shown that phosphorylated p38 enhances osteoclast survival, but not function, under inflammatory conditions in vivo (70). As far c-fms/M-CSF interaction is concerned, c-fms, the only MCSF receptor, is a tyrosine kinase that is autophosphorylated when occupated, and activates ERK1/2 and PI3K/AKT signaling pathways (26,27). Finally, $a_v\beta_3$ integrins, essential for osteoclasts adhesion to matrix and resorptive capacity, utilize ERK signaling pathway (34). Collectively, these data introduce a possible role of a MAP3K, such as Tpl2, in the signaling pathway downstream RANK/RANKL and c-fms/M-CSF and prior to ERK activation, that led as to study the osteoclast differentiation in Tpl2 knockout mice.

1.4.1 NALP3 – NALP3 KNOCKOUT MICE

The cells of the immune system have developed several mechanisms in order to recognize and fight a number of exogenous pathogenic microorganisms. The pattern recognition receptors (PRRs) are expressed on cells of the innate immunity, recognize components of the intruding microorganisms and activate mechanisms of the innate and the adaptive immunity. PRRs include Toll like Receptors (TLRs) that patrol the extracellular but also the intracellular environment from several pathogenic motifs (71), and also NLRs [nucleotide-oligomerization domain (NOD), leucine-rich repeat (LRR) containing Receptors] that act by activating the transcription of pro-inflammatory genes (72). Several members of the NLRs family, in response to stimulation, form a cytoplasmic complex of proteins, called inflammasomes that activate pro-inflammatory caspases, which, in turn, catalyze the maturation of the active forms of the target-cytokines (73-76). It is important that NLRs recognize not only pathogenic motifs (PAMPs), but also danger signals produced from the organism itself, such as DNA, RNA, uric acid, that are released from dying cells (77). NALP3 represents the most studied and major receptor that can recognize such signals.

Figure 4: Structure of NALP3 protein (78)

NALP3 inflammasome processes mainly interleukin-1β (IL-1β) and interleukin-18 (IL18) that are synthesized as immature, inactive precursors, and need a catalyzing process by caspase-1 to become active. IL-1β and IL-18 are pro-inflammatory cytokines, implicated in infections and trauma, and their overproduction is linked to septic shock and autoimmune diseases (79). The recruitment of caspase-1 to NALP3 inflammasome and its activation is the starting step of the production of these cytokines.

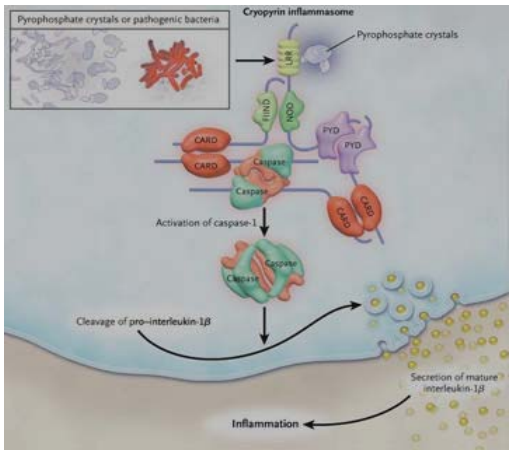

Figure 5: Signaling cascade of NALP3 inflammasome. NALP3 protein cooperates, through PYD-PYD interactions, with the adaptor-protein ASC, which contains the CARD region, necessary for the recruitment of caspase-1. Caspase -1 cleaves pro-interleukin-1β into mature interleukin-1β, resulting to inflammation.

Mutations in the CIAS1 gene that encodes for Nalp3 protein have been linked to three human autosomal dominant autoinflammatory syndromes, called cryopyrin-associated periodic syndromes. These are the Muckle-Wells syndrome (MWS), the Familial Cold Autoinflammatory Syndrome (FCAS) and the Neonatal Onset Multisystemic Inflammatory Disease (NOMID) (80, 81). These syndromes are characterized by periodical fevers, cutaneous rashes and arthritis. Monocytes isolated from these patients produce high levels of IL-1β (82), and actually patients respond well to the treatment with the IL-1 receptor antagonist (Anakinra) (83, 84).

In fact, most of the fourty mutations identified in the Nalp3 gene are located on the NACHT region (figure 4), which is implicated in the formation of the inflammasome. These mutations do not inactivate Nalp3 protein, but most likely cause a decrease in the threshold of the inflammasome formation and activation. Thus, a higher tendancy of inflammasome formation is correlated well with the increase of the IL-1β produced in these patients (85). To this end, Nalp3 knockout mice have been generated (86), with the constitutive depletion of the Nalp3 gene, which do not produce any specific phenotype, but are protected partially from septic shock, caused by low doses of LPS, but lose this protection in higher doses. Finally, mice that lack either ASC or NALP3 have impaired

contact-hypersensitivity responses to skin irritants. Such chemicals can trigger the release of IL-1β in keratinocytes and dendritic cells, which implies that these irritants might directly activate NALP3. These findings demonstrate an involvement of the NALP3 inflammasome in the T-cell-dependent contact-hypersensitivity response and provide further support for a role of NLRs in linking innate and adaptive immune responses (86,87).

1.4.2 Nalp3 and osteoclastogenesis

To this end, taking together the characteristics of NALP3 related human syndromes and the effect of IL-1 on osteoclast differentiation, we decided to study osteoclastogenesis in NALP3 knockout mice. IL-1β is produced in large amounts in the periphery of patients with cryopyrin-related syndromes, and this may be due to a facilitated inflammasome formation. As mentioned above, IL-1 is a potent inducer of osteoclasts differentiation, produced in high levels in inflammatory situations, such as rheumatoid arthritis, autoinflammatory syndromes etc. Actually, IL-1 mediates the TNFα-induced RANKL expression, thus promotion of osteoclastogenesis and bone erosion (48). In the contrary, in the NALP3 knockout mouse model, IL-1β is expected to be produced in lower levels, since NALP3 is one of the most potent caspase-1 activators, leading to the cleavage of pre-IL-1β into IL-1β. In addition, it was very recently shown that murine and human osteoblasts express NALP3, and that the level of expression of this cytosolic receptor is modulated after bacterial challenge. Furthermore, it was demonstrated that NALP3 expression is needed for caspase-1 activation and maximal induction of apoptosis in osteoblasts after infection with Salmonella, related with increased bone erosion in sites of infection. Therefore, it would be interesting to study the effect of the NALP3 absence in the pathway of osteoclast formation, in both processes of normal and under LPS-induced stress osteoclastogenesis (88).

2. OBJECTIVES

In the present study, we sought to determine whether Tpl2 and Nalp3 proteins are in some way implicated in the differentiation program of osteoclasts, since as described above, there are several findings that indicate a possible role of any of the two proteins in this process. Although, both Tpl2 knockout and NALP3 knockout mice do not present a phenotype that is related to a severe osteoclast deficiency (osteopetrosis) or hyperactivity (osteopenia/osteoporosis), it is not known whether each of the proteins participates in the signaling pathway of osteoclastogenesis.

Specific aim 1: To this end, we first established the differentiation process towards osteoclasts in C57BL/6 (wild type) mice from bone marrow derived cells. Then, we sought to confirm the same differentiation process in $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice.

Specific aim 2: In order to examine whether Tpl2 protein is implicated in the signaling pathways of factors regulating osteoclast differentiation (RANK/RANKL, GM-CSF/c-Fos or integrins) we assessed the levels of ERK phoshporylation upon LPS stimulation of osteoclasts differentiated from BMDMs from $Tpl2^{-/-}$ mice.

Secific aim 3: To delineate whether Tpl2 or NALP3 proteins are required for the differentiation process towards osteoclasts and whether osteoclastogenesis is affected in $Tpl2^{-/-}$ or $NALP3^{-/-}$ mice, we looked at the mRNA levels of osteoclast-specific genes, such as NFATc1, TRAP, Cathepsin-K, MMP-9, RANKL, OPG and OSCAR, in mature osteoclasts derived from BMDMs of both knockout mouse strains.

In order to assess the first objective, the osteoclasts were differentiated using a differentiation protocol from bone marrow derived cells, from WT mice and also in $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice. Cell cultures were stained with the osteoclast-specific stain TRAP on day 9 of the differentiation process. For the second objective, osteoclasts, in several days of the differentiation process, were used for protein extraction, after LPS and RANKL stimulation for 30 minutes, in order to examine the phosphorylation status of ERK. Finally, RNA were extracted from differentiated mature osteoclasts from WT, $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice, in order to perform real-time PCR for the osteoclast-specific genes and detect differences between wild-type and knockout mice.

3. MATERIALS AND METHODS

3.1 CELL CULTURES:

3.1.1 Isolation of Bone Marrow Cells

Bone Marrow cells are extracted from the femurs and tibia of one mouse, using a needle of 18G diameter, with 20ml of Phosphate Buffer Saline (PBS) in order to wash out bone marrow from the dissected ends of the bones. Cell aggregates are resuspended by well pipetting cell suspension with the needle.

3.1.2 Culture Medium

- MEM-a
- FBS
- Penicillin/Streptomycin
- L-Glutamine
- M-CSF
- RANKL

All cultures were performed in MEM-alpha medium (a-MEM), supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100µg/ml) and 2mM L-Glutamine (all from Gibco, Carlsbad, CA).

Specific growth factors for the osteoclastogenesis were used; 20ng/ml murine recombinant M-CSF (Peprotech) and 100ng/ml murine recombinant RANKL (Peprotech).

3.1.3 Osteoclasts differentiation from Bone Marrow Derived Cells

Cell suspension of bone marrow cells, isolated as described above are subsequently subjected to red blood cell (RBC) lysis, using sterile double destiled water for 4-5 seconds, and 10x HBSS (Hank's Balanced Salt Solution) to recuperate concentration. Cells are then washed with 10ml 1x HBSS and centrifuged for 10 minutes at 1400rpm. Cell pellet was resuspended in complete medium at a concentration of 3×10^5 cells/ml and plated in a 10cm diameter petri plate. Cells were cultured for 1 day with complete media, supplemented with 20ng/ml MCSF. After 1 day MCSF stimulation, non-adherent osteoclast precursors were collected and counted. Cells were plated at a concentration of

2 x 10^5 cells/ml in a 24 well-plate and media were supplemented with 20ng/ml MCSF and 100ng/ml RANKL. Cells were cultured for an additional 6 days, with addition of fresh media every 3 days. In two wells, round glass cover slips were added before plating the cells, in order to be stained on day 9 using TRAP staining kit (Sigma).

3.1.4 TRAP Staining of Osteoclasts

TRAP staining was performed using manufacturer's protocol (Sigma). Briefly, 2 wells were used for the staining, where media were removed before adding fixative solution in room temperature for 30 seconds. Cells were then washed thoroughly with prewarmed at 37ºC double destiled water, without drying. Then prewarmed solutions, consisting of Fast Garmet GBC, Sodium Nitrite solution, Napthol, acetate and with or without tartrate were respectively added in the two wells. Cells were incubated for 1 hour in a 37 ºC/5%CO_2 incubator and then rinsed thoroughly with ddH_2O. Cells were stained with Hematoxylin solution for 2 minutes and rinsed several times with alkaline tap water to blue nuclei. After air drying, cover slides were removed and evaluated microscopically.

3.2 FLOW CYTOMETRY

Flow cytometry is a technique for counting, examining, and sorting cells, suspended in a stream of fluid, based on the expression of surface and intacellular markers. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. Cells collected from cultures or immediately isolated from mice or humans can be suspended in a suspension consisting of PBS supplemented with 2,5% FBS, in order to be stained with fluorochromes conjugated on antibodies against intracellular or surface markers of the cells to be studied. Cells are incubated with the fluorescent-conjugated antibodies for 30 minutes on ice and in dark. The antibodies used were: anti-a_v-integrin-PE (CD51) (eBioscience) and IgG_{2b}-isotype-PE (BD Pharmingen). Then, cells are washed with 3ml PBS and centrifuged for 10 minutes at 1400rpm. Cell pellet is resuspended in 200μl PBS/FBS and flow cytometry was performed with an Epics Elite model flow cytometer (Coulter). Data were analysed using WinMDI software.

3.3 WHOLE CELL EXTRACTS PREPARATION

3.2.1 Stimulation of differentiating osteoclasts

Cells were collected from cell cultures after removing supernatants, using a scraper and 1ml of media. They were then centrifuged for 10 minutes at 1400rpm and then divided into three eppendorfs (approximately 10^6cells/condition). In the first condition, osteoclasts were not stimulated, in the second they were stimulated with 10ng/ml LPS and in the third with 100ng/ml RANKL for 30 minutes in 37ºC. For the last 5 minutes 5μg/ml pefabloc was added.

3.3.2 Whole Cell Protein Extraction

Cells were either collected from plates using a scraper or upon stimulation – as described above – were immediately centrifuged at 1400rpm for 10 minutes. Supernatants were discarded and cell pellet was resuspended in 200μl of protein lysis buffer, that causes the rupture of the cell-membrane and the nuclear membrane.

We used a lysis buffer consisting of: RIPA solution, 1x Protease Inhibitors, 1mM Na-Vanadate and 0,1M DTT. RIPA extraction buffer consists of:

10% glycerol	5% Na-deoxycholate
1M Tris-HCl pH 7,5	5M NaCl
10% NP-40	0,5M EDTA

Cells were incubated with the RIPA lysis buffer for 15 minutes on ice and then centrifuged for 15 minutes at 10000rpm at 4ºC. Supernatants, that are the protein extracts, were collected in a new eppendorf and stored at -80 ºC.

3.3.3 Quantification of protein extracts with a spectrophotometer

- Biorad Protein assay Dye Reagent (Biorad Lab. Ltd., Hemel Hempsted, UK)
- Cuvettes

The quantification of proteins concentration is performed using the spectrophotometer and the Biorad Protein assay Dye Reagent that alters its colour from light to dark blue, depending on the concentration of proteins. In eppendorfs, 1.2ml ddH_2O, 0.3ml Dye Reagent and 0.005ml of protein extracts are mixed well to prepare a homogenous suspension that is transferred into cuvettes. Samples are counted in a spectrophotometer at 595nm and proteins concentration is calculated according the equasion of the standard curve (y=0.01655x), that links density (OD) (=y) and proteins amount in mg (=x).

3.4 WESTERN BLOT

3.4.1 SDS-PAGE ELECTROPHORESIS

Protein electrophoresis in a denaturating polyacrylamide gel results to the separation of proteins based on their molecular weight. It consists of two gels, a stacking gel, where protein samples are loaded, and a resolving gel, where proteins are separated. A concentration of 10% of the resolving gel is usually used, but depends on the molecular weight of the proteins to be studied.

The resolving gel is prepared first using the electrophoresis apparatus (Biorad), in 1mm thikness usually. Small layer of isopropanol is added on top, to bring into line the polyacrylamide gel, and it is left to rest for 10-15 minutes, in order to be polymerized. Then, the stacking gel is prepared and added on top of the running gel (isoporopanol has been removed) and combs are placed in the gel, quickly before it is thickened. The gel is left to gelatinate and after that it is transferred into the tank filled with 1lt of running buffer. Combs are removed and wells are thoroughly washed with 1x running buffer. Components of the gels and running buffer are listed below.

Resolving gel (10% polyacrylamide):

- ddH_2O
- 30% polyacrylamide mix (29:1)
- 1,5M Tris (pH 8,8)
- 10% SDS
- 10% ammonium persulfate
- TEMED

Stacking gel:

- ddH_2O
- 30% polyacrylamide
- 1M Tris (pH 6,8)
- 10% SDS
- 10% ammonium persulfate
- TEMED

Running Buffer (10x):

- 0.25M Tris-HCl, pH=8.3
- 1.92M glycine
- 1% (w/v) SDS

Protein extracts are supplemented with the gel sample buffer that helps to denaturate proteins.

Gel Sample Buffer:
- 187.5 mM Tris-HCl,
- 150 mM β-mercaptoethanol
- 6% (w/v) SDS
- 0.03% (w/v) bromophenol blue

Protein samples are then boiled in the Heat Block for 10 minutes at 100ºC, so as proteins to be denaturated in their primary structure, and immediately placed on ice, to reduce temperature. Samples are centrifuged at 14000rpm for 1 minute and are next loaded in wells. A protein marker containing the following molecular weights: 181 KD, 115.5 KD, 82.2 KD, 64.2 KD, 48.8 KD, 37.1 KD, 25.9 KD, 19.4 KD (Invitrogen) is also loaded. The voltage of the apparatus is set at 100-120Volts and running takes place for 90-120 minutes, so as the proteins have run the gel and have been well separated. Molecular weight determination is based on the bands of the protein ladder.

3.4.2 Western Blot Analysis

Following the protein polyacrylamide gel electrophoresis, proteins must be blotted on a nitrocellulose transfer membrane. The gel is transferred from the previous apparatus into a different one consisting of a ‘cassette’ that includes two 3mm Whatmann papers and a nitrocellulose transfer membrane. Casettes are placed in a tunk full of 2lt Transfer buffer, in the correct direction, so as the proteins can move from the negative to the positive pole. Transfer of the proteins needs approximately 60-90 minutes. Then, the nitrocellulose membrane is placed in wash solution (TBST) supplemented with 10% milk, for blocking non-specific bindings, for 1 hour. Next, the membrane is washed three times with TBST, for 10 minutes and the primary antibody is added at the indicated concentration overnight, on a rocking apparatus, at 4ºC. The next day, the membrane is

washed thoroughly, three times, for 10 minutes each, and then the secondary antibody is added, in the indicated concentration, for 1 hour at room temperature on a rocking apparatus. The washing steps are repeated and finally the membrane is ready to be developed, using Enhanced Chemo Luminescence system (ThermoScientific), on an auto-radiography film (Fuji). The membrane can be re-used for the detection of a different protein, using another primary antibody. For this purpose, the membrane must be first subjected to 'stripping', for 10 minutes in a 50ºC water-bath.

Buffers used for this step of Western blotting are:

Transfer buffer:

- 25mM Tris-HCl, pH 8,3
- 0,2M glycin
- ddH_2O up to 800ml
- 200ml methanol

TBST:

- 60ml NaCl 5mM
- 20ml Tris-HCl 1M, pH 8,0
- 2ml Tween
- ddH_2O up to 2lt

Strip solution:

- 100mM 2-mercaptoethanol
- 2% SDS
- 62,5mM Tris-HCl pH 6,7

Primary antibodies used were: anti-phospho-ERK1/2 Ser217/Ser221 (Sigma) at 1:500 concentration and its corresponded anti-mouse-HRP secondary antibody (Sigma) (1:2000) and anti-tubulin at 1:1000 concentration and the same secondary antibody.

3.5 RNA EXTRACTION FROM OSTEOCLASTS

Cells from differentiation cultures (~10^6 cells) were collected after supernatants were removed, with 1ml of PBS. Samples were centrifuged for 10 minutes at 1400rpm and cell pellets were resuspended in 1ml of Trizol reagent (Sigma). Samples were store at -80ºC until use or immediately used for RNA extraction. 200µl of chloroform were added, tubes were shaken for 15 seconds and left to rest for 3-5 minutes. Then, they were centrifuged for 15 minutes at 13.000rpm at 4ºC in order to separate the organic from the aqueous phase that contains RNA. The aqueous phase is transferred in a new eppendorf and incubated with equal amount of isopropanol for 10 minutes and thes is centrifuged for 10 minutes, at 12000rpm at 4ºC. The pellet consists of the RNA, that is washed twice with 1ml ethanol 75%, left to air dry, and is resuspended in 50µl H_2O.

In order to calculate the RNA quantity and purity, we counted optical density at 260nm and 280nm, using nanoDrop technologie. The ratio of the two absorbances provides the purity of RNA (1,8-2 ratio is required for a pure RNA sample) and the quantity is calculated based on the optical absorbance at 260nm (1nm corresponds to 40µg/ml RNA). RNA samples are stored at -80ºC or immediately subjected to reverse transcription into cDNA.

3.6 REVERSE TRANSCRIPTION-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is an in vitro method for amplifying a defined piece of mRNA from a cell population. First, DNA remnants are removed by a DNase-I treatment for 20 minutes at room temperature, and EDTA addition for 10 minutes at 65ºC. Ideally, we start from an amount of 2µg of RNA to transcript into cDNA

A mix of RNA (2µg), 2µl dNTPs mix (10mM) and 1µl of Random Hexamers (Invitrogen) is prepared and incubated for 5 minutes at 65ºC, in order for the RNA to be denaturated and secondary structures or aggregates to be destroyed. Next, a mixture of cDNA buffer, DTT, H_20, RNease out (ribonuclease inhibitors) and reverse transcriptase (ThermoscriptRT, Invitrogen) is prepared and added in the initial mix of RNA. Sample is incubated for 60 minutes at 50ºC and ended by incubation for 5 minutes at 85ºC, in order to deactivate reverse transcriptase.

A cDNA sample was also prepared from differentiated osteoclasts that express the genes that were studied, in order to be used as a reference sample and provide a standard curve of decreasing RNA amount.

3.7 REAL-TIME PCR

Real Time polymerase chain reaction (real-time PCR) is performed in a special Thermal Cycler (ABI-Prism 7000 Real Time PCR). The double stranded DNA produced in each cycle of the reaction can be calculated by the counts of the DNA-binding agent used, that in this case was Sybr-green, that binds non-specifically in the dsDNA, but not in single-stranded DNA.

cDNA produced from the reverse transcription is diluted 10 times in DEPC water. In each reaction we use 5μl of the diluted cDNA sample to serve as a template. cDNA is incubated with a set of gene specific primers, dNTPs 10mM, $MgCl_2$, PCR master mix, Sybr green enriched with ROX dye, in a reaction of 20μl final volume.

The quantification of the target-gene product is based on the quantification of a reporter gene, constituvely expressed in the same samples where we examine the expression of osteoclast-specific genes. To achieve a relevant quantification of the target-gene, both reference gene and gene under examination are amplified in the same time, using gene-specific primers. In the experiment under investigation murine β-actin, a cytoskeleton protein was used. The data were processed using the relative quantification of genes expressed, by serial dilutions of a sample positive for genes studied, thus creating a standard curve, for both control and target gene. The expression of the target gene is calculated by the ratio of the amount of the reference gene expressed (actin) divided to the amount of the target gene (normalized).

Analysis is performed on a ABI-Prism 7000 to create standard curves and relative quantifications. Osteoclast-specific genes that were examined are: TRAP, NFATc1, MMP9, Cathepsin K, RANKL, OSCAR and OPG. The temperature profile consisted of an initial 95° C step for 10 minutes (for Taq activation), followed by 40 cycles of 95° C for 15 sec and 72° C for 30 seconds. Sequences of the gene-specific primers used are shown below:

mu actin forward:	GGAGATTACTGCTCTGGCTC
mu actin reverse:	GGACTCATCGTACTCCTGCT
muNFATc1 forward:	CGCAGAACACTACAGTTATG
muNFATc1 reverse:	ATTGGCTGAAGGAACAG

muRANKL forward:	CGGGTTCCCATAAAGTC
muRANKL reverse:	GGCGTACAGGTAATAGAAGC
muTRAP forward:	TGCGACCATTGTTAGCCA
muTRAP reverse:	ACATAGCCCACACCGTTCTC
muCATHEPSIN K forward:	GGCCAGGATGAAAGTTGTA
muCATHEPSIN K reverse:	CTTCAGGGCTTTCTCGTT
muMMP9 forward:	CATTCGCGTGGATAAGGA
muMMP9 reverse:	TCACACGCCAGAAGAATTTG
muOSCAR forward:	GGTCTCAGCCCAGTAATGT
muOSCAR reverse:	TCATGCCCGGTATGC
muOPG forward:	TGAGGAAGGGCGTTAC
muOPG reverse:	AAACTGTGTTTCGCTCTG

4. RESULTS

4.1 Differentiation of Osteoclasts from Bone Marrow Cells

The first experimental approach was to establish the differentiation process from bone marrow derived cells into osteoclasts, in wild type mice (C57BL/6) and then reproduce the differentiation in $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice. To this end, cells in the late differentiation stage (day 9 of differentiation protocol) were stained with the osteoclast-specific histochemical stain TRAP (Tartrate-Resistant Acidic Phosphatase) and pictures were obtained using the light microscope. The expression of TRAP, is regarded as an osteoclast marker, although there are other cells of the macrophage/dendritic cell family that express TRAP under certain conditions (89). TRAP is mostly characterized by its biochemical property, i.e., through its acid phosphatase function, which cannot be blocked by tartrate. Figure 6 represents the pictures obtained from differentiated osteoclasts from BL6 mice (A) and NALP3 KO mice (B) on day 9 of differentiation. Cells with two or more nuclei can be recognised, that is a characteristic feature of osteoclasts.

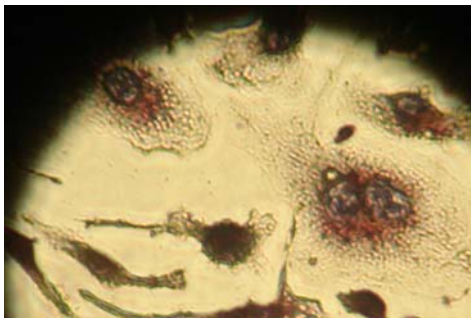

Figure 6A: TRAP staining of osteoclasts differentiated from C57BL/6 bone marrow cells

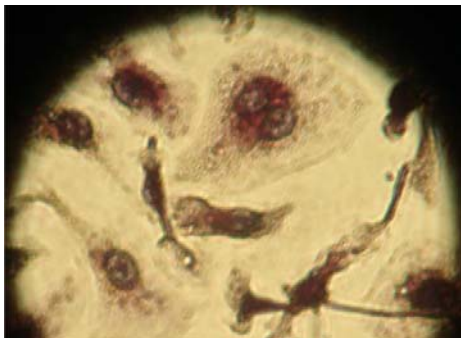

Figure 6B: TRAP staining of osteoclasts differentiated from NALP3 KO bone marrow cells

4.2 Osteoclasts characterization by flow cytometry

The integrin $\alpha_v\beta_3$ is strongly expressed by osteoclasts and is required for the adherence of the osteoclast to the bone matrix. The binding of the osteoclast to the bone matrix is essential for its activation and initiation of resorptive function. Integrins are transmembrane proteins that combine α and β subunits and realize cell to cell and cell to matrix interactions, necessary for osteoclast activation. In fact, mice lacking β_3 integrins are osteosclerotic, due to dysfunctional osteoclasts.

Therefore, in order to confirm that bone marrow cells have been differentiated into osteoclasts, flow cytometry was performed for the expression of α_v integrin on the surface of osteoclasts, at the end of the differentiation process.

Thus, cells on day 9 of the differentiation, were collected, washed with PBS and stained with anti-a_v integrin antibody, conjugated with the fluorochrome phytoerythrine (PE). Osteoclasts are characterized as a_v-expressing cells.

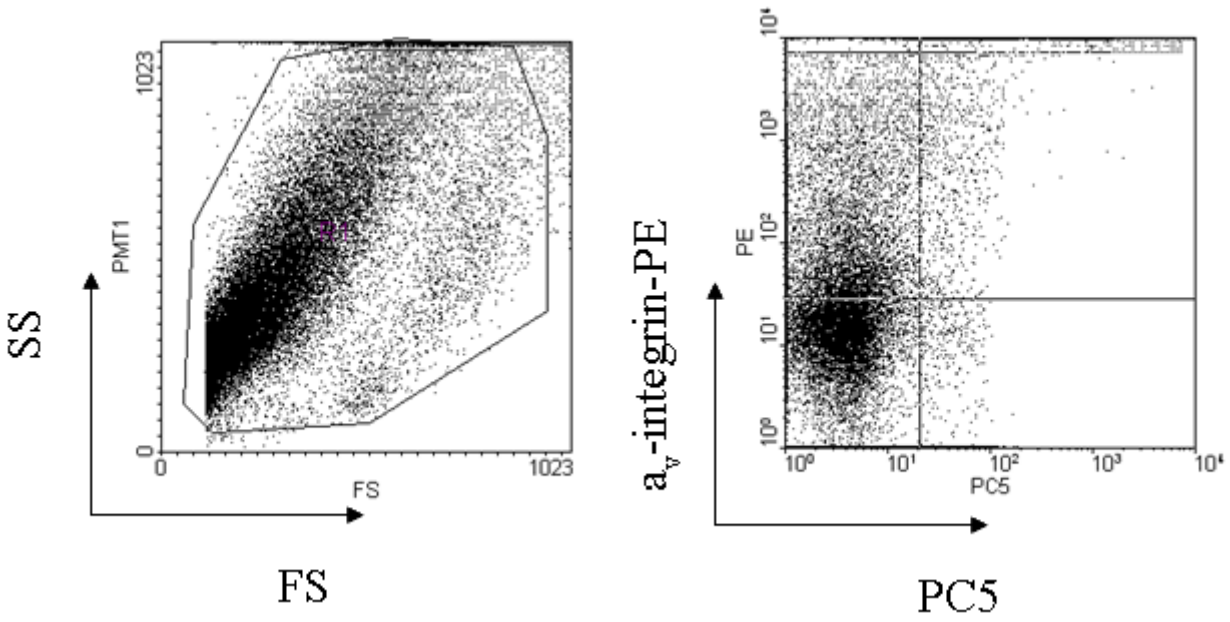

Figure 7: FACS plots depicting cells upon differentiation process

The above FACS plots depict cells upon osteoclastogenesis. The left plot shows the total cell population and the panel on the right shows cells stained for anti-a_v-integrin-PE conjugated. Cells positive for the expression of a_v integrin at a percentage of at least 60% can be thus characterized as osteoclasts.

4.3 Tpl2 protein involvement in osteoclast differentiation

In order to study whether Tpl2 protein is implicated in the differentiation process of osteoclasts, we isolated bone marrow derived cells from Tpl2$^{-/-}$ and wild type (BL6) mice and cultured them with 20ng/ml MCSF and 100ng/ml RANKL, following the osteoclastogenesis protocol, as described in Materials and Methods (3.1.3). Cells from both Tpl2$^{-/-}$ KO and WT mice were collected on day 5 of the differentiation protocol and were divided in three conditions. In the first condition, cells were not stimulated, in the second condition cells were stimulated with 10ng/ml LPS for 30 minutes and in the third condition with 100ng/ml RANKL for 30 minutes. Cells were lysed with a protein extraction buffer (RIPA) for 15 minutes on ice and whole protein extracts were collected. Protein extracts were quantified and 40μg of each sample were loaded on a 10% polyacrylamide gel to proceed into Western Blotting.

The phosphorylation status of ERK1/2 was examined, induced upon LPS stimulation, using an anti-pERK antibody in 1:1000 dilution. As a loading control, an anti-actin murine antibody was used in a 1:1000 dilution, developed from the same blot. As shown in figure 7, it is observed that the phosphorylation of ERK is not induced upon stimulation of osteoclasts with either LPS or RANKL, and most importantly, it does not differ when Tpl2$^{-/-}$ KO cells are compared to WT cells. Thus, it can be assumed that Tpl2 protein might not be implicated in osteoclasts differentiation, and this was examined in an early phase of osteoclastogenesis.

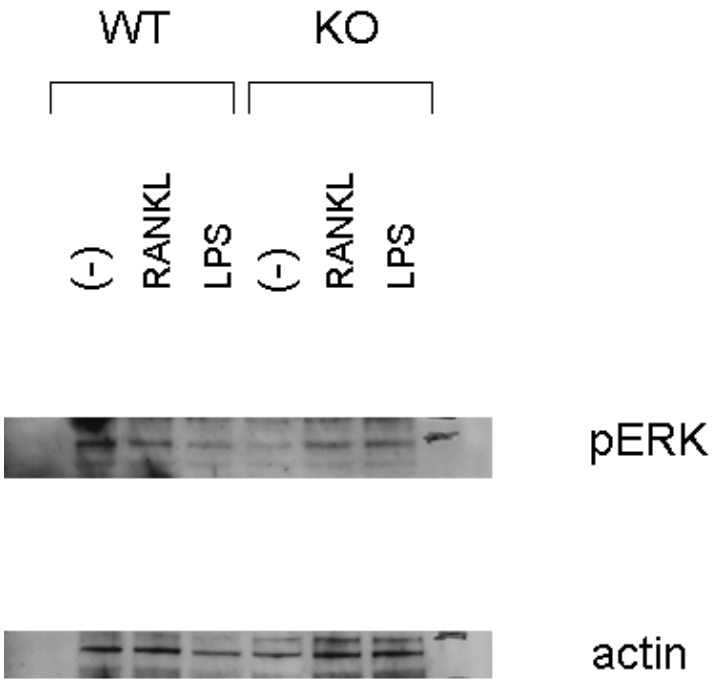

Figure 8: Western Blotting of osteoclasts stimulated with LPS or RANKL, in order to examine ERK phosphorylation status.

4.4 Implication of Tpl2 and NALP3 proteins in the differentiation process towards osteoclasts

In order to further study whether Tpl2 and NALP3 proteins are involved in the differentiation process towards osteoclasts, we examined the expression of several osteoclast-specific genes, such as TRAP, NFATc1, Cathepsin K and MMP9, in osteoclasts derived from $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice. Bone marrow cells cultured without the addition of RANKL and differentiated osteoclasts (cultured with MCSF and RANKL, as described in Materials and Methods) from both knockout strains and also from wild-type mice were collected, in order to be subjected to RNA extraction.

RNA was quantitated, reversally transcripted into cDNA and used for real-time PCR, in order to examine the up-regulation of osteoclast-specific genes in cells treated for osteoclastogenesis as compared to untreated, and also to detect differences in osteoclast-gene-expression between WT and the two KO strains. The expression of murine β-actin was used as a reference gene.

Following, diagrams presented show the induction of the genes: TRAP (figure 9), cathepsin K (figure 10), NFATc1 (figure 11) and MMP9 (figure 12), in osteoclasts from WT, $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice, as compared to cells non-treated for osteoclastogenesis, from the same three mouse strains.

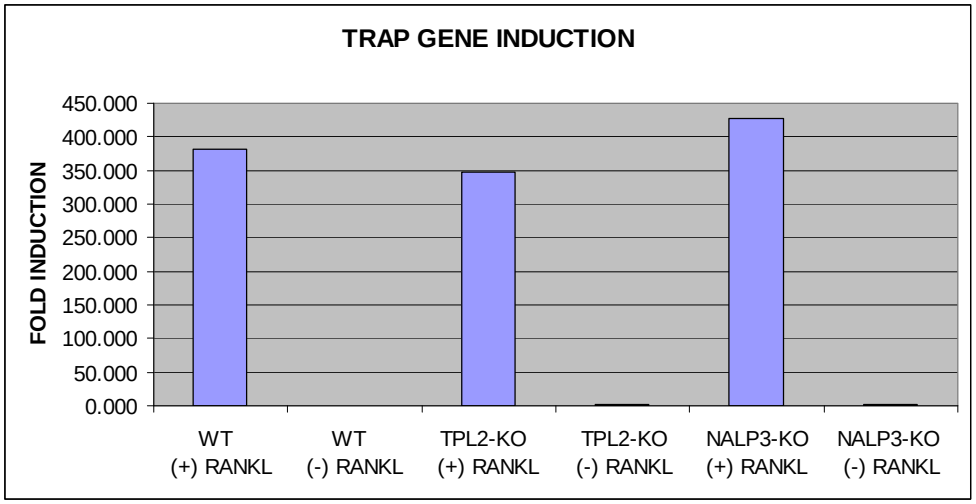

Figure 9: Induction of TRAP mRNA levels in cells treated with RANKL, from WT, $Tpl2^{-/-}$ and $NALP3^{-/-}$ mice

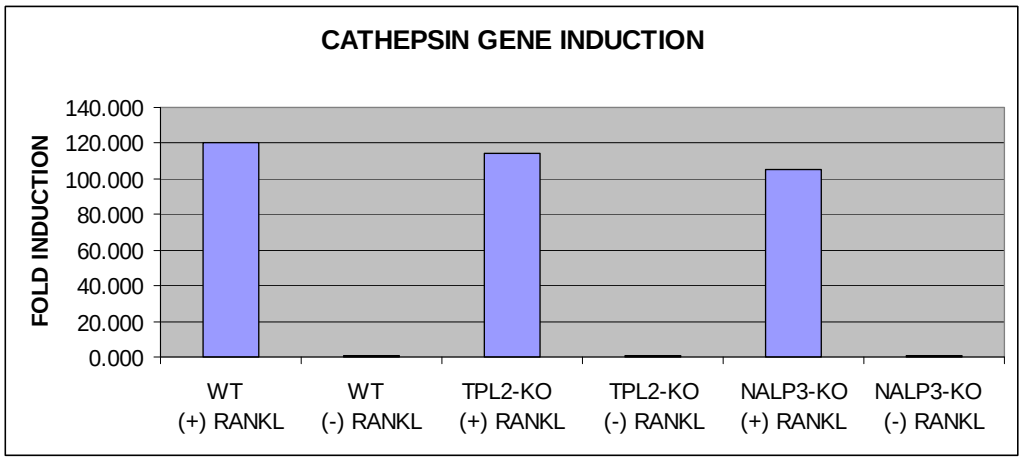

Figure 10: Induction of cathepsin-K mRNA levels in cells treated with RANKL, from WT, Tpl2$^{-/-}$ and NALP3$^{-/-}$ mice

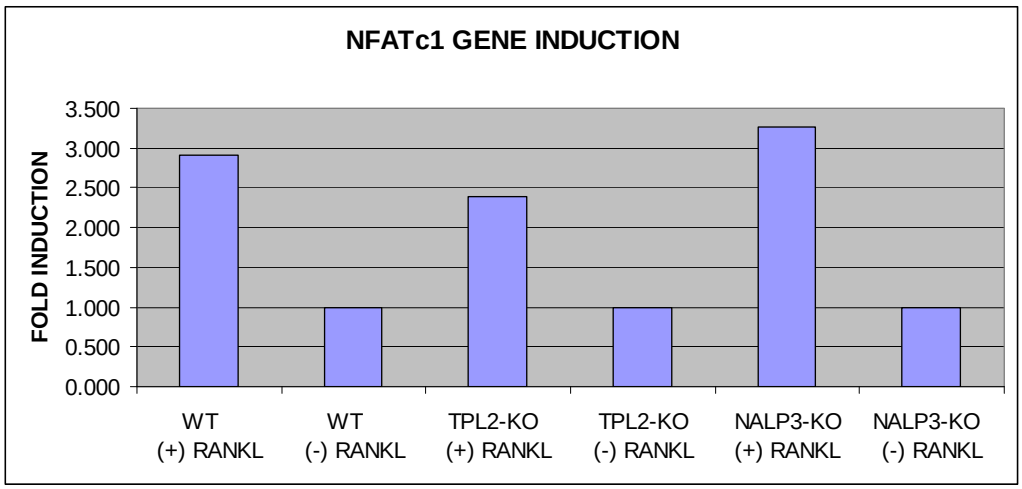

Figure 11: Induction of NFATc1 mRNA levels in cells treated with RANKL, from WT, Tpl2$^{-/-}$ and NALP3$^{-/-}$ mice

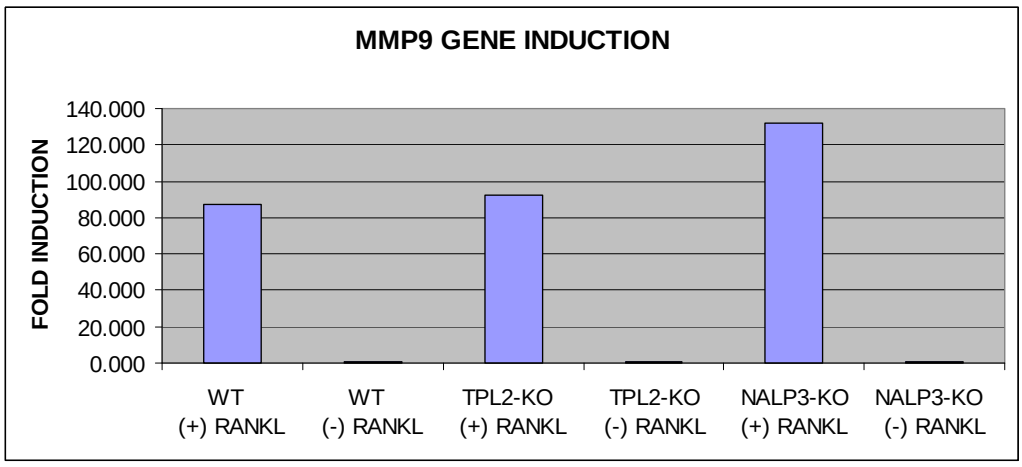

Figure 12: Induction of MMP9 mRNA levels in cells treated with RANKL, from WT, Tpl2$^{-/-}$ and NALP3$^{-/-}$ mice

As observed, the addition of RANKL does induce the expression of all the osteoclast-specific genes, TRAP, cathepsin K, NFATc1 and MMP9. In fact, TRAP gene is up-regulated 300 times in RANKL-treated cells, and this is independent of the mouse strain. There is also a minimum 100 fold induction of the expression of cathepsin K and MMP9 in cells treated with RANKL as compared to untreated cells. NFATc1 is also up-regulated (3 fold induction) upon RANKL treatment, in all three groups. Most importantly, the up-regulation of all genes is in comparable levels among WT and both KO mice. Taken together, these data indicate that neither Tpl2 nor NALP3 proteins are implicated in the differentiation process towards osteoclasts, since both $Tpl2^{-/-}$ and $NALP3^{-/-}$ cells up-regulate, in levels similar to WT cells, all osteoclast-specific genes.

5. CONCLUSIONS – DISCUSSION

The osteoclast is characterized as the 'sole' bone-resorbing cell, and this is due to its unique characteristics, that provide osteoclast with the ability to dissolve organic and mineral bone. These characteristics include its size, multiple nuclei, the actin ring, the sealing zone, the ruffled border with the acidid pH and the enzymes that osteoclasts produce. Thus, osteoclasts are responsible for the bone resorption, during two main physiological processes, bone modeling – during development – and bone remodeling, a lifelong process intended for the renewal of tissue. In these situations, osteoclasts' activity is well balanced with osteoblasts' activity, the cell population responsible for the synthesis of bone, in order to maintain bone matrix in stable condition. Another important feature of the osteoclast is the fact that osteoclasts' development and function are largely controlled by the microenvironment, especially by pro-inflammatory cytokines such as IL-1 and TNFa. Therefore, osteoclast formation and function are enhanced in inflammatory situations, such as rheumatoid arthritis, leading to the clinical feature of osteopenia and osteoporosis, a condition of systemic reduced bone mass. Finally, studying molecules associated with the differentiation and function of osteoclasts can reveal the identification of new targets in the therapy of osteoclast-associated bone loss, a common feature of inflammation related diseases.

In this context, Tpl2 and Nalp3 protein are involved, in different ways, in inflammatory conditions. Tpl2 as a serine/threonine kinase of the MAP3Ks family, mediates the activation of ERK1/2, JNK and p38 kinases and also ERK5, NFkB and NFAT (50-53). Tpl2 is activated downstream of several receptors, including TLRs (TLR2,3,4,7,9) and TNFR (TNFR1 and CD40), that are important for the immune response and inflammation, by signals such as LPS (for TLR4), produced by gram (-) bacteria, and TNFa, abundant in situations of inflammation (57, 58). It is expected that the lack of Tpl2, through decreased phosphorylation of MEK/ERK upon LPS or TNFa stimulation, will result in decreased levels of pro-inflammatory cytokines. ERK kinase, that can be downstream of Tpl2 activation, is implicated in three major steps of osteoclast differentiation: RANK/RANKL interaction, c-fms/M-CSF binding and integrins signaling pathway. To this end, it is important to delineate the role of Tpl2 in different cell types, such as osteoclasts, that are closely linked to the immune response. Nalp3 protein is involved in the immune response, through its participation in the formation of the multi-protein complex of inflammasome, that mainly mediates the production of IL-

1b and IL-18, major pro-inflammatory cytokines. Nalp3 inflammasome is activated following several danger signals, such as change in the pH, DNA or RNA release and uric acid production. Nalp3 inactivation can lead to a decreased production of IL-1b and IL-18, and therefore a positive result in the context of an inflammatory condition. Osteoclasts are highly influenced, and positively affected in terms of differentiation and activation, by pro-inflammatory cytokines, and most importantly IL-1 and TNFa. Thus, the manipulation of Nalp3 could possibly affect the differentiation or function of osteoclasts.

Collectively, the data from the literature presented above led us to examine the possible role of Tpl2 and Nalp3 proteins in the differentiation process towards osteoclasts. We differentiated osteoclasts from bone marrow cells isolated from $Tpl2^{-/-}$, $Nalp3^{-/-}$ and wild type mice and controlled the differentiation process with the osteoclast-specific TRAP staining, with the assesement of ERK phosphorylation on $Tpl2^{-/-}$ osteoclasts and basically with the analysis of mRNA levels of osteoclast-specific genes in all three mouse strains. From the results obtained, both with western blotting and real-time PCR, it was apparent that not significant differences in osteoclast formation appeared in the two knock-out mouse strains. Phosphorylation of ERK was not different in $Tpl2^{-/-}$ mice as compared to WT, upon the activation of osteoclasts with LPS or RANKL. Next, and most importantly, from the real-time PCR data, it was clearly shown that the levels of expression of osteoclast-specific genes, were comparable between all three mouse strains and for all genes studied (TRAP, NFATc1, MMP9 and cathepsin K). Subsequently, it was concluded that neither Tpl2 nor Nalp3 proteins are involved in the differentiation process of osteoclasts, since osteoclasts formation was not impaired in both $Tpl2^{-/-}$ and $Nalp3^{-/-}$ knock-out mice, and this was examined in different stages of the differentiation process.

REFERENCES:

1. Steven L. Teitelbaum**,** Rous-Whipple Award Lecture, Osteoclasts: What Do They Do and How Do They Do It? Osteoclasts in Health and Disease 429, *AJP February 2007, Vol. 170, No. 2*

2. Coccia PF, Krivit W, Cervenka J, Clawson C, Kersey JH, Kim TH, Nesbit ME, Ramsay NK, Warkentin PI, Teitelbaum SL, Kahn AJ, Brown DM: Successful bone-marrow transplantation for infantile malignant osteopetrosis. *N Engl J Med 1980*, 302:701–708

3. Zvi Bar Shavit, The osteoclast: a multinucleated, hematopoietic-origin, bone resorbing osteoimmune cell, *J Cellular Biochemistry (2007)* 102: 1130-1139

4. Suda, T., et al. 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.

5. Davies J, Warwick J, Totty N, Philp R, Helfrich M, Horton M The osteoclasts functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J Cell Biol (1989)* 109:1817–1826

6. Nakamura I, Duong LT, Rodan SB, Involvement of $a_v\beta_3$ integrins in osteoclast function, *J Bone Miner Metab (2007)* 25:337-344

7. Kevin P. McHugh, Kairbaan Hodivala-Dilke, Ming-Hao Zheng, Noriyuki Namba, Jonathan Lam, Deborah Novack, Xu Feng, F. Patrick Ross, Richard O. Hynes, and Steven L. Teitelbaum, Mice lacking b3 integrins are osteosclerotic because of dysfunctional osteoclasts, *J Clin Invest 2000,* vol 105, 4

8. Vaananen K. Mechanism of osteoclast mediated bone resorption-Rationale for the design of new therapeutics. *Adv Drug Deliv Rev 2005* 57: 959–971.

9. William J. Boyle, W. Scott Simonet & David L. Lacey, Osteoclast differentiation and activation, *Nature 2003*, Vol 423

10. Gowen M, Lazner F, Dodds R, Kapadia R, Field J, Tavaria M, Bertoncello I, Drake F, Zavarselk S, Tellis I, Hertzog P, Debouck C, Kola I. Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. *J Bone Miner Res 1999,* 14: 1654–1663.

11. Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M.. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J Biol Chem 1996* 271: 12511–12516.

12. FP Ross and AM Christiano, Nothing but skin and bone, *J Clin Invest, 2006*, 116: 1140-1149

13. Matthew T Gillespie, Impact of cytokines and T lymphocytes upon osteoclast differentiation and function, *Arthritis Research & Therapy, 2007*, 9: 103

14. Weinstein RS. 1995. The rate of bone resorption in Paget's disease proceedings of the second international symposium on Paget's disease of Bone-Sydney. Australia.

15. Roodman GD, Windle JJ. Paget disease of bone. *J Clin Invest 2005* 115:200–208.

16. SL Teitelbaum and FP Ross, Genetic regulation of osteoclast development and function, *Nature Reviews, Genetics, 2003,* vol 4

17. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/ osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Natl Acad Sci 1998* USA 95:3597–3602.

18. Kapur RP, Yao Z, Iida MH, Clarke CM, Doggett B, Xing L, Boyce BF: Malignant autosomal recessive osteopetrosis caused by spontaneous mutation of murine Rank. *J Bone Miner Res 2004*, 19:1689-1697.

19. BF Boyce and L Xing, Biology of RANK, RANKL and osteoprotegerin, *Arthritis Research & Therapy 2007* 9/S1

20. Asagiri M, Takayanagi H, The molecular understanding of osteoclast differentiation, *Bone* 40 *(2006)* 251-264

21. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. *Cell 1997* 89:309–319.

22. Yao, G.-Q., Sun, B. H., Weir, E. C. & Insogna, K. L. A role for cell-surface CSF-1 in osteoblast-mediated osteoclastogenesis. *Calcif. Tissue Int. (2002)* 70, 339–346.

23. Yoshida, H. et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature (1990)* 345, 442–444.

24. Insogna, K. L. et al. Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts. *(1997) J. Clin. Invest.* 100, 2476–2485.

25. J.MW Quinn and M.T Gillespie, Modulation of osteoclast formation, *Bioch and Biophys Research Commun 328 (2005)* 739-745

26. Faccio R, Zallone A, Ross FP, Teitelbaum SL: c-Fms and the $a_v\beta_3$ integrin collaborate during osteoclast differentiation. *J Clin Invest 2003*, 111:749–758

27. H. Takayanagi, Mechanistic insight into osteoclast differentiation in osteoimmunology, *J Mol Med, (2005)* 83: 170-179

28. Kim, N., Takami, M., Rho, J., Josien, R. & Choi, Y. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. *J. Exp. Med. (2002)*, 195, 201–209

29. Satoru Ishikawa, Noriko Arase, Tadashi Suenaga, Yoshitomo Saita, Masaki Noda, Takayuki Kuriyama, Hisashi Arase and Takashi Saito, Involvement of FcRγ in signal transduction of osteoclast-associated receptor (OSCAR) *International Immunology 2004*, Vol. 16, No. 7, pp. 1019-1025

30. Estelle Merck, Blandine de Saint-Vis, Mathieu Scuiller, Claude Gaillard, Christophe Caux, Giorgio Trinchieri, and Elizabeth E. M. Bates, Fc receptor γ-chain activation via hOSCAR induces survival and maturation of dendritic cells and modulates Toll-like receptor responses, *Blood 2005,* Vol. 105, No. 9, pp. 3623-3632.

31. Estelle Merck, Claude Gaillard, Mathieu Scuiller, Patrizia Scapini, Marco A. Cassatella, Giorgio Trinchieri, and Elizabeth E. M. Bates, Ligation of the FcRγ Chain-Associated Human Osteoclast-Associated Receptor Enhances the Proinflammatory Responses of Human Monocytes and Neutrophils**,** *The Journal of Immunology,* 2006, 176: 3149–3156.

32. Kabsun Kim, Jung Ha Kim, Junwon Lee, Hye-Mi Jin, Seoung-Hoon Lee, David E. Fisher, Hyun Kook, Kyung Keun Kim, Yongwon Choi, and Nacksung Kim, Nuclear Factor of Activated T Cells c1 Induces Osteoclast-associated Receptor Gene Expression during Tumor Necrosis Factor-related Activation-induced Cytokine-mediated Osteoclastogenesis, *J. Biol. Chem., 2005*, Vol. 280, Issue 42, 35209-35216

33. Feng X et al, Tyrosines 559 and 807 in the cytoplasmic tail of the m-csf receptor play distinct roles in osteoclast differentiation and function, *Endocrinology, 2002,* 143: 4868-4874

34. Roberta Faccio, Sunao Takeshita, Alberta Zallone, F.Patrick Ross and Steven L.Teitelbaum, c-Fms and $\alpha_v\beta_3$ integrin collaborate during osteoclast differentiation. *J Clin Invest 2003*, vol 111, number 5: 749-758

35. Shintaro Nomura, Takahiko Sakuma, Yuji Higashibata, Keisuke Oboki, and Motohiko Sato, Molecular cause of the severe functional deficiency in osteoclasts by an arginine deletion in the basic domain of Mi transcription factor, *J Bone Miner Metab (2001)* 19:183–187

36. Kabsun Kim, Junwon Lee, Jung Ha Kim, Hye Mi Jin, Bin Zhou, Soo Young Lee, and Nacksung Kim, Protein Inhibitor of Activated STAT 3 Modulates Osteoclastogenesis by Down-Regulation of NFATc1 and Osteoclast-Associated Receptor, *J Immunology 2007 178: 5588–5594.*

37. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *(1999) Genes Dev* 13:1015–1024

38. Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue J Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *(1999) Genes Cells* 4:353–362

39. Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ, Wagner EF Fos1 is a transcriptional target of c-Fos during osteoclast differentiation. *(2000) Nature Genet* 24:184–187

40. McHugh, K. P. *et al.* Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J. Clin. Invest.* 105, 433–440 (2000).

41. Manhui Pang , Ariel F. Martinez , Isabel Fernandez, Wayne Balkan, Bruce R. Troen, AP-1 stimulates the cathepsin K promoter in RAW 264.7 cells, *Gene (2007)* 403 151–158

42. J.P. David, M. Rincon, L. Neff, W.C. Horne and R. Baron, Carbonic Anhydrase II Is an AP-1 Target Gene in Osteoclasts, *J. Cell. Physiol. 2001,* 188: 89-97

43. Kim N, Odgren PR, Kim DK, Marks SC Jr, Choi Y. Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. *Proc Natl Acad Sci USA 2000.* 97:10905–10910

44. Zou W, Bar-Shavit Z., Dual modulation of osteoclast differentiation by lipopolysaccharide. *J Bone Miner Res 2002* 17:1211–1218

45. Abu-Amer, Y., Ross, F.P., Edwards, J., and Teitelbaum, S.L.. Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its p55 receptor. *J. Clin. Invest. 1997,* 100:1557–1565

46. Jonathan Lam, Sunao Takeshita, Jane E. Barker, Osami Kanagawa, F. Patrick Ross, and Steven L. Teitelbaum, TNF-a induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand, *J Clin Invest 2000*, volume 106, 12:1481-1488

47. Hideki Kitaura, Ping Zhou, Hyun-Ju Kim, Deborah V. Novack, F. Patrick Ross, and Steven L. Teitelbaum, M-CSF mediates TNF-induced inflammatory osteolysis*, J Clin Invest, 2005,* 115, 12: 3418-3427

48. Shi Wei, Hideki Kitaura, Ping Zhou, F. Patrick Ross, and Steven L. Teitelbaum, IL-1 mediates TNF-induced Osteoclastogenesis, *J Clin Invest 2005*, vol 115, 2: 282-290

49. Patriotis, C., A. Makris, S.E. Bear, and P.N. Tsichlis. (1993). Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. *Proc Natl Acad Sci U S A* 90:2251-2255.

50. Tsatsanis, C., C. Patriotis, and P.N. Tsichlis. (1998). Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB. *Oncogene* 17:2609-2618.

51. Tsatsanis, C., C. Patriotis, S.E. Bear, and P.N. Tsichlis. (1998). The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. *Proc Natl Acad Sci U S A* 95:3827-3832.

52. Patriotis, C., A. Makris, J. Chernoff, and P.N. Tsichlis.(1994). Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase. *Proc Natl Acad Sci U S A* 91:9755-9759.

53. Ceci, J.D., C.P. Patriotis, C. Tsatsanis, A.M. Makris, R. Kovatch, D.A. Swing, N.A. Jenkins, P.N. Tsichlis, and N.G. Copeland. (1997). Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. *Genes Dev* 11:688-700.

54. Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, and P.N. Tsichlis.(2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103:1071-1083.

56. Eliopoulos, A. G., Wang, C. C., Dumitru, C. D., and Tsichlis, P. N. (2003).Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. *EMBO J.* 22: 3855–3864.

57. Banerjee A., Gugasyan R., McMahon M. & Gerondakis S. (2006). Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. *PNAS* 103: 3274–3279.

58. Anderson, K.V. (2000).Toll signaling pathways in the innate immune response, *Current.Opin.Immonol* 12,13-19.

59. Matthew S. Hayden and Sankar Ghosh1.(2004). Signaling to NF-κB, *Genes & Development, 18: 2195–2224.*

60. Belich, M. P., Salmeron, A., Johnston, L. H., and Ley, S. C. (1999) *Nature 397, 363–368.*

61. Waterfield, M. R., Zhang, M., Norman, L. P., and Sun, S. C. (2003) *Mol. Cell* 11,685–694.

62. Heissmeyer, V., Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1999) *EMBO J.* 18, 4766–4778.

63. Waterfield, M., Jin, W., Reiley, W., Zhang, M., and Sun, S. C. (2004) *Mol. Cell. Biol.* 24,6040–6048.

64. Cho, J., Melnick, M., Solidakis, G. P., and Tsichlis, P. N. (2005) *J. Biol. Chem.* 280,20442–20448.

65. Caivano, M., Rodriguez, C., Cohen, P., and Alemany, S. (2003) *J. Biol. Chem.* 278,52124–52130. 41

66. Aristides G. Eliopoulos, Santasabuj Das, and Philip N. Tsichlis Syk Regulates TNF-induced Tpl2 Activation Signals (2006) *Journal of Biological Chemistry* 281:1371–1380.

67. Lawrence P. Kane, N. Mollenauer, Zheng Xu,1 Christoph W. Turck, and Arthur Weiss.(2002). Akt-Dependent Phosphorylation Specifically Regulates Cot Induction of NF-κB-Dependent Transcription *Molecular and Cellular Biology.* 22: 5962–5974.

68. Aristides G.Eliopoulos, Chun-Chi Wang, Calin D.Dumitru and Philip N.Tsichlis, Tpl2 transduces CD40 and TNF signals that activate, ERK and regulates IgE induction by CD40, *The EMBO Journal Vol. 22, 2003*

69. Hotokezaka, H. *et al.* U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. *J. Biol. Chem.* 2002, **277,** 47366–47372

70. Teruhito Yamashita, Yasuhiro Kobayashi, Toshihide Mizoguchi, Mariko Yamaki, Toshiki Miura, Sakae Tanaka, Nobuyuki Udagawa, Naoyuki Takahashi, MKK6–p38 MAPK signaling pathway enhances survival but not bone-resorbing activity of osteoclasts, *Biochemical and Biophysical Research Communications, (2008)* 365 252–257

71. Takeda K. and Akira S., 2005, Toll-like receptors in innate immunity, *Int Immunol 17 :1-14*

72. Martinon F, K Burns and Tschopp F, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-1b. *Mol. Cell* 10: 417–426

73. Harton, J.A., Linhoff, M.W Zhang, J., and Ting, J.P. (2002) Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. *J. Immunol*. *169*: 4088–4093

74. Martinon, F., and Tschopp, J. (2005) NLRs join TLRs as innate sensors of pathogens. *TRENDS in Immunology*. 26: 447–454

75. McGonagle, D., Savic, S., McDermott, M. F (2007) The NLR network and the immunological disease continuum of adaptive and innate immune-mediated inflammation against self. *Semin Immunopathol* 29:303-313

76. Mariathasan, S., and Monack. D. M. (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat. Rev. Immunol.* 7: 31–40

77. Matzinger, P. (2002) The danger model: a renewed sense of self. *Science, 296: 301-305*

78. Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K. Molineaux, S.M. Weidner, J.R., Aunins J et al (1992) A novel

heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356: 768–774

79. Dinarello, C.A. (1998). Interleukin-1b, interleukin-18, and the interleukin-1β converting enzyme. *Ann. N. Y. Acad. Sci.* 856, 1–11

80. Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A., Kolodner, R. D., (2001), Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome, *Nat Genet 29: 301-305*

81. Aganna, E., Martinon, F., Hawkins, P. N., Ross, J.B., Swan, D.C., Booth, D.R., Lachmann H.J et al, (2002), Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. *Arthritis Rheum* 46:2445–2452

82. Agostini, L., Martinon, F., Burns, K., McDermott, M. F., Hawkins, P. N., and Tschopp, J. (2004) NALP3 Forms an IL-1β-Processing Inflamassome with Increased Activity in Muckle-Wells Autoinflammatory Disorder. *Immunity, 20: 319–325*

83. Hawkins, P. N., Lachmann, H.J., McDermott, M. F, (2003) Interleukin- 1-receptor antagonist in the Muckle-Wells syndrome, *N Engl J Med 348: 2583-2584*

84. Deepti Verma, Maria Lerm, Robert Blomgran Julinder, Per Eriksson, Peter Soderkvist, and Eva Sarndahl Gene Polymorphisms in the NALP3 Inflammasome Are Associated With Interleukin-1 Production and Severe Inflammation Relation to Common Inflammatory Diseases?, *Arthritis and Rheumatism, 2008*

85. Gattorno, M., Tassi, S., Carta, S., Delfino, L., Ferlito, F., Pelagatti, M.A., et al. (2007) Pattern of interleukin-1β secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum* 56:3138–48

86. Sutterwala, F.S., Ogura, Y., Szczepanik, M., Tejero, M. L., Lichtenberger, Grant, E. P., Bertin, J. Coyle, A. J., Galan, J. E., Askenase, P. W., and Flavell, R. A. (2006) Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1, *Immunity* 24: 317-327

87. Watanabe, H., Gaide, O., Petrilli, V., Martinon, F., Contassot, E., Roques, S., Kummer, J. A., Tschopp, J., French, L. E. (2007) Activation of the IL-1β processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 127:1956–1963

88. Samuel H McCall, Mahnaz Sahraei, Amy B Young, Charles S Worley, Joseph A. Duncan, Jenny Pan Yun Ting, Ian Marriott, 2008, Osteoblasts Express NLRP3, a Nucleotide-Binding Domain and Leucine-Rich Repeat Region Containing Receptor Implicated in Bacterially Induced Cell Death, *Journal of Bone and Mineral Research*

89. Walsh NC, Cahill M, Carninci P, Kawai J, Okazaki Y, Hayashizaki Y, Hume DA, et al. (2003) Multiple tissue-specific promoters control expression of the murine tartrate-resistant acid phosphatase gene. *Gene* 307:111–123