



(51) International Patent Classification:

A61K 38/00 (2006.01) *A61P 19/10* (2006.01)
A61P 19/00 (2006.01)

(21) International Application Number:

PCT/IB2016/052903

(22) International Filing Date:

18 May 2016 (18.05.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

102015000018209 27 May 2015 (27.05.2015) IT

(71) Applicant: UNIVERSITÀ DEGLI STUDI DEL
PIEMONTE ORIENTALE "AMEDEO AVOGADRO"
[IT/IT]; via Duomo, 6, 13100 Vercelli (IT).

(72) Inventors: DIANZANI, Umberto; corso Ferrucci, 56,
10138 Torino (IT). GIGLIOTTI, Casimiro Luca; via
Isonzo, 1/e, 28100 Novara (IT). BOGGIO, Elena; via Um-
berto I, 2, 28040 Mezzomerico (Novara) (IT).

(74) Agent: FREYRIA FAVA, Cristina; c/o Buzzi, Notaro &
Antonielli d'Oulx S.r.l., Via Maria Vittoria, 18, 10123
Torino (IT).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: LIGANDS OF B7H RECEPTOR IN THE TREATMENT OF OSTEOPENIA AND OSTEOPOROSIS

(57) Abstract: A novel use of ligands of B7h receptor in the treatment of osteoporosis or osteopenia is disclosed as well as the use of B7h receptor as target for the screening of pharmaceutical active agents useful in the treatment of osteopenia or osteoporosis.



WO 2016/189428 A1

"Ligands of B7h receptor in the treatment of osteopenia
and osteoporosis"

* * *

5 FIELD OF THE INVENTION

The present invention concerns a novel use of ligands of B7h receptor in the treatment of osteopenia and osteoporosis.

10 BACKGROUND OF THE INVENTION

Osteoclasts are giant cells formed by cell-cell fusion of monocyte-macrophage precursors and characterized by multiple nuclei, abundant vacuoles and lysosomes; they play a key role in bone development and
15 remodelling, which involves also osteoblasts and osteocytes. Osteoclasts differentiate from monocytes under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor k-B ligand (RANKL).

20 Osteoclasts function is stimulated by triggering of receptor activator of nuclear factor-kappa B (RANK) expressed on the membrane of osteoclasts by RANKL. In the healthy bone, the main source of RANKL are osteoblasts expressing it as a surface receptor in
25 response to bone-resorbing factors and it is cleaved into a soluble molecule (sRANKL) by metalloproteinase (MMPs). Moreover, RANKL is expressed also by stromal cells, lymphocytes, and macrophages which can support osteoclasts function during inflammation.
30 Osteoprotegerin (OPG) is a soluble receptor of RANKL and is secreted by osteoblasts and stromal cells to inhibit RANK stimulation and osteoclastogenesis induced by RANKL. The binding of M-CSF to its colony-stimulating factor 1 receptor (c-fms) on osteoclasts
35 progenitors upregulates expression of RANK in these

cells and promotes osteoclastogenesis. Osteoclasts differentiation includes cell polarization with formation of ruffled membrane and sealing of the osteoclasts to the bone to form a sealing zone, or
5 clear zone, that separates the resorption lacunae from the surround. This is the secretion site of acid, tartrate resistant acid phosphatase (TRAP), cathepsins, and MMPs leading to demineralization of the inorganic component of the bone and hydrolysis of its organic
10 components. Then, coupling mechanisms promote the differentiation and recruitment of osteoblasts at the resorption lacunae, where they secrete the organic component of bone which is then mineralized by hydroxyapatite. Some osteoblasts entrapped within the
15 matrix become osteocytes and secrete sclerostin inhibiting osteoblasts function and terminating the remodelling cycle. Sclerostin expression is inhibited when osteocytes are exposed to mechanical forces, which targets bone remodeling to areas of maximal strain.

20 An excess of osteoclasts activity leads to pathological bone loss and can be detected in conditions such as osteoporosis, rheumatoid arthritis and other autoimmune diseases, in which a key role has been ascribed to inflammatory cytokines and adaptive
25 immunity. Moreover, some neoplasia involving immune cells, such as multiple myeloma, are characterized by intense focal bone erosions ascribed to high expression of RANKL by stromal cells and, possibly, myeloma cells. Bone metastases of solid cancer, too, may be osteolytic
30 and prostatic cancer may promote bone resorption through the expression of a soluble form of RANKL.

Several inflammatory cytokines, such as TNF- α , Interleukin (IL)-1, IL-6, and M-CSF upregulate RANKL expression and stimulate osteoclasts function. A key
35 role is played by type 17 T helper (Th17) cells

secreting IL-17 that induces the expression of RANKL in osteoblasts and synovial cells. Moreover, IL-17 supports recruitment of several types of immune cells which contribute to the bone damage and produce
5 cytokines and other proinflammatory molecules supporting osteoclasts differentiation and activity.

B7h (CD275, also known as B7H2, B7-RP1, ICOSL, GL50) belongs to the B7 family of surface receptors and it binds ICOS (CD278), which belongs to the CD28
10 family¹⁻⁵. ICOS is selectively expressed by activated T cells, whereas B7h is expressed by a wide variety of cell types, including B cells, macrophages, dendritic cells, and a subset of T cells. However, B7h is also expressed by cells of non haemopoietic origin such as
15 vascular endothelial cells, epithelial cells, and fibroblasts, and in many primary tumors and tumor cell lines. The main known function of B7h is triggering of ICOS, which functions as a costimulatory molecule for activated T cells by modulating their cytokine
20 secretion and, particularly, increasing secretion of Interferon (IFN)- γ (in humans), IL-4 (in mice), and IL-10, IL-17, and IL-21 (in both species). However, recent reports have shown that the B7h:ICOS interaction can trigger bidirectional signals able to modulate also the
25 response of the B7h-expressing cells. In mouse dendritic cells, this B7h-mediated "reverse signalling" induces partial maturation with prominent augmentation of IL-6 secretion. In human dendritic cells, it was found that it modulates cytokine secretion, promotes
30 capacity to cross-present endocytosed antigens in class I MHC molecules, and inhibits adhesiveness to endothelial cells and migration. B7h stimulation also inhibits adhesiveness and migration of endothelial cells and tumor cell lines. These effects are
35 accompanied by decreased phosphorylation of ERK and p38

in endothelial cells; decreased phosphorylation of FAK and down-modulation of β -Pix in endothelial cells and tumor cells. Finally, B7h triggering inhibits development of lung metastases upon injection of NOD-
5 SCID-IL2R γ null mice with CF-PAC1 cells, and C57BL/6 mice with B16-F10 cells.

OBJECT AND SUMMARY OF THE INVENTION

The present description concerns expression of B7h
10 receptor in osteoclasts and B7h receptor triggering to reduce and/or inhibit osteoclasts differentiation, maturation and/or activity.

The object of the present invention is to provide new compounds for the treatment of osteoporosis or
15 osteopenia.

According to the invention, the above object is achieved thanks to the method specified in the ensuing claims, which are understood as forming an integral part of the present description.

20 In an embodiment, the instant disclosure discloses a novel use of ligands of B7h receptor in the treatment of osteoporosis and osteopenia.

In a further embodiment, the present description concerns use of B7h receptor as target for the
25 screening of pharmaceutical active agents useful in the treatment of osteopenia and osteoporosis, wherein the pharmaceutical active agent interferes with osteoclasts differentiation, maturation and/or function, thus inhibiting osteoclastogenesis.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described, by way of example only, with reference to the enclosed figures of drawing, wherein:

- **Figure 1: Morphological analysis and CD14, B7h,
35 and Catepsin-K expression in monocytes-derived**

osteoclasts (MDOCs). **A)** Cells were photographed at day 0 (T0), day 14 (T14) and day 21 (T21) of culture in the presence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) by phase-contrast microscopy. **B)** CD14, B7h, and Catepsin-K expression were assessed by flow cytometry in MDOCs at T0, T14 and T21. Numbers in each panel indicate the % of positive cells. Panels are representative of 5 experiments.

- Figure 2: Effect of ICOS-Fc on osteoclasts differentiation. Monocytes were induced to differentiate to MDOCs in the presence and absence of ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) added at day 0 (T⁰⁻²¹ treatment). **A)** Cells were photographed at T10 and T21 by phase-contrast microscopy. **B)** CD14 and Catepsin-K expression were assessed by flow cytometry in MDOCs at T10 and T21. Numbers in each panel indicate the % of positive cells vs the internal negative control. Panels are representative of 5 experiments.

- Figure 3: Effect of ICOS-Fc on osteoclasts differentiation. Monocytes were induced to differentiate to MDOCs in the presence and absence of ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) added from day 14 (T¹⁴⁻²¹ treatment). **A)** Cells were photographed at T21 by phase-contrast microscopy. **B)** CD14 and Catepsin-K expression were assessed by flow cytometry in MDOCs at T21. Numbers in each panel indicate the % of positive cells vs the internal negative control. Panels are representative of 3 experiments. **C)** The bar graphs show the number of nuclei counted in each field at day 21 (mean from 5 fields); data are expressed as the mean ± standard error of the mean (SEM) from 3 independent experiments (*: p<0.01 versus the control).

- Figure 4: Effect of ICOS-Fc on osteoclasts

differentiation. Monocytes were induced to differentiate to MDOCs in the presence and absence of ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) added from day 7 (T⁷⁻²¹ and T⁷⁻¹⁴ treatments).

5 **A)** Cells were photographed at T21 by phase-contrast microscopy. **B)** CD14 and Catepsin-K expression were assessed by flow cytometry in MDOCs at T21. Numbers in each panel indicate the % of positive cells vs the internal negative control. Panels are representative of
10 3 experiments.

- **Figure 5: Effect of ICOS-Fc on differentiated osteoclasts.** After 21 days of differentiating culture, MDOCs were cultured in the presence and absence of ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc
15 (1 µg/ml) for 3 other days (T²¹⁻²⁴ treatment), washed and then cultured for other 4 days (T25-T28). **A)** Cells were photographed at T24, T25 and, T28 by phase-contrast microscopy. **B)** CD14 and Catepsin-K expression were assessed by flow cytometry in MDOCs at T24, T25 and,
20 T28. Numbers in each panel indicate the % of positive cells vs the internal negative control. Panels are representative of 3 experiments.

- **Figure 6: Effect of ICOS-Fc on TRAP staining.** MDOCs differentiating from monocytes were treated with
25 or without ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) as described in Figure 2 (T¹⁴⁻²¹ treatment). At day 21, the cell layer was stained with the Fast Garnet GBC Base Solution assessing TRAP activity. **A)** Microphotographs of the staining area were
30 taken to monitor the TRAP positive cells (n=3). **B)** The bar graphs show the % of the TRAP⁺ cells; data are expressed as the mean±SEM of the percentage of inhibition versus the control from 3 independent experiments (*: p<0.01 versus the control).

35 - **Figure 7: Effect of B7h triggering on actin**

remodelling. MDOCs differentiating from monocytes were treated with or without ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) as described in Figure 2 (T¹⁴⁻²¹ treatment). **A)** Cells were stained with fluorescein isothiocyanate (FITC)-phalloidin marking actin and were photographed by a fluorescent microscope at Day 21. **B)** The bar graphs show the % of the perinuclear F actin ring positive cells; data are expressed as the mean±SEM of the percentage of increase versus the control from 3 independent experiments (*: p<0.05 versus the control).

- **Figure 8: Effect of B7h triggering on the osteoclasts osteolytic activity.** Monocytes were plated on Osteo Surface plates and were induced to differentiate towards osteoclasts in the presence and absence of ICOS-Fc (1 µg/ml), ICOS-msFc (1 µg/ml), or ^{F119S}ICOS-Fc (1 µg/ml) as described in Figure 2 (T¹⁴⁻²¹ treatment). At Day 21, culture supernatants were harvested and examined for calcium release. Data represent the mean±SEM of the percentage of inhibition versus the control from 4 independent experiments performed in duplicate. (*: p<0.05 vs the control).

- **Figure 9: Effects of treatment with ICOS-Fc in a mouse model of osteoporosis.** Seven-week-old female C57BL/6 mice were injected with sRANKL 1 mg/Kg intraperitoneally (i.p.) at 24-hours (hrs) intervals for 3 days together with either mouse ICOS-Fc (msICOS-huFc) (n=3) 100 µg/ml, or human ^{F119S}ICOS-Fc (n=3) 100 µg/ml or phosphate buffered saline (PBS, control group, n=3). Mice were sacrificed 4 hrs after the last injection. **A)** Staining of undecalcified sections of tibia and femurs in the different-injected groups. **B)** Histogram showing the % mean±SEM of calcified bone in the cortical region(**: p<0.01 or ***: p<0.001 versus control mice receiving no treatments).

- **Figure 10: Amino acid sequence of ICOS-Fc construct** (SEQ ID No.: 1).

- **Figure 11: Amino acid sequence of human ICOS** (SEQ ID No.: 29); the underlined amino acids correspond
5 to the extracellular portion of ICOS (SEQ ID No.:2).

- **Figure 12: Amino acid sequence of human B7h receptor** (SEQ ID No.: 27).

- **Figure 13: Effect of anti-B7h antibodies on TRAP staining.** MDOCs were treated with or without ICOS-Fc
10 (1 µg/ml) or α-B7h/eBioscience(1 µg/ml) or α-B7h/R&D Systems (1 µg/ml) added at day 14 (T¹⁴⁻²¹ treatment) or at day 21 (T²¹⁻²⁴ treatment). At day 21, the cell layer was stained with the Fast Garnet GBC Base Solution assessing TRAP activity. **A)** Microphotographs of the
15 staining area were taken to assess TRAP positive cells. **B)** Data are expressed as mean±SEM of the percentage of the TRAP⁺ cells from four different fields (*: p<0.05, **:p<0.01 versus the control).

- **Figure 14: Effect of ICOS reagents and anti-B7h antibodies on B7h signaling in MDOCs.** Differentiated
20 MDOCs were treated or not with **A)** either ICOS-Fc or ICOS-msFc or ^{F119S}ICOS-Fc or **B)** α-B7h/eBioscience or α-B7h/R&D Systems antibodies (5 µg/ml) for 30 min at day 21. Then, expression of phospho-p38 was assessed by
25 western blot. The same blots were also probed with anti-p38 antibody as a control. Data are expressed as the mean±SEM of the percentage of the phospho-p38 expression from 3 independent experiments (*: p<0.05 versus the control).

30 - **Figure 15: Effects of treatment with ICOS-Fc in mouse osteoporosis induced by OVX.** Mice were subjected to OVX and, 24 hours later, treated with ICOS-Fc (msICOS-msFc) (n=3) or PBS (control group, n=3) for 4 weeks. **A)** Representative images of cortical bone from
35 mice treated with PBS or ICOS-Fc. **B)** Bar graphs of

proportion of calcified bone in the cortical region evaluated in 6 sections from each mouse (3 sections/leg). Data are expressed as mean $\pm$ SEM (**: p<0.01 versus the control).

5

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail, by way of non limiting example, with reference to a novel use of ICOS, natural ligand of B7h receptor, for the treatment of osteoporosis and osteopenia.

It is clear that the scope of this description is in no way limited to the use of ICOS only; other ligands of B7h receptor (like for example antibodies, either monoclonal antibodies, and genetically engineered/humanized antibodies and fragments thereof) may be used in the treatment of osteoporosis and osteopenia, wherein such ligands are able to reduce/inhibit osteoclasts differentiation, maturation and/or function thus inhibiting bone loss induced by osteoclasts being able to trigger B7h receptor activity in osteoclasts.

In the following description, numerous specific details are given to provide a thorough understanding of embodiments. The embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the embodiments.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various

places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

The headings provided herein are for convenience only and do not interpret the scope or meaning of the embodiments.

The present description concerns a novel use of ligands of B7h receptor in the treatment of osteoporosis and osteopenia, such ligands being able to trigger/stimulate B7h receptor activity, preferably in osteoclasts.

In an embodiment, the ligand of B7h receptor useful in the treatment of osteoporosis and osteopenia is selected from:

a) a human ICOS protein having the amino acid sequence set forth in SEQ ID No.: 29;

b) a homologue of human ICOS protein having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 29;

c) a human ICOS portion of the amino acid sequence set forth in SEQ ID No.: 29 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of native ICOS protein;

d) a human ICOS extracellular domain having the amino acid sequence set forth in SEQ ID No.: 2;

e) a human ICOS extracellular domain portion of the amino acid sequence set forth in SEQ ID No.: 2 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2; or

f) a homologue of human ICOS extracellular domain

having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 2 and having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2.

In an embodiment, the present description concerns use of B7h receptor as target for the screening of pharmaceutical active agents useful in the treatment of osteopenia and osteoporosis, wherein the pharmaceutical active agent interferes with osteoclasts differentiation, maturation and/or function, binds to B7h receptor and triggers B7h receptor activity.

In a further embodiment, the present description provides a pharmaceutical composition for use in the treatment of osteoporosis and osteopenia comprising at least one ligand of B7h receptor and a pharmaceutical acceptable vehicle.

In an embodiment, the pharmaceutical composition comprises at least one ligand of B7h receptor selected from:

- a) a human ICOS protein having the amino acid sequence set forth in SEQ ID No.: 29;
- b) a homologue of human ICOS protein having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 29;
- c) a human ICOS portion of the amino acid sequence set forth in SEQ ID No.: 29 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of native ICOS protein;
- d) a human ICOS extracellular domain having the amino acid sequence set forth in SEQ ID No.: 2;
- e) a human ICOS extracellular domain portion of the amino acid sequence set forth in SEQ ID No.: 2

having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2; or

- 5 f) a homologue of human ICOS extracellular domain having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 2 and having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation,
10 maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2.

In a still further embodiment, the present description provides a method of identifying a pharmaceutical active agent suitable for use in the
15 treatment of osteopenia and osteoporosis, comprising the steps of: a) providing a test agent, b) putting in contact the test agent with osteoclasts expressing B7h receptor, c) testing the ability of the test agent to reduce osteoclasts maturation, differentiation and/or
20 function, d) selecting the test agent that reduces osteoclasts maturation, differentiation and/or function as the active agent useful in the treatment of osteopenia and osteoporosis, wherein the active agent binds to B7h receptor and triggers B7h receptor
25 activity. Preferably, the method provides for measuring secretion of tartrate-resistant acid phosphatase (TRAP) by osteoclasts, cell actin cytoskeleton organization in osteoclasts, and/or calcium release by osteoclasts.

In a different embodiment, the present description
30 concerns a method of treating osteoporosis or osteopenia comprising the steps of: providing a patient suffering from osteoporosis or osteopenia, administering to the patient a medicament comprising a ligand of B7h receptor, thereby reducing osteoporosis
35 or osteopenia in the patient.

Bone remodelling is a complex process managed by osteoblasts and osteoclasts, and the immune system is involved in regulating the function of these cells through the activity of cytokines and surface
5 receptors.

The present description discloses a novel pathway involved in the lymphocyte/bone cell interactions by showing that binding of ICOS, expressed by activated T cells, to its ligand B7h, expressed by osteoclasts,
10 inhibits osteoclasts maturation and function. These effects were detected using ICOS-Fc, a recombinant soluble form of ICOS, and were specific since they were not displayed by ^{F119S}ICOS-Fc, a mutated form of ICOS-Fc incapable of binding B7h.

15 The effect on osteoclasts differentiation was detected by treating cells with ICOS-Fc during the in vitro differentiation of monocytes to osteoclasts driven by M-CSF and RANKL. ICOS-Fc almost completely blocked the differentiation when treatment was started
20 at the beginning of the three weeks differentiating culture, but it arrested the differentiation also when the treatment was started in the last week, as shown by the decreased cell multinuclearity and the arrest of acquirement of the osteoclasts morphologic and
25 phenotypic features induced by treatment with ICOS-Fc. This effect was not due to cell toxicity since cell survival was normal even when cultures were prolonged for a fourth week.

Moreover, the effect was reversible since
30 interruption of the treatment in the last week of culture allowed cells to restart the osteoclasts differentiation path. The arrest of differentiation was accompanied by an altered organization of the actin cytoskeleton which, in ICOS-Fc treated cells, displayed
35 a perinuclear distribution in a F-actin ring without

the signs of polarization typical of the sealing zone delimiting the erosive lacuna detected on osteoclasts. In line with these data, cells treated with ICOS-Fc displayed decreased expression of TRAP and decreased
5 osteolytic activity in vitro.

A second key point was the effect of ICOS-Fc on already differentiated osteoclasts, in which treatment with ICOS-Fc induced a striking decrease of the cell and nuclei sizes without substantial effects on cell
10 viability. Again, the effect was reversible since cells reenlarged and reassumed the osteoclasts phenotype upon interruption of the treatment.

These effects on *in vitro* osteoclasts differentiation and function were supported by the *in vivo* results showing that treatment with ICOS-Fc strikingly inhibits the systemic bone resorption induced in mice by treatment with high doses of soluble RANKL, which is a mouse model of osteoporosis comparable to the ovariectomy model in terms of
15 20 decrease in bone density.

The immune system is capable to modulate bone formation, and bone loss is a common feature of several chronic inflammatory and autoimmune diseases. Indeed, the risk of osteoporosis is increased in patients with
25 rheumatoid arthritis, inflammatory bowel disease or systemic lupus erythematosus, and aggressive localized bone destruction can be a feature of certain autoimmune diseases, cancers, and infections.

Rheumatoid arthritis patients display three types
30 of bone involvement: osteoporosis, periarticular bone loss, and joint erosions. Periarticular bone loss and joint erosions are specific of rheumatoid arthritis and involve the sites hit by the autoimmune response. Inflammatory cytokines such as IL-1, IL-6 and TNF- α are
35 abundant in the synovial fluid and synovium and can

induce RANKL on synovial fibroblasts and stromal cells. Moreover, RANKL is expressed by T and B cells in the synovial tissue and fluid of rheumatoid arthritis patients and can be involved in osteoclasts activation, and antibodies against citrullinated proteins, which are a rheumatoid arthritis marker, may have a stimulating effect on osteoclasts. By contrast, osteoporosis is not specific of rheumatoid arthritis but is a common finding in chronic inflammatory diseases such as psoriasis, ankylosing spondylitis, systemic lupus erythematosus, multiple sclerosis, inflammatory bowel diseases, pemphigus vulgaris, and others. It is also typical in chronic inflammation associated with transplantation and aging. In chronic inflammation, osteoporosis is ascribed to multiple factors including high levels of inflammatory cytokines in the blood and, often, steroid therapy.

Menopausal estrogen deficiency may involve increased osteoclasts activity due to removal of an inhibitory effect of estrogens on osteoclasts differentiation and function, but also increased production of inflammatory cytokines such as TNF- α and IL-17 and increased RANKL expression of B and T cells.

In the immune control of bone formation, a key role has been ascribed to T helper cells. Th1 and Th2 cells secrete IFN- γ and IL-4, respectively, which are anti-osteoclastogenic cytokines. By contrast, Th17 cells express high levels of RANKL and secrete IL-17 inducing expression of RANKL on mesenchymal cells and recruitment of inflammatory cells. Moreover, Th17 cells secrete also IL-22 that may induce osteoblast differentiation enhancing bone formation at sites of inflammation. The cells may act also by use of surface receptors since their CD40L can stimulate CD40 on stromal cells inducing these cells to express RANKL and

suppressing expression of OPG downregulating the activity of the RANKL/RANK axis. Moreover, osteoclastogenesis can be inhibited also by regulatory T cells (Treg) through release of transforming growth factor β (TGF β) and surface expression of cytotoxic T lymphocyte antigen 4 (CTLA4) that is capable to inhibit osteoclasts formation by binding its ligands B7.1 and B7.2 expressed on monocytes and preventing their differentiation to osteoclasts. This finding is intriguing since both CTLA4 and ICOS belong to the CD28 family of costimulatory receptors and bind surface receptors belonging to the B7 family. Moreover, ICOS too is involved in Treg function since, in appropriate conditions, ICOS triggering can induce Treg differentiation and TGF β secretion from naïve Th cells, and subsets of Treg cells express high levels of ICOS. The effect displayed by B7h triggering on osteoclasts seems to be wider than that displayed by B7.1/B7.2 triggering since it also reversibly inhibits the function of differentiated osteoclasts.

Human B7h receptor has the amino acid sequence set forth in SEQ ID No.: 27.

Human ICOS protein, the natural ligand of B7h receptor, has the amino acid sequence set forth in SEQ ID No.: 29.

The ligands of B7h receptor useful for the treatment of osteoporosis and osteopenia can be selected from the ligands of B7h receptor of natural, synthetic, or recombinant origin, like for example ICOS or portions thereof, small molecule compounds, aptamers, antibodies, peptides, B7h receptor dominant positive, wherein such ligands bind to B7h receptor and are able to trigger B7h receptor activity.

The ligands of B7h receptor may be fused or conjugated to one or more stabilizing molecules in

order to reduce or prevent degradation, to increase half-life and/or solubility, to reduce toxicity, and/or immunogenicity.

Use of molecules able to stabilize a protein for its administration to a mammal is a technique widely known in the art.

The B7h receptor ligand may be conjugated to an antibody or an antibody fragment in order to increase specificity, pharmacokinetics, and/or biodistribution, exploiting the antibody properties of specifically binding to an osteoclast or another bone component.

Stabilizing molecules that can be conjugated to a B7h receptor ligand can be selected i.a. from:

- Polyethylene glycols (PEGs) or derivatives thereof. PEG derivatives able to link amino groups present on the B7h receptor ligands are i.a. epoxide PEG, aldehyde PEG, nitrophenyl carbonate PEG and succinimidyl ester PEG; PEG derivatives able to link thiol groups present on the B7h receptor ligands are i.a. orthopyridyl disulfide PEGs; PEG derivatives able to link hydroxyl groups present on the B7h receptor ligands are i.a. PEG-COOH activated with N-hydroxysuccinimide or hydroxybenzotriazole. Other PEG derivatives are represented by PEG-polyacetal with pH-dependent hydrolysis, and PEG-dextrin (polymer-masking-unmasking-protein therapy, PUMPT).

- poly-L-lysine citramide (via a lysine or an ethylcarbamate spacer), styrenemaleic acid anhydride and poly-hydroxypropylmetacrylamide.

- human Fc antibody domain.

In order to increase the delivery of the B7h receptor ligand to the site of treatment, namely osteoclasts expressing B7h receptor, the B7h receptor ligands may also be hyperglycosylated or conjugated to mannose residues.

Hyperglycosylation may be performed by either in situ chemical reactions or site-directed mutagenesis resulting in either N-linked or O-linked protein glycosylation. In N-linked glycosylation, the
5 saccharide chain is attached to asparagine of tripeptide sequence Asn-X-Ser/Thr, where X represents an amino acid other than proline. Polysialic acid (PSA) is often used for hyperglycosylation. Large-molecular-weight PSAs are suitable for the delivery of low-
10 molecular-weight drugs and peptides, while lower molecular weight PSAs could be used for large proteins as well as particulate drug-delivery systems.

The conjugation to mannose residues exploits binding of the conjugate to mannose receptors, which
15 are reported to be expressed on Kupffer cells, macrophages, alveolar, monocyte-derived dendritic cells and subsets of vascular and lymphatic endothelial cells. Mannosylated proteins can be recognized by mannose-specific lectins, namely, mannose receptors and
20 MBPs.

The delivery of B7h receptor ligand may also be increased exploiting colloidal drug delivery systems known in the art, wherein the colloidal carriers can be selected i.a. from microparticles, nanoparticles and
25 liposomes. Microparticles are generally made of biodegradable polymers like for example starch, alginate, collagen, poly(lactide-co-glycolide) (PLGA), polycaprolactones (PCL). Nanoparticles are usually manufactured from natural or synthetic polymers, such
30 as chitosan, alginate, PCL, polylactic acid (PLA), poly(glycolide), PLGA. Liposomes may also be surface modified with PEG in order to interfere with recognition and uptake by reticulo endothelial system and to extend circulation time. Moreover, in situ

thermosensitive hydrogels undergo sol-gel phase transition in response to changes in temperature.

Materials and Methods

Cells

5 Peripheral Blood Mononuclear Cells (PBMCs) were obtained by density gradient centrifugation from human blood samples obtained from healthy donors who signed their written informed consent. Osteoclasts were prepared from CD14⁺ monocytes, isolated from PBMCs with
10 the EasySep™ Human CD14 Negative Selection Kit (cod. 19059, StemCells Technologies, Vancouver, BC, Canada). In particular, 0.5x10⁶ monocytes were plated in a 24 wells plate (cod. 662160, Greiner Bio-One, Capital DriveMonroe, NC, USA) and cultured for 21 days in
15 differentiation medium, composed of DMEM (cod. 41966-029 Invitrogen, Burlington, ON, Canada, USA), and 10% Fetal Bovine Serum (FBS; cod. 10270, Invitrogen), supplemented with recombinant human M-CSF (25 ng/ml, cod. 216-MC, R&D Systems, Minneapolis, MN, USA), and
20 RANKL (30 ng/ml, cod. 390-TN, R&D Systems). The differentiation medium was changed every 3 days. At different times, cells were treated with 1 µg/ml of either ICOS-Fc (SEQ ID No.: 1 - a fusion protein containing the extracellular portion of the human ICOS
25 (SEQ ID No.: 2)) fused to the human IgG1 Fc (SEQ ID No.: 3)) or ICOS-msFc (SEQ ID No.: 4 - a chimeric molecule containing the extracellular portion of the human ICOS (SEQ ID No.: 2) fused to the mouse IgG1 Fc (SEQ ID No.: 5)). Controls were performed using
30 ^{F119S}ICOS-Fc (SEQ ID No.: 6 - wherein the mutated form of the extracellular portion of the human ICOS (SEQ ID No.: 7) carrying the F119S substitution is fused to the human IgG1 Fc (SEQ ID No.: 3)) and msICOShuFc (SEQ ID No.: 24 - a fusion protein containing the extracellular

portion of the mouse ICOS (SEQ ID No.: 28)) fused to the human IgG1 Fc (SEQ ID No.: 3)).

ICOS cloning and production

The extracellular portion of the human or mouse ICOS was cloned into a modified eukaryotic expression vector derived from pCDNA3.1/Hygro(+) plasmid (cod. V870-20, Invitrogen) and reported as p-Minibody (pMB-SV5) by Di Niro R. et al.⁶, PubMed ID: 17678525. This vector differs from the original one by: the Kozak sequence (5' CCACATGG 3' - SEQ ID No.: 8) which is required for the initiation of the translation in eukaryotic cells; the secretory leader sequence (5' GCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTGGCTACA 3' - SEQ ID No.: 9) which was introduced to allow the release of the protein in the culture supernatants; the mini intron sequence (5' GTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATATG GGTGACAATGACATCCACTTTGCCTTCTCTCCACAG 3'- SEQ ID No.: 10) to increase the level of the protein expression. A tag sequence to target the produced-protein was introduced and it is derived from Simian Virus-5 (SV5 tag) (5' GGCAAACCAATCCCCAAACCCACTGCTGGGCCTGGATAGTACT 3'- SEQ ID No.: 11) and it is useful for monoclonal antibody recognition of the protein. This vector allowed to clone the fragments of interest in frame with the coding sequence of the human or mouse constant fragment of the immunoglobulin IgG1 (Fc) domain, having nucleotide sequences set forth in SEQ ID No.: 30 and 31, respectively.

To generate the ICOS-Fc construct (SEQ ID No.: 1), the nucleotide sequence encoding the extracellular portion of the human ICOS (SEQ ID No.: 12) was amplified with specific primers: ICOS forward BsshII
35 primer (5' GGCGCGCATGCCGAAATCAATGGTTCTGCC 3' - SEQ ID

No.: 13, Sigma-Genosys, The Woodlands, TX, USA) and ICOS reverse NheI primer (5' GCTAGCAAGTTGTGATTCATAAATATGC 3' - SEQ ID No.: 14, Sigma-Genosys). The amplified fragments were digested
5 with BssHII (cod. R0199S, New England Biolabs inc, Ipswich, MA, USA) and NheI (cod. R0131S, New England Biolabs inc) enzymes. The double digested fragments were cloned into the previously described pMB-SV5 plasmid. The nucleotide sequence was determined by
10 sequencing.

The nucleotide sequence of the expression vector coding for ICOS-Fc is set forth in SEQ ID No.:21.

In the case of ICOS-msFc (SEQ ID No.: 4) cloning, the same protocol described above was followed, except
15 that the pMB-SV5 vector contained the coding sequence of the mouse Fc domain (SEQ ID No.: 31).

The nucleotide sequence of the expression vector coding for ICOS-msFc is set forth in SEQ ID No.: 22.

To generate the human mutated ^{F119S}ICOS-Fc
20 construct (SEQ ID No.: 6), the mutation F119S in the extracellular portion of human ICOS was introduced with a forward primer (5' TCAATTTTGTGATCCTCCTCCTTCTAAAGTAACTCTTACAGG 3'- SEQ ID No.: 15, Sigma-Genosys) annealing at BseRI digestion
25 site and ICOS human reverse NheI primer (5' GCTAGCAAGTTGTGATTCATAAATATGC 3'- SEQ ID No.: 14, Sigma-Genosys). The nucleotide sequence of the extracellular portion of human mutated ^{F119S}ICOS is set forth in SEQ ID No.: 26. The mutated fragments were cloned into the
30 pMB-SV5 plasmid with the coding sequence of the human Fc domain, after digestion with BseRI (cod. R0581, New England Biolabs inc) and NheI enzymes.

The nucleotide sequence of the expression vector coding for ^{F119S}ICOS-Fc is set forth in SEQ ID No.:23.

35 To generate the mouse ICOS fused with the human Fc

(msICOS-huFc) construct (SEQ ID No.: 24), the nucleotide sequence encoding the extracellular portion of mouse ICOS (SEQ ID No.: 16) was amplified with specific primers: ICOS mouse forward BsshII primer (5' TTGGCGCGCATGCCGAAATCAATGGCTCGGCCGATC 3' - SEQ ID No.: 17, Sigma-Genosys) and ICOS mouse reverse NheI primer (5' CTAGCTAGCTAGCCAGAGCTTCAGCTGGC 3' - SEQ ID No.: 18, Sigma-Genosys). The vector used in this cloning was the pMB-SV5 with the coding sequence of the mouse Fc domain.

The nucleotide sequence of the expression vector coding for msICOS-huFc is set forth in SEQ ID No.:25.

The plasmid DNA was transformed into One Shot® TOP10 Chemically Competent *Escherichia Coli* bacterial cells (*E. coli*; cod. C4040-03, Life Technologies, Carlsbad, CA, USA). The resulting colonies were screened using specific primers: P-Hygro sense (5' CTGCTTACTGGCTTATCG 3' - SEQ ID No.: 19, Sigma-Genosys) and P-Hygro antisense (5' CAGATGGCTGGCAACTAG 3' - SEQ ID No.: 20, Sigma-Genosys) and the construct was confirmed by sequencing. Finally, the plasmid DNA was transfected using FreeStyle™ MAX Reagent (cod. 16447100, Life technologies) into Chinese Hamster Ovarian-suspension cell line (CHO-s) (cod. R8/00-07, Invitrogen). The stable clones were obtained thanks to the presence of Hygromycin resistance in the vector; to this end the clones were grown under selection with Hygromycin-B (cod. 10687-010, Invitrogen) at the concentration of 0.2 mg/ml that allow full selection of transfected cells. The cells were grown in serum free IMDM medium (cod. BE12-915F01, Lonza, Basel, Switzerland) and the serum free culture supernatants were purified using Protein G Sepharose™ 4 Fast Flow columns (cod. 17-0618-01, GE Healthcare, Piscataway Township, NJ, USA).

Immunofluorescence

The osteoclasts phenotype was assessed by immunofluorescence and flow cytometry (BD, Bioscience, San Diego, CA, USA) using the appropriate FITC-, PE-, and APC-conjugated monoclonal antibodies (mAb) to CD14 (cod. 21270146, Immunotools, Friesoythe, Germany), Catepsin-K (cod. BS1611R-FITC, Bioss Inc., Woburn, MA, USA), and B7h (cod. FAB165P, R&D Systems). Levels of Catepsin-K were evaluated after cell permeabilization using the FIX and PERM kit (cod. GAS003, Invitrogen) following the manufacturer's instructions.

Actin staining was performed on cells fixed on glass coverslip with 4% paraformaldehyde (cod. 76240, Sigma-Aldrich, Saint Louis, MO, USA), washed and then permeabilized with a solution containing 5% FBS, 1% bovine serum albumin (BSA, cod. 05479-250G, Sigma-Aldrich) and 0.1% Triton X-100 (cod. T9284, Sigma-Aldrich) for 1 hrs at room temperature. Then, the coverslip were stained with FITC-conjugated phalloidin (cod. F432, Invitrogen) in a solution of 0.1% Triton X-100, 1% BSA, 2% FBS. After 2 hrs, cells were washed with PBS plus 0.1% Triton X-100 for 10 minutes and observed by a phase-contrast microscope Axiovert 40 CFL (Carl Zeiss, Oberkochen, Germany), photographed with a Retiga 200R digital camera (QImaging, Surrey, BC, Canada), and analyzed with the Image Pro Plus Software for micro-imaging (Media Cybernetics, version 5.0, Bethesda, MD, USA).

30

Tartrate-resistant acid phosphatase (TRAP) assay

TRAP activity was assessed on cells fixed on glass coverslips with a commercial kit (cod. 387A-1KT, Sigma-Aldrich) composed of 25% Citrate Solution (citric acid 18 mmol/l, sodium citrate 9 mmol/l, sodium chloride 12

35

mmol/l, pH 3.6), 65% acetone and 10% formaldehyde at 37%, for about 30 seconds. Then, the coverslips were washed with deionized water, stained with Fast Garnet GBC Base Solution (7 mg/ml, Sigma-Aldrich), and
5 observed by contrast phase microscopy. The TRAP positivity was analyzed with an imaging system (Image-Pro Plus).

Calcium release assay

10 Monocytes (0.5×10^6) were plated on 24 well Osteo Assay Surface culture plates (cod. CLS3987, Corning Inc., Corning, NY, USA), and differentiated to MDOCs as described above adding the ICOS reagents at day 14. At day 21 of culture, cells were washed and incubated for
15 other 24 hrs with fresh medium. Supernatants were then collected and the calcium level was evaluated by a calcium colorimetric assay Kit (cod. MAK022-1KT, Sigma-Aldrich).

20 In vivo analysis

Soluble RANKL (cod. GWB-P0945I, GenWay Biotech, Inc, San Diego, CA, USA; 1 mg/kg) was injected i.p. daily for 3 days into 7-week-old C57BL/6 female mice (cod. 057, Harlan Laboratories, Indianapolis, IN,
25 USA), as reported by Tomimori Y. et al.⁷, alone or in combination with 100 µg of msICOS-huFc (a fusion protein containing the extracellular portion of the mouse ICOS (SEQ ID No.: 28)) fused to the human IgG1 Fc (SEQ ID No.: 3), or ^{F119S}ICOS-Fc. Control mice were
30 injected with PBS or 100 µg ICOS-msFc, or ^{F119S}ICOS-Fc but not with RANKL. The mice were sacrificed 4 hrs after the last injection, and blood samples, tibias, and femora were harvested for analysis. Mice were bred under pathogen-free conditions in the animal facility
35 of the Department of Health Sciences and were treated

in accordance with the University Ethical Committee.

Samples of the tibia and femora were fixed at room temperature for 2 days in Concentrated neutral buffered formalin diluted to 4% in PBS pH 6.9 (cod. F0033, Diapth, Martinengo, BG Italy) and dehydrated in ascending concentrations of ethanol (cod. 02860-2.5L, Sigma-Aldrich) for one night before performing a three-step impregnation in methylmethacrylate (MMA) monomer (cod. 8005902500, Merck, Darmstadt, Germany) for at least 3 days. For embedding, specimen blocks were impregnated in 80% (vol/vol) stabilized MMA, 20% (vol/vol) Plastoid N (cod. 5866, Rohm Pharma, Germany) for 2 hrs in uncapped vials under vacuum and embedded in capped 10mL glass vials (water bath, cod. BR778012, Sigma-Aldrich) at 37 °C overnight. After polymerization, the glass vials were removed and moistened sections (50 mm) were cut on a Leica SP 1600 Saw Microtome with a rotating diamond saw blade for high-quality sample preparation of hard materials for microscopical analysis and mounted on polyethylene slides. Cut was performed on the long axis of the bone and the sections were stained using light-green (cod. 1159410025, Merck) and basic fuchsine (cod. 47860, Sigma-Aldrich) for histological evaluation. The sections were then examined histomorphologically and morphometrically by an investigator blinded to the identity of the material. These measurements were performed using a light microscope and analyzed with Leica imaging software (DFC320 Leica digital camera and software Leica QWin Plusv2.6). All measurements were performed at a magnification of 20X. Cortical bone morphology included tissue volume, medullary volume (Me.v) and bone volume ($Bv = \text{tissue volume} - \text{Me.v}$). Endocortical and periosteal bone surface were also measured.

Western blot

MDOCs at T21 were treated with either ICOS-Fc or ICOS-msFc or ^{F119S}ICOS-Fc or two different anti-B7h
5 antibodies: anti human B7h (cod. BMS16-5889-82, eBioscience, Inc. San Diego, CA, USA; clone MIH12) and anti human B7h (cod. MAB1651, R&D Systems; clone 136716).

MDOCs were lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl
10 (cod. S7653 Sigma-Aldrich), 5 mM EDTA (cod. E6758 Sigma Aldrich), 1% NP-40 (cod. 74385 Sigma-Aldrich) with phosphatase and protease inhibitor cocktails (cod. P2850 and cod. P8340, Sigma-Aldrich). Then, 30 µg of proteins were run on 10% SDS PAGE gels and transferred
15 onto Hybond-C extra nitrocellulose membranes (cod. 10600016, Ge Healthcare, Piscataway, NJ, USA). The membranes were then probed with antibodies to phospho-p38-MAPK (cod. 9211, Cell Signaling Technology, Danvers, MA, USA) and p38-MAPK (cod. 9212, Cell
20 Signaling Technology), followed by anti-rabbit HRP-conjugated secondary antibody (cod. A0545, Sigma-Aldrich). The bands were detected via chemiluminescence using the VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and densitometric
25 analysis was performed with the Multi-Analyst software (version 1.1, Bio-Rad Laboratories).

Ovariectomy

Bilateral ovariectomy (OVX) was performed in 6/8-weeks-
30 old female C57BL/6 mice anesthetized with a mix of Zoletil® (60mg/kg) and Xilazina® (20mg/kg) i.p. One day after surgery, mice were treated with seven i.p. injections (1 every 4 days for 4 weeks) of either PBS or msICOS-msFc (400µg). They were sacrificed 4 days
35 after the last injection and organs and bones were

collected for analysis.

Data analysis

Statistical analysis were performed using ANOVA
5 with Dunnett's test. $p < 0.05$ was considered
significant. The statistical analysis were performed
with GraphPad InStat software (GraphPad Software, San
Diego, CA, USA).

10 Results

B7h expression in osteoclasts

Monocyte-derived osteoclasts (MDOCs) were obtained
by culturing CD14⁺ monocytes for 21 days in
differentiation medium containing M-CSF and RANKL. In
15 order to assess the MDOCs differentiation and monitor
B7h expression, the present inventors evaluated the
cell morphology by optical microscopy and expression of
surface CD14, marking monocytes, intracellular
Cathepsin K, marking osteoclasts, and B7h, by three-
20 color immunofluorescence and flow cytometry performed
at the beginning (day 0, T0) and the end (day 21, T21)
of the MDOCs differentiation culture and in the
intermediate day 14 (T14) (Figure 1).

25 Effects of B7h triggering on differentiating osteoclasts

Since B7h is expressed during the MDOCs
differentiation culture, the present inventors
evaluated the effect of B7h triggering on
30 differentiating MDOCs using ICOS-Fc. To assess the
specificity of the ICOS effect, cells were also treated
with either ^{F119S}ICOS-Fc, that is a mutated form of ICOS
incapable to bind B7h, or ICOS-msFc, in which the human
ICOS is fused with a mouse Fc γ portion to minimize
35 interaction with the human Fc γ receptors. Treatment of

differentiating MDOCs was started at either T0 or T14 of the culture by adding the ICOS reagents to the differentiating medium. The culture was continued up to T21 to perform the T^{0-21} and T^{14-21} treatments.

5 Results showed that the T^{0-21} treatment with ICOS-Fc or ICOS-msFc potentially inhibited MDOCs differentiation. At day 10 (T10) cells displayed a round shape and at T21 they acquired a spindle-like morphology; moreover, they showed defective CD14
10 downregulation and cathepsin K upregulation which mark osteoclast differentiation. By contrast, cells treated with F119S ICOS-Fc did not display any difference from untreated cells showing the typical progression toward the MDOCs morphology and phenotype (Figure 2).

15 The T^{14-21} treatment with either ICOS-Fc or ICOS-msFc showed a substantial slowing down of MDOCs differentiation since, at T21, cells displayed decreased cell size and nuclei pyknosis; decreased ability to adhere to the culture wells; increased
20 number of cells with one nucleus only and decreased number of cells with >3 nuclei compared to untreated cells; and defective cathepsin K upregulation and, especially, CD14 downregulation. Moreover, several cells displayed a star-like morphology that was not
25 detected in untreated cells. By contrast, cells treated with F119S ICOS-Fc were similar to untreated cells (Figure 3).

To assess reversibility of the ICOS-Fc effect, cells were treated with ICOS-Fc, ICOS-msFc, or
30 F119S ICOS-Fc at day 7 (T7) washed at T14 and then incubated to T21 in the presence (T^{7-21} treatment) or absence (T^{7-14} treatment) of ICOS-Fc, ICOS-msFc, or F119S ICOS-Fc. Results showed that the T^{7-21} treatment with ICOS-Fc or ICOS-msFc induced a morphology and phenotype
35 similar to those described above for the T^{14-21}

treatment. The cells treated with $F119S$ ICOS-Fc did not display any difference from untreated cells. By contrast, the T^{7-14} treatment induced a morphology and phenotype converging to that displayed by untreated
5 cells (Figure 4).

Effects of B7h triggering on differentiated osteoclasts

Treatment of already differentiated MDOCs was
10 performed by treating cells at T21 with the ICOS reagents and analyzing them after 3 days (T24) to perform the T^{21-24} treatment. Results showed that the T^{21-24} treatment with ICOS-Fc or ICOS-msFc induced striking decrease of the cell and nuclei sizes, and
15 decreased expression of cathepsin K compared to untreated cells. Analysis of cell viability by the Trypan blue exclusion test showed that cells were viable. By contrast, the T^{21-24} treatment with $F119S$ ICOS-Fc did not display any effect (Figure 5).

20 To assess reversibility of the ICOS effect, T^{21-24} -treated cells were washed at T24 and incubated for 1 (T25) or 4 days (T28) in differentiation medium in the absence of ICOS-Fc. Results showed that cells treated with ICOS-Fc or ICOS-msFc and then grown in the absence
25 of ICOS-Fc started to enlarge and upregulated cathepsin K at T25, and displayed a MDOCs-like morphology, converging to that displayed by untreated cells, at T28 (Figure 5). Analysis of cell viability by the Trypan blue exclusion test showed that cells were viable. By
30 contrast, cells that had been untreated or treated with $F119S$ ICOS-Fc maintained their morphology and phenotype at T25 and T28.

Effect of B7h triggering on osteoclasts function

35 Since the bone lytic activity of osteoclasts is

related to their ability to secrete the content of intracytoplasmic granules containing several lytic enzymes including TRAP, the present inventors assessed the effect of the T¹⁴⁻²¹ treatment with the ICOS reagents on TRAP expression assessed by a TRAP enzyme assay. Results showed that MDOCs treated with ICOS-Fc or ICOS-msFc display lower TRAP activity in terms of number of TRAP⁺ cells and their staining intensity than untreated cells and cells treated with ^{F119S}ICOS-Fc. This result was mostly evident in the cells with >3 nuclei (Figure 6).

Since a key aspect of osteoclasts function is cytoskeleton organization to form the ruffle border at the erosion area delimited by the sealing zone, the present inventors analyzed the effect of the ICOS reagents on the cell actin organization by intracellular staining of T¹⁴⁻²¹-treated MDOCs cells with FITC-phalloidin. Results showed that, in untreated MDOCs, actin was polarized with a pattern typical of the sealing zone delimiting the erosive lacuna of osteoclasts. By contrast, in cells treated with ICOS-Fc or ICOS-msFc, actin displayed a perinuclear distribution in a typical F-actin ring without signs of polarization. Cells treated with ^{F119S}ICOS-Fc displayed a pattern similar to that of untreated cells (Figure 7).

To assess the effect of ICOS on the osteolytic activity of MDOCs, the present inventors evaluated their ability to promote calcium release from crystalline calcium phosphate *in vitro*. MDOCs differentiation was induced in wells coated with a synthetic surface made of an inorganic crystalline calcium phosphate mimicking living bone material in the presence and absence of the ICOS reagents using a T¹⁴⁻²¹ protocol. At T21, cells were washed and cultured for

further 24 hrs in fresh medium, and release of calcium was then assessed in the culture supernatants using a colorimetric assay. Results showed that the T¹⁴⁻²¹ treatment with ICOS-Fc or ICOS-msFc, significantly
5 decreased the calcium release compared with untreated MDOCs, whereas ^{F119S}ICOS-Fc did not display any effect (Figure 8).

Finally, the present inventors assessed the effect of B7h triggering *in vivo* using a model of osteoporosis
10 induced by treating mice with high doses of soluble RANKL. Female C57BL/6 mice (7-week-old) were injected i.p. daily for 3 days with RANKL (1 mg/kg) alone or in combination with either msICOS-huFc (formed by the mouse ICOS and human Fc) or ^{F119S}ICOS-Fc (100 µg/mouse).
15 The mice were sacrificed 4 hrs after the last injection. Histological analysis of tibias stained with Fuchsin and light green showed a marked bone loss in the RANKL-injected mice, which was significantly inhibited by co-treatment with msICOS-huFc but not with
20 ^{F119S}ICOS-Fc (Figure 9).

Effects of B7h triggering by anti-B7h antibodies on TRAP staining

The present inventors evaluated the effect of B7h
25 triggering using two different anti-B7h antibodies (α-B7h/eBioscience and α-B7h/R&D Systems) on differentiating (T¹⁴⁻²¹ treatment) and already mature (T²¹⁻²⁴ treatment) MDOCs, which were stained for TRAP. The T¹⁴⁻²¹ treatment showed that untreated MDOCs
30 appeared as giant cells with a dense TRAP staining. Treatment with α-B7h/eBioscience showed a substantial inhibition of MDOCs differentiation with decreased formation of multinuclear TRAP positive cells, comparable to the results obtained with ICOS-Fc. By
35 contrast, treatment with α-B7h/R&D Systems displayed a

minimal inhibitory effect. The T²¹⁻²⁴ treatment showed that untreated MDOCs appeared as giant cells with a dense TRAP staining. Treatment with α -B7h/eBioscience showed a substantial inhibition of MDOCs size and a decreased positivity of multinuclear TRAP positive cells comparable to the results obtained with ICOS-Fc. By contrast, treatment with α -B7h/R&D Systems displayed no inhibitory effect. The results are shown in Figure 13.

Effect of B7h triggering on MDOCs signaling

To investigate whether the inhibitory effect of B7h ligands on MDCOs differentiation and activity correlated with their agonistic activity on B7h, the present inventors assessed their effect on B7h signaling evaluated as induction of phosphorylation of p38 in MDOCs. MDOCs at T21 were treated in the absence and presence of either ICOS-Fc or ICOS-msFc or ^{F119S}ICOS-Fc or α -B7h/eBioscience or α -B7h/R&D Systems (5 μ g/ml) for 30 minutes, and the expression level of phospho-p38 MAPK was then assessed by western blot; expression of total p38 MAPK was assessed as a control. Results showed that, compared to untreated cells, phospho-p38 was upregulated by treatment with ICOS-Fc and ICOS-msFc but not with ^{F119S}ICOS-Fc. The results obtained with the anti-B7h antibodies showed that phospho-p38 was upregulated by treatment with α -B7h/eBioscience, at a level comparable to that obtained with ICOS-Fc. By contrast, treatment with the α -B7h/R&D displayed a minimal effect. The results are shown in Figure 14.

Effects of treatment with ICOS-Fc in a chronic mouse model of osteoporosis

6/8-weeks-old female C57BL/6 mice received OVX

and, after 24 hours, were injected i.p. every 4 days for 4 weeks with either PBS or a total mouse ICOS-Fc (formed by the mouse ICOS and the mouse Fc). The mice were sacrificed 4 days after the last injection.

5 Morphometric measurements of mineralized bone tissue showed a marked bone loss in the PBS-injected mice and the bone loss was significantly inhibited by the treatment with ICOS-Fc. The results are shown in Figure 15.

10 Naturally, while the principle of the invention remains the same, the details of construction and the embodiments may widely vary with respect to what has been described and illustrated purely by way of example, without departing from the scope of the
15 present invention.

References

1. Swallow et al. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNF- α . *Immunity* 5 1999;11:423-432.
2. Redoglia et al., Characterization of H4: a mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor. *Eur J Immunol* 1996;26:2781-9.
- 10 3. Buonfiglio et al. Characterization of a novel human surface molecule selectively expressed by mature thymocytes, activated T cells and subsets of T cell lymphomas. *Eur J Immunol*. 1999;29:2863-74.
4. Hutloff et al. ICOS is an inducible T cell co- 15 stimulator structurally and functionally related to CD28. *Nature* 1999; 397:263-266.
5. Buonfiglio et al. The T cell activation molecule H4 and the CD28-like molecule ICOS are identical. *Eur J Immunol* 2000;30:3463-7.
- 20 6. Di Niro et al. Construction of miniantibodies for the in vivo study of human autoimmune diseases in animal models. *BMC Biotechnology* 2007;7:46.
7. Tomimori et al. Evaluation of pharmaceuticals with a novel 50-hour animal model of bone loss. *J Bone* 25 *Miner Res*. 2009;24:1194-205.

CLAIMS

1. Ligand of B7h receptor for use in treatment of a subject suffering from osteopenia or osteoporosis.

5

2. Ligand of B7h receptor for use according to claim 1, wherein the ligand is selected from:

a) a human ICOS protein having the amino acid sequence set forth in SEQ ID No.: 29;

10 b) a homologue of human ICOS protein having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 29;

c) a human ICOS portion of the amino acid sequence set forth in SEQ ID No.: 29 having the ability of
15 binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function;

d) a human ICOS extracellular domain having the amino acid sequence set forth in SEQ ID No.: 2;

e) a human ICOS extracellular domain portion of
20 the amino acid sequence set forth in SEQ ID No.: 2 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2; or

25 f) a homologue of human ICOS extracellular domain having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 2 and having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation,
30 maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2.

3. Ligand of B7h receptor for use according to claim 1 or 2, wherein the ligand is in soluble form.

35

4. Ligand of B7h receptor for use according to any one of claims 1 to 3, wherein the ligand is to be administered by injection, infusion.

5 5. Ligand of B7h receptor for use according to any one of claims 1 to 4, wherein the ligand is fused or conjugated to a stabilizing molecule.

10 6. Ligand of B7h receptor for use according to any one of claims 1 to 4, wherein the ligand is hyperglycosylated or conjugated to mannose residues.

15 7. Use of B7h receptor as target for the screening of pharmaceutical active agents useful in the treatment of osteopenia or osteoporosis.

20 8. Use according to claim 7, wherein the pharmaceutically active agent interferes with osteoclasts differentiation, maturation and/or function.

25 9. Use according to claim 7 or claim 8, wherein the pharmaceutically active agent binds to B7h receptor.

30 10. Pharmaceutical composition comprising at least one ligand of B7h receptor and a pharmaceutical acceptable vehicle for use in the treatment of osteoporosis and osteopenia.

35 11. Pharmaceutical composition according to claim 10, wherein the at least one ligand of B7h receptor is selected from:

 a) a human ICOS protein having the amino acid sequence set forth in SEQ ID No.: 29;

b) a homologue of human ICOS protein having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 29;

5 c) a human ICOS portion of the amino acid sequence set forth in SEQ ID No.: 29 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function;

d) a human ICOS extracellular domain having the amino acid sequence set forth in SEQ ID No.: 2; or

10 e) a human ICOS extracellular domain portion of the amino acid sequence set forth in SEQ ID No.: 2 having the ability of binding to B7h receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in
15 SEQ ID No.: 2; or

f) a homologue of human ICOS extracellular domain having at least 80%, preferably at least 90% sequence homology to the amino acid sequence set forth in SEQ ID No.: 2 and having the ability of binding to B7h
20 receptor and inhibiting osteoclasts differentiation, maturation and/or function of the amino acid sequence set forth in SEQ ID No.: 2.

12. Method of identifying a pharmaceutical active
25 agent suitable for use in the treatment of osteopenia or osteoporosis, comprising the steps of:

a) providing a test agent,

b) putting in contact the test agent with osteoclasts expressing B7h receptor,

30 c) testing the ability of the test agent to reduce osteoclasts maturation, differentiation and/or function,

d) selecting the test agent that reduces osteoclasts maturation, differentiation and/or function
35 as the active agent useful in the treatment of

osteopenia and osteoporosis, wherein the active agent binds to B7h receptor.

13. Method according to claim 12, wherein the
5 testing step c) comprises measuring secretion of tartrate-resistant acid phosphatase (TRAP) by osteoclasts, cell actin organization in osteoclasts, and/or calcium release by osteoclasts.

10 14. A method of treating osteoporosis or osteopenia, comprising the steps of: providing a patient suffering from osteoporosis or osteopenia, administering to the patient a medicament comprising a ligand of B7h receptor, thereby reducing osteoporosis
15 or osteopenia in the patient.

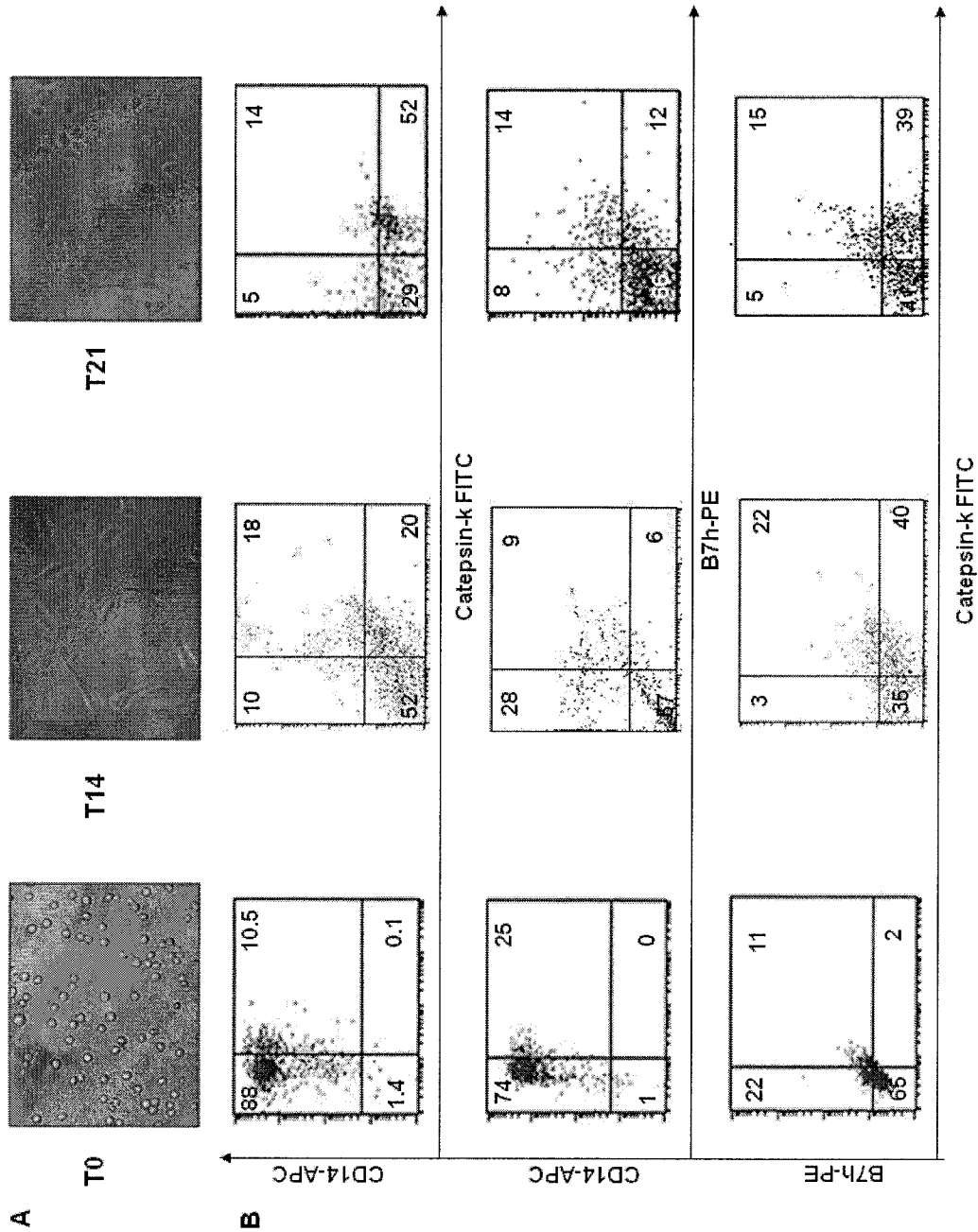


Figure 1

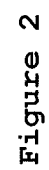


Figure 2

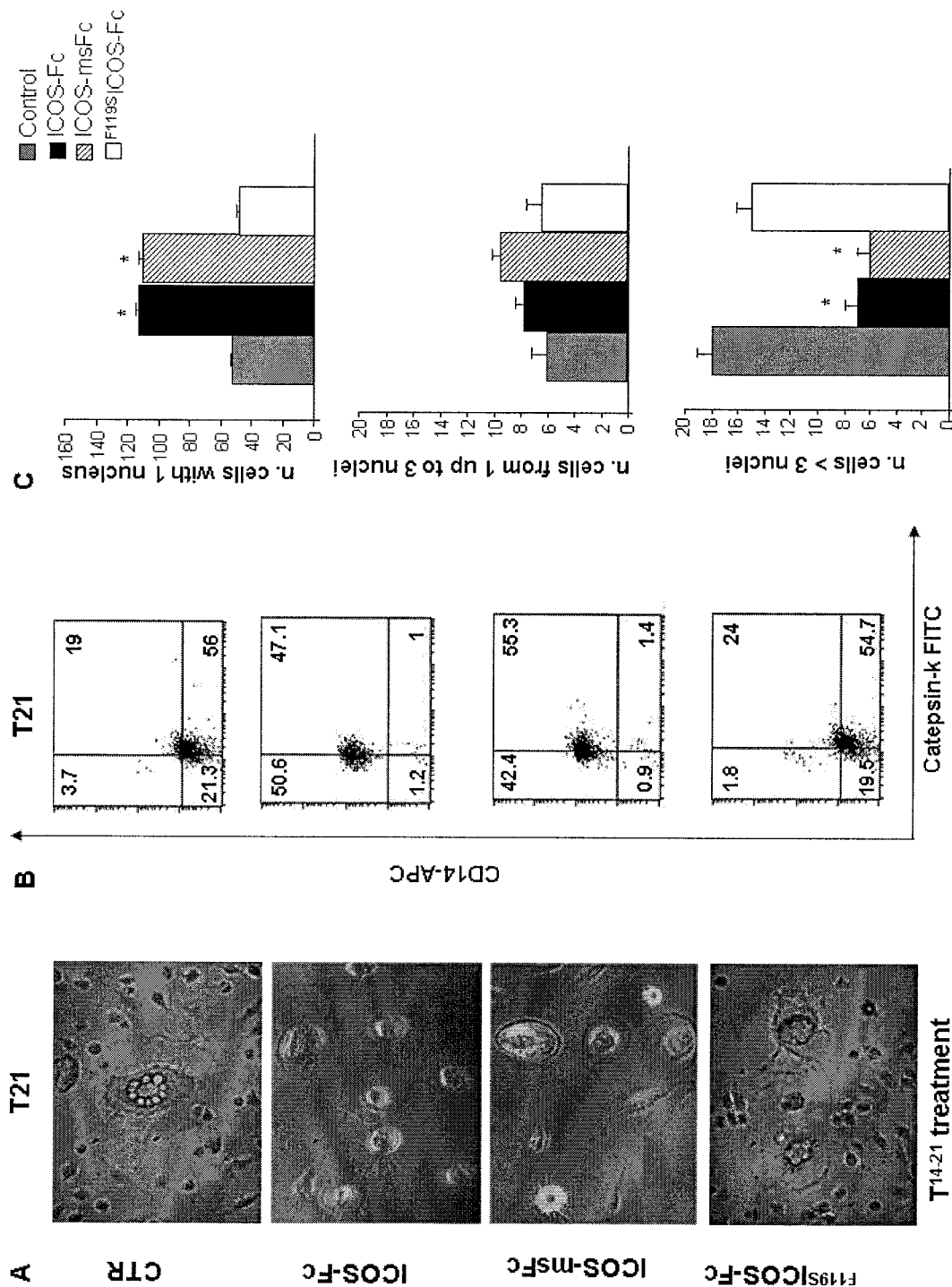


Figure 3

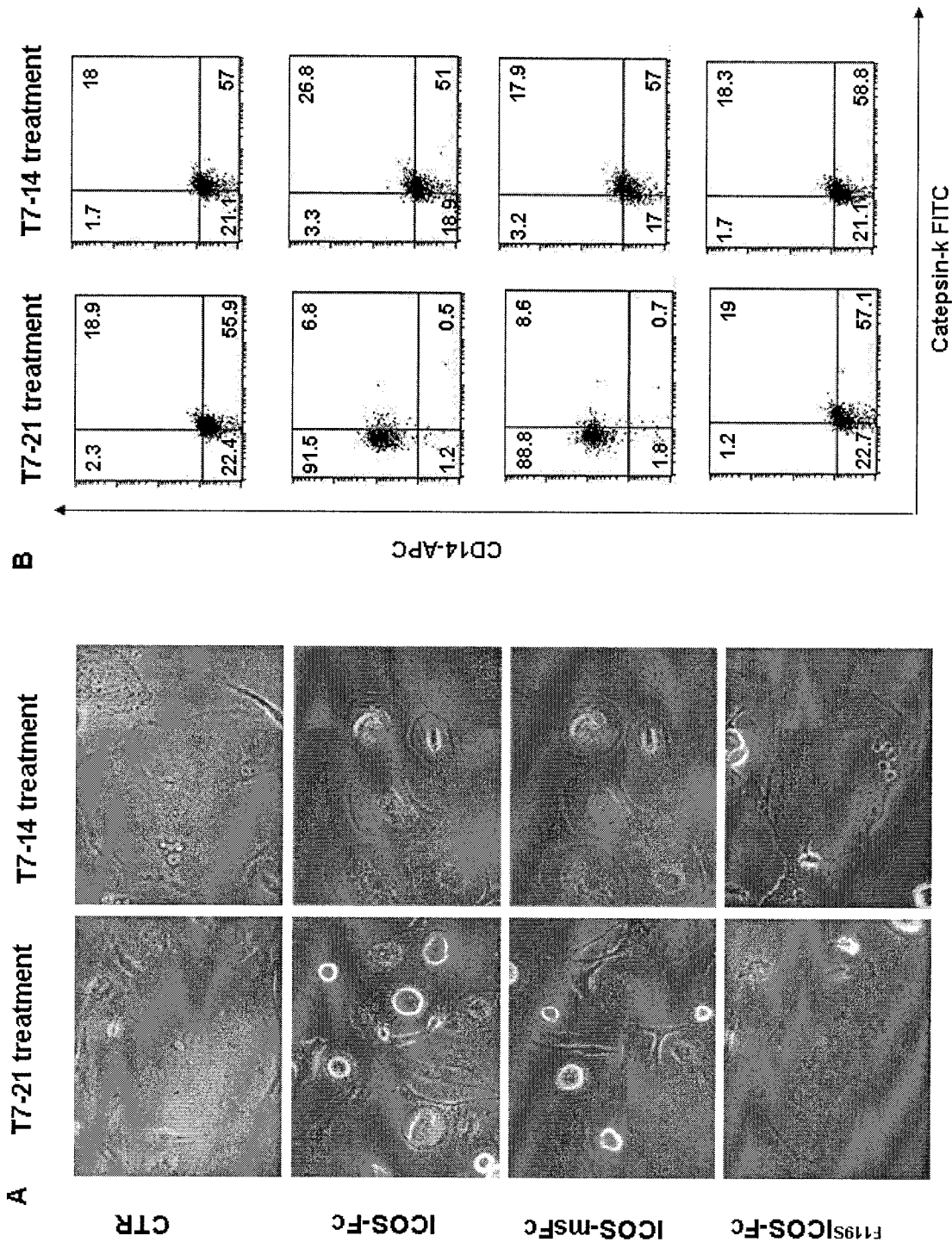


Figure 4

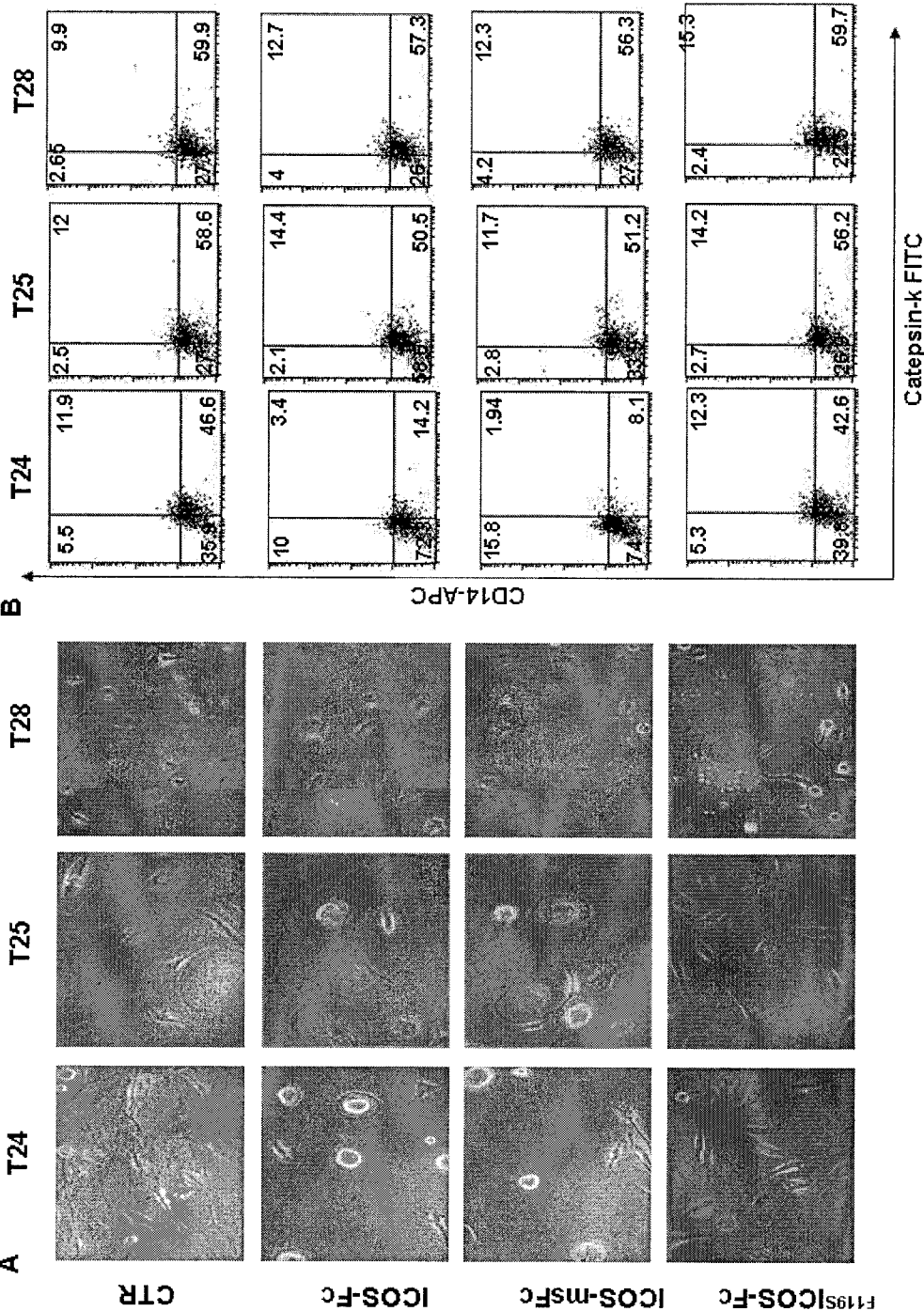


Figure 5

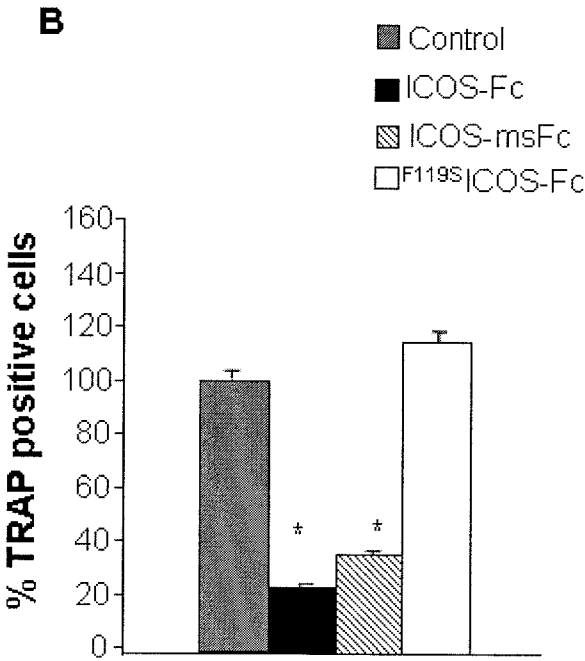
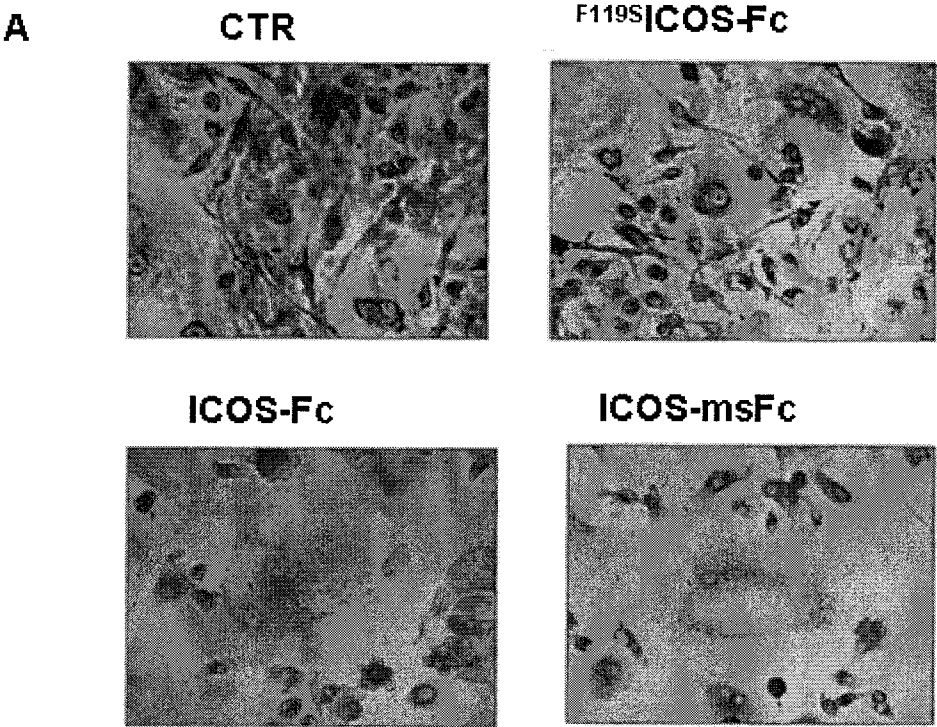


Figure 6

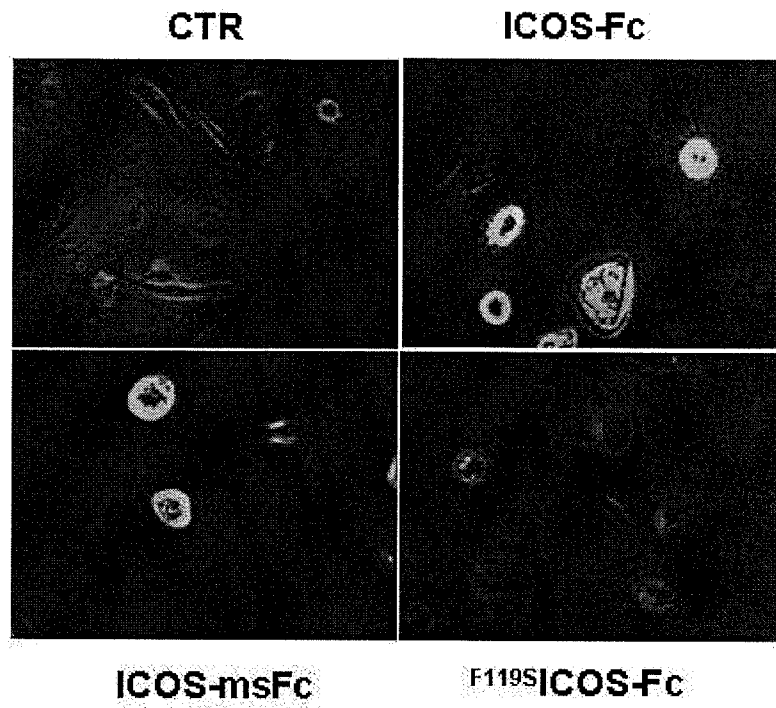
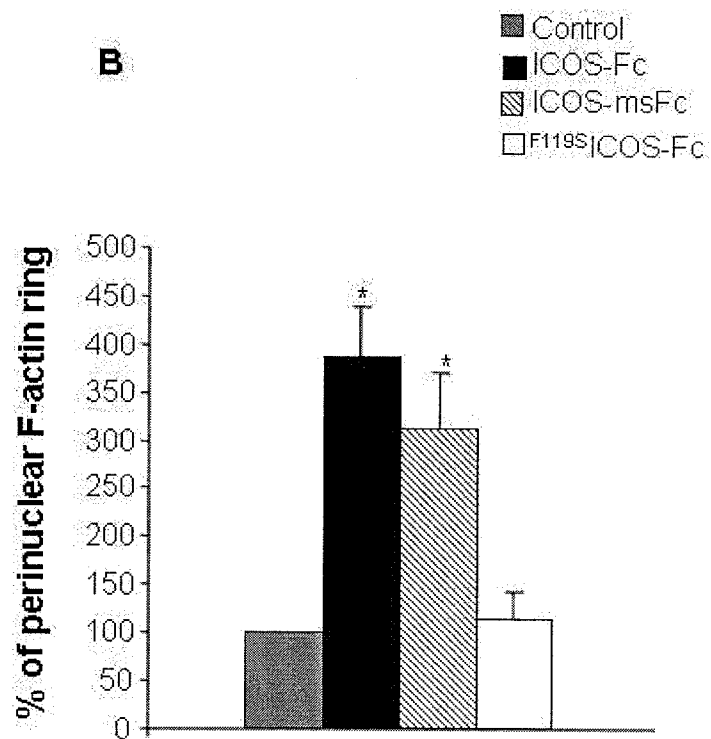
A**B**

Figure 7

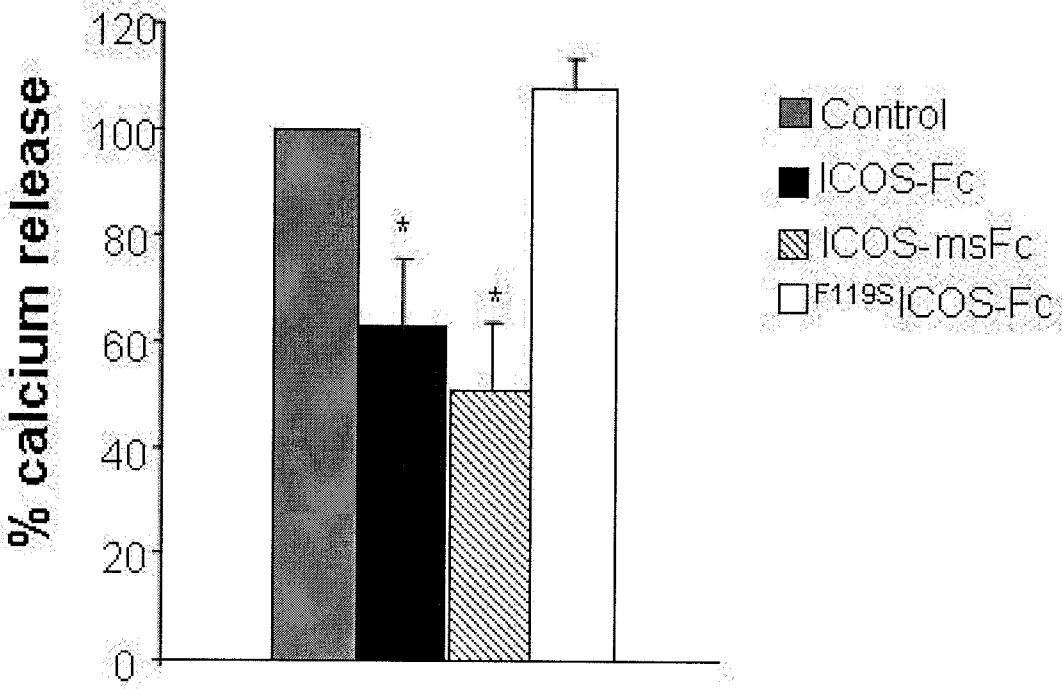
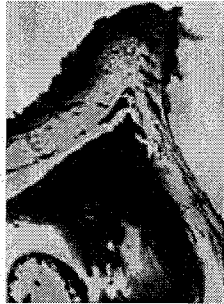


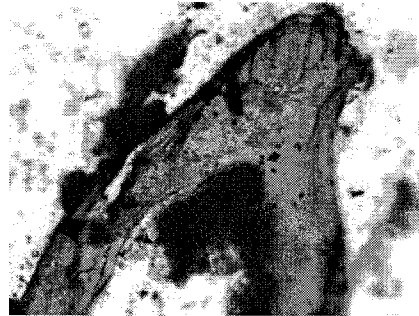
Figure 8

A

CTR



msICOS-huFc



RANKL



RANKL+msICOS-huFc



RANKL+F119S|COS-Fc



F119S|COS-Fc



Figure 9

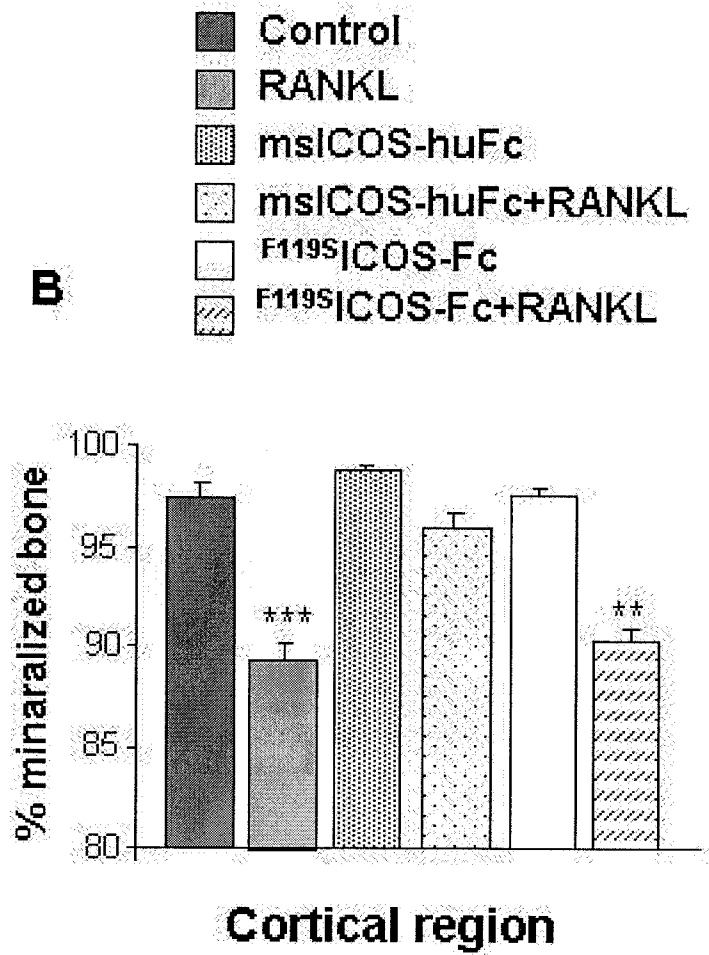


Figure 9 - cont.

10	20	30	40	50	60
MGWSLILLFL	VAVATGGAHA	EINGSANYEM	FIFHNGGVQI	LCKYPDIVQQ	FKMQLLKGGQ
70	80	90	100	110	120
ILCDLTCTKG	SGNTVSIKSL	KFCHSQLSNN	SVSFFLYNLD	HSHANYYFCN	LSIFDPPPFK
130	140	150	160	170	180
VTLTGGYLHI	YESQLASDKT	HTCPPCPAPE	LLGGPSVFLF	PPKPKDTLMI	SRTPEVTCVV
190	200	210	220	230	240
VDVSHEDPEV	KFNWYVDGVE	VHNAKTKPRE	EQYNSTYRVV	SVLTVLHQDW	LNGKEYKCKV
250	260	270	280	290	300
SNKALPAPIE	KTISKAKGQP	REPQVYTLPP	SREEMTKNQV	SLTCLVKGFY	PSDIAVEWES
310	320	330	340	350	360
NGQPENNYKT	TPPVLDSDGS	FFLYSKLTVD	KSRWQQGNVF	SCSVMHEALH	NHYTQKSLSL
370	380				
SPGKTSGKPI	PNPLLGLDST				

Figure 10

10	20	30	40	50
MKSGLWYFFL	FCLRIKVLTG	<u>EINGSANYEM</u>	<u>FIFHNGGVQI</u>	<u>LCKYPDIVQQ</u>
60	70	80	90	100
<u>FKMQLLKGGQ</u>	<u>ILCDLTCTKG</u>	<u>SGNTVSIKSL</u>	<u>KFCHSQLSNN</u>	<u>SVSFFLYNLD</u>
110	120	130	140	150
<u>HSHANYYFCN</u>	<u>LSIFDPPPFK</u>	<u>VTLTGGYLHI</u>	<u>YESQLCCQLK</u>	<u>FWLPIGCAAF</u>
160	170	180	190	
VVVCILGCIL	ICWLTKKKYS	SSVHDPNGEY	MFMRVNTAK	KSRLTDVTL

Figure 11

10	20	30	40	50
MRLGSPGLLF	LLFSSLRADT	QEKEVRAMVG	SDVELSCACP	EGSRFDLNDV
60	70	80	90	100
YVYWQTSESK	TVVTYHIPQN	SSENVDSRY	RNRALMSPAG	MLRGDFSLRL
110	120	130	140	150
FNVTPQDEQK	FHCLVLSQSL	GFQEVLSVEV	TLHVAANFSV	PVVSAPHSPS
160	170	180	190	200
QDELTFTCTS	INGYPRPNVY	WINKTDNSLL	DQALQNDTVF	LNMRGLYDVV
210	220	230	240	250
SVLRIARTPS	VNIGCCIENTV	LLQQNLTVGS	QTGNDIGERD	KITENPVSTG
260	270	280	290	300
EKNAATWSIL	AVLCLLVVVA	VAIGWVCRDR	CLQHSYAGAW	AVSPETELTG

Figure 12

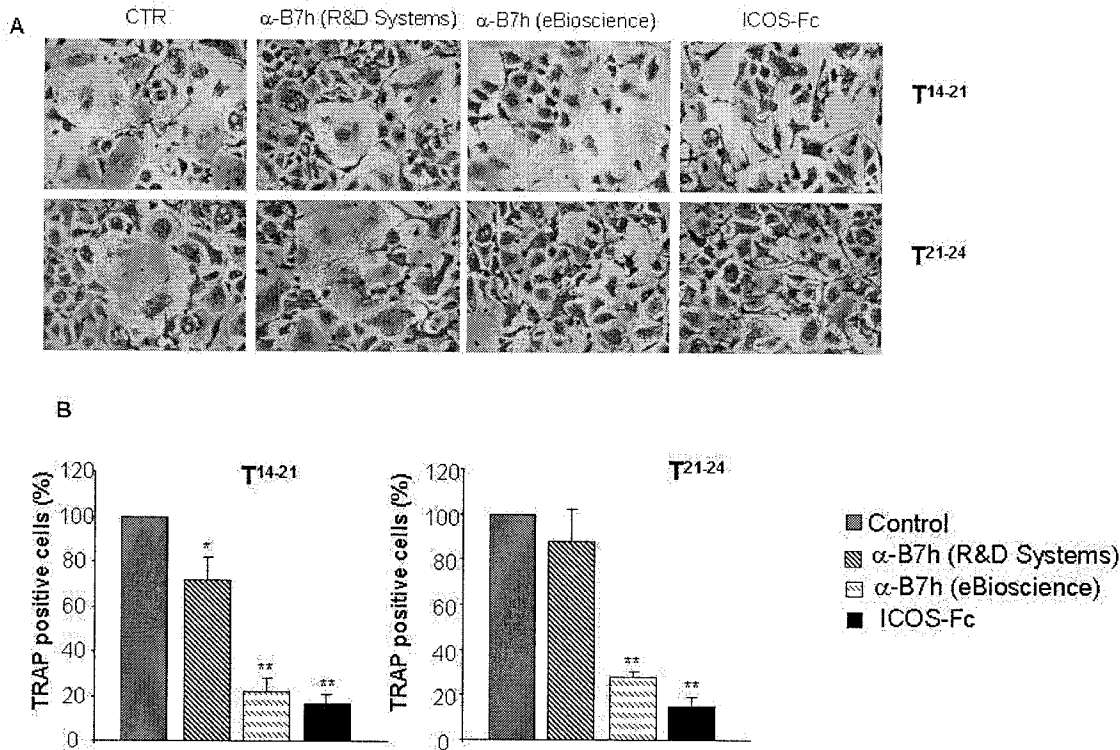


Figure 13

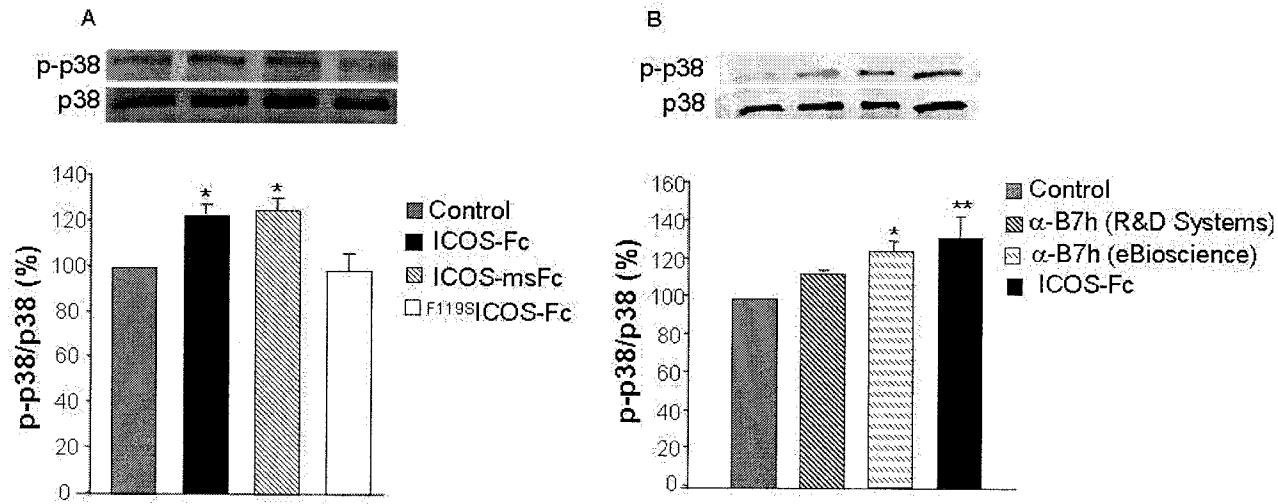


Figure 14

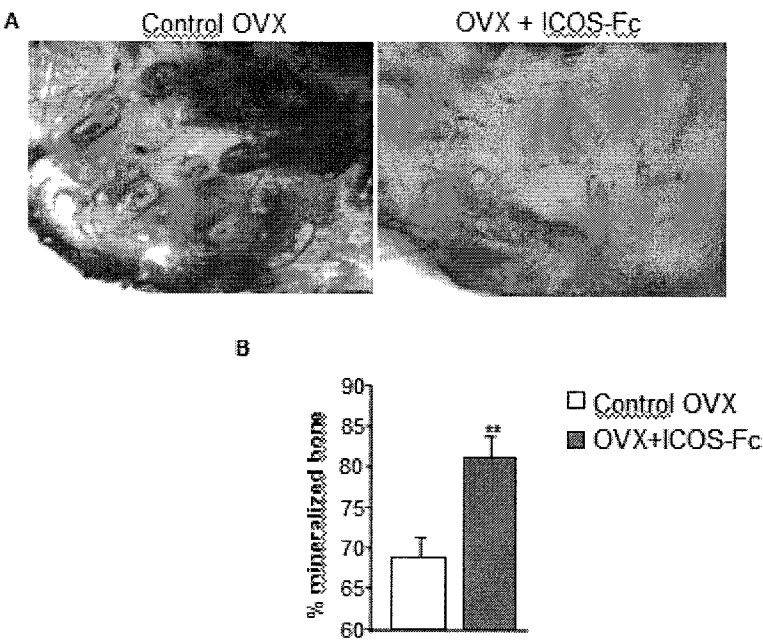


Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/052903

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/00 A61P19/00 A61P19/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, BIOSIS, INSPEC, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IWAI HIDEYUKI ET AL: "BLOCKADE OF ICOS-B7H PATHWAY AMELIORATES MURINE COLLAGEN-INDUCED ARTHRITIS", THE FASEB JOURNAL, FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY, BETHESDA; US, vol. 16, no. 4, 20 March 2002 (2002-03-20), page A713, XP008073616, ISSN: 0892-6638	1,3,4, 10,14
Y	abstract	1-14
X	WO 2007/011941 A2 (AMGEN INC [US]; SIU GERALD [US]; SHEN WENYAN [US]; YOSHINAGA STEVEN KI) 25 January 2007 (2007-01-25)	1,3,4, 10,14
Y	the whole document claims; examples	1-14
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 July 2016

Date of mailing of the international search report

02/08/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Orlando, Michele

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/052903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/159725 A1 (AMGEN INC [US]) 2 October 2014 (2014-10-02)	1,3,4, 10,14
Y	the whole document claims; examples	1-14
X	WO 2014/173975 A1 (UNIV ABERDEEN [GB]) 30 October 2014 (2014-10-30)	1,3,4, 10,14
Y	the whole document page 40, lines 19-29	1-14
Y	S. OCCHIPINTI ET AL: "Triggering of B7h by the ICOS Modulates Maturation and Migration of Monocyte-Derived Dendritic Cells", THE JOURNAL OF IMMUNOLOGY, vol. 190, no. 3, 28 December 2012 (2012-12-28), pages 1125-1134, XP055224216, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.1201816 the whole document page 1133, left-hand column	1-14
Y	C. DIANZANI ET AL: "B7h Triggering Inhibits the Migration of Tumor Cell Lines", THE JOURNAL OF IMMUNOLOGY, vol. 192, no. 10, 11 April 2014 (2014-04-11), pages 4921-4931, XP055224661, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.1300587 the whole document	1-14
A	C. DIANZANI ET AL: "B7h Triggering Inhibits Umbilical Vascular Endothelial Cell Adhesiveness to Tumor Cell Lines and Polymorphonuclear Cells", THE JOURNAL OF IMMUNOLOGY, vol. 185, no. 7, 3 September 2010 (2010-09-03), pages 3970-3979, XP055223879, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.0903269 the whole document	1-14
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/052903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J.-S. PARK ET AL: "JAK2-STAT3 Blockade by AG490 Suppresses Autoimmune Arthritis in Mice via Reciprocal Regulation of Regulatory T Cells and Th17 Cells", THE JOURNAL OF IMMUNOLOGY, vol. 192, no. 9, 31 March 2014 (2014-03-31), pages 4417-4424, XP055223861, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.1300514 the whole document -----	1-14
A	QIAN ZHANG ET AL: "Interleukin-10 Inhibits Bone Resorption: A Potential Therapeutic Strategy in Periodontitis and Other Bone Loss Diseases", BIOMED RESEARCH INTERNATIONAL, vol. 20, no. 7, 16 January 2014 (2014-01-16), pages 764-5, XP055224310, US ISSN: 2314-6133, DOI: 10.1111/j.1365-2141.2008.07278.x the whole document -----	1-14
A	N. TAKAHASHI ET AL: "Impaired CD4 and CD8 Effector Function and Decreased Memory T Cell Populations in ICOS-Deficient Patients", THE JOURNAL OF IMMUNOLOGY, vol. 182, no. 9, 20 April 2009 (2009-04-20), pages 5515-5527, XP055223898, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.0803256 the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2016/052903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007011941	A2	25-01-2007	
		AU 2006270009 A1	25-01-2007
		BR PI0614040 A2	09-03-2011
		CA 2614972 A1	25-01-2007
		CA 2854576 A1	25-01-2007
		CN 101321783 A	10-12-2008
		CR 9745 A	22-07-2008
		DK 1915398 T3	04-07-2016
		EA 200800355 A1	29-08-2008
		EP 1915398 A2	30-04-2008
		EP 2388276 A2	23-11-2011
		EP 2388277 A2	23-11-2011
		EP 2397496 A2	21-12-2011
		EP 2397497 A2	21-12-2011
		EP 2397498 A2	21-12-2011
		EP 2397499 A2	21-12-2011
		ES 2572177 T3	30-05-2016
		IL 188642 A	29-01-2015
		JP 5142998 B2	13-02-2013
		JP 2009501549 A	22-01-2009
		KR 20080049014 A	03-06-2008
		KR 20100101016 A	15-09-2010
		KR 20130023356 A	07-03-2013
		KR 20140097582 A	06-08-2014
		NZ 604090 A	25-07-2014
		NZ 625807 A	27-11-2015
		SG 164369 A1	29-09-2010
		SG 196835 A1	13-02-2014
		UA 102667 C2	12-08-2013
		US 2008166352 A1	10-07-2008
		US 2011104757 A1	05-05-2011
		US 2013171695 A1	04-07-2013
		US 2016145345 A1	26-05-2016
		WO 2007011941 A2	25-01-2007
		ZA 200801137 B	26-05-2010
WO 2014159725	A1	02-10-2014	
		AU 2014244498 A1	24-09-2015
		CA 2904992 A1	02-10-2014
		CN 105324127 A	10-02-2016
		CR 20150544 A	01-12-2015
		EA 201591720 A1	29-01-2016
		EP 2968491 A1	20-01-2016
		JP 2016512546 A	28-04-2016
		KR 20150130343 A	23-11-2015
		PE 17722015 A1	17-12-2015
		PH 12015502074 A1	25-01-2016
		SG 11201507208V A	29-10-2015
		TW 201446797 A	16-12-2014
		US 2016024225 A1	28-01-2016
		UY 35396 A	30-09-2014
		WO 2014159725 A1	02-10-2014
WO 2014173975	A1	30-10-2014	
		AU 2014257549 A1	26-11-2015
		AU 2014257565 A1	26-11-2015
		CA 2909921 A1	30-10-2014
		CA 2909923 A1	30-10-2014
		CN 105492610 A	13-04-2016
		CN 105531370 A	27-04-2016
		EP 2989203 A2	02-03-2016

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2016/052903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		EP 2989204 A1	02-03-2016
		KR 20160010466 A	27-01-2016
		KR 20160015220 A	12-02-2016
		US 2016068600 A1	10-03-2016
		US 2016176951 A1	23-06-2016
		WO 2014173959 A2	30-10-2014
		WO 2014173975 A1	30-10-2014
