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MULTINUCLEATE GIANT CELLS FORMED FROM CAPRINE PERIPHERAL BLOOD MONONUCLEAR CELLS ARE FUNCTIONALLY ACTIVE OSTEOCLASTS.

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Introduction

It is well recognized that cells of the monocyte/macrophage lineage are capable of fusion to form multinucleated giant cells (MGCs) both *in vivo* and *in vitro*. Depending on the environment, MGCs are giant cells from mycobacterium-induced granulomas, giant cells tumors of bone, foreign body giant cells, and osteoclasts (OCs).

MGCs are observed in caprine macrophages after infection with Small Ruminant Lentivirus (SRLV: caprine arthristis-encephalitis virus and maedi-visna virus), in this case syncitia are a typical cytopathic effect of lentiviral replication after long incubation [1]; hence MGCs formation from goats PBMC cultures is generally considered a sign of SRLV infection.

It has been demonstrated that circulating osteoclast precursors exist primarily within the monocytic fraction of peripheral blood [2-4] and that, in certain human diseases, peripheral

blood mononuclear cells (PBMCs) may form OCs in culture without the addition of osteoclastogenic cytokines [4-6]. Excessive OCs formation from PBMCs has been observed in diseases characterized by bone loss, reviewed in [7]. OCs appears as large multinucleated cells that express typical markers and that are able to resorbe bone.

Here we demonstrated that MGCs are spontaneously formed in PBMCs cultures of healthy goats and that these cells are functionally active OCs, hence the formation of MGCs cannot be considered as cytopathic effect of SRLV.

MATERIALS AND METHODS

Animal Selection

Eight Alpine goats (1 buck and 7 does), selected from a dairy farm free from Small Ruminant Lentivirus and mycoplasma infection, were sampled in September 2009. All animals were healthy and aged 2-4 years. The females were in the seasonal estrus period, not lactating and not pregnant. Peripheral blood samples (100 ml) were collected in CPDA-1 blood bags with citrate solution (Terumo, Rome, Italy) from each animal.

Isolation of PBMCs

PBMCs were obtained from all the subjects with density gradient method as previously described [8]. Anticoagulated blood was diluted 1:1 with Hank's buffer solution (Invitrogen, Milan, Italy) and completely layered on an identical volume of Lymphocyte Separation Medium LSM 1077 (M-Medical S.r.l., Milan, Italy) with a density of 1.077 ± 0.001 g/ml. Samples were centrifuged for 30 min at 700 × g and 20°C without applying a brake. The PBMCs interface was carefully removed by pipetting and was washed twice with Hank's buffer solution by stepwise centrifugation for 15 min at 300 × g and for 10 min at 90 × g for platelet removal.

Freshly isolated PBMCs were directly seeded at $4x10^5$ cells/well in triplicate in 96-well plates (Becton Dickinson & Co., Bedford, MA), at $2x10^6$ cells in $10cm^2$ Lab-Tek flaskettes with glass slide and at $5x10^6$ cells in $25cm^2$ flasks (Nalge Nunc International, Milan, Italy) using alpha minimal essential medium (αMEM, Invitrogen, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere and medium was changed every 2-3 days of incubation.

Commento [D1]: ho tolto questa parte xchè non diamo i dati! l'ho messa nel pit formation assay di cui, invece, diamo i dati!

Multinucleated Giant Cells Staining

PBMCs were plated in 96-well plates and were fed every 3 days. On the 1^{st}, 7^{th}, 14^{th} and 21^{st} day, adherent cells were air dried and stained with May-Grunwald Giemsa (Merck Chemicals, Milan, Italy) or fixed with 3.7% formaldehyde in PBS and stained for Tartrate Resistant Acid Phosphatase (TRAP) (Acid Phosphatase, Leukocyte staining kit, Sigma Diagnostics, Milan, Italy). In the TRAP assay, the number of TRAP-positive multinucleated cells (3 or more nuclei per cell) was measured in each well, and a mean value ± SD was calculated.

Rhodamine Phalloidin Staining

For staining of cytoplasmatic F-actin, PBMCs were plated onto flaskette with glass slides. Cells were incubated for 7-14 days at 37 °C in a humidified 5% CO2 atmosphere until MGCs formation. Subsequently cells were fixed in 2% paraformaldehyde for 15 minutes at room temperature. Cells were then rinsed for 20 minutes with PBS containing 0.1 M glycine and permeabilized with PBS containing 0.3% Triton X-100 for 15 minutes. Incubation with rhodamine phalloidin (Molecular Probes, Invitrogen, Milan, Italy) diluted 1:300 in PBS was carried out for 30 minutes RT. Slides were then washed in PBS for 45 minutes and coverslips were mounted with Vectashield mounting medium with DAPI

(Vector Labs, DBA Italia S.R.L., Milan, Italy). Fluorescence staining was analyzed by Video Confocal microscopy (ViCo, Nikon).

αVβ3 Immunofluorescence

For immunofluorescence staining, PBMCs were plated onto flaskette with glass slides. Cells were incubated for 7-14 days at 37 °C in a humidified 5% CO2 atmosphere until MGCs formation. Subsequently cells were fixed in 5 minutes with ice cold methanol, and air dried. After permeabilization with PBS Tween 20 (1:1000), cells were treated for one hour RT with anti-αVβ3 FITC- conjugated monoclonal antibody (Lifespan Bioscience, San Diego, CA, dilution 1:1000). Hoechst 33258 (Sigma Aldrich, Milan Italy) was added for nuclear staining. Coverlips were mounted in anti-fading solution (Sigma Aldrich, Milan Italy) and observed using a Leica DC300F fluorescent microscope.

RNA Isolation

For RNA isolation, freshly isolated PBMCs were directly seeded in 25cm^2 flasks at 5x10^6 cells density. Cells were incubated from 1 to 21 days at 37 °C in a humidified 5% CO2 atmosphere. At 1st and 21st day of culture non adherent cells were removed with repeated PBS (Invitrogen, Milan, Italy) washing. Total RNA was extracted from adherent cells with TRIzol reagent (Invitrogen, Milan, Italy) and eluted in molecular grade water. RNA samples were treated with DNAse I (New England Biolabs, Milan, Italy) and analyzed with an Agilent Bioanalyzer 2100 for quantification and evaluation of quality and purity (only high quality RNA with RIN greater than 7 and A260/280 ratio greater than 1.8 was used in real-time PCR analyses).

One µg of total RNA was retrotranscribed using SuperScript III RNase H-free reverse transcriptase (Invitrogen, Milan, Italy) and oligo-dT according to manufacturer's instruction.

Detection of OCs gene markers transcripts

The consensus sequence of calcitonin receptor (CTR), cathepsin K (CTK), integrin αVβ 3 and matrix metallopeptidase 9 (MMP9) from different species (Bos taurus, Ovis aries, Sus scrofa and Homo sapiens when available) were used to design gene specific primers since the caprine sequences were not available. Partial nucleotide sequence of the caprine CTR, CTK, integrin αVβ 3 and MMP 9 were obtained by RT-PCR using total RNA extracted from goat blood. The amplified fragments were cloned with the TOPO TA Cloning Kit (Invitrogen, Milan, Italy) and sequenced by BMR Genomics (Padova, Italy) on an ABI377 sequencer by using the ABI PRISM dye-terminator cycle sequencing ready reaction kit with Amplitaq DNA polymerase (Perkin-Elmer, Applied Biosystems), thus confirming the detection of caprine CTR, CTK, integrin αVβ 3 and MMP9 transcripts (data not shown).

Real Time PCR

Real-time PCR was performed in the MJ Research Opticon 2 (BioRad, Milan, Italy) using SYBR Green PCR Master Mix (Applied Biosystems, Milan, Italy). The GAPDH housekeeping gene was chosen as an internal control for normalization of expression data using primers previously published [9] Among several measured endogenous control genes, we chose GAPDH for normalization across different genes based on preliminary experiments that indicated that this gene showed the most relatively constant expression in different samples.

The primers for osteoclast marker genes were designed based on corresponding caprine transcript sequences: MMP9f (TTATGGTCTCTGGGCAAAGG) and MMP9r (GTGGTGCAGGAGGAGTAGGA); integrin αVβ 3 f (GGCTTCAAAGACAGCCTCAC) and integrin αVβ 3 r ACACCCCACACTCAAAGGTC); CTRf (AGCGGGTGACTCTGCATAAG)

and CTRr (CCTTTTCACGAGCTCTCCAT); CTKf (AAAAACCAGGGTCAATGTGG) and CTKr (TGCCGGTTTTCTTCTTGAGT).

All PCR reactions were optimized for an annealing temperature of 60°C by varying primer concentration. This allows for a single plate assay of all genes under identical thermal cycling conditions. Primer concentrations were between 200 and 300nM in a final volume of 20 μl. The optimization of primer concentrations also minimizes artifacts and primer dimer formation. The primer pair efficiency was obtained from a standard curve experiment where a series of dilution of the same sample was correlated to the Ct values.

Each reaction contained 2 μl of cDNA, 200 – 300nM of the sense and antisense specific primers, 10 μl of 2× SYBR Green PCR Master Mix in a final volume of 20 μl. The amplification program consisted of a preincubation step for denaturation of the template cDNA and activation of AmpliTaq Gold (10 min, 95 °C), followed by 40 cycles consisting of a denaturation step (15 s, 95 °C) and an annealing and extension step (1 min, 60 °C). After each cycle, fluorescence was measured. A negative control without cDNA template was run in each assay. Samples were run in duplicate. PCR products were subjected to a melting curve analysis and subsequently 2% agarose/TAE gel electrophoresis to confirm amplification specificity, Tm and amplicon size, respectively. The results obtained were compared using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The amount of target gene expressed in 21 days samples, normalized to an endogenous housekeeping gene and relative to the 1 day samples, is then given by $2^{-\Delta\Delta C}{}_T$, where $\Delta\Delta C_T = \Delta C_T$ (21 days sample) - ΔC_T (1 day sample), and ΔC_T is the C_T of the target gene subtracted from the C_T of the housekeeping gene.

Pit Formation Assay

In order to evaluate functional activity of OCs, PBMCs cultures were plated in triplicate on dentin slices (supplied by Pantec, Italy) $1x10^6$ cells/well with or without Macrophage

Colony Stimulating factor (M-CSF, [25 ng/ml], Chemicon, Italy) and Receptor Activator of Nuclear Factor κB Ligand (RANKL, [30 ng/ml], Chemicon, Italy), these factors stimulates osteoclastogenesis On the 21st day of culture dentine slices were removed from the wells, rinsed in PBS, and placed in 0.25% trypsin for 15 minutes; washed in distilled water and left overnight in 0.25 M ammonium hydroxide. The slices were then washed in distilled water and stained with 0.5% (w/v) toluidine blue. In order to avoid aspecific staining the dentine slices were left in NaOH 2 N for few seconds and examined by light microscope. The total surface of each dentine slice was inspected and the resorption areas were photographed with a digital camera (Nikon Coolpix) attached to an inverted research microscope was used to photograph the surface of each well. Lacunar resorption, determined by examining each micrograph with a dedicated computer image analysis system developed in our lab (patent n Italian Patent Application n TO2006A00565), was expressed as the total percentage of the surface reabsorbed [10].

Statistical Analysis

All statistical analyses were computed by using statistical software (SPSS 17.0, SPSS Inc., Chicago, USA). OCs growth over time (OCs /well) at 1, 7, 14 and 21 days of culture and differential gene expression at 21 days compared to 1 day culture were tested with Shapiro-Wilk test to assess normal distribution of data. OCs were not normally distributed; therefore a non parametric Friedman test was used.

Differential gene expression data were normally distributed; therefore a parametric T test for Paired Samples was used. Statistical significance was accepted at $P < 0.05$.

RESULTS

MGCs spontaneously formed in PBMCs cultures of healthy goats are TRAP positive.

Multinucleate giant cells started to develop from 7^{th} day of culture. Development and growth of MGCs was not influenced by the presence or absence of M-CSF and RANKL factors. MGCs were large (250-500 µm), roundish with fairly distinct cell borders, abundant, light blue, finely granular cytoplasm characterized by variably sized clear vacuoles and darker areas in association with single or double, central to paracentral clusters of round to oval, variable-sized (10-25 µm) nuclei (up to 12 nuclei) with one or two prominent nucleoli. Some multinucleated giant cells were characterized by single or multiple, 50-300 µm long cytoplasmic projections (Fig. 1A).

Intense TRAP positivity (brownish-red stain) was detected exclusively around the nuclei of the multinucleated cells (Fig.1B). TRAP positive cells progressively increased over time (Fig.1C).

The MGCs.cytoskeleton has the typical organization of OCs one.

It is well recognized that OCs has a typical cytoskeleton organization with F-actin clustered in a ring around the cell periphery corresponding to the sealing zone; OCs are able to attach to bone and resorbe it by this structure. Figure 2 shows the F-actin distribution as it appears at confocal microscopy in caprine MGCs (A) and in mononucleated cells (macrophages) from the same culture (B). MGCs were characterized by the presence of an actin ring at the cell periphery whereas macrophages showed uniform distribution of podosomes in the cytoplasm and the actin ring is not present.

Since the $\alpha V\beta 3$ is expressed in OCs, cells cultured on glass were fixed and immunostained with anti-$\alpha V\beta 3$ mAb (figure 2C). We observed specific intense staining all over the osteoclast and not in single nucleated cells.

MGCs express OCs markers.

In order to evaluate if caprine MGCs expressed typical osteoclasts genes after 21 days of culture in absence of osteoclastogenic cytokines we carried on a real time RT-PCR for CTK, CTR, MMP9 and integrin $\alpha V\beta$ 3 at baseline and after 21 days of cultures. The analyzed genes significantly increased after the residence in cultures (Fig. 3), this datum confirms the morphological and functional data.

MCGs resorb dentine in culture.

After 21 days of culture we observe the presence of multiple resorption pits on dentine slices both in the presence and in the absence of M-CSF and RANKL (Fig.4 BC). There were no significant differences in the amount of bone resorbed in presence or absence of osteoclastogenic cytokines (Fig. 4D).

DISCUSSION

SRLV causes chronic inflammatory disease in the brain, lungs, joints, and mammary glands [11] and are widely distributed in the caprine population. Following infection, animals produce anti-viral antibodies, though seroconversion may occur any time from a few weeks to several months. The main cells infected in vivo are monocytes and macrophages, with the bone marrow serving as a reservoir of infection [1]. Infected monocytes, after transformation in macrophages, develop a typical cytopathic effect in culture and fuse to form MGCs, hence the formation of MGCs in culture from caprine PBMCs has been widely used as a sign of SRLV infection.

Here we demonstrated that caprine PBMCs spontaneously form MGCs in culture without infection, moreover we demonstrated that these MGCs are active osteoclasts.

The OC is a multinucleated cell uniquely specialized in bone resorption. It has been shown that osteoclast precursors comprise 1-4% of circulating monocytes and that osteoclasts can be formed from CD14+ monocyte/macrophage precursors [12, 13]. Osteoclast formation involves a multistep process during which there is loss of monocyte/macrophage markers and gain of osteoclast markers [14] in the presence of two essential cytokines, M-CSF and RANKL. Furthermore, PBMC-derived T cells are able to support directly and indirectly osteoclast formation and activation from blood monocytes [4, 15]. However recent studies demonstrated that OCs formation can occur spontaneously from PBMCs in certain conditions. In particular, osteoclastogenesis occurs even in the absence of M-CSF and RANKL in PBMCs cultures from patients affected by bone lytic diseases as postmenopausal osteoporosis [4, 5], cancer [16, 17], rheumatic diseases [18-20], periodontitis [21, 22] and chronic liver disease with osteopenia [23].

In this study we observed the formation of MGCs from PBMCs cultures of goats free from viruses' infection. The formation of these cells starts after 7 days and their number increases after 15 and 21 days of culture. In order to characterize these cells we employed morphological and functional assays and we tested the expression of genetic markers of OCs.

Throughout the differentiation process OCs express a series of markers, such as TRAP, CTK, MMP-9, CTR and the integrin $\alpha V\beta 3$, which, along with multinucleation, characterise the OC phenotype. TRAP is inserted in the ruffled membrane and is secreted within the sealing zone to degrade bone matrix [24, 25]; the expression of this enzyme is a fundamental step in OCs differentiation. CTK is a cysteine protease that is selectively expressed in OCs and leads to degradation of bone matrix proteins, deficiency in this protein results in the human disease pycnodysostosis characterized by osteosclerosis and decreased bone resorption [26]. MMP-9 is highly expressed in OCs and is essential for

their recruitment into bone [27, 28]. CTR belongs to the family of seven-transmembrane–domain G-protein–coupled receptors and specifically binds calcitonin [29]; it is involved in the regulation of mineral metabolism by inhibiting bone resorption and increasing calcium renal excretion [30]. The expression and biologic role of CTR are directly correlated to the bone resorbing activity of OCs [31]. αVβ3 integrin or vitronectin receptor is present on the surface of OCs and is required for their attachment with bone matrix proteins, that is an important initial step in bone resorption [32, 33]. Various studies have indicated that αVβ3 is the most abundant integrin in OCs and that OCs adhesion with extracellular matrix is inhibited by blocking αVβ3 integrin [34].

Here we demonstrated that the above mentioned markers, which are specifically expressed by OCs and are fundamental for their function, are progressively more expressed in PBMCs cultures after MGCs formation.

A fundamental trait of OCs differentiation is their typical cytoskeleton structure that allows the cells to adhere to several substrates on which they form distinct F-actin structures. Mature OCs exhibit podosomes similar to monocyte-derived cells such as macrophages or dendritic cells in vitro. Podosomes are formed by an F-actin core, surrounded by several focal adhesion proteins, such as integrins, vinculin, paxillin, and talin [35, 36]. In mature OCs, podosomes are arranged at the cell periphery as a characteristic belt [37]. Podosomes are linked to the extracellular matrix through adhesion receptors as αVβ3 [35]. Based on in vitro observations, it has been proposed that podosomes are essential for extracellular matrix degradation, invasion and migration in vivo [38]. In our culture MGCs exhibit the typical cytoskeleton organization of OCs with actin ring formation, podosomes and the expression of αVβ3 on cell membrane.

Besides the morphological aspects, the hallmark of OCs is their unique ability to resorbe mineralised calcium apatite or carbonate substrates such as bone, dentin or nacre [4, 5, 39, 40]; here we showed that MGCs formed in PBMCs cultures are able to resorbe dentine in vitro. The addition of M-CSF and RANKL do not significantly increase the ability of these cells to resorbe bone. This is in line with the data obtained in humans and in plenty of studies there was not a significant increase in OC formation and activity following the addition of M-CSF and RANKL, reviewed by De Vries and Everts [7].

In conclusion we suggest that the formation of MGCs from caprine PBMCs occurs also in the absence of SRLV infection and hence could not be considered as a virus' cythopatic effect. We rather suggest that caprine PBMCs are able to spontaneously form OCs in cultures. These cells can be considered as fully differentiated OCs as they express the typical OCs markers and are able to resorbe bone in vitro.

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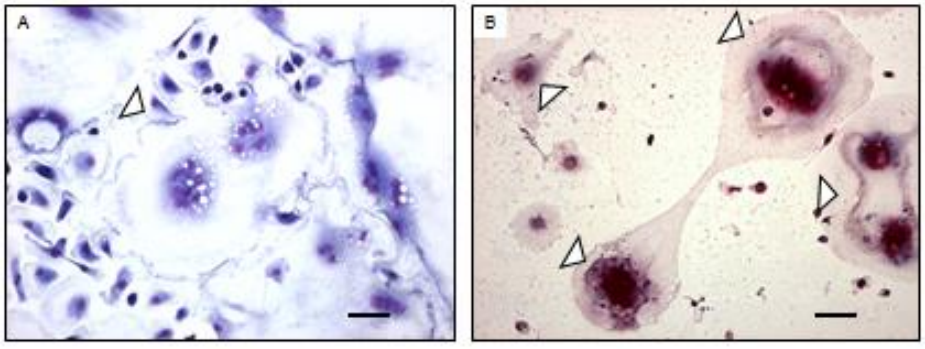
FIGURE LEGENDS

Figure 1. PBMCs from healthy goats spontaneously form TRAP positive MGCs in culture. A) Giemsa staining of MGCs (10X, indicated by arrow heads), MGCs are characterized by the presence of more than three nuclei in the central region of the cell. B) TRAP staining of MGCs (10X, indicated by arrow heads), brownish-red stain (positivity) was detected exclusively around the nuclei of the multinucleated cell whereas the mononucleated cells (macrophages) resulted negative. The scale bar represents 40 μm. C) The box and whiskers plot shows the number of multinucleated (3 or more nuclei) TRAP+ cells at different time points. P values were calculated with Friedman test: p=0.000.



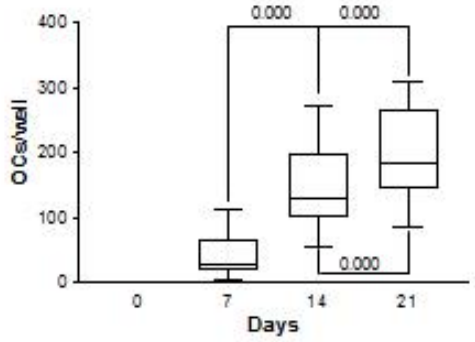


Figure 2. MGCs from caprine PBMCs displays an F-actin ring and are positive for αVβ3.. Actin cytoskeleton was visualized by rhodamine phalloidin staining. MGCs showed the formation of an actin ring at the cell periphery (A, 10X). Actin was uniformly distributed in the cytoplasm of mononucleated cells/macrophages (B, 20X). Specific intense staining for αVβ3 (C, 10X) was observed all over the MGCs and not in mononucleated cells/macrophages. The scale bars represent 40 μm.

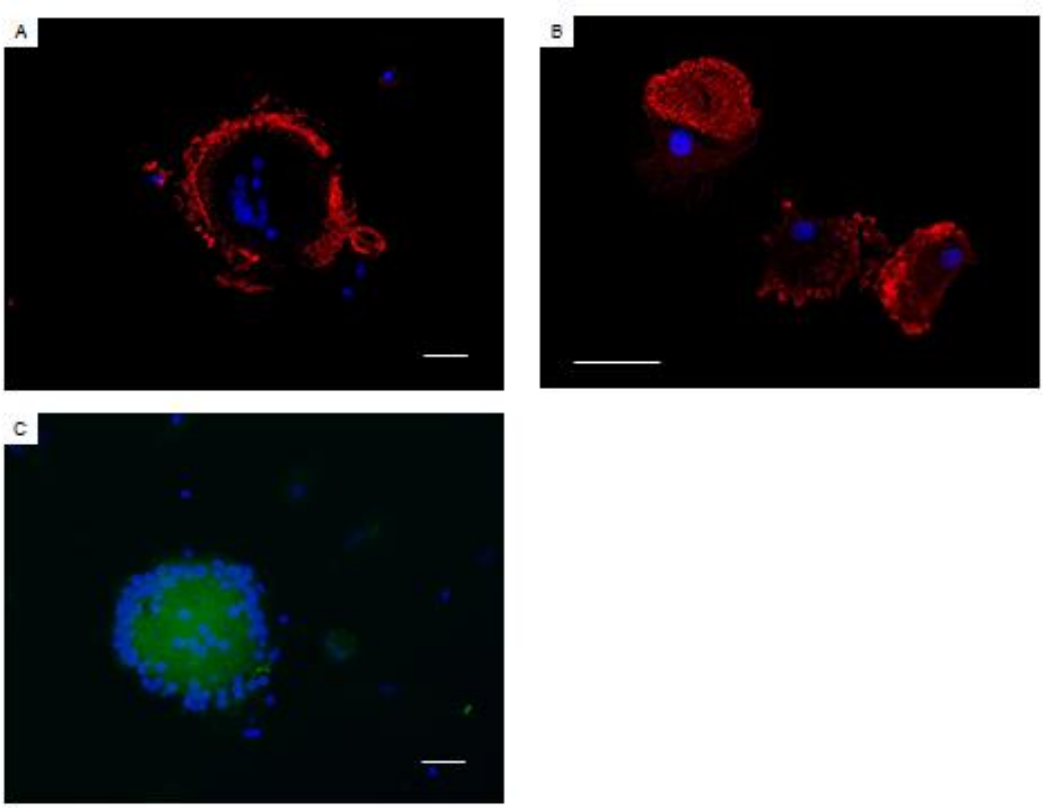


Figure 3. Relative fold expression of osteoclast specific gene markers (CTK, CTR, MMP9 and integrin αVβ 3) increases after 21 days of cultures. The graph shows the amount of target gene expressed in 21 days samples, normalized to the GAPDH endogenous housekeeping gene and relative to the 1 day samples, is then given by $2^{-\Delta\Delta C}{}_{T}$ method.
Bars represent mean and standard deviation, * P < 0.001, p values were calculated by Student's paired T test.

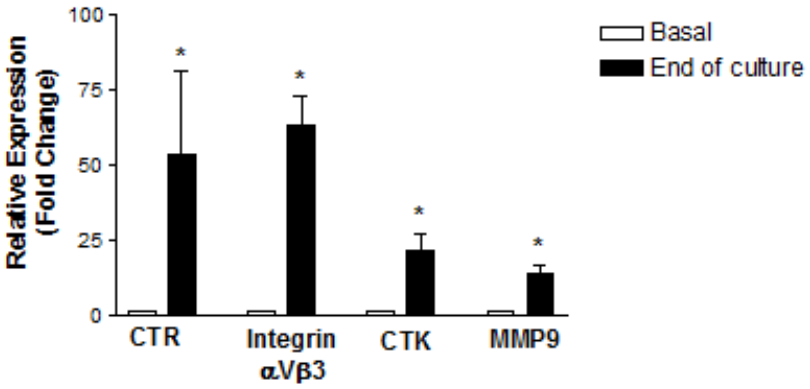
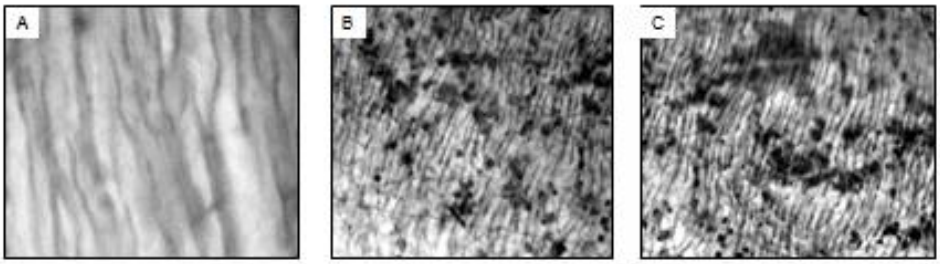
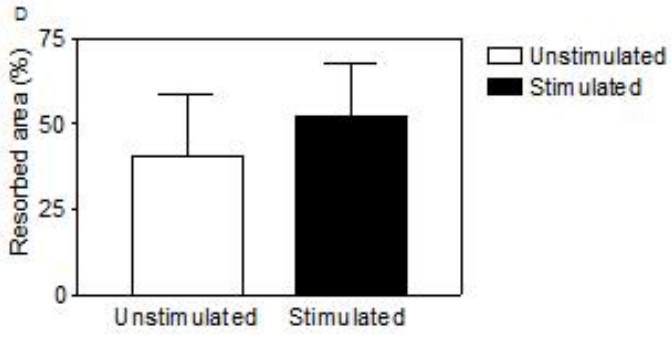



Figure 4. MGCs from caprine PBMCs are able to resorbe dentine slices. Micrographs (10X) show the pits formation after 21 days of culture without (B) or with (C) the addition of M-CSF and RANKL, panel A shows dentine without pits, cells were removed after 24 hrs of culture. Pits (darker areas) are demonstrated by toluidine blue staining of dentine slices after cells removal. No significant difference was observed in total percentage of the surface reabsorbed with or without the addition of M-CSF and RANKL (C).