

Cell-specific effects of TNF- α and IL-1 β on alkaline phosphatase: implication for syndesmophyte formation and vascular calcification

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Tumor necrosis factor (TNF)- α and interleukin (IL)-1 β stimulate tissue non-specific alkaline phosphatase (TNAP) activity and mineralization in cultures of vascular smooth muscle cells (VSMCs). They are, therefore, considered as stimulators of vascular calcification in the context of atherosclerosis and diabetes type 2. In contrast, although ankylosing spondylitis (AS) leads to the formation of syndesmophytes, which are ectopic ossifications from entheses (where ligaments, tendons and capsules are attached to bone), anti-TNF- α therapies fail to block bone formation in this disease. In this context, our aims were to compare the effects of TNF- α and IL-1 β on TNAP activity and mineralization in enthesal cells and VSMCs. Organotypic cultures of mouse ankle entheses were treated or not with TNF- α and IL-1 β for 5 days. Micro-computed tomography was performed to determine trabecular bone parameters, and histology to assess TNAP activity and mineralization. Human mesenchymal stem cells cultured in pellets in chondrogenic conditions and human VSMCs were also used to determine the effects of cytokines on TNAP activity and expression, measured by quantitative PCR. In organotypic cultures, TNF- α and IL-1 β significantly reduced the tibia BV/TV ratio. They also inhibited TNAP activity in enthesal chondrocytes *in situ*, and in mouse and human chondrocytes *in vitro*. In contrast, TNF- α stimulated TNAP expression and activity in human VSMCs. These differences were likely due to cell-specific effects of peroxisome proliferator-activated receptor γ (PPAR γ), which is inhibited by TNF- α . Indeed, in human chondrocytes and VSMCs, the PPAR γ inhibitor GW-9662 displayed the same opposite effects as TNF- α on TNAP expression. In conclusion, whereas TNF- α and IL-1 β stimulate TNAP activity in VSMCs, they inhibit it in enthesal cells *in situ* and on chondrocytes *in vitro*. The identification of PPAR γ as a likely mediator of cytokine effects deserves consideration for future research on the mechanisms of ectopic ossification.

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Ankylosing spondylitis (AS) is a chronic inflammatory disease of unknown origin affecting the axial skeleton including the sacroiliac joints and the spine and also the peripheral joints, leading to ankylosis of joints and poor physical function.¹ Although the pathogenesis of AS is poorly understood, the fibro-cartilage in the entheses (the sites of attachment of tendons and ligaments into bone) is the primary site of the disease and where new bone formation is initiated.¹ New bone formation from entheses progresses along tendons and ligaments, and eventually leads to bone fusions, which contribute to ankylosis. These newly formed

bone spurs are called syndesmophytes when they develop in the spine and enthesophytes when they occur in peripheral joints.

To date, our understanding of the mechanisms leading to spur formation has been almost exclusively provided by the important pioneering studies of Luyten on one hand, and Benjamin and McGonagle on the other hand. The Luyten's group has characterized the spondyloarthropathy-like arthritis that spontaneously develops in aging DBA/1 mice.^{2,3} In this mouse model, new bone formation starts at the enthesis and occurs through endochondral ossification.²

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On the other hand, Benjamin and McGonagle have thoroughly studied the age-related enthesophyte formation in the Achilles tendon in both rodents and humans.^{4,5} Although obviously it remains unclear whether formation of syndesmophytes (which occur in the inflammatory context of AS) and enthesophytes (which may mainly result from mechanical factors during aging⁶) follow the same molecular pathways, the Achilles tendon is also affected in AS in humans,⁷ and is fundamentally similar to the entheses principally affected in AS.⁴ Consequently, Benjamin *et al*^{4,5} reasonably assumed that the mechanisms governing syndesmophyte formation in the spine and enthesophyte development in the Achilles tendon may be similar. Their studies revealed that the process of ossification starting at an enthesis is slightly different from endochondral ossification of a cartilage growth plate. Indeed, whereas in a cartilage growth plate the growth is ensured by chondrocyte proliferation, at an enthesis, the ossification is governed by the metaplasia of fibro-chondrocytes into mineralization-competent chondrocyte-like cells.^{4,5} These chondrocyte-like cells then mineralize their extracellular matrix (ECM), and this calcified matrix, is like the calcified growth plate cartilage, vascularized, resorbed, and eventually replaced by bone. Importantly, in this model, the initial event in the formation of enthesophytes and syndesmophytes is therefore the ECM calcification by enthesal chondrocyte-like cells.

Despite the fact that the mechanisms governing ossification from an enthesis have been thoroughly dissected, the links between inflammation, inflammatory cytokines and spur formation during AS remain obscure.^{8–10} Some studies reported that new syndesmophytes arose more often at sites where active inflammation was present at baseline, but the majority of syndesmophytes arose from sites without detectable inflammation on MRI at baseline.¹⁰ Moreover, although anti-tumor necrosis factor α (anti-TNF- α) therapies have been shown to improve most symptoms in AS patients, they have proven unsuccessful to block the progression of bone spurs both in patients with AS,^{7,10} and in the DBA/1 model of spondyloarthritis.³ The fact that anti-TNF treatments do not significantly alter the course of syndesmophyte formation may be due to the fact that TNF- α likely displays both stimulatory and inhibitory effects, as it does in endochondral ossification during bone healing.^{11–13} It is also possible that other cytokines are involved in syndesmophyte formation. In this regard, genome-wide association study of AS has identified interleukin (IL)-1 cytokine pathway in disease susceptibility.¹⁴ Interestingly, IL-1 β levels in peripheral blood monocytes are elevated in patients with AS,¹⁵ and clinical and MRI studies have shown that the anti-IL-1 anakinra was effective in active AS,^{16,17} even though the changes appeared less dramatic than those evident on anti-TNF- α agents. Since TNF- α and IL-1 β appear to display important non-redundant effects in bone remodeling,¹⁸ we chose to investigate the effects of both cytokines on enthesis mineralization.

To avoid the interference of indirect, systemic effects of cytokines on enthesal chondrocyte-like cells, we developed organotypic cultures of mouse bones and joints, where enthesal cells were directly exposed *in situ* to the action of cytokines. Particular attention was paid to the Achilles tendon, which is prone to ossification during AS,⁷ and for which ossification has been thoroughly studied and illustrated.⁴ In addition to mineralization, we particularly investigated the effects of cytokines on the expression and activity of tissue non-specific alkaline phosphatase (TNAP). Indeed, TNAP is an enzyme that hydrolyzes pyrophosphate ions, which are potent mineralization inhibitors, and thereby is able to induce the calcification in tissues containing a fibrillar collagen.^{19,20} Recently, we showed that TNF- α and IL-1 β stimulate TNAP expression and activity independently of the master osteoblast transcription factor RUNX2, and hypothesized that these cytokines may be able to induce calcification in tissues enriched in a fibrillar collagen, such as the enthesal fibro-cartilage.²¹ This hypothesis was conceivable since TNF- α stimulates TNAP expression in vascular smooth muscle cells (VSMCs),^{22,23} and since blockade of TNF- α with infliximab specifically inhibits vascular calcification in the *Ldlr*-/- diabetes mellitus mouse model, without reducing obesity, hypercholesterolemia or hyperglycemia.²⁴ In this hypothesis, cytokines may stimulate ectopic collagen calcification by the mere stimulation of TNAP activity, and this calcified matrix, may in turn induce the ossification process, in the arterial wall,²⁵ and in enthesal fibro-cartilage during AS.^{4,5}

MATERIALS AND METHODS

Chemicals

Cell culture plastic ware was purchased from D Dutscher (Brumath, France). Dulbecco's minimum essential medium (DMEM), α -MEM, fetal calf serum (FCS), insulin, L-glutamine, penicillin, streptomycin (P/S), trypsin/EDTA, and Extract-All reagents were from Eurobio (Les Ulis, France). Vitamin C, 1,25(OH)₂VD₃, β -glycerophosphate (β -GP) were obtained from Sigma-Aldrich Corporation (St Quentin Fallavier, France). DNase I, Taq DNA polymerase and SYBR green mix were from Roche Diagnostics (Meylan, France). Random primers were obtained from TibMolBiol (Berlin, Germany). Superscript II reverse transcriptase and dNTPs were purchased from Invitrogen (Cergy Pontoise, France). Human and/or mouse TNF- α , IL-1 β and TGF- β 1 were purchased from R&D Systems (Lille, France). GW-9662 was from Tebu-bio (Le Perray en Yvelines, France).

Organotypic Cultures of Whole Mouse Joints

To avoid that indirect, systemic effect of both cytokines interfere with their direct ones, we developed organotypic cultures of mouse bones. Nine 6-week-old C57BL6 mice from Charles River were euthanized and skinned. Posterior legs were harvested, and the muscles thoroughly discarded, leaving the entheses in the knees and ankles intact. In particular, the soleus

muscle was discarded at distance from the Achilles tendon insertion into the calcaneus bone. Bones were gently washed in sterile PBS containing antibiotics and placed in FCS-free DMEM supplemented with antibiotics. The left explants were left untreated and the right ones were treated with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β for 5 days. After the incubation period, explants were immediately immersed in 4% phosphate-buffered formalin. Analysis of architectural parameters of tibias from six mice was done using the high-resolution X-ray micro-computed tomography (micro-CT) system SkyScan-1072 (SkyScan, Belgium). Analyses were performed at 60 kV, 9 W and 0.68°. Relative volume (BV/TV) of the tibia and femora (trabecular bone) was quantified in the group treated with cytokines and compared with that of the untreated group. Other trabecular variables (number, thickness and separation) were similarly obtained using the SkyScan CtAn software. For histological analysis, bones from the three other mice were embedded in glycol methyl methacrylate and 5- μ m thick sections were performed as previously published.²⁶ Von kossa staining was performed by incubating slides in 1% silver nitrate and then in 0.5% hydroquinone after washing. Staining of alkaline phosphatase activity was performed overnight by slide incubation in BCIP in a tris-maleate buffer containing nitroblue tetrazolium chloride.

Cell Cultures

Human bone marrow mesenchymal stem cells (MSCs) from three donors were used. Cells consisted in purified MSCs from two healthy donors (a 34-year-old female and a 36-year-old male (Lonza, Walkersville, USA; certified positive for CD29, CD44, CD105 and CD166; and negative for CD14, CD34 and CD45)) and also in MSCs obtained from trabecular bone explants prepared from the iliac crest bone harvested during pelvic osteotomy in one child with Legg-Perthes-Calve disease, as previously described.²¹ In accordance with our regional ethic committee, which authorized us to use bone explants for research purposes ('Comité Consultatif de la Protection des Personnes dans le Recherche Biomédicale de Lille,' CCPPRB, 2000/10/03), surgeons asked informed consent to the children's parents.

MSCs were seeded at a density of 5000 cells/cm² and routinely cultured in DMEM containing 10% FCS, 1% P/S and 1% L-glutamine. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in air. Cells were subcultured at ~80–90% confluence with trypsin/EDTA. To induce osteoblast differentiation, MSCs were seeded at 5000 cells/cm² in DMEM and at confluence, medium was replaced by an osteogenic medium, consisting of DMEM with 10% FCS, containing 10⁻⁸ M 1,25(OH)₂VD₃, 50 μ M vitamin C and 10 mM β -GP.²¹ Cells were treated with cytokines on day 14 for the indicated times. To induce chondrogenic differentiation, human MSCs were cultured in pellets containing 500 000 cells per condition. Pellets were cultured for 21 days in FCS-free DMEM supplemented with 10 μ g/ml of bovine insulin, 50 μ M of ascorbic acid, 10⁻⁸ M of dexamethasone

and 10 ng/ml of TGF- β 1, as detailed elsewhere.²⁷ To induce calcification, pellets were then cultured in α -MEM containing 5% FCS, 10 μ g/ml of insulin, 10⁻⁸ M 1,25(OH)₂VD₃ and 5 mM β -GP. Pellets were finally treated on day 24 with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β for 2 days for PCR analysis or 7 days to assess TNAP activity. To evaluate TNAP activity, chondrocyte pellets were fixed, embedded in glycol methyl methacrylate, sectioned and stained as described above for organotypic cultures.

Human VSMCs were cultured from explants of human aorta in DMEM containing 15% FCS, 1% P/S and 1% L-glutamine. Briefly, the cells were prepared from explants of tissue and were confirmed as VSMCs by positive staining with monoclonal antibodies against α -smooth muscle actin (sc-32251). Cells were maintained in DMEM containing 15% FCS, 1% glutamax[®] (Sigma), 1% penicillin/streptomycin (Sigma) and at 37°C with atmospheric air and 5% CO₂. Cells were subcultured after treatment with 0.25% trypsin and 0.02% EDTA. To determine the effect of 1 ng/ml of TNF- α on TNAP expression, cells were cultured in α -MEM containing 15% FCS, 1% P/S, 1% L-glutamine, 10⁻⁸ M 1,25(OH)₂VD₃ and 10 mM β -GP.

Murine ATDC5 chondrocytes were cultured as previously described in details.²⁶ Briefly, cells were seeded at 15 000 cells/cm² and grown in DMEM/F12 supplemented with insulin. On day 21, DMEM/F12 was replaced with α -MEM and cells were treated on day 24, with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β for 48 h.

RNA Extraction, Reverse Transcription and PCR

Total RNA was extracted using Extract-All reagent according to the manufacturer's instructions. Briefly, lysis of the cells in Extract-All was followed by centrifugation at 12 000 g for 15 min, at 4°C in the presence of chloroform. The upper aqueous phase was collected, and the RNA was precipitated by addition of isopropanol and centrifugation at 12 000 g for 10 min, at 4°C. RNA pellets were washed with 75% ethanol, dried and reconstituted in sterile water. Total RNA was quantified by spectrophotometer at 260 nm wave length and the integrity of RNA was controlled by the 28S/18S rRNA ratio after agarose gel electrophoresis. Contaminating DNA was removed from RNA samples in a 30 min digestion at 37°C with DNase I. In all, 6 μ g of each RNA sample was then used for reverse transcription performed under standard conditions with Superscript II reverse transcriptase and random hexamer primers in a 20- μ l final volume. The reaction was carried out at 42°C for 30 min and stopped with incubation at 99°C for 5 min. The RT reactions were then diluted to 100 μ l in water. In all, 1 μ l of stock cDNA template was used in subsequent PCRs.

Quantitative PCR Experiments

Quantitative PCR was performed using a LightCycler system (Roche Diagnostics) according to the manufacturer's instructions. Reactions were performed in 10 μ l volume with

0.3 μ M primers, 4 mM MgCl₂ and 1 μ l of LightCycler-FastStart DNA Master SYBR Green I mix. Protocol consisted of a hot start step (8 min at 95°C) followed by 40 cycles including a 10-s denaturation step (95°C), a 10-s annealing step and an elongation step at 72°C varying from 15 to 40 s. The primer sequences and PCR conditions for each cDNA were 5'-CAAA GGCTTCTTCTTGCTGGT-3' (forward) and 5'-AAGGGCT TCTTGCCGTGTC-3' (reverse) generating a 257-bp fragment with an annealing temperature (Ta) of 60°C for human and mouse *TNAP*; 5'-GTTCCAATATGATTCCACCC-3' (forward) and 5'-AGGGATGATGTTCTGGAGAG-3' (reverse) generating a 487-bp fragment with a Ta of 55°C for human *GAPDH*; 5'-TGGAAAGCTGTGGCGTGATG-3' (forward) and 5'-CCAAAGAGGTCCGCCGTACA-3' (reverse) generating a 180-bp fragment with a Ta of 60°C for mouse *GAPDH*; 5'-CGACCTGGAAGTCCAACACTAC-3' (forward) and 5'-AGCA ACATGTCCCTGATCTC-3' (reverse) generating a 289-bp fragment with a Ta of 62°C for human *RPLP0*. Quantification data represented the mean of duplicate conditions. Relative quantification analyses were performed by RelQuant 1.01 Software (Roche Diagnostics).

Analytical Methods

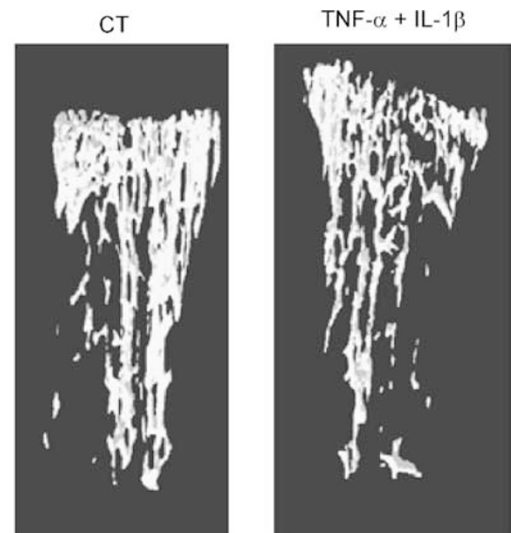
For determination of TNAP activity, cells cultured in 6-well plates were harvested in 0.2% Nonidet P-40 and disrupted by sonication. The homogenate was centrifuged at 1500g for 5 min, and TNAP activity in the supernatant was determined by the method of Lowry as previously described.²¹ In the same lysates, the protein content was determined with an assay from Bio-Rad. Results are shown as nmol paranitrophenol (pNP)/min/mg protein.

Statistical Analysis

All experiments were performed in triplicates and repeated at least twice with each cell type. Results are expressed as mean \pm s.e.m. For statistical analysis, a Mann-Whitney test was used. A difference between experimental groups was considered to be significant when $P < 0.05$.

RESULTS

To assess the relevance of our *ex vivo* culture model, we first determined with micro-CT the effects of cytokines on tibia bone parameters. Micro-CT analysis showed that cultures treated with TNF- α and IL-1 β had a significantly decreased trabecular BV/TV ratio, associated with a significantly lower number of trabeculae (Figure 1). These results confirmed the well-acknowledged effect of TNF- α and IL-1 β in inducing bone loss, and therefore indicated that our organotypic cultures were relevant in mimicking the catabolic effect of cytokines. Moreover, these results indicated that cytokines were able to induce significant changes in bone architectural parameters in a 5-day culture period and thus suggested that this period might be long enough for cytokines to modulate ossification from entheses.



BV/TV (%)	17.15 \pm 1.22	11.61 \pm 1.19 #
Tb Th (mm)	64.80 \pm 2.40	58.63 \pm 3.44
Tb Nb (/mm)	2.63 \pm 0.15	1.93 \pm 0.22 #
Tb Sp (mm)	184.64 \pm 3.58	193.92 \pm 10.40

Figure 1 TNF- α and IL-1 β induce bone loss in organotypic cultures of whole mouse joints. Micro-CT determination of bone trabecular architectural parameters after treatment for 5 days with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β as compared with untreated cultures; # $P < 0.05$ as compared with untreated bones.

We harvested and cultured the tibias leaving the Achilles tendon insertion into the calcaneus as intact as possible (Figure 2a). We then performed alkaline phosphatase staining (Figure 2b) and von kossa staining (Figure 2c). Interestingly enough, in both treated and untreated organ cultures, TNAP activity was much stronger in enthesial chondrocyte-like cells than in osteoblasts from the neighboring calcaneus (Figure 2b). Moreover, the fact that in entheses, TNAP activity was localized at the boundary between mineralized and non-mineralized tendon (Figure 2b and c) strongly suggests that it was involved in the ongoing mineralization. Furthermore, Figure 2c reveals that bone formation from the calcaneus into the Achilles tendon was preceded by a phase of tendon fibro-cartilage mineralization.

Concerning the effects of cytokines on enthesial chondrocytes, we observed that TNF- α and IL-1 β reduced alkaline phosphatase staining in organotypic culture (Figure 2b). To obtain quantitative data on this inhibitory effect, we first performed quantitative PCR experiments in mouse chondrocytes. We used the mouse ATDC5 chondrocyte cell line, which recapitulates the successive phases of chondrocyte differentiation,²⁶ and observed that TNF- α and IL-1 β strongly decreased the levels of *TNAP* transcripts in these cells (Figure 3). We then aimed at determining whether this inhibitory effect on TNAP expression and activity is

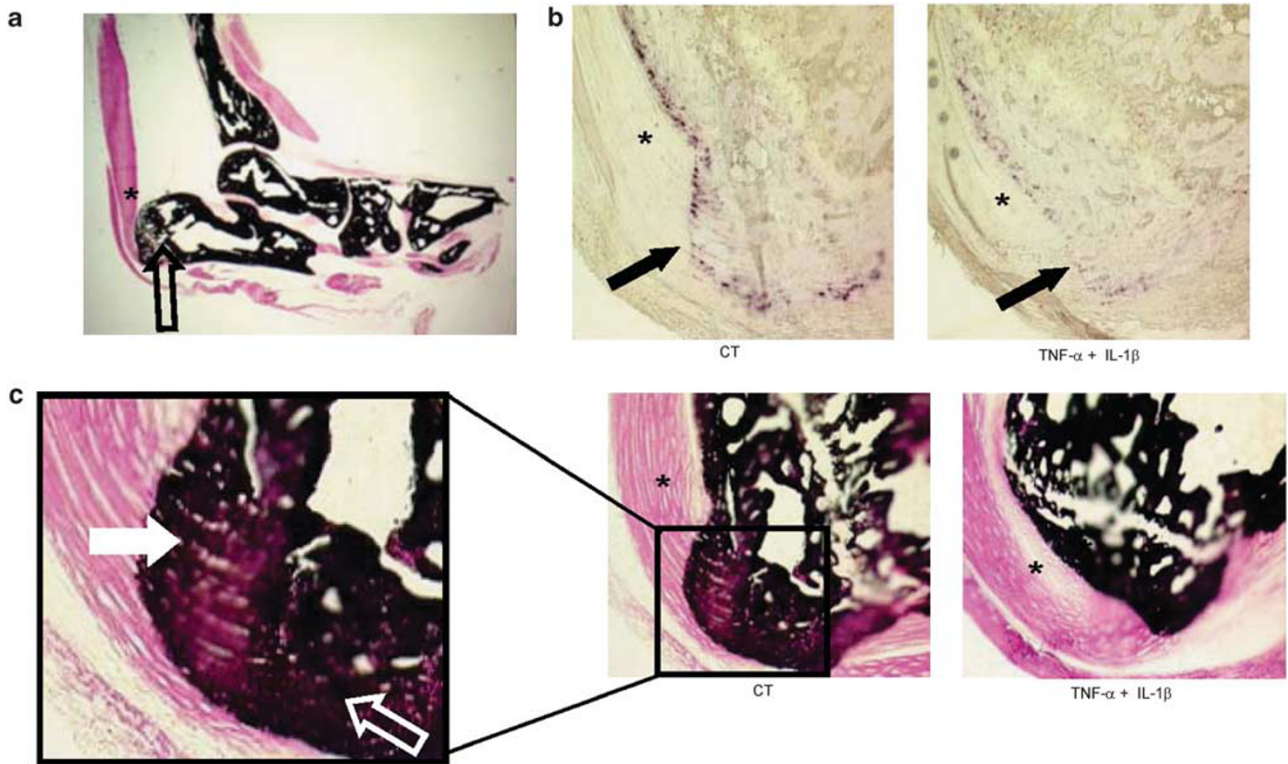


Figure 2 TNF- α and IL-1 β inhibit TNAP activity in mouse entheses *in situ*. (a) Von kossa staining of an ankle joint section after 5 days of culture; * indicates the Achilles tendon and the empty black arrow shows the calcaneus bone. (b) Staining of alkaline phosphatase activity in ankle joint sections after treatment with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β as compared with untreated cultures. * indicates the Achilles tendon and the black arrows show the alkaline phosphatase activity in enthesal chondrocytes. (c) Von kossa staining of ankle joint sections after treatment with 1 ng/ml of TNF- α and 0.1 ng/ml of IL-1 β as compared with untreated cultures. On all samples examined, a zone of calcified tendon was clearly noticeable (white arrow) in contact with the mineralized calcaneus bone (empty white arrow).

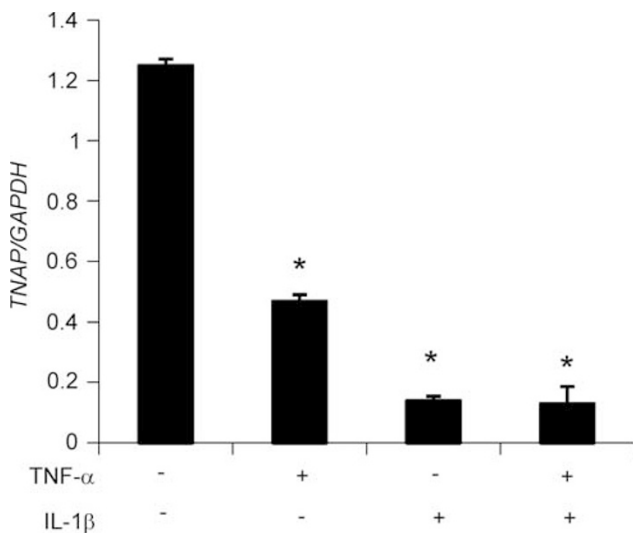


Figure 3 TNF- α and IL-1 β decrease the levels of TNAP transcripts in ATDC5 chondrocytes. In ATDC5 cells, TNF- α at 1 ng/ml and/or IL-1 β at 0.1 ng/ml decreased the levels of TNAP after 48 h of treatment, as measured by quantitative PCR. * P < 0.05 as compared with untreated cells.

restricted to mouse chondrocytes or can be extended to human chondrocytes. To address this issue, we treated chondrocytes differentiated in pellets from human MSCs with TNF- α and IL-1 β for 2 days to measure TNAP mRNA levels, and for 7 days to stain alkaline phosphatase activity. Figure 4 clearly indicates that, like in mouse chondrocytes, TNF- α and IL-1 β also dramatically inhibited TNAP expression (Figure 4a) and activity (Figure 4b) in human chondrocytes. In conclusion, and since TNAP activity is absolutely required for mineralization,²⁸ the fact that TNF- α and IL-1 β decreased TNAP expression and activity in both human and mouse chondrocytes indicates that these cytokines may inhibit enthesal calcification.

The data presented above indicate that TNF- α and IL-1 β decrease TNAP expression and activity in chondrocytes and enthesal chondrocyte-like cells, and therefore might exert inhibitory effects on ectopic bone formation during AS. This is in marked contrast with the published effects of TNF- α on TNAP expression and activity in VSMCs^{22,23} and on vascular calcification in diabetic mice.²⁴ In this context, we aimed at deciphering the mechanisms responsible for the different

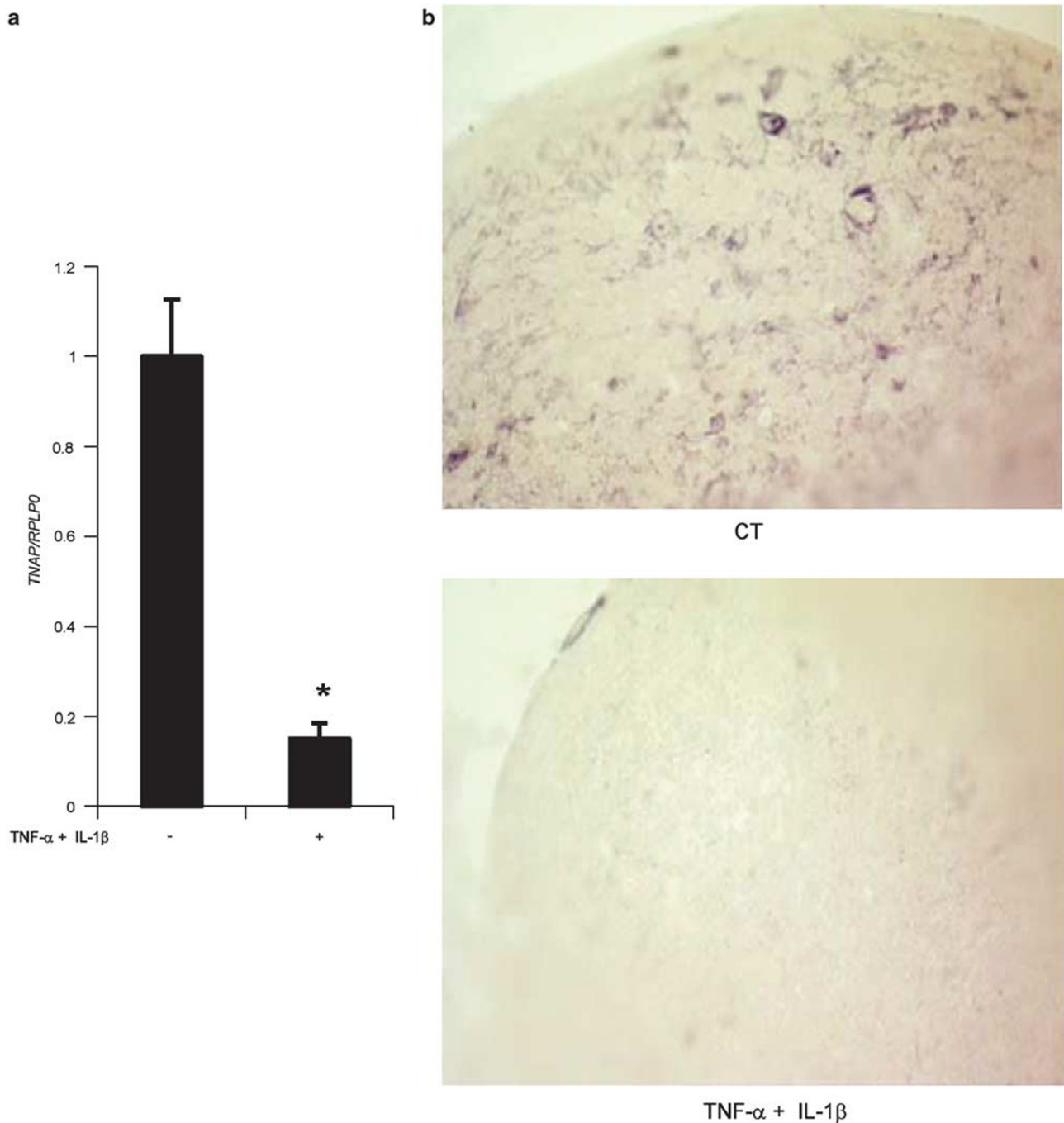


Figure 4 TNF- α and IL-1 β inhibit TNAP in human MSC-derived chondrocytes. In human chondrocytes cultured in pellets as detailed in 'Materials and methods', TNF- α at 1 ng/ml and IL-1 β at 0.1 ng/ml decreased the levels of *TNAP* transcripts after 48 h of treatment, as measured by quantitative PCR (a), and reduce TNAP activity after 7 days of treatment, as revealed by staining of alkaline phosphatase activity of pellet sections (b). * $P < 0.05$ as compared with untreated cells.

effect of TNF- α on TNAP in chondrocytes and VSMCs. We first confirm that TNF- α stimulates both TNAP expression and activity in human VSMCs. Indeed, quantitative PCR experiments revealed a two-fold increase in *TNAP* mRNA levels after a 48-h treatment (Figure 5a). This increase in the levels of *TNAP* transcripts in VSMCs led to a significant

stimulation of TNAP activity measured after 7 days of treatment (Figure 5b).

We then investigated the molecular mechanisms responsible for these intriguing opposite effects of TNF- α in chondrocytes and VSMCs. We focused our efforts on the likely involvement of peroxisome proliferator-activated

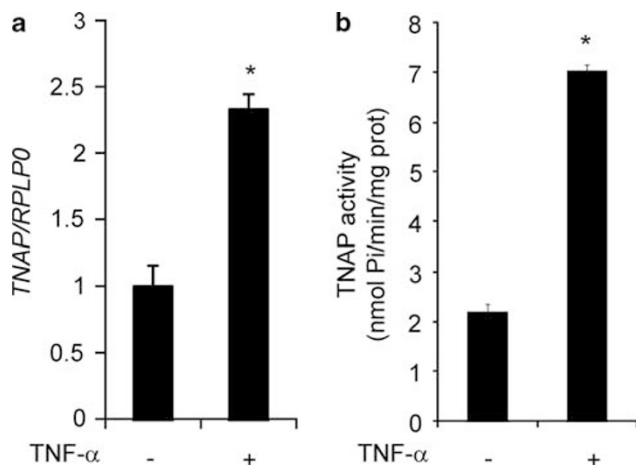


Figure 5 TNF- α stimulates TNAP in human VSMCs. In human VSMCs cultured as detailed in 'Materials and methods', TNF- α at 1 ng/ml increased the levels of TNAP at 48 h, as measured by quantitative PCR (a) and TNAP activity after 7 days, as measured by the method of Lowry (b). * $P < 0.05$ as compared with untreated cells.

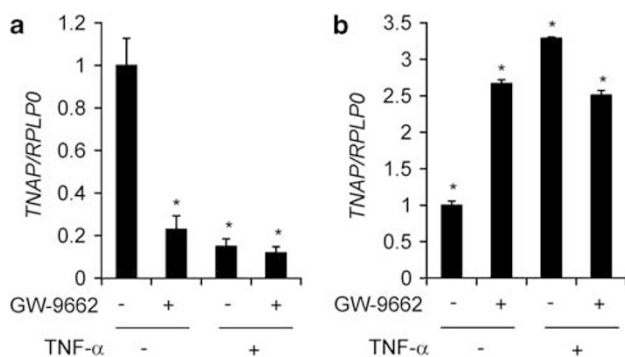


Figure 6 PPAR γ inhibition results in a decrease in TNAP levels in human chondrocytes, but to a stimulation in human VSMCs. (a) In human chondrocytes differentiated in pellets from MSCs, TNF- α at 1 ng/ml and GW-9662 at 100 μ M decreased TNAP levels after 48 h as measured by quantitative PCR. (b) In VSMCs, TNF- α at 1 ng/ml and GW-9662 at 100 μ M stimulated TNAP levels after 48 h as measured by quantitative PCR. * $P < 0.05$ as compared with untreated cells.

receptor γ (PPAR γ). Indeed, we recently observed that TNF- α increases the levels of TNAP transcripts through inhibition of PPAR γ expression and activity in human osteoblasts.²⁹ Moreover, TNF- α likely inhibits PPAR γ activity in each cell type expressing PPAR γ , including human VSMCs and chondrocytes.³⁰ Furthermore, in VSMCs PPAR γ has already been shown to inhibit TNAP expression.³¹ To decipher whether PPAR γ modulates TNAP expression in human VSMCs and chondrocytes, we used the selective inhibitor GW-9662 at 100 μ M. Indeed, our recent study showed that this concentration was as potent as 1 ng/ml TNF- α in inhibiting PPAR γ activity and stimulating TNAP expression.²⁹ Results shown in Figure 6 indicate that GW-9662 mimicked the effects of TNF- α on TNAP expression in both VSMCs and chondrocytes. In human chondrocytes, both TNF- α and

GW-9662 dramatically inhibited the levels of TNAP transcripts (Figure 6a), whereas in human VSMCs, they strongly increased these levels (Figure 6b). These results indicate that PPAR γ inhibition increases TNAP mRNA levels in human VSMCs but has the opposite effects in human chondrocytes.

DISCUSSION

In the present study, we found that TNF- α and IL-1 β inhibited alkaline phosphatase activity in mouse entheses *in situ*, as well as in mouse and human chondrocytes. Since TNAP activity is required for collagen mineralization,^{20,28} these results reveal that the direct effect of cytokines on enthesal fibro-cartilage mineralization is inhibitory. Together with the acknowledged negative effect of both cytokines on osteoblast differentiation and bone formation,^{32,33} our study indicates that TNF- α and IL-1 β are potent direct inhibitors of syndesmophyte formation in AS.

This inhibitory effect may help explain why anti-TNF- α therapies are unsuccessful to block the progression of bone spurs both in patients with AS,^{7,10} and in the DBA/1 model of spondyloarthropathy,³ whereas they improve symptoms in AS patients.^{7,10} Regarding more precisely the time course of syndesmophyte formation in AS patients, our results seem to suggest that early in the disease cytokines may indirectly stimulate syndesmophyte formation, but afterwards new bone formation is more likely to occur in sites where inflammation has resolved. Interestingly enough, it has been shown that new syndesmophytes occur mainly at sites where active inflammation has completely resolved, but rarely at sites with persistent inflammation.⁹ With regard to therapeutic considerations, our data fit well with the hypothesis of Schett and Rudwaleit¹⁰ who proposed that a rapid and persistent control of inflammation in the early phase of disease could prevent structural damage. Such a strategy needs, however, to be started before the molecular switch to an anabolic bone response has occurred.

Nonetheless, several questions remain unanswered. The mechanisms through which inflammation stimulates spur formation in patients with AS likely represent the most important one. In this regard, recently published data suggest that cytokines may stimulate new bone formation indirectly through several bone anabolic factors, such as canonical Wnts. Canonical Wnts are indeed potent stimulators of bone formation, and expression of Dkk-1, an inhibitor of canonical Wnt signaling, may be decreased in patients with AS.³⁴ More importantly, blockade of Dkk-1 in a mouse model of rheumatoid arthritis has been shown to induce new bone formation from joints, highlighting a possible initiating role for canonical Wnts.³⁴ Bone morphogenetic proteins (BMPs) also represent attractive candidates that may induce new bone formation in AS. BMPs were originally identified as factors that can induce new bone formation when ectopically implanted,³⁵ and their roles in cartilage and bone development have been studied extensively.³⁶ Several studies have shown that TNF- α and/or IL-1 β stimulate the expression of

BMP-2 by chondrocytes,^{37,38} suggesting that enthesal inflammation in AS may induce the local secretion of BMP-2, leading to syndesmophyte formation. The putative interest of targeting BMPs in AS has been suggested by Lories and collaborators, who showed that overexpression of noggin, a BMP antagonist with broad BMP ligand interaction, inhibits both the onset and severity of disease in the DBA/1 model of spondyloarthritis, in a preventive and therapeutic strategy.³⁹

Interestingly enough, our histological studies revealed the presence of calcified fibro-cartilage surrounding the characteristic fibro-chondrocyte rows (Figure 2c), which therefore demonstrates that mineralization of a pre-existing ECM is an early event in the bone formation proceeding from the calcaneus into the Achilles tendon. Consequently, TNAP expression by enthesal cells is likely a key event that initiates ossification (Figure 2).²⁰ In this regard, TNAP likely represents a very interesting therapeutic target in the treatment of ossification in patients with AS, as it has also been proposed in the treatment of vascular calcification.^{25,40} Moreover, the fact that an intense staining was observed in calcifying entheses while only a faint one could be noticed in osteoblasts in the neighboring calcaneus indicates that inhibition of TNAP may have minor undesirable effects on bone formation (Figure 2). Nevertheless, selective inhibition of bony overgrowth and ankylosis without interfering with physiological bone formation obviously appears preferable. To achieve this goal, the key hurdle to overcome is to dissect the molecular mechanisms leading to excessive bone formation, and first of all, to determine the effects of pro-inflammatory cytokines such as TNF- α and IL-1 β in this process. In this regard, the identification of the molecular factors involved downstream of cytokine receptors, such as PPAR γ , appears important.

Finally, our results suggest that inflammatory cytokines may display distinct effects on ectopic bone formation depending on the site. Indeed, whereas TNF- α and IL-1 β may be direct inhibitors of ectopic bone formation in patients with AS, TNF- α is strongly suspected to be a direct activator of vascular calcification that develops in association with diabetes mellitus,²⁴ and probably also in the context of atherosclerosis.^{41,42} Indeed, several independent groups have shown that TNF- α stimulates the expression and activity of TNAP in VSMCs.^{22–24} Since TNAP overexpression in mouse under the control of the $\alpha 2\text{coll1}$ promoter of the collagen type I induces vascular calcification,²⁰ we have recently proposed that TNF- α may induce vascular calcification by the mere stimulation of TNAP.²⁵ In the present study, we first confirm that TNF- α stimulates TNAP expression and activity in human VSMCs,^{22–24} and further suggest that like in human osteoblasts,²⁹ TNF- α may act by inhibiting PPAR γ activity. This finding is consistent with the reported inhibitory effect of PPAR γ on alkaline phosphatase activity and mineralization in culture of bovine VSMCs.³¹ However, the reason why PPAR γ inhibition stimulates TNAP expression in human

VSMCs but decreases it in human chondrocytes remains obscure. Identification of the involved mechanisms is today under intense investigation.

In conclusion, the present study indicates that TNF- α and IL-1 β inhibit TNAP activity in mouse entheses *in situ* and in human and mouse chondrocytes *in vitro*, and therefore are likely potent direct inhibitors of new bone formation in AS. These data may therefore provide a molecular explanation to understand why anti-TNF therapies are unable to block new bone formation in AS. On the opposite, we confirm that TNF- α stimulates TNAP activity in human VSMCs and suggest that, like in human osteoblasts, this effect is mediated by PPAR γ inhibition.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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