Effects of interleukin-17A on osteogenic differentiation of isolated human mesenchymal stem cells

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Objectives: Rheumatoid arthritis (RA) is characterized by defective bone repair and excessive destruction and ankylosing spondylitis (AS) by increased ectopic bone formation with syndesmophytes. Since TNF-α and IL-17A are involved in both diseases, this study investigated their effects on the osteogenic differentiation of isolated human bone marrow-derived mesenchymal stem cells (hMSCs).

Methods: Differentiation of hMSCs into osteoblasts was induced in the presence or absence of IL-17A and/or TNF-α. Matrix mineralization (MM) was evaluated by alizarin red staining and alkaline phosphatase (ALP) activity. mRNA expression was measured by qRT-PCR for bone morphogenetic protein (BMP)-2 and Runx2, genes associated with osteogenesis, DKK-1, a negative regulator of osteogenesis, Schnurri-3 and receptor activator of nuclear factor kappa B ligand (RANKL), associated with the cross talk with osteoclasts, and TNF-α receptor type I and TNF-α receptor type II (TNFRII).

Results: TNF-α alone increased both MM and ALP activity. IL-17A alone increased ALP but not MM. Their combination was more potent. TNF-α alone increased BMP2 mRNA expression at 6 and 12 h. These levels decreased in combination with IL-17A at 6 h only. DKK-1 mRNA expression was inhibited by TNF-α and IL-17A either alone or combined. Supporting an imbalance toward osteoblastogenesis, RANKL expression was inhibited by TNF-α and IL-17A. However, TNF-α but not IL-17 alone decreased Runx2 mRNA expression at 6 h. In parallel, TNF-α but not IL-17 alone increased Schnurri-3 expression with a synergistic effect with their combination. This may be related to an increase of TNFRII overexpression.

Conclusion: IL-17 increased the effects of TNF-α on bone matrix formation by hMSCs. However, IL-17 decreased the TNF-α-induced BMP2 inhibition. Synergistic interactions between TNF-α and IL-17 were seen for RANKL inhibition and Schnurri-3 induction. Such increase of Schnurri-3 may in turn activate osteoclasts leading to bone destruction as in RA. Conversely, in the absence of osteoclasts, this could promote ectopic bone formation as in AS.

Keywords: mesenchymal stem cell, IL-17A, TNF-α, rheumatoid arthritis, ankylosing spondylitis

INTRODUCTION

Interleukin-17A (IL-17A) is a pro-inflammatory cytokine that contributes to the pathogenesis of several inflammatory diseases (1, 2). In rheumatoid arthritis (RA), there is excessive bone destruction and defective bone repair leading to massive joint damage, while in ankylosing spondylitis (AS) there is excessive but ectopic ossification leading to syndesmophytes combined with systemic bone loss (1, 3–5). Moreover, increased levels of IL-17 in both diseases have suggested its contribution to these bone defects (4). Furthermore, IL-17A is considered as a new target for treatment of both RA and AS, as previously shown for TNF-α inhibition (6–11). Moreover, we have previously found that addition of inhibitors of

IL-17A and TNF-α alone or even better in combination, decreases bone destruction in an *ex vivo* model of RA (12).

The mechanisms used by IL-17A to promote bone loss include activation of osteoclastogenesis, which occurs both directly and also through expression of receptor activator of nuclear factor kappa B ligand (RANKL) and M-CSF by stromal cells (4, 13). Moreover, IL-17A can induce target cells to produce pro-inflammatory cytokines such as IL-6, IL-1, CXCL8, TNF, and matrix metalloproteinases (2, 14–17). On the other hand, TNF-α inhibits osteoblastogenesis through increased expression of Dickkopf 1 (DKK-1) (18, 19) and induces bone loss by degradation of bone morphogenetic protein (BMP)-2 signaling via Smad ubiquitin regulatory factor (Smurf)1 and NF-kB (20–22). In contrast to their classical effects on bone loss, some studies have indicated that IL-17A and TNF-α possibly could increase osteogenesis (23–30). IL-17A can induce proliferation and differentiation of human mesenchymal stem cells (hMSCs) in a manner dependent

Abbreviations: AS, ankylosing spondylitis; BMP2, bone morphogenetic protein 2; DKK-1, dickkopf 1; MSC, mesenchymal stem cell; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor kappa B ligand; Runx2, runt-related transcription factor-2; Shn3, Schnurri-3.

on the generation of reactive oxygen species (ROS) (31). Moreover, IL-17A can significantly increased leptin production that inhibits adipogenesis and promotes osteogenesis on human bone marrow-derived mesenchymal stem cells (hMSCs) via JAK/STAT signaling (23). TNF-α can promote osteogenic differentiation through triggering NF-kB and enhancing the expression of BMP2 and RUNX2 (24, 25, 28).

Due to these conflicting results, our objective was to examine whether IL-17A alone and/or TNF-α, positively or negatively modulate osteogenic differentiation in hMSCs. To study these aspects, we focused on key genes involved in bone turnover: BMP2, Runx2, DKK-1, RANKL, and Schnurri-3, a gene recently associated with bone resorption in mice (32–34), but with a paucity of data on its role in the human context.

MATERIALS AND METHODS

CELL CULTURE, OSTEOGENIC INDUCTION, AND EXPERIMENTAL DESIGN

hMSCs were provided by the cell therapy department. They were obtained from residues of quality controls of bone marrow for transplantation harvested from adult donors after signing an informed consent. A fibroblast colony-forming unit (CFU-F) was used to optimize culture and expansion of hMSCs. Cells were cultured at 37°C in α-MEM (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS-Hyclone, Thermo scientific, Saint Aubin, France), 2 mM L-glutamine, 100 U/ml penicillin, streptomycin. Cells were used between passage 3 and 6 at which cells were >99% stained negative for CD34 and CD45 and positive for CD73 and CD90 (antibodies obtained from PharMingen). For osteogenic differentiation, hMSCs were plated at a density of 5×10^3 cells/cm^2 and cultured in stem Xvivo Osteogenic/adipogenic base Medium (R&D systems, Lille, France), supplemented with 100 nM dexamethasone (Sigma, saint Quentin-Fallavier, France), 10 mM β-glycerophosphate (Sigma), and 50 μM ascorbic acid (Sigma). hMSCs were differentiated for 21 days in the absence or presence of 1 ng/ml TNF-α (R&D systems, Lille, France) and/or 50 ng/ml IL-17A (R&D systems) (23, 24, 31). Half of the medium was changed every 3 days.

MINERALIZATION ASSAY

Cells were washed twice with PBS, fixed with 70% cooled ethanol for 1 h, and then washed with water. Cells were stained for 20 min at ambient temperature with alizarin red (pH: 4.2, 40 min, Sigma) and examined under light microscope. The red color obtained referred to calcium deposit.

ALKALINE PHOSPHATASE ASSAY

hMSCs seeded in 12-well plates were lysed with the assay buffer (Abcam, Paris, France). The protein contents in the lysates were determined using the Bradford protein assay (Sigma). Ten microliters from the remaining lysate was mixed with 20 μl of MUP, used as a substrate (Abcam) in a 96-well plate, and incubated at room temperature for 30 min. Fluorescence intensity was measured at extension/emission of 360/440 nm. The alkaline phosphatase (ALP) activity was normalized to protein content and expressed as unit per microgram protein.

QUANTITATIVE RT-PCR ANALYSIS

RNA was purified using RNeasy kits (Qiagen, Les Ulis, France). The concentration of RNA was quantified by spectrophotometry (SmartSpec™ 3000, Biorad, Hercules, CA, USA). Five hundred nanograms of total RNA was reverse transcribed with the Quanti Tec Reverse Transcription (Qiagen Kit) into cDNA. PCR amplification was performed on a Light Cycler (Roche Diagnostics, Switzerland) using Fast-Start™ DNA Master SYBR Green I real-time PCR kit (Roche Molecular Biochemicals, Switzerland). The expression of the genes was normalized to the expression of human cyclophilin B (CPB) (Qiagen; 5'tgtggtgtttggcaaagttc3'; 3'gtttatcccggctgtctgtc5'). The list of primers (Qiagen) is as follows: BMP2 (5'ccaccatgaagaatctttgga3'; 3'gagttggctgttgcaggttt5'), RUNX2 (5'gtggacgaggcaagagttt3'; 3'tggggtctgtaatctgactc5'), DKK-1 (5'ccttggatgggtattccaga3'; 3'tccatgagagccttttctcc5'), RANKL (5'accagcatcaaaatcccagg3'; 3'ccccaaagtatgttgcatcc5'), Shn 3 (5'ccctg agccataaccctgaa 3'; 3'gtaggacttggcgttggtgt 5'), TNF-α receptor type II (TNFRII) (5' ggtctccttgctgctgtttc3'; 3'ccggagattctcaaatccaa5'), and TNF-α receptor type I (TNFRI) (5' accaagtgccacaaaggaac 3'; 3'ctgcaattgaagcactggaa 5').

STATISTICAL ANALYSIS

Analysis was performed using a Wilcoxon test from Graphpad Prism. p-Values were determined for every analysis. p-Values <0.05 were considered significant.
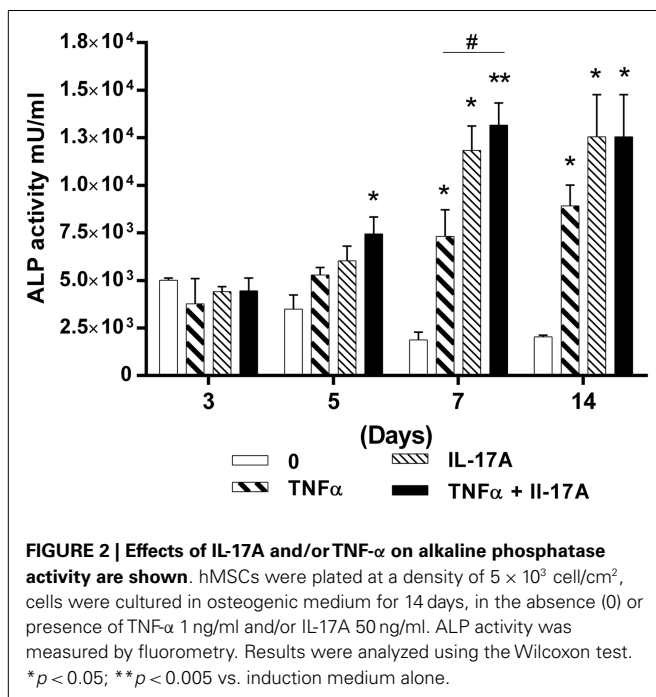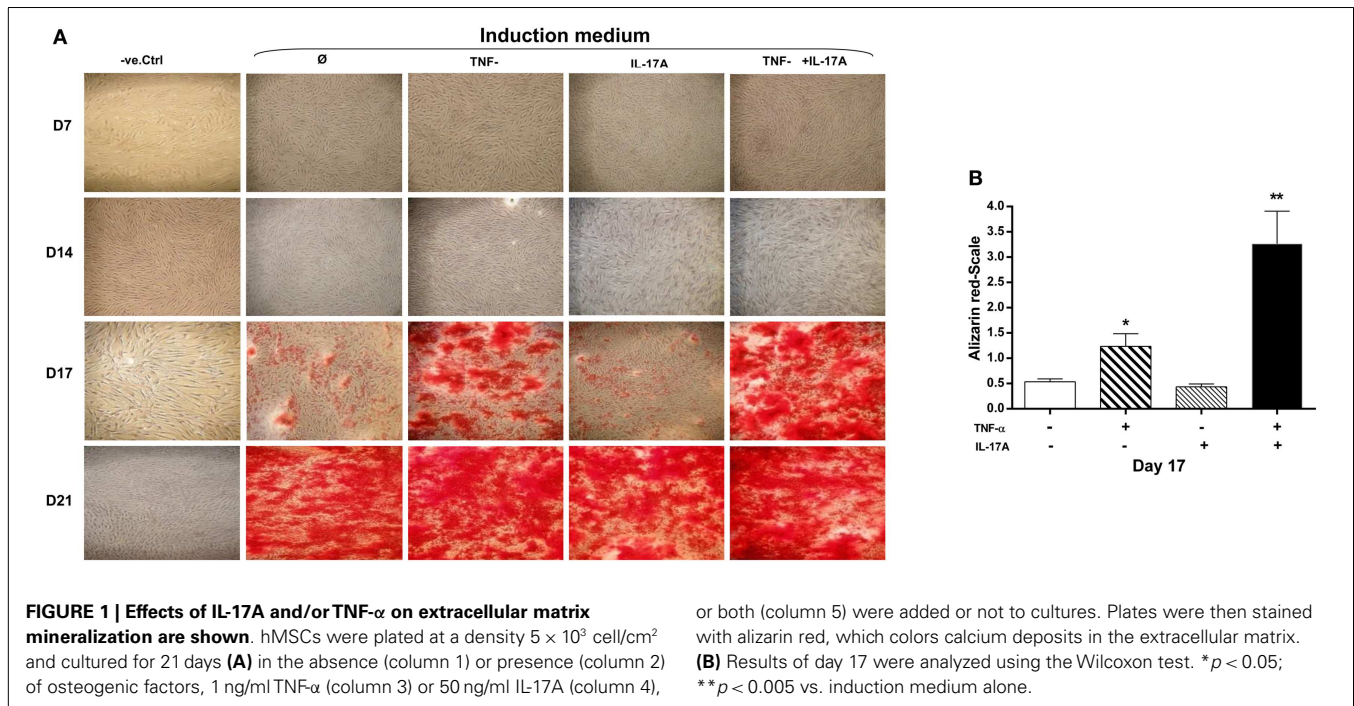
RESULTS

SYNERGISTIC EFFECTS OF IL-17A AND TNF-α ON EXTRACELLULAR MATRIX MINERALIZATION

A key characteristic of MSCs is their ability to differentiate into cells of different tissue lineages. Mineralization of the extracellular matrix is a marker of hMSCs differentiation into osteoblasts. To evaluate the effects of IL-17A and/or TNF-α on MM, hMSCs were cultured for 21 days in a medium supplemented with osteogenic factors with and without cytokines. Alizarin red staining was used to visualize mineralization. As shown in **Figure 1A**, column 2, culture of MSC with osteogenic factors alone induced a weak MM, which appeared at day 17 and reached its maximum level at day 21. Addition of IL-17A alone did not modify this mineralization (**Figure 1A**, column 3; **Figure 1B**). Addition of TNF-α enhanced this mineralization at day 17 (**Figure 1A**, column 4; **Figure 1B**, *$p < 0.05$), which was further enhanced in the presence of the two cytokines (**Figure 1A**, column 5; **Figure 1B**, **$p < 0.005$). At day 21, maximum levels were observed in each condition except in the negative control. Thus, these results show that TNF-α but not IL-17A alone enhanced bone mineralization, which was further potentiated but without acceleration by IL-17A.

SYNERGISTIC EFFECTS OF IL-17A AND TNF-α ON ALKALINE PHOSPHATASE ACTIVITY

Since TNF-α and IL-17A increased mineralization of the extracellular matrix, we next investigated their effects on ALP, an enzyme, which is essential for bone mineralization. ALP activity was measured at days 3, 5, 7, and 14 (**Figure 2**). At day 3, no difference in the presence or absence of cytokine was detected. Increased levels of ALP activity were detected at day 5 when the two cytokines were combined (4.4×10^3 U/ml at day 3 vs. 7.4×10^3 U/ml at

FIGURE 1 | Effects of IL-17A and/or TNF-α on extracellular matrix mineralization are shown. hMSCs were plated at a density 5×10^3 cell/cm^2 and cultured for 21 days **(A)** in the absence (column 1) or presence (column 2) of osteogenic factors, 1 ng/ml TNF-α (column 3) or 50 ng/ml IL-17A (column 4), or both (column 5) were added or not to cultures. Plates were then stained with alizarin red, which colors calcium deposits in the extracellular matrix. **(B)** Results of day 17 were analyzed using the Wilcoxon test. $^*p < 0.05$; $^{**}p < 0.005$ vs. induction medium alone.

FIGURE 2 | Effects of IL-17A and/or TNF-α on alkaline phosphatase activity are shown. hMSCs were plated at a density of 5×10^3 cell/cm^2, cells were cultured in osteogenic medium for 14 days, in the absence (0) or presence of TNF-α 1 ng/ml and/or IL-17A 50 ng/ml. ALP activity was measured by fluorometry. Results were analyzed using the Wilcoxon test. $^*p < 0.05$; $^{**}p < 0.005$ vs. induction medium alone.

day 5, $^*p < 0.05$) or at day 7 in the presence of each cytokine alone (**Figure 2**). Moreover, the combination of the two cytokines resulted in a further increase of ALP activity, the maximum level being achieved at day 7 (1.8×10^3 U/ml without cytokine vs. 1.3×10^4 U/ml with both cytokines, $^{**}p < 0.005$). Day 14 shows almost the same results as day 7.

Therefore, these results support those of the alizarin red staining, by showing that the combined action of IL-17A and

TNF-α resulted in synergistically increasing and accelerating osteoblast differentiation from hMSCs. However, ALP activity appeared more sensitive to the effects of IL-17A alone.

TNF-α BUT NOT IL-17A ALONE INCREASES THE EXPRESSION LEVELS OF BMP2 mRNA

To better understand the effects of IL-17A and TNF-α on the differentiation of hMSCs, mRNA expression levels of BMP2 were measured at 6, 12, 24, and 72 h. BMP2 plays an important role in the development of bone and cartilage and induces osteoblast differentiation in a variety of cell types (35). BMP2 mRNA expression was increased at 6 h with TNF-α alone (16-fold with TNF-α as compared with control without cytokine, $^{**}p < 0.05$), with a kinetic curve demonstrating a decrease upon time (**Figure 3**). IL-17A alone did not change BMP2 mRNA expression levels. The combination of the two cytokines resulted in a significant decrease of BMP2 mRNA expression as compared with the effects of TNF-α alone at 6 h (16-fold with TNF-α vs. 9-fold with TNF-α + IL-17A, $^{\#}p < 0.05$). This inhibitory effect was not seen at 12 h (5.0-fold TNF-α vs. 5.4-fold IL-17A + TNF-α, NS). Overall, the combination of these two cytokines resulted in a significant increase of BMP2 mRNA expression as compared with controls (ninefold with TNF-α + IL-17A vs. control, $^*p < 0.05$, at 6 h, and 5.4-fold at 12 h). Therefore, these results showed that TNF-α increased the expression of BMP2 mRNA levels, an effect, which was inhibited by IL-17A.

IL-17A AND TNF-α INCREASE THE EXPRESSION LEVEL OF Shn3 mRNA BUT NOT THOSE OF Runx2

Schnurri-3 (Shn3) is a zinc finger protein, which plays a key regulatory role in skeletal remodeling in mouse (36). In hMSCs, Shn3-mRNA expression levels increased significantly only at early 6 h

in the presence of TNF-α alone (twofold with TNF-α, $*p < 0.05$) with a kinetic curve demonstrating a decrease upon time. IL-17A alone did not change Shn3-mRNA expression levels (**Figure 4A**). The combination of the two cytokines resulted in a significant increase of Shn3-mRNA expression as compared with the effects of TNF-α alone at 6 h (fivefold with IL-17A + TNF-α vs. twofold with TNF-α, $^{\#\#}p < 0.005$). No significant change of Shn3-mRNA expression levels was observed in the presence of either one or two cytokines between 12 and 24 h.

Runx2 is a key transcription factor that has been associated with osteogenesis (37). However, its mRNA expression levels showed no increase but rather unexpectedly a significant decrease at 6 h in the

FIGURE 3 | Effects of IL-17A and TNF-α on BMP2 are shown. hMSCs were cultured in osteogenic medium in the presence or absence of TNF-α 1 ng/ml and/or IL-17A 50 ng/ml. Osteogenic gene expression of BMP2 was measured by qRT-PCR at early time points of (6, 12, 24, and 72 h). Results were analyzed using the Wilcoxon test. $*p < 0.05$; $**p < 0.005$ vs. induction medium alone (0), $^{\#}p < 0.05$ TNF-α alone vs. IL-17A + TNF-α.

presence of TNF-α alone or combined to IL-17A as compared with control (0.5-fold with TNF-α or IL-17A+, TNF-α $*P < 0.05$). No significant change of Runx2 mRNA expression levels was observed in the presence of either one or two cytokines between 12 and 72 h (**Figure 4B**).
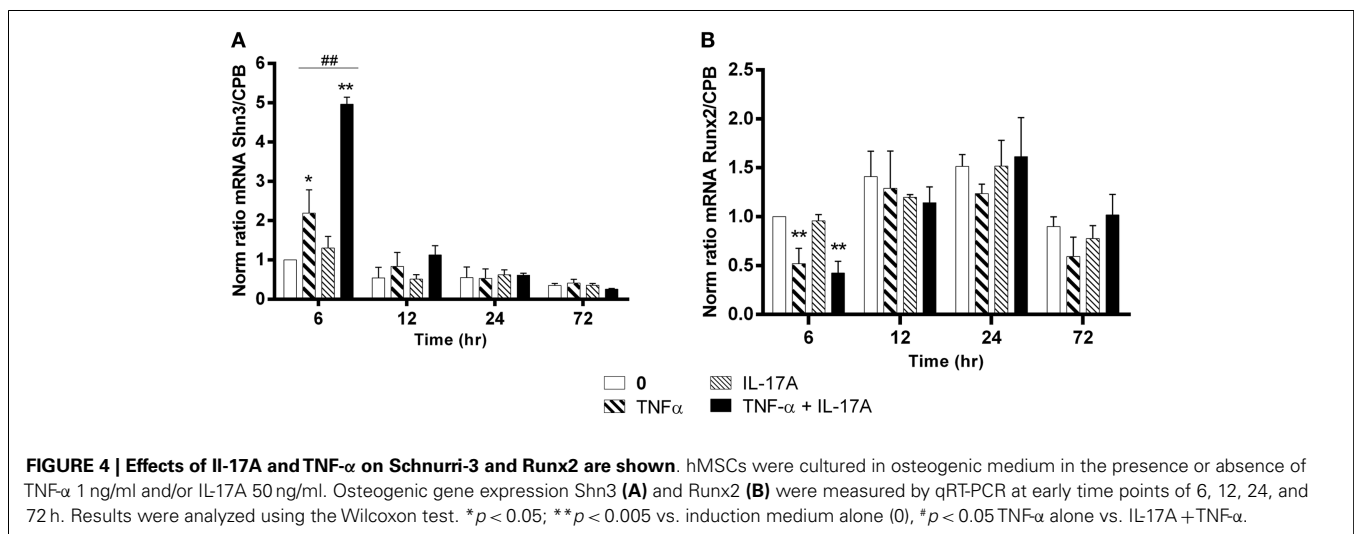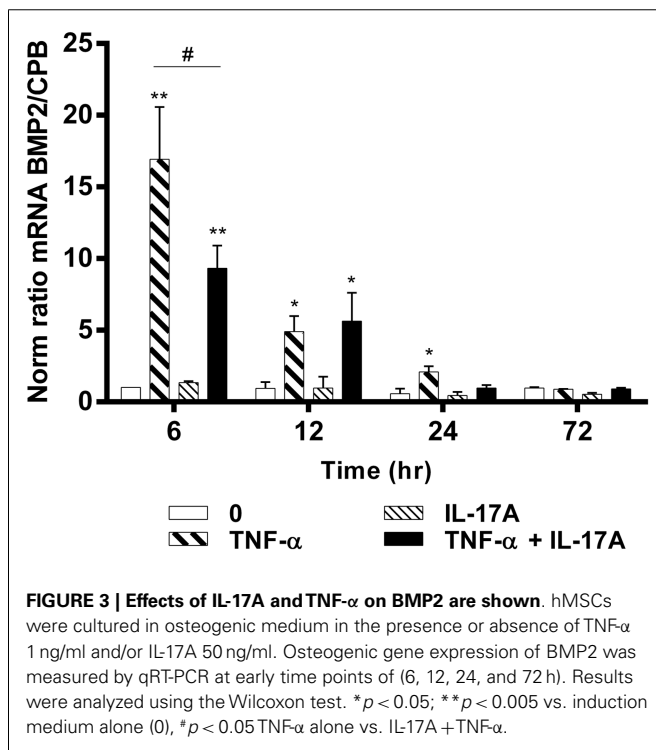
Therefore, TNF-α alone and even more in combination with IL-17A increased Shn3-mRNA levels and decreased those of RUNX2 at early time points, while IL-17A alone showed no effects.
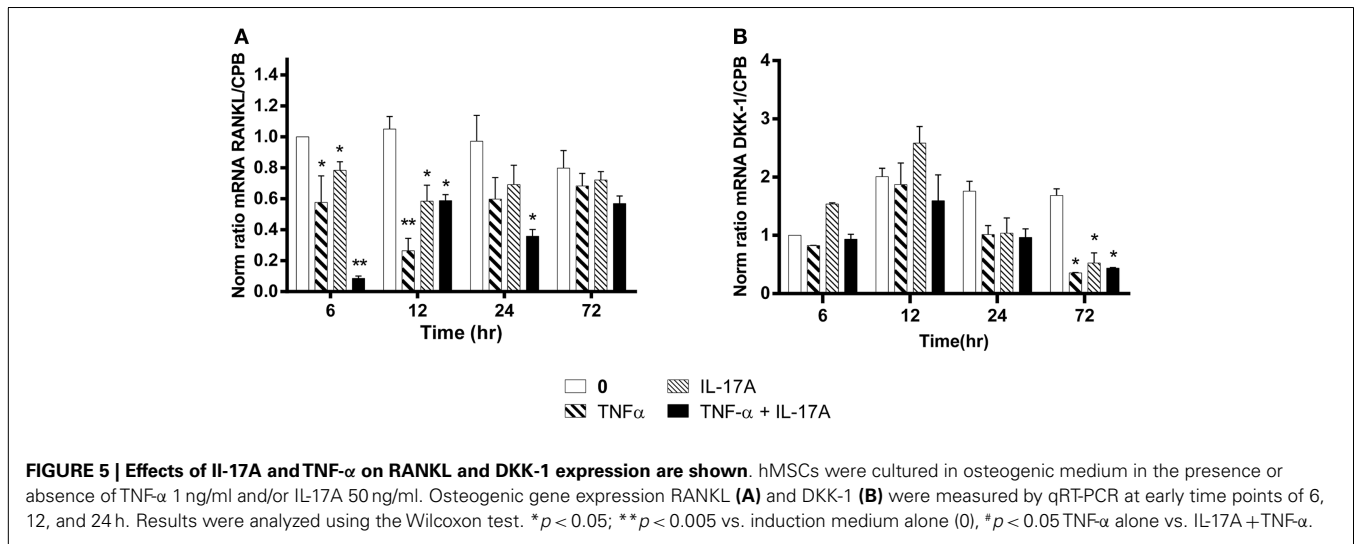
IL-17A AND TNF-α DECREASE RANKL AND DKK-1 mRNA EXPRESSION LEVELS

The RANKL produced by osteoblasts plays a key role in osteoclast differentiation and activation (38). RANKL mRNA expression levels without cytokines remained stable over time, i.e., from 6 to 72 h (**Figure 5A**). In contrast, RANKL mRNA levels were significantly reduced as early as 6 h, when either IL-17A or TNF-α added alone (0.6-fold with TNF-α, 0.8-fold with IL-17A vs. 1-fold without cytokine, $*p < 0.05$). The combined action of the two cytokines resulted in a more profound decrease of RANKL mRNA levels (0.1-fold with IL-17A combined to TNF-α vs. 1-fold without cytokine, $**p < 0.005$). Moreover, this decrease was sustained upon time, since it was still observed at 24 h (0.35-fold with TNF-α combined to IL-17A vs. 1-fold without cytokine, $*p < 0.05$). This result shows that these two cytokines might enhance osteogenesis by reducing RANKL expression.

Among the several molecules that negatively regulate Wnt signaling, DKK-1 prevents the activation of the Wnt signaling pathway (39). Upon differentiation of MSC into osteoblasts, DKK-1 mRNA expression levels without cytokines remained stable over time, i.e., from 12 to 72 h. In contrast, DKK-1 mRNA levels were significantly reduced at 72 h, when either IL-17A or TNF-α or both were added (0.3-fold with TNF-α, IL-17A, alone or combined vs. 1.6-fold without cytokine, $*p < 0.05$) (**Figure 5B**). This suggests that these two cytokines might enhance osteogenesis by overcoming the negative modulation mediated by DKK-1.

Therefore, these results show a combined action of TNF-α and IL-17A on hMSCs increased osteogenesis through an inhibition of DKK-1 and RANKL gene expression.
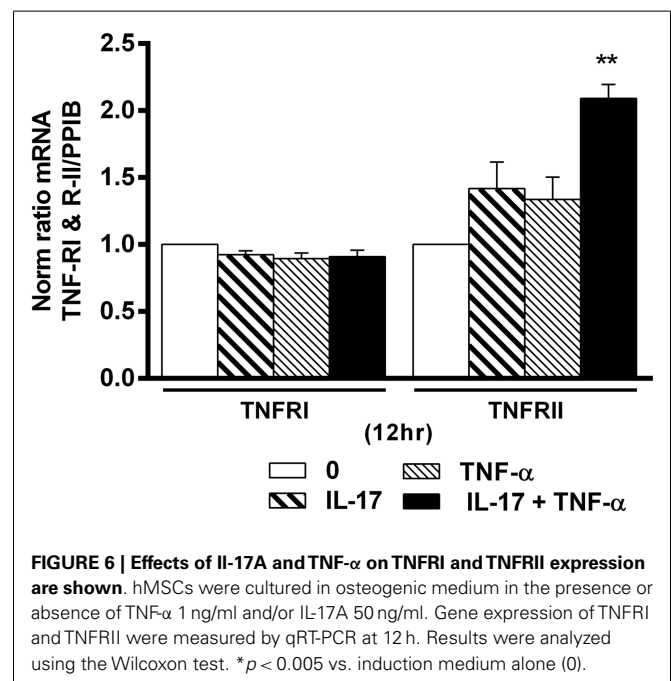

FIGURE 4 | Effects of Il-17A and TNF-α on Schnurri-3 and Runx2 are shown. hMSCs were cultured in osteogenic medium in the presence or absence of TNF-α 1 ng/ml and/or IL-17A 50 ng/ml. Osteogenic gene expression Shn3 **(A)** and Runx2 **(B)** were measured by qRT-PCR at early time points of 6, 12, 24, and 72 h. Results were analyzed using the Wilcoxon test. $*p < 0.05$; $**p < 0.005$ vs. induction medium alone (0), $^{\#}p < 0.05$ TNF-α alone vs. IL-17A + TNF-α.

FIGURE 5 | Effects of Il-17A and TNF-α on RANKL and DKK-1 expression are shown. hMSCs were cultured in osteogenic medium in the presence or absence of TNF-α 1 ng/ml and/or IL-17A 50 ng/ml. Osteogenic gene expression RANKL **(A)** and DKK-1 **(B)** were measured by qRT-PCR at early time points of 6, 12, and 24 h. Results were analyzed using the Wilcoxon test. $*p < 0.05$; $**p < 0.005$ vs. induction medium alone (0), $#p < 0.05$ TNF-α alone vs. IL-17A + TNF-α.

COMBINATION OF IL-17A AND TNF-α INCREASE TNFRII BUT NOT TNFRI EXPRESSION

TNF-α receptor type I and TNFRII play an important role in cell proliferation, survival, and death (40, 41). Since IL-17 increased the effects of TNF-α in hMSCs, we looked at a possible effect on TNF-R expression. Addition of IL-17A and TNF-α alone or in combination had no effect on TNFRI mRNA expression levels (**Figure 6**). On the other hand, IL-17A and TNF-α alone had a very modest effect on TNFRII mRNA expression levels, but the combination induced a clear increase (2.0-fold with IL-17A + TNFα vs. 1-fold without cytokine at 12 h ($*p < 0.005$). Therefore, these results show that the combined action of TNF-α and IL-17A on hMSCs may result from TNFRII but not TNFRI overexpression.

DISCUSSION

The goal of this study was to evaluate the effects of cytokines on the differentiation of human MSCs into bone forming cells, focusing on TNFα classical target for treatment and on IL-17, an emerging one, these two cytokines being known to interact. The first conclusion is the effect of TNF-α to promote osteogenic differentiation of hMSCs by increased deposition of calcium into the extracellular matrix, enhanced ALP activity, enhanced expression BMP2 mRNA levels, and decreased expression of RANKL and DKK-1 mRNA levels with an increase of Schnurri-3. As it is often the case, the effects of IL-17A alone were absent or limited in similar assays. The combination of TNF and IL-17 showed complex interactions with a net effect on enhanced matrix formation, ALP activity, and Schnurri-3 expression with reduced RANKL expression. A key surprising effect was the inhibition of the TNF-enhanced BMP2 expression when IL-17 was added.

Previous studies in human and murine systems have already shown an effect of IL-17A or TNF-α used alone on osteogenic induction (23–29, 31, 42). However, the classical view describes TNFα as an inhibitor of bone formation, more particularly with reference to RA. Among those studies, TNF-α has been reported to activate through TNF receptor-associated factor-2, a cascade of biochemical events involving NF-κB, AP-1, and MAPKs, leading to the activation of osteoclastic resorption and inhibition of

FIGURE 6 | Effects of Il-17A and TNF-α on TNFRI and TNFRII expression are shown. hMSCs were cultured in osteogenic medium in the presence or absence of TNF-α 1 ng/ml and/or IL-17A 50 ng/ml. Gene expression of TNFRI and TNFRII were measured by qRT-PCR at 12 h. Results were analyzed using the Wilcoxon test. $*p < 0.005$ vs. induction medium alone (0).

osteoblast proliferation and matrix synthesis (43–45). Like TNF-α, IL-17A also activates NF-κB and AP-1 but through TRAF protein (46, 47). IL-17A inhibitory effect on matrix production in chondrocytes and osteoblasts leads to joint damage. It activates the production and function of MMPs, and a combination of IL-17A to TNF leads to irreversible cartilage damage in a murine model (3). IL-17A seems to increase ALP activity during osteogenesis and this is in harmony with two previous studies in the human system (23, 31).

While TNF-α was shown to increase BMP2 expression in different cell types like chondrocytes, endothelial cells, and dental pulp cells (48–50), little is known about the effects of TNF-α on BMP2 expression in hMSCs during the process of osteogenic

differentiation. Here, TNF-α significantly increased BMP2 mRNA expression in hMSCs within 24 h of treatment (**Figure 3**) suggesting that TNF-α alone induces osteogenesis by increasing BMP2 expression. Much less is known on the effects of IL-17. IL-17A alone showed no effect. Instead of the expected potentiation with the combination with TNF, a reduction was observed at least at early time points.

Another factor involved in the regulation of osteogenesis is DKK-1, a major inhibitor of the Wnt signaling pathway (39). DKK-1 promotes internalization of the receptor complex and dampens the Wnt signal (51). This inhibition is potentiated in the presence of TNF-α (18, 19). However, our findings suggest that DKK-1 mRNA expression significantly decreased in the presence of TNF-α, which was associated with osteogenic induction. This discordance could be related to the use of MSCs, since opposite results have been obtained with synoviocytes (18). Moreover, serum levels of DKK-1 decreased following anti-TNFα therapy in RA patients (19) but not in AS patients (52). This suggests that the effects of TNFα on DKK-1 may not be direct, but may depend on factors associated with the disease status. Nevertheless, whatever the effect of TNF-α on DKK-1, we observed a strong correlation between the decrease of DKK-1 expression and the increase of bone mineralization. This observation is supported by Kowena et al. in a review on signaling (51).

Receptor activation of NF-κB ligand, a type II membrane protein of the TNF superfamily, is expressed in osteoblasts, osteocytes, and some activated T cells, specifically the Th17 cells (53, 54). RANKL is implicated in bone homeostasis, with TNFα known to induce its expression, and IL-17 having a modest effect. Additive effects of the combination TNFα + IL-17 on RANKL expression have been observed in human synoviocytes by us and others (55, 56). With hMSCs, opposite results were observed, since RANKL mRNA expression significantly decreased in the presence of IL-17A and/or TNF-α, which was associated with osteogenic induction.

Shn3 expression in mouse MSCs directly controls osteoblastic bone formation via Runx2, while indirectly regulating osteoclastic bone resorption (32, 33). Shn3-deficient mice show increased bone mass. However, there is a relative paucity of data on the role of Shn3 in the human context and its regulation with these cytokines has not been studied before. Our results showed that TNF-α alone or combined with IL-17A increased Shn3-mRNA expression levels at early time. Such increase would be a signal for osteoclast activation leading to bone destruction. This inter-cellular bridge would depend on whether osteoclasts are in close proximity with MSCs or osteoblasts. This difference could explain the different patterns of bone remodeling observed in RA and AS.

Since IL-17 increased the effects of TNF-α on hMSCs, we examined the possibility of an effect on TNF-R expression in order to explain the increased activity often observed with the combination of the two cytokines. TNFRI is ubiquitously expressed on nearly all cells but TNFRII has a more restricted expression, specifically on human lymphocytes (57–59) and MSCs (60, 61). Here, IL-17 and TNF alone or in combination had no effect on TNFRI expression. In contrast, TNFRII expression was regulated by the two cytokines in combination, with a modest not significant effect when used alone. These results are in line with those

previously observed with synoviocytes, where a regulatory effect on TNFRII was also observed following exposure to TNF and IL-17. In this later case, however, IL-17 alone was able to increased TNFRII expression (62). Our results show that TNF-α and IL-17A may increase induce osteogenesis via TNFRII overexpression.

Our finding that TNFα and IL-17A activate osteogenesis, with IL-17A potentiating the effect of TNFα, may explain the mechanisms of ligament ossification with ectopic bone formation as observed in AS. It has been shown that the molecular pathways underlying AS recapitulate the presence of endochondral bone formation, where BMPs play a key role (63). Similar to their role in artery plaque formation during atherosclerosis, type II diabetes, and aortic valve disease, inflammatory cytokines are strongly suspected to induce ectopic bone formation (64–66). However, the conditions are different for the local and systemic bone destruction that is observed in RA. Both *in vitro* and *in vivo* results with TNF inhibitors are in line with the net effect of TNF on bone loss. Furthermore, the combined inhibition of TNF-α, IL-1, and IL-17 was more effective than the inhibition of a single cytokine to control inflammation and bone resorption in an *ex vivo* RA model (12).

Application to RA and AS pathogenesis and clinical manifestations indicates a key difference. In RA, the combination of TNF-α and IL-17 induces bone loss from massive osteoclast activation in sites, where osteoblasts and osteoclasts interact locally such as in juxta-articular sites (12). In contrast, AS is characterized by the ectopic bone formation from MSC derived from tendons and ligaments. At this particular site, osteoclasts are not directly presented and activated and the combination of TNF-α and IL-17 may then lead to ectopic ossification (1). Massive bone loss in whole bone such as vertebra and femoral neck is observed in AS as in RA.

In summary, this is the first study demonstrating that the two pro-inflammatory cytokines IL-17A and TNF-α can interact to induce osteogenic differentiation of human MSCs. Through complex interactions, IL-17A can potentiate the effects of TNF-α. An important new finding in the human context is the effect on Schunnri3, where the induction by TNF-α is further increased by IL-17A in a synergistic fashion. Such effect would provide an activation signal to osteoclasts. A close proximity to osteoclasts would be seen in whole bone as in juxta-articular sites of RA or vertebra in RA and AS. In tendons and ligaments where osteoclasts are not present, the lack of inter-cellular connection would lead to osteogenesis. At the site of tendon insertion to bone, the destructive pathway would be seen leading to erosion.

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