BMP-2 and TGF-β1 inhibit the expression of the pro-inflammatory cytokine IL-34 in rheumatoid arthritis synovial fibroblasts

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Running head: BMP-2 and TGF-β inhibit IL-34 expression in RA

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Potential conflicts of interest: NO

Abstract

Interleukin-34 (IL-34) is a pro-inflammatory cytokine implicated in rheumatoid arthritis (RA). The present study aimed to assess the IL-34 expression in response to two members of the TGF-β family, TGF-β1 and BMP-2, in synovial fibroblasts from RA patients. IL-34, TGF-β1 and BMP-2 productions were measured in patient synovial fluids by ELISA. IL-34 mRNA levels were quantified by qPCR in human synovial fibroblasts and murine mesenchymal stem cells (mMSCs). Pharmacological inhibitions were used to determine the involvement of ALK1 and ALK5 downstream TGF-β1 and BMP-2. IL-34, TGF-β1 and BMP-2 were expressed in synovial fluids from RA patients. We found a significant correlation between IL-34 and TGF-β1 expressions. Levels of both IL-34 and TGF-β1 were thus correlated with the total leukocyte counts in the synovial fluids. TGF-β1 and BMP-2 decreased IL-34 expression in the synovial fibroblasts or in mMSC in a dose- and time-dependent manner through respectively ALK5 and ALK1 pathways. In addition, TGF-β1 antagonized TNFα-induced IL-34 gene expression. This work identifies TGF-β1 and BMP-2 as potent inhibitors of IL-34 expression in RA synovial fibroblasts. These cytokines, as upstream inhibitors of IL-34, may thus contribute to antagonize inflammation and bone erosions in RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by a chronic inflammation of the synovial tissue that leads to progressive joint destruction.[1] Among the cells located in the inflamed joint, synovial fibroblasts are crucial players driving inflammation and bone erosion.[2]

IL-34,[3] basically described as promoting monocyte proliferation and survival, and osteoclast differentiation,[4] is expressed by synovial fibroblasts of RA patients. Its expression, correlated with inflammation, the number of leucocytes and the severity of the synovitis, is upregulated by TNFα and IL-1β.[5]

TGF-βs and BMPs, are proteins involved in control of many biological processes such as cell proliferation and differentiation. The importance of TGF-β for the pathogenesis of arthritis is emphasized by several observations made in patients and in animal models.[6] TGF-β1 and its receptors TβRII were detected at high levels in the synovial fibroblasts of RA patients and in RA synovial fluids.[7-9] In this context, numerous studies have demonstrated the beneficial effects of TGF-β in RA.[6] TGF-β1 suppresses acute and chronic arthritis in experimental models, and a strong increase of TGF-β1 expression was measured in the remission state of disease.[10] Much less data exist regarding the role of BMP during the pathogenesis of RA. However, increasing evidences suggest that BMPs, known to play a crucial role in skeletal development, could play a major role in RA to restore function of synovial joints.[11] In this study, we hypothesised that IL-34 expression could be modulated by TGF-β1 and BMP-2.

Materials and Methods

Synovial fluid

Synovial fluids were obtained in the Rheumatologist Unit of Nantes University Hospital. The clinical characteristics of patients are summarized in Table 1. The study was approved by the local ethic committee and by the French Research Ministry (n° 2008-402). IL-34, BMP-2 and TGF-β levels were measured in synovial fluids by ELISA assay (antibodies-online-GmbH for IL-34, R&D System for BMP-2 and TGF-β1).

Cell cultures

Synovial fibroblasts, obtained from the synovial tissue of RA patients, and mouse C3H10T1/2 mesenchymal stem cells (MSCs), purchased from the ATCC (CCL-226), were cultured in alpha-MEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Perbio). TGF-β1 and BMP-2 were purchased from R&D System. ALK1 inhibitor (LDN 212854) and ALK5 inhibitor (SB431542) were purchased from Sigma.

Reverse transcription PCR analysis

RNA was extracted using the NucleoSpin RNAII kit (Macherey-Nagel). One microgram of total RNA was used for first strand cDNA synthesis using ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). DNase I treatment (25 units, 15 min) of total RNA was performed to eliminate genomic contamination.. Real-time PCR was performed with a Chromo4 instrument (Biorad) using SYBR Green Supermix reagents (Biorad). Primer sequences were described in Table 2.

Western blot

Western Blot analyses were performed as previously described.[4] Briefly, cells were lysed in a lysis buffer (SDS 1%, Tris pH 7.4 10mM, Sodium orthovanadate 1 mM) and protein concentration was determined by BCA kit (Sigma). Twenty micrograms of total protein in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) were separated by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Membranes were immunoblotted with anti phospho-Smad3 and Smad3 (Millipore), or phospho-Smad1/5 and Smad1/5 (Cell Signaling) antibodies.

Confocal microcopy experiments

Cell cultures, treated with TNF-α (10 ng/mL), and TGF-β1 (10 ng/mL) for 24h, were fixed and stained with the primary antibody against IL-34 (Diaclone, INSERM UMR 957) as previously described.[12]

Statistical analysis

The Spearman test was use to look for a correlation between IL-34 expression and the diagnosis. Kruskal Wallis test was used to assess the change in gene expression. An alpha level of 0.05 was chosen to assess statistical significance.

Results

Increased production of IL-34 and TGF-β1 in RA synovial fluids

The expression of IL-34, TGF-β1 and BMP-2 was detected in synovial fluids of RA patients (n=44, Figure 1A). IL-34 and TGF-β1 levels were associated with the inflammation intensity measured by the leukocyte counts (Figure 1B, respectively r=0.4, p=0.01 and r=0.36, p=0,02). A positive correlation between Il-34 and TGF-β levels in synovial fluids was found (Figure 1C, r=0.63, p<0.0001). No significant association was found between BMP-2 and IL-34 expression (Figure 1C), and between BMP-2 and the inflammation intensity measured by the leukocyte counts (Figure 1B).

TGF-β1 and BMP-2 decrease Il-34 expression in human RA synovial fibroblasts and mMSCs

The IL-34 mRNA steady-state levels following TGF-β1 or BMP-2 treatment were next investigated by quantitative RT-PCR. Both TGF-β1 (10 ng/ml) and BMP-2 (100 ng/ml) significantly decreased IL-34 mRNA steady state levels in synovial fibroblasts from three different RA patients (Figure 2A, left panel) and in other mesenchymal cells such as mMSC (Figure 2A, right panel). The decrease of IL-34 expression in the presence of TGF-β1 or BMP-2 resulted in a time- and dose-dependent inhibition of IL-34 mRNA (Figure 2B and 2C). Only TGF-β1 down regulated M-CSF in human synovial cells (Figure 2D).

ALK5 and ALK1 receptors are involved in the inhibition of IL-34 expression driven by TGFβ1 or BMP-2

Since BMP-2 and TGF-β1 preferentially signal from the membrane to the nucleus via respectively ALK1 and ALK5 receptors, we used validated chemical inhibitors of ALK1 (LDN 212854) and ALK5 (SB431542). We first demonstrated that BMP-2 and TGF-β1 stimulate respectively the ALK1/Smad1-5 and the ALK5/Smad2-3 signaling pathways (Figure 3A, left panels). We secondly demonstrated that LDN 212854 and SB431542 specifically inhibit the ALK1/Smad1-5 and the ALK5/Smad2-3 signaling pathways in mMSC (Figure 3A, middle and right panels). Then, we observed that ALK1 and ALK5 inhibitors block the response to respectively BMP-2 and TGF-β1 in mMSC (Figure 3B). Similarly, in fibroblasts of two RA patients (P1 and P2), we observed that the ALK5 inhibitor reverses the effect of TGF-β1 on IL-34 gene expression and that the ALK1 inhibitor partially reverses the effect of BMP-2 (Figures 3C and 3D). Interestingly, TGF-β1 stimulates BMP-2 mRNA steady state level in RA synovial fibroblasts (Supplemental Figures S1).

TGF-β1 inhibits IL-34 expression in inflammatory conditions

Since pro-inflammatory cytokines such as IL-1β and TNF-α play a crucial role in RA and since we previously demonstrated that TNF-α induces IL-34 expression,[5] we studied the effect of TGF-β1 and BMP-2 in inflammatory conditions. To this end, cells were treated with TNF-α. As shown in Figure 4, TGF-β1 (Figure 4A) and BMP-2 (Figure 4B) significantly reduce the response of the cells to TNF-α. Specifically, TGF-β1 and BMP-2 repress the basal levels of IL-34 rather than directly the response of the cells to TNF-α. Confocal microscopy analyses confirmed that IL-34 production

paralleled the modulation of IL-34 mRNA (Figure 4C). Specifically, TNF-α treatment resulted in an increase of IL-34 production in contrast to TGF-β1 which markedly reduced it. To ascertain this results on protein level, we quantified the IL-34 production by ELISA approach. As shown in Figure 4D, …..

Discussion

Scientific studies have focused on the complex role of cytokines in the development and progression of RA. During the last decade, it has been demonstrated that pro-inflammatory cytokines such as TNF-α or IL-1β play a crucial role in the pathophysiology of RA. These cytokines indeed promote inflammation and osteoclastogenesis in the arthritic joint leading to the destruction of cartilage and bone.[13] In this context, neutralizing TNF-α has proven successful to control RA in many cases.[13] During the last years, new pro-inflammatory cytokines, such as IL-32, IL-34 or IL-35 have been studied for their role in the pathogenesis RA. Recently, we demonstrated that IL-34, as a downstream effector of TNFα and IL-1β, is involved in the pathogenesis of RA.[5]

A better understanding of the mechanisms regulating the expression of this cytokine should allow to development new therapeutic approaches. We identified here two cytokines, BMP-2 and TGF-β1 able to inhibit IL-34 gene expression. We specifically demonstrated that TGF-β1 and BMP-2 inhibit IL-34 expression in synovial fibroblasts and in mMSC via respectively the ALK1 and ALK5 receptors. BMP-2 may indirectly reduce bone resorption by inhibiting the expression of IL-34 known to be able to stimulate osteoclastogenesis.[4] BMP-2 could thus prevent the bone degradation in RA by directly stimulating bone formation and indirectly by inhibiting bone resorption.

The role of TGF-β, identified as a pro-fibrotic agent in many tissues,[14] remains unclear in RA. It appears that this cytokine, involved in the pannus development,[9] could act as a pro-fibrotic agent in RA. In this context, some authors demonstrated the beneficial effects of TGF-β in RA.[6] Indeed, systemic administration of TGF-β1

antagonized the development of polyarthritis in susceptible rats.[15] In addition, the inhibition of TGF-β signaling increased the clinical severity of arthritis in collagen-induced arthritis model.[10]

To resume, we describe for the first time, the regulation of IL-34 expression by BMP-2 and TGF-β in the context of inflammatory stimulation. Such control loops appear to play a key role in the control of inflammation and bone resorption in RA (Figure 5). Future pre-clinical investigations are necessary to assess the potential clinical application of targeting IL-34 in RA. In addition, since BMP-2 and TGF-β1 have pleiotropic effects in different cells types, targeting these cytokines in RA need more investigations to define therapeutic treatments.

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Figure legends

Figure 1. ***IL-34, TGF-β1 and BMP-2 production in synovial fluid.***

Synovial fluid samples of RA patients were collected (n=44). **A:** Detection of IL-34, TGF-β1 and BMP-2 in synovial fluids. Each plot represents an individual sample. **B:** Correlation between levels of IL-34, TGF-β1, BMP-2 and total leukocyte count (mean +/-SD, each plot represents an individual sample). **C:** Correlation between levels of IL-34 and TGF-β, and of IL-34 and BMP-2. Each plot represents an individual sample.

Figure 2. ***BMP-2 and TGF-β1 repress IL-34 mRNA expression.***

A: Synovial fibroblasts from three patients (left panel, P1 to P3) and mMSC (right panel) (means ± S.D., n=3, *p<0.05, ***p<0.001) were incubated in presence or absence of BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) for 8h. IL-34 mRNA levels were determined by RT-qPCR.

B: mMSC (means ± S.D., n=3, *p<0.05, ***p<0.001) were stimulated with BMP-2 (0, 25, 50, 75, 100 or 200 ng/mL) for 8h (left panel), or with 100 ng/mL BMP-2 for either 8, 24 or 48h (right panel). IL-34 mRNA levels were determined by qRT-PCR.

C: mMSC (means ± S.D., n=3 *p<0.05, **p<0.01, ***p<0.001, ***p<0.001) were stimulated with TGF-β1 (0, 0.1, 1, 2.5, 5, or 10 ng/mL) for 8h (left panel), or with 10 ng/mL TGF-β1 for either 8, 24 or 48h (right panel). IL-34 mRNA levels were determined by qRT-PCR.

D: Synovial fibroblasts (P1 , P2 , P3) or mMSC (means ± S.D., n=3) were stimulated with BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) for 8h. M-CSF mRNA levels were determined by qRT-PCR.

Figure 3. ***ALK5 and ALK1 receptors are involved in the inhibition of IL-34 expression driven by TGFβ1 or BMP-2***

A: mMSC were incubated with SB431542 (10 μM) or LDN212854 (10 nM). 1h after, BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) was added for 1h. Phospho-Smad levels were detected by Western Blot. Histogramms represent the ratio of the phosphoSmad to the total Smad protein levels.

B: mMSC were incubated with SB431542 (10 μM) or LDN212854 (10 nM). 1h after, BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) was added for 8h. Il-34 mRNA levels were determined by RT-qPCR. (means ± S.D., n=3, *p<0.05, ***p<0.001).

C and D: synovial fibroblasts from two patient (P1 and P2) were incubated with SB431542 (10 μM, C) or LDN212854 (10 nM, D). 1h after, BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) was added for 8h. Il-34 mRNA levels were determined by RT-qPCR.

Figure 4. ***TGF-β1 antagonizes TNF-α-induced IL-34.***

A and B: Synovial fibroblasts from three patients (P1 to P3) were stimulated with TNF-α (10 ng/mL) together with TGF-β1 (A, 10 ng/mL) or BMP-2 (B, 100 ng/mL) for 8h. IL-34 mRNA levels were determined by RT-qPCR.

C: Synovial fibroblasts cultured on labtek chamber slides were treated with TNFα (10 ng/mL), TGF-β1 (10 ng/mL) or both for 24 h. IL-34 expression (pink), and nuclei stained by Hoechst reagent (blue) were observed by confocal microscopy. A representative experiment (X40) is shown.

D: ELISA

Figure 5. Schematic representation of the regulation of IL-34 expression in RA

Table 1 : Characteristics of patients included in the study

Leukocyte number/ mm3	DMARS/ Biotherapy	Corticosteroids	Sex	Age (yrs)	Duration of disease (months)	Antibody status		CRP(mg/l)
						ACPA	RF	
High inflammatory fluids								
27000	Abatacept	+	F	59	360	+	+	ND
22500	Tocilizumab	+	M	59	408	-	+	45
21000	Tocilizumab/ MTX	+	M	65	3	+	+	217
33600	Tocilizumab	-	M	53	132	+	+	<5
30900	Tocilizumab/ MTX	+	M	71	36	-	-	<5
25320	Leflunomid	+	M	42	96	+	-	39.8
44100	MTX	+	M	40	10	-	+	109
48000	-	+	F	71	456	-	ND	ND
43080	-	-	F	74	84	-	-	ND
39900	-	-	M	77	36	+	+	64
38300	-	-	F	74	72	-	-	23
28000	-	-	F	81	120	+	+	33
22080	-	+	M	52	108	+	+	59
Moderate inflammatory Fluids								
14200	Abatacept	+	F	84	8	+	+	95
10710	Tocilizumab	+	F	66	216	+	+	30
19920	Rituximab	+	F	55	228	-	+	ND
17000	Adalimumab/ MTX	+	M	53	144	ND	+	62
13000	MTX	+	F	66	ND	ND	ND	ND
11600	MTX/Hydroxy chloroquine	+	F	64	1	+	=	47
Low inflammatory Fluids								
2280	Abatacept/ MTX	+	F	72	>200	+	+	6
9120	Tocilizumab	+	F	66	48	-	-	31
ND	Tocilizumab/ MTX	+	F	61	240	+	+	ND
5600	Tocilizumab	+	F	44	108	-	+	104
2070	Adalimumab/ MTX	-	F	43	192	+	-	10
6600	Adalimumab	+	M	64	300	+	+	7.9
1700	Adalimumab/ MTX	+	M	51	132	+	+	<3
1080	Infliximab/ MTX	ND	M	60	96	+	+	ND
5280	Infliximab/ MTX	-	M	50	160	+	+	4
3600	Infliximab/ MTX	+	F	51	84	+	+	58
ND	MTX	+	F	73	204	+	+	ND
ND	MTX	-	M	48	36	+	+	15
7440	MTX	+	F	52	20	+	-	ND
7420	MTX	+	F	83	36	+	+	24.7
4500	MTX	+	F	37	ND	-	-	ND
3500	MTX	+	F	76	ND	ND	+	ND
9000	-	+	F	48	144	+	+	ND
1200	-	-	F	68	140	-	-	29.8
4800	-	+	F	73	9	+	+	30
1380	-	+	F	45	120	+	+	182
2640	-	+	F	45	120	+	+	182
1500	ND	ND	F	55	ND	ND	ND	ND
4590	-	-	M	71	4	-	-	87.7
9120	-	-	M	46	1	+	+	35
ND	ND	ND	F	59	ND	ND	ND	ND

MTX: Methotrexate; RF: Rheumatoïd Factor, ACPA: Anti-citrullinated Protein Antibody

Table 2: Sequences of primers

human IL34 sens	5'-CTTACGAGGGGGTGTTCAGA-3'
human IL34 antisens	5'-ACCAAGACCCACAGATACCG-3'
mouse IL34 sens	5'-GGACACACTTCTGGGGACA-3'
mouse IL34 antisens	5'-CCAAAGCCACGTCAAGTAGG-3'
human GAPDH sens	5'-TGGGTGTGAACCATGAGAAGTATG-3'
human GAPDH antisens	5'-GGTGCAGGAGGCATTGCT-3'
mouse Hprt sens	5'-TCCTCCTCAGACCGCTTTT-3'
mouse Hprt antisens	5'-CCTGGTTCATCATCGCTAATC-3'

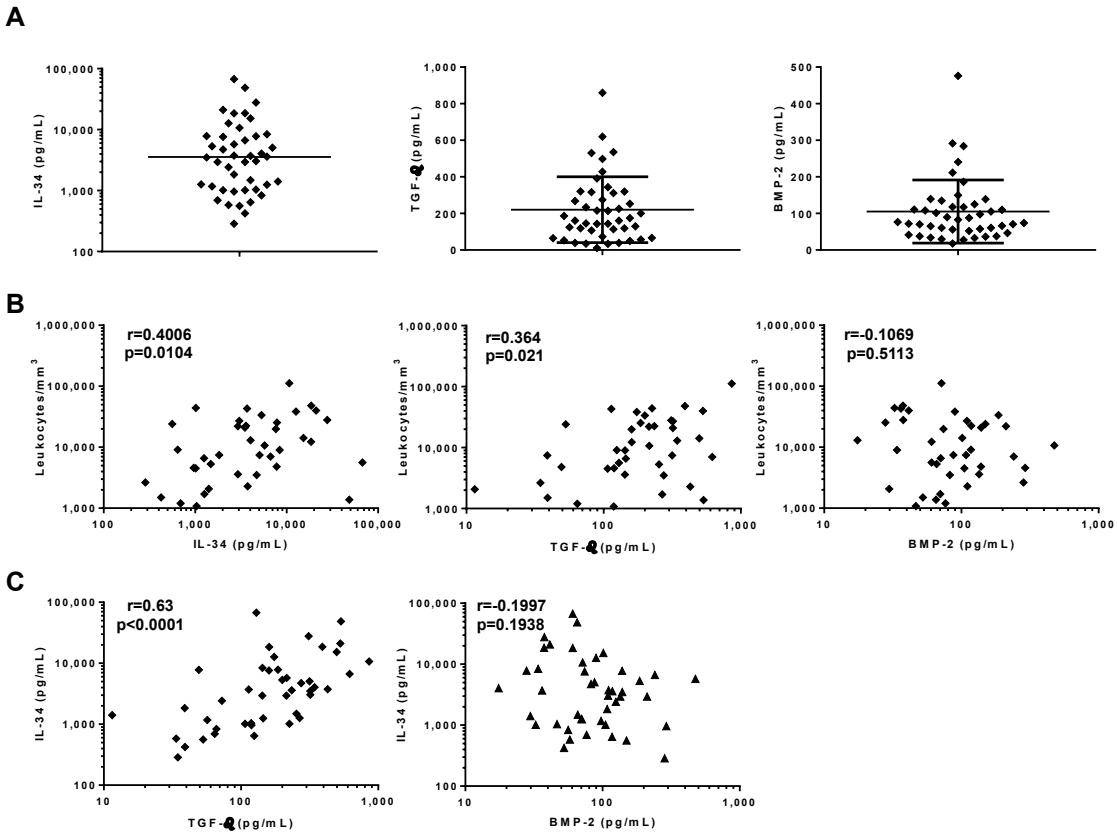

A
IL-34 (pg/mL)
TGF-β (pg/mL)
BMP-2 (pg/mL)
B
r=0.4006
p=0.0104
Leukocytes/mm3
IL-34 (pg/mL)
r=0.364
p=0.021
TGF-β (pg/mL)
r=-0.1069
p=0.5113
BMP-2 (pg/mL)
C
r=0.63
p<0.0001
TGF-β (pg/mL)
r=-0.1997
p=0.1938
BMP-2 (pg/mL)

Figure 1

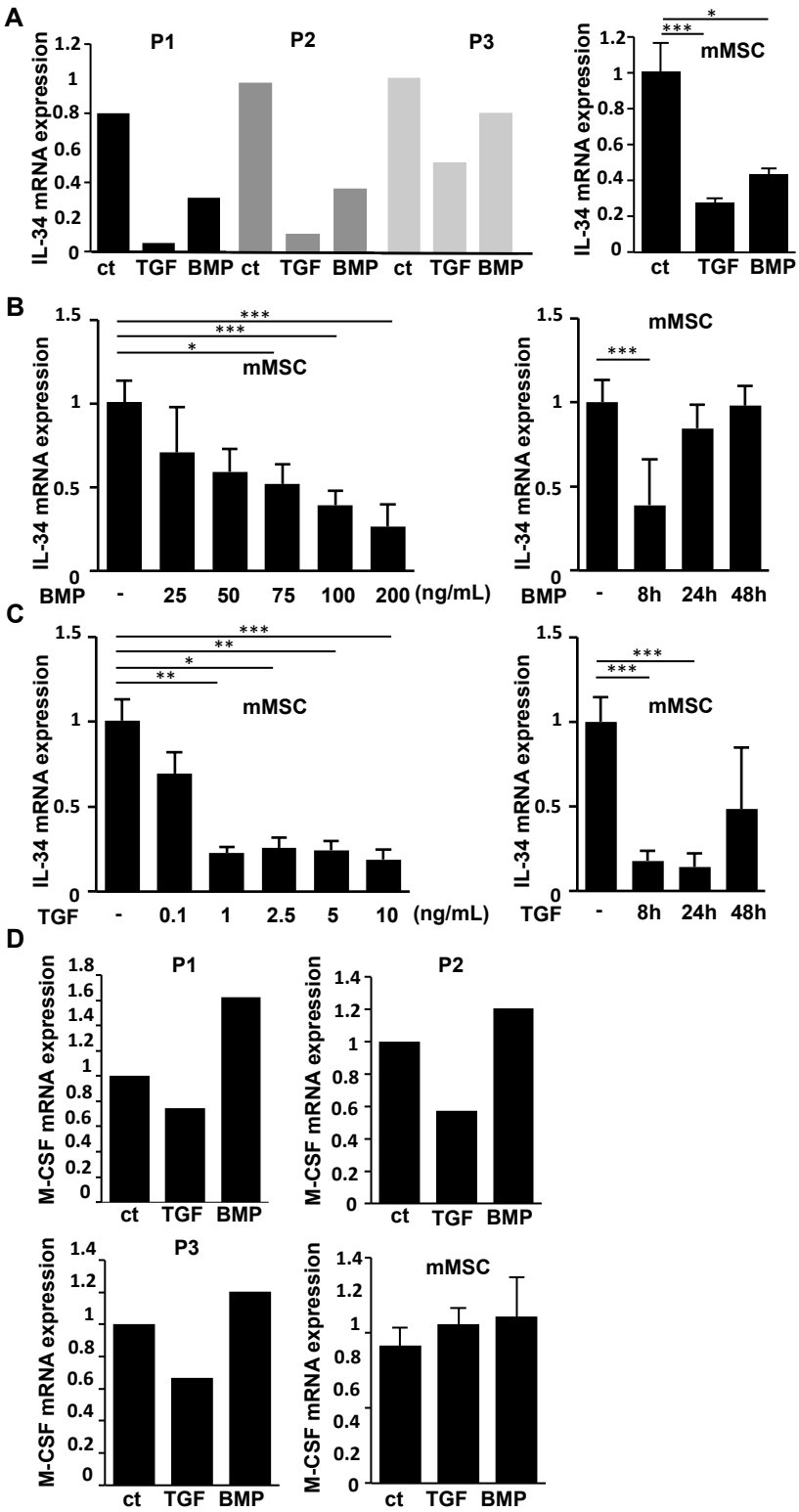

A
P1
P2
P3
IL-34 mRNA expression
ct TGF BMP ct TGF BMP ct TGF BMP
mMSC
ct TGF BMP
B
mMSC
BMP - 25 50 75 100 200 (ng/mL)
BMP - 8h 24h 48h
C
mMSC
TGF - 0.1 1 2.5 5 10 (ng/mL)
TGF - 8h 24h 48h
D
P1
P2
P3
mMSC
M-CSF mRNA expression
ct TGF BMP

Figure 2

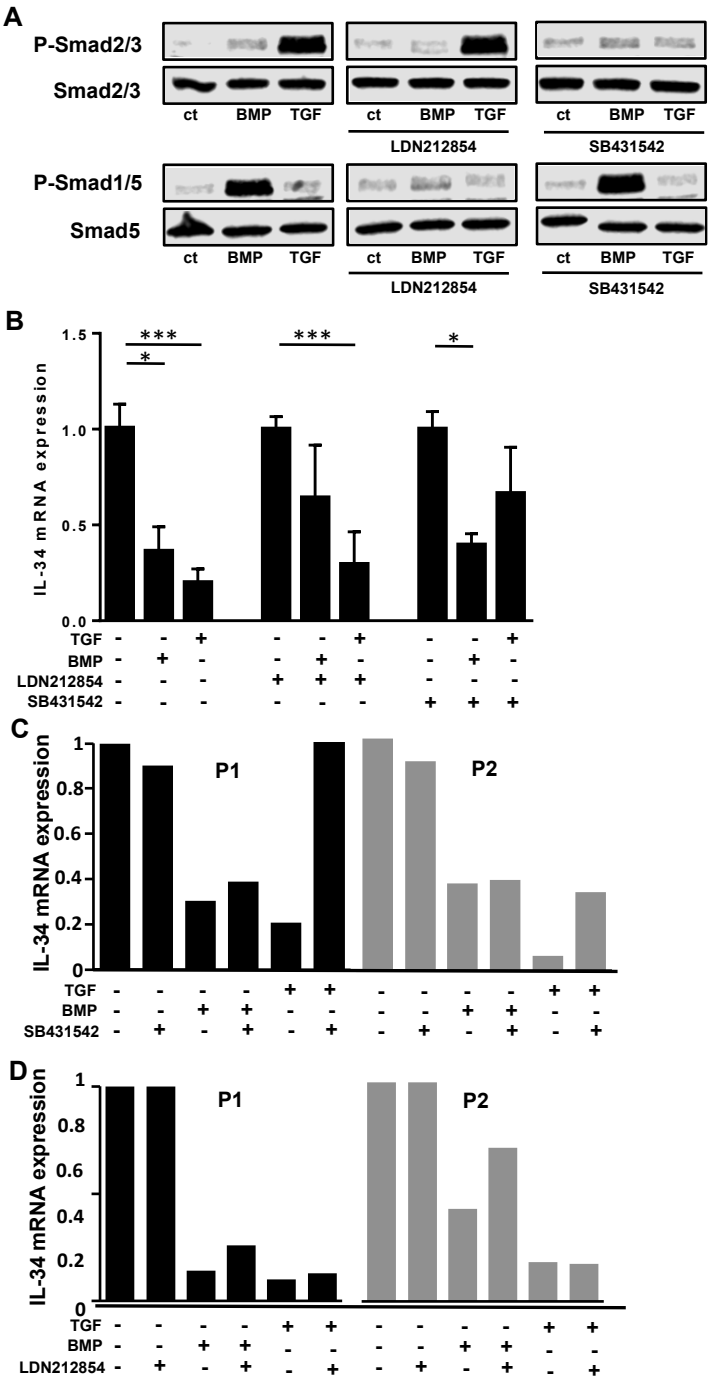

A
P-Smad2/3
Smad2/3
ct
BMP
TGF
LDN212854
SB431542
P-Smad1/5
Smad5
B
IL-34 mRNA expression
1.5
1.0
0.5
0.0
***
*
TGF
BMP
LDN212854
SB431542
C
IL-34 mRNA expression
P1
P2
1
0.8
0.6
0.4
0.2
0
D
IL-34 mRNA expression
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P2
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0.8
0.6
0.4
0.2
0

Figure 3

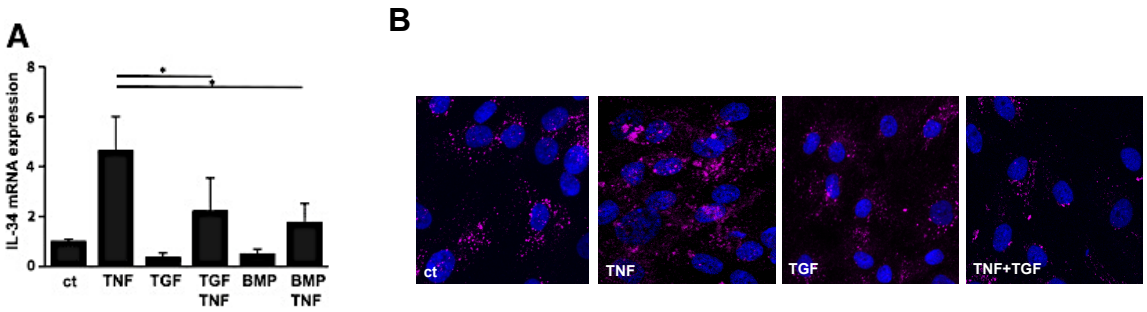
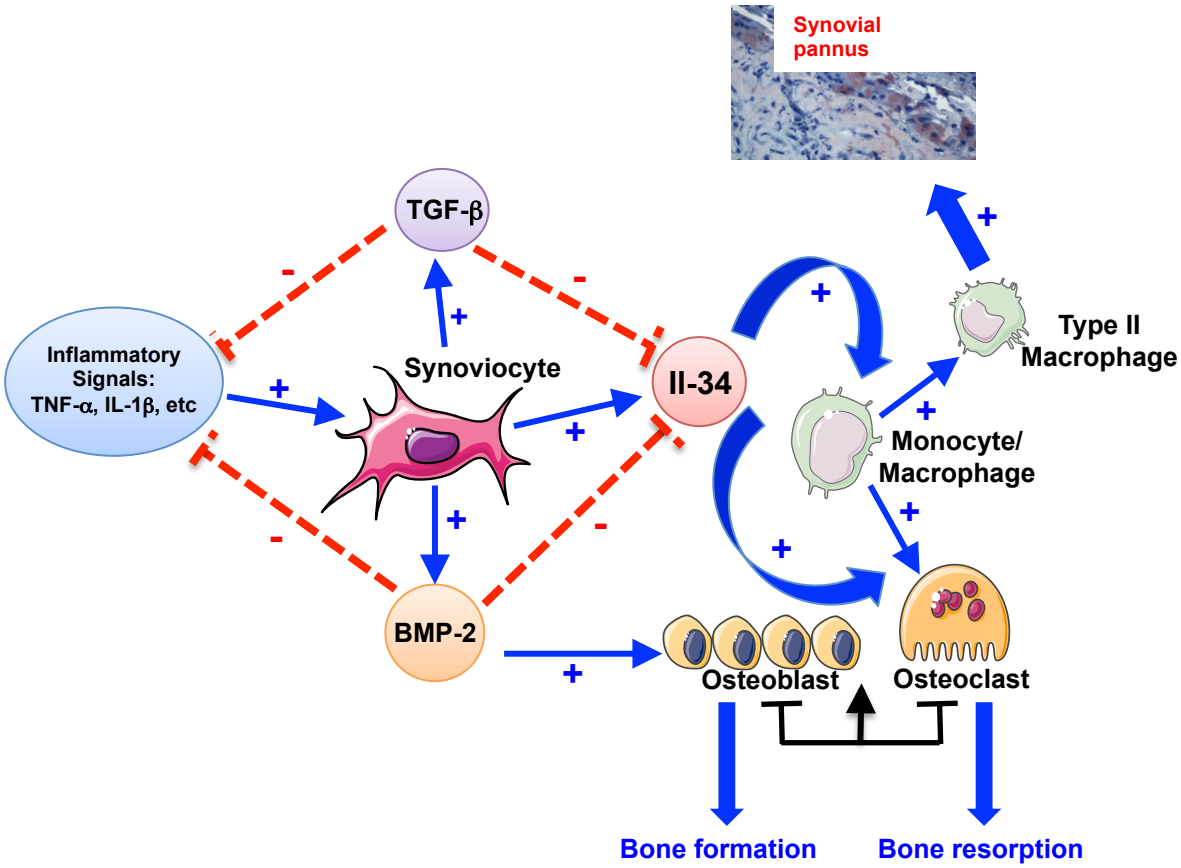
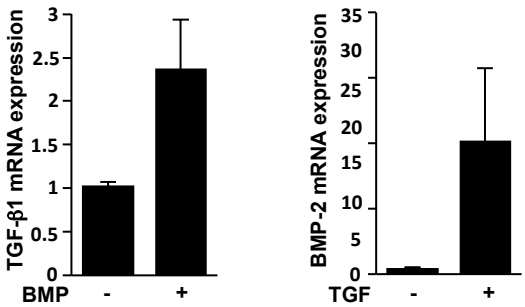

A
IL-34 mRNA expression
8
6
4
2
0
ct
TNF
TGF
TGF TNF
BMP
BMP TNF
B
ct
TNF
TGF
TNF+TGF

Figure 4


Synovial pannus
TGF-β
Inflammatory Signals: TNF-α, IL-1β, etc
Synoviocyte
Il-34
Type II Macrophage
Monocyte/ Macrophage
BMP-2
Osteoblast
Osteoclast
Bone formation
Bone resorption

Figures 5



Supplemental Figures S1

Supplemental Figure legends

Supplemental Figures 1. ***BMP-2 and TGF-β1 effect on TGF-B1 and BMP-2 mRNA expression***

RA synovial fibroblast were incubated with BMP-2 (100 ng/mL) or TGF-β1 (10 ng/mL) for 8h. TGF-β1 (left panel) or BMP-2 (right panel) mRNA levels were determined by qRT-PCR.