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Abstract

Objectives

Interleukin-34 (IL-34) is a new cytokine implicated in macrophage differentiation and osteoclastogenesis. The present study assessed the IL-34 expression in the tissue of patients with rheumatoid arthritis.

Methods

Immunohistochemistry was performed in synovial biopsy from patients suffering from rheumatoid arthritis (n=20), osteoarthritis (n= 3) or other inflammatory arthritis (n=4). IL-34 was detected in the synovial fluid by ELISA and its mRNA expression was studied by qPCR in rheumatoid synovial fibroblasts after stimulation by TNF-α and IL-1β. Wild type, *jnk1*$^{-/-}$ *-jnk2*$^{-/-}$ and *nemo* $^{-/-}$ murine fibroblasts and pharmacological inhibitions were used to determine the involvement of NFκB and JNK in that effect.

Results

IL-34 was expressed in 24/27 biopsies with 3 samples from RA patients being negative. We found a significant association between IL-34 expression and the synovitis severity. Levels of IL-34 and the total leukocyte count in the synovial fluid were correlated. TNF-α and IL-1β stimulated Il-34 expression by the synovial fibroblasts in a dose/time dependant manner through the NFκB and JUNK pathway.

Conclusion

This work identify for the first time IL-34 expression in the synovial tissue of arthritic patients. This cytokine, as a downstream effector of TNF-α and IL-1β, may contribute to the inflammation and bone erosions in RA.

MESH Keywords Adult ; Aged ; Aged, 80 and over ; Arthritis, Rheumatoid ; complications ; genetics ; metabolism ; Cells, Cultured ; Dose-Response Relationship, Drug ; Female ; Fibroblasts ; drug effects ; metabolism ; Gene Expression Regulation ; drug effects ; Humans ; Interleukin-1beta ; pharmacology ; Interleukins ; genetics ; metabolism ; MAP Kinase Signaling System ; physiology ; Male ; Middle Aged ; NF-kappa B ; physiology ; Osteoarthritis ; genetics ; metabolism ; RNA, Messenger ; genetics ; Synovial Fluid ; metabolism ; Synovitis ; etiology ; genetics ; metabolism ; Tumor Necrosis Factor-alpha ; pharmacology

Author Keywords Interleukin-34 ; Arthritis ; Rheumatoid Arthritis ; Osteoarthritis ; inflammation

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial tissue that leads to progressive joint destruction. Among the cells located in the inflamed joint, synovial fibroblasts are important players driving inflammation and bone erosion.[1] They are recognized as a source of cytokines such as IL-6 or RANKL which activate immune response and osteoclastogenesis. There is evidence that the Colony Stimulating Factors (CSFs) play a major role in inflammation and bone destruction in RA.[2] Macrophage-CSF (M-CSF) is the primary regulator of the biology of mononuclear phagocytes and is also essential for osteoclastogenesis.[3] In the synovial tissue of RA patients, it is expressed by the macrophages but also by the synovial fibroblasts and is up-regulated by inflammatory cytokines like TNF-α.[2 ,4] Furthermore, M-CSF knockout mice are protected against collagen-induced arthritis, and M-CSF administration exacerbates inflammation and joint destruction.[2 , 5] In this context, therapeutic targeting of M-CSF is currently being developed.[2]

IL-34 is a newly discovered cytokine which plays a role in macrophage differentiation and proliferation.[6] IL-34 is expressed in various tissues and it is most abundant in the spleen. This cytokine shares numerous common features with M-CSF, especially its receptor (MCSF-R) explaining partly their functional overlap. However, their interaction with MCSF-R, the signalling pathway activated and their expression patterns differ during the embryonic development underlining their probable specific biological function.[7] Recently, we have

shown that IL-34 could be substituted for M-CSF to promote osteoclastogenesis *in vitro* .[8] Its role in inflammation is also likely as IL-34 increase IL-6 and chemokine level in human whole blood.[9] Although numerous studies have underlined the role of M-CSF in arthitis, no data are currently available on the expression of IL-34 in RA.

In this study, we hypothesized that IL-34 could be expressed by the synovial fibroblasts of RA patients and this expression could be modulated by TNF–α and IL-1β.

METHODS

Synovial fluid and biopsies

Synovial biopsies were obtained surgically at the time of an arthroplasty and were summarized in Table S1 . All patients enrolled have given their formal consent. The study was approved by the local ethics committee and by the French Research Ministry (N°2008-402). The mean (+/−SD) duration of the RA was 11+/−5 years and the mean synovitis score was 4.4+/−1.9 (range 2–8). The mean score of synovial hyperplasia, stroma activation and inflammatory infiltrates was 1.3+/−1.2, 1.5+/−0.6 and 1.6+/−0.5 respectively (Table S1). IL-34 expression was detected by immunohistochemistry performed as previously described.[8] The histopathological severity of synovitis was graded as described by Krenn et al.[11] IL-34 levels were measured in synovial fluids by ELISA assay (antibodies-online-GmbH, USA) according the manufacturer's recommendations.

Cell cultures

Synovial fibroblasts, obtained from the synovial tissue of RA patients, human fibroblasts (WI-26) and murine wild type, *jnk1*$^{-/-}$ *-jnk2*$^{-/-}$ and *nemo* $^{-/-}$ fibroblast cell lines were cultured as previously described.[11 –14]

Reverse Transcription-PCR analysis

Total RNA was extracted using NucleoSpin RNAII kit (Macherey-Nagel, France) and one microgram of total RNA was used for first strand cDNA synthesis using Thermoscript kit (Invitrogen, France).[8] Real-time PCR was performed using SYBRGreen Supermix (Biorad, France) and primers described in Table S2 .

Statistical analysis

Mann-Whitney test was use to look for an association between Il-34 expression and the histological characteristics. Correlation between level of IL-34 and leukocytes count in the synovial fluid was measured using the nonparametric Spearman rank order test. Student t-test was used to assess the change in gene expression. $p<0.05$ was considered as statistically significant.

RESULTS

IL-34 is expressed in the synovial tissue of RA and OA patients

We first assessed the expression of *IL-34* in the synovial tissue in synovial biopsy from patients suffering from rheumatoid arthritis (n= 20), osteoarthritis (n=3) or other inflammatory arthritis (n=4) (Table S1). According to the grading system of all the biopsies, 11 had slight synovitis, 9 had moderate synovitis, and 7 had strong synovitis. IL-34 was detected in 24 of the 27 biopsies with 3 samples from RA patients being negative. In the synovial tissue, IL-34 was expressed in the synovial lining layer by the synoviocytes and macrophages (Fig. 1A, B) as confirmed by the double immmunostaining for IL-34 and CD68 (Fig. S1) and in the sub-lining layer by endothelial cells and fibroblasts (Fig. 1C, D). In RA patients, a significant association was found between IL-34 expression in the synovial lining layer and the histological severity of the synovitis with a mean score of synovitis of 5.8+/−1.9 and 3.7+/−1.6 in the IL-34 positive and negative biopsies respectively (p=0.022) (Fig. 1E). IL-34 expression within the synovial lining layer is also associated with the synovial hyperplasia/enlargement: the mean score of hyperplasia was 2.2+/−0.9 and 0.6+/−1 in the biopsies with and without IL-34 positive cells respectively (p=0.004) (Fig. 1F). No significant association was found between IL-34 expression and the diagnosis. Interestingly, IL-34 levels were significantly higher in synovial fluids of RA patients compared to OA patients ($p<0.05$, Fig. 1G) and were associated with the inflammation intensity measured by the leukocyte counts ($r=0.82$, $p<0.001$)(Fig. 1H).

TNF-α and Il-1β increase IL-34 gene expression in rheumatoid synovial fibroblasts

We next assessed the expression of IL-34 by the synovial fibroblasts *in vitro* and its regulation by TNF-α and Il-1β. IL-34 mRNA was detectable in non stimulated cells. Stimulation with TNF-α resulted in a significant dose-dependent induction of IL-34 mRNA with a plateau from 25ng/mL and a maximum of induction after 6 hours with 10ng/mL (Fig. 2A). The time course study using 10ng/mL TNF-α shows that this effect remained stable until 24 hours (data not shown). Similarly, Il-1β also increased dose-dependently IL-34 mRNA expression with a peak reached at 6h then decreased quickly thereafter (Fig. 2B). Confocal microscopy (Fig. 2C) and flow cytometry (Fig. S2) analyses confirmed that TNF-α and Il-1β upregulated the expression by synovial fibroblasts of IL-34 at the protein level compared to the untreated cells

JNK and NF-κB activities are required for TNF-α and IL-1β to stimulate IL-34 mRNA levels in fibroblasts and synoviocytes

TNF-α and IL-1β treatment of WI-26 fibroblasts led to a time- and dose-dependent increase in IL-34 mRNA levels (Fig. 3A,B). Rapid and persistent induction of IL-34 mRNA levels was observed in response to TNF-α (10ng/mL) (Fig. 3A) and IL-1β (10ng/mL) (Fig. 3B) peaking at 6 and 10h respectively, and remaining at levels significantly higher than their basal expression state up to 24h and 48h. To understand the mechanisms by which TNF-α and IL-1β are able to stimulate IL-34 mRNA levels, the role played by the JNK and NF-κB pathways was examined respectively in immortalized *jnk*$^{-/-}$ and *nemo* $^{-/-}$ fibroblasts (Fig. 3B,C), and in synoviocytes (Fig. 3D) with or without specific inhibitors of the JNK and NF-κB pathways. Results shown in Figure 3C (left panel) indicate that the effects of TNF-α on *IL-34* gene expression was significantly inhibited both in *nemo*$^{-/-}$ and *jnk*$^{-/-}$ cells 6h Finally, treatment of synoviocytes cultures with the specific IKKβ inhibitor or JNK inhibitor significantly inhibited (around 88% in presence of 10 μM IKKV and 28% in presence of 10μM SP600125) the effect of 10ng/mL TNF-α (Fig. 3D) after treatment of the cells with 10ng/ml of TNF-α (Fig. 3C) or IL-1β (data not shown). Confocal microscopy analyses confirmed the effects of signaling pathway inhibitors at the protein level (Fig. S3).

DISCUSSION

Pro-inflammatory cytokines, promoting inflammation and osteoclastogenesis in the arthritic joint, are fundamental to RA pathophysiology.[15] In this study we demonstrated that a newly discovered cytokine, IL-34, is expressed by the synovial fibroblasts and that TNF-α and IL-1β stimulate its expression. The present report shows for the first time that Il-34 is expressed in the synovial tissue of arthritic patients, mainly by the cells of the synovial lining layer and to al lesser extent by the fibroblasts and endothelial cells in the sub-lining layer. Our data support that synovial fibroblasts are a source of this cytokine as revealed by the IL-34 mRNA and protein expression *in vitro* . Importantly, our data showed that the expression of IL-34 in the synovial lining layer was associated with the severity of synovitis. IL-34 may have a pro-inflammatory effect, promoting macrophages differentiation and proliferation in the synovial tissue. On the other hand, we have shown that pro-inflammatory cytokines are able to increase IL-34 expression by synovial fibroblasts explaining this association.

Pro-inflammatory cytokines are known to induce expression of a large range of cytokines, chemokines or metalloproteases in synovial fibroblasts.[1] We have shown that TNF-α and IL-1β are able to enhance IL-34 expression in RA synovial fibroblasts. These results were confirmed in a human lung fibroblasts cell line (WI-26), showing that the expression of IL-34 may extend to other fibroblastic cells. We have shown that JNK and NF-κB activities, the two main signaling pathways activated by TNF-α and IL-1β,[16 ,17] are required for these cytokines to stimulate IL-34 mRNA levels in fibroblasts and synoviocytes. Thus, IL-34 could be a downstream effector of IL-1β and TNF-α, mediating their effect on inflammation and osteoclastogenesis.

In view of the role of IL-34 in osteoclastogenesis and inflammation, this cytokine is likely to play a role in the pathogenesis of RA and therefore could constitute a new therapeutic target. Further explorations are needed to delineate the exact role of IL-34 in the inflammatory process associated with RA.

Acknowledgements:

This work was supported by the ARTHRITIS Fondation Courtin (to Dr J.M. Berthelot) We thank the MicroPICell platform (Nantes University, IFR26) for confocal microscopy and Philippe Hulin for his technical assistance.

Footnotes:

Competing Interest: None declared

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Figure 1

IL-34 is expressed in the synovial tissue of patients with OA and RA

Representative immunohistochemical staining for IL-34 (red staining) (ab75723, Abcam, France) in synovial biopsies samples from patients with RA. (**A–D**) IL-34 was expressed in the synovial lining layer by synoviocytes (open arrow)(**A**), multinucleated giant cells (open triangle) and macrophages (Asterix) (**B**). In the sublining layer, endothelial cells (dotted arrow), inflammatory cells (triangle) and fibroblasts (arrow) were also positive for IL-34 **(C, D).** Comparison of the mean synovitis score (from 0, slight synovitis to 9, strong synovitis) (**E**) and mean hyperplasia of the lining layer score (from 0, no hyperplasia to 3, major hyperplasia (**F)** according to the expression of IL-34 in the synovial lining layer; *p<0.05; **p<0.01 compared to the IL-34$^-$ group. **(G)** Comparative levels of IL-34 measured by ELISA assay (ref. ABIN455583) in synovial fluids in OA and RA patients, each plot being an individual sample, *** p<0.001. (H) Correlation between IL-34 levels and total leukocyte counts in the synovial fluids of patients with RA and OA, r = 0.82, p<0.0001.

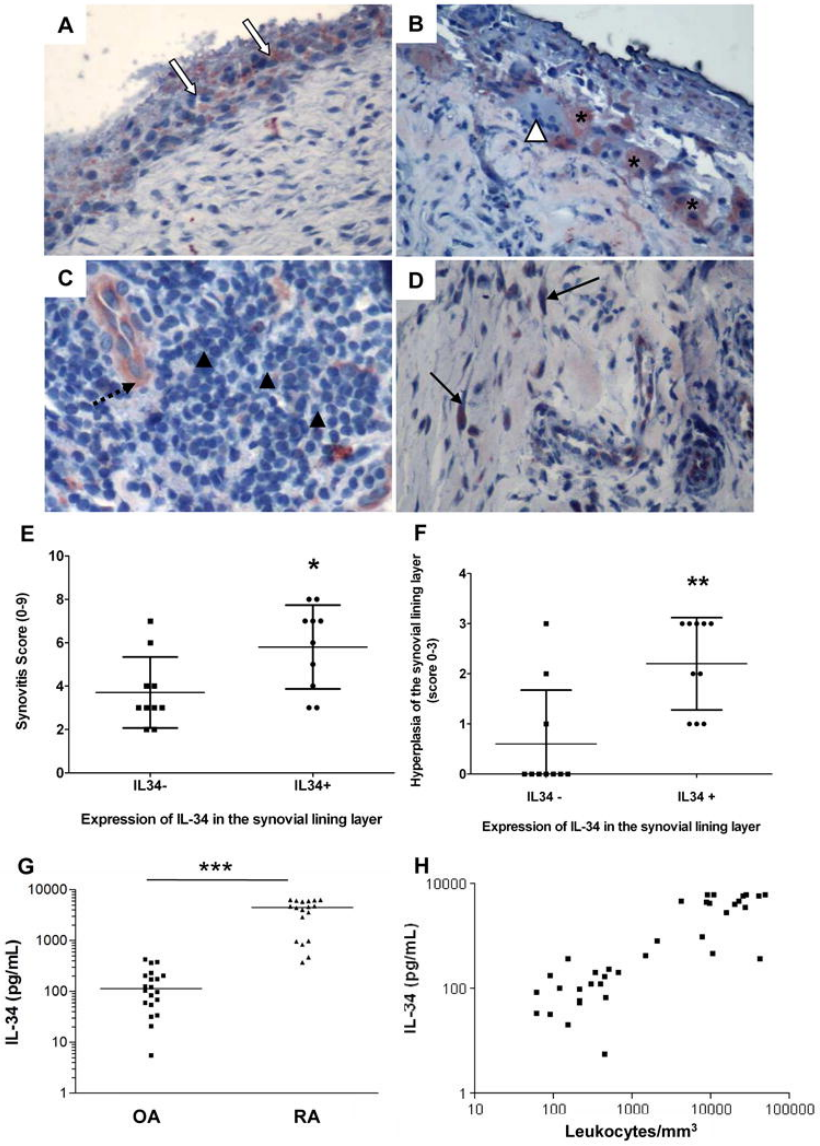

Figure 2

TNF–α and IL-1β induce IL-34 mRNA expression in RA synovial fibroblasts

Synovial fibroblasts from RA patients (n=3) were stimulated with **(A)** increased dose of TNF-α (1 to 50 ng/mL) for 6h hours or with TNF–α 10 ng/mL for 2h, 6h, 10h and 24h and **(B)** increased dose of IL-1β (1 to 25 ng/mL) for 6 hours or with IL-1β 10 ng/mL for 2h, 6h, 10h and 24h. After incubations, IL-34 mRNA levels were determined by real time RT-PCR, normalized to GAPDH **(C)** Synovial fibroblasts cultured on labtek chamber slides (Millipore, France) were treated or not with TNF–α (10 ng/mL) or IL-1β (10 ng/mL) for 24h. IL-34 expression (green), actin filaments detected by alexa fluor 546-conjugated phalloidin (red), and nuclei stained by DAPI (blue) were observed by confocal microscopy. A representative experiment was shown.

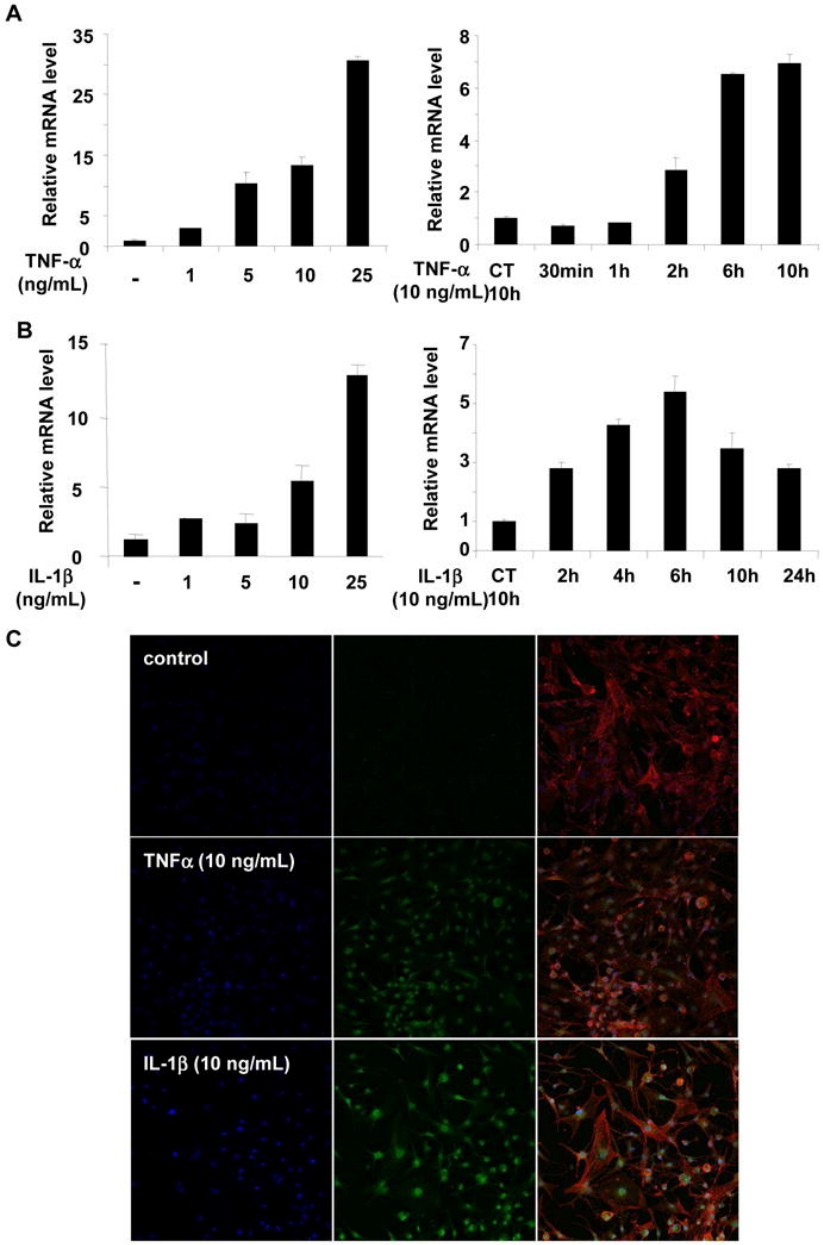

Figure 3

JNK and NF-κB activities are required for TNF-α and IL-1β to stimulate IL-34 mRNA levels in fibroblasts and synoviocytes

WI-26 fibroblast cells were treated either with 10 ng/mL TNF-α (**A**) or 10 ng/mL IL1β (**B**) for 2h, 6h, 10h, 24h and 48h (right panels) or with various concentrations of TNF-α or IL-1β (1, 5, 10, 25 and 50 ng/mL) for 24h, as indicated. After incubations, IL-34 mRNA steady-state levels were determined by real time RT-PCR. The expression of the houskeeping gene GAPDH was used as control. (**C**) Wild type (wt) nemo$^{-/-}$ and jnk$^{-/-}$ fibroblasts were cultured in the presence or the absence of 10 μM JNK inhibitor (JNK II) or 10 μM IKKβ inhibitor V (IKKV). One hour later 10 ng/ml TNF-α or IL-1β were added for 6h. IL-34 mRNA steady-state levels were then determined by real time RT-PCR. Bars indicate mean ± S.D. of two independent experiments performed, each with duplicate samples. *p<0.05; ***p<0.001 compared to the control. (**D**) Human synovial fibroblasts were treated with with or without 10 μM JNK inhibitor or IKKβ inhibitor V. One hour later 10 ng/mL TNF-α or IL-1β were added for 6h. After incubations, *IL-34* mRNA steady-state levels were determined by real time RT-PCR. A representative experiment was shown.