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# IL-17RA and IL-17RC Receptors Are Essential for IL-17A-Induced ELR<sup>+</sup> CXC Chemokine Expression in Synoviocytes and Are Overexpressed in Rheumatoid Blood<sup>1</sup>

Saloua Zrioual, Myew-Ling Toh, Anne Tournadre, Yuan Zhou, Marie-Angélique Cazalis, Alexandre Pachot, Vincent Miossec, and Pierre Miossec<sup>2</sup>

**IL-17A is a cytokine secreted by the newly described Th17 cells implicated in rheumatoid arthritis (RA). Less is known about its receptors in synoviocytes. IL-17RA and IL-17RC were found to be overexpressed in RA peripheral whole blood and their expression was detected locally in RA synovium. In vitro, IL-17A synergized with TNF- $\alpha$  to induce IL-6, IL-8, CCL-20, and matrix metalloproteinase-3. Using microarrays, a specific up-regulation of Glu-Leu-Arg<sup>+</sup> CXC chemokines was observed in IL-17A-treated synoviocytes. Using both posttranslational inhibitions by silencing interfering RNA and extracellular blockade by specific inhibitors, we showed that both IL-17RA and IL-17RC are implicated in IL-17A-induced IL-6 secretion, whereas in the presence of TNF- $\alpha$ , the inhibition of both receptors was needed to down-regulate IL-17A-induced IL-6 and CCL-20 secretion. Thus, IL-17A-induced IL-6, IL-8, and CCL20 secretion was dependent on both IL-17RA and IL-17RC, which are overexpressed in RA patients. IL-17A-induced pathogenic effects may be modulated by IL-17RA and/or IL-17RC antagonism. *The Journal of Immunology*, 2008, 180: 655–663.**

The newly characterized IL-17-secreting T cells (1, 2), named Th17, play a pathogenic role in rheumatoid arthritis (RA).<sup>3</sup> Th17 can now be placed alongside the classically known Th1 and Th2 cells, these three cell types developing by way of distinct lineages. In mouse models, TGF- $\beta$  and IL-6 are implicated in the differentiation of Th17 cells from naive T cells (3, 4), and IL-23 contributes to their survival and proliferation. The crucial role of Th17 in chronic inflammatory disorders comes also from studies in mice showing that Th17 and regulatory T cells result from reciprocal differentiation pathways (3). The important role of IL-17/IL-23 axis in diseases such as RA is supported by substantial anti-inflammatory and joint protective effects of IL-17 antagonism (soluble IL-17Rf fusion protein and neutralizing anti-IL-17 Abs) in murine and human models of arthritis. In addition, IL-17 deficient mice show markedly diminished collagen-induced arthritis (5).

In RA patients, high local levels of IL-17A are present in both synovium and synovial fluid (6, 7). IL-17A induces proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6) (8, 9) and CXC chemokines

which recruit neutrophils into the joint (10, 11), stimulate angiogenesis (12, 13), and are implicated in joint degradation (14). The relevance of this cytokine has been further underlined by in vitro studies performed in the presence of TNF- $\alpha$  and IL-1 $\beta$ , two key pathogenic proinflammatory cytokines overexpressed in RA joints (8, 15, 16). IL-17A often acts synergistically with these two cytokines to induce inflammatory mediators in synoviocytes, human osteoblasts, myoblasts, and chondrocytes (8, 17–19). Furthermore, a recent clinical study demonstrated that synovial IL-17A expression is associated with joint destruction (20).

Chemokines are small cytokines which have been classified into four groups according to their structure. C, CC, CXC, or CX3C chemokines differ by the presence of conserved cysteines. Moreover, CXC chemokines are grouped into Glu-Leu-Arg or ELR<sup>+</sup> chemokines depending on the presence or absence of a sequence of three amino acids (Glu-Leu-Arg) in their N terminus. This ELR motif is implicated in CXC chemokine-associated regulatory effects on angiogenesis, with ELR<sup>+</sup> chemokines having angiogenic properties and non-ELR chemokines acting mainly as angiostatic factors (21, 22). The neutrophil attractant characteristics of CXC chemokines have also been linked to the presence of the ELR motif (23). To study the contribution of IL-17A in RA pathogenesis, we analyzed its regulatory effect on chemokine expression by synoviocytes using microarrays and focusing on CCL20/MIP-3 $\alpha$ , which is implicated in the recruitment of activated lymphocytes and immature dendritic cells (18, 24).

Although IL-17A has affinities with IL-17RA, binding assays have suggested the involvement of additional receptor components (25). To date, four additional receptors have been identified in the IL-17R family based on sequence homology to IL-17R (IL-17Rh1, IL-17RC, IL-17RD, and IL-17RE) and among them, IL-17RC has been shown recently to physically associate with IL-17RA, suggesting that it may be a functional component in the IL-17R complex (26). Nevertheless, the final structure of the functional IL-17 receptor and the mechanisms of interactions with IL-17A remain unknown.

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<sup>3</sup> Abbreviations used in this paper: RA, rheumatoid arthritis; siRNA, silencing interfering RNA; OA, osteoarthritis; HV, healthy volunteer; DAS, disease activity score; MMP, matrix metalloproteinase; HPRT1, hypoxanthine phosphoribosyltransferase 1; AU, arbitrary unit; ELR, Glu-Leu-Arg; PPIB, peptidylprolyl isomerase B.

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We analyzed the expression of IL-17RA and IL-17RC in peripheral whole blood and demonstrated their overexpression in RA patients. In RA synoviocytes, we observed that both IL-17RA and IL-17RC are required to transduce IL-17A signals leading to increased expression of critical genes for RA pathogenesis. Our data suggest that IL-17RA and IL-17RC overexpression may play an important role in RA pathogenesis as shown by their modulation using silencing interfering RNA (siRNA) delivery or extracellular inhibitors.

## Materials and Methods

### Patients and healthy volunteers

Forty RA patients fulfilling the American College of Rheumatology 1987 revised criteria for RA (27) and 19 healthy volunteers (HV) were enrolled for determination of gene expression profiles in whole peripheral blood using U133A microarrays (Affymetrix). Clinical indices and biological markers in this study included age, sex, disease duration, right Larsen wrist x-ray index, rheumatoid factor, C-reactive protein, and number of Disease Modifying Anti-Rheumatic Drugs. Patients were divided into two groups according to the right Larsen wrist x-ray index: destructive RA (Larsen wrist score  $\geq 2$ ) and nondestructive arthritis (Larsen wrist score  $< 2$ ). On the basis of the modified disease activity score 28 (DAS 28) joint index, 31 were evaluated as severe RA (DAS 28  $> 3.2$ ) and 9 as moderate RA (DAS 28  $\leq 3.2$ ). All the participants gave their written informed consent. The protocol was approved by the committee for protection of persons participating in biomedical research.

### Immunohistochemistry

Synovium pieces kept for immunohistochemistry analysis were directly fixed in 10% phosphate-buffered formaldehyde. After paraffin embedding, samples were cut into 4- $\mu$ m serial sections, mounted on glass slides, and treated for removal of paraffin (OTTIX Plus; DiaPath). To detect Ag expression in paraffin-embedded sections, Ag retrieval procedure was performed by incubation in citrate buffer (pH 6) for 40 min at 99°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min before the application of primary Ab. The sections were then incubated for 1 h with primary Abs (R&D Systems): 10  $\mu$ g/ml mouse monoclonal anti-IL-17A (IgG2b), 10  $\mu$ g/ml mouse monoclonal anti-IL-17RA (IgG1), or 10  $\mu$ g/ml goat polyclonal anti-IL-17RC Abs. In negative control sections, irrelevant Ab (mouse IgG2b, mouse IgG1, or goat serum, respectively) was applied at the same concentration as the primary Ab. After washing, the sections were incubated with biotinylated anti-mouse and anti-goat Igs for 15 min, followed by streptavidin-peroxidase complex for 15 min and 3,3' diaminobenzidine chromogen solution (DakoCytomation). The sections were then counterstained with Mayer's hematoxylin.

### Cell culture

RA synoviocytes were obtained from synovium tissue of RA patients undergoing joint surgery who fulfilled the American College of Rheumatology criteria for RA as previously described (27). In brief, synovium tissue was minced into small pieces and then incubated for 2 h at 37°C with proteolytic enzymes (1 mg/ml collagenase and hyaluronidase from Sigma-Aldrich). Synoviocytes were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, streptomycin at 37°C in a humidified 5% CO<sub>2</sub> incubator as previously described (28). Experiments were performed in synoviocytes at passages 3–7, which were  $>99\%$  negative for CD45, CD1, CD3, CD19, CD14, and HLA-DR and were positive for the expression of CD44 (Abs obtained from BD Pharmingen).

### Cell separation

PBMCs cells were isolated from RA patients by Ficoll-Paque plus density gradient centrifugation of heparinized blood (Amersham Biosciences). CD14<sup>+</sup> monocytes, CD3<sup>+</sup> lymphocytes, or CD19<sup>+</sup> lymphocytes were then isolated from PBMC by positive selection using MACS isolation kits (Miltenyi Biotec). The purity of the CD14<sup>+</sup> monocyte subset (90–95%), CD3<sup>+</sup> lymphocyte subset ( $>95\%$ ), or CD19<sup>+</sup> lymphocyte subset ( $>90\%$ ) were determined by flow cytometry analysis (FACS scan; BD Biosciences) using specific mAbs against human PE-conjugated CD14, human PE-conjugated CD3, human PE-conjugated CD19 (BD Pharmingen), or isotype control Abs (BD Pharmingen) as per the manufacturer's instructions as described (28).

### Cytokines, Abs, and ELISAs

Human recombinant TNF- $\alpha$  was purchased from Sigma-Aldrich. Human rIL-17A, the mAb against human IL-17RA and the polyclonal goat Ab against IL-17RC, were purchased from R&D Systems. RA synoviocytes seeded in 96-well plates ( $1 \times 10^4$  cells/well), were stimulated with IL-17A or IL-17F 50 ng/ml alone or in combination with 0.5 ng/ml TNF- $\alpha$  for 36 h. IL-6, IL-8, and CCL20 levels were quantified in removed supernatants by ELISA (eBioscience; Diaclone; R&D Systems, respectively).

### RNA extraction and purification

Whole blood samples were collected in PAXgene Blood RNA tubes (Pre-AnalytiX) to minimize postsampling activation of mRNA (29) and total RNA extracted using the PAXgene Blood RNA kit (PreAnalytiX). Synoviocytes seeded in 6-well plates ( $5 \times 10^5$  cells/well) were serum deprived for 2 h and then stimulated for 12 h with IL-17A 50 ng/ml alone or in combination with 0.5 ng/ml TNF- $\alpha$  in DMEM/10% FCS. RNA from synoviocytes and blood cell subsets (CD3<sup>+</sup>, CD14<sup>+</sup>, CD19<sup>+</sup>) were extracted using TRIzol reagents (Invitrogen Life Technologies), according to the manufacturer's instructions. RNA was purified using RNeasy kits (Qiagen). The concentration of RNA was quantified by spectrophotometry at 260 nm (SmartSpecTM3000; Bio-Rad).

### Real-time quantitative RT-PCR analysis

One microgram of total RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen Life Technologies). Briefly, total RNA was denatured by incubating for 5 min at 65°C with 4  $\mu$ M oligo(dT) primer and then reverse transcribed by using a final concentration of 0.5 mM dNTP, 40 U/ $\mu$ l RNase OUT, 0.01 M DTT, and 10 U/ $\mu$ l of ThermoScript reverse transcriptase. Reverse transcription was performed by incubation at 50°C for 60 min followed by 85°C for 5 min. The obtained cDNA was diluted 1/10 with distilled water and 10  $\mu$ l were used for amplification.

Specific primer sets for IL-6, CXCL8/IL-8, matrix metalloproteinase (MMP)-3, RANTES/CCL5, GAPDH, and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were optimized for the LightCycler instrument (Roche Molecular Biochemicals), and purchased from Search-LC. Primer sets for IL-17RA (GenBank accession no. NM\_014339) and IL-17RC (GenBank accession no. NM\_153461) were synthesized by Eurogentec: IL-17RA forward: 5'-AGACACTCCAGAACCAATTCC-3', IL-17RA reverse: 5'-TCTTAGAGTTGCTCTCCACCA-3', IL-17RC forward: 5'-AC CAGAACCTCTGGCAAGC-3', IL-17RC reverse: 5'-GAGCTGTTCAC CTGAACACA-3. The PCR was performed using the LightCycler FastStart DNA SYBR Green I kit (Roche Molecular Biochemicals) according to the protocol provided with the parameter-specific kits (45 amplification cycles, denaturation at 96°C, primer annealing at 68°C with touchdown to 58°C, amplicon extension at 72°C), except for IL-17RC amplification (45 amplification cycles, denaturation at 99°C, primer annealing at 68°C with touchdown to 58°C, amplicon extension at 72°C). Because of the large number of isoforms ( $>90$ ) generated from the *IL-17RC* gene (30), IL-17RC amplification products have been sequenced (Genome Express). The designed primers led to the amplification of a 191-bp sequence which corresponds to exons 11 through 13. No amplification products corresponding to deletion of exon 12 were detected. The copy number of target mRNA was normalized by the housekeeping gene *GAPDH* or *HPRT1*.

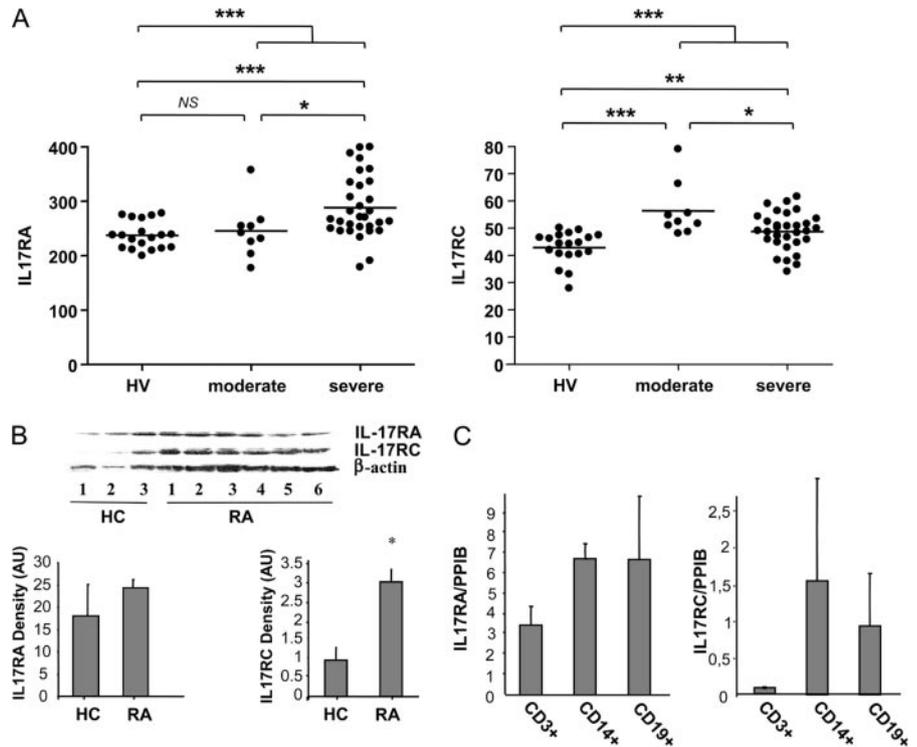
### mRNA microarray hybridization and analysis

Five micrograms of RNA from peripheral whole blood (31 RA patients and 19 healthy volunteers) and 2  $\mu$ g of RNA from in vitro experiment done in RA synoviocytes were analyzed using HG-U133A arrays (Affymetrix), according to the manufacturer's instructions. RNA integrity number was assessed using RNA 6000 nanochips and the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was used to prepare double-stranded cDNA containing the T7 promoter sequence. cRNA was synthesized and labeled with biotinylated ribonucleotide (GeneChip IVT Labeling kit; Affymetrix). The fragmented cRNA was hybridized onto HG-U133A oligonucleotide arrays (22,283 probe sets). The arrays were washed and stained using the fluidic station FS450 (Affymetrix) (protocol EukGE-WS2v4). The array was scanned with the Agilent G2500A GeneArray Scanner. The NETAFFX web site (www.affymetrix.com) was used to select candidate genes.

### Silencing interfering RNA

A mixture of four siRNA duplexes (siGENOME SMARTpool siRNA) specific for IL-17RA (NM\_014339) and IL-17RC (NM\_032732) were purchased from Dharmacon. RA synoviocytes were seeded at a density of  $5 \times 10^5$  cells/60-mm plate before transfection and used at 70–80% confluence. Cells were transfected with control siRNAs (siCONTROL siRNA as a

**FIGURE 1.** IL-17RA and IL-17RC mRNA expression in blood. **A**, IL-17RA and IL-17RC mRNA expression in peripheral whole blood from 40 RA patients (31 patients with severe RA and 9 with moderate RA) and from 19 healthy volunteers (HV), as determined by microarray analysis. Results are presented as fluorescent intensity (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.0005$  by Mann-Whitney  $U$  test). **B**, IL-17RA or IL-17RC were detected in whole blood from HV ( $n = 3$ ) or RA patients ( $n = 6$ ) by Western blotting using actin as a loading control. Density ratios of IL-17RA or IL-17RC were normalized to actin and expressed as AU (\*,  $p < 0.05$  by Mann-Whitney test). **C**, IL-17RA and IL-17RC mRNA expression in peripheral blood subsets from three RA patients. CD14<sup>+</sup> monocytes, CD3<sup>+</sup> lymphocytes, and CD19<sup>+</sup> lymphocytes were isolated from PBMC by positive selection. IL-17RA and IL-17RC mRNA expression were assessed by real-time RT-PCR. PPIB was used to normalize gene expression. Values represent the mean  $\pm$  SEM expression from three RA patients (\*,  $p < 0.05$  by Mann-Whitney test).



negative control and siGLO peptidylpropyl isomerase B (PPIB) (cyclophilin B) as a positive control) or target siRNAs (siGENOME SMARTPool IL-17RA siRNA and IL-17RC siRNA) by nucleofection (Amaxa) according to the manufacturer's instructions (program U23; Human Dermal Fibroblast Nucleofector kit). Dose- and time-response experiments were performed to determine the best time point and the lowest suitable concentration of siRNA duplexes needed for efficacious RNA silencing. Thus, cells were nucleofected with 0.5 or 0.05  $\mu\text{g}$  of either IL-17RA or IL-17RC siRNA duplexes, respectively, per  $5 \times 10^5$  cells. Forty-eight hours posttransfection, the cells were stimulated for 12 or 36 h with IL-17A, IL-17F, or IL-17A plus TNF- $\alpha$ . Some of the results performed with siGENOME SMARTPool reagents were confirmed with the new ON-TARGET plus SMARTPool reagents used to reduce off-target effects (Dharmacon).

#### Western blotting

IL-17RA and IL-17RC expression was measured by Western blot using mouse anti-human mAb against IL-17RA or goat anti-human polyclonal Ab against IL-17RC (R&D Systems) as described previously (31). Protein concentration was measured using the BCA Protein assay reagent kit (Pierce). Eighty micrograms of protein were separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore). Membranes were stripped and serially reprobed with Abs against actin (Chemicon International). Western blots were scanned and densitometry ratios of IL-17RA or IL-17RC were normalized to actin content and expressed as arbitrary units (AU) using Image Gauge software (version 3.46).

#### Statistical analysis

Protein levels are expressed as mean  $\pm$  SEM. mRNA expression of target genes was normalized with GAPDH or HPRT1 mRNA expression in synoviocytes and PPIB mRNA expression in whole blood. Data from functional analysis are expressed as the fold induction compared with untreated controls. For functional experiments in RA synoviocytes, statistical analysis was done using one-way ANOVA and post-hoc comparisons were conducted using Dunnett's  $t$  test. In ex vivo studies, Mann-Whitney's statistical test was used to compare two groups, whereas correlations were done using Spearman's statistical test. Differences resulting in  $p$  values  $< 0.05$  were considered to be statistically significant.

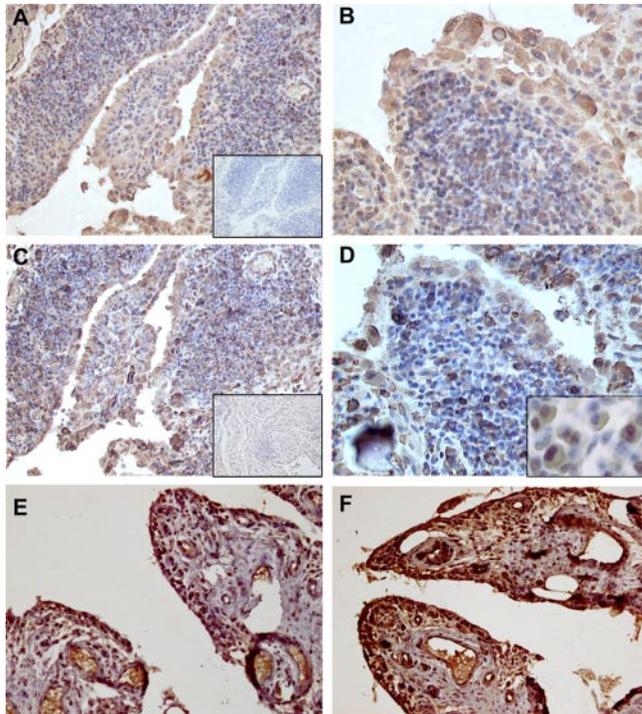
## Results

### IL-17RA and IL-17RC are overexpressed in peripheral whole blood from RA patients

Although the expression of IL-17A has already been shown in peripheral blood from RA patients (32), data concerning its receptors is missing. Thus, to determine whether IL-17RA and IL-17RC expression could be differentially expressed in RA patients when compared with healthy volunteers, we examined their expression at the mRNA level in whole blood using microarrays (Fig. 1A). A significant up-regulation of both IL-17RA and IL-17RC mRNA expression was observed in RA patients ( $n = 40$ ) when compared with healthy volunteers ( $n = 19$ ) (IL-17RA median: 262.5 vs 237.2;  $p = 0.003$  and IL-17RC median: 50.46 vs 44.29;  $p = 0.0002$ ). For IL-17RA mRNA expression, such difference was in fact the consequence of a specific overexpression in patients with severe RA (IL-17RA median in HV vs moderate or severe RA patients: 237.2 vs 241.9 or 270.2, respectively;  $p = 0.8$  or  $p = 0.0004$ ). This was not the case for IL-17RC mRNA expression which was higher in moderate vs severe RA patients (IL-17RC median in HV vs moderate or severe RA patients: 44.3 vs 52.4 or 49.1, respectively;  $p < 0.0001$  or  $p = 0.003$ ).

Because it was recently suggested that IL-17RA and IL-17RC act as a heterodimer to transduce IL-17A signals (26), we analyzed whether the expression of these two receptors was correlated in whole blood cells ( $n = 59$ ). We failed to demonstrate a correlation between the mRNA expression of IL-17RA and IL-17RC ( $R = -0.098$ ,  $p = 0.5$ ). Moreover, it should be noted that IL-17RA mRNA levels were 5-fold higher than those of IL-17RC.

To determine whether IL-17RA and IL-17RC overexpression in RA patients could have a pathophysiological significance, we analyzed their association with clinical activity or severity markers of RA. No correlation was found between IL-17RA or IL-17RC mRNA and DAS 28 index, C-reactive protein, rheumatoid factor, shared



**FIGURE 2.** Detection of IL-17RA and IL-17RC in RA synovium. Immunostaining of paraffin-embedded serial sections using mouse anti-IL-17RA mAb (A and B, respectively,  $\times 200$  and  $\times 400$ ) and goat anti-IL-17RC polyclonal Ab (C and D, respectively,  $\times 200$  and  $\times 400$ ) shows a diffuse positive staining (brown) in both the lining and sublining area. Control staining was performed using mouse IgG1 and goat serum ( $\times 200$ ) (insets in A and C). Immunostaining of IL-17RA and IL-17RC were also performed in OA synovium (E and F, respectively;  $\times 200$ ). Some of the IL-17A-producing cells have a plasmacytoid-like appearance as suggested by the remote nucleus in a large cytoplasm (inset in D,  $\times 600$ ).

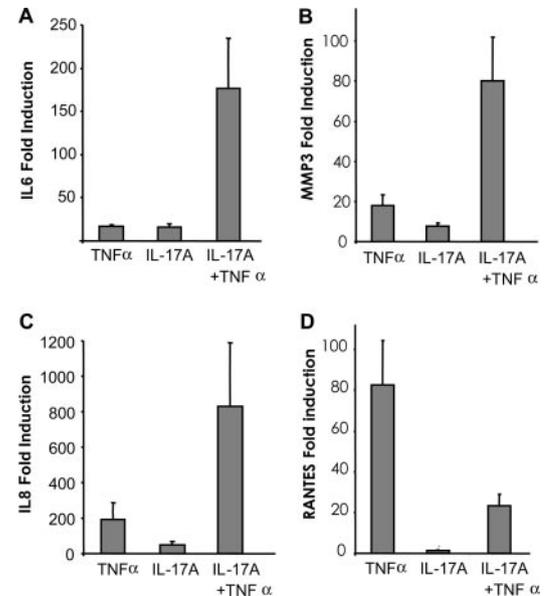
epitope (33), Larsen wrist score, or patient age, sex, or disease duration (data not shown).

IL-17RA and IL-17RC expression was also analyzed at the protein level by Western blotting (Fig. 1B). As for the mRNA level, IL-17RA levels were found to be significantly higher than those of IL-17RC (IL-17RA vs IL-17RC median at the mRNA ( $n = 59$ ) or protein level ( $n = 9$ ): 253.7 vs 48.2;  $p < 0.0001$  or 22.8 vs 2.5;  $p < 0.0001$ , respectively). Moreover, a significant up-regulation of IL-17RC but not of IL-17RA protein expression was observed in whole blood from RA patients when compared with healthy volunteers (IL-17RC median: 0.9 vs 2.9;  $p = 0.02$  and IL-17RA median: 13.0 vs 23.1;  $p = 0.5$ , respectively).

Members of the IL-17 family are known to be widely expressed (14, 34). Nevertheless, we wondered which cell type in RA peripheral blood could express IL-17RA and/or IL-17RC. Thus, we investigated IL-17RA and IL-17RC mRNA expression in T cells, B cells, and monocytes from RA patients by RT-PCR. Both IL-17RA and IL-17RC were expressed in CD14<sup>+</sup> monocytes and CD19<sup>+</sup> lymphocytes. In contrast, lower levels of IL-17RA and IL-17RC were measured in CD3<sup>+</sup> cells (Fig. 1C).

#### *IL-17RA and IL-17RC are broadly expressed in whole synovium tissue from RA patients*

Although IL-17RA and IL-17RC expression has been demonstrated *in vitro* in RA synoviocytes (35, 36), their expression *in situ* in the joint remains unclear. We performed immunohistochemical analysis to investigate whether IL-17Rs were expressed in synovium tissues from RA patients. Both IL-17RA and IL-17RC re-



**FIGURE 3.** Effect of IL-17A, alone or in combination with TNF- $\alpha$ , on the mRNA expression of proinflammatory mediators. After serum deprivation, RA synoviocytes were stimulated for 12 h with IL-17A (50 ng/ml), alone or in combination with TNF- $\alpha$  (0.5 ng/ml). Total RNA was extracted and reverse transcribed. IL-6 (A), MMP-3 (B), IL-8 (C), and RANTES (D) mRNA levels were quantified by real-time RT-PCR. The values were normalized with GAPDH mRNA expression (mean  $\pm$  SEM in untreated cells: 0.20  $\pm$  0.12, 0.08  $\pm$  0.02, 0.05  $\pm$  0.02, 0.0004  $\pm$  0.0001, respectively) and are expressed as fold induction compared with the untreated condition. Values represent the mean  $\pm$  SEM of four independent experiments (\*,  $p < 0.05$  by Dunnett's test).

ceptors were found to be broadly expressed within the synovium of RA patients (Fig. 2, A–D). Moreover, both receptors displayed overlapping staining within the synovium, as demonstrated using serial sections. Such diffuse staining was also observed in osteoarthritis (OA) synovium tissues (Fig. 2, E and F), where IL-17RA and IL-17RC expression was detected in the lining and sublining area. This diffuse staining confirmed the ubiquitous expression of IL-17RA and IL-17RC, both by stromal cells and infiltrating immune cells. We next examined expression of IL-17A in the same samples. IL-17A-positive cells were detected in lymphocytic infiltrates. When studied at high magnification, these IL-17-producing cells had a plasma cell-like morphology with a remote nucleus in a large cytoplasm (inset of Fig. 2D), as we have previously described (37).

#### *IL-17A regulates key inflammatory mediators*

The regulatory effect of IL-17A on RA synoviocytes was examined using microarrays, and confirmed by RT-PCR for four major genes involved in RA pathogenesis: IL-6, MMP-3, IL-8, and RANTES. After 12 h of stimulation, IL-17A stimulation up-regulated TNF- $\alpha$ -induced IL-6, MMP-3, and IL-8/CXCL8 mRNA expression and inhibited TNF- $\alpha$ -induced RANTES/CCL5 mRNA expression. For confirmation, we analyzed target gene expression from independent experiments done with synoviocytes obtained from four different RA patients using RT-PCR (Fig. 3). We confirmed that IL-17A or TNF- $\alpha$  alone significantly induced IL-6 and MMP-3 mRNA expression (15.8  $\pm$  4.1- and 17  $\pm$  2.8-fold induction, respectively, for IL-6,  $p < 0.05$  and 7.6  $\pm$  1.9- and 18.0  $\pm$  4.8-fold induction, respectively, for MMP-3,  $p < 0.05$ ). In the presence of TNF- $\alpha$ , IL-17A synergistically induced IL-6 and MMP-3 mRNA expression (175.9  $\pm$  57.7- and 80.1  $\pm$  22.0-fold

Table I. Effect of IL-17A and/or TNF- $\alpha$  on chemokine mRNA expression in RA synoviocytes, as determined by microarrays<sup>a</sup>

ID	Symbol	Fold Change			p Value		
		IL-17A	TNF- $\alpha$	IL-17A + TNF- $\alpha$	IL-17A	TNF- $\alpha$	IL-17A + TNF- $\alpha$
<b>CXC chemokines</b>							
204470_at	CXCL1	++	+	+++	0.00002	0.000027	0.00002
209774_x_at	CXCL2	+++	++	++++	0.00002	0.00002	0.00002
207850_at	CXCL3	+++	++	++++	0.00002	0.00003	0.00002
215101_s_at	CXCL5	+	++	+++	0.094279	0.000774	0.00002
206336_at	CXCL6				0.00002	0.00002	0.00002
205592_at	CXCL8/IL8				0.5	0.030967	0.5
211506_s_at	CXCL8/IL8	0			0.00002	0.00002	0.00002
203915_at	CXCL9	0	++	+	0.532344	0.078937	0.5
204533_at	CXCL10	0	+	+	0.838962	0.00006	0.000241
210163_at	CXCL11	0	0	0	0.5	0.00002	0.018128
209687_at	CXCL12	0	0	0	0.980376	0.999899	0.999932
205242_at	CXCL13	0	0	0	0.998664	0.958799	0.999886
218002_s_at	CXCL14	0	0	0	0.454766	0.441923	0.284967
<b>CC chemokines</b>							
207533_at	CCL1	0	0	0	0.161038	0.5	0.284967
216598_s_at	CCL2	0	+	+	0.00002	0.00002	0.00002
204103_at	CCL4	0	0	0	0.645558	0.39129	0.065566
204655_at	CCL5	0	+	0	0.998923	0.000101	0.013078
208075_s_at	CCL7	+	+	+	0.00002	0.00002	0.00002
214038_at	CCL8	0	+	+	0.000692	0.000114	0.00002
210133_at	CCL11	0	0	0	0.5	0.185981	0.274048
216714_at	CCL13	0	0	0	0.657563	0.118009	0.633407
205392_s_at	CCL14///CCL15	0	0	0	0.757415	0.5	0.5
207354_at	CCL16	0	0	0	0.153232	0.868657	0.596188
207900_at	CCL17	0	0	0	0.5	0.596188	0.846768
32128_at	CCL18	0	0	0	0.326797	0.5	0.799953
210072_at	CCL19	0	0	0	0.532344	0.5	0.786812
205476_at	CCL20	+++	+++	+++++	0.00002	0.00002	0.00002
204606_at	CCL21	0	0	0	0.342437	0.065566	0.138386
207861_at	CCL22	0	0	0	0.5	0.747149	0.5
210548_at	CCL23	0	0	0	0.041201	0.021224	0.213188
221463_at	CCL24	0	0	0	0.138386	0.467656	0.37888
206988_at	CCL25	0	0	0	0.805199	0.921063	0.958799
207955_at	CCL27	0	0	0	0.596188	0.830902	0.5
<b>CX3C chemokines</b>							
823_at	CX3CL1	0	+	+	0.5	0.000014	0.002991
<b>XC chemokines</b>							
206365_at	XCL1	0	0	0	0.725952	0.961585	0.846768
206366_x_at	XCL2	0	0	0	0.366593	0.232549	0.145682

<sup>a</sup> The fold changes are normalized to untreated fibroblast-like synovial cells. RA synoviocytes were stimulated for 12 h with IL-17A (50 ng/ml), alone or in combination with TNF- $\alpha$  (0.5 ng/ml). The expression of chemokines, which were grouped according to the structural-based classification, and is represented as fold induction when compared to the untreated group (fold induction: 0, 0–2; +, 2–10; ++, 10–100; +++, 100–500; +++++, 500–1000) (HG-U133A microarrays are lacking the probe sets specific for: CXCL4, CXCL7, CCL3, CCL6, CCL9, CCL10, CCL12, CCL26, and CCL28).

induction, respectively). IL-17A alone up-regulated IL-8 mRNA levels by RA synoviocytes ( $47.1 \pm 21.7$ -fold induction) and it synergized with TNF- $\alpha$  to induce a 800-fold induction ( $829.0 \pm 358.1$ -fold induction for IL-17A plus TNF- $\alpha$ ). In contrast, IL-17A inhibited TNF- $\alpha$ -induced RANTES mRNA expression (mean  $\pm$  SEM by TNF- $\alpha$  compared with TNF- $\alpha$  plus IL-17A,  $82.5 \pm 21.9$  compared with  $23.4 \pm 5.8$ ).

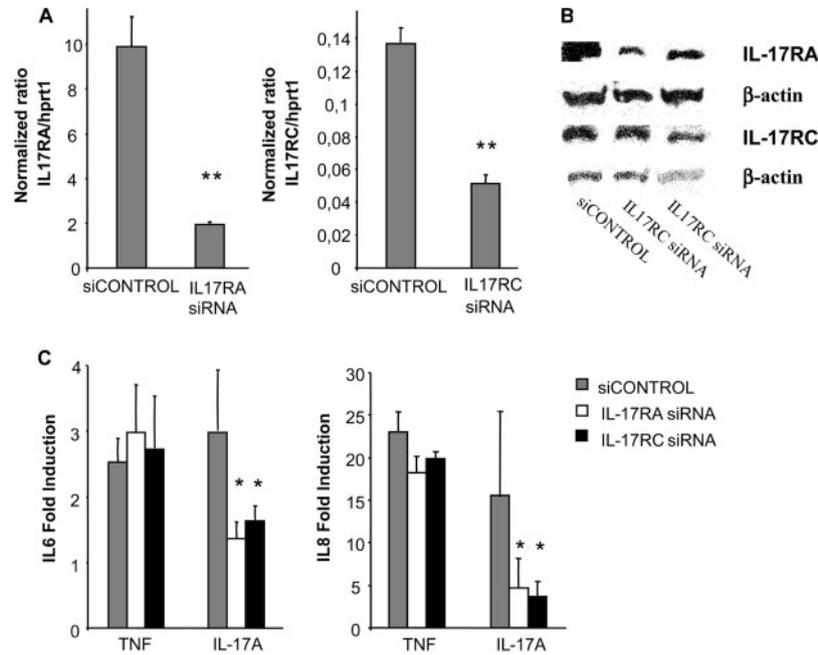
#### IL-17A specifically induced ELR<sup>+</sup> CXC chemokines

To extend the results observed with IL-8, we next analyzed by microarrays the regulatory effect of IL-17A on chemokine expression (Table I). We observed a specific up-regulation of all ELR<sup>+</sup> CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8). In contrast, IL-17A had no effect on the expression of non-ELR CXC chemokines (CXCL9, CXCL10 and CXCL11). Moreover, IL-17A inhibited TNF- $\alpha$ -induced CXCL10 and CXCL11 mRNA expression. Among the CC chemokines, IL-17A had a positive effect on CCL7 and particularly on CCL20 mRNA levels whereas it inhibited CCL2 and CCL5 mRNA levels. Finally, IL-17A inhibited TNF- $\alpha$ -induced CX3CL1 mRNA expression,

whereas it had no effect on C chemokine expression (XCL1 and XCL2).

#### Both IL-17RA and IL-17RC are needed for IL-17A-induced IL-6 secretion

Among the described IL-17R family members, IL-17RA and IL-17RC appear the most critical in mediating cellular responsiveness to IL-17A. Both receptors are expressed by synoviocytes, as demonstrated by baseline expression assessed by real-time RT-PCR. As observed in PBMCs, levels of IL-17RA were 5-fold higher than those of IL-17RC. We investigated the functional contribution of IL-17RA and IL-17RC in mediation of IL-17A biological effects by using siRNA. We first determined the lowest suitable dose of target siRNA to efficiently diminish IL-17RA and IL-17RC expression. A total of 80 and 62% specific knockdown of IL-17RA and IL-17RC mRNA expression were observed at 24 h posttransfection using 0.5 and 0.05  $\mu$ g of siRNA, respectively (Fig. 4A). The knockdown efficiency was also confirmed at the protein level by Western blotting (Fig. 4B). Indeed, we observed a reduced immunostaining using anti-IL-17RA Ab in synoviocytes transfected



**FIGURE 4.** Effect of IL-17RA siRNA and IL-17RC siRNA on IL-17A- or IL-17F-induced IL-6 and IL-8 secretion by RA synoviocytes. RA synoviocytes were transfected with IL-17RA siRNA and IL-17RC siRNA at 0.5 and 0.05  $\mu\text{g}$ , respectively. A scrambled nonsilence siRNA, siCONTROL, was used as a negative control. **A**, Knockdown efficiency of IL-17RA siRNA and IL-17RC siRNA was assessed by real-time RT-PCR. The values represent data obtained 24 h posttransfection and are expressed as mean  $\pm$  SEM of three independent experiments (\*,  $p < 0.05$  by Dunnett's test). **B**, Knockdown efficiency of IL-17RA siRNA and IL-17RC siRNA was assessed by Western blotting 48 h posttransfection, using actin as a loading control. A representative western blot among three separate experiments is shown. **C**, Effect of IL-17RA siRNA and IL-17RC siRNA on IL-17A-induced IL-6 and IL-8 secretion by RA synoviocytes. Forty-eight hours after siRNA delivery (siCONTROL, IL-17RA siRNA, or IL-17RC siRNA), RA synoviocytes were stimulated for 12 h with IL-17A at 50 ng/ml or TNF- $\alpha$  at 0.5 ng/ml. IL-6 and IL-8 levels were quantified in removed supernatants by ELISA. The values are expressed as fold induction compared with the untreated condition (mean  $\pm$  SEM in untreated cells transfected with siCONTROL siRNA: 2.4  $\pm$  0.8 ng/ml and 367  $\pm$  39.6 pg/ml, respectively) and are represented the mean  $\pm$  SEM of three independent experiments (\*,  $p < 0.05$  by Dunnett's test).

with IL-17RA siRNA duplexes, and a reduced immunostaining using anti-IL-17RC Ab in synoviocytes transfected with IL-17RC siRNA duplexes. Forty-eight hours after transfection with IL-17RA siRNA, IL-17RC siRNA or the scrambled negative control (siCONTROL), RA synoviocytes were stimulated with IL-17A or IL-17F for 12 h and supernatants analyzed for IL-6 and IL-8 levels by ELISA. Stimulation with TNF- $\alpha$  alone was used as a control. As shown in Fig. 4C, TNF- $\alpha$ -induced IL-6 or IL-8 secretion by RA synoviocytes was not significantly affected by IL-17RA or IL-17RC knockdown (mean  $\pm$  SEM by IL-17RA siRNA or IL-17RC siRNA compared with siCONTROL siRNA transfected cells: 2.9  $\pm$  0.7 or 2.7  $\pm$  0.7 compared with 2.5  $\pm$  0.4 respectively,  $p$  was NS for IL-6 secretion and 12.8  $\pm$  1.8 or 19.8  $\pm$  1.2 compared with 23.0  $\pm$  2.4 respectively,  $p$  was NS for IL-8 secretion), whereas IL-17A-induced IL-6 or IL-8 secretion was significantly reduced after both IL-17RA knockdown and IL-17RC knockdown (mean  $\pm$  SEM by IL-17RA siRNA and IL-17RC siRNA compared with siCONTROL siRNA: 1.3  $\pm$  0.2 and 1.6  $\pm$  0.3 compared with 3  $\pm$  0.9, respectively,  $p < 0.05$  for IL-6 secretion and 4.7  $\pm$  3.6 and 3.6  $\pm$  1.8 compared with 15.6  $\pm$  9.9, respectively,  $p < 0.05$  for IL-8 secretion) (Fig. 4B). However, the contribution of both receptors to IL-17F response could not be estimated because of the low induction of proinflammatory cytokines by IL-17F alone (IL-6 and IL-8-fold induction compared with the untreated situation  $\pm$  SEM, 1.52  $\pm$  0.48 and 1.28  $\pm$  0.28).

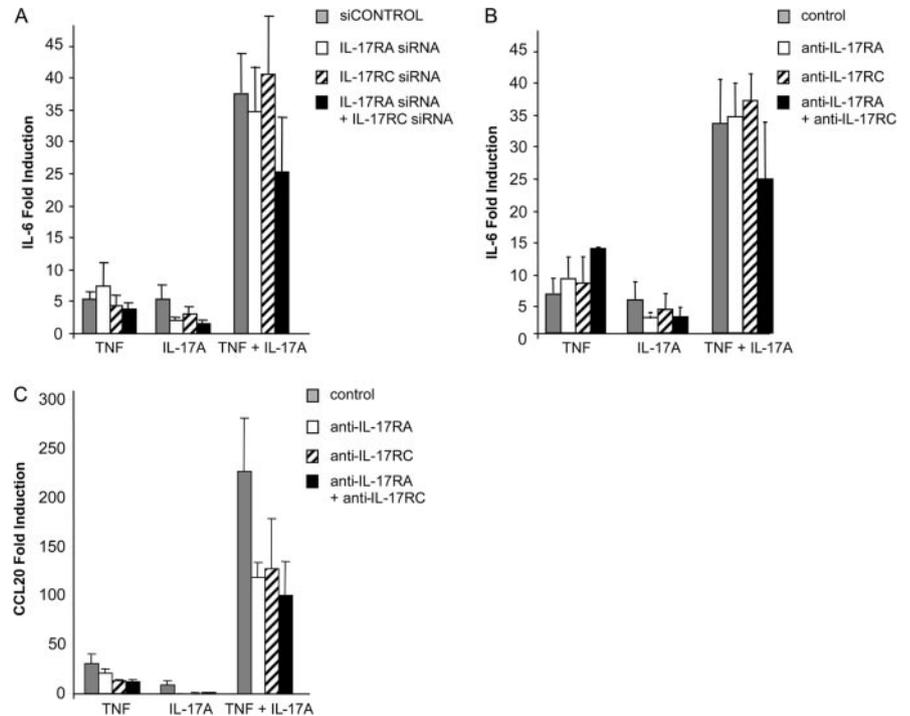
*In the presence of TNF- $\alpha$ , the combined inhibition of IL-17RA and IL-17RC is needed to diminish IL-17A-induced IL-6 or CCL20 secretion by RA synoviocytes*

We then investigated the efficiency of IL-17RA and IL-17RC inhibition on IL-17A-induced IL-6 and CCL20 secretion in the pres-

ence of TNF- $\alpha$ , this latest cytokine being critical to consider with clinical applications in mind. Thus, transfected RA synoviocytes were stimulated with IL-17A alone or in combination with TNF- $\alpha$ , and supernatants were analyzed for IL-6 levels by ELISA. As shown in Fig. 5A, after 36 h of stimulation, IL-17RA or IL-17RC knockdown alone had no effect on IL-17A plus TNF-induced IL-6 secretion (mean  $\pm$  SEM by IL-17RA siRNA and IL-17RC siRNA compared with siCONTROL siRNA-transfected cells, 33.6  $\pm$  5.1 and 36.1  $\pm$  4.1 compared with 32.6  $\pm$  6.6;  $p > 0.9$ ), whereas the combination of IL-17RA siRNA and IL-17RC siRNA had an inhibitory effect (mean  $\pm$  SEM by IL-17RA siRNA plus IL-17RC siRNA compared with siCONTROL siRNA transfected cells, 23.9  $\pm$  8.8 compared with 32.6  $\pm$  6.6;  $p < 0.05$ ).

The effect of IL-17RA knockdown by siRNA was then compared with its extracellular blockade using neutralizing Abs against IL-17RA or IL-17RC. RA synoviocytes were preincubated with anti-IL-17RA and/or anti-IL-17RC Abs for 2 h and then stimulated for 36 h with IL-17A alone or in combination with TNF- $\alpha$ . IL-6 and CCL20 levels were analyzed by ELISA (Fig. 5, B and C). Results similar to those observed using siRNA were obtained: anti-IL-17RA and -IL-17RC Abs decreased IL-17A-induced IL-6 or CCL20 secretion by RA synoviocytes (mean  $\pm$  SEM by anti-IL-17RA or -IL-17RC Ab compared with the situation without inhibitors, 2.0  $\pm$  0.7 or 3.0  $\pm$  1.1 compared with 5.2  $\pm$  1.8;  $p < 0.05$  for IL-6 secretion and 0.0  $\pm$  0.2 and 1.9  $\pm$  0.8 compared with 8.8  $\pm$  4.6;  $p < 0.05$  for CCL20 secretion), but had no suppressive effects in the presence of TNF- $\alpha$  (mean  $\pm$  SEM by anti-IL-17RA Ab and -IL-17RC Ab compared with the situation without inhibitors, 34.7  $\pm$  6.8 and 40.6  $\pm$  9.1 compared with 37.6  $\pm$  6.3; NS for IL-6 secretion and 20.5  $\pm$  4.4 and 12.5  $\pm$  1.2 compared with 20.6  $\pm$  9.7; NS for CCL20 secretion). Moreover, the combination

**FIGURE 5.** Effect of IL-17RA and/or IL-17RC inhibition by siRNA or extracellular inhibitors, on IL-17 plus TNF- $\alpha$ -induced IL-6 secretion. **A**, Efficiency of posttranscriptional inhibition of IL-17RA and/or IL-17RC using siRNA. RA synoviocytes from the three cell populations (transfected with siCONTROL, IL-17RA siRNA, and IL-17RC siRNA) were stimulation for 36 h with IL-17A (50 ng/ml), alone or in combination with TNF- $\alpha$  (0.5 ng/ml). IL-6 levels were quantified in removed supernatants by ELISA. The values represent the mean  $\pm$  SEM of three independent experiments (\*,  $p < 0.05$  by Dunnett's test). **B** and **C**, Efficiency of extracellular inhibition using Abs. RA synoviocytes were preincubated for 2 h (37°C, 5% CO<sub>2</sub>) with anti-IL-17RA Ab (10  $\mu$ g/ml) and/or anti-IL-17RC Ab (10  $\mu$ g/ml). The cells were then stimulated with IL-17A (50 ng/ml), alone or in combination with TNF- $\alpha$  (0.5 ng/ml). IL-6 and CCL20 levels were quantified in removed supernatants by ELISA. The values represent the mean  $\pm$  SEM of three independent experiments (mean  $\pm$  SEM in untreated cells: 1.7  $\pm$  0.6 ng/ml and 1.4  $\pm$  1.6 pg/ml, respectively) (\*,  $p < 0.05$  by Dunnett's test).



of anti-IL-17RA and IL-17RC Abs induced a decrease of IL-17A plus TNF- $\alpha$ -induced IL-6 secretion (mean  $\pm$  SEM by anti-IL-17RA Ab plus anti-IL-17RC Ab compared with the situation without inhibitors, 25.3  $\pm$  8.6 compared with 37.6  $\pm$  6.3 for IL-6 secretion and 226.7  $\pm$  54.4 pg/ml compared with 100.6  $\pm$  59.7;  $p < 0.05$  for CCL20 secretion).

## Discussion

RA is a systemic inflammatory disease characterized by chronic synovium inflammation leading to matrix destruction. RA synovium and synovial fluid are highly infiltrated by immune cells. Among them, activated CD4<sup>+</sup> T cells contribute to RA pathogenesis with a pivotal role of the newly characterized Th17 cells, which secrete IL-17A and IL-17F, IL-22, TNF- $\alpha$ , and IL-6 (38–41).

We showed that IL-17A synergized with TNF- $\alpha$  to induce IL-6 mRNA expression by RA synoviocytes. IL-6 is an important proinflammatory cytokine, regulating both local and systemic inflammation. Moreover, IL-6 was recently demonstrated to be a major mediator of Th17 differentiation. This differentiation pathway presents unique features because of its relationship with regulatory T cell developmental program. Indeed, in mouse models, the differentiation of CD4<sup>+</sup> naive T cells into regulatory T cells in the presence of TGF- $\beta$  is completely inhibited by IL-6, whereas it promotes Th17 cells. The developmental program induced by IL-6 in the presence of TGF- $\beta$  induces the expression of the nuclear receptor ROR $\gamma$ t which controls Th17 differentiation leading to IL-17A and IL-17F secretion (42). Thus, IL-17A-induced IL-6 secretion by RA synoviocytes results in a positive feedback which could be involved in the promotion of local Th17 induction.

One key property of IL-17A is its orchestral role in mediating the migration of inflammatory cells, which takes a central place in RA pathogenesis (43). The list of chemokines has been growing extensively, a condition where the use of extensive gene analysis is well-suited to compare levels. Chemokine secretion by activated RA synoviocytes regulates different phases in this multistep pro-

cess: recruitment through the activation of endothelial cells, local retention and activation of immune cells, and transmigration to the synovial fluid. Data obtained using microarrays suggested that IL-17A promotes the selective expression of ELR<sup>+</sup> CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8) in RA synoviocytes, especially in the presence of TNF- $\alpha$ , whereas it has no effect or an inhibitory effect on the expression of ELR<sup>-</sup> CXC chemokines (CXCL9, CXCL10, CXCL11, and PF4). This specific regulatory effect of IL-17A highlights its role in neutrophil recruitment into the joint, but also supports its specific promoting effect on angiogenesis. Indeed, the ELR motif has been implicated in the neutrophil attractant properties of CXC chemokines, and in their angiogenic effects. IL-17A regulatory effects on angiogenic (ELR<sup>+</sup>) vs angiostatic (non-ELR) chemokines are just opposite to those of IFN- $\gamma$ . Indeed, it was demonstrated that IFN- $\gamma$  inhibits angiogenesis in part through the inhibition of ELR<sup>+</sup> CXC chemokines (44).

In the group of CC chemokines, we observed an inhibitory effect of IL-17A on TNF- $\alpha$ -induced CCL2, CCL5, and CX3CL1 mRNA expression in RA synoviocytes, which suggests a negative effect on the migration of mononuclear cell types including activated T cells, macrophages, NK cells, and regulatory T cells. In contrast, IL-17A up-regulated the expression of the CC chemokines, CCL20 and CCL7, which are both expressed in RA joint (45–47). CCL20 is implicated in the local recruitment of CCR6-positive cells, which include immature dendritic cells, naive B cells, and memory T cells (18, 24, 46, 47). Indeed, we have previously demonstrated the expression of CCR6 in subsets of IL-17- and IFN- $\gamma$ -producing cells within RA synovium (45).

Among IL-17R family members, we confirmed the expression of IL-17RA and IL-17RC by RA synoviocytes. Using both siRNA delivery and extracellular inhibitors, we showed that the inhibition of IL-17RA or IL-17RC alone was sufficient to induce a significant reduction in IL-17A-induced IL-6, IL-8, and CCL20 secretion, demonstrating that both IL-17RA and IL-17RC are implicated in transducing IL-17A signals in RA synoviocytes. This result is consistent with the current concept of a functional receptor composed

of IL-17RA and IL-17RC subunits (26). Nevertheless, we observed that the inhibition of IL-17RA or IL-17RC when used alone failed to significantly reduce IL-17A and TNF- $\alpha$ -induced IL-6 or CCL20 secretion. In that case, the combined inhibition of IL-17RA and IL-17RC was needed for a significant decrease in IL-17A and TNF- $\alpha$ -induced IL-6 or CCL20 secretion. At the mRNA level, IL-17RA had a 5-fold higher expression than IL-17RC systemically and locally. However, although there was a trend to more potent inhibition of synovial IL-17A-induced IL-6 by IL-17RA antagonism compared with IL-17RC inhibition, this was not statistically significant. This suggests that despite increased expression of IL-17RA compared with IL-17RC, their absolute expression levels may be less important compared with functional outcomes following antagonism.

In conclusion, we demonstrated that IL-17RA and IL-17RC are overexpressed systemically in RA patients. This overexpression could make synovial cells and immune cells more sensitive to IL-17, a major regulatory cytokine implicated in RA pathogenesis. Finally, we showed that both IL-17RA and IL-17RC are implicated in IL-17A-induced inflammatory signals in RA synovial cells. These receptors could be targets for therapeutic intervention, in combination with conventional anti-TNF treatment.

## Disclosures

The authors have no financial conflict of interest.

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