New pro-inflammatory cytokine IL-40 is produced by activated neutrophils and plays a role in the early stages of seropositive rheumatoid arthritis

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ABSTRACT

Objective Interleukin (IL)-40 is a new cytokine related to immune system function and malignancies. Recently, an association of IL-40 with rheumatoid arthritis (RA) and externalisation of neutrophil extracellular traps (NETosis) was found. As neutrophils are implicated in RA development, we investigated IL-40 in early stages of RA (ERA).

Methods IL-40 was determined in serum of treatment naive patients with ERA at baseline (n=60) and 3 months after initiation of conventional therapy and in healthy controls (HC; n=60). Levels of IL-40, cytokines and NETosis markers were measured by ELISA. NETosis was visualised by immunofluorescence. In vitro experiments were performed on peripheral blood neutrophils from ERA patients (n=14). Cell-free DNA was analysed in serum and supernatants.

Results Serum IL-40 was elevated in ERA compared with HC (p<0.0001) and normalised after 3 months of therapy (p<0.0001). Baseline serum IL-40 correlated with rheumatoid factor (IgM) (p<0.01), anti-cyclic citrullinated peptide (p<0.01) autoantibodies and NETosis markers (proteinase 3; neutrophil elastase (NE); myeloperoxidase) (p<0.0001). Levels of NE significantly decreased after therapy (p<0.01) and correlated with the decrease of serum IL-40 (p<0.05). In vitro, neutrophils enhanced IL-40 secretion following NETosis induction (p<0.001) or after exposure to IL-1β, IL-8 (p<0.05), tumour necrosis factor or lipopolysaccharide (p<0.01). Recombinant IL-40 up-regulated IL-1β, IL-6 and IL-8 (p<0.05 for all) in vitro.

Conclusion We showed that IL-40 is significantly up-regulated in seropositive ERA and decreases after conventional therapy. Moreover, neutrophils are an important source of IL-40 in RA, and its release is potentiated by cytokines and NETosis. Thus, IL-40 may play a role in ERA.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by persistent synovial inflammation and articular damage.1 During the last two decades, characterisation of new cytokines and their signalling pathways has helped to develop new treatment strategies in RA2; however, there is still a considerable number of patients who do not respond to the standard-of-care therapy or progress rapidly after the onset of symptoms.3 Timely diagnosis offers the opportunity of starting the targeted therapy at an earlier stage of arthritis within the ‘window of opportunity’ and thereby preventing the irreversible joint damage and disability.4 As patients with early RA (ERA) show a specific cytokine profile, which is transient and no longer
present in long-standing RA,\textsuperscript{5,6} it is urgent to search for new cytokines of the early phase of RA that could potentially serve as predictive biomarkers of RA development or treatment response.

Interleukin (IL)-40 (encoded by the C17orf99 gene) represents one of the newly described cytokines implicated in autoimmune inflammation.\textsuperscript{7–12} IL-40 was identified in 2017 as a small (27 kDa) secreted protein with a unique structure distinct from any known cytokine family.\textsuperscript{7} Initially, IL-40 was detected in organs associated with the immune system such as the fetal liver, bone marrow or activated B-cells potentiated by IL-4 and transforming growth factor (TGF\(\beta\))-1.\textsuperscript{7,8,13} Recent data show that IL-40 is also expressed by other leucocytes or fibroblasts.\textsuperscript{9,14} In mice, IL-40 expression peaks with lactation, and C17orf99/ mice exert microbiome alternations, reduced IgA levels and impaired B-cell functions.\textsuperscript{7} In addition, a link between IL-40 and malignancy has been proposed based on the differential expression of IL-40 found in B-cell lymphomas.\textsuperscript{7,14,15}

To date, very little is known about the involvement of IL-40 in immune processes. In 2012, IL-40/C17orf99 was identified as one of the specific markers distinguishing autoimmune hepatitis from healthy individuals.\textsuperscript{16} A recent study by Gao et al revealed that IL-40 expression decreases under anti-inflammatory conditions in vitro.\textsuperscript{17} Moreover, emerging evidence shows an implication of IL-40 in the pathogenesis of autoimmune rheumatic diseases.\textsuperscript{9–12} Rizzo et al demonstrated that IL-40 is elevated in the serum and salivary glands of patients with primary Sjögren syndrome (SS), and the tissue expression of IL-40 correlates with the expression of pro-fibrotic molecules IL-4 and TGF-\(\beta\).\textsuperscript{7} In addition, recent data imply that altered expression of IL-40 may be associated with lupus nephritis.\textsuperscript{10} Very recently, an upregulation of IL-40 in RA was demonstrated.\textsuperscript{11,12} Based on our study on RA,\textsuperscript{11} the elevation of IL-40 in RA is associated with disease activity, autoantibodies and the externalisation of neutrophil extracellular traps (NETosis). We also demonstrated that elevated serum levels of IL-40 significantly decreased following B-cell depletion therapy (rituximab) in RA patients. According to our in vitro studies, IL-40 acts as an inducer of chemokines, and metalloprotease release by RA synovial fibroblasts.\textsuperscript{11}

Based on the above-mentioned considerations, we aimed to assess the implication of IL-40 in the early phase of RA development by analysing serum levels of IL-40 in treatment-naïve ERA patients and investigating the relationship between IL-40 and neutrophils.

**MATERIALS AND METHODS**

**Patients**

The study enrolled 60 treatment naïve ERA patients (40 women and 20 men) with a mean (SD) age of 52.4 (15.9) years and a symptom duration of up to 6 months. Serum from ERA patients was obtained in two intervals: at baseline and 3 months after the introduction of conventional pharmacological therapy. The patients fulfilled the 2010 American College of Rheumatology/EULAR criteria.\textsuperscript{18} The control group consisted of 60 sex-matched and age-matched healthy individuals (healthy controls (HC), 40 women and 20 men) with a mean (SD) age of 52.4 (12.9) years. The characteristics of all participants are listed in table 1. For all in vitro experiments, peripheral blood from ERA patients was obtained (n=14 in total).

**Laboratory measurements**

Serum samples from ERA patients (n=60) and HC (n=60) were obtained and immediately stored at −80°C.

**Table 1** Laboratory and clinical parameters of ERA patients and healthy controls (HC)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ERA (n=60)</th>
<th>HC (n=60)</th>
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<tbody>
<tr>
<td>0 months (baseline)</td>
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<td></td>
</tr>
<tr>
<td>Patients, n</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Females/males, n</td>
<td>40/20</td>
<td>40/20</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.39 (2.06)</td>
<td>52.43 (1.66)</td>
</tr>
<tr>
<td>DAS28 ESR</td>
<td>5.33 (0.19)</td>
<td>2.89 (0.20)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>17.58 (2.90)</td>
<td>5.91 (0.20)</td>
</tr>
<tr>
<td>Anti-CCP positivity, n (%)</td>
<td>30 (50%)</td>
<td>–</td>
</tr>
<tr>
<td>RF positivity, n (%)</td>
<td>38 (63%)</td>
<td>–</td>
</tr>
<tr>
<td>csDMARDs/GCs, n (%)</td>
<td>–</td>
<td>54/51 (90/85%)</td>
</tr>
<tr>
<td>Methotrexate, n (%)</td>
<td>–</td>
<td>44 (73%)</td>
</tr>
<tr>
<td>Sulfasalazine, n (%)</td>
<td>–</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Leflunomide, n (%)</td>
<td>–</td>
<td>1 (1.5%)</td>
</tr>
<tr>
<td>3 months (after treatment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients, n</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Females/males, n</td>
<td>40/20</td>
<td>40/20</td>
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<td>Age, years</td>
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<td>DAS28 ESR</td>
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<td>CRP, mg/L</td>
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<td>Anti-CCP positivity, n (%)</td>
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<td>RF positivity, n (%)</td>
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<tr>
<td>csDMARDs/GCs, n (%)</td>
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<td>Methotrexate, n (%)</td>
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<td>Sulfasalazine, n (%)</td>
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<tr>
<td>Leflunomide, n (%)</td>
<td>–</td>
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</tbody>
</table>

Data are expressed as mean (SEM).

anti-CCP, anti-cyclic citrullinated peptide antibody; CRP, C reactive protein; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; DAS28 ESR, disease activity score in 28 joints with erythrocyte sedimentation rate; ERA, early stages of rheumatoid arthritis; RF, rheumatoid factor.
Disease activity was assessed by the Disease Activity Score for 28 joints (DAS28) using erythrocyte sedimentation rate. C reactive protein (CRP) was measured by turbidimetry (Olympus Biochemical Analyser; Olympus, Tokyo, Japan), and the levels of serum anti-cyclic citrullination peptide (anti-CCP) antibodies and IgM rheumatoid factor (IgM-RF) were evaluated by ELISA kits (both from TestLine Clinical Diagnostics, Brno, Czech Republic). Antinuclear antibodies (ANA) were detected by indirect immunofluorescence (Immuno Concepts, Sacramento, CA, USA) and further characterised by the Line Immuno Assay (LIA) method (IMTEC, Wiesbaden, Germany).

In vitro experiments on neutrophils from patients with ERA

Neutrophils were separated from the peripheral blood of ERA patients using Ficoll gradient separation and dextran sedimentation, followed by hypotonic lysis of erythrocytes as previously described. The viability and the cell count of isolated neutrophils were determined by Countess II FL Automated cell counter (Thermo Fisher, Waltham, MA, USA) using Trypan Blue staining (mean 95%). The purity of isolated neutrophils was assessed by flow cytometry (CD45, CD15 and CD16 positive cells, mean 98%). As isolation technology may affect neutrophil function, we reproduced part of our in vitro experiments on neutrophils purified using negative selection by magnetic beads (EasySep Magnet and EasySep Direct Human Neutrophil Isolation Kit, StemCell Technologies, San Diego, CA, USA). All isolated neutrophils were transferred into 6-well plates (1×10⁶ per well) in RPMI-1640 culture medium (Thermo Fisher) containing 10% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France), 0.2% GlutaMax (Thermo Fisher) and 0.2% HEPES solution (both from Sigma-Aldrich, St. Louis, MO, USA).

To assess the effect of extracellular IL-40 on neutrophils in vitro, neutrophils were stimulated with recombinant IL-40 (10, 50, 100 and 250 ng/mL, Aviva Systems Biology, San Diego, CA, USA) and with lipopolysaccharide as a positive control (LPS, 100 ng/mL, LPS from E. coli O26:B6, Sigma-Aldrich) for an interval of 4 hours and/or 24 hours at 37°C in a humidified atmosphere of 5% CO₂. Simultaneously, to induce NETosis, neutrophils were treated with phorbol 12-myristate-13-acetate (PMA; 100 nM, Sigma-Aldrich) for 4 hours under the conditions described above. In order to evaluate the potential inducers of IL-40, neutrophils were treated with recombinant proteins IL-1β, IL-6 and IL-8, tumour necrosis factor (TNF) (all 10 ng/mL, R&D Systems, Minneapolis, MN, USA), and LPS for 24 hours as described above. Cell supernatants were collected, centrifuged at 86 g for 10 min at room temperature (RT), and stored at −80°C until use. LPS serotype from E. coli O26:B6 (Sigma-Aldrich) was selected as a positive control that induces an inflammatory response but not NETosis. The IL-40 dose was selected based on the serum and synovial fluid levels found in patients with long-standing RA.

Immunofluorescence visualisation of NETosis

Following isolation, ERA neutrophils were seeded onto poly-L-lysine (Sigma-Aldrich) coated coverslips (3×10⁴ cells per coverslip) which were placed into the 12-well plates with 500 µL of cultivation medium and treated by IL-40 (250 ng/mL) and PMA (100 nM) in concentrations and conditions described above. After 4 hour, neutrophils were fixed with 4% formalin solution (Sigma-Aldrich) for 30 min at RT. 2x washed with PBS and incubated with wheat germ agglutinin (WGA) Alexa Fluor 488 conjugate (5 ng/mL, Thermo Fisher) for 10 min at RT. Consequently, cells were 3x washed with PBS and permeabilized with 0.2% Triton X (Sigma-Aldrich) for 1 min at RT. Next, cells were 3x washed with PBS and incubated with 2% bovine serum albumin (BSA) in PBS for 1 hour at RT. Slides were incubated with mouse anti-human myeloperoxidase (MPO) monoclonal antibody over night at 4°C (1:200, Invitrogen, Carlsbad, CA, USA) and C17orf99 rabbit anti-human polyclonal antibody (1:700, Sinobiological, Eschborn, Germany) for 3 hours at RT. Following 3x wash in PBS, slides were treated with secondary antibodies goat anti-mouse Abberior STAR RED and goat anti-rabbit Abberior STAR 580 (both 1:500, Abberior Instruments GmbH, Göttingen, Germany) for 1 hour at RT. Finally, slides were 3x washed with PBS, treated with 4’,6-diamidino-2-phenylindole (DAPI, 1:10000, Invitrogen) for 10 min at RT, samples were 3x washed in PBS and 1x in distilled water and mounted with Abberior Mount Liquid Antifade (Abberior Instruments GmbH). All antibody dilutions were made in 2% BSA in PBS. All incubations were performed in humid chamber.

Microscopy analysis

The images were obtained using a combination of confocal and superresolution microscopy, DAPI and WGA 488 were acquired in confocal mode and images of MPO (STAR RED) and IL-40 (STAR 580) in cell detail were acquired using stimulated emission depletion (STED) microscopy. The imaging was performed on an Abberior Expert Line STED system (Abberior Instruments GmbH) equipped with a Nikon Eclipse Ti-E body (Nikon, Tokyo, Japan), QUAD beam scanner and Nikon CFI Plan Apo 60x/NA 1.40 oil-immersion objective (Nikon). The samples were illuminated with 405 nm, 485 nm, 561 nm and 640 nm lasers. The fluorescence excited with 561 and 640 nm laser was depleted by a pulsed 775 nm STED laser in 2D donut shape. The fluorescence signal passed the pinhole set to 1 AU, was filtered by emission filters (422–467 nm, 506–594 nm, 580–630 nm and 650–720 nm) and the signal was detected with single-photon counting detectors (Excitellas Technologies, Waltham, MA, USA). The images were obtained by the Inspector software (Abberior Instruments GmbH).

Analysis of IL-40, cytokines and NETosis markers

IL-40 levels in the serum and supernatants were assessed by a commercially available ELISA kit (MyBioSource, San Diego, CA, USA) with a detection limit of 0.25 ng/
mL and inter-assay and intra-assay reliability <15%. The levels of NETosis-related markers, such as MPO, neutrophil elastase (NE) (both Abcam, Cambridge, UK) and proteinase 3 (PR3) (BioVendor, Brno, Czech Republic), were measured using commercially available ELISA kits in the serum and supernatants. Similarly, the levels of TNF, IL-1β, IL-6 and IL-8 were analysed by ELISA kits in the supernatants (Invitrogen). All ELISA tests were performed according to the manufacturer’s instructions, and the analysis was performed at 450 nm using a Sunrise ELISA reader (Tecan, Salzburg, Austria).

**Analysis of cell-free DNA**

Circulating cell-free DNA (cfDNA) was isolated from the serum of patients with ERA and from neutrophil supernatants by use of QIAamp Circulating Nucleic Acid Kit (Quiagen, Hilden, Germany). The concentration of cfDNA was analysed by Agilent High Sensitivity DNA Kit on Agilent 2100 Bioanalyzer (both Agilent Technologies, Santa Clara, CA, USA).

**Statistical analysis**

Data are presented as mean (SEM) unless stated otherwise. The normal distribution was determined using the Kolmogorov-Smirnov test. Wilcoxon matched-pairs signed rank test was used to compare IL-40 levels in ERA patients at baseline and after 3 months of therapy. Fisher’s exact test was used to determine if the proportion of patients with decreasing IL-40 significantly differs between double seropositive and seronegative patients. It was also used for in vitro experiments to compare cytokines levels in treated versus non-treated neutrophils. Mann-Whitney test was used to assess the differences in IL-40 levels between HC and ERA patients. The association of IL-40 with laboratory and clinical parameters and cytokines was determined by Spearman correlation. The results were considered significant when p was less than 0.05 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). For data analysis, GraphPad Prism 6 software was used (GraphPad Software, San Diego, CA, USA).

**RESULTS**

**Increased IL-40 correlates with autoantibody levels and decreases after conventional treatment in the serum of ERA patients**

The levels of IL-40 were elevated in the serum of ERA patients (n=60) compared with HC (n=60) (4.51 (0.91) vs 1.47 (0.09) ng/mL, p<0.0001) and were normalised after 3 months of conventional treatment (4.51 (0.91) vs 1.98 (0.24) ng/mL; p=0.0001) (figure 1A). Moreover, levels of IL-40 were significantly higher in double-seropositive (RF (IgM)’anti-CCP’ (n=23) compared with double-seronegative (RF (IgM)’anti-CCP’ (n=26) patients (7.77 (2.15) vs 1.95 (0.23) ng/mL; p=0.0001) and also in double-seronegative patients compared with HC (1.95 (0.23) vs 1.47 (0.09) ng/mL, p<0.01) (see colour coding in figure 1A). The decrease of IL-40 levels following the therapy was more pronounced in double seropositive (p=0.0001) in contrast to double seronegative (p<0.05) patients (figure 1B,C). Using Fisher’s exact test, we showed that the proportion of patients with decreasing IL-40 in both groups is significant (p=0.026).

We were interested in whether patients with high and low IL-40 levels have a different treatment response. Therefore, we divided the group of seropositive patients into two subgroups: high and low IL-40 (cut-off value 6 ng/mL) and analysed the effect of treatment on inflammation and disease activity in each subgroup. When evaluating the treatment response in double seropositive RA patients with high and low IL-40 levels, we found no significant difference in the decrease of CRP levels (p<0.01 for both). However, double seropositive patients with high IL-40 levels appeared to have a better statistically significant improvement in clinical disease activity as assessed by DAS28 (p<0.01) compared with patients with low IL-40 levels (p<0.05). Thus, it can be speculated that elevated IL-40 levels in the context of seropositivity may represent a suitable biomarker of treatment response.

In all ERA patients, serum IL-40 strongly correlated with the levels of both autoantibodies RF (IgM) (r=0.461, p<0.01) and anti-CCP (r=0.407, p<0.01) (n=60) (figure 1D,E). In addition, 16 out of 60 patients with ERA tested positive for ANA autoantibodies; however, the levels of IL-40 were comparable between ANA positive and ANA negative patients (figure 1F). IL-40 did not correlate with any other clinical or laboratory parameters of interest, with the exception of a weak positive correlation (r=0.290, p=0.035) of IL-40 with CRP levels after 3 months of therapy (online supplemental data 1).

**Serum IL-40 is associated with NETosis markers and with the changes of serum NE levels after therapy in patients with ERA**

Given our previous finding of the link between IL-40 and NETosis in RA,11 we sought to analyse this phenomenon in the early phase of RA. Here we show that the levels of NETosis markers MPO, PR3 and NE were up-regulated in all ERA patients at baseline (n=60) in contrast to HC (n=60) MPO: 125.80 (16.01) versus 43.59 (2.95) ng/mL; p<0.0001, PR3: 204.30 (18.05) versus 103.80 (6.55) ng/mL; p<0.0001 and NE: 360.00 (35.63) versus 122.90 (9.43) ng/mL; p<0.0001) (figure 2A). At baseline, the levels of IL-40 correlated with peripheral blood neutrophil count (r=0.351, p<0.05) (figure 2B) and with the surrogate NETosis markers (n=60) such as neutrophil granule enzymes PR3 (r=0.581, p<0.0001), NE (r=0.613, p<0.0001), MPO (r=0.593, p<0.0001) (figure 2C–E) and with the levels of cfDNA (r=0.314, p=0.066) (n=35) (figure 2F).

Moreover, the levels of prime neutrophil granule enzymes MPO and NE positively correlate with the increased levels of cfDNA (n=35) (MPO: r=0.355, p=0.036; NE: r=0.450, p=0.007), which rules out possible causes of their upregulation other than NETosis. Only a trend towards the association between PR3 and cfDNA was found (r=0.291, p=0.090). To assess whether the therapy modulates the serum IL-40 levels simultaneously with
NETosis, we analysed levels of NE, as one of the NETosis parameters, in ERA patients both before and after the therapy. We showed a significant decrease of the serum NE after therapy (361.80 (35.08) vs 213.40 (23.40) ng/mL; p=0.002), which was positively associated with the downregulation of serum IL-40 (r=0.360, p<0.05) (n=46).

**Activated neutrophils increase IL-40 secretion, which, in turn, can promote a positive inflammatory feedback loop**

The association of IL-40 with activated neutrophils is underlined by our in vitro experiments showing an abundant release of IL-40 by ERA neutrophils undergoing NETosis (n=11) on PMA stimulation compared with untreated neutrophils (3873 (677) vs 1361 (286) pg/mL; p<0.001) (figure 3A). This is further confirmed by the immunofluorescence staining demonstrating the externalisation of IL-40 during PMA-induced NETosis (figure 4). This analysis provided additional data regarding the possible subcellular localisation of IL-40 in neutrophils. Based on our observation, IL-40 was localised in the cytoplasm and within the cytoplasmic membrane (WGA) in unstimulated cells. In PMA-treated cells, a partial overlap of IL-40 with membrane was detected. Interestingly, IL-40 did not colocalise with either MPO or DNA in both, unstimulated or PMA-treated cells.

In addition to NETosis as a trigger for IL-40 release, we also demonstrated that neutrophils activated by proinflammatory cytokines such as IL-1β, IL-8 and TNF, but not IL-6, increased IL-40 secretion compared with untreated cells (n=9) (IL-1β: 1770 (263) vs 1296 (299) pg/mL, p<0.05; TNF: 3816 (671) vs 1296 (299) pg/mL, p<0.01; IL-8: 1902 (207) vs 1296 (299) pg/mL, p<0.05). Similarly, neutrophils exposed to LPS significantly enhanced IL-40 production in contrast to untreated cells (8163 (924) vs 1296 (299) pg/mL, p<0.01) (figure 3B). Finally, we show that recombinant IL-40 has the potential to trigger the release of pro-inflammatory cytokines IL-1β (100 ng/mL

**Figure 1** The levels of IL-40 are significantly elevated in the serum of ERA patients at baseline (n=60) compared with HC (n=60) and normalise after 3 months of therapy (A). The decrease in IL-40 levels is more prominent in double seropositive (B) compared with double seronegative (C) patients in response to the therapy. Serum IL-40 is associated with serum levels of RF (IgM) (D) and anti-CCP (E) autoantibodies in all ERA patients at baseline (n=60). There was no significant difference between serum levels of IL-40 in ANA positive and negative ERA patients at baseline (F). Horizontal lines represent the mean (SEM). The association of IL-40 with autoantibodies was analysed using Spearman correlation. *p<0.05; ****p<0.0001. ANA, antinuclear antibodies; anti-CCP, anti-cyclic citrullinated peptides antibodies; ERA, early rheumatoid arthritis; HC, healthy control; IL-40, interleukin 40; ns, non-significant; RF (IgM), rheumatoid factor IgM.
Figure 2  Serum levels of neutrophil extracellular traps markers (MPO, PR3, NE) are up-regulated in ERA patients (n=60) at baseline compared with HC (n=60) (A). Serum IL-40 significantly correlates with the peripheral blood neutrophil count (B), with serum levels of PR3 (C), NE (D), MPO (E) (n=60) and with cfDNA on the border of statistical significance (F) (n=35). Horizontal lines represent the median. The association of IL-40 with variables was analysed using Spearman correlation. ****p<0.0001. cfDNA, cell-free DNA; ERA, early rheumatoid arthritis; HC, healthy controls; IL-40, interleukin 40; MPO, myeloperoxidase; NE, neutrophil elastase; ns, non-significant; PR3, protease 3.
Neutrophils from patients with early stages of rheumatoid arthritis (ERA) (n=11) significantly enhanced the release of IL-40 following neutrophil extracellular traps induction by PMA (A). Exposure to various inflammatory stimuli such as IL-1β, TNF, IL-8 and LPS led to significant release of IL-40 by ERA neutrophils (n=9) (B). Recombinant IL-40 induced secretion of IL-1β (C), IL-6 (D) and IL-8 (E), but not TNF (F) in a dose-dependent manner by ERA neutrophils (n=4–11). The data are presented as the mean (SEM). *p<0.05; **p<0.01; ***p<0.001. ctrl, untreated cells; IL, interleukin; LPS, lipopolysaccharide; NS, non-significant; PMA, phorbol 12-myristate-13-acetate; TNF, tumour necrosis factor.

Figure 3
of IL-40: 0.337 (0.089) vs 0.116 (0.026) pg/mL, p<0.05; 250 ng/mL of IL-40: 0.564 (0.123) vs 0.116 (0.026) pg/mL, p<0.01), IL-6 (50 ng/mL of IL-40: 2.055 (0.557) vs 0.169 (0.139) pg/mL; p<0.05, 100 ng/mL of IL-40: 6.462 (1.490) vs 0.169 (0.139) pg/mL; p<0.01, 250 ng/mL of IL-40: 9.160 (1.764) vs 0.169 (0.139) pg/mL; p<0.001) or IL-8 (250 ng/mL of IL-40: 27.13 (1.21) vs 22.44 (1.44) pg/mL; p<0.05) but not TNF by neutrophils (n=4–11) compared with untreated cells (figure 3C–F). Recombinant IL-40 in the central part of the cell does not merge with MPO. IL-40 in detail of PMA-treated cells were obtained by STED microscopy. Scale bar: 8 µm. ctrl, unstimulated cells; ERA, early rheumatoid arthritis; IL-40, interleukin-40; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate-13-acetate; STED, stimulated emission depletion; WGA, wheat germ agglutinin.

**Figure 4** Immunofluorescence staining of IL-40 release during externalisation of neutrophil extracellular traps (NETosis) in ERA neutrophils (n=4). The representative images show fluorescent staining of DNA (blue), wheat germ agglutinin that binds to the plasma membrane (WGA, magenta), and immunofluorescent staining of neutrophil marker myeloperoxidase (MPO, red) and IL-40 (green) and their merged images. In non-NETotic neutrophils (ctrl), IL-40 partially overlaps with the intact plasmatic membrane (white arrow). A small proportion of IL-40 signal can also be observed in the cytoplasmic area (white arrow heads). IL-40 in the central part of the cell does not merge with MPO. PMA-treated neutrophils release decondensed chromatin that does not colocalise with IL-40 (green arrows). Similarly, DNA-appendant MPO does not merge with IL-40. There is a partial overlap in IL-40 signal and plasma membrane remnants (blue arrows). The images were obtained using confocal microscopy, MPO and IL-40 in detail of PMA-treated cells were obtained by STED microscopy. Scale bar: 8 µm. ctrl, unstimulated cells; ERA, early rheumatoid arthritis; IL-40, interleukin-40; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate-13-acetate; STED, stimulated emission depletion; WGA, wheat germ agglutinin.

**DISCUSSION**

In this study, we demonstrate for the first time that IL-40 is produced by activated neutrophils and is implicated in the early stage of RA development. IL-40 is elevated in the serum of ERA patients and correlates with the levels of autoantibodies, neutrophil count and NETs formation. Our in vitro experiments show increased IL-40 secretion in neutrophils on exposure to pro-inflammatory cytokines or IL-8 (250 ng/mL of IL-40: 27.13 (1.21) vs 22.44 (1.44) pg/mL; p<0.05) but not TNF by neutrophils (n=4–11) compared with untreated cells (figure 3C–F). Recombinant IL-40 in the central part of the cell does not merge with MPO. IL-40 in detail of PMA-treated cells were obtained by STED microscopy. Scale bar: 8 µm. ctrl, unstimulated cells; ERA, early rheumatoid arthritis; IL-40, interleukin-40; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate-13-acetate; STED, stimulated emission depletion; WGA, wheat germ agglutinin.

Finally, we proved that recombinant IL-40 does not induce NETosis in neutrophils from ERA patients, as demonstrated by immunofluorescence staining (figure 5A) and analysis of cfDNA and MPO levels (figure 5B,C) in neutrophils exposed to IL-40.

**Figure 4** Immunofluorescence staining of IL-40 release during externalisation of neutrophil extracellular traps (NETosis) in ERA neutrophils (n=4). The representative images show fluorescent staining of DNA (blue), wheat germ agglutinin that binds to the plasma membrane (WGA, magenta), and immunofluorescent staining of neutrophil marker myeloperoxidase (MPO, red) and IL-40 (green) and their merged images. In non-NETotic neutrophils (ctrl), IL-40 partially overlaps with the intact plasmatic membrane (white arrow). A small proportion of IL-40 signal can also be observed in the cytoplasmic area (white arrow heads). IL-40 in the central part of the cell does not merge with MPO. PMA-treated neutrophils release decondensed chromatin that does not colocalise with IL-40 (green arrows). Similarly, DNA-appendant MPO does not merge with IL-40. There is a partial overlap in IL-40 signal and plasma membrane remnants (blue arrows). The images were obtained using confocal microscopy, MPO and IL-40 in detail of PMA-treated cells were obtained by STED microscopy. Scale bar: 8 µm. ctrl, unstimulated cells; ERA, early rheumatoid arthritis; IL-40, interleukin-40; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate-13-acetate; STED, stimulated emission depletion; WGA, wheat germ agglutinin.
cytokines or during NETosis. Moreover, IL-40 positively regulates inflammation in neutrophils via secretion of IL-1β, IL-6 and IL-8.

Here, we show that IL-40 is up-regulated in the serum of ERA patients compared with healthy individuals, which is in agreement with studies on patients with RA and SS and further highlights the role of IL-40 in the pathogenesis of autoimmune inflammation. As this study was performed simultaneously with the study on RA with sample replicates to assess plate-to-plate consistency, we can state that the levels of IL-40 in patients with ERA are approximately two times lower compared with long-standing RA. This could be partly explained by the fact that early acute stages of RA are accompanied by a specific cytokine milieu in concentrations that are transitional and different from those present during chronic inflammation. Similar to long-standing RA, IL-40 levels were higher in double seropositive compared with seronegative patients. However, as the seronegative subgroup still shows upregulation of IL-40 compared with healthy individuals, we believe that the occurrence of IL-40 is not strictly related to the presence of autoantibodies alone. Accordingly, downregulation of IL-40 after therapy was more pronounced in double seropositive patients; however, also significant for the seronegative subgroup. Interestingly, there was no association of

Figure 5  Recombinant IL-40 does not induce externalisation of neutrophil extracellular traps in ERA neutrophils (n=4) (A). The representative image shows fluorescent staining of DNA (blue), wheat germ agglutinin that binds to the plasma membrane (WGA, magenta) and immunofluorescent staining of neutrophil marker myeloperoxidase (MPO, red) and their merged images. The quantification of cfDNA (B) and MPO (C) release by IL-40 stimulated neutrophils further supports the abovementioned. The images were obtained using confocal microscopy. Scale bars: 2.9 µm for ctrl and IL-40-stimulated cells, 16.7 µm for PMA-stimulated cells. cfDNA, cell-free DNA; ctrl, unstimulated cells; ERA, early rheumatoid arthritis; IL-40, interleukin 40; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate-13-acetate; WGA, wheat germ agglutinin.
IL-40 with parameters of inflammation or disease activity, except for a weak correlation with CRP after 3 months of therapy, which should be interpreted with caution, as no analogous correlation of these two parameters were found at baseline or during the therapy. Moreover, serum IL-40 levels positively correlate with peripheral blood neutrophil count in ERA, which is in line with the neutrophil-related expression of IL-40 found in the synovial fluid and tissue of long-standing RA. It is well established that neutrophils play an essential role in the initiation and progression of RA; furthermore, dysregulation of NETosis in RA has been described. Our findings of elevated NETosis markers in the serum of ERA patients and their association with cfDNA and serum IL-40 further highlight the link between IL-40 and activated neutrophils in the early phase of RA. As the presence of autoantibodies increases the NETs production in RA and IL-40 is related to both autoantibodies and NETosis in ERA, we can speculate that IL-40 may be involved in the pathological crosstalk between neutrophils and B-cells. This is underscored by the fact that the upregulation of serum IL-40 and NETosis markers is more pronounced in double seropositive compared with seronegative patients (data not shown). However, this requires further investigation. In addition to that, we found a positive correlation between the decrease in serum IL-40 and the selected NETosis marker NE following conventional pharmacological therapy in ERA patients. In this context, studies suggested an indirect inhibition of NETs production via downregulation of reactive oxygen species following conventional therapy with either methotrexate or glucocorticoids. Thus, based on the abovementioned evidence and on the parallel downregulation of IL-40 and NE, we believe that neutrophils undergoing NETosis are a significant source of IL-40 in treatment-naïve ERA patients. In support of this, our in vitro data demonstrate that PMA-challenged neutrophils undergoing NETosis abundantly release IL-40. Indeed, we showed that pro-inflammatory cytokines involved in RA immunopathology, TNF in particular, could stimulate neutrophil secretion of IL-40 in vitro. Although TNF was the most potent inducer of IL-40 in neutrophils among all the studied cytokines, anti-TNF therapy does not seem to have significant effect on the levels of IL-40 in RA patients. Considering the novelty and the lack of data on IL-40, TNF may represent only one of many IL-40 inducing factors just as neutrophils may be only one fraction of its potential cellular sources.

The observed extracellular release of IL-40 from activated neutrophils is in line with the presence of IL-40 in the cytoplasm of neutrophils detected by immunofluorescence. All this is consistent with the description of IL-40 as a soluble secreted protein. Nevertheless, we also demonstrated that IL-40 associates with the plasma membrane, which is agreement with study of Zhang et al, who predicted IL-40 to be an 'intrinsic component of the plasma membrane'. It should be noted that the colocalisation of IL-40 with WGA (plasma membrane marker) in NETotic cells must be interpreted with caution, as WGA may have also affinity for some intracellular carbohydrates that are exposed during NETosis. Overall, we could speculate that IL-40 may potentially exist as two variants, a soluble and a membrane-bound form. Future studies should therefore focus on addressing some of the questions raised by these results.

Given the accumulation of IL-40 in the circulation of patients with ERA, we sought to explore the interaction between IL-40 and neutrophils in vitro. We showed that extracellular IL-40 potentiates neutrophils to release cytokines IL-1β, IL-6 and IL-8, some of which could in turn stimulate the secretion of IL-40 by neutrophils, and thereby perpetuate the inflammatory loop. Importantly, IL-1β, IL-6 and IL-8 are among the key cytokines dominating RA from the very beginning. In addition, the potential of IL-40 to induce IL-8 was previously observed in RA synovial fibroblasts. Nevertheless, it is important to note that the pro-inflammatory effect of IL-40 on neutrophils was observed at the concentrations involved in RA immunopathology.
of IL-40 (50–250ng/mL) that rather reflect the inflammatory milieu in the joint than in the blood circulation. Our data on pro-inflammatory role of IL-40 are further supported by the study of Gao et al who explored the anti-inflammatory effects of IL-38 on poly (I:C)-induced lung inflammation in vitro and showed that IL-38 reduces IL-40 gene expression. Collectively, our findings imply that IL-40 may be involved in the neutrophils associated inflammatory response in the early phase of RA development (figure 6).

However, this study has some limitations. First, a long-term longitudinal study would be beneficial to follow the kinetics of IL-40 in relation to disease activity and radiographic progression. Moreover, our study involved the analysis of IL-40 in the serum but not in the synovial fluid samples, which altogether would provide a more complete overview of the implication of IL-40 in RA development and progression. Since IL-40 seems to be more linked to the autoantibody-positive form of RA, studies elucidating the cross talk between B-cells and neutrophils should be performed in the future. Finally, the molecular mechanisms underlying the pro-inflammatory effect of IL-40 on neutrophils need to be investigated. Despite these limitations, this study provides a better understanding of the role of recently discovered cytokine IL-40 in the pathogenesis of RA and demonstrates that activated neutrophils are an important source of IL-40 in early RA.

CONCLUSIONS

Taken together, this study brings a new insight into the contribution of IL-40 to the pathogenesis of seropositive RA. IL-40 is elevated in the serum of patients with ERA and correlates with levels of autoantibodies, neutrophil count and markers of NETosis, indicating its role in the acute flare-up and impaired immune response. More importantly, we demonstrate that IL-40 is a neutrophil-derived cytokine, whose secretion requires a specific pro-inflammatory environment that seems to be met in the ERA. In addition, extracellular IL-40 exerts pro-inflammatory effects on neutrophils and thereby participates in the amplification of the immune reaction in RA.

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Contributors

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Competing interests

None declared.

Patient consent for publication

Not applicable.

Ethics approval

This study involves human participants and was approved by the Ethic Ethics Committee of the Institute of Rheumatology in Prague, Czech Republic. Reference number of the ethics approval is 6485/2020. Written informed consent was obtained from all participants. Participants gave informed consent to participate in the study before taking part.

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Supplemental material

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