Impaired microRNA processing in neutrophils from rheumatoid arthritis patients confers their pathogenic profile. Modulation by biological therapies

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ABSTRACT

The aim of this study was to investigate the microRNA expression pattern in neutrophils from rheumatoid arthritis patients and its contribution to their pathogenic profile and to analyze the effect of specific autoantibodies or inflammatory components in the regulation of microRNAs in rheumatoid arthritis neutrophils and its modulation by biological therapies.

Neutrophils were isolated from paired peripheral blood and synovial fluid samples of 40 patients with rheumatoid arthritis and from peripheral blood of 40 healthy donors. A microRNA array was performed using nCounter technology. Neutrophils from healthy donors were treated *in vitro* with antibodies to citrullinated protein antigens isolated from rheumatoid arthritis patients and tumor necrosis factor-α or interleukin-6. A number of cytokines and chemokines were analyzed. *In vitro* treatments of rheumatoid arthritis-neutrophils with tocilizumab or infliximab were carried out. Transfections with pre-microRNAs and DICER downregulation were further performed.

Rheumatoid arthritis-neutrophils showed a global downregulation of microRNAs and genes involved their biogenesis, alongside with an upregulation of various potential mRNA targets related to migration and inflammation. Decreased levels of microRNAs and DICER correlated with autoimmunity, inflammation and disease activity. Citrullinated protein antigens and tumor necrosis factor-α decreased the expression of numerous microRNAs and their biogenesis-related genes, increasing their potential mRNA targets. Infliximab reversed those effects. Transfections with pre-miRNAs-223, -126 and -148a specifically modulated genes regulating inflammation, survival and migration. DICER depletion influenced the neutrophils inflammatory profile.

Taking together rheumatoid arthritis neutrophils exhibit a global low abundance of microRNAs induced by autoantibodies and inflammatory markers, which might contribute to their pathogenic activation. microRNA biogenesis is significantly impaired in rheumatoid arthritis-neutrophils and further associated with a greater downregulation of microRNAs mainly related to migration and inflammation in synovial neutrophils. Finally, anti-tumor necrosis factor-α and anti-interleukin-6 receptor treatments can modulate microRNA levels in the neutrophils, minimizing their inflammatory profile.

INTRODUCTION

Several immune cells including T and B lymphocytes, macrophages, synovial fibroblast and neutrophils are known to be relevant in the Rheumatoid Arthritis (RA) pathogenesis .[1] Among them, RA neutrophils are activated cells, characterized by a prolonged lifespam, increased migratory capacity and production of inflammatory molecules and reactive oxygen species (ROS). In severe acute inflammation, synovium accumulates a great number of these cells in a more activated state, promoting cartilage destruction and joint damage. [2]

Antibodies to citrullinated protein antigens (ACPAs) are currently considered the most specific autoantibodies in RA, being related to the activity of the disease and poorer prognosis.[3] ACPAs have been shown able to induce neutrophils to produce high levels of inflammatory mediators, ROS and to generate NETosis. [2,4]

Epigenetic modifications contribute to the development of RA, affecting disease susceptibility and severity. [5, 6] Among them, several microRNAs have been linked to the chronic inflammation in RA. [5] MicroRNAs (miRNAs) are short noncoding RNAs present in all multi-cellular organisms involved in a broad range of cellular processes. They cause posttranscriptional and posttranslational gene silencing, by recognizing a specific sequence of mRNA, binding to it and inhibiting its translation to protein. [7] The miRNA is first transcribed to long primary miRNA of several kb in length (pri-miRNA). This pri-miRNA is then processed by Drosha in a precusor miRNA (pre-miRNA) of 70-nucleotide approximately. The pre-miRNA transported out of the nucleus by exportin 5 (XPO-5) and then processed by DICER to a mature double stranded miRNA of approximately 22 nucleotides. RNA-induced silencing complex (RISC) (composed by the transactivation-responsive RNA-binding protein (TRBP) and Argonaute (AGO)) removes the complementary strand. DICER binds to RISC, forming the core of RISC-loading complex. DICER is considered a crucial factor in the miRNA processing since its presence is necessary to stimulate RNA processing by AGO. [8,9]. Functional miRNA is able to bind to the 3'-untranslated region (UTR) of the target mRNA, causing mRNA cleavage or translational repression.[10]

Several studies, mainly been conducted on lymphocytes, monocytes, macrophages and synovial fibroblast, have reported the role of various miRNAs in the pathogenesis of RA, being critical for the increased expression of inflammatory cytokines and prolonged cell survival.[5, 11]

We undertook this study to evaluate the miRNA profile and the proteins involved in miRNA processing in circulating and synovial neutrophils from RA patients, in order to gain insight about its role in the different activation states of these cells. The effects of

ACPAs or inflammatory components and biological therapies in the expression of miRNAs in neutrophils was further assessed.

METHODS

On line supplemental methods is available.

RA patients and healthy donors

Forty RA patients and 40 healthy donors (HD) were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) and achieved a total score of 6 or greater according to 2010 criteria. The patients were taking the following treatments: corticosteroids (50.0%), leflunomide (42.5%), hydroxychloroquine (45.0%), NSAIDs (80.0%) and methotrexate (65%). All patients were tested for the presence of ACPAs and rheumatoid factor (RF) by clinical laboratory routine analysis. All participants enrolled were Caucasian, recruited at the department of Rheumatology, and gave their written informed consent approved by the ethical committee of the Reina Sofia Hospital (Cordoba, Spain).

Clinical details of RA patients and HD are shown in table 1. Peripheral blood was withdrawn from all the RA patients and the HD. Synovial fluid (SF) from RA patients was obtained through arthrocentesis. Study design is displayed on a flow chart (Supplementary figure 1).

Isolation of neutrophils from PB and SF

Neutrophils from peripheral blood of HD and paired synovial fluid and peripheral blood samples of RA patients were isolated (after centrifugation to obtain buffy coat and osmotic lysis of the pellet) by immunomagnetic positive selection with human anti-CD15 microbeads (Miltenyi Biotec S.L, Bergisch Gladback, Germany) using AUTOMACs (Miltenyi Biotec). [12]

miRNA expression profiling

The nCounter miRNA Assay (NanoString Technology, Seattle, WA, USA) detects simultaneously 800 human miRNAs in each sample. 100 ng of RNA, pooled samples of neutrophils from PB of 10 HD, neutrophils from PB of 10 RA patients and neutrophils from SF of 10 RA patients were prepared by ligating a specific DNA tag (miR-tag) onto the 3´end of each mature miRNA followed by 16-20h hybridization (65ºC) to nCounter Reporter and Capture probes. The rest of the protocol was performed following the manufacturer's recommendations (NanoString Technologies; Seattle, WA). Data were normalized by the geometric mean of top 100 miRNAs detected using the nSolver software. This microRNA array was performed in pooled samples of the 10 RA patients that best represented the mean values of age, gender, disease activity, evolution time and autoimmunity of the clinical validation cohort.

IgG-ACPAs isolation from RA patients

IgGs from serum of 5 different RA patients with high titers of ACPAs and negative for RF (enriched IgG-ACPAs) and 5 HD ((IgG- normal human serum (NHS)) were isolated using HiTrap protein G HP columns (GE Healthcare).

In vitro treatments of neutrophils

Neutrophils purified from 5 RA patients (having DMARDs and not taking any biological therapies) were pre-treated with FCRII blocking Reagent (Miltenyi Biotec) for 15 min and subsequently incubated with IFX (100 μg/ml) or TCZ (20 μg/ml) for 6 hours. That selection of these patients allowed to isolate neutrophils that assumingly were activated, which meant increased expression of inflammatory cytokines, so that the effects of miRNAs transfection could be demonstrated by proving the reduction in the levels of those molecules.

Neutrophils purified from 5 HD were treated in vitro with IgG-NHS or enriched IgGs-ACPAs (500 ug/ml), TNF-α and IL-6 (10 ng/ml) for 6 hours. Samples were processed for RT-PCR analyses.

RESULTS

Global decrease in miRNA levels of neutrophils from RA patients.

Among the 800 miRNAs analyzed, levels of 133 miRNAs were detected in neutrophils. Using a fold change cut-off of >2, 94 miRNAs were reduced in PB-RA neutrophils comparing to PB-HD, and 3 of them were elevated (Supplementary table 1, Figure 1A). Besides, synovial neutrophils showed 34 miRNAs even more reduced compared to its paired PB sample (fold change cut-off of >2) (Figure 1B). IPA software uncovered the main enriched biological functions and pathways on which those miRNAs are involved, including immune disease, inflammatory response and connective disorders (Figure 1D).

Low abundance of miRNA levels in RA neutrophils might be due to a defect in the miRNA processing.

Eight altered miRNAs were identified by IPA as the main regulators of proteins involved in the abnormal activation of neutrophils in RA, including miRNA -126, -148a, -29c, let-7b, -30c, -17, -21 and 223 (Figure 2). The expression of these miRNAs was validated in all the samples separately. A technical validation was performed separately in the 10 samples previously used for the pool. In addition, a clinical validation was carried out separately in the 30 remaining samples (Supplementary figure 2). Thus, levels of most of the selected miRNAs were significantly reduced in PB-RA neutrophils compared to PB-HD neutrophils. A greater reduction in the expression of miR-148a, miR-29c and

let-7b in the SF paired samples was observed (Figure 3A). In addition, there was not significant differences in the reduced levels of miRNAs among patients treated or not treated with methotrexate (Supplementary figure 3).

There was a significant reduction in the expression of genes involved in the miRNA processing (DICER and AGO-1) in neutrophils from PB-RA patients compared to PB-HD. Of note, DICER, AGO-1, AGO-2 and XPO-5 were diminished in neutrophils from synovial fluid of RA patients (Figure 3B).

Bioinformatic identification and expression of the putative targets of reduced miRNAs in RA neutrophils.

Seven putative mRNA targets were chosen in based on their recognized role in the pathogenesis of RA, being key factors in inflammation (TNF-α, IL-1β, IL-6R), cell adhesion (VEGF-A), migration (IL-8) and survival (STAT3 and AKT). These targets were significantly upregulated in PB-RA neutrophils (Figure 3C). A greater alteration was observed in SF neutrophils.

Using enrichment analysis of those selected targets, enriched pathways mainly related to inflammatory processes were revealed. This included a broad range of secondary chemokines and cytokines which are indirectly connected with the eight selected mRNA targets, amplifying the inflammatory cascade (Supplementary figure 4). Thus, a human cytokine array was performed in neutrophils from RA patients (PB and SF) and HD (PB).

Neutrophils from PB of RA patients showed increased protein expression of CCL5, CD40L, CXCL1, CXCL2, IL-1ra, IL-16, IL-18, IL-32a, PAI-1 and TREM-1 compared to HD (Figure 3D). A differential proteome profile was observed in neutrophils from synovial fluid of RA patients compared to HD and PB paired samples (MIP-1α/1β, CCL5, CD40L, C5/5a, CXCL1, CXCL12, ICAM-1, IL-1β, IL-1ra, IL-8, IL-13, IL-16, IL-18, MIF and TREM-1) (Figure 3D).

Reduced levels of miRNAs in RA neutrophils are related to autoimmunity, clinical and serological parameters

Decreased levels of both, miRNAs and DICER significantly correlated with the activity of the disease, levels of ACPAs and clinical inflammatory markers. Elevated serum levels of TNFα correlated with low levels of DICER. However, there was not association between the levels of miRNAs and serum TNF-α (Figure 4A).

ACPAs reduces miRNA levels in healthy neutrophils

Enriched IgG-ACPAs downregulated the expression of the 8 selected miRNAs in healthy neutrophils (Figure 4B). Accordingly, a significant reduction of genes involved in the miRNA biogenesis was observed (Figure 4C). Enriched IgG-ACPAs also increased the expression of the selected mRNA targets (Figure 4D). Finally, enriched IgG-ACPAs promoted a significant upregulation of secondary chemokines and cytokines indirectly related with the eight selected mRNA targets (CCL1, CCL2, MIP-1α/β, CCL5, CD40L, CXCL1, CXCL12, G-CSF, GM-CSF, IFN-γ and IL-8) (Figure 4E).

Inflammatory mediators decrease the expression of miRNAs in neutrophils, which might be restored by IFX or TCZ.

TNF-α and IL-6 levels were significant elevated in serum from RA patients; a further increase was observed in SF from those RA patients (Figure 5A).

In vitro, TNF-α downregulated the levels of the 8 selected miRNAs alongside with a decrease in the expression of DICER and AGO-2 in healthy neutrophils (Figure 5B). Treatment with IL-6 reduced the levels of miR-126, let-7b, miR-17, AGO1 and AGO2 (Figure 5C).

We observed that fresh neutrophils from RA patients had significantly higher levels of TNF-α and IL-6 mRNA compared to freshly isolated neutrophils from healthy donors (Supplementary figure 5A). In addition, after 6 hours of in vitro culture, levels of TNFα and IL6 were elevated in the culture media of RA neutrophils (Supplementary figure 5B).

In vitro treatment of active neutrophils purified from RA patients with IFX restored the low levels of the 8 selected miRNAs while TCZ only up-regulated the expression of miR-148a (Figure 5D). Accordingly, IFX upregulated the expression levels of AGO1. Regarding mRNA targets, IFX reduced the mRNA expression of VEGF-A, TNF-α, IL-1β, IL-8 and STAT3. Treatment with TCZ also diminished the expression of various of these mRNA targets such as IL-1β, IL-8, IL-6R and STAT3 (Figure 5E). In addition, protein release of TNF-α, IL-8 and IL-1β was reversed in RA neutrophils after treatments with both, IFX and TCZ (Figure 5F).

Overexpression of miR-126, miR-148a and miR-223 in RA neutrophils decreases specific targets involved in inflammation, migration and cell survival.

We selected three downregulated miRNAs to evaluate their role in migration, proinflammatory profile and cell survival of RA neutrophils: miR-223 was the most abundant in neutrophils, miR-148a and miR-126 have several potential and demonstrated mRNA targets involved in inflammation. As seen in figure 6, miR-126, miR-148a and miR-223 overexpression led to a downregulation of their specific mRNA

targets: miR-126 overexpression induced a significant downregulation of VEGF-A protein expression (Figure 6A), and miR-223 overexpression promoted a significant decrease in the protein expression of IL-8 and IL-1β. Interestingly, miR-223 transfection induced a significant increase of VEGF mRNA and protein (Figure 6C). On the other hand, miR-148a overexpression reduced gene and protein expression levels of TNF-α (Figure 6B).

DICER downregulation in neutrophils might exacerbate their inflammatory profile.

Using a low number of lentiviral particles, 25% of DICER expression was inhibited in HL-60 neutrophil-like cells (Figure 6D). This reduction promoted a significant decrease in all the selected miRNAs (Figure 6E). Protein levels of a number of cytokines and chemokines were significantly upregulated in neutrophils after DICER downregulation (Figure 6F).

DISCUSSION

This study reports for the first time on the altered miRNA expression profile in neutrophils from RA patients, describing a defect in miRNAs processing machinery responsible for a global low abundance of miRNAs, mediated by ACPAs and inflammatory mediators, promoting the high inflammatory profile of these cells in RA.

Several miRNAs have been shown increased in PB and inflamed joints in RA patients, correlating with disease activity and promoting the production of inflammatory mediators involved in the synovitis. [13, 14] Likewise, various single nucleotide polymorphism (SNPs) have been studied in miRNAs in RA .[15]

Our study shows a global downregulation of the miRNAs expression in RA neutrophils, more marked in synovial neutrophils, suggesting that it might contribute to the abnormal activated profile of these cells in the synovium.

In this sense, global downregulation of miRNAs has been shown in human alveolar macrophages induced by cigarettes smoking, responsible for the changes in gene expression associated with the disease.[16] In autoimmune disorders, we recently described a global downregulation of the miRNA levels in neutrophils from patients with systemic lupus erythematosus and antiphospholipid syndrome, which may indicate that chronic inflammation and/or autoimmunity is associated to a reduction of miRNAs in neutrophils. [17] In the present study, we demonstrate that either ACPAs or inflammatory mediators, especially TNF-α can modulate the miRNA expression profile, through a reduction of several proteins involved in its processing, which might be translated into an increase of genes that might be involved in inflammation, cell survival and migration.

Up to date, no study has reported the effect of ACPAs in the expression of miRNAs. In our hands, the reduced levels of miRNAs and DICER in RA neutrophils correlated with elevated levels of ACPAs. Accordingly, our in vitro studies demonstrated a direct involvement of these autoantibodies in the deregulation of various miRNAs -and their specific protein targets- globally related to the pathogenesis of RA.

We further demonstrated that the global downregulation of the miRNAs expression in RA neutrophils was associated, at least partially, to the reduced levels of DICER. A recent study suggested the role of DICER in neutrophils differentiation, where the DICER inhibition attenuated the activation of autophagy, a process that is needed for proper neutrophil differentiation.[12] DICER plays a crucial role in miRNA biogenesis. Thus, it has been suggested that mRNA and protein levels of DICER must be strictly controlled since small changes can initiate various pathological processes. [18] Here, we prove a novel role for DICER in neutrophils, showing that little reductions can induce a proinflammatory profile in neutrophils by downregulating several miRNAs and, hence, a number of putative targeted cytokines and chemokines.

Currently, little is known about the miRNAs regulating the neutrophil function. Several miRNAs have been recently involved in the development and function of the neutrophils and in various pathological states, including miRNA-155, miRNA-34a, miRNA-223, miRNA-142, miRNA-452 and miRNA-466L.[19] Overexpression of miRNA-155 and miRNA-34a in neutrophils from patients with myelodysplastic syndrome has been shown to contribute to an alteration of the migration.[20] In addition, decreased levels of both, the miRNA-145 and the miRNA-143 have been shown in AML, which were responsible for the blockade of the differentiation process of the neutrophils.[21]

Alongside with previous evidence, here we show that miRNA-223 is one of the most abundant miRNA on neutrophils.[22] It has recently been demonstrated that the miRNA-223 is an important regulator blocking the infiltration of neutrophils in alcoholic hepatic disease.[22] Supporting a role for this miRNA in the infiltration capacity of the neutrophils, in the present work we demonstrated how the overexpression of the miRNA-223 in the neutrophils of RA patients reduced specifically the expression of IL-1β and IL-8, molecules involved in inflammation and migration.

The role of the miRNA-126 in the vascular integrity has been also evidenced.[23] We observed reduced levels of the miRNA-126 in RA neutrophils, while its induced overexpression in RA neutrophils reduced significantly the levels of VEGF, pointing out the role of this miRNA in the neutrophil adhesion and migration.

Multiple functions have been attributed to miRNA-148a in several diseases. Thus, low levels of miRNA-148 were related to less survival time and increased recurrence risk in bladder cancer.[24] In addition, miRNA-148 has been related to innate and adaptive

immune responses.[25] Our data is in agreement with such studies, since we found reduced levels of the miRNA-148 in RA neutrophils, associated to increased levels of TNF-α, a key inflammatory protein driving the RA disease.

Others miRNAs found decreased in RA neutrophils, such as miRNA-21, Let-7 and miRNA-30, have previously been reported to be altered in different types of tumors, thus playing a relevant role in tumorigenesis, invasion and metastasis of cancer cells.[26-29] In addition, Let-7 and miRNA-17 regulate the response of the T cells.[27, 30] Finally, a recent study demonstrated that the levels of all the members of the miRNA 29 family were decreased in PBMC and CB34+ cells of bone marrow of AML patients. The normalization of their levels partially inhibited the abnormal proliferation of the blasts, blocked the myeloid differentiation and repressed the apoptosis.[31]

TNF-α and IL-6 are key inflammatory effectors in RA, whose levels are elevated in RA serum and even more increased in RA SF. We found a marked effect of TNF-α on neutrophils, reducing genes related to miRNA processing (including DICER and AGO-1) and downregulating the 8 miRNAs selected. By contrast, IL-6 had not that stronger effect but was able to reduce the levels of miRNA-126, let-7b and miRNA-17 alongside with the expression of AGO-1 and AGO-2. Treatment of active RA neutrophils with IFX or TCZ reduced the inflammatory profile, downregulating gene expression of VEGFA, TNF-α, IL-1β, IL8, IL6R and STAT3. However, only IFX was able to restore the global levels of selected miRNAs, alongside with genes involved in their processing in RA neutrophils, an effect that might be expected after the stronger effect of TNF-α observed in reducing the miRNAs levels, thus suggesting that IFX might specifically minimize the abnormal profile of the RA neutrophils through the inhibition of TNFα, which directly acts reducing the expression of miRNAs. In agreement with these results, we recently demonstrated that in vivo treatment with anti-TNFα drugs during 6 months regulated the levels of several miRNAs in plasma of RA patients. Moreover, miRNA-23 and miRNA-223 were identified as potential biomarkers of therapy effectiveness.[32]

Altogether, our study shows that neutrophils from RA patients have a defect in the miRNA biogenesis machinery, more marked in synovial neutrophils, and induced by ACPAs and inflammatory mediators. This defect might be directly associated with the abnormal neutrophil activation, increasing their proinflammatory profile, observed by the higher expression of a number of chemokines and cytokines.

Among the miRNAs altered in RA neutrophils, we demonstrate that the miRNA-223, miRNA-126 and miRNA-148 are involved in the modulation of genes involved in processes such as migration, inflammation and cell survival in neutrophils. Finally,

biological therapies would be able to improve miRNAs processing, upregulating the levels of miRNAs, which might reduce the activation of the neutrophil.

Beyond the regulation of miRNAs in RA neutrophils, there should be other epigenetic mechanisms that might contribute to the abnormal activation of these cells in RA context, such as chromatin modification.

Limitation of the study: This is a cross-sectional study where consecutive patients from standard clinical practice were recruited. Those patients were being treated with standard therapy, including immunosuppressants, by the time of the samples and clinical details collection. Thus, the effects of specific treatments in the expression levels of miRNAs or the molecules involved in their biogenesis in neutrophils could not be analyzed.

The isolation of neutrophils with anti-CD15 microbeads could be considered as a potential limitation of this work. Up to date there is not a worldwide accepted method, so that neutrophil isolation techniques have shown either some activation or functional impairment of the cells and presence of small amounts of contaminating cells.

Choosing an adequate method to isolate neutrophils from synovial fluid is challenging. In our hands, after testing potential priming/activation and percentage of contaminating cells, isolation of neutrophils with anti-CD15 microbeads was proven to be a suitable approach to obtain enough number of inactivated neutrophils. Nevertheless, the consensus in the selection of the right isolation method that allows to compare neutrophil paired samples from synovium and peripheral blood is still needed.

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