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

Ivan Arias de la Rosa,[1*] Carlos Perez-Sanchez,[2*] Patricia Ruiz-Limon,[3*] Alejandra Patiño-Trives,[1] Carmen Torres-Granados,[1] Yolanda Jimenez-Gomez,[1] Maria del Carmen Abalos-Aguilera,[1] Irene Cecchi,[4] Rafaela Ortega,[1] Miguel Angel Caracuel,[1] Jerusalem Calvo-Gutierrez,[1] Alejandro Escudero-Contreras,[1] Eduardo Collantes-Estevez,[1#] Chary Lopez-Pedrera[1#] and Nuria Barbarroja[#1,5]

**IAR, CPS and PRL contributed equally as co-first authors.*

#ECE, CLP and NB contributed equally as co-senior authors.

[1]Rheumatology service, Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC)/Reina Sofia Hospital/University of Cordoba, Cordoba, Spain; [2]Deparment of Medicine, University of Cambridge, School of Clinical Medicine, Addenbroke's Hospital, Cambridge Institute for Medical Research, Cambridge, UK; [3]Biomedical Research Institute (IBIMA), Service of Endocrinology and Nutrition, Malaga Hospital Complex (Virgen de la Victoria), Malaga, Spain; [4]Department of Clinical and Biological Sciences, Center of Research of Immunopathology and Rare Diseases-Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases, Turin, Italy and [5]CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

 doi:10.3324/haematol.2018.205047

Received: August 22, 2018.

Accepted: January 10, 2020.

Pre-published: January 16, 2020.

Correspondence: *NURIA BARBARROJA* - nuria.barbarroja.exts@juntadeandalucia.es

METHODS

Purity and priming of isolated granulocytes

Purity of the neutrophil fraction was evaluated via flow cytometry (FACSCalibur cytometer), by analysing the size and complexity of the population (forward and size scatters) and using specific antibodies anti-human CD15, anti-human CD14 and anti-human CD66b (Immunostep, Salamanca, Spain) and staining with Wright-Giemsa. By these methods, 98.5 ± 2.5 viable neutrophils were obtained (**Supplementary figure 6**).
In addition, as neutrophils could be activated or primed by the isolation method, ROS production induced by fMLP (1μM for 15 min) (Sigma-Aldrich) and CD66b expression were evaluated before and after isolation of CD15+ cells with anti-CD15 microbeads from peripheral blood of healthy donors and RA patients and synovial fluid of RA patients. Thus, ROS production and CD66b expression (measured by flow cytometry) were not different in neutrophils from buffy coat and neutrophils isolated with anti-CD15 microbeads (**Supplementary figure 7 and 8**).

RNA isolation.

Total RNA from neutrophils was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations.

Target gene prediction and integrated analysis by IPA.

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA; https://analysis.ingenuity.com).

In silico analysis software reveals enrichment for molecular networks and signaling pathways. For this purpose, all differentially regulated miRNAs and fold changes were imported into IPA. The right-tailed Fisher's exact test was used to calculate a p-value determining the statistical probability that association between a set of molecules and a pathway or function might be due to chance alone. Additionally, specific targets (experimentally observed and predicted with high bioinformatics confidence) regulated by the differentially expressed miRNAs were also identified by using the different database integrated in IPA software.
Enrichment analysis of the potential targets of the altered miRNAs was performed using the Enrich web-server tool (http://amp.pharm.mssm.edu/Enrichr/).

Gene expression and validation miRNA arrays through quantitative real-time reverse transcriptase PCR.

Gene expression was assessed by real time PCR using a LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA).

For the validation of the miRNA array**,** RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Life Technologies, Madrid, Spain). The reaction was conducted in a GeneAmp PCR System 9700 (Life Technologies) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The RT product was combined with 5 µl of Taqman 2x Universal PCR Master Mix, No AmpErase UNG, 0,5 µl of specific 20X Taqman miRNA Assay and 0,5 µl of water to generate a PCR of 10 µl of total volume. Real-time PCR was carried out on a LightCycler Thermal Cycler System (Roche Diagnostics) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were normalized to the mean of miRNA U6. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta\Delta Ct}$. Samples were analysed in triplicate and negative controls were included in all the reactions.

Protein extraction, western blot and human cytokine array

Neutrophils were lysed on ice for 15 minutes in NP-40 lysis buffer, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM Na2-ethylene glycol tetraacetic acid, 0.1 mM Na2−ethylene diamine tetraacetic acid, 1 mM sodium orthovanadate, 1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and phosphate and protease inhibitor cocktails (Sigma-Aldrich, St. Louis, Missouri, USA). Cytoplasmic lysates were pelleted by centrifugation at 15,000g for 5 minutes at 4°C. The supernatant was recovered and frozen at −80°C.

Forty micrograms of cytoplasmic extracts were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose membranes. Membranes were stained with human monoclonal anti-DICER (Abcam, Cambridge, UK). Visualization of immune complexes was performed using secondary antibodies conjugated to HRP and the Luminol Reagent detection system (Santa Cruz Biotechnology Inc. Data are presented as integrated optical density (IOD) and expressed in arbitrary units (AU).

250 µg of neutrophil cytoplasmic lysates was subjected to human cytokine array to detect 36 human cytokines, chemokines, and acute phase proteins simultaneously (R&D Systems, Minneapolis, USA), following the manufacturer's recommendations. Data are

presented as integrated optical density (IOD) and expressed in arbitrary units (AU) (a map displaying the coordinate and the protein of each spot is shown in **supplementary figure 9).**

Inflammatory protein levels

Levels of protein in culture supernatants, serum and synovial fluid (TNF-α, IL-8, IL-6, VEGF and IL1β) were analyzed through ELISA (Bionova, Madrid, Spain) following the manufacturer's recommendations. Some modifications regarding time of incubation, washing steps and sample dilution were included in order to avoid non-specific binding.[1]

Apoptosis

Rates of apoptosis in neutrophils after in vitro treatments were analysed using annexin V or Draq7-propidium iodide (ThermoFisher, Waltham, MA, USA; Biostatus, Shepshed, UK) following the manufacturer's recommendations by flow cytometry (**Supplementary figure 10**). No significant apoptosis was observed.

Transfection of RA neutrophils with pre-miRNAs

Transient transfection of primary neutrophils purified from RA patients was performed with 100 nM miRNA mimics (Life Technologies) (miR-126, miR-148a and miR-223 separately and a non-specific control (scrambled)), using 2ul of siPORT$_{TM}$NeoFX$_{TM}$ transfection agent (Life Technologies) for 10^6 cells/500ul medium (RPMI1640 supplemented with 10% fetal bovine serum).

Transfection efficiency was controlled by flow cytometry using a Cy3™ Dye-Labeled Pre-miR scrambled control (ThermoFisher scientific) and was estimated to be between 96-100 %. Apoptosis was also tested after transfection using Annexin V/Propidium iodide by flow cytometry (**Supplementary figure 11**). Transfections had no significant effect in neutrophil apoptosis.

After 12 hours, cells were collected to analyze potential targets of these miRNAs. Data were expressed as relative changes to the values of the cells transfected with scrambled control.

Downregulation of DICER in neutrophils

Since human neutrophil lifespan is short, downregulation of DICER was performed on human neutrophils derived from a cell line, HL60. HL-60 cells were differentiated to

neutrophils with DMSO (1.25%) during 6 days. Neutrophil differentiation was evaluated through flow cytometry using human anti-CD11b antibody (BD Bioscience, New Jersey, USA). After 6 days of treatment the 65% of HL-60 cells were differentiated to neutrophils. Knockdown of DICER was performed as previously described.[12]

pLKO.1 lentiviral vectors, expressing shRNAs targeting DICER1 (shDICER1_ 3155 NM_030621 TRCN0000290426) or non-targeting shRNA were purchased from Sigma-Aldrich. Cells ($2x10^5$) were seeded in 1 ml of medium with low number of viral particles ($6x10^5$) and polybrene (8 μg/ml) on a 6-well plate. Plate was centrifuged at 1000g, 32ºC for 1h and incubated overnight. Thereafter, the medium was replaced with fresh medium and cells were incubated at 37ºC, 5% CO2 for 48h. Transfection was carried out for 48 hours and with lower number of lentiviral particles in order to avoid apoptosis.

Transduction efficiency was evaluated with a green fluorescence protein lentivirus positive control by flow cytometry (Sigma-Aldrich). Eighty percent of the cells were infected.

Statistical analysis

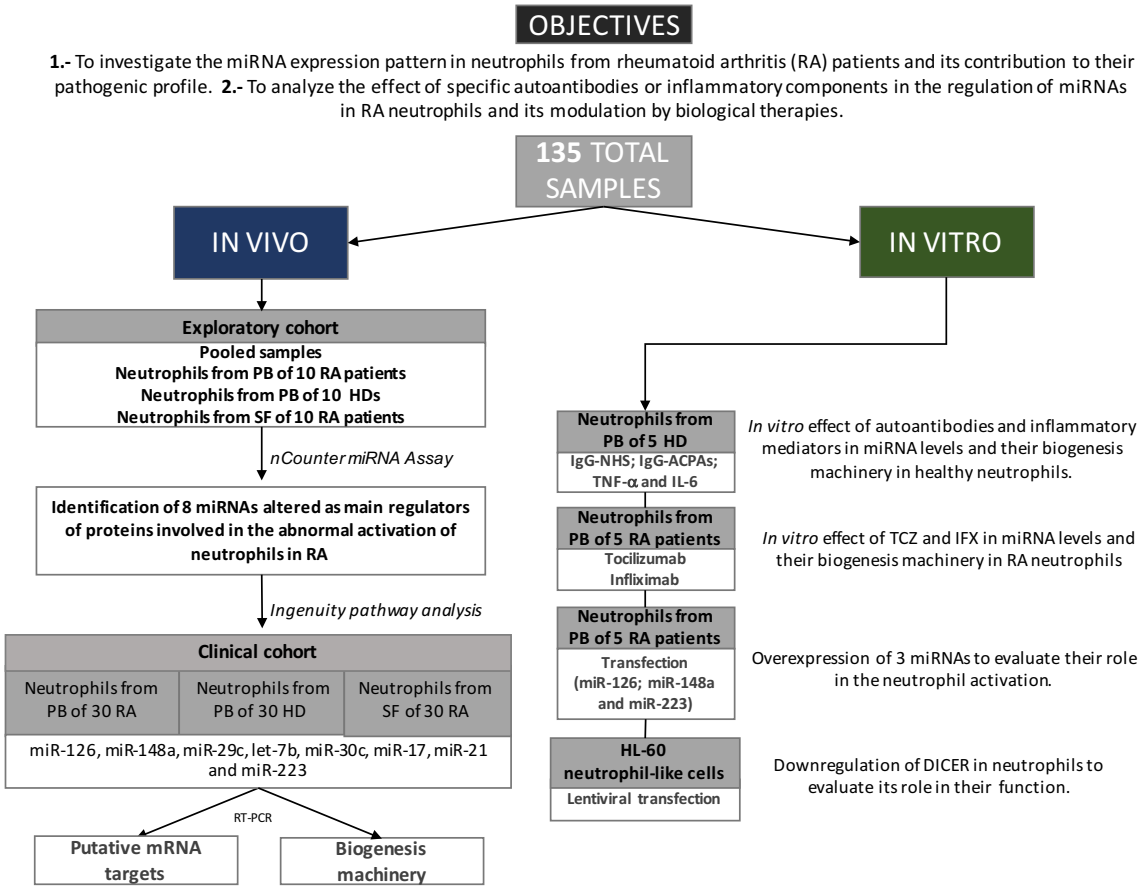
Statistical analyses were performed with SSPS 17.0 (SPSS Inc., Chicago, IL, USA). Following normality and equality of variance tests, variables were compared using paired Student's t test or alternatively by a non-parametric test (Mann–Whitney rank sum test). Paired samples within the same subjects were compared by Wilcoxon signed-rank test. Spearman correlation was calculated to estimate the linear correlations between variables. P-values <0.05 were considered statistically significant.

Supplementary table 1. Expression levels of miRNAs in neutrophils from peripheral blood of RA patients and healthy donors.

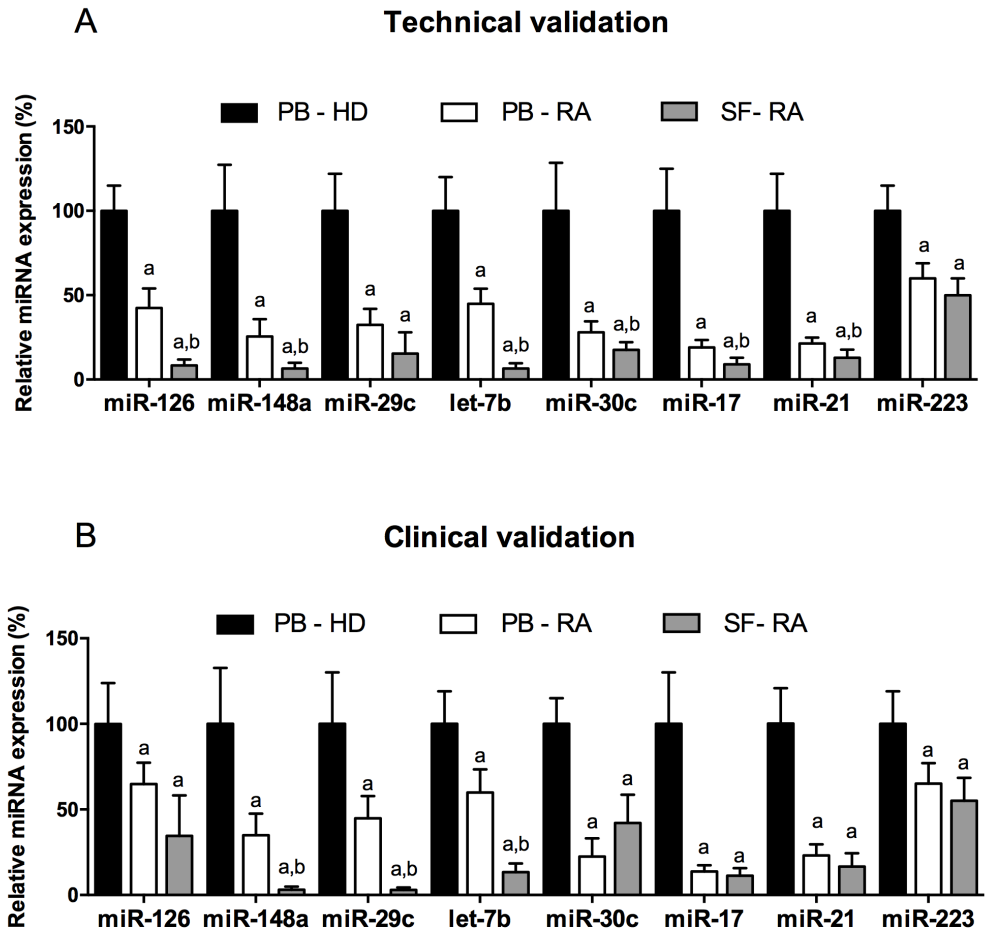
	Normalized counts		Fold change
miRNA	**RA**	**HD**	
hsa-miR-142-3p	2043,03	119134,67	-58,31
hsa-miR-4454	174,56	9965,90	-57,09
hsa-miR-21-5p	35,00	1481,20	-42,32
hsa-let-7a-5p	332,96	10813,64	-32,48
hsa-miR-29b-3p	250,53	7767,26	-31,00
hsa-miR-720	35,00	993,26	-28,38
hsa-miR-26a-5p	88,90	2363,70	-26,59
hsa-let-7g-5p	329,73	8346,42	-25,31
hsa-miR-374a-5p	105,06	2119,00	-20,17
hsa-let-7d-5p	61,42	1234,33	-20,10
hsa-miR-150-5p	210,12	3203,48	-15,25
hsa-miR-191-5p	471,97	6659,62	-14,11
hsa-miR-106b-5p	93,75	1308,18	-13,95
hsa-miR-30b-5p	35,00	450,30	-12,87
hsa-let-7f-5p	40,41	498,80	-12,34
hsa-miR-107	48,49	579,16	-11,94
hsa-miR-26b-5p	202,04	2102,35	-10,41
hsa-miR-450a-5p	109,91	1042,49	-9,48
hsa-miR-106a	114,76	1046,83	-9,12
hsa-miR-17-5p	114,76	1046,83	-9,12
hsa-miR-29a-3p	143,85	1290,08	-8,97
hsa-miR-130a-3p	38,79	317,09	-8,17
hsa-miR-1537	43,64	340,98	-7,81
hsa-miR-126-3p	174,56	1350,17	-7,73
hsa-miR-301a-3p	35,00	246,87	-7,05
hsa-miR-125a-5p	100,21	693,54	-6,92
hsa-miR-340-5p	139,00	949,82	-6,83
hsa-miR-181a-5p	140,62	935,34	-6,65
hsa-miR-32-5p	101,83	666,76	-6,55
hsa-miR-374b-5p	35,00	220,08	-6,29
hsa-miR-98	35,00	215,01	-6,14
hsa-miR-143-3p	35,00	214,29	-6,12
hsa-miR-199a-3p	64,65	393,83	-6,09
hsa-miR-199b-3p	64,65	393,83	-6,09
hsa-miR-140-5p	150,32	904,94	-6,02
hsa-miR-20a-5p	179,41	1075,07	-5,99
hsa-miR-20b-5p	179,41	1075,07	-5,99
hsa-miR-15b-5p	1928,27	11179,96	-5,80
hsa-miR-15a-5p	1550,05	8802,51	-5,68
hsa-miR-186-5p	58,19	317,09	-5,45
hsa-miR-29c-3p	189,11	959,23	-5,07
hsa-let-7i-5p	161,63	805,03	-4,98
hsa-miR-28-5p	46,87	231,66	-4,94
hsa-miR-423-5p	56,57	273,65	-4,84
hsa-miR-582-5p	193,96	916,52	-4,73
hsa-miR-223-3p	82619,83	385315,19	-4,66
hsa-miR-144-3p	1124,96	5190,00	-4,61
hsa-miR-148b-3p	221,44	1001,95	-4,52
hsa-miR-148a-3p	520,46	2263,79	-4,35
hsa-miR-145-5p	224,67	974,44	-4,34
hsa-miR-454-3p	35,00	150,58	-4,30
hsa-miR-93-5p	538,23	2315,19	-4,30
hsa-miR-24-3p	53,34	224,42	-4,21
hsa-miR-342-3p	168,10	691,37	-4,11
hsa-miR-16-5p	2240,22	9019,69	-4,03
hsa-miR-338-3p	214,97	853,54	-3,97
hsa-miR-199b-5p	109,91	425,68	-3,87
hsa-let-7e-5p	35,00	134,65	-3,85
hsa-miR-27b-3p	30,71	113,66	-3,70
hsa-miR-197-3p	153,55	568,30	-3,70
hsa-miR-30e-5p	164,86	602,33	-3,65
hsa-miR-320e	42,02	153,48	-3,65
hsa-miR-19b-3p	242,45	881,77	-3,64
hsa-miR-30c-5p	63,04	221,53	-3,51
hsa-miR-22-3p	163,25	563,23	-3,45
hsa-miR-363-3p	35,00	120,18	-3,43

	Normalized counts		Fold change
miRNA	**RA**	**HD**	
hsa-miR-590-5p	40,41	136,83	-3,39
hsa-miR-361-3p	63,04	209,22	-3,32
hsa-miR-361-5p	147,09	487,94	-3,32
hsa-miR-92a-3p	53,34	176,64	-3,31
hsa-miR-346	45,26	148,41	-3,28
hsa-let-7b-5p	720,88	2330,40	-3,23
hsa-miR-23a-3p	2511,76	7869,34	-3,13
hsa-miR-185-5p	129,31	398,17	-3,08
hsa-miR-423-3p	30,71	93,39	-3,04
hsa-miR-664-3p	35,00	103,52	-2,96
hsa-miR-23b-3p	61,42	180,26	-2,93
hsa-miR-132-3p	35,56	102,08	-2,87
hsa-miR-193a-5p	35,56	99,18	-2,79
hsa-miR-505-3p	40,41	110,76	-2,74
hsa-miR-25-3p	1205,77	3300,49	-2,74
hsa-miR-222-3p	185,88	508,21	-2,73
hsa-miR-4516	665,92	1818,56	-2,73
hsa-miR-142-5p	35,00	91,94	-2,63
hsa-miR-221-3p	37,18	97,01	-2,61
hsa-miR-425-5p	127,69	332,29	-2,60
hsa-miR-194-5p	35,00	90,49	-2,59
hsa-miR-30d-5p	96,98	239,63	-2,47
hsa-miR-200c-3p	35,56	86,87	-2,44
hsa-miR-199a-5p	35,00	83,98	-2,40
hsa-miR-181c-5p	35,00	83,25	-2,38
hsa-miR-424-5p	71,12	167,23	-2,35
hsa-miR-19a-3p	145,47	325,78	-2,24
hsa-miR-99b-5p	40,41	81,81	-2,02
hsa-miR-1260a	35,00	68,05	-1,94
hsa-miR-324-5p	35,00	66,60	-1,90
hsa-miR-451a	41408,51	67873,22	-1,64
hsa-miR-4443	35,00	57,19	-1,63
hsa-miR-27a-3p	35,00	56,47	-1,61
hsa-miR-146a-5p	35,00	53,57	-1,53
hsa-let-7c	38,79	59,36	-1,53
hsa-miR-34c-5p	37,18	54,30	-1,46
hsa-miR-365a-3p	35,00	50,68	-1,45
hsa-miR-128	35,00	47,78	-1,37
hsa-miR-769-5p	35,00	47,06	-1,34
hsa-miR-190a	35,00	46,33	-1,32
hsa-miR-30a-5p	35,00	46,33	-1,32
hsa-miR-378a-3p	35,00	45,61	-1,30
hsa-miR-378i	35,00	45,61	-1,30
hsa-miR-421	35,00	44,88	-1,28
hsa-miR-216b	42,02	51,40	-1,22
hsa-miR-140-3p	30,71	36,92	-1,20
hsa-miR-494	35,00	41,27	-1,18
hsa-miR-28-3p	35,00	39,82	-1,14
hsa-miR-7-5p	35,00	39,82	-1,14
hsa-miR-151a-3p	35,00	39,09	-1,12
hsa-miR-33a-5p	35,00	38,37	-1,10
hsa-miR-299-3p	35,00	37,65	-1,08
hsa-miR-495	35,00	37,65	-1,08
hsa-miR-147b	35,00	36,92	-1,05
hsa-miR-4286	77,58	76,01	1,02
hsa-miR-192-5p	50,11	48,50	1,03
hsa-miR-378e	116,38	94,11	1,24
hsa-miR-515-5p	37,18	35,00	1,24
hsa-miR-215	37,18	35,00	1,24
hsa-miR-216a	37,18	35,00	1,24
hsa-miR-411-5p	38,79	35,00	1,29
hsa-miR-4531	40,41	35,00	1,35
hsa-miR-188-5p	42,02	35,00	1,40
hsa-miR-579	69,50	35,00	1,88
hsa-miR-302d-3p	113,14	43,44	2,60
hsa-miR-548aa	145,47	35,00	4,85
hsa-miR-548ai	214,97	35,00	7,17

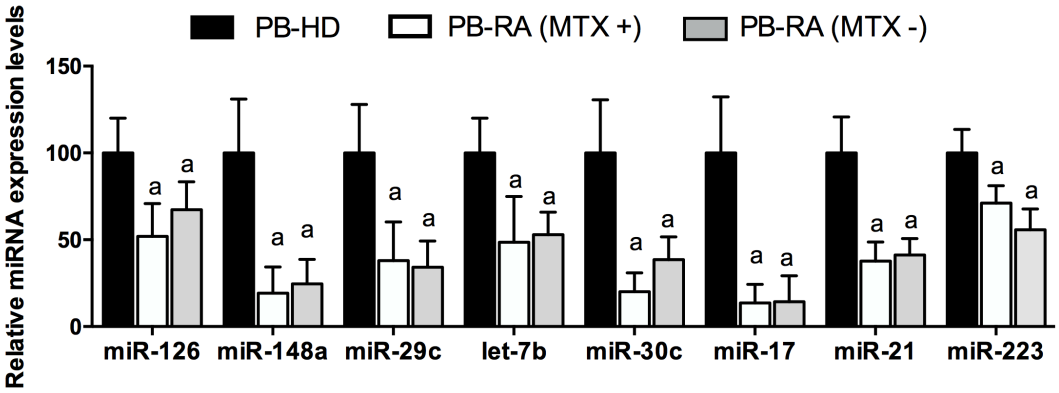
The list of the miRNAs detected after the nCounter miRNA Assay in neutrophils from peripheral blood of healthy donors (HD) and rheumatoid arthritis (RA) patients are shown in the 1st and 5th columns of the table. The normalized NanoString counts for the different miRNAs in RA patients and HD are displayed in the 2nd, 3rd, 6th and 7th columns of the table, respectively. miRNAs are sort depending on the fold change expression value between RA patients and HD as indicated in the 4th and 5th columns of the table.



Supplementary figure 1. Flowchart of the study design**.** One hundred thirty-five human samples were included in this study. Firstly, nCounter miRNA assay was performed on pooled samples of neutrophils from PB of 10 HD, neutrophils from PB of 10 RA patients and neutrophils from SF of 10 RA patients. Eight altered miRNAs were identified by IPA as the main regulators of proteins involved in the abnormal activation of neutrophils in RA, the expression of these 8 miRNAs, genes involved in miRNA biogenesis and putative mRNA targets of those miRNAs were analyzed individually in neutrophils from 40 PB-RA samples, 40 PB-HD and 40 SF-RA. In addition, neutrophils isolated from PB of 5 HD were treated in vitro with ACPAs isolated from RA patients, TNF-α or IL-6 for 6 hours. Neutrophils isolated from PB of 5 RA patients with high disease activity and no taking any biological therapies were treated in vitro with Tocilizumab or Infliximab. Additionally, overexpression of miR-126, miR-148a and miR-223 was performed in neutrophils from PB of 5 RA patients with high disease activity. HL-60 cell line was differentiated to neutrophils using DMSO 1.25% during 6 days. DICER1 expression was reduced on HL-60 neutrophil-like cells through lentiviral transfection. MicroRNA, miR; Peripheral Blood, PB; Rheumatoid Arthritis, RA; Healthy donor, HD; Synovial fluid, SF; Antibodies to citrullinated protein antigens, ACPAs; Tumor necrosis factor alpha, TNFα; Infliximab, IFX; Tocilizumab: TCZ.

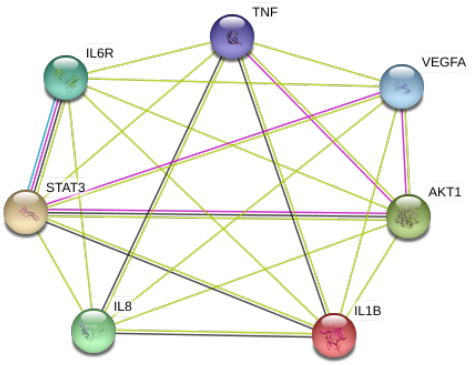


Supplementary figure 2. (A) Technical validation: relative miRNA expression levels in neutrophils of paired peripheral blood (PB) and synovial fluid samples of 10 patients with RA and from PB of 10 healthy donors previously used pooled in the miRNA array. (B) Clinical validation: relative miRNA expression levels in neutrophils of paired peripheral blood (PB) and synovial fluid samples of 30 patients with RA and from PB of 30 healthy donors by RT-PCR. ap<0.05 vs PB-HD.



Supplementary figure 3. Relative miRNA expression levels in neutrophils from peripheral blood (PB) of healthy donors (HD) (n=40) and PB of rheumatoid arthritis (RA) patients with methotrexate (MTX) (n=28) or without MTX (n=12). [a]p<0.05 vs PB-HD.

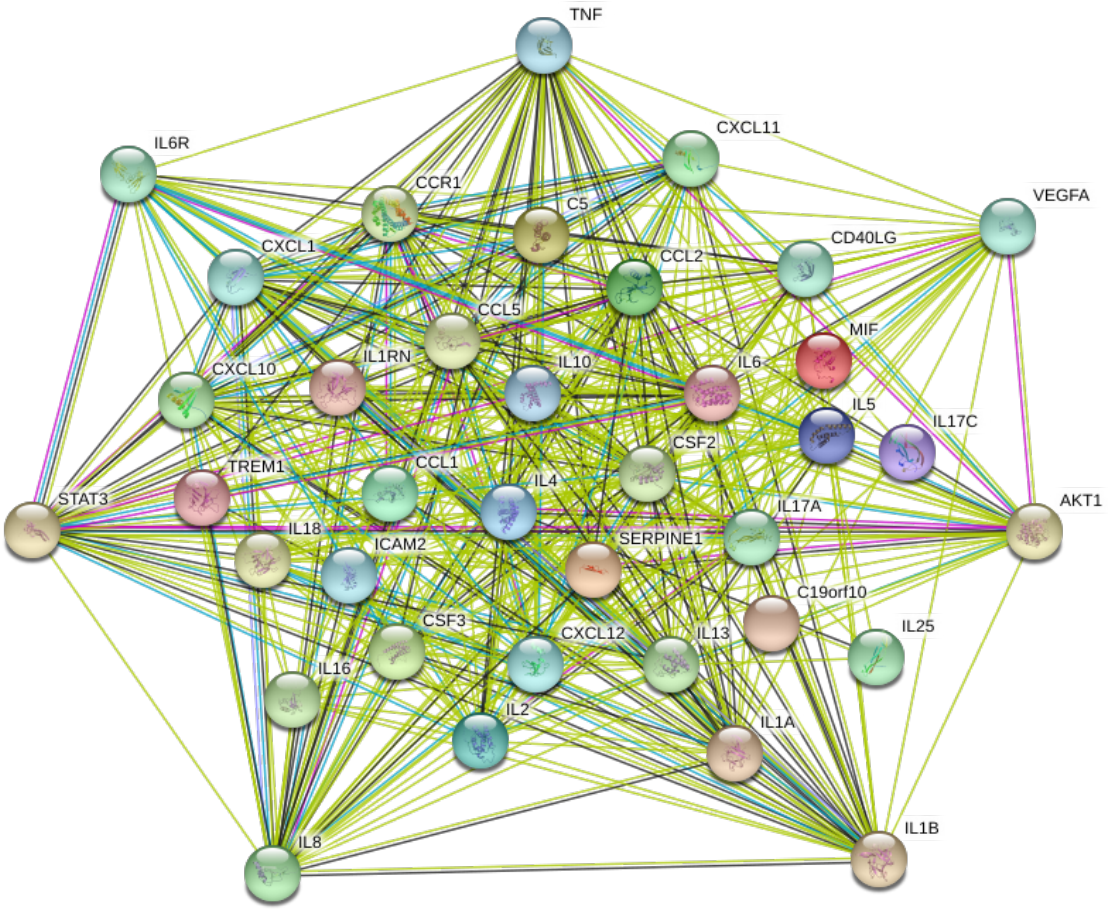
A



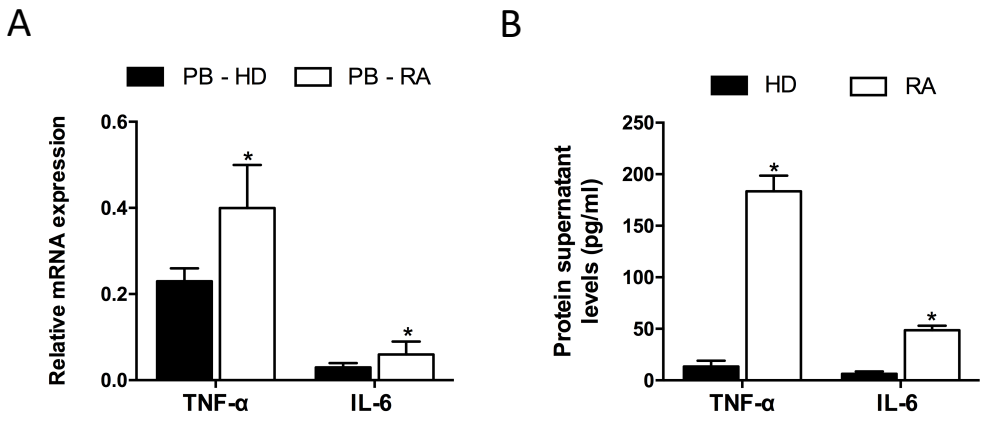
B

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	Interleukin signaling pathway_Homo sapiens_P00036	0.0003785	0.003028	-1.61	12.69
2	JAK/STAT signaling pathway_Homo sapiens_P00038	0.004890	0.01956	-1.05	5.60
3	Ras Pathway_Homo sapiens_P04393	0.02391	0.06169	-1.47	5.48
4	EGF receptor signaling pathway_Homo sapiens_P00018	0.03754	0.06169	-1.47	4.84
5	PDGF signaling pathway_Homo sapiens_P00047	0.03855	0.06169	-1.27	4.13
6	Angiogenesis_Homo sapiens_P00005	0.04866	0.06398	-1.18	3.58
7	CCKR signaling map ST_Homo sapiens_P06959	0.05635	0.06398	-1.23	3.55
8	Inflammation mediated by chemokine and cytokine signaling pathway_Homo sapiens_P00031	0.06398	0.06398	-1.12	3.09

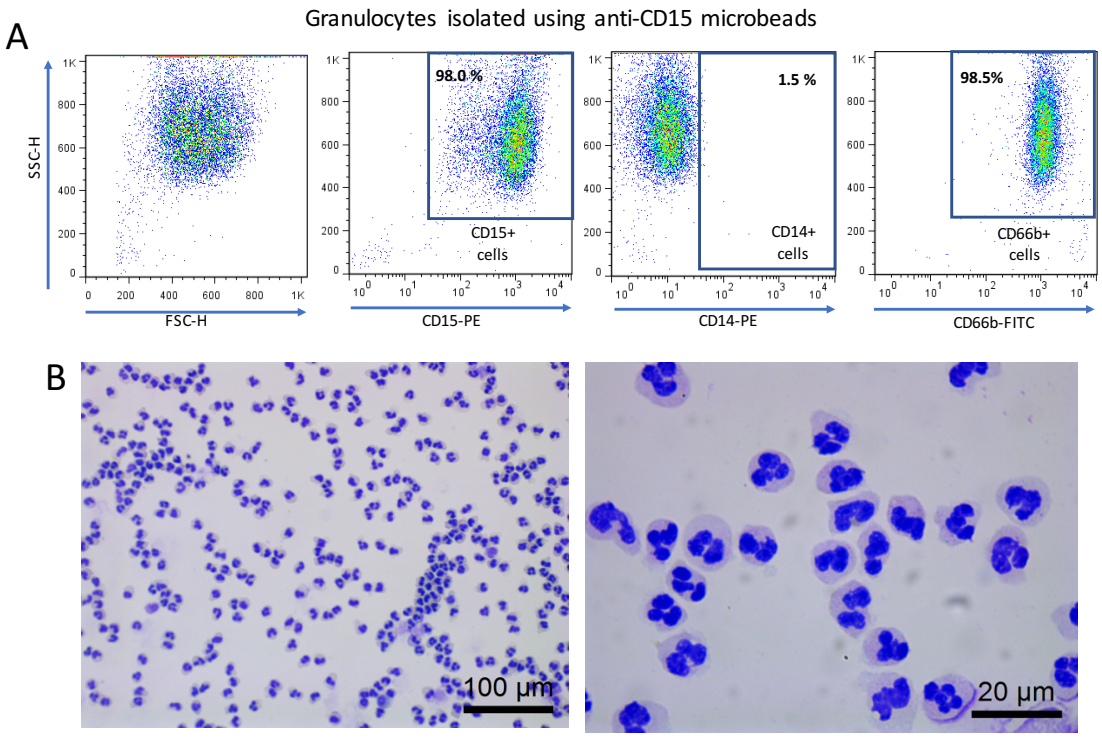
C



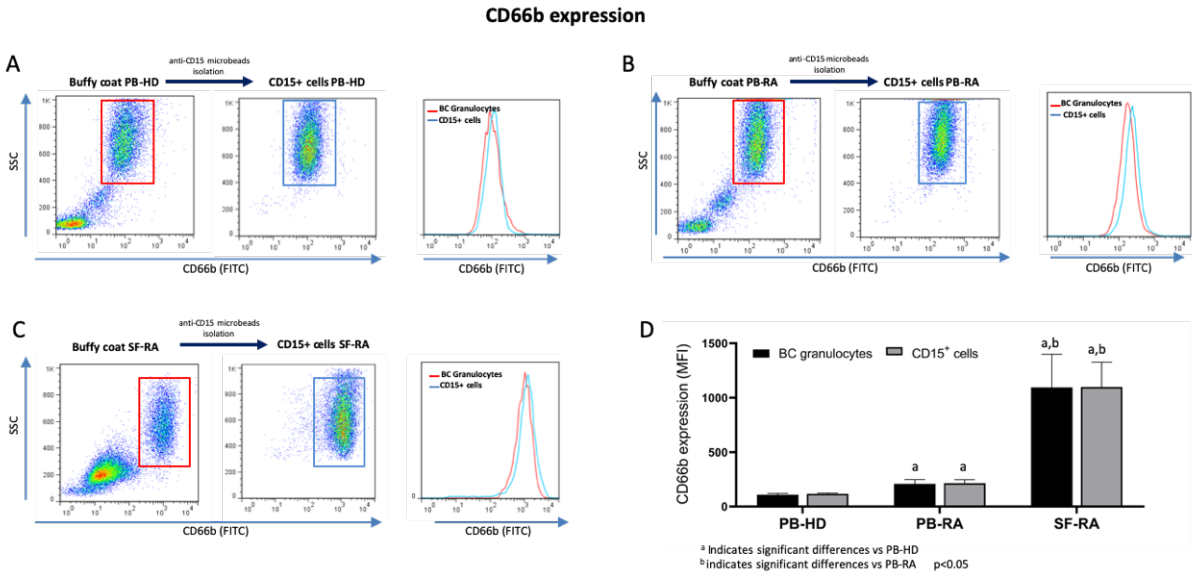
Supplementary figure 4. Functional analysis of the selected potential targets associated with the altered miRNAs of RA neutrophils. (A) Protein-protein interaction network among the selected putative targets of the 8 altered miRNAs in RA neutrophils. The network was generated through the database STRING (http://string-db.org/)**,** which includes known and predicted functional associations between proteins. **(B)** Enrichment analysis of the selected potential targets of the altered miRNAs using the Enrich web-server tool (http://amp.pharm.mssm.edu/Enrichr/). The results displayed in the table show that the enriched pathways are mainly related to inflammatory processes. **(C**) Protein-protein interaction network between the selected potential targets of the 8 altered miRNAs in RA neutrophils and the proteins included in the human cytokine array. The network shows that the selected potential targets of the altered miRNAs are directly connected with a broad range of secondary molecules involved in the amplification of the inflammatory cascade.



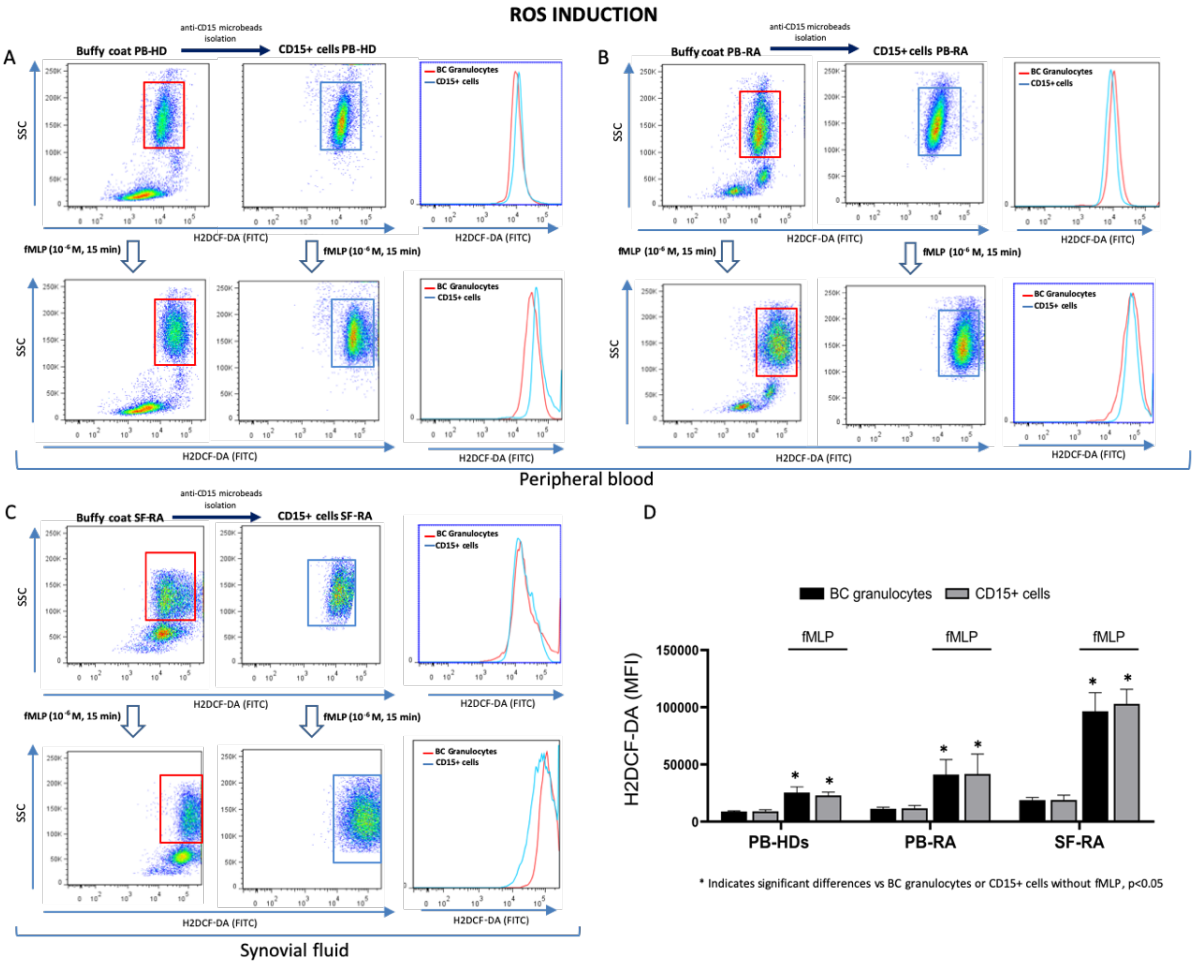
Supplementary figure 5. (**A**) Relative mRNA expression levels of TNF-α and IL-6 in neutrophils isolated from peripheral blood of RA patients and healthy donors. (**B**) Supernatant levels of TNF-α and IL-6 in the culture media of neutrophils from peripheral blood of RA patients and healthy donors.



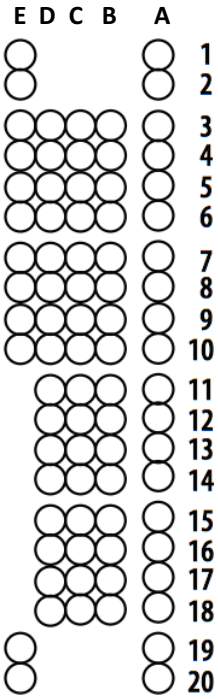
Supplementary figure 6. (A) Percentage of granulocytes isolated from peripheral blood of a RA patient using anti-CD15 microbeads (AUTOMACs) by flow cytometry. (B) Wright-Giemsa in granulocytes isolated from peripheral blood using anti-CD15 microbeads (AUTOMACs).



Supplementary figure 7: Analysis of CD66b expression on granulocytes before and after anti-CD15 isolation procedure in peripheral blood of healthy donors (n=10) and RA patients (n=10) and in synovial fluid of RA patients (n=10). (A) Representative dot plot and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a healthy donor. (B) Dot plot and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from peripheral blood of RA patients. (C) Dot plot and histograms showing the expression of CD66b in granulocytes from buffy coat (before isolation) and CD15+ cells (after isolation with anti-CD15 microbeads) from synovial fluid of RA patients. (D) CD66b expression measured as median fluorescence intensity. [a] indicates significant differences vs PB-HD, [b] indicates significant differences vs PB-RA, p <0.05.



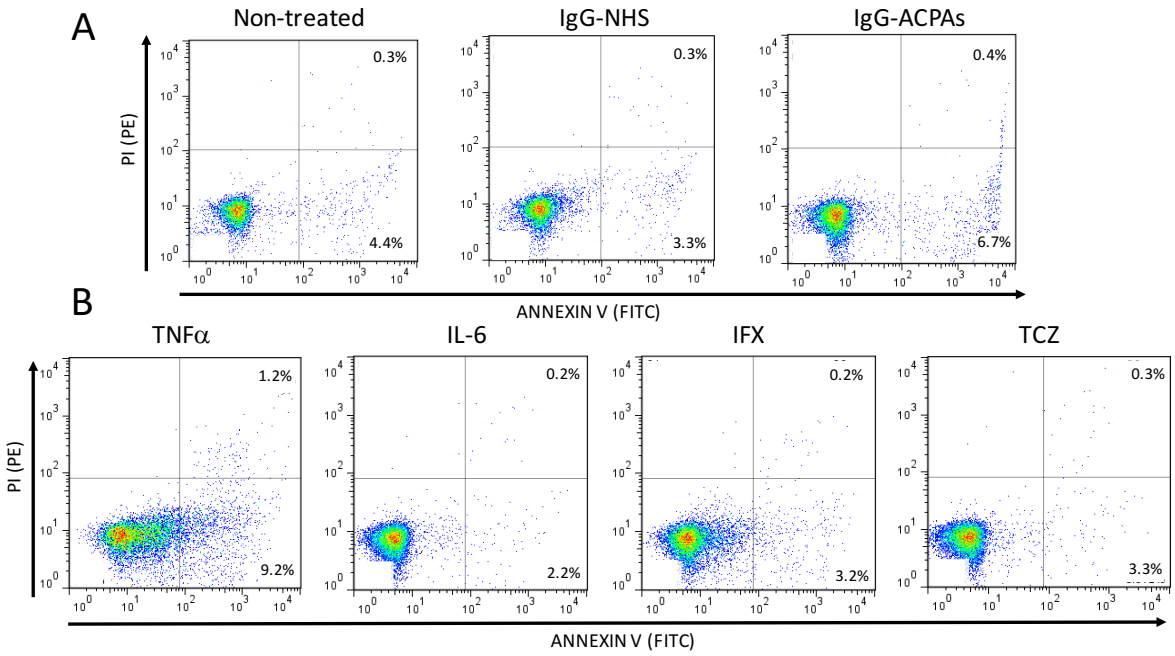
Supplementary figure 8: Analysis of ROS production before and after anti-CD15 isolation procedure in peripheral blood of healthy donors (n=10) and RA patients (n=10) and in synovial fluid of RA patients (n=10). Cells were treated without or with fMLP 1μM for 15 min. ROS production was measured using H2DCF-DA by flow cytometry. (A) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and $CD15^+$ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a healthy donor treated with or without fMLP. (B) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and $CD15^+$ cells (after isolation with anti-CD15 microbeads) from peripheral blood of a RA patient treated with or without fMLP. (C) Representative dot plot and histograms showing the ROS production in granulocytes from buffy coat (before isolation) and $CD15^+$ cells (after isolation with anti-CD15 microbeads) from synovial fluid of a RA patient treated with or without fMLP. (D) ROS production measured as median fluorescence intensity. * indicates significant differences vs non treated cells (without fMLP), $p < 0.05$.



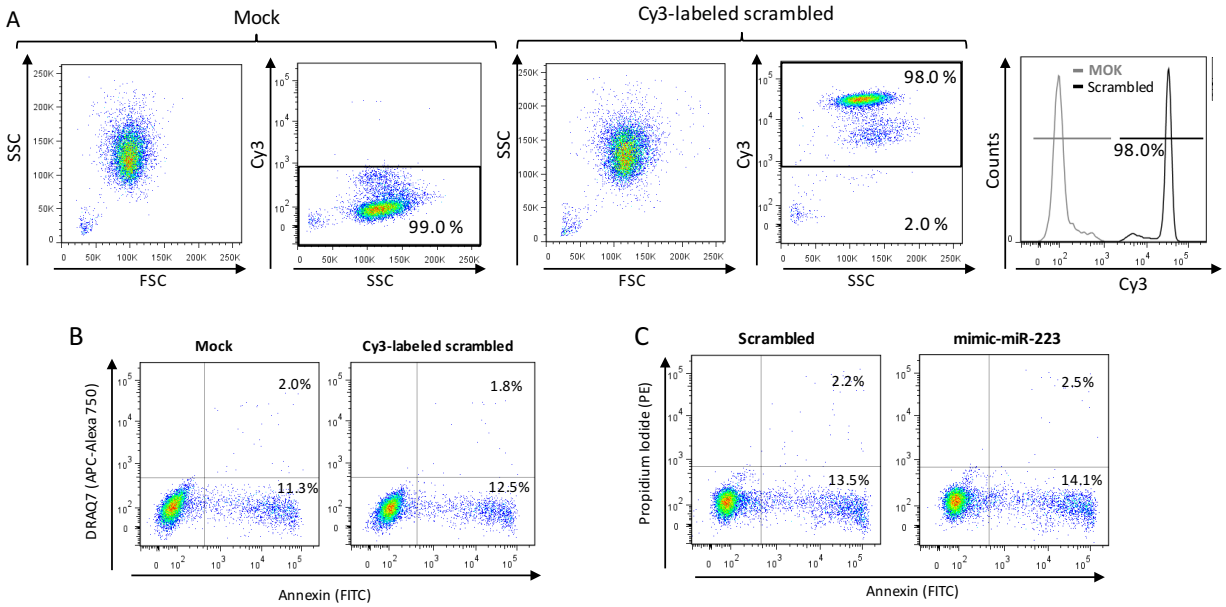
Coordinate	Target/Control
A1, A2	Reference Spots
A3, A4	CCL1/I-309
A5, A6	CCL2/MCP-1
A7, A8	MIP-1a/MIP-1b
A9, A10	CCL5/RANTES
A11, A12	CD40 Ligand/TNFSF5
A13, A14	Complement Component C5/C5a
A17, A18	CXCL10/IP-10
A19, A20	Reference Spots
B3, B4	CXCL11/I-TAC
B5, B6	CXCL12/SDF-1
B7, B8	G-CSF
B9, B10	GM-CSF
B11, B12	ICAM-1/CD54
B13, B14	IFN-g
B15, B16	IL-1a/IL-1F1
B17, B18	IL-1b/IL-1F2
C3, C4	IL-1ra/IL-1F3
C5, C6	IL-2
C7, C8	IL-4

Coordinate	Target/Control
C9, C10	IL-5
C11, C12	IL-6
C13, C14	IL-8
C15, C16	IL-10
C17, C18	IL-12 p70
D3, D4	IL-13
D5, D6	IL-16
D7, D8	IL-17A
D9, D10	IL-17E
D11, D12	IL-18/IL-1F4
D13, D14	IL-21
D15, D16	IL-27
D17, D18	IL-32a
E1, E2	Reference Spots
E3, E4	MIF
E5, E6	Serpin E1/PAI-1
E7, E8	TNF-a
E9, E10	TREM-1
E19, E20	Negative Control

Supplementary figure 9. Human cytokine array map. Figure displaying the coordinate and the target protein of each spot of the array.



Supplementary figure 10. (A) Levels of apoptosis in neutrophils isolated from healthy donors treated with IgGs purified from healthy donors (IgG-NHS) and RA patients (enriched IgG-ACPAs) for 6 hours measured by Annexin V/Propidium iodide (PI) through flow cytometry. (B) Levels of apoptosis in neutrophils isolated from healthy donors treated with TNF-α or IL-6 and neutrophils from RA patients treated with IFX or TCZ for 6 hours, measured by Annexin V/Propidium iodide (PI) through flow cytometry.



Supplementary figure 11. Transfection efficiency and apoptosis in primary neutrophils isolated from RA patients after 12 hours of transfection with miRNA mimics. (A) Levels of Cy3 fluorescence in neutrophils isolated from RA patients transfected with cy3-labeled scrambled for 12 hours. Mock, cells incubated with siPORT, was used as negative control. (B) Levels of apoptosis in neutrophils from RA patients transfected with cy3-labeled scrambled (C) Levels of apoptosis in neutrophils from RA patients transfected with scrambled and mimic miR-223.

REFERENCES

1. Güven, E., Duus, K., Lydolph, M.C., Jørgensen, C.S., Laursen, I., Houen, G. Non-specific binding in solid phase immunoassays for autoantibodies correlates with inflammation markers. J Immunol Methods. 2014; 403 (1-2): 26-36