Tocilizumab (TCZ) Decreases Angiogenesis in Rheumatoid Arthritis through its Regulatory Effect on miR-146a-5p and EMMPRIN/CD147

Devy Zisman (✉ devyzisman@gmail.com)

Carmel Hospital <https://orcid.org/0000-0003-0523-2292>

Mirna Safieh

Carmel Hospital

Elina Simanovich

Carmel Hospital

Joy Feld

Carmel Hospital

Amalia Kinarty

Carmel Hospital

Liron Zisman

Carmel Hospital

Tal Gazitt

Carmel Hospital

Amir Haddad

Carmel Hospital

Muna Elias

Carmel Hospital

Itzhak Rosner

Carmel Hospital

Lisa Kaly

Carmel Hospital

Michal A. Rahat

Carmel Hospital

Research Article

Keywords: Rheumatoid Arthritis (RA), Tocilizumab (TCZ), Angiogenesis, EMMPRIN/CD147, miR-146a-5p, Thrombospondin-1 (Tsp-1)

Posted Date: February 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-222740/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Frontiers in Immunology on December 15th, 2021. See the published version at <https://doi.org/10.3389/fimmu.2021.739592>.

Abstract

Background

Angiogenesis is an important contributor to the development of Rheumatoid arthritis (RA). Tocilizumab (TCZ), an anti-IL-6 receptor antibody, is used in the treatment of RA patients, and has been shown to exert anti-inflammatory effects. However, its effects on angiogenesis are not fully elucidated, and the molecular mechanisms regulating this effect are unknown.

Methods

We evaluated the concentrations of several pro- and anti-angiogenic factors and the expression levels of several microRNA molecules that are associated with RA and angiogenesis in serum samples obtained from 40 RA patients, before and 4 months after the initiation of TCZ treatment. Additionally, we used an *in vitro* co-culture system of fibroblasts (the HT1080 cell line) and monocytes (the U937 cell line) to explore the mechanisms of TCZ action.

Results

Serum samples from RA patients treated with TCZ exhibited reduced levels of EMMPRIN/CD147, enhanced expression of miR-146a-5p and miR-150-5p, and reduced angiogenesis as was manifested by the reduced number of tube-like structures formed by EaHy926 endothelial cell line. *In vitro*, the accumulation of the pro-angiogenic factors EMMRPIN, VEGF and MMP-9 in the supernatants was increased by co-culturing the HT1080 fibroblasts and the U937 monocytes, while the accumulation of the anti-angiogenic factor thrombospondin-1 (Tsp-1) and the expression levels of miR-146a-5p were reduced. Transfection of HT1080 cells with the miR-146a-5p mimic, decreased the accumulation of EMMPRIN, VEGF and MMP-9. When EMMPRIN was neutralized with a blocking antibody, supernatants derived from these co-cultures exhibited reduced migration, proliferation and tube formation in functional assays.

Conclusions

Our findings implicate miR-146a-5p in the regulation of EMMPRIN and propose that TCZ affects angiogenesis through its effects on EMMRPIN and miR-146a-5p.

1. Introduction

Tocilizumab (TCZ) in RA treatment

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes joint inflammation, damage and bone erosion, as well as many systemic manifestations. The pathophysiology of RA is based on a complex network of pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α) [1, 2]. The binding of IL-6 to its membranal receptor (*cis* activation) or its soluble receptor (*trans* activation) and the subsequent binding of the complex to the gp130 receptor chains evokes pro-

inflammatory signals, primarily through JAK/STAT3 activation, but also through the activation of the MAPKs and PI3K/Akt pathways [3]. These pathways, together with TNF α and IL-1 β , synergistically activate pro-inflammatory and pro-angiogenic molecules [4]. This accounts for the pro-inflammatory effects of IL-6 including neutrophil and monocyte recruitment, endothelial cell activation, B cell stimulation leading to autoantibody production, and induction of acute phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA) [3]. Furthermore, IL-6 together with TNF α drive the proliferation of fibroblast-like synoviocytes (FLS) and promote their local secretion of a myriad of cytokines [5], as well as their stimulation of angiogenesis through phosphorylation of STAT3 and induction of vascular endothelial growth factor (VEGF) production [6, 7].

The humanized anti-IL-6 receptor monoclonal antibody tocilizumab (TCZ) blocks both the *cis* and *trans* signaling pathways of IL-6. Although it is known to lead to the accumulation of IL-6 in the serum of treated patients [2], TCZ reduces the production of pro-inflammatory cytokines, reduces CRP and SAA levels, and ameliorates the systemic manifestation of RA, such as pain, fatigue, and anemia [1, 2, 8]. While the effects of TCZ on angiogenesis have yet to be investigated in depth, TCZ has been shown to reduce VEGF serum levels in complete Freund's adjuvant (CFA)-induced arthritic rats [9], and to reduce the mean vessel density (MVD) in the synovium of RA patients as evaluated by immunohistochemical staining for the endothelial marker CD31 [10]. However, the detailed mechanisms of action, which enable TCZ to inhibit angiogenesis, are not yet understood.

The increased metabolic need for oxygen by proliferating FLS and infiltrating immune cells results in local hypoxia, which stimulates the production of pro-angiogenic factors (e.g., VEGF). Thus, an excess of pro-angiogenic relative to anti-angiogenic mediators is generated, and this imbalance switches on angiogenesis and increases blood vessel density to help sustain pannus progression [6]. VEGF, which promotes endothelial cell migration, proliferation, and tube formation, [11] is the most potent pro-angiogenic factor known. Additional important pro-angiogenic factors are matrix metalloproteinases (MMPs) which degrade the basement membrane and allow endothelial cell migration; EMMPRIN/CD147 which can induce both VEGF and MMPs secretion [12]; and the neutrophil gelatinase associated lipocalin (NGAL), which can bind to and protect MMP-9 from degradation [13]. On the other hand, thrombospondin-1 (Tsp-1) is an example of a potent endogenous inhibitor of angiogenesis [14, 15].

MicroRNAs (miRNAs) are small (20–23 nucleotides long) non-coding RNA molecules, which function in post-transcriptional regulation of gene expression via base-pairing with complementary mRNA sequences leading to their silencing by cleavage or inhibited translation. Thus, miRNAs have been implicated in the regulation of many cellular processes, including angiogenesis, particularly in the context of malignancy [16–18]. For instance, we have previously linked the regulation of EMMPRIN expression to miR-146a-5p in cancer cells [19, 20]. However, little is known about the involvement of miRNAs in angiogenesis in the context of RA.

The goal of the research presented here was to evaluate the effects of TCZ on levels of pro- and anti-angiogenic factors found in sera of RA patients, as well as on the accumulation of several circulating

miRNAs known to be associated with angiogenesis. Our findings including the regulatory role of miR-146a-5p on angiogenesis were then corroborated in an *in vitro* co-culture system of monocyte and fibroblast cell lines.

2. Methods

2.1 Patients: The study cohort included 40 patients diagnosed with RA, who fulfilled the 2010 EULAR and ACR classification criteria for RA [21] with active disease according to the disease activity score in 28 joints (DAS28-CRP) score ($\text{DAS} \geq 3.2$) [22] and initiating treatment with tocilizumab infusion (8mg/kg every 4 weeks). The patients were recruited consecutively from the Rheumatology Clinic in Carmel Medical Center and Bnei Zion Medical Center, Haifa, Israel, after failure of conventional disease modifying anti-rheumatic drugs (cDMARDs). All other medical decisions were at the physicians' discretion. All patients were examined and blood samples were obtained before TCZ infusion at enrollment (referred to as "Before") and 4 months after the beginning of treatment (referred to as "After 4 m"). Blood was immediately centrifuged, and serum samples were stored at -80°C until further analysis. Response to treatment was assessed according to the EULAR response criteria [22]. We further stratified our patients to "responders"- those patients who experienced an improvement in their DAS-28-CRP score ≥ 1.2 - and their total DAS28-CRP score was < 5.1 , and to "non-responders" - patients who demonstrated no change or a change < 1.2 in DAS-28-CRP score [22].

Patients diagnosed with additional inflammatory disease or active neoplastic diseases were excluded from the study. The study was approved by Carmel Hospital Institutional Review Board (Helsinki committee CMC-0018-11) and all patients signed an informed consent form.

2.2 Sandwich Enzyme-Linked Immunosorbent Assay (ELISA): Concentrations of EMMPRIN, VEGF, MMP-9, IL-6, NGAL, and Tsp-1 were measured using commercial DuoSet ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Duplicate serum and supernatant samples were diluted according to preliminary calibration experiments. To determine cytokine concentrations in serum samples, samples tested for EMMPRIN, MMP-9 and NGAL were diluted 1:100; samples tested for VEGF and IL-6 were diluted 1:4; and samples tested for Tsp-1 were diluted 1:1000. To determine the cytokine concentrations in supernatants derived from cultured cells, samples were diluted 1:100, except for Tsp-1 (1:1000). The human high sensitivity CRP (hsCRP) was measured by ELISA kit (AssayPro, St. Charles, MO), and samples were diluted 1:4,000.

2.3 Quantitative real-time PCR (qPCR) analyses: Total RNA was extracted from 200 μl of serum derived from the RA patients at the different time points or from 4×10^5 HT1080 cells, using the total RNA purification kit (Norgen Biotek, Ontario, Canada) according to the manufacturer's instructions. To assess the expression of specific miRNAs, 350 ng of total RNA were reverse transcribed at 37°C for 1 hour using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific/Applied Biosystems, CA) and a mixture of the 5xRT primers for each of the miRNA examined (Thermo Fisher Scientific/Applied Biosystems). The miRNAs measured (miR-16-5p, miR-21-5p, miR-132-3p, miR-146a-5p, miR-150-5p, miR-

155-5p, miR-203a-3p, miR-221-3p, and miR-323a-3p) and the RNU6B (U6) small RNA endogenous control were amplified in triplicates using the TaqMan microRNA assay kit (Thermo Fisher Scientific/Applied Biosystems) according to the manufacturer's instructions. The reaction was carried out for 40 cycles, each of 15 sec at 95°C and 60 sec at 60°C, using the StepOne real-time PCR (Thermo Fisher Scientific/Applied Biosystems). The comparative method ($2^{-\Delta\Delta CT}$) was used for relative quantification, and serum samples from the first visit (before the initiation of TCZ treatment) served as a calibrator in each experiment.

To date, there is still a debate as to which miRNA or small RNA molecule is best to use for normalization strategy for circulating miRNAs [23]. Since miR-16 and U6 are two of the most frequently used reference genes, we compared their stability in the serum samples of patients before and 4 months after initiation of TCZ treatment, and chose U6 which demonstrated better stability for the normalization (median U6 C_T values of 30.4, IQR 31.4, 28.9, $n = 76$; miR-16 median CT value 25.95, IQR 27.91, 23.95, $n = 76$) (**Fig S1**).

2.4 Cultured Cells: To study the interactions between fibroblasts and monocytes, we co-cultured the human fibrosarcoma cell line HT1080 (ATCC CCL-12012) and the monocyte-like U937 cells (ATCC 1593). HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, IL), 10% fetal calf serum (FCS), 1% amphotericin B, 1% L-glutamine, 1% non-essential amino acids (NEAA), and 1% antibiotics, with the addition of 25% conditioned medium supplement derived from the human promyelocytic leukemic cell line HL60 (ATCC CRL-240) which secretes fibroblast growth factor-2 (FGF-2). U937 cells were cultured in RPMI-1640 medium, 10% FCS, amphotericin B (27 μ M) and 1% antibiotics. The human endothelial cell line EaHy926 (ATCC CRL-2922) was cultured in DMEM with 10% FCS, 1% glutamine, 2% HAT, and 1% antibiotics. All cell lines were split twice a week at a ratio of 1:4. To avoid the masking of signals, after cells were seeded in plates and allowed to adhere, medium was replaced with serum-starved medium with 0.1% BSA for the duration of the experiment. All cell lines were regularly tested for morphological changes and presence of mycoplasma.

HT1080 (4×10^5 cells) or U937 (4×10^5 cells) were cultured separately or in co-culture, with or without TNF α (1ng/mL), and after 48 hours, supernatants were collected for further analysis. In some experiments, increasing amounts of the recombinant EMMPRIN protein (R&D systems, Minneapolis, MN) was added as indicated, or alternatively, the anti-EMMPRIN blocking antibody (2 ng/ml, Biolegend, San Diego, CA) was added to some of the wells. In experiments where RNA was extracted, HT1080 and U937 cells were separated by an insert (0.4 μ m pore size) allowing exchange of soluble nutrients and proteins but precluding cell-cell contact.

2.5 Wound assay (in vitro): EaHy926 cells were seeded (10^5 cells/well) in 96-well plates and cultured to confluency. The monolayer was then scratched using a toothpick, and the non-adherent cells were removed by washing with PBS. At this point, supernatants from the HT1080 and U937 co-cultures (diluted 1:2) with or without the addition of the anti-EMMPRIN antibody (2 ng/ml), were added to the endothelial layer. Images of the scratch site were acquired immediately after scratching the cell monolayer (T0) and 24 h later (T24) (Moticam 2MP, magnification x4), and the wound area was measured at both times using

the ImagePro plus 4.5 software (Media Cybernetics, Inc., Rockville, MD). The subtraction of the area at T0 from the area measured at T24 reflected the area to which the endothelial cells migrated in wound closure.

2.6 Tube formation assay (in vitro): EaHy926 cells (8×10^4 cells/well) were plated in triplicates in DMEM with 2% FCS on 96-well plates which were previously coated at 4°C with Coultrex® reduced growth factor basement membrane extract (Travigen, Gaithersburg, MD) and polymerized at 37°C for 2 hours. Serum samples (diluted 1:4) or supernatants from the HT1080 and U937 co-cultures (diluted 1:2) were added. Images were obtained after 6 hours of incubation (Moticam 2MP, magnification x4), and the number of closed lumens were counted in two separate fields.

2.8 Transfection of HT1080 cells with miR-146-5p mimic: HT1080 cells (10^4 cells) were seeded in a 96-well plate in 100µl of full medium and incubated overnight. The Lipofectamine RNAi MAX (Ambion, Austin, TX) was diluted 1:25 in Opti-MEM medium and combined with an Opti-MEM medium containing 30 nM of miRNA-146a-5p mimic, or its negative control (NC mimic, both from Ambion) to create miRNA-lipid complex that was added to each well and incubated overnight. Cells were then washed with PBS, and were incubated with serum-starved medium with 0.1% BSA, with or without addition of U937 cells for additional 48 hours, before collecting the supernatants for further analysis.

2.7 Statistics: All values are presented as means $\pm$ standard error of measurement (SEM). The nonparametric Kruskal-Wallis analysis of variance (ANOVA) test was used to compare multiple groups, followed by the Dunn's multiple post-hoc comparison test. Two groups were compared with the two-tailed Mann-Whitney U test, or if paired, with the Wilcoxon matched-paired signed rank test. P values exceeding 0.05 were not considered significant.

3. Results

3.1 Study population: The age of the study population was 57.5 ± 11.1 years, with disease duration of 7.7 ± 5.6 years, 33 (82.5%) were female and 53.9% were positive for rheumatoid factor. The demographic and clinical data of the patients participating in the study are summarized in Table 1. Following 4 months of TCZ treatment, 25/40 (62.5%) patients were classified as “responders” according to EULAR criteria. Notably, after 4 months of treatment, the mean DAS-28 CRP dropped from 5.47 (IQR 6.1, 4.75) to 3.52 (IQR 4.81, 2.8), tender joint count decreased from 12.5 ± 1.02 to 5.9 ± 0.82 , and the swollen joint count decreased from 9.25 ± 0.85 to 4.07 ± 0.66 .

Table 1
Demographic, clinical characteristics, underlying diseases, and treatment of the study groups

	Non- responding patients	Responding patients	Total RA patients	P values: Responding vs. non-responding
No. participants	15	25	40	
Sex: Female (%)	14 (93.3%)	19 (76%)	33 (82.5%)	ns
Age (years) ± SD	56.9 ± 3.3	57.9 ± 2.1	57.53 ± 11.1	ns
Disease Duration	8 ± 1	7.52 ± 1.3	7.7 ± 5.6	ns
Tobacco use (%)	3 (20%)	7 (28%)	10 (25%)	ns
RF positive (%)	8 (53.3%)	13 (52%)	21 (53.9%)	ns
Anti-CCP positive (%)	3 (20%)	9 (36%)	12 (30%)	ns
Comorbidities:				
Hypertension	3 (20%)	9 (36%)	12 (30%)	ns
Hyperlipidemia	8 (53.3%)	11 (44%)	19 (47.5%)	ns
Diabetes mellitus (DM)	5 (33.3%)	3 (12%)	8 (20%)	ns
Chronic obstructive pulmonary disease (COPD)	0 (0%)	1 (4%)	1 (2.5%)	ns
Ischemic heart disease (IHD)	0 (0%)	1 (4%)	1 (2.5%)	ns
Prior malignancy	1 (6.6%)	0 (0%)	1 (2.5%)	ns
Medications (at baseline):				
Methotrexate (MTX)	12 (80%)	10 (40%)	27 (67.5%)	0.0217
Sulfasalazine (SSZ)	1 (6.6%)	4 (16%)	5 (12.5%)	ns
Hydroxychloroquine (HCQ)	1 (6.6%)	3 (12%)	4 (10%)	ns
Leflunomide (LEF)	0 (0%)	3 (12%)	3 (7.5%)	ns
Corticosteroid dose in milligrams (mean + SD)	3 ± 5.9	7.2 ± 10.5	5.62 ± 9.2	ns
* anti-CCP, anti-cyclic citrullinated peptide; ns, not significant; RF, rheumatoid factor; SD, standard deviation.				

As expected, serum IL-6 levels were increased after 4 months of treatment (**Fig S2A**) with higher levels in responders than in non-responders (**Fig S2C**). The hsCRP levels were higher in the RA patients before treatment, and were significantly reduced after 4 months of treatment. However, no difference in hsCRP levels was found between responders and non-responders (**Fig S2B, S2D**).

3.2 TCZ affects the concentrations of pro-angiogenic factors

Comparing the levels of pro-angiogenic factors, we noted a drop in EMMPRIN after 4 months of TCZ (Fig. 1A), an increase in NGAL (Fig. 1C), and no significant change in MMP-9 or VEGF levels (Fig. 1B, 1D). Likewise, no change occurred in the serum levels of MMP-3 and MMP-7 (data not shown). The serum levels of the anti-angiogenic factors Tsp-1 (Fig. 1E) and endostatin (data not shown) were also unchanged. Because the angiogenic switch is turned on when the concentrations of pro-angiogenic factors exceed those of anti-angiogenic factors, we calculated the ratio between the pro-angiogenic factor EMMPRIN and the anti-angiogenic factor Tsp-1 for each patient before and 4 months after initiating TCZ, and found a significant decrease following 4 months of treatment (Fig. 1F).

To show that TCZ affected the balance between serum levels of pro- and anti-angiogenic factors, we assessed their angiogenic potential directly on endothelial cells using the tube formation assay. Serum samples before and 4 months after initiation of TCZ treatment were incubated with the endothelial cell line EaHy926, and the number of closed lumens generated, reflecting the angiogenic potential, was quantified. We show that in accordance with the EMMPRIN levels and the EMMPRIN/Tsp-1 ratio, the endothelial cells generated a reduced number of closed lumens after 4 months of TCZ treatment with thicker layers of cells between the lumens, demonstrating reduced angiogenesis (Fig. 1G, 1H).

3.3 Patients responding to TCZ treatment demonstrate reduced EMMPRIN/Tsp-1 ratio. To investigate the correlation between the effects of TCZ on angiogenic factors and the clinical response which was observed in treated patients, we stratified the patients into responders and non-responders, resulting in 15 RA patients who did not respond to TCZ treatment and 25 RA patients who responded well to TCZ treatment according to EULAR criteria [22]. Only NGAL levels were increased in the responders relative to the non-responders (Fig. 2C). Levels of VEGF, MMP-9 and Tsp-1, and surprisingly even EMMPRIN levels, were not different between responders and non-responders (Fig. 2A, 2B, 2D, 2E). However, although each one of these factors separately did not reveal a difference between responders and non-responders, the ratio between EMMPRIN and Tsp-1 was reduced in the responding patients (Fig. 2F), indicating the usefulness of this ratio in evaluating the state of angiogenesis in treated patients.

3.4 TCZ affects the serum expression of miR-146a-5p and miR-150-5p. We next asked whether miRNAs are involved in the regulation of angiogenesis in RA and whether TCZ affects their expression. We selected 9 miRNAs whose expression has been linked to angiogenesis in previous studies and which were also shown to have dysregulated expression in RA [24], and followed their expression in RA patients before and after initiation of TCZ treatment. We chose to examine the level of circulating miRNAs, as

those are known to be stable and protected from RNase activity within exosomes or when complexed with serum proteins [18]. We show that no change occurred in the levels of the miRNAs tested except for the levels of miR-146a-5p and miR-150-5p which were significantly increased after 4 months of TCZ treatment relative to treatment initiation (Fig. 3). However, no difference was found in the levels of all the serum miRNAs, including miR-146a-5p and miR-150-5p, between patients considered responders and non-responders to TCZ (data not shown).

3.5 The pro-angiogenic factors are increased by the co-culture, and the anti-angiogenic factor Tsp-1 is reduced. To explore the mechanisms responsible for the changes observed in the patient serum samples after 4 months of TZC treatment, we turned to an *in vitro* co-culture system of HT1080 fibroblasts and U937 monocytes. Levels of secreted EMMPRIN, VEGF and MMP-9 in the U937 single cultures were minimal (Fig. 4A-C). In co-cultures, EMMPRIN and VEGF levels were synergistically elevated after 48 hours of incubation without TNF α relative to the levels in the single culture of HT1080 (both by about 1.7 fold, $p < 0.05$), whereas MMP-9 levels showed no significant change. The addition of TNF α increased MMP-9 levels in the co-culture (by 2.2 fold, $p < 0.001$), but not those of EMMPRIN or VEGF (Fig. 4A-C), consistent with the known inducing activity of TNF α on MMP-9 [25]. Levels of the anti-angiogenic factor Tsp-1 were reduced in the co-culture relative to the single culture of HT1080 (by 2 fold, $p < 0.001$), and the presence of TNF α further reduced them (by 1.9 fold, $p < 0.05$, Fig. 4D). The ratio between EMMPRIN and Tsp-1 was increased by the co-culture relative to the single culture of HT1080 cells (by 2 fold, $p < 0.05$), and the addition of TNF α further increased it (by 2.3 fold, $p < 0.05$, Fig. 4E)

3.6 EMMPRIN expression promotes VEGF and MMP-9 expression *in vitro*, and neutralization of EMMPRIN activity reduces angiogenesis. We next asked whether EMMPRIN is directly involved in the pro-angiogenic effects of the co-culture. To this end, we incubated each cell type alone with increasing amounts of human recombinant EMMPRIN protein. In both cell lines, the addition of TNF α was necessary to elevate MMP-9 levels, and a significant increase in MMP-9 level (by about 2 fold, $p < 0.05$, Fig. 5A, 5C) was observed upon adding a concentration of 500 ng/ml of recombinant EMMPRIN relative to the addition of TNF α alone to each cell line. On the other hand, TNF α had no influence on the expression of VEGF, and the addition of 500 ng/ml of recombinant EMMPRIN increased VEGF in HT1080 cells (about 2 fold, $p < 0.01$), but not in U937 cells (Fig. 5B, 5D).

The neutralizing anti-EMMPRIN antibody was added to the two cell types co-cultured in the presence of TNF α , and after 48 hours of incubation, the accumulation of VEGF and MMP-9 in the supernatants was significantly reduced (by 1.8 and 1.4 respectively, $p < 0.05$, Fig. 5E, 5F).

Next, the overall contribution of EMMPRIN to the angiogenic potential of the supernatants was examined in functional *in vitro* assays. Conditioned media (CM) were collected from the TNF α -induced fibroblast-monocyte co-cultures, and EMMPRIN's activity was neutralized by the addition of the anti-EMMPRIN antibody. These treated CM were then incubated with EaHy926 endothelial cells, and the effect was compared to the non-neutralized CM. Neutralization of EMMPRIN activity reduced the ability of

endothelial cells to form tube-like structures (by 1.6 fold, $p = 0.071$, Fig. 6A, 6C), or to migrate and close the gaps formed by a scratch (by 1.4, $p = 0.008$, Fig. 6B, 6C).

3.7 TCZ regulates miR-146a-5p which in turn regulates EMMPRIN expression in HT1080 cells. Both miR-146a-5p and miR-150-5p were elevated in the serum of RA patients following 4 months of TCZ treatment. Since we have previously demonstrated that miR-146a-5p regulates EMMPRIN expression in tumor cells, we chose to focus on this miRNA and to explore whether it regulates EMMPRIN expression in fibroblasts and monocytes, and whether TCZ treatment affects EMMPRIN and angiogenesis through this regulatory pathway. We evaluated the expression of miR-146a-5p in each cell type in single cultures and in co-cultures in the presence of TNF α , compared to the single cultures without TNF α , which served as calibrators (indicated by the dashed line). For HT1080 cells, the co-culture reduced the levels of miR-146a-5p expression (by 3.5 fold, $p = 0.0024$), while U937 showed no significant change (Fig. 7A).

Next, we asked whether overexpression of miR-146a-5p would affect the expression levels of EMMPRIN, VEGF and MMP-9. HT1080 cells were transfected either with the miR-146a-5p mimic or with a scrambled sequence (NC mimic), and after 24 hours the transfected cells were incubated in co-culture with TNF α . Transfection of the miR-146-5p mimic reduced EMMPRIN, VEGF and MMP-9 (by 1.3, 2.3 and 2.2 fold, respectively, $p < 0.05$, Fig. 7B-D), whereas the negative control did not differ from the co-culture with the non-transfected cells.

To assess the effects of TCZ on angiogenesis, we added increasing amounts of the drug to the co-cultured cells with TNF α , and observed that EMMPRIN, VEGF and MMP-9 were all increased (by 1.5 fold, $p < 0.05$) at a concentration of 500 ng/ml relative to co-cultured cell without the drug (Fig. 7E). Lastly, we show that TCZ at 500 ng/ml reduced miR-146a-5p expression levels in the HT1080 cells (by 1.6 fold, $p < 0.001$), but increased it in U937 cells (by 1.4 fold, $p < 0.001$). This inverse expression pattern was also qualitatively reflected by the increase in EMMPRIN expression in HT1080 cells and decreased expression in U937 cells as demonstrated by the western blot analysis (Fig. 7G).

4. Discussion

Angiogenesis is an important process in the pathophysiology of RA [26], but the mechanisms regulating it are yet unclear. TCZ is a biologic agent indicated for the treatment of RA [2], but its effects on pathological angiogenesis have not been sufficiently studied. Here we demonstrate that EMMPRIN (known to be a pro-angiogenic factor in the tumor microenvironment [27]) is involved in angiogenesis in RA patients and in a co-culture of fibroblasts and monocytes *in vitro*. Furthermore, we demonstrate that TCZ affects the angiogenic process, at least partially, through its effects on pro-angiogenic factors, particularly EMMPRIN and its regulator miR-146a-5p. We also demonstrate that the ratio between EMMPRIN and Tsp-1 levels is a useful measure of the angiogenic state in RA patients.

The effects of TCZ on our RA patient cohort concurred with the known effects of TCZ, showing clinical improvement in arthritis, and causing reductions in DAS28 scores and high sensitivity CRP levels. Also, in

accordance with previous observations, IL-6 serum levels increased in RA patients following initiation of TCZ treatment [2, 28].

We show here that after 4 months of treatment, TCZ reduced EMMPRIN serum levels as well as the EMMPRIN/Tsp-1 ratio. With the help of the functional tube-formation assay, we were able to demonstrate a direct effect of EMMPRIN on endothelial cells. However, despite the reduction in EMMPRIN, with its known ability to induce VEGF and MMPs [12, 27], no parallel reduction in the serum levels of VEGF or MMP-9 occurred in the sera of RA patients. This finding may be explained by the presence of alternative signaling pathways to that of EMMPRIN which may induce VEGF and MMP-9 secretion, such as TNF α , a known inducer of MMP-9 [25] or tissue hypoxia, a known inducer of VEGF [29, 30]. Thus, the inhibition of the IL-6 signaling pathway by TCZ may not be sufficient to reduce the serum concentrations of these mediators in the synovial microenvironment, which is rich in pro-inflammatory cytokines.

Next, we demonstrate that EMMPRIN is directly involved in the regulation of angiogenesis using an *in vitro* co-culture system, as its levels were increased in the media of co-cultured fibroblasts and monocytes together with those of VEGF and MMP-9, and the anti-EMMPRIN antibody reduced these levels while recombinant EMMPRIN increased them. This involvement was further established by its direct effects on migration, proliferation, and tube-formation of the endothelial cells in the scratch and tube formation assays, and the ability of an anti-EMMPRIN antibody to reduce them. The obvious inconsistency between the unchanged serum levels of VEGF and MMP-9 in the RA patients and their elevated levels in the *in vitro* system may suggest the involvement of additional factors in their regulation, including other cell types and multiple cytokines, which were not present in the isolated *in vitro* system.

Although no difference was detected in EMMPRIN, VEGF, MMP-9 or Tsp-1 serum levels between patients who responded well to TCZ treatment and those who were unresponsive to therapy, the ratio between EMMPRIN and Tsp-1 was reduced in the responding patients compared to the non-responders. These results suggest that use of the EMMPRIN/Tsp-1 ratio, which takes into account small changes in the balance between pro- and anti-angiogenic factors, might be a reliable way to assess the angiogenic status in patients. However, additional studies are necessary to establish the validity of this proposed parameter in assessing the effects of conventional or biologic DMARDs on angiogenesis in RA patients.

Among the pro-angiogenic factors we tested, NGAL was significantly elevated in TCZ-treated patients, as well as in responding patients relative to non-responders. NGAL is known to form heterodimers with MMP-9, thus protecting the latter from degradation. In addition, NGAL has been shown to regulate VEGF expression and to promote angiogenesis [31]. Previous reports demonstrated higher levels of NGAL in the serum of RA patients compared to healthy controls, suggesting that it promotes angiogenesis [32]. In this regard, the observation of elevated levels of NGAL in RA patients after TCZ administration is contrary to the general anti-angiogenic effects mediated by TCZ. However, notably, NGAL plays many other pleiotropic roles unrelated to its role in angiogenesis, such as in protecting against bacterial infection by acting as an iron-carrying protein, in modulating oxidative stress, in promoting neutrophil chemotaxis, and in regulating thermogenesis and lipid metabolism by acting as an adipokine [10, 30]. Moreover,

although NGAL normally protects MMP-9 from degradation, we did not observe any elevation in MMP-9 serum levels in RA patients. This finding further supports the notion that the primary role played by NGAL in RA is unrelated to angiogenesis, and that its elevation after initiation of TCZ treatment may be related to its role as an adipokine. This possibility must be carefully explored in a follow-up study.

Several miRNAs have previously been implicated in the pathogenesis of RA, some of which are also known to be involved in the regulation of angiogenesis [16, 18, 24, 33, 34]. Of these, we selected 9 miRNAs to examine the effect of TCZ on their circulating levels and observed that only miR-146a-5p and miR-150-5p were elevated after 4 months of treatment. Previous studies have implicated these two miRNAs in the inflammation that is driving RA. The long non-coding RNA LINC01197 which exhibits low levels in RA patients, normally acts as a sponge that binds miR-150-5p, thereby leading to enhanced thrombospondin-2 levels and reduced inflammation [35]. In agreement with our findings, previous studies have shown increased levels of miR-146a-5p in peripheral blood mononuclear cells (PBMC) derived from RA patients [36], and the increased miR-146a-5p levels found in synovial fluid and in PBMC derived from RA patients were linked to decreased apoptosis in CD4⁺ T cells derived from RA patients [37]. However, we are unaware of any previously published studies examining the effects of TCZ on the expression of the particular miRNAs we chose to examine in our study, and only one study demonstrated an increase in the level of a different miRNA - miR-148a - by TCZ in neutrophils isolated from RA patients *in vitro* [38].

Since we have already previously shown that miR-146a-5p participates in the regulation of EMMPRIN expression in tumor cells [19, 20], we suspected that this miRNA was also involved in EMMRPIN regulation in fibroblasts, and therefore focused on this miRNA in our *in vitro* experiments. We demonstrate that transfection of the fibroblast cell line HT1080 with the miR-146a-5p mimic reduced the secretion of EMMPRIN, and subsequently of VEGF and MMP-9, implicating this miRNA in the regulation of EMMPRIN in fibroblasts.

We note that TCZ decreased miR-146a-5p levels and increased EMMPRIN levels in fibroblasts *in vitro*, whereas an opposite effect was observed in the monocytic cell line U937. Furthermore, while TCZ decreased miR-146a-5p in fibroblast cells, it increased its levels in the serum samples from treated RA patients. These inconsistencies may be explained by differing effects exerted by TCZ on different cell types, as observed *in vitro* for the fibroblasts and monocytes. Alternatively, the serum may reflect the state in the synovium, where interactions between fibroblasts and other cell types may generate a balance different from that observed in the *in vitro* co-culture system involving only two cell-lines.

The induction of miR-146a-5p is mostly attributed to stimulators activating the NF-κB pathway, such as TNFα and IL-6 [39, 40], and accordingly its levels have been shown to increase in RA patients [40]. The presence of TNFα in our *in vitro* system can explain the upregulation of miR-146a-5p compared to non-stimulated cells, and the inhibitory effect of TCZ on the expression of miR-146a-5p might suggest a disruption of this pathway. This assumption is corroborated by the cooperation between TNFα-induced NF-κB and the JAK/STAT3 pathway which has recently been demonstrated in brain pericytes [41], and by the inhibitory effects of TCZ on NF-κB in a rat model of sepsis [42]. Thus, we propose that TCZ helps

regulate the TNF α -induced expression of miR-146a-5p through interference with the NF- κ B pathway, and consequently controls the expression of EMMPRIN and thereby of angiogenesis. Further investigation is required to map the exact nature of this interference.

5. Conclusion

In summary, we establish an important role for EMMPRIN in mediating pro-angiogenic signals in RA patients and demonstrate a strong link between miR-146a-5p expression and the regulation of EMMPRIN secretion. Importantly, we show that TCZ reduces angiogenesis in RA patients, and we suggest that this is partially due to the ability of TCZ to interfere with the expression of miR-146a-5p, leading to changes in EMMPRIN levels. We also suggest that the ratio between EMMPRIN and Tsp-1 may reflect the angiogenic status in RA patients more accurately than any one factor alone.

Abbreviations

bDMARDs, biologic disease modifying anti-rheumatic drugs

cDMARDs, conventional disease modifying anti-rheumatic drugs

CRP, C-reactive protein

EMMPRIN/CD147, extracellular matrix metalloproteinase inducer

FLS, fibroblast-like synoviocytes

IL, interleukin

JAK, Janus Kinase

MAPK, mitogen activated protein kinase

miRNA, microRNA

MMPs, matrix metalloproteinases

MVD, mean vessel density

NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells

NGAL, neutrophil gelatinase associated lipocalin (lipocalin-2)

RA, Rheumatoid arthritis

SAA, serum amyloid A

STAT, signal transducers and activators of transcription

VEGF, vascular endothelial growth factor

TCZ, tocilizumab

TNF α , tumor necrosis factor alpha

Tsp-1, thrombospondin-1

Declarations

Ethics approval and consent to participate: The research was reviewed and approved by the local Institutional Review Board at Carmel Medical Center (CMC-0018-11) and all participants signed informed consent before enrollment.

Consent for publication: Not applicable

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

Declarations of interest: none.

Funding: This research was partially supported by a young physician scholarship from the Israeli Society of Rheumatology.

Acknowledgements: Not applicable

Authors' contributions: MS, ES, AK, and LZ performed the experiments; JF, TG, AH, ME, IR, LK and DZ recruited the patients; DZ, JF secured the funding; TG, JF, AH, ME, IR, LK reviewed and edited the paper; MAR and DZ designed the work, analyzed and interpreted the data, and drafted the paper.

Acknowledgement: Not applicable

References

1. Biggioggero M, Crotti C, Becciolini A, Favalli EG. Tocilizumab in the treatment of rheumatoid arthritis: An evidence-based review and patient selection. *Drug Des Devel Ther.* 2019;13:57–70.
2. Ogata A, Kato Y, Higa S, Yoshizaki K. IL-6 inhibitor for the treatment of rheumatoid arthritis: A comprehensive review. *Mod Rheumatol [Internet].* Taylor & Francis; 2019;29:258–67. Available from: <https://doi.org/10.1080/14397595.2018.1546357>

3. Rose-John S. Interleukin-6 signalling in health and disease [version 1 ; peer review : 3 approved]. F1000Research. 2020;9:1013 (11 pages).
4. Nakahara H, Song J, Sugimoto M, Hagihara K, Kishimoto T, Yoshizaki K, et al. Anti-interleukin-6 receptor antibody therapy reduces vascular endothelial growth factor production in rheumatoid arthritis. *Arthritis Rheum.* 2003;48:1521–9.
5. Srirangan S, Choy EH. The role of Interleukin 6 in the pathophysiology of rheumatoid arthritis. *Ther Adv Musculoskelet Dis.* 2010;2:247–56.
6. Marrelli A, Cipriani P, Liakouli V, Carubbi F, Perricone C, Perricone R, et al. Angiogenesis in rheumatoid arthritis: A disease specific process or a common response to chronic inflammation? *Autoimmun Rev.* 2011;10:595–8.
7. Tartour E, Pere H, Maillere B, Terme M, Merillon N, Taieb J, et al. Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. *Cancer Metastasis Rev* [Internet]. 2011/01/21. 2011;30:83–95. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21249423>
8. Favalli EG. Understanding the Role of Interleukin-6 (IL-6) in the Joint and Beyond: A Comprehensive Review of IL-6 Inhibition for the Management of Rheumatoid Arthritis. *Rheumatol Ther* [Internet]. Springer Healthcare; 2020;7:473–516. Available from: <https://doi.org/10.1007/s40744-020-00219-2>
9. Abdel-Maged AES, Gad AM, Abdel-Aziz AK, Aboulwafa MM, Azab SS. Comparative study of anti-VEGF Ranibizumab and Interleukin-6 receptor antagonist Tocilizumab in Adjuvant-induced Arthritis. *Toxicol Appl Pharmacol* [Internet]. Elsevier; 2018;356:65–75. Available from: <https://doi.org/10.1016/j.taap.2018.07.014>
10. Hirohata S, Abe A, Murasawa A, Kanamono T, Tomita T, Yoshikawa H. Differential effects of IL-6 blockade tocilizumab and TNF inhibitors on angiogenesis in synovial tissues from patients with rheumatoid arthritis. *Mod Rheumatol.* 2017;27:766–72.
11. Elshabrawy HE, Chen Z, Volin M V, Ravella S, Virupannavar S, Shahrara S. The Pathogenic Role of Angiogenesis in Rheumatoid Arthritis. *Angiogenesis.* 2015;18:433–48.
12. Muramatsu T. Basigin (CD147), a multifunctional transmembrane glycoprotein with various binding partners. *J Biochem.* 2016;159:481–90.
13. Santiago-Sánchez GS, Pita-Grisanti V, Quiñones-Díaz B, Gumpfer K, Cruz-Monserrate Z, Vivas-Mejía PE. Biological Functions and Therapeutic Potential of Lipocalin 2 in Cancer. *Int J Mol Sci.* 2020;21.
14. Sherbet G V. Endogenous inhibitors of angiogenesis. In: Sherbet G V., editor. *Mol Approach to Cancer Manag.* London: Academic Press, Elsevier Inc; 2017. p. 113–30.
15. Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, Roberts DD. Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. *J Biol Chem.* 2010;285:38923–32.
16. Salinas-Vera YM, Marchat LA, Gallardo-Rincón D, Ruiz-García E, Astudillo-De la Vega H, Echavarría-Zepeda R, et al. AngiomiRs: MicroRNAs driving angiogenesis in cancer (Review). *Int J Mol Med.*

2019;43:657–70.

17. Sun LL, Li WD, Lei FR, Li XQ. The regulatory role of microRNAs in angiogenesis-related diseases. *J Cell Mol Med*. 2018;22:4568–87.
18. Wang Y, Wang L, Chen C, Chu X. New insights into the regulatory role of microRNA in tumor angiogenesis and clinical implications. *Mol Cancer*. Molecular Cancer; 2018;17:1–10.
19. Simanovich E, Brod V, Rahat MM, Rahat MA. Function of miR-146a-5p in Tumor Cells As a Regulatory Switch between Cell Death and Angiogenesis: Macrophage Therapy Revisited. *Front Immunol* [Internet]. 2018;8:article 1931, 16 pages. Available from: <http://journal.frontiersin.org/article/10.3389/fimmu.2017.01931/full>
20. Perske C, Lahat N, Sheffy Levin S, Bitterman H, Hemmerlein B, Rahat MA. Loss of inducible nitric oxide synthase expression in the mouse renal cell carcinoma cell line RENCA is mediated by microRNA miR-146a. *Am J Pathol* [Internet]. 2010 [cited 2016 Jan 27];177:2046–54. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2947298&tool=pmcentrez&rendertype=abstract>
21. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum*. 2010;62:2569–81.
22. Fransen J, van Riel PLCM. The Disease Activity Score and the EULAR Response Criteria. *Rheum Dis Clin North Am* [Internet]. Elsevier Ltd; 2009;35:745–57. Available from: <http://dx.doi.org/10.1016/j.rdc.2009.10.001>
23. Donati S, Ciuffi S, Brandi ML. Human circulating miRNAs real-time qRT-PCR-based analysis: An overview of endogenous reference genes used for data normalization. *Int J Mol Sci*. 2019;20:1–19.
24. Churov A V, Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: Altered expression and diagnostic potential. *Autoimmun Rev* [Internet]. Elsevier B.V.; 2015;14:1029–37. Available from: <http://dx.doi.org/10.1016/j.autrev.2015.07.005>
25. St-Pierre Y, Couillard J, Van Themsche C. Regulation of MMP-9 gene expression for the development of novel molecular targets against cancer and inflammatory diseases. *Expert Opin Ther Targets*. 2004;8:473–89.
26. Cantatore FP, Maruotti N, Corrado A, Ribatti D. Anti-angiogenic effects of biotechnological therapies in rheumatic diseases. *Biol Targets Ther*. 2017;11:123–8.
27. Grass GD, Toole BP. How, with whom and when: an overview of CD147-mediated regulatory networks influencing matrix metalloproteinase activity. *Biosci Rep* [Internet]. 2016;36:Article e00283 (16 pages). Available from: <http://bioscirep.org/cgi/doi/10.1042/BSR20150256>
28. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood*. 2008;112:3959–64.

29. Metzger CS, Koutsimpelas D, Brieger J. Transcriptional regulation of the VEGF gene in dependence of individual genomic variations. *Cytokine* [Internet]. Elsevier Ltd; 2015;76:519–26. Available from: <http://dx.doi.org/10.1016/j.cyto.2015.07.015>
30. Berra E, Milanini J, Richard DE, Le Gall M, Viñals F, Gothié E, et al. Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem Pharmacol*. 2000;60:1171–8.
31. Yang J, McNeish B, Butterfield C, Moses MA. Lipocalin 2 is a novel regulator of angiogenesis in human breast cancer. *FASEB J*. 2013;27:45–50.
32. Gulkesen A, Akgol G, Poyraz AK, Aydin S, Denk A, Yildirim T, et al. Lipocalin 2 as a clinical significance in rheumatoid arthritis. *Cent Eur J Immunol*. 2017;42:269–73.
33. Fu H, Hu D, Zhang L, Tang P. Role of extracellular vesicles in rheumatoid arthritis. *Mol Immunol* [Internet]. Elsevier; 2018;93:125–32. Available from: <https://doi.org/10.1016/j.molimm.2017.11.016>
34. Madanecki P, Kapoor N, Bebok Z, Ochocka R, Collawn JF, Bartoszewski R. Regulation of angiogenesis by hypoxia: The role of microRNA. *Cell Mol Biol Lett*. 2013;18:47–57.
35. Zhao F, Dong J, Guo J, Bi L. Inhibiting role of long non-coding RNA LINC01197 in inflammation in rheumatoid arthritis through the microRNA-150/THBS2 axis. *Exp Cell Res* [Internet]. Elsevier Inc.; 2020;394:112136. Available from: <https://doi.org/10.1016/j.yexcr.2020.112136>
36. Pauley KM, Satoh M, Chan AL, Bubbs MR, Reeves WH, Chan EKL. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res Ther*. 2008;10:1–10.
37. Li J, Wan Y, Guo Q, Zou L, Zhang J, Fang Y, et al. Altered microRNA expression profile with miR-146a upregulation in CD4⁺T cells from patients with rheumatoid arthritis. *Arthritis Res Ther*. 2010;12:1–12.
38. de la Rosa IA, Perez-Sanchez C, Ruiz-Limon P, Patiño-Trives A, Torres-Granados C, Jimenez-Gomez Y, et al. Impaired microRNA processing in neutrophils from rheumatoid arthritis patients confers their pathogenic profile. Modulation by biological therapies. *Haematologica*. 2020;105:2250–61.
39. Paterson MR, Kriegel AJ. MiR-146a/b: A family with shared seeds and different roots. *Physiol Genomics*. 2017;49:243–52.
40. Bae SC, Lee YH. MiR-146a levels in rheumatoid arthritis and their correlation with disease activity: a meta-analysis. *Int J Rheum Dis*. 2018;21:1335–42.
41. Matsumoto J, Dohgu S, Takata F, Machida T, Bölükbaşı Hatip FF, Hatip-Al-Khatib I, et al. TNF- α -sensitive brain pericytes activate microglia by releasing IL-6 through cooperation between I κ B-NF κ B and JAK-STAT3 pathways. *Brain Res*. 2018;1692:34–44.
42. Ibrahim YF, Moussa RA, Bayoumi AMA, Ahmed ASF. Tocilizumab attenuates acute lung and kidney injuries and improves survival in a rat model of sepsis via down-regulation of NF- κ B/JNK: a possible role of P-glycoprotein. *Inflammopharmacology* [Internet]. Springer International Publishing; 2020;28:215–30. Available from: <https://doi.org/10.1007/s10787-019-00628-y>

Figures

Figure 1

Angiogenic factors in serum samples of RA patients before and 4 months after initiation of TCZ treatment. Box plot representing the concentrations of angiogenic mediators in the serum of RA patients before and 4 months after initiation of TCZ treatment. (A) EMMPRIN, (B) VEGF, (C) NGAL, (D) MMP-9, (E) thrombospondin-1 (Tsp-1), and (F) the ratio between EMMPRIN and Tsp-1 as a suggested measure of enhanced angiogenesis. (G) Tube formation assay with (H) closed lumen quantitation, to assess the direct angiogenic potential of serum samples on EaHy926 endothelial cell (n=4).

Figure 2

The change in the disease activity score DAS28 is not associated with a difference in the concentration of angiogenic factors, except for the ratio EMMPRIN/Tsp-1. Patients were stratified into responders and non-responders according to the change in their DAS28 score to assess the effects of TCZ on the concentrations of the angiogenic factors. (A) EMMPRIN, (B) VEGF, (C) NGAL, (D) MMP-9, (E) Tsp-1, and (F) the ratio between EMMPRIN and Tsp-1 as a suggested measure of enhanced angiogenesis.

Figure 3

TCZ treatment increases the serum expression of miR-146a-5p and miR-150. Total RNA was extracted from serum samples before and 4 months after initiation of TCZ treatment, and the expression levels of 9 different microRNA known to be involved in angiogenesis and in RA were determined using comparative CT method for relative quantification, where each patient served as its own control. (A) miR-16-5p, (B) miR-21-5p, (C) miR-132-3p, (D) miR-146a-5p, (E) miR-150-5p, (F) miR-155-5p, (G) miR-203a-3p, (H) miR-221-3p, (I) miR-323a-3p.

Figure 4

Co-culture enhances angiogenesis. HT1080 cells (4×10^5 cells) were cultured alone or in co-culture with U937 monocytes at a ratio of 1:1, in the absence or presence of TNF α (1ng/mL). Supernatants were collected after 48h of incubation and the concentrations of (A) EMMPRIN, (B) VEGF, (C) MMP-9, and (D) Tsp-1 were determined by ELISA. (E) The EMMPRIN/Tsp-1 ratio was calculated for each repetition ($n=6-7$ in all groups).

Figure 5

EMMRPIN regulates the increase in VEGF and MMP-9. Cells were seeded in single cultures (2×10^4 cells for HT1080 or U937), with or without TNF α (1 ng/mL), and increasing concentrations of recombinant human EMMRPIN or the IgG Fc fragment (Fc, at 200 ng/mL) were added as indicated. After 48 h of incubation, supernatants were collected and concentrations of (A, C) MMP-9 and (B, D) VEGF were determined by ELISA ($n=5-6$ in all groups). * $p<0.05$, ** $p<0.01$, compared to cells with TNF α and without

recombinant EMMPRIN. Moreover, the involvement of EMMPRIN was demonstrated by adding the blocking anti-EMMPRIN antibody (2 ng/ml) to a co-culture of HT1080 and U937 cells (2x10⁴ each) in the presence of TNF α (1 ng/mL). After 48h, the concentration of (E) VEGF and (F) MMP-9 were estimated by ELISA (n=7 in each group).

Figure 6

EMMPRIN mediates the angiogenic function of the co-culture. Supernatants derived from the HT1080 and U937 co-culture experiments were collected, and diluted 1:2 in full medium with or without the addition of blocking anti-EMMPRIN antibody (2 ng/mL). This mixture was added to EaHy926 endothelial cells (8×10^4 cells per well) seeded on a Coulterx® layer, and incubated for 6 hours. (A) Representative images (magnification x20), and (C, left panel) quantification of the closed lumen tube-like structures ($n=10$). Alternatively, confluent monolayer of EaHy926 cells was scratched with a toothpick, detached cells were washed away, and cells were allowed to migrate for 24 hours to close the wound. (B) Representative images obtained at the beginning of the experiment (0 h) and after 24 hours (magnification x20). (C, right panel) The area of the scratch at T24 was subtracted from the area at T0, to calculate the area endothelial cells migrated to ($n=9$).

Figure 7

TCZ affects the expression levels of miR-146a and EMMPRIN. (A) Total RNA was extracted from HT1080 or U937 cells that were co-cultured with or without TNF α (1 g/ml), and the expression levels of miR-146a-5p were determined in each of the cell types in single culture without TNF α (dashed line, n=5 in each group). (B-D) HT1080 cells (104 cells) were transfected with miR-146a-5p mimic or its scrambled negative control (NC mimic). After transfection, cells were co-cultured with U937 cells with the addition of

TNF α (1 ng/ml), and 48 hours later the concentrations of (B) EMMPRIN, (C) VEGF and (D) MMP-9 were determined by ELISA (n=5-6 for each group). (E) Increasing amounts of TCZ were added to HT1080 and U937 co-cultures with TNF α (1 ng/ml), and the concentrations of EMMPRIN, VEGF and MMP-9 were determined by ELISA (n=13 in each group). (F) Expression levels of miR-146a-5p in co-cultures of HT1080 and U937 cells with or without the addition of TCZ (500 ng/ml) were determined in each cell type (n=9-10 in each group). (G) EMMPRIN protein expression levels were visualized by western blot analysis in HT1080 or U937 separately after their co-culturing with and without the addition of TCZ (500 ng/ml).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigS1.tif](#)
- [sfig2.jpg](#)