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Extracellular microRNAs induce dendritic cell-dependent joint inflammation and potentiate osteoclast differentiation via TLR7/8 engagement

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ABSTRACT

Objectives: Monocyte-derived dendritic cells (DCs) are key players in the induction of inflammation, autoreactive T cell activation and loss of tolerance in rheumatoid arthritis (RA), but the precise mechanisms underlying their activation remain elusive. Here, we hypothesized that extracellular microRNAs released in RA synovial fluids may represent a novel, physiological stimulus triggering unwanted immune response via TLR8-expressing DC stimulation.

Methods: Human monocyte-derived DCs were stimulated with a mixture of GU-rich miRNAs upregulated in RA tissues and released in synovial fluids (Ex-miRNAs). Activation of DCs was assessed in terms of NF-κB activation by Western blot, cytokine production by ELISA, T cell proliferation and polarization by allogeneic mixed lymphocyte reaction. DC differentiation into osteoclasts was evaluated in terms of tartrate-resistant acid phosphatase production and formation of resorption pits in dentine slices. Induction of joint inflammation in vivo was evaluated using a murine model of DC-induced arthritis. TLR7/8 involvement was assessed by specific inhibitors.

Results: Ex-miRNAs activate DCs to secrete TNFα, induce joint inflammation, start an early autoimmune response and potentiate the differentiation of DCs into aggressive osteoclasts.

Conclusions: This work represents a proof of concept that the pool of extracellular miRNAs overexpressed in RA joints can act as a physiological activator of inflammation via the stimulation of TLR8 expressed by human DCs, which in turn exert arthritogenic functions. In this scenario, pharmacological inhibition of TLR8 might offer a new therapeutic option to reduce inflammation and osteoclast-mediated bone destruction in RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by destructive changes in bone and cartilage of multiple joints, in which both the activation of autoimmune T cells and the increased production of inflammatory cytokines, especially TNFα, play central pathogenetic roles [1].

Dendritic cells (DCs) are a family of innate immune cells involved in cytokine production, antiviral response, antigen presentation and tolerance, with DCs of the myeloid subset specialized in bridging innate and adaptive immunity. Depending on their activation status, DCs discriminate between appropriate and excessive immune responses possibly leading to autoimmunity. Myeloid DCs deriving from infiltrating monocytes are emerging key players in RA as proinflammatory cells as well as crucial inducers of autoimmunity via the fueling of adaptive immune responses and breaking of self-tolerance [2]. Aside of

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TNFα production and priming of autoreactive T cells, myeloid DCs participate to RA pathogenesis also by differentiating into aggressive osteoclasts (OCs), thus contributing to the imbalance of bone homeostasis and exacerbating bone disruption of RA joints. Despite RA synovial fluid, rich in inflammatory and autoimmune mediators, is known to play a role in this process [3], the precise mechanisms activating both immune and non-immune functions of DCs in RA remain poorly characterized [2].

In several pathological conditions, including autoimmunity, fluctuations in the expression of microRNAs (miRNAs) play a role in disease pathogenesis and exacerbation, such as induction of joint inflammation and destruction in the case of RA [4,5]. MiRNAs are short, noncoding single stranded RNAs that are present in the cell cytoplasm but are also abundantly released in extracellular fluids. By reaching neighboring cells, extracellular miRNAs work as intercellular regulators, both via "classical" post-transcriptional mechanisms [6] and via the triggering of innate immune receptors (namely, Toll-like Receptors 7 and 8, TLR7 and TLR8) which, in turn, activate the secretion of proinflammatory mediators [5,7]. At difference with epigenetic regulation, requiring the accumulation of several hundreds of copies of one or few selected miRNAs in every recipient cell, TLR triggering is exerted by any miRNA, or miRNA pool, containing a sufficient amount of guanosines and uridines (GU-rich miRNAs), without any requirement of sequence specificity [7,8]. In addition, the induction of proinflammatory mediators potently amplifies the regulatory potential of even few miRNAs. These aspects are relevant since they overcome the hypothetical limitations of the regulatory potential of extracellular miRNAs related to their low concentration in extracellular fluids. Indeed, TLR activation by extracellular miRNA is emerging as a new trigger of undesired immune activation and tissue damage in different contexts where the pool of extracellular miRNAs is altered, including autoimmune diseases [7].

Based on these premises, we investigated whether GU-rich extracellular miRNAs upregulated in RA (Ex-miRNAs) [9–12] may activate monocyte-derived DCs via TLR7/8 in terms of TNFα secretion, T cell activation and OC differentiation, the three main contributions of DCs to RA pathogenesis and progression.

2. Materials and Methods

2.1. An extended materials and methods section can be found in supplemental materials

2.1.1. Ex-miRNA preparation and DC stimulation

Ex-miRNAs were generated by mixing equal amount of synthetic hsa-miR21–5p, hsa-miR203a-3p, hsa-miR574–5p and hsa-let7b-5p stabilized with a phosphorothioate linkage between each base (Integrated DNA Technologies). These miRNAs were selected as representative GU-rich miRNAs among published miRNA increased in RA tissues and synovial fluids [9–12]. miR210–5p was used as a non-GU-rich negative control [13]. Human and murine DCs were obtained as described in supplemental materials and stimulated at 2×10^6/ml with 10 μg/mL of Ex-miRNAs complexed with DOTAP (Roche Diagnostics), or DOTAP alone as negative control. Complexation of Ex-miRNAs with DOTAP was performed as previously described [14]. Where indicated DCs were pre-treated for 1 h with 3 μM CU-CPT9a (Cayman Chemical) or 1 μM Enpatoran (MedChemExpress).

2.1.2. In vivo induction and assessment of DC-induced inflammatory arthritis (DCIA)

DCIA was induced in 8–12 weeks old male DBA/1 mice (Envigo) according to the protocol published by Leung and colleagues with the following minor modifications [15]. 5×10^5 DCs, pretreated or not for 1 h with Enpatoran, were matured with Ex-miRNAs (10 μg/mL) and simultaneously pulsed with 50 μg/mL of bovine type II collagen (Sigma-Aldrich). After 24 h of stimulation, DCs were administrated subcutaneously into each hind footpad. Joint inflammation was monitored in terms of paw thickness using a caliper (Mitutoyo) before the injection and regularly from day 5 after the injection. Clinical paw scores were recorded by 2 independent observers that were blinded as to the group allocation. Draining lymph nodes were removed at the end of the experiments (day 14) and single-cell suspensions were obtained for each mouse. Procedures involving animal handling and care conformed to protocols approved by the University of Brescia in compliance with national (D.L. N.116, G.U., suppl.40, 18-2-1992 and N.26, G.U. March 4, 2014) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; NIH Guide for Care and Use of Laboratory Animals, National Academies Press, 2011). The study was approved by the Italian Ministry of Health (approval 144/2020-PR). All efforts were made to minimize the number of animals used and their suffering. A completed ARRIVE checklist was included in Supplemental Materials.

2.1.3. OC differentiation and TRAP assay

Human DCs were seeded at 5×10^5/mL on glass tissue culture chamber (Sarsted) in α-MEM (SIGMA) (supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin, and streptomycin: all from Gibco, Thermo Fisher Scientific) in the presence of 100 ng/ml RANKL (Peprotech) and 25 ng/mL M-CSF (Miltenyi Biotec). From day 3 of differentiation cells were also stimulated with 10 μg/mL of Ex-miRNAs or vehicle alone or pretreated for 1 h with Enpatoran every 3 days. The stimulation with M-CSF alone was used as negative control. Stimuli and medium were changed every 3 days for 12 days of culture. At day 12 of differentiation tartrate-resistant acid phosphatase (TRAP) activity was assessed using the TRAP Staining Kit (Cosmo-Bio), both on OCs and supernatants. Representative images were captured using Axio Imager. A2 microscope and elaborated through AxioVision 3D software (Zeiss).

2.2. Bone resorption

To assess OC resorption activity, human DCs were seeded at 5×10^5/ml and differentiated on dentine slices (Pantec) for 21 days. From day 3 of differentiation and every 3 days, cells were stimulated with 10 μg/mL of Ex-miRNAs or DOTAP alone as previously described. At day 21, OCs were destroyed by overnight incubation with 0.25 M NH_4OH and dentine slices were stained with toluidine blue to detect resorption pits. Representative images were captured using Axio Imager. A2 microscope and elaborated through AxioVision 3D software. Resorbed areas were quantified using ImageJ software.

2.2.1. Statistical analysis

Sample group normality was confirmed by Shapiro-Wilk test before application of parametric statistical analysis. Statistical significance among the experimental groups was determined using paired or unpaired 2-tailed Student's *t*-test, 1-way ANOVA with Dunnett's post-hoc test, or Tukey's multiple comparison test as appropriated (GraphPad Prism 9). $P < 0.05$ was considered significant.

3. RESULTS and discussion

3.1. Ex-miRNAs induce activation of innate and adaptive immune functions of human DCs via the engagement of TLR8

Based on previously published works we identified, synthesized and mixed four GU-rich miRNAs (let7b, miR574, miR21 and miR203) upregulated in RA and detected in RA synovial fluids to obtain a pool mimicking the GU-rich miRNA content of RA synovial fluids, which will be indicated here as "Ex-miRNAs" [9–12]. Previous work by our group [13] and Supplemental Fig. 1 show that each of these miRNAs bears the potential to activate TLR7-and TLR8-expressing transfectants. Stimulation of human monocyte-derived DCs with Ex-miRNAs induced a rapid phosphorylation of the inflammatory master transcription factor NF-κB

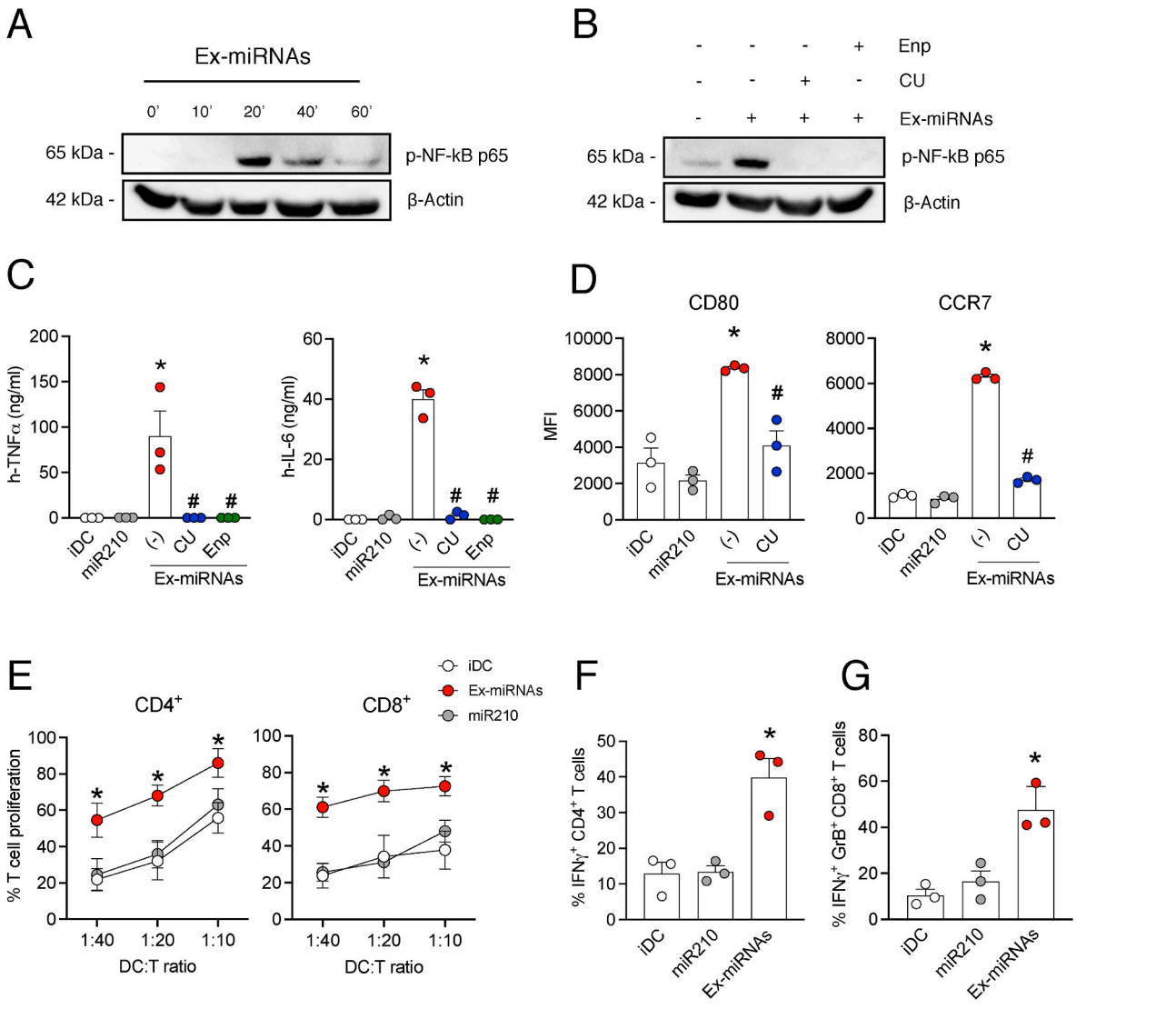

Fig. 1. Ex-miRNAs activate innate and adaptive immune functions of human DCs via the engagement of TLR8. (A-B) Human monocyte derived DCs were stimulated with Ex-miRNAs (10 μg/m) as indicated **(A)** or pretreated with CU-CPT9a (CU, 3 μM) or Enpatoran (Enp, 1 μM) for 1 h and then stimulated with Ex-miRNAs for 20 min **(B)**. Total proteins were blotted against p–NF–κB p65 and β-actin. One representative fluorogram out of 3 is shown. **(C–D)** Human DCs were pretreated for 1 h with the indicated inhibitors and then stimulated with Ex-miRNA or miR210 as a negative non-GU-rich control or vehicle alone (iDC) for 24 h. **(C)** TNFα and IL-6 production was evaluated by ELISA. **(D)** Surface expression of CD80 and CCR7 was assessed by flow cytometry. Data are expressed as mean ± SEM (n = 3). *P < 0.05 vs iDC and #P < 0.05 vs (−) by 1-way ANOVA followed with Tukey's test. **(E)** DCs stimulated as in **C** were cocultured for 6 days with CFSE-stained allogenic naïve $CD4^+$ T cells or $CD8^+$ T cells at the indicated DC:T cell ratio. Data are expressed as mean ± SEM (n = 3) of the percentage of proliferating T cells. **(F–G)** DCs were cocultured for 6 days with allogenic naïve $CD4^+$ T cells **(F)** or $CD8^+$ T cells **(G)** at a DC:T cell ratio 1:40. Intracellular IFNγ **(F)** or IFNγ and Granzyme B (GrB) **(G)** were evaluated by flow cytometry. **(E–G)** Data are expressed as mean ± SEM (n = 3). *P < 0.05 vs iDC by 1-way ANOVA followed with Dunnett's post hoc test.

(Fig. 1A). This activation was strongly inhibited in the presence of Enpatoran, a dual TLR7/8 inhibitor, as well as of CU-CPT9a, a specific TLR8 inhibitor (Fig. 1B). These inhibitors are well known for their low toxicity [16,17] and did not affect DC viability (Supplemental Fig. 2A). Of note, Enpatoran is currently undergoing a Phase II clinical trial to manage Lupus Erythematosus (https://clinicaltrials.gov/study/NCT05162586). Ex-miRNA-stimulated DCs, but not DCs activated by a non-GU-rich miRNA (miR210 [13]), secreted the proinflammatory cytokine TNFα (Fig. 1C), a key pathogenetic player in RA [1], which release was abolished by the pretreatment with both inhibitors. In addition to TNFα, Ex-miRNAs also induced the secretion of IL-6, IL-12 and IFNβ, all of which were blocked by TLR inhibitors (Fig. 1C and Supplemental Fig. 2B). These results demonstrate that DC activation is triggered by TLR8 and confirm a dominant role for TLR8 in the activation of monocyte-derived DCs in response to ssRNAs, as previously described [14].

Since RA depends on both induction of local inflammation and on priming of autoreactive T cells, we asked whether DCs activated by Ex-miRNAs acquire the mature phenotype and the capability to stimulate T cell proliferation and polarization. Ex-miRNAs, but not miR210, induced DC maturation in terms of CD80 and CCR7 upregulation, which was TLR8-dependent as demonstrated by its impairment upon CU-CPT9a treatment (Fig. 1D). DC phenotypical maturation also included the upregulation of CD86 and MHC class I and II (Supplemental Fig. 2C). By contrast, Ex-miRNA-stimulated DCs decreased the uptake of dextran, a function that is typical of immature DCs (Supplemental Fig. 2D). The functional capability of Ex-miRNA-activated DCs to stimulate T cells was investigated in coculture experiments with allogeneic naïve $CD4^+$ or $CD8^+$ T cells. Ex-miRNA-activated DCs, but not miR210-activated DCs, induced the proliferation of both naïve $CD4^+$ and $CD8^+$ T cells (Fig. 1E)

which was significantly decreased when stimulation was performed in the presence of CU-CPT9a (Supplemental Fig. 2E). In accordance, only Ex-miRNA-activated DCs induced a functional skewing of proliferating $CD4^+$T cells towards a Th1 phenotype, known to be involved in RA pathogenesis [2], as measured by the production of IFNγ (Fig. 1F). Of note, CU-CPT9a significantly reduced the production of IFNγ (Supplemental Fig. 2F). This effect is in line with the above-described production of the Th1-polarizing cytokines IL-12 and IFNβ. Consistently, activated $CD8^+$ T cells increased the production of the Th1-orienting cytokine IFNγ and of the cytotoxic molecule Granzyme B (Fig. 1G), all of which were significantly reduced by TLR8 blockade (Supplemental Fig. 2G).

We previously demonstrated that synthetic GU-rich miRNAs, as well as GU-rich miRNA-containing exosomes from plasma of systemic lupus erythematosus patients, induced activation and phenotypical maturation of the plasmacytoid subset of human DCs via TLR7 triggering [7,13]. We also showed that a mixture of TLR-activating miRNAs, each used at suboptimal concentrations, induced cytokine release [7,13], indicating that miRNA pools can activate TLRs regardless of the relatively low concentration of each miRNA. The present results confirm and extend these findings to TLR8-expressing myeloid DCs stimulated with a mix of GU-rich miRNAs mimicking the extracellular pool of miRNAs that may activate monocyte-derived DCs in RA synovia. In addition, they show for the first time that Ex-miRNA-mediated DC maturation induce T cell proliferation, Th1 polarization and cytotoxic properties, all of which are functional to RA pathogenesis [2].

3.2. Ex-miRNA-stimulated DCs induce DC-dependent inflammatory arthritis

The capability of Ex-miRNA-stimulated DCs to induce joint inflammation was assessed by performing a murine model of DC-induced inflammatory arthritis (DCIA) [14]. To this end, we preliminarily assessed the ability of Ex-miRNAs to activate murine DCs, as expected based on the similar selectivity of human and murine ssRNA-sensing TLRs [15]. DCs derived from $CD34^+$ staminal cells of DBA/1 mice responded to Ex-miRNAs, but not to miR210, by secreting TNFα and IL-6 (Fig. 2A). Because TLR8 activity in mice is controversial and TLR7 is thought to take over its functions, Enpatoran was used to confirm the involvement of TLR7 in murine DC activation (Fig. 2A). No effect on DC viability was observed in the presence of Enpatoran (Supplemental Fig. 3). Fig. 2B shows that murine DC stimulated with Ex-miRNAs also acquired the phenotypical markers of maturation CD86 and CD40 in a TLR7-dependent fashion.

Fig. 2C shows that collagen-pulsed DCs activated by Ex-miRNAs, but not immature DCs, induced an early increase in paw thickness into congenic recipient mice, indicating the onset of RA-mimicking joint inflammation which mostly depends on the production of TNFα [14]. This effect was prevented by Enpatoran pretretament, thus confirming the involvement of murine TLR7 (Fig. 2C). Previous work showed that DCIA also recapitulates the adaptive phase of RA when collagen-specific T cells contributes to tissue damage [14]. In accordance, T cells recovered from paw draining lymph nodes (collected at the end of the experiment, day 14) of mice treated with Ex-miRNA-stimulated DCs promptly proliferated when restimulated *in vitro* with collagen, while T cells from other experimental groups did not (Fig. 2D). Proliferating T cells also showed an activated phenotype, as measured by the increased expression of the activation markers CD69 and CD44 (Fig. 2E) and a potential Th1 skewing, as assessed by a consistent release of IFNγ (Fig. 2F).

The role of DCs in the establishment and maintenance of RA is unquestioned, but the mechanisms underlying their activation, especially in the early phases of the disease, remain poorly characterized [2]. Here, we show that a mixture of GU-rich miRNAs selected from miRNAs upregulated in RA activates DCs to induce inflammatory arthritis. The fact that these same miRNAs are present in extracellular vesicles of RA-synovial fluids [9–12] strongly support their potential role as intercellular regulators. Future experiments using RA synovial fluids and/or miRNAs purified from RA synovial fluids will provide the final confirmation. Unfortunately, these experiments were not feasible in our hands due to practical limitations in the availability of such biologic material. To our knowledge, this is the first evidence that extracellular miRNAs increased in RA may work as unwanted proinflammatory stimuli activating DCs to secrete TNFα, induce joint inflammation and triggering an autoreactive T cell response.

3.3. Ex-miRNAs potentiate the differentiation of DCs into aggressive OCs

DCs contribute to RA pathogenesis also by differentiating into OCs, which are responsible for bone erosion. Thus, experiments were performed to investigate the potential contribution of Ex-miRNAs in the DC-OC differentiation process induced by M-CSF and RANKL [3]. Fig. 3A shows that OCs differentiated in the presence of Ex-miRNAs appeared bigger and more nucleated, with thicker actin rings in the podosomes as compared to OC differentiated with M-CSF + RANKL alone. As expected, cells differentiated with M-CSF alone remained small and mononucleated, maintaining a DC-like morphology. To confirm the differentiation towards OCs, we evaluated the expression of Tartrate-resistant acid phosphatase (TRAP), a typical OC marker involved in bone and collagen homeostasis and also in immune functions, including Th1 polarization [18]. As expected, OCs exhibited an intense TRAP staining, although M–CSF–DCs also expressed it basally, as previously described [18] (Fig. 3B). Interestingly, OCs differentiated in the presence of Ex-miRNAs showed a higher release of TRAP in the supernatant, which was significantly inhibited when Enpatoran was maintained in the culture during differentiation (Fig. 3C). Because increased release of TRAP correlates with a more aggressive resorptive behavior [18], we performed pit assay to assess OC functional capabilities. OCs differentiated in the presence of Ex-miRNAs induced a resorption pit area that was significantly higher as compared to OCs differentiated with M-CSF + RANKL alone (Fig. 3D), confirming that Ex-miRNAs have the potential to foster the differentiation of DCs into more aggressive OCs. We are aware that the use of miRNAs purified from RA synovial fluids could provide more translational relevance to our findings. However, obtaining miRNAs sufficient to treat DCs for the whole process of OC differentiation implies the availability of consistent amounts of synovial fluids, which is unfeasible in our hands as stated above.

Since Enpatoran could bring TRAP levels back to those observed in the absence of Ex-miRNAs, we conclude that Ex-miRNA activity largely depend on TLR stimulation, in line with what recently shown for monocyte-OC transition [10]. However, we cannot exclude that other miRNAs may also exert “classical” post-transcriptional effects, as described in other systems [19]. The two mechanisms are not contrasting nor mutually exclusive: on the contrary, they may cooperate and reinforce each other. Thus, the possibility that miRNAs influence DC-OC transition via combined classical and non-classical regulation remains an attractive possibility.

4. Conclusion

Despite the limitation due to the use of synthetic miRNAs vehiculated by DOTAP, we believe that this work represents the proof of concept that the extracellular miRNA pool overexpressed in RA synovia can act as a physiological activator of inflammation via the stimulation of TLRs expressed by DCs. Activated DCs, in turn, contribute to RA pathogenesis via different mechanism including TNFα production, naïve T cell priming and OC transition. Unconventional regulation via TLR7/8 triggering by extracellular miRNAs is now recognized as a possible pathogenetic and amplification mechanism in several inflammation-dependent pathological conditions, including cancer growth and metastasis, neurodegeneration, cardiovascular disease and autoimmunity [7]. In RA, it was previously demonstrated that Let-7b, which is also

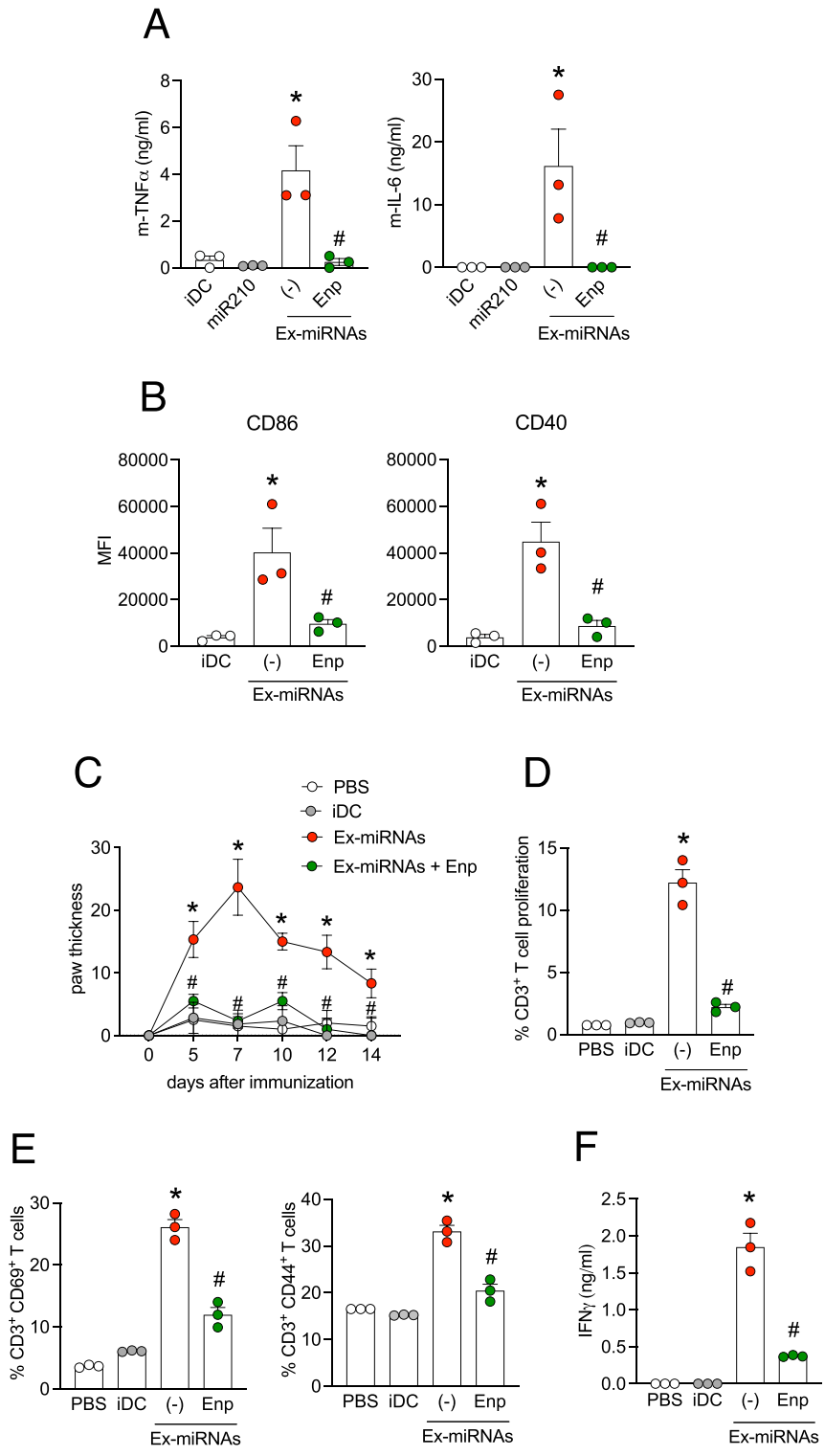

Fig. 2. Ex-miRNA-activated murine DCs induce inflammatory arthritis and T cell activation via TLR7 triggering. (A–B) Bone marrow-derived DCs were pretreated 1 h with Enpatoran and then stimulated with Ex-miRNAs or miR210 or vehicle alone (iDC) for 24 h. **(A)** TNFα and IL-6 production was evaluated by ELISA. **(B)** Surface expression of CD86 and CD40 was assessed by flow cytometry. Data are expressed as mean ± SEM (n = 3). *P < 0.05 vs iDC and #P < 0.05 vs (−) by 1-way ANOVA followed with Tukey's test. **(C)** 5×10^5 mouse DCs, pretreated or not with Enpatoran, were stimulated with Ex-miRNAs or with vehicle alone (iDC) in the presence of collagen and injected into each hind footpad of DBA\1 mice. PBS-treated mice were used as control (PBS). Delta thickness of each paw **(C)** was measured and expressed as mean ± SEM (n = 3) of one representative experiment out of 2. **(D–F)** Cells from draining lymph nodes of the indicated experimental conditions were cultured *in vitro* for 72 h. $CD3^+$T cell proliferation **(D)** and the expression of activating markers on $CD3^+$T cells **(E)** were assessed by flow cytometry. **(F)** IFNγ production was evaluated by ELISA. Data are expressed as mean ± SEM (n = 3). *P < 0.05 vs PBS and #P < 0.05 vs (−) by 1-way ANOVA followed with Tukey's test.

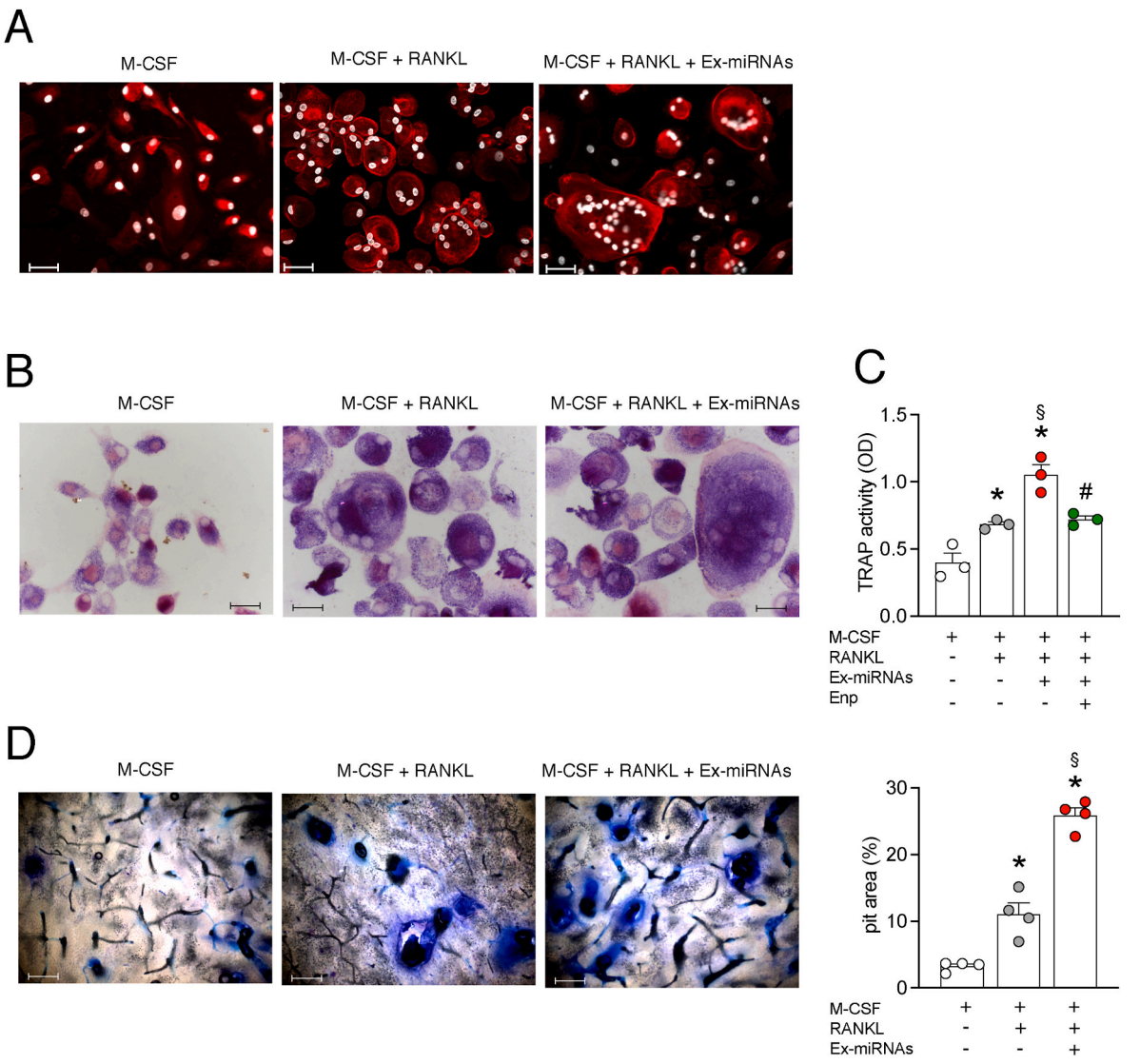

Fig. 3. Ex-miRNAs induce DC-OC differentiation. (**A-B-C**) Human DCs were differentiated for 12 days in the presence of M-CSF plus RANKL, or M-CSF alone as negative control. Starting from day 3 and every 3 days, cells were stimulated with Ex-miRNAs in the presence or not of Enpatoran. (**A**) At day 12, OCs were stained with phalloidin (red) and DAPI (white). Magnification x20, scale bars = 50 μm. (**B–C**) TRAP activity was assessed both on OCs **(B)** and in supernatants **(C)**. Magnification x40, scale bars = 10 μm. (**C**) Data are shown as mean ± SEM (n = 3) of Optical Density (OD), *P < 0.05 vs M-CSF and §P < 0.05 vs M-CSF + RANKL and $^{\#}$P < 0.05 vs M-CSF + RANKL + Ex-miRNAs by 1-way ANOVA with Tukey's test. (**D**) DCs were differentiated for 21 days on dentine slices and Ex-miRNAs were added starting from day 3. Bone resorption activity of OCs were shown as representative images (left panels, magnification x10, scale bars = 10 μm) with quantification of results (right panel). Data are expressed as the mean ± SEM (n = 4) of the percentage of resorption pit area, *P < 0.05 vs M-CSF and §P < 0.05 vs M-CSF + RANKL by 1-way ANOVA with Tukey's test.

included in Ex-miRNAs, induced proinflammatory M1 macrophage polarization via murine TLR7 ligation which, in turn, induced joint swelling in a model of arthritis [9]. More recently, extracellular vesicles from RA synovial fluid containing miR-574–5p were shown to bind TLR7/8 expressed by monocytes and to promote their differentiation into OCs [10]. These findings indicate that, in RA, deregulated extracellular miRNAs can activate different types of TLR7/8-expressing cells, which all contribute to exacerbated inflammation and tissue damage. From a translational point of view, it is of particular relevance that Enpatoran is undergoing Phase II clinical trial for the treatment of autoimmune conditions (https://clinicaltrials.gov/study/NCT05162586). Indeed, pharmacological inhibition of TLR7/8 might represent a forthcoming novel therapeutic option to reduce inflammation and osteoclast-mediated bone destruction in RA.

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CRediT authorship contribution statement

Carolina Gaudenzi: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Tiziana Schioppa:** Investigation, Methodology, Validation. **Mauro Passari:** Data curation, Investigation. **Giovanni Zucchi:** Investigation, Visualization. **Laura**

Tiberio: Investigation. **Yasmin Vahidi:** Investigation, Visualization. **Sara Scutera:** Methodology. **Tiziana Musso:** Methodology. **Silvano Sozzani:** Funding acquisition, Writing – review & editing. **Annalisa Del Prete:** Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Valentina Salvi:** Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. **Daniela Bosisio:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that no conflict of interests exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103189.

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