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Liposomal AntagomiR-155-5p restores anti-inflammatory macrophages and improves arthritis in pre-clinical models of Rheumatoid arthritis

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Abbreviations: RA, rheumatoid arthritis; CIA, collagen-induce arthritis; STA, Serum transfer arthritis; PEG-liposome, PEGylated-liposome; M2, alternative activated macrophages; BMDM, bone-marrow derived macrophages.

Abstract

Introduction: We previously reported an increased expression of miR-155 in rheumatoid arthritis (RA) patients blood monocytes that could be responsible for impaired monocyte polarization to anti-inflammatory M2-like macrophages. In this study, we employed two pre-clinical models of RA: the Collagen-Induce-Arthritis (CIA) and the K/BxN Serum-Transfer-Arthritis (STA), to examine the therapeutic potential of antagomiR-155-5p entrapped within PEGylated (PEG)-liposomes in resolution of arthritis and re-polarization of monocytes towards anti-inflammatory M2 phenotype.

Methods: AntagomiR-155-5p or antagomiR-control were encapsulated in PEG-liposomes of 100 nm in size and -10mV in zeta potential with high antagomiR loading efficiency (above 80%). Mice were injected intravenously with 1,5nmol/100 μ L PEG-liposomes containing antagomiR-155-5p or control after induction of arthritis.

Results: We demonstrated the biodistribution of fluorescently tagged-PEG-liposomes to inflamed joints 1 hour after injection of fluorescently tagged-PEG-liposomes and as well as their subsequent liver's accumulation after 48 hours, indicative of hepatic clearance, in arthritic mice. Injection of PEG-liposomes containing antagomiR-155-5p decreased arthritis score and paw swelling as compared to PEG-liposomes containing antagomiR-control or systemic delivery of free antagomiR-155-5p. Moreover, treatment with PEG-liposomes containing antagomiR-155-5p lead to the restoration of bone marrow monocytes defect in anti-inflammatory macrophage differentiation without any significant functional change in other immune cells including splenic B and T cells.

Conclusion: The injection of antagomiR-155-5p encapsulated in PEG-liposomes allows delivering small RNA to monocytes/macrophages, reduced joint inflammation in murine models of RA, providing a promising strategy in human disease.

Introduction

Monocytes-macrophages are key players in the pathogenesis and therapeutic response in rheumatoid arthritis (RA) ⁽¹⁻⁵⁾. Numbers of studies have found an increase of pro-inflammatory macrophages recruitment and activation in synovium in both human and mouse models ^{(4;7) (8;9)}.

On the other hand, overexpression of some micro-RNAs (miR) associated with inflammation has been detected in blood and synovium of RA patients. Among these miR, an up-regulation of miR-155 expression has been shown in RA synovial macrophages, fibroblasts as well as in peripheral blood and synovial fluid CD14⁺ monocytes ^(10;11). We have previously demonstrated that overexpression of miR-155 in RA blood monocytes, lead to a defect of monocytes polarization in anti-inflammatory macrophages ⁽⁶⁾.

The collagen-induced-arthritis (CIA) is the most commonly used murine model, in which breaking tolerance induces an immune-mediated inflammatory attack of the joints. It is an acute model that fulfills a few of the RA classification criteria, such as chronic inflammation and erosive arthritis. It is also highly variable depending on the quality of the type II collagen (CII) used, the environmental factors and works efficiency only on the strain DBA/10IaHsd. In contrast, K/BxN serum-transfer-arthritis (STA) in naïve C57BL/6 mice tends to be a fast-onset inflammatory model that mimics only the effector phase of RA. The STA model is a robust and reproducible model in many mice strains with manifestations of arthritis occurring a few days after serum injection. The inflammatory response is driven by autoantibodies against the ubiquitously expressed self-antigen, glucose-6-phosphate isomerase (G6PI), leading to the formation of immune complexes that drive activation of different innate immune cells. Interestingly, both mouse models exhibit pro-inflammatory macrophages infiltration in synovium that plays a key role in the pathophysiology. ^{(12-15) (8;9)}

AntagomiRs can be used for neutralizing actions of miR. However, they are relatively unstable *in vivo* and chemical modifications may alter their biological properties. Furthermore, similar to conventional anti-inflammatory drugs, antagomiR-155-5p may cause severe non-myeloid cells effects side effects such as reduced protective immunity, fibrosis, or liver steatosis ⁽¹⁶⁾. Recently, partners of our group described in the CIA model an efficient strategy in which dexamethasone palmitate (DXP) formulated as PEGylated nanoparticles can extravasate through leaky endothelium in inflamed joints and be phagocytosed by monocytes and macrophages ⁽¹⁷⁾. This occurred after a significant accumulation of labeled PEGylated

nanoparticles, as compared to low score joints ⁽¹⁷⁾. Moreover, lipid nanoparticles such as liposomes can protect all types of nucleic acids against degradation ⁽¹⁸⁾.

Based on robust evidence of the pathogenic role of miR-155 in human RA ⁽⁶⁾, we have explored if excess of miR-155-5p could drive inflammation in CIA and STA models and consequently, evaluated the therapeutic utility of an antagomiR-155-5p encapsulated in pegylated liposomes in these 2 mouse models.

Materials and Methods

Mice

C57BL/6J mice were purchase from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred in-house. DBA/1OlaHsd were purchase from Envigo (France). All mice were housed and maintained in a specific-pathogen-free facility at the University of Glasgow for C57BL/6J mice and Faculty of Pharmacy Châtenay-Malabry for DBA/1OlaHsd under a 12-hour light/dark cycle in a temperature-controlled room with free access to water and food. *In vivo* experiments were performed under the protocol apafis#2842-2015110914248481v5.

For CIA model, 10-week-old male DBA/1OlaHsd mice were injected intradermally at the base of the tail with an emulsion of grade chick type II collagen and complete Freud's adjuvant. At day 21, mice received a boost injection of the same composition. We determined arthritic score and paw volume every two days since D21 and sacrificed mice at D37. For STA model, 8-weeks-old male C57BL/6J mice were injected at days 0 and 2 intraperitoneally with 150 μ L of arthritogenic serum (K/BxN mice) to induce arthritis. We determined arthritic score and paw size every two days since D0 and sacrificed mice at D5 during the strong inflammation phase. Clinical arthritis scores in CIA and STA were evaluated, based on a visual scoring scale of the paws with evaluation of the erythema, swelling and ankylosis of each paw with these following criteria: 0 = normal, 1 = swelling and/or redness of paw or 1 digit, 2 = 2 digits swelling and/or redness, 3 = 3 digits swelling and/or redness, and 4 = severe arthritis of the entire paw and digit with skin shedding and wrist/ankle swelling. The total score was based on all four paws, with a maximum score of 16 for each mouse.

PEG-Liposomes production and characterization

PEG-liposomes were prepared using a thin lipid film-hydration method. The first step consisted in mixing together chloroform solutions of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cholesterol and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine with conjugated polyethylene glycol 2000 (DSPE - mPEG) in 62:35:3M ratio, and 1% of 1,1'- dioctadecyl - 3,3,3',3'-tetramethylindotricarbocyanine perchlorate (DID) for fluorescently-tagged liposomes. The chloroform present in the mixture was evaporated by rotary evaporator to form a dry lipid film. The resulting lipid film was hydrated with 10mL of RNase-Dnase-free water, the solution was vortex-mixed for 10 min following by 15min of sonication in ice.

Those empty PEG-liposomes were then concentrated using Millipore columns Amicon (Merck, Germany) with 100 kDa cutoff to obtain 500 μ L after several centrifugations at 15°C - 20min - 4000 rpm before characterization. PEG-liposomes were characterized by their size,

polydispersity index (Pdl), and zeta potential, measured by dynamic light scattering using Nano ZS from Malvern (173° scattering angle) at 25°C. Samples were diluted 1/20 in water for the size and Pdl, or in 1 mM NaCl solution for the zeta potential determination. Measurements were performed in triplicate. Phospholipid concentration within PEG-liposome suspensions were also measured by enzymatic colorimetric method (Sigma -Aldrich -Merck, Germany) and PEG -liposome suspensions were diluted to obtain a final concentration of 2.5mg for antagomiR encapsulation. Briefly, antagomiR/protamine polyplexes were formed by mixing 2nmol of antagomiR-control, control-cy³ or 155-5p (MirVANA inhibitor second generation - Life Technologies, Waltham, MA #4464088) with 10nmol of protamine (Merck, Germany). The antagomiR/protamine polyplexes were then mixed with 2.5mg of PEG-liposomes, and 5 cycles of 1min in liquid nitrogen following by 2min in water bath at 60°C were performed. To remove unencapsulated antagomiR, liposomes suspensions were incubated with 10 µL of EDTA-trypsin (Thermo Fisher Scientific, Waltham, MA) at 37°C for 10min to break down any non-encapsulated antagomiR/protamine polyplexes. Free antagomir and protamine were then removed by 3 successive centrifugal ultrafiltration PBS washes using 100 kDa cutoff Amicon columns (Merck, Germany).

For encapsulation efficiency, we determined the unencapsulated antagomiR by Quantifluor (Promega, France) using antagomiR as standards. We obtained a range of 70.2 – 81.3% of encapsulated polyplexes.

Cell culture and PEG-liposomes uptake

Murine macrophage cell line RAW 264.7 obtained from ATCC was cultured in Dulbeco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin-streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The uptake of PEG-liposomes containing AntagomiR-control or AntagomiR-155-5p in RAW 264.7 cells was measured by RT-qPCR. Cells were seeded in 12-well plates at 8×10⁴ cells/well and incubated 24 h to reach 80% confluency. PEG-liposomes were added at 1X (1.5 nmol/100 µL) – 2.5X and 5X and plates were incubated for 48h. The medium was then removed, and cells were washed with PBS1X and frozen at -80°C until RNA extraction and RT-qPCR analysis.

Epifluorescence imaging for biodistribution in CIA mice

Tagged DID 1%-liposome as described above were diluted at 2.5 in PBS1X to optimize fluorescent in mice. For imaging, mice were anesthetized in an induction chamber with 3%

isoflurane and maintained with 2% isoflurane. DBA/10IaHsd control and arthritis induced mice were injected with 100 μ L by retroorbital injection at D27 and imaging 1h-2h-4h-24h and 48h post-injection on dorsal and ventral positions. At D29, animals were euthanized, and liver, spleen, kidney, heart, lung and intestine were harvested. The epifluorescence of the different organs was then analyzed using an IVIS Lumina III machine (Caliper Life Science, Hopkinton, MA) equipped with a heated stage. Setting for imaging were automatic phototime exposition, medium binning, excitation wavelength of 640nm, and emission wavelength of 710nm. Living Image Software (version 4.5.5, Caliper Life Science, Hopkinton, MA) was used to visualize the fluorescence images and quantify intensity using regions of interest (ROI) and subtraction of the background fluorescence. Background fluorescence was determined on the oral mucosa, skin of the feet and prepuce.

Therapeutic treatment of antagomiR-155-5p in CIA and STA mice

For systemic injection, DBA/10IaHsd-CIA mice were injected on D22, D29, D36 and D41, with 43.4 nmol per injection of antagomiR-155-5p, antagomiR-control, or PBS.

DBA/10IaHsd-CIA and C57BL/6J-STA mice were injected on D30, D32 and D34 (CIA) or on D1, D2, D3 and D4 (STA) and received intravenously 1.5 nmol per injection of antagomiR-155-5p or antagomiR-control encapsulated in PEG-liposomes.

Mice were evaluated every 2-day, based on a visual scoring scale of the paws with evaluation of the erythema, swelling and ankylosis of each paw (score 1 to 4).

Cells isolation – monocytes differentiation in macrophages – synovium tissues

Single-cell suspensions were prepared by standard mechanical disruption for liver and spleen and filtered through respectively 70 μ m or 40 μ m nylon cell strainers. Splenocytes were counted and monocytes, B and T cells were isolated using CD11b microbeads, B cells isolation kit and Pan T cells isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) to achieve a purity of >75%. Crushed liver or isolated splenocytes were stored at -80°C until analysis.

To generate bone-marrow derived macrophages (BMDM), bone marrow cells from femurs and tibias of mice were harvested and isolated by centrifugation. Briefly, femurs and tibias were folded in 0.2 mL Eppendorf holed by a 23G needle and centrifuged at 4°C-3000g-1min in a 1.5 mL Eppendorf containing 200 μ L of PBS1X. Cells were filtrated on 70 μ m nylon cell strainer and red blood cells were lysed in RBC buffer (Biolegends, San Diego, CA). Cells were washed and bone-marrow monocytes were isolated using a monocytes isolation kit for BM according

to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany) to achieve a purity of >80%. Then monocytes were washed, counted, stored at -80°C. For experimental works, 250 000 cells/mL were seeded in 6-well plates and cultured in IDMEM supplemented with 100IU/mL penicillin-streptomycin, 10% of FBS and M-CSF (10ng/mL) for 5 days. IL-4 (20ng/mL) was then added for 24h treatment. Cells were washed for analysis and supernatant were harvested and stored at -80°C until analysis.

Mouse synovia were harvested and transferred in Eppendorf coating with 0.4% BSA, with 1mL of RPMI 1640 media supplemented with 100IU/mL penicillin-streptomycin and 10% L-Glutamine, 15µL liberase™ (5mg/mL Roche, France) and 100µL DNaseI (10µg/mL Roche, France). After 45min incubation under 150 rpm shaking at 37°C. Cells were filtered through 100µm nylon cell strainer, washed, and stained.

Flow cytometry

For bone-marrow monocytes purity, cells were saturated with FcBlock and incubated for 30min at 4°C with anti-CD11b and anti-Ly6C antibodies. After monocytes polarization in macrophages by M-CSF+IL-4 for M2 phenotype, cells were harvested in 100% FBS and scratched, washed once with PBS 1x, saturated with FcBlock, and incubated 30min at 4°C with anti-F4/80, anti-CD11b and anti-CD206 (gating strategy in **Supplementary Figure 1 and Supplementary Figure 3A**).

For monocytes, B, and T cells spleen purity, cells were saturated with FcBlock and incubated for 30min at 4°C with anti-B220 for B cells, anti-CD3, anti-CD4 and anti-CD8 for T cells, anti-CD11b and anti-Ly6C antibodies for monocytes (gating strategy in **Supplementary Figure 3C-D**).

For mice synovial tissues staining, cells were saturated with FcBlock and incubated for 30min at 4°C with anti-CD45, Dump channel anti-CD3-CD19-NK.1.1-Ly6G, anti-CD11b, anti-F4/80, anti-CD64, anti-ly6C, anti-MHC-II, anti-MerTk, anti-CD163, anti-CD206 and anti-CX3CR1 (gating strategy in **Supplementary Figure 3B**).

For blood analysis staining, 50µL of blood are harvest in 1X PBS solution containing 1nM EDTA. Cells were pre-treated with red-blood cell lysis (RBC) at 1X for 5min at room temperature, following by saturation with FcBlock and incubated for 30min at 4°C with anti-CD45, Dump channel anti-CD3-CD19-NK.1.1-Ly6G-CD11c, anti-CD11b and anti-ly6C.

The indicated antibodies and isotype-matched antibodies used were obtained from Biolegend (San Diego, Californie). Stained cells were acquired by using BD FACSCantoI, BD LSRII or SORP BD Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed with

Flowjo V10. Doublets were excluded by appropriate FSC/SSC gates while dead cells were excluded using Fixable viability dye eFluor 780 (ref 65-0865-18 life technologies) for BMDM and cells purity or Live-Dead BUV496 (ref L23105 life technologies) for blood and synovial staining.

ELISA

IgG-IgM and IgA concentration in plasma samples and IL-10 concentration in cell-free supernatants after a 6-day culture of BMDM were measured according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA).

Quantitative PCR

Total RNA from RAW 264.7 - liver - spleen monocytes, B cells, T cells - bone marrow monocytes and monocytes polarization in M1 or M2 phenotype macrophages was extracted with the GeneJET kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer specifications, for miR-155-5p, SOCS-1, CEBP β , MARCO, YM-1, PU.1, SHIP-1, PRDM1, IFN- γ and IL-4 detection. The quantification of mRNA expression was determined by TaqMan real-time PCR and miRNA by miScript II SYBR Green PCR kit according to the manufacturer's instructions. The amounts of SOCS-1, CEBP β , MARCO, YM-1, PU.1, SHIP-1, PRDM1, IFN- γ and IL-4 were normalized to the endogenous GAPDH, and miR-155-5p expression was normalized to miR-25 and RNU6⁽³¹⁾. Efficiency of PCR using Taqman probes was performed using a serial dilution of amplicons and verification of the melting curve for SYBR Green. Calculations of mRNA and miRNA expression levels were performed using the comparative cycle threshold method ($\Delta\Delta Ct$).

Statistical analysis

The data were analyzed by using Graph Pad Prism V9.3.1. Data were tested by unpaired t-test for two groups and the Kruskal-Wallis test with multiple comparisons for multiple groups, expressed as mean \pm SEM with plot individual values.

Results

Impaired maturation of bone marrow monocytes polarization in anti-inflammatory macrophages in arthritic mice.

To explore whether the defect of monocyte polarization in anti-inflammatory macrophages is present in CIA mice, 10-week-old male DBA/10Jahsd mice were injected with an emulsion of grade chick type II collagen and complete Freund's adjuvant to induce arthritis, and we analyzed bone marrow monocytes polarization at D36. For the STA model, 8-week-old male C57BL/6J mice were injected intraperitoneal at D0 and D2 with 150 μ L of arthritogenic serum, and we analyzed monocytes polarization at D5 (**Figure 1A**). After culture in medium supplemented with M-CSF for 5 days and 24h of IL-4 stimulation for M2 polarization (**gating strategy in figure Supp 1A and B**), we did not find any change in expression of pan-macrophages markers CD11b-F4/80 in CIA and STA mice (**Figure 1B**). After validation of M2 and M1 markers of macrophages in these two mouse models, we demonstrated a defect of M2 polarization in arthritic mice with a lower CD206 expression (**Figure 1C**), decrease of IL-10 production (**Figure 1D**) and decrease of YM-1 relative expression (**Figure 1E**) compared to controls. Thus, CIA and STA models impaired maturation of bone marrow monocytes in anti-inflammatory macrophages similar to that observed in RA patients.

miR-155-5p is overexpressed in monocytes and M2-macrophages in arthritis mice.

To follow up our previous publication on the link between miR-155 overexpression in monocytes/macrophages and the defect of monocytes polarization in anti-inflammatory macrophages in human RA ⁽⁶⁾, we analyzed mature miR-155-5p expression and demonstrated up-regulation in CIA M2 macrophages (**Figure 1F**) and in STA monocytes and M2 macrophages (**Figure 1G**) compared to controls. Moreover, we also found down-regulation of two major mRNA target of miR-155-5p, and regulator of monocytes polarization in anti-inflammatory macrophages, SOCS-1 and CEBP β in arthritic mice (**Figure 1H-I**). These results confirmed the translational potential of findings observed in human RA toward arthritis murine models and suggested the utility of these mouse models for testing specific therapeutic approaches such as antagomiR-155-5p encapsulated in PEG-liposomes for correcting such abnormalities.

PEG-Liposome accumulate on joints of arthritic mice.

We chose liposomes as potent vectors to deliver fragile hydrophobic molecules such as antagomiRs. Once injected in the blood circulation, liposomes passively extravasate and accumulate at the sites of inflammation⁽¹⁹⁾. This improved drug distribution typically leads to an increase in drug efficacy and a reduction of side effect and toxicity⁽²⁰⁻²²⁾. We produced PEG-liposomes labelled with 1% of lipophilic tracer dialkylcarbocyanine (DID), with fluorescence excitation and emission spectra that allow optical imaging in the near infrared window. CIA or control DBA/10IaHsd mice were injected at D27 with an empty DID1%-PEG-liposomes. Dorsal and ventral position imaging was performed at 1, 2, 4, 24 and 48 hours after liposomes injection using an *in vivo* imaging system (**Figure 2A-B**). Images obtained demonstrated that DID 1%-PEG-liposomes can infiltrate the inflamed paw joints and accumulate in highly inflamed paws (score 2, 3 and 4) compared to mildly inflamed paws (score 1) and regular paws (score 0 / healthy) (**Figure 2B and C**). Moreover, DID 1%-PEG-liposomes were still detected in paws joint and in some organs such as liver and spleen after 48 hours post-injection (**Figure 2D and E**), suggesting prolonged action.

PEG-Liposomes encapsulating antagomiR-155-5p ameliorate arthritis

In the CIA mice model a preliminary experiment consisting in the systemic delivery of 43,4nmol per injection of free antagomiR-155-5p at D22-D29-D36 and D41 did not change the arthritic score. (**Figure Supp 2A**). Since PEG-liposomes were nicely distributed in inflamed joints, we proceeded with its encapsulation in this formulation. We first determined the efficiency of antagomiR encapsulated in PEG-liposomes *in vitro* in cells. We incubated murine macrophages (RAW 264.7) with PEG-liposome encapsulating antagomiR-control or antagomiR-155-5p for 24 h at 37°C, with the rate 70.2 – 81.3% of polyplex-antagomiR entrapped in liposomes. We determined the relative normalized expression of SOCS-1 and CEBP β by RT-qPCR. We demonstrated the same efficiency to decrease relative expression of SOCS-1 and CEBP β with 1X (1.5 nmol/100 μ L), 2.5X and 5X (**Figure Supp 2B**). We showed in a CIA model with high inflammation, that therapeutic injection at D30 -D32 and D34 of antagomiR-155-5p encapsulated in PEG-liposomes (1.5 nmol/100 μ L) reduced arthritis score and paw volume significantly only at D36 but not at other time points (**Figure 3A-C**). The absence of difference at other time points might be due to the fact that the arthritis score was very high and that the concentration of 1.5nmol of antagomiR-155-5p in PEG-liposome could

be limited in case of high inflammation. We confirmed in the STA mice model, that early injection of PEG-liposomes containing antagomiR-155-5p (1.5 nmol/100 μ L) led to a significant reduction of arthritis score and paw size (**Figure 3D-F**).

Restoration of monocyte defect in differentiation into anti-inflammatory macrophages after treatment in-vivo with PEG-Liposomes encapsulating antagomiR-155-5p.

We next investigated whether the decrease of arthritis score and paw volume/size in CIA and STA mice could be associated with restoration of the monocyte polarization into anti-inflammatory macrophages. For this purpose, we isolated monocytes from bone marrow of arthritis mice treated with PEG-liposome encapsulating antagomiR-control or antagomiR-155-5p. We found no significant change in the pan-macrophages marker CD11b-F4-80 expression (**Figure 4A and Supp 2D**). We observed a restoration of monocytes polarization into M2 macrophage with increase of CD206, IL-10 secretion and YM-1 expression (anti-inflammatory markers) (**Figure 4B and Supp 2E**) and a decrease of MARCO (pro-inflammatory marker) (**Figure 4C and Supp 2F**). This suggests efficient delivery of antagomiR-155-5p by PEG-liposomes to monocytes and macrophages (**Figure Supp 2C and G**).

In-vivo treatment with PEG-Liposomes encapsulating antagomiR-155-5p impacts synovial homeostasis.

To determine if the inhibition of miR-155-5p had an impact on resident macrophages and migratory monocytes in synovial tissue, we extracted on D5 the synovial tissue of STA mice (**gating strategy in figure Supp 3A-B**). We demonstrated that treatment with antagomiR-155-5p loaded in PEG-liposomes induced a decrease in CD45⁺ immune cell infiltration (**Figure 4D**) and of the pool of B, T, NK and Neutrophils cells (**Figure 4F**). Interestingly, we did not find any change in the synovial infiltration of the CD45⁻ cells, probably reflecting epithelium-fibroblast cells (**Figure 4E**).

There are two main subsets of blood monocytes in mice: inflammatory monocytes, which express CD11b⁺Ly6C^{Hi}, and patrolling monocytes that express CD11b⁺Ly6C^{Lo}. We demonstrated a significant decrease in synovial tissue of Ly6C⁺ inflammatory monocytes that comes from the blood after treatment with PEG-liposomes (cluster CD45⁺DUMP⁻Ly6C⁺CD11b⁺F4/80⁻CD64⁻) (**Figure 4G**). Moreover, in mice treated with PEG-liposomes encapsulating antagomiR-155-5p a decrease in CD11b⁺F4/80⁺ macrophages population was

observed (**Figure 4H**). In CD11b⁺F4/80⁺ macrophages population, there was no change in lining resident macrophages CX3CR1⁺ and no restoration of the protective lining macrophages CX3CR1⁺MerTK⁺ after treatment (**Figure 4I-K**) compared to control mice (**Figure Supp 3B**). We observed an increase of the tissue infiltrating macrophages CD11b⁺F4/80⁺CX3CR1⁻MerTK⁻ after treatment with liposomes encapsulating antagomiR-155-5p (**Figure 4L**). In the tissue infiltrating macrophages population, we found new populations of CD11b⁺F4/80⁺CD206^{high} / CD11b⁺ F4/80⁺CD163^{high} cells probably deriving from blood monocytes (**Figure 4J-M**) and suggesting a restoration of the monocyte defect in M2 macrophages polarization after treatment with an antagomir-155-5p encapsulated in PEG-liposome.

Internalization of antagomiR-155-5p tagged cy³ on blood monocytes in arthritis mice

We explored the impact of PEG-liposomes encapsulating antagomiR labelled with Cyanine-3 (cy³) on blood monocytes at D2 and on synovial macrophages at D5 (**Figure 5A**). We detected cy³ signal only in blood monocytes CD11b⁺Ly6C⁺ and not in other cells (DUMP⁺ cells) or non-injected mice (**Figure 5B and E**). Moreover, we found more cy³ signal in blood monocytes in STA mice than in control mice, probably due to the shift of Ly6C fluorescence intensity in STA mice. Thus, we suggest that PEG-liposomes are more directed to the Ly6C^{high} monocytes population.

At D5, we harvested bone-marrow and synovium tissues. In bone marrow, we detected cy³ signal in CD11b⁺ monocytes/macrophages population without any difference between control mice and STA (**Figure 5C and F**). In synovium, we demonstrated a slight cy³ signal in STA macrophages F4/80⁺CD11b⁺, no cy³ signal in the resident macrophages CX3CR1⁺ and in the protective population CX3CR1⁺MerTK⁺. As the tissue infiltrating macrophages are present only in STA mice, we demonstrated the presence of a cy³ signal in these macrophages (**Figure 5D and G**).

Altogether, these results suggest that PEG-liposome are first internalized by blood monocytes CD11b⁺Ly6C^{high} and hitchhike within these monocytes which go into synovium and polarized in macrophages CD11b⁺F4/80⁺ (23-24).

In-vivo treatment with PEG-liposomes encapsulating antagomiR-155-5p has limited impact on other immune cells.

The objective of the encapsulation of antagomiRs within PEG-liposomes was to specifically direct them to monocytes/macrophages (linked to phagocytosis) and to spare other immune cells, and as much as possible liver and spleen macrophages. As antagomiR-155-5p may cause severe non-myeloid cells effects side effects on others immune cells, we analyzed the impact of antagomiR-155-5p or antagomiR-control effect on Kupffer cells, sorted CD11b⁺ monocytes, B and T cells of spleen (**Figure Supp 3D-F**). We were able to demonstrate that treatments with antagomiR-155-5p encapsulated in PEG-liposomes had no impact on the percentage of these cells in CIA (**Figure Supp 3C-F**) or on their absolute number. Likewise, antagomiR-155-5p encapsulated in PEG-liposomes had no impact on the main targets of miR-155 in sorted T cells, such as IFN- γ – IL-4 – SOCS-1 and CEBP β (**Figure 6C and Figure Supp 4C**). In sorted B cells antagomiR-155-5p encapsulated in PEG-liposomes did not impact the relative expression of PU.1 – SHIP-1 – PRDM1 - SOCS-1 - CEBP β (**Figure 6D and Figure Supp 4D**) and immunoglobulin secretion in mice plasma cells (**Figure 6E and Figure Supp 4E**), suggesting that antagomiR-155-5p encapsulated in PEG-liposomes have no or minor effect on T and B cells.

Then, we studied the effect antagomiR-155-5p encapsulated in PEG-liposomes on liver and spleen monocytes/macrophages of CIA and STA mice. We found a paradoxical decrease in SOCS-1 and CEBP β in liver macrophages of CIA mice and a trend in STA mice (**Figure 6A and Supp 4A**). Conversely, we found a trend for increase of SOCS-1 and CEBP β , which was the expected action of the antagomir-155-5p in spleen monocytes (**Figure 6B and Supp 4B**). Thus, encapsulated in PEG-liposomes, antagomiR-155-5p displayed only a limited impact on spleen monocytes and liver macrophages, despite the high accumulation in liver observed by optical imaging (**Figure 2D and E**).

Discussion

Using reverse translational science, we confirmed our previous observations in human RA ⁽⁶⁾ and found in two pre-clinical mouse models of RA, a defective bone-marrow monocyte polarization into anti-inflammatory macrophages (M2), with a decrease of CD206, YM-1 expression and secretion of IL-10. As in humans, we found that this defect was associated with an increase of miR-155-5p in monocytes and anti-inflammatory macrophages in both mouse models. As BMDMs and synovial macrophages have different transcriptional profile ⁽²⁵⁾, we also studied subsets of synovial macrophages and BMDMs. Subsequently, we demonstrated that treatment with an antagomiR-155-5p encapsulated in PEG -liposomes was able to decrease joint inflammation, to restore bone marrow monocytes polarization into anti-inflammatory macrophages, to reduce immune cells infiltration in synovial tissue, to increase the CD206⁺ and CD163⁺ tissue infiltrating macrophages and to decrease expression of mRNAs target of miR-155-5p.

miRs indeed represent a class of small endogenous non-coding RNA molecules which play an important role in post-transcriptional regulation of gene expression. The critical role of miR-155 in RA has been further demonstrated in mice, as subcutaneous injection of collagen in *miR-155*^{-/-} mice did not lead to the development of clinical signs of arthritic such as increase in paw volume and swelling ⁽²⁶⁾. AntagomiR-155-5p are chemically modified nucleic acids designed to bind miR-155-5p (100% coverage of miRBase V21). Here, we demonstrated that antagomiR-155-5p encapsulated in PEG-liposomes to treat CIA and STA mice lead to a reduction in arthritis score, a restoration of the defect of monocytes polarization in anti-inflammatory macrophages with increase of CD206, YM-1, IL-10 secretion and decrease of MARCO. Moreover PEG-liposomes were specifically taken up by blood and bone marrow monocytes CD11b⁺Ly6C^{high}, which were increased in STA mice, and had no or minor effect on liver macrophages, spleen monocytes, B cells, T cells and synovial residents macrophages. Similarly, to the most preclinical experiments in mouse models of arthritis ⁽²⁷⁾, we found that prophylactic injection of PEG-liposome containing antagomiR-155-5p was more efficient to decrease inflammation and arthritic score than a curative strategy. However, in both arthritis models, prophylactic or curative treatment with a small concentration of antagomiR-155-5p (1.5 nmol/100 μ L) induced a restoration of monocyte defect in anti-inflammatory macrophages and had an impact on infiltrating macrophages, while the treatment as not impact on residents macrophages and on CD45 negative cells, probably reflecting epithelium-fibroblast cells. The

relative expression of miR-155-5p in monocytes/macrophages, is more important in the high inflammatory STA than in the CIA mice model, could be linked to the difference of inflammation between both models. This difference could be also explained by the timing of the analysis where monocytes were harvested for STA near to the exponential phase of the disease/inflammation as compared to CIA where monocytes harvest was performed on the plateau of the disease/inflammation.

PEG-liposomes are ideal vectors to improve the addressing of antagomiR to cells for several reasons: 1- they protect antagomiR from enzymatic degradation ⁽¹⁸⁾, 2- they combine the advantages of being biocompatible and nontoxic, 3- they are predominantly captured by activated phagocytic blood monocytes and macrophages that are present at the site of inflammation. Using targeting ligands could even increase this specificity to monocytes/macrophages. But until now, no specific receptor of monocytes/macrophages has been established, that could increase the delivery in phagocytic monocytes/macrophages. Liposomes have also the advantage to be quickly available and have a good safety profile for clinical translation as shown by the high number of liposomal drugs on the market. For example, Daunorubicin entrapped in liposome (DaunoXome) has an improved pharmacokinetic profile compared with free Daunorubicin, is well tolerated, and had a significant antitumor activity in patients with AIDS-KS (AIDS related Kaposi's sarcoma) ⁽²⁸⁾. Moreover, in 2018 Patisiran (Onpattro) a lipid nanoparticle formulation of siRNA (LNPs) for the treatment of hereditary transthyretin-mediated (hATTR) amyloidosis was clinically approved ⁽²⁹⁾. More recently in 2020, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has extended throughout the world and resulted in an unprecedented global public health crisis. In the emergency, six mRNA vaccines have been used to prevent SARS-CoV-2 infection with lipid formulations via intramuscular injection ⁽³⁰⁾.

Treatment of RA has been markedly improved over the past 20 years with the use of five TNF inhibitors, two IL-6 receptor inhibitors, one co-stimulation antagonist, one B-cell targeted therapy and four Jak inhibitors ⁽³¹⁾. However, none of these treatment targets a specific pathophysiologic pathway specific of RA. Thus, these treatments may lead to some adverse events as recently illustrated by the increased risk of cancer and of major cardio-vascular events with Jak inhibitors compared to anti-TNF ⁽³²⁾.

Our approach with antagomiR-155-5p encapsulated in PEG-liposomes has the advantage to target a cellular abnormality, the defect of polarization of monocytes into M2 anti-inflammatory

macrophages specific of RA since we did not find it in other inflammatory diseases ⁽⁶⁾. In this study, we showed that this defect also exists in two different models of RA. Actually, targeting miR-155 could not be specific of monocytes/macrophages. Indeed, several studies proved the involvement and the key role of miR-155 on CD4⁺T cells on activation, differentiation, function, and apoptosis ⁽³³⁾, but also on B cells by PU.1 regulation that lead to the survival and proliferation of B blast, plasmablast and consequently antibody production ⁽³⁴⁾. However, thanks to its encapsulation in liposomes, antagomiR-155-5p had no impact of mRNA-targets of miR-155-5p in other immune cells (CD4, CD8, B cells and plasma cells) than monocytes/macrophages and a limited impact on liver and spleen monocytes/macrophages, thus limiting the possible induction of side effects due to action on these cells. Indeed, phagocytosis is requested for the availability of the encapsulated antagomir and is made by monocytes and macrophages. The encapsulated antagomiR-155-5p was present in both cellular types and acts probably at the stage of blood monocytes for re-orienting him to a correct differentiation into anti-inflammatory macrophages.

In summary, the encapsulation of antagomiR-155-5p in PEG-liposomes appears to be an effective strategy to deliver small RNA to monocytes/macrophages and reduce joint inflammation in mouse models of RA. The repolarization of unbalanced macrophages by PEG-liposome encapsulating antagomiR-155-5p could be a promising, specific and safe therapeutic strategy to treat human RA.

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Competing interests: All authors have no conflict of interest

Data and Materials availability: The data that support the findings of this study are available on request from the corresponding author: audrey.paoletti@inserm.fr

WHAT IS ALREADY KNOWN ABOUT THIS TOPIC

- Increased expression of miR-155 in monocytes of patients with rheumatoid arthritis could be responsible for the defect of monocyte polarization to anti-inflammatory macrophages.

WHAT THIS STUDY ADDS

- Treatment with an antagomiR-155-5p entrapped in PEG-liposomes in two mice models of arthritis mice led to restoration of the defect of blood monocytes polarization toward an anti-inflammatory macrophage in synovial tissue, reduction of immune cell infiltration in synovial tissue and improvement of arthritis, without any side effect on other immune cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This study is a proof of concept demonstrating that it is possible, thanks to liposome encapsulation, to address an antagomiR in monocytes/macrophages in joints at the site of inflammation, which can avoid systemic sides effects. This strategy is promising in human disease.

Figures and Legends

Figure 1: ex-vitro monocytes polarization in CIA and STA mice and miR-155-5p implication in monocytes polarization in M2. Experimental timeline of immunization procedure and development of arthritis in 2 pre-clinical model. CFA, Freund's complete adjuvant, CII, bovine collagen type II (A). The differentiation of sorted bone-marrow monocytes to BMDM-M2 was assessed in CIA (n=11 red dots) and STA (n=5 blue dots) arthritic model with respective control mice (n=11 for CIA, n=5 for STA black dots), by flow cytometry with anti-CD11b and anti-F4/80 antibodies (B). Specific markers of M2 macrophage polarization were assessed: CD206 (C) IL-10 secretion in cell culture supernatant (D) and YM-1 relative expression (E). miR-155-5p expression on bone-marrow monocytes and BMDM-M2 was determined by quantitative real-time PCR on CIA (F) and STA (G) mice model. Target mRNA SOCS-1 and C/EBP- β relative expression on CIA monocytes (H) and STA monocytes (I). Data are shown as symbols and mean \pm SEM and were compared by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 2: Accumulation in-vivo of DID 1%-PEG-liposomes in inflamed joints. Experimental timeline of immunization and DID-PEG-liposomes (A). 10-week-old male DBA/1OlaHsd mice were injected intradermally at the base of the tail with an emulsion of grade chick type II collagen and complete Freud's adjuvant. At day 21, mice received a boost injection of the same composition. After IV injection at day 27 of fluorescent PEG-liposomes the evolution of the NIR signal (dorsal view) in healthy mice (n=3) and CIA mice (n=6) was monitored according to the arthritis score for each paw. *In vivo* representative images of one mouse per group before and after injection with DID 1%-PEG-liposomes at 1h, 2h, 4h 24 h and 48 hours. The score of each paw is indicated in white squares on the "before injection" image (B). Average Radiant Efficiency 4 h after IV injection with DID 1%-PEG-liposomes in paws segregated based on their arthritis score (C). *In vivo* representative images of one CIA mouse after 48 hours injection with DID 1%-PEG-liposomes. The score of each paw is indicated in white squares on the "before injection" image. *ex-vivo* organs representative images of the same CIA mice with the same sequence and scale (D) and Average Radiant Efficiency on CIA mice (n=6) demonstrated the comparison between paw, liver, spleen, kidneys, heart, lung and intestine bio-distribution of DID 1%-PEG-liposomes (E). Data are shown as symbols and mean \pm SEM and were compared by ordinary one-way ANOVA with multiple comparisons. ****p<0.0001.

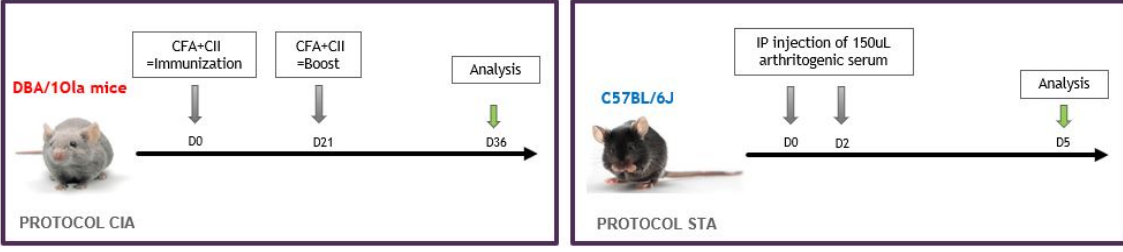
Figure 3: Treatment of CIA and STA mice with PEG-liposomes containing antagomiR-control or antagomiR-155-5p. Experimental timeline of immunization and PEG-liposomes injection in CIA model (A) or STA (D). For CIA mice (red dots), antagomiR-control or 155 entrapped in PEG-liposomes are intravenously injected on days 30, 32 and 34, for STA (blue dots) PEG-liposome are intravenously injected on days 0, 1, 2, 3, and 4. Hind paw volume was measured with a plethysmometer for CIA model (B) or caliper for STA (E). Mice were evaluated every 2-day, based on a visual scoring scale of the paws with evaluation of the erythema, swelling and ankylosis of each paw (score 1 to 4) (C and F). Data are shown as symbols and mean and error \pm SEM and were compared by ordinary two-way ANOVA with multiple comparisons or by one-way ordinary ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. We use classical normalization formula where data mean of D29 for CIA and D2 for STA mice of each group of injection is equal to 100%.

Figure 4: ex-vitro monocytes polarization in STA mice and ex-vivo synovial homeostasis after treatment with PEG-Liposomes containing an antagomiR-155-5p. The differentiation of sorted bone-marrow monocytes to BMDM-M2 was assessed in STA (blue dots panel A-C) arthritic model with respective control mice (black dots), by flow cytometry with anti-CD11b and anti-F4/80 antibodies (A). Specific markers of M2 macrophage polarization were assessed: CD206, IL-10 secretion in cell culture supernatant and YM-1 relative expression (B). Relative expression of M1 macrophages marker MARCO was determined by Taqman probes (C). Synovial tissue cells were assessed by flow cytometry analysis (panel D-N) using anti-CD45, DUMP channel: CD19-CD3-NK1.1-LY6G, anti-CD11b, anti-F4/80, anti-MHCII, anti-CD64, anti-LY6C, anti-MerTK, anti-CD163, anti-CD206 and anti-CX3CR1. Determination of CD45⁺ cells (D), CD45⁻ (E), DUMP channel⁺ cells (B-T-NK and Neutro) (F), inflammatory monocytes (G) and macrophages (H) infiltration in synovial tissue. Exploration of residents CX3CR1⁺ (I and K), protective lining MerTK⁺ (I and L), tissue infiltrating (I and M) or tissue infiltrating CD206⁺ or CD163⁺ macrophages (J and N). Data are shown as symbols and mean \pm SEM and were compared by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

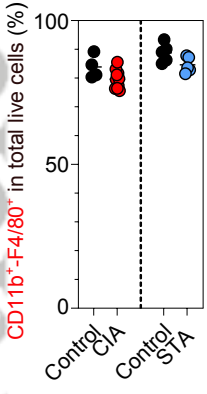
Figure 5: Specific bio-distribution ex-vivo of PEG-Liposomes in monocytes-macrophages cells. Experimental timeline of immunization and PEG-liposomes containing antagomiR-control-cy3 injection in STA model (A). Determination of cy3 staining in blood monocytes (B), bone marrow CD11b⁺ monocytes (C) or synovial tissues cells and macrophages (D). Data are shown as symbols and mean ± SEM and were compared by unpaired t-test. **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 6: CIA mice treatment in-vivo of PEG-Liposomes containing an antagomiR-155-5p has limited impact on other immune cells. qPCR on liver pellets (Kupffer cells) of SOCS-1, CEBP-β and GAPDH (A). qPCR on sorted spleen monocytes of SOCS-1, CEBP-β and GAPDH (B). qPCR of spleen on T cells sorted of IFN-γ, IL-4, SOCS-1, CEBP-β and GAPDH (C). qPCR of spleen sorted B cells of PU.1, SHIP-1, PRDM1, SOCS-1, CEBP-β and GAPDH (D). ELISA on mice serum for determination of IgG – IgA and IgM (E).

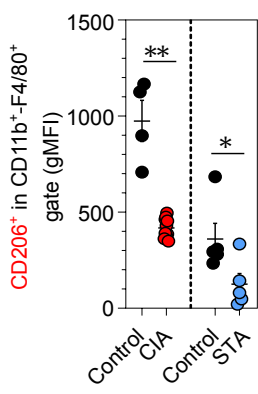
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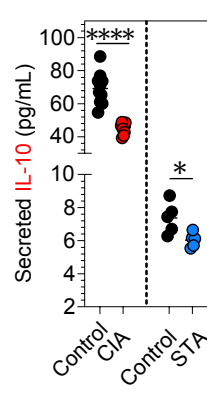
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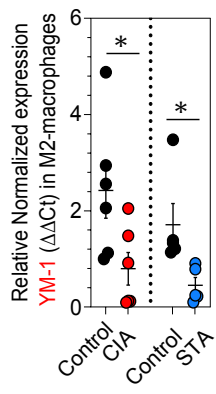
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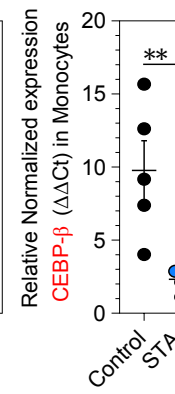
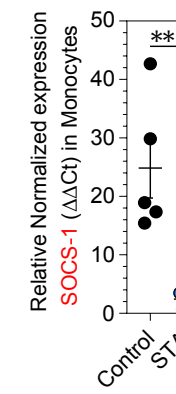
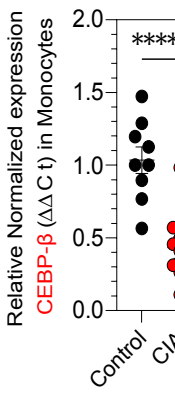
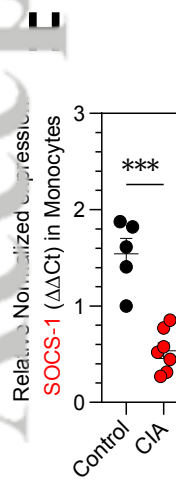
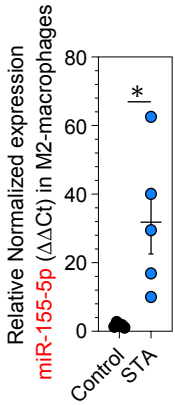
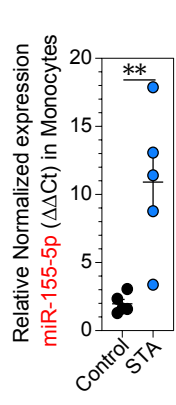
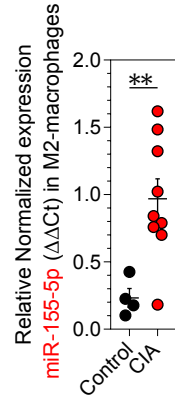
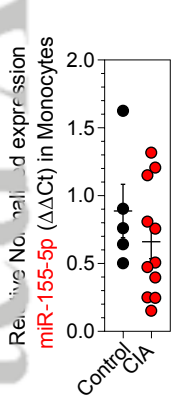
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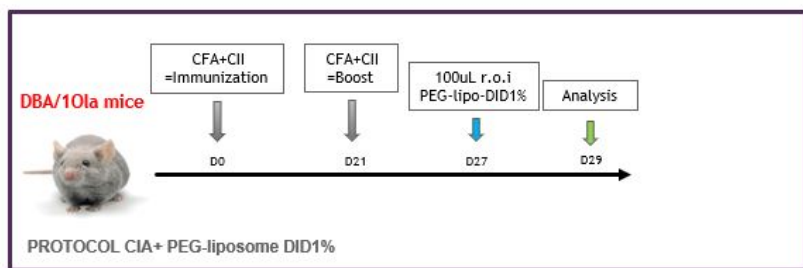


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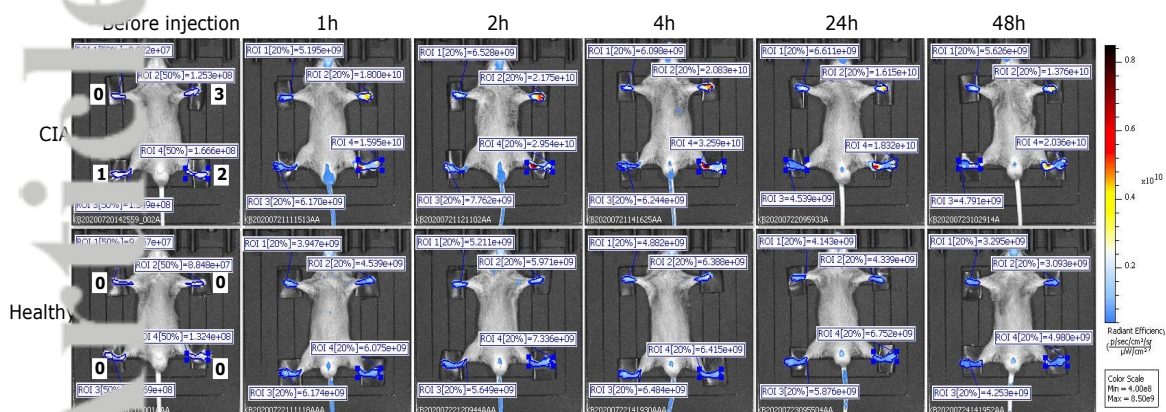


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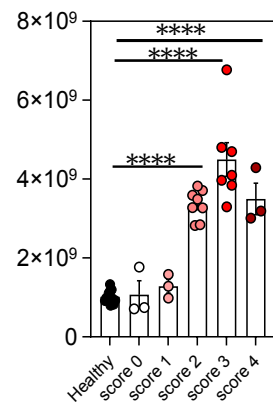


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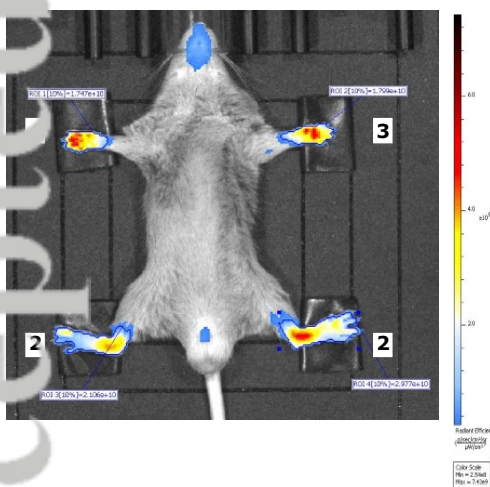


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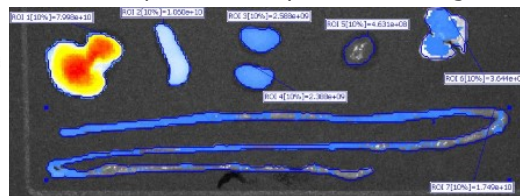
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D

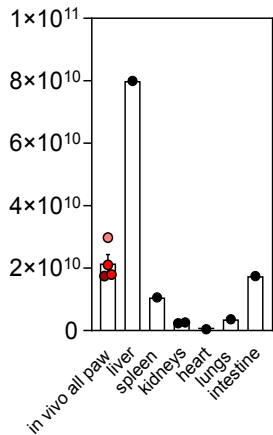


Liver Spleen Kidneys Heart Lungs

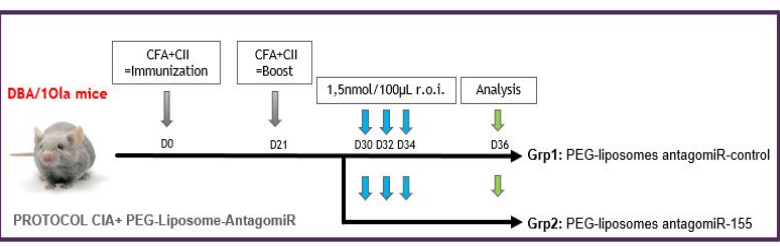


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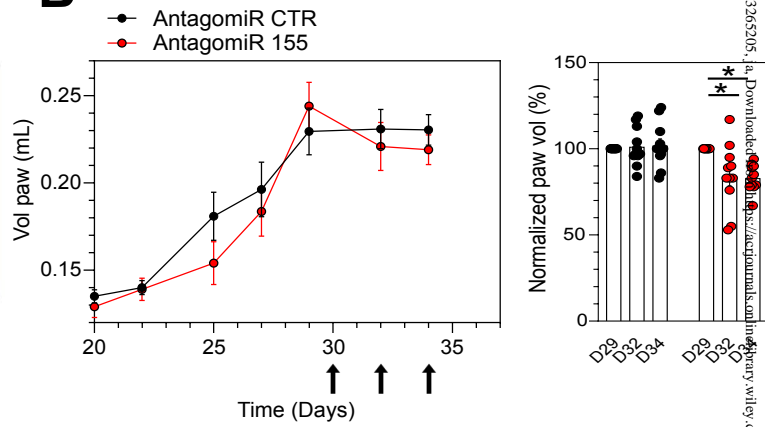
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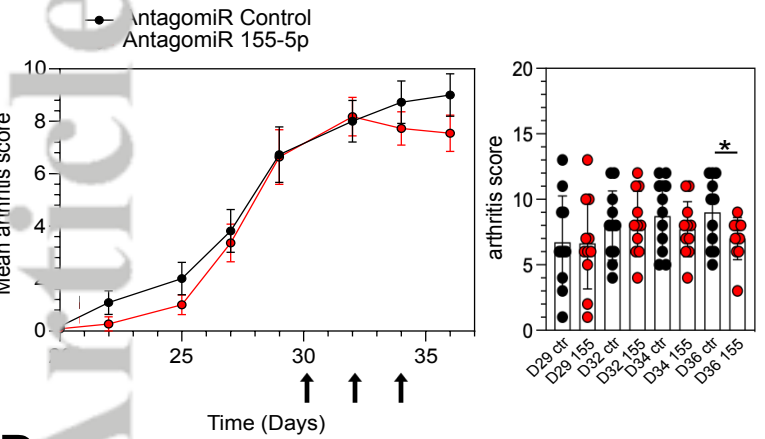
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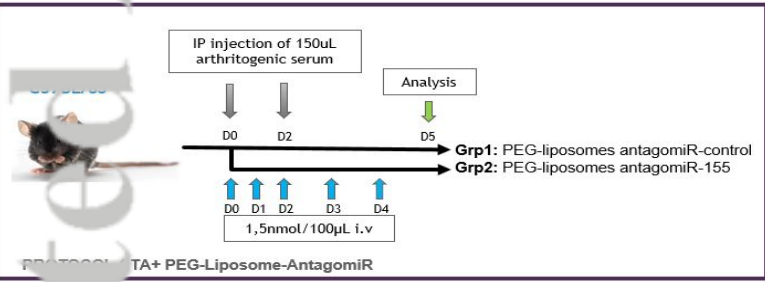
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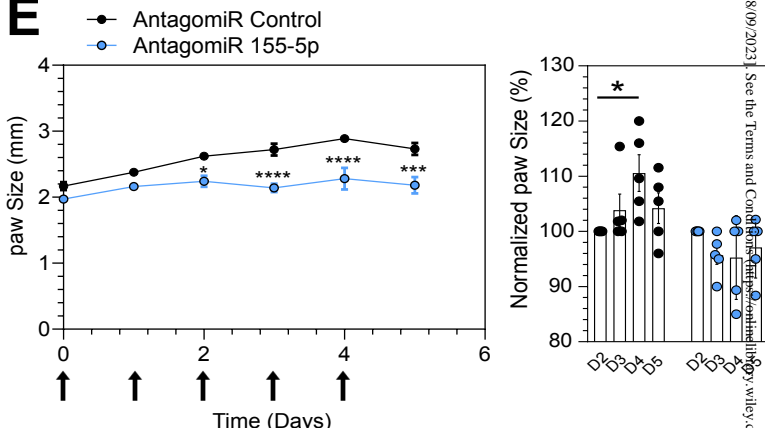
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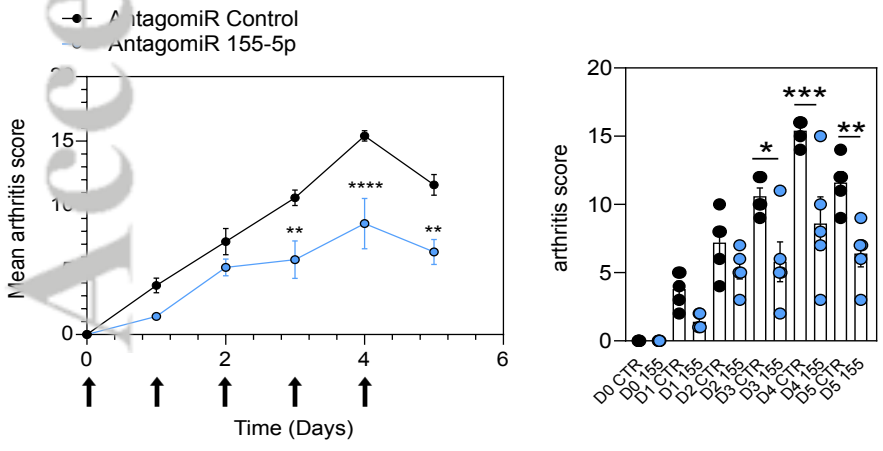
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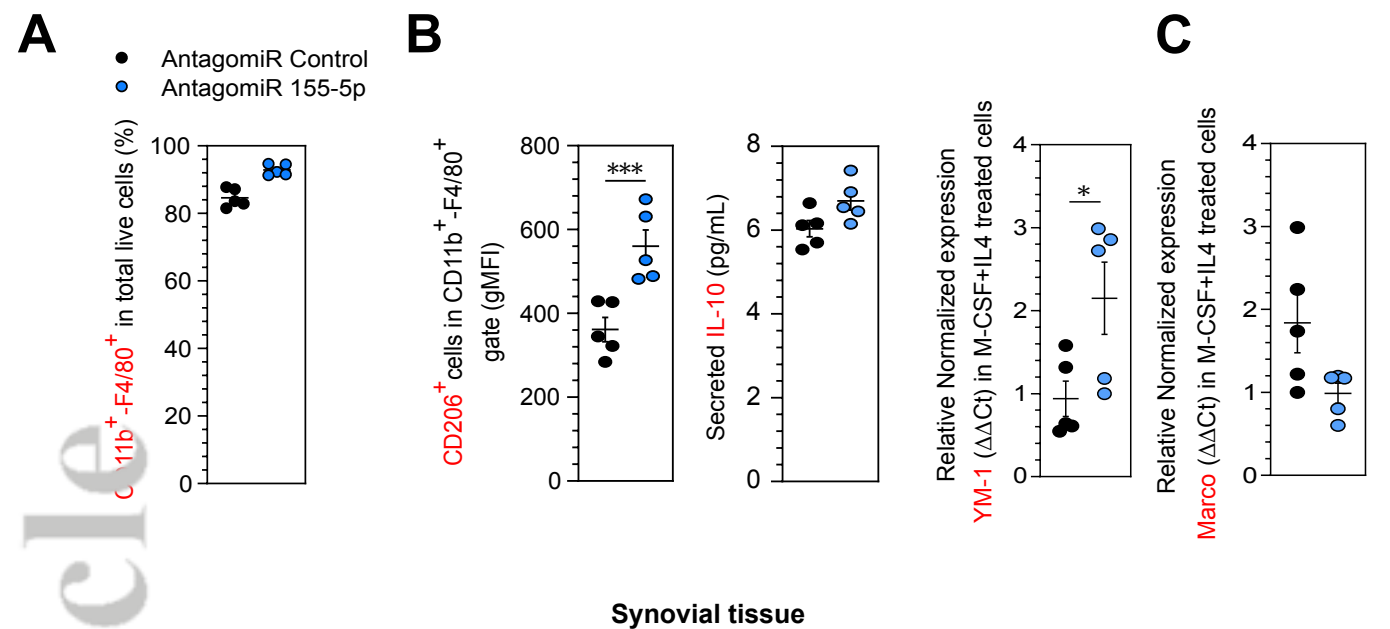


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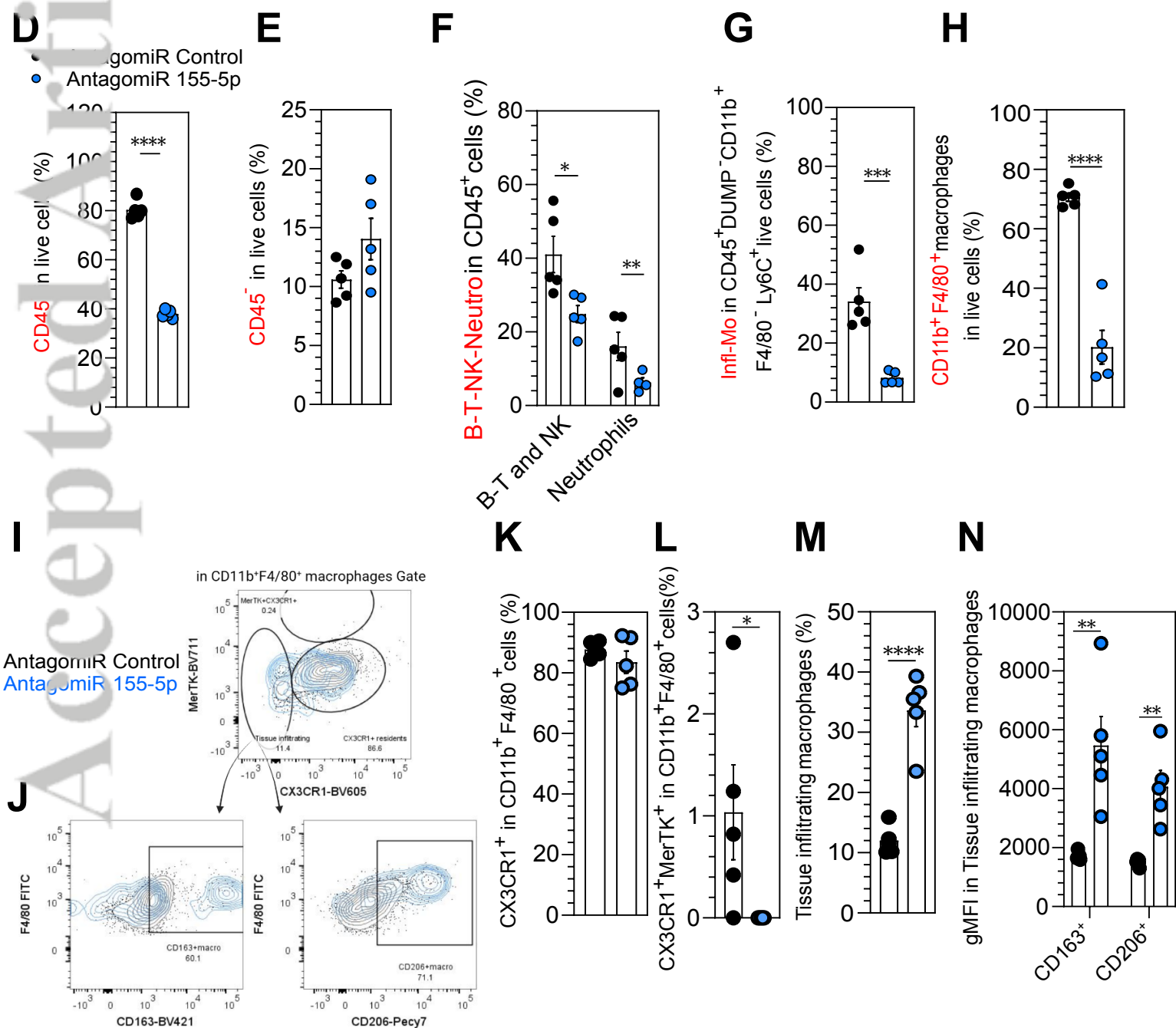


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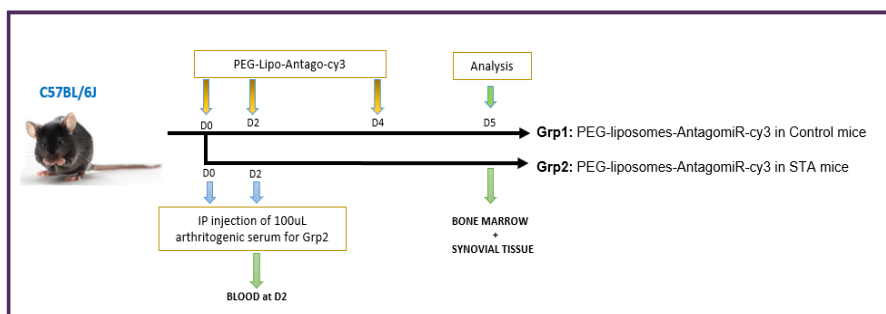




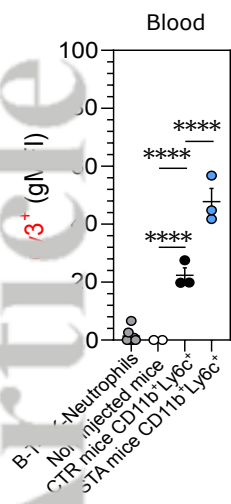
Synovial tissue



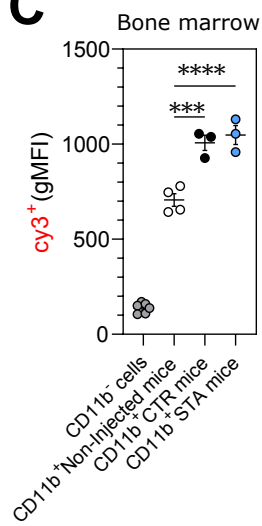
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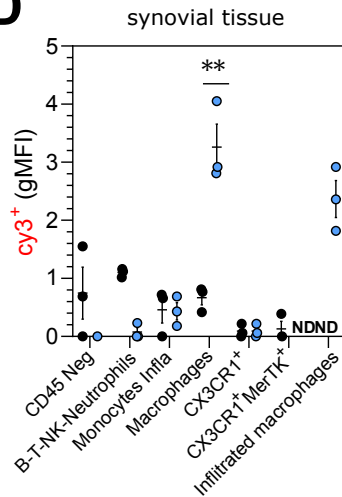
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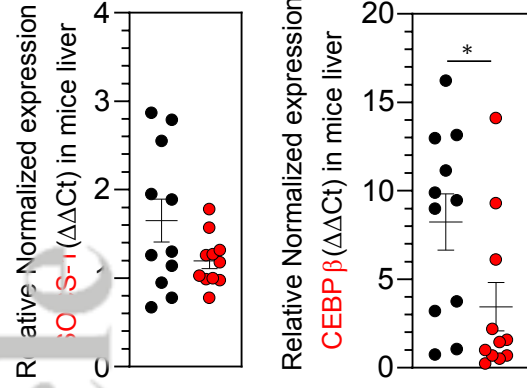


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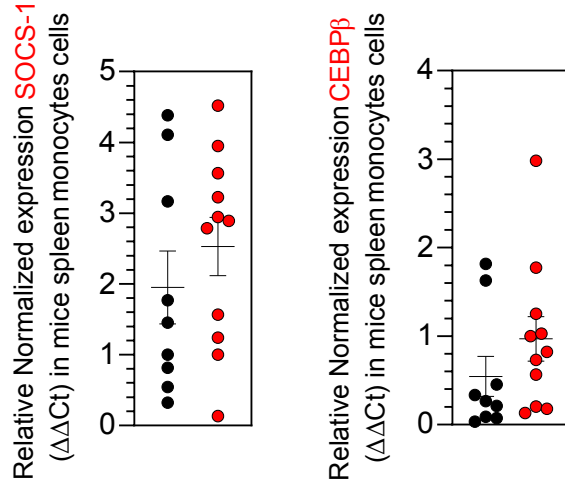


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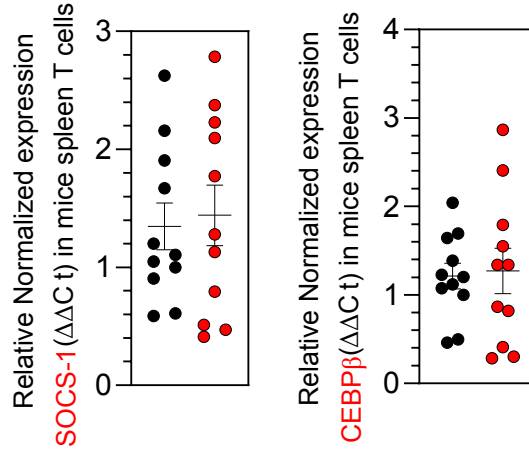
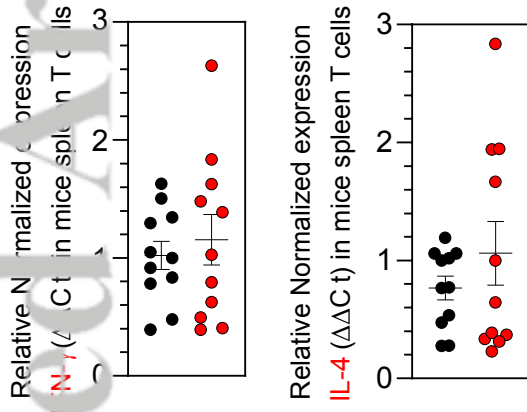
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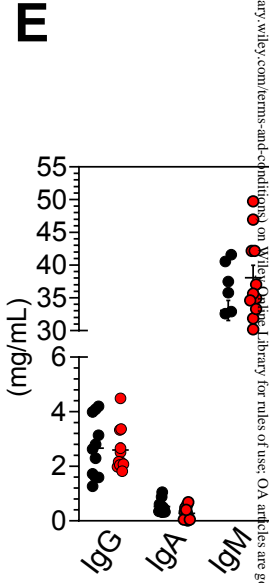
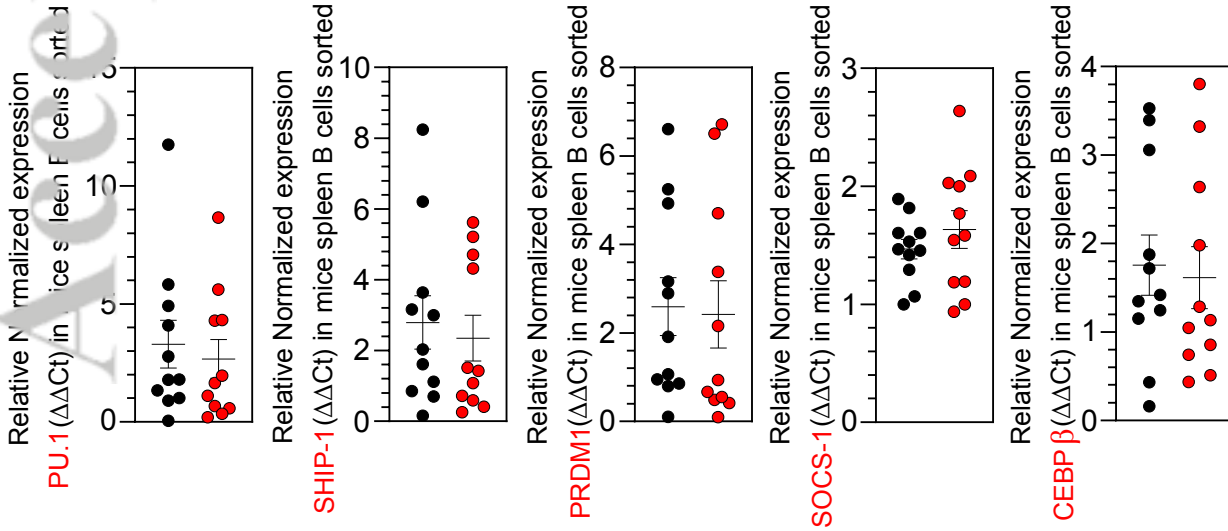
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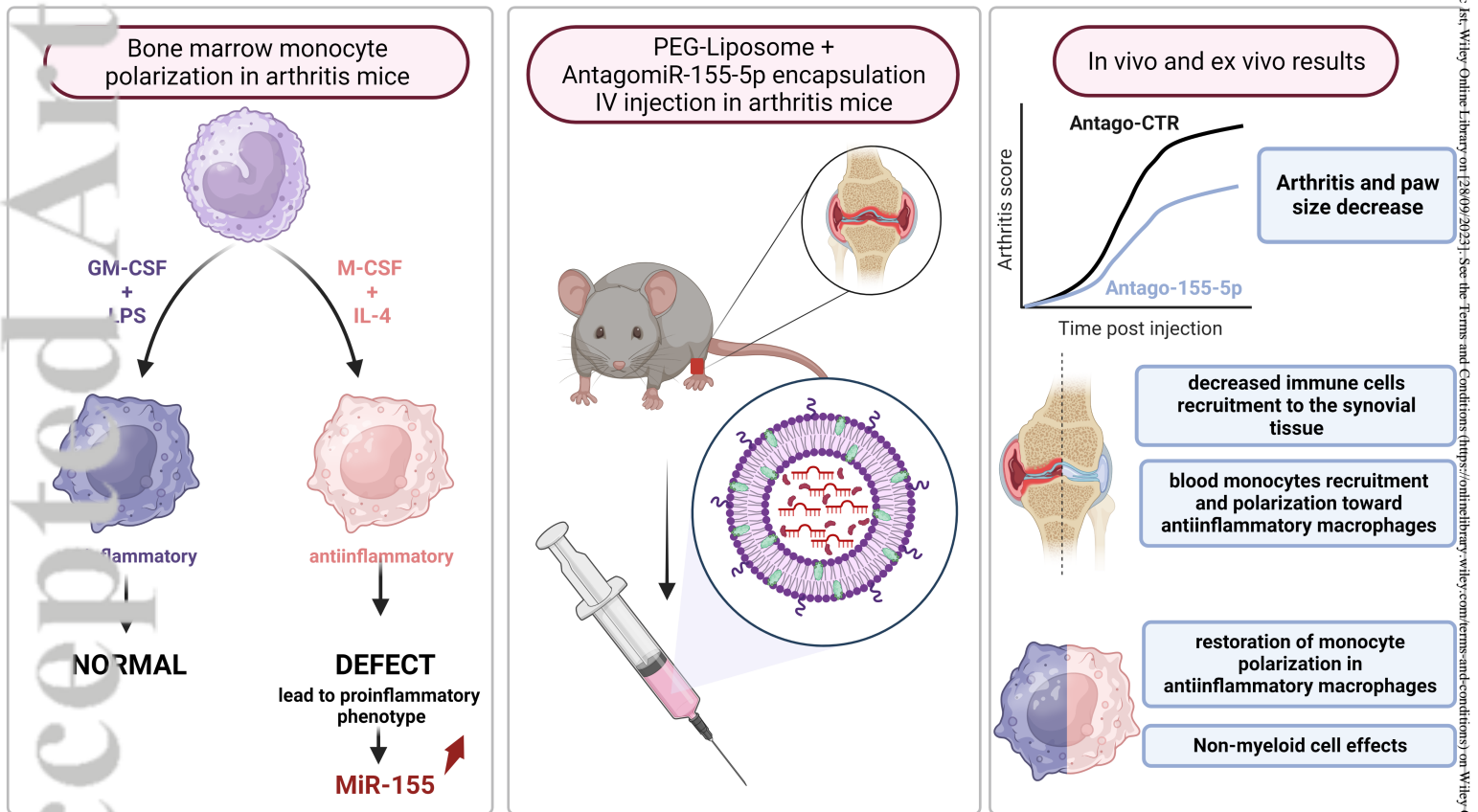
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● AntagomiR 155-5p



D ● AntagomiR Control
● AntagomiR 155-5p



PEG-Liposome Injection Containing an AntagomiR-155-5p Restores Antiinflammatory Macrophages and Improves Arthritis in Preclinical Models of Rheumatoid Arthritis



Paoletti A, et al, Cailleau C, et al. Liposomal AntagomiR-155-5p restores antiinflammatory macrophages and improves arthritis in preclinical models of rheumatoid arthritis. *Arthritis Rheumatol* 2023.

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