

Magnetic nanoparticle priming of mesenchymal stromal cells generates MRI- detectable extracellular vesicles with enhanced immunomodulatory activity

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Abstract

Extracellular vesicles (EV) derived from mesenchymal stromal cells are emerging as promising cell-free therapeutics for immune modulation and tissue repair. However, strategies enabling both functional control and in vivo tracking of EV remain limited. Here, we developed a nanoparticle-based preconditioning strategy to generate magnetically responsive EV while maintaining compatibility with scalable production processes. Citrate-coated maghemite ($\gamma\text{-Fe}_2\text{O}_3$) nanoparticles were incorporated into a three-dimensional EV production platform based on microcarrier culture and controlled turbulent stimulation of human adipose-derived stromal cells. Short-term nanoparticle exposure did not affect cell viability, adhesion to microcarriers, or vesicle production, demonstrating compatibility with scalable EV manufacturing. Magnetic resonance imaging revealed strong T_2 signal attenuation in nanoparticle-labelled EV preparations, confirmed by electron paramagnetic resonance quantification of iron content. Cryo-electron microscopy further revealed nanoparticle clusters within vesicular structures and in co-isolated protein fractions, suggesting incorporation during vesicle biogenesis. Functionally, nanoparticle preconditioning enhanced EV immunomodulatory activity in a THP1-Dual-derived macrophage model. Magnetic field exposure further amplified these effects, promoting coordinated transcriptional reprogramming of macrophages and shifting inflammatory phenotypes toward a more regulatory state. Together, these findings demonstrate that iron oxide nanoparticle preconditioning enables scalable production of magnetically responsive EV and introduces a strategy for magnetically controllable and image-guided EV-based immunotherapies.

1. Introduction

Inflammation is a fundamental physiological response of the body to infection and tissue injury. This complex process involves the coordinated activation of immune cells and the release of pro- and anti-inflammatory mediators to restore homeostasis. However, excessive, persistent, or uncontrolled inflammation can become detrimental, contributing to the development of chronic pathologies such as autoimmune diseases, allergies, and cancer. Controlling the delicate balance between effective defence and pathological dysregulation has therefore become a major therapeutic challenge, where immunomodulation emerges as a promising strategy to restore immune homeostasis.

In this context, extracellular vesicles (EV) have gained considerable attention as key mediators of intercellular communication. These nanosized, cell-derived biological nanoparticles, typically ranging from 30 to 1000 nm depending on their subtype, are secreted as part of the cellular secretome and transport a wide array of bioactive molecules, including proteins, lipids, and RNA, capable of modulating immune responses¹. Among them, mesenchymal stromal cell-derived EV (MSC-EV) stand out due to their potent immunomodulatory and regenerative properties. They have been shown to suppress T cell proliferation², promote the expansion of regulatory T cells³, and drive macrophage polarisation toward the anti-inflammatory M2 phenotype⁴. By fostering a tolerogenic and pro-repair microenvironment, MSC-EV emerge as strong candidates for the treatment of chronic inflammatory and autoimmune diseases^{5–}

⁸, while avoiding the risks associated with cell transplantation (immunogenicity, tumorigenesis, thrombosis and availability).

In parallel, superparamagnetic iron oxide nanoparticles (ION) have rapidly expanded their applications in biomedicine. Initially developed as contrast agents for magnetic resonance imaging⁹ (MRI) and as tools for targeted drug delivery or tumour hyperthermia^{10,11}, these nanoparticles have also been extensively explored for cell labelling and tracking applications. Over the past two decades, our group has contributed to the development of robust strategies for cellular labelling using citrate-stabilised maghemite nanoparticles synthesised via the Massart procedure^{12–15}. These studies demonstrated efficient and biocompatible internalisation of ION within cell endosomes across a wide range of cell types¹², establishing this approach as a versatile and widely applicable method for magnetic cell labelling and MRI-based cell tracking. Beyond imaging applications, magnetically labelled cells have also enabled the development of magnetic targeting strategies aimed at improving cell delivery to specific tissues. More recently, these approaches have been extended to immune cell therapies, including CAR-T cells, where nanoparticle labelling allows both non-invasive monitoring and magnetically enhanced delivery of therapeutic cells to tumour sites¹⁶. Additionally, our group have further demonstrated that EV released by magnetically labelled cells contained ION^{17–20}. These findings indicate that nanoparticles can be incorporated into EV through intracellular trafficking or vesicle biogenesis pathways prior to secretion. Such observations suggest that EV derived from nanoparticle-labelled cells may inherit magnetic properties from their parental cells, opening new opportunities for engineering magnetically responsive EV.

Beyond their well-established use for labelling and imaging applications, ION have also been shown to directly influence immune responses. Several studies have demonstrated their ability to influence their ability to modulate inflammatory processes, including the reprogramming of tumour-associated macrophages toward a pro-inflammatory phenotype to inhibit tumour growth²¹, the reduction of inflammatory protein and the inhibition of mast cell infiltration in a rat model of atopic dermatitis^{22,23}. These findings suggest an emerging role for ION in regulating inflammatory processes, opening new avenues for the treatment of immune-mediated chronic diseases.

Taken together, the integration of EV and ION within the field of immunomodulation represents an innovative therapeutic approach for inflammatory disorders. While EV provide the specificity and biological complexity of cell-derived signals, ION offer unique physical and pharmacological properties. Their combined use may act synergistically to restore immune homeostasis and support tissue regeneration.

However, for such approach to be clinically relevant, robust and scalable EV production are required. To address this challenge, our group previously developed a patented EV production process based on three-dimensional culture of mesenchymal stromal cells on microcarriers combined with controlled turbulent stimulation, enabling active and GMP-compatible EV production²⁴. This system not only

supports scalable manufacturing but also enhances vesicle secretion through biomechanical stimulation of producer cells, allowing efficient EV production within four hours compared with conventional serum-starvation approaches, which typically require 24 to 72 hours of starvation. The translational relevance of this platform has been demonstrated in several *in vivo* studies, where MSC-EV produced using this approach showed therapeutic efficacy in multiple preclinical models of tissue regeneration^{25–27}. Importantly, such a reproducible and scalable production platform²⁸ also provides an opportunity to integrate EV engineering strategies, such as ION labelling, within the manufacturing process itself. While most current strategies for generating magnetically responsive EV rely on post-production association of vesicles with iron oxide nanoparticles^{29–31}, we instead explored a cell preconditioning strategy based on our previous studies, in which EV-producing mesenchymal stem cells were exposed to ION prior to vesicle release within the 3D culture system. Integrating nanoparticle priming directly into a scalable EV production process allowed us to investigate how magnetic preconditioning of producer cells influences both the biophysical characteristics, MRI detectability, magnetic responsiveness and the immunomodulatory potency of the resulting EV. These effects were evaluated using a macrophage-based functional assay previously established in our group³².

2. Results

2.1. Preconditioning of stromal cells with ION in a stirred-tank bioreactor

Maghemite labelling was integrated into a standardised three-dimensional culture method for EV production, as illustrated in Fig. 1A. Briefly, primary human adipose-derived stem cells (hASC) were first expanded in conventional 2D culture flasks before being detached and seeded into a 1 L spinner flask containing Cytodex 1 microcarriers. Initial cell attachment was performed using an intermittent agitation cycle consisting of 45 minutes without agitation followed by 5 minutes of agitation, repeated 15 times. This protocol allowed efficient cell adhesion to the microcarriers, after which cells were cultured for at least three days until reaching a density of approximately 15 cells per microcarrier, as determined by microscopic imaging. At this stage, spinner flasks were thoroughly washed with PBS to remove residual serum that could interfere with iron oxide nanoparticle (ION) labelling. Cell preconditioning was then carried out directly in the bioreactor for 30 minutes in serum-free RPMI medium containing 0.5 mM γ -Fe₂O₃ nanoparticles and an equivalent concentration of sodium citrate under gentle agitation, followed by a 10-minute chase period. After preconditioning, cells were washed again with PBS to remove any remaining conditioning medium before initiating turbulent EV production. For control conditions, spinner flasks were similarly washed with PBS to remove serum prior to the addition of production medium. At this point (*t* = 0h), samples were collected to assess cell viability and determine the basal level of particles present in the culture medium using nanoparticle tracking analysis (NTA), which determines particle size and concentration by analysing the Brownian motion of individual particles larger than 50 nm, a size range that include EV.

To evaluate the impact of superparamagnetic iron oxide nanoparticle (ION) preconditioning on EV production, we first assessed its effect on cell viability prior to EV release. As shown in Fig. 1B, cell viability remained above 90% under both conditions, with no significant difference observed between control cells (Ctrl-hASC) and ION-exposed cells (ION-hASC) within the bioreactor. Cell attachment to microcarriers (Fig. 1C) was also not affected by ION, despite the additional PBS washing steps that could potentially interfere with cell adhesion and viability.

2.2. Influence of ION preconditioning on EV production by turbulence in a stirred-tank bioreactor

Following medium replacement and assessment of cell viability, turbulent EV production was initiated for 4 hours at a defined Kolmogorov length scale of 34 μm (Fig. 2A). This parameter corresponds to the size of the smallest turbulent eddies generated within the bioreactor flow. The formation of these vortices induces an energy cascade that generates hydrodynamic shear stresses at the cell surface, thereby promoting the active release of extracellular vesicles

After 4 hours of turbulence, samples were collected to assess cell viability and particle release ($t = 4\text{h}$). Cell viability was found to be higher than 85% systematically following turbulence production (data not shown). The EV-enriched conditioned medium (CM) is then clarified by centrifugation followed by filtration through a 0.45 μm membrane. The clarified medium is subsequently purified and concentrated using tangential flow filtration (TFF), during which the buffer is exchanged from production medium to PBS to enable long-term storage. An additional ultrafiltration (UF) step is performed to further concentrate the EV preparation prior to downstream *in vitro* assays.

As shown in Fig. 2B, particle release at $t = 0\text{h}$ was minimal and comparable under both conditions (below 5 000 particles per cell). At this stage, particle concentrations were below the detection limit of NTA, suggesting that the few detected events are likely attributable to background noise rather than EV. This confirms efficient rinsing and indicates that particle production observed after 4 hours is associated with EV release during the turbulent production phase. After four hours of turbulent stimulation, particle release per cell was similar between the two conditions, reaching 37827 ± 14830 particles per cell for EV-Ctrl (non-preconditioned cells) and 46834 ± 4254 particles per cell for EV-ION (preconditioned cells) (Fig. 2B), indicating that ION preconditioning did not significantly affect particle release. Following TFF, a concentration factor, defined as the ratio of particle concentration after processing to that before processing, of approximately 30 was achieved, and the subsequent UF step further increased particle concentration by approximately 10-fold (Fig. 2C). This downstream processing enabled efficient particle concentration while maintaining similar concentration factors for both EV-Ctrl and EV-ION samples, confirming the reproducibility of the purification workflow across conditions. Moreover, size distribution analysis by NTA before TFF (Pre-TFF) and after TFF and UF (Post-UF) showed no significant difference between the two EV type (Fig. 2D). The median particle diameter was approximately 90 nm for both conditions, while the mean diameter was around 140 nm. These results indicate that ION preconditioning did not alter EV physical properties and that the downstream processing steps did not

affect particle size distribution. Finally, the presence of typical EV structures was confirmed in both conditions by transmission electron microscopy (TEM) (Fig. 2E). The identity of EV was further validated by Western blot analysis, which revealed positive staining for the EV-associated tetraspanins CD63 and CD81 in both EV-Ctrl and EV-ION samples (Fig. 2F). Tetraspanins are transmembrane proteins commonly enriched in the membrane of extracellular vesicles and are widely used as canonical markers to confirm EV identity.

2.3. Analysis of magnetic properties of EV-ION

Having established that ION preconditioning does not impair cell viability or EV release, we next investigated whether ION-preconditioned cells produced EV containing nanoparticles. To address this, EV preparations were first characterized by MRI using a preclinical 4.7T MRI system through the analysis of longitudinal (T_1) and transverse (T_2) relaxation times, followed by quantitative assessment using electron paramagnetic resonance (EPR).

For MRI analysis, two independent batches of EV-ION obtained from identical production processes were analysed. For each batch of EV-ION, EV-Ctrl samples without ION were used as controls and tested at comparable concentration (8.6×10^{10} and 6.7×10^{10} particles for batches 1 and 2, respectively). Samples were homogeneously dispersed in 0.3% agarose gel. As shown in Figs. 3A and 3B, T_1 -weighted MRI revealed subtle differences in signal intensity between EV-Ctrl and EV-ION samples. However, quantitative analysis demonstrated a slight shortening of T_1 relaxation times in both EV-ION batches (2410 ± 66 ms and 2310 ± 56 ms) compared to their respective EV-Ctrl controls (2540 ± 89 ms and 2549 ± 76 ms), with a more pronounced effect observed in batch 2 (Fig. 3E).

Consistently, T_2 -weighted imaging showed a strong signal attenuation in EV-ION samples relative to EV-Ctrl (Figs. 3C and 3D), indicative of enhanced transverse relaxation. This attenuation was more pronounced in batch 2 compared to batch 1. Multi-echo analysis confirmed a significant reduction in T_2 relaxation times in both EV-ION batches (119 ± 2.4 ms and 99 ± 4.6 ms) compared to controls (196 ± 1.1 ms and 194 ± 1.3 ms) (Fig. 3E).

Then, to further confirm the presence of iron oxide nanoparticles within the two EV-ION preparations, electron paramagnetic resonance (EPR) analysis was performed on 2 μ L of undiluted samples, each containing 2×10^8 particles quantified by NTA. As shown in Figs. 3F and 3G, EPR quantification revealed a higher concentration of γ -Fe₂O₃ nanoparticles in the second EV-ION batch (107.2 μ M) compared to the first batch (70.7 μ M). This difference is consistent with the MRI results, which indicated more pronounced reduction of magnetic resonance relaxation times in batch 2 relative to batch 1. No detectable EPR signal was observed in EV-Ctrl samples (data not shown). Based on these measurements, the iron content was estimated to be 3.96×10^{-5} pg Fe per EV particle for batch 1 and 6.0×10^{-5} pg Fe per EV particle for batch 2, corresponding to approximately 14 and 21 iron oxide nanoparticles per EV, respectively. Combining MRI and EPR quantification, the transverse relaxivity (r_2) of

the nanoparticles associated with EV-ION was estimated to be approximately $480 \text{ mM}^{-1} \cdot \text{s}^{-1}$. Finally, cryo-electron microscopy revealed clusters of iron oxide nanoparticles (approximately 10 nm in diameter size) located both within EV structures and in co-isolated protein fractions (Fig. 3H and 3I).

2.4. EV immunomodulatory potency and multivariate analysis of macrophage responses

We next investigated whether ION preconditioning influence the immunomodulatory potency of EV compared with non-primed EV. To address this, both EV were tested at three particle doses, as determined by NTA (10000, 20000, and 80000 particles per cell), using a macrophage inflammation model previously described by our group³². Briefly, THP1-Dual monocytes were first differentiated into resting M0-like macrophages and subsequently polarised into either M1-like (pro-inflammatory) or M2-like (anti-inflammatory) macrophages prior to lipopolysaccharide (LPS) stimulation in the presence or absence of EVs. Co-treatment with dexamethasone and LPS was used as a negative control for inflammation. In addition, exploiting the magnetic properties of EV-ION, an external magnetic field gradient was applied during EV-ION treatment to determine whether their magnetic responsiveness and accumulation can further modulate immunomodulatory activity (Fig. 4A). Macrophage responses were assessed at both signalling and transcriptional levels. Activation of the NF- κ B and IRF pathways, two key regulators of inflammatory responses, was quantified through the secretion of SEAP and Lucia reporter proteins in the culture supernatant, enabled by the dual reporter system of THP1-Dual cells. In parallel, transcriptional responses were evaluated by measuring the expression of six genes involved in macrophage inflammatory regulation, including pro-inflammatory mediators associated with M1 activation (IL-1 β , IL-6, TNF α and iNOS), which promote cytokine production and nitric oxide-mediated inflammatory responses, and regulatory markers associated with alternative macrophage polarisation (CCL22 and IL-10), which contribute to immune regulation and resolution of inflammation. Then, to obtain an integrated view of macrophage responses across the different treatments, these signalling and transcriptional parameters were subsequently combined in a principal component analysis (PCA) (Fig. 4). This multivariate approach reduces complex datasets into a limited number of principal components, thereby facilitating the visualisation of coordinated changes in macrophage responses and the identification of treatment-dependent shifts in inflammatory phenotypes.

We first evaluated the effects of LPS and LPS co-treated with dexamethasone to establish the baseline inflammatory response of macrophages (Fig. 4B-G) and their corresponding displacement in the PCA analysis after 24 h of stimulation (Fig. 4H). LPS treatment induced a strong activation of both the IRF and NF- κ B signalling pathways in M1- and M2-like macrophages. Notably, IRF activation was more pronounced in M2-like macrophages compared with M1-like macrophages, which already displayed a higher basal inflammatory signalling state. Co-treatment with dexamethasone markedly reduced the activation of both pathways, bringing macrophage responses closer to those observed in unstimulated cells. Analysis of gene expression (Figs. 4F and 4G) revealed that LPS stimulation increased the expression of all analysed genes, including both pro-inflammatory and regulatory markers, with the

exception of iNOS, whose induction remained minimal in M2-like macrophages. Among the genes examined, IL-6 showed the strongest induction in both macrophage populations and was particularly elevated in M2-like macrophages. Dexamethasone treatment attenuated most LPS-induced transcriptional responses. However, iNOS expression was unexpectedly increased following LPS and dexamethasone treatment. Importantly, no differences in gene expression were observed between unstimulated macrophages cultured with or without magnetic field exposure, indicating that the magnetic field alone did not influence baseline macrophage activation (Supplementary Figure S1).

To obtain an integrated view of these signalling and transcriptional responses, all measured parameters were subsequently combined in a principal component analysis (PCA) (Fig. 4H). PCA of signalling and gene expression parameters revealed distinct clustering patterns across macrophage polarisation states and treatment conditions. Untreated (blue) M2-like macrophages were located in the neutral upper left region of the PCA scores plot (cluster (a)), while untreated M1-like macrophages formed a distinct cluster (a'), indicating baseline differences between macrophage polarisation states. Upon LPS stimulation (red), M2 macrophages shifted towards the right along the PC1 axis (cluster (b)), whereas M1 macrophages exposed to LPS shifted towards the lower right region of the plot, along both the PC1 and PC2 axes (cluster (b')). Moreover, upon dexamethasone co-treatment (green), the corresponding clusters shifted towards regions associated with reduced inflammatory activation (clusters (c) for M2-like macrophages and (c') for M1-like macrophages). The orientation of these clusters was consistent with the contribution of individual variables identified in the PCA loading plot. Accordingly, treatments that attenuated inflammatory signalling, such as dexamethasone, resulted in a repositioning of macrophage clusters towards regions of the PCA space associated with reduced contributions from the pro-inflammatory variables.

We then evaluated the impact of EV treatment at three different doses (10000, 20000 and 80000 particles per cells) on pro-inflammatory signalling pathways in M1- and M2-polarised macrophages (Fig. 5). Across all three treatments conditions, EV preparations showed a tendency to attenuate LPS-induced IRF pathway activation in a dose-dependent manner in M1-like macrophages (Fig. 5A). A similar trend was observed for NF- κ B activation. However, the application of an external magnetic field further strengthened this effect, with EV-ION reducing LPS-induced NF- κ B activation at all tested concentrations (Fig. 5B). In contrast to M1 macrophages, a significant reduction in IRF pathway activation was observed in M2-like macrophages only when cells were treated with EV-ION under an external magnetic field gradient (Fig. 5C). By comparison, the effects on NF- κ B activation were similar to those observed in M1 macrophages (Fig. 5D).

To further characterize the effects of EV on macrophage responses, we next assessed the expression of key inflammation-related genes 24 h after treatment. In M1-like macrophages, among the six genes analysed, the most pronounced effects were observed for IL-6, CCL22, TNF α and IL-10 (Fig. 5E). In the absence of an external magnet, both EV-Ctrl and EV-ION attenuated LPS-induced expression of IL-6 and CCL22 in macrophages. In contrast, the application of an external magnetic field markedly enhanced the expression of these genes, as well as IL-10. Under magnetic field exposure, IL-6 expression increased by

approximately 200-fold and reached up to 600-fold at the highest EV-ION dose, while CCL22 expression increased by approximately 200-fold compared to unstimulated M0 macrophages. By contrast, EV-Ctrl induced a markedly lower increase in CCL22 expression, approximately tenfold less than that observed with EV-ION treatment under a magnetic field. In parallel, IL-10 expression was enhanced following EV-ION treatment under a magnetic field gradient, with an increase of at least five-fold relative to controls. In parallel, transcriptional analysis in M2-like macrophage revealed differential regulation of inflammatory genes (Fig. 6F). Under magnetic field exposure, EV treatment reduced LPS-induced expression of IL-1 β and IL-6, while EV-ION without magnetic stimulation selectively decreased IL-6 expression. Consistent with observations in M1 macrophages, EV-ION under a magnetic field strongly increased CCL22 expression (up to 200-fold compared to naïve M0 macrophages). In contrast, EV-Ctrl induced a modest increase in CCL22 but primarily enhanced IL-10, IL-1 β , and IL-6 expression. Notably, the induction of IL-1 β and IL-6 was specific to EV-Ctrl and was not observed with EV-ION.

Given the complexity of these transcriptional responses, we next performed a PCA to evaluate the global impact of EV treatments on macrophage inflammatory states (Fig. 6). PCA plots for each EV treatment condition are presented in the supplementary figures S2, S3 and S4. As shown in Fig. 6, EV-Ctrl treatment (purple) did not alter the clustering associated with the inflammatory state of M1-like macrophages (cluster (b')). In contrast, M2-like macrophages (cluster (b)) displayed a dose-dependent displacement towards less inflammatory regions of the PCA space. Similarly, EV-ION treatment (orange) did not affect the clustering of M1-like macrophages. However, M2-like macrophages exhibited a stronger dose-dependent shift towards the cluster associated with reduced inflammatory activation compared with EV-Ctrl, indicating a more pronounced anti-inflammatory effect. Upon exposure to a magnetic field (light blue), EV-ION treatment resulted in a pronounced displacement of both M1-like and M2-like macrophages towards clusters associated with reduced inflammatory activation ((a), (a') and (c)). Importantly, distinct responses were observed between the two macrophages populations. M1-like macrophages shifted towards higher PC2 and negative PC1 values (upper left region of the PCA space), whereas M2-like macrophages primarily shifted along the negative PC1 axis.

Taken together, these findings demonstrate that ION-based preconditioning enables the generation of magnetically responsive EV while preserving scalable production and intrinsic vesicle properties. This approach not only allows efficient nanoparticle loading of EV during biogenesis but also enhances their immunomodulatory activity in a controllable manner, particularly under magnetic field gradient exposure. These results support the concept that combining biological vesicle engineering with physical modulation provides a powerful strategy to regulate EV-mediated cellular responses.

3. Discussion

In the present study, we developed a nanoparticle-based preconditioning strategy to engineer magnetically responsive EV.

We were able to integrate 8 nm citrate coated-maghemite nanoparticle exposure into a well-established three-dimensional EV production platform based on microcarrier culture and controlled turbulent stimulation. This approach allowed us to investigate whether magnetic preconditioning of EV-producing cells could be implemented without compromising cell viability, adhesion to microcarriers, or vesicle production efficiency. Importantly, the short-term exposure of hASC to citrated maghemite nanoparticles synthesized by Massart procedure did not affect cell viability or attachment to microcarriers, as both parameters remained comparable between primed and non-primed cells. These observations are consistent with our previous reports indicating that superparamagnetic iron oxide nanoparticles are generally well tolerated by mammalian cells when appropriately stabilised in serum-free RPMI medium supplemented with sodium citrate and used within controlled concentration ranges^{12–14,33,34}. The compatibility of nanoparticle preconditioning with the microcarrier-based culture system is particularly relevant in the context of scalable EV manufacturing. Microcarrier cultures are widely used in bioprocessing because they enable high cell densities while maintaining favourable mass transfer and mechanical properties. Our results indicate that the additional washing and labelling steps required for nanoparticle preconditioning do not interfere with cell adhesion or expansion within this three-dimensional environment of bioreactor. This suggests that magnetic preconditioning can be incorporated into existing EV production workflows in stirred-tanks bioreactor without compromising culture stability. Importantly, nanoparticle priming did not significantly affect vesicle release under these conditions. Particle production per cell after four hours of turbulent stimulation remained comparable between primed and non-primed cells, indicating that intracellular exposure to maghemite nanoparticles does not impair the ability of hASC to generate extracellular vesicles. This observation suggests that the cellular mechanisms responsible for mechanically induced vesicle release remain intact following nanoparticle internalisation by the parental cells in their endosomes. Downstream processing steps, including tangential flow filtration and ultrafiltration, further confirmed the robustness of the production workflow. Both EV preparations exhibited similar concentration factors during purification, demonstrating that nanoparticle preconditioning did not alter the behaviour of the secretome during filtration-based concentration. Maintaining comparable recovery and concentration efficiencies between conditions is essential for ensuring batch-to-batch reproducibility and for enabling reliable comparison of EV preparations in downstream functional assays. Similarly, nanoparticle priming did not appear to influence the global physical characteristics of the secretome. Size distribution analysis performed after purification revealed comparable particle size profiles for EV-Ctrl and EV-ION preparations, with median diameters around 90 nm and mean diameters around 140 nm. These values fall within the typical size range reported for small extracellular vesicles and suggest that maghemite exposure does not significantly modify vesicle biogenesis or the overall size distribution of released particles. Finally, structural and molecular analyses confirmed the presence of extracellular vesicles in both preparations. Transmission electron microscopy revealed vesicular structures consistent with EV morphology, while Western blot detection of the canonical EV-associated tetraspanins CD63 and CD81 further validated vesicle identity. These two tetraspanins are widely recognised as key markers of extracellular vesicles due to their enrichment in the vesicle membrane and their role in membrane organisation and vesicle formation. Taken together, these findings demonstrate that citrate-coated ION preconditioning can be

successfully integrated into a scalable EV production process without compromising cell viability, vesicle release, or the structural characteristics of the resulting EV-enriched secretome. This compatibility is a critical prerequisite for the development of magnetically responsive EV-based therapeutic strategies.

Although our primary objective was to enhance EV immunomodulatory potency, we also provided clear evidence of nanoparticle incorporation within the purified EV product. MRI analysis revealed a pronounced T_2 signal attenuation in EV-ION preparations compared with control samples. The marked reduction in T_2 relaxation times observed in both batches indicates that ION were effectively incorporated into the EV-enriched secretome and retained their magnetic properties after EV secretion and purification. The stronger T_2 effects observed in the second batch further suggest that variations in nanoparticle loading can directly influence the magnetic contrast generated by EV preparations. Additionally, EPR analysis provided quantitative confirmation of iron oxide nanoparticles incorporation into EV. The detection of $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles signals exclusively in EV-ION samples confirmed that the magnetic signal detected by MRI was indeed associated with the presence of iron oxide nanoparticles. Moreover, the higher iron concentration measured in batch 2 was consistent with the stronger T_2 signal attenuation observed in MRI measurements, indicating good agreement between both analytical techniques. Based on these measurements, the estimated nanoparticle loading corresponded to approximately 14 to 21 iron oxide nanoparticles per EV, which is markedly higher than the levels we previously reported when iron oxide nanoparticles were incorporated into THP1-derived macrophages and EV were produced using conventional secretion methods³⁵. Although this loading remains lower than that achieved with post-isolation EV labelling strategies, such as those described by Wang et al.³¹, the resulting EV preparations still generated a detectable MRI signal. Additionally, the calculated transverse relaxivity ($r_2 \approx 480 \text{ mM}^{-1}\cdot\text{s}^{-1}$) further confirms the strong magnetic efficiency of the nanoparticle-containing EV preparations. Such relaxivity values fall within the range reported for clustered superparamagnetic ION³⁶ and indicate that the magnetic behaviour of the nanoparticles is largely preserved following their association with EV. This suggests that vesicle-associated nanoparticles remain magnetically active and capable of generating detectable MRI contrast. Moreover, cryo-electron microscopy further revealed nanoparticle clusters both within vesicular structures and in co-isolated protein fractions, suggesting that incorporation may occur through endocytic trafficking and vesicle biogenesis pathways rather than solely by post-secretion adsorption. These observations are consistent with our previous reports demonstrating intercellular transfer of iron oxide nanoparticles via extracellular vesicles¹⁷⁻¹⁹, supporting a biogenesis-associated mechanism of nanoparticle incorporation.

We next investigated whether nanoparticle priming translated into enhanced immunomodulatory potency using the THP1-Dual macrophage model previously established in our group³². Notably, the effects of EV treatment were dependent on macrophage polarisation state. While EV-Ctrl exerted modest regulatory effects, particularly in M2-like macrophages, M1-like macrophages remained comparatively less responsive to treatment. In contrast, EV-ION treatment resulted in a more pronounced modulation of inflammatory responses, with M2-like macrophages displaying greater sensitivity to nanoparticle-primed

EV. At the transcriptional level, these effects were reflected by differential regulation of IL-6, TNF- α , CCL22 and IL-10. In the absence of magnetic stimulation, both EV preparations attenuated LPS-induced IL-6 expression in M1-like macrophages, whereas EV-ION exerted a stronger modulatory effect in M2-like macrophages. Magnet exposure markedly reshaped these responses in a polarisation-dependent manner. In M2-like macrophages, magnetic stimulation promoted a strong induction of CCL22 alongside a decrease in IL-6 expression, consistent with reinforcement of a regulatory phenotype. In M1-like macrophages, although baseline responsiveness to EV treatment was more limited, magnetic stimulation induced a broader transcriptional reorganisation, characterised by concomitant modulation of TNF- α , IL-6, CCL22 and IL-10 expression. Rather than reflecting a simple increase in pro-inflammatory markers, this coordinated pattern suggests a complex rebalancing of inflammatory and regulatory pathways. As confirmed by PCA, these changes resulted in a clear phenotypic displacement of M1-like macrophages towards a less inflammatory state, indicating that magnetically responsive EV can partially reprogram even strongly pro-inflammatory macrophages.

These transcriptional changes must be interpreted within the complex regulatory network governing macrophage polarisation. Although IL-6 and TNF- α are classically associated with pro-inflammatory activation, IL-6 also exerts pleiotropic effects, including promotion of alternatively activated macrophage phenotypes and induction of IL-10 expression³⁷, while TNF- α can synergise with IL-6 to remodel chemokine production and influence macrophage recruitment dynamics³⁸. In parallel, CCL22 and IL-10 represent central mediators of regulatory macrophage function. The coordinated modulation of these cytokines therefore does not reflect simple suppression of inflammation but rather a reorganisation of the inflammatory-regulatory balance. The amplification observed under magnetic field gradient exposure may arise from enhanced local concentration of magnetically loaded EV at the cell surface, increased cellular uptake, or multivalent clustering effects that potentiate receptor engagement and downstream signalling pathways. Such mechanisms could explain the concerted reshaping of IL-6 and TNF- α response alongside the marked induction of CCL22 and IL-10. Importantly, the differential sensitivity observed between M1- and M2-like macrophages may reflect intrinsic differences in receptor expression, endocytic capacity, or baseline signalling thresholds, rendering M2-like macrophages more responsive to EV-mediated modulation in the absence of magnetic stimulation. Crucially, principal component analysis provided an integrative view of these effects. Rather than reflecting changes in individual genes, magnetic stimulation of EV-ION induced a global shift in macrophage inflammatory phenotype, as revealed by PCA.

Beyond the mechanistic insights provided here, our findings have important translational implications. The ability to magnetically modulate EV-mediated immunoregulation introduces a controllable physical parameter into cell-free therapeutic strategies. Unlike conventional EV engineering approaches based on post-isolation functionalisation, nanoparticle priming at the producer cell level enables the generation of intrinsically magnetically responsive vesicles while maintaining compatibility with scalable production workflows. Importantly, the demonstration of measurable magnetic properties suggests that such vesicles may not only be functionally amplified under external magnetic stimulation but could also, in

principle, be magnetically guided *in vivo*. The use of iron oxide nanoparticles further offers the advantage of MRI-based tracking, a clinically established and non-invasive imaging modality³⁹, in contrast to fluorescence-based approaches limited by tissue penetration and translational applicability. This dual functionality, combining magnetic responsiveness with imaging capability, raises the prospect of spatially controlled and image-guided EV therapies. The capacity to externally enhance or direct EV activity through magnetic stimulation may therefore open new opportunities for targeted immunomodulation, particularly in pathological contexts characterised by dysregulated macrophage polarisation, such as chronic inflammatory diseases, tissue injury, or tumour-associated macrophage reprogramming.

Building on these findings, further investigation of the intracellular trafficking pathways underlying nanoparticle incorporation during EV biogenesis would provide deeper mechanistic insight and may enable optimisation of magnetic loading efficiency. Such advances could facilitate the rational development of magnetically guided and image-monitored EV-based immunotherapies.

4. Conclusions

This study demonstrates that nanoparticle-based preconditioning of EV-producing cells enabled the generation of magnetically responsive extracellular vesicles without compromising production efficiency or cell viability. Magnetic iron oxide incorporation occurs during vesicle biogenesis and confers measurable magnetic properties, while preserving compatibility with scalable manufacturing workflows. Functionally, nanoparticle priming enhanced the immunomodulatory activity of EV in a macrophage polarisation-dependent manner. Magnetic field exposure further amplifies these effects, promoting coordinated transcriptional reprogramming and repositioning of macrophage phenotypes within the inflammatory landscape. Rather than inducing simple suppression of inflammatory signalling, magnetically responsive EV drive a reorganisation of the inflammatory–regulatory balance. Collectively, these findings introduce a strategy that integrates biological engineering with physical modulation, enabling externally controllable and potentially image-guided EV-based immunotherapies. Nanoparticle priming therefore represents a promising platform for the development of magnetically steerable and functionally tuneable extracellular vesicle therapeutics.

5. Materials and Methods

5.1. Cell Culture

Primary human adipose-derived stem cells (hASC; Cell-Easy, Toulouse, France) were cultured in complete α -MEM medium supplemented (Sigma-Aldrich) with sodium bicarbonate (Sigma-Aldrich) and 10% foetal bovine serum (FBS; Fisher Scientific). Upon reaching 70% confluence, cells were detached with 0.05% trypsin-EDTA (Fisher Scientific) and transferred in a 1L spinner flask containing Cytodex 1 (Sigma-Aldrich) for 3D production. For 3D culture, cell seeding was done at a ratio of five cells for one microcarrier in complete α -MEM medium. Following a standardised protocol²⁵, cells were submitted to 3

min of mixing at 32 rpm followed by 45 min of rest to obtain homogenous cell adhesion. Intermittent cycle was performed for at least 18h and followed by continuous gentle mixing at 34 rpm for 3D expansion.

5.2. Cell Viability for 3D cell culture

Total cell quantification was calculated using a NucleoCounter® NC-200™ (Chemometec A/S) according to the manufacturer's instructions. Briefly, 200 µL of homogenised cell suspension recovered from the culture medium was treated with lytic buffer (Reagent A, Chemometec) to detach cells from microcarriers, followed after 2 min by the addition of stabilising buffer (Reagent B, Chemometec). A calibrated volume of the resulting suspension was loaded into the counting cassette containing acridine orange (AO) and DAPI for discrimination of total and dead cells, respectively. Dead cell concentration was determined from the non-lysed cell suspension, and cell viability was automatically calculated by the instrument. Cell density was subsequently estimated by dividing the total number of viable cells in the spinner flask by the total number of microcarriers, yielding the average number of cells per bead.

Live/Dead staining was performed to assess cell attachment on microcarriers. 200 µl of homogenised cell suspension is incubated directly on microcarriers using the ReadyProbes™ Viability Kit (ThermoFisher). One drop of NucBlue® Live and NucGreen® Dead are added to the cell suspension. Cells are incubated for 20min under agitation at 37°C. Then, 100µl of cell suspension is deposited on a glass slide and a cover slip is added on top of it. Image acquisition is performed using EVOS M5000 Imaging System (Fisher Scientific), equipped with epi-fluorescent channels for DAPI and GFP signal detection. Image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

5.3. Cell preconditioning and turbulence production

Superparamagnetic iron oxide nanoparticles were provided by Dr Florent Carn from "Matière et Systèmes Complexes" laboratory (UMR 7057, Paris, France). Briefly, nanoparticles were prepared according to the Massart procedure⁴⁰, which consists of the alkaline co-precipitation of iron chloride salts in aqueous solution to form magnetite nanoparticles, followed by controlled oxidation to obtain maghemite ($\gamma\text{-Fe}_2\text{O}_3$). Then, the nanoparticles are chelated with citrate, which confers negative surface charges (COO^-) at pH 7 and stability in biological media. The core diameter was determined to be approximately 8 nm, and iron concentration was quantified by inductively coupled plasma mass spectrometry (ICP-MS).

Maghemite cell preconditioning was performed when a density of 15 cells per microcarrier was reached. Cells were rinsed five times with PBS (Fisher Scientific) before adding the conditioning medium consisting of 0.5 mM superparamagnetic iron oxide nanoparticles (ION) suspended in RPMI medium (Fisher Scientific) supplemented with an equivalent concentration of sodium citrate (Sigma-Aldrich) to ensure electrostatic stability. Cells were incubated at 37°C for 30 min under gentle agitation, followed by a 10 min chase period. After conditioning, cells were washed five times with PBS to remove residual nanoparticles before the addition of the EV production medium.

Turbulence EV production was performed when a density of 15 cells per microcarrier was reached. Cells were rinsed five times with PBS, and the complete culture medium was replaced with serum-free, phenol red-free α -MEM. Cells were then exposed to a turbulent flow characterized by a Kolmogorov length of 34 μm for 4 h to stimulate EV production²⁴. Following EV production, EV-rich secretome was clarified by centrifugation (10 min, 2,000 x g). The supernatant was subsequently passed through a 0.45 μm membrane filtration unit (Fisher Scientific) and kept at 4°C until purification.

5.4. EV purification and concentration

To purify and concentrate EV from both secretome, the latter was processed by tangential flow filtration (TFF) using a cellulose T-Series cassette (Cytiva). Feed and retentate pressures were continuously monitored using pressure sensors connected to a PressureMAT unit (PendoTECH), and fluid circulation was driven by a MasterFlex™ pump system (Fisher Scientific). Prior to filtration, the TFF membrane was rinsed with 0.1 M NaOH (Sigma-Aldrich) for 30 min, followed by extensive washing with autoclaved distilled water until neutral pH was reached. The membrane was subsequently equilibrated with serum-free, phenol red-free α -MEM before loading the EV-rich CM. Filtration was initiated by opening the permeate outlet, allowing continuous removal of the permeate. The retentate was then diafiltered against PBS and recovered in a collection tube. To maximize EV recovery, the system was flushed with PBS to collect residual material retained within the circuit. The concentrate and flush fractions were pooled and passed through a 0.22 μm filtration unit.

To further concentrate the EV preparation, an additional ultrafiltration step was performed using UV-sterile Amicon Ultra-15 centrifugal filters (100kDa, Sigma-Aldrich). Samples were centrifuged at 3000xg for 45 min, after which the concentrates were recovered, aliquoted into low protein-binding microtubes (Sarstedt), and stored at – 80°C for long-term preservation.

5.5. EV characterisation

Particle concentration and size distribution were determined by nanoparticle tracking analysis (NTA) using a ZetaView QUATT instrument (Particle Metrix). The ZetaView system was calibrated using 96 nm polystyrene beads (Particle Metrix), with laser sensitivity and shutter values set to 60 and 200, respectively. Samples were diluted in PBS to reach an optimal concentration range of 1×10^7 to 5×10^7 particles/mL. Measurements were performed at 25°C with laser sensitivity and shutter values set to 82 and 100, respectively. Mean particle concentration was calculated from the average of measurements acquired at eleven distinct positions within the measurement chamber.

Western Blot analysis of EV samples was performed using 0.3 $\mu\text{g}/\mu\text{L}$ of protein quantified by Pierce™ BCA protein assay kit (ThermoScientific) and diluted in 1X Laemmli sample buffer (Bio-Rad). Proteins were separated on 4–15% Mini-PROTEAN TGX precast gels (Bio-Rad) and transferred onto 0.2 μm PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 15 min at room temperature in EveryBlot Blocking Buffer (Bio-Rad) and subsequently incubated overnight at 4°C with primary antibodies against CD63 and CD81 (Invitrogen), diluted 1:1000 in Tris-buffered saline

(Biorad) containing 0.1% Tween-20 (TBS-T, Sigma-Aldrich) and 5% bovine serum albumin (BSA, Sigma-Aldrich). After washing with TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated anti-mouse IgG secondary antibodies diluted 1:2000 in blocking buffer. Protein bands were revealed using SuperSignal™ West Pico Plus Chemiluminescent Substrate (ThermoFisher) and visualised with a Syngene PXi imaging system. Image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Transmission electron microscopy (TEM) images of EV samples and AF4 fraction were acquired using a Hitachi HT 7700 microscope operated at 120 kV (ISC MIMA2, INRAE, Jouy-en-Josas). Samples were deposited onto plasma-treated copper grids (Sigma-Aldrich) and negatively stained with 2% uranyl acetate for 1 min at room temperature. Image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Cryo-electron microscopy (Cryo-EM) images of EV samples was performed by depositing a 5 µL drop of the initial suspension onto Quantifoil® carbon-coated grids (Quantifoil Micro Tools GmbH, Germany). Excess liquid was blotted using filter paper, and grids were rapidly plunge-frozen in liquid ethane using a homemade mechanical cryo-plunger to form a thin vitreous ice layer. Grids were then transferred to a Gatan 626 cryo-holder cooled with liquid nitrogen and inserted into the microscope for observation at –180°C. Cryo-TEM images were acquired using a JEOL JEM-2100 microscope (JEOL, Japan) operated at 200 kV and equipped with a LaB₆ filament and a low-dose system (Minimum Dose System, JEOL) to minimize electron beam damage. Images were recorded with an Ultrascan 1000 CCD camera (2k × 2k pixels; Gatan, USA). Cryo-TEM was performed at “Plateforme Microscopie électronique en transmission” (IMPMC, Sorbonne Université). Image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Magnetic Resonance Imaging (MRI) acquisitions were performed at the imaging facility of the Université Paris Cité “Plateforme Imageries du Vivant” (PARCC-HEGP, Inserm UMR 970, Paris, France) with a preclinical 4.7 T MRI system (Bruker BioSpec 47/40 USR, Ettlingen, Germany) combined with a 35 mm inner diameter resonator and the ParaVision 360 software. Five 0.2 mL tubes containing 150 µL of samples diluted in 0.3% low-melting-point agarose (ref) were grouped on the sample cradle and imaged simultaneously within a single axial slice of 1 mm thickness. The T1 parameters of the samples were calculated using a spin echo sequence with eight different TR values: 200, 400, 800, 1500, 3000, 5000, 8000, 15000 ms at TE 8 ms (FOV 20 x 20 mm, matrix 256 x 192, planar resolution 78 x 104 µm, taq: 11 min). The T2 parameters were calculated using a multi echo sequence with fifteen different TE values ranging from 12 to 180 ms at TR 2500 ms (echo spacing 12 ms, FOV 2 x 2 cm, matrix 192 x 192, planar resolution 104 x 104 µm, taq 8 min). T1 and T2 weighted images were performed with two spin echo TurboRARE sequences with respectively TR/TE = 6000/10 ms and TR/TE = 15000/121 ms (FOV 15 x 15 mm, matrix 150 x 150, planar resolution 100 x 100 µm, taq 10 min each).

Electron paramagnetic resonance (EPR) measurements were performed using an Elexsys E500 spectrometer (Bruker, Wissembourg, France) operating at X-band (9.8 GHz) and equipped with an SHQ

high-sensitivity cavity. A 2 μL aliquot of each sample was loaded into glass capillary tubes (1–5 μL , Drummond). Capillary tubes were sealed using Critoseal clay (Leica) and placed inside an EPR tube. EV samples were analysed undiluted and compared to $\gamma\text{-Fe}_2\text{O}_3$ nanoparticle standards prepared at various concentrations, with a stock concentration of 1.24 M diluted in 0.5 mM citrate solution. Typical acquisition parameters were as follows: microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; receiver gain, 45 dB; time constant, 40.96 ms; conversion time, 40.96 ms; 2048 data points; sweep width, 700 mT; and sweep time, 83.89 s. Data acquisition and processing were carried out using Xepr software (Bruker).

5.6. Immunomodulatory Assay

Macrophage polarisation was performed by treating THP1-Dual cells (Invivogen) with 5ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 24 hours at 37°C for M0 macrophage polarisation. Then, cells were washed twice with PBS and polarised for 24 hours in M1 macrophages with 100 ng/ml LPS (Invitrogen) and 20 ng/ml IFN- γ (Peprotech) or in M2 macrophages using 25 ng/ml IL-4 and 25 ng/ml IL-13 (Peprotech). Following incubation, cells were treated either with PBS (untreated cells), or 100 ng/ml LPS (positive control for inflammation) or LPS with 20 $\mu\text{g}/\text{ml}$ dexamethasone (negative control for inflammation). Additionally, macrophages were treated with LPS with EV at different concentrations determined by NTA (10 000, 20 000 or 80 000 particles per cells). After 24 hours of treatment, cell supernatant was collected and stored at -20°C and RNA was extracted for subsequent gene analysis.

Magnetic immunomodulatory assay was performed on macrophages following the same polarization protocol, using glass-bottom six-well plates. For cell treatment, culture plates were placed on a custom-built magnetic device consisting of six neodyme magnetic discs (35 mm in diameter, Supermagnete) mounted on parallelepiped neodyme magnets (40 $\times$ 10 $\times$ 5 mm, Supermagnete). The assembly was positioned beneath the culture plate to apply a static magnetic field during cell exposure (calculated to be in the range of 150–220 mT at the cell plane; Fig. 7). Macrophages were treated as described previously, except that only EV–ION were used for cell stimulation.

Quanti-Blu™ and Quanti-Luc assay™ were performed to monitor the activation of NF- κB and IRF pathway respectively. NF- κB activation was assessed by measuring SEAP activity in cell supernatants using Quanti-Blu™ reagent (InvivoGen), prepared according to the manufacturer's instructions. Supernatants were incubated with Quanti-Blu™ solution (1:10, final volume 100 μL) in 96-well plates, and absorbance was measured at 620 nm. IRF pathway activation was evaluated by quantifying Lucia luciferase activity in supernatants using Quanti-Luc™ reagent (InvivoGen) prepared for flash detection. Luminescence was recorded immediately after mixing supernatants with Quanti-Luc™ solution (1:6, final volume 60 μL).

RT-qPCR analysis of macrophage gene expression was performed following total RNA extraction with the NucleoSpin™ RNA Plus kit (Macherey-Nagel). Reverse transcription was carried out using 500 ng of total RNA with SuperScript™ II Reverse Transcriptase (Invitrogen) and RNase inhibitor (Promega). Quantitative PCR was performed using PowerUp™ SYBR Green Master Mix (Applied Biosystems) and gene-specific primers (Eurogentec; Table 1). Thermal cycling conditions consisted of an initial

denaturation step of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. RPLP0 was used as the reference gene for normalization, and relative mRNA expression levels were calculated using the threshold cycle method.

Table 1
Primers used for RT-qPCR

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
RPLP0	TGCATCAGTACCCCATTCTATCAT	AAGGTGTAATCCGTCTCCACAGA
IL-1β	TCAGCCAATCTTCATTGCTCAA	TGGCGAGCTCAGGTACTTCTG
IL-6	CGGGAACGAAAGAGAAGCTCTA	GGCGCT TGTGGA GAAGGA
TNF-α	TCAATCGGCCCGACTATCTC	CAGGGCAATGATCCCAAAGT
iNOS	CCCCTTCAATGGCTGGTACA	GCGCTGGACGTCACAGAA
CCL22	CGGCGCCAACATGGAA	CAGACGGTAACGGACGTAATCA
IL-10	TGAGAACAGCTGCACCCACTT	TCGGAGATCTCGAAGCATGTTA

5.7. Statistical Analysis

Data are presented as mean ± standard deviation, except for RT-qPCR results, which are expressed as mean ± standard error of the mean (SEM). Differences among multiple groups were assessed using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. Statistical significance was defined as follows: *p < 0.03; **p < 0.002; ***p < 0.0002; ****p < 0.0001. Multivariate statistical analysis was performed using principal component analysis (PCA). All statistical analyses were conducted using GraphPad Prism software (version 10.4.1; San Diego, CA, USA).

Declarations

Conflicts of interest

Florence Gazeau is co-founder and shareholder of the spin-off EverZom and Exocyris. Florence Gazeau is also co-founder of Evora Biosciences.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

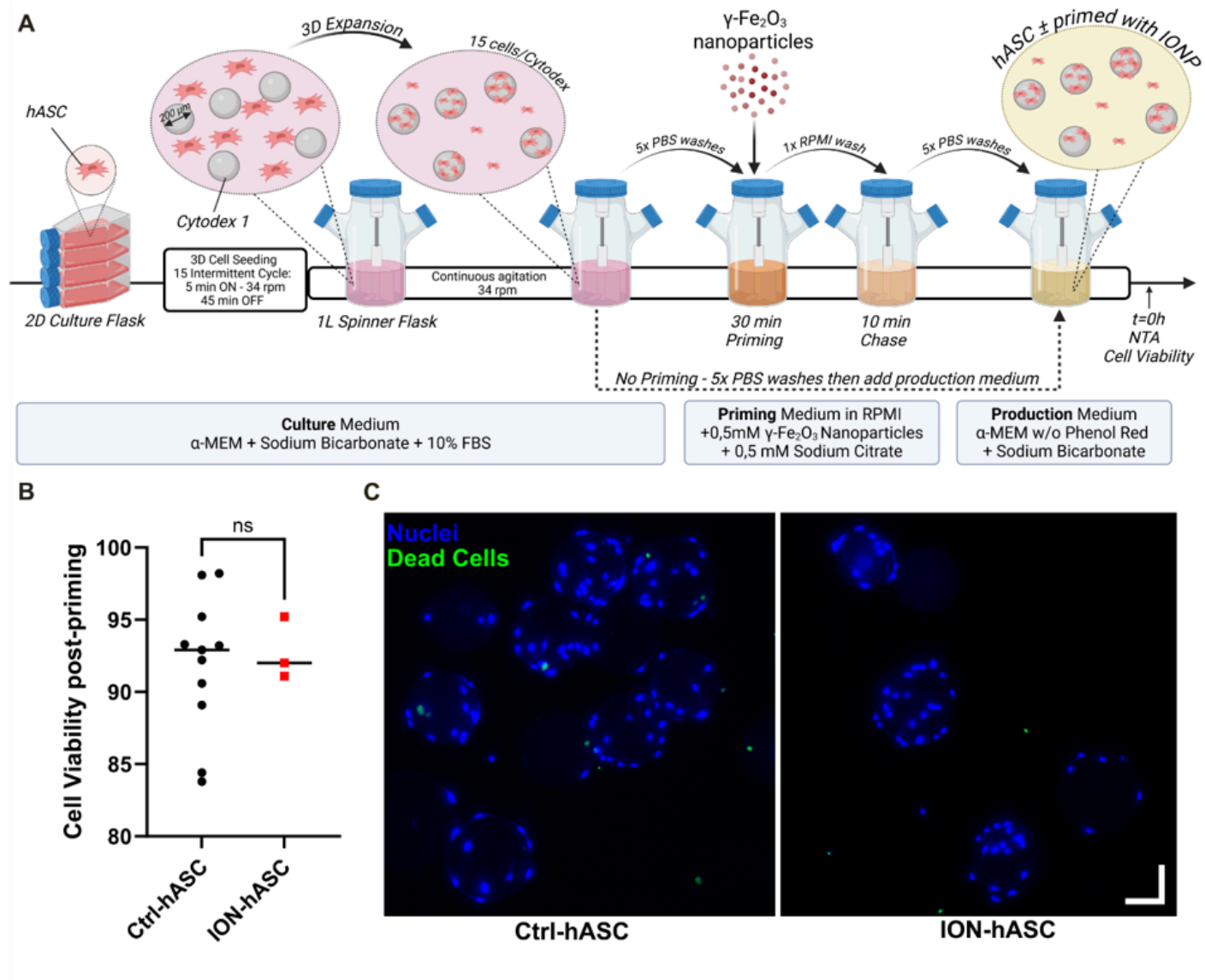


Figure 1

Cell labelling strategy for maghemite preconditioning in a 3D culture system (A) Schematic representation of the ION preconditioning protocol. (B) Cell viability assessed by NucleoCounter measurement following ION priming and prior to EV production. Viability is expressed as the percentage of live cells relative to the total number of counted cells. (C) Representative microscopic images of producer cells on microcarriers following Live/Dead staining prior to the induction of turbulent flow for EV production. Blue: nuclei of total cells; Green: nuclei of dead cells. Magnification = 4X. Scale bar = 100 μm (A) Created on Biorender.com

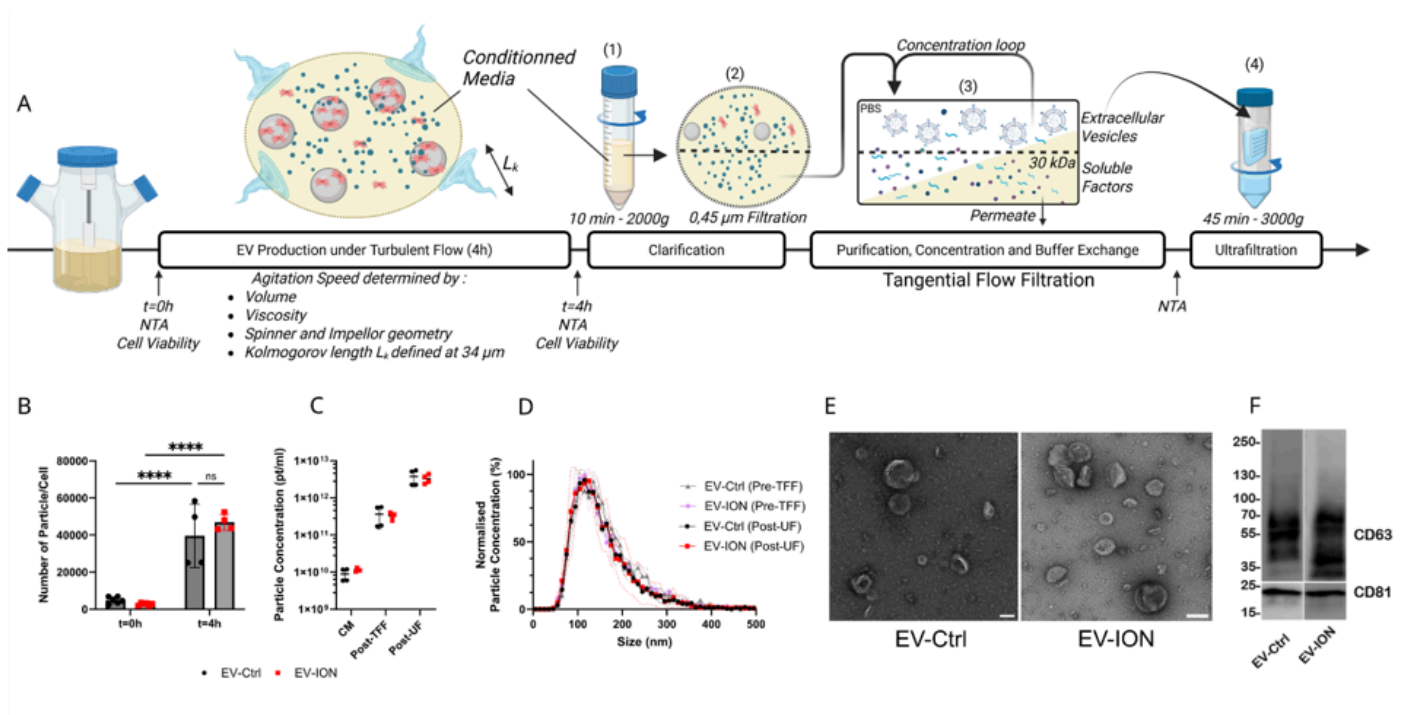


Figure 2

Production and downstream workflow for EV purification and concentration (A) Number of particles produced per cell before ($t=0h$) and after four hours of production ($t=4h$). (C) Particle concentration after tangential flow filtration (Post-TFF) and ultrafiltration (Post-UF). (B-C) Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis was performed using one-way ANOVA (**** $p < 0.0001$). (D) Size distribution analysis of EV-ION compared to EV-Ctrl measured by NTA prior TFF (Pre-TFF) and after TFF and UF (Post-UF). (E) Negative staining of EV observed by transmission electron microscopy. Scale bar = 100 nm (F) Western blot analysis of the EV-associated tetraspanins CD63 and CD81 in EV-Turb and EV-ION samples, confirming the presence of EVs in the cell secretome. (A) Created on Biorender.com

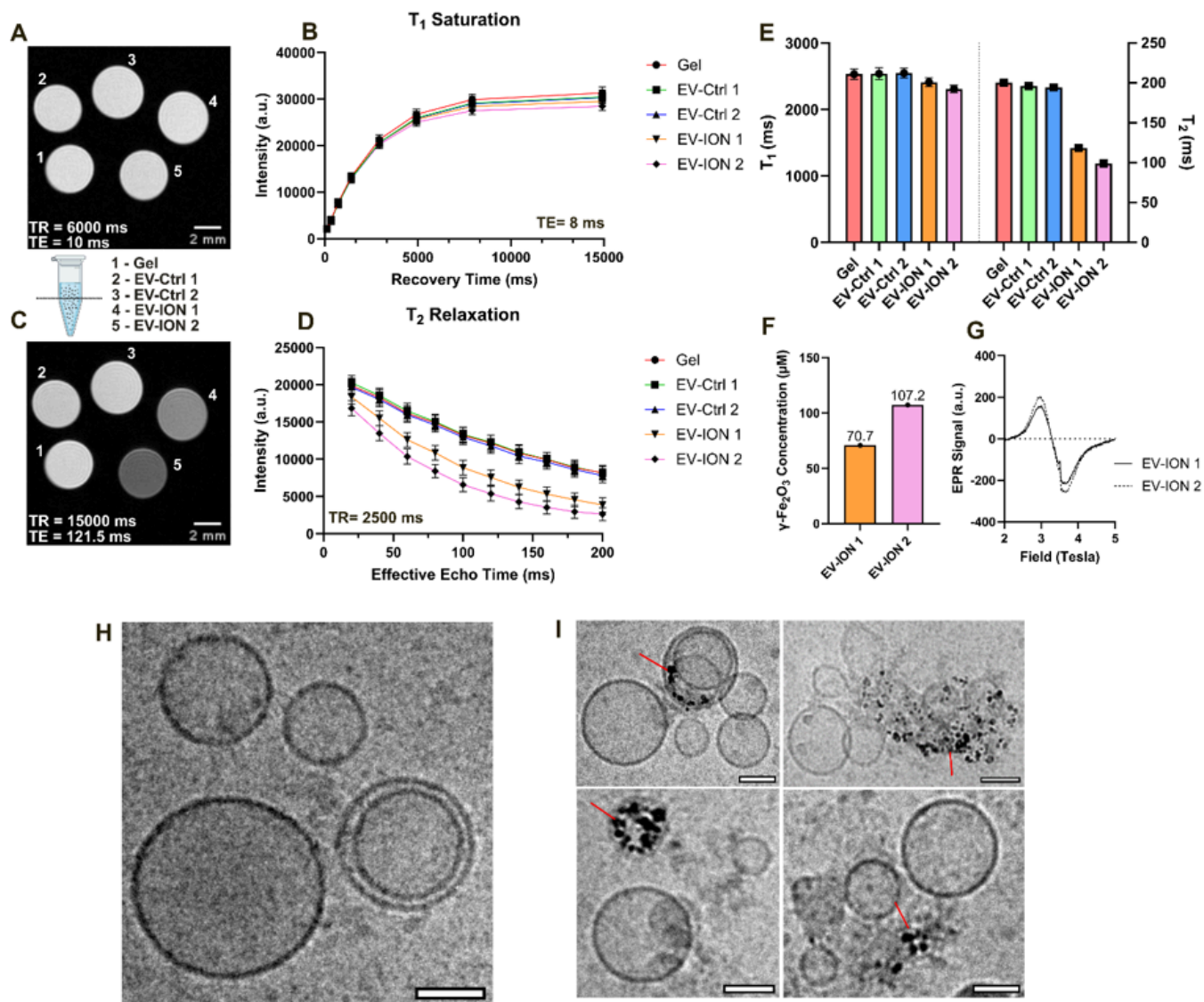


Figure 3

Magnetic characterisation of EV following ION preconditioning. Two independent batches of EV-ION were analysed and embedded in 0.3% low-melting point agarose (total volume = 150 μ l). (A) Transverse T1-weighted MR images of the different experimental conditions at TR=6000 ms and TE = 10 ms. (B) T1 saturation recovery curves obtained from MRI analysis at TE= 8 ms. (C) T2-weighted MR images of the tested conditions at TR = 15000 ms and TE = 121.5 ms. (D) T2 relaxation decay curves derived from multi-echo MRI acquisition at TR = 2500 ms. (E) Quantitative analysis of T1 and T2 relaxation times. (F) Quantification of γ -Fe₂O₃ content in EV samples measured by electron paramagnetic resonance (EPR). (G) Representative EPR spectra of EV-ION samples. (H-I) Cryo-electron microscopy of EV-Ctrl (H) and EV-ION (I). Scale bar = 100 nm.

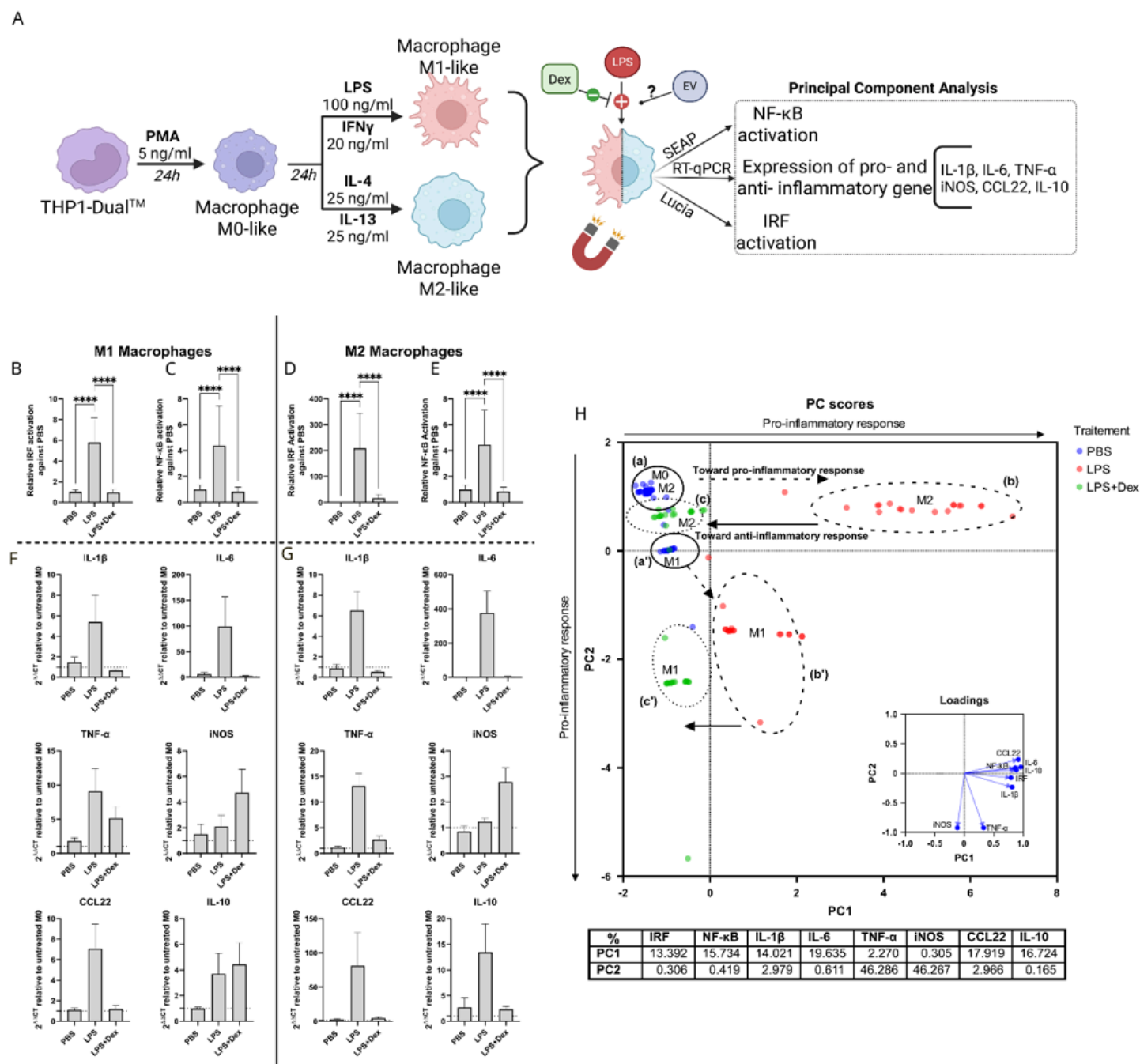


Figure 4

Immunomodulatory effects of control treatments on macrophage inflammatory responses. (A) THP1-Dual are polarised in M0 macrophage then in M1 and M2 macrophages. Macrophages were treated with PBS (unstimulated control), LPS (positive inflammatory control), or LPS combined with dexamethasone (LPS + Dex; anti-inflammatory control) or EV. (B-C) Relative activation of the IRF and NF- κ B pathways in M1-like macrophages compared with unstimulated cells. (D-E) Relative activation of the IRF and NF- κ B pathways in M2-like macrophages compared with unstimulated cells. (F) RT-qPCR analysis of inflammatory gene expression in M1-like macrophages relative to unstimulated M0-like macrophages. (G) RT-qPCR analysis of inflammatory gene expression in M2-like macrophages relative to unstimulated

M0-like macrophages. Data are presented as mean $\pm$ SD for signalling assays and mean $\pm$ SEM for gene expression analysis. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (**** $p < 0.0001$). N=3. (H) PCA scores plot showing the clustering of unstimulated M2-like macrophages (cluster (a)) and M1-like macrophages (cluster (a')). LPS stimulation induced a shift of M2-like macrophages from cluster (a) to cluster (b), and M1-like macrophages from cluster (a') to cluster (b'). (LPS + Dex) resulted in a shift of M2-like macrophages from cluster (b) towards cluster (c), and a shift of M1-like macrophages from cluster (b') towards cluster (c'). PCA loading plot indicating the contribution of individual signalling and gene expression variables to PC1 and PC2. (I) Relative contribution of each gene to the principal components. (A) Created on Biorender.com.

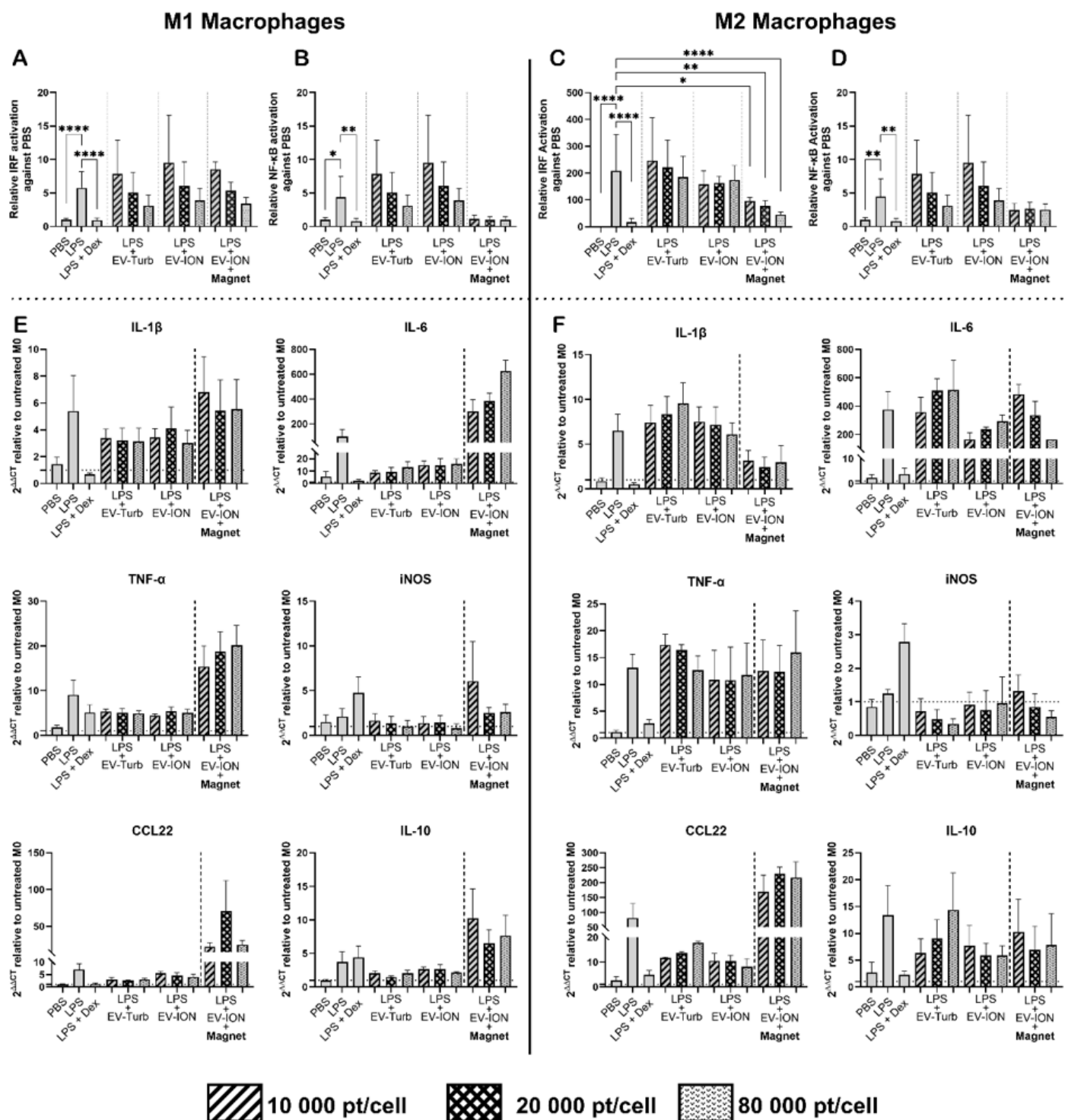


Figure 5

Immunomodulatory effects of EV treatments on macrophage inflammatory responses. Macrophages were treated with PBS (unstimulated control), LPS (positive inflammatory control; 100 ng/ml), or LPS combined with dexamethasone (LPS + Dex; anti-inflammatory control; 100ng/ml + 20µg/ml). EV were tested in LPS-containing medium at three particle doses (10 000, 20 000 and 80 000 particles per cell). EV-ION samples were additionally evaluated under an external magnetic field. (A–B) Relative activation

of the IRF and NF- κ B pathways in M1-like macrophages compared with unstimulated cells. (C–D) Relative activation of the IRF and NF- κ B pathways in M2-like macrophages compared with unstimulated cells. (E) RT-qPCR analysis of inflammatory gene expression in M1-like macrophages relative to unstimulated M0-like macrophages. (F) RT-qPCR analysis of inflammatory gene expression in M2-like macrophages relative to unstimulated M0-like macrophages. Data are presented as mean $\pm$ SD for signalling assays and mean $\pm$ SEM for gene expression analysis. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s multiple comparison test (* $p < 0.03$, ** $p < 0.002$, *** $p < 0.0002$, **** $p < 0.0001$). N=3.

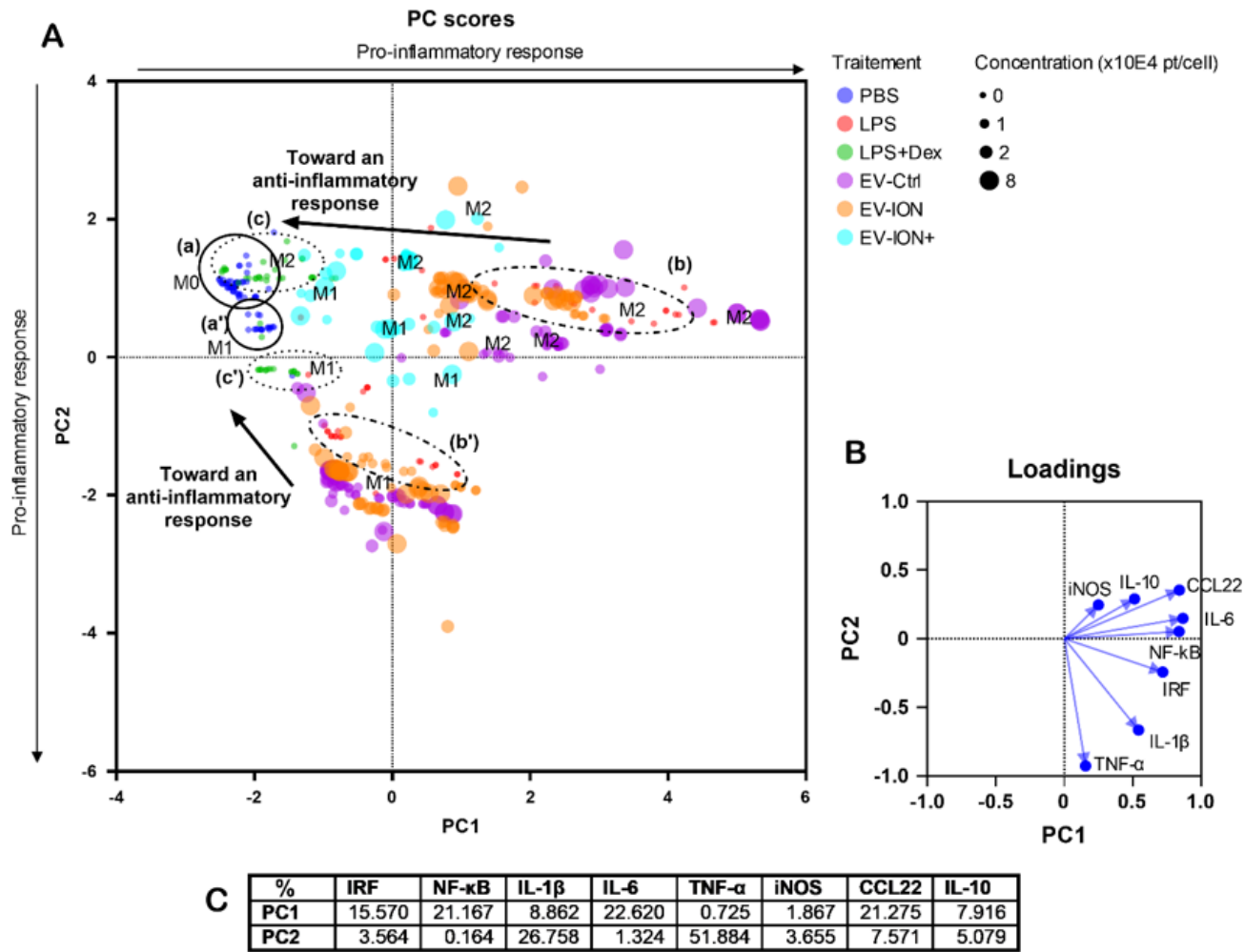


Figure 6

Principal component analysis (PCA) of macrophage inflammatory responses under different treatment conditions. PCA was performed on combined signalling (NF- κ B and IRF activation) and gene expression parameters. (A)PCA scores plot showing the clustering of unstimulated macrophages (clusters (a) and (a')), LPS-stimulated macrophages (clusters (b) and (b')), and dexamethasone-treated LPS-stimulated macrophages (clusters (c) and (c')). EV treatment conditions at different particle doses (1 x 10⁴, 2 x 10⁴, and 8 x 10⁴ particles per cell) were compared with the corresponding control clusters (a, a', b, b', c, c').

EV-ION+ denotes EV-ION treatment performed under an external magnetic field. (B) PCA loading plot indicating the contribution of individual signalling and gene expression variables to PC1 and PC2. (C) Relative contribution of each gene to the principal components.

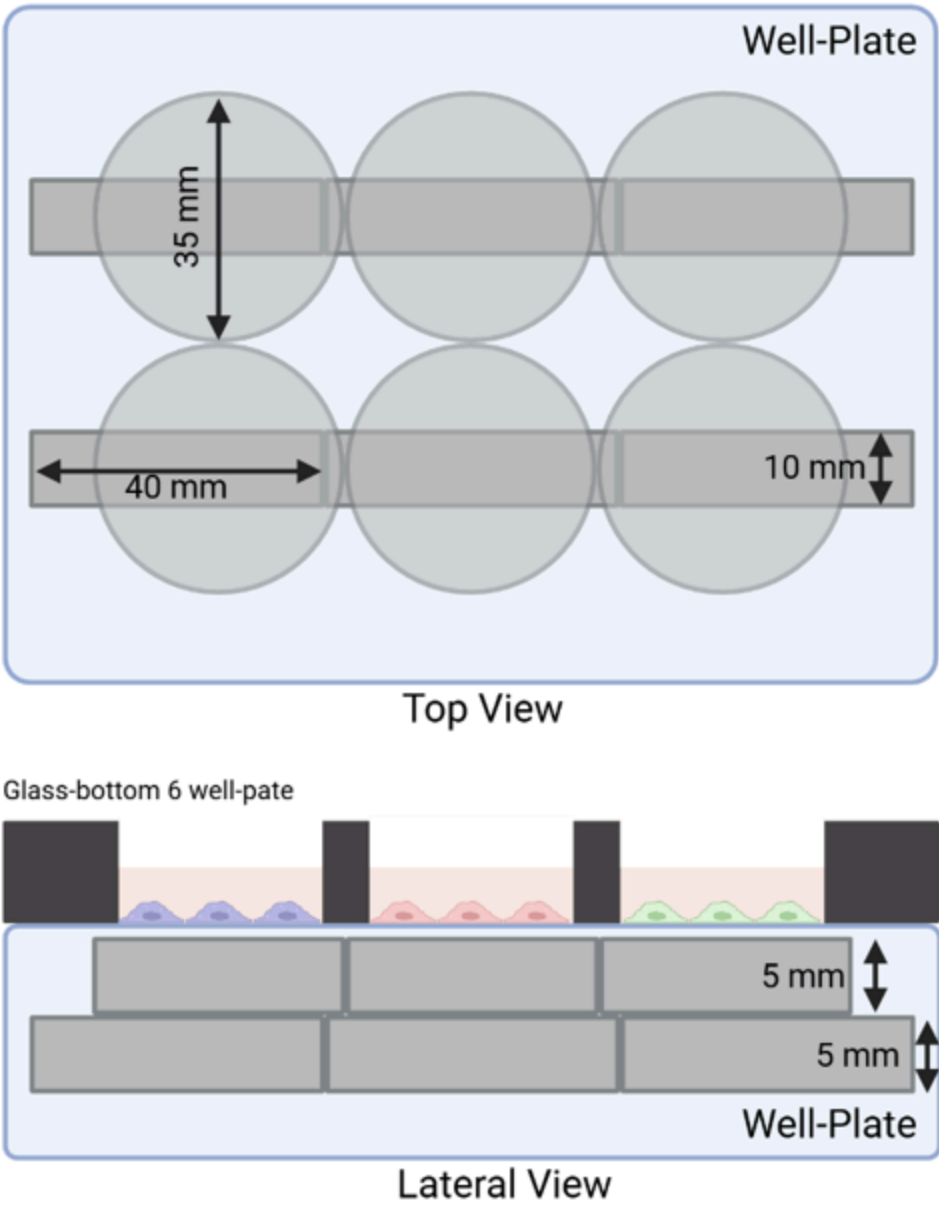


Figure 7

Schematic representation of the magnetic exposure device used for macrophage treatment. Top and lateral views of the custom-built magnetic assembly positioned beneath a glass-bottom 6-well plate. The device consisted of six magnetic discs (35 mm diameter) mounted on parallelepiped magnets (40 x 10 x 5 mm). Created on Biorender.com

Supplementary Files

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