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Immunomodulatory function of licensed human bone marrow mesenchymal stromal cell-derived apoptotic bodies

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ABSTRACT

Background: Mesenchymal stromal cells (MSCs) show great potential for immunomodulatory and anti-inflammatory treatments. Clinical trials have been performed for the treatment of Type 1 diabetes, graft-versus-host disease and organ transplantation, which offer a promise of MSCs as an immunomodulatory therapy. Nevertheless, their unstable efficacy and immunogenicity concerns present challenges to clinical translation. It has emerged that the MSC-derived secretome, which includes secreted proteins, exosomes, apoptotic bodies (ABs) and other macromolecules, may have similar therapeutic effects to parent MSCs. Among all of the components of the MSC-derived secretome, most interest thus far has been garnered by exosomes for their therapeutic potential. However, since MSCs were reported to undergo apoptosis after in vivo transplantation and release ABs, we speculated as to whether ABs have immunomodulatory effects. In this study, cytokine licensing was used to enhance the immunomodulatory potency of MSCs and ABs derived from licensed MSCs in vitro were isolated to explore their immunomodulatory effects as an effective non-viable cell therapy.

Results: IFN-γ and IFN-γ/TGF-β1 licensing enhanced the immunomodulatory effect of MSCs on T cell proliferation. Further, TGF-β1 and IFN-γ licensing strengthened the immunomodulatory effect of MSC on reducing the TNF-α and IL-1β expression by M1 macrophage-like THP-1 cells. Additionally, we discovered the immunomodulatory effect mediated by MSC-derived apoptotic bodies. Licensing impacted the uptake of ABs by recipient immune cells and importantly altered their phenotypes.

Conclusion: ABs derived from IFN-γ/TGF-β1-licensed apoptotic MSCs significantly inhibited T cell proliferation, induced more regulatory T cells, and maintained immunomodulatory T cells but reduced pro-inflammatory T cells.

1. Introduction

Mesenchymal stromal cells (MSCs) have been reported as very promising immunomodulatory [1,2] and anti-inflammatory [3–5] therapies, in conditions such as rheumatoid arthritis, type 1 diabetes and uveitis. Amongst other immunoregulatory activities, MSCs can inhibit T cell proliferation [6] and also promote regulatory T cells (Tregs) [7]. However, the limited efficacy [8] and immunogenicity problems [9–12] following MSC administration are still critical hurdles that currently impede the wider therapeutic application of MSCs.

The secretome of MSCs consists of soluble factors including growth factors, chemokines, and cytokines as well as extracellular vesicles (EVs) such as exosomes and apoptotic bodies (ABs) [13]. It has been established that the MSC-secretome is immunomodulatory [14–17]. Conditioned medium derived from human amniotic membrane MSCs was reported to suppress allogeneic T cell proliferation, induce T

Abbreviations: AB, apoptotic body; ANOVA, one-way analysis of variance; BCA, bicinchoninic acid; EV, extracellular vesicle; FACS, Flow Cytometry Staining Buffer; FBS, fetal bovine serum; IDO1, indoleamine 2,3-dioxygenase 1; LPS, lipopolysaccharide; M1φ, M1 macrophage-like; MSC, mesenchymal stromal cell; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD-L1, Programmed death-Ligand 1; PFA, paraformaldehyde; Treg, regulatory T cell.

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lymphocytes with a regulatory phenotype, and reduce pro-inflammatory cytokine secretion [15]. The MSC secretome was also shown to inhibit B cell proliferation and block B cell differentiation, with an increase in the proportion of mature B cells, and a reduction of antibody-secreting cell formation [16]. Furthermore, through intraperitoneal injection, conditioned medium derived from mice adipose MSCs led to increased induction of Tregs in vivo, together with higher and lower levels of circulating IL-10 and IL-17 levels respectively, and ultimately ameliorating the colitis in these treated mice [17].

EVs demonstrate a similar therapeutic effect to their parental cells [18–20]. EVs are small lipid-bilayer delimited vesicles released by almost all cells and have gained interest in the past years for their cell-to-cell communication properties. Based on their biogenesis, EVs can be categorized into exosomes, ectosomes/ microvesicles or apoptotic bodies (ABs). Exosomes are released from live cells when multi-vesicular bodies fuse with the membrane [21–23]. In contrast, ABs or apoptosomes are produced by apoptotic cells which includes condensation of the nuclear chromatin, membrane blebbing, and disintegration of the cellular content into distinct membrane-enclosed vesicles. ABs were verified to have the same therapeutic effects as their parental non-apoptotic cells [24–27] on the basis of similar proteomic [27] and transcriptional [26] signatures. More importantly, it has been reported that MSCs undergo apoptosis in vivo after transplantation [28] which was required for their therapeutic function [29]. Therefore, it is interesting to speculate whether AB generated in vivo can replace live MSC delivery. AB therapy (cell-free therapy) is of low immunogenicity [30–32] in theory and is more convenient for storage and transportation compared with live cells. Thus far, MSC-derived ABs have been verified to be effective in models of wound healing [33] and myocardial infarction [34]. The current study represents the first to investigate the immunomodulatory efficacy of (licensed) MSC-derived ABs.

Pre-activation or licensing of MSCs, a convenient and effective way to strengthen their immunomodulatory potential, has been described [35,36]. First, it was reported that IFN-γ, a pro-inflammatory cytokine, can induce MSCs to be more immunomodulatory [35] through upregulation of Programmed death-Ligand 1 (PD-L1, CD274) [37,38] and indoleamine 2,3-dioxygenase 1 (IDO1) [39,40]. Meanwhile, other pro-inflammatory cytokines such as IL-1β [41,42] and TNF-α [43,44] were also studied to enhance the potency of MSCs. Interestingly, our previous study demonstrated that licensing with TGF-β1, an anti-inflammatory cytokine, also can induce a stronger immunomodulatory effect of murine MSCs compared to untreated MSCs to modulate corneal allograft rejection [45].

In addition to their effects on T cells, MSCs and their EVs have also profound effects on innate immune responses such as activation or polarization of macrophages [46–48]. MSCs can promote an anti-inflammatory and highly phagocytic macrophage phenotype through EV-mediated mitochondrial transfer [47]. Moreover, MSC-derived EVs alone can depolarize lipopolysaccharide (LPS)-induced pro-inflammatory macrophages [49].

Herein, we licensed MSCs using TGF-β1, IL-1β, TNF-α and IFN-γ. We found that both IFN-γ and dual IFN-γ/TGF-β1 licensing enhanced the immunomodulatory effect of MSCs on T cell proliferation. These licensing strategies also strengthened the effect of MSCs on reducing the expression of TNF-α and IL-1β by M1 macrophage-like (M1φ) THP-1 cells. ABs derived from apoptotic, licensed MSCs led to an inhibition of T cell proliferation, induction of Tregs, a continuance of immunomodulatory CD73+ T cells and reduction of activated CD69+ T cells. Lastly, we show that the efficacy of ABs is at least partly related to their uptake efficiency. These findings suggest licensing strategies for enhancing MSC immunomodulation and apoptotic body therapy.

2. Materials and methods

2.1. Isolation, culture, and characterization of MSC from human bone marrow

Human MSCs were isolated from the bone marrow of three healthy volunteers at Galway University Hospital under an ethically approved protocol according to a standardized procedure. Written consent was obtained from the volunteers. Briefly, bone marrow cell suspensions were layered onto a Ficoll density gradient, and the nucleated cell fraction was collected, washed, and resuspended in an MSC culture medium. After 24 h of cultivation, nonadherent cells were removed, fresh medium was added, and individual colonies of fibroblast-like cells were allowed to expand and approach confluence prior to passage.

Human MSCs were cultured in α-MEM (BioSciences, Dublin, Ireland) with 1% penicillin/streptomycin (Sigma-Aldrich, Wicklow, Ireland), 10% fetal bovine serum (FBS) (Sigma-Aldrich, Wicklow, Ireland), and 1 ng/mL FGF-2 (Sigma-Aldrich, Wicklow, Ireland).

MSCs were characterized for the expression or absence of specific cell surface markers (CD73, CD44, CD45, CD90, CD11b and HLA-DR) by flow cytometry and for their differentiation capacity. At first, MSCs were harvested and incubated with anti-human antibodies (see Table S1 for additional details) diluted in flow cytometry staining buffer (FACS buffer). Samples were analyzed using a flow cytometer (Cytek Northern Lights™ 3000). Flow cytometry data were analyzed using FlowJo analysis software version 10 (Tree Star Inc.) Differentiation of MSC was performed according to previous methods [50]. MSCs from three donors (donor 1, 2 and 3) were characterized respectively.

2.2. Peripheral blood mononuclear cells (PBMC) isolation and culture

PBMCs were isolated by density-gradient centrifugation from whole blood samples after written informed consent was obtained from four healthy volunteers (NUIG Research Ethics Committee). Freshly drawn peripheral blood was collected in 5 mL ethylene diamine tetra-acetic acid (EDTA) Vacutainer® tubes (BD Medical Supplies, Crawley, UK). PBMCs were isolated by layering 3 mL of anti-coagulated blood over 3 mL endotoxin-free Ficoll-Premium (Sigma-Aldrich, Wicklow, Ireland) density-gradient solution in a 15 mL tube. Samples were then centrifuged at 400 g for 22 mins at 18 °C. Using a plastic Pasteur pipette, the visible "buffy coat" layer of mononuclear cells was removed. PBMCs were transferred into fresh 15 mL tubes, washed twice with 10 mL DPBS (ThermoFisher Scientific) and centrifuged at 400 g for 5 min at 25 °C. The total number of live cells was determined by Trypan Blue exclusion.

PBMCs were cultured in RPMI-1640 medium (BioSciences, Dublin, Ireland) with 1% penicillin/streptomycin (Sigma-Aldrich, Wicklow, Ireland), 10% fetal bovine serum (FBS) (Sigma-Aldrich, Wicklow, Ireland).

2.3. Cytokine licensing

After the plastic adherence of MSCs (density: $3.7 \sim 4.2 \times 10^6$ cells/T175 flask), the medium was removed and replaced with basal medium containing single recombinant human cytokines such as 50 ng/mL TGF-β1, 50 ng/mL, IL-1β, 20 ng/mL TNF-α and 50 ng/mL IFN-γ or a combination of any two of the aforementioned cytokines (all from Peprotech) for a period of 24 h. Cells were then washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) and used in subsequent assays. MSC of passages one to five were used in this study. Non-licensed MSC was called naïve MSC in the later chapters.

2.4. Macrophage-like THP-1 cell culture

THP-1 cells and human peripheral blood cells were cultured in RPMI-1640 (Sigma-Aldrich, Wicklow, Ireland) with 10% heat-inactivated FBS (Thermo Fisher Scientific, Dublin, Ireland), 1% L-Glutamine (Sigma-

Aldrich, Wicklow, Ireland), and 1% penicillin/streptomycin (Sigma-Aldrich, Wicklow, Ireland).

THP-1 cells were incubated with 100 ng/mL PMA (Sigma-Aldrich, Wicklow, Ireland) for 24 h to differentiate macrophage-like THP-1 cells. After 24 h, the differentiated macrophage-like THP-1 cells were attached to the flask. After washing off the suspended undifferentiated cells, macrophage-like THP-1 cells were incubated with LPS (100 ng/mL) (Sigma-Aldrich, Wicklow, Ireland) and IFN-γ (20 ng/mL) (Peprotech, London, UK)) for another 24 h to induce M1 macrophage-like (M1φ) THP-1 cells.

2.5. AB preparation and characterization

AB isolation was optimized from previous studies [33,34,51]. Firstly, MSCs were treated with staurosporine (Sigma-Aldrich, Wicklow, Ireland) at 0.5 μM for 12 h to induce apoptosis. The supernatant was collected and centrifuged at 1000 g for 10 min at 4 °C to remove cells and debris. Then, the supernatant was further centrifuged at 16,000 g for 30 min at 4 °C, and the pellet was washed twice in phosphate-buffered saline (PBS). The washing fluid was also collected. The isolated ABs were suspended in 100 μL PBS and stored at − 80 °C and used up within three days.

For AB characterization, we chose to analyze PD-L1 because PD-L1 was found to be highly expressed in IFN-γ-licensed MSCs [37,52] and PD-L1 is known for its immunosuppressive effect [53,54]. ABs derived from three different human donor MSCs were suspended in FACS (DPBS supplemented with 1% FBS and 0.05% sodium azide) buffer and incubated with PerCP/Cyanine5.5 anti-human PD-L1 (Biolegend, California, USA) for 30 min. After staining, the sample was centrifuged at 16,000 g for 30 min at 4 °C to pellet the stained ABs. Then, stained ABs were resuspended in fresh FACS buffer for analysis using the Northern Lights™ 3000 flow cytometer (Cytek). The total protein content of ABs was quantified by using the Bicinchoninic acid assay (Thermo Fisher Scientific, Dublin, Ireland) after lysed by RIPA lysis buffer (Pierce™, Thermo Fisher Scientific, Dublin, Ireland). The size of ABs was assessed through the Zetasizer Nano ZS90. 10 μL of samples fixed in 2% PFA and incubated on 200 mesh gold formvar carbon-coated electron microscopy grids (Aquilent) for 20 mins to allow attachment. Samples were incubated with 1% glutaraldehyde (Sigma) followed by negative staining with 2% phosphotungstic acid (Sigma) for 15 s. Samples were analysed using a Hitachi 7500 electron microscope, at a magnification of 50,000X for wide-field view, 100,000X for morphological assessment, all at an accelerating voltage of 75 kV.

2.6. PBMC/MSC & PBMC/ MSC-AB assay

PBMCs were stained with the CellTrace™ Violet proliferation kit (Thermo Fisher Scientific, Dublin, Ireland) according to the manufacturer's protocol and seeded in 96 well-round bottom plates (Sarstedt) at a concentration of 1×10^5 cells/100 μL of complete medium with or without 2×10^4 Human T-Activator CD3/CD28 Dynabeads® (Thermo Fisher Scientific, Dublin, Ireland). In the T cell proliferation assay, 1×10^4 beads were used. PBMCs were subsequently co-cultured with MSCs as depicted in Fig. 1A. MSCs were added to wells of lymphocytes at a concentration of $1x10^4$ cells/100 μL (ratio of 1:10 MSCs: lymphocytes). MSCs from three donors and PBMCs from three or four donors were used in this assay.

To assess the impact of ABs on T cell proliferation, 0.125, 0.25, 0.5, 1 or 2 μg ABs were incubated with PBMCs. For the rest of the assessment of ABs on PBMCs, 500 ng ABs were incubated with PBMC. ABs from donor 1′s MSC and PBMC from three or four donors were used.

After 96 h, cells were harvested and incubated with anti-human antibodies (see Table S1 for additional details) diluted in FACS buffer. eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Dublin, Ireland) was used for FOXP3 staining. Samples were analyzed using a flow cytometer (Cytek). Flow cytometry data were analyzed using FlowJo analysis software version 10 (Tree Star Inc.).

2.7. THP-1/MSC & THP-1/ MSC-AB assay

Human THP-1 cells have been shown to serve well as a model for primary human monocytes/macrophages [55]. We used THP-1 cell-differentiated macrophages to study the effect of MSCs/MSC-derived ABs on macrophages.

2.5×10^5 M1φ THP-1 cells and 5×10^4 MSCs (or 0.2, 0.4, 0.8, 1.6 or 5 μg ABs) were seeded into a 24-well plate. 48 h later, cells were harvested and incubated with anti-human antibodies (see Table S1 for additional details) diluted in FACS buffer. eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Dublin, Ireland) was used for TNF-α and IL-1β staining. MSCs and ABs from three donors were used in this assay (Fig. 1E). In addition, 2.5×10^5 macrophage-like THP-1 cells were incubated with 5 μg ABs to test the safety. 48 h later, the cells were harvested and stained by Annexin-V and sytox AADVANCED.

2.8. AB uptake assay

ABs were suspended in FACS buffer and incubated with CellTrace™ CFSE (Thermo Fisher Scientific, Dublin, Ireland) for 30 min. After staining, the sample was centrifuged at 16,000 g for 30 min at 4 °C to pellet the stained AB. Stained ABs were resuspended in fresh MSC basal medium or PBMC medium for 5 min at 37 °C. Then, the sample was centrifuged at 16,000 g for 30 min at 4 °C to pellet the stained ABs and the unbound dye was removed. Finally, the CFSE-stained ABs were suspended in fresh THP-1 or PBMC medium.

For PBMC up-take assessment of ABs, 1×10^5 PBMCs were seeded in the 96-well round bottom plate. 50 ng or 500 ng ABs stained with CFSE were incubated with PBMC for 8 h or 24 h respectively. Cells were then washed three times with PBS and analyzed using a flow cytometer (Cytek). ABs from three donors and PBMC from three or four donors were used in this assay.

For assessment of M1φ THP-1-cell phagocytosis of ABs, 1×10^5 macrophage-like THP-1 cells were seeded in a 96-well round bottom plate. After they were differentiated into an M1 phenotype, 400 ng ABs stained with CFSE were incubated with M1φ THP-1 cells for 8 h. For the blocked uptake assay, M1 cells were incubated with 1 μM wortmannin (Sigma-Aldrich, Wicklow, Ireland) 30 min (based our previous pilot study) prior to the incubation with CFSE-stained ABs. In this assay, the incubation time with ABs was also 8 h (without wortmannin). Cells were washed three times with PBS and analyzed using a flow cytometer (Cytek). For immunocytochemistry staining, 2 h following the AB incubation, cells were fixed with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific, Dublin, Ireland) for 15 mins and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Wicklow, Ireland) for 15 mins. Then the permeabilized cells were stained with DAPI (Biolegend, California, USA) and Flash Phalloidin™ Red 594 (Biolegend, California, USA). The pictures were captured through the Olympus CKX53 microscope and analyzed with cellSens imaging software. ABs from three donors were used in this assay.

2.9. Statistics

Data are presented as mean ± SD. Most comparisons between groups were done by analysis of one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. AB immunomodulatory effects on T cell proliferation (Fig. 3B-D) was performed by analysis of two-way ANOVA with Sidak's multiple comparisons test. Differentiation capacity (supplementary material 1 D and F),viability (supplementary material Fig. 4) and blocked uptake assay (Fig. 6H) was performed by two-tailed unpaired *t*-test. Differences were considered significant for $p < 0.05$. p > but significance close to 0.05 was labelled in the figure.

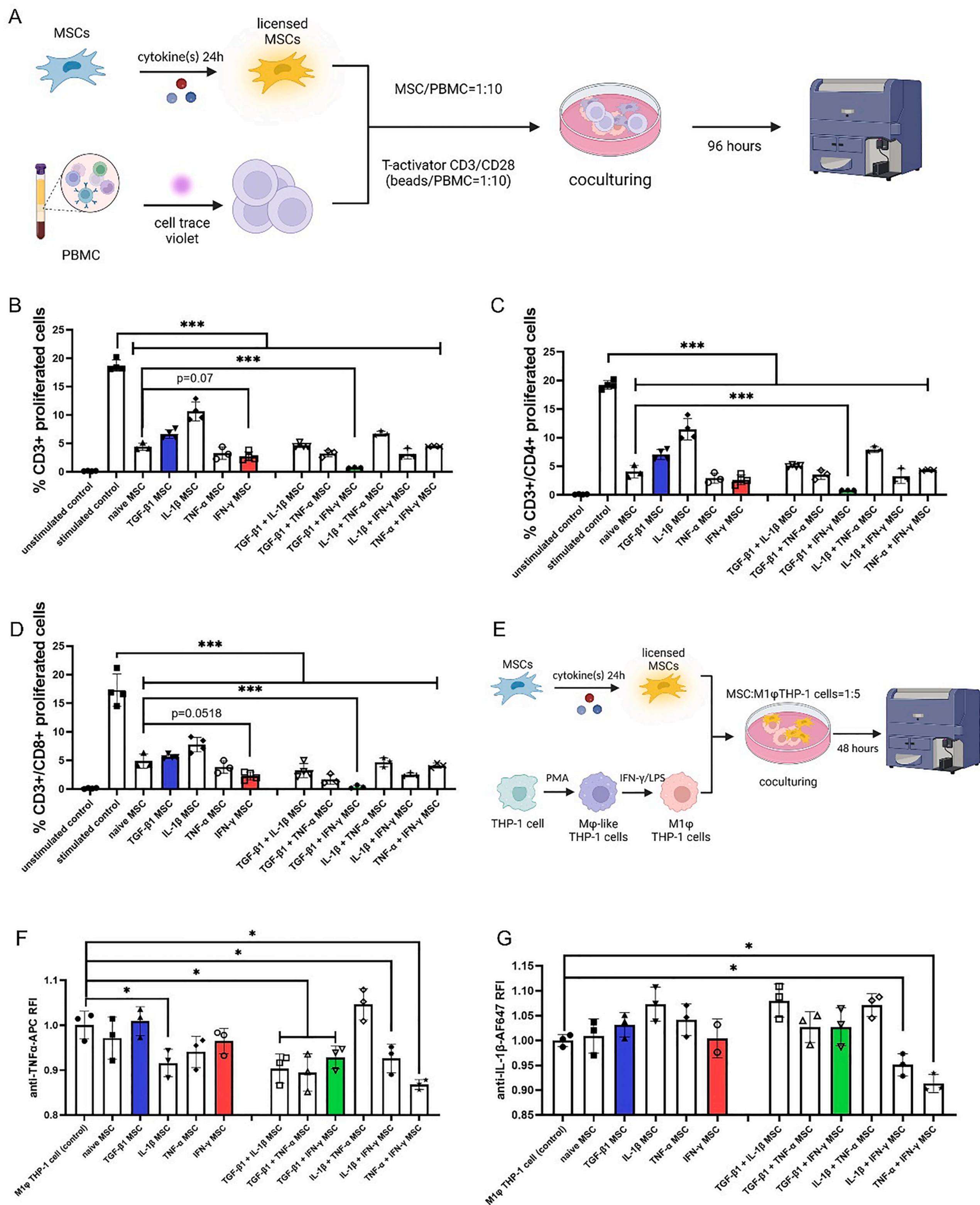

Fig. 1. The immunomodulatory efficacy of mesenchymal stromal cells (MSCs) can be strengthened by anti/pro-inflammatory cytokines licensing. A. Schematic depicting the experimental set-up of MSC/peripheral blood mononuclear cell (PBMC) co-cultures. B-D. Percentage of proliferated CD3+, CD3+/CD4 + and CD3+/CD8+ T cells. All groups, except the unstimulated control, were stimulated with Human T-Activator CD3/CD28 Dynabeads. E. Schematic illustrating the experimental overview of MSC/THP-1 coculturing assay. F-G. TNF-α and IL-1β expression of M1φ THP-1 cells after being treated with MSCs. Relative fluorescence intensity (RFI) in F and G was normalized by M1φ THP-1 cells, which is the control group. Flow cytometry was used to assess the proliferation and protein expression. Representative results of three or four (as dots shown) independent experiments are shown ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Statistical analysis was performed using GraphPad Software (8.0.2).The donor heterogenicity details was summarized in supplementary material Table 2.

3. Results

3.1. Characterization of human MSCs

MSCs from three healthy donors were used in this study. Flow cytometry was used to identify protein expression on the cell surface of MSCs (supplementary material Fig. 1A (positive markers) and supplementary material Fig. 1B (negative markers)). CD90, CD73 and CD44 were all highly expressed on MSCs from all of the donors, in contrast, CD11b, CD45 and HLA-DR were all absent. The differentiation capacity (adipogenic and osteogenic) of MSCs from all three donors was verified as shown in supplementary material Fig. 1 C-F. All the MSCs can differentiate into adipocytes and osteocyte/osteoblasts.

3.2. Licensing of MSCs with pro-/anti-inflammatory cytokines inhibited allogeneic T cell proliferation in MSC-PBMC co-cultures

For the licensing strategy, we chose one anti-inflammatory cytokine, TGF-β1, and three pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ. TGF-β1 was shown in our previous study to induce stronger immunomodulatory murine MSCs compared to untreated MSCs to modulate corneal allograft rejection [45]. Furthermore, for the aforementioned pro-inflammatory cytokines, sole licensing of IL-1β [41], TNF-α [43] or IFN-γ [35] can induce anti-inflammatory and immunomodulatory MSC phenotype. However, the combination of these cytokines was included here to investigate a potential synergistic effect. MSCs and allogeneic CD3/CD28-activated peripheral blood mononuclear cells (PBMCs) were co-cultured to evaluate their efficacy in a T cell proliferation assay which is widely used to test the immunomodulatory potency of MSCs [38,45,52].

The results of co-culture assays of allogeneic (PBMCs) and licensed MSCs are presented in Fig. 1. Flow cytometry gating strategy for identification of proliferated total T cells (CD3+), effector CD4+ T cells (CD3+/CD4+) and effector CD8+ T cells (CD3+/CD8+) are shown in supplementary material Fig. 2A. As shown in Fig. 1B, C and D, both naïve and licensed MSCs inhibited the proliferation of CD3+, CD3 + CD4 + and CD3 + CD8+ T cells. In particular, IFN-γ and dual TGF-β1/IFN-γ licensing had a very profound inhibitory effect on T cell proliferation. Notably, sole licensing with TGF-β1 or IL-1β alone appears to weaken the immunomodulatory effect of MSCs, despite our finding that TGF-β1 licensing enhances the immunomodulatory capacity of mice MSCs in vitro and in vivo [45]. Nevertheless, its combination with IFN-γ further strengthens the originally strong inhibitory effect of IFN-γ licensing and the dual treatment shows a synergistic effect. TNF-α licensing maintains the original immunomodulatory effect of MSCs.

3.3. Licensing of MSCs with pro-/anti-inflammatory cytokines reduced M1φ THP-1 cell expression of TNF-α and IL-1β

Next, we investigated the effect of MSC-licensing on innate immune cell activation, namely macrophages and their capacity to express pro-inflammatory cytokines TNF-α and IL-1β. THP-1 cells, as a human leukemia monocytic cell line, have been extensively used to study monocyte/macrophage functions [55,56]. An M1 macrophage-like (M1φ) THP-1 cell model was established to investigate the effect of MSCs on reducing THP-1 expression of pro-inflammatory cytokines. The gating strategy is shown in supplementary material Fig. 2B. TNF-α and IL-1β protein expression were detected through intracellular staining and flow cytometry analysis. For TNF-α protein expression, we found that naïve MSCs had no effect on TNF-α production by THP-1 cells at the ratio of 5:1 (THP-1: MSC). However, licensing of MSCs achieved immunomodulation. Although the sole use of TGF-β1 did not lead to the inhibition of TNF-α expression, TGF-β1 combined with any other cytokine suppressed TNF-α expression (Fig. 1F). Interestingly, licensing of MSCs with IFN-γ (plus IL-1β or TNF-α) strongly reduced TNF-α expression. Other licensing groups, including TNF-α and TNF-α/IL-1β, kept the original TNF-α level. In all, TGF-β1 and IFN-γ licensing may enhance the capacity of MSCs to reduce TNF-α expression. Regarding modulation of IL-1β production by (licensed) MSCs, we could show that only licensing of MSCs with IL-1β/IFN-γ and TNF-α/IFN-γ led to a significant inhibition. Neither naïve nor other tested licensing strategies seem to be able to modulate IL-1β production by THP-1 cells (Fig. 1G).

In summary, IFN-γ and TGF-β1/IFN-γ licensing were able to enhance the inhibitory effect of MSCs on allogeneic T cell proliferation. In addition, TGF-β1 and IFN-γ licensing enhanced the ability of MSCs to reduce pro-inflammatory cytokines expressed by M1φ THP-1. Therefore, these two cytokines were used to prepare licensed ABs in the subsequent experiments.

3.4. AB characterization

In the subsequent experiments, only TGF-β1, IFN-γ and their combination were used to stimulate the MSCs. We prepared naïve and licensed MSC-derived ABs as shown in Fig. 2A and as previously described [33,34]. Flow cytometry was used to characterize apoptosis (Sytox AADCANCED-/Annexin-V+) of MSCs and the PD-L1 expression on ABs derived from apoptotic cells.

For AB preparation, at the end of the isolation procedure, the washing fluid was collected to confirm that our AB sample did not contain any staurosporine traces or that the staurosporine concentration was not sufficient to induce apoptosis. In subsequent experiments (See T cell proliferation assay using ABs), we detected T cells with strong proliferative ability in the presence of our TGF-β1 AB sample, which also shows that the staurosporine of our AB samples is very less and not enough to induce apoptosis. We also tested if treatment of MSCs with cytokines may induce apoptosis. As Fig. 2B shows, staurosporine induced apoptosis in MSCs as expected. Neither pre-treatment of MSCs with cytokines nor incubation of fresh MSCs with washing fluid from staurosporine-treated MSCs induced apoptosis, which suggested that our AB sample did not contain residual staurosporine. A Bicinchoninic acid protein (BCA) assay was performed to quantify the protein yield of each group of ABs (Fig. 2C). 24-hour licensing with TGF-β1, IFN-γ or both did not affect AB yield. $1x10^6$ naïve or licensed human MSCs produced approximately 8.37 μg ABs. To analyze ABs, we used the Cytek flow cytometer which can sensitively detect small particles [57].

The gating strategy of ABs is shown in Fig. 2D. With the equal 150 μL injection, 94 particles from FACS buffer and 1910 particles from the 16000 g pellet of conditioned medium were recorded in the A gate. This suggests that there are some particles in FACS buffer and a conditioned medium having similar size and granularity with ABs which cannot be excluded by the current strategy. They might be EVs or other protein impurities. Nevertheless, a significantly higher quantity, 13,798 particles of AB size and granularity, from 16000 g pellet of apoptosis medium were recorded. We found that IFN-γ-licensed MSC-derived ABs (IFN-γ ABs, pellet of IFN-γ apoptosis medium) highly expressed PD-L1 (Fig. 2E), which was consistent with previously reported data that IFN-γ licensing upregulates PD-L1 on MSCs [37,38,52].

In addition, we also measured the size of both naïve and cytokine-licensed ABs. We show that the majority of ABs are larger than 500 nm and there is no significant difference between both naïve and licensed ABs. The size of ABs, as measured by zeta potential and size analyzer is shown in Fig. 2F and G. And we finally visualized the AB by transmission electron microscope as shown in Fig. 2H.

3.5. MSC-derived ABs inhibited allogeneic CD3+, CD3+/CD4+ and CD3+/CD8+ T cell proliferation

Next, we investigated if ABs derived from either naïve or licensed

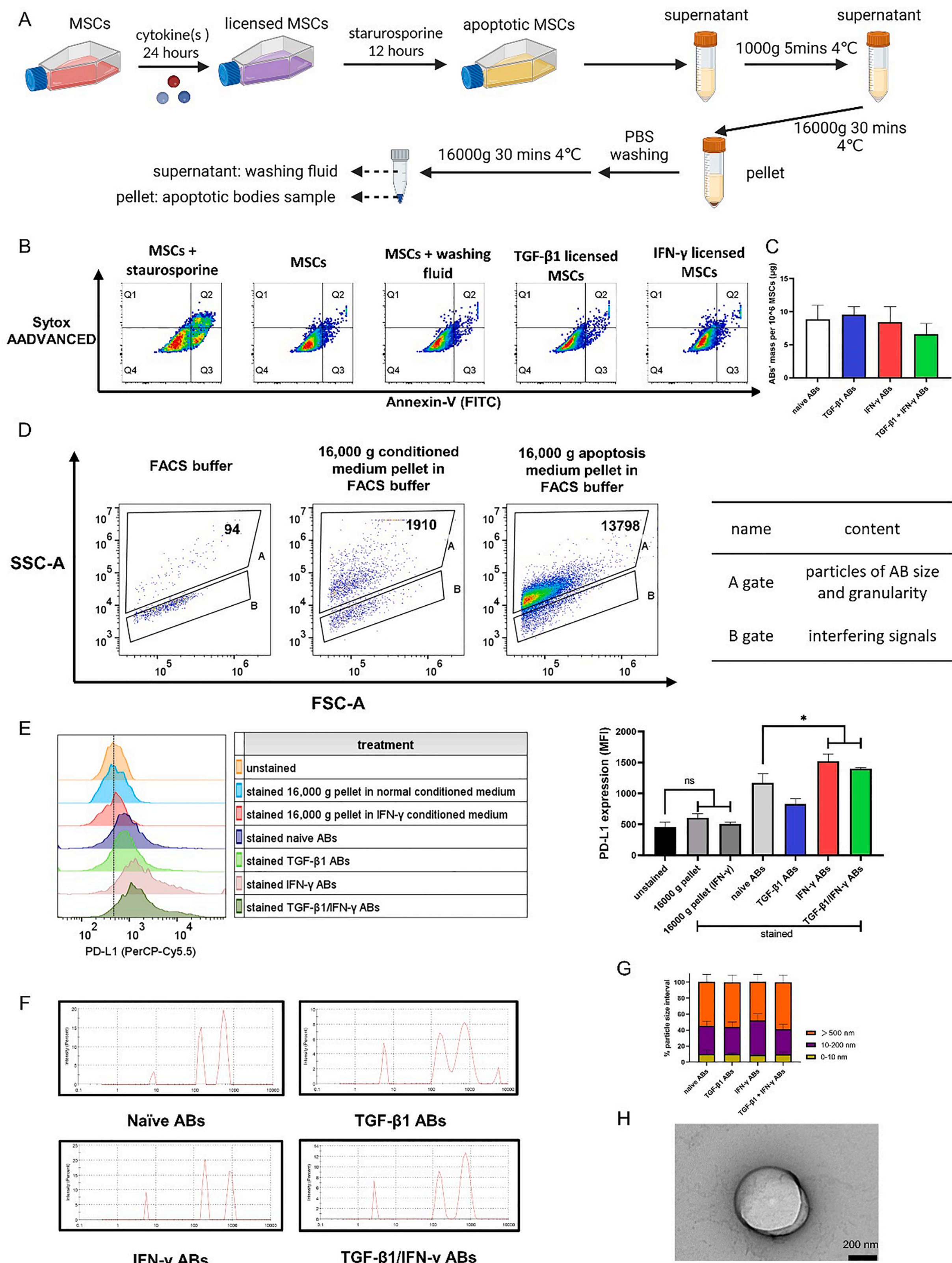

Fig. 2. Preparation and characterization of apoptotic bodies (ABs). A. Experimental overview of AB induction and isolation. B. Characterization of MSC apoptosis. Flow cytometry was used to assess the percentage of apoptotic cells (Annexin V+/Sytox AADVANCED-). C. Yield of ABs by quantifying the protein via bicinchoninic acid (BCA) assay. D. Gating strategy of particles of AB size and granularity with equal 150 μL injection of each group. E. Representative histograms of PD-L1 expression assessed through flow cytometry. F-G. The size distribution of ABs was assessed through Zeta Potential Analyzer. H. The representative transmission electron microscope image of AB. Representative results of three independent experiments are shown ± SD and analyzed by one-way analysis of variance with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

MSCs can modulate T cell proliferation. PBMC incubation with various amounts of ABs was performed as shown in Fig. 3A. The gating strategy is shown in supplementary material Fig. 2A. Total proliferated T cells (CD3+ T cells) were quantified (Fig. 3B). We found a strong inhibition of T cell proliferation when treated with either naïve ABs or ABs from TGF-β1/IFN-γ licensed MSCs. Sole licensing with IFN-γ but not TGF-β1 also showed a strong inhibitory effect. Even when incubating PBMC with a low dose of naïve or TGF-β1/IFN-γ ABs, hardly any proliferating T cells were detected. This very strong inhibitory effect was also seen on CD4 + effector T cells (Fig. 3C) and CD8 + effector T cells (Fig. 3D). In other words, in the presence of either naïve or TGF-β1/IFN-γ ABs, T cell proliferation is strongly inhibited.

When analyzing the inhibitory effect resulting from the lowest dose of 125 ng ABs (Fig. 3E-J), both naïve and dual-licensed ABs have a strong inhibitory effect on CD3+, CD3+/CD4 + and CD3+/CD8+ T cell proliferation. Even though the proliferated cells percentage caused by dual-licensed AB was lower compared with naïve AB, it did not reach statistical significance. While TGF-β1 ABs and also IFN-γ ABs individually significantly inhibited the CD3 + and CD3+/CD8+ T cell proliferation, the inhibitory effect caused by these two licensing treatments was weakened compared to naïve ABs. TGF-β1 licensing abrogated the inhibitory effect of ABs on T cell proliferation. In summary, ABs from both naïve and dual-licensed MSCs had a strong immunomodulatory effect on T cell proliferation even at a very low dose (125 ng ABs: 10^5 PMBC).

3.6. Naïve and dual-licensed MSC-derived ABs increased the percentage of Treg, maintained the ratio of CD73 + immunosuppressive T cells and lowered CD69 + activated T cells

Next, we investigated if incubation of PBMCs with ABs modulated the T cell phenotype. At first, the percentage of Tregs after incubating PBMC with ABs was investigated. Tregs are a specialized subpopulation of T cells that act to suppress the immune response, thereby maintaining homeostasis and self-tolerance. For the detection of Tregs induced by ABs, the gating strategy is shown in supplementary material Fig. 2C. The result (Fig. 4A and D) of this experiment was consistent with the T cell proliferation assay shown in Fig. 3. Both naïve ABs and TGF-β1/IFN-γ ABs induced a higher ratio of Tregs and the Treg induction by TGF-β1/IFN-γ ABs was slightly stronger than naïve ABs.

For the detection of T cell activation markers, the gating strategy is shown in supplementary material Fig. 3A. CD73 is an immunosuppressive marker, which can degrade adenosine triphosphate to adenosine and initiate anti-inflammation [58,59]. CD69 is an activation marker of T cells involved in the production of IL-2, TNF-α, and nitric oxide [60]. Our results show that no modulation of CD73 expression was detected between the different groups or compared to controls (Fig. 4B and E). However, ABs significantly reduced the ratio of CD69+ T cells in total CD3+ T cells (Fig. 4C and F), which may indicate a reduced activation of T cells. Moreover, the reduction in CD69 expression by naïve and sole licensed ABs is more pronounced than with dual licensed ABs.

In summary, ABs were able to strongly inhibit allogeneic T cell proliferation at a very low dose. They induced a higher ratio of Tregs which can be further enhanced by dual TGF-β1/IFN-γ licensing. Interestingly, ABs maintained the ratio of immunosuppressive CD73+ T cells but at the same time reduced the ratio of activated CD69+ T cells.

3.7. ABs were primarily taken up by CD14 + monocytes in PBMC and IFN-γ licensing may impede AB uptake

To better understand the immunomodulatory mechanism of ABs we studied the potential uptake of ABs by PBMCs. The gating strategy is shown in supplementary material Fig. 3B. Two models were established to investigate this in detail. Firstly, (Fig. 5A, short time model) using a low dose of ABs, we observed that CFSE + stained ABs were preferentially phagocytosed by CD14 + monocytes in the PBMC population (Fig. 5B-C). When incubating 50 ng ABs with PBMC, after 8 h, about 8% of CD14 + monocytes were CFSE+, indicating ~ 8% of monocytes phagocytosed ABs. In contrast, only approximately 1.5% of CD14-/CD3- cells had taken up ABs, and ABs were rarely taken up by CD3+/CD14- T cells. In this short-time and low-dose assay, T cells did not phagocytose ABs. Therefore, to clarify whether T cells can also take up ABs, we extended the time and also used a higher dose (Fig. 5A, long time model). The uptake capacity of each PBMC subtype of different ABs was also quantified in this model. When incubating PBMCs with a high dose of ABs for an extended period (24 h), all subpopulations of PBMCs became CFSE+, indicating that all cells are able to take up ABs, including CD3+/CD14- T cells, CD14+/CD3- monocytes and CD14-/CD3- cells (Figure D-F). Interestingly, the CFSE MFI of cells treated with IFN-γ ABs was the lowest (Figure G-I), indicating that IFN-γ licensing may impede the uptake of ABs.

3.8. TGF-β1 ABs were the most efficient at reducing TNF-α and IL-1β expression by M1φ THP-1 cells, which may attribute to their high uptake efficiency

So far, we showed that MSCs have an immunomodulatory effect on M1φ THP-1 cells and described the potential of monocytes to phagocytose ABs. We also wanted to investigate the effect of ABs on M1φ THP-1 cells and uptake efficacy.

The ABs/THP-1 assay was performed as shown in Fig. 6A. As shown in Fig. 6B-D, ABs had a strong effect on downregulating protein expression of both TNF-α and IL-1β. Interestingly, TGF-β1 ABs and TGF-β1/ IFN-γ ABs were the most effective. The sole use of IFN-γ showed a relatively strong effect, but it showed no benefit compared with naïve ABs. Finally, the uptake of ABs by M1φ THP-1 cells was studied. At first, through immunocytochemistry staining, Fig. 6E showed M1φ THP-1 cells are phagocytosing ABs. The M1φ THP-1 cells were also analyzed by flow cytometry for more quantitative detection. As demonstrated in Fig. 6F and G (gating strategy supplementary material Fig. 3B), naïve ABs and TGF-β1 ABs were more phagocytosed to a higher extent after 8 h compared to IFN-γ and IFN-γ/TGF-β1 ABs, which was consistent with our previous result on PBMC/ABs up-take assay. This may also be the reason why TGF-β1 ABs were the most effective in reducing M1φ THP-1 cell expressing pro-inflammatory cytokines. In the blocked-uptake assay, wortmannin was used to study the uptake mechanism. As shown in Fig. 6H-I, wortmannin blocked the uptake significantly. Because wortmannin is a phosphoinositide 3-kinase inhibitor [61] which is relevant to the phagocytosis of large particles [62], this assay suggests that the uptake of ABs is partly dependent on the phosphoinositide 3-kinase pathway at least. In addition, 2.5×10^5 macrophage-like THP-1 cells were incubated with 5 μg naïve ABs (the highest ratio in this chapter) to test whether the ABs are cytotoxic. As shown in supplementary material Fig. 4, compared with the control group, ABs did not induce low viability and apoptosis, which indicates the ABs are not cytotoxic.

4. Discussion

The T cell proliferation assay is probably the most commonly used method to assess the immunomodulatory potency of MSCs [63,64]. Therefore, the result of this part is given special. In the MSC/PBMC co-culture assays, we verified that IFN-γ and dual IFN-γ/TGF-β1 licensing strengthens the inhibitory effect of MSCs on T cells. This is the crucial reason why the subsequent experiment also continued the work of TGF-β1/IFN-γ licensing even if this combination is merely a relatively powerful one in MSC/macrophage co-culture assay. Interestingly, sole TGF-β1 licensing did not have the same inhibitory effect (Fig. 1B, C and D) which is different to what we have previously observed in the mouse [45].

When we used ABs instead of MSCs, the strong inhibitory effect by dual IFN-γ/TGF-β1 licensing was maintained, as were the trends

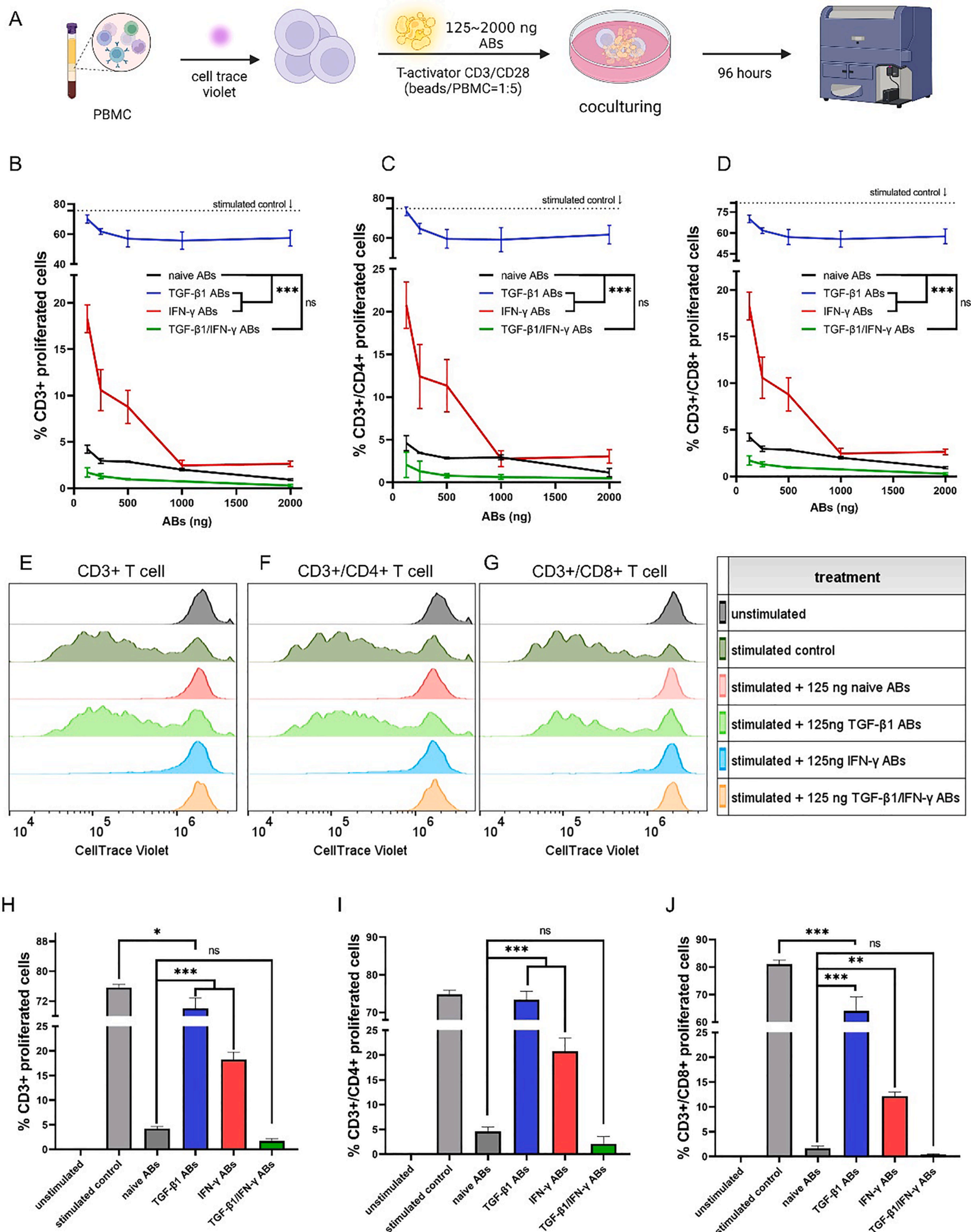

Fig. 3. Mesenchymal stromal cell (MSC)-derived apoptotic bodies (ABs) inhibit allogeneic T cell proliferation. A. Schematic of the experimental overview of AB/PBMC T cell proliferation assay. All groups, except the unstimulated control, were stimulated with Human T-Activator CD3/CD28 Dynabeads. B-D. Percentage of proliferated CD3+, CD3+/CD4 + and CD3+/CD8+ T cells when treated with each group of ABs in a gradient concentration. E-G. Representative histograms of Celltrace Violet (CTV) of CD3+, CD3+/CD4 + and CD3+/CD8+ T cells when treated with 125 ng ABs in each group. H-J. Percentage of proliferated CD3+, CD3+/CD4 + and CD3+/CD8+ T cells when treated with 125 ng each group of AB. Flow cytometry was used to assess the proliferation. Representative results of three independent experiments are shown ± SD. B-D were analyzed by analysis of two-way ANOVA with Sidak's multiple comparisons test. H-J were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

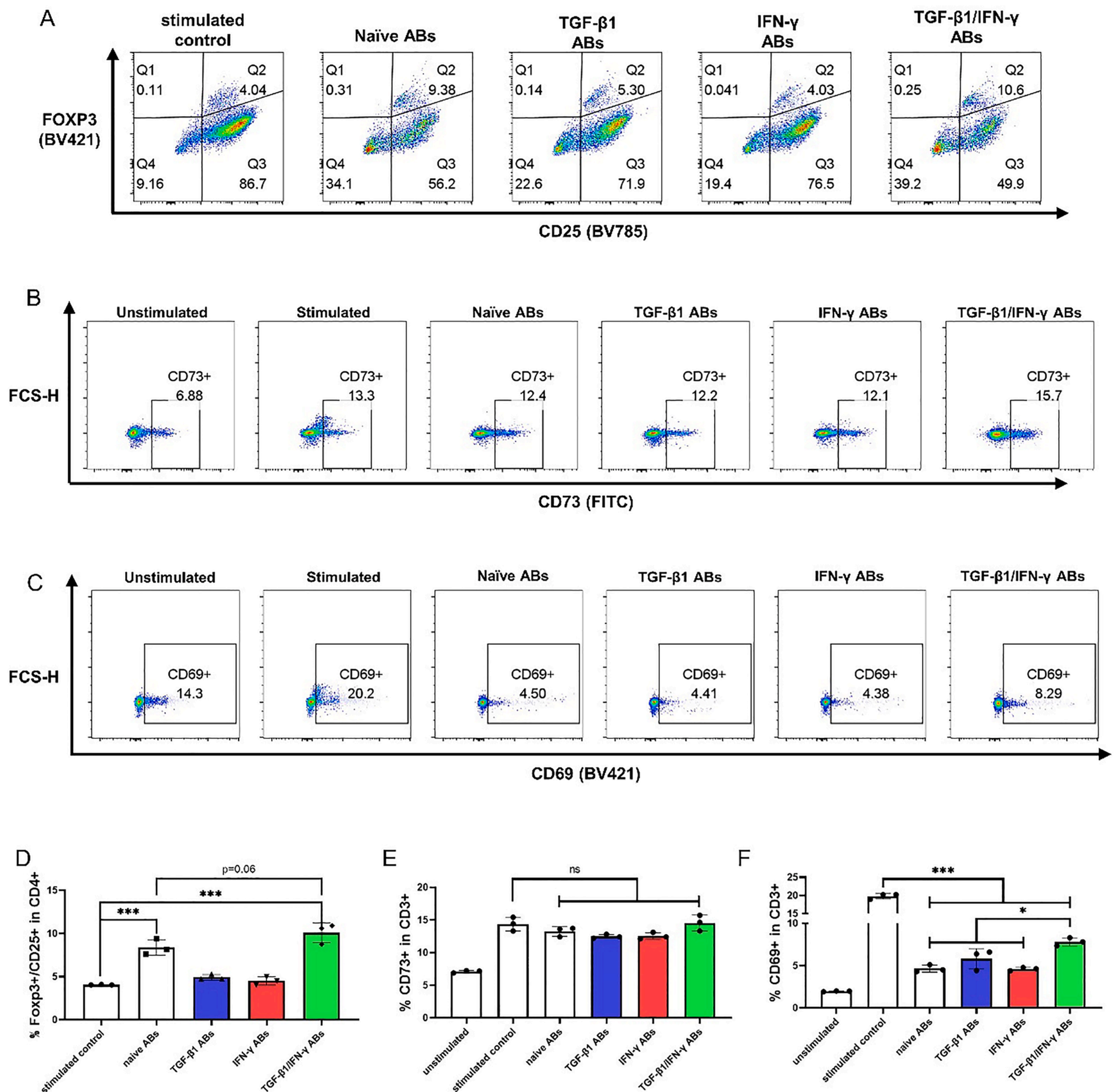

Fig. 4. Naïve and dual-licensed mesenchymal stromal cell (MSC)-derived apoptotic bodies (ABs) altered the T cell immunomodulatory subtype. A-C. Representative pseudocolor plots of FOXP3/CD25 + cells (Tregs), CD73+ T cells and CD69+ T cells. D-F. Percentage of Tregs, CD73+ T cells and CD69+ T cells. Flow cytometry was used to assess the cell subtype. All groups, except the unstimulated control, were stimulated with Human T-Activator CD3/CD28 Dynabeads. Representative results of three independent experiments are shown ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

observed with TGF-β1 licensing. Interestingly, IFN-γ licensing did not enhance the immunomodulatory efficiency of ABs compared to ABs from naïve MSCs (Fig. 3C, E and F) which was unexpected. We expected that each post-cytokine-licensed AB would have a similar effect as their parent cells, but we found that both sole TGF-β1 and IFN-γ licensed ABs had a reduced inhibitory effect on T cell proliferation, compared with naïve ABs. We suggest that the low phagocytosis of IFN-γ ABs resulted in a reduction of the originally strong effect (Fig. 5F). If the uptake efficiency of IFN-γ ABs were as high as that of naïve ABs, they may inhibit the T cell proliferation to a greater extent. With regards to TGF-β1 licensed ABs, we believe that their effect is weak because the effect of TGF-β1-licensed MSC was weak. Therefore, even if phagocytosis of TGF-β1 ABs was high, this might contribute less. In summary, we speculate that IFN-γ ABs should have strong effects on inhibiting T cell proliferation but the effects were weakened by their low uptake (Fig. 5G). TGF-β1 ABs have a weak effect on inhibiting T cell proliferation as TGF-β1-licensed cells (Fig. 1B-D), so even if their uptake is high, their effects are weak. IFN-γ/TGF-β1 ABs have a slightly strengthened effect than naïve ABs because this combined licensed strategy both has strengthened biological effect from IFN-γ licensing (Fig. 1B-D) and keeps intermediate uptake from TGF-β1 licensing (Fig. 5G).

Previous studies have found PD-L1 is upregulated in IFN-γ-licensed MSCs [37,52]. We found PD-L1 is also upregulated in IFN-γ-licensed MSC-derived ABs. This consistent protein expression of molecules in the

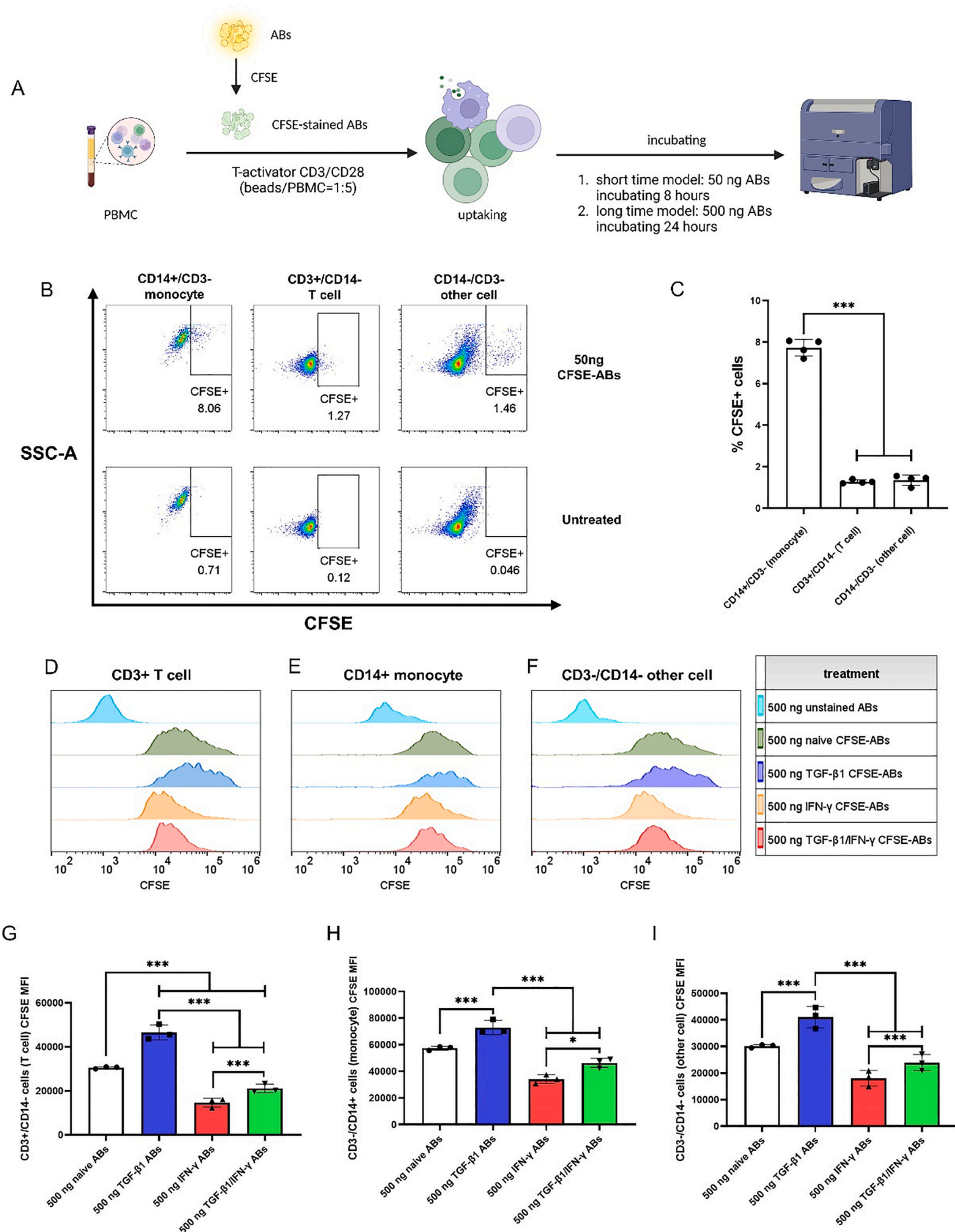

Fig. 5. Uptake of apoptotic bodies (ABs) by peripheral blood mononuclear cells (PBMC). A. Experimental overview of AB uptake assay showing that ABs were labelled with CFSE before treatment onto PBMCs. All groups were stimulated with Human T-Activator CD3/CD28 Dynabeads. B. Representative pseudocolor plots of CFSE (Carboxyfluorescein succinimidyl ester) + cells. C. Percentage of CFSE + cells. D-F. Representative histograms of CFSE level in CD3+/CD14- cells (T cell), CD3-/CD14 + cells (monocyte) and CD3-/CD14- cells (other cell). G-I. CFSE median fluorescence intensity (MFI) of CD3+/CD14- cells (T cell), CD3-/CD14 + cells (monocyte) and CD3-/CD14- cells (other cell). Representative results of three or four (as dots shown) independent experiments are shown ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

vesicle membrane and the cell membrane is common in engineered EVs [65] and exosomes [66]. Our result demonstrated that IFN-γ ABs retain the high PD-L1 upregulation of their parental MSC. Furthermore, based on the current gating strategy, other interfering particles of AB size and granularity caused by conditioned medium (like Fig. 2D middle) might result in PD-L1 false positive high PD-L1 readings. However, we tested these particles and confirmed that they are PD-L1 low (Fig. 2E).

It is reasonable to speculate that licensing probably influences the uptake because this present and previous study has shown that licensing can alter the expression of the membrane surface proteins [37,52]. This alteration of protein expression may be retained in the ABs, and thus through protein binding to the target cell may affect the uptake.

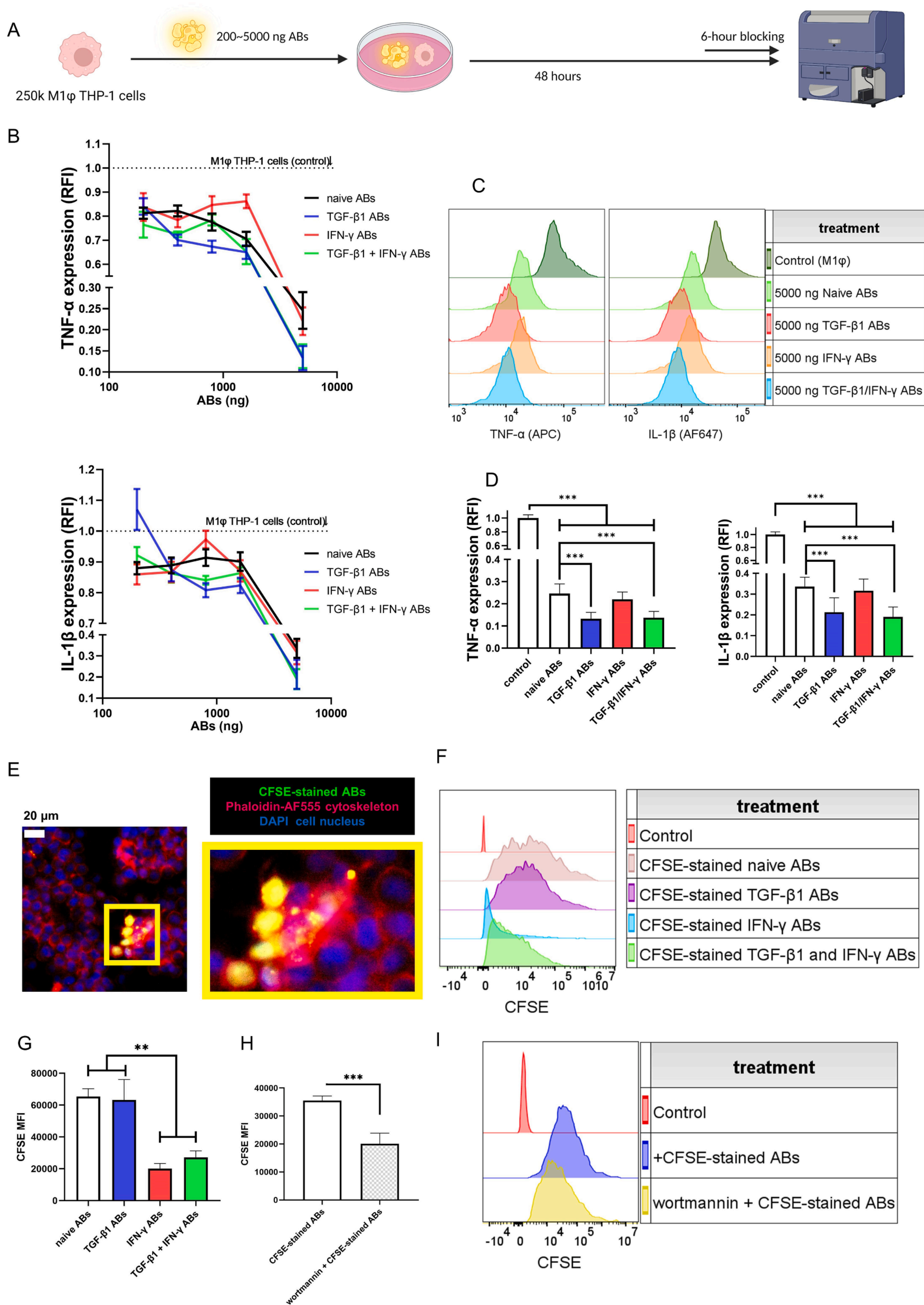

Fig. 6. Effect of apoptotic bodies (ABs) on M1 macrophage-like (M1φ) THP-1 cells expression of pro-inflammatory cytokines. A. Experimental overview of THP-1 incubating with ABs. B. TNF-α and IL-β expression of M1φ THP-1 cells when treated with a gradient dosage of ABs. C-D. TNF-α and IL-β expression of M1φ THP-1 cells when treated with 5000 ng ABs. E. Immunocytochemistry staining picture of M1φ THP-1 when incubating CFSE-stained ABs for 2 h. F-G. CFSE fluorescent intensity of M1φ THP-1 cells after incubating CFSE-stained ABs for 8 h. H-I. CFSE fluorescent of blocked uptake assay. The relative fluorescence intensity (RFI) in B and D were normalized by the control group, which was M1φ THP-1 cells. Protein expression was assessed through flow cytometry. Representative results of three independent experiments are shown ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Staurosporine is widely used to induce apoptosis and prepare ABs [33,34,67]. Three main results reveal that our AB samples did not contain staurosporine or that the concentration was negligible: 1) the washing fluid, which was obtained by washing the AB sample, did not induce apoptosis (Fig. 2A).; 2) the T cell proliferation caused by TGF-β1 ABs was robust, suggesting there is no residual staurosporine inside the AB (Fig. 3B); 3) large dosage of ABs did not result in THP-1 apoptosis and low viability (supplementary material Fig. 4).

We reported that all groups of licensed ABs maintain the expression of CD73 but downregulate CD69 on T cells. CD73 can degrade adenosine triphosphate to adenosine and reduce inflammation [58,59]. The adenosinergic immunosuppression pathway is crucial [68,69]. For example, CD73+ T cells or their EVs were reported to be available to CD4 + CD39+ Tregs for the production of immunomodulatory adenosine [70]. In addition, in an inflammatory model caused by *Trypanosoma gondii*, CD73 was downregulated which reduced adenosine [71]. This verified that CD73 may be downregulated during inflammation. CD69 is considered an activated marker of T cells on both pro- and anti-inflammation [72,73]. For pro-inflammation, CD69+ T cells were poised for the high production of pro-inflammatory cytokines [74]. The increase of CD4+/CD69+ T cells possibly represents inflammation [75] and aggravates autoimmune disease [76,77]. Moreover, the anti-inflammatory function of CD69 is often linked to Tregs. It was reported that CD69 expression is required to maintain the immune tolerance mediated by Foxp3+ Tregs [78]. And CD69 enhanced the immunomodulatory function of Tregs [79] and reduce the immune damage [80].

A new M1φ THP-1 cell-based model was established to investigate the immunomodulatory potential of MSCs and their ABs, which was also inspired by the direct interaction between monocyte [81] /macrophage [48,82] and MSCs. We concluded that ABs were mostly phagocytosed by monocytes and less so by T cells. By quantifying the expression of pro-inflammatory cytokines TNF-α and IL-1β, this model is very valuable to detect the immunomodulatory effects of MSCs or ABs. We found that sole licensing with TGF-β1 enhanced the capacity of MSC and its ABs to modulate M1φ THP-1 cells. Similarly, with AB inhibiting T cell proliferation, we speculated that each kind of AB has a similar effect as their parent cell. In other words, sole TGF-β1 or IFN-γ ABs should be more effective to affect M1 polarization than naïve ABs because both TGF-β1 and IFN-γ licensing enhanced the effect of MSCs on M1 polarization. However, we observed that IFN-γ licensing weakened this effect of ABs. We speculated that the low uptake efficacy of IFN-γ ABs limited its originally powerful effect (Fig. 6G). On the contrary, the high uptake of TGF-β1 ABs enhanced its originally powerful effect. Therefore, we conclude that TGF-β1 ABs were the most favorable AB to reduce pro-inflammatory cytokine expression. In terms of combined licensing of IFN-γ and TGF-β1, the effect was the same with sole TGF-β1 (Fig. 6D). We speculated in this assay the strengthened effect of TGF-β1 is very strong so that the reduced uptake caused by IFN-γ has little effect on the final result.

In our work, we did not detect any effect of naïve MSCs on THP-1 cells which may be because of the ratio of MSCs to THP-1 cells (THP-1:MSC = 5:1) used in our experiments. We did not use a high ratio of MSC because it might cause a powerful effect on reducing M1 macrophage expressing IL-1β and TNF-α so we might not see the noticeable strengthened effect induced by licensing. Some studies suggested reducing the ratio of MSCs (macrophage: MSC = 2:1 [83] or 1:1 [84,85]) which mediated a strong depolarizing effect on M1 macrophage. ABs were shown to have great potential for immunomodulation, especially the TGF-β1/IFN-γ ABs. They strongly inhibited T cell proliferation and simultaneously maintained a higher ratio of Tregs. In the M1φ THP-1 cell assay, naïve ABs, as well as all groups of licensed ABs, were able to inhibit pro-inflammatory cytokine expression. Lastly, AB lowered the ratio of CD69+ T cells activated and at the same time maintained the ratio of CD73+ T cells (immunosuppressive).

5. Conclusions

In all, we reported that IFN-γ licensing enhanced the inhibitory effect of MSCs on T cell proliferation. Moreover, TGF-β1 and IFN-γ licensing enhanced the effect of MSCs on M1φ THP-1 cells. We also found that licensing influences the uptake of ABs by PBMCs and also modulates their impact on recipient cell phenotype. TGF-β1/IFN-γ ABs strongly inhibited T cell proliferation and simultaneously kept a high ratio of Tregs. TGF-β1/IFN-γ ABs also maintained the T cell ratio between a low pro-inflammatory and high immunomodulatory phenotype. Besides, ABs were proven to reduce M1φ THP-1 cells expressing the pro-inflammatory cytokines TNF-α and IL-1β.

CRediT authorship contribution statement

Jiemin Wang: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Ellen Donohoe:** Methodology, Writing – review & editing. **Aoife Canning:** Methodology, Writing – review & editing. **Seyedmohammad Moosavizadeh:** Writing – review & editing. **Fiona Buckley:** Methodology, Writing – review & editing. **Meadhbh Á. Brennan:** Methodology, Writing – review & editing, Supervision. **Aideen E. Ryan:** Writing – review & editing, Supervision. **Thomas Ritter:** Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2023.111096.

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