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Original Research

Immunomodulatory potential of cytokine-licensed human bone marrow-derived mesenchymal stromal cells correlates with potency marker expression profile

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Abstract

Cytokine(s) pre-activation/licensing is an effective way to enhance the immunomodulatory potency of mesenchymal stromal cells (MSCs). Currently, IFN-γ licensing received the most attention in comparison with other cytokines.

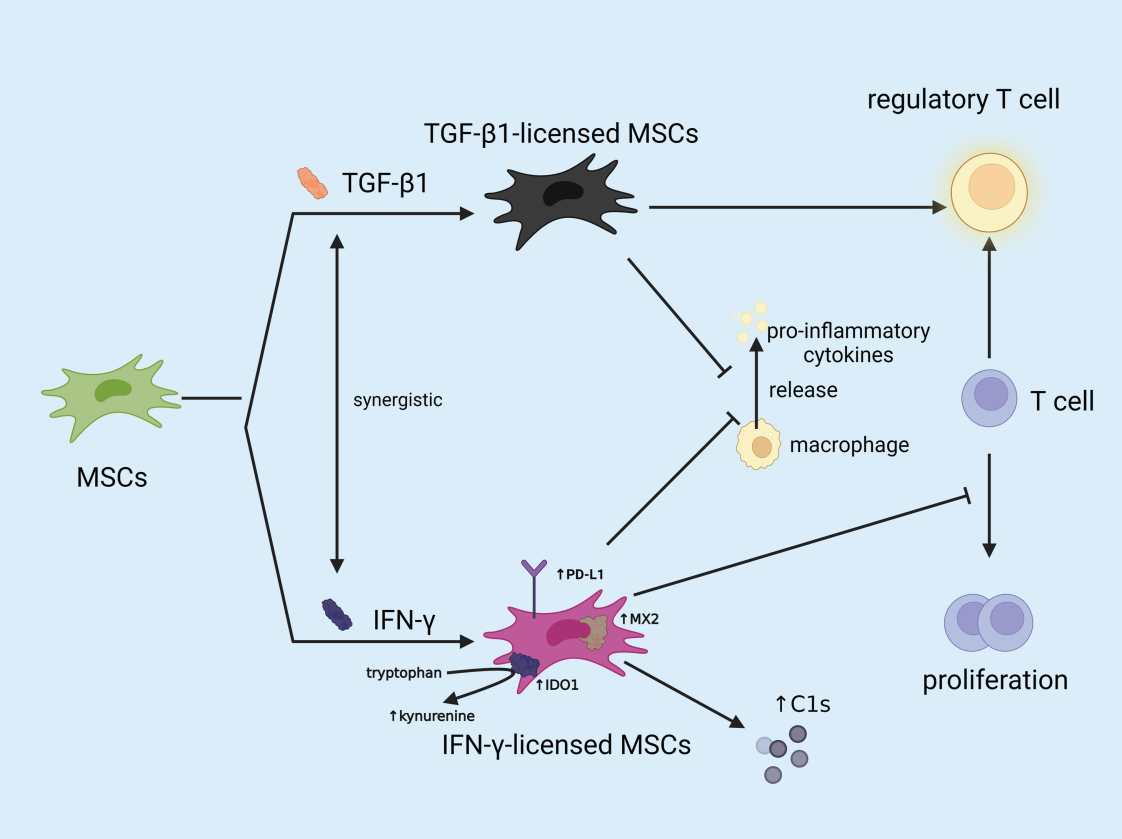
After licensing human bone marrow-derived MSCs with pro-/anti-inflammatory cytokines IFN-γ, IL-1β, TNF-α, TGF-β1 alone or in combination, the in vitro immunomodulatory potency of these MSCs was studied by incubating with allogeneic T cells and macrophage-like THP-1 cells. In addition, immunomodulation-related molecules filtered by bioinformatics, complement 1 subcomponent (C1s), and interferon-induced GTP-binding protein Mx2 (MX2), were studied to verify whether to reflect the immunomodulatory potency.

Herein, we reported that different cytokines cause different effects on the function of MSC. While TGF-β1 licensing enhances the capacity of MSCs to induce T cells with an immunosuppressive phenotype, IFN-γ-licensing strengthens the inhibitory effect of MSC on T cell proliferation. Both TGF-β1 and IFN-γ licensing can enhance the effect of MSC on reducing the expression of pro-inflammatory cytokines by M1 macrophage-like THP-1 cells. Interestingly, IFN-γ upregulates potential potency markers extracellular C1s and kynurenine (KYN) and intracellular MX2. These 3 molecules have the potential to reflect mesenchymal stromal cell immunomodulatory potency. In addition, we reported that there is a synergistic effect of TGF-β1 and IFN-γ in immunomodulation.

Key words: mesenchymal stromal cells; licensing; immunomodulation; potency assay; IFN-γ; TGF-β1.

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Graphical Abstract



Significance statement

This study first revealed different licensing strategies for strengthened mesenchymal stromal cell immunomodulation. Transforming growth factor β1 and interferon-γ licensing can strengthen the immunomodulatory capacity of mesenchymal stromal cells from different pathways respectively. Then the study selected 2 genes from open open-access dataset and 3 evidence-based markers to verify whether these are involved in mesenchymal stromal cell activation. The study also found indoleamine 2,3-dioxygenase-1-secreted kynurenine was highly related to the mesenchymal stromal cell immunomodulatory property. Finally, Interferon-γ can upregulate the selected markers but TGF-β1 cannot, which suggests TGF-β1 probably has a different pathway to strengthen the immunomodulatory capacity.

Introduction

Currently, mesenchymal stromal cells (MSCs) are being investigated for their potential as novel immunosuppressive[1,2] and anti-inflammatory[3-5] treatments. Briefly, in terms of mechanism, MSCs are shown to inhibit T-cell proliferation[6] and also promote regulatory T cells (Tregs).[7] Moreover, MSCs also can polarize macrophages to M2-like anti-inflammatory phenotype.[8] Due to these immunomodulatory properties MSCs were applied in many clinical trials,[9] however, their limited therapeutic efficacy in patients[10] has prevented a more widespread application. Therefore, alternative techniques need to be developed to make MSCs more efficacious and to identify MSCs with higher therapeutic potential.

Licensing, which is also known as cytokine-activating, is a convenient and effective way to enhance the immunomodulatory potential of MSCs.[11,12] It was reported that IFN-γ, a pro-inflammatory cytokine, can induce MSCs to be more immunomodulatory.[11] IFN-γ licensing was verified to enhance the inhibitory effect of MSC on T-cell proliferation. Currently, researchers have primarily prepared good manufacturing practice manufactured IFN-γ licensed MSCs for clinical trials.[13] Besides, other pro-inflammatory cytokines like IL-1β[14,15] and TNF-α[16,17] were also studied to enhance the potency of MSCs. Lastly, in addition to pro-inflammatory cytokines, our previous study using murine MSCs demonstrated that TGF-β1, as an anti-inflammatory cytokine, also can induce stronger immunomodulatory MSCs in vitro and in a corneal allograft rejection model.[18] Other groups also reported TGF-β1 licensing enhances CXCR3-mediated MSC engraftment to the liver and strengthens anti-inflammatory efficacy.[19]

IFN-γ licensing can strengthen the immunomodulatory potency of MSCs, however, it is important to have assays developed for potency testing of clinical-grade MSC products.[20,21] This would satisfy the mechanistic research of MSC and its final registration for clinical research.[22-24] Up to now, in terms of potency assays, IDO1[20,25,26] and PD-L1[20,27] are often regarded as markers of MSC immunomodulatory potency. Meanwhile, IFN-γ is verified to upregulate these 2 immunosuppressive proteins PD-L1[11,12] and IDO1[14,15] of MSCs. Next, metabolized by IDO1,[28] kynurenine (KYN) is enriched

by IDO1-upregulated cells[29,30] and we speculated that this amino acid is elevated in MSC conditioned medium. These 3 markers are evidence-based and have the potential to reflect the MSC immunomodulatory potency. We investigated whether these 3 markers can be influenced by other cytokines. In addition, by comparing publicly available sequencing data, we filtered complement C1s subcomponent (C1s) and interferon-induced GTP-binding protein Mx2 (MX2) may be upregulated in strong immunomodulatory MSCs. These 2 genes/proteins are selected as the new candidate markers to reflect the immunomodulatory potency.

Herein, by comparing different licensing strategies, we report the different immunomodulatory effects induced by TGF-β1 and IFN-γ in MSCs. TGF-β1-licensing strengthens the effect of MSC on altering the T cell to the high ratio of regulatory T cells (Tregs), $CD73^{hi,}$ and $CD69^{low}$, while IFN-γ licensing enhances the effect of MSC on inhibiting T-cell proliferation. Both TGF-β1 and IFN-γ licensing can enhance the effect of MSC on reducing the expression of TNF-α and IL-1β by M1 macrophage-like (M1φ) THP-1 cells. Lastly, IFN-γ upregulates extracellular protein C1s, KYN, and intracellular MX2. These 3 new molecules have the potential to reflect the immunomodulatory potency of MSC.

Materials and methods

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

Human MSCs were isolated from the bone marrow of 3 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 hours 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) with 1% penicillin/streptomycin (Sigma-Aldrich), 10% fetal bovine serum (FBS) (Sigma-Aldrich), and 1 ng/mL FGF-2 (Sigma-Aldrich).

Macrophage-like THP-1 cell culture

THP-1 cells and human peripheral blood cells were cultured in RPMI-1640 (Sigma-Aldrich) with 10% heat-inactivated FBS (Thermo Fisher Scientific), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich).

THP-1 cells were incubated with 100 ng/mL PMA (Sigma-Aldrich) for 24 hours to differentiate to macrophage-like THP-1 cells. After 24 hours, 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) and IFN-γ (20 ng/mL) (Peprotech) for another 24 hours to induce M1 macrophage-like (M1φ) THP-1 cells.

Cytokine licensing

After the plastic adherence of MSCs (density: 3.7–4.2 × 10^6 cells/ T175 flask), the medium was removed and replaced with basal medium containing single recombinant human cytokines including 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 2 of the aforementioned cytokines (all from Peprotech) for a period of 24 hours. Cells were then washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) and used in subsequent assays. MSC of passages 1–5 were used in this study. Non-licensed MSC was called naïve MSC in the later chapters.

Peripheral blood mononuclear cell (PBMC)/MSC coculture assay

PBMCs were stained with the CellTrace Violet proliferation kit (Thermo Fisher Scientific) 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). PBMCs were subsequently co-cultured with MSCs as depicted in Figure 1A. MSCs were added to wells of lymphocytes at a concentration of 1 × 10^4 cells/100 µL (ratio of 1:10 MSCs: lymphocytes). MSCs from 3 donors and PBMCs from 3 or 4 donors were used in this assay.

THP-1/MSC coculture assay

Human THP-1 cells have been used as a representative model for primary human monocytes/macrophages. We used THP-1 cell-differentiated macrophages to study the effect of MSC on macrophages as described in methods.

1 × 10^5 M1φ THP-1 cells and 5 × 10^4 MSCs were seeded into a 24-well plate. Forty-eight hours later, cells were harvested and incubated with anti-human antibodies (see Supplementary Table for additional details) diluted in FACS buffer. eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) was used for TNF-α and IL-1β staining. MSCs from 3 donors were used in this assay (Figure 2A).

Bioinformatics analysis

Human MSCs immunomodulatory capacity comparison-related series (from the NCBI Gene Expression Omnibus database) were selected for bioinformatics analysis. They were GSE126461, GSE117901, GSE117901, GSE68610, GSE142816, and GSE122091. The details of the series are shown in Table 1. The data were analyzed and extracted from GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). In differential gene screening, *P*-value less than 0.05 (–logP greater 1.3) and LogFC greater than 1 (upregulation) or less than 1 (downregulation) were defined as candidate differential genes. Gene ontology enrichment analysis, heatmaps, and Volcano plots were performed by R (4.2.2). A Venn diagram was generated via JVenn.[31]

Statistics

Data are presented as mean ± SD. The majority of comparisons between groups were done by analysis of 1-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Figure 6C-E is done by analysis of 2-way ANOVA with Sidak's multiple comparisons test. Differences were considered significant for $P < .05$. $P >$ but closed to .05 was labeled in the figure.

Univariate linear regression models were performed on R to explore the correlation (Figure 6F and supplementary materal Figrue 7) between selected potency markers and immunomodulatory properties (proliferated T-cell ratio). The regression results were displayed as slope and *P*-values were set at < .05 for statistical significance.

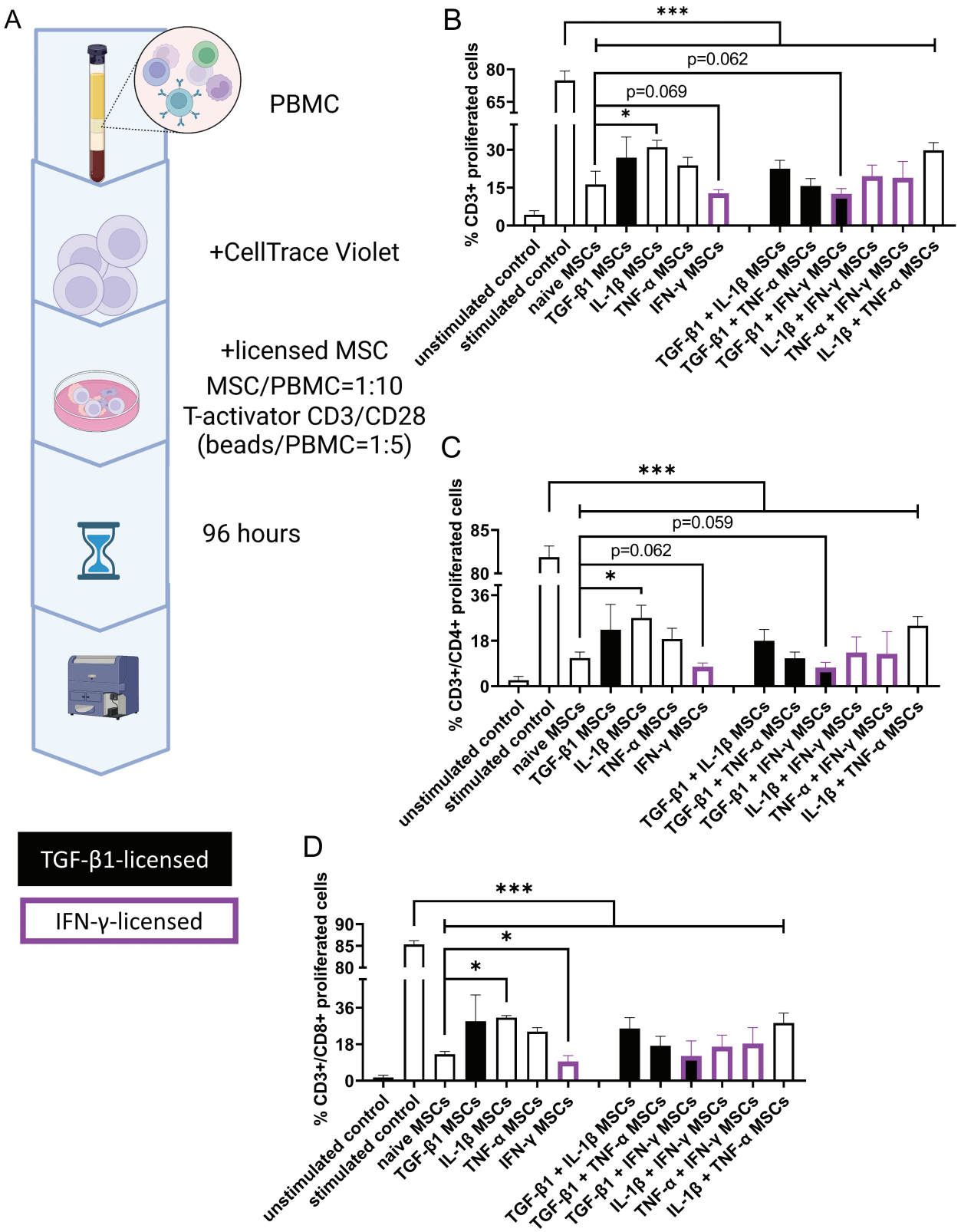


Figure 1. The inhibitory effect of (licensed) mesenchymal stromal cell (MSC) on T-cell proliferation. A. Schematic depicting the experimental set-up of MSC/peripheral blood mononuclear cell (PBMC) co-cultures. B-D. Percentage of proliferated CD3+, CD3+/CD4+ and CD3+/CD8+ T cells. All groups, except the unstimulated control, were stimulated with Human T-Activator CD3/CD28 Dynabeads. Flow cytometry was used to assess the proliferation. The proliferated ratio of each group is shown. Representative results of 3 independent experiments are shown ±SD and analyzed by 1-way ANOVA with Tukey's multiple comparisons test. $^{*}P < .05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

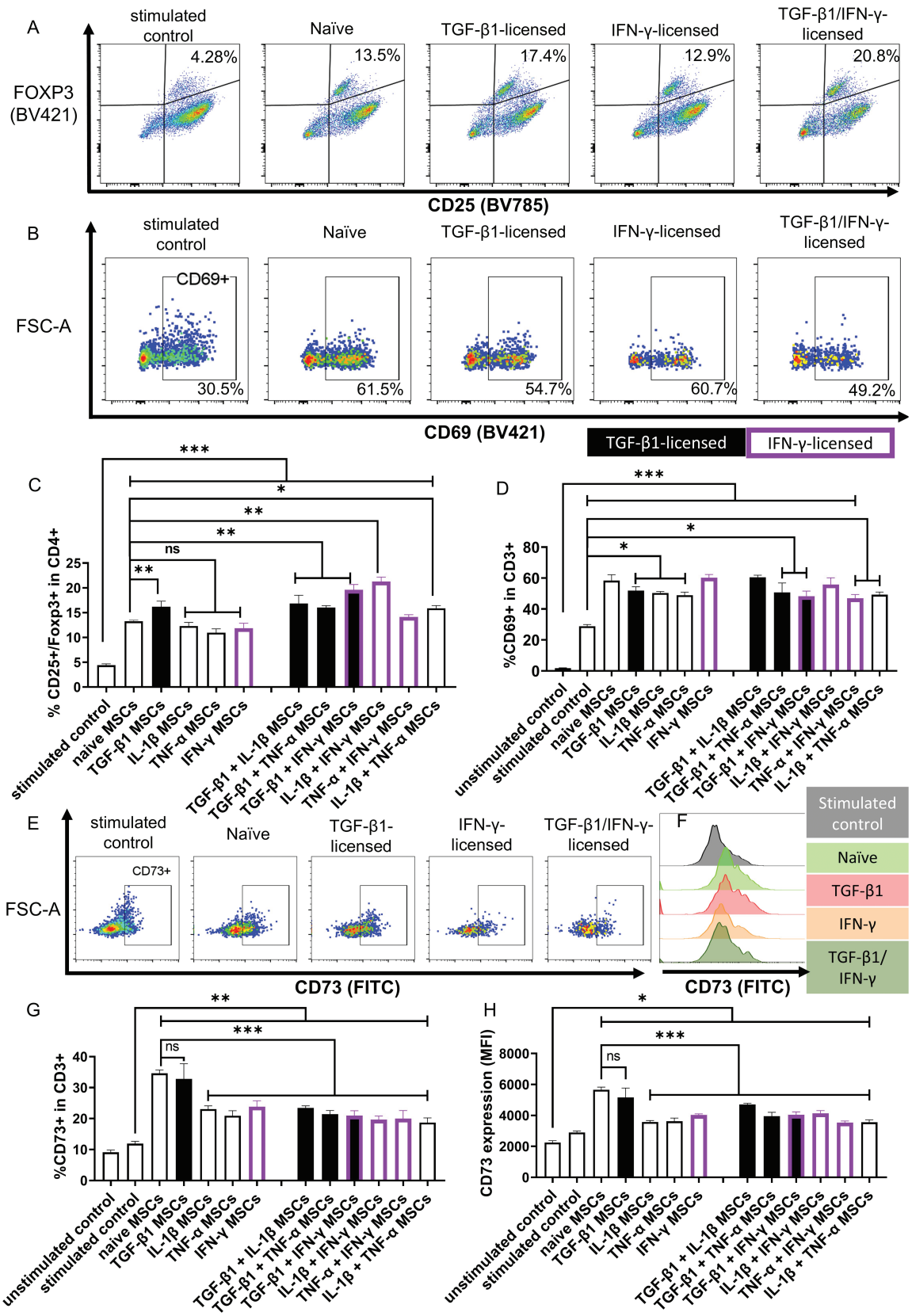


Figure 2. The T-cell phenotype is altered after coculturing with (licensed) mesenchymal stromal cell (MSC). A-B. The representative pseudocolor plots of FOXP3/CD25+ cells (regulatory T cells, Tregs), CD69+ T cells. C-D. The percentage of Tregs and CD69+ T cells. E. The representative pseudocolor plots of CD73+ T cells. F. The representative histograms of CD73 expression. G. The percentage of CD73+ T cells. H. The expression of CD73 (MFI, median fluorescence intensity). All groups, except the unstimulated control, were stimulated with Human T-Activator CD3/CD28 Dynabeads. Flow cytometry was used to assess the protein expression. Representative results of 3 independent experiments are shown ±SD and analyzed by 1-way ANOVA with Tukey's multiple comparisons test. *$P < .05$; **$P < .01$; ***$P < .001$.

Table 1. Detail of information of selected bioinformatic series.

Series	Cell	interfere
GSE126461	Human atrial appendage derived cells (human cardiac mesenchymal stromal-like cells)	Activated by IFN-γ
GSE117901	Human umbilical cord derived MSCs	Early passage vs. late passage;
GSE117901	Human umbilical cord derived MSCs	Activated by TNF-α, IFN-γ
GSE68610	Human MSCs	Activated by IL-1β, TNF-α, and IFN-γ
GSE142816	Human bone marrow-derived MSCs	Activated by IFN-γ and TNF-α
GSE122091	Human embryonic stem cells derived MSCs	Activated by IFN-γ

Statistical analysis was performed using GraphPad Software (8.0.2).

Other

The methods of MSC characterization, peripheral blood mononuclear cell (PBMC) isolation and culture, RT-qPCR, dot-blotting, and KYN quantification are illustrated in the supplementary material. The instruction of mesenchymal stromal cell and peripheral blood mononuclear cell origin in each assay are summarized in Supplementary Table S2.

Results

MSC characterization: IFN-γ but not TGF-β licensing leads to upregulation of HLA-DR on human bone marrow-derived MSCs

Human bone marrow-derived MSCs from 3 healthy donors were used in this study. All the MSCs can differentiate into adipocytes and osteocytes/osteoblasts. Flow cytometry was used to identify protein expression on the cell surface of MSCs (Supplementary Figure S1A negative markers and Supplementary Figure S1B positive markers. CD11b and CD45 were not expressed on MSCs and this did not change following licensing. In addition, CD90, CD73, and CD44 markers were all highly expressed on MSCs from all of the donors and also did not change after licensing. HLA-DR is expressed lowly in all groups of MSCs except IFN-γ-licensed MSCs (Supplementary Figure S1C). HLA-DR upregulation is regarded as enhancing immunosuppressive capacity.[32] This is also consistent with others' work.[33,34] The differentiation capacity (adipogenic and osteogenic) of MSCs from all 3 donors was verified as shown in Supplementary Figure S2A and B. All the MSCs can differentiate into adipocytes and osteocytes/osteoblasts.

IFN-γ and TGF-β1/IFN-γ licensing strengthens the effect of MSCs on inhibiting allogeneic T-cell proliferation

At first, various cytokine-licensed MSCs were cocultured with allogeneic PBMCs to investigate the inhibitory effect on T-cell proliferation, which is regarded as the gold standard for assessing the immunomodulatory potency of MSCs.[35,36] The coculture assay of PBMCs and MSCs is performed as shown in Figure 1A. Flow cytometry was used to assess the T-cell proliferation. The gating strategy of proliferated T cells (CD3+), effector CD4 + T cells (CD3+/CD4+), and effector CD8 + T cells (CD3+/CD8+) are shown in Supplementary Figure S3A. As shown in Figure 1B-D, all MSC treatment groups efficiently inhibit the proliferation of CD3+, CD4+, and CD8+ T cells. Among the single cytokine licensing groups, IFN-γ licensed MSCs showed the strongest inhibitory effect in terms of numbers, even if the *P*-value is not significant. Interestingly, 50 ng/mL IL-1β licensing significantly weakened the inhibitory effect of naïve MSCs on T-cell proliferation. Meanwhile, among the combined cytokine licensing groups, and although not significant, TGF-β1/IFN-γ-licensed MSCs showed the strongest inhibitory effect on T-cell proliferation in terms of numbers. Finally, the percentage of proliferated T cells is less following coculture with TGF-β1/IFN-γ-licensed MSCs (CD3+ and CD3/CD4+) compared to sole IFN-γ-licensed MSCs. This demonstrates that TGF-β1 and IFN-γ may have additive immunomodulatory effects on MSC-licensing.

TGF-β1 licensing of MSCs increases allogeneic regulatory T-cell numbers following coculture assays and induces T-cell immunosuppressive phenotype

Next, the subtype of T cells following MSC coculture was investigated. The percentage of regulatory T cells (Tregs) was first focused on. The gating strategy of Tregs is shown in Supplementary Figure S3B. As shown in Figure 2A and C, all MSC-groups, including naïve and licensed, induced more Tregs than stimulated allogeneic control. TGF-β1-licensed MSCs (black bars) induced more Tregs than the naïve (non-licensed) MSC group and other sole cytokine-treated MSC groups, which suggested TGF-β1 licensing enhances the efficiency of MSCs to induce Tregs. Among dual licensing strategies, TGF-β1 plus another cytokine can induce more Tregs, including IL-1β plus IFN-γ or TNF-α. In terms of Tregs induction, TGF-β1 licensing is the most effective. And TGF-β1/IFN-γ-licensing induced more Tregs than sole use of TGF-β1 in terms of numbers, which demonstrates again that TGF-β1 and IFN-γ may have a synergistic immunomodulatory effect.

Then, we investigated the CD69 expression of T cells. Highly expressed CD69 represents the activation of T cells. The gating strategy is shown in Supplementary Figure S3C. As shown in Figure 2B and D, coculture with allogeneic MSCs significantly increased the ratio of CD69+ T cells, indicating that allogeneic MSC coculture activates T cells. However, compared with naïve MSCs, licensing with either TGF-β1, IL-1β, and TNF-α reduced the CD69 expression as well as a combination of TNF-α plus any other cytokine and TGF-β1 plus IFN-γ. Interestingly, IFN-γ licensing alone did not reduce the number of CD69+ cells only in combination with TGF-β1. In summary, TNF-α is the most effective cytokine because both single and combined use of TNF-α decreased the percentage of CD69+ T cells. Besides, TGF-β1 is also powerful except TGF-β1 plus IL-1β, which possibly demonstrates

IL-1β and TGF-β1 licensing may have an antagonistic effect in CD69 reduction. Specifically, TGF-β1 plus IFN-γ still have the most powerful effect in terms of numbers. This fact further suggests the synergistic effect of TGF-β1 and IFN-γ.

In terms of the T-cell phenotype study, we lastly focused on CD73 expression. It is regarded as an immunosuppressive protein and high CD73 expression represents strong immunomodulatory function. The gating strategy of CD73 expression and CD73+ T cells is shown in Supplementary Figure S3C. The results are shown in Figure 2E-H. Coculture with MSCs increased both CD73 expression and the number of CD73+ T cells. However, compared with naïve MSCs, all of the licensing strategies cannot keep the CD73 expression on a high level except sole TGF-β1 licensing. This fact suggests that only TGF-β1 licensing can maintain the capacity of naïve MSC keeping CD73 highly expressed.

Both TGF-β1 and IFN-γ licensing enhances the effect of MSCs on reducing the TNF-α and IL-1β expression of M1φ THP-1 cells

As described above, IFN-γ licensing was found to enhance the inhibitory effect of MSCs on T-cell proliferation and TGF-β1 licensing to strengthen the effect of MSCs on inducing an immunomodulatory T-cell phenotype. Therefore, these 2 licensing strategies were used to investigate their role modulation of innate immune cells in this MSC/THP-1 co-culture assay. THP-1 cells, as a human leukemia monocytic cell line, have been extensively used to study monocyte/macrophage functions[37,38]

We assessed the expression of 2 pro-inflammatory cytokines TNF-α and IL-1β by M1φ THP-1 cells. The experiment was performed as shown in Figure 3A and cytokine expression is assessed by flow cytometry via intracellular staining. The gating strategy of cytokine expression is shown in Supplementary Figure S4A. As shown in Figure 3B and C, naïve MSCs exhibited the capacity to reduce expressions of both TNF-α and IL-1β by M1φ THP-1 cells. However, both single TGF-β1 and IFN-γ-licensing strengthened the capacity. Moreover, in terms of TNF-α expression, dual TGF-β1, and IFN-γ-licensed MSCs can reduce its expression to a great degree, which suggests that TGF-β1 and IFN-γ are synergistic in reducing TNF-α expression.

Next, we concentrated on the crosstalk between MSCs and M1φ THP-1 cells to primarily address the mechanism of the above effect. The assay was performed as shown in Figure 3D using fluorescently labeled (CTV) naïve or licensed MSCs. The ratio of the MSCs to THP-1 cells was adjusted to 1:1 because when we applied the same ratio as in the previous experiment, the CD11b-negative population (MSCs) were very less, which is possibly due to that MSCs are phagocytosed by M1φ THP-1 cells. As shown in Figure 3E-G, CTV+/CD11b + (Q2 in Figure 3G) represents THP-1 cells that have phagocytosed MSCs. And CTV+/CD11b– represents MSCs that have not crosstalked with THP-1 cells. We found that TGF-β1-licensed MSCs preferentially got in touch with THP-1 cells (Figure 3E) and IFN-γ licensing impeded the crosstalk Figure 3F. This may be why TGF-β1 licensing strengthened the capacity of reducing TNF-α/IL-1β expression, while this cannot explain the mechanism of IFN-γ licensing.

As shown in Figure 3E-G, THP-1 cells that were co-cultured with MSCs are represented by CTV+/CD11b+ (Q2 in Figure 3G). Conversely, CTV+/CD11b– represents MSCs that have not engaged in crosstalk with THP-1 cells. Our findings indicate that TGF-β1-licensed MSCs displayed a preference for interacting with THP-1 cells (Figure 3E), while the crosstalk was impeded by IFN-γ licensing, as depicted in Figure 3F. This difference may explain why TGF-β1 licensing enhanced the capacity to reduce TNF-α/IL-1β expression, whereas the mechanism behind the effects of IFN-γ licensing remains unexplained.

Bioinformatics analysis: potential new potency markers C1s and MX2 are upregulated in strong immunomodulatory MSC groups

After we found the enhanced therapeutic effect of MSCs following cytokine licensing with TGF-β1 and IFN-γ, we wanted to primarily explore the molecular mechanism of these 2 licensing strategies. This mechanism also will contribute to the establishment of MSC immunomodulatory potency. The identification of functional markers of potency and easily deployable standardized and quantifiable methods are necessary.[22] At first, 6 datasets were selected comparing the genome difference between strong and weak immunomodulatory MSCs of MSC-like cells. In this chapter, the 6 selected open-access datasets were analyzed.[39-42]

As shown in Figure 4A, the volcano plot showed that compared to weak immunomodulatory control, in strong immunomodulatory groups, there were plenty of gene upregulation (red dots) and downregulation (blue dots). The twenty most differentially expressed genes were listed as shown in supplementary material Figure 6. IDO1, which was the evidence-based marker, was also significantly upregulated in GSE126461, GSE117901, GSE6861, and GSE142816 with large fold change. In the above standard (logFC > 1 and $P < .05$) the number and name of upregulated genes were counted in each dataset (Figure 4B). Finally, there were 2 genes identified, which were C1s and MX2 and were observed to be upregulated in all datasets. This indicates an association of C1s and MX2 with strong immunomodulatory MSCs. The *P*-value and logFC of C1s and MX2 in each dataset are shown in Figure 4C.

A gene ontology analysis was performed on the basis of the above-upregulated genes (Supplementary Figure S5). The enriched pathways in each dataset were shown. However, none of the pathways was enriched in every group. Relatively, the frequently enriched pathways were the regulation of the immune effector process (belongs to biological process), peptide binding (molecular function), amide binding (molecular function), intrinsic component of organelle (cellular component), and endocytic vesicle (cellular component). These pathways were enriched in the 5 cytokine(s)-licensing groups (GSE126461 activated by IFN-γ, GSE117901 activated by TNF-α, and IFN-γ, GSE68610 activated by IL-1β, TNF-α, and IFN-γ, GSE142816 activated by IFN-γ and TNF-α and GSE122091 activated by IFN-γ).

IFN-γ (or combined with another cytokine) licensing upregulated PD-L1, IDO1, MX2, and C1s from gene to protein

Based on the previous results, 2 bioinformatics-filtered genes and 2 evidence-based genes were identified as the candidate genes to reflect the immunomodulatory capacity of MSCs. These 4 markers are investigated from gene to protein expression. qPCR was performed to quantify the gene expression and several techniques including

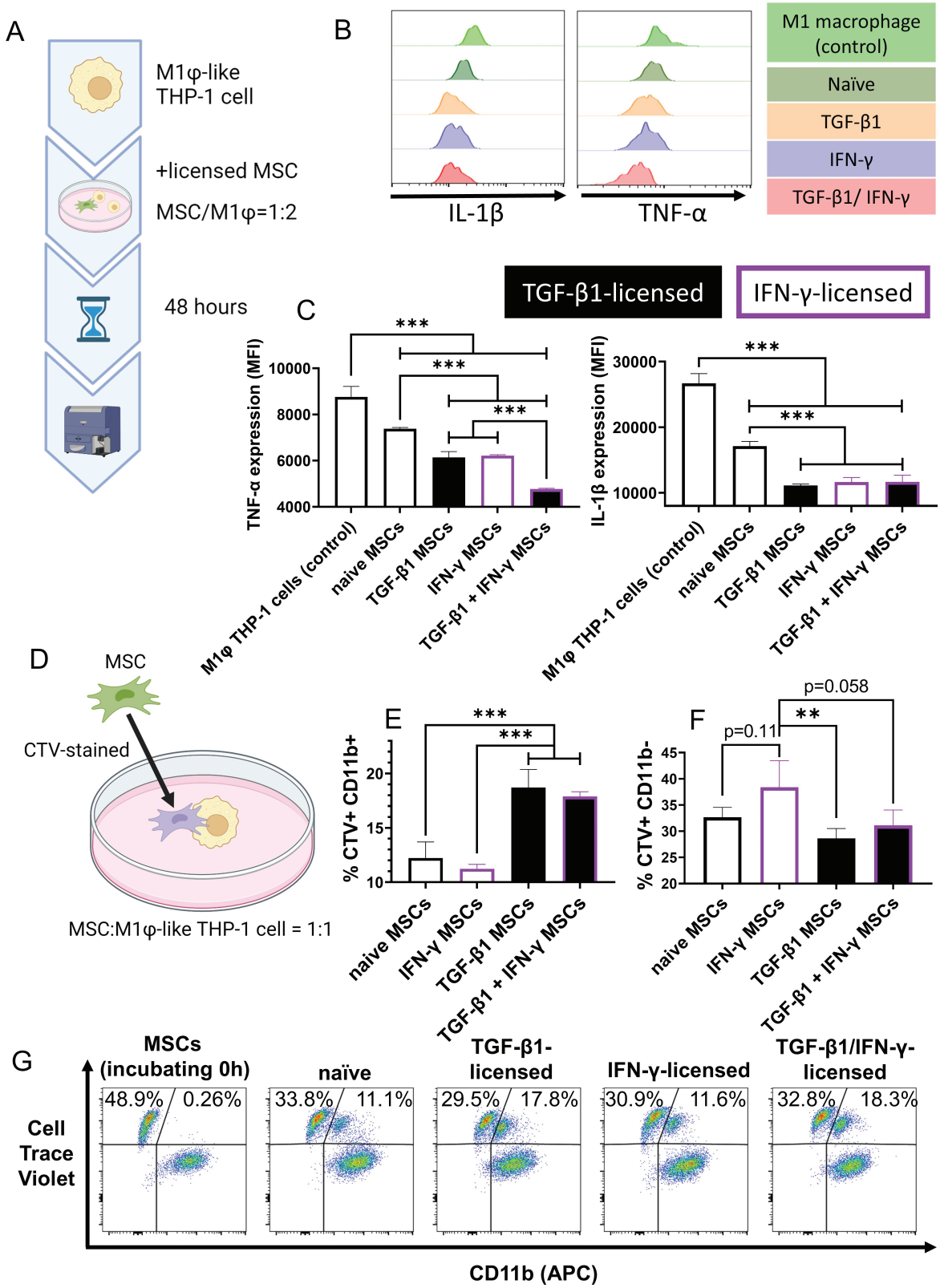


Figure 3. Both TGF-β1 and IFN-γ licensing enhances the effect of mesenchymal stromal cells (MSCs) on reducing the TNF-α and IL-1β expression of M1φ THP-1 cells. A. Schematic illustrating the experimental overview of MSC/THP-1 coculturing assay. B. The representative histograms IL-1β and TNF-α by THP-1 cells. C. The expression of IL-1β and TNF-α by THP-1 cells (MFI, median fluorescence intensity). D. Schematic illustrating the experimental overview of MSC/THP-1 coculturing assay to focus on their crosstalk. E. The percentage of THP-1 cells crosstalk with MSCs (Q2 in Figure 3G). F. The percentage of MSCs which has not crosstalked with THP-1 cells(Q1 in Figure 3G). G. The representative pseudocolor plots of MSC/THP-1 crosstalk assay. Flow cytometry was used to assess the protein and crosstalk. Representative results of 3 independent experiments are shown ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

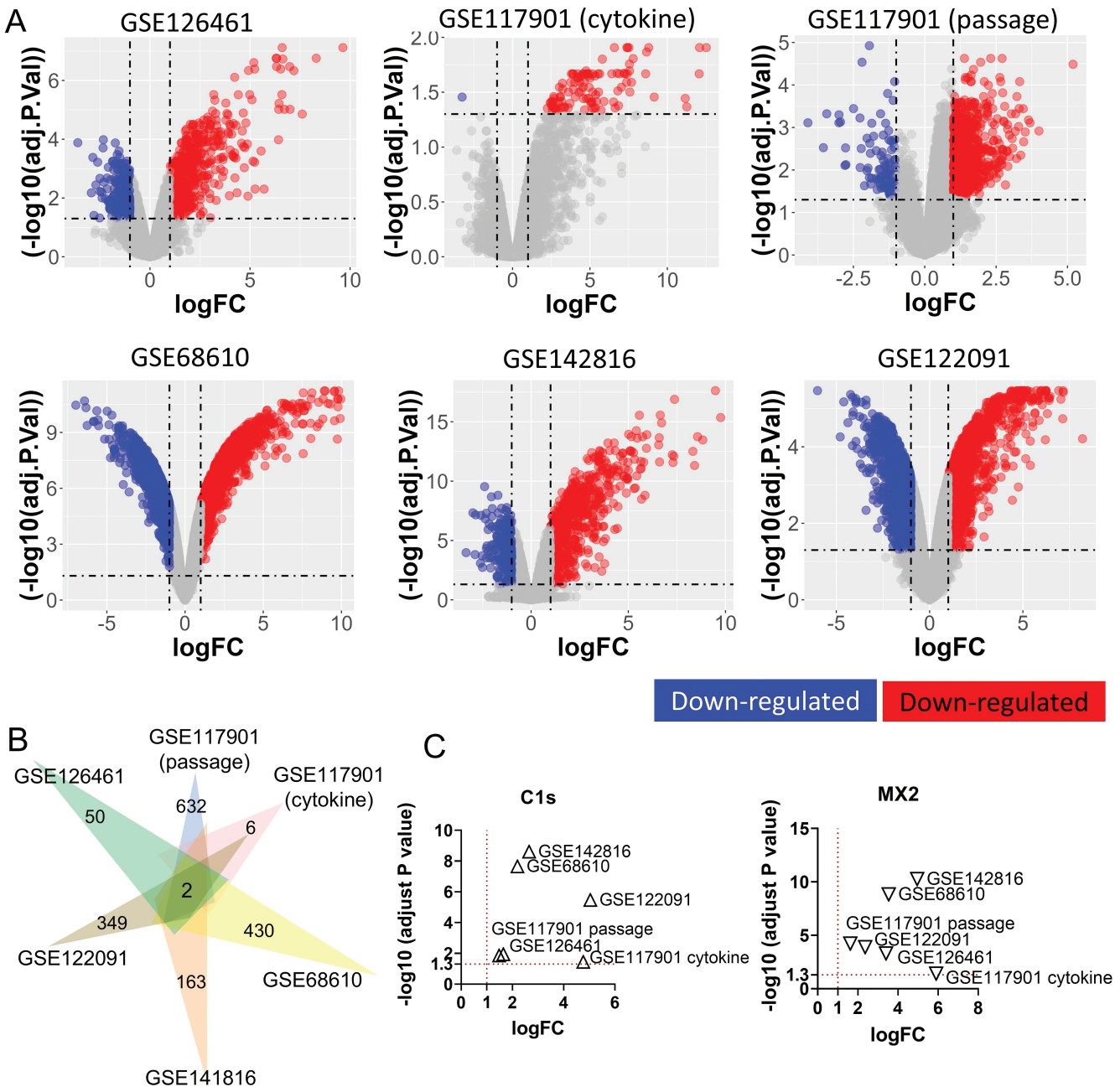


Figure 4. The bioinformatics analysis of strong immunomodulatory mesenchymal stromal cells (MSCs). A. The volcano plots of each datasets. B. The Venn diagram of significant upregulated genes in each dataset. Complement 1 subcomponent (C1s) and interferon-induced GTP-binding protein Mx2 (MX2) are upregulated in all the datasets. C. The fold change and adjust P value of C1s and MX2 in each dataset.

dot-blotting, flow cytometry, and ELISA were performed to quantify the protein expression according to the location of the protein.

First of all, as shown in Figure 5A, B, we focused on PD-L1. PD-L1 was upregulated in RNA and protein by IFN-γ. Interestingly, in terms of RNA, PD-L1 was drastically upregulated by TNF-α/IFN-γ, while TGF-β1/IFN-γ and IL-1β/IFN-γ only upregulated PD-L1 to the same extent as sole IFN-γ. However, in terms of protein, TGF-β1/IFN-γ licensing upregulated PD-L1 to a great extent but the other IFN-γ combinations maintain the effect of sole IFN-γ. IL-1β also upregulated PD-L1 significantly while the effect is weaker than IFN-γ. Besides, other licensing strategies have no effect on PD-L1 upregulation. This indicates a synergistic effect of TGF-β1/IFN-γ licensing on PD-L1 protein expression on MSCs.

Second, in terms of IDO1, as shown in Figure 5C, D, IFN-γ also upregulated IDO1 in RNA and protein. In detail, IFN-γ plus IL-1β or TNF-α had a stronger effect on increasing IDO1 mRNA than IFN-γ plus TGF-β1. Similarly, as for protein level, TGF-β1 combining with IFN-γ inhibited the IDO1 upregulation by sole IFN-γ, while IL-1β or TNF-α combining maintain the upregulation. Other licensing strategies have no effect on IDO1 upregulation.

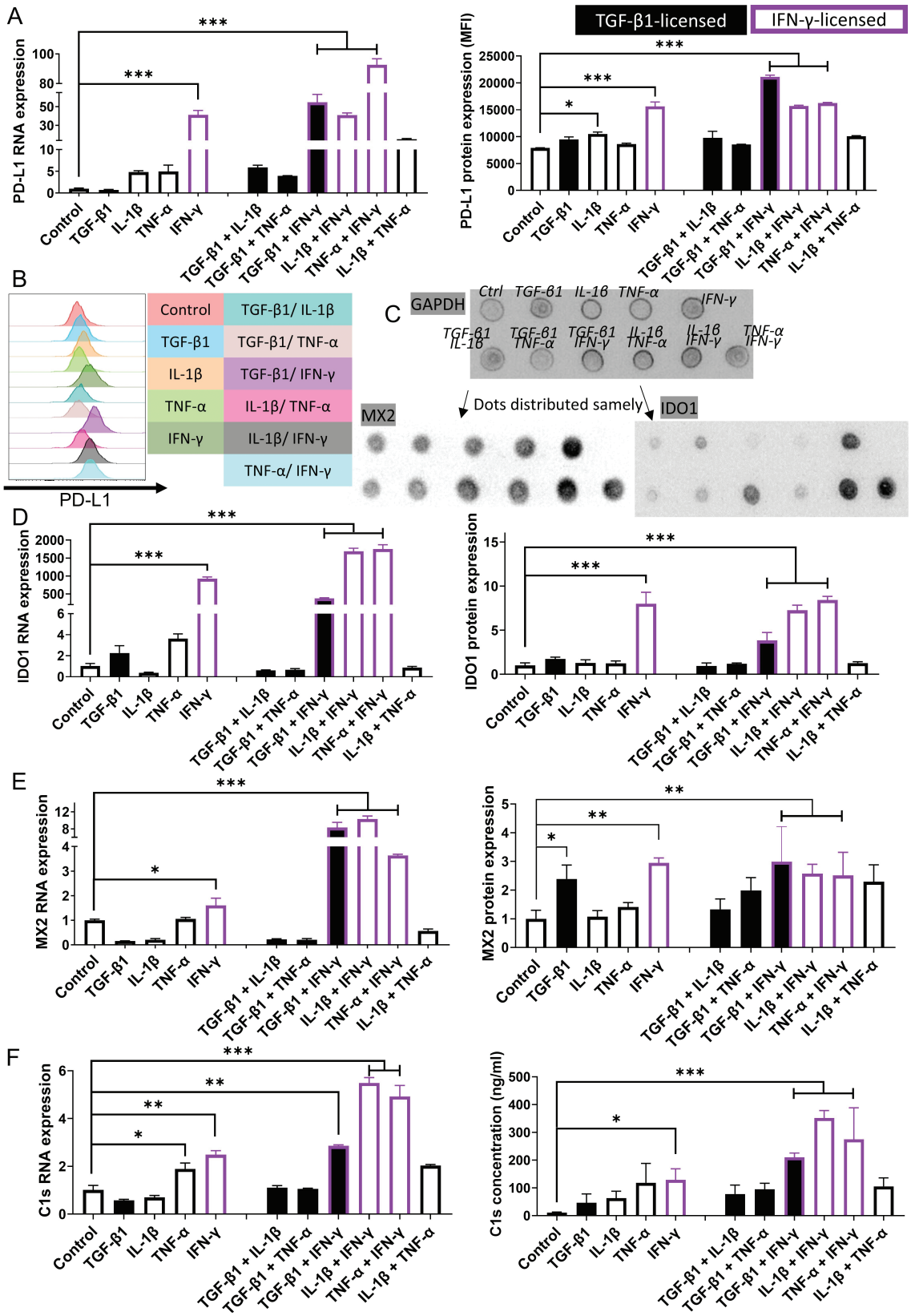


Figure 5. The gene and protein alteration of IFN-γ-licensed mesenchymal stromal cells (MSCs). A. PD-L1 expression in RNA and protein. B. The representative histograms of PD-L1. C. The representative dots of MX2, GAPDH, and IDO1 via dot-blotting in each group. D. IDO1 expression in RNA and protein. E. MX2 expression in RNA and protein. F. C1s expression in RNA and protein. The IDO1 and MX2 protein expressions are assessed by dot-blotting. The PD-L1 protein expression is assessed by flow cytometry. The C1s protein concentration is assessed by ELISA. All of the gene quantification is assessed by qPCR. Representative results of 3 independent experiments are shown ±SD and analyzed by one-way ANOVA with Tukey's multiple comparisons test. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < 0.001$.

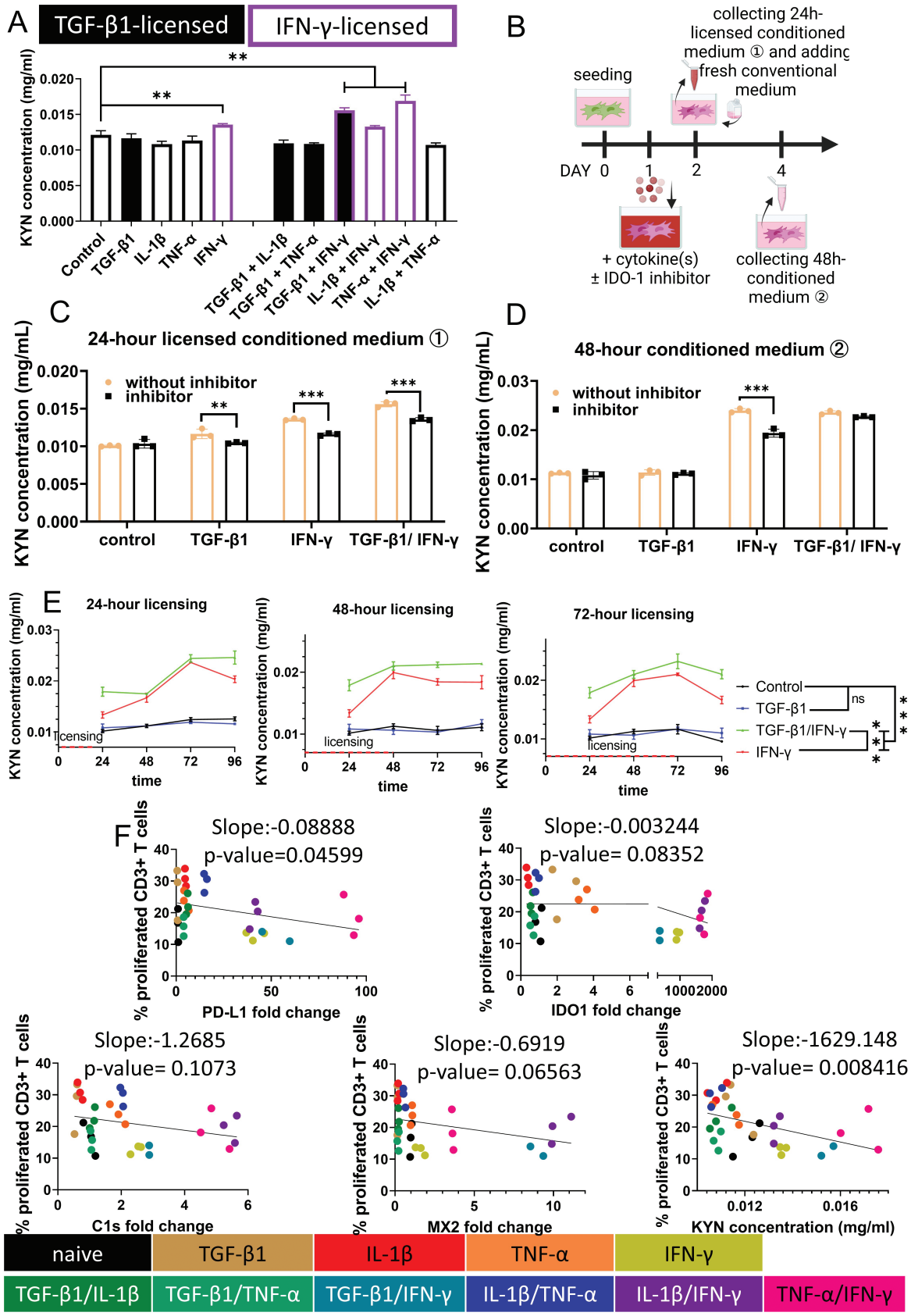


Figure 6. IFN-γ licensing increases IDO1-dependent kynurenine (KYN) concentration and KYN has the strongest negative correlation with the proliferated T-cell ratio. A. KYN concentration in each 24-hour licensing (or not) group. B. Schematic depicting the experimental set-up of KYN IDO1-dependence assay. C-D. KYN concentration corresponds to different time points. E. Time-dependence of KYN concentration. F. The correlation study between selected potency markers and immunomodulatory properties. The slope and *P*-value were labeled. A was analyzed by 1-way ANOVA with Tukey's multiple comparisons test. C-E were done by analysis of 2-way ANOVA with Sidak's multiple comparisons test. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < 0.001$. *F* was analyzed by univariate linear regression models.

For the bioinformatics-filtered MX2, as shown in Figure 5C, D. IFN-γ is still the most effective cytokine to upregulate MX2 in RNA and protein. Other licensing strategies have no effect on this upregulation. As for the combined licensing strategies, IFN-γ plus any other cytokine have a stronger effect on upregulating MX2 in RNA than sole IFN-γ. We performed an extra mRNA qPCR to confirm the upregulation (Supplementary Figure S4C).

Lastly, as shown in Figure 5F, IFN-γ also can upregulate C1s in both RNA and protein levels. As for RNA, IFN-γ plus IL-1β or TNF-α had a stronger effect on upregulation than sole IFN-γ. IFN-γ plus TGF-β1 maintain the upregulation. TNF-α also had a slight but significant effect on C1s RNA upregulation. As for protein, the result is approximately consistent with C1s qPCR. IFN-γ plus IL-1β or TNF-α had a stronger effect on C1s protein upregulation than sole IFN-γ.

IFN-γ licensing increases IDO1-dependent KYN concentration

In terms of immunomodulatory potency assays, characteristics that would be beneficial are simplification and convenience. The above paragraph mainly leveraged qPCR and immune blotting to quantify the gene and protein candidates, respectively. In addition to gene and protein, KYN, as an amino acid generated by IDO1, is of great significance to be a potency marker because this KYN assay is based simple and cost-saving spectrometry-based methodology. This method was verified and the standard curve is shown in Supplementary Figure S4D.

We first quantify the KYN in the 24-hour licensed MSC conditioned medium. As shown in Figure 6A, KYN concentration was increased by IFN-γ, but the synergistic effect caused by combined licensed strategies was not obvious. Other licensing strategies did not have significant effects.

Meanwhile, LW106, an inhibitor of IDO1, was used to confirm the IDO1-dependence of KYN. In the reports, LW106 only inhibits the IDO1 function but maintains its invariable expression.[43] The KYN IDO1-dependence assay is performed as shown in Figure 6B. After 24-hour licensing (Figure 6C), the KYN concentration became lower in the presence of the inhibitor. This indicates that the KYN originated from IDO1. After the licensed MSCs above underwent a 48-hour culturing with fresh medium (Figure 6D), the KYN concentration in a single IFN-γ licensed group was still low. But TGF-β1/IFN-γ licensed MSCs restored more secreted KYN, which possibly demonstrated that TGF-β1/IFN-γ licensing is stronger to IDO1 than a single use of IFN-γ. Considering that IDO1 expression caused by TGF-β1/IFN-γ is lower than sole IFN-γ but TGF-β1/IFN-γ licensing resulted in a higher concentration of KYN, the dual licensed strategy possibly not only increased the expression but also activated the function.

Lastly, the time dependence of KYN secretion is shown in Figure 6E. The KYN concentration peak appeared 72 hours after the beginning of the licensing. And no matter how much time the licensing, the KYN concentration caused by TGF-β1/IFN-γ was significantly higher than sole use of IFN-γ, which suggests the synergistic effect of TGF-β1/IFN-γ at least on IDO1 function.

The correlation study between selected potency markers and immunomodulatory properties

This part is to investigate the correlation between markers (widely accepted PD-L1 and IDO1 and newly found MX2, C1s, and KYN) and immunomodulatory properties. In the presented study, the gene level of C1s, MX2, IDO1, and PD-L1 and the concentration of KYN were regarded as the property of MSCs (independent variable), and the proliferated T-cell ratio in 3 types was regarded as the immunomodulatory capacity of MSCs (dependent variable). We selected the gene level of the markers but not the protein because the gene level in the study was more sensitive. The univariate linear regression models were performed to analyze the correlation. As displayed in Figure 6F and supplementary material Figure 7, all of the highly expressed markers reflected the low-proliferated T-cell ratio, which represents stronger immunomodulatory capacity, and lowly expressed markers reflected the high-proliferated T-cell ratio. Among all the selected markers, the correlation of the widely accepted PD-L1 and the proliferated T-cell ratio was strong with *P*-values less than .05 in all types of T cells. However, the correlation between the IDO1 gene level and the proliferated T-cell ratio was weaker with *P*-values more than 0.05 in CD3+ and CD3/CD4 + T cells. Both C1s and MX2 had a weaker correlation with CD3+ T-cell ratio but strong with CD3/CD4+ and CD3/CD8+ proliferated T-cell ratio. Most importantly, KYN concentration had the strongest correlation with the proliferated T-cell ratio with a very significant statistical difference. This suggests that secreted KYN concentration best reflects the inhibited proliferation of MSCs on T cells. KYN may be the key factor in MSC immunomodulation.

Discussion

Nowadays, co-incubation with allogeneic T cells and thus the quantification of proliferated T cells is the most commonly used method to measure the immunomodulatory potency of MSCs.[35,36] However it requires human blood samples and the consumption of MSCs themselves. In this study, high expressions of MX2, PD-L1, C1s, IDO1, and KYN reflected the strong immunomodulation of MSC to some degree. These detections do not require PBMC. More importantly, C1s and KYN are extracellular. Their detections would not consume MSCs, this is significant in saving MSC samples for the therapy. In other words, C1s and KYN are highly concentrated in the conditioned medium. The MSCs from which they originated can still be used for future experiments. Moreover, as shown in Figure 6F and supplementary material Figure 7, the KYN concentration better reflects the immunomodulatory capacity of MSCs than widely accepted PD-L1 and IDO1. This suggests that secreted KYN concentration best reflects the inhibited proliferation of MSCs on T cells. In the future application of MSCs, the extracellular KYN concentration may be quantified to predict the immunomodulatory capacity of MSCs to achieve standardization.

CD69 and CD73 were selected as 2 markers to define the phenotype of T cells. CD73 is an immunosuppressive molecule, which can degrade adenosine triphosphate to adenosine, which is verified as a strong immunosuppressive substance,[44,45] and initiates anti-inflammatory effects.[46,47] In addition, CD73+ T cells or their extracellular vesicles were reported to be available to CD4+ CD39+ Tregs for the production of immunosuppressive adenosine.[48] In an inflammatory model caused by *Trypanosoma gondii*, CD73 was downregulated which reduced adenosine.[49] This verified that CD73 may be downregulated during inflammation. In terms of CD69, it is an activation marker[50] for T cells for both pro and anti-inflammation. CD69+ T cells were reported for the high production of pro-inflammatory cytokines.[51]

The increase of CD4+/CD69+ T cells triggered inflammation[52] and aggravated autoimmune disease.[53,54] For the anti-inflammation side, CD69^{hi} Tregs were hyperreactive Tregs which are immunosuppressive.[55] However, the proportion of Tregs in T cells is very small. Therefore we believe the total effect of CD69^{hi} T cell is pro-inflammatory. In general, CD73 was selected as the immunosuppressive marker and CD73^{hi} represented strong immunomodulatory capacity. CD69 was selected as the activation marker and CD69^{hi} represented activated inflammatory T cell. We found that high expression of immunosuppressive CD73 can be kept by sole TGF-β1 licensing and sole IFN-γ licensing is not conductive to decrease inflammatory CD69^{hi} ratio.

In single cytokine licensing, the effect of TGF-β1 is limited in upregulating the potency markers. Because TGF-β1 did not upregulate selected markers and drastically inhibited T-cell proliferation. IFN-γ is the most effective cytokine to enhance MSC immunomodulation because IFN-γ licensing strengthens the inhibitory effect of MSC on T-cell proliferation. Interestingly, TGF-β1/IFN-γ combined licensing could be more immunomodulatory. This combined licensing synergistically induced higher expression of MX2, PD-L1, C1s, and KYN than a single use of IFN-γ. More importantly, this combining licensed MSCs were the most effective way both to induce immunosuppressive Tregs and inhibit T-cell proliferation even if the *P*-value is not very significant.

For newly found IFN-γ licensing-related markers, C1s was also verified upregulation in the IFN-γ response-related clear cell renal cell carcinoma model.[56] Besides, C1s is a component of the complement system. The complement system is involved in phagocytosis,[57] including the phagocytosis of MSC.[58] However, C1s was reported to have a limited impact on the clearance of early apoptotic cells and post-phagocytosis immunosuppressive properties. It only affects the phagocytosis of late apoptotic cells.[59] In terms of KYN, its immunosuppressive property is widely recognized. Recent studies revealed that tryptophan 2,3-dioxygenase 2-induced increase in KYN played a pivotal role in glioma development through immunosuppressive effects.[60] In autoimmunity, KYN also can serve as an effective immunomodulatory adjuvant candidate for type 1 diabetes vaccines.[61] Lastly for MX2, up to now, there is not any published study correlating MX2 with immunomodulation.

In terms of potency markers for TGF-β1 licensing, the presented study failed to find an independent one. Future studies should focus on identifying a potency marker for TGF-β licensing of MSCs. This could be informed by appropriate RNA sequencing datasets and immunological assay signaling molecules. The present study is the first to find TGF-β1 licensing increases the crosstalk between MSCs and macrophages. In the above results, IFN-γ has an obviously different pathway to activate the MSCs when compared to TGF-β1. In the case that TGF-β1 do not upregulate our 5 selected potency candidate markers, it is reasonable to speculate that TGF-β1 facilitates the MSC crosstalk with macrophages. This licensing pathway is compatible with the licensing caused by IFN-γ. Therefore, in many aspects, TGF-β1 and IFN-γ have a synergistic effect.

Conclusion

Human bone marrow-derived MSCs can be licensed by TGF-β1 and IFN-γ. TGF-β1 licensing enhanced the effect of MSCs on inducing the T cells to an immunosuppressive phenotype with CD25^{hi}/FOXP3^{hi}, CD69^{low}, and CD73^{hi}, while IFN-γ licensing strengthened the inhibitory effect on T-cell proliferation. Both licensing strategies help MSC to inhibit M1 macrophages expressing TNF-α and IL-1β. As for the molecular mechanism of cytokine licensing, TGF-β1 and IFN-γ target different pathways leading to differential expression of potential potency markers. IFN-γ can upregulate PD-L1, MX2, C1s, and IDO1 from gene to protein, and upregulated IDO1 secrets more KYN, which can best reflect the inhibited proliferation of MSCs on T cells. However, TGF-β1 licensing might work by increasing the cellular crosstalk. Lastly, these 2 cytokines have a synergistic effect in some immunomodulatory mechanisms.

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Author contributions

Jiemin Wang: conception, design, experiment and manuscript writing. Yingying Zhou: design and data analysis. Ellen Donohoe, Aoife Canning, Seyedmohammad Moosavizadeh : methodology. Aideen E Ryan: conception and supervision. Thomas Ritter: conception, financial support, supervision.

Conflicts of interest

The authors declared no potential conflicts of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary material

Supplementary material is available at *Stem Cells* online.

References

1. Castro LL, Kitoko JZ, Xisto DG, et al. Multiple doses of adipose tissue-derived mesenchymal stromal cells induce immunosuppression in experimental asthma. *Stem Cells Transl Med*. 2020;9(2):250-260. https://doi.org/10.1002/sctm.19-0120

2. Poggi A, Varesano S, Zocchi MR. How to hit mesenchymal stromal cells and make the tumor microenvironment immunostimulant rather than immunosuppressive. *Front Immunol*. 2018;9:262.
3. Sukho P, Hesselink JW, Kops N, et al. Human mesenchymal stromal cell sheets induce macrophages predominantly to an anti-inflammatory phenotype. *Stem Cells Dev*. 2018;27(13):922-934. https://doi.org/10.1089/scd.2017.0275
4. Ge XH, Shi K, Hou J, et al. Galectin-1 secreted by bone marrow-derived mesenchymal stem cells mediates anti-inflammatory responses in acute airway disease. *Exp Cell Res*. 2021;407(1):112788. https://doi.org/10.1016/j.yexcr.2021.112788
5. Ortiz-Virumbrales M, Menta R, Pérez LM, et al. Human adipose mesenchymal stem cells modulate myeloid cells toward an anti-inflammatory and reparative phenotype: role of IL-6 and PGE2. *Stem Cell Res Therapy*. 2020;11(1):462. https://doi.org/10.1186/s13287-020-01975-2
6. Marinescu CI, Preda MB, Burlacu A. A procedure for in vitro evaluation of the immunosuppressive effect of mouse mesenchymal stem cells on activated T cell proliferation. *Stem Cell Res Therapy*. 2021;12(1).
7. Azevedo RI, Minskaia E, Fernandes-Platzgummer A, et al. Mesenchymal stromal cells induce regulatory T cells via epigenetic conversion of human conventional CD4 T cells in vitro. *Stem Cells*. 2020;38(8):1007-1019. https://doi.org/10.1002/stem.3185
8. Treacy O, Lynch K, Murphy N, et al. Subconjunctival administration of low-dose murine allogeneic mesenchymal stromal cells promotes corneal allograft survival in mice. *Stem Cell Res Therapy*. 2021;12(1):227. https://doi.org/10.1186/s13287-021-02293-x
9. Squillaro T, Peluso G, Galderisi U. Clinical trials with mesenchymal stem cells: an update. *Cell Transplant*. 2016;25(5):829-848. https://doi.org/10.3727/096368915X689622
10. Oliver-Vila I, Ramírez-Moncayo C, Grau-Vorster M, et al. Optimisation of a potency assay for the assessment of immunomodulative potential of clinical grade multipotent mesenchymal stromal cells. *Cytotechnology*. 2018;70(1):31-44. https://doi.org/10.1007/s10616-017-0186-0
11. Klinker MW, Marklein RA, Lo Surdo JL, Wei C-H, Bauer SR. Morphological features of IFN-gamma-stimulated mesenchymal stromal cells predict overall immunosuppressive capacity. *Proc Natl Acad Sci USA*. 2017;114(13):E2598-E2607. https://doi.org/10.1073/pnas.1617933114
12. Patrick MD, Annamalai RT. Licensing microgels prolong the immunomodulatory phenotype of mesenchymal stromal cells. *Front Immunol*. 2022;13:987032. https://doi.org/10.3389/fimmu.2022.987032
13. Guess AJ, Daneault B, Wang R, et al. Safety profile of good manufacturing practice manufactured interferon gamma-primed mesenchymal stem/stromal cells for clinical trials. *Stem Cells Transl Med*. 2017;6(10):1868-1879. https://doi.org/10.1002/sctm.16-0485
14. Liu HC, et al. IL-1 beta-primed mesenchymal stromal cells exert enhanced therapeutic effects to alleviate Chronic Prostatitis/Chronic Pelvic Pain Syndrome through systemic immunity. *Stem Cell Res Therapy*. 2021;12(1).
15. Aussel C, Baudry N, Grosbot M, et al. IL-1 beta primed mesenchymal stromal cells moderate hemorrhagic shock-induced organ injuries. *Stem Cell Res Therapy*. 2021;12(1):438. https://doi.org/10.1186/s13287-021-02505-4
16. Shin TH, Ahn J-S, Oh S-J, et al. TNF-alpha priming elicits robust immunomodulatory potential of human tonsil-derived mesenchymal stem cells to alleviate murine colitis. *Biomedicines*. 2020;8(12):561. https://doi.org/10.3390/biomedicines8120561
17. Mead B, Chamling X, Zack DJ, Ahmed Z, Tomarev S. TNF alpha-mediated priming of mesenchymal stem cells enhances their neuroprotective effect on retinal ganglion cells. *Investig Ophthalmol Visual Sci*. 2020;61(2):6. https://doi.org/10.1167/iovs.61.2.6
18. Lynch K, Treacy O, Chen X, et al. TGF-beta1-licensed murine MSCs show superior therapeutic efficacy in modulating corneal allograft immune rejection in vivo. *Mol Ther*. 2020;28(9):2023-2043. https://doi.org/10.1016/j.ymthe.2020.05.023
19. Garg A, Khan S, Luu N, et al. TGF beta(1) priming enhances CXCR3-mediated mesenchymal stromal cell engraftment to the liver and enhances anti-inflammatory efficacy. *J Cell Mol Med*. 2023;27(6):864-878. https://doi.org/10.1111/jcmm.17698
20. Guan Q, Li Y, Shpiruk T, Bhagwat S, Wall DA. Inducible indoleamine 2,3-dioxygenase 1 and programmed death ligand 1 expression as the potency marker for mesenchymal stromal cells. *Cytotherapy*. 2018;20(5):639-649. https://doi.org/10.1016/j.jcyt.2018.02.003
21. Nolta JA, Galipeau J, Phinney DG. Improving mesenchymal stem/stromal cell potency and survival Proceedings from the International Society of Cell Therapy (ISCT) MSC preconference held in May 2018, Palais des Congres de Montreal, Organized by the ISCT MSC Scientific Committee. *Cytotherapy*. 2020;22(3):123-126. https://doi.org/10.1016/j.jcyt.2020.01.004
22. Galipeau J, Krampera M, Barrett J, et al. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. *Cytotherapy*. 2016;18(2):151-159. https://doi.org/10.1016/j.jcyt.2015.11.008
23. Robb KP, Fitzgerald JC, Barry F, Viswanathan S. Mesenchymal stromal cell therapy: progress in manufacturing and assessments of potency. *Cytotherapy*. 2019;21(3):289-306. https://doi.org/10.1016/j.jcyt.2018.10.014
24. Schneider RS, Vela AC, Williams EK, et al. High-throughput on-chip human mesenchymal stromal cell potency prediction. *Adv Healthcare Mater*. 2022;11(2):e2101995. https://doi.org/10.1002/adhm.202101995
25. Gonzalez-Pujana A, Vining KH, Zhang DKY, et al. Multifunctional biomimetic hydrogel systems to boost the immunomodulatory potential of mesenchymal stromal cells. *Biomaterials*. 2020;257:120266. https://doi.org/10.1016/j.biomaterials.2020.120266
26. Skibber MA, Olson SD, Prabhakara KS, Gill BS, Cox CS. Enhancing mesenchymal stromal cell potency: inflammatory licensing via mechanotransduction. *Front Immunol*. 2022;13:874698. https://doi.org/10.3389/fimmu.2022.874698
27. He HP, Takahashi A, Mukai T, et al. The immunomodulatory effect of triptolide on mesenchymal stromal cells. *Front Immunol*. 2021;12.
28. Salminen A. Role of indoleamine 2,3-dioxygenase 1 (IDO1) and kynurenine pathway in the regulation of the aging process. *Ageing Res Rev*. 2022;75:101573. https://doi.org/10.1016/j.arr.2022.101573
29. Torosyan R, Huang S, Bommi PV, et al. Hypoxic preconditioning protects against ischemic kidney injury through the IDO1/kynurenine pathway. *Cell Rep*. 2021;36(7):109547. https://doi.org/10.1016/j.celrep.2021.109547
30. Bellucci M, Pompa A, De Marcos Lousa C, et al. Human Indoleamine 2,3-dioxygenase 1 (IDO1) expressed in plant cells induces kynurenine production. *Int J Mol Sci*. 2021;22(10):5102. https://doi.org/10.3390/ijms22105102
31. Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer. *BMC Bioinf*. 2014;15(1):293. https://doi.org/10.1186/1471-2105-15-293
32. van Megen KM, van 't Wout ET, Lages Motta J, et al. Activated mesenchymal stromal cells process and present antigens regulating adaptive immunity. *Front Immunol*. 2019;10:694. https://doi.org/10.3389/fimmu.2019.00694
33. Lenero C, Bowles AC, Correa D, Kouroupis D. Characterization and response to inflammatory stimulation of human endometrial-derived mesenchymal stem/stromal cells. *Cytotherapy*. 2022;24(2):124-136. https://doi.org/10.1016/j.jcyt.2021.07.005
34. Grau-Vorster M, Laitinen A, Nystedt J, Vives J. HLA-DR expression in clinical-grade bone marrow-derived multipotent

mesenchymal stromal cells: a two-site study. *Stem Cell Res Therapy*. 2019;10(1):164. https://doi.org/10.1186/s13287-019-1279-9
35. Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. *Front Immunol*. 2015;6:560. https://doi.org/10.3389/fimmu.2015.00560
36. De Wolf C, Van De Bovenkamp M, Hoefnagel M. Regulatory perspective on in vitro potency assays for human mesenchymal stromal cells used in immunotherapy. *Cytotherapy*. 2017;19(7):784-797. https://doi.org/10.1016/j.jcyt.2017.03.076
37. Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. *Int Immunopharmacol*. 2014;23(1):37-45. https://doi.org/10.1016/j.intimp.2014.08.002
38. Forrester MA, Wassall HJ, Hall LS, et al. Similarities and differences in surface receptor expression by THP-1 monocytes and differentiated macrophages polarized using seven different conditioning regimens. *Cell Immunol*. 2018;332:58-76. https://doi.org/10.1016/j.cellimm.2018.07.008
39. Diedrichs F, Stolk M, Jürchott K, et al. Enhanced immunomodulation in inflammatory environments favors human cardiac mesenchymal stromal-like cells for allogeneic cell therapies. *Front Immunol*. 2019;10:1716. https://doi.org/10.3389/fimmu.2019.01716
40. Fang XH, Abbott J, Cheng L, et al. Human mesenchymal stem (stromal) cells promote the resolution of acute lung injury in part through Lipoxin A4. *J Immunol*. 2015;195(3):875-881. https://doi.org/10.4049/jimmunol.1500244
41. Mazzoni A, Maggi L, Montaini G, et al. Human T cells interacting with HNSCC-derived mesenchymal stromal cells acquire tissue-resident memory like properties. *Eur J Immunol*. 2020;50(10):1571-1579. https://doi.org/10.1002/eji.202048544
42. Selich A, Zimmermann K, Tenspolde M, et al. Umbilical cord as a long-term source of activatable mesenchymal stromal cells for immunomodulation. *Stem Cell Res Therapy*. 2019;10(1):285. https://doi.org/10.1186/s13287-019-1376-9
43. Fu R, Zhang Y-W, Li H-M, et al. LW106, a novel indoleamine 2,3-dioxygenase 1 inhibitor, suppresses tumour progression by limiting stroma-immune crosstalk and cancer stem cell enrichment in tumour micro-environment. *Br J Pharmacol*. 2018;175(14):3034-3049. https://doi.org/10.1111/bph.14351
44. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. *Nat Rev Cancer*. 2017;17(12):709-724. https://doi.org/10.1038/nrc.2017.86
45. Faraoni EY, Singh K, Chandra V, et al. CD73-dependent adenosine signaling through Adora2b drives immunosuppression in ductal pancreatic cancer. *Cancer Res*. 2023;83(7):1111-1127. https://doi.org/10.1158/0008-5472.CAN-22-2553
46. Zhuang RL, Meng Q, Ma X, et al. CD4(+)FoxP3(+)CD73(+) regulatory T cell promotes cardiac healing post-myocardial infarction. *Theranostics*. 2022;12(6):2707-2721. https://doi.org/10.7150/thno.68437
47. Eltzschig HK, Thompson LF, Karhausen J, et al. Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. *Blood*. 2004;104(13):3986-3992. https://doi.org/10.1182/blood-2004-06-2066
48. Schuler PJ, Saze Z, Hong C-S, et al. Human CD4(+)CD39(+) regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73(+) exosomes or CD73(+) cells. *Clin Exp Immunol*. 2014;177(2):531-543. https://doi.org/10.1111/cei.12354
49. Francois V, Shehade H, Acolty V, et al. Intestinal immunopathology is associated with decreased CD73-generated adenosine during lethal infection. *Mucosal Immunol*. 2015;8(4):773-784. https://doi.org/10.1038/mi.2014.108
50. Cibrian D, Sanchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol*. 2017;47(6):946-953. https://doi.org/10.1002/eji.201646837
51. Wienke J, Veldkamp SR, Struijf EM, et al. T cell interaction with activated endothelial cells primes for tissue-residency. *Front Immunol*. 2022;13:827786. https://doi.org/10.3389/fimmu.2022.827786
52. Kimura MY, Hayashizaki K, Tokoyoda K, et al. Crucial role for CD69 in allergic inflammatory responses: CD69-Myl9 system in the pathogenesis of airway inflammation. *Immunol Rev*. 2017;278(1):87-100. https://doi.org/10.1111/imr.12559
53. Kilmartin DJ, Fletcher ZJ, Almeida JA, et al. CD69 expression on peripheral CD4(+) T cells parallels disease activity and is reduced by mycophenolate mofetil therapy in uveitis. *Investig Ophthalmol Vis Sci* 2001;42(6):1285-1292.
54. Peixoto TV, Carrasco S, Botte DAC, et al. CD4(+)CD69(+) T cells and CD4(+)CD25(+)FoxP3(+) Treg cells imbalance in peripheral blood, spleen and peritoneal lavage from pristane-induced systemic lupus erythematosus (SLE) mice. *Adv Rheumatol*. 2019;59(1).
55. Yu L, Yang F, Zhang F, et al. CD69 enhances immunosuppressive function of regulatory T-cells and attenuates colitis by prompting IL-10 production. *Cell Death Dis*. 2018;9(9).
56. Liu LX, Du X, Fang J, et al. Development of an interferon gamma response-related signature for prediction of survival in clear cell renal cell carcinoma. *J Inflamm Res*. 2021;14:4969-4985. https://doi.org/10.2147/JIR.S334041
57. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res*. 2010;20(1):34-50. https://doi.org/10.1038/cr.2009.139
58. Gavin C, Meinke S, Heldring N, et al. The complement system is essential for the phagocytosis of mesenchymal stromal cells by monocytes. *Front Immunol*. 2019;10.
59. Colonna L, Parry GC, Panicker S, Elkon KB. Uncoupling complement C1s activation from C1q binding in apoptotic cell phagocytosis and immunosuppressive capacity. *Clin Immunol*. 2016;163:84-90. https://doi.org/10.1016/j.clim.2015.12.017
60. Zhong CH, Peng L, Tao B, et al. TDO2 and tryptophan metabolites promote kynurenine/AhR signals to facilitate glioma progression and immunosuppression. *Am J Cancer Res*. 2022;12(6):2558-2575.
61. Sun J, Shi J, Li J, et al. The effect of immunosuppressive adjuvant kynurenine on type 1 diabetes vaccine. *Front Immunol*. 2021;12.