EXTENDED REPORT

PPARβ/δ directs the therapeutic potential of mesenchymal stem cells in arthritis

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ABSTRACT

Objectives To define how peroxisome proliferator-activated receptor (PPAR) β/δ expression level in mesenchymal stem cells (MSCs) could predict and direct both their immunosuppressive and therapeutic properties. PPARβ/δ interacts with factors such as nuclear factor-kappa B (NF-κB) and regulates the expression of molecules including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. Since these molecules are critical for MSC function, we investigated the role of PPARβ/δ on MSC immunosuppressive properties.

Methods We either treated human MSCs (hMSCs) with the irreversible PPARβ/δ antagonist (GSK3787) or derived MSCs from mice deficient for PPARβ/δ (PPARβ/$\delta^{-/-}$ MSCs). We used the collagen-induced arthritis (CIA) as model of immune-mediated disorder and the MSC-immune cell coculture assays.

Results Modulation of PPARβ/δ expression in hMSCs either using GSK3787 or hMSCs from different origin reveals that MSC immunosuppressive potential is inversely correlated with *Ppard* expression. This was consistent with the higher capacity of PPARβ/$\delta^{-/-}$ MSCs to inhibit both the proliferation of T lymphocytes, in vitro, and arthritic development and progression in CIA compared with PPARβ/$\delta^{+/+}$ MSCs. When primed with proinflammatory cytokines to exhibit an immunoregulatory phenotype, PPARβ/$\delta^{-/-}$ MSCs expressed a higher level of mediators of MSC immunosuppression including VCAM-1, ICAM-1 and nitric oxide (NO) than PPARβ/$\delta^{+/+}$ MSCs. The enhanced NO_2 production by PPARβ/$\delta^{-/-}$ MSCs was due to the increased retention of NF-κB p65 subunit on the κB elements of the inducible nitric oxide synthase promoter resulting from PPARβ/δ silencing.

Conclusions Our study is the first to show that the inhibition or knockdown of PPARβ/δ in MSCs primes their immunoregulatory functions. Thus, the regulation of PPARβ/δ expression provides a new strategy to generate therapeutic MSCs with a stable regulatory phenotype.

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INTRODUCTION

Peroxisome proliferator-activated receptor (PPAR)β/δ displays a variety of biological functions. Indeed, in addition to its role in lipid and glucose metabolism, cell terminal differentiation and proliferation, PPARβ/δ possesses anti-inflammatory activities including inhibition of cytokine production, nuclear factor-kappa B (NF-κB) signalling and cell adhesion molecule expression.[1–3]

PPARβ/δ is expressed by mesenchymal stem cells (MSCs),[4 5] which beyond their role in tissue repair, wound healing and haematopoiesis support, are able to modulate the immune system.[6 7] Although incompletely understood and subject to controversy, the mechanisms involved in MSC immunosuppressive properties follow multiple redundant pathways.[6 8–12] MSCs do not display innate immunosuppressive properties rather upon stimulation with proinflammatory cytokines they become immunosuppressive in a dose-dependent manner and through both contact-dependent mechanisms and soluble factors.[13] The soluble molecules produced by MSCs involved in their immunosuppressive properties, might differ according to the species they originate from. Some factors are produced both by murine and human MSCs, such as prosprostaglandin E2 (PGE2), interleukin (IL)-10, programmed cell death 1 ligand 1 and IL-6,[6 14–19] other are specific for human or mouse. Murine MSCs use inducible nitric oxide synthase (iNOS) producing nitric oxide (NO), which is highly immunosuppressive at high concentrations through largely undefined mechanisms.[6 17 20 21] Adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 cooperate with suppressive molecules including NO to mediate MSC immunosuppressive properties.[22] Indeed, adhesion molecules promote T cells anchoring MSCs, where high concentrations of immunosuppressive factors such as NO are produced and inhibit T cell proliferation. ICAM-1 and VCAM-1 inhibition significantly reversed MSC immunomodulatory function both in vitro and in vivo.[23 24]

Through their potent immunomodulatory functions, MSCs appear as candidate of choice for the treatment of inflammatory/autoimmune diseases. However, although MSC therapy is considered safe, phase III clinical trials for the treatment of inflammatory/autoimmune diseases using MSCs did not show any therapeutic effect.[25] This inconclusive effect related to the use of MSCs in large-scale clinical trials might be due to the absence of an appropriate cytokine environment that MSCs face in vivo to stimulate their immunosuppressive functions. Therefore, the therapeutic effect of MSCs in vivo needs to be enhanced for clinical applications either by controlling the in vivo microenvironment of MSCs or by preconditioning the cells.

With the objective to achieve an efficient clinical application of MSCs and to enhance their

therapeutic potential to treat inflammatory diseases, we addressed the role of PPARβ/δ in the ability of MSCs to control immune responses. Since PPARβ/δ displayed potent anti-inflammatory activities, we investigated whether the modulation of PPARβ/δ expression in MSCs could modify both their immunosuppressive and therapeutic properties. Using MSCs derived from PPARβ/δ-deficient mice, we showed that PPARβ/δ is pivotal for MSC immunomodulatory effect both in vitro and in vivo. Thus, our findings improve our knowledge on the mechanisms underlying MSC properties on immune responses and identify PPARβ/δ as a promising target for enhancing the therapeutic effect of MSCs on inflammatory/autoimmune diseases.

METHODS

Isolation and characterisation of MSCs

For human bone marrow-derived (hBM) MSCs, menstrual blood stromal stem cells (Men-SCs) and umbilical cord (UC)-MSCs isolation, we used the published protocols.[26 27] Murine MSCs were isolated as previously described[17] from BM of C57BL/6 mice, *Ppard*$^{fl/fl}$ sox2cre^{tg} PPARβ/δ-deficient mice referred as PPARβ/δ$^{-/-}$ MSCs and their wild-type *Ppard*$^{fl/+}$ littermates referred as PPARβ/δ$^{+/+}$ MSCs provided by Béatrice Desvergne.[5] MSCs were characterised according to the surface expression of CD44, CD29 and CD105 and the non-expression of haematopoietic markers such as CD45 and CD11b.

Proliferation assays

Freshly isolated splenocytes or T-CD4^{+} from DBA/1 mice were labelled with CellTrace Violet (CTV) (Life-Technology, Saint Aubin, France) and activated with 5 µg/mL of concanavalin A (ConA) (Sigma-Aldrich) or CD3/CD28 beads (Invitrogen), respectively. Then, splenocytes were cultured alone or in the presence of MSCs at a cell ratio of 1 MSC per 10 splenocytes or 1–50 in mixed lymphocyte reaction (MLR) media. When indicated, PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs were treated with the inhibitor of NF-κB activity (Bay 11–7082; Sigma-Aldrich) for 20 min at a concentration of 1 µg/mL before activation with 20 ng/mL interferon-γ (IFN-γ) and 10 ng/mL tumour necrosis factor-α (TNF-α; R&D Systems, Lille, France) during 24 h. For neutralisation of VCAM and ICAM, specific capture antibodies (BD Biosciences, Le Pont de Claix, France) were added to the coculture while for inhibition of NO production, the chemical inhibitor L-NAME (Sigma-Aldrich) was used. After 72 h, proliferation was quantified by flow cytometry.

Measurement of cell adherence under flow conditions

PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs were labelled with CellTracker Red CMTPX (Life Technologies) and freshly isolated splenocytes from DBA mice were stained with carboxyfluorescein succinimidyl ester (CFSE) Green Dye (Life Technologies) following manufacturer's recommendations. Concentration of 7.5×10^4 MSCs were seeded in a µ-Slide I 0.2 Luer ibiTreat (Biovalley, Marne-la-Vallée, France) and activated with 20 ng/mL of IFN-γ and 10 ng/mL TNF-α for 24 h. Labelled splenocytes were activated with ConA for 24 h before the flow assay was performed. During flow assay, 4×10^6 splenocytes were diluted in 4 mL of MLR medium and placed in circulation as a bolus at 1 dyne/cm^2 through the µ-Slide until T cells entered the chamber as previously described.[28] Then the flow was reduced to 0.2 dyne/cm^2 for 1 min to allow T cells accumulation followed by an increase of 0.75 dyne/cm^2 using a peristaltic pump Minipuls 3 (Gilson, Villiers le Bel, France). Quantification of splenocyte adhesion on MSCs was performed every 2 s shots during 5 min at 37°C in a Carl Zeiss LSM 5 live duo microscope. After imaging, attached splenocytes were followed and counted during 5 min over 100 MSCs using the ImageJ software.

NF-κB activity

MSCs were transfected with either plasmid containing (NF-κB) firefly luciferase (pNF-κB.Luc) or plasmid that contains the promoter region from the herpes simplex virus thymidine kinase promoter without enhancer elements (pTAL.Luc) used as negative control. Thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK) was used to assess the transfection efficiency and for the normalisation (BD Biosciences Clontech). Transfection was performed using the lipofectamine reagent (Life Technologies) Opti-MEM medium (Gibco). Twenty-four hours after transfection, cells were activated for 3 h with 20 ng/mL IFN-γ and 10 ng/mL TNF-α. Then cells were lysed and protein concentrations were determined using the Bradford protein assay kit (Sigma-Aldrich). Relative NF-κB activity was calculated according to the relative luciferase activity that was measured using the dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions and normalised to protein concentration. Data were expressed as a mean of four independent experiments performed in triplicate and were represented as a percentage of the relative NF-κB activity.

Chromatin immunoprecipitation

MSCs were treated with 20 ng/mL IFN-γ and 10 ng/mL TNF-α. After 2 h of incubation, the cells were harvested and chromatin immunoprecipitation (ChIP) analysis was performed with a ChIP-IT High Sensitivity Kit (Active Motif, La Hulpe, Belgium) according to manufacturer's instructions. More details in online supplementary material.

Arthritis induction and measurement

DBA/1 male mice (9–10 weeks old) were immunised with 100 µg of chicken collagen II (ChCII) (Thermo Scientific, Rockford, Illinois, USA) as previously described.[29] In the preventive protocol, 5×10^5 MSCs per mouse were injected intravenously before the onset of the disease at days 18 and 24 after mouse immunisation. In the curative approach, we administrated a single dose of 5×10^5 MSCs. MSCs were injected intravenously when mice reached a 2–4 score of arthritis. The experiments were divided in different groups: (untreated) collagen-induced arthritis (CIA) group and groups treated with either C57BL/6 MSCs, PPARβ/δ$^{+/+}$ MSCs, PPARβ/δ$^{+/+}$ MSCs preincubated 24 h in culture with the potent, selective, irreversible PPARβ/δ antagonist GSK3787 (PPARβ/δ$^{+/+}$-GSK3787) and PPARβ/δ$^{-/-}$ MSCs. Preventive experiments were performed at least twice with 8–10 mice per experimental group in each separate experiment, while curative experiments were performed on 5 mice with a 2–4 score of arthritis per experimental group. Animal experiments were performed in accordance with the Ethical Committee for animal experimentation of the Languedoc-Roussillon (Approval CEEA-LR-1042). Signs of arthritis were assessed by measuring the swelling of the hind paws or the arthritic score was defined using an extended scoring protocol as we previously described.[29] At euthanasia, or when indicated, blood and draining lymph nodes (dLNs) were collected for immune cell analysis by cytometry and the hind limbs for X-ray micro-CT (µCT) and histological analysis (H&E–safranin O staining).

Bone analysis

Hind paws were fixed in 4% formaldehyde and scanned in an X-ray μCT at 18 μm voxel size (Skyscan 1176, Bruker microCT, Kontich, Belgium). During scanning, paws were placed in the scanner with the long axis aligned with the axis of the scanner bed. Image acquisition required 6 min using the following parameters: 50 kV, 500 μA, 0.5 mm aluminium filter, 180° scan, rotation step 0.7° and frame averaging of 1. Dataset were reconstructed and analysed using NRecon and CTAn softwares, respectively (Skyscan). A region of interest was drawn to contain only the navicular bone. The ratio of bone volume/tissue volume was calculated for each bone and compared between control mice (CIA).

Statistical analysis

Results were expressed as the mean±SEM. All in vitro experiments were performed using three different biological replicates at least three independent times. For the in vivo studies relying on the preventive protocol, 8–10 animals were used for each experimental or control groups and repeated for at least twice. For the curative protocol, we used five mice for each experimental or control groups. The p values were generated using non-parametric analysis using the Mann–Whitney U test to compare between two groups; $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) were considered statistically significant. All the analyses were performed using the GraphPad Prism TM 6 software (Graphpad Software, San Diego, California, USA).

RESULTS

PPARβ/δ regulates the immunosuppressive properties of MSCs

To determine whether PPARβ/δ is critical for MSC immunosuppressive properties, we modulated its expression in hBM-MSCs. Cells were pretreated with the selective and irreversible PPARβ/δ antagonist GSK3787 for 24 h and cocultured with peripheral blood mononuclear cells (PBMCs) stained with CTV and activated with phytohemagglutinin (PHA) for 3 days. As revealed by the percentage of proliferating cells quantified by fluorescence-activated cell sorting (FACS) on the basis of CTV dilution, PPARβ/δ inhibition significantly increased the suppressive effect of hBM-MSCs (figure 1A). Since we recently showed that human menstrual fluids-derived MSCs (Men-SCs) display lower immunosuppressive potential than hBM-MSCs,[26] we addressed whether *Ppard* mRNA expression level was correlated with their immunomodulatory properties. First, in a proliferation test using PBMCs activated with PHA for 3 days, we confirmed that hBM-MSCs displayed a more potent inhibitory effect on activated PBMCs than Men-SCs (figure 1B). In addition, when compared with these two types of MSCs, we showed that a third source of MSCs, UC-MSCs, possessed a similar suppressive effect than BM-MSCs (figure 1B). Using reverse transcription (RT)-PCR, we observed that the three types of MSCs expressed *Ppard*, although to a significantly higher extent in Men-SCs than in BM-MSCs and UC-MSCs (figure 1C). This result suggests a possible inverse correlation between *Ppard* mRNA expression level and the immunosuppressive potential of MSCs.

PPARβ/δ directs the therapeutic potential of MSCs in the CIA model

To further confirm the role of PPARβ/δ on MSC suppressive potential, we isolated MSCs from the BM of PPARβ/δ-deficient mice (PPARβ/$\delta^{-/-}$) and their control littermates (PPARβ/$\delta^{+/+}$). First, we showed that PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs were negative for CD11b and CD45 and although to a different extent they were positive for markers expressed on MSCs such as CD44, Sca-1, CD29 and CD105 (see online supplementary

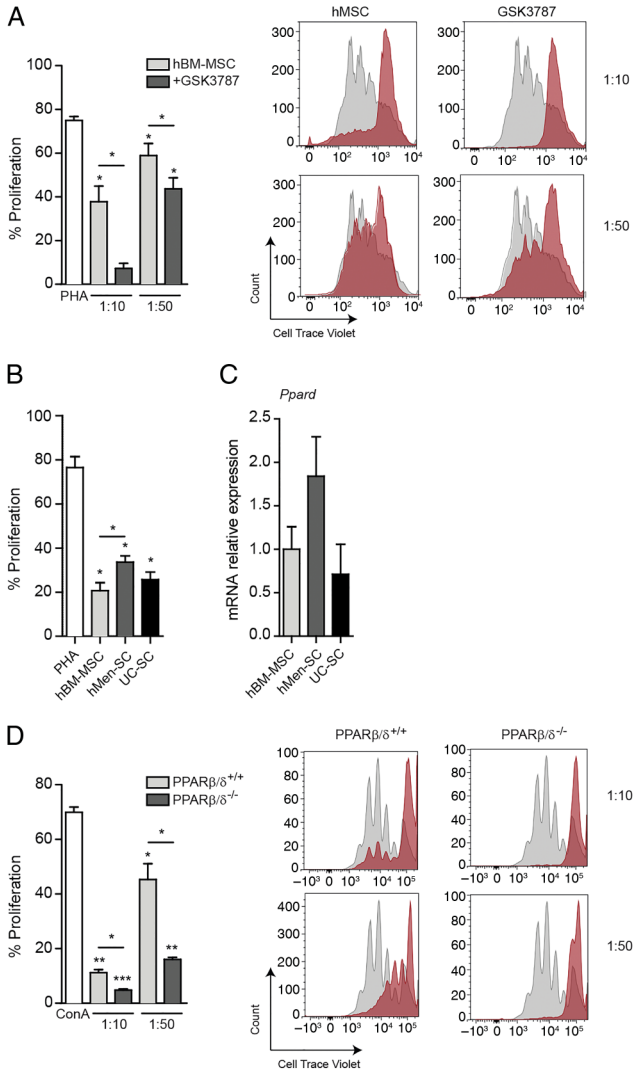

Figure 1 Inhibition of PPARβ/δ expression enhances the suppressive effect of human and murine MSCs. (A) PHA-activated human PBMCs labelled with CellTrace Violet were cultured in the absence or presence of hBM-MSCs treated or not with 1 μM of GSK3787, an inhibitor of PPARβ/δ, for 24 h at different MSCs:PBMCs ratios (1:10 and 1:50). On the left panel, grey histograms represent PHA-activated PBMCs and red histograms represent activated PBMCs cocultured with hBM-MSCs either untreated or treated with GSK3787. (B) Proliferation test using PHA-activated human PBMCs labelled with CellTrace Violet and cocultured with either hBM-MSCs, hMen-SC or hUC-MSCs. (C) Quantification of *Ppard* mRNA expression level by RT-PCR in hBM-MSCs, hMen-SC and hUC-MSCs. (D) Murine-activated splenocytes labelled with CellTrace Violet were cultured in the presence of either PPARβ/$\delta^{+/+}$ MSCs or PPARβ/$\delta^{-/-}$ MSCs at different ratios of MSC: splenocyte (1:10 and 1:50). On the left panel, grey histograms represent ConA-activated splenocytes and red histograms represent activated splenocytes cocultured with either PPARβ/$\delta^{+/+}$ or PPARβ/$\delta^{-/-}$ MSCs. Results are represented as mean±SEM of at least three independent experiments and three different PBMC donors or mice. ConA, concanavalin A; hBM-MSCs, human bone marrow-derived-MSCs; hMen-SC, human menstrual blood stromal stem cells; hUC-MSCs, human umbilical cord-MSCs; MSC, mesenchymal stem cell; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPARβ/δ, peroxisome proliferator activated receptor.

figure S1). Then, we compared the ability of PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs to inhibit ConA-stimulated splenocyte proliferation. At cell ratios of 1:10 and 1:50, both PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs significantly suppressed the proliferation of splenocytes, but to a higher extent for PPARβ/δ$^{-/-}$ MSCs (figure 1D). Going further, we assessed the effect of PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs in the CIA model and demonstrated that PPARβ/δ$^{+/+}$ MSCs administration before the onset of the disease did not exert any effect on the development of the disease (figure 2A). In contrast, PPARβ/δ$^{-/-}$ MSCs significantly reduced the clinical signs of arthritis of the injected mice compared with mice of the control group (CIA) or the group treated with PPARβ/δ$^{+/+}$ MSCs (figure 2A). In the same experiment, we observed that MSCs isolated from C57BL/6 mice were able to decrease the clinical score of arthritis as previously reported (figure 2A).[17 29] As expected on the basis of the clinical score, histological analysis of the synovial membrane, bone and cartilage revealed the presence of large amounts of inflammatory cells and areas of bone erosion in control and PPARβ/δ$^{+/+}$ MSCs-treated mice and not in C57BL/6 and PPARβ/δ$^{-/-}$ MSCs-treated mice (figure 2B). The protective effect of C57BL/6 and PPARβ/δ$^{-/-}$ MSCs against bone erosion at the hind paw as compared with control or PPARβ/δ$^{+/+}$ MSCs-treated mice was shown by μCT analysis (figure 2C). The bone volume density of the navicular bone, quantitatively analysed among other tarsal bones due to previous studies,[30–32] was significantly higher in C57BL/6 and PPARβ/δ$^{-/-}$ MSCs-treated mice as compared with CIA or PPARβ/δ$^{+/+}$ MSCs-injected animals (figure 2C). We then assessed the T cell response following MSC treatment. At day 25, we observed a significant decrease of the percentage of CD19^{-}CD138^{+} plasmablasts in the blood of C57BL/6 and PPARβ/δ$^{-/-}$ MSCs-treated mice as compared with CIA or PPARβ/δ$^{+/+}$ MSCs-treated animals (figure 2D). At euthanasia, while the frequency of proinflammatory T helper

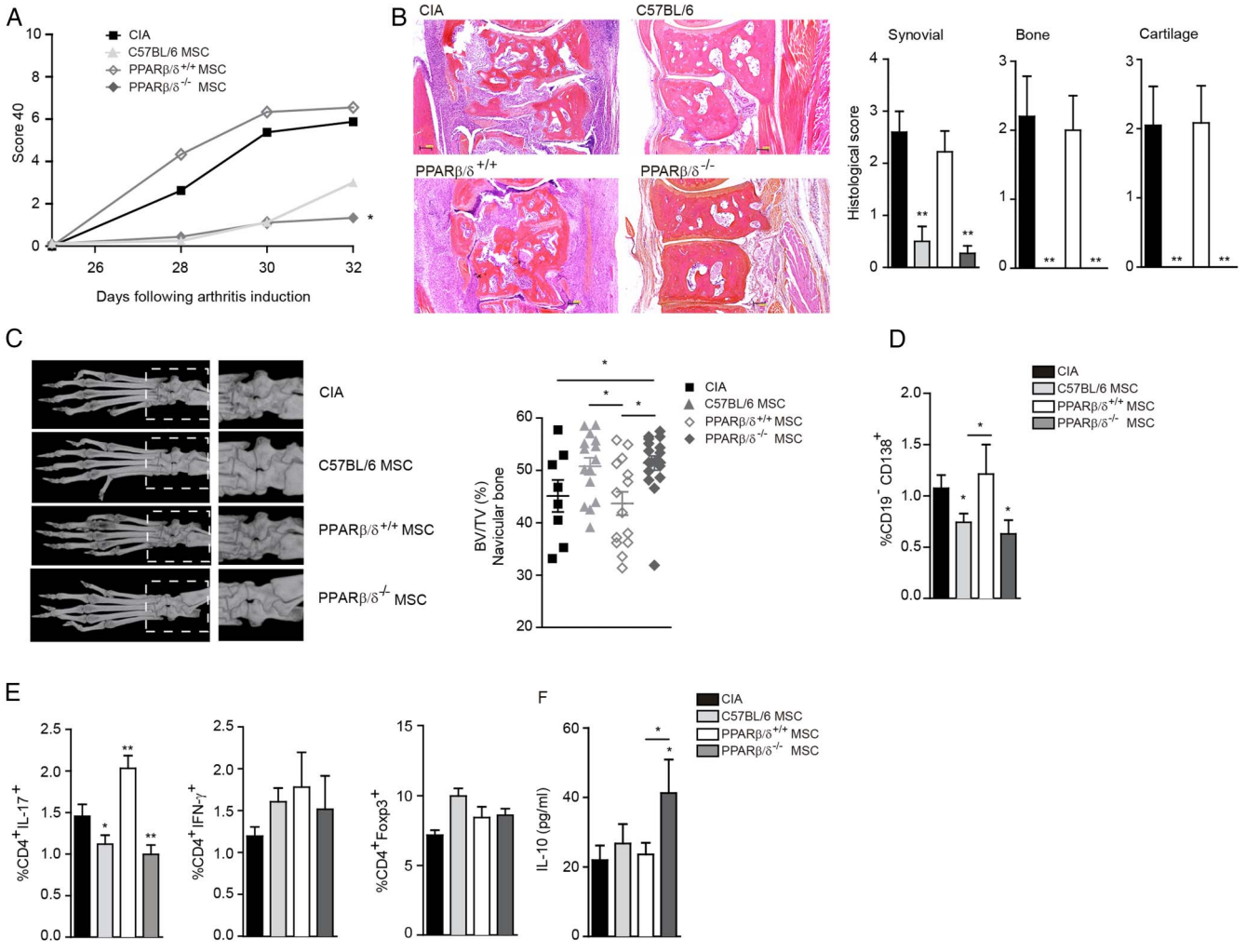

Figure 2 PPARβ/δ knockdown increases the therapeutic benefit of MSCs in the experimental model of arthritis when injected before the onset of the disease. (A) Clinical score was determined every each day from day 25. (B) On the left, representative pictures of H&E staining of ankle joints from control or MSCs-treated mice and on the right, the synovial membrane, bone and cartilage histological score (0–4). (C) On the left, representative pictures of bone erosion in ankles from CIA mice or mice treated with C57BL/6, PPARβ/δ$^{+/+}$ or PPARβ/δ$^{-/-}$ MSCs using micro-CT scans. On the right, evaluation of the navicular bone volume density (BV/TV) in the different groups of mice. (D) Mean percentage of plasmablasts in the blood of CIA mice or mice treated with C57BL/6, PPARβ/δ$^{+/+}$ or PPARβ/δ$^{-/-}$ MSCs. (E) Proinflammatory Th17 and Th1 cell as CD4^{+}Foxp3^{+} regulatory T cells (Treg) cell frequency was assessed in dLNs of mice in each experimental groups at the day of euthanasia. (F) IL-10 detection using ELISA in the supernatants of dLNs activated with 25 μg/mL of chicken collagen II during 48 h. Bars show the mean±SEM of 8–10 mice per experimental group. BV, bone volume; CIA, collagen-induced arthritis; ConA, concanavalin A; dLN, draining lymph node; IFN-γ, interferon-γ; IL, interleukin; MSC, mesenchymal stem cell; PPARβ/δ, peroxisome proliferator activated receptor; TV, trabecular bone volume.

(Th)17 cells was significantly higher in the dLNs of PPARβ/δ$^{+/+}$ MSCs-treated mice than in those of CIA mice, it was significantly lower in the dLNs of mice treated with C57BL/6 or PPARβ/δ$^{-/-}$ MSCs (figure 2E). In contrast, no difference in the Th1 proinflammatory response was observed in mice treated with MSCs as compared with CIA mice (figure 2E). The treatment of mice with PPARβ/δ$^{-/-}$ MSCs did not impact the percentage of CD4^{+}Foxp3^{+} regulatory T cells (Treg) cells when compared with CIA (figure 2E). However, a higher production level of IL-10 was measured in the supernatants of ChCII-activated dLN from PPARβ/δ$^{-/-}$ MSCs-treated mice as compared with CIA mice or mice treated with PPARβ/δ$^{+/+}$ MSCs (figure 2F). Going further, we investigated whether the preincubation of PPARβ/δ$^{+/+}$ MSCs with the selective and irreversible PPARβ/δ antagonist GSK3787 for 24 h before their injection (PPARβ/δ$^{+/+}$-GSK3787) would induce their therapeutic effect in the CIA model. As revealed by the clinical score of arthritis, PPARβ/δ$^{+/+}$-GSK3787 massively repressed the development and the progression of the disease contrary to the untreated PPARβ/δ$^{+/+}$ MSCs (see online supplementary figure 2A). This potent therapeutic effect of PPARβ/δ$^{+/+}$-GSK3787 was associated with a significant decrease of the frequency of both proinflammatory Th17 cells and plasmablasts (see online supplementary figure 2B). The treatment of mice with PPARβ/δ$^{+/+}$ MSCs or PPARβ/δ$^{+/+}$-GSK3787 MSCs did not affect the percentage of Th1 and CD4^{+}Foxp3^{+} Treg cells when compared with CIA (see online supplementary figure 2B). Finally, a significant decrease in the percentage of CD8^{+}IFN-γ^{+} and B220^{+}IL-10^{+} cells was observed in the dLNs of CIA and PPARβ/δ$^{+/+}$ MSCs-treated mice (see online supplementary figure 2C). These data demonstrate the critical role of PPARβ/δ in the preventive properties exerted by MSCs in the CIA model was associated with a significant decrease of Th17 cells and plasmablasts.

MSCs administration in established arthritis massively reduces the severity of the disease in a PPARβ/δ-dependent manner

We investigated the therapeutic potential of MSCs and the role of PPARβ/δ in the CIA model, once the immunised mice have developed arthritis. To that aim, we performed a single intravenous injection of either PPARβ/δ$^{+/+}$, PPARβ/δ$^{+/+}$-GSK3787 or PPARβ/δ$^{-/-}$ MSCs in mice with a score of 2–4. Both PPARβ/δ$^{+/+}$-GSK3787 and PPARβ/δ$^{-/-}$ MSCs significantly prevented arthritis progression and limited arthritic symptoms as monitored by the clinical score from day 2 to 3 after MSC administration (figure 3A). Of note, from day 3, mice treated with PPARβ/δ$^{+/+}$ MSCs displayed a significantly lower arthritic score compared with the CIA mice. Nevertheless, at day 3, the score severity was significantly lower in mice treated with PPARβ/δ$^{-/-}$ MSCs compared with mice treated with PPARβ/δ$^{+/+}$ MSCs (figure 3A). Moreover, we assessed the T cell response following curative MSC treatment. Two days after MSC injection in mice with arthritis, we observed a significant decrease of the percentage of Th17 cells in the blood of mice treated with both PPARβ/δ$^{+/+}$-GSK3787 MSCs and PPARβ/δ$^{-/-}$ MSCs as compared with CIA or PPARβ/δ$^{+/+}$ MSCs-treated mice (figure 3B). The frequency of Th1 cells was significantly lower only in the blood of mice treated with PPARβ/δ$^{-/-}$ MSCs as compared with the three other groups of mice. Since pathogenic Th17 cells are also characterised by their capacity to produce granulocyte-macrophage colony-stimulating factor (GM-CSF),[29 33] we quantified GM-CSF in the supernatant of bCII-stimulated dLN cells isolated from untreated CIA control mice or mice treated with MSCs. The cells from the dLNs of mice treated with either PPARβ/δ$^{+/+}$-GSK3787 MSCs or PPARβ/δ$^{-/-}$ MSCs produced significantly lower levels of GM-CSF than those of CIA mice or mice treated with PPARβ/δ$^{+/+}$ MSCs (figure 3C). PPARβ/δ is pivotal in the curative properties of MSCs in arthritis targeting the generation of Th17 cells.

PPARβ/δ expression level governs MSC immunosuppressive function in a contact-dependent manner

To further demonstrate the role of PPARβ/δ on the immunosuppressive properties of MSCs and comprehensively study the underlying molecular mechanism, we carefully compared the properties of PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs in vitro. Since PPARβ/δ regulates the expression level of adhesion molecules, we investigated whether cell–cell adhesion was required for immunosuppression mediated by PPARβ/δ$^{-/-}$ MSCs. PPARβ/δ$^{+/+}$ MSCs were cocultured with fresh splenocytes stimulated with ConA in the presence or absence of a Transwell system. When splenocytes were not in contact with MSCs, the enhanced immunosuppressive effect of PPARβ/δ$^{-/-}$ MSCs compared with PPARβ/δ$^{+/+}$ MSCs was lost suggesting the requirement of cell–cell contact mechanisms (figure 4A). In parallel, we observed that PPARβ/δ$^{-/-}$ MSCs expressed significantly higher levels of ICAM-1 and VCAM-1 than PPARβ/δ$^{+/+}$ MSCs in basal

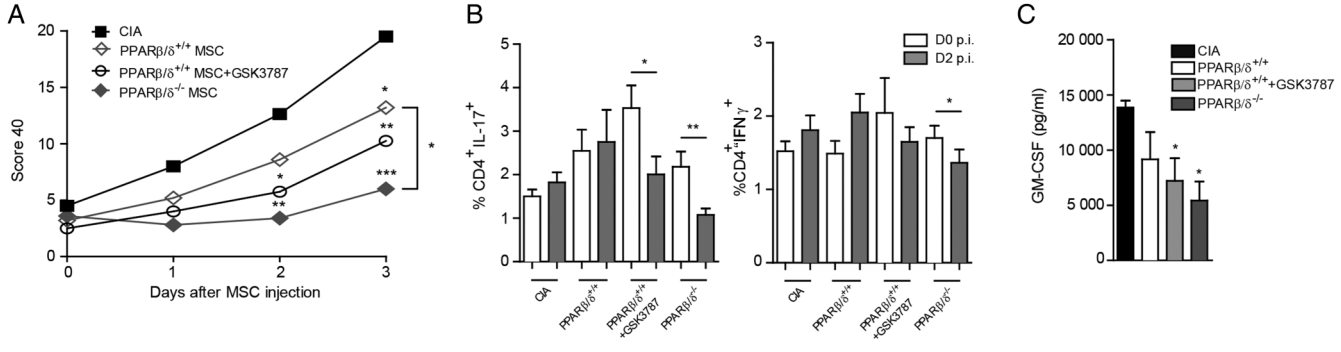

Figure 3 PPARβ/δ inhibition or knockdown increases the therapeutic benefit of MSCs when injected in mice with established arthritis. (A) Arthritis score was assessed daily from the day of MSC injection (p.i.) until the sacrifice of the mice. When indicated, a single dose of 5×10^{5} MSCs per mouse was injected intravenously after the onset of the disease, that is, once the immunised mice reached a 2–4 score of arthritis. (B) Mean percentage of proinflammatory Th17 and Th1 cell frequency was assessed in the blood of mice in each experimental groups 2 days after MSC injection. (C) GM-CSF detection using ELISA in the supernatants of dLNs activated with 25 μg/mL of chicken collagen II during 48 h. Bars show the mean±SEM of five mice per experimental group. CIA, collagen-induced arthritis; dLN, draining lymph node; IFN-γ, interferon-γ; IL, interleukin; MSC, mesenchymal stem cell; p.i., postinjection; PPARβ/δ, peroxisome proliferator activated receptor; Th, T helper.

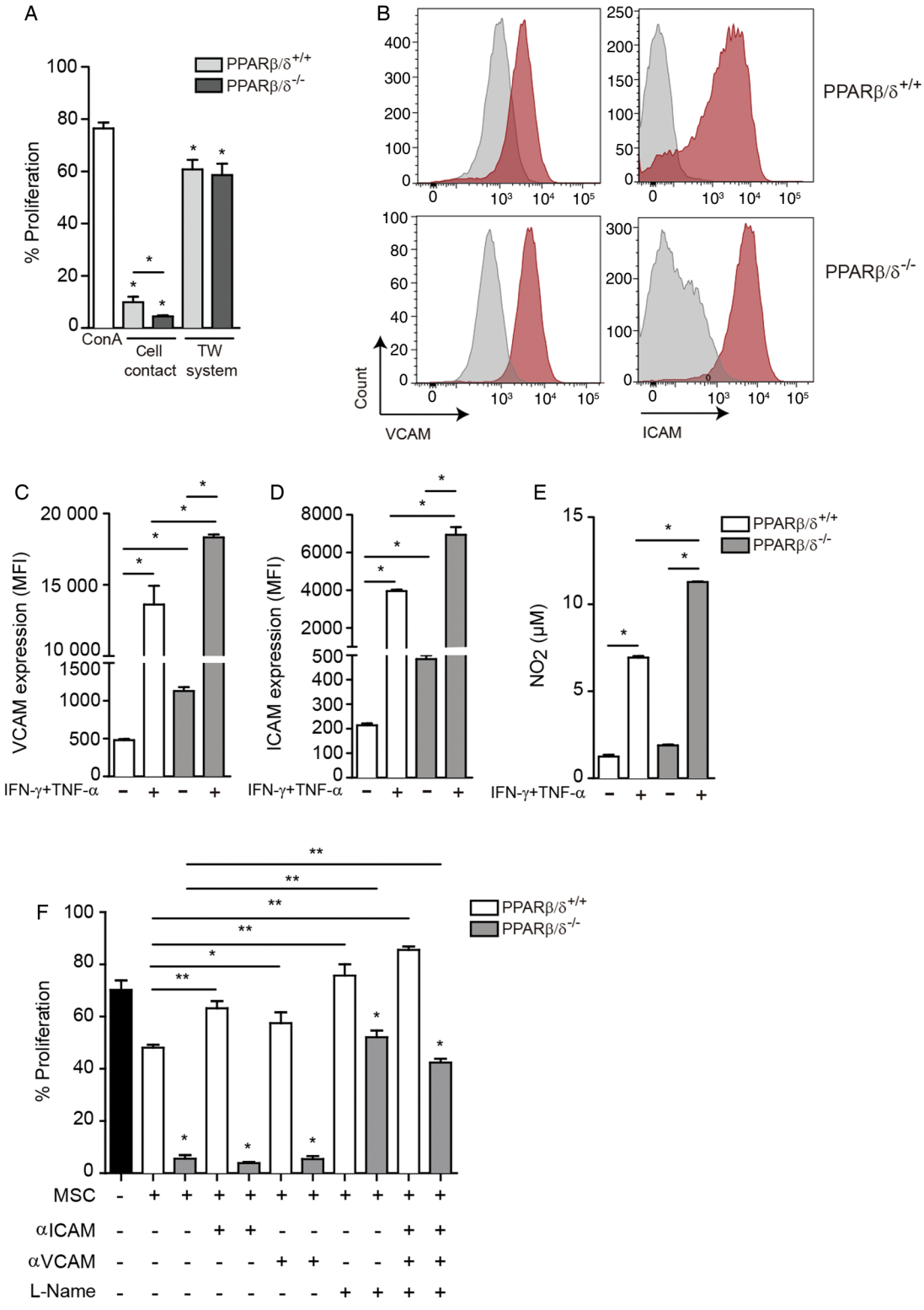

Figure 4 PPARβ/δ inhibition increases the expression of adhesion molecules and nitric oxide production by MSCs. (A) Murine-activated splenocytes labelled with CellTrace Violet were cocultured with or without PPARβ/$\delta^{+/+}$ or PPARβ/$\delta^{-/-}$ MSCs in the presence or absence of a TW system. (B) Representative FACS histogram for ICAM and VCAM expression by PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs. Grey histograms represent the unactivated MSCs and the red histograms represent MSCs activated with IFN-γ and TNF-α. (C) MFI values reflecting VCAM expression levels on unactivated and IFN-γ-activated and TNF-α-activated PPARβ/$\delta^{+/+}$ MSCs and PPARβ/$\delta^{-/-}$ MSCs for 24 h. (D) MFI values reflecting ICAM expression levels on unactivated and IFN-γ-activated and TNF-α-activated PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs for 24 h. (E) Quantification of NO_2 production using a modified Griess reagent in the supernatants of unactivated and activated PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs for 24 h. (F) CD3/CD28-activated $CD4^+$ T cells labelled with CellTrace Violet were cocultured with or without PPARβ/$\delta^{+/+}$ (white) or PPARβ/$\delta^{-/-}$ (grey) MSCs. When specified, anti-ICAM (αICAM), anti-VCAM (αICAM) neutralising antibodies or L-NAME, an inhibitor of NO, were added in the cultures alone or in combination. Results are represented as mean±SEM of at least three independent experiments. ConA, concanavalin A; IFN-γ, interferon-γ; MFI, median fluorescence intensity; MSC, mesenchymal stem cell; NO, nitric oxide; PPARβ/δ, peroxisome proliferator activated receptor; TNF-α, tumour necrosis factor-α; TW, Transwell.

conditions (figure 4B–D). Then, we treated PPARβ/δ$^{-/-}$ and PPARβ/δ$^{+/+}$ MSCs with IFN-γ and TNF-α cytokines well described to enhance MSC immunosuppressive potential. We showed a substantial increase of ICAM-1 and VCAM-1 on MSCs (figure 4C,D) as well as a higher production of NO_2 by PPARβ/δ$^{-/-}$ MSCs compared with PPARβ/δ$^{+/+}$ cells (figure 4E). Then to address the role of ICAM-1, VCAM-1 and NO_2 on the immunosuppressive properties of MSCs, we cultured activated CD4$^+$ T cells alone or in presence of either PPARβ/δ$^{+/+}$ MSCs or PPARβ/δ$^{-/-}$ MSCs and when indicated, anti-ICAM or anti-VCAM neutralising antibodies or L-NAME, a direct inhibitor of nitric oxide synthesis, were added alone or in combination (figure 4F). While the addition of neutralising antibodies or L-NAME alone or in combination significantly reversed the immunosuppressive properties of PPARβ/δ$^{+/+}$ MSCs, a loss of PPARβ/δ$^{-/-}$ MSCs inhibitory potential was noticed only when the secretion of NO_2 was inhibited (figure 4G). All together, these results suggest that PPARβ/δ might play a critical role in the immunosuppressive properties of MSCs likely by enhancing the expression of adhesion molecules and the production of NO_2.

Role of PPARβ/δ expression in the interaction between MSCs and splenocytes

In order to compare the rate of T cell adhesion on PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$MSCs, we seeded MSCs on a flow chamber and activated them with proinflammatory cytokines for 24 h prior to add ConA-activated splenocytes (figure 5A). The percentage of adherent splenocytes was determined by evaluating representative planes of the time-lapse recording with at least 100 MSCs for each condition. Our results revealed that circulating splenocytes attached at a significantly higher rate on PPARβ/δ$^{-/-}$ MSCs than PPARβ/δ$^{+/+}$ MSCs (figure 5B). These data correlated with the significantly higher expression levels of VCAM and ICAM in PPARβ/δ$^{-/-}$ MSCs compared with PPARβ/δ$^{+/+}$ MSCs (figure 4B,C). Moreover, real-time visualisation of the interaction between splenocytes and MSCs revealed that activated splenocytes adhered preferentially to PPARβ/δ$^{-/-}$ MSCs as shown by a significantly higher number of green cells (see online supplementary movies 1 and 2 and figure 5C). All together, these results demonstrate the critical role of PPARβ/δ in the interactions between MSC and T cells.

PPARβ/δ orchestrates the immunosuppressive effect of MSCs through the modulation of NF-κB activity

Since PPARβ/δ acts as a negative regulator of NF-κB activity,[2] and that immunosuppression is mediated by activation of NF-κB in MSCs,[34] we examined the activity of NF-κB in both PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs. Using the luciferase-based reporter system, we demonstrated that PPARβ/δ$^{-/-}$ MSCs displayed a significantly higher NF-κB activity than PPARβ/δ$^{+/+}$ MSCs (figure 6A). We then investigated the effect of NF-κB activity using Bay, widely used as an irreversible chemical inhibitor of NF-κB, and showed a decrease of NF-κB activity (figure 6B). Bay treatment significantly reduced the expression levels of ICAM and VCAM and impaired the production of NO_2 by PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs, but to a lesser extent on PPARβ/δ$^{-/-}$ MSCs (figure 6C–E). Moreover, while the treatment with Bay was sufficient to reverse the immunosuppressive effect of PPARβ/δ$^{+/+}$ MSCs on splenocytes proliferation at a high MSC:splenocyte ratio (1:10), it was not on PPARβ/δ$^{-/-}$ MSCs. In contrast, at a lower ratio (1:50), the inhibition of NF-κB activity impaired the suppressive effects of PPARβ/δ$^{-/-}$ MSCs while it did not on PPARβ/δ$^{+/+}$ MSCs (figure 6F).

To further analyse the role of PPARβ/δ and NF-κB activity on NO production, we performed ChIP experiments to investigate the recruitment of the p65 subunit of NF-κB on *inos* promoter. At steady state, we observed that the PPARβ/δ$^{-/-}$ MSCs showed an increased level of p65 binding on *inos* promoter, indicating an activated status of these cells per se (figure 6G). Moreover, we observed that the activation of PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ MSCs by IFN-γ and TNF-α induced a massive recruitment of p65 subunit to the *inos* promoter in the PPARβ/δ-deficient MSCs, in comparison to PPARβ/δ$^{+/+}$ MSCs (figure 6G,H).

DISCUSSION

Although PPARβ/δ activation or overexpression have been shown to lead to a decrease of inflammation,[35] our study demonstrates that its repression enhances the therapeutic benefit of MSCs in arthritis by upregulating the mediators involved in their immunosuppressive effects.

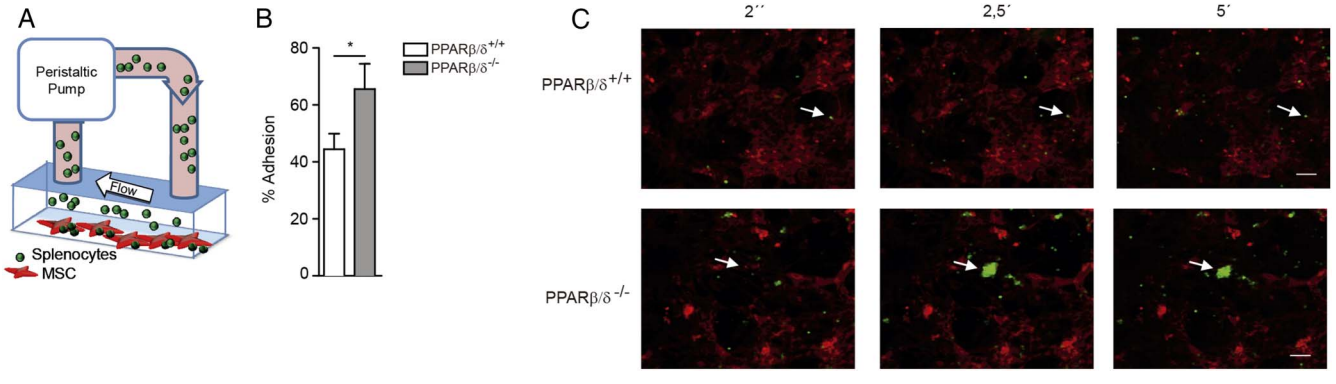

Figure 5 Activated splenocytes highly adhere to PPARβ/δ$^{-/-}$ MSCs compared with PPARβ/δ$^{+/+}$ MSCs. (A) Schematic representation of the flow assay. MSCs were activated with IFN-γ and TNF-α during 24 h and then stained with the CellTracker Red CMTPX and cultured in a flow chamber. carboxyfluorescein succinimidyl ester (CFSE)-labelled activated splenocytes were inserted in the circulation through a flow chamber at a speed of 0.6 mL/min using a peristaltic pump. (B) Percentage of adherent cells was calculated considering representative planes of the time-lapse with at least 100 MSCs for each condition and the number of splenocytes that attached to the MSCs was counted during 5 min with the ImageJ software. (C) Representative time-lapse pictures show the interaction between splenocytes and MSCs every 2 s during 5 min. Scale bar: 50 μm. Adhesion rate results are represented as mean±SEM of at least five independent experiments. IFN-γ, interferon-γ; MSC, mesenchymal stem cell; PPARβ/δ, peroxisome proliferator activated receptor; TNF-α, tumour necrosis factor-α.

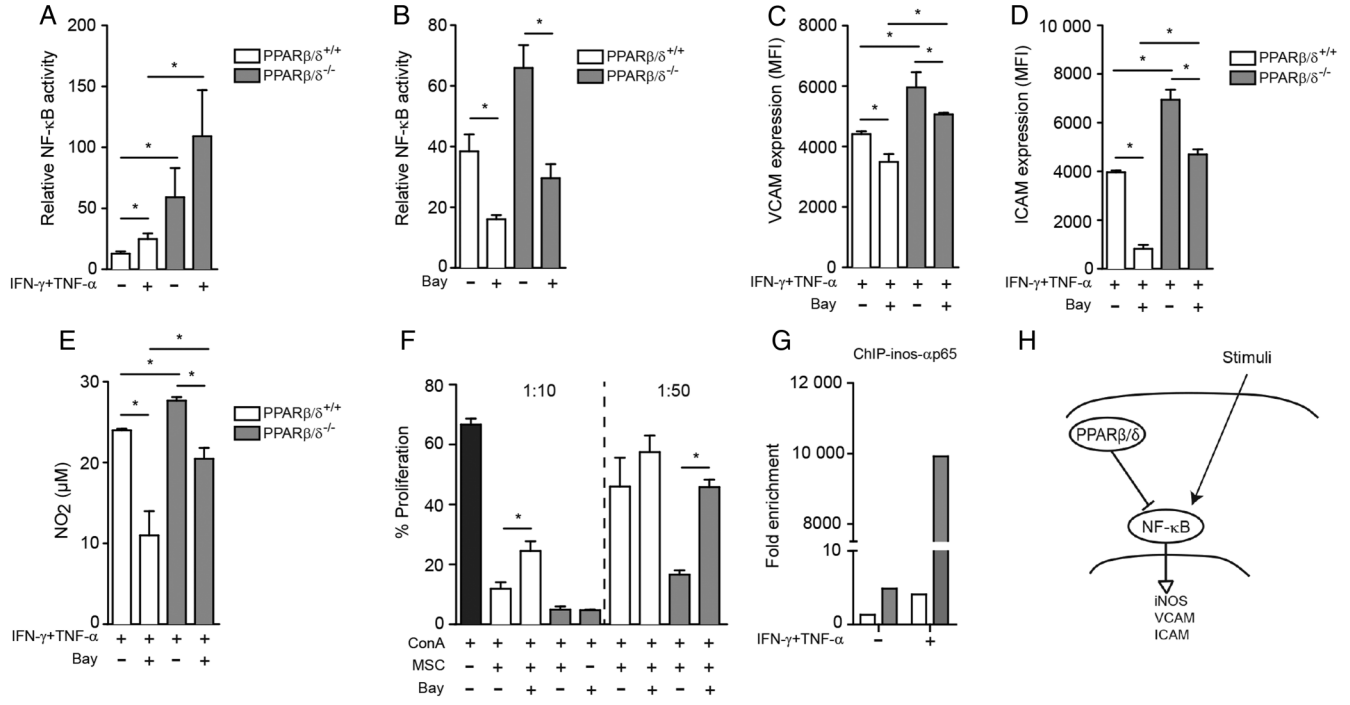

Figure 6 PPARβ/δ modulates NF-κB activity and orchestrates the immunosuppressive mechanism mediated by MSCs. (A) NF-κB activity assessment in PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs without or with a 3 h activation. (B) Inhibition of NF-κB activity in PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs using Bay. (C and D) VCAM and ICAM expression levels on activated PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs. (E) NO_2 quantification in the supernatants of IFN-γ and TNF-α activated PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs treated or not with Bay. (F) Proliferation assay with ConA-activated splenocytes cultured in the absence or presence of PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs treated or not with Bay at different MSC:splenocyte ratios of 1:10 and 1:50. Results are represented as mean±SEM of at least three independent experiments. (G) Binding of p65 on *inos* promoter was analysed by chromatin immunoprecipitation in PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ MSCs, 2 h after IFN-γ and TNF-α stimulation. (H) PPARβ/δ is involved in the suppressive effect of MSCs by blocking NF-κB activity and modulating the expression of VCAM, ICAM and iNOS transcription. ChIP, chromatin immunoprecipitation; CIA, collagen-induced arthritis; ConA, concanavalin A; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; MFI, median fluorescence intensity; MSC, mesenchymal stem cell; PPARβ/δ, peroxisome proliferator activated receptor; TNF-α, tumour necrosis factor-α.

PPARβ/δ, a well-known regulator of inflammation that acts through transactivation of anti-inflammatory genes or transrepression of proinflammatory genes is expressed by MSCs.[2 35] Here, we show that PPARβ/δ irreversible inhibition enhances the immunosuppressive potential of both human and murine MSCs in vitro. This paradoxical role of PPARβ/δ on MSC function is in line with the fact that the activation with proinflammatory cytokines is required to induce MSC-mediated immunosuppression. Indeed, activation by signals from a proinflammatory environment enhances the immunosuppressive properties of MSCs render primed as a negative feedback loop.[36–38] Our results reveal for the first time that the silencing of an anti-inflammatory mediator, PPARβ/δ, primes MSCs towards an immunoregulatory phenotype. This unexpected effect of PPARβ/δ on the immune response mediated by MSCs was associated with an increase of the adhesion molecules such as ICAM and VCAM as well as NO_2 production by MSCs deficient for PPARβ/δ compared with their wild-type counterpart. Moreover, we demonstrate that PPARβ/$\delta^{-/-}$ MSCs with enhanced immunosuppressive capacities display a significantly higher NF-κB activity both at steady state and upon activation with proinflammatory cytokines. This is consistent with the inhibitory action of PPARs on NF-κB signalling pathways described in immune cells that is associated with their anti-inflammatory properties[2] and the TNF-α-mediated NF-κB activation involved in the immunomodulatory activity of MSCs.[34] In this latter study, the authors described that the impairment of NF-κB activation antagonises the inhibitory effect of MSCs on T cell proliferation. Here, we show a massive recruitment of p65 subunit of NF-κB transcription complex on *inos* promoter in the PPARβ/$\delta^{-/-}$ MSCs when activated with IFN-γ and TNF-α compared with PPARβ/$\delta^{+/+}$ MSCs. This result suggests that in MSCs, PPARβ/δ represses the binding of NF-κB p65 subunit on *inos* promoter and therefore the capacity of MSCs to produce NO_2. All together, these results reveal a fundamental mechanism though which PPARβ/δ is an upstream regulator of mediators involved in MSC immunomodulatory properties and induced by proinflammatory cytokines.

Moreover, we demonstrate that PPARβ/$\delta^{+/+}$ MSCs did not exhibit any preventive or therapeutic effect in the CIA model, while PPARβ/δ deficiency enhanced the therapeutic effect of MSCs. The absence of therapeutic potential of PPARβ/$\delta^{+/+}$ MSCs likely results from their genetic background as previously discussed.[39] However, we demonstrated that the potent, selective and irreversible inhibition of PPARβ/δ in PPARβ/$\delta^{+/+}$ using GSK3787 induced both a preventive and curative therapeutic potential to these cells in the CIA model. This beneficial effect observed following the injection of MSCs deficient for PPARβ/δ was associated with a lower frequency of Th17 cells as compared with mice treated with PPARβ/$\delta^{+/+}$ MSCs. Our finding reveals that MSCs deficient for PPARβ/δ exhibit a strong regulatory phenotype and protect from inflammation.

All together, our results provide new insight into the mechanisms that mediate the immunosuppressive properties of MSCs and highlight the role of PPARβ/δ as a potential means for enhancing MSC therapeutic potential in inflammatory disorders.

Therefore, strategies to prime MSC based on PPARβ/δ inhibition should enhance their therapeutic potential and optimise their clinical use. Pretreatment of MSCs with GSK3787 enhances MSC immunosuppressive properties and therefore could provide a new strategy to generate therapeutic MSCs with a stable regulatory phenotype. Such licensed MSCs might be considered in clinic as a cellular drug for autoimmune and inflammatory disorders.

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