Received: 7 January 2021 | Accepted: 28 July 2021

DOI: 10.1002/sctm.21-0008

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

Authored by a member of CORD BLOOD ASSOCIATION

STEM CELLS TRANSLATIONAL MEDICINE

IFN-γ and PPARδ influence the efficacy and retention of multipotent adult progenitor cells in graft vs host disease

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Funding information
Science Foundation Ireland (SFI), Grant/Award Numbers: 12/RI/2346 (2), 13/SIRG/2172; HRB Translational Medicine Postdoctoral Research Fellowship, Grant/Award Number: PDTM/2011/21; EU Career Integration Grant, Grant/Award Number: 321697; Irish Research Council Enterprise Partnership Scheme Scholarship part funded by ReGenesys BVBA, Grant/Award Number: EPSPG/2013/581

Abstract

Cell-based therapy for the treatment of inflammatory disorders has focused on the application of mesenchymal stromal cells (MSCs) and multipotent adult progenitor cells (MAPCs). Despite the recent positive findings in industry-sponsored clinical trials of MSCs and MAPCs for graft vs host disease (GvHD), cell therapy is efficacious in some but not all patients, highlighting the need to identify strategies to enhance cell-based therapeutic efficacy. Here, we demonstrate the capacity for interferon (IFN)-γ licensing to enhance human MAPC efficacy and retention following early administration in a humanized mouse model of acute GvHD (aGvHD). Activation of the nuclear receptor peroxisome proliferator-activated receptor delta (PPARδ) negatively influenced the retention and efficacy of human MAPCs as well as IFN-γ-licensed MAPCs in the aGvHD model. PPARδ antagonism significantly enhanced the efficacy of human MAPCs when administered early in the humanized aGvHD model. COX-2 expression in human MAPC was significantly decreased in IFN-γ licensed MAPCs exposed to a PPARδ agonist. Importantly, MAPC exposure to the PPARδ antagonist in the presence of a COX-2 inhibitor indomethacin before administration significantly reduced the efficacy of PPARδ antagonized MAPCs in the aGvHD humanized mouse model. This is the first study to demonstrate the importance of PPARδ in human MAPC efficacy in vivo and highlights the importance of understanding the disease microenvironment in which cell-based therapies are to be administered. In particular, the presence of PPARδ ligands may negatively influence MAPC or MSC therapeutic efficacy.

KEYWORDS
COX-2, graft vs host disease, IFN-γ, in vivo biodistribution, mesenchymal stromal cells, multipotent adult progenitor cells, PPARδ

Significance statement

This study provides a new insight to effects that peroxisome proliferator-activated receptor (PPAR)-delta activation or inhibition has on human multipotent adult progenitor cell (MAPC) immunomodulation. This study demonstrates that PPAR-delta inhibition enhances MAPC efficacy in a preclinical model of acute graft vs host disease.

This study highlights the importance of understanding the disease microenvironment and how factors such as PPAR-delta ligands may negatively influence MAPC efficacy.

1 | INTRODUCTION

Multipotent adult progenitor cells (MAPCs) are a population of postnatal cells isolated from the human bone marrow with pro-reparative and immunomodulatory capacities. MAPCs and mesenchymal stromal cells (MSCs) have suppressive effects on T-cell proliferation and function in vitro and in vivo[1-5] and have demonstrated therapeutic efficacy in mouse models of acute graft vs host disease (aGvHD).[6,7] Both MSCs and MAPCs have demonstrated safety in clinical trials with the incidence and severity of GvHD reduced.[8,9] Despite improvements in clinical trial (including primary endpoint) design and more focused patient stratification, not all patients respond to MSC therapy.[10,11] Recent findings from an industry-sponsored phase-III clinical trial has demonstrated an overall response rate of 69%,[12] highlighting the need to better understand the influence of the disease microenvironment on cell activation and function and to identify ways to enhance cell therapeutic efficacy. For example, our group has improved the efficacy of human bone marrow-derived MSCs administered to a humanized model of aGvHD by licensing cells with interferon-γ (IFN-γ).[13] IFN-γ and subsequent STAT1 phosphorylation are required for the therapeutic effects of MSCs in a number of in vitro and in vivo settings.[14-18] Thus, MSCs fail to exert immunosuppression when IFN-γ concentrations are low in their microenvironment, or when they exhibit poor responsiveness to IFN-γ activation.[14-19]

Apart from cytokines like IFN-γ, induced expression or activation of other genes or transcription factors may play an important role in MSC immunomodulatory capacity.[20-22] The activation of peroxisome proliferator-activated receptor-delta (PPARδ) in mouse MSCs has been shown to impair MSC efficacy in a mouse model of arthritis, whereas knockdown or antagonism of PPARδ enhanced mouse MSC efficacy via enhanced nitric oxide (NO) production.[20] PPARδ is a ligand-activated transcription factor that regulates key cellular metabolic functions.[23] Natural ligands for PPARδ include free fatty acids, eicosanoids, and oxysterols,[24] which have been shown to increase in plasma of GvHD patients in addition to being predictive of GvHD development.[25,26] Notably, there are important differences in mechanisms of action mediated by mouse and human MSC. Although mouse MSC function has been associated with NO production, human MSC do not produce NO and their immunomodulatory functions are more closely associated with factors like IDO and PGE-2.[27] Given the capacity for the potential negative effects that PPARδ ligand activation may have on MAPCs or IFN-γ-licensed MAPCs in GvHD and that the influence of IFN-γ licensing or PPARδ activation/inhibition on human MAPC therapeutic effects in vivo has not been investigated, we examined the effect of PPARδ agonism and antagonism in human MAPCs and the capacity for IFN-γ licensing to enhance human MAPC efficacy in aGvHD. Furthermore, we investigated the influence of PPARδ on human MAPC immunomodulation in aGvHD. Using a humanized model of aGvHD, specific PPARδ chemical agonists/antagonists and novel 3D cryo-imaging, this study demonstrates that IFN-γ stimulation of human MAPCs prior to administration improves their efficacy in vivo, and that PPARδ activation inhibits the immunosuppressive effects of human MAPCs and of IFN-γ-licensed human MAPCs. Thus, these findings increase our understanding of the potential negative effects that PPARδ ligands present in the disease microenvironment may have on human MAPC and demonstrates two pathways by which MAPC efficacy can be enhanced.

2 | MATERIALS AND METHODS

2.1 | MAPC isolation and generation

Human MAPCs used in this study were clinical grade MAPCs isolated from the bone marrow of healthy donors by Athersys as previously described.[28] MAPCs were cultured on flasks coated with 1X Fibronectin (Sigma-Aldrich, Wicklow, Ireland) in complete MAPC media containing 50% Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland), 30% MCDB-201 (Sigma-Aldrich) supplemented with 18% FBS (Atlas Biolegend, Fort Collins, Colorado), insulin transferrin selenium (Lonza), linoleic acid-albumin (Sigma-Aldrich), penicillin-streptomycin (Sigma-Aldrich), 2-phospho-l-ascorbic acid (Sigma-Aldrich), dexamethasone (Sigma-Aldrich), epidermal growth factor (R&D Systems, Abingdon, UK), and platelet-derived growth factor (R&D Systems) at a density of 2000 cells/cm^2.

MAPCs stimulated with IFN-γ (γMAPCs) were cultured with 50 ng/mL recombinant human IFN-γ (Peprotech, London, UK) for 24 hours prior to in vivo administration. MAPCs treated with the PPARδ agonist (MAPC PPAR[+]) were treated with 1 μM GW0742 (Tocris, Abingdon, UK) for 48 hours, whereas MAPCs treated with the antagonist (MAPC PPAR[−]) were treated with 1 μM GSK3787 (Tocris) for 48 hours. MAPCs treated with both the agonist and IFN-γ (γMAPC PPAR[+]) or antagonist and IFN-γ (γMAPC PPAR[−]) were treated with their respective agonist or antagonist for 48 hours with the addition of IFN-γ at 50 ng/mL for the following 24 hours. A concentration of 1 μM of the agonist and antagonist was used based on previous publications using human macrophages.[29] In some cases, MAPCs were treated

with 1 μM GSK3787 (Tocris) and 10 μM indomethacin (Sigma-Aldrich) for 48 hours. For imaging experiments, MAPCs were washed in complete MAPC media and incubated at 10×10^6 cells/mL for 1 hour with Qtracker 625 cell label (Life Technologies, (Part of ThermoFisher Scientific, Horsham, UK)) followed by two washes in MAPC media and three washes in phosphate-buffered saline (PBS; Sigma-Aldrich). All MAPC cultures were counted, washed twice in sterile PBS, and administered intravenously (i.v.) to the mice.

2.2 | Humanized model of aGvHD

The humanized mouse model of aGvHD was set up as previously described.[5,30,31] NOD-SCID IL-2rγ^{null} mice (NSG) (Jackson Laboratories, Bar Harbour, Maine) (6-18 weeks old) were exposed to a conditioning dose of 2.4 Gray (Gy) whole-body gamma irradiation. Both male and female mice were used and sex-matched mice were used in the experiments. Human peripheral blood mononuclear cells (PBMC) from three healthy donors (buffy coats supplied by the Irish Blood Transfusion Service) were isolated by Ficoll-density centrifugation and washed three times in PBS before being administered to irradiated NSG mice (8×10^5/g) via i.v. injection 4 hours following irradiation. Negative control mice received PBS. In some groups, subcultured MAPCs (6.4×10^4/g) between passage 2 and 5 from three donors were administered i.v. on either day 0 or day 7. For tissue analysis, mice were culled on day 12.

2.3 | Cryo-imaging

For imaging of organs, mice were culled 24 hours following MAPC administration by cervical dislocation. Organs of interest were harvested and put onto a thin layer of black optimal cutting temperature (OCT) compound (Bioinvision Inc, Cleveland, Ohio) in peel away molds kept on ice. Organs were covered in OCT and frozen on a metal block chilled in liquid nitrogen. Once OCT had solidified, samples were transferred to −80°C. Sectioning and imaging were carried out using the automated CryoViz imaging system (BioInvision Inc). Images were then processed to form a 3D image of the organs of interest. Images were then analyzed using CryoViz processing and a cell-detection software (BioInvision Inc).

2.4 | Histopathological analysis and scoring

GvHD target organs (the lung, liver, and gut) were harvested on day 12. Tissue was fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 hours before being immersed in 70% ethanol at 4°C for 24 to 72 hours. Tissue was then processed for histology using an automated processor (Shandon Pathcentre, Runcorn, UK) and embedded in paraffin wax. Five micrometers of tissue sections were stained with hematoxylin and eosin (H&E) and examined blindly for damage and mononuclear cell infiltration using a semiquantitative scoring system as previously described.[5]

2.5 | Western blotting

Total protein lysates were extracted in radioimmunoprecipitation assay buffer and added to 6X sample buffer before being separated on a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel at 80 V for up to 2 hours. Protein was transferred to polyvinylidene difluoride membranes in a Hoefer TE 70 Semiphor semi-dry transfer unit (GE Healthcare, Dublin, Ireland). Membranes were blocked with 5% BSA or nonfat milk before incubation with primary antibodies (STAT1, pSTAT1, and Actin [Cell Signaling, London, UK]) under agitation at 4°C overnight. Membranes were then incubated with a rabbit secondary antibody conjugated to HRP (Cell Signaling) and developed using BM Chemiluminescence Western Blotting Substrate (POD) (Roche, Dublin, Ireland) before being imaged using a G:BOX (Syngene, Cambridge, UK). Densitometry was carried out using ImageJ open-source software (National Institutes of Health).

2.6 | Flow cytometry

MAPCs were cultured, transferred to v bottom 96-well plates and washed twice in flow cytometry staining buffer (PBS containing 2% FBS). Cell pellets were dissociated briefly by vortexing and antibodies for CD105 APC, ICAM1 PE, or PDL1 PE (eBioscience, Part of ThermoFisher Scientific) were added. Cells were incubated with surface antibodies for 30 minutes at 4°C, and then washed in flow cytometry staining buffer. Cells were then ready to acquire by flow cytometry on an Accuri C6. For intracellular staining, cells were incubated with 1X Brefeldin A (eBioscience, Part of ThermoFisher Scientific) for 4 hours before harvest. The intracellular FoxP3 kit was used per manufacturer's instructions (eBioscience, Part of ThermoFisher Scientific) to prepare cells for intracellular staining. Cells were blocked with 2% rat serum (eBioscience, Part of ThermoFisher Scientific) for 15 minutes to prevent nonspecific staining, and either IDO or COX-2 antibodies (BD Biosciences, Berkshire, UK) were then added for 45 minutes. Cells were then washed in flow cytometry staining buffer and acquired using the Accuri C6 (BD Biosciences).

2.7 | Ethics

All procedures involving the use of animals or human materials were carried out by licensed personnel. Ethical approval for all work was granted by the biological research ethics committee of Maynooth University (BRESC-2014-002). Bone marrow aspirates were obtained with informed consent from healthy adults under a Research Bone Marrow Donor Program (Lonza,

Walkersville, Maryland). Project authorization was received from the scientific animal protection unit of the health products regulatory agency under AE19124/P006 whereby the terms of the animal experiments within this project were outlined and adhered to. NOD.Cg-Prkdc^{scid}IL2^{tmlWjl}/Szj (Jackson Labs) mice were used in these studies.

2.8 | Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, California). The Mann-Whitney *U* test was used when statistical analysis was required between two experimental groups. One-way analysis of variance (ANOVA) was used when comparisons between multiple groups were required. The Mantel-Cox test was used to compare survival between treatment groups. Two-way ANOVA with repeated measures was used to analyze data with two variables such as time and treatment. Data are presented as the ± standard error of the mean (SEM). *P*-values of <.05 (*), <.01 (**), <.001 (***), or <.0001 (****) were considered statistically significant.

3 | RESULTS

3.1 | MAPCs significantly delay GvHD onset when administered on day 7, but not day 0

Mouse MAPCs have previously been shown to increase survival in mouse models of GvHD when administered directly to the spleen but not via intracardiac injection.[6] Given that MAPCs are administered i.v. in clinical trials to treat indications such as GvHD, it is important to investigate if i.v. administered human MAPCs are efficacious in a GvHD model system where disease pathology is driven by human immune cells. We have previously shown that human MSCs prolong survival in a humanized aGvHD model, when administered i.v. on day 7 but not on day 0.[5] This is associated with the difference in the level of proinflammatory cytokines (like IFN-γ) present in the microenvironment to activate human MSCs to become immunosuppressive. First, we sought to determine if human MAPCs acted in a similar manner.

Human MAPCs administered i.v. on day 0 did not significantly improve the survival of aGvHD mice, while MAPCs administered on day 7 significantly prolonged the survival and reduced the aGvHD disease score of aGvHD mice (Figure 1A,B). Mice receiving MAPCs on day 7 had a median survival time of 26 days, with 30% of the group surviving until the end of the study (following a single dose of MAPCs) (Figure 1B). In line with this, MAPCs administered on day 7 significantly reduced the pathological score in the small intestine (Figure 1C) and liver (Figure 1F) and reduced the pathological score in the lung (Figure 1E). In contrast, MAPCs administered on day 0 reduced the pathological score in the liver (Figure 1F) but not in the small intestine, colon, or lung (Figure 1C-E).

3.2 | Prelicensing MAPCs with IFN-γ significantly enhances the efficacy and retention of MAPCs administered on day 0 in the humanized aGvHD model

The immunosuppressive capacity of human MSCs in the aGvHD model is enhanced following pretreatment with IFN-γ.[5,31] Here, we investigated the effects of prestimulation on the efficacy of human MAPCs in the humanized aGvHD model. Mice that received IFN-γ prelicensed MAPCs (γMAPCs) on day 0 had a lower aGvHD score on day 14 than those that received PBMC only (Figure 2A). Similarly, γMAPCs, but not MAPCs, significantly prolonged survival following i.v. administration on day 0 (Figure 2B). The pathological score in the small intestine and colon (Figure 2C,D) were significantly reduced, while the pathological score in the lung (Figure 2E) was reduced (but not with statistical significance) in aGvHD mice that received γMAPCs but not unstimulated MAPCs on day 0 (Figure 2C-E). In line with the findings from Figure 1F, unstimulated MAPCs but not γMAPCs significantly reduced the pathological score in the liver (Figure 2F).

Because of the enhanced survival of aGvHD mice following treatment with γMAPCs, we sought to investigate the retention of γMAPCs in comparison to unstimulated MAPCs. IFN-γ stimulation of MSCs increases expression of adhesion molecules such as ICAM1, and chemokines such as CXCL9 and CXCL12.[17] Therefore, we hypothesized that if γMAPCs have a similar expression pattern to IFN-γ-stimulated MSC, that they may be better equipped to escape entrapment in the lungs and upon reaching sites of injury, they may be retained there in greater numbers than unstimulated MAPCs. Both MAPCs and γMAPCs were labeled with the Qtracker 625 labeling kit before being administered to the aGvHD model on day 0, along with PBMCs. Twenty-four hours after PBMC and MAPCs administration, the lung, liver, and spleen were harvested and samples were sectioned and imaged using the CryoViz. There was no significant difference in the number of MAPCs or γMAPCs detected in the lungs of aGvHD mice (Figure 3A). In the spleen and liver, the number of γMAPCs detected was significantly increased compared with MAPC (Figure 3B,C). Therefore, these data might suggest that IFN-γ prelicensing of MAPCs may improve migration or perhaps retention of MAPCs in aGvHD target organs.

3.3 | PPARδ agonism impairs the efficacy of MAPCs in aGvHD

In addition to cytokine licensing, other genes such as the transcription factors TWIST1 and STAT1 have been shown to play an important role in MSC immunomodulatory capacity.[20,32] The transcription factor PPARδ has been shown to play a key role in mouse MSC immunomodulation in vivo.[21] The impact of PPARδ on human MSC or indeed MAPC immunosuppressive function in vivo has not yet been reported. Here, we

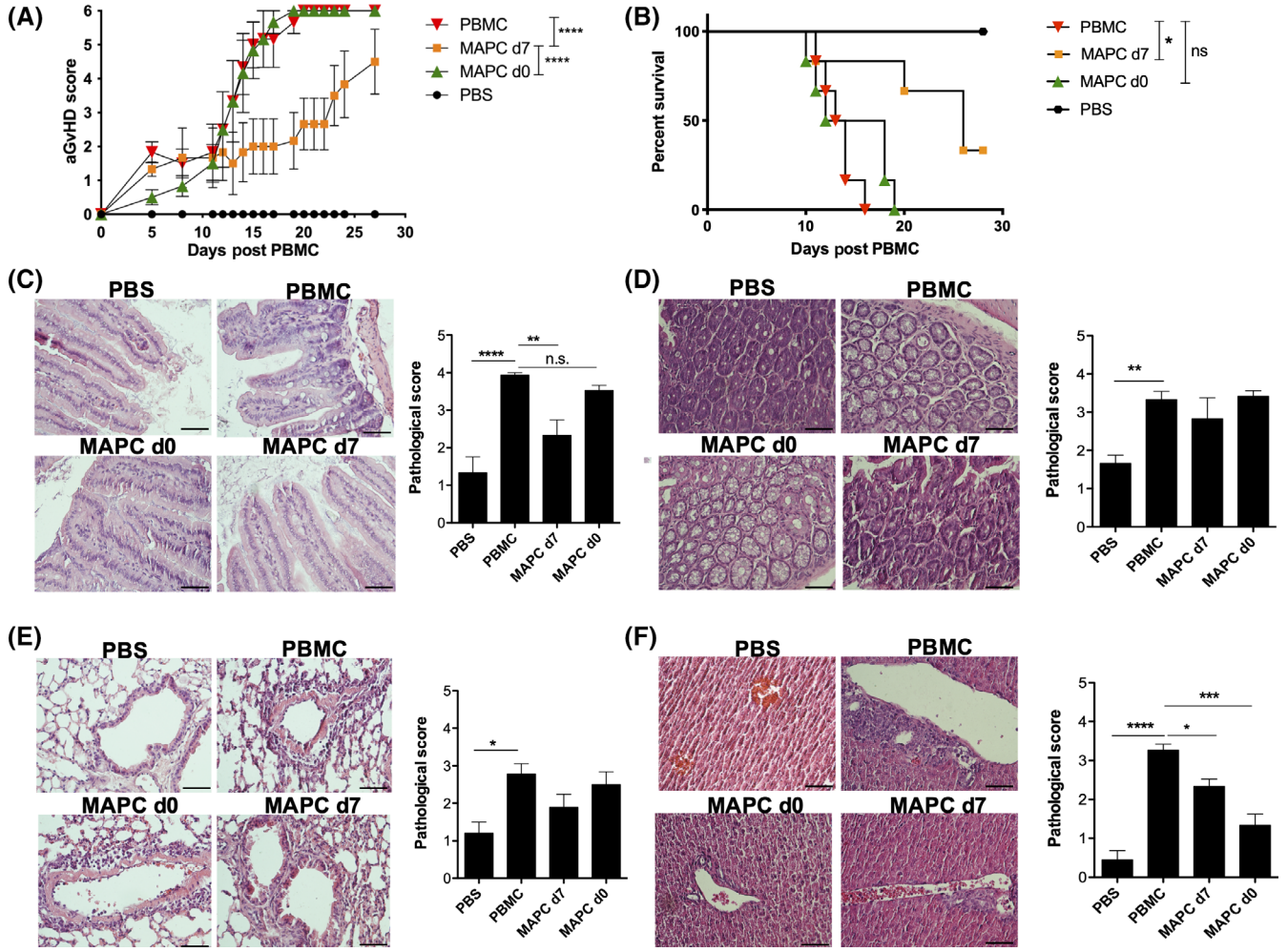

FIGURE 1 Multipotent adult progenitor cells (MAPCs) administered on day 7 but not day 0 inhibit acute graft vs host disease (aGvHD) development. 8×10^5 human PBMC per gram were administered via i.v. to irradiated NOD-SCID IL-2rγ^{null} (NSG) mice on day 0. 6.4×10^4 human MAPCs per gram were administered i.v. along with PBMC on day 0 or 7 days later. Mice were monitored regularly for symptoms of aGvHD and scores were recorded (A). Mice with a pathological score of 5 or higher were humanely euthanized by cervical dislocation and survival was graphed (B). Small intestine (C), colon (D), lung (E), and liver (F) were harvested on day 12, examined for signs of GvHD using H&E staining and given pathological scores based on the appearance of tissue destruction and mononuclear cell infiltration (C-F), scale bar = 20 μm, magnification 400×. Statistical analysis of the survival curve was carried out using a Mantel Cox test and pathological scores were analyzed using one-way ANOVA with Tukey's multiple comparison test or two-way ANOVA with repeated measures where *≤.05, ***≤.01, ***≤.001, and ****≤.0001. Experiments were repeated twice using two PBMC donors and two MAPC donors, n = 6/group

investigated the effects of PPARδ agonism and antagonism on human MAPC immunomodulatory function in the humanized aGvHD model. MAPCs were cultured with or without a PPARδ agonist for 48 hours prior to administration to the humanized aGvHD model on day 7. MAPCs treated with the PPARδ agonist (MAPC d7 PPAR[+]) lost the ability to reduce the aGvHD score (Figure 4A) and prolong survival in the aGvHD mice (Figure 4B).

Following on from this, we investigated the capacity for PPARδ antagonism to enhance MAPCs efficacy when administered on day 0 to the aGvHD model. PPARδ antagonized MAPCs (MAPC d0 PPAR[−]) but not MAPCs administered on day 0 reduced the aGvHD disease score (Figure 4C) and significantly prolonged survival in the aGvHD model (Figure 4D).

In addition to understanding if PPARδ agonism or antagonism can reduce or enhance MAPC efficacy, it was also important to determine if PPARδ agonism had any negative effects on IFN-γ MAPC prelicensing. To this end, MAPCs were exposed to the PPARδ agonist (GW0742) for 48 hours in culture. rhIFN-γ was added at a concentration of 50 ng/mL for the last 24 hours prior to harvest to generate γMAPCs. Cells were counted and washed three times with sterile PBS before being administered via i.v. on day 0. Although γMAPCs reduced the aGvHD score (Figure 4E) and significantly prolonged survival (Figure 4F), PPARδ agonized IFN-γ-licensed MAPCs (γMAPC d0 PPAR[+]) did not significantly reduce the aGvHD score or prolong survival (Figure 4E,F). These data highlight the capacity for PPARδ antagonism to enhance MAPC immunomodulatory function but also the potential

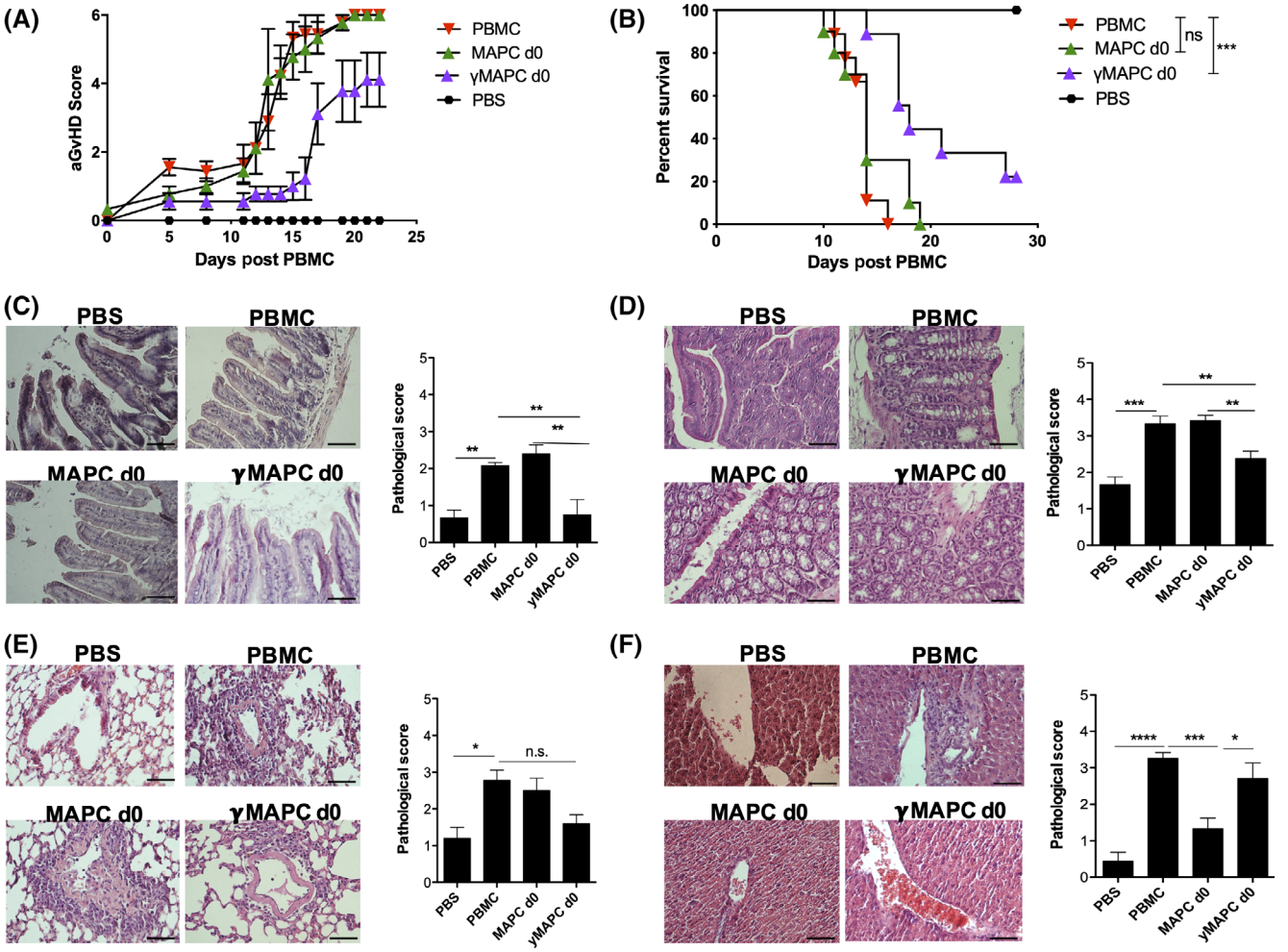

FIGURE 2 Interferon (IFN)-γ prelicensing improves the efficacy of multipotent adult progenitor cells (MAPCs) administered on day 0. 8×10^5 human PBMC per gram were administered to irradiated NOD-SCID IL-2rγ^{null} (NSG) mice on day 0. 6.4×10^4 γMAPCs or unlicensed MAPCs per gram were administered i.v. along with PBMC on day 0. Mice were monitored regularly for symptoms of acute graft vs host disease (aGvHD) and scores were assigned (A). Mice with an aGvHD score of 5 or higher were humanely euthanized by cervical dislocation and survival was graphed (B). γMAPCs significantly prolonged survival in the aGvHD model, while unlicensed MAPCs had no effect. Small intestine (C), colon (D), lung (E), and liver (F) were harvested on day 12, examined for signs of GvHD using H&E staining and given pathological scores based on the appearance of tissue destruction and mononuclear cell infiltration (C-F), scale bar = 20 μm, magnification 400×. Statistical analysis of the survival curve was carried out using a Mantel Cox test and pathological scores were analyzed using one-way ANOVA with Tukey's multiple comparison test where *≤.05, ***≤.01, ***≤.001, and ****≤.0001. Experiments were repeated twice using two PBMC donors and two MAPC donors, n = 6/group

negative impact that PPARδ ligand activation may have on human MAPCs in vivo.

3.4 | PPARδ negatively impacts the retention of MAPCs in aGvHD

Since treatment with the PPARδ agonist hindered the therapeutic efficacy of MAPCs, and PPARδ knockout mouse MSCs showed increased expression of adhesion molecules such as ICAM1 and VCAM1 in response to proinflammatory cytokines,[21] we hypothesized that PPARδ agonism may impact the retention of MAPCs in aGvHD target organs. Qtracker-labeled MAPCs, γMAPCs, PPARδ-agonized MAPCs and PPARδ-agonized γMAPCs administered to aGvHD mice on day 7 via i.v. were imaged 24 hours later in the lung, liver, and spleens using CryoViz imaging. Although there was no difference in the number of MAPCs compared with PPARδ-agonized MAPCs (MAPC PPAR[+]) in the lungs (Figure 5A), the number of PPARδ-agonized MAPCs detected in the spleen and liver was significantly reduced (Figure 5B,C). Similarly, the number of PPARδ-agonized γMAPCs (γMAPC PPAR[+]) detected were significantly reduced in the lung and spleen and reduced in the liver compared with the number of γMAPCs detected in mice that received PBMCs (Figure 5D-F). These data might suggest that PPARδ agonism negatively impacts the retention of MAPCs and IFN-γ-licensed MAPCs in GvHD target organs.

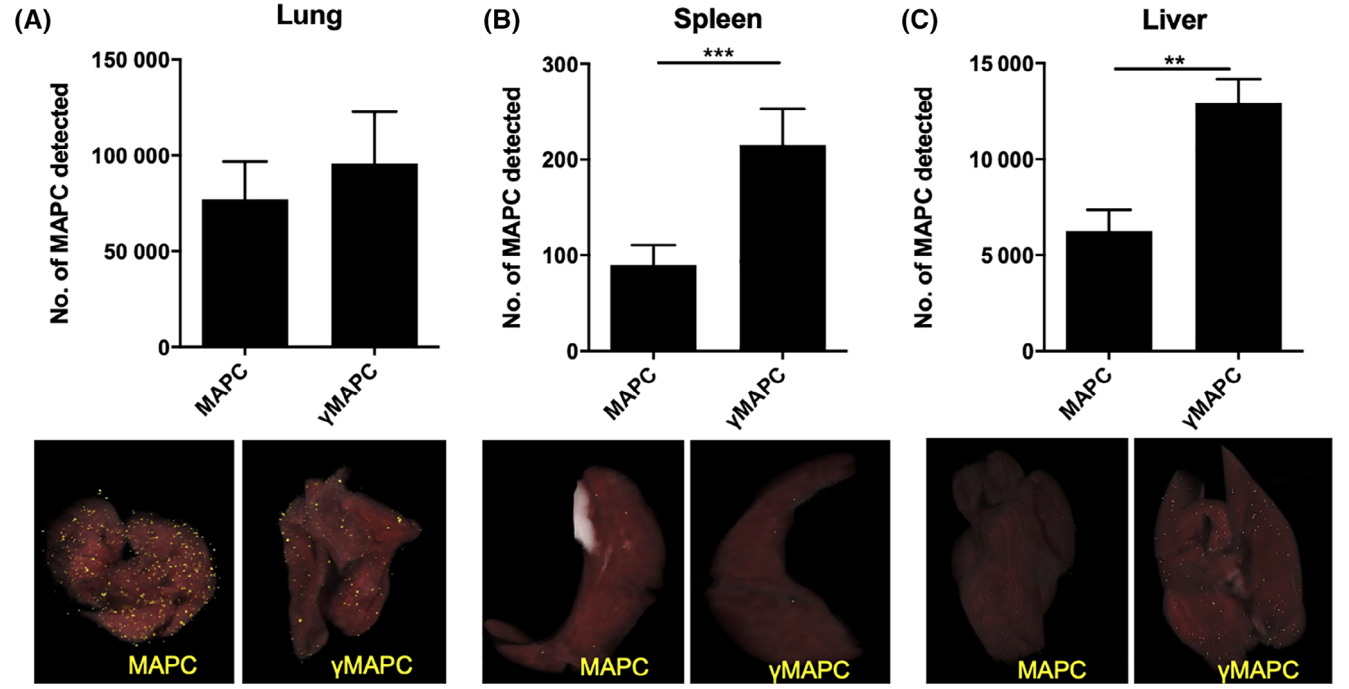

FIGURE 3 Interferon (IFN)-γ prelicensing improves the retention of multipotent adult progenitor cells (MAPCs) in acute graft vs host disease (aGVHD) target organs administered on day 0. MAPCs and γMAPCs were labeled with Qtracker® 625 and administered to the aGvHD model on day 0 as described in figure legend 2. Lung (A), spleen (B), and liver (C) were harvested and snap frozen 24 hours after MAPC administration. Tissue was imaged and processed using CryoViz™ technology. Statistical analysis was carried out using the Mann-Whitney *U* test to compare between two groups; where *≤.05, **≤.01, and ***≤.001. n = 5 per group

3.5 | PPARδ agonism or antagonism has no effect on the STAT1 target genes ICAM-1, PDL1, or IDO in human MAPCs

PPARβδ$^{-/-}$ mouse MSCs exhibit an enhanced response to proinflammatory cytokines in vitro with increased expression of ICAM, VCAM, and iNOS compared with their wild-type counterparts.[21] Similarly, PPARδ$^{-/-}$ mouse MSCs display increased NF-κB activity in response to IFN-γ and TNF-α stimulation.[21] These data, taken together with our in vivo data, suggest that PPARδ may suppress IFN-γ signaling in MSCs and MAPCs. Since the immunosuppressive properties of MSCs are dependent on the presence of IFN-γ in the microenvironment,[20,33] we hypothesized that PPARδ agonism might suppress IFN-γ signaling in MAPCs. Exposure of both human and mouse MSCs to IFN-γ results in STAT1 induction and phosphorylation. In mouse MSCs, STAT1 knockdown inhibits mRNA levels of PDL1, NOS2, and IL18bp, and reduces the immunosuppressive capacity of these MSCs in a T-cell proliferation assay, whereas in human MSC, STAT1 knockdown reduces IDO production.[15,18] Therefore, since PPARδ agonism abrogated the immunosuppressive capacities of MAPCs, we examined the impact of PPARδ agonism and antagonism on STAT1 signaling in human MAPCs.

IFN-γ stimulation increased the protein levels of both STAT1 and pSTAT1 compared with unstimulated cells. Neither PPARδ agonism nor antagonism significantly altered STAT1 or pSTAT1 in MAPCs or IFN-γ-stimulated MAPCs (Figure 6A,B). IFN-γ-stimulated MAPCs expressed or produced significantly increased levels of the STAT1 target genes ICAM-1 and PDL1 and increased levels of IDO. However, expression or production of ICAM-1, PDL1, and IDO was not influenced by PPARδ agonism or antagonism in MAPC or γMAPCs (Figure 6C-E).

Along with IDO, PGE2 is the most widely reported mediator of human MSC[34] and MAPC immunosuppression, and has been reported to be required for MSC and MAPC efficacy in GvHD.[1,3] PGE2 is converted from arachidonic acid by the enzyme COX-2 and both factors are constitutively expressed by both mouse and human MSCs; however, their expression is further increased by exposure of MSCs to IFN-γ or TNF-α.[2,35] Moreover, we have recently demonstrated an important role for PGE-2 in human MAPC suppression of homeostatic T-cell proliferation in vivo.[1] Since COX-2 is an NF-κB-regulated protein, and Luz-Crawford et al reported increased NF-κB activity following PPARδ knockdown,[21] we next hypothesized that PPARδ agonism and antagonism may alter COX-2 expression by MAPCs. The percentage of COX-2 expressing MAPCs was significantly upregulated following IFN-γ stimulation (Figure 7A,B). PPARδ agonism significantly reduced the percentage of COX-2 expressing γMAPCs (γMAPC PPAR[+]), while PPARδ antagonism did not affect the percentage of COX-2 expressing γMAPCs (γMAPC PPAR

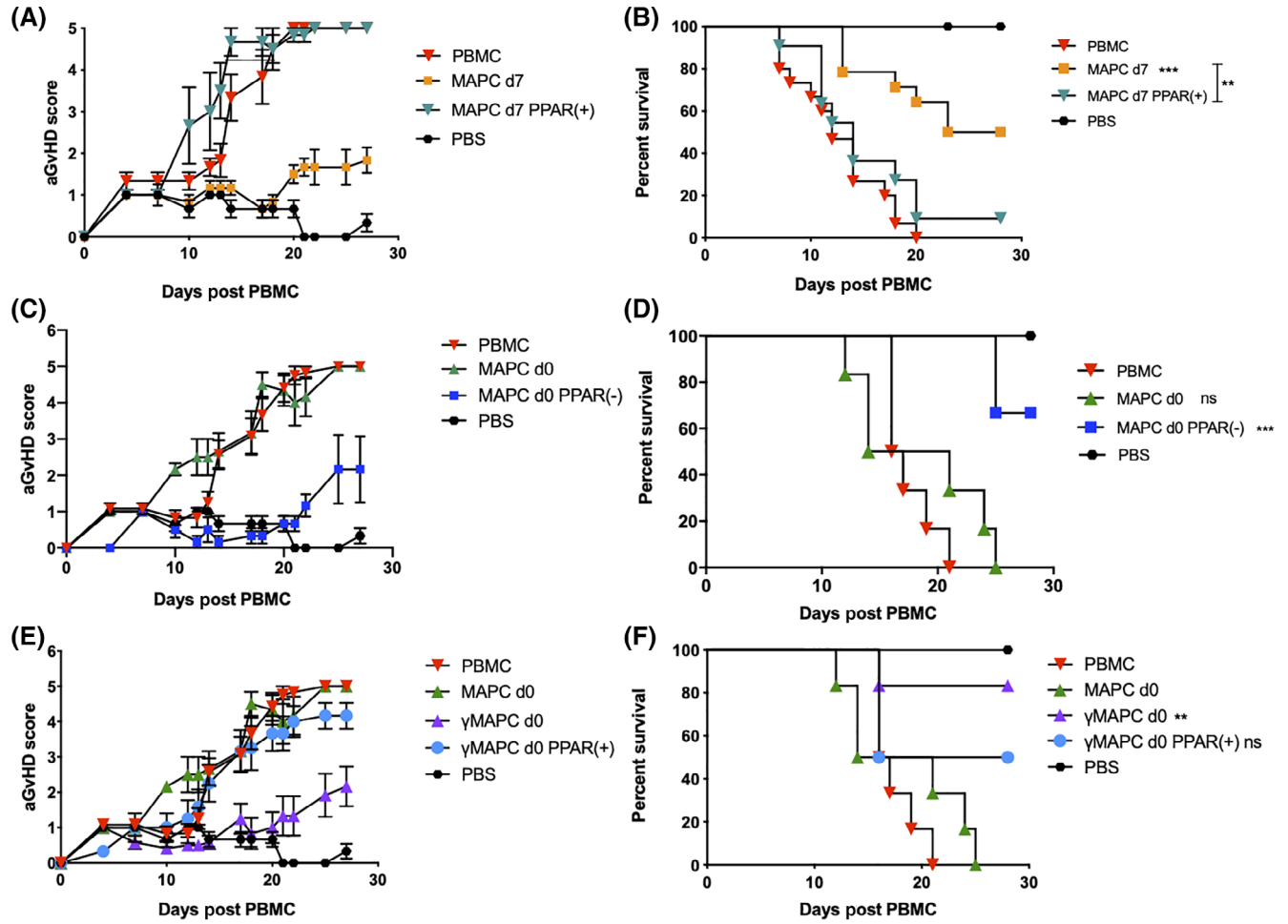

FIGURE 4 Peroxisome proliferator-activated receptor-delta (PPARδ) agonism reduces the efficacy of multipotent adult progenitor cell (MAPC) and γMAPCs in the acute graft vs host disease (aGvHD) model. 8×10^5 human PBMC per gram were administered to irradiated NOD-SCID IL-2rγ^{null} (NSG) mice on day 0. 6.4×10^4 per gram MAPCs, PPARδ agonist-treated MAPCs (MAPC PPAR [+]), PPARδ antagonist-treated MAPCs (MAPC PPAR[−]), interferon (IFN)-γ-licensed MAPCs (γMAPC), or PPARδ agonist-treated γMAPCs (γMAPC PPAR[+]) were administered to the aGvHD model on day 0 or day 7. MAPC d7 cells significantly prolonged survival in the aGvHD model, while MAPC d7 PPAR(+) cells had no effect (A, B). MAPC d0 cells did not prolong survival in the aGvHD model, while MAPC d0 PPAR(−) cells significantly prolonged survival (C, D). γMAPC d0 cells but not MAPC d0 or γMAPC PPAR(+) cells significantly prolonged survival in the aGvHD model (E, F). Statistical analysis of the survival curve was carried out using a Mantel Cox test where **≤.01 and ***≤.001. n = 6 mice/group

[−]) (Figure 7A,B). In line with this, agonism of PPARδ reduced (but not significantly) PGE-2 production by γMAPC (Supplementary Figure 1). We further investigated the role of COX-2 in PPARδ-antagonized MAPC efficacy in aGvHD using an inhibitor to suppress COX-2 activity in PPARδ-antagonized MAPCs in vitro before administration to the aGvHD mice on day 7. PPARδ antagonism significantly enhanced the efficacy of MAPCs administered on day 7 (Figure 7C). Importantly, the exposure of PPARδ-antagonized MAPC to the COX-2 inhibitor indomethacin before administration significantly reduced the efficacy of PPARδ-antagonized MAPC in the aGvHD-humanized mouse model (Figure 7C). These data suggest that COX-2 may play an important role in MAPC efficacy in aGvHD and that PPARδ antagonism/agonism may play an important role in modulating COX-2 expression in MAPCs.

4 | DISCUSSION

In 2004, Le Blanc et al published a landmark study reporting the successful use of haploidentical MSCs in a young patient with steroid refractory aGvHD.[36] Since then, investigators have been excited about the potential of using "off the shelf" cells as a therapy to prevent or treat GvHD. However, in the first major industry-sponsored phase-III clinical trial, MSCs failed to meet the primary endpoint of durable complete response lasting at least 28 days.[37] Importantly, the most recent Mesoblast phase-III clinical trial with informed selective patient enrolment focusing on pediatric GvHD successfully met the primary end point. Within this trial, the day-28 overall response rate was 69%,[12] highlighting that even with patient stratification, MSC therapy can successfully treat some but

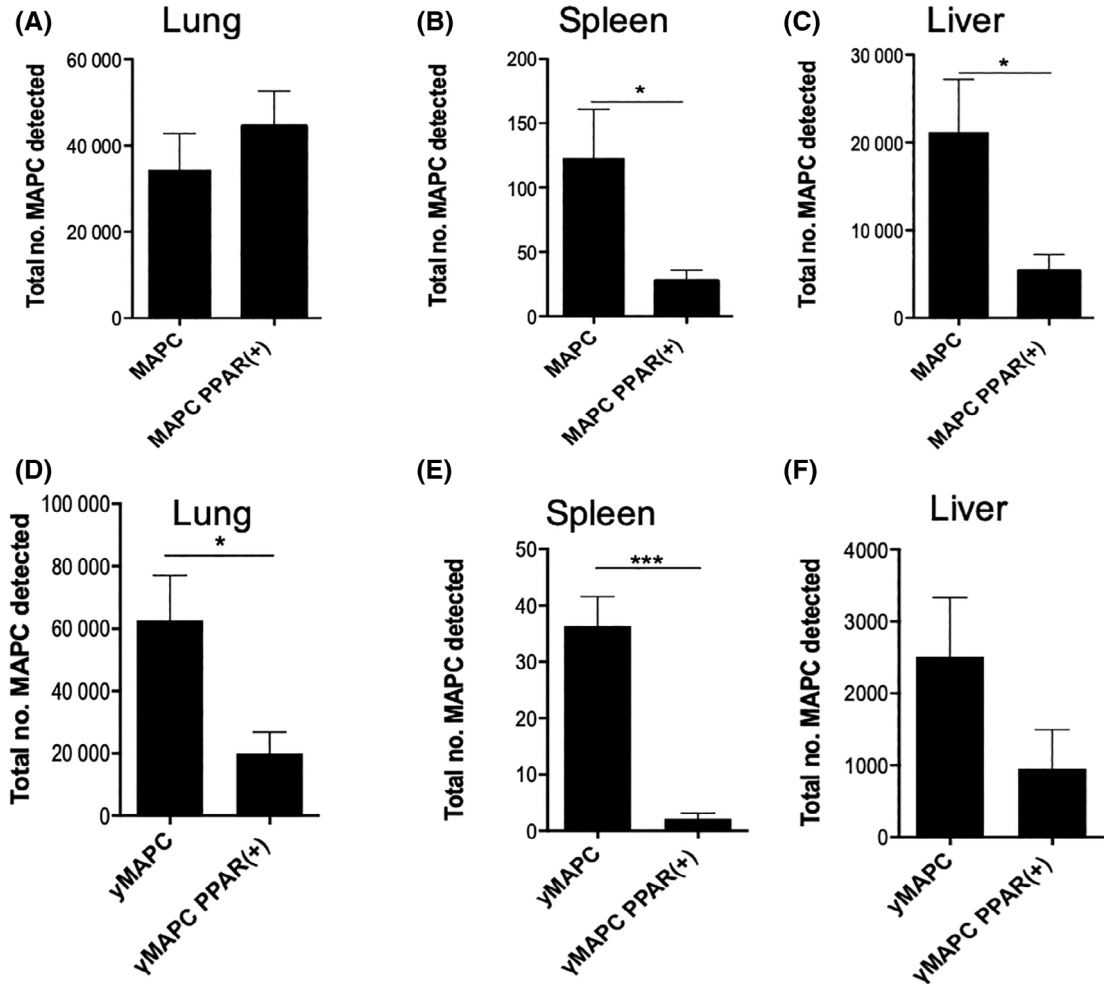

FIGURE 5 Peroxisome proliferator-activated receptor-delta (PPARδ) agonism impairs the retention of multipotent adult progenitor cell (MAPC) and γMAPCs in the acute graft vs host disease (aGvHD) model. MAPCs and PPARδ agonist-treated MAPCs (MAPC PPAR[+]) cells were labeled with Qtracker® 625 and administered to the aGvHD model on day 7. Mice were humanely euthanized and lung, spleen, and liver were harvested 24 hours after MAPCs administration. Tissue was processed and imaged using CryoViz™ technology. The number of MAPCs detected in the lung was unchanged (A) but was reduced in the spleen (B) and liver (C) when MAPCs were treated with PPARδ agonist prior to administration. γMAPCs and PPARδ agonist-treated γMAPCs (γMAPC PPAR[+]) were labeled with Qtracker® 625 and administered to the aGvHD model on day 0. Mice were humanely euthanized and lung, liver, and spleen harvested 24 hours after MAPC administration. The number of γMAPCs detected in the lung, spleen, and liver was reduced when MAPCs were treated with PPARδ agonist prior to administration (D-F). Statistical analysis was carried out using the Mann-Whitney *U* test to compare between two groups; where *≤.05 and ***≤.001. n = 6/group

not all patients. It is clear from these results that further elucidation of MSC and MAPC biology require clarification in order to maximize the therapeutic potential of these cells. Thus, this study sought to identify pathways through which MAPC efficacy could be enhanced, to understand the modes of action of these pathways, and efficacy and retention of MAPCs in a clinically relevant, humanized mouse model of aGvHD. Our data indicate that the efficacy and retention of MAPCs in aGvHD may be improved by IFN-γ stimulation and that agonism of PPARδ negatively influences MAPC and γMAPC retention. Importantly, PPARδ activation may inhibit the therapeutic activity of MAPCs and IFN-γ-licensed MAPCs in inflammatory environments.

One major concern surrounding the use of MSCs or MAPCs in the clinic is the timing of administration. Mouse models have shed some light on this issue. For example, Highfill et al[6] found that mouse MAPCs had no effect on GvHD prevention when infused systemically on the same day as bone marrow transplantation; however, intrasplenic delivery of MAPCs was beneficial. Similarly, we have previously shown that human MSCs administered i.v. on day 0 were ineffective at alleviating GvHD symptoms.[5] Overall, MAPCs administered on day 7 significantly improved the disease score, survival time, and tissue pathology of aGvHD animals. While MAPCs were more potent at alleviating cellular infiltration and tissue damage in the lung and GI tract when

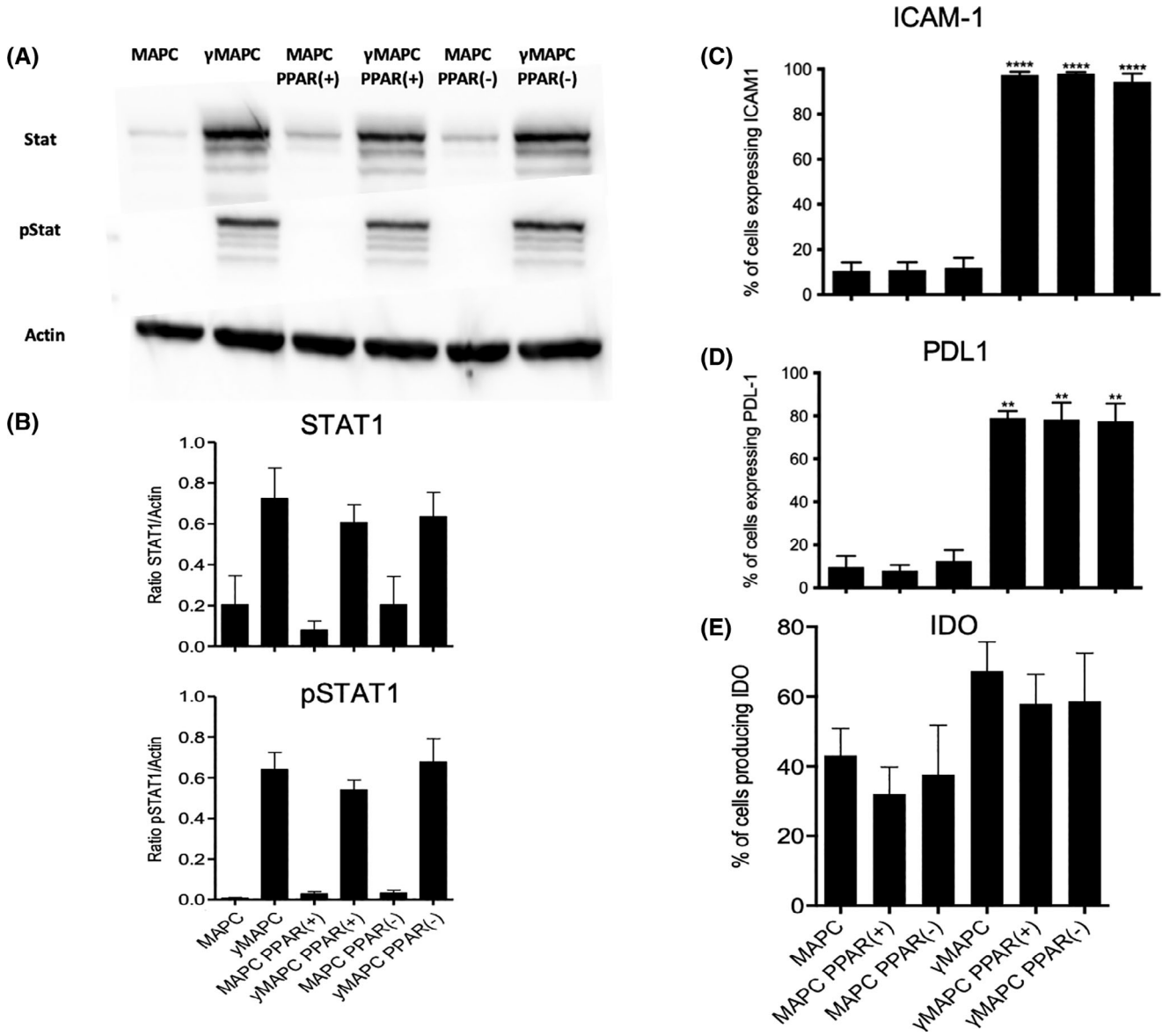

FIGURE 6 Peroxisome proliferator-activated receptor-delta (PPARδ) agonism or antagonism has no influence on STAT-regulated interferon (IFN)-γ-stimulated immunomodulatory factors by multipotent adult progenitor cells (MAPCs). MAPCs, PPARδ agonist-treated MAPCs (MAPC PPAR[+]), and PPARδ antagonist-treated MAPCs (MAPC PPAR[−]) were stimulated with IFN-γ for 24 hours before being collected and examined for protein levels of STAT1 and pSTAT1 by Western blot, and IDO, PDL1, and ICAM1 by flow cytometry. Representative image and densitometry of STAT1 and pSTAT1 levels show that PPARδ agonism or antagonism has no impact on STAT1 in response to IFN-γ stimulation (A). Actin was used as a loading control, and the ratio of proteins of interest to actin was measured using densitometry (B). PPARδ agonism or antagonism had no effect on ICAM1 or PDL1 expression or IDO production by MAPCs (C-E). Statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison test where *≤.05, ***≤.01, ***≤.001, and ****≤.0001. n = 3/group

administered on day 7 compared with day 0, surprisingly MAPCs delivered on day 0 were superior at reducing signs of GvHD in the liver. This may associated with the capacity for MAPCs to promote an anti-inflammatory monocyte/macrophage population in the liver[38] and other organs following phagocytosis of the MAPCs as has been reported for MSCs.[39-41]

Prestimulation with IFN-γ before administration improves the therapeutic effect of MSCs delivered to GvHD animals at early time points.[5,16] Here, we demonstrate that γMAPCs delivered to the aGvHD model on day 0 were superior at alleviating the disease score, prolonging survival, and reducing tissue damage in the gut and lungs than unlicensed MAPCs. In line with the data showing a greater reduction of pathological score in the liver by MAPCs day 0, there was no significant reduction of liver pathology in mice that received γMAPCs on day 0. This may be explained by an accumulation of immune cells in response to γMAPCs retained in the liver in

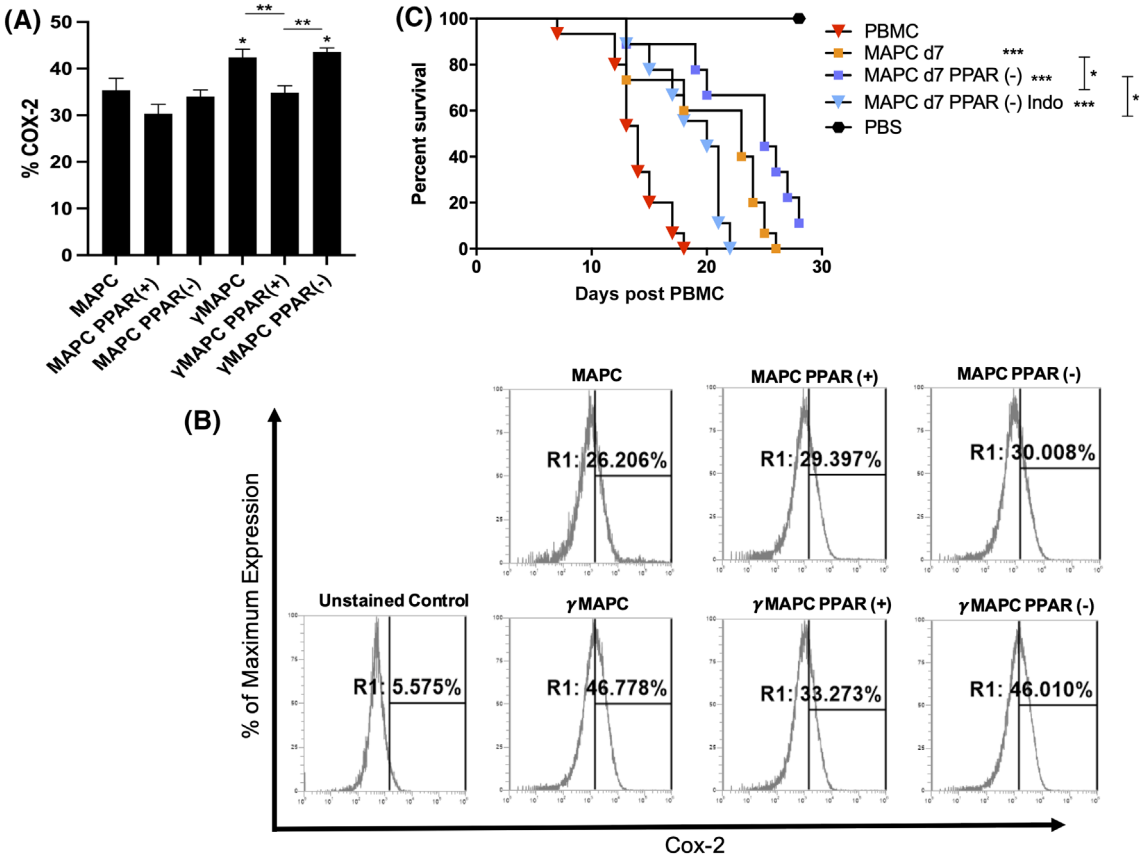

FIGURE 7 Peroxisome proliferator-activated receptor-delta (PPARδ) antagonism enhances COX-2 production by multipotent adult progenitor cells (MAPCs). MAPCs, PPARδ agonist-treated MAPCs (MAPC PPAR[+]), and PPARδ antagonist-treated MAPCs (MAPC PPAR[−]) were stimulated with interferon (IFN)-γ for 24 hours before being collected and examined for protein levels of COX-2 by intracellular flow cytometry. IFN-γ stimulation increased COX-2 expression by MAPCs and this effect was significantly inhibited by PPARδ agonism. Expression of COX-2 by γMAPCs was not inhibited when PPARδ was antagonized (A, B) (n = 5/group). 8×10^5 human PBMC per gram were administered to irradiated NOD-SCID IL-2rγ^{null} (NSG) mice on day 0. 6.4×10^4 MAPCs, PPARδ antagonist-treated MAPCs (MAPC d7 PPAR[−]), or PPARδ antagonist-treated MAPCs exposed to the COX-2 inhibitor indomethacin (Indo) (MAPC d7 PPAR[−] Indo) per gram were administered to the acute graft vs host disease (aGvHD) model on day 7. There was a significant difference in the capacity for MAPC vs MAPC PPAR(−) and between MAPC PPAR(−) and MAPC PPAR(−) Indo in prolonging survival of the aGvHD mice (C). A, Statistical analysis was carried out using the Mann-Whitney *U* test to compare between two groups; C, statistical analysis of the survival curve was carried out using a Mantel Cox test where *<.05, **<.01, and ***<.001. C, Experiments were repeated twice (n = 9-15 per group)

comparison to unlicensed MAPCs, as Hoogduijn and colleagues have previously reported the presence of MSCs in the liver following phagocytosis by phagocytic cells.[38] Overall, these data align well with previous studies of MSCs in aGvHD.[5,16] Furthermore, these data support the proposal that licensing MAPCs with IFN-γ prior to administration may improve their therapeutic efficacy when delivered at sub-optimal time points.

Next, we sought to examine whether γMAPCs would show increased distribution toward GvHD target organs compared with unlicensed MAPCs. MAPCs and MSCs stimulated with IFN-γ show increased expression of adhesion molecules VCAM and ICAM, migratory, and chemotactic mediators (such as CCL2/CXCL9/CXCL10)[42,43] and both MSCs and MAPCs licensed with IFN-γ are less susceptible to natural killer cell lysis.[28,44] Therefore, we hypothesized that γMAPCs may demonstrate increased distribution toward GvHD target organs or persist for longer compared with unlicensed MAPCs. At 24 hours post administration, the number of γMAPCs detected in the spleen and liver of aGvHD mice was significantly higher than the number of unlicensed MAPCs detected. The number of cells detected in the lung was unchanged, which suggests that γMAPCs were not persisting longer than unlicensed MAPCs in the lungs, but that more cells may have migrated toward or were retained for longer in distal target organs.

As PPARδ expression/activation has been shown to negatively influence mouse MSC immunomodulatory capacity in a mouse model of arthritis,[21] we examined the effect of PPARδ agonism and antagonism in human MAPCs and γMAPCs in our clinically relevant humanized mouse aGvHD model. Antagonism of PPARδ significantly enhanced the efficacy of

MAPCs administered on day 0. Activation of PPARδ using a selective PPARδ agonist significantly impaired the ability of MAPCs administered on day 7, and γMAPCs administered on day 0 to prolong survival and reduce disease score compared with untreated MAPCs.

Since PPARδ$^{-/-}$ MSCs demonstrated increased expression of ICAM1 and VCAM1 when stimulated with IFN-γ and TNF-α,[21] and PPARδ activation hindered the immunosuppressive capacity of MAPCs in the aGvHD model, we hypothesized that PPARδ agonism would impair the retention of MAPCs. The number of PPARδ agonist-activated MAPCs detected in the liver and spleen was significantly lower than untreated MAPCs, while the number of PPARδ agonist-activated γMAPCs detected in the liver was lower and significantly lower in the spleen compared with γMAPC. While there was no difference in the number of PPARδ-agonized MAPCs detected in the lung compared with MAPCs, PPARδ activation significantly reduced the number of γMAPCs detected in the lung 24 hours following administration. Therefore, in general these data suggest that PPARδ agonism reduces the retention of MAPC and γMAPCs in vivo in GvHD target organs.

Murine MSCs lacking PPARδ demonstrated increased efficacy in a murine model of arthritis,[21] and responded to cytokine stimulation with enhanced expression of immunomodulatory factors such as VCAM1, ICAM1, and NOS due to increased NF-κB activity. Little else is known about the role of PPARδ in MSCs, however, in macrophages PPARδ activation inhibits STAT1 and NF-κB activation.[29,45-47] Since STAT1 activation is required for the therapeutic efficacy of MSCs,[18,48] we examined the role of PPARδ agonism or antagonism on the expression or production of immunomodulatory factors in IFN-γ-stimulated human MAPCs. Here, we found that PPARδ agonism or antagonism had no effect on the increased expression of STAT1, phospho STAT1, ICAM1, PDL1, or IDO driven by IFN-γ stimulation in MAPCs. Recently, we have shown that COX-2 and PGE-2 play a key role in MAPC suppression of homeostatic T-cell proliferation in vitro and in vivo. Importantly, the increased COX-2 expression in response to IFN-γ stimulation was suppressed following PPARδ agonism. This alteration of COX-2 expression may be STAT1 independent, as COX-2 is generally considered to be an NF-κB target gene, and NF-κB can be activated by IFN-γ independent of STAT1.[49] Since PPARδ is known to interfere with NF-κB activity[50] and PPARδ agonism suppresses STAT3 target gene expression in human macrophages,[29] the effects seen in MAPCs here may be due to impaired NF-κB rather than STAT1 signaling following IFN-γ stimulation. Finally, using the aGvHD model, we demonstrate that inhibition of COX-2 via the inhibitor indomethacin in PPARδ-antagonized MAPCs significantly reduces the capacity for PPARδ-antagonized MAPCs to prolong survival. The COX-2 data suggest that the effects of PPARδ agonism on the potency of MAPCs in our model may be due to impaired COX-2 activity in response to inflammation. The pattern of MAPC biodistribution observed aligns with published studies on MSC fate following phagocytosis by lung macrophages.[38,51] The observation that MAPC-derived COX-2 is important for efficacy links strongly to a number of exciting studies in the field showing that in vivo macrophage polarization following MSC administration plays an important role in mediating MSC protective effects in vivo. These studies demonstrate the important role for mouse resident macrophages or phagocytes in inflammatory bowel disease,[41] asthma,[52] and in aGvHD.[40] Moreover, studies have elegantly shown the important role for MSC-derived COX-2 in shaping mouse-resident macrophages,[53,54] key players in facilitating MSC protective effects. It is possible that MAPC-derived COX-2 may shape lung-resident macrophages/phagocytes leading to the protective effects observed and that the presence of PPARδ ligands may negatively impact on this macrophage polarizing process via reduction in COX-2. However, this theory would need to be tested using clodronate liposomes in the presence or absence of the PPARδ agonist/antagonist.

In conclusion, this study improves our understanding of the modes of action, efficacy, and retention of MAPCs in a humanized model of aGvHD. This work demonstrates for the first time that PPARδ plays a significant role in human MAPC immunomodulatory capacity in vivo and highlights the importance of understanding the disease microenvironment and how factors such as PPARδ ligands may negatively influence MAPC efficacy in vivo.

ACKNOWLEDGMENTS

Fiona Carty was supported by an Irish Research Council Enterprise Partnership Scheme Scholarship part funded by ReGenesys BVBA (EPSPG/2013/581). The research leading to these results received financial support in the form of an EU Career Integration Grant to Karen English (Grant Number 321697), a HRB Translational Medicine Postdoctoral Research Fellowship to Karen English (Grant number PDTM/2011/21), and from Science Foundation Ireland (SFI) under Grant Number 13/SIRG/2172 through a Starting Investigator Research Grant awarded to Dr. Karen English. This publication has emanated from research supported in part by a research grant from Science Foundation Ireland (SFI) under Grant Number 12/RI/2346 (2); an infrastructure award supporting the CryoViz.

CONFLICT OF INTEREST

Fiona Carty was funded under an Irish Research Council Enterprise Partnership Scheme Scholarship part funded by ReGenesys BVBA. Karen English, Hazel Dunbar, and Ian James Hawthorne declared no competing interests. Anthony E. Ting, Samantha R. Stubblefield, and Wouter Van't Hof are employees of Athersys Inc. and have compensated stock options from Athersys, Inc.

AUTHOR CONTRIBUTIONS

F.C.: performed the studies, analyzed the data, and co-wrote the paper; H.D.: analyzed the data and co-wrote the paper; I.J.H.: performed experiments and analyzed the data; A.E.T., S.R.S., W.V.H.: provided MAPCs and reagents, and contributed intellectually to the study, analyzed the data, and co-wrote the paper; K.E.: designed and supervised the study, analyzed the data, and wrote the paper.

DATA AVAILABILITY STATEMENT

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

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