Mesenchymal stromal cells encapsulated in licensing hydrogels exert delocalized systemic protection against ulcerative colitis via subcutaneous xenotransplantation

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ARTICLE INFO

ABSTRACT

The ability of mesenchymal stromal cells (MSCs) to release a plethora of immunomodulatory factors makes them valuable candidates to overcome inflammatory bowel diseases (IBD). However, this cell therapy approach is still limited by major issues derived from nude MSC-administration, including a rapid loss of their immunomodulatory phenotype that impairs factor secretion, low persistence and impossibility to retrieve the cells in case of adverse effects. Here, we designed a licensing hydrogel system to address these limitations and thus, obtain a continuous delivery of bioactive factors. IFNγ-loaded heparin-coated beads were included in injectable *in situ* crosslinking alginate hydrogels, providing a 3D microenvironment that ensured continuous inflammatory licensing, cell persistence and implant retrievability. Licensing-hydrogel encapsulated human MSCs (hMSCs) were subcutaneously xenotransplanted in an acute mouse model of ulcerative colitis. Results showed that encapsulated hMSCs exerted a delocalized systemic protection, not presenting significant differences to healthy mice in the disease activity index, colon weight/length ratio and histological score. At day 7, cells were easily retrieved and *ex vivo* assays showed fully viable hMSCs that retained an immunomodulatory phenotype, as they continued secreting factors including PGE2 and Gal-9. Our data demonstrate the capacity of licensing hydrogel-encapsulated hMSCs to limit the *in vivo* progression of IBD.

1. Introduction

Inflammatory bowel diseases (IBD) – comprising ulcerative colitis and Chron's disease – cause inflammatory processes in the gastrointestinal tract, leading to ulcerations, diarrhea or abdominal pain, among others [1]. The etiology of IBD is multifactorial: multiple genetic and environmental factors cause an immune dysregulation that results in the aforementioned inflammatory state [1]. Currently available treatments – mainly based on anti-inflammatory drugs, immunosuppressants and biologic agents – are only focused on amelioration of major symptoms

and prevention of complications. Moreover, their lack of specificity requires the employment of high doses, aggravating their troublesome side effect profile [2,3]. Thus, conventional therapies are far from optimal and there is an urgent need for the development of alternative treatments [4].

In this context, mesenchymal stromal cells (MSCs) are attractive treatment candidates in IBD because of their immunomodulatory properties. Indeed, MSCs have been reported to interfere in a range of inflammatory pathways such as JAK/STAT [5], PI3K/AKT [6] or NF-κB [7]. MSCs regulate both, innate and adaptive immunity by interacting

with multiple immune cells, including macrophages, dendritic cells or T cells [8]. Their secretome – composed of extracellular vesicles and soluble factors such as galectin-9 (Gal-9) or prostaglandin E2 (PGE2) – is considered the main responsible for MSC-mediated immunomodulation [9–11]. However, for MSCs to release immunomodulatory factors, they must exhibit a MSC2 anti-inflammatory phenotype, which is highly dependent on the local microenvironment. To overcome this limitation, multiple pre-conditioning strategies are being explored, including inflammatory licensing or hypoxic culture. However, these protocols are performed prior to MSC implantation and they only achieve transient effects that vanish rapidly after administration [11–13].

Among the hundreds of early phase studies and dozens of advanced human clinical trials conducted with MSCs – a high number focused on inflammatory and immune disorders – the intravenous (IV) route is predominant [14,15]. However, to date, the only MSC-based product approved by the European Medicines Agency is Darvadstrocel (Alofisel®), intended for local subdermal use in Crohn related enterocutaneous fistula [16–19]. This fact highlights that the important limitations of IV delivery hold back the clinical translation of MSC-based products. After IV infusion, an important fraction of MSCs is rapidly cleared and the rest gets trapped in the lungs – where recent studies report that they persist no>3 days – [20,21]. The low persistence is directly correlated to unsuccessful outcomes [22]. Moreover, despite IV MSC delivery is supported by significant safety experience, there are still concerns about the risk of thrombosis and potential adverse inflammatory effects [23]. In this vein, it is important to highlight that once IV implanted, MSCs are no longer retrievable, disabling their withdrawal in case of adverse events. From a practical standpoint, the maximum tolerated MSC dose in IV bolus is limited (1×10^6 cells) [22], leading to the need of repeated dosing. This is not only a drawback for patient comfort and compliance, but may lead to complications too. Indeed, MHC mismatch can result in immunization following initial dosing, compromising the effectivity of multidosing protocols [24].

In the search of alternative delivery routes, the intraperitoneal (IP) route of administration has gained special attention in preclinical studies for IBD treatment. Interestingly, recent research points out to a systemic effect of IP administered MSCs [25]. Nevertheless, the risk of intra-abdominal organ injury or infection remains. In light of these observations, such systemic effect could be induced via a less invasive and safer route, such as the subcutaneous (SC). Despite the latter has not been extensively explored in IBD, it may represent a promising approach since it is simple, efficient and inexpensive and thus, commonly employed to deliver multiple drugs [26,27]. In any case, the use of these alternative routes does not overcome the major drawback: the poor cell persistence with the consequent loss of effectiveness.

In this regard, MSC encapsulation in 3D hydrogels represents a valuable strategy, not only to ensure cell persistence, but also to provide a microenvironment that modulates the secretory profile of MSCs in a sustained fashion [12]. Moreover, it enables to retrieve the implant in case of adverse events, significantly enhancing the biosafety of the therapy. In the present work, our aim was to explore the *in vivo* effects of SC administered MSC-laden hydrogels in the context of IBD. With that aim, we developed an injectable hydrogel system with intrinsic characteristics intended to promote and prolong the immunomodulatory effects of adipose tissue-derived human MSCs (hMSCs) – from here on licensing hydrogels –. In particular, IFNγ-loaded heparin-coated beads were included in *in situ* crosslinking alginate (isCA) hydrogels, which provide continuous inflammatory licensing. The resulting IFNγ-isCA hydrogels were SC implanted in a xenogeneic acute dextran sodium sulfate (DSS) colitis mouse model. Clinical outcomes were assessed during the *in vivo* study and the severity of the disease was histopathologically analyzed in the excised colon tissue. At the end of the *in vivo* study licensing hydrogels were retrieved and both MSC viability and immunomodulatory capacity were evaluated.

2. Materials and methods

2.1. Cell culture

Adipose tissue-derived hMSCs were purchased from ATCC® (Cat. No: PCS-500–011) and cultured in Mesenchymal Stem Cell Basal Medium (ATCC®, Cat. No: PCS-500- 030™) supplemented with the Mesenchymal Stem Cell Growth Kit (ATCC®, Cat. No: PCS-500–040). Cells were cultured at 37 °C in a 5% CO$_2$ / 95% air atmosphere and passaged every 4–6 days at 70–90% confluence. For all experiments, cells were used at passage 4–7.

2.2. Licensing hydrogel fabrication

Prior to licensing hydrogel fabrication, materials and hMSCs were prepared. Briefly, a 20 % CaSO$_4$ slurry in ddH$_2$O and a 2 % alginate solution in no supplemented DMEM w/o Ca^{+2} and Mg^{+2} (Life technologies) were prepared. hMSCs intended for encapsulation were overnight licensed (≈ 16 h) by supplementing their culture media with 20 ng/mL IFNγ and 10 ng/mL TNFα (Sigma-Aldrich). After this inflammatory preconditioning, cells were retrieved from culture and suspended at a 4×10^6 cells/mL rate in the 2 % alginate solution. Afterwards, agarose beads coated with heparin (BioRad) were included to the suspension. Such beads – 232.35 ± 34.19 μm in diameter – were previously loaded with IFNγ by being incubated for 1 h with a 80 ng/mL solution of the cytokine. The resulting suspension was transferred to LuerLock syringes (BD) and maintained on ice until hydrogel fabrication. To form the hydrogels, a dual-syringe system was employed. A Luerlock syringe containing the hMSC-alginate-IFNγbead mixture was connected to another containing the CaSO$_4$ slurry by means of a fluid dispensing connector (Braun) and the contents were gently mixed. Prior to hydrogel gelation, hydrogels were casted in tissue culture plates for *in vitro* studies – and maintained at 37 °C, 5% CO$_2$ in hMSC complete culture media – or SC administered for *in vivo* studies.

2.3. Evaluation of hMSC viability, metabolic activity and secretion of immunomodulatory factors

To assess hMSC viability, a Live/Dead staining of hMSCs encapsulated in licensing hydrogels was performed. With that aim, hydrogels were washed with PBS (w/Ca^{+2} and Mg^{+2}) (Gibco) and dyed with a Live/Dead kit (Fisher Scientific) following manufacture's indications. After a 30 min incubation at RT, an epi-fluorescence microscope (Nikon) equipped with a DSD2 confocal modulus was employed to capture Z-stacks, from which the confocal maximum intensity projection was selected.

Metabolic activity was assessed by means of the Cell Counting Kit-8 (CCK-8) (Sigma Aldrich). Licensing hydrogels were washed with PBS (w/Ca^{+2} and Mg^{+2}) (Gibco) and afterwards incubated at 37 °C for 4 h in hMSC complete culture media supplemented with 10% CCK-8 reagent. Plates were read using an Infinite M200 TECAN plate reader (GMI Inc.) at 450 nm, with reference wavelength at 650 nm.

To evaluate the ability of licensing hydrogel-encapsulated hMSCs to release immunomodulatory factors, hydrogels were incubated for 24 h in complete hMSC media. The resulting conditioned media was assayed for Gal-9 and PGE2 determination by means of ELISA (Fisher Scientific, Abcam, respectively), following the manufacturer's instructions.

2.4. In vivo evaluation of licensing hydrogels in a murine acute colitis model

In vivo studies were performed following the ethical guidelines established by the institutional animal care and use committee of the University of Basque Country UPV/EHU (approved protocol M20/2018/004). Six week-old male C57BL/6 mice (Janvier Laboratories) with an average weight of 18–20 g were housed under standard conditions, with

food and tap water supplied *ad libitum*. To induce acute colitis, 3% (w/v) DSS was administered via drinking water for 5 consecutive days, when the solution was replaced by regular tap water [28]. Mice were SC administered with the corresponding treatment at day 0, attending to their study group (n = 5): healthy group (control animals without DSS exposition), untreated group (DSS exposure without treatment), nude hMSCs group (DSS + 2×10^6 nude hMSCs), isCA-hMSCs (DSS + 2×10^6 isCA-hydrogel encapsulated hMSCs – with no addition of IFNγ loaded beads –) and IFNγ-isCA-hMSCs (DSS + 2×10^6 IFNγ isCA-hydrogel encapsulated hMSCs).

2.4.1. Disease activity index (DAI) determination

During the conduction of the *in vivo* study, weight loss was monitored daily. At day 7, stool consistency was evaluated and rectal bleeding was determined by means of Hemoccult (Sensa), a test for the detection of occult fecal blood. These 3 parameters were considered to score the DAI of each mouse on a scale of 0–4, as described by Wirtz *et al.* [28] and shown in Table 1.

2.4.2. Colon weight/length ratio evaluation

Colon weight/length ratio was determined for being a sensitive and reliable indicator of the disease severity and extent inflammatory status associated to colitis [29]. On day 7, animals were sacrificed by cervical dislocation and colon tissue was excised. After rinsing the luminal contents with PBS, colons were weighed and measured to calculate the ratio between them.

2.4.3. Histological evaluation of colonic tissue

The distal region of the colon was excised and fixed in 4% formaldehyde for histological analyses. For each sample, 3 different sections were performed and stained with hematoxylin-eosin (H&E). Sections for each mouse were blindly evaluated by a pathologist, following the score system previously described by Geboes *et al.* [30], which is described in Table 2.

2.5. Data analysis and statistics

Results are expressed as the mean ± standard deviation. Statistical computations were performed using SPSS 23 (IBM). Data normality was evaluated by means of the Shapiro-Wilk test. For two-group comparisons, normally distributed data was assessed by the parametric Student's *t*-test while for non-normally distributed data the non-parametric Mann–Whitney *U* test was employed. For multiple comparisons data with a normal distribution, one-way ANOVA was performed, using Levene's test to assess the homogeneity of variances. If homogeneous, the Bonferroni post-hoc test was employed, and if not, the Tamhane post-hoc test was used instead. For multiple comparison of non-normally distributed data the Kruskal-Wallis test was performed. P-values<0.05 were assumed to be significant in all analyses.

Table 1
Clinical severity evaluation of colitis. Disease activity index (DAI) scoring system [28].

Score	Weight loss	Stool consistency	Blood
0	None	Normal	Negative hemocult
1	1–5%	Soft but still formed	Negative hemocult
2	6–10%	Soft	Positive hemocult
3	11–18%	Very soft; wet	Blood traces in stool visible
4	>18%	Watery diarrhea	Gross rectal bleeding

3. Results and discussion

3.1. Development of licensing hydrogels and in vitro characterization with adipose tissue-derived hMSCs

First, injectable licensing hydrogels were developed as a multifunctional platform that acts not only as an optimized administration system but also interacts with hMSCs to enhance their anti-inflammatory MSC2 phenotype. We previously reported that sustained inflammatory licensing overcomes the transient effects of conventional preconditioning strategies, prolonging the release of key immunomodulatory factors, such as Gal-9 by hMSCs [12]. Therefore, here, we followed the same strategy and incorporated IFNγ-loaded heparin-coated agarose beads but in this case, to injectable isCA hydrogels. These isCA hydrogels consist of a 3D alginate network that crosslinks *in situ* once implanted, enabling a rapid and efficient *in vivo* administration of hMSCs. Moreover, their preparation benefits from a simple elaboration method, which is performed under mild conditions that ensure hMSC viability and could be easily scaled-up.

Adipose tissue-derived hMSCs – previously overnight preconditioned with IFNγ / TNFα – were immobilized in the resulting IFNγ-isCA hydrogels. As a control, blank isCA hydrogels – without IFNγ-loaded beads – were included in the study (Fig. 1A). Macro- and microscopic images of licensing hydrogels are shown in Fig. 1B.

Prior to *in vivo* studies, *in vitro* assays were carried out to determine the impact of the inclusion of IFNγ-loaded heparin coated-beads in hMSC metabolism and viability for the extension of the *in vivo* study. Regarding cell metabolism, the CCK-8 test revealed a higher metabolic activity in hMSCs encapsulated in IFNγ-isCA hydrogels at days 1 and 7 after cell encapsulation (Fig. 1C). These results are in line with the literature, where licensing has been reported to induce a higher metabolic activity that enables the cells to produce the immunomodulatory factors [31].

Furthermore, Live/Dead staining showed a majority of viable cells in licensing hydrogels 7 days after encapsulation (Fig. 1D). This fact confirms that the continuous exposure to IFNγ does not have a detrimental effect on hMSC viability. Moreover, our results indicate that licensing hydrogels enable to harbor a high hMSC dose (4×10^6 MSCs / mL) and that such confluence does not compromise cell viability. In line with previous studies, no cell proliferation was observed in the gels [12,32].

3.2. In vivo effects of licensing hydrogel-encapsulated hMSCs SC administered in DSS-induced murine acute colitis

We next evaluated the *in vivo* effects of SC administered hMSC-laden licensing hydrogels. To do so, a single dose of xenogeneic adipose tissue-derived hMSCs was SC administered in an acute mouse model of ulcerative colitis with documented similarity to the human disease [28,33]. The system developed here was hypothesized adequate for the treatment of IBD, since it presents key advantages that result helpful in the therapy, including the maintenance of cell persistence and the promotion of viability – which reduce the dosing frequency, improving patient comfort – the activation of the MSC2 immunomodulatory phenotype and the accessible and minimally invasive administration route.

The model was induced by exposing C57BL/6 immunocompetent mice to 3 % DSS via drinking water during 5 consecutive days. As shown in Fig. 2A, 2×10^6 overnight IFNγ / TNFα licensed hMSCs – either nude or encapsulated in isCA / IFNγ-isCA hydrogels – were SC implanted on day 0. Additionally, a healthy control group – with no exposure to DSS – and an untreated control group – which was exposed to DSS but no treatment was administered – were included in the study. The DSS solution was replaced by regular water for 2 additional days. Supplementary Fig. S1 shows that all mice in the study ingested the same DSS dose during the study – except for the healthy group, not exposed to the DSS solution –. The clinical outcome of the untreated control group

Table 2
Histopathological severity evaluation of colitis. The total histological score was calculated by the addition of the scores of architectural distortion, inflammatory infiltrate, neutrophil presence in lamina propia and epithelium, crypt destruction and erosion / ulceration. Adapted with slight modifications from [30].

Score	Architectural distortion	Inflammatory infiltrate	Neutrophils in *Lamina propia*	Neutrophils in epithelium	Crypt destruction	Erosion or ulceration
0	No abnormality	No increase	None	None	None	No erosion, ulceration, or granulation tissue
1	Mild abnormality	Mild but unequivocal increase	Mild but unequivocal increase	< 5% crypts involved	Probable—local excess of neutrophils in part of crypt	Recovering epithelium + adjacent inflammation
2	Mild / moderate diffuse or multifocal abnormalities	Moderate increase	Moderate increase	< 50% crypts involved	Probable —marked attenuation	Probable erosion —focally stripped
3	Severe diffuse or multifocal abnormalities	Marked increase	Marked increase	> 50% crypts involved	Unequivocal crypt destruction	Unequivocal erosion

confirmed that DSS successfully induced colitis in mice, presenting the typical symptoms of the disease including weight loss, loose feces and bloody stool.

During the *in vivo* study, animal weight was daily followed-up for being a key indicator of DSS-colitis development [34]. Results proved the expected effects from the chemical induction of acute colitis in the DSS-groups compared to the healthy control group, observing weight loss in all of them once DSS was withdrawn at day 5 (Fig. 2B). At day 7, stool consistency was examined, observing that only the untreated and nude hMSCs groups presented statistically significant differences with the healthy control (Fig. 2C). Moreover, at such time point, the presence of blood in stool was evaluated by means of a fecal occult blood test. Results of weight loss, stool consistency and fecal bleeding were gathered to score the disease activity index (DAI), which assesses the severity of colitis (Table 1). Once again, the trend previously observed in stool consistency was repeated: the groups treated with hydrogel-encapsulated hMSCs did not show significant differences to the healthy control, while the untreated and nude hMSCs groups did (Fig. 2D). It is interesting to highlight that, whereas an 80% of the isCA hMSCs-treated mice and a 60% of IFNγ-isCA hMSCs-treated mice presented a DAI of 2, an 80 % in the nude hMSCs group scored 3 or higher (Fig. 2E). These results are in line with previous studies that relate increased cell persistence to improved clinical outcomes [22].

On day 7, mice were sacrificed and the colonic tissue was retrieved (Fig. 2F). Excised colon tissue from each individual mouse are shown in Supplementary Fig. S2. The colon weight/length ratio (Fig. 2G) followed the same trend abovementioned showing only statistically significant differences in the untreated and nude hMSCs groups in comparison to healthy mice.

Overall, these results indicate that the SC implantation of hydrogel-encapsulated hMSCs exerted a delocalized systemic protective effect that was involved in slowing down the progression of the disease.

3.3. Histological appearance evaluation of colonic tissue

The histological evaluation of colitis associated inflammatory injury in the excised colonic tissue supported the symptomatic results previously observed. In Fig. 3A representative micrographs of H&E stained colon sections are displayed. Sections from all mice are shown in Supplementary Fig. S3. The total histological score (Fig. 3B) was calculated attending to inflammation, presence of polymorphonuclear neutrophils (PMNN) in *lamina propia* and epithelium, architectural distortion, crypt destruction and tissue erosion, as described in Table 2 (Fig. 3C) [30].

In the untreated control group, we confirmed the correct colitis induction showing that DSS caused important inflammation, eroded and distorted the colon tissue architecture reducing the protective epithelial layer, destructed the crypts and increased the infiltration of immune cells. This negative control group showed statistically significant differences with the healthy control group. Interestingly, this was also observed in the nude hMSC-treated group, in contrast to the groups where hMSCs were hydrogel-encapsulated, which did not. Indeed, we

observed a restored histological appearance of the colon mucosa in a 40% of the isCA hydrogel-treated mice and a 60% of IFNγ-isCA hydrogel-treated mice. In particular, these subpopulations scored below 5, resembling the native tissue characteristics observed in the healthy control group. These results indicate that in these mice, hMSC-laden hydrogels were able to systemically exert a protective effect that drastically reduced the extension and severity of DSS-associated inflammatory injury. In contrast, all mice in the untreated control group showed the severe DSS-driven effects and the nude hMSCs treated mice presented intermediate outcomes (Fig. 3D).

In line with our observations, it has been proposed that extravascular delivery routes alternative to the IV, such as IP, are effective in treating DSS colitis in mice by evoking a systemic effect acting upon host macrophages [25]. Indeed, same authors reported that the IP or SC administration of 10×10^6 nude bone marrow-derived MSCs provided systemic effects that reduced DSS colitis severity in mice more effectively than the maximum tolerated IV bolus dosing (1×10^6 MSCs) [22]. Our results with nude cells do not correlate with their positive outcomes, which could be due to differences in dosage – they employed a 5 times higher dose –, or cell origin – they administered allogeneic bone marrow-derived murine MSCs while here, a xenogeneic implant of adipose tissue-derived hMSCs was performed –.

Together with the abovementioned, to the extent of our notice, there is only another study in which SC administration of MSCs was employed to treat DSS-derived colitis in mice [35]. Despite using different colitis generation protocols and hMSC source – bone marrow –, they also presented positive outcomes in colon length and inflammation. However, as *in vivo* imaging demonstrated, they attributed such results to the capacity of nude hMSCs to remain in the SC tissue and act as a depot, gradually entering the systemic circulation to migrate to the inflamed colon where they exerted a local effect [35].

The present study is, to the best of our knowledge, the first administering SC a single dose of xenogeneic hMSCs encapsulated in a hydrogel platform that continuously activates the MSC2 immunomodulatory phenotype. We relate the protective effect of this novel approach – observed both, clinically and histologically – to a delocalized systemic effect driven by the immunomodulatory factors released by the encapsulated cells, which persist in the SC tissue until the end of the study.

3.4. Viability and immunomodulatory activity of explanted xenogeneic hMSCs

An additional – and pivotal – advantage offered by hydrogel encapsulation of hMSCs is the increased cell residence time. A recent publication proved that the prolonged *in vivo* persistence of allogeneic MSCs, IP or SC administered, directly correlated with their pharmaceutic activity in DSS colitis in mice. In this line, implanted cells with limited viability were suggested to be inefficient at improving therapeutic outcomes due to a shortened *in vivo* persistence and accelerated phagocytic clearance, especially when administered IV [22]. Apart from ensuring retention, hydrogel encapsulation of hMSCs offers the
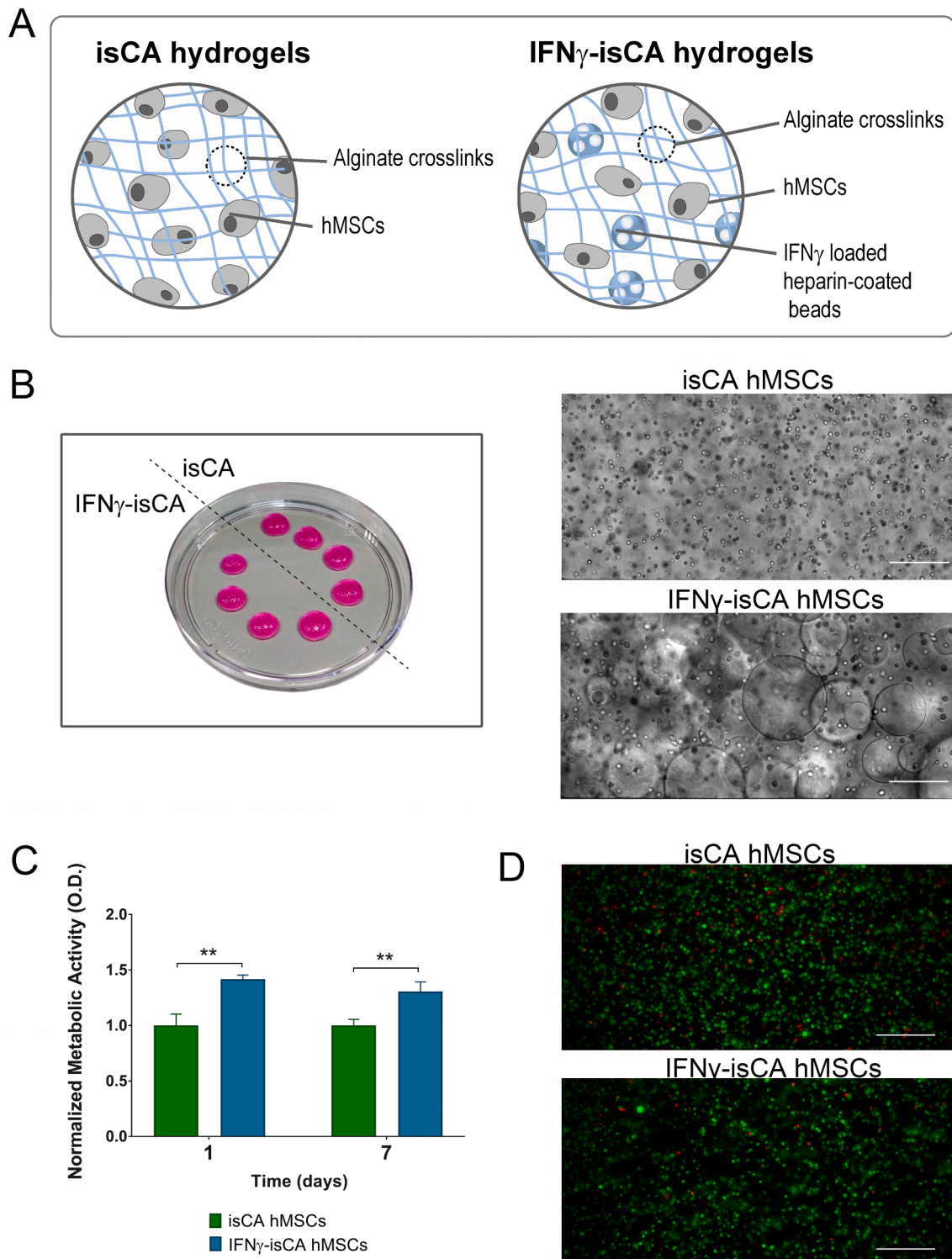
Fig. 1. Licensing hydrogel development. (A) Schematic representation of *in situ* crosslinking alginate (isCA) injectable hydrogels designed in the study to *in vivo* subcutaneously administer adipose derived hMSCs. **(B)** Macroscopic and bright-field microscopic images of the resulting hydrogels. **(C)** Metabolic activity of hMSCs on days 1 and 7 after hydrogel encapsulation. **(D)** Live/Dead staining of hMSCs 7 days after hydrogel encapsulation. Calcein in green for live cells, propidium iodide in red for dead cells. Results are shown as the mean ± standard deviation (n = 3). Scale bars = 200 μm. Statistical significance: **p < 0.01. hMSCs: human mesenchymal stromal cells.

possibility of retrieving the implant. In this context, the SC space is an accessible site that allows a simple extraction of the hydrogel. This does not only significantly escalate the biosafety of the therapy, but also enables to study hMSC fitness once the preclinical studies are over. Hence, in our study, whereas nude hMSCs could not be recovered,

hydrogel encapsulated hMSCs were retrieved after animal sacrifice (Fig. 4A). Despite being xenogeneic implants, hydrogels were easily retrieved at day 7 post-implantation and no thick fibrotic capsule surrounding the implant was observed by naked eye visualization. All explants are shown in Supplementary Fig. S4.
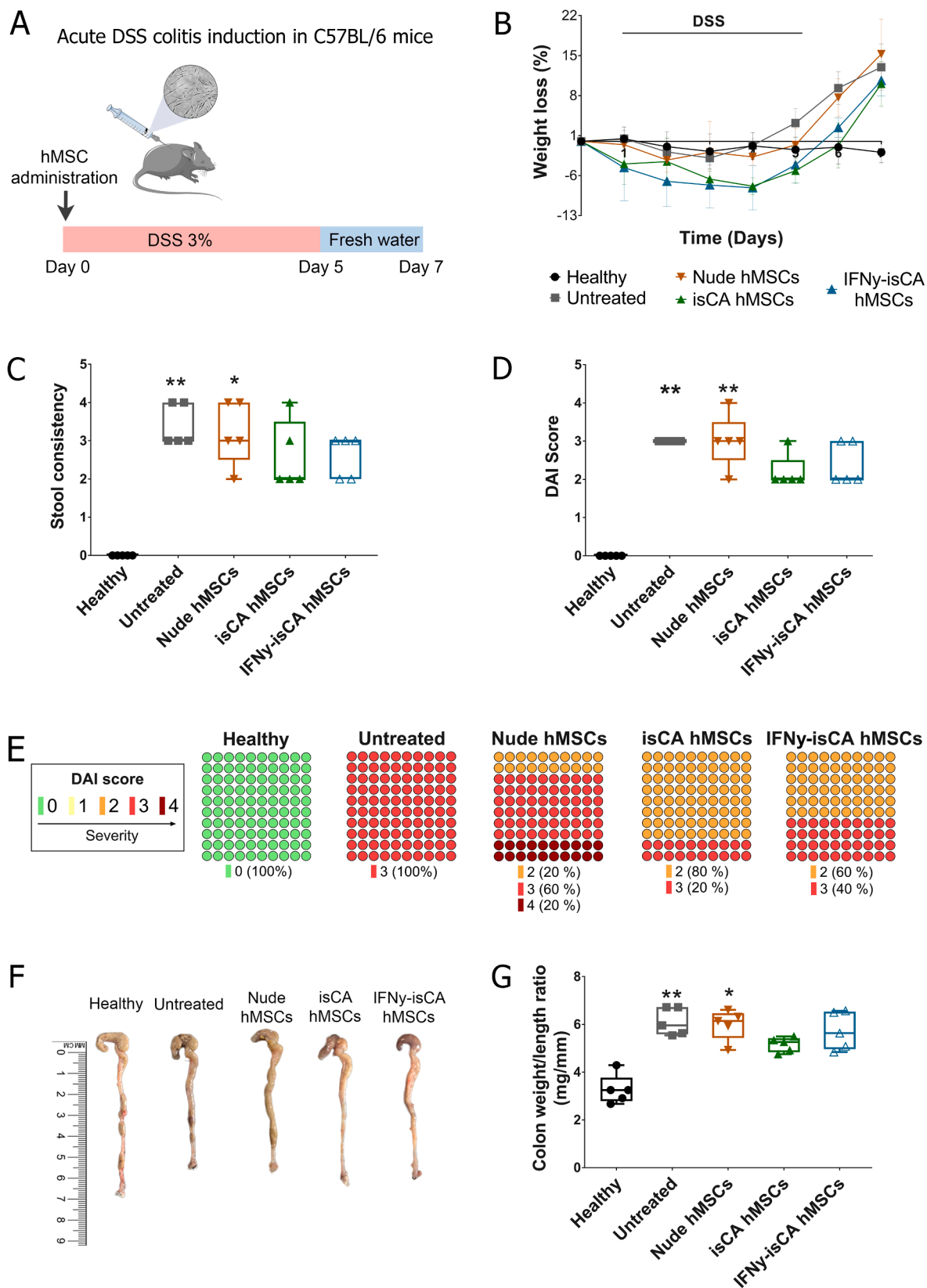
Fig. 2. In vivo evaluation of licensing hydrogel-encapsulated hMSCs in murine acute colitis. (A) Experimental layout of dextran sodium sulfate (DSS) acute colitis induction in mice and hMSC administration. Clinical outcomes of colitis were assessed by measuring **(B)** body weight changes, **(C)** stool consistency, **(D-E)** disease activity index (DAI), **(F)** colon length and **(G)** colon weight/length ratio. Results are shown as the mean ± standard deviation (n = 5). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to Healthy control group. $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 in comparison to untreated control group. $^{++}$p < 0.01 in comparison to nude hMSCs. hMSCs: human mesenchymal stromal cells.
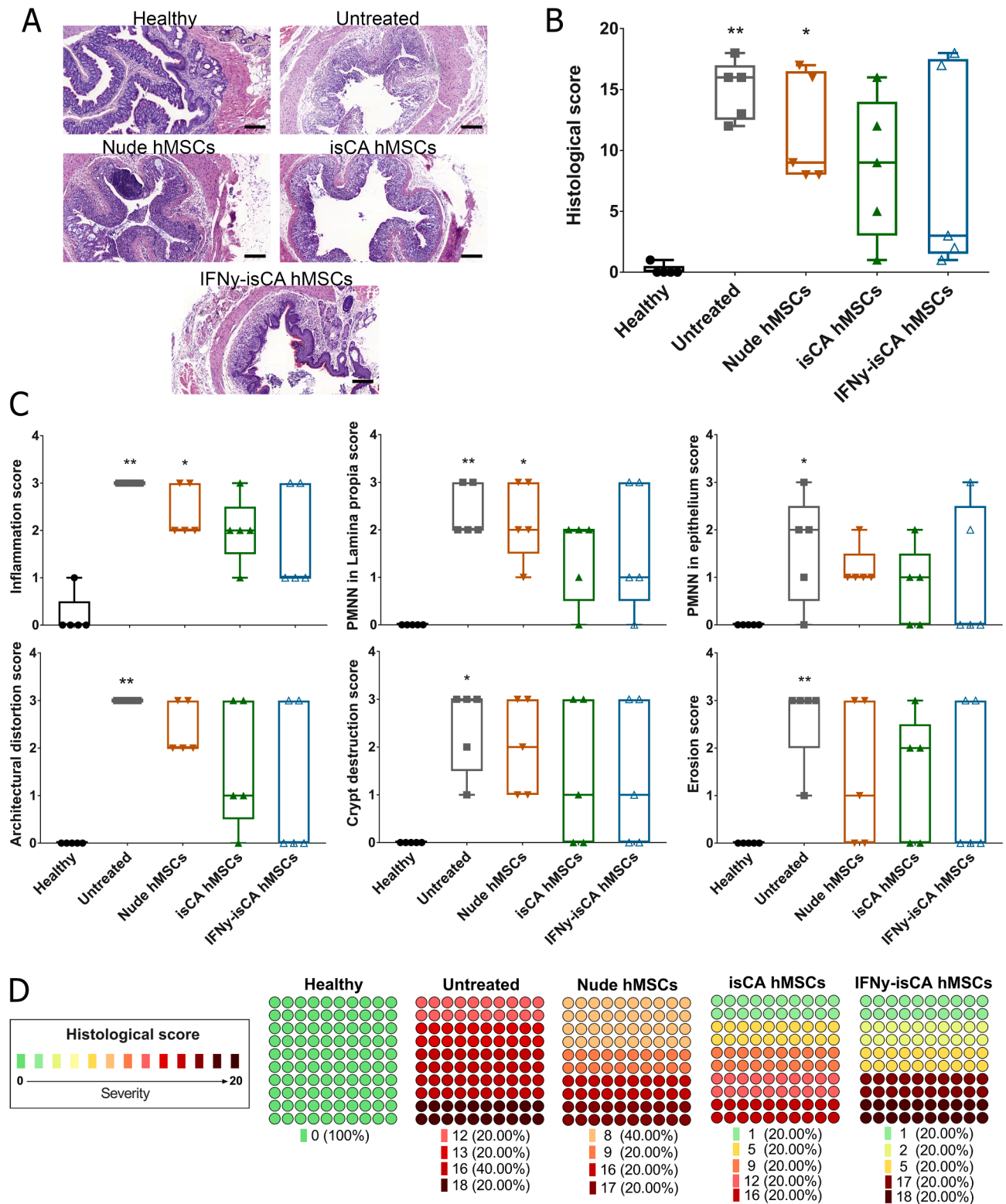
Fig. 3. Histopathological effects of licensing hydrogel-encapsulated hMSCs in murine acute colitis. **(A)** Histological sections of the colon stained with hematoxylin-eosin. **(B)** Histological score in mice with DSS-induced colitis was determined analyzing **(C)** colonic inflammation, inflammatory cell infiltration in the *lamina propia* and epithelium, architectural distortion, crypt destruction and tissue erosion. **(D)** Parts of a whole dot plots show the percentage of mice scores for each group. Results are shown as the mean ± standard deviation (n = 5). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to Healthy control group. ##p < 0.01, ###p < 0.001 in comparison to untreated control group. ++p < 0.01 in comparison to nude hMSCs. hMSCs: human mesenchymal stromal cells. PMNN: polymorphonuclear neutrophils. Scale bars: 200 μm.
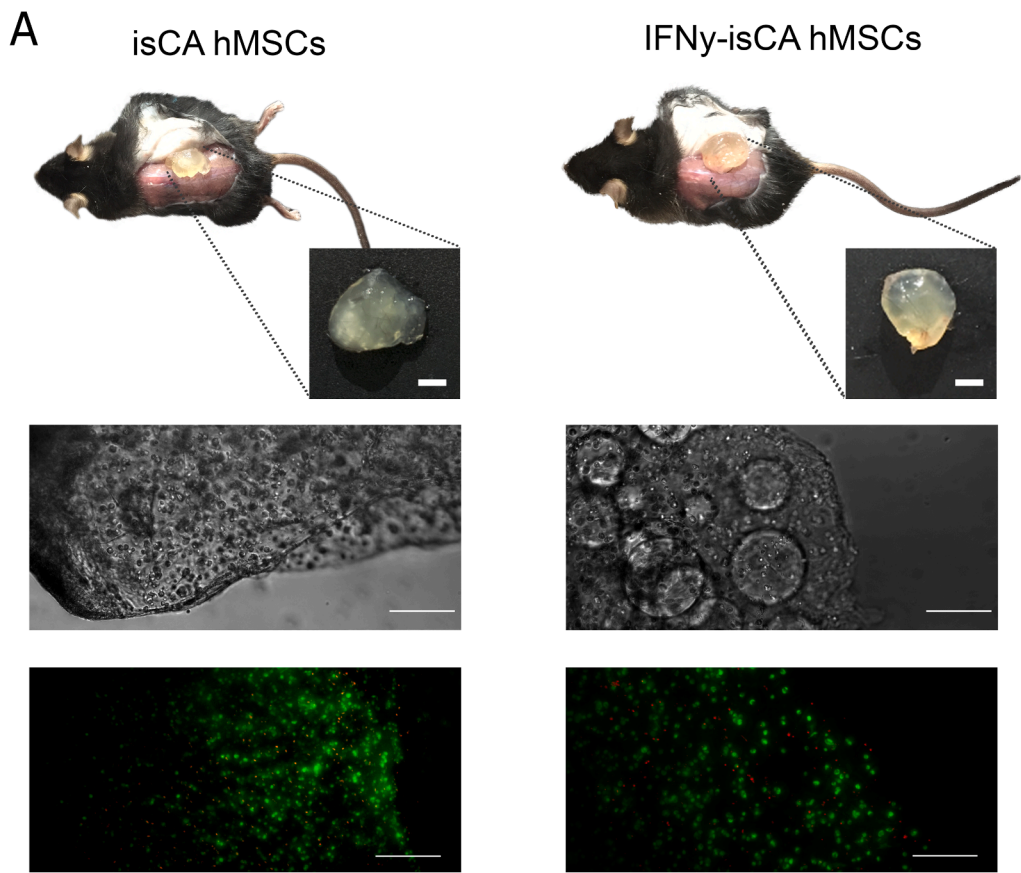
Fig. 4. Viability and immunomodulatory activity of explanted xenogeneic hMSCs. (A) Licensing hydrogels were easily retrieved after 7 days of *in vivo* implantation in mice (scale bars = 0.5 cm) and microscopically analyzed under bright-field and live/Dead staining. Calcein in green for live cells, propidium iodide in red for dead cells (scale bars 200 μm). PGE2 and Gal-9 secretion **(B-C)** 24 h post-encapsulation and **(D-E)** 24 h post *in vivo* explantation were determined to evaluate the immunomodulatory capacity of hMSCs. Results are shown as the mean ± standard deviation (n = 3–5). Statistical significance: *p < 0.05, ***p < 0.001.
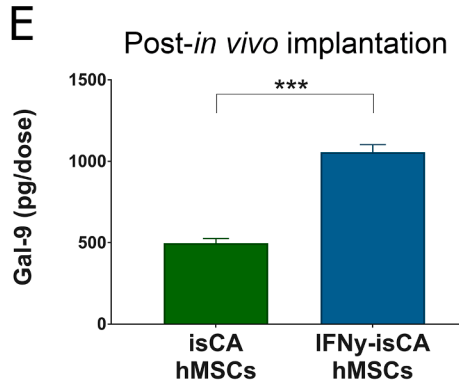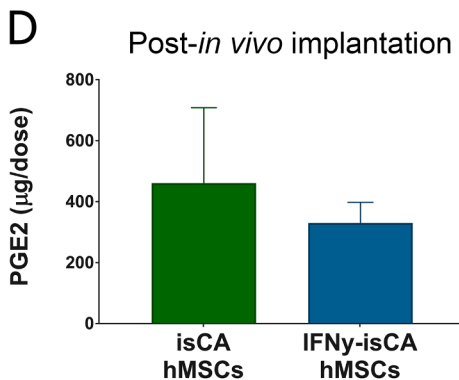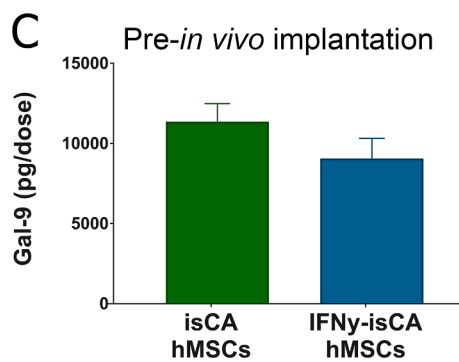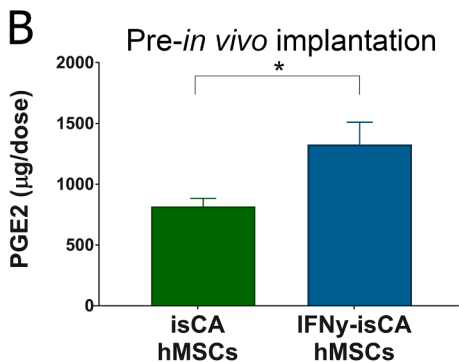
At a microscopic scale, bright-field images showed homogeneity of cells in gels. Indeed, the single cell distribution was maintained during the study and we did not observe the formation of cell aggregates. Furthermore, Live/Dead staining revealed a majority of viable cells after the 7-day *in vivo* implantation, which is an especially relevant result considering the xenogeneic origin of hMSCs, and also crucial for obtaining a positive therapeutic outcome [22].

It is important to highlight that we obtained the same *in vivo* protective effect in both isCA and IFNγ-isCA hydrogel-treated groups. The fact that we did not observe a superior effect of IFNγ-isCA hydrogels could be attributed to the acute colitis model employed here not being sensible enough, but also to a failure in the maintenance of the sustained licensing effect. To dismiss the latter, we next assessed the immuno-modulatory capacity of the explanted hMSCs. To do so, we evaluated the presence of PGE2 and Gal-9 in the conditioned media resulting from culturing the hydrogels for 24 h. In particular, we monitored the secretion of those immunomodulatory factors in hydrogels pre-implantation at day 0 and post *in vivo* explantation at day 7. Results showed that prior to implantation, encapsulated hMSCs produced both PGE2 and Gal-9, being the secretion of the former significantly higher in IFNγ-isCA hydrogels (Fig. 4 B–C). Despite after 7 days of *in vivo* implantation hMSCs in both groups maintained an active MSC2 anti-inflammatory phenotype still secreting PGE2 and Gal-9, cells encapsulated in IFNγ-isCA hydrogels produced significantly higher levels of the latter (Fig. 4 D–E). These results indicate that the inclusion of IFNγ-loaded heparin-coated beads does not only present an *in vitro* effect as previously reported [12], but it is also able to initially boost PGE2 secretion and afterwards prolong the Gal-9 production up to at least 7 days of *in vivo* implantation. Therefore, once proving that the sustained licensing effect was successful, we may attribute the lack of superiority of IFNγ-isCA hydrogels to the DSS acute colitis mouse model employed here.

Despite the acute 7-day model employed here has been demonstrated adequate to study innate immune mechanisms in the context of colitis and subsequent tissue repair – being therefore, appropriate to prove the hypotheses followed in this work – [28], it may not present the suitable duration in order to detect the differences in effect that sustained licensing could evoke. Hence, future research is guaranteed to further explore if chronic IBD models could benefit from IFNγ-isCA hydrogels, which are precisely designed to maintain hMSC immunomodulation in the long-term. In this line, chronic models would also be valuable to thoroughly study the foreign body response, which may not be adequately observed in acute models.

4. Conclusions

Injectable licensing hydrogels enabled a rapid and efficient *in vivo* implantation of hMSCs in the SC space of a xenogeneic acute DSS-colitis mouse model, also allowing an easy retrieval at the end of the study, which did not only ensure cell persistence, but also promoted the biosafety of the therapy. Our results indicate that a delocalized systemic effect of licensing hydrogel-encapsulated hMSCs conferred a protective effect *in vivo*, limiting the progression of colitis. Licensing hydrogels not only promoted cell viability, but also boosted and prolonged the MSC2 immunomodulatory phenotype, as demonstrated after 7 days of *in vivo* implantation, when the retrieved hMSCs presented a high viability and retained the ability to release bioactive factors including PGE2 and Gal-9. Overall, our results demonstrate the injectable licensing hydrogels developed here as an optimized platform to enhance the immunomodulatory cell therapy mediated by hMSCs.

Declaration of Competing Interest

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

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2022.01.007.

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