

# Cryopreserved apoptotic mesenchymal stromal cells retain functional efficacy in suppressing an allergic inflammation in a murine model

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## Abstract

Mesenchymal stromal cell (MSC) apoptosis is required for in vivo immunosuppression. However, the induction of apoptosis is heavily dependent on the recipient's immune system. In graft-versus-host disease (GvHD), patients who fail to respond to MSCs are in fact those whose immune cells are unable to induce MSC apoptosis ex vivo. The information is critical to explain why responses in clinical trials vary even though the same sources of MSC products are infused. More importantly, it highlights the need for an alternative MSC treatment for the nonresponders. By using a mouse model of ovalbumin (OVA)-induced allergic inflammation, we demonstrated that we could generate apoptotic MSCs (ApoMSCs) in vitro and use them to successfully reduce allergic airway inflammation. In order to address the logistics of their potential future clinical application, we have shown that ApoMSCs could be cryopreserved without impairing efficacy compared to freshly generated ApoMSCs. We have also highlighted that MSCs need to undergo complete apoptosis before cryopreservation to retain their immunosuppressive activity. The cryopreserved ApoMSCs could serve as a potential future off-the-shelf cellular product, in particular for patients who suffer from inflammatory conditions yet do not harbor the immune capacity to induce MSC apoptosis in vivo. Our data provide proof-of-concept that under laboratory conditions, ApoMSCs can be successfully frozen and thawed without affecting their anti-inflammatory activity, as tested in a murine model of allergic inflammation.

**Key words:** mesenchymal stem cells (MSCs); apoptosis; cellular therapy; clinical translation; immunosuppression; immunotherapy; allergic inflammation.

## Significance Statement

In this study, we have provided proof-of-concept data under laboratory conditions supporting the development of an off-the-shelf cellular product that may benefit the patients who are classified as nonresponders to the conventional MSC therapy. Our in vitro-generated ApoMSCs successfully demonstrated anti-inflammatory effects in the asthma mouse model. More importantly, we showed that ApoMSCs retained their therapeutic effects after cryopreservation, suggesting that they could be seamlessly implemented in the clinical logistics. Overall, our data provide valuable insights that can contribute to the development of next-generation MSC therapies for better clinical outcomes.

## Introduction

Human mesenchymal stromal cell (MSC) therapy has been proven clinically safe and highly efficacious in inflammatory diseases such as graft-versus-host disease (GvHD)<sup>1,2</sup> and Crohn's disease (CD).<sup>3</sup> There are now 10 approved MSC products in Europe, Japan, South Korea, India, Canada, and New Zealand respectively.<sup>4,5</sup> Despite this increasing therapeutic application, the mechanism of action underpinning the anti-inflammatory activity in vivo has remained unclear.

Consequently, the poor definition of their target profile and patient selection have prevented the achievement of meaningful clinical results and undermined the selection of the best therapeutic applications.

Following intravenous infusion, MSCs become trapped within the lung microvasculature and undergo apoptosis.<sup>6–10</sup> Whilst previous studies have suggested a small portion of remaining viable cells were responsible for the immunosuppressive activity through the release of paracrine factors

or extracellular vesicles,<sup>11,12</sup> more recent research has demonstrated that MSC apoptosis itself is responsible for the anti-inflammatory effects in vivo.<sup>8,9,13</sup> Apoptotic MSC (ApoMSCs) are efferocytosed by tissue phagocytes<sup>7,8</sup> that are reprogrammed to ultimately deliver the anti-inflammatory activity.<sup>8,9,14</sup> This explanation has resolved the perceived paradox between MSC efficacy and their lack of engraftment. Complementing the efferocytosis mechanism, caspase activation in ApoMSCs induces an immunosuppressive secretome that impairs T-cell activity and further contributes to the anti-inflammatory effects of MSC therapy.<sup>15</sup> Furthermore, Apoptotic caspase activity could also reduce the immunogenicity of dying cells by neutralizing danger-associated molecular patterns.<sup>16</sup> In line with these observations, recent investigation has discussed the therapeutic potential of MSCs which undergo caspase activation and complete apoptosis could be better than heat-inactivated or metabolically inactive MSCs in vivo.<sup>8,9,14</sup>

Whilst the anti-inflammatory activities of MSCs have been associated to ApoMSCs, a key clinical challenge to MSC therapy is that not all patients respond effectively to MSC therapy. Galleu et al.<sup>8</sup> highlighted a translational breakthrough in GvHD, where peripheral blood mononuclear cells (PBMCs) isolated from patients unresponsive to MSC therapy failed to induce MSC apoptosis ex vivo whilst only those PBMCs isolated from the responders could. Similar findings have also been confirmed in Crohn's disease.<sup>15</sup> These studies suggest that not all patients possess the capability to induce MSC apoptosis post-infusion and host factors are more important than MSC cell preparation to elicit a therapeutic response. Although the underlying biology remains to be elucidated, this observation corroborates discrepancies found in the clinical responses of patients receiving the same source and batch of MSC preparation.<sup>3,17</sup> To circumvent the uncertainty of host factors, a therapeutic product consisting of either apoptotic MSCs or MSCs primed to undergo apoptosis could have a wider therapeutic application.

Therefore, in this study, we aim to test the anti-inflammatory effects of in vitro-generated apoptotic MSCs through Fas stimulation,<sup>8,14,15</sup> in a murine model of ovalbumin (OVA)-induced pulmonary allergic inflammation. Additionally, we have investigated the impacts of freeze-thawing processes on the activity of those in vitro-generated apoptotic MSCs as cryopreservation therefore validating the stability and retained efficacy of this potential cell-based therapy.<sup>18</sup>

## Methods

Details of MSC and ApoMSCs preparation are presented in [Supplementary Material](#).

### Murine model of OVA-induced pulmonary allergic inflammation

All animal procedures were performed under the authority and approval of the UK Home Office outlined in the Animals (Scientific Procedures) Act 1986, 2012 revisions with local ethical approval from King's College London (Project Licence: P11C06933). A murine model of allergic inflammation was established to evaluate the anti-inflammatory capabilities of ApoMSC treatment as previously reported.<sup>8</sup> Briefly, Female BALB/c mice (10–25 g; Charles River, UK) were sensitized with chicken egg ovalbumin (30 µg/0.4 mL administered intraperitoneally) on days 0 and 7 with control

animals treated with vehicle only. On days 14, 15, and 16, groups were challenged with an aerosolized solution of OVA (3%) for 30 minutes. On day 16, 1 hour post the final challenge mice were treated with ApoMSCs ( $2 \times 10^5$  cells/mouse) intravenously. Twenty-four or 48 hours post final allergen challenge (day 17 or 18) mice were terminally anaesthetized with 25% w/v Urethane, bronchoalveolar lavage fluid was collected and processed for total and differential counts, as previously described.<sup>8,19</sup>

### Apoptosis measurements via flow cytometry

For in vitro studies measuring apoptotic cell death, ApoMSC preparations were obtained by plating  $5 \times 10^5$  cells per well in a 96 round-bottom well plate and stimulated with human anti-Fas for 15 minutes at 37 °C, 5% CO<sub>2</sub>. Post stimulation, ApoMSCs were washed to remove free anti-Fas and frozen at –80 °C in cryopreservation buffer (FBS 10% v/v DMSO). Frozen ApoMSCs were then thawed and incubated for 24 hours at 37 °C, 5% CO<sub>2</sub> or analyzed immediately. Apoptosis was measured using Annexin V apoptosis detection kits (BD Biosciences) and CellEvent caspase 3/7 flow cytometry assay kit (Thermo Fisher Scientific Scientific) according to the manufacturer's protocol. The percentage of apoptotic cells was calculated as Annexin V+ cells and caspase 3/7+ cells on the dot plots.

### Statistical analysis

Results are expressed as mean ± SEM. One-way ANOVA and Dunnett's multiple comparisons test were used to compare 3 or more mean values. A probability of null hypothesis less than 5% ( $P < .05$ ) was considered statistically significant.

## Results

### Fas-induced apoptotic MSCs (ApoMSCs) are immunosuppressive in a murine model of allergic inflammation

We have previously reported that MSCs made apoptotic in vitro via exposure to anti-Fas and GrB significantly reduced allergen (OVA) induced eosinophil recruitment in T<sub>H</sub>2-mediated murine model of pulmonary allergic inflammation.<sup>8</sup> In order to simplify the procedure with a view of making a future clinically feasible product, we investigated whether the anti-inflammatory effects of ApoMSCs required both Fas stimulation and GrB. We compared the anti-inflammatory effects of ApoMSCs induced by pretreatment with both anti-Fas antibody and GrB, or with anti-Fas antibody alone. Both anti-Fas/GrB (green circles) and anti-Fas alone (purple circles) treated MSC preparations induced a significant reduction in OVA-induced total pulmonary leukocyte recruitment ( $P < .001$ , [Figure 1B](#)) and eosinophil infiltration ( $P < .01$ , [Figure 1C](#)) into the BAL with no observable differences detected between the different ApoMSC preparations. These results indicate that anti-Fas treatment alone is sufficient for the anti-inflammatory effects of ApoMSCs in a T<sub>H</sub>2 model of allergic inflammation.

We next performed a dose titration of anti-Fas treatment of MSCs to identify the optimal concentration required to produce anti-inflammatory ApoMSCs. In vitro, we confirmed that treatment with 0.1, 1, and 10 µg/mL anti-Fas induced significant apoptosis ([Supplementary Figure S1A](#)), with complete apoptosis (~80% Annexin V expression) being achieved 24 hours post stimulation ([Supplementary Figure](#)

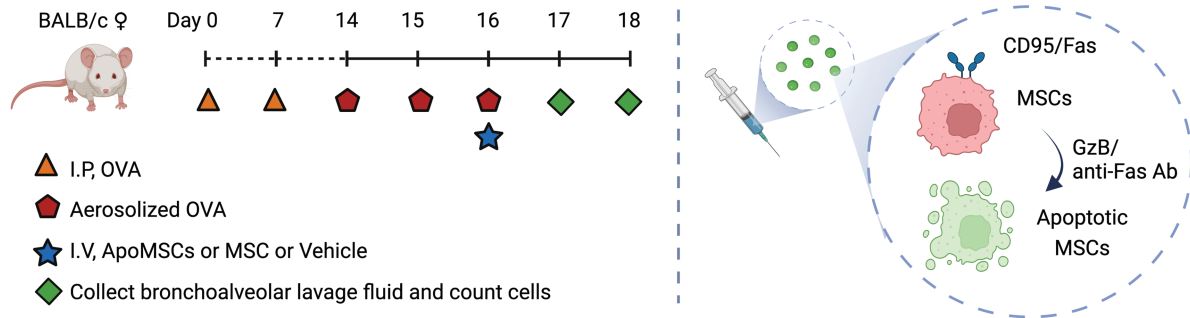
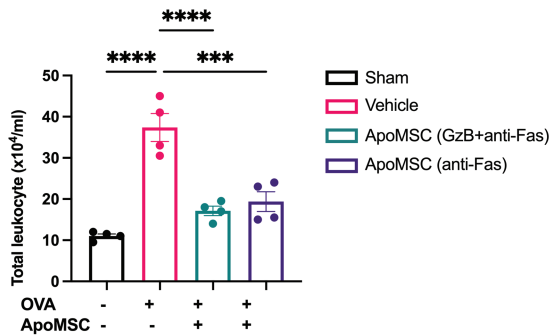
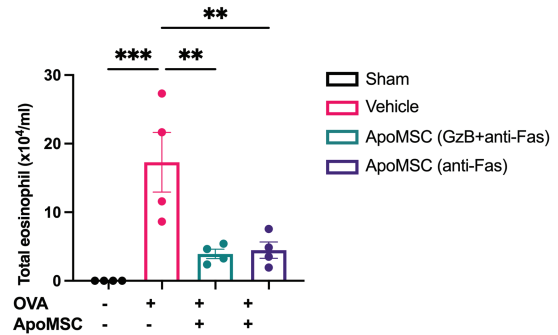
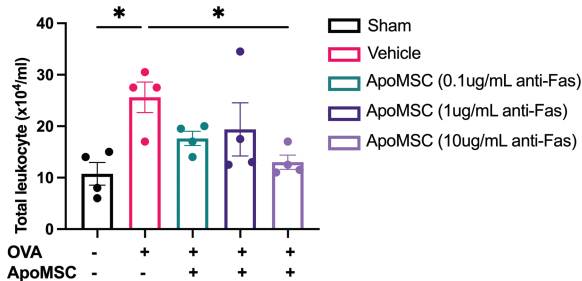
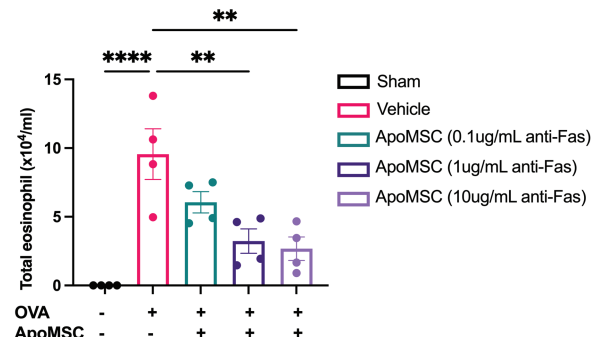
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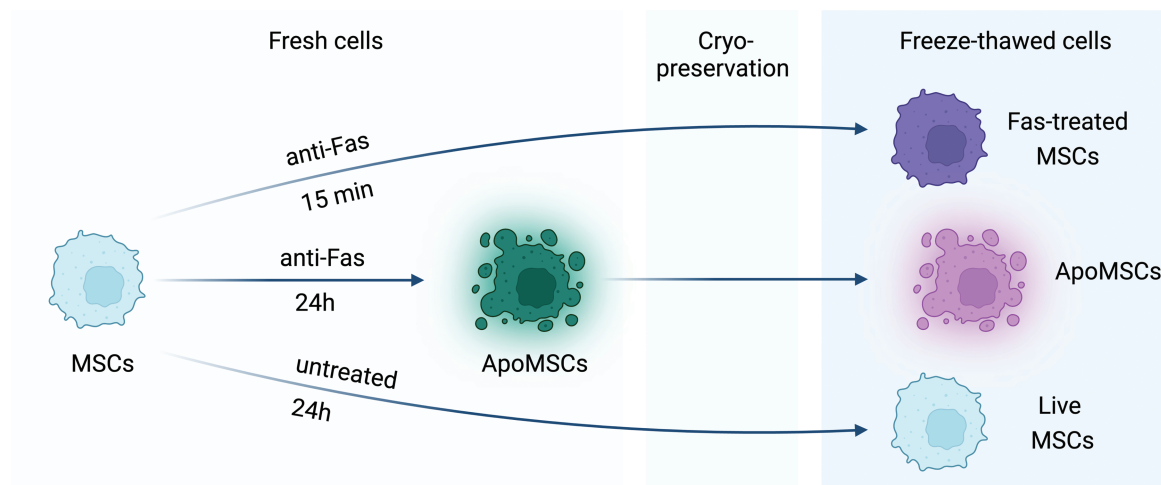
**Figure 1.** Human anti-Fas treatment is sufficient for inducing ApoMSCs with anti-inflammatory effects in a Th2 model of allergic inflammation. (A) Female BALB/c mice were immunized i.p. with 30 µg/mouse OVA on days 0 and 7 and subsequently challenged with OVA through aerosol on days 14, 15, and 16. Live or ApoMSCs were administered intravenously 1 hour post final allergen challenge and the bronchoalveolar lavage (BAL) fluid was collected on day 17 or 18 for cell count analysis. Anti-inflammatory effects of ApoMSCs generated by treatment with either GrB + anti-Fas or anti-Fas alone versus vehicle control were quantified through changes in total pulmonary leukocyte recruitment (B) or eosinophil recruitment (C) into the lavage fluid. In further studies, ApoMSCs were generated with either vehicle, 0.1, 1, or 10 µg/mL anti-Fas prior to infusion. Anti-inflammatory effects were quantified through changes in total pulmonary leukocyte recruitment (D) or eosinophil recruitment (E) into the lavage fluid. Results are shown as mean ± SEM (\* $P < .05$ , \* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$ ),  $n = 4$  per group. Each data point indicates an individual mouse.

S1B). We therefore evaluated the anti-inflammatory effect of MSCs treated with 0.1, 1, and 10 µg/mL anti-Fas in vivo. We observed a dose-dependent reduction in both pulmonary leukocyte (Figure 1D) and pulmonary eosinophil recruitment following anti-Fas stimulation with significant reductions in eosinophil recruitment achieved by ApoMSCs produced via stimulation with 1 ( $P < .01$ ) and 10 µg/mL ( $P < .01$ ) anti-Fas (Figure 1E). As 10 µg/mL anti-Fas antibody treatment produced the greatest reduction in pulmonary eosinophil recruitment with the least variability, we therefore selected these conditions for the induction of ApoMSCs in all future studies.

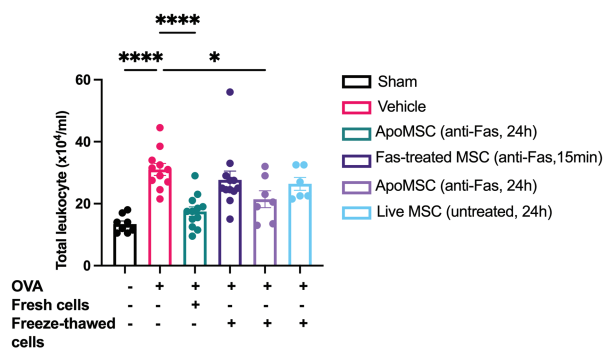
### ApoMSCs can be cryopreserved without impairing their anti-inflammatory activity in vivo

When inducing MSC apoptosis, cells are stimulated with 10 µg/mL anti-Fas for 15 minutes, washed and incubated for a further 24 hours prior to administration in vivo. As an important step to identify whether the Fas-induced ApoMSCs could be used as a future “off-the-shelf” therapy, we compared the anti-inflammatory activity of ApoMSCs that had been frozen at  $-80^{\circ}\text{C}$  prior to infusion versus those prepared fresh on the day of administration (Figure 2A). Here, we observed that ApoMSCs frozen

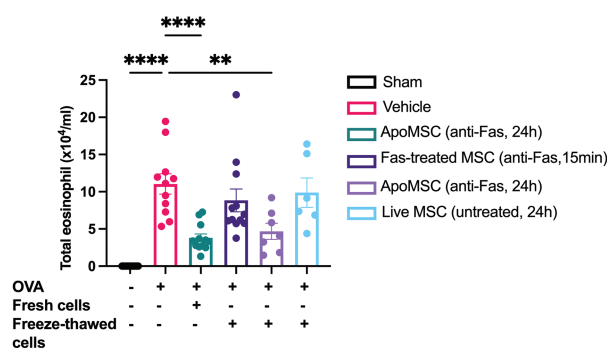
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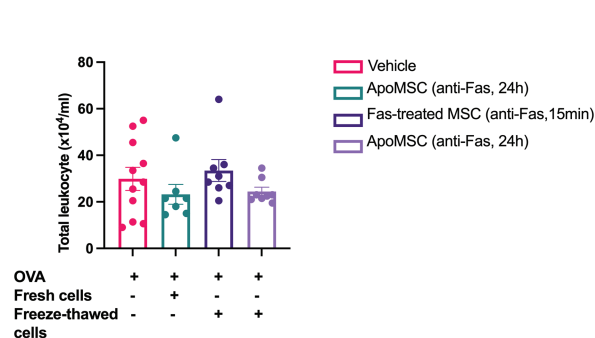
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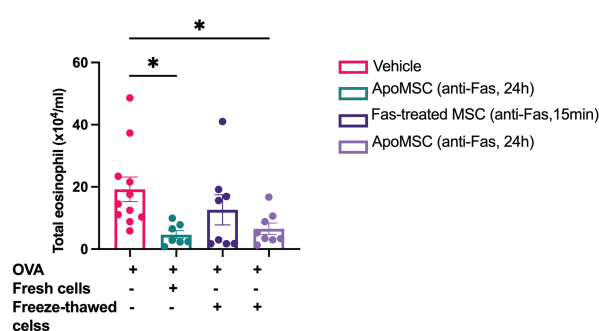


**Figure 2.** ApoMSC need to undergo complete apoptosis prior to cryopreservation to exert in vivo immunosuppression. Female BALB/c mice were immunized i.p. with 30 µg/mouse OVA on days 0 and 7 and subsequently challenged with OVA through aerosol on days 14, 15, and 16. One hour post final allergen challenge, mice were treated with freshly prepared anti-Fas treated ApoMSCs, ApoMSCs generated by anti-Fas treatment for 15 minutes prior to a single freeze/thaw cycle, ApoMSCs generated by anti-Fas for 24 hours prior to a single freeze/thaw cycle or untreated MSCs subjected to a single freeze/thaw cycle. (A) represents a schematic overview of ApoMSC preparation. Anti-inflammatory effects were quantified at 24 (B, C) or 48 hours (D, E) post final allergen challenge through changes in total pulmonary leukocyte (B, D) and eosinophil (C, E) recruitment. Results are shown as mean ± SEM (\* $P$  < .05, \* $P$  < .01, \*\*\*\* $P$  < .0001).  $N$  = 6 = 12 over 3 independent experiments. Each data point indicates an individual mouse.

in cryopreservation buffer 24 hours post stimulation with anti-Fas and subsequently thawed prior to in vivo administration retained significant suppression of OVA-induced total leukocyte ( $P$  < .05, lilac circles, Figure 2B) and pulmonary eosinophil recruitment ( $P$  < .01, lilac circles Figure 2C) 24 hours post final allergen challenge when compared to vehicle control. Importantly, this reduction was comparable to the reductions in total leukocyte ( $P$  < .001, green circles,

Figure 2B) and eosinophil recruitment ( $P$  < .01, green circles, Figure 2C) from Fas-induced ApoMSCs prepared fresh (not subject to a single freeze-thaw cycle). Furthermore, we also found that the levels of the  $T_H2$  cytokines IL-4 and IL-5 in the bronchoalveolar lavage fluid were lower in mice treated with fresh and freeze-thawed ApoMSCs when compared to vehicle control, and whilst this did not reach statistical significance, the trend was consistent with the profile observed

in pulmonary eosinophil recruitment (Supplementary Figure S2). Importantly the anti-inflammatory effects of ApoMSCs frozen following complete apoptosis remained similar to those prepared fresh even when assessed at 48 hours post final allergen challenge as measured through pulmonary total leukocyte (Figure 2D) and eosinophil (Figure 2E) recruitment, further corroborating that cryopreservation had no impact on ApoMSCs that had been allowed to undergo complete apoptosis over a 24-hour incubation period. Of note, unstimulated MSCs (those not stimulated with anti-Fas) subjected to the same single freeze-thaw cycle did not demonstrate any significant reduction in either total pulmonary cell recruitment or pulmonary eosinophil recruitment at any time point (blue circles). Furthermore, these untreated MSCs failed to demonstrate any significant apoptosis as measured by Annexin V expression or caspase 3/7 activation in vitro (Supplementary Figure S1C, S1D). This confirms that the anti-inflammatory effects of ApoMSCs are dependent on specifically Fas stimulation-induced apoptosis.

### ApoMSCs need to undergo complete apoptosis prior to cryopreservation to retain anti-inflammatory activity in a $T_H2$ model of allergic inflammation

Following the identification that Fas stimulation is sufficient for the production of anti-inflammatory ApoMSCs and that a single freeze-thaw cycle did not impair therapeutic efficacy, we next sought to investigate whether complete in vitro apoptosis was required prior to administration to retain anti-inflammatory activity.

Our early in vitro studies measuring the induction of apoptosis revealed that complete apoptosis in MSCs is achieved only after 24 hours of anti-Fas stimulation (Supplementary Figure S1B). Further experiments showed only basal Annexin V expression and caspase 3/7 activation in MSCs stimulated with anti-Fas for 15 minutes prior to being frozen when compared to those cultured for 24 hours post stimulation, confirming that the full 24-hour incubation period following Fas stimulation is required for full apoptosis to occur (Supplementary Figure S1C, S1D). To investigate whether complete apoptosis was required for anti-inflammatory activity we therefore treated mice with either MSCs frozen immediately post 15-minute stimulation with anti-Fas and thawed prior to infusion, or those frozen and thawed (once) 24 hours post-Fas stimulation to ensure complete induction of MSC apoptosis. Fas-stimulated MSCs administered after only 15-minute stimulation failed to demonstrate any significant reductions in either pulmonary total leukocyte (Figure 2B, D) or eosinophil recruitment (Figure 2C, D) following allergen challenge (dark purple circles) at any timepoint, whilst this was retained in mice treated with Fas-stimulated MSCs and allowed to undergo complete apoptosis over 24 hours prior to in vivo administration (green and lilac circles). As animals treated with MSCs stimulated with anti-Fas for only 15 minutes failed to demonstrate any anti-inflammatory activity in vivo, we conclude that the anti-inflammatory activity observed from ApoMSCs requires complete Fas-induced apoptosis for therapeutic activity rather than Fas stimulation alone. Therefore, we suggest that whilst ApoMSCs can retain therapeutic activity following a single freeze-thaw cycle, cells must be allowed to undergo complete apoptosis over a 24-hour incubation period prior to being frozen.

## Discussion

The recent discovery that in vivo MSC apoptosis is critical for the in vivo induction of anti-inflammatory activity offers a novel prospect to better harness the therapeutic benefits of MSC.<sup>8,9,13</sup> Our research, confirmed by others, has demonstrated that the recipient's activated cytotoxic cells induce in vivo MSC apoptosis.<sup>8,9</sup> These apoptotic MSCs are then efferocytosed by monocytes/macrophages and subsequently converted into anti-inflammatory cells. Identifying a strategy that circumvents the need for the presence of cytotoxic cells in MSC recipients would have the great advantage of broadening the number of patients who could benefit from MSC-based therapies. Therefore, the administration of ex vivo-generated ApoMSCs emerges as an attractive approach. Our previous studies have demonstrated that ApoMSCs possess anti-inflammatory properties in mouse models of OVA-induced allergic inflammation and GvHD.<sup>8</sup> Similarly, other studies have reported therapeutic effects of ApoMSCs in mouse models of allergic airway inflammation, sepsis, acute liver and lung injury, and colitis.<sup>9,20-24</sup>

One of the major hurdles in delivering ApoMSC as a future clinical product is how ApoMSCs could be delivered to the bedside. In this study, we have addressed how to optimize the protocol for the production of Fas-stimulated ApoMSCs, with a particular focus on developing a cryopreserved product that would be feasible for immediate administration post thawing. ApoMSCs generated ex vivo and subsequently frozen retained their anti-inflammatory efficacy after thawing prior to in vivo infusion (Figure 2). However, our data also suggest that the freezing process could compromise the ability of MSCs to undergo complete apoptotic cell death as MSCs treated with anti-Fas for only 15 minutes prior to being frozen showed low activation of caspase 3/7 and low expression of externalized phosphatidylserine after thawing and were not effective in alleviating inflammation. Therefore, it is critical for the Fas-activated MSCs to undergo complete apoptotic cell death through a caspase-mediated process prior to the freezing process to achieve optimal anti-inflammatory activity in vivo.

Whilst further research is required to confirm the wider immunosuppressive benefits of ApoMSCs in patients, these findings provide a significant step forward in their application in clinical practice. Firstly, our studies demonstrate that complete apoptosis prior to any freeze/thaw cycle is required to achieve maximal anti-inflammatory activity. Secondly, the ability to generate a therapeutically active frozen ApoMSC product under laboratory conditions is a significant step forward to shed light on the design of GMP-grade ApoMSCs. If this activity is reproduced in ApoMSCs produced under GMP conditions, this would offer significant benefits for any future clinical application as it would allow this proposed pre-prepared product to be subsequently transported to and stored at the site of application. This would provide significant benefits over requiring the induction of MSC apoptosis at the bedside. Future studies are also warranted to compare the impact of cryopreservation at  $-80^{\circ}\text{C}$  versus liquid nitrogen, as well as short-term versus long-term storage.

In conclusion, our study provides data under laboratory conditions that unveil a significant step forward in the process of demonstrating the therapeutic potential of apoptotic MSC therapy in the treatment of allergic inflammation and provides valuable insights for the development of a standardized cellular product in next-generation ApoMSC therapy.

## Author Contributions

Richard T. Amison, Tik S. Cheung, Chiara Giacomini, Antonio Galleu, Francesco Dazzi designed the study; Richard T. Amison, Tik S. Cheung, Chiara Giacomini, Antonio Galleu, Yanira Riffo-Vasquez collected data; Richard T. Amison, Tik S. Cheung, Chiara Giacomini, Roberto Savoldelli, Anna Kozłowska analyzed data; Richard T. Amison, Tik S. Cheung, Chiara Giacomini, Francesco Dazzi, Roberto Savoldelli, Anna Kozłowska, Ryan Hicks wrote the manuscript. All authors reviewed and approved the final manuscript.

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## Conflict of interest

All authors declared no potential conflicts of interest.

## Data Availability

The data generated in this study are available within the article and its [supplementary data](#) files. All raw data are available upon request.

## Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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