Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells

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Abstract

Background: Adult bone marrow-derived mesenchymal stem cells (MSC) possess potent immune modulatory effects which support their possible use as a therapy for immune-mediated disease. MSC induce regulatory T cells (Treg) in vitro although the in vivo relevance of this is not clear.

Objective: This study addressed the hypothesis that adult bone marrow derived-MSC would prevent the pathology associated with allergen-driven airway inflammation, and sought to define the effector mechanism.

Methods: The influence of allogeneic MSC was examined in a model system where Treg induction is essential to prevent pathology. This was tested using a combination of a model of ovalbumin-driven inflammation with allogeneic MSC cell therapy.

Results: Systemic administration of allogeneic MSC protected the airways from allergen-induced pathology, reducing airway inflammation and allergen-specific IgE. MSC were not globally suppressive but induced CD4+FoxP3+ T cells and modulated cell-mediated responses at a local and systemic level, decreasing IL-4 but increasing IL-10 in bronchial fluid and from allergen re-stimulated splenocytes. Moderate dose cyclophosphamide protocols were used to differentially ablate Treg responses; under these conditions the major beneficial effect of MSC therapy was lost, suggesting induction of Treg as the key mechanism of action by MSC in this model. In spite of the elimination of Treg a significant reduction in airway eosinophilia persisted in those treated with MSC.

Conclusion: These data demonstrate that MSC induce Treg in vivo and reduce allergen-driven pathology. Multiple Treg dependent and independent mechanisms of therapeutic action are employed by MSC.

Abbreviations
BALF, bronchoalveolar lavage fluid; CY, cyclophosphamide; GVHD, graft-versus-host-disease; H&E, haematoxylin and eosin; MSC, mesenchymal stem cells; OVA, ovalbumin; PAS, periodic acid Schiff; PBS, phosphate buffered saline; PFA, paraformaldehyde; PGE-2, prostaglandin E2; Treg, regulatory T cells; TGF-β, transforming growth factor-β.
Recent advances in immunological understanding have re-evaluated the role of suppression and demonstrated that pathogenic T cells can be actively countered by regulatory CD4+CD25+FoxP3+ T cells (Treg) in murine models (11). In these situations the development of allergic airway inflammation is due to inadequate, defective or overwhelmed Treg responses. These models provide powerful tools to study the influence of the suppressive or trophic function of MSC. If MSC induction of CD4+CD25+ FoxP3+ cells in vitro (12, 13) is mirrored in vivo, then the ovalbumin (OVA) sensitization model offers a means to test the biological significance of MSC as cell therapeutic inhibitors of allergic airway pathology.

This study addressed the hypothesis that adult bone marrow derived-MSC would prevent the pathology associated with allergen-driven airway inflammation, and sought to define the effector mechanism. Adult bone marrow derived allogeneic MSC actively prevented the induction of allergen-driven pathology in a murine model via induction of Treg suggesting a novel cell therapy for allergic human disease.

Materials and methods

Animals

Allergen sensitization was as previously described (14) using 8- to 12-week old, female BALB/cOlaHsd (H-2d) mice (Harlan, Oxon, UK), whereas FVB/NHanHsd (H-2q) male mice were the source of allogeneic MSC. Mice were maintained according to the regulations of the Irish Department of Health, and the institutional research ethics committee. Mice were sensitized by intra-peritoneal injection of 100 µg/ml ovalbumin (OVA) emulsified in aluminum hydroxide (AlumImject™) (Pierce, IL, USA) on days 0, 7 and 14. Mice were challenged intra-nasally with OVA (50 µg/ml) or sterile PBS (sham) on days 14, 25, 26 and 27 (Fig. 1A).

Isolation and culture of bone marrow derived mesenchymal stem cells

Bone marrow from of FV/BN mice was resuspended in Mesencult Basal Medium, supplemented with 10% (v/v) Mesencult supplement (Stem Cell Technologies, Vancouver, Canada). Cells were maintained as previously described (15), and used between passages 4 and 9 with rigorous purification and quality control to ensure purity as previously described (15). All MSC used were capable of differentiation to the three major mesenchymal lineages (13), and were MHC class I+, Sca-1+, CD44low, CD106low, MHC-II+, CD11b+, CD11c+, CD34+, CD45+ and CD117+. For paraformaldehyde (PFA)-fixed MSC, cells were pelleted and resuspended in 50 ml PFA (0.5% in PBS) for 20 min at room temperature, before extensive washing and use.

MSC therapy

Allogeneic H-2k MSC (or fixed control cells) were washed twice with PBS and resuspended at 5 x 10^6 cells/ml. A preliminary investigation was carried out to ascertain whether MSC-induced expansion of Treg was dose-dependent and determine the optimum dose. 100 µl of MSC at 5 x 10^6, 5 x 10^5 or 5 x 10^4 cells/ml were administered i.v. and the expression of FoxP3 by CD4+ T cells was quantified by flow cytometry 0, 4, 8 and 12 days following treatment (Fig. S1). Treg induction reflected MSC dose and was greatest in mice receiving 0.5 x 10^6 cells/mouse (Fig. S1); therefore, subsequent experiments utilized this dose. 0.5 x 10^6 MSC, fixed MSC or PBS were delivered via tail (100 µl, i.v.) on days 7 and 14 to H-2k mice (n = 8) as follows: (i) sham sensitized-PBS alone; (ii) OVA-sensitized mice-PBS; (iii) OVA-sensitized mice-MSC; (iv) OVA-sensitized mice + PFA-fixed MSC; (v) control PBS sham sensitized mice infused with

![Figure 1 Study design. (A) OVA sensitization, (B) CY depletion of Treg. CY (150 mg/kg) was delivered via i.p. injection on day 8, 12 and 15.](image-url)
MSC; (vi) control sham PBS-sensitized with PFA-fixed MSC (Fig. 1A). MSC locating to the airways within 24 h as previously documented (16) and data not shown. At 28 days, bronchoalveolar lavage fluid, and histopathological studies were performed, serum collected and splenocytes re-stimulated in vitro (14). All experiments were performed at least twice.

**Histopathology and airway physiology**

At 28 days BALF was collected (14) and differential cell counts performed as described (14). Nonlavaged lungs were fixed, sectioned and stained with hematoxylin/eosin (H&E), or combined Discombes/Alcian blue/periodic acid-Schiff (PAS) stain (14). Pathology was scored using a semi-quantitative scoring system as mild, moderate or severe and shown for convenience in Supporting information (Fig. S2). Lung function was assessed by unrestrained plethysmography and expressed as the surrogate index of enhanced pause (PenH) as previously described (14). This approach has limitations and was used as supporting rather than a definitive indicator of airway hyper-responsiveness.

**Measurement of cytokines and antibody response**

IL-4, IL-10 and IL-13 from BALF or antigen re-stimulated or control splenocyte supernatants were analyzed by flow cytometry (Becton-Dickinson, New Jersey, USA), using Cytometric Bead Array Flex Sets (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer’s instructions. Standard curves and raw data were generated for each cytokine using FCAP Array v1.0.1 software (BD Biosciences). OVA-specific serum IgE was measured by ELISA as previously optimized (17).

**Figure 2** Representative morphological changes in bronchiolar transverse sections of lungs and BAL composition at day 28 from (A & E) Nonsensitized, (B & F) OVA-sensitized, (C & G) OVA-sensitized, MSC treated, (D & H) OVA-sensitized, PFA-fixed MSC treated. Airway inflammation was detected using H&E (A-D) (magnification ×100) and combined Discombes/Alcian blue/PAS (E-H) staining (magnification ×400). p and h indicate perivascular inflammation and bronchiolar epithelial hypertrophy, respectively. g and m indicate goblet cell hyperplasia and mucus secretion, respectively. Negative controls were sham infected/sensitized with saline. The data are representative of three experiments; in each case, at least five animals were assessed. Results are expressed as mean ± SEM of cell number (* *P < 0.05, **P < 0.01).
**T<sub>reg</sub>** depletion and assessment

In T<sub>reg</sub> studies, mice were sensitized with OVA on day 0, 7 and 14, and MSC delivered on days 7 and 14. An established model of pharmacological T<sub>reg</sub> depletion was employed (18). Mice received 3 low doses of 150 mg/kg cyclophosphamide (CY) (Sigma) intraperitoneally on day 8, 12 and 15 (Fig. 1B). On day 19, splenocytes and lung leukocytes were prepared from representative animals. Single cell suspensions were prepared by dissociating tissue with collagenase D (Sigma) and labeled for surface CD4, CD25 and intracellular FoxP3 as previously described (13). The effectiveness of depletion by dose and time was established in preliminary experiments and verified by the absence of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> populations in spleen or lungs of test animals by flow cytometry at the time points selected (Fig. S3). Remaining mice were challenged with OVA (50 μg in 30 μl PBS) intranasally on day 25, 26 and day 27 and assessed as above (Fig. 1B).

**Statistical analysis**

Values for all measurements were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GRAPHPAD PRISM<sup>TM</sup> software (GraphPad, San Diego, CA, USA). Comparison was made using the Kruskal–Wallis test, or the Mann–Whitney test as appropriate. Significance was denoted by<sup>P</sup>-value < 0.05.

**Results**

**Allogeneic MSC therapy reduces allergen-driven airway pathology**

The influence of MSC cell therapy was examined in a murine model of allergic pathology. Nonsensitized control mice exhibited no allergen-driven airway inflammation as expected (Fig. 2A), whereas OVA-sensitized mice exhibited typical peribronchial and perivascular inflammation (Fig. 2B). In contrast, MSC therapy resulted in markedly decreased pathology, with decreased peribronchial inflammation (Fig. 2C). Consistent with these data were physiological observations of a surrogate of airway hyper-responsiveness suggesting that MSC therapy reduced bronchial hyper-reactivity compared to OVA-sensitized mice (Fig. S3). However MSC needed to be viable as delivery of PFA-fixed MSC to allergen sensitized mice resulted in more severe pathology when compared to OVA-sensitized mice, displaying strong perivascular inflammation and bronchiolar epithelial hypertrophy (Fig. 2D). Thus MSC cell therapy reduces classical allergen-driven pathology in this model. A consistent feature of asthma is the production of mucus blockage of the peripheral airways (19). MSC delivery reduced airway mucus, whereas PFA-fixed MSC exacerbated goblet cell hyperplasia and mucus secretion in allergen-sensitized mice (Fig. 2E–H). Thus, live, but not fixed, MSC therapy reduced multiple characteristic aspects of allergen-driven airway pathology.

**Allogeneic MSC therapy protects against allergen-driven lung eosinophilia**

A cardinal feature of allergen-driven airway inflammation is the elevated number of inflammatory cells in the lungs, particularly eosinophils (9). Control mice showed minimal cellularity in bronchoalveolar lavage (Fig. 2), whereas OVA sensitization resulted in significant infiltration (<sup>P</sup> < 0.05). Total cellular infiltration was decreased in OVA-sensitized mice that received MSC, whereas it remained high in sensitized animals treated with PFA-fixed MSC. BALF from control mice had few cells, other than macrophages; however OVA sensitization/challenge resulted in eosinophilic inflammation. Airway eosinophilia was significantly reduced in OVA sensitized mice following MSC delivery (<sup>P</sup> < 0.05), whereas PFA-fixed MSC treatment caused a considerable increase in airway eosinophilia when compared to OVA sensitized mice (Fig. 2). The number of macrophages in BALF was similar to controls. These findings demonstrated that live allogeneic MSC have wide ranging therapeutic influence on allergen-driven airway inflammation and in particular eosinophilic inflammation.

**Allogeneic MSC cell therapy induces T<sub>reg</sub> in vivo and modulates allergen-specific immunity**

IgE induction is a feature of allergen-driven pathologies and OVA sensitization induces IgE and an allergen-specific Th2 response (20). The capacity for MSC to influence IgE induction was examined by measuring OVA-specific IgE in serum from OVA-sensitized mice in which MSC were used therapeutically. Allogeneic MSC therapy suppressed the allergen-specific IgE response (Fig. 3), in comparison to mice sens-
sitized to OVA alone ($P < 0.05$) (Fig. 3). Sensitized mice that received PFA-fixed MSC showed similar levels of OVA specific IgE to OVA sensitized mice.

The phenomena described above might be explained by MSC-mediated global, nonspecific immune suppression or by MSC interference in immune induction. Therefore, the effect of allogeneic MSC on T cell mediated immunity was examined. Particular attention was given to IL-4 and IL-13 induction as these play well defined roles in allergen-driven pathology (21). As expected, IL-4 and IL-13 in both BALF and splenocyte supernatants were significantly higher in the OVA compared to sham-sensitized mice (Fig. 4). However, a marked reduction in IL-4 and IL-13, but a significant increase in IL-10 (Fig. 4) ($P < 0.05$) was observed in BALF (Fig. 5B) ($P < 0.05$). Similarly, when spleen cells were re-stimulated ex vivo with allergen, IL-10 was increased but a reduction in IL-13 and IL-4 was observed. This was an important observation as it shows that the protective effect of MSC was a result of targeted, specific modulation of local immunity rather than a global suppression of the immune response.

The induction of local and systemic IL-10 strongly suggested that MSC were inducing a regulatory T cell population. Therefore, the generation and expansion of Treg subsets was assessed in the lungs and spleens of OVA-sensitized mice, and mice that received MSC therapy. MSC induced or expanded a population of regulatory cells; most notably a CD4$^{+}$FoxP3$^{+}$ population was observed in both the lungs

**Figure 4** Spleens and BALF were harvested on day 28. Splenocytes were cultured in the presence of media alone (□) or OVA (200 μg/ml) (●) for 72 h. Cytokine responses from similar cultures are shown for (A & C) IL-4, (B & D) IL-13 and (C & E) IL-10. Responses are representative of triplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± SEM ($*P < 0.05$).
and spleen from sensitized, MSC-treated mice (Fig. 5). A significant increase in FoxP3 expression ($P < 0.05$) was observed in CD4+ T cells from MSC-treated compared to untreated sensitized mice providing evidence that MSC induce Treg populations in vivo.

Regulatory T cells are required for MSC mediated inhibition of allergic airway inflammation

Detection of Treg does not necessarily equate to an essential function. To investigate whether MSC exerted their immunosuppressive function via induction of Treg, these suppressor cells were depleted and the effect of MSC delivery on airway pathology was examined. Pharmacological depletion based on cyclophosphamide (CY) administration has been widely used to examine the effect of Treg depletion in disease models (22) as it both impairs functionality and depletes Treg in vivo (23). Fortunately MSC are ALDH+ (24) and thus, resistant to CY (25), and showed no impairment in differentiation capacity to CY during in vitro exposure (data not shown). A protocol consistent with previous studies (18) was chosen to allow OVA-specific effector cell induction but which depleted Treg (Fig. 1B). Contrary to earlier findings, MSC did not confer protection when Treg were depleted. In the absence of Treg, MSC-treated OVA-sensitized mice displayed significant cellular infiltration including peribronchial inflammation at day 28 (Fig. 6) comparable to allergen sensitized mice. Delivery of MSC in the absence of Treg also resulted in more pronounced levels of mucus production with some airways showing profound obstruction (Fig. 6). When mice were depleted of Treg, there was no observable difference in IgE between those that received MSC and positive controls (Fig. 6). These data support a model where atopic responses are moderated in vivo by the suppressive influence of constitutive Treg, and more importantly that Treg were required for the MSC-mediated reduction of pathology and allergen specific IgE.

MSC cell therapy in Treg-depleted allergen sensitized mice did not alter the Th2 profile (of IL-4 or -13) in BALF (Fig. 6) and did not elevate IL-10, in direct contrast to when Treg were not depleted (Fig. 4). Similarly, MSC therapy in Treg-depleted mice did not impact on allergen-specific Th2 responses in the spleen. Taken together these data strongly suggest that the mechanism of beneficial action by MSC in this model is dependent on the induction of Treg. Thus MSC modulate allergen specific local and systemic immunity through a Treg mechanism. A feature of CY treatment in this model is enhanced pathology and increased airway eosinophilia (18). Similar findings are reported here (Fig. 6). However, MSC treatment significantly reduced eosinophilia even in the absence of Treg (Fig. 6), but did not influence other indices or improve overall pathology. Together these data indicate that whilst Treg induction is required to moderate Th2-driven inflammation, an alternative Treg independent mechanism might also be employed by MSC.

Discussion

This study demonstrated that adult bone marrow-derived allogeneic MSC actively prevent the induction of allergen-driven pathology through a Treg-dependent mechanism. Systemic administration of MSC protected the airways from OVA-induced pathology evidenced by reduced lung pathology and cellular inflammation in BALF and reduced allergen specific IgE. MSC were not globally immunosuppressive but rather immunomodulatory, inducing splenic OVA recall responses dominated by IL-10, a cytokine also elevated in BALF by MSC therapy. MSC therapy induced populations of CD4+FoxP3+ T cells in the lung and spleen. Depletion of Treg ablated the protective effect of MSC therapy in terms of the major indices of pathology, and restored class switching to IgE. Thus Treg are required for the protective effect of MSC therapy in this model, however MSC continued to affect eosinophilia indicating that MSC also use Treg-independent mechanisms to modulate effector function.

The mechanisms of MSC effector action (and hierarchy) in vivo are poorly understood. MSC can induce direct repair, may be cytoprotective, pro-angiogenic, anti-fibrotic or act through other paracrine effects (6, 7, 16). We have previously outlined mechanisms by which MSC induce Treg in vitro, defining roles for cell contact, TGF-β and PGE-2 (13). This
study selected a system where prevention of pathology could be directly linked to Treg induction in vivo. Increased CD4+FoxP3+ T cells and elevated IL-10, locally (lung/ BALF) and systemically (spleen) following MSC therapy was circumstantial evidence for a role for Treg as the mechanism for MSC-mediated protection in this model. Although the increase in FoxP3+ Treg was modest, it is quantitatively similar to recent studies where similar induction was associated with decreased pathology (26). MSC-mediated immunosuppression has been suggested by the expansion of CD4+FoxP3+ cells in vitro (8, 26). CD4+CD25+FoxP3+ Treg are critical for control of antigen-specific inflammation [for review (27)] and their recruitment into the airways can suppress allergic airway inflammation (28). Recently, IL-10 production by Treg was shown to be essential to control immune responses in the lung (29). The current study revealed that MSC therapy increased CD4+FoxP3+ T cells in lung and spleen which was associated with elevated IL-10 supporting the findings of Rubstov with regard to the importance of Treg/IL-10, but more importantly strongly suggesting that MSC induction of Treg was not simply an in vitro phenomenon.

Treg cell induction in vitro or even in vivo by MSC is an important and interesting finding but its significance depends on the functional contribution to reduced pathology. Here, the contribution of Treg, induced by MSC, to exert functional

Figure 6 Representative morphological changes at 28 days in bronchiolar transverse sections of lungs from Treg-depleted, sham-sensitized, Treg-depleted (CY-PBS), OVA-sensitized (CY-OVA) and Treg-depleted, OVA-sensitized, MSC-treated (CY-OVA MSC). (A) Airway inflammation detected using haematoxylin and eosin (H&E) and combined Discombes/Alician blue/PAS staining of fixed lung sections. Original magnification, top panels x 100, bottom panels x 400. (B) OVA-specific IgE in serum elicited in response to OVA sensitization in Treg depleted mice. (C) Cellular composition of BAL from Treg depleted mice 24 h after final OVA exposure. (D) Cytokine profile of BALF elicited by OVA sensitization. All sections are representative of at least three animals.
protection was studied using the cyclophosphamide depletion model. Alternative approaches such as CD25 depletion could not be used as these would interfere with T cell activation and Th2 induction and confound interpretation, whereas this regime differentially depletes functional Treg (30). Using this model it was shown that Treg induced by allogeneic MSC therapy were required for the reduction in pathologic score, mucus secretion and allergen-induced IgE. Thus, this study goes beyond demonstrating Treg induction in vivo by MSC to show a biological significance for that process.

Treg depletion ablated most of the beneficial effects of MSC therapy, indicating the mechanism of protection in this model. However MSC supported a significant reduction in airway eosinophilia despite Treg depletion (Fig. 6). This observation was important for two reasons: firstly it indicated that MSC could modulate effector cell function by alternative Treg-independent mechanisms; secondly as reduced eosinophilia is seen here whilst the pathology is impaired, it suggests that altered eosinophilia cannot account for the MSC-mediated effects seen in Fig. 2, and that the primary mechanism by which MSC reduce pathology is via Treg induction. We and others have previously shown that MSC express a variety of immunosuppressive cytokines including hepactocyte growth factor (HGF) at concentrations that can suppress allogenic responses in vitro (31). HGF negatively regulates allergic airway inflammation and hyper-responsiveness (32) via direct attenuation of eosphinil chemotactic function. The expression of HGF by MSC (31, 33) and reduced eosinophilia is consistent with such a Treg-independent role, and consistent with human clinical studies where reduced airway eosinophilia had little impact on pathology (34). The implication of an alternative MSC mechanism of action is that MSC therapy may allow a targeting of complex multi-factorial diseases that involves both fibrotic and inflammatory processes and Treg-dependent and independent aspects. Allogeneic MSC possess specific immunomodulatory properties that target critical pathogenic features for the development of allergic asthma. Here we demonstrate the efficacy of MSC based cell therapy in a well characterized murine model. We also illustrate the mechanism of action by which protection is mediated as a proof of concept for MSC based immunotherapy for a broad range of diseases where chronic inflammation results in pathology.

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