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

H. Kavanagh & B. P. Mahon

Cellular Immunology Laboratory, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

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Correspondence
Bernard P. Mahon, Cellular Immunology Laboratory, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland.
Tel.: +353-1-708-3835
Fax: +353-1-708-3845
E-mail: bp.mahon@nuim.ie

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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 (T_{reg}) *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 T_{reg} 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 T_{reg} responses; under these conditions the major beneficial effect of MSC therapy was lost, suggesting induction of T_{reg} as the key mechanism of action by MSC in this model. In spite of the elimination of T_{reg}, a significant reduction in airway eosinophilia persisted in those treated with MSC.
Conclusion: These data demonstrate that MSC induce T_{reg} *in vivo* and reduce allergen-driven pathology. Multiple T_{reg} dependent and independent mechanisms of therapeutic action are employed by MSC.

Mesenchymal stem or stromal cells (MSC) are a heterogeneous population of cells (1), readily isolated from bone marrow. In addition to potential for tissue repair, MSC possess potent anti-proliferative and anti-inflammatory effects (2, 3) which support their therapeutic use for immune-mediated diseases. Preclinical models of autoimmune/inflammatory conditions have demonstrated a beneficial role for MSC in graft tolerance (4), rheumatoid arthritis (5), and multiple sclerosis (6). Protective roles have also been described in models of acute lung injury, including pulmonary fibrosis (7) and more recently allergic rhinitis (8). However, the precise mechanism by which MSC mediate protection is less clear.

Allergic asthma is an inflammatory disease of the airways, driven in part by Th2- cell induction, eosinophil and mast cell activation and mediator release (9), to establish an inflammatory response combined with profound airway remodeling. Current therapies for allergic asthma include the use of glucocorticoids, however, even long-term pharmacotherapies do not re-program the underlying immune deviation which drives pathology (10). There is an unmet need to develop strategies to reverse immunologic reactivity and chronic airway inflammation.

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; $T_{reg,}$ regulatory T cells; TGF-β, transforming growth factor-B.

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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 (T_{reg}) in murine models (11). In these situations the development of allergic airway inflammation is due to inadequate, defective or overwhelmed T_{reg} 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 T_{reg} 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-2^d) mice (Harlan, Oxon, UK), whereas FVB/NHanHsd (H-2^q) 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^+, $CD44^{low}$, $CD106^{low}$, 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-2^q MSC (or fixed control cells) were washed twice with PBS and resuspended at 5×10^6 cells/ml. A preliminary investigation was carried out to ascertain whether MSC-induced expansion of T_{reg} was dose-dependent and determine the optimum dose. 100 μl of MSC at 5×10^6, 5×10^5 or 5×10^4 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). T_{reg} induction reflected MSC dose and was greatest in mice receiving 0.5×10^6 cells/mouse (Fig. S1); therefore, subsequent experiments utilized this dose. 0.5×10^6 MSC, fixed MSC or PBS were delivered via tail (100 μl, i.v.) on days 7 and 14 to H-2^d 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

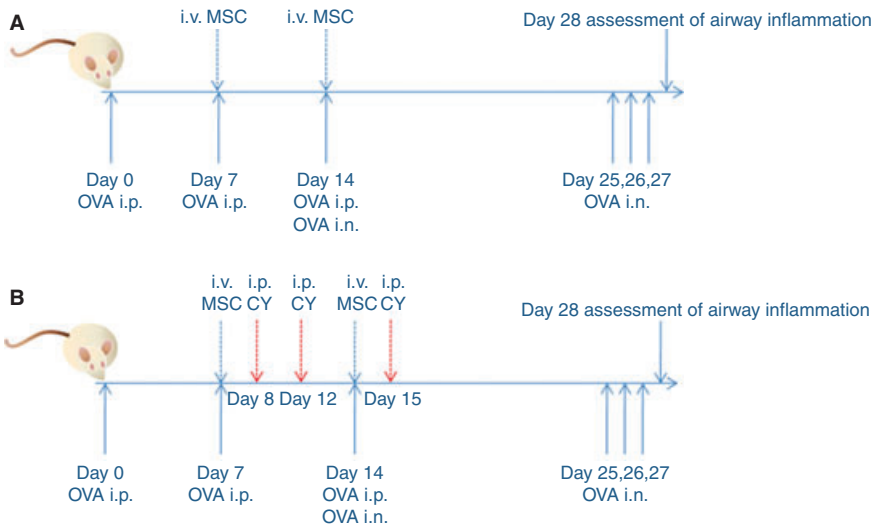

Figure 1 Study sesign. (A) OVA sensitization, (B) CY depletion of T_{reg}. CY (150 mg/kg) was delivered via i.p. injection on day 8, 12 and 15.

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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).

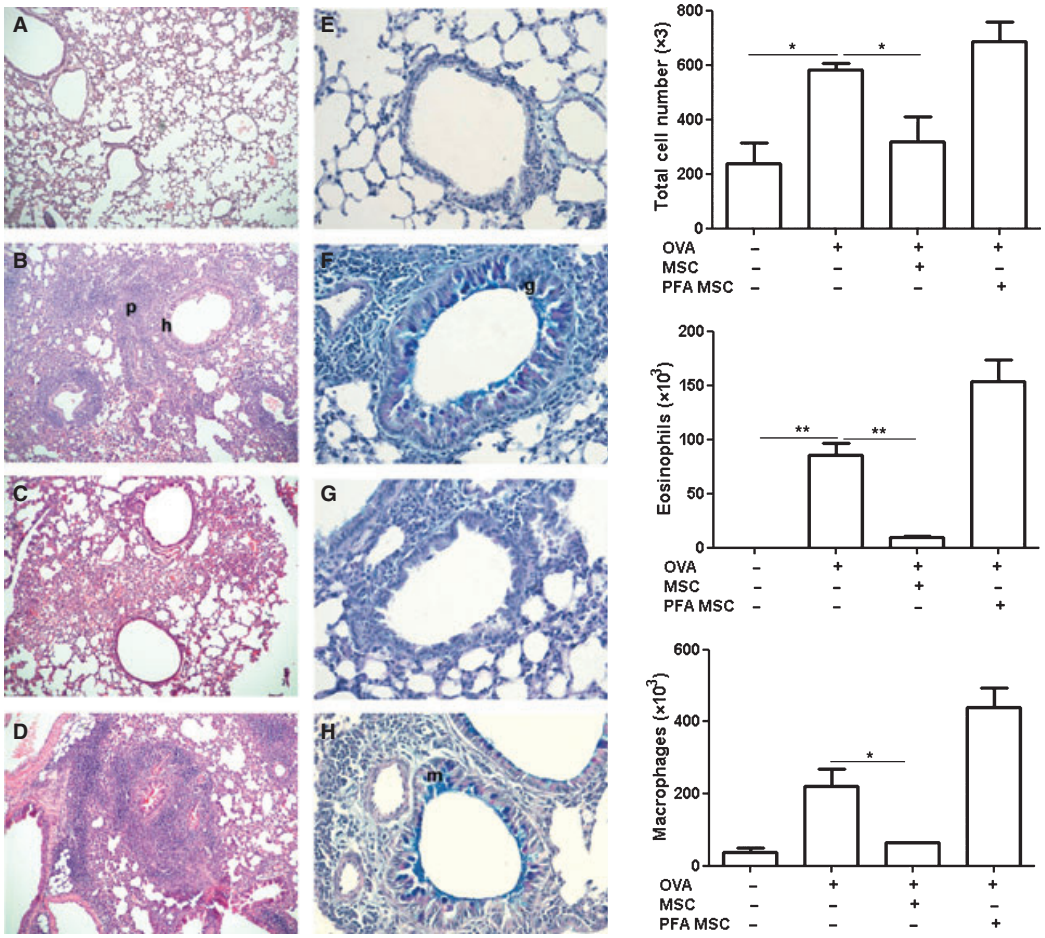

Figure 2 Representative morphological changes in bronchiolar transverse sections of lungs and BAL composition at day 28 from (A & E) Nonsensitized, (B & F) OVA-sensitized, (C & G) OVA-sensitized, MSC treated, (D & H) OVA-sensitized, PFA-fixed MSC treated. Airway inflammation was detected using H&E (A–D) (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 (*$P < 0.05$, **$P < 0.01$).

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T_{reg} depletion and assessment

In T_{reg} 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_{reg} 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 $CD4^+$ $CD25^+FoxP3^+$ 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™ 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 P-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. 2 C). 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 ($P < 0.05$). 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 ($P < 0.05$), 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_{reg} *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 sen-

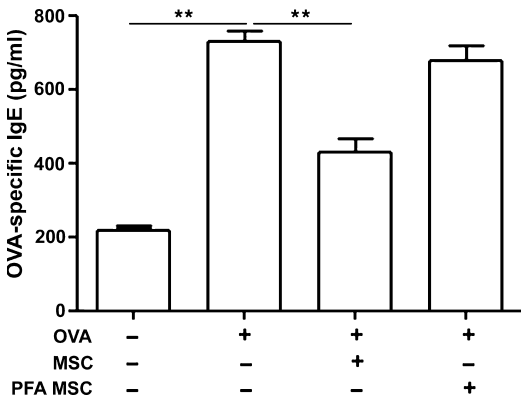

Figure 3 OVA-specific IgE in serum elicited in response to OVA sensitization. Sera were collected on day 28 and OVA-specific serum IgE levels were measured by ELISA. The data are representative of three experiments; in each case, at least five animals were assessed, and each individual assessment was performed independently in triplicate. Concentrations below 200 pg/ml were considered negative. Results are expressed as mean antibody concentrations ± SEM ($^{**}P < 0.01$, $^{***}P < 0.001$).

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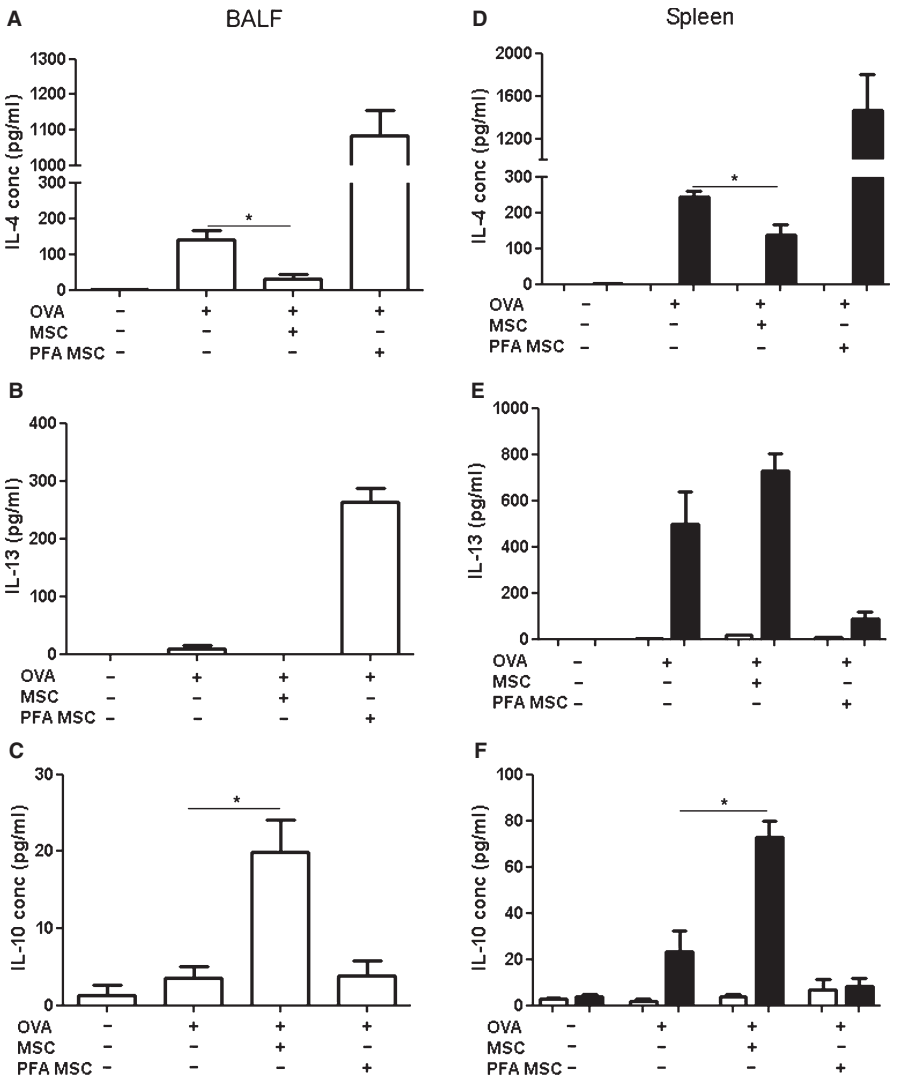

Figure 4 Spleens and BALF were harvested on day 28. Splenocytes were cultured in the presence of media alone (▭) or OVA (200 μg/ml) (■) for 72 h. Cytokine responses from similar cultures are shown for (A & C) IL-4, (B & D) IL-13 and (C & E) IL-10. Responses are representative of triplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± SEM (*$P < 0.05$).

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 restimulated *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 T_{reg} 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

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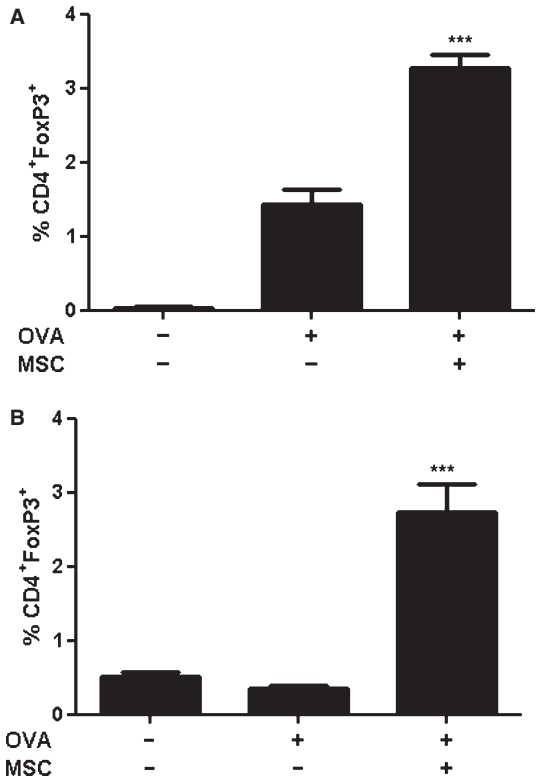

Figure 5 Expression of $CD4^+FoxP3^+$ in lymphoid cells isolated from the lungs (A) and spleen (B) of either OVA-sensitized or OVA-sensitized MSC-treated mice. Cells were intracellularly stained with PE-conjugated anti-FoxP3. Responses are representative of duplicate experiments, each of which was determined independently from at least four mice per group.

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 T_{reg} populations *in vivo*.

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

Detection of T_{reg} does not necessarily equate to an essential function. To investigate whether MSC exerted their immunosuppressive function via induction of T_{reg}, 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 T_{reg} depletion in disease models (22) as it both impairs functionality and depletes T_{reg} *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 T_{reg} (Fig. 1B). Contrary to earlier findings, MSC did not confer protection when T_{reg} were depleted. In the absence of T_{reg}, 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 T_{reg} also resulted in more pronounced levels of mucus production with some airways showing profound obstruction (Fig. 6). When mice were depleted of T_{reg}, 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 T_{reg}, and more importantly that T_{reg} were required for the MSC-mediated reduction of pathology and allergen specific IgE.

MSC cell therapy in T_{reg}-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 T_{reg} were not depleted (Fig. 4). Similarly, MSC therapy in T_{reg}-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 T_{reg}. Thus MSC modulate allergen specific local and systemic immunity through a T_{reg} 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 T_{reg} (Fig. 6), but did not influence other indices or improve overall pathology. Together these data indicate that whilst T_{reg} induction is required to moderate Th2-driven inflammation, an alternative T_{reg} 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 T_{reg}-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 T_{reg} ablated the protective effect of MSC therapy in terms of the major indices of pathology, and restored class switching to IgE. Thus T_{reg} are required for the protective effect of MSC therapy in this model, however MSC continued to affect eosinophilia indicating that MSC also use T_{reg}-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 T_{reg} *in vitro*, defining roles for cell contact, TGF-β and PGE-2 (13). This

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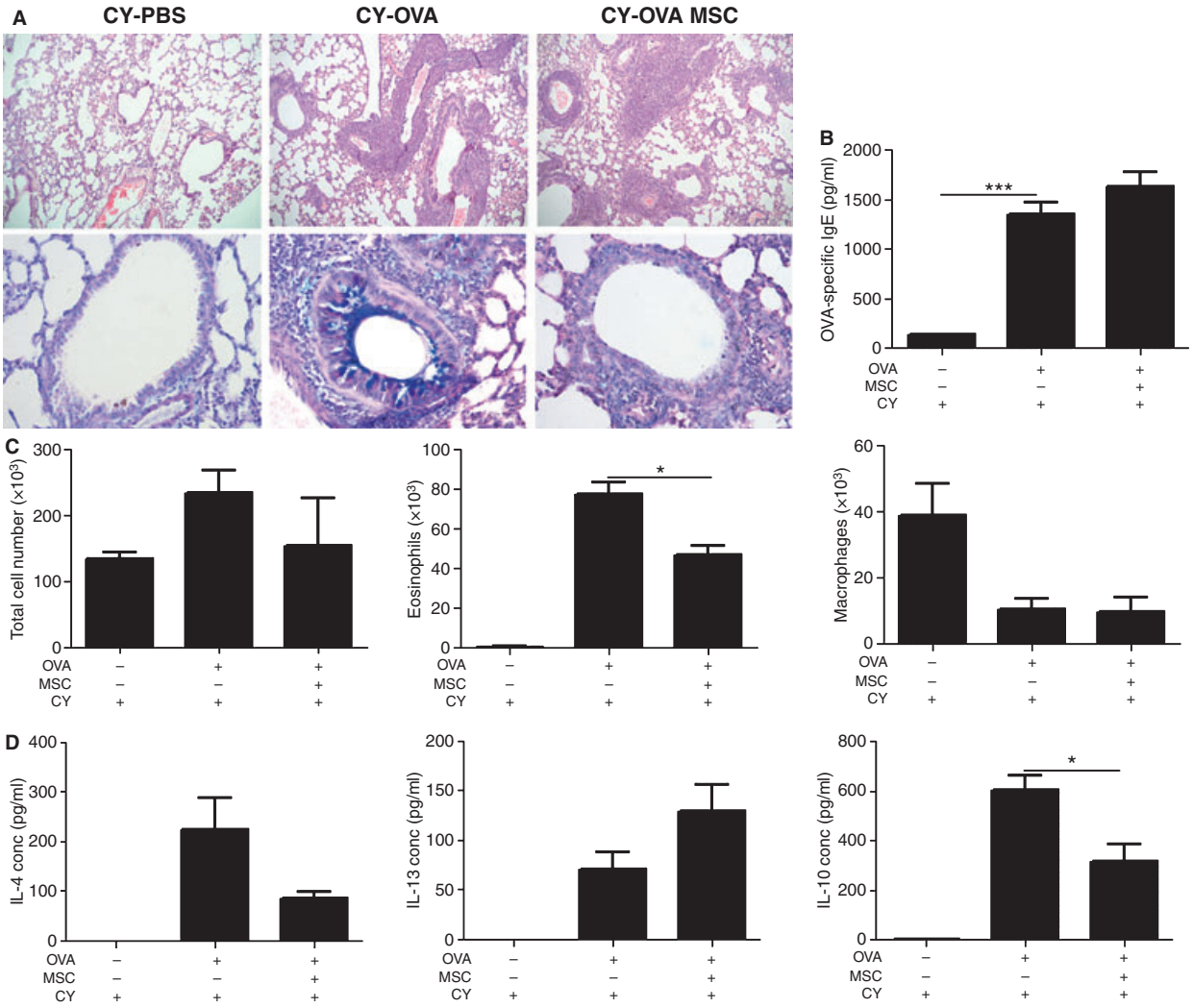

Figure 6 Representative morphological changes at 28 days in bronchiolar transverse sections of lungs from T_{reg}-depleted, sham-sensitized, T_{reg}-depleted (CY-PBS), OVA-sensitized (CY-OVA) and T_{reg}-depleted, OVA-sensitized, MSC-treated (CY-OVA MSC). (A) Airway inflammation detected using haematoxylin and eosin (H&E) and combined Discombes/Alcian blue/PAS staining of fixed lung sections. Original magnification, top panels × 100, bottom panels × 400. (B) OVA-specific IgE in serum elicited in response to OVA sensitization in T_{reg} depleted mice. (C) Cellular composition of BAL from T_{reg} 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.

study selected a system where prevention of pathology could be directly linked to T_{reg} 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 T_{reg} as the mechanism for MSC-mediated protection in this model. Although the increase in $FoxP3^+$ T_{reg} 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). $CD4^+CD25^+FoxP3^+$ T_{reg} 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 T_{reg} 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 T_{reg}/IL-10, but more importantly strongly suggesting that MSC induction of T_{reg} was not simply an *in vitro* phenomenon.

T_{reg} 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 T_{reg}, induced by MSC, to exert functional

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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 T_{reg} (30). Using this model it was shown that T_{reg} 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 T_{reg} induction *in vivo* by MSC to show a biological significance for that process.

T_{reg} 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 T_{reg} depletion (Fig. 6). This observation was important for two reasons: firstly it indicated that MSC could modulate effector cell function by alternative T_{reg}-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 T_{reg} induction. We and others have previously shown that MSC express a variety of immunosuppressive cytokines including hepatocyte growth factor (HGF) at concentrations that can suppress allogeneic responses *in vitro* (31). HGF negatively regulates allergic airway inflammation and hyper-responsiveness (32) via direct attenuation of eosinophil chemotactic function. The expression of HGF by MSC (31, 33) and reduced eosinophilia is consistent with such a T_{reg}-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 T_{reg}-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.

Acknowledgment

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Supporting Information

Additional Supporting Information may be found in the online version of this article at www.wileyonlinelibrary.com:

Figure S1. OVA sensitized mice received 100 μl MSC i.v. of 5×10^3 (open), 5×10^4 (horizontal shading) or 5×10^5 (black bar) cells/ml. On day 4 or 8 following treatment, lungs were harvested and digested with collagenase/DNase I for 1 h followed by intra/extracellular labelling with PE-conjugated anti-FoxP3/Cy5-conjugated anti-CD4. Results are expressed as mean values ± SD obtained from groups of at least three mice and are representative of two independent experiments. $*P < 0.05$; $**P < 0.005$.

Figure S2. Pathology scoring of H&E-stained lung sections. Original magnification × 100. Perivascular and peribronchial inflammation was evaluated as (A) mild, (B) moderate, and (C) severe. (A) Mild peribronchial inflammation (center) surrounded by clear alveoli and cross-sectioned bronchioles (bottom left, right). (A) Moderate peribronchial inflammation surrounding bronchiole (top right, bottom right) and moderate alveolar inflammation. (C) Severe perivascular and peribronchial inflammation with bronchial epithelial hypertrophy (center, center right).

Figure S3. The effect of CY treatment on $CD4^+FoxP3^+$ expression. Mice were given 150 mg/kg of CY intraperitoneally at day 0. Spleen cells were isolated followed by intra/extracellular labeling of anti-FoxP3-PE/anti-CD4-Cy5 then analyzed by flow cytometry. Results are expressed as mean values ± SD obtained from groups of at least three mice and are representative of two independent experiments. $*P < 0.05$; $**P < 0.005$.

Figure S4. Airway responsiveness was assessed on day 37 by methacholine induced airflow obstruction from conscious mice using whole-body plethysmography in conjunction with the BioSystem XA software (Buxco Electronics, USA) as previously described (14). (A) Nonsensitized (-○-), OVA-sensitized (-■-), OVA-sensitized + MSC-treated (-▼-), OVA-sensitized + PFA-fixed MSC treated (-●-). Results are expressed as mean enhanced pause (PenH) ± SEM. Where no error bars are visible, error bars are shorter than the size of the data point symbol.

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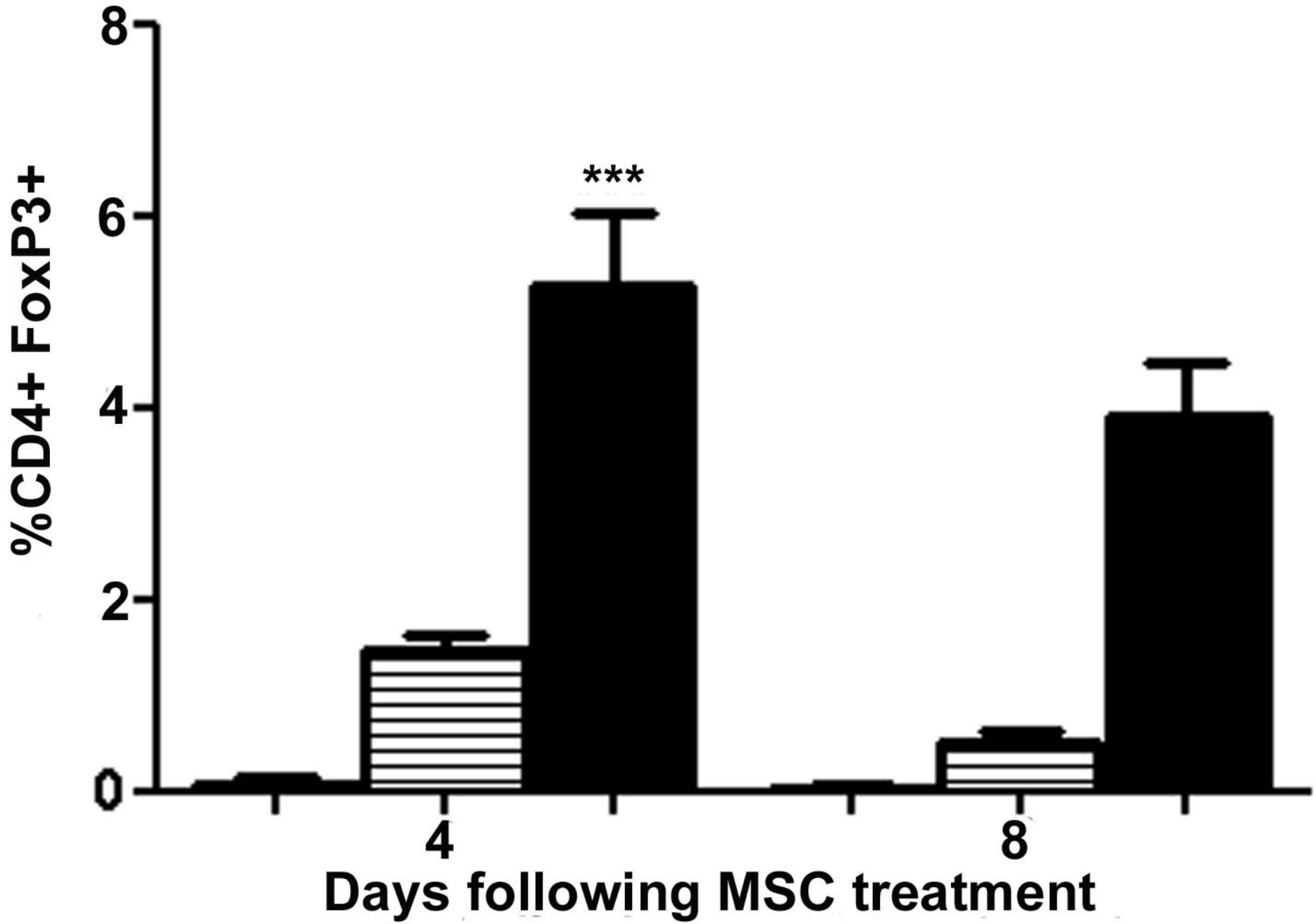
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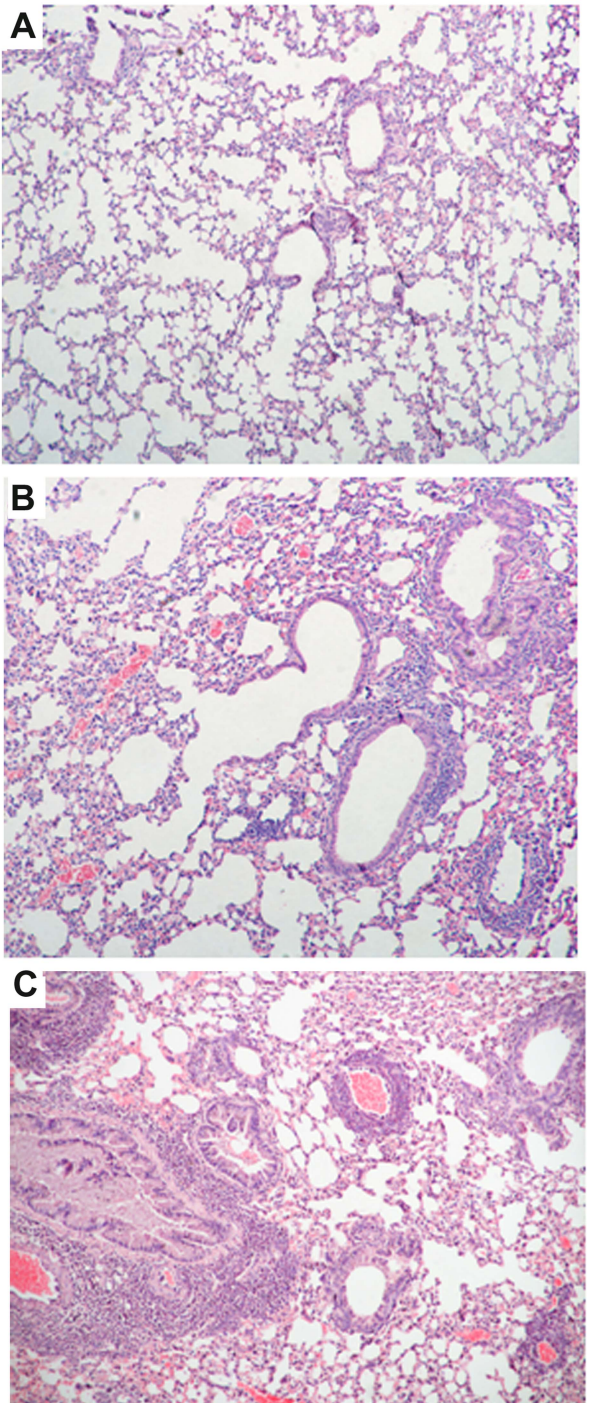
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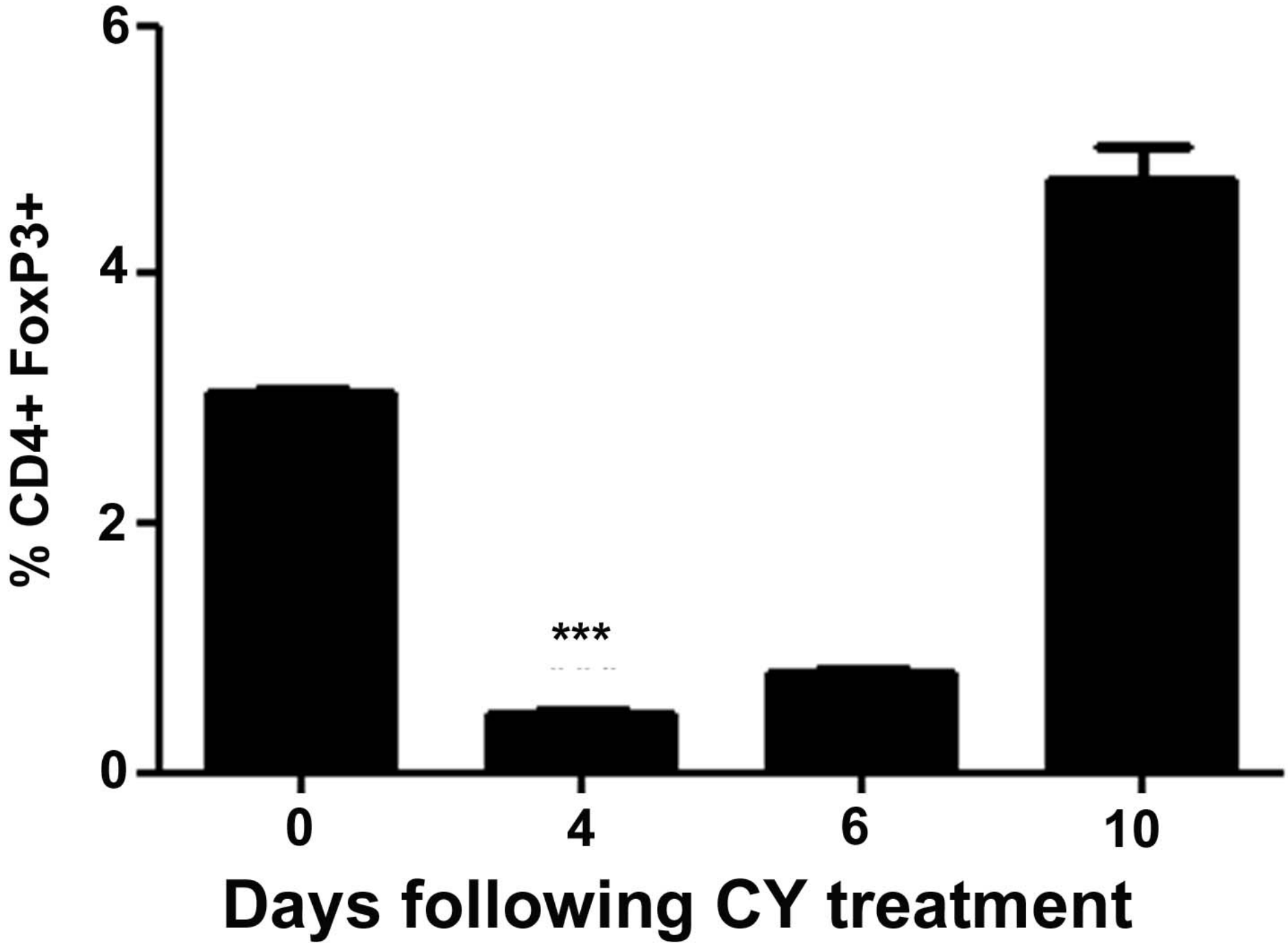
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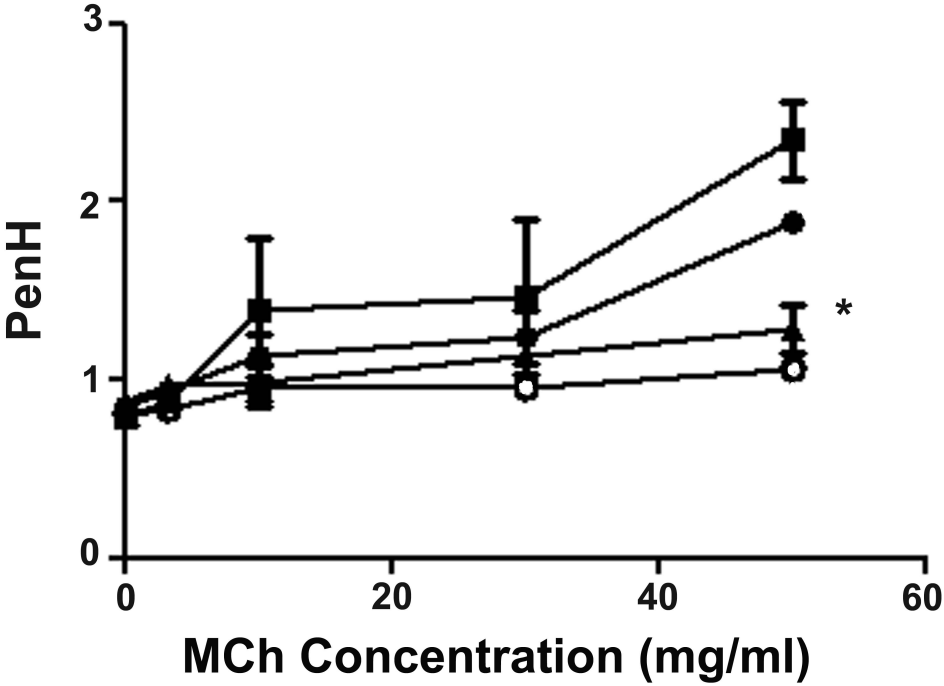
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