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Modulation of Alveolar Macrophage Inflammation in Sarcoidosis by Bone Marrow-Derived MSCs

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Abstract

Sarcoidosis is a severe inflammatory disorder that can involve multiple organs, particularly the lungs and lymph nodes. Bone marrow-derived mesenchymal stromal cells (MSCs) have the ability to "reprogram" various macrophage populations toward an anti-inflammatory phenotype. This study investigated whether alveolar macrophages from sarcoidosis patients exhibit a similar anti-inflammatory response when co-cultured with MSCs. Bronchoscopy and bronchoalveolar lavage (BAL) were performed on fifteen sarcoidosis patients and eight healthy controls. Unselected BAL cells, comprising 70–94% macrophages, were isolated and cultured with or without MSCs derived from healthy donors. After stimulating the cultures with lipopolysaccharide, supernatants were collected to measure levels of interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α). In two additional sarcoidosis patients, flow cytometry was used to evaluate intracellular cytokines and surface markers to validate the findings. In nine out of eleven sarcoidosis samples, co-culture with MSCs resulted in decreased TNF-α (indicative of pro-inflammatory M1 activity) and increased IL-10 (indicative of anti-inflammatory M2 activity). BAL cells from control subjects displayed minimal changes in cytokine production. Flow cytometric analysis in the additional patients confirmed a shift of alveolar macrophages from a pro-inflammatory (M1) to an anti-inflammatory (M2) state following MSC co-culture. These findings indicate that alveolar macrophages, like other macrophage populations, can adopt an anti-inflammatory phenotype in response to MSC interaction. We propose that administering MSCs to the airways could potentially reduce lung inflammation and lower the need for corticosteroids in patients with sarcoidosis.

Keywords: Sarcoidosis, Bone marrow stromal cells, Alveolar macrophages, Cell therapy

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Introduction

Bone marrow-derived stromal cells, commonly referred to as mesenchymal stromal cells (MSCs), are key players in regenerating skeletal tissues within the bone marrow niche and supporting hematopoiesis. MSCs represent a highly heterogeneous population [1] capable of differentiating into various mesenchymal lineages, including osteogenic, myogenic, and adipogenic pathways [2]. Their immunomodulatory properties have spurred extensive research into their therapeutic potential for inflammatory disorders, such as T-cell-mediated conditions like graft-versus-host disease (GVHD) [3], as well as granulomatous

diseases, including Crohn's disease [4]. MSCs mitigate inflammation and promote tissue repair both through direct cell–cell interactions, including mitochondrial transfer [5], and via paracrine signaling mechanisms [6,7]. Clinical studies to date have shown that MSCs possess an excellent safety profile [8,9].

It is widely recognized that MSCs can induce activated macrophages to transition from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype [10–13]. Cyclooxygenase-2 (COX-2) signaling plays a central role in this reprogramming and was initially demonstrated in a mouse sepsis model [14]. Inflammatory triggers, such as lipopolysaccharide (LPS) and TNF-α, engage toll-like receptors on MSCs, activating nuclear factor kappa B (NF-κB), which upregulates COX-2 expression. COX-2, in turn, promotes the synthesis of prostaglandin E2 (PGE2) [14], which interacts with macrophage receptors to enhance IL-10 secretion while reducing TNF-α production. This immunomodulatory behavior appears consistent across MSCs derived from multiple tissue sources [13]. Recent studies also indicate that phagocytosis of apoptotic MSCs by macrophages further amplifies the production of immunosuppressive factors, revealing an additional mechanism of action [15].

Sarcoidosis is a systemic granulomatous inflammatory disorder primarily driven by dysregulated macrophage TNF-α production and enhanced proliferation of T-helper-1 (Th1) and Th17 cells [16]. The disease frequently affects the lungs and lymphatic system, though its precise etiology remains largely undefined [17, 18]. Oligoclonal expansion of patient T-cells suggests that sarcoidosis may involve an antigen-driven immune process in genetically susceptible individuals [19–22]. Macrophage activation is central to disease pathogenesis [23], and macrophage-mediated recruitment of T-helper cells is recognized as a key contributor [24]. Current treatment strategies—glucocorticoids, cytotoxic agents, and anti-TNF-α monoclonal antibodies—carry significant risk–benefit considerations [25].

We hypothesized that human MSCs could exert a beneficial effect on sarcoidosis by reprogramming alveolar macrophages (AMs) toward an anti-inflammatory state. To test this hypothesis in vitro, we collected bronchoalveolar lavage (BAL) samples from biopsy-confirmed sarcoidosis patients and healthy controls for co-culture experiments with human MSCs. While it is established that pro-inflammatory macrophages from various sources adopt an anti-inflammatory phenotype upon MSC co-culture, our results demonstrate for the first time that AMs from sarcoidosis patients respond similarly. MSCs induced increased IL-10 and decreased TNF-α production in AMs, suggesting that MSC therapy may represent a safe adjunct or alternative to conventional pharmacological treatment.

Methods

Clinical procedure

Seventeen adult patients with sarcoidosis (Scadding stages 0–IV) who were receiving minimal or no anti-inflammatory therapy, along with eight healthy controls, were enrolled at the NIH under protocol 96-H-0100 (NCT00001532) to undergo bronchoscopy with bronchoalveolar lavage (BAL). Prior to the procedure, all participants underwent comprehensive medical evaluations, including laboratory testing and pulmonary function assessments. Procedures were performed under intravenous conscious sedation with topical anesthesia of the airways. BAL was conducted using 270–300 mL of sterile saline in 30 mL aliquots, primarily targeting the right and left upper lobes. Recovered fluid was collected into Lukens traps and kept on ice.

Approximately 10 mL of BAL fluid (BALF) was used to assess cell viability and perform cytometric analysis for white blood cell counts. Viability was determined via Trypan Blue staining, and differential cell counts were obtained using cytocentrifugation followed by Diff-Quik™ staining. The remaining BALF was utilized for in vitro co-culture experiments with MSCs. Detailed information regarding culture and co-culture conditions, as well as the ELISA protocol, is provided in the online supplementary data.

Cell culture

Clinical-grade human MSCs, obtained from iliac crest biopsies of healthy adult donors under NIH protocol 10-CC-0053 (NCT01071577), were previously expanded and cryopreserved in aliquots containing 1–4 million cells. For experiments, MSCs were thawed from liquid nitrogen storage and maintained in MEM-α supplemented with 10 percent FBS, 1 percent GlutaMax, and 1 percent Penicillin-Streptomycin (termed "MSC medium"). For alveolar macrophages (AMs), RPMI 1640 containing 10% FBS, 1% Penicillin-Streptomycin, and 0.00035% beta-mercaptoethanol was used as macrophage medium. Cells were stored frozen in a mixture of 50% relevant culture medium, 40 percent FBS, and 10 percent DMSO. All cultures were grown at 37 °C with 5 percent CO_2 and 20% O_2.

Upon receipt, unselected BAL cells (70–94 percent AMs) were filtered and prepared for co-culture assays. Cells were centrifuged, resuspended in macrophage medium, counted, and plated overnight in 96-well plates, either alone or together with passage 4–6 MSCs. The standard co-culture setup involved 100,000 BAL cells per 10,000 MSCs in 200 µL of medium, replicated eight times. Half of the wells were stimulated with 1 µg/mL LPS the next morning, and supernatants were collected at 6 and 24 hours to quantify TNF-α and IL-10 by ELISA.

For additional experiments, passage 5 MSCs from two donors were pooled and seeded at 3–4 × 10^4 cells per well in 6-well UpCell™ plates (Nunc™, ThermoFisher Scientific). After overnight incubation under standard conditions, MSC medium was replaced with 3 mL of macrophage medium, either with 3–4 × 10^5 BAL cells or without them as a control. Plates were incubated for 16 hours at 37 °C, 5% CO_2, and 20% O_2.

Osteogenic and adipogenic differentiation

To confirm the multipotent nature of MSCs, in vitro differentiation assays were conducted following established protocols [26]. MSCs were plated in 6-well plates and maintained in either osteogenic or adipogenic differentiation media for 16 days. At the end of the culture period, cells were fixed and stained with Alizarin Red to detect calcium deposits for osteogenesis or with Oil Red O to visualize lipid accumulation for adipogenesis. Representative images are provided in Figure S1.

ELISA

Initial pilot studies were performed to determine optimal MSC-to-BAL cell ratios and appropriate time points for cytokine measurement. After co-culture, plates were centrifuged, and supernatants were collected into low-binding plates for immediate ELISA analysis or stored at −20 °C. To avoid exceeding the ELISA standard range, supernatants were diluted 1:5.

Cytokine levels of human IL-10 and TNF-α were measured using DuoSet ELISA kits (DY217B, DY210, DY417, DY410; R&D Systems) according to the manufacturer's protocol. Absorbance at 450 nm was read using a Turner BioSystems Modulus Microplate Reader with TMB substrate. Samples exceeding the top standard or showing unusually high variability among replicates were reanalyzed.

Flow cytometry

For immunophenotyping, a panel of antibodies was utilized, including anti-CD68 (APC), anti-CD11c (PE and PerCP-Cy5.5), anti-CD80 (PE-Cy5), anti-CD163 (Brilliant Violet 711), anti-CD206 (APC/Fire 750), anti-TNF-α (Brilliant Violet 785), and anti-IL-10 (PE) (BioLegend, San Diego, CA, USA).

Surface staining was performed under non-enzymatic conditions to preserve sensitive M2 markers. Cells were gently detached by placing plates at 4 °C for 30 minutes, followed by careful pipetting. Fc receptor-mediated nonspecific binding was blocked using Human TruStain FcX (BioLegend) for 10 minutes at room temperature. Cells were then incubated with surface antibodies in the dark at 4 °C for 20 minutes and washed three times with PBS containing 5% FBS. Cell viability was assessed using DAPI staining. Single-stain compensation controls were prepared with UltraComp eBeads (Invitrogen, Waltham, MA, USA). Data acquisition was conducted immediately on a BD LSRFortessa (BD Biosciences, San Jose, CA, USA), and analysis was performed with FlowJo software (v10.5.3).

For intracellular cytokine detection, Monensin and Brefeldin A were evaluated, and Monensin was chosen as the most effective inhibitor. It was added to cultures at a final concentration of 2 μM four hours prior to harvesting to block cytokine secretion. Cells were stained for viability with Zombie UV™ and Fc receptors were blocked before applying surface antibodies as above. After staining, cells were fixed at room temperature for 20 minutes and permeabilized using the Intracellular Fixation and Permeabilization Buffer Set (eBioscience). Antibodies specific to TNF-α and IL-10 were then applied for 30 minutes at 4 °C in the dark, followed by washes. Single-stain controls for compensation were prepared in parallel, and FMO controls were used to set gating parameters.

Results

In preliminary experiments, we observed that cytokine modulation increased proportionally with the number of MSCs present in the culture (Figure S2). A co-culture ratio of one MSC per ten BAL mononuclear cells produced robust and reproducible changes in TNF-α and IL-10 levels and was therefore used throughout the study.

Seventeen patients with biopsy-confirmed sarcoidosis and compatible clinical presentations were enrolled. Most subjects were receiving little or no anti-inflammatory therapy at the time of BAL collection **(Table 1)**. All Scadding stages were represented in the cohort: Stage 0 (3 patients, 17.6 percent), Stage I (3 patients, 17.6 percent), Stage II (1 patient, 5.9 percent), Stage III (6 patients, 35.3 percent), and Stage IV (4 patients, 23.5 percent). Eight healthy adults served as control participants. Compared to controls, sarcoidosis patients exhibited lower mean values of forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and diffusion capacity adjusted for hemoglobin (DLCO adj.) (Table S1). Additionally, sarcoidosis subjects had higher serum ACE levels and peripheral monocyte counts (Tables S2 and S3). While peripheral lymphocyte numbers were similar between groups, BAL fluid from sarcoidosis patients contained a significantly higher proportion of lymphocytes **(Table 2)**.

Table 1. Demographics and Basic Clinical Features

Parameter	Controls (*n* = 7) Mean Value	Sarcoidosis (*n* = 15) Mean Value	*p*-Value
Total Participants			
Female (number)/(%)	4/(50%)	11/(73.3%)	NS
Age (years)	45.4	53.1	NS
Race			
Black (number)/(%)	5/(62.5)	8/(53.3)	NS

White (number)/(%)	2/(25)	5/(33.3)	NS
Asian (number)/(%)	1/(12.5)	0	NS
Multiracial (number)/(%)	0	2/(13.3)	NS
Height (cm), (SD)	174, (0.91)	168, (0.36)	NS
Modification of MRC Dyspnea Scale	0	0.9, (1.36)	0.027
Inhaled Steroid (number/(%)), (SD)	0	5/(33.3%), (0.49)	0.019
Prednisone (number / (%)), (SD)	0	2/(20%), (0.35)	NS

Abbreviations: Medical Research Council (MRC). SD: standard deviation. NS: not significant.

Table 2. Bronchoalveolar Lavage Cell Counts

Parameter	Controls (n = 7)		Sarcoidosis (n = 15)		p-Value
	Mean Value	SD	Mean Value	SD	
BAL Cell count (× 10^7)	21	9.7	22.6	14.8	NS
BAL Lymphocytes (%)	6.57	6.4	16.96	12.5	0.02
BAL Macrophage (%)	80.25	33.2	70.21	23.7	NS
BALF % return (%)	48.6	8.2	51.67	12.2	NS
Viability of cells (%)	80.57	15.7	84.6	8.0	NS

Abbreviation: NS: not significant.

ELISA assays were used to evaluate cytokine release (TNF-α and IL-10) from BAL cells cultured either alone or together with human MSCs. Culture supernatants were collected at 6 and 24 hours following LPS stimulation of MSC–BAL cell co-cultures obtained from both sarcoidosis patients and healthy controls. Analysis of these samples revealed that BAL cells derived from sarcoidosis patients exhibited a marked reduction in TNF-α secretion ($p = 0.029$) along with a significant rise in IL-10 levels ($p = 0.011$), whereas BAL cells from controls did not show these alterations **(Figure 1A,B)**. These findings suggest a phenotypic transition of sarcoidosis BAL cells from a pro-inflammatory M1 profile toward an anti-inflammatory M2 state.

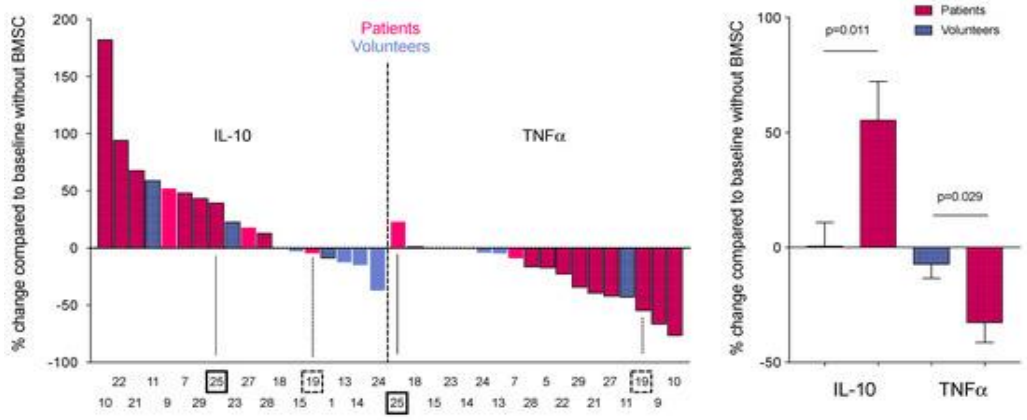

Figure 1. Percent changes in cytokine output from co-cultures of bone marrow stromal cells (MSCs) and bronchoalveolar lavage (BAL) cells following lipopolysaccharide (LPS) stimulation. (A) IL-10 (left) and TNF-α (right) concentrations were quantified from culture supernatants of MSC–BAL co-cultures derived from sarcoidosis patients (red) and healthy controls (blue). Among the 11 samples tested, two sarcoidosis cases (19 and 25) deviated from the overall pattern in either IL-10 or TNF-α levels, as indicated by arrows and asterisks. In contrast, BAL cells from control subjects exhibited minimal changes without a consistent pattern. (B) The average percentage changes for IL-10 (anti-inflammatory) and TNF-α (pro-inflammatory) are presented, showing that BAL cells from sarcoidosis cases consistently shifted toward an anti-inflammatory profile (increased IL-10 coupled with reduced TNF-α), whereas control samples showed no such trend (n = 9 sarcoidosis; n = 7 controls; unpaired Student's t-test; values shown as mean ± SEM; $p = 0.011$ for IL-10 and $p = 0.029$ for TNF-α)

Cytokine release was also assessed in co-cultures not exposed to LPS. In these cultures, IL-10 and TNF-α levels were absent or barely detectable. MSCs cultured alone failed to secrete measurable amounts of either cytokine regardless of LPS exposure, confirming that the cytokines originated from BAL cells.

To further validate this and identify the responsible cell type, intracellular IL-10 and TNF-α expression was examined in alveolar macrophages (AMs) using flow cytometry, along with macrophage-associated surface markers. The sensitivity of cytokine detection in these cells eliminated the need for LPS stimulation. BAL samples from two additional sarcoidosis patients were analyzed: subject 16, who displayed greater clinical symptoms and impaired function, suggestive of active disease (Table S4), and subject 17, who had quiescent disease with fewer AMs and a reduced CD4+/CD8+ ratio (Tables S4 and S5). BAL cells from these subjects were

cultured with and without MSCs for 16 hours, harvested, and analyzed by flow cytometry, focusing on CD206+ cells (identified as AMs [27, 28]).

In subject 16, co-culture with MSCs doubled the proportion of IL-10–producing AMs (22.1% vs. 11.6%) **(Figure 2A, B)** while simultaneously reducing TNF-α production (2.27% vs. 1.07%) **(Figure 2C, D)**, yielding a fourfold rise in the IL-10/TNF-α ratio **(Figure 3)**. AMs from subject 17, however, were less responsive to MSCs: IL-10 production more than doubled (3.05 percent to 8.67percent) **(Figure 4A, B)**, but TNF-α showed a slight increase (0.94 percent to 1.25 percent) **(Figure 4C, D)**, resulting in only a twofold increase in the IL-10/TNF-α ratio **(Figure 3B)**.

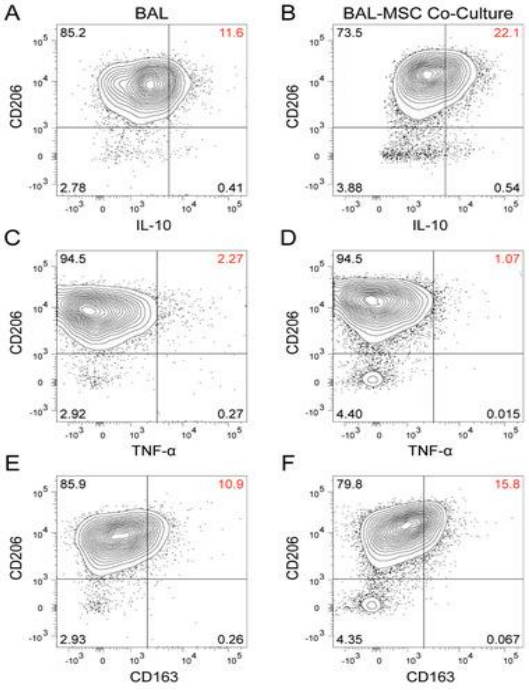

Figure 2. Flow cytometry data from mononuclear cells (approximately 90% macrophages) freshly isolated from a sarcoidosis patient (subject 16). BAL cells were cultured for 16 hours either alone (A,C,E) or together with bone marrow stromal cells (MSCs) (B,D,F). Alveolar macrophages (AMs) were identified using the surface marker CD206, and intracellular staining was used to evaluate cytokine expression. When BAL cells were co-cultured with MSCs, AMs displayed higher IL-10 production (A,B) and reduced TNF-α expression (C,D). Additionally, levels of CD163, a surface marker associated with anti-inflammatory activity, were elevated (E,F). Collectively, these observations indicate that MSC interaction drives AMs away from a pro-inflammatory phenotype and toward a more anti-inflammatory profile

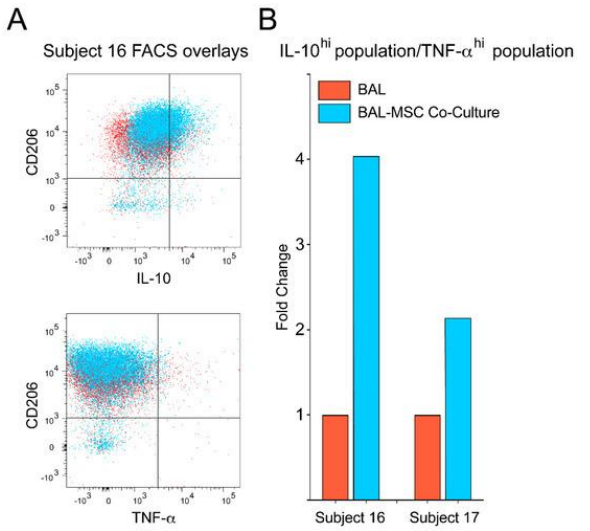

Figure 3. Intracellular cytokine ratios in alveolar macrophages (AMs), highlighting a transition from an M1-like pro-inflammatory profile toward an M2-like anti-inflammatory phenotype after co-culture with bone marrow stromal cells (MSCs). (A) BAL cells alone are shown in red, whereas BAL cells from MSC co-cultures are shown in blue. The co-cultured cells exhibit a rightward shift, reflecting higher mean fluorescent intensity (MFI) of IL-10+ cells, along with an upward shift, corresponding to increased CD206 expression. Because relatively few cells produced TNF-α, a leftward displacement was less noticeable, although an upward shift in CD206 expression remained apparent. (B) The fold-change in the IL-10/TNF-α ratio is presented for both sarcoidosis cases (subjects 16 and 17). BAL cells grown without MSCs are shown as red bars (baseline ratio set to 1), while BAL cells cultured with MSCs are shown as blue bars, representing the fold increase relative to baseline. The data demonstrate that, in both subjects, BAL cells adopted a more anti-inflammatory profile, though the magnitude of the shift varied between individuals

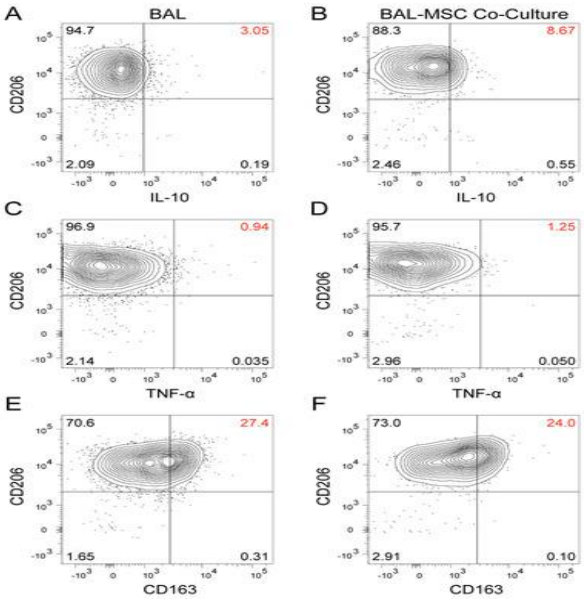

Figure 4. Flow cytometric evaluation was carried out on freshly isolated mononuclear cells obtained from a sarcoidosis case (subject 17), where macrophages represented about 73% of the population. Bronchoalveolar lavage (BAL) cells were plated and incubated for 16 hours either in the presence (B, D, F) or absence (A, C, E) of bone marrow stromal cells (MSCs). Alveolar macrophages (AMs) were identified using the surface marker CD206, and intracellular staining was employed to assess cytokine output. When BAL cells were co-cultured with MSCs, IL-10 levels nearly tripled (A, B), while TNF-α secretion showed minimal change (C, D). Interestingly, the surface marker CD163, often associated with an anti-inflammatory phenotype, was reduced under these conditions (E, F). Collectively, these observations imply that MSC interaction may promote a reorientation of AMs from a pro-inflammatory profile toward one with more anti-inflammatory properties

In agreement with earlier studies [27, 28], our findings further confirmed that CD206 was broadly expressed among AMs in sarcoidosis subjects **(Figure 2 and Figure 4)**. The fluorescence intensity of CD206 rose consistently after MSC co-culture in both individuals tested **(Figure 2, Figure 3A, and Figure 4)**. For CD163, which is typically considered an M2 macrophage indicator, divergent trends were seen: subject 16 exhibited an increase (10.9% to 15.8%) **(Figure 2E, F)**, whereas subject 17 showed a decline (27.4% to 24%) **(Figure 4E, F)**. Notably, no heterogeneity of CD163 expression was detected in either subject **(Figure 2E, F; Figure 4E, F)**.

With respect to M1 macrophage-associated markers, subject 16 demonstrated marked increases in CD11c (25.3% to 40.1%) and CD80 (20.9% to 41.1%) upon MSC co-culture (Figure S3). Conversely, in subject 17, these markers slightly declined—CD11c from 56.9% to 55.2% and CD80 from 49.8% to 41.9%—relative to AMs cultured alone (Figure S4). Comparable trends were observed in both fixed and unfixed preparations.

Discussion

This work provides evidence that alveolar macrophages derived from an inflammatory setting, such as sarcoidosis, undergo phenotypic alterations when exposed to MSCs, adopting traits consistent with an anti-inflammatory state. While MSC-mediated modulation of macrophages has been documented in other tissue contexts, the observation that AMs from sarcoidosis patients display this responsiveness highlights its potential clinical implications, supporting the idea that MSC-based cellular therapy should be investigated further.

Our earlier studies established that the inflammatory context critically shapes the immunomodulatory behavior of MSCs. Soluble inflammatory mediators—including cytokines, prostaglandins, and nitric oxide—can enhance MSC immunosuppressive activity by stimulating the release of regulatory factors [14]. AMs obtained from sarcoidosis patients are typically activated and characterized by elevated TNF-α secretion compared with those from healthy controls [29]. When MSCs encounter this sarcoidosis-associated inflammatory environment, as opposed to a balanced control milieu, they appear more effective at driving AMs toward an anti-inflammatory phenotype. This interaction is likely to result in reduced macrophage-derived TNF-α and an enhanced production of IL-10.

Bone marrow stromal cells are recognized for their broad immunomodulatory effects, one of which is the ability to reprogram macrophages. Through this process, MSCs shift macrophages from an activated, pro-inflammatory phenotype (M1) toward a more regulatory, anti-inflammatory phenotype (M2) [14]. This conversion depends on the COX-2 pathway in MSCs, which boosts the release of PGE2. The PGE2 molecule then binds to EP2 and EP4 receptors on macrophages, triggering enhanced secretion of IL-10. Beyond influencing macrophages, MSCs can dampen T-cell responses and promote immune tolerance by interfering with dendritic cell maturation [30], a process thought to involve STAT-3 signaling and direct cell-to-cell interactions [31]. Based on this evidence, we hypothesized that MSCs might be capable of reducing pulmonary inflammation in sarcoidosis.

Sarcoidosis is a systemic disorder marked by the presence of noncaseating granulomas within a Th1/Th17-driven cytokine milieu, characterized by elevated IL-2, IL-12, IL-17, TNF-α, and IFN-γ [24]. Therapeutic strategies under consideration include TNF-α neutralizing antibodies and other inhibitors of pro-inflammatory pathways [32]. A small phase I clinical study tested intravenous administration of placental mesenchymal-like cells in four patients with chronic stage II–III sarcoidosis. Each patient received two infusions in one week and was followed for two years. Remarkably, two patients discontinued prednisone use and showed notable radiographic improvement [33].

Since no suitable animal model exists, we pursued this question using an “ex-vivo” approach. Primary BAL cells from patients were cultured with and without healthy human MSCs for a short period to simulate their interaction. We then assessed both cytokine output and surface marker profiles of the alveolar macrophages (AMs) [34].

While cytokine measurements revealed that IL-10 increased and TNF-α decreased in the presence of MSCs **(Figure 1B, Figure 3B)**-suggesting a shift toward an anti-inflammatory phenotype-the surface marker expression

did not fully match a classic M2 profile (Suppl. Figures S2A–D, S3A–D). This discrepancy could be due to different timing in cytokine secretion versus marker expression. Recent reports argue that AMs may not conform strictly to M1 or M2 phenotypes but instead adopt hybrid features [27, 28]. Thus, cytokine data may offer a more reliable indication of AM inflammatory status, and the conventional M1/M2 framework may need reevaluation for this macrophage subtype [35].

Our ELISA analysis of co-culture supernatants showed two sarcoidosis cases that deviated from the general trend of reduced TNF-α and elevated IL-10. In one subject (19 in **Figure 1**), both cytokines decreased after BAL cells were co-cultured with MSCs in the presence of LPS, likely influenced by an active sinus infection at the time of BAL sampling. In another subject (25 in **Figure 1**), TNF-α rose modestly while IL-10 increased more substantially. This individual had continued steroid therapy, unlike the other participants. Even in these atypical cases, the overall balance still leaned toward an anti-inflammatory effect, with the ratio shifting in favor of IL-10 over TNF-α. Flow cytometry from one of these two patients revealed a similar pattern: a small elevation in TNF-α paired with a larger IL-10 increase, consistent with clinically inactive disease at that time. These findings suggest that MSCs may require macrophage-derived pro-inflammatory cues to maximize their modulatory effects [36].

Recent reviews have examined the use of MSCs in treating pulmonary disorders [37, 38]. We proposed that MSC therapy, delivered locally through the airways, could reduce lung inflammation in patients with sarcoidosis. The route by which MSCs are administered may influence therapeutic outcomes and determine the molecular pathways activated for immunomodulation. For airway diseases like sarcoidosis, MSCs can be delivered intravenously or directly into the bronchial tree. In rodent models, intravenously infused MSCs are largely trapped within the pulmonary vasculature, resulting in a high concentration in the lungs [39]. In humans, however, MSCs transiently localize to the lungs after intravenous infusion before redistributing rapidly to organs of the reticuloendothelial system, particularly the liver and spleen [40]. Intravascular delivery triggers the instant-blood-mediated inflammatory reaction (IBMIR), a complex inflammatory cascade that ultimately clears both living and apoptotic MSCs from the capillaries, potentially inducing a sustained anti-inflammatory state mediated by monocytes and macrophages [41,42].

In contrast, intrabronchial delivery bypasses IBMIR and allows MSCs to interact directly with alveolar inflammatory cells, including macrophages and lymphocytes. Although it remains unclear which method provides superior efficacy, intrabronchial administration may achieve a more concentrated effect within the lungs, potentially enhancing therapeutic outcomes while requiring fewer cells and minimizing systemic, IBMIR-related thrombo-inflammatory risks [41].

Our findings support further investigation of MSCs as a therapy for pulmonary sarcoidosis, given their potential to reduce inflammation and possibly decrease steroid dependence. MSCs have been employed in clinical studies for over a decade. While outcomes vary depending on the disease context and trial design, the overall consensus is that MSC therapy is safe [9, 43, 44]. A pilot study in preterm infants demonstrated that intratracheal MSC delivery is feasible and free from major adverse events [45]. Endobronchial MSC administration is currently under evaluation in idiopathic pulmonary fibrosis (NCT01919827), with results pending. Phase 1 trials using MSCs derived from bone marrow [46], placenta [47], and adipose tissue [48] have shown a safe profile, without evidence of treatment-induced fibrosis. Although idiopathic pulmonary fibrosis differs from sarcoidosis, these findings are relevant, as M2 macrophages have been implicated in promoting pulmonary fibrosis [49]. The net effect of MSCs on fibrosis in humans remains uncertain, though animal studies suggest both pro- and antifibrotic potential [50].

In mouse models, macrophage origin appears critical for fibrosis development [51]. Thus, targeting specific macrophage subsets to modify their profibrotic behavior or limit lung infiltration could be a promising strategy. However, the absence of a sarcoidosis-specific mouse model limits direct translation of these findings to patients. Several approaches have been explored to improve MSC delivery in respiratory disease [52]. For instance, Kardia *et al.* [53] used fibroblast-like cells from rabbits to show that cells cultured in vitro can be aerosolized without detectable stress, with most cells surviving and proliferating. More recent work indicates that MSCs maintain higher viability when delivered via compressed nebulization compared with ultrasonic or mesh devices [54]. Beyond delivery technique, other variables such as dosing and timing also require optimization. One advantage of MSCs is their ability to be banked in frozen aliquots. While some concerns exist regarding potential loss of efficacy after cryopreservation [55], in many clinical contexts this may not pose a major issue [56]. Because freezing may reduce MSC immunomodulatory activity [44], our study utilized freshly trypsinized MSCs. The extent to which cryopreservation alters MSC effects on alveolar macrophages remains unknown and will need to be clarified in future clinical trials.

Considering the established safety profile of MSC therapy and its effectiveness in counteracting inflammation, MSCs could provide a steroid-sparing approach or potentially replace systemic steroids in the management of sarcoidosis.

Supplementary Materials

The following are available online at https://www.mdpi.com/2077-0383/9/1/278/s1. Figure S1: In vitro differentiation of MSCs; Figure S2: Optimization of coculture cell ratios; Figure S3: Flow cytometry analysis of M1 and M2 macrophage markers in subject 16; Figure S4: Flow cytometry analysis of M1 and M2 markers in subject 17. Table S1: Pulmonary functional results; Table S2: Laboratory results; Table S3: Peripheral blood lymphocyte phenotype; Table S4: Summary of clinical data of subjects 16 and 17; Table S5: BAL counts and peripheral blood lymphocyte phenotype in subjects 16 and 17.

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