Lung Resident Mesenchymal Stem Cells Isolated from Human Lung Allografts Inhibit T Cell Proliferation via a Soluble Mediator

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Development of allograft rejection continues to be the major determinant of morbidity and mortality postlung transplantation. We have recently demonstrated that a population of donor-derived mesenchymal stem cells is present in human lung allografts and can be isolated and expanded ex vivo. In this study, we investigated the impact of lung resident mesenchymal stem cells (LR-MSCs), derived from allografts of human lung transplant recipients, on T cell activation in vitro. Similar to bone marrow-derived MSCs, LR-MSCs did not express MHC II or the costimulatory molecules CD80 or CD86. In vitro, LR-MSCs profoundly suppressed the proliferative capacity of T cells in response to a mitogenic or an allogeneic stimulus. The immunosuppressive function of LR-MSCs was also noted in the absence of direct cell contact, indicating that LR-MSCs mediated their effect predominantly via a soluble mediator. LR-MSCs isolated from lung transplant recipients demonstrated PGE2 secretion at baseline (385 ± 375 pg/ml), which increased in response to IL-1β (1149 ± 1081 pg/ml). The addition of PG synthesis inhibitors (indomethacin and NS-398) substantially abrogated LR-MSC-mediated immunosuppression, indicating that PGE2 may be one of the major soluble mediators impacting T cell activity. This is the first report to demonstrate that human tissue-derived MSCs isolated from an allogeneic environment have the potential to mediate immunological responses in vitro. The Journal of Immunology, 2008, 181: 4389–4396.

Materials and Methods

Isolation and identification of lung-derived MSCs

Mesenchymal stem cells were obtained from BAL of lung transplant recipients as previously described under a protocol approved by the Institutional Review Board of the University of Michigan Health Care System.
Biotec) according to manufacturer’s instructions. LR-MSCs/well (2 × 10^5; irradiated 30 Gy) were plated into 96-well, flat-bottom plates in complete DMEM 1640 supplemented with 10% FCS 4 h before the addition of 2 × 10^5 responder cells/well (Pan T cells derived from healthy volunteers). Cells were cocultured for 5 days with the addition of [^3]H]thymidine in the last 18 h of culture. Values represent thymidine uptake by proliferating cells (average cpm). No significant difference was noted in the T cell vs T cell populations isolated from five healthy volunteers. For allogeneic stimulation, 2 × 10^5 responder T cells were incubated with variable dilutions of MSC:T cells in a 96-well plate 4 h before the addition of 2 × 10^5 responder T cells (Pan T cells). Data were presented as a percentage of the T cell proliferation determined in the absence of LR-MSCs (100%). Irradiated MSCs (30 Gy) were plated at 1/10, 1/50, 1/100, 1/500, and 1/1000 dilution of MSC:T cells in a 96-well plate 4 h before the addition of 2 × 10^5 Pan T cells (constant in all wells) and PHA. Data represent mean ± SEM of 10 separate experiments.

PBMC isolation

PBMCs from healthy volunteers were fractionated by density centrifugation using Lymphoprep gradient (Axis-Shield). Theuffy coat was isolated and subpopulations of T cells (Pan T cell, CD4^+, CD4^+CD25^+, and CD8^+) were purified using the relevant magnetic MicroBead kits (Miltenyi Biotec) according to manufacturer’s instructions.

Proliferation assays and cytokine measurement

LR-MSCs (2 × 10^5; 30 Gy irradiated) were plated per well in a 96-well flat-bottom culture-treated plates 4 h before the addition of Pan T cells or subpopulations of T cells (2 × 10^5 cells/well) in complete DMEM supplemented with 10% FCS. For mitogenic stimulation, the cultures were stimulated with PHA (12.5 μg/ml; Sigma-Aldrich) for 5 days. Eighteen hours before harvest, [^3]H]thymidine (1 μCi) was added.[^3]H]Thymidine incorporation is expressed as the mean of triplicates in cpm. For dose-response experiments, 2 × 10^5 responder T cells were incubated with various numbers of MSCs. For allogeneic stimulation, 2 × 10^5 MSCs/well were plated in 96-well, round-bottom, culture-treated plates 4 h before the addition of the stimulator (1 × 10^5 PBMC/well; 15 Gy irradiated) and 1 × 10^5/10^6 cells/well of third party responder PBMCs or responder Pan T cells. Cells were cocultured in complete RPMI 1640 supplemented with 10% FCS for 8 days with the addition of [^3]H]thymidine for the last 18 h of culture. To mimic APCs, CD28/CD3/CD2 Ab-coated beads (Miltenyi Biotec) were used to induce proliferation according to the manufacturer’s instructions at a ratio of 1:2 bead to T cell. The cells were cultured in the presence and absence of LR-MSCs and assessed for proliferation 3 days later. For cytokine measurement, third party 30 Gy-irradiated MSCs (8 × 10^5 MSC/well) were plated into 24-well, flat-bottom plates in DMEM 4 h before the addition of 4 × 10^5 responder T cells/well in the presence or absence CD28/CD3/CD2 Ab-coated beads. After 72 h, cell-free supernatants were collected and analyzed for IL-2 and IL-10 levels using a highly specific enzyme immunoassay technique (eBioscience and R&D Systems, respectively).

Transwell experiments

LR-MSCs (30 Gy irradiated) (2 × 10^5 cells/well) were plated in the upper chamber of a 96-well Transwell plate with a 0.5-μm pore size membrane (Corning) 4 h before the addition of Pan T cells (2 × 10^5 cells/well) to the lower chamber. Cultures were stimulated by the addition of 12.5 μg/ml PHA and [^3]H]thymidine was added for the last 18 h of culture.

Determination of PGE2 synthesis and immunoblot analysis

To measure PGE2 in BAL, lipids were extracted from BAL fluid samples via solid phase extraction using Sep-Pak C-18 cartridges (Waters). Eluted sample was reconstituted in antibody buffer and PGE2 was quantified in using a highly sensitive and specific enzyme immunoassay kit from Cayman Chemicals according to the manufacturer’s suggestions. For measurement of PGE2 synthesis by LR-MSCs, LR-MSCs were cultured in the absence or presence of IL-1β (10 ng/ml) for 24 h. PGE2 was quantified in the supernatant by ELISA. In separate experiments, protein was isolated and cyclooxygenase (COX)-1 and COX-2 proteins were analyzed by Western blotting using antibodies to COX-1 and COX-2.

FIGURE 1. A, Lung-derived MSCs do not elicit a Pan T cell immunological response in vitro. LR-MSCs/well (2 × 10^5; irradiated 30 Gy) were plated into 96-well, flat-bottom plates in complete DMEM 1640 supplemented with 10% FCS 4 h before the addition of 2 × 10^5 responder cells/well (Pan T cells derived from healthy volunteers). Cells were cocultured for 5 days with the addition of[^3]H]thymidine in the last 18 h of culture. Values represent thymidine uptake by proliferating cells (average cpm). No significant difference was noted in the T cell vs T + LR-MSC group (p = 0.53). Data represent the mean ± SEM of 10 separate experiments with LR-MSCs derived from 10 lung transplant recipients and peripheral Pan T cells purified from five healthy volunteers. B, Immunophenotyping of LR-MSCs by flow cytometric analysis demonstrates absence of costimulatory molecules CD80 and CD86. Histograms show specific mAbs in purple and control isotype-specific IgGs in green.

(18) BAL samples were used only if a bronchoscopy was performed for routine surveillance within 6 mo of transplantation with no evidence of acute rejection or infection on transbronchial biopsies and microbiological cultures, respectively. Cells were maintained in culture in DMEM with penicillin/streptomycin and 10% FCS at 37°C in 5% CO2 and used at passages 2–6. LR-MSCs obtained from individual BAL samples were treated as separate cell lines.

PBMC isolation

PBMCs from healthy volunteers were fractionated by density centrifugation using Lymphoprep gradient (Axis-Shield). Theuffy coat was isolated and subpopulations of T cells (Pan T cell, CD4^+, CD4^+CD25^+, and CD8^+) were purified using the relevant magnetic MicroBead kits (Miltenyi Biotec) according to manufacturer’s instructions.
FIGURE 3. Lung-derived MSCs can inhibit the proliferative response of subpopulations of T cells and do not require preexisting Tregs. A and B, LR-MSCs/well (2 × 10^5; irradiated 30 Gy) were plated into 96-well, flat-bottom plates 4 h before the addition of 2 × 10^5 responder cells/well (CD4^- (A) or CD8^- T cells (B)) in the presence (+) or absence (-) of PHA (12.5 μg/ml). The cells were cultured for 5 days with the addition of [3H]thymidine in the last 18 h of culture. Values represent [3H]thymidine uptake by proliferating cells (average cpm). Data represent the mean ± SEM of experiments with LR-MSCs derived from three lung transplant recipients; C, CD4^+CD25^- T cells were depleted using a Miltenyi Biotec CD4^+CD25^- isolation kit. The CD4^+CD25^- T cells were plated as described for CD4^+T cells. Data represent the mean ± SEM of experiments with LR-MSCs derived from three lung transplant recipients. *, p < 0.05 vs T cells stimulated with PHA in the absence of LR-MSCs.

Statistical analysis

Data are presented as mean values ± SEM. Statistical significance was analyzed using the GraphPad Prism 3 software. Significance was assessed with a Student’s t test for comparisons of two groups or with ANOVA and a post hoc Bonferroni test for three or more groups, p < 0.05 was considered significant.

Results

LR-MSCs derived from human lung allografts fail to stimulate allogeneic T cells

We have previously demonstrated that donor LR-MSCs can be isolated as late as 11 years postlung transplantation, clearly indicating that these cells can survive in an allogeneic environment (18). Because donor LR-MSCs are exposed to recipient circulating cells, we first investigated whether LR-MSCs cultured with third party T cells lead to T cell proliferation in vitro. No proliferation was noted when Pan T cells were cultured with irradiated allogeneic LR-MSCs, 2,125 ± 178 cpm and 2,311 ± 211.9 cpm mean T cell [3H]thymidine incorporation in the absence and presence of MSCs, respectively; p = 0.53; Fig. 1A). Similar results were noted when LR-MSCs were cocultured with peripheral blood PBMCs and a subpopulation of T cells (CD4 and CD8 T cells; data not shown). We have previously demonstrated that LR-MSCs express HLA-I but not HLA II (DR or DQ) (18). Examination of costimulatory molecules demonstrated that, in addition, LR-MSCs do not express CD80 or CD86, indicating that they do not function as classical APCs (Fig. 1B).

LR-MSCs inhibit T cell proliferation in response to mitogen stimulation in vitro in a dose-dependent manner

Because LR-MSCs were not immunogenic, we next sought to determine whether, similarly as BM-MSCs, they had an immunosuppressive impact on lymphocytes in response to mitogenic stimulation in vitro. LR-MSCs were cocultured for 4 days with peripheral blood T cells of third party healthy volunteers in the presence of PHA at a ratio of 1:10 LR-MSCs to T cells. Upon examination of the coculture under light microscopy, we observed that wells containing T cells alone in the presence of PHA displayed typical T cell clustering, indicative of a robust proliferative response. In contrast, wells containing a monolayer of LR-MSCs in addition to T cells and PHA lacked T cell clustering, with the T cells appearing to be distributed singularly on top of the LR-MSC monolayer. Additionally, T cells in the coculture appeared alive and healthy, and their viability was confirmed by trypan blue exclusion (not shown). Upon quantitative analysis obtained by [3H]thymidine incorporation, there was a significant reduction in PHA-induced T cell proliferation in the presence of LR-MSCs (84 ± 7%; p = 0.0001; Fig. 2A and B). Lower inhibition was noted with a decreasing ratio of LR-MSCs to responder T cells (Fig. 2C).

LR-MSCs inhibit both CD4 and CD8 T cell populations in vitro and do this independently of preexisting regulatory T cell (Treg) populations

Because both CD4^+ and CD8^+ T cells have been shown to mediate allograft rejection, we aimed to determine whether LR-MSCs can block T cell proliferation of these individual cell populations in vitro. Both CD4^+ and CD8^+ T cells stimulated by PHA exhibited a markedly decreased proliferative capacity when cocultured with LR-MSCs, with [3H]thymidine incorporation reduced to unstimulated baseline levels (p < 0.001 and 0.002, respectively; Fig. 3, A and B). To determine whether LR-MSCs require the presence of preexisting CD4^+CD25^- regulatory T cells to mediate this effect, peripheral blood CD4^+ T cells populations were depleted of CD4^-CD25^- T cells, cocultured with LR-MSCs, and stimulated with PHA as previously described. LR-MSCs significantly inhibited the proliferation of the CD4^-CD25^- population, indicating...
that preexisting regulatory T cells are not necessary for LR-MSCs to inhibit T cell activation in vitro \( (p < 0.001; \text{Fig. 3C}) \).

**LR-MSCs suppress T cell proliferation in response to allogeneic stimuli**

Although LR-MSCs were effective at regulating T cell activation in response to mitogenic stimulation, allogeneic stimulation may be more relevant to the transplanted lung. To examine this in vitro, we performed a MLR assay in which T cell-depleted and irradiated PBMCs were used to stimulate allogeneic Pan T cells in the presence or absence of third party MSCs (LR-MSCs). The cells were cultured for 8 days with addition of \(^{3}H\)thymidine in the last 18 h of culture. Values represent thymidine uptake by proliferating cells (average cpm). Data represent the mean ± SEM of experiments with LR-MSCs derived from five lung transplant recipients. \( *, p < 0.001 \) vs T cells; \( **, p < 0.001 \) vs T cells plus PBMC or beads.

**Soluble mediators play an important role in mediating T cell suppression by LR-MSC**

To determine whether MSCs require direct contact with effector T cells to abrogate their proliferative response, we cultured the two populations in a Transwell apparatus that separated the two populations by a porous membrane. In the absence of direct cell contact, LR-MSCs were still able to inhibit T cell proliferation in response to PHA, albeit to a lesser extent (T cell proliferation was reduced by 58 ± 10%; \( Fig. 5A \)). This inhibition was lower than what was seen with direct cell-cell contact (inhibition of 84 ± 7%). Transwell experiments were repeated with the use of both irradiated and nonirradiated LR-MSCs \( (n = 3) \), and no significant differences were noted \( (p = 0.33; \text{Fig. 5B}) \). These data suggest that
soluble mediators are important for the T cell-inhibitory affects of LR-MSCs but that direct cell contact further facilitates the suppressive ability of LR-MSCs.

Prostanoid production by LR-MSCs contributes to their immunosuppressive potential. A, LR-MSCs demonstrate PGE₂ synthetic capacity. LR-MSCs isolated from BAL of 15 lung transplant recipients were cultured in the presence (+) or absence (−) of IL-1β (10 ng/ml) for 24 h before cell-free supernatants were collected and analyzed for PGE₂ levels using a highly specific enzyme immunoassay technique. Data represent mean ± SEM. B, LR-MSCs cultured in the presence (+) or absence (−) of IL-1β were scraped into lysis buffer and analyzed by Western blotting for the expression of COX-2. COX-2 was expressed at baseline and the expression was increased in the presence of IL-1β (*, p < 0.05). C and D, Exogenous PGE₂ inhibits T cell proliferation in response to both mitogenic (C) and allogeneic (D) stimulation. E, LR-MSC mediated T cell suppression is reversed by inhibitors of PG synthesis. Irradiated LR-MSCs were cocultured with PHA-stimulated T cells in the presence (+) of indomethacin (10 μM) or NS398 (5 μM) (*, p < 0.05 compared with T cells plus PHA). The presence of indomethacin and NS398 significantly reversed suppression of T cell proliferation by LR-MSCs (*, p < 0.05 compared with T cells plus LR-MSC; n = 4 experiments).
effect was demonstrated in the context of both mitogen and alloantigen stimulation and across CD4 and CD8 T cell populations and did not require the presence of preexisting CD4^+CD25^+ T cells. LR-MSC-mediated suppression of T cells was partially contact dependent and largely explained by the actions of a soluble mediator. COX inhibitors blocked the immunosuppressive potential of LR-MSCs on T cells, suggesting that PGE_2 may be a major mediator of the immunomodulatory effects of LR-MSC in vitro. This is the first report demonstrating that MSCs isolated from a transplanted lung have immunosuppressive capacity in vitro, a finding that contributes to our understanding of the immunosuppressive potential of tissue resident MSCs.

Transplantation offers the only definitive therapy for a variety of end stage lung diseases. However, long-term outcomes for lung transplant recipients are poor, with a 10-year lung survival rate of only 26% (25). A major challenge in lung transplantation continues to be allograft rejection orchestrated by recipient-derived-inflammatory cells, primarily T cells, which infiltrate the graft during acute rejection (1). Recipient-derived T lymphocytes also play a seminal role in the pathogenesis of BOS. Lymphocytic bronchitis, a condition characterized by lymphocytic infiltration of the small airways, is considered to be a harbinger of BOS in humans (26) and precedes the development of luminal obliteration in animal models of tracheal transplantation (27–29). Although host responses to allograft are very well characterized, the role of graft-derived cells in a transplant milieu remains to be elucidated. Our study suggests that resident donor-derived cells might have a role in modulating the local microenvironment in the lung allograft, a novel paradigm that begets further investigation.

We demonstrate that lung resident MSCs derived from allografts possess immunosuppressive potential. MSCs are a unique, well-characterized population of progenitor cells characterized by their ability to differentiate into multiple mesenchymal lineages (30). Although an emerging body of data indicates that MSCs possess immunomodulatory properties both in vitro and in vivo (12, 31, 32), our study is unique in several ways. As we have clearly demonstrated that MSCs isolated from allografts are donor derived and hence resident in a human adult lung (18), this is the first study to report that tissue-resident populations of MSCs possess immunoregulatory potential. It is important to note that we failed to isolate meaningful numbers of BM-MSCs from the BAL of lung allograft recipients, indicating that they were not major contributors to the lung MSC population (18). The facts that LR-MSCs possess immunoregulatory potential, are the major population isolated from a lung allograft, and can be easily isolated from BAL make them an attractive therapeutic option for prolonging lung allograft acceptance. Further, our study demonstrates for the first time that MSCs isolated from an allogeneic milieu retain their immunosuppressive potential. The majority of prior reports on the immunoregulatory role of BM-MSCs have used cells isolated from healthy volunteers. In a single report, Bacigalupo et al. demonstrated that BM-derived MSCs from patients with sickle cell anemia were deficient in their ability to down-regulate T cell proliferation (33). In this study we examined cells isolated from lungs posttransplantation and demonstrate their ability to suppress T cell proliferation. Variability was noted in the degree of suppression by LR-MSCs isolated from different donors, signifying the need to investigate further the mechanism of this variability and its clinical implications. Furthermore, it should be noted that the cells used in this study were isolated early posttransplantation in the absence of histological evidence of rejection. Whether the T cell suppressive

**Discussion**

In this study we examined interactions between T cells and donor-derived LR-MSCs isolated from the BAL of human lung transplants. We demonstrate that, in vitro, LR-MSCs significantly inhibited proliferation of third party, HLA-mismatched T cells. This

![Prostanoid production by LR-MSCs modulates T cells cytokine secretion](http://www.jimmunol.org/)

**FIGURE 7.** LR-MSCs modulate cytokine secretion by T cells. Supernatants from T cells stimulated with CD28/CD3/CD2 Ab-coated beads for 72 h in the presence (+) and absence (−) of LR-MSCs and indomethacin were analyzed for IL-2 and IL-10 by ELISA. A. Significant decrease in IL-2 was noted in presence of LR-MSCs (p < 0.001). Inhibition of PG secretion by the addition of the COX inhibitor indomethacin completely reversed IL-2 inhibition (p < 0.001). B. Significant increase in IL-10 was noted in the presence (+) of LR-MSCs (p < 0.001), which was significantly but not completely ameliorated by indomethacin (p < 0.01). Data represent the mean ± SEM of experiments performed in triplicate with three separate LR-MSC cell lines.
ability of LR-MSCs changes over time and predicts the development of acute and chronic allograft rejection in human lung transplant recipients remains to be established. The ability to isolate LR-MSCs with a minimally invasive procedure used routinely in the management of lung transplant recipients (bronchoscopy and BAL) offers a unique opportunity to study these cells in vitro and correlate their phenotypic properties to clinical outcomes in future studies.

The mechanisms by which MSCs mediate T cell suppression remain to be entirely elucidated (12, 31). Various studies, including our own, support the idea that a significant contributor to the immunosuppressive effects of MSC is the production of soluble mediators (13, 16). PGE$_2$, an immunomodulatory lipid mediator, is presently one of the leading candidates for MSC-induced immune suppression (16, 20). PGE$_2$ is a well-established inhibitor of T cell proliferation (34, 35). It has been demonstrated that PGE$_2$ inhibits IL-2 secretion and the subsequent proliferation of T cells via a cAMP (protein kinase A)-dependent mechanism (36, 37). Similarly, by increasing intracellular cAMP, PGE$_2$ has been demonstrated to augment IL-10 expression by T cells (38). The G protein-coupled receptors EP2 and EP4 are thought to be important in demonstrating the need to study the contribution of local graft-derived soluble mediators such as PGE$_2$. These findings point to a possibly interesting to note that this degree of reversal of inhibition is similar by secretion of PGE$_2$ likely explain the in vivo capacity of LR-MSCs to survive in an allogeneic environment. These results are consistent with direct contact among LR-MSCs and T cells remains to be entirely elucidated (12, 31). Various studies, including our own, support the idea that a significant contributor to the immunosuppressive effects of MSC is the production of soluble mediators (13, 16). PGE$_2$, an immunomodulatory lipid mediator, is presently one of the leading candidates for MSC-induced immune suppression (16, 20). PGE$_2$ is a well-established inhibitor of T cell proliferation (34, 35). It has been demonstrated that PGE$_2$ inhibits IL-2 secretion and the subsequent proliferation of T cells via a cAMP (protein kinase A)-dependent mechanism (36, 37). 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