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Soluble Signals from Mechanically Disrupted Lung Tissue Induce Lung-Related Gene Expression in Bone Marrow-Derived Cells In Vitro

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ABSTRACT

Differentiation of bone marrow (BM)-derived cells into lung epithelial cells has been reported in vivo in animal models of lung injury. Most studies have used cytokeratin or surfactant protein expression as markers of BM-to-lung cell differentiation. However, concerns as to whether fusion rather than differentiation is the mechanism involved, verification of BM-derived lung cells, and inconsistent findings with different injury models mean that the differentiation potential of BM-derived cells remains unclear. We used a co-culture system, in which BM cell–lung cell fusion is prevented, to examine the ability of 'damage' signals released from mechanically disrupted lung tissue to induce expression of lung-related genes in BM-derived cells in vitro. BM-derived hematopoietic progenitor cells (BM-HPCs) were co-cultured with mechanically disrupted lung tissue. Liver tissue and medium-only co-cultures were also studied as controls. BM-HPCs differentiated into myeloid cells in culture. BM-HPCs proliferated in response to soluble lung damage signals and differentiated into suspension and adherent populations with dendritic cell- and Langerhans cell-like characteristics, respectively. Induction or up-regulation of cytokeratins 7 and 18 and surfactant protein B mRNA expression occurred in the suspension, dendritic cell (DC)-like population during co-culture with lung tissue. In contrast, these genes were not induced or up-regulated in medium-only or liver co-cultures. Up-regulation of E-cadherin mRNA and protein expression also occurred in response to lung damage signals. These results confirm that signals released from damaged lung tissue can induce lung-related gene expression in BM-derived DC-like cells in the absence of cell fusion.

INTRODUCTION

IT HAS BEEN REPORTED that bone marrow (BM)-derived cells can engraft in various tissues in vivo, including lung, heart, liver, kidney, and skin, and differentiate into host tissue-type cells (reviewed in ref. 1). Most lung studies have involved delivery of donor BM-derived cells to recipient mice that have some form of lung injury. Subsequent apparent co-expression of donor markers, such as the Y chromosome or green fluorescent protein (GFP), with lung epithelial cell markers, usually cytokeratins and surfactant proteins, has been reported and proposed to imply differentiation of BM cells into lung epithelial cells (2–14). However, issues such as identification of BM-derived epithelial cells, the types of BM-derived cells involved, differentiation versus fusion, and relevance of the mode of lung injury have yet to be clarified. As a result, BM cell involvement in reconstitution of the lung epithelium in vivo remains controversial.

In 2001, Krause et al. reported that a single BM-derived donor hematopoietic stem cell (HSC) could give rise to bronchial and type II cells in lethally irradiated recipient mice, as determined by co-localization of the Y chromosome with cytokeratin protein and surfactant protein B

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(SP-B) mRNA (2). In the same year, Kotton et al. demonstrated that plastic-adherent BM cells could engraft in lungs of bleomycin-injured mice (3). Expression of the type I cell protein T1α in BM-derived cells and binding of the type I cell-specific lectin *Lycopersicon esculentum* was reported. No engraftment as type II cells was detected. Krause and co-workers subsequently used whole BM that was either male donor-derived (10) or contained a GFP reporter gene (4) to demonstrate BM cell differentiation into type I and type II cells in lethally irradiated mice, again using cytokeratin protein and SP-B mRNA as lung markers. Other studies reported that BM side population (SP) cells give rise to alveolar (5) and tracheal cells (6) that express cytokeratin proteins in injured, but not in healthy, mice that $Flk1^+/CD31^-/CD34^-$ cells from human fetal BM can contribute to lung epithelial regeneration in irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (7) and that BM-derived mesenchymal stem cells (MSCs) can give rise to type II cells (8) and also type I cells and lung fibroblasts during bleomycin-induced lung injury (13).

Other reports have refuted some of these findings however. In contrast to Krause et al. (2), Wagers and coworkers reported that single GFP-labeled HSCs did not contribute appreciably to nonhematopoietic tissues, including lung, in lethally irradiated recipients (14). Debate also centers on the techniques used to identify putative BM-derived lung cell phenotypes in situ. Studies using deconvolution microscopy and lineage-specific reporter systems to identify BM-derived SP-C-expressing lung cells reported overlapping or tightly juxtaposed cells that could have appeared as false-positive donor-derived epithelial cells by conventional microscopy (15,16). Problems may also be associated with GFP reporter systems. It has been suggested that the low level of GFP expression detectable in donor-derived lung cells in vivo may be due to fusion and subsequent donor gene inactivation (6). However, GFP expression levels can vary in the tissues of different strains of GFP donor mice, with some strains containing only approximately 40% GFP-positive mononuclear cells in the marrow (17). Furthermore, in one strain of reporter mice that use the β-actin promoter to drive GFP expression, offspring usually die a few months after birth, indicating that unknown detrimental effects of GFP expression exist that may affect studies using cells derived from these animals (17). Robust demonstration of efficacy of reporter systems such as GFP is therefore essential.

It has been proposed that the mode and extent of tissue injury influences the extent to which BM-derived cells engraft and contribute to regeneration (17). Presumably, signals from damaged lung tissue enhance homing and engraftment of donor BM cells to the lungs and their differentiation into lung epithelial cells (18). As well as depleting BM cells in recipient animals, irradiation causes lung damage. Recently, it has been shown that BM-to-lung cell differentiation only occurs if irradiation levels are above the threshold that induces lung damage (19). However, inconsistent findings between animal studies involving irradiation have been reported (2,14).

Although differentiation of BM cells into lung epithelial cells is predicted to result in co-expression of donor markers with lung epithelial cell markers, fusion of BM cells to host cells could result in the same phenotype. Fusion has been shown to take place in the liver, brain, and heart in vivo (20–22). It is not clear if fusion or differentiation or both occur in the lung. Krause and co-workers found no evidence of fusion in an in vivo model using whole BM (23) whereas Spees et al. observed both differentiation and fusion in vitro using BM-derived MSCs (24). A subsequent study by this group, however, using a different co-culture system, found no evidence of fusion of MSCs with lung epithelial cells (12). Neither is it clear whether donor cells that engraft in the lungs arrive directly following injection, which is unlikely in the case of a single-donor HSC, or are recruited via the bone marrow. In the latter case, the cell type that ultimately engrafts in the lung may not be the same as the original donor population. It will be essential to determine which, if any, BM-derived cells are capable of expressing lung-related genes if they are to be used for gene delivery or targeted repair.

In this study, we tested the hypothesis that soluble lung 'damage' signals, such as those released from disrupted lung cells and extracellular matrices, can induce lung-related gene expression in BM-derived cells in the absence of fusion. An in vitro co-culture system was used in which BM cells were physically separated from mechanically macerated lung tissue obtained from otherwise healthy animals. Induction of cytokeratin and surfactant genes, as reported from in vivo studies, was determined. The co-culture system addressed three key issues associated with the in vivo studies. First, only soluble lung-derived signals could interact with the BM cells and potential BM cell/lung cell fusion was prevented. Second, the BM-derived cells could be characterized before and after co-culture to identify the cell types involved. And third, because only BM-derived cells were harvested for analysis, doubts regarding the true identity of cells expressing lung-related genes were eliminated. Using this system, we have demonstrated induction of lung-related gene expression in BM-derived DC-like cells in vitro in response to soluble signals released from damaged lung tissue.

MATERIALS AND METHODS

BM cell isolation and lineage depletion

BM cells were isolated from 6- to 8-week-old female C3H-Hen mice (Harlen, UK). Care of animals was in ac-

cordance with institutional guidelines. Following cervical dislocation, whole bone marrow was obtained by flushing the femurs and tibias with an ice-cold solution of phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Red blood cells were lysed using an RBC Lysis Solution (Biolegend, San Diego, CA). A population enriched for hematopoietic progenitor cells was obtained from the remaining cell suspension using a negative-selection cell isolation system according to manufacturer's instructions (R&D Systems, Abingdon, UK). Briefly, cells were labeled with a cocktail of biotinylated antibodies against a panel of hematopoietic lineage marker antigens (CD5, CD45R, CD11b, Gr-1, 7-4, and Ter-119) and then incubated with anti-biotin magnetic beads. The magnetically labeled lineage-positive cells were separated by retention in a 6-ml tube within a magnetic field, whereas the unlabeled cells were eluted. Magnetic separation was carried out three times. Unlabeled cells were finally eluted and retained for culture or characterization. These cells are referred to as BM-derived hematopoietic progenitor cells (BM-HPCs).

Co-culture system

BM culture medium comprised of RPMI-1640 (Sigma, Tallaght, Ireland), 10% conditioned medium from Giant Cell Tumour (Sigma), 13.5% heat-inactivated fetal bovine serum (FBS; Gibco, Poole, UK), 2 mM L-Glut (Gibco), and 50 mg/L gentamicin sulfate (Sigma). Lung and liver tissues used in co-culture experiments were harvested from the same animals used to obtain BM cells or, in GFP co-culture experiments, from GFP-expressing FVB.Cg-~Tg (GFPU) 5Nagy/J mice (Jackson Laboratories). Four grams wet weight of each tissue was chopped into approximately 1-mm^2 cubes and mixed with 6 ml of BM culture medium. Next, 800 μl of the resulting suspension was placed in a well of a 24-well tissue culture plate. A total of 1.5×10^5 HPCs in 330 μl of BM culture medium were seeded into tissue culture inserts that had a 0.4-μm membrane pore size. Inserts were placed into wells containing the disaggregated tissue suspension or control medium. Plates were incubated at 37°C, 5% CO_2 for up to 3 days. Cells were harvested at day 2 and day 3. Suspension cells were analyzed separately from the adherent population.

Dendritic cell isolation and co-culture

BM cells were obtained by flushing the leg bones of 6- to 8-week-old female C3H-Hen mice, as above. Dendritic cells (DCs) were cultured as previously described (25) with minor modifications. Nonadherent immature DCs were harvested at day 7 and either analyzed by flow cytometry or co-cultured as follows: 5×10^5 DCs in either 1 ml of BM culture medium or 1 ml of DC medium (21) were seeded into six-well tissue culture plate inserts. Inserts were placed into six-well plates containing 2 ml of control medium, lung tissue, or liver tissue per well. Adherent and suspension cells were harvested at day 3 of co-culture for subsequent RT-PCR analysis.

Cell counts

Suspension cell numbers were determined using an ethidium bromide/acridine orange staining solution and viewing with a UV microscope. Adherent cells were counted using a DAPI nuclear stain and a fluorescent microscope. A two-tailed *t*-test was used to determine signifcance.

Characterization of cells by flow cytometry

The extent of progenitor cell enrichment in the BM-HPC population was determined by flow cytometry. Lineage-depleted cells, already labeled with biotinylated lineage marker antibodies during the separation procedure, were resuspended in PBS with 1% BSA and incubated with phycoerythrin (PE)-conjugated or PE-Cy7-conjugated anti-mouse-CD117, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD34, biotinylated anti-mouse CD45, FITC-conjugated anti-mouse Sca1, and FITC-conjugated anti-mouse CD90 (eBiosciences, San Diego, CA). Cells were also relabeled with biotinylated lineage marker antibodies to ensure adequate labeling during separation. Biotinylated antibodies were detected with subsequent addition of Streptavidin-APC (eBiosciences). At day 3 of culture, suspension cells harvested from the co-culture system were labeled with PE-conjugated anti-mouse CD11b antibody, FITC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse F4/80, PE-conjugated MHC Class II, and biotinylated anti-mouse Gr-1 antibody with subsequent incubation with Streptavidin-APC (eBiosciences). Immature DCs harvested at day 7 were used as controls. Isotype antibody controls (eBiosciences) were performed for all of the antibodies used. Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson, Cowley, UK) and analyzed using CellQuest software (Becton Dickinson).

Immunofluorescence

Adherent cells were fixed in ice-cold methanol for 5 min. Primary antibodies used were rat anti-mouse CD11b, rat anti-mouse CD11c, rat anti-mouse F4/80 (eBiosciences), mouse anti-mouse E-cadherin (BD Pharmingen, San Diego, CA). Secondary antibodies used were Alexafluor 488 and Alexafluor 568 (Molecular Probes, Invitrogen). Immunofluorescence was detected with a fluorescent microscope. Phase-contrast images

were taken at 20× magnification using an Olympus digital camera, model C310.

RT-PCR

Total RNA was prepared from both suspension and adherent cells harvested at day 2 and day 3 using TriReagent (Sigma) according to the manufacturer's instructions. Samples were treated with DNase I, Amp Grade (Invitrogen, Paisley, UK) for 30 min and cDNA was synthesized using Moloney murine leukemia virus (M-MLV) Reverse Transcriptase (Invitrogen). PCR primers were designed using 'Primer 3' Web software (Whitehead Institute for Biomedical Research, MA). PCR consisted of 35 cycles 94°C for 45 sec (denaturation), 58°C (unless otherwise stated) for 1 min (annealing), and 72°C for 2 min (extension) using a PTC-100 programmable thermal cycler (MJ Research, Inc., Bio-Rad, Waltham, MA). The PCR primers were as follows: SP-A, 5′-AAG TGC AAT GGG ACA GAA-3′ and 5′-AAG TTG ACT GAC TGC CCA-3′, product 389 bp; SP-B, 5′-CTA CCT GCC CCT GGT TAT-3′ and 5′-GAC TTG CAG AAA TGG CAC-3′, product 549 bp; SP-C, 5′-GGT GAT TGT AGG GGC TCT-3′ and 5′-AAG AAT CGG ACT CGG AAC-3′, product 365 bp; SP-D, 5′-TGC CTT CTC CCA CTA TCA-3′ and 5′-CAC AGA TAA CAA GGC GCT-3′, 55°C annealing temperature, product 392 bp; CC-10, 5′-ATC GCC ATC ACA ATC ACT-3′, 5′-GAA TCT TAA ATC TTG CTT ACA CAG AGG-3′, 55°C annealing temperature, product 282 bp; cytokeratin 7, 5′-GCA GGA TGT GGT GGA AGA TT-3′ and 5′-TCT GCG ATG CTG GAC TCT AA-3′, product 507 bp; cytokeratin 8, 5′-CTG ACC GAC GAG ATC AAC-3′ and 5′-GCT CTG CAG CTC CTC ATA-3′, product 225 bp; cytokeratin 18, 5′-AGA CTG GGG CCA CTA CTT CA-3′ and 5′-CTG ATT TCG GCA GAC TTG GT-3′, product 498 bp; cytokeratin 19, 5′-TGA TCG TCT CGC CTC CTA CT-3′ and 5′-AAT CCA CCT CCA CAC TGA CC-3′, product 452 bp; ECAD, 5′-GGC TGG ACC GAG AGA GTT-3′ and 5′-CTG CTT GGC CTC AAA ATC C-3′, product 350 bp; GAPDH, 5′-CTG CAC CAC CAA CTG CTT AG-3′ and 5′-TGA GGG AGA TGC TCA GTG TT-3′, 55°C annealing temperature, 30 PCR cycles, product 487 bp. Absence of contaminating genomic DNA was confirmed for each RT-PCR experiment using controls that omitted M-MLV. GAPDH was used as a housekeeping gene and cDNA template was equalized to give equivalent bands of GAPDH. Mouse airway epithelial cells (AECs) were isolated as previously described (26) and used as a positive control for RT-PCR.

Flow cytometry for protein expression in suspension cells

Suspension cells were analyzed at day 3 for lung-related protein expression by flow cytometry. Cells were harvested, washed twice in ice-cold PBS, and fixed and permeabilized as previously described (27). Briefly, cells were fixed in 0.25% paraformaldehyde for 1 h at 4°C, centrifuged, and permeabilized in 0.2% Tween-20 for 15 min at 37°C. Cells were then centrifuged, incubated in blocking serum for 20 min at room temperature and primary antibody for 30 min at 4°C. Following two wash steps, cells were blocked again and incubated with secondary antibody. Cells were washed twice again in 0.2% Tween-20, and once in PBS, 2% FBS, and 0.1% sodium azide, before resuspension in PBS, 2% FBS, and 0.1% sodium azide. Primary antibodies used were mouse anti-mouse E-cadherin (BD Pharmingen), rabbit anti-sheep surfactant protein-B (Chemicon, Hampshire, UK), and mouse anti-mouse Pan-keratin (Sigma). The secondary antibody used was Alexafluor 488 (Molecular Probes, Invitrogen). Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson, Cowley, UK) and analyzed using CellQuest software (Becton Dickinson).

RESULTS

Initial experiments using whole BM

In vivo studies using whole BM have reported differentiation of BM cells into lung epithelial cells (4). Therefore, we began by determining the effects of soluble signals from mechanically macerated lung tissue on lung-related gene expression in whole BM cultures. Co-cultures with medium alone or with liver tissue were used as controls. Following co-culture, BM cells were harvested and examined for induction of SP-B, CC-10, cytokeratin 7, cytokeratin 18, and cytokeratin 19 mRNA expression using RT-PCR. Induction of these mRNAs was detectable in some samples at some timepoints in response to soluble lung-derived signals, but not in medium-only or liver controls. However, no consistent pattern of gene expression was determinable (results not shown). It was hypothesized that large populations of mature, lineage-positive (lin^+), whole BM cells were obscuring visualization of lung-related gene expression in smaller stem cell subpopulations. Therefore, a population of cells enriched for lineage-negative progenitor cells, BM-HPCs, was isolated and examined in co-culture.

Characterization of BM-HPCs after lineage depletion

Separation of mature hematopoietic cells (T cells, B cells, monocytes/macrophages, granulocytes and erythrocytes and their committed precursors) from whole BM was carried out. First, red cell lysis reduced the number of whole BM cells by approximately one third. Sub-

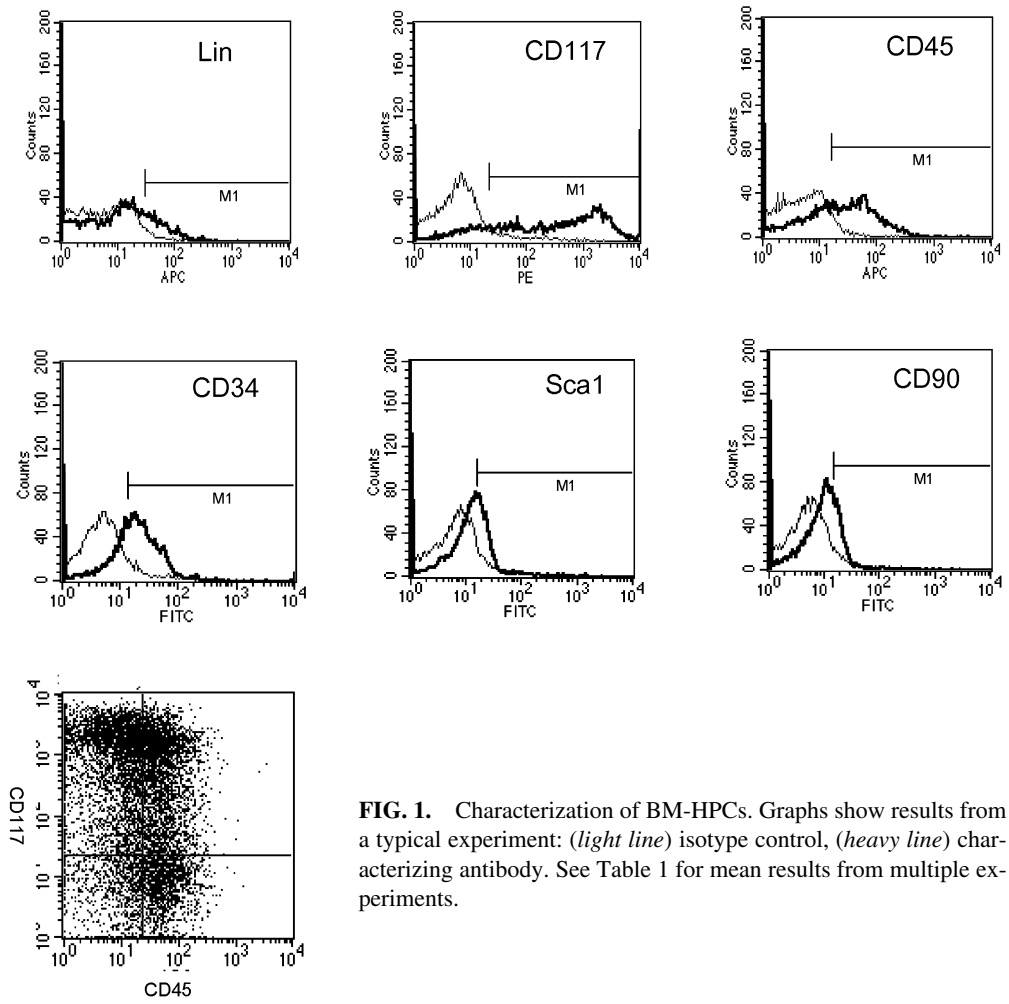

FIG. 1. Characterization of BM-HPCs. Graphs show results from a typical experiment: (*light line*) isotype control, (*heavy line*) characterizing antibody. See Table 1 for mean results from multiple experiments.

sequently, 0.93 % of the remaining cell population was harvested following separation ($n = 3$). On average, 91% of this BM-HPC population was lin$^-$ as determined by relabeling with lineage antibodies. These cells were further characterized to be 72% CD117$^+$, 59% CD34$^+$, 51% CD45$^+$, 25% Sca1$^+$, and 9% CD90$^+$. Approximately 10% were CD117$^-$ CD45$^-$ indicating that approximately 90% of the BM-HPC population were of hematopoietic lineage ($n = 2$). Results of a representative experiment are shown in Fig. 1. Data averaged from three separate experiments are shown in Table 1.

F1
T1

Proliferation of BM-HPCs in co-culture

BM-HPCs were cultured in complete medium containing 10% Giant Cell Tumour conditioned medium. This was used to promote the growth of hematopoietic cells due to its combination of colony stimulating factors—granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF), amongst other factors. Therefore, the BM-HPCs differentiated once placed in culture, and we will refer to cultured BM-HPCs as BM-derived myeloid cells, BM-MCs, from this point onward.

Although most BM-MCs remained in suspension during co-culture, approximately 24%, 17%, and 14% adhered to the tissue culture inserts in medium-only, lung, and liver co-cultures, respectively, by day 3. Suspension

TABLE 1. CHARACTERIZATION OF FRESHLY ISOLATED BM-HPCs

Characterizing antibody	*Percentage cells*
Lin$^-$	90.63% ± 6.35
CD117$^+$	71.63% ± 2.57
CD45$^+$	50.51% ± 5.03
CD34$^+$	58.75% ± 5.01
Sca1$^+$	24.78% ± 4.57
CD90$^+$	8.67% ± 4.17
*CD117$^-$ ± CD45$^-$	10.83% ± 2.13

Table shows mean results from three individual experiments (*$n = 2$).

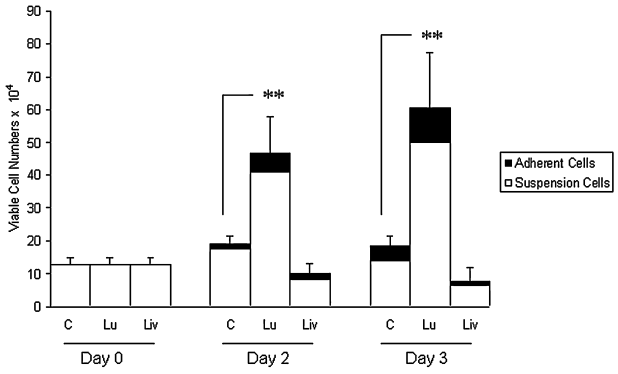

FIG. 2. Proliferation of BM-HPCs in co-culture. Graph represents mean of three separate experiments. Co-culture with lung tissue resulted in a 4.6-fold increase in BM-HPC cell number. Cell numbers were significantly higher than those in medium-only controls at day 2 and day 3 (** = $p < 0.01$). Cells in medium-only remained relatively constant whereas cell numbers declined in co-culture with damaged liver tissue. Adherent cells represent approximately 24%, 17%, and 14% of control (C), lung (Lu), and liver (Liv) co-cultures by day 3.

and adherent populations were analyzed separately from this point onward.

Suspension and adherent cells were counted separately and a total cell count was determined. Although total cell numbers remained relatively constant when cultured with medium alone, co-culture with lung tissue resulted in a 4.7-fold increase in total BM-MC cell number compared to seeding density (Fig. 2). In contrast, a decline in total BM-MC cell number occurred during co-culture with liver tissue.

F2

Effects of co-culture on BM-MC phenotype

The effects of co-culture on BM-MC phenotype, with medium-only and in the presence of soluble signals from lung tissue, were determined. Results of a typical experiment are shown in Fig. 3. Mean values from multiple experiments are shown in Table 2. Suspension BM-MCs were harvested for characterization by flow cytometry. By day 3 of co-culture, BM-MCs co-cultured with medium alone had become $CD11b^{hi}$ $CD11c^{-}$ $Gr\text{-}1^{lo}$ $F4/80^{lo}$ MHC II^{-}. In contrast, BM-MCs co-cultured with lung tissue showed up-regulation of CD11c and were MHC II^{lo}. For comparison, CD11c expression in control immature DCs was examined. BM-MCs co-cultured with lung were 37% $CD11c^{+}$ whereas immature DCs were 67% $CD11c^{+}$. These expression profiles indicate that soluble signals from lung tissue induced BM-MCs to differentiate toward a DC-like phenotype.

F3
T2

Adherent BM-MCs were analyzed by immunofluorescence for CD11b, CD11c, F4/80, and E-cadherin expression at day 3 of co-culture (Fig. 4A). Adherent cells, whether co-cultured with medium alone or with lung or liver tissue, were over 97 % $CD11b^{+}$ and negative for CD11c. Adherent cells in medium-only co-cultures were very weakly positive for F4/80 and did not express E-cadherin protein. In contrast, 72% of adherent cells co-cultured with lung tissue were strongly positive for F4/80 and expressed high levels of E-cadherin, which was localized to the cell membrane and at regions of cell-to-cell contact. This expression pattern of is indicative of a Langerhans-like cell type. Phase-contrast images (Fig. 4B) show a marked change in morphology from control cells to cells co-cultured with lung tissue.

F4

Effects of co-culture on lung-related gene expression in BM-MCs

Lung-related gene expression was analyzed by RT-PCR. Suspension and adherent BM-MCs were harvested at day 3 and analyzed for expression of E-cadherin, CC-10, surfactant protein, and cytokeratin mRNA. Total RNA extracted from primary mouse AECs was used a positive control.

SP-B gene expression was not detected in suspension BM-MCs co-cultured with either control medium or liver tissue (Fig. 5). In contrast, SP-B gene expression was induced in suspension BM-MCs co-cultured with lung tissue. Expression of SP-A, SP-C, SP-D, and CC-10 mRNA was not detected in any BM-MC co-cultured cells (results not shown). Low-level cytokeratin 18 mRNA expression was evident in day-0 freshly isolated BM-MCs as expected. Although BM cells do not contain cytokeratin proteins, cytokeratin mRNA transcripts are present in these cells (28). Up-regulation of cytokeratin 18 gene expression occurred in BM-MCs co-cultured with lung tissue, but not in medium-only or liver co-cultures. Induction of cytokeratin 7 expression occurred in BM-MCs co-cultured with lung tissue at day 3, but not in control- or liver co-cultures. Induction of cytokeratin 19 was also detected only in BM-MCs co-cultured with lung tissue, but expression was inconsistent. There was no induction of cytokeratin 8 mRNA (results not shown). E-cadherin mRNA expression was up-

F5

FIG. 3. Characterization of suspension BM-MCs at day 3 of co-culture. Graphs are representative of three separate experiments. **(A)** Characterization of BM-MCs in medium-only co-culture. Cells are $CD11b^{hi}$ $CD11c^{-}$ $Gr\text{-}1^{lo}$ $F4/80^{lo}$ $MHCII^{-}$, indicative of BM-derived myeloid cells. **(B)** Characterization of BM-MCs in co-culture with lung tissue. Cells are $CD11b^{hi}$ $CD11c^{lo}$ $Gr\text{-}1^{lo}$ $F4/80^{lo}$ $MHCII^{lo}$, indicating that BM-MCs are differentiating toward a DC-like phenotype. **(C)** Characterization of immature DCs (Imm DCs) for comparison.

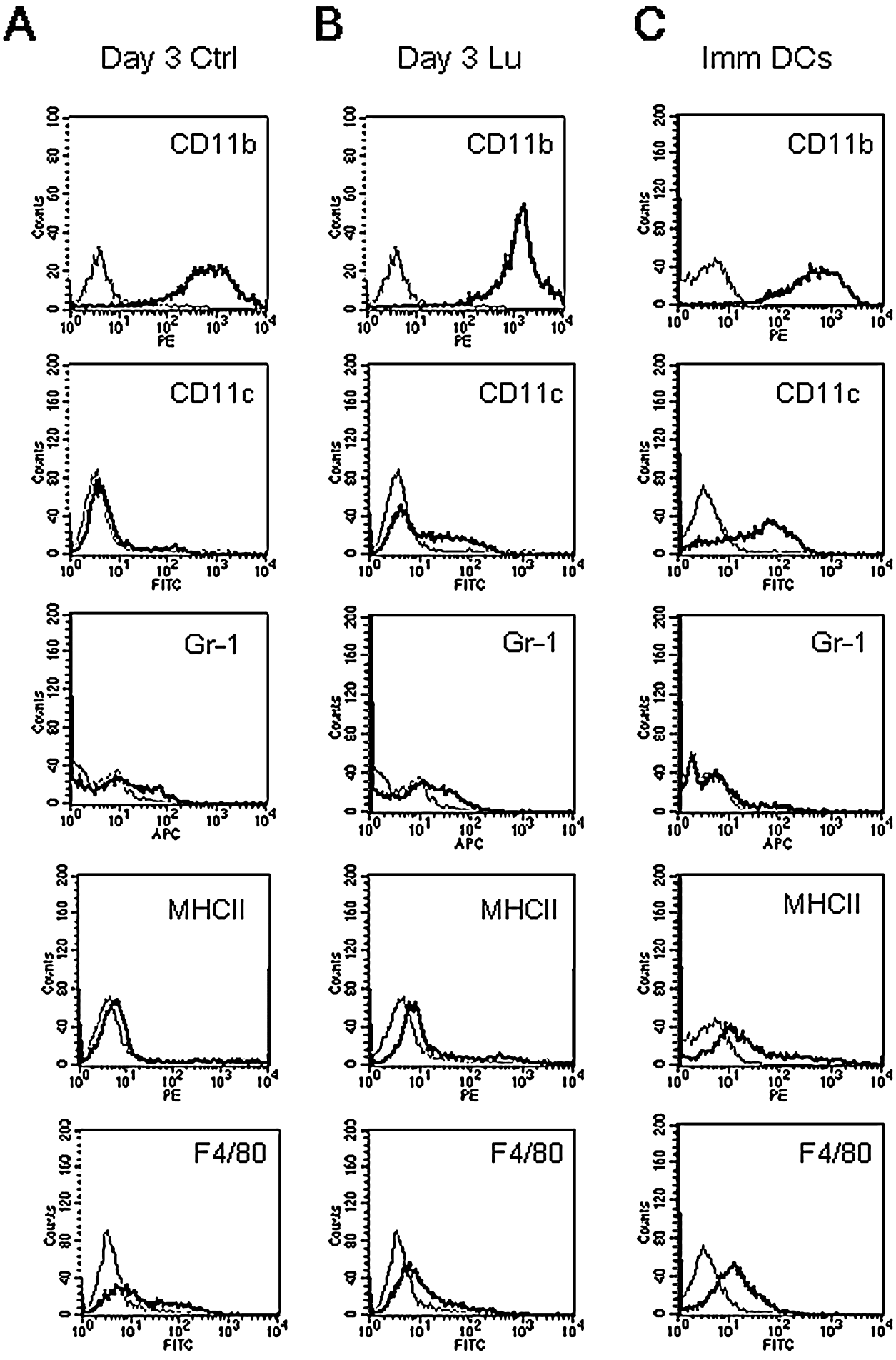
A
Day 3 Ctrl
B
Day 3 Lu
C
Imm DCs
CD11b
CD11c
Gr-1
MHCII
F4/80
Counts
PE
FITC
APC

Table 2. Characterization of Suspension BM-MCs at Day 3 of Co-Culture

AU1

	Day-3 control	*Day-3 Lu*	*Imm DCs*
CD11b	89.7% ± 2.9	91.8% ± 2.5	95.27% ± 0.8
CD11c	6.1% ± 4.5	36.9% ± 3.0	67.17% ± 2.3
Gr-1	19.6% ± 12.6	17.0% ± 8.3	9.13% ± 3.6
F4/80	18.7% ± 13.2	13.3% ± 3.3	47.48% ± 9.4
MHC II	0.8% ± 1.3	9.5% ± 4.5	34.57% ± 8.2

Table shows mean results from three individual experiments using medium-only and lung tissue co-cultures. Immature DCs (Imm DCs) were used as a comparison ($n = 2$).

regulated in BM-MCs co-cultured with lung tissue compared to medium-only co-cultures.

Adherent cells were analyzed for expression of the same genes but only E-cadherin mRNA expression was detected (Fig. 5). Cytokeratin 18 mRNA was detected occasionally, but not consistently, in BM-MCs co-cultured with lung tissue.

It is highly unlikely that tissue-derived RNA or cells crossed into the inserts to give rise to false-positive RT-PCR results in BM-MCs. First, liver cells contain cytokeratin 7, 18, and 19 mRNA transcripts and proteins, yet cytokeratin expression was never detected by RT-PCR in BM-HPCs co-cultured with damaged liver tissue. Second, no RT-PCR bands were detected in controls experiments where medium without BM-HPCs was co-cultured with lung or liver tissue extracts (results not shown). In addition, co-cultures using BM-HPCs from wild-type mice and lung tissue from FVB.Cg-~Tg (GFPU) 5Nagy/J mice ubiquitously expressing GFP were carried out. No GFP mRNA was detected in harvested BM-MCs cells and no GFP-positive adherent cells were present on the tissue culture insert when viewed by microscopy ($n = 2$, results not

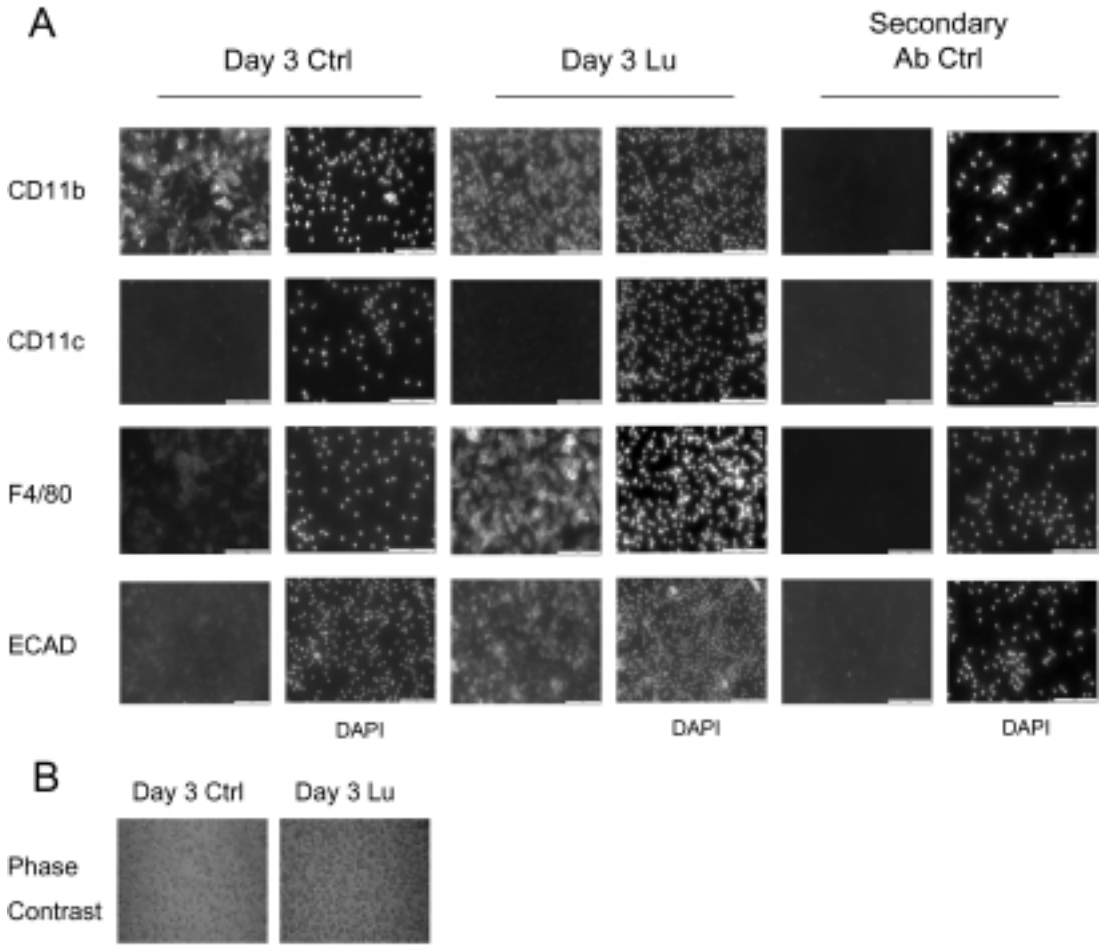

FIG. 4. **(A)** Characterization of adherent BM-MCs at day 3 of co-culture. Photomicrographs are representative of three separate experiments. DAPI is used as a nuclear counterstain. BM-MCs in medium-only co-culture (Ctrl) are $CD11b^{+}$ $CD11c^{-}$ $F4/80^{lo}$ $ECAD^{-}$. BM-MCs in co-culture with lung tissue are $CD11b^{+}CD11c^{-}$ $F4/80^{+}$ $ECAD^{+}$, indicating a Langerhans cell-like phenotype. Secondary antibody controls, omitting primary antibodies, demonstrate lack of nonspecific binding. **(B)** Phase-contrast images taken at 20× magnification show a marked change in morphology.

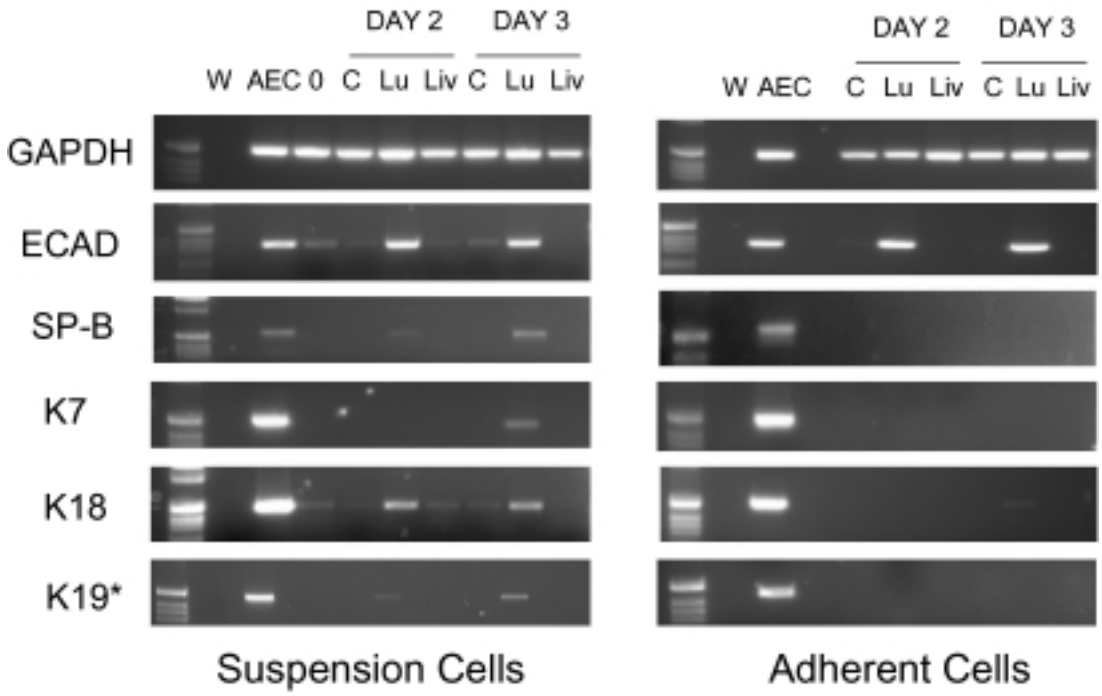

FIG. 5. RT-PCR analysis of suspension cells and adherent BM-MCs at day 2 and day 3 of co-culture. W, no cDNA control; AEC, airway epithelial cell; 0, day zero freshly isolated BM-HPCs; C, BM-MCs co-cultured in medium-only; Lu, BM-MCs co-cultured with lung tissue; Liv, BM-MCs co-cultured with liver tissue; ECAD, E-cadherin; SP-B, surfactant protein B; K7, cytokeratin 7; K18, cytokeratin 18; K19, cytokeration 19 (*inconsistent result). SP-B, K7, and K19* were induced in co-culture with lung tissue whereas K18 and E-cadherin were up-regulated.

shown). Furthermore, immunofluorescence for E-cadherin was carried out using a green fluorescent secondary antibody to confirm that cells expressing E-cadherin were not GFP-positive and therefore were not derived from lung tissue below the insert (results not shown).

Induction of lung-related protein expression

Because SP-B, cytokeratin and E-cadherin mRNA expression was induced or up-regulated in suspension BM-MCs co-cultured with lung tissue, expression of corresponding proteins was examined by flow cytometry. No SP-B or cytokeratin protein was detected in suspension BM-MCs co-cultured with either medium alone or with lung tissue. Expression of E-cadherin protein was induced in approximately 11% of BM-MCs co-cultured with lung tissue compared to 2.8% of BM-MCs co-cultured with medium only (Fig. 6). Although E-cadherin is not lung-specific and is expressed by DCs, it is involved in integration into epithelium and therefore may play a role in BM-derived cell engraftment during regeneration.

F6

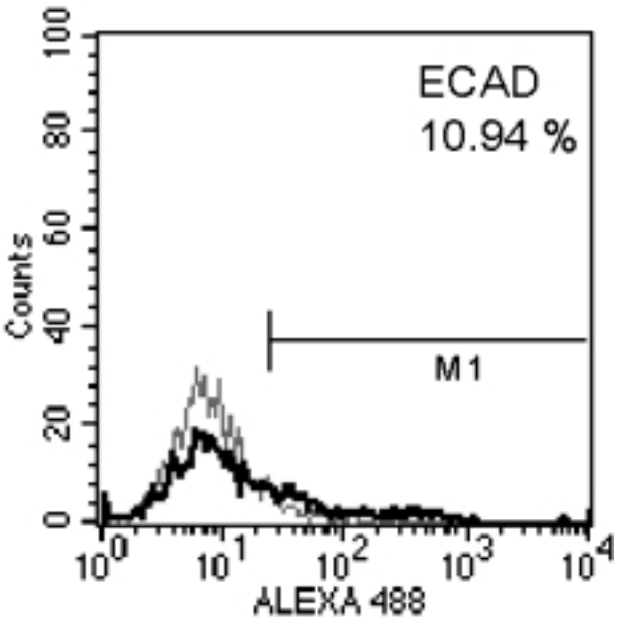

FIG. 6. E-cadherin protein expression in BM-MCs co-cultured with lung tissue at day 3. Graph is representative of three separate experiments. (*Light line*) isotype control, (*heavy line*) E-cadherin antibody. Approximately 11% of cells co-cultured with lung tissue expressed E-cadherin compared with 2.8% of BM-MCs in medium only controls.

Co-culture using immature DCs

Co-culture of BM-HPCs with lung tissue produced a DC-like phenotype capable of co-expressing lung-related

genes. Therefore, we examined whether soluble signals from lung tissue could induce lung-related gene expression in immature DCs derived in vitro from cultured BM. No induction of lung-related genes was detected in co-cultured DCs grown in either BM culture medium or DC culture medium (results not shown).

DISCUSSION

In vivo studies indicate that BM-derived cells can contribute to regeneration of injured lung epithelia. However, a number of concerns relating to these studies mean that the phenomenon of BM-to-lung epithelial cell differentiation remains controversial. We endeavored to verify in vivo findings of lung-related gene expression in BM-derived cells by using an in vitro system that eliminated many of the confounding factors associated with animal studies. The co-culture system prevented BM cell–lung cell fusion and facilitated retrieval of BM-derived cells for characterization and analysis of lung-related gene expression. Using this system, we have confirmed that soluble 'damage' signals from mechanically disrupted lung tissue can induce or up-regulate expression of several of those genes used as markers of BM-to-lung epithelial cell differentiation in vivo, namely cytokeratins and surfactant protein B.

The co-culture system enabled us closely to examine separate BM-derived subpopulations as they emerged in culture in response to soluble lung damage signals. The BM-HPCs used here proliferated in response to these soluble lung damage signals and differentiated into suspension and adherent populations with DC- and Langerhans cell-like characteristics, respectively. Interestingly, under current conditions, lung-related gene expression occurred only in the BM-MC suspension, DC-like population. However, when in vitro-derived immature DCs were co-cultured with lung tissue, no lung-related gene expression was detected. This may indicate that a more immature myeloid precursor population, capable of expressing cytokeratin and surfactant protein B, was present in the BM-MC population, but not in the in vitro-derived DC population.

Expression of cytokeratin and surfactant proteins did not occur in BM-MCs. This may reflect differences in soluble and nonsoluble factors present in vitro and in vivo, such as cell–cell contact with epithelial cells, for example. Alternatively, the cells used in this study may not be capable of ultimately expressing lung-related proteins. It is possible however that the time points looked at in these experiments did not allow for protein synthesis.

It is interesting to note that DCs are recruited to the lung during inflammation and integrate into the epithelium via E-cadherin interactions with epithelial cells (29). Therefore, DCs would appear to be ideal candidates for BM-derived cell engraftment and regeneration of injured lung epithelia. In addition, type II pneumonocytes have certain features in common with DCs. They express major histocompatibility complex II (MHC II) and are among the few nonprofessional antigen-presenting cell types in the body (30). Type II cells also express CD208/dendritic cell-lysosomal associated membrane protein (DC-LAMP), a protein originally described as specific to DCs (31,32). Therefore, there may be a closer relationship between type II cells and DCs than currently understood.

In summary, we have demonstrated that BM-derived DC-like cells are capable of expressing lung-related genes, specifically those used as markers of BM-to-lung cell differentiation in vivo. Induction or up-regulation of these genes appears to occur in response to soluble signals from damaged lung tissue and in the absence of cell fusion.

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