

# Characterization of Murine Lung Dendritic Cells: Similarities to Langerhans Cells and Thymic Dendritic Cells

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## Summary

Dendritic cells (DC) are potent accessory cells (AC) for the initiation of primary immune responses. Although murine lymphoid DC and Langerhans cells have been extensively characterized, DC from murine lung have been incompletely described. We isolated cells from enzyme-digested murine lungs and bronchoalveolar lavages that were potent stimulators of a primary mixed lymphocyte response (MLR). The AC had a low buoyant density, were loosely adherent and nonphagocytic. AC function was unaffected by depletion of cells expressing the splenic DC marker, 33D1. In addition, antibody and complement depletion of cells bearing the macrophage marker F4/80, or removal of phagocytic cells with silica also failed to decrease AC activity. In contrast, AC function was decreased by depletion of cells expressing the markers J11d and the low affinity interleukin 2 receptor (IL-2R), both present on thymic and skin DC. AC function was approximately equal in FcR<sup>+</sup> and FcR<sup>-</sup> subpopulations, indicating there was heterogeneity within the AC population. Consistent with the functional data, a combined two-color immunofluorescence and latex bead uptake technique revealed that lung cells high in AC activity were enriched in brightly Ia<sup>+</sup> dendritic-shaped cells that (a) were nonphagocytic, (b) lacked specific T and B lymphocyte markers and the macrophage marker F4/80, but (c) frequently expressed C3b1R, low affinity IL-2R, FcRII, and the markers NLDC-145 and J11d. Taken together, the functional and phenotypic data suggest the lung cells that stimulate resting T cells in an MLR and that might be important in local pulmonary immune responses are DC that bear functional and phenotypic similarity to other tissue DC, such as Langerhans cells and thymic DC.

Ia<sup>+</sup> cells with a dendritic shape are widely distributed in many nonlymphoid tissues such as skin and lung, as well as in all lymphoid tissue, i.e., spleen, lymph nodes, thymus, and Peyer's patches (1-14). An Ia<sup>+</sup> dendritic cell (DC)<sup>1</sup> has been implicated as the principal cell responsible for priming virgin and resting T lymphocytes in studies that used a primary allogeneic MLR as a measure of accessory cell (AC) activity (9, 15, 16).

The lung has a large surface area exposed to the environment, and local pulmonary immune responses are essential to protect the host from inhaled microorganisms. It follows, therefore, that effective APC should exist in the lung. Alveolar macrophages (AM) are abundant, but are poor APC in most species, including mouse and human (17-19). Studies have demonstrated AC activity in cell populations derived

from minced, enzyme-digested rat and human lung, and from tracheal epithelial digests in the mouse (5, 6, 20-22). In all of these studies, evidence strongly suggested that the AC were more like splenic DC than macrophages. This evidence is consistent with the identification of Ia<sup>+</sup>, dendritic-shaped cells in rat, human, and mouse lung tissue sections (5, 7, 23). Ia<sup>+</sup> DC have not been isolated from whole murine lung, nor have they been examined for phenotypic characteristics other than Ia. Therefore, their relationship to splenic DC and other types of DC is unclear. An examination of this relationship is important because it was recently proposed that there are two distinct populations of DC based on surface markers and tissue location. DC in one population, represented by splenic DC, express 33D1, but not the interdigitating cell (IDC) marker, NLDC-145, or the B cell/thymocyte marker, J11d. These DC are likely to be located in the marginal zone of the spleen (24). Peyer's patch DC and nonadherent thymic DC also express 33D1. The second group of DC, typified by epidermal Langerhans cells, lack 33D1, but express NLDC-145 and J11d (12, 25). Collagenase digestion released a loosely adherent population of DC from the thymus and an addi-

<sup>1</sup> *Abbreviations used in this paper:* AC, accessory cell; AM, alveolar macrophages; BAC, bronchoalveolar cells; DC, dendritic cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; IDC, interdigitating cells; FAd, firmly adherent cells; LAd, loosely adherent cells; NAd, nonadherent cells.

tional small population of DC from the spleen, which express these same two markers and lack 33D1 (12). Recent studies suggest there are functional differences between the two types of DC with regard to antigen processing and the ability to prime resting T lymphocytes (26).

In the current study a mouse pulmonary AC with potent MLR-stimulating capacity was obtained by bronchoalveolar lavage from airways and by collagenase digestion from lung interstitium. Like splenic DC, these lung cells had a low buoyant density, were loosely adherent, and nonphagocytic. However, a substantial population more closely resembled Langerhans cells and loosely adherent thymic DC phenotypically, i.e., they expressed J11d and NLDC-145, but not 33D1.

## Materials and Methods

**Mice.** C57BL/6 and CBA mice, 7–15 wk old and of both sexes were from The Jackson Laboratories (Bar Harbor, ME) or Sasco (Houston, TX).

**Antibodies.** The mAbs M5/114, which recognizes several Ia haplotypes, including IA<sup>b</sup> (27) and M1/70 (28), were from Boehringer Mannheim (Indianapolis, IN). The hybridomas GK1.5 (29), 30-H12 (30), 7D4 (31), 14.8 (32), 25-5-16S (33), and J11d (34) were from the American Type Culture Collection (Rockville, MD). The hybridomas 33D1 (35) and NLDC-145 (36) were gifts from Dr. Ralph Steinman (The Rockefeller University, New York, NY); the hybridomas 2.4G2 (37) and F4/80 (38) were gifts from Dr. Pam Witte (Loyola University Medical Center, Chicago, IL); and YTS 169.4 (39, 40) was a gift from Dr. H. Waldmann (University of Cambridge, Cambridge, UK). Antibody specificities are given in Table 2. All mAbs were rat IgG2b except for NLDC-145 (rat IgG2a); J11d and 7D4 (rat IgM); and 25-5-16S (mouse IgM). Control antibodies included affinity-purified rat IgG and the irrelevant mouse IgM mAb H59, a gift from Dr. Dorothy Yuan (University of Texas Southwestern Medical Center, Dallas, TX; 41).

**Preparation of Lung Cells.** Mice were injected intraperitoneally with 150 units heparin (Upjohn Company, Kalamazoo, MI) and 7.5 mg Nembutal (Abbott Laboratories, North Chicago, IL) and exsanguinated. Bronchoalveolar cells (BAC) were obtained by inserting a catheter into the trachea and washing the lungs with at least five 1-ml aliquots of PBS containing 0.6 mM EDTA. The pulmonary vasculature was perfused with sterile saline to remove peripheral blood cells. The lavaged, perfused lungs were removed, minced, and incubated at 37°C for 90 min in collagenase and 30 µg/ml DNase (Type IV bovine pancreatic DNase I; Sigma Chemical Co., St. Louis, MO). In initial studies, three sources of collagenase were used: (a) Sigma Chemical Co., 150 U/ml; (b) Worthington Biochemical Corp. (Type I; Freehold, NJ), 150 U/ml; and (c) Boehringer-Mannheim Biochemicals, (Indianapolis, IN; Collagenase A), 0.7 mg/ml. Different lots of collagenase resulted in inconsistent yields; therefore, we used the collagenase sampling program offered by Boehringer-Mannheim, and in most studies used this source of collagenase. Digested lung tissue was tapped through a wire screen, particulate matter was allowed to settle or was removed by rapid filtration through a nylon wool plug, and cells were washed twice in HBSS (Inland Laboratories, Austin, TX). Lung cells were separated into density fractions on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Piscataway, NJ) by suspending cells in the highest density Percoll, overlaying them with the lower density Percoll, and centrifuging at 400 g for 20 min. Various fractions were tested for AC activity in an MLR. Pooled

low density fractions (<1.075 g/ml) were further separated into three adherence fractions. Cells were incubated in tissue culture dishes for 2 h at 37°C, nonadherent cells (NAd) were removed, and adherent cells were incubated overnight. The cells that spontaneously detached during the overnight culture were called loosely adherent cells (LAd). The cells that remained attached after the overnight culture were termed firmly adherent cells (FAd).

**Silica Treatment.** To determine if phagocytic cells were responsible for AC activity in the lung, LAd were treated with silica particles (0.1 µm; a gift from Dr. K. Robock, Stein Kohlenberg-Bauverian, 43 Essen-Krey, FRG). LAd were incubated at 37°C for 2–4 h with 250 µg/ml silica in RPMI 1640, 25 mM Hepes (Inland), plus 20% FCS (Gibco Laboratories, Grand Island, NY). Cells were layered on a Nycodenz cushion (Accurate Chemical and Scientific Corp., Westbury, NY; 1.15 g/ml) and centrifuged for 15 min at 1,000 g. Viable, nonphagocytic cells were recovered at the interface; the pellet contained silica-laden cells, dead cells, and free silica.

**EA Rosetting.** LAd were fractionated into FcR<sup>+</sup> and FcR<sup>-</sup> cells by rosetting with antibody-coated sheep erythrocytes (EA). A 2.5% solution of SRBC was incubated with a subagglutinating dose of IgG anti-SRBC antibodies (Cordis, Miami, FL) for 30 min at 37°C. The SRBC were washed twice with PBS and resuspended to make a 0.5% solution. Equal volumes of spleen or lung LAd (10<sup>6</sup>/ml) and the SRBC suspension were mixed and centrifuged at 200 g. This mixture was incubated on ice for 30 min, gently resuspended, layered on top of a Percoll cushion (1.075 g/ml), and centrifuged at 400 g for 20 min. The rosetted cells (FcR<sup>+</sup>) and free SRBC were in the pellet, while the majority of nonrosetted cells (FcR<sup>-</sup>) were collected at the interface. SRBC in the FcR<sup>+</sup> fraction were lysed by treatment with Tris-ammonium chloride and residual cells were washed in HBSS before use.

**Depletion of LAd with mAb and Complement Treatment.** Cells were incubated with 33D1, J11d, 7D4, F4/80, or one of the two control antibodies for 45 min on ice, washed, and treated for 1 h with baby rabbit serum (Pel-Freez Clinical Systems, Brown Deer, WI) as a source of complement. Cells were washed in HBSS before use. When depleting LAd of F4/80-bearing cells, 25 µg/ml rabbit anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was included in the second incubation.

**Morphology and Immunofluorescence of Lung LAd.** To determine the phenotype of cells in lung and BAC populations, cells were first incubated with latex particles to identify phagocytic cells, then stained for Ia alone or in combination with several other mAbs as shown in Table 1. The cells were incubated for 2 h at 37°C in a 0.1% solution of 1.1 µm latex particles (Sigma Chemical Co.) in RPMI containing 20% FCS. Free latex was separated from the cells by centrifugation over FCS. Cell surface markers were detected by indirect or two-color immunofluorescence. The cells were first stained with a panel of mAb followed by fluorescein-conjugated F(ab')<sub>2</sub> mouse anti-rat IgG, which also recognized the light chains of the IgM mAb (Jackson ImmunoResearch Laboratories). For two-color staining, the cells were then incubated with biotinylated M5/114, followed by staining with avidin-Texas Red (Boehringer-Mannheim Biochemicals). The cells were examined for latex uptake and red and green fluorescence using a Zeiss epifluorescent photomicroscope.

**Mixed Leukocyte Reaction.** Nylon wool-passed spleen cells (93% Thy-1.2<sup>+</sup>, 6% Ia<sup>+</sup>) from CBA mice (3 × 10<sup>5</sup>/well) were incubated with various numbers of irradiated AC (2,000 rad) in triplicate in 200 µl complete RPMI (2 mM L-glutamine, 5 × 10<sup>-5</sup> M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin) supplemented with 5% heat-inactivated FCS in flat-bottomed microtiter wells (Costar, Cam-

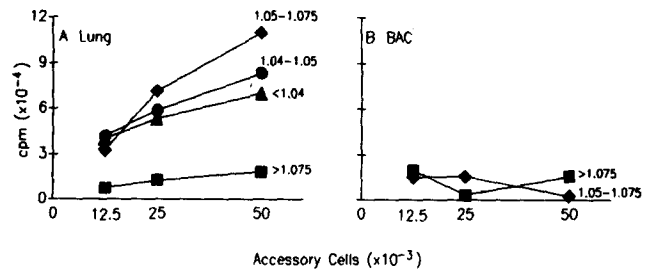
bridge, MA) at 37°C, 5% CO<sub>2</sub> for 4 d. All cultures contained 1 µg/ml indomethacin (Sigma Chemical Co.) and 250 U/ml catalase (Worthington Biochemical Corp.). 18 h before harvesting, 0.5 µCi [<sup>3</sup>H]TdR (Amersham Corp., Arlington Heights, IL) was added to each well. Cultures were harvested with an automated cell harvester and analyzed in a liquid scintillation counter.

## Results

**Density Gradient Fractionation of AC from Minced, Enzymatically Digested Lungs.** Ia<sup>+</sup> DC have been identified in frozen sections of rat, human, and mouse lungs and subsequently partially enriched in enzymatically digested lungs from rat and man (5–7, 42). We attempted to isolate DC from mouse lung by exploiting characteristics described for murine splenic DC (8). BAC were obtained by bronchoalveolar lavage and the pulmonary vasculature was perfused to remove blood cells. The remaining lung tissue was carefully dissected free of the trachea and hilar region, including the mainstem bronchi, before preparing the cell suspensions to avoid contamination with LN cells. Cell suspensions were fractionated on discontinuous Percoll density gradients. Four fractions were tested for stimulatory activity in an MLR. The high density fraction of lung cells ( $\rho > 1.075$  g/ml), which consisted primarily of lymphocytes, had little stimulatory activity (Fig. 1 A). All three fractions with densities  $< 1.075$  g/ml were enriched in AC activity; thus, in subsequent experiments a single low density fraction (1.035–1.075 g/ml) was collected. This low density fraction contained ~35% of the total lung cells. When BAC were fractionated, cells were present in only the two high density fractions ( $> 1.075$  and 1.050–1.075 g/ml). No enrichment was seen in either of these fractions (Fig. 1 B).

**Adherence Properties of the Lung AC.** Differential adherence properties have been useful in separating murine DC from other cell types, including macrophages and lymphocytes. Mouse splenic DC are loosely adherent while mouse Peyer's patch DC and Langerhans cells are nonadherent (12, 14, 43, 44). The low density minced lung cells or the unfractionated BAC were separated into NAd, LAd, and FAd and tested for AC activity in an MLR. LAd from both minced lung and BAC were enriched in AC activity (Fig. 2). LAd comprised ~10% of both the low density lung cells and total BAC. NAd were also somewhat enriched in AC activity, while the FAd had little activity. There was a concern that scraping cells with a rubber policeman might damage them and reduce AC activity; therefore, in some experiments (including the one shown in Fig. 2), FAd that had not been mechanically dislodged were used. The low density lung cells or BAC were initially plated in microtiter plates and incubated overnight. All nonadherent cells were removed, counted, and subtracted from the number of cells originally placed in the wells to determine the number of FAd remaining in the wells. Results were similar to other experiments where FAd were obtained by scraping the tissue culture dishes with a rubber policeman.

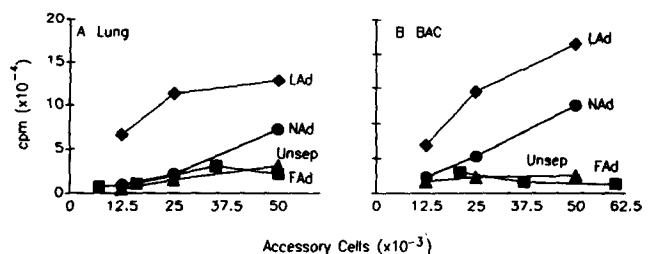
**Composition of Lung and BAC Populations.** Unfractionated cells and LAd from lung and BAC were examined by phase



**Figure 1.** Comparison of the Percoll density fractions from lung and BAC in stimulating an MLR. A representative experiment from a total of five is shown. T cell-enriched spleen cells ( $3 \times 10^5$ /well) from CBA mice were used as responders. Lung cells from enzymatically digested lungs (A) and BAC (B) were fractionated on discontinuous Percoll gradients. Fractions were:  $< 1.04$ , 1.04–1.05, 1.05–1.075, and  $> 1.075$ . Cells from BAC were found in only the two high density fractions. [<sup>3</sup>H]TdR incorporation for responder cells alone was 7,000 cpm.

and fluorescence microscopy to determine the cell types present in each population. It was essential to characterize the Ia<sup>+</sup> cells because they are the principal stimulators of an MLR. Cells were incubated with latex particles to distinguish macrophages from other cell types and stained for Ia by indirect immunofluorescence. There was an increased percentage of Ia<sup>+</sup> cells in LAd from both lung and BAC as compared to unfractionated cells (Table 1). The majority of Ia<sup>+</sup> cells in both lung and BAC LAd were nonphagocytic dendritic-shaped cells. These cells were easily distinguished from other Ia<sup>+</sup> cells, not only by their characteristic dendritic shape, but also by the extreme intensity of the Ia staining (Fig. 3). Because of the low numbers of LAd obtained from BAC, further studies concentrated on identifying and characterizing the AC from minced lung rather than BAC.

**Silica Treatment.** Because phagocytosis of latex particles distinguished lung macrophages and DC, silica was used to deplete macrophages (16, 45, 46). Lung LAd were incubated with silica for 2–4 h. Preliminary studies demonstrated that shorter incubation times did not alter AC activity of lung LAd but were also insufficient to deplete phagocytic cells. Nonphagocytic LAd were enriched in AC activity as compared with untreated LAd (Fig. 4). Indirect immunofluorescent staining demonstrated a two- to fourfold enrichment



**Figure 2.** Comparison of adherence fractionated cells from lung and BAC in stimulating an MLR. A representative experiment from a total of four is shown. Low density lung cells (A) or BAC (B) were compared with NAd, LAd, and FAd. [<sup>3</sup>H]TdR incorporation for responder spleen cells alone was 7,642 cpm.

in the percentage of Ia<sup>+</sup> DC after silica treatment (Untreated LAd, 19 ± 7% DC; silica-treated LAd, 40 ± 9% DC; n = 4). Thus, silica treatment confirmed that the cells in LAd expressing AC function were not classical macrophages.

**Fractionation of Lung AC Using Fc Receptor Expression.** DC in the spleen do not express FcR, thus, antibody-coated SRBC have been used to separate the FcR<sup>-</sup> DC from macrophages, which are FcR<sup>+</sup> (43). In contrast, Langerhans cells express FcR, and mouse tracheal DC have been reported to be FcR<sup>+</sup> using the mAb 2.4G2, which recognizes the murine FcRII (5, 47). Lung cells were fractionated into FcR<sup>+</sup> and FcR<sup>-</sup> populations by rosetting lung LAd with IgG-coated SRBC (EA). Spleen LAd were treated similarly to verify the reliability of the method. FcR<sup>-</sup> cells in the spleen were enriched in AC activity, while FcR<sup>+</sup> cells were relatively depleted (Fig. 5). In four experiments, no difference in AC activity was seen when comparing FcR<sup>+</sup> and FcR<sup>-</sup> subpopulations from lung LAd. Indirect immunofluorescence demonstrated similar percentages of Ia<sup>+</sup> DC in rosetted and nonrosetted populations. In a representative experiment, 79% of the rosetted DC stained with 2.4G2, compared with only 7% of the nonrosetted DC, verifying that the majority of FcR<sup>+</sup> DC rosetted and the majority of FcR<sup>-</sup> DC did not. These results indicated that lung DC were heterogeneous in the expression of FcR.

**Effect of Depleting LAd of Cells that Express 33D1, F4/80, J11d, and Low Affinity IL-2R on AC Function.** The mAb 33D1, in the presence of complement, depletes AC activity from splenic and Peyer's patch cells, but has no effect on the AC activity of Langerhans cells (14, 15, 44). Treatment of lung LAd with 33D1 and complement failed to deplete AC activity, although similar treatment markedly decreased the capacity of splenic LAd to stimulate an MLR (Fig. 6). The mAb F4/80 recognizes freshly isolated Langerhans cells (as well as most macrophages), but not splenic or thymic DC. When lung LAd were incubated with F4/80 followed by a secondary antibody (to facilitate complement fixation) and complement, AC activity was slightly increased (Fig. 7). In a typical experiment, F4/80 and complement treatment decreased F4/80<sup>+</sup> cells from 44 to 9% and increased the per-

centage of DC from 8 to 17%. However, in the experiments using F4/80, cell numbers were not readjusted after antibody and complement treatment and the absolute numbers of Ia<sup>+</sup> DC were unchanged. Therefore, the slight increase in the MLR demonstrated that the cells killed by the treatment had no positive effect on the MLR. Although B cells made up a very small percentage of LAd (typically 2–7%), it was important to demonstrate these cells were not contributing to the AC activity in lung LAd. Murine B cells are recognized by the mAb 14.8. Removal of B cells by panning or complement lysis with this mAb did not change AC activity. Both methods were effective at depleting Ig<sup>+</sup> cells to undetectable levels (data not shown). Thus, lung AC did not express typical markers for splenic DC (33D1), freshly isolated Langerhans cells or macrophages (F4/80), or B cells (14.8).

The mAb J11d recognizes a cell surface marker on many cells including most B cells, thymocytes, thymic DC and Langerhans cells, but not splenic DC. When lung LAd were treated with J11d and complement, AC activity was decreased 40%, suggesting that a portion of the lung AC express the J11d marker (Fig. 8). In one experiment, J11d and complement treatment was shown to decrease the percentage of Ia<sup>+</sup> DC from 23 to 9%. As expected, splenic AC activity was unaffected by this treatment (data not shown). Both splenic and thymic DC express low affinity IL-2R, recognized by the mAb 7D4. When lung LAd were treated with 7D4 and complement, AC activity was decreased 63% (Fig. 8). In this set of experiments, treatment of LAd with anti-Ia and complement as a control resulted in a 68% decrease in the MLR. In one experiment, treatment with 7D4 or anti-Ia and complement reduced Ia<sup>+</sup> DC from 23% to 5 and 7%, respectively. In summary, the majority of lung AC activity was expressed by J11d and low affinity IL-2R<sup>+</sup> cells.

**Phenotype of Lung DC.** The percent of Ia<sup>+</sup> DC that expressed various markers previously used to characterize macrophages, DC, and other leukocytes was determined (Table 2). Two-color immunofluorescence showed that the Ia<sup>+</sup> DC did not express the macrophage/Langerhans cell marker F4/80, the T lymphocyte markers, Thy-1.2, CD4, and CD8, or the B cell marker recognized by the mAb 14.8. Over half the

**Table 1.** Percentage of Ia<sup>+</sup> Cells in Lung and BAC Populations

	Cell yield per mouse*	Total Ia <sup>+</sup>	Ia <sup>+</sup> Macrophages <sup>‡</sup>	Ia <sup>+</sup> DC <sup>§</sup>	Ia <sup>+</sup> Other <sup>  </sup>	MLR stimulation <sup>¶</sup>
Lung	10 <sup>7</sup>	15 ± 6	1 ± 1	1 ± 2	13 ± 5	+
Lung LAd	2 × 10 <sup>5</sup>	30 ± 7	4 ± 1	18 ± 3	7 ± 5	+++
BAC	10 <sup>6</sup>	2 ± 1	1 ± 1	1 ± 2	<1	-
BAC LAd	10 <sup>5</sup>	8 ± 3	<1	6 ± 3	2 ± 1	+++

The data shown are the percentages of Ia<sup>+</sup> cells in each population as determined by indirect immunofluorescence, n = 3.

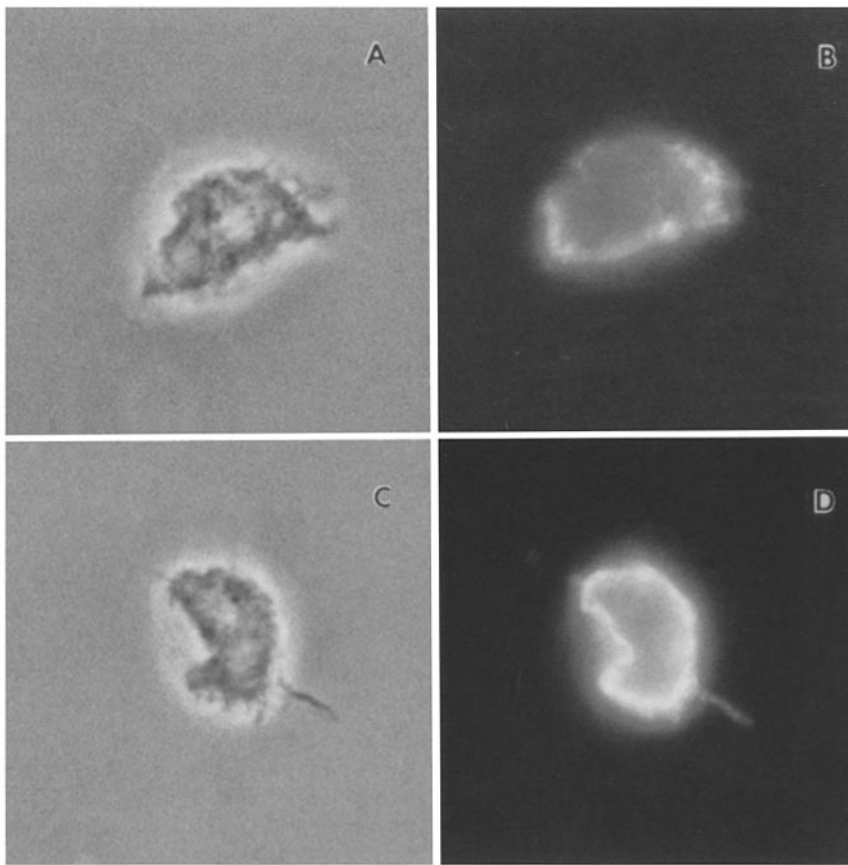
\* Average number of cells obtained per mouse.

‡ Determined by uptake of three or more latex particles.

§ Nonphagocytic, dendritic-shaped cells.

|| Non-phagocytic, non-dendritic-shaped cells, which likely include B lymphocytes and weakly phagocytic macrophages.

¶ Relative capacity to serve as stimulators of a primary allogeneic MLR. See Figs. 1 and 2.

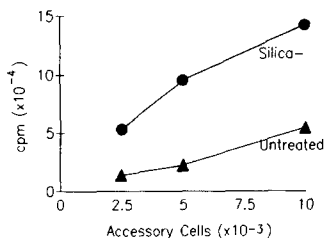


**Figure 3.** Phase and fluorescent micrographs of two typical pulmonary DC. LAd were treated with latex particles for 2 h, followed by indirect immunofluorescence to detect Ia. The phase micrographs (A and C) show the dendritic shape and absence of phagocytosed latex particles. The fluorescent micrographs (B and D) demonstrate Ia staining in fields identical to A and C, respectively ( $\times 900$ ).

Ia<sup>+</sup> DC expressed J11d (67%), FcR2 (53%), C3biR (61%), and the low affinity IL-2R (81%). The IDC marker NLDC-145 was clearly detected on 40% of the Ia<sup>+</sup> DC. Because NLDC-145 staining was particularly faint, it was difficult to distinguish from the bright Ia staining; thus, 40% is a minimal percentage.

### Discussion

This study demonstrated that enzyme-digestion of murine lung released cells that expressed a potent primary MLR-stimulating capacity. Various fractionation procedures designed to separate the potential AC candidates led to the conclusion



**Figure 4.** Comparison of untreated and silica-treated lung LAd. A representative experiment of six is shown. Lung LAd were incubated with silica particles and live cells collected on a Nycodenz gradient. [<sup>3</sup>H]TdR incorporation for responder cells alone was 618 cpm.

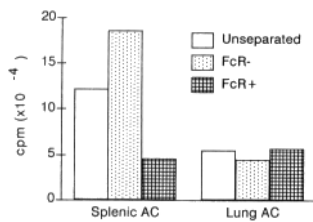
that DC, not macrophages, were responsible for the potent AC activity. AM, which are poor AC, were removed by bronchoalveolar lavage before the lungs were processed. These results confirmed that AM are poor AC; however, a small

**Table 2.** Cell Surface Antigen Expression by Lung LAd

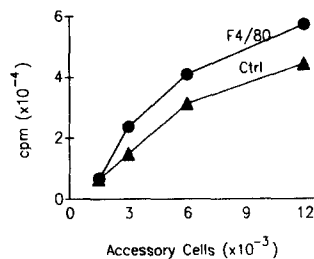
Hybridoma	Specificity	Percent lung DC
2.4G2	FcR2	53 ± 5*
M1/70	C3biR	61 ± 13
F4/80	MO	2 ± 3
NLDC-145	IDC	40 ± 9
7D4	IL-2R (p55)	81 ± 10
J11d	B, thymocytes	67 ± 21
14.8	CD45 (B cells)	<2
30-H12	Thy-1.2	<2
GK1.5	CD4	<2
YTS169.4	CD8	<2

Combined two-color immunofluorescence and latex bead uptake was used to determine a panel of surface markers on Ia<sup>+</sup>, nonphagocytic DC.

\* Average ± SD of at least three separate determinations.



**Figure 5.** Comparison of FcR<sup>+</sup> and FcR<sup>-</sup> subpopulations from spleen and lung LAd in stimulating an MLR. A representative experiment of four is shown. Spleen and lung LAd were incubated with EA, then rosetted and nonrosetted cells were separated on a Percoll gradient. Data are shown for  $2.5 \times 10^4$  AC/well. [<sup>3</sup>H]TdR incorporation of responder spleen cells alone was 5,942 cpm.

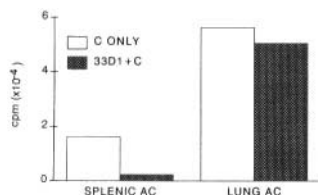


**Figure 7.** Effect of depleting F4/80-bearing cells on accessory cell activity in lung LAd. A representative experiment from four is shown. LAd were treated with either F4/80 or rat IgG for 45 min, washed, and incubated with rabbit anti-rat IgG and C' for 1 h at 37°C. The remaining cells were tested for AC activity in an MLR. [<sup>3</sup>H]TdR incorporation for responder cells alone was 1,565 cpm.

subpopulation of BAC was shown to contain potent stimulators of an MLR. Depletion of additional macrophages from the lung cells due to their continued adherence to plastic resulted in enrichment of AC activity. FAd were relatively devoid of AC activity. Further depletion of macrophages by treatment with silica or with F4/80 and complement resulted in even greater MLR-stimulating capacity. Thus, it was unlikely that typical macrophages were responsible for the AC activity. In contrast to macrophages, DC are generally loosely adherent, nonphagocytic, and lack F4/80; therefore, we examined LAd for Ia<sup>+</sup> dendritic-shaped cells.

Using a combined latex bead uptake and immunofluorescence technique, nonphagocytic, brightly Ia<sup>+</sup> dendritic-shaped cells were identified in lung LAd. These DC were virtually undetectable in the unfractionated lung cells, but comprised ~17% of lung LAd and 40% of silica-treated LAd. Two-color immunofluorescence revealed that the majority of Ia<sup>+</sup> lung DC shared several markers with thymic DC and epidermal Langerhans cells (12, 25). Simultaneous enrichment for Ia<sup>+</sup> DC and AC activity strongly implicated DC as the lung AC, and depletion studies with mAb and complement supported these observations.

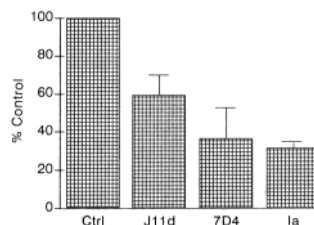
Depletion of J11d<sup>+</sup> cells, which included 67% of the DC, resulted in a 40% decrease in AC activity. Depletion of low affinity IL-2R<sup>+</sup> cells, which included 84% of Ia<sup>+</sup> lung DC, resulted in a 63% decrease in MLR-stimulating capacity, a reduction in activity comparable to treatment with anti-Ia and complement. In contrast to the spleen, lung AC activity



**Figure 6.** Effect of the mAb 33D1 and complement on the AC activity of spleen and lung LAd. A representative experiment from eight is shown. Spleen and lung LAd were treated with 33D1 and complement or complement alone. The remaining cells were tested for AC activity in an MLR using  $2.5 \times 10^4$  AC/well. [<sup>3</sup>H]TdR incorporation of the responder spleen cells alone was 2,212 cpm.

was not decreased by treatment with 33D1 and complement, consistent with reports that many DC found outside the spleen do not express 33D1 (12). These results raise three important questions: (a) Why are lung DC heterogeneous? (b) Why are lung DC difficult to purify to homogeneity? and (c) What is the role for DC in pulmonary immunity?

The heterogeneity in surface marker expression among lung DC is shown in Table 1. One trivial explanation for this heterogeneity is incomplete re-expression of protease-sensitive markers after collagenase treatment. However, some of the markers, such as FcR2, are not protease-sensitive (48, 49). Therefore, we propose that the phenotype of a lung DC depends on two factors: (a) the developmental state of the DC; and (b) the location of the DC within the lung. Studies by others indicate that both of these factors are important (23, 25). Developmental or maturational changes in phenotype have been documented for Langerhans cells (discussed in detail below). DC isolated from epidermis (Langerhans cells) or tracheal epithelium are likely in the same stage of development, and are, therefore, homogeneous. In contrast, DC isolated from whole lung likely include not only resident DC, but also cells migrating into or out of the lung, which could contribute to heterogeneity. Relative to site within the lung, Holt and Schon-Hegrad observed differential expression of two macrophage markers, ED1 and ED2, by DC in the re-



**Figure 8.** Effect of depleting J11d, IL-2R, and Ia-expressing cells on AC activity of lung LAd. LAd were treated with J11d, 7D4, 25-5-16S, or H59 as a control plus complement. The remaining cells were tested for AC activity in an MLR using  $2.5 \times 10^4$  AC/well. Data are expressed as percent of control response (Ab + C'-treated cpm/control cpm  $\times 100$ )  $\pm$  SEM in order to compare results of multiple experiments. Data from two to four experiments were combined: for anti-Ia,  $n = 2$ ; for 7D4,  $n = 3$ ; for J11d,  $n = 4$ . [<sup>3</sup>H]TdR incorporation for responder cells alone was <10% of control in each experiment.

spiratory tract of the rat (23). Specifically, ED1 stained DC in tracheal epithelium and mucosa, in bronchus-associated lymphoid tissue, and in airway epithelium; ED2 stained DC in alveolar walls and the pleura. Both ED1<sup>+</sup> and ED2<sup>+</sup> DC were observed in peribronchial and perivascular areas. Within each of these two subpopulations, there was additional heterogeneity in expression of other markers, such as CD4, C3b1R, and OX41. Thus, lung DC isolated in the present study may include cells analogous to the upper and lower respiratory tract DC described in the rat as well as DC in transit to or from a relatively fixed position in the lung, such as in epithelium and/or loosely organized lymphoid tissue.

The heterogeneity was a hindrance in the attempt to purify lung DC. Many DC shared markers with lung macrophages, such as FcRII and C3b1R. In addition, AM expressed the IDC marker NLDC-145 and lacked C3b1R. Attempts to purify lung DC using anti-Ia reagents were frustrated because macrophages and DC tended to aggregate (likely because macrophage FcR bound the Ig-coated DC). Methods that resulted in the death of one or the other cell type, such as silica or mAb and complement treatment were the most effective ways of analyzing the respective roles of macrophages and DC in MLR-stimulation, but these methods did not allow comparison of positively and negatively selected cells. Nevertheless, when a large percentage of DC were depleted, AC activity was greatly decreased, and when a large percentage of macrophages were depleted with silica, AC activity was markedly increased.

A role for DC in the lung can be inferred by: (a) comparing lung DC to other types of DC, particularly Langerhans cells; (b) considering the location of DC within the lung; and (c) analyzing what is known about the initiation of immune responses in the lung. Phenotypically, the majority of lung DC most closely resemble epidermal Langerhans cells and a recently isolated population of thymic DC (12, 25). Crowley et al. (12) suggested there are two types of DC: (a) a "fixed" DC (33D1<sup>-</sup>, J11d<sup>+</sup>, NLDC-145<sup>+</sup>) located in tissues such as thymus and skin, as well as in the T cell areas of spleen and lymph node (IDC); and (b) a "migrating" DC (33D1<sup>+</sup>, J11d<sup>-</sup>, NLDC-145<sup>-</sup>), typified by the major population of DC isolated from the spleen and thought to be located in the marginal zone (24). Lung AC are 33D1<sup>-</sup> and many are J11d<sup>+</sup> and NLDC-145<sup>+</sup>, resembling the first group of DC. Among the fixed or interdigitating type of DC, epidermal Langerhans cells have been best characterized. Langerhans cells are thought to process exogenous antigens in the skin and migrate to draining LN where they activate antigen-specific T lymphocytes (50). In vitro studies that supported this concept demonstrated that both the phenotype and function of Langerhans cells were altered during culture, presumably analogous to changes seen in vivo during migration to draining LN. Romani et al. demonstrated that freshly isolated Langerhans cells were efficient at processing and presenting intact myoglobin to T cell clones, but were poor stimulators of a primary MLR (26). However, after 3 d in culture with keratinocytes or granulocyte/macrophage CSF (GM-CSF), the Langerhans cells lost their ability to process myoglobin, and became potent stimulators of a primary MLR.

Ia expression was dramatically increased within the first 12–18 h of culture, and FcRII and F4/80 were lost during the first 1–2 d (25). The authors suggested that secretion of GM-CSF by keratinocytes could initiate this maturation process in vivo after Langerhans cells came into contact with exogenous antigens. Thus, following antigen processing, Langerhans cell Ia expression would increase, resulting in a high density of specific antigen/Ia complex on the cell surface. Subsequently, the Langerhans cell would lose its capacity to process antigens, including self antigens, and acquire the capacity to activate virgin and resting T cells in the draining LN. This sequence of events would provide a very potent and specific activation signal.

We suggest a similar role for DC in the lung because of their phenotypic similarities to Langerhans cells. Although we have not been able to directly address the effect of GM-CSF on lung DC, we have observed that, similar to bulk epidermal cell cultures, overnight incubation of lung cells results in an increased capacity to stimulate an MLR (Data not shown). Addition of GM-CSF during this incubation did not further increase the AC function. However, it is possible that optimal amounts of GM-CSF are made by the lung cells, thus additional GM-CSF has no effect. Studies using anti-GM-CSF during the overnight culture will be required to directly address the effect of GM-CSF on lung DC.

The fact that both skin and lung have large surface areas exposed to the environment results in the need for efficient stimulation of local immune responses. DC in the epithelium lining the airways and alveoli, as well as DC in lung parenchyma and bronchus-associated lymphoid tissue, may survey their environment for antigens that have traversed the epithelium. The hypothesis that lung DC carry processed, exogenous antigens to draining LN to stimulate immune responses is consistent with a body of evidence concerning antibody responses after lung immunization (51). In several species, when SRBC were instilled into the lung a primary antibody response occurred predominantly in the draining LN, rather than within pulmonary parenchyma or in systemic lymphoid tissue. Although both AM and polymorphonuclear leukocytes can carry antigen to draining LN, neutrophils cannot stimulate immune responses and, except in the guinea pig, there is little evidence that AM can stimulate primary immune responses (52–54). In contrast, DC are capable of stimulating primary immune responses, as demonstrated by their capacity to stimulate both an autologous and a primary allogeneic MLR (15, 55).

The ability of murine lung DC to process and present antigens has not been thoroughly examined, although mouse tracheal DC have been shown to present antigen to T cell hybridomas (5). Preliminary results suggest that lung LAD (which have been effectively cultured for 24 h) are superior to splenic DC in presenting both soluble and particulate antigens to primed LN T cells. However, further purification of DC will be necessary to determine whether the same cells that stimulate the MLR are responsible for presenting antigen. It will also be important to determine whether freshly isolated lung AC, like freshly isolated Langerhans cells, differ in function from the overnight-cultured cells.

This work was supported by National Institutes of Health grants AI-21951 and CA-09082, and by a grant from Texas Department, Ladies Auxiliary of the Veterans of Foreign Wars.

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Received for publication 28 December 1989 and in revised form 8 March 1990.

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