

Intraepithelial Airway Dendritic Cells: A Distinct Subset of Pulmonary Dendritic Cells Obtained by Microdissection

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Summary

Dendritic cells (DC), in general, and pulmonary DC, in particular, are a heterogeneous population of cells, their phenotype and function being dependent on their anatomic location, their state of activation, and the regulatory effect of locally secreted cytokines. Using a novel microdissection technique, the epithelium from the trachea and entire airway system was harvested, and the contained DC isolated at >90% purity. The phenotype and function of these airway DC (ADC) was compared to DC isolated, at >90% purity, from the parenchyma of the same lung. In contrast to lung DC (LDC), ADC did not express intercellular adhesion molecule 1 (ICAM-1) in situ, the amount of immune associated antigen (Ia) expressed was less (as determined by immunoperoxidase staining and immunopanning), and >50% of ADC displayed Fc receptors (FcR). The majority of LDC were ICAM-1⁺, <5% expressed FcR, and all were intensely Ia⁺. Airway DC were most numerous in tracheal epithelium, but they were also present in small numbers in the epithelium of the most distal airways. Their numbers increased in all segments of the tracheobronchial epithelium in response to the administration of IFN- γ . ADC were consistently more effective than LDC in presenting soluble (hen egg lysozyme) and particulate (heat-killed *Listeria monocytogenes*) antigens to antigen-sensitized T cells. By contrast, LDC were significantly more efficient in stimulating the proliferation of nonsensitized T cells in an autologous mixed leukocyte reaction. These data suggest that in normal animals, intraepithelial DC of airways share many attributes with Langerhans cells of the skin. Interstitial LDC, by contrast, reside in an environment where they may be exposed to a different set of regulatory factors and where they have progressed to a more advanced stage of differentiation than ADC. Both groups of DC are, however, heterogeneous, reflecting the continuous turnover that these cells undergo in the lung.

A wide variety of antigens continuously impinge on the epithelial surfaces of the skin, gastrointestinal tract, and lung. In the lung, a number of defense mechanisms, including mucociliary clearance (1), secreted antibodies in the alveolar lining fluid (2), phagocytosis by macrophages (3), and the barrier function of epithelial tight junctions (4), minimize the penetration of these antigens beyond the epithelium. Once these barriers are breached, however, the respiratory immune system must discriminate between pathogenic (viruses, bacteria) and innocuous (pollen) antigens. Regulation of an appropriate T cell response to foreign antigens is mediated, in part, by a population of dendritic cells (DC)¹ localized

within the airway epithelium (5, 6), within the alveolar septae, and in the connective tissue around airways and large vessels (7–9). DC, first discovered in the spleen (10), are highly efficient accessory cells that constitutively express high levels of surface MHC class II glycoprotein (11). Derived from the bone marrow (12), they are widely distributed throughout most organs (11, 13, 14), where they exist in various states of differentiation (15) and activation (16).

A phenotypically diverse population of DC appears to exist in the lung. Cytochemical studies indicate that DC in airway epithelium express Fc receptors (FcR) (5) and are organized, by way of slender dendritic processes, into a contiguous network similar to that formed by Langerhans cells in the skin (6). Furthermore, recent reports suggest that ~50% of pulmonary DC, obtained after enzymatic digestion of whole lung, express FcR (9, 17). In none of these studies, however, was the lung parenchyma freed of airway epithelium with

¹ Abbreviations used in this paper: ADC, airway DC; DC, dendritic cells; FcR, Fc receptors; HEL, hen egg lysozyme; HKL, heat-killed *Listeria monocytogenes*; Ia, immune associated antigen; LDC, lung DC; NCS, newborn calf serum; P/S, penicillin/streptomycin.

its contained subset of DC, nor has it been possible to obtain highly enriched DC specifically from the airway epithelium in order to examine their accessory cell capabilities. Recent studies indicate that the state of differentiation of accessory cells may be regulated, in part, by cytokines such as granulocyte/macrophage-CSF (18–20). As these cells mature, they generate additional accessory signals, including cell adhesion molecules, which are required for their effective interaction with naive T cells (16). It is, therefore, of considerable interest to establish whether subsets of DC are preferentially localized to specific compartments of the lung, where their state of activation might be subject to the control of locally secreted cytokines.

In the present study, a novel microdissection method (21) was modified in order to exclusively isolate DC from airway epithelium and to compare the phenotype and function of these cells with those separated from the parenchyma of the same lung. The results indicate that DC in airway epithelium constitute a distinct subset of pulmonary DC that share a number of characteristics with Langerhans cells of the skin (22, 23).

Materials and Methods

Animals. Pathogen-free, 6–8-wk-old female Lewis rats (160–180 g) were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals, housed in restricted access research animal care facilities at Massachusetts General Hospital, were permitted free access to food and water and underwent monthly monitoring for viral infections.

Reagents and Antibodies. BSA, fraction V, for density gradients was obtained from Armour Pharmaceutical Co. (Kankakee, IL). Enzymes used were as follows: dispase, grade II (Boehringer Mannheim Biochemicals, Indianapolis, IN); collagenase (Worthington Biochemical Corp., Freehold, NJ); and DNAase I (Sigma Chemical Co., St. Louis, MO). Low melting temperature agarose Sea-Plaque GtG was from FMC BioProducts (Rockland, ME). Cytokines used were TNF- α (Genzyme Corp., Boston, MA), rIFN- γ (a gift of Genentech, Inc., South San Francisco, CA), and rIL-2 (a gift of Hoffmann-La Roche, Nutley, NJ). Embedding medium was O.C.T. compound (Tissue-Tek, Miles Inc., Elkhart, IN). Cell culture reagents included RPMI 1640 medium (JRH Biosciences, Lenexa, KS), FCS, HBSS and penicillin/streptomycin (P/S) (Gibco Laboratories, Grand Island, NY), and newborn calf serum (NCS) (Hyclone Laboratories, Inc., Logan, UT). SRBC (Whittaker Bioproducts, Walkersville, MD) were used. mAbs (their specificities are indicated in Table 3), included rabbit anti-SRBC IgG (Diamedix Corp., Miami, FL), OX-1 (24), OX-22 (25), OX6 (26), W3/13 (27), W3/25 (28), OX8 (28), OX41 (29), and OX43 (30), all from Accurate Chem. & Sci. Corp. (Westbury, NY). mAbs 1A29, WT.1 (31), and WT.3 (31) were from Dr. M. Miyasaka. mAb C11b (32) was a generous gift from Drs. E. M. Goodell (Mary Imogene Bassett Hospital, Cooperstown, NY) and W. E. Bowers (University of South Carolina School of Medicine, Columbia, SC). RMA mAb (33) was produced in this laboratory. They were all used at 1:100 and 1:400 dilution, except for C11b, which was used at 8–16 μ g/ml, and 1A29, WT.1, and WT.3, which were used at 20 μ g/ml.

Microdissection Procedure. A modification of the microdissection procedure developed by Plopper et al. (21) was used. Briefly, female

Lewis rats were anesthetized with sodium pentobarbital, 5 mg/100 g body weight. After exsanguination via the abdominal aorta, the diaphragm was incised and the lungs lavaged with six 5-ml aliquots of 10 mM Na₂EDTA in PBS. The last 5 ml was left in the airway for 15 min at 20°C to open epithelial cell tight junctions and to facilitate subsequent access of dispase to the underlying basement membrane. It was withdrawn from the lung before initiating the next step. The thorax was opened, the left ventricle incised, and the pulmonary vasculature perfused via the pulmonary artery with HBSS until the lung blanched completely. A volume of 8–10 ml of agarose solution (1% agarose, 2.5 U/ml dispase, 50 U/ml DNAase, 2% dialyzed FCS) at 37°C was gently infused into the airways and lungs. After the trachea were tied off, the lung and attached trachea were removed and immersed for 30–60 min in ice-cold PBS to solidify the agarose. Under sterile conditions, the heart was removed and the lung parenchyma carefully dissected from the tracheobronchial tree. In this manner, the entire tracheobronchial tree was separated intact from the lung (Fig. 1 a). DC were then isolated separately from the lung fragments and tracheobronchial tree as described below.

Interstitial Lung DC. The pieces of lung were further minced into 1-mm³ fragments and incubated in an enzyme solution, 4 ml/g lung tissue, containing 150 U/ml of collagenase in RPMI 1640 medium with 10% FCS, 1% P/S, 5 mM Hepes, and 5×10^{-5} M 2-ME (complete medium) as previously described (34). Low-density cells were harvested by equilibrium density centrifugation (see below).

Airway Epithelial DC. After removal of the lung, the tracheobronchial tree was incubated in complete RPMI medium with 5% FCS for 1 h in a humidified incubator with 95% air, 5% CO₂. During this step, dispase, dispersed in the agarose, permeated the airway epithelium and cleaved the latter from the underlying basement membrane (Fig. 1, b–d). The tracheobronchial tree was gently perfused with three 35-ml aliquots of PBS, 5% NCS. The cell suspension was passed through four layers of gauze and washed twice with PBS, 5% NCS. To separate DC from airway epithelium, the cell suspension was concentrated by centrifugation into 10 ml of 10 mM EDTA in Earle's balanced salt solution and incubated for 30 min at 37°C in a shaking water bath. The single-cell suspension was washed twice in PBS and 5% NCS, and the low-density cells were separated by equilibrium density centrifugation.

Separation of Low-Density Cells. BSA solutions were prepared by the procedure of Steinman and Cohn (10), to yield a pH of 7.35 ± 0.5 and a density of 1.082 g/cm³, as determined by refractometry (model ABBE-3L; Bausch & Lomb Inc. Instruments & Systems Div., Rochester, NY). Approximately 4×10^7 cells/ml of lung or airway cells were mixed with the dense BSA solution, and 2.5-ml aliquots were distributed into separate tubes. These were overlaid with 1 ml of diluted BSA solution (density 1.048 g/cm³ and centrifuged at 10,000 g for 30 min at 4°C. Low-density cells at the interface were harvested and washed twice with PBS. They were plated in a 100-mm plastic petri dish in complete medium for 2 h. Nonadherent cells were replated in a second petri dish for 2 h, after which nonadherent cells were discarded and the adherent cells in both petri dishes were incubated overnight \pm TNF- α , 80 U/ml, in 5% CO₂, 95% room air at 37°C.

Immunopanning for Immune-associated Antigen-positive (Ia⁺) Cells. The next day, nonadherent lung and airway cells were harvested from the plastic petri dishes by gently washing with 3% FCS in PBS. Preliminary experiments showed that, whereas coating petri dishes with a 1:400 dilution of OX-6 (anti-Ia) mAb was optimal for lung DC (LDC), a dilution of 1:100 was required for recovery of airway DC (ADC). Panning was for 1 h at 4°C, at

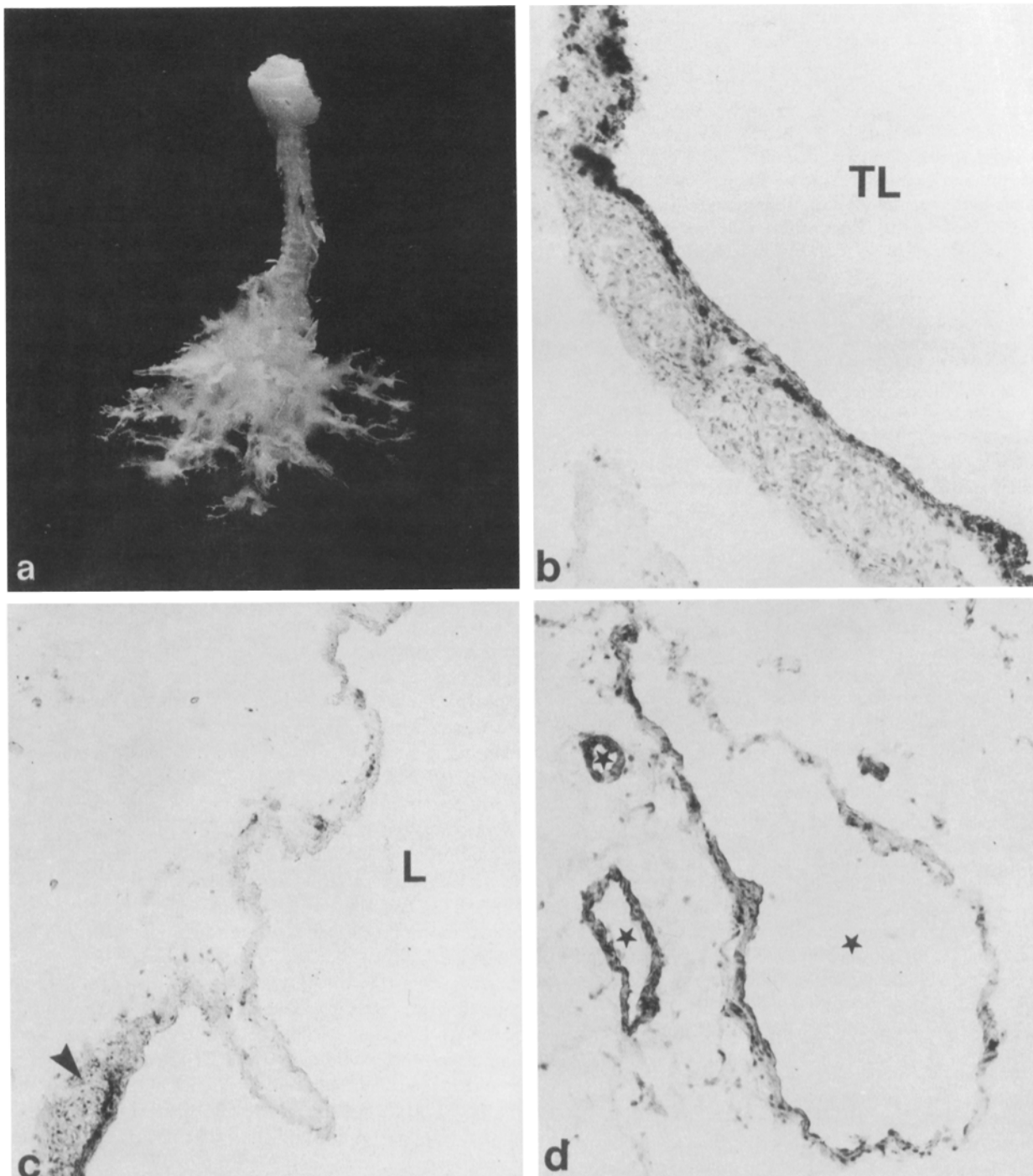


Figure 1. (a) Tracheobronchial tree dissected free of lung tissue before the isolation of ADC. (b) Segment of trachea, stained for Ia, after enzymatic detachment of epithelial cells. Ia⁺ cells below the basement membrane remain. (c) Segment of bronchus, with a portion of cartilage in the wall (arrowhead), from the same lung as in a. (d) Small airways in the distal portion of the tracheobronchial tree (asterisks). As in more proximal segments, only epithelium with the contained DC has been removed. (a) $\times 2$; (b and d) $\times 138$; (c) $\times 87$.

which time nonadherent cells were subjected to two additional 1-h cycles of OX-6 panning. All adherent cells were pooled by gently scraping with a plastic cell scraper (Nunc, Inc., Naperville, IL), counted, and viability assessed by trypan blue dye exclusion.

Immune T Cell Isolation. Immune T cells were prepared to either a particulate antigen, heat-killed *Listeria monocytogenes* (HKL),

or to a soluble antigen, hen egg lysozyme (HEL) by immunizing adult Lewis rats with HKL (10^7 organisms) or HEL ($100 \mu\text{g}$) in CFA in the base of the tail as described (35). Immune T cells were cultured in the presence of IL-2, stimulated every 3 wk with antigen, and shown by flow cytometry to be $>95\%$ W3/13⁺, W3/25⁺.

Proliferation Assays. Airway and pulmonary DC were irradiated (1,000 rad), washed, and plated (10^4 /well) in triplicate in 96-well flat-bottomed culture plates (Becton Dickinson & Co., Lincoln Park, NJ) in complete medium. HEL or HKL immune T lymphoblasts (5×10^4 /well) were added with either HEL (100 μ g/ml) or HKL (10^7 /well) (34). A primary one-way MLR was evaluated using irradiated airway or pulmonary DC (10^4 /well) from Lewis rats and splenic T cells (5×10^4 /well) obtained from normal Long Evans rats by separation on a nylon wool column (Fenwal Inc., Deerfield, IL). Residual Ia⁺ cells were removed by panning on OX6-coated plates (35). By immunocytofluorometry, the lymphocytes obtained were 98% W3/13⁺ and <1% of the cells were Ia⁺. Incubation time for antigen-mediated assays was 72 h and that for the primary MRL was 6 d before harvest. Wells were pulsed with [³H]thymidine (1 μ Ci/well; sp act of 50–80 Ci/mmol; New England Nuclear, Boston, MA) for 6 h, harvested using a cell harvester (Skatron AS, Lierbyen, Norway), and counted in a Tri-Carb liquid β -scintillation spectrometer (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL).

Detection of FcR. Sheep E were coated with a subagglutinating dose of rabbit anti-E-IgG as described (36), except that the IgG-coated erythrocytes and DC were centrifuged for 2 min at 200 g and incubated for 1.5 h at 4°C. A minimum of 100 cells was evaluated for rosetting in each preparation, and counts were obtained from three separate isolations. Alveolar macrophages served as positive controls, and noncoated E as negative controls.

Immunocytochemical and Nonspecific Esterase Staining. Frozen sections, 4- μ m thick, of lung and trachea were prepared. Some tracheal sections were cut at the level of and parallel to the plane of the epithelium (6). Frozen sections and cytocentrifuge preparations of isolated DC were air dried and fixed in acetone. They were stained by an indirect avidin-biotin immunoperoxidase method (Vector Laboratories, Inc., Burlingame, CA) with appropriate dilutions of the primary mAb (34). Air dried, cytocentrifuged cells were stained for nonspecific esterase, using α -naphthyl acetate as substrate (37).

For immunoelectron microscopy, lung and trachea were fixed in periodate-lysine-paraformaldehyde fixative (38) for 4 h at 4°C and quenched in 0.1 M NH₄Cl/PBS. Sections, 100- μ m thick, were stained by an indirect avidin-biotin method using similar antibody dilutions to those utilized on frozen sections (34). Ultrathin sections were cut on an Ultratome V (LKB Instruments, Inc., Bromma, Sweden) and examined with an electron microscope (model 301; Philips Corp., Eindhoven, The Netherlands).

Morphometry. Two groups of three rats each were injected intraperitoneally for 5 d with either IFN- γ , 2×10^5 U/d, or PBS. After anesthetizing the rats, the lungs were removed and filled with a small amount of O.C.T. Compound. Sections, 1-mm thick, were cut perpendicular to the long axis of each lobe and frozen. Frozen sections, 4- μ m thick, were stained for Ia (34). Using the Bioquant System V (R & M Biometrics, Nashville, TN), the perimeter of each airway was measured and the number of Ia⁺ cells within and immediately underneath the epithelium was enumerated by touch counting. The number of ADC per millimeter of epithelium was calculated and plotted against the minimum diameter of the airways.

Statistical Methods. Data were analyzed using an unpaired *t* test available in the statistical software package INSTAT (Graphpad, San Diego, CA). Data are expressed as mean \pm SD.

Results

Isolation and Recovery of ADC and LDC. Recovery of DC was monitored by cell counts at each step of the isolation procedure, and the purity of the final preparation was evalu-

Table 1. Recovery and Purity of ADC and LDC

	Total cell yield per lung*	Ia ⁺ DC per lung†	Percent yield Ia ⁺ DC per lung	Purity‡
	$\times 10^{-6}$	$\times 10^{-4}$		%
Airway	3.2 ± 1.0	0.7 ± 0.2	0.23 ± 0.1	89.0 ± 4.6
Lung	55.0 ± 18.0	7.6 ± 2.7	0.14 ± 0.06	90.0 ± 3.5

* The data are derived from nine isolations and are expressed as the mean \pm SD.

† Cells retrieved after three to four sequential Ia panning steps.

‡ The purity was determined by immunoperoxidase staining of cytocentrifuged cells retrieved after the final Ia panning step. A minimum of 500 cells was counted in each preparation. All of the Ia⁺ cells had a dendritic cell morphology.

ated by cell counts on cytocentrifuge preparations stained for Ia. From a total of nine isolations, the average yield, based on the starting single cell suspension, was $0.23 \pm 0.1\%$ for ADC and $0.14 \pm 0.06\%$ for LDC (Table 1). In each of four cytocentrifuge preparations, a total of 500 cells was evaluated for the number of Ia⁺ cells with DC morphology. The results indicated that the mean purity of the isolated ADC was $89 \pm 4.6\%$ and for LDC it was $90 \pm 3.5\%$. Correcting for purity, the average yield of DC, therefore, was 6.2×10^3 DC/tracheobronchial tree and 6.8×10^4 DC/lung. To obtain sufficient numbers of DC to assay their accessory cell function, 10–12 rats were used per isolation.

Although insufficient numbers of cells were available for flow cytometry, it was observed that in order to retrieve Ia⁺ ADC by immunopanning, a higher concentration of OX6 mAb (1:100) was required than was necessary to recover LDC (1:400). This, together with the observation that the intensity of immunoperoxidase staining for Ia was generally less on ADC, suggested that the surface density of Ia on ADC was lower than that on LDC. However, the long, delicate cytoplasmic processes were a characteristic feature of both isolated ADC and LDC (Fig. 2, *a* and *b*).

Initial attempts to culture purified ADC failed, which suggested that these cells require the presence in vitro of specific cytokines. TNF- α was selected because of its ability to maintain the viability of Langerhans cells in culture without stimulating maturation (39). Addition of TNF- α to isolated ADC maintained not only their viability, but also the expression of FcR (Table 2). Furthermore, TNF- α , added to cultures of purified LDC, had little effect on their phenotype or function (Tables 3 and 4).

Fc Receptors. Expression of FcR by ADC, LDC, and alveolar macrophages, isolated in the presence of TNF- α from the same lung, was measured by rosetting with IgG-coated sheep Es (IgG-E) (Fig. 3, *a* and *b*). As indicated in Table 2, $56 \pm 14\%$ of ADC, in contrast to only $3.8 \pm 0.9\%$ of LDC, expressed FcR; $>94 \pm 3\%$ of alveolar macrophages formed IgG-E rosettes. In the course of these experiments, a batch of infected rats was mistakenly sent by Charles River Breeding

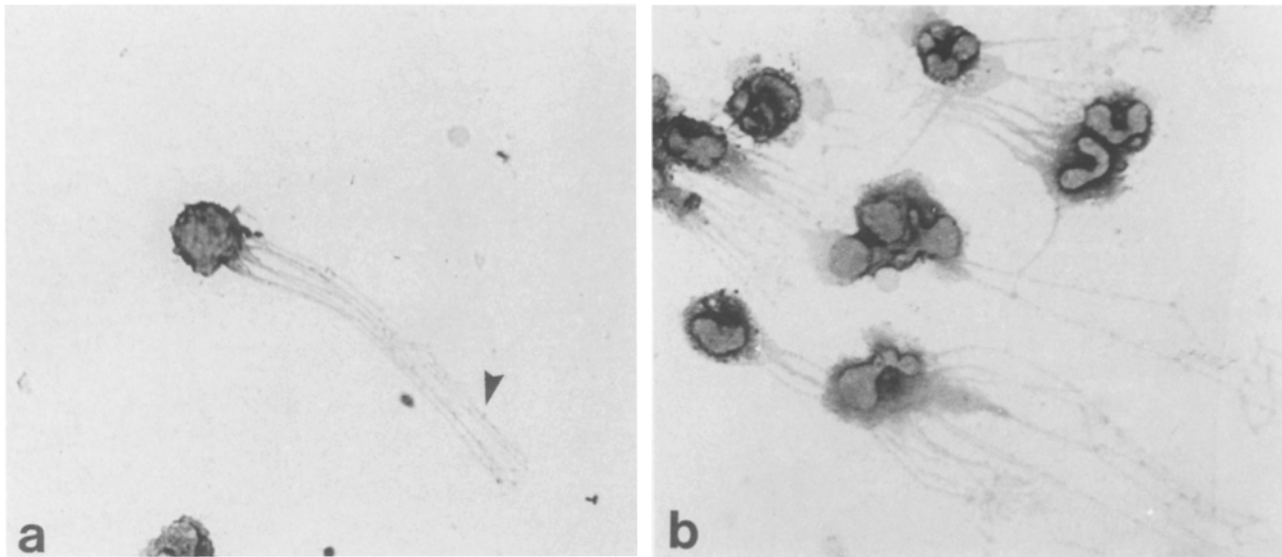


Figure 2. (a) Cytochrome preparation of ADC stained for Ia. Note the long, tenuous cell processes (arrowheads). (b) LDC stained for Ia. Note the multilobulated nucleus and the long, tenuous cell processes. $\times 538$.

Laboratories from their Wilmington facilities. Interestingly, 87% of ADC and 27% of LDC cells isolated from these rats expressed FcR, suggesting that during an ongoing pulmonary infection, there is an augmented traffic of recently released FcR⁺ DC to the lung. Alternatively, local proliferation of such cells cannot be ruled out.

After counts of IgG-E rosettes were completed at 4°C, the remaining cells were warmed to 37°C for 40 min and the number of cells displaying phagocytosis of E was counted. Erythrophagocytosis was detected in 40% of alveolar macrophages, in 3% of ADC, and in none of the LDC preparations (Table 2).

Phenotype of ADC. The phenotype of ADC was determined in situ by immunoperoxidase staining of frozen sections of trachea cut parallel to the surface of the epithelium

(6). The characteristic long cytoplasmic processes of intraepithelial DC afforded a means for their identification (Fig. 4). Unequivocal identification of DC in the lung parenchyma, however, was hampered by the presence of other Ia⁺ blood-borne cells. Their phenotype was, therefore, determined by immunoperoxidase staining of freshly isolated LDC (Table 3). Because of the low cell yield, parallel isolates of ADC were stained only for Ia, CD4, and intercellular adhesion molecule 1 (ICAM-1).

ADC in situ were uniformly Ia⁺ (Fig. 2), and their number per unit area of tracheal epithelium was used to normalize all the other cell counts. Although 100% of ADC were CD45R⁺, only 55% of these cells were recognized by C11b (a mAb that detects a subset of mature DC) (32). In contrast to recent observations (6), only 14% of ADC in Lewis rats reacted with OX41, and 33% and 35% of these cells stained for CD4, in situ and in fresh isolates, respectively. They were negative for ICAM-1 in situ, but at the end of the 2-day isolation procedure, performed in the presence of TNF- α , ~43% of isolated ADC expressed ICAM-1 (Table 3). Phenotypically, freshly isolated LDC were similar to ADC, except that the staining for Ia appeared to be more intense, and, most strikingly, 79% of LDC expressed ICAM-1.

Cytochemical reaction for nonspecific esterase was strongly positive in 100% of the isolated alveolar macrophages. By contrast, 38% of isolated ADC contained small juxtanuclear, esterase positive cytoplasmic vacuoles, whereas LDC isolated from the same lung were negative (data not shown).

Morphometry of ADC and Response to IFN- γ . ADC were most numerous in the tracheal epithelium, and their number decreased in the peripheral portions of the tracheobronchial tree (Fig. 5). Administration of IFN- γ resulted in a two- to fivefold increase in the number of ADC in the tracheobronchial epithelium, indicating that this lymphokine stimulates

Table 2. Expression of FcR and Erythrophagocytosis by ADC and LDC and Alveolar Macrophages

Assay	ADC	LDC	Alveolar macrophage
Percent IgG-E rosettes (4°C)*	56 \pm 14	4 \pm 0.9	93 \pm 4
Percent erythrophagocytosis (37°C)†	3	0	40

* Percent IgG-E rosettes formed at 4°C. All three cell types were obtained from the same three separate isolations. The data represent the mean \pm SD.

† Percent of cells that had ingested more than two E after incubation for 40 min at 37°C. The data represent the mean of two determinations.

Table 3. Phenotype of Rat ADC and LDC as Determined by Immunoperoxidase Staining

Antibody		Specificity	ADC in situ*	ADC isolate†	LDC isolate§
			%	%	%
OX-6	MHC class II antigen (26) (DC, B cells, some macrophages)		100	92	100
OX-1	CD45R (24) (all hematopoietic cells)		100	–	100
C11b	Unknown (32) (DC subset)		55	–	46
1A29	CD54 (ICAM-1)		0	43	79
WT.1	CD11a (31) (LFA-1)		0	78	62
WT.3	CD18 (31) (β 2 subunit of integrin)		0	–	–
OX-41	Unknown (30) (macrophages)		14	–	<10
OX-43	Unknown (29) (macrophages)		0	–	<10
RMA	Unknown (33) (activated macrophages)		0	–	0
OX-22	CD45Rb (25) (naive T cells)		0	–	–
W3/13	CD43 (27) (T cells)		0	–	–
W3/25	CD4 (28) (class II MHC-restricted T cells)		33	35	25
OX-8	CD8 (28) (class I MHC-restricted T cells)		0	–	0
Anti- μ -chain	IgM (B cells)		0	–	0

* Immunoperoxidase staining was performed on frozen sections of trachea, sectioned parallel to the plane of the epithelial cell surface. Only cells displaying long cytoplasmic processes, typical of ADC, were graded. Counts obtained by staining for Ia were used to normalize the remaining counts.

† These data represent the percent of positively stained cells present in fresh isolates of ADC. Because of the small numbers of ADC obtained in each isolation and because these cells could be readily identified in situ within the airway epithelium, only selected antibodies were tested on freshly isolated ADC.

§ Because it was difficult to distinguish LDC in situ from other intrapulmonary cells, immunoperoxidase staining was performed on TNF- α -treated, freshly isolated LDC.

^{||} See Materials and Methods.

the migration of DC not only to lung parenchyma (34), but also to all segments of the airway epithelium.

Ultrastructure. DC in airway epithelium projected delicate cell processes between epithelial cells and up to, but not beyond, the epithelial cell tight junctions (Fig. 6). The lobulated nucleus and cytoplasm, containing small mitochondria,

endoplasmic reticulum, a Golgi apparatus, and a few vesicular structures, were similar in appearance to LDC (7). Examination of multiple tissue blocks of airways failed to reveal Birbeck granules in these cells.

Functional Studies. The accessory cell activity of both ADC and LDC, isolated by the methods outlined, was excellent.

Table 4. Comparison of Accessory Cell Function of ADC with LDC

	ADC* + TNF- α	LDC* – TNF- α	LDC [§] + TNF- α
	cpm	cpm	cpm
+ HEL [†]	192,215 \pm 20,742	79,158 \pm 5,834	–
– HEL	28,803 \pm 9,276	9,614 \pm 931	–
+ HKL [†]	573,789 \pm 36,219	249,497 \pm 37,594	241,455 \pm 48,611
– HKL	20,492 \pm 6,384	12,840 \pm 11,875	6,765 \pm 4,669
Autologous MLR [†]	420,318 \pm 14,529	825,634 \pm 30,028	788,174 \pm 38,436
Syngeneic MLR	108,501 \pm 20,098	106,621 \pm 28,082	106,680 \pm 7,978

* 10⁴ cells/well.

† 5 \times 10⁴ cells/well of either HEL-immune, HKL-immune T cells or unprimed splenic T cells. See Materials and Methods for amounts of antigen added.

Representative data from one of three experiments expressed as cpm \pm SD of triplicate wells. Background counts due to T cells alone were subtracted from all values. These ranged from 241 to 1,600 cpm. It should be noted that throughout these experiments 1 μ Ci/well of [³H]thymidine with a sp act of 50–80 Ci/mmol was used. This likely accounts for the high counts obtained.

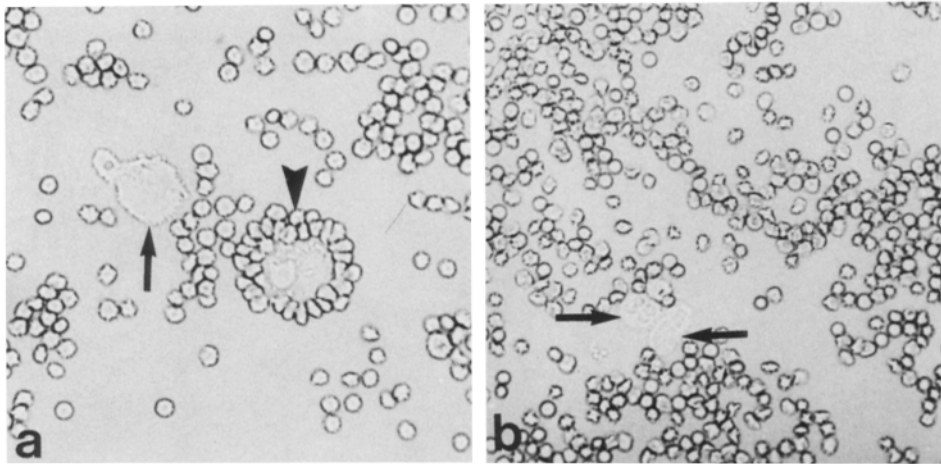


Figure 3. (a) Two ADC, of which one formed a rosette with IgG-coated sheep Es (IgG-E) (arrowhead) and the other did not (arrow). (b) LDC lack FcR for IgG-E (arrows). $\times 473$.

When the accessory cell function of these two populations of DC, derived from two distinct compartments of the same lung, was compared, however, a significant difference was apparent (Table 4 and Fig. 7). ADC were consistently more effective in presenting both soluble (HEL) and insoluble (HKL) antigen to antigen-sensitized T cells, whereas LDC were considerably more competent in stimulating naive or resting T cells in a one-way MLR assay. Although ICAM-1 expression could not be demonstrated in ADC *in situ*, ICAM-1 was present on 43% of ADC at the end of the 2-d isolation procedure. This observation is further supported by the fact that T cells readily formed clusters with both airway and lung DC in all the functional assays used (data not shown). In fact, by days 2 and 3 of the antigen presentation assays, T cell/ADC clusters were larger than T cell/LDC clusters.

In additional control experiments, DC isolated from both

airways and lungs were incubated for 30 min in the presence of dispase to determine whether this enzyme might adversely affect their accessory cell function. No difference was detected, in a one-way MLR, between dispase-treated DC and controls.

Discussion

DC originate in the bone marrow (12), from which they migrate, via the bloodstream, to most tissues, except the brain (11, 13, 14). In nonlymphoid tissues, at steady state, DC undergo continuous, albeit slow, turnover (40), resulting in a phenotypically and functionally heterogeneous population, depending on the anatomical compartment in which they reside (11), their stage of differentiation (35), and their state of activation in response to locally secreted cytokines (11).

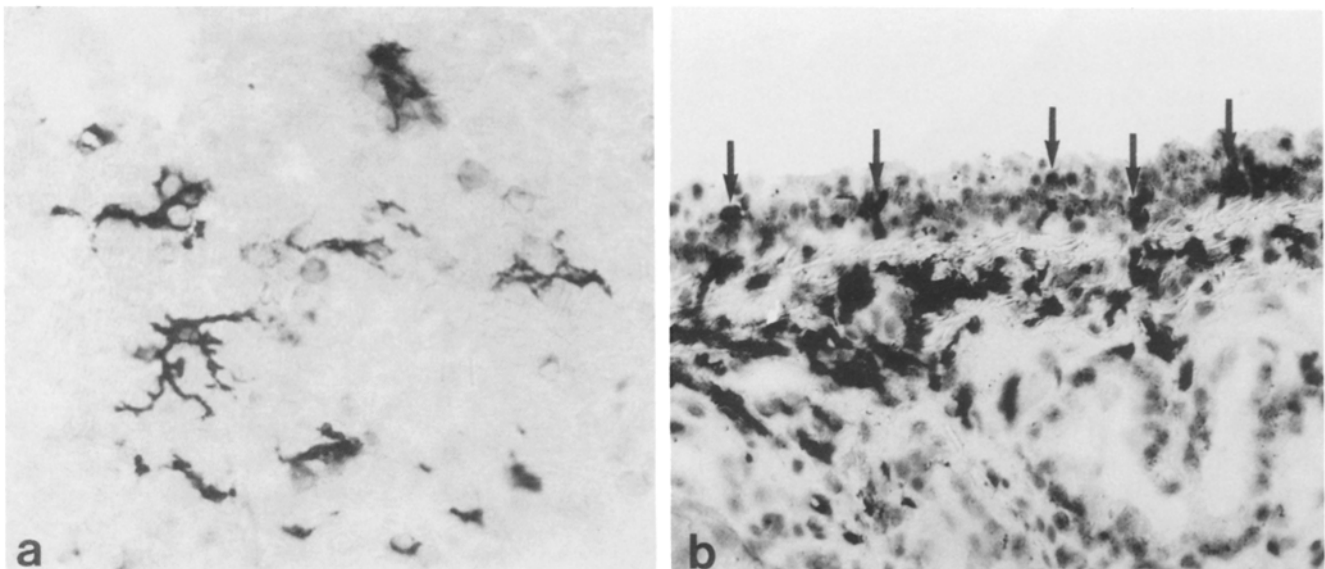


Figure 4. Frozen section of tracheal epithelium cut parallel (a) and perpendicular (b) to the plane of the epithelium and stained for Ia. Ia⁺ ADC with long, arborizing cell processes are intercalated between epithelial cells (arrows). $\times 390$.

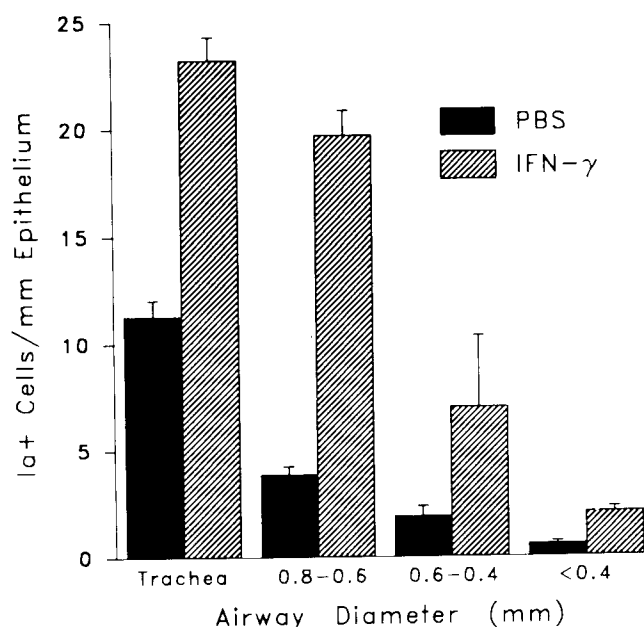


Figure 5. Histogram relating the number of Ia⁺ ADC per millimeter of airway epithelium in control (PBS; solid bars) and IFN- γ injected (hatched bars) rats. See Materials and Methods for details.

Once they are induced to migrate to local lymph nodes or the spleen, they do not recirculate (40, 41).

In the lung, DC are localized to several distinct anatomical compartments, including the airway epithelium (5, 6); the interstitial connective tissue of airways, vessels, alveolar septae, and pleura (5, 34); and the pulmonary vasculature (34). To specifically examine the DC in airway epithelium, the strategy in the present study was to devise a method whereby the entire tracheobronchial tree was cleanly separated from the lung parenchyma before DC were isolated from either compartment. To this end, a modification of a novel microdissection technique (21) was combined with the use of dispase, a neutral protease that cleaves at the level of the basement membrane (42), to enable the exclusive isolation of DC from airway epithelium. Since the pulmonary vasculature had been thoroughly perfused prior to the isolation procedure, the lung parenchyma dissected from these airway preparations provided a source of interstitial LDC, the phenotype and function of which were then compared with those of ADC.

Previous studies indicated that ADC, like their counterpart in the skin (43), express FcR, and that crude epithelial cell isolates from airways contained cells capable of antigen presentation to T cell hybridomas (5). These observations suggested that pulmonary DC, like Langerhans cells of the skin (22), might exist in two functionally distinct forms. The first, by virtue of their antigen-processing capabilities, could activate antigen-sensitized T cells via high-affinity T cell receptors, whereas the second, by upregulating surface ligands and accessory signals, could stimulate naive or unprimed T cells. That DC in the lung are heterogeneous is suggested by recent studies of both murine (9) and rat lung DC (17). Although the trachea and extrapulmonary bronchi were re-

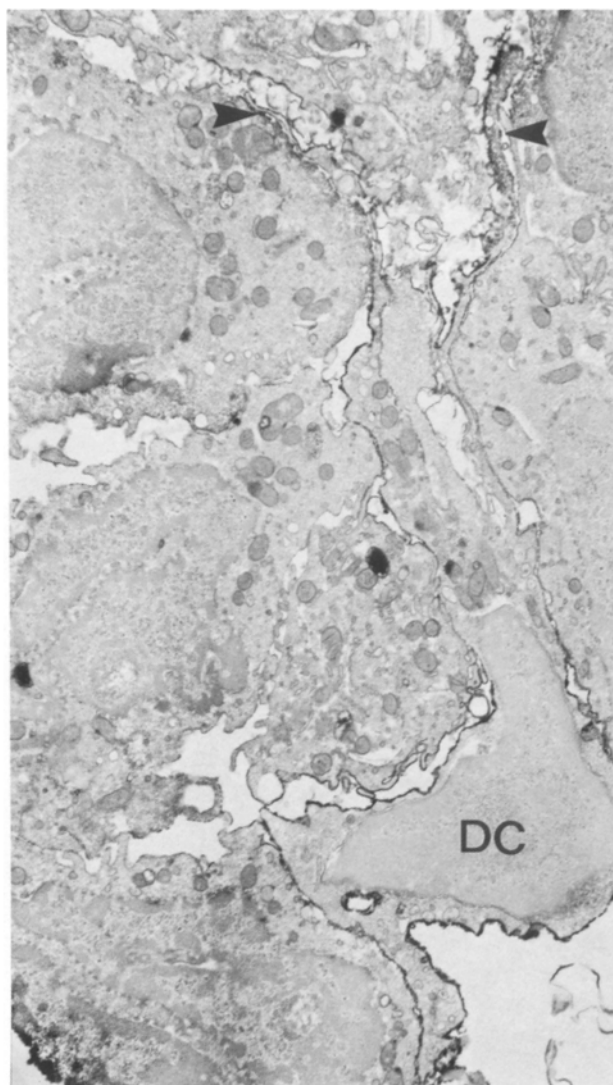


Figure 6. Electron micrograph of an ADC intercalated between airway epithelial cells. Arrowheads indicate slender Ia⁺ DC processes extending between epithelial cells. $\times 8,500$.

moved before lung DC were isolated, it is likely that a significant number of ADC were included in these preparations. Approximately half of the isolated LDC in both species were reported to express FcR. In mice, no difference in the ability to stimulate a one-way MLR was noted between FcR⁺ and FcR⁻ DC (9). This was not the case in the present study; the predominantly FcR⁺ ADC were less effective as accessory cells in the MLR than LDC, which were virtually FcR⁻ and predominantly ICAM-1⁺. It is unclear whether this difference is the result of the isolation procedures or a species difference.

The lack of FcR⁺ cells in the LDC fractions was unexpected. Since DC undergo continuous turnover (40, 41), it is reasonable to expect that DC at varying stages of differentiation should be present throughout the lung. A number of factors may have contributed to this outcome, among them being the strict use of pathogen-free animals. Under such

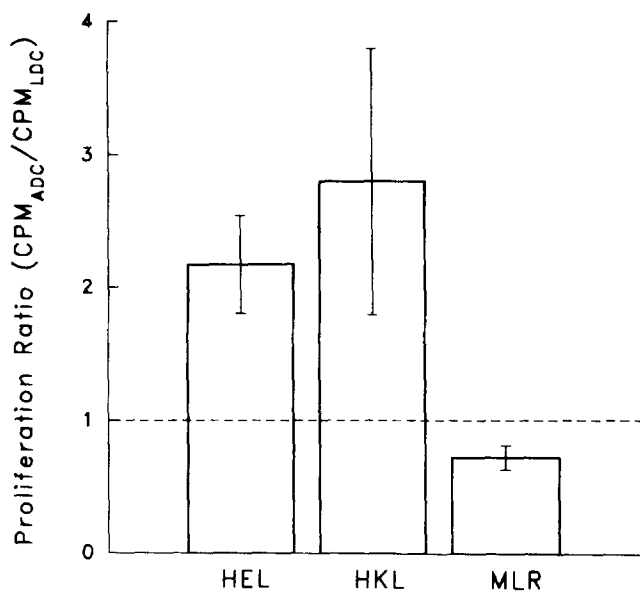


Figure 7. Graph relating the proliferation of immune T cells (HEL or HKL) or allogeneic splenic T cells (MLR) induced by ADC to that induced by LDC. The bars that extend above the dotted line indicate the greater stimulatory capacity of ADC relative to LDC in antigen presentation. By contrast, LDC are superior to ADC in stimulating T cell proliferation in the one-way allogeneic MLR. The data are derived from the mean \pm SD of a minimum of three separate isolations for each of the three functional assays.

control conditions, there would be little stimulus, either via endothelial cell activation or intrapulmonary chemoattractive gradients, to induce circulating DC to emigrate to the lung. This is supported by the observation that in an infected group of rats, an increased number of FcR⁺ DC was detected in both the lung parenchyma and airway epithelium. Another contributing factor is the fact that in these preparations the connective tissue compartment around the tracheobronchial tree was not included (Fig. 1, *b* and *c*). In this compartment, DC may include a significant population of cells that are either emigrating from the lung via lymphatics or are FcR⁺ DC newly arrived from the bloodstream. This possibility is supported by observations made in rat lungs with the use of mAbs to two macrophage markers, ED1 and ED2, that are expressed differentially on DC (44). DC in tracheal epithelium stained preferentially with ED1, whereas those in alveolar walls and pleura stained with ED2. Interestingly, in the peribronchial and perivascular connective tissue, both ED1⁺ and ED2⁺ DC were observed. Finally, the long period of time required to isolate these cells from both ana-

tomical locations may have contributed to the loss of FcR from LDC and a reduction in FcR expression by ADC, despite the presence of TNF- α .

Expression of ICAM-1 on LDC suggests that they may be in a more advanced stage of differentiation or activation than ADC. ICAM-1 (45), a cell-adhesion molecule that interacts with the leukocyte integrin LFA-1, participates in the heterologous clustering of T cells with DC (46) and is expressed on many LDC. By contrast, ADC *in situ*, like Langerhans cells in the skin (43), do not express ICAM-1. At the end of the 2-d isolation procedure, however, 43% of ADC and 79% of LDC expressed ICAM-1. In contrast to LDC, ADC appeared to be more fragile and required the presence of TNF- α throughout the isolation procedure. It is possible that TNF- α contributed to the upregulation of ICAM-1 expression by ADC (47); however, addition of TNF- α to LDC had little effect on their accessory cell function (Table 4). The relatively reduced expression of ICAM-1 by ADC might account for the somewhat fewer initial DC/T cell clusters observed in the functional assays. However, by the second day of the antigen presentation assay, the heterologous clusters involving ADC were considerably larger than those involving LDC, suggesting perhaps further upregulation of adhesion molecules and/or Ia on the surface of the ADC in ADC/T cell clusters.

Intratracheal administration of bacterial lipopolysaccharide elicited an acute inflammatory response within the airway epithelium of rats and resulted in augmented migration of DC to the epithelium at all levels of the airway (6). Parenteral administration of IFN- γ similarly resulted in a two- to threefold rise in ADC throughout the tracheobronchial epithelium, but without an acute inflammatory response. The seemingly directed migration of DC to airway epithelium may be the result of IFN- γ -stimulated upregulation of endothelial integrin expression and/or release of cytokines from the airway epithelium, which may, in turn, act as chemoattractants for DC.

In summary, results of the present study suggest that ADC resemble Langerhans cells of the skin both in phenotype and function. Located in the epithelium, these cells are strategically placed to take up and process both soluble and particulate antigens that may have traversed the epithelial airway barrier. In the milieu of the epithelium they are, however, inefficient stimulators of naive, unprimed T cells and must, therefore, migrate to local LN, where, either in transit or in the LN itself, they differentiate into cells expressing the necessary accessory signals to stimulate naive, nonsensitized T cells.

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References

1. Sleight, M.A., J.R. Blake, and N. Liron. 1988. The propulsion of mucus by cilia. *Am. J. Respir. Dis.* 137:726.
2. Bignon, J., M.C. Jaurand, M.C. Pinchon, C. Sapin, and J.M. Warnet. 1976. Immunoelectron microscopic and immunochemical demonstration of serum proteins in the alveolar lining material of the rat lung. *Am. J. Respir. Dis.* 113:109.
3. Crystal, R.G. 1991. Alveolar macrophages. In *The Lung* Scientific Foundations. R.G. Crystal and J.B. West, editors. Raven Press, New York. 527-539.
4. Schneeberger, E.E., and R.D. Lynch. 1992. Tight junctions in the lung. In *Tight Junctions*. M. Cereijido, editor. CRC Press, Boca Raton, FL. 337-351.
5. Sertl, K., T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner, and E. Schevach. 1986. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. *J. Exp. Med.* 163:436.
6. Schon-Hegrad, M.A., J. Oliver, P.G. McMenamin, and P.G. Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* 173:1345.
7. Schneeberger, E.E., M. DeFerrari, M.J. Skoskiewicz, P.S. Russell, and R.B. Colvin. 1986. Induction of MHC-determined antigens in the lung by interferon-gamma. *Lab. Invest.* 55:138.
8. Rochester, C.L., E.M. Goodell, J.K. Stoltenborg, and W.E. Powers. 1988. Dendritic cells from rat lung are potent accessory cells. *Am. Rev. Respir. Dis.* 138:121.
9. Pollard, A.M., and M.F. Lipscomb. 1990. Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J. Exp. Med.* 172:159.
10. Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. *J. Exp. Med.* 137:1142.
11. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271.
12. Bowers, W.E., and M.R. Berkowitz. 1986. Differentiation of dendritic cells in cultures of rat bone marrow cells. *J. Exp. Med.* 163:872.
13. Hart, D.N.J., and J.W. Fabre. 1981. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J. Exp. Med.* 153:347.
14. Austyn, J.M. 1987. Lymphoid dendritic cells. *Immunology* 62:161.
15. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* 161:526.
16. Streilein, J.W., S.F. Grammer, T. Yoshikawa, A. Demidem, and M. Vermeer. 1990. Functional dichotomy between Langerhans cells that present antigen to naive and to memory/effector T lymphocytes. *Immunol. Rev.* 117:159.
17. Xia, W., E.E. Schneeberger, K.M. McCarthy, and R.L. Kradin. 1991. Accessory cells of the lung: II. Ia⁺ pulmonary dendritic cells display cell surface antigen heterogeneity. *Am. J. Respir. Cell Molec. Biol.* 5:276.
18. Witmer-Pack, M.D., W. Olivier, J. Valinsky, G. Schuler, and R.M. Steinman. 1987. Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* 166:1484.
19. Heufler, C., F. Koch, and G. Schuler. 1987. Granulocyte-macrophage colony-stimulating factor and interleukin-1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.* 167:700.
20. MacPherson, G.G. 1989. Properties of lymph-borne (veiled) dendritic cells in culture. I. Modulation of phenotype, survival and function: partial dependence on GM-CSF. *Immunology* 68:102.
21. Plopper, C.G., A.M. Chang, A. Pang, and A.R. Buckpitt. 1991. Use of microdissected airways to define metabolism and cytotoxicity in murine bronchiolar epithelium. *Exp. Lung Res.* 17:197.
22. Streilein, J.W., and S.F. Grammer. 1989. In vitro evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. *J. Immunol.* 143:3925.
23. Romani, N., G. Schuler, and P. Fritsch. 1991. Identification and phenotype of epidermal Langerhans cells. In *Epidermal Langerhans Cells*. G. Schuler, editor. CRC Press, Boca Raton, FL. 50-86.
24. Sunderland, C.A., W.R. McMaster, and A.F. Williams. 1979. Purification with monoclonal antibody of a predominant leukocyte common antigen and glycoprotein from rat. *Eur. J. Immunol.* 9:155.
25. Spickett, G.P., M.R. Brandon, D.W. Mason, A.F. Williams, and G.R. Woolett. 1983. MRC OX22; a monoclonal antibody that labels a new subset of T-lymphocytes and reacts with the high molecular weight form of the leukocyte common antigen. *J. Exp. Med.* 158:795.
26. McMaster, W.R., and A.F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* 9:426.
27. Williams, A.F., G. Galfre, and C. Milstein. 1977. Analysis of cell surfaces by xenogenic myeloma-hybrid antibodies. Differentiation of rat lymphocytes. *Cell* 12:633.
28. White, R.A.H., D.W. Mason, A.F. Williams, G. Galfre, and C. Milstein. 1978. T-lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. *J. Exp. Med.* 148:664.
29. Robinson, A.P., T.M. White, and D.W. Mason. 1986. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies, MRC OX41 and MRC OX42, the latter recognizing the complement receptor type 3. *Immunology* 57:239.
30. Robinson, A.P., T.M. White, and D.W. Mason. 1986. MRC OX43: a monoclonal antibody that reacts with all vascular endothelium in the rat except brain capillaries. *Immunology* 57:231.
31. Tamatani, T., M. Kotani, and M. Miyasaka. 1991. Characterization of the rat integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur. J. Immunol.* 21:627.

32. Holowachuk, E.W., J.K. Stoltenberg, W.E. Bowers, and E.M. Goodell. 1991. Mouse anti-rat dendritic cell-specific monoclonal antibodies: characterization of antigens. *FASEB J.* 5:A996.
33. Yamin, M., D. Lazarus, E.E. Schneeberger, K.M. McCarthy, W. Xia, and R.L. Kradin. 1990. Anti-RMA: a murine monoclonal antibody that activates rat macrophages. I. Distribution and characterization of the RMA antigen. *Am. J. Respir. Cell Mol. Biol.* 2:207.
34. Kradin, R.L., K.M. McCarthy, W. Xia, D. Lazarus, and E.E. Schneeberger. 1991. Accessory cells of the lung. Interferon-gamma increases Ia+ dendritic cells in the lung without augmenting their accessory activities. *Am. J. Respir. Cell Mol. Biol.* 4:210.
35. McCarthy, K.M., J.L. Gong, J.R. Telford, and E.E. Schneeberger. 1992. Ontogeny of Ia-positive accessory cells in the fetal and newborn rat lung. *Am. J. Respir. Cell Molec. Biol.* 6:349.
36. Kradin, R.L., K.M. McCarthy, and E.E. Schneeberger. 1986. Opsonic receptor function is reduced on the surface of newborn alveolar macrophages. *Am. Rev. Respir. Dis.* 133:238.
37. Lennert, K. 1978. Malignant lymphomas other than Hodgkin's disease. Springer-Verlag, New York Inc., New York. p. 81.
38. McClean, I.W., and P.K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077.
39. Koch, F., C. Heufler, E. Kampgen, D. Schneeweiss, G. Bock, and G. Schuler. 1990. Tumor necrosis factor alpha maintains the viability of murine epidermal langerhans cells in culture, but in contrast to granulocyte/macrophage colony-stimulating factor, without inducing their functional maturation. *J. Exp. Med.* 171:159.
40. Metlay, J.P., E. Pure, and R.M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45.
41. Larsen, C.P., P.J. Morris, and J.M. Austyn. 1990. Migration of dendritic leukocytes from cardiac allografts into host spleens. *J. Exp. Med.* 171:307.
42. Fogarty, W.M., and P.J. Griffin. 1973. The neutral protease of *Bacillus polymyxa*. *Biochem. Soc. Trans.* 1:400.
43. Stingl, G. 1990. Dendritic cells of the skin. *Dermatol. Clin.* 8:673.
44. Holt, P.G., and M.A. Schon-Hegrad. 1987. Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies. *Immunology.* 62:349.
45. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425.
46. Inaba, K., and R.M. Steinman. 1987. Monoclonal antibodies to LFA-1 and to CD4 inhibit the mixed leukocyte reaction after the antigen-dependent clustering of dendritic cells and T lymphocytes. *J. Exp. Med.* 165:1403.
47. Springer, T.A. 1990. The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annu. Rev. Cell Biol.* 6:359.