Downregulation of the Antigen Presenting Cell Function(s) of Pulmonary Dendritic Cells In Vivo by Resident Alveolar Macrophages

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Summary

Class II major histocompatibility complex (Ia)-bearing dendritic cells (DC) from airway epithelium and lung parenchyma express low-moderate antigen presenting cell (APC) activity when freshly isolated. However, this function is markedly upregulated during overnight culture in a manner analogous to epidermal Langerhans cells. The in vitro "maturation" process is inhibited by coculture with pulmonary alveolar macrophages (PAM) across a semipermeable membrane, and the degree of inhibition achieved can be markedly increased by the presence of tumor necrosis factor α. In addition, PAM-mediated suppression of DC function is abrogated via inhibition of the nitric oxide synthetase pathway. Functional maturation of the DC is accompanied by increased expression of surface Ia, which is also inhibited in the presence of PAM. Prior elimination of PAM from DC donors via intratracheal administration of the cytotoxic drug dichloromethylene diphosphonate in liposomes, 24–72 h before lung DC preparation, achieves a comparable upregulation of APC activity, suggesting that (consistent with the in vitro data) the resident PAM population actively suppresses the APC function of lung DC in situ. In support of the feasibility of such a regulatory mechanism, electron microscopic examination of normal lung fixed by intravascular perfusion in the inflated state (which optimally preserves PAM in situ), revealed that the majority are preferentially localized in recesses at the alveolar septal junctions. In this position, the PAM are in intimate association with the alveolar epithelial surface, and are effectively separated by as little as 0.2 μm from underlying interstitial spaces which contain the peripheral lung DC population. A similar juxtaposition of airway intraepithelial DC is demonstrated with underlying submucosal tissue macrophages, where the separation between the two cell populations is effectively the width of the basal lamina.

Previous studies from this laboratory initially drew attention to the role of dendritic cells (DC)[1] analogous to those described by Steinman and Nussenzweig (1), as the principal resident APC population in parenchymal lung tissue of rat (2). These observations were confirmed and extended by other investigators in a variety of species including human (3–9), and were further extended to the epithelium of the conducting airways where class II MHC antigen (Ia)-bearing DC were shown to form a tightly meshed network comparable to that of epidermal Langerhans cells (10–12).

The epithelial surfaces within the respiratory system occupied by these DC are continuously exposed to an array of pathogenic and nonpathogenic airborne antigens from the environment, and the maintenance of local homeostasis requires fine control of immunological processes, particularly those involving T cell activation. Active suppression of T cell responses to inert antigens plays an important role in local immunoregulation, at the antigen-specific level through the induction of immunological tolerance (13, 14) analogous to the phenomenon of oral tolerance to dietary antigens (15), and also via the generalized lymphocytostatic effects of endogenous tissue macrophages, in particular the pulmonary alveolar macrophage (PAM) population (for review see reference 16). The latter inhibit T cell proliferation induced by lung and airway DC, prompting the suggestion that acces-

[1] *Abbreviations used in this paper:* DC, dendritic cell; DPDP, dichloromethylene diphosphate; LNC, lymph node T cell; MMA, monomethylarginine; PAM, pulmonary alveolar macrophage; RLN, regional lymph node; VC, veil cell.

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sory cell function may be an additional target for the immunomodulatory effects of local macrophages (2, 6).

Previous studies on DC from the epidermis and from lymph suggest that the antigen presentation functions of these cells are indeed actively regulated in vivo, as they perform poorly as APC when freshly isolated, but markedly upregulate their activity during overnight culture, with concomitant loss of processing capacity (17–19). It has been suggested that this in vitro maturation process mimics the changes that normally occur during the migration of DC from tissue sites to regional LN, i.e., their prime function changes from antigen sampling, processing, and storage at the periphery, to antigen presentation in the lymphoid tissues (20).

In the study below, we present direct evidence for the operation of such a control mechanism in the lung and airway mucosa, and further demonstrate that the functional phenotype of the DC is actively controlled by neighboring cells within these tissue microenvironments. Thus, DC freshly isolated from the tracheal epithelium and lung parenchyma express only modest T cell stimulatory activity in MLR assays and in presentation of soluble protein antigens, and they demonstrate up to a 100-fold increase in stimulatory activity after overnight culture. Additionally, two lines of evidence indicate that the low APC activity of lung DC is actively maintained in situ via diffusible mediators from resident PAM. First, selective in vivo elimination of PAM by intratracheal administration of a macrophage cytotoxic drug encapsulated in liposomes (21, 22) leads to the rapid enhancement of the APC activity of resident lung DC to approximate the high levels achieved by in vitro culture; and second, the in vitro maturation of APC activity in these cells is inhibited by coculture with PAM across a semipermeable membrane. The latter inhibition is amplified by TNF-α, and is abrogated in the presence of an inhibitor of macrophage nitric oxide production.

Materials and Methods

Animals. Specific pathogen-free BN and WAG rats were obtained from the Animal Resources Centre, Murdoch University (Perth, Western Australia). They were barrier housed until used.

Antibodies. The mAbs, ED1 against an intracellular marker on macrophages and some DC (23), and ED2 against mature tissue macrophages (23) were provided gratis by Dr. C. Dijkstra, Department of Histology, Vrije Universiteit, and the mAbs Ox6 and Ox12 (anti-class II MHC and anti-κ L chains, respectively [24]) by Professor A. Williams (Medical Research Council Cellular Immunology Unit, Oxford, UK). Biotinylated sheep anti-mouse IgG and streptavidin–horseradish peroxidase (HRP) conjugates were from Amersham (Sydney, Australia).

Cytokines and Blocking Reagents. Purified natural murine GM-CSF, recombinant human TNF-α, human IL-1α, and antimurine TNF-α/β blocking antibody were from Genzyme Corp. (Boston, MA). Anti-TGF-β blocking antibody was from R & D Systems, Inc., (Minneapolis, MN). Recombinant human IL-6 was from Boehringer Mannheim (Mannheim, Germany). Antimurine GM-CSF blocking antibody (25) was a kind gift from Drs. K. Takeda and K. Kumagai, Department of Microbiology, Tohoku University (Sendai, Japan). Indomethacin, NG-monomethyl-arginine (MMA), and DMSO were from Calbiochem Corp. (La Jolla, CA).

Visualization of Cells in Tissue Sections. Frozen sections of trachea and lung tissue were prepared and immunostained as described in detail in our earlier publications (6, 12), except that tissue fixation with ethanol was performed via intravascular perfusion through the pulmonary artery (at 40 cm H_2O) after inflation of the lungs with air to 30 cm H_2O. Lungs were then slowly deflated to 12 cm H_2O during perfusion. This technique has been shown to optimally fix PAM in situ on the alveolar surface (26, 27). For electron microscopy, 2.5% cacodylate-buffered glutaraldehyde was substituted for ethanol in the fixation process. Tissue blocks were processed in the conventional fashion and embedded in Araldite™ (TAAB, Reading, UK). Ultra-thin sections (600–800 nm) were strained with uranyl acetate and lead citrate before examination in a transmission electron microscope (model 410_x; Phillips, Eindhoven, The Netherlands).

Cell Preparation. The preparation of enriched DC from collagenase-digested parenchymal lung and tracheal epithelial sheets was as previously described (6). The procedure involves rigorous initial depletion of endogenous monocytes/macrophages and B cells via slow elution from nylon wool, and finally, selection for ultra low-density cells on Percoll, yielding populations containing up to 35% Ia^+ cells, many of which demonstrate overtly pleiomorphic or veil cell (VC)-like morphology (6, 28). mAbs against surface κ chains (for B cells) or the ED2 marker (tissue macrophages) stained <2% of the enriched DC populations. PAM were prepared by bronchoalveolar lavage as described (6).

Preculture of DC. Enriched DC populations were cultured overnight in a variety of media including RPMI 1640, alone or supplemented with 10% FCS and Con A-activated T cell supernatant (28). As reported previously (28), overall cell recovery from the cultures were variable (median 35%) and were lowest in cultures containing RPMI alone. Except where stated, the culture medium employed in preculture experiments reported below comprised RPMI 1640 plus 10% FCS. In some experiments, DC were incubated overnight in 24-h supernatant from PAM cultured at 2×10^6/ml in RPMI plus 10% FCS, or in FCS-supplemented RPMI containing a variety of cytokines (vide infra), and no consistent variations in DC recovery after overnight incubation were observed. In one series of experiments, DC were cultured in modified Boyden chambers (10^6 in 200 μl medium, separated by a membrane (Millipore, Sydney, Australia; pore size 0.22 μm) from PAM (2×10^6 in 300 μl medium). A proportion of this phase of the study was subsequently repeated employing culture chamber inserts (Millicell CM; 0.4-μm pore size; Millipore) to separate the DC and PAM populations.

Antigen Presentation. The assay for presentation of soluble protein antigens is as described previously (6), employing lymph node T cells (LNC) from WAG rats immune to either BSA or OVA or *Der p* I antigen (low molecular weight allergen from the house dust mite; 29). LNC were initially depleted of endogenous APC before each assay, and their capacity to respond to soluble antigen without readdition of exogenous DC was checked within each experiment (6), and data was rejected if APC depletion was found to be incomplete. The assays employed 4×10^5 immune LNC in 200 μl RPMI 1640 supplemented with 5% syngeneic normal rat serum and 5×10^{-5} M 2-ME in triplicate wells, and varying numbers of antigen-pulsed enriched DC (see below). The DC were X-irradiated (1,500 rad) immediately before addition to the T cell cultures. Proliferation was measured as [^{3}H]thymidine incorporation into DNA at 96 h, and expressed as median Δ dpm per culture after subtraction of background controls. Interculture variation was minimal with SE of replicates being within 10% of means. Crossover specificity controls were performed with BSA-immune T cells as previously described (6). The small number of experi-

ments in which significant autologous MLR were observed with unpulsed DC were rejected from the analyses.

In vitro antigen pulsing of DC was performed via incubation in vitro at 2–500 μg/ml (for *Der p* I) or 1.0 mg/ml (BSA or OVA), followed by three washings in medium. In vivo antigen pulsing was performed by exposure of WAG rats to an aerosol generated from a solution of 2.0 mg/ml OVA as described in (6).

The rat MLR system employed is as detailed (30) with slight modification (28), and involved coculture of 5×10^5 responder cells (mesenteric LNC from WAG rats) with graded numbers of enriched X-irradiated lung DC from BN rats.

In Vivo Depletion of PAM. The procedure involved intratracheal instillation of a suspension of liposomes containing dichloromethylene diphosphonate (DMDP-liposomes), as described (21, 22). The liposomes spread rapidly throughout the surfactant layer, and within 12 h ≥90% of PAM contain >5 droplets. The drug becomes cytotoxic after release in the phagolysozomes and kills the PAM, depleting the resident population by up to >90% within 24–48 h of liposome administration (21, 22). No translocation of DMDP or labeled liposomes below the epithelium has been observed, and morphometric analysis of immunostained lung sections from treated animals indicates that the procedure does not alter the density/surface phenotype of interstitial cell populations (21, 22). No consistent changes in cell yield (including the enriched DC fraction) have been detected in lung digests from the liposome-treated animals.

Flow Cytometry. Cell surface expression of class II MHC (Ia) antigen was compared on fresh/cultured lung DC after immunostaining with an Ox6–FITC conjugate, employing a flow cytometer (Epics; Coulter Corp., Hialeah, FL).

Results

APC Functions of Lung DC After In Vivo Depletion of PAM. In the experiments in Fig. 1 *A*, anesthetized BN rats were intratracheally inoculated with 200 μl of liposomes containing DMDP or PBS, or an equivalent volume of PBS alone, 48 h before preparation of lung DC. These preparations, together with equivalent cells from intact controls, were assayed for APC activity in a one-way MLR as detailed in Materials and Methods, and it can be seen that the activity of DC from PAM-depleted animals was on the order of fivefold greater than controls.

The experiments of Fig. 1 *B* illustrate the degree of enhancement of the APC activity of lung DC, as a function of time post DMDP-liposome administration. The PAM population in these animals was reduced to ~30% of controls by the 24 h time point falling to 10% by 48–72 h, as in earlier studies (22), and it is evident that the maximal effects of PAM elimination occur within 24 h.

In Fig. 2, the effects of in vivo PAM elimination are examined in relation to presentation of soluble protein antigens. In Fig. 2 *A*, control and PAM-depleted WAG rats were ex-

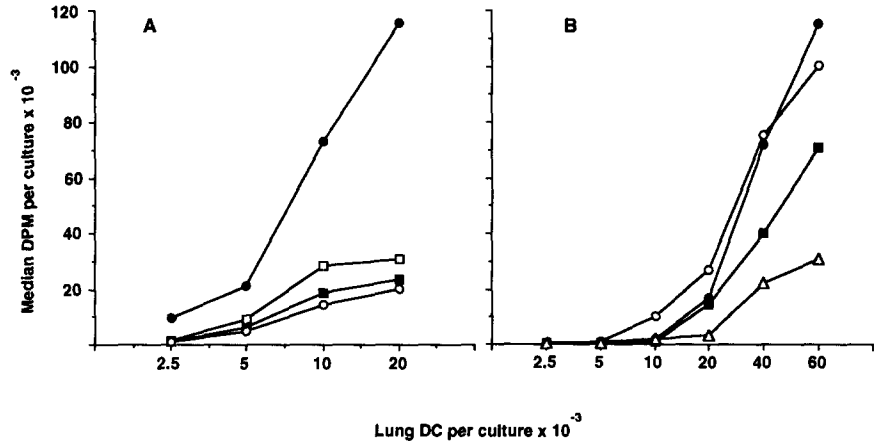

Figure 1. Antigen presentation by lung DC in a primary MLR. Effect of prior depletion of PAM in DC donors. Semipurified lung DC from groups of control or treated BN rats ($n = 3$) were titrated into cultures of WAG LN cells, and resulting DNA synthesis determined as incorporation of [^{3}H]thymidine at the 120-h time point. (*A*) Lung DC pools were from untouched controls (■), and animals intratracheally inoculated 48 h previously with either PBS (○) or liposomes containing PBS (□) or DMDP (●). (*B*) MLR stimulatory activity of lung DC prepared from BN rats at varying periods after intratracheal administration of DMDP-liposomes. Zero-time control (△); 24 h (●), 48 h (○), and 72 h (■) after administration.

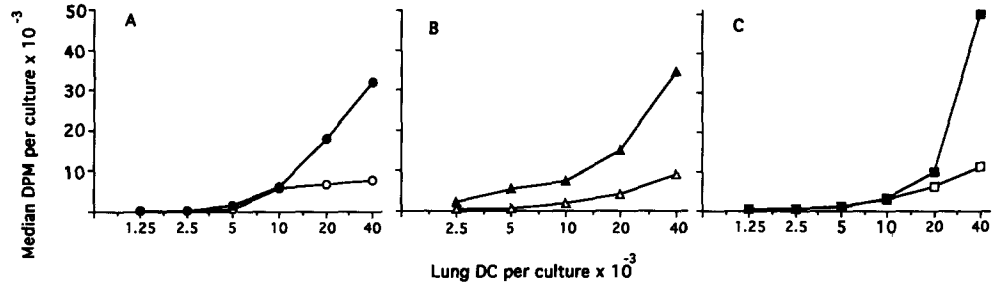

Figure 2. Presentation of a protein antigen to immune T-cells by lung DC. (*A*) Normal control WAG rats (○) and WAG rats intratracheally inoculated 48 h previously with DMDP-liposomes (●) were exposed for 30 min to an aerosol of OVA, and 1 h later lung DC were prepared from both groups and titrated into cultures of purified syngeneic OVA-immune T cells. Resulting OVA- induced T cell proliferation was determined as ^{3}H-DNA synthesis at the 96-h time point and is shown as median Δdpm per culture. (*B*) Lung DC from control (△) and PAM-depleted (▲) WAG rats were pulsed in vitro with OVA antigen for 90 min, before titration into cultures of syngeneic OVA-immune T-cells. Data as per *A*. (*C*) Lung DC from intact WAG rats were prepared 1 h after antigen pulsing in vivo via 30 min exposure of the donor animals to aerosolized OVA. The DC were titrated onto cultures of syngeneic OVA-immune T cells immediately after preparation (□) or after overnight preincubation in serum-supplemented growth medium (■). Data as per *A*.

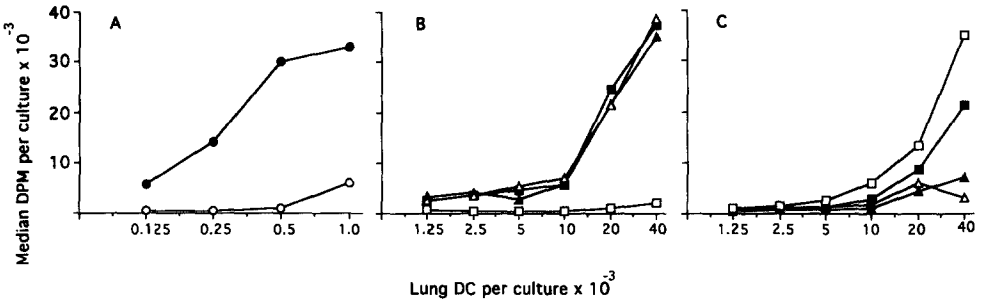

Figure 3. Modulation of airway and lung DC function(s) in vitro. Stimulation of primary MLR responses of WAG T cells by DC from BN lung and airways. (*A*) Tracheal epithelial DC titrated into allogeneic T cell cultures fresh (○) or after overnight preculture (●). (*B*) Lung DC into T cell cultures fresh (□), or after overnight incubation in RPMI (▲), RPMI supplemented with 10% FCS (△), or Con A-activated T cell culture supernatant (■). (*C*) Lung DC into T cell cultures fresh (▲), after overnight incubation in medium (□), or in PAM culture supernatant (■), or after preincubation in the presence of live AM across a semipermeable membrane (△).

posed to an aerosol of OVA for 30 min, and 1 h later, lung DC were prepared from both groups and titrated into cultures of syngeneic OVA-immune T cells. DC from the PAM-depleted animals in these experiments displayed on the order of fourfold increased APC activity relative to intact controls. Comparable differences were also observed comparing lung DC from intact and PAM-depleted animals that were pulsed with OVA antigen in vitro (Fig. 2 *B*).

Enhancement of APC Activity of Lung DC by Overnight Culture: Inhibition by Diffusible Mediators from PAM. In Fig. 2 *C*, normal WAG rats were exposed to aerosolized OVA for 30 min. Lung DC were prepared from these animals 1 h later and cultured overnight in RPMI 1640 plus 10% FCS. A second group of animals were aerosol-exposed 1 d later, and lung DC again prepared 1 h after exposure. These two lung DC preparations were titrated in parallel into cultures of OVA-immune syngeneic T cells, and ^{3}H-DNA synthesis determined 4 d later. It can be seen in Fig. 2 *C* that overnight culture of the antigen-exposed lung DC in the absence of macrophages leads to marked enhancement of APC activity comparable to that observed following in vivo PAM depletion.

In vitro maturation of APC functions is also demonstrable employing primary MLR as the readout, for both airway intraepithelial DC (Fig. 3 *A*) and their counterparts in parenchymal lung tissue (Fig. 3 *B*; see also preliminary report in reference 28). Additionally, the maturation process is inhibitable by coculture with PAM across a semipermeable membrane (Fig. 3 *C*). Whereas the degree of stimulation of APC activity afforded via in vitro culture and the corresponding degree of sensitivity to PAM are variable from experiment to experiment, the overall phenomenon per se is highly reproducible (Fig. 5; *left*). However, attempts to duplicate the inhibitory effects of coculture with live PAM employing 24-h PAM culture supernatants met with extremely variable results, the best of which (equivalent to ~50% suppression) is shown in Fig. 3 *C*. In the majority of experiments, the supernatants were ineffective.

The experiment in Fig. 4 demonstrates the activity of PAM in relation to processing and presentation of a soluble protein antigen. Thus, lung DC from normal BN rats were pulsed overnight with *Der p* I antigen in the presence and absence of PAM (transmembrane) and/or recombinant human TNF-α, before titration into cultures of *Der p* I-immune syngeneic T cells. Overnight incubation of DC with *Der p* I clearly facilitates maturation of APC function(s), as evidenced by the high levels of T cell proliferation induced by the pulsed cells. Coculture with PAM (transmembrane) reduces overall T cell stimulatory activity by ~50% in this experiment, whereas in several others (Fig. 5), complete suppression was observed. The addition of high levels of TNF-α (1,000 U/ml) to the DC cultures in this experiment resulted in a small reduction (23%) in T cell stimulation, and synergized with transmembrane PAM leading to ablation of APC activity. This synergistic effect of TNF-α was demonstrable over a series of experiments, whereas the effects of TNF-α alone were highly variable, ranging from zero (in three quarters of ex-

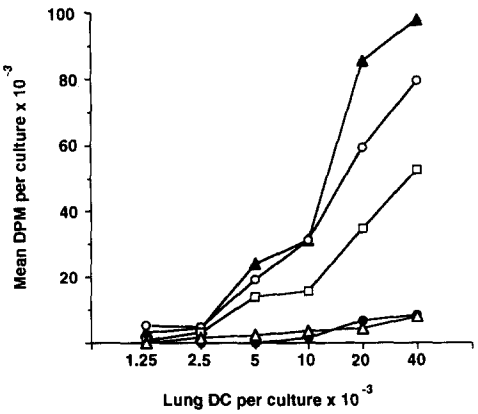

Figure 4. Presentation of *Der p* I antigen to immune T cells by lung DC: modulation by AM and TNF-α. Lung DC were prepared from naive BN rats, and titrated onto cultures of purified syngeneic P1-immune T cells after overnight incubation in medium alone (●), 500 μg/ml P1 antigen (▲), P1 antigen plus TNF-α at 1,000 U/ml (○), P1 antigen plus transmembrane PAM (□), or P1 plus TNF-α plus transmembrane PAM (△).

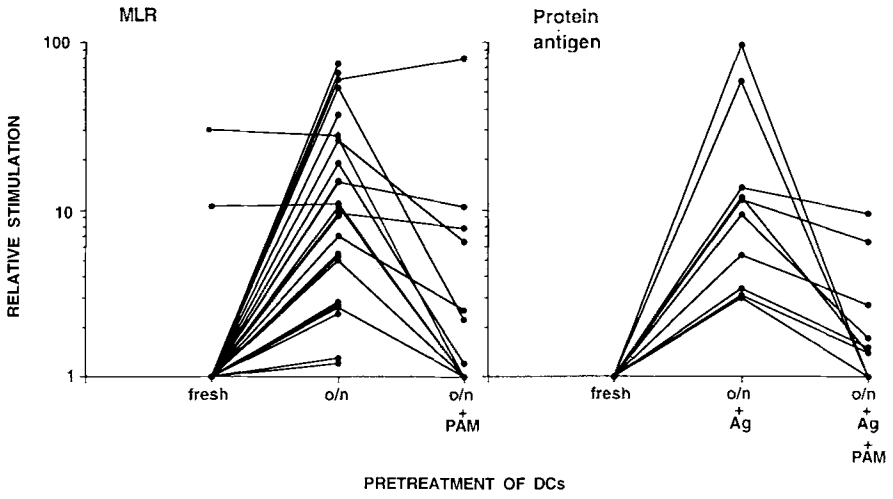

Figure 5. Upregulation of the APC activity of lung DC by overnight preculture, and inhibition by transmembrane AM. Summary data from a series of experiments examining presentation of alloantigen and soluble protein antigens. (*MLR*) Format as per Fig. 3 *C*, and within each experiment stimulation indices (SI) for DC incubated overnight (*o/n*) with or without PAM were normalized against that achieved with fresh DC. On rare occasions lung DC preparation from ostensibly normal animals appeared preactivated in vivo (note two examples in this series shown with SI = 10 and 30 when *fresh*) and this activity was also inhibited by PAM. (*Protein antigen*) Bracket of experiments involving presentation of OVA or *Der p* I antigen to immune T cells. Format as per Fig. 4. Data for each experiment again normalized against SI observed with DC incubated overnight in medium alone (*o/n*).

periments) up to a maximum of 35% reduction, leading to the conclusion that this cytokine is one of a series of mediators which act in concert to downregulate the APC activity of the lung DC.

Attempts to identify other possible regulatory cytokines in these coculture systems employing purified or recombinant IL-1, IL-6, GM-CSF, and blocking antibodies against GM-CSF and TGF-β, have failed to detect significant activity either alone or in various combinations. Moreover, the addition of high levels of blocking antibody against TNF-α/β to the PAM chamber did not abrogate suppression, reinforcing the view that this cytokine does not act alone in this system. Additionally, inclusion of 10 μg/ml of indomethacin in the cocultures or pretreatment of PAM donors by a combination of feeding and injection (employing a protocol previously demonstrated by us to modulate other function of PAM [31]), failed to block the suppressive effects of PAM in this system, arguing against a significant role for prostaglandins.

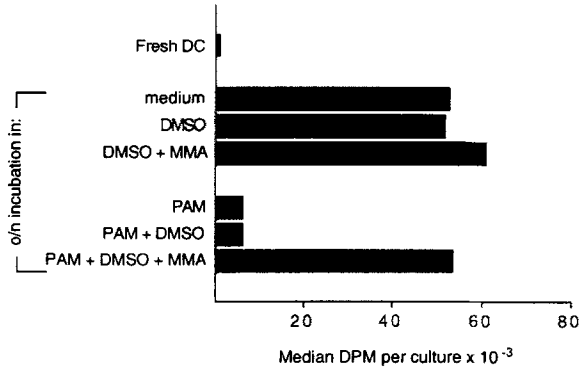

Figure 6. Abrogation of the suppressive activity of PAM via inhibition of the nitric oxide synthetase pathway. Stimulation of primary MLR responses of WAG T cells by BN lung DC after overnight coculture with PAM in the presence or absence of MMA. The MMA was dissolved in DMSO and diluted 500 times into the cultures to a final concentration of 0.5 mM. Controls contained medium or DMSO alone. Culture format as per Fig. 3 *C*. The data shown were replicated in three successive experiments.

Abrogation of the Suppressive Effects of PAM Via Inhibition of the Nitric Oxide Synthetase Pathway. Recent reports from a number of laboratories indicate that the in vitro "suppressive" activity of macrophages from lymphoid organs (32) and from lung (33) are attributable in part to the release of nitric oxide. The representative experiment shown in Fig. 6 employed the same methodology, involving preculture of DC in the presence of transmembrane PAM, with or without the nitric oxide synthetase inhibitor MMA or the vehicle (DMSO) employed to solubilize the latter. It can be seen that incubation of lung DC in the presence of PAM inhibits the maturation of MLR-stimulating activity, and this inhibition is abrogated by the inclusion of MMA in the coculture system. MMA modulation of macrophage suppression is reversible by L-arginine but not D-arginine (data not shown) as detailed (32).

Surface Expression of Ia on Lung DC during Overnight Culture. Table 1 illustrates two consecutive experiments involving overnight culture of lung DC in the presence or absence of transmembrane PAM. As noted above, Ia expression in these preparations is restricted to a subpopulation of cells which display characteristic VC- or DC-like morphology. Analysis

Table 1. *Modulation Ia Expression on Lung DC During Overnight Culture*

	Surface Ia expression		
Experiment	Fresh	After overnight incubation alone	After overnight incubation with PAM
1	2.81	4.68	2.93
2	2.31	4.03	3.21

Lung DC were immunostained with Ox6 (anti-Ia)-FITC immediately after preparation (fresh), or after overnight incubation in one well of a Boyden chamber (10^6 cells in 200 μl) adjacent to a second chamber containing medium (alone) or PAM (2×10^6 in 300 μl). Data shown are mean fluorescence intensity.

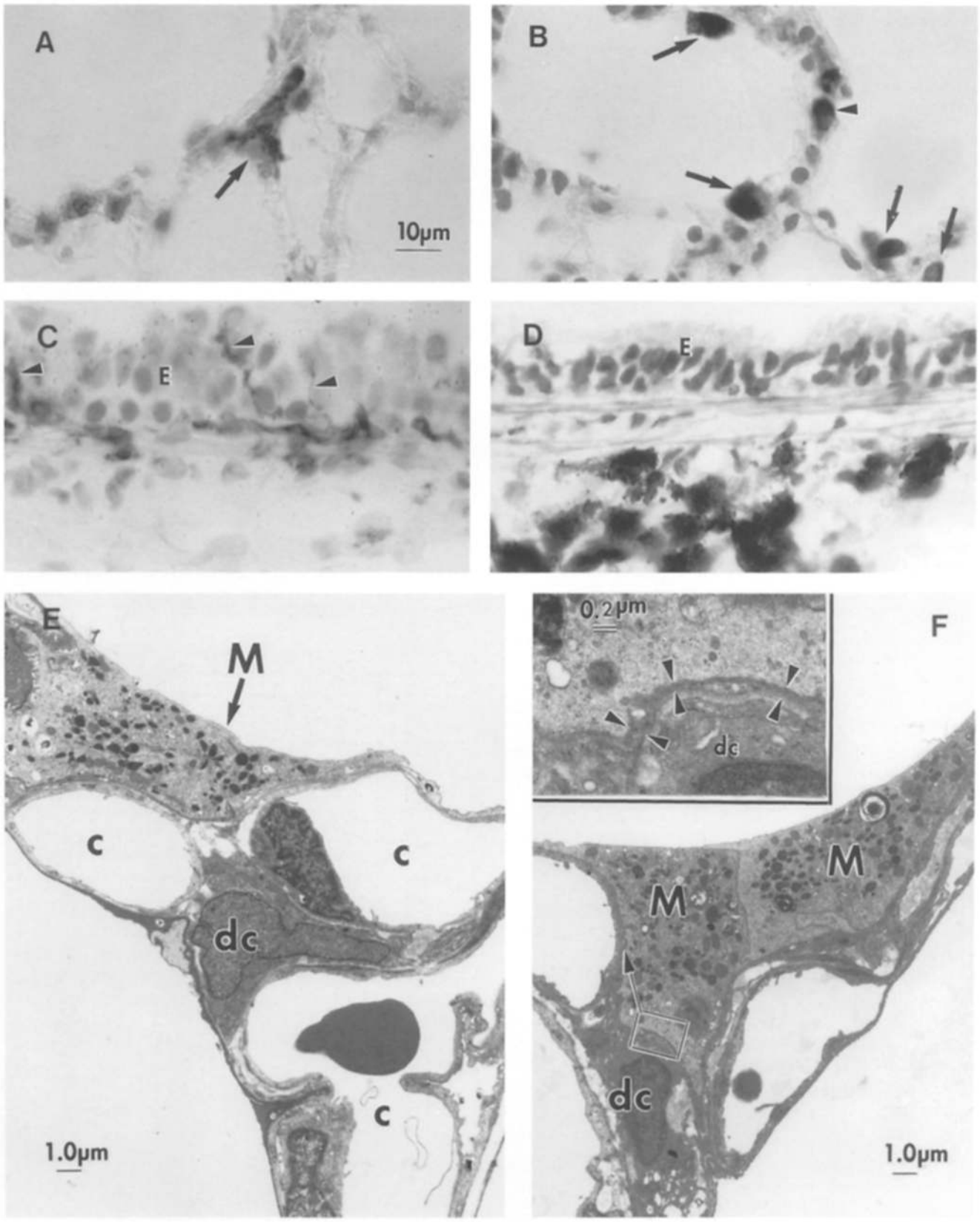

Figure 7. Juxtaposition of DC and macrophage populations in rat lung and airway tissues. (*A*) Interstitial Ia$^+$ DC (*arrow*) straddling an alveolar septal junction. (*B*) ED1$^+$ macrophages (*arrows*) on the lumenal surface of the alveolar space. A small interstitial macrophage (*arrowhead*) by contrast is situated in a septal wall, possibly within the vasculature. (*C*) Ia$^+$ DC within the tracheal epithelium (E). The cell bodies lie close to the basement membrane and their processes (*arrows*) interdigitate between the epithelial cells. (*D*) Subepithelial macrophages in trachea heavily stained via the ED2

of fluorescence intensity profiles derived from the latter after immunostaining with the mAb Ox6-FITC indicated increased Ia expression after overnight incubation consistent with that reported for epidermal Langerhans cells (34), and inhibition of this increase occurred in the presence of PAM.

In Vivo Relationship between DC and Macrophages in Respiratory Tract Tissues. Fig. 7 comprises a series of photomicrographs which illustrate the juxtaposition of DC and macrophage populations in the airway mucosa and the terminal air spaces. Figs. 7, *A* and *B* illustrate immunostained sections from the alveolar region of the rat lung fixed by vascular perfusion. Fig. 7 *A* demonstrates an $Ox6^+$ (Ia^+) cell exhibiting typical pleiomorphic DC-like morphology, localized within the interstitium at the junction of three alveolar septa with cell processes extending into adjacent septal walls. Previous observations indicate that these cells do not express the pan-macrophage markers ED1 or ED2 (6, 28) confirming their identity as DC, and observation of Ox6-stained sections from a series of animals indicated that >90% occur either within or directly adjacent to these alveolar septal junctional zones (data not shown). Fig. 7 *B* illustrates $ED1^+$ PAM adherent to the lumenal surface of the alveolar epithelium adjacent to alveolar septal junctions. A variable but significant proportion of PAM detected in perfused-fixed rat lungs (in the range of 50–80%) appeared in these areas. This predilection of PAM to localize at septal junctional areas has also been reported in human lung (27).

Fig. 7, *C* and *D* show sections of airway mucosa stained with Ox6 and ED2, respectively. Note the $Ox6^+$ (Ia^+) DC within the airway epithelium (Fig. 7 *C*), and the heavily stained $ED2^+$ mature tissue macrophages which are restricted to the adjacent submucosa, immediately beneath the epithelial basement membrane. We have previously demonstrated that DC represent the sole source of Ia-staining in normal airway epithelium of unstimulated specified pathogen free (SPF) rats (10, 12) and have confirmed by double staining that the ED2 marker is restricted to mature tissue macrophages in rat respiratory tract (28) as has been reported for other tissues (23).

Electron microscopic analysis of lungs fixed via intravascular perfusion (Fig. 7, *E* and *F*) confirmed that the majority of PAM (readily identifiable by their numerous electron-dense intrathymic inclusions) are located at the alveolar junctional zones. The optimal preservation of morphology afforded by this method further revealed that PAM are spread out upon the type I epithelial lining and form part of the natural rounded contour of the alveolus by "filling" the crevices and recesses of the junctional zones (Fig. 7, *E* and *F*). Indeed, often these recesses are occupied by two or more PAM (Fig. 7 *F*), separated from the underlying interstitial cell population (which, based on immunoperoxidase staining, are known to be rich in DC) by only a thin attenuated covering of type I epithelium. As demonstrated in Fig. 7 *F*, the spatial separation between PAM and adjacent interstitial DC (such as the "candidates" identified in Fig. 7, *E* and *F*) would be 0.2–1.0 μm.

Discussion

The primary function of DC is believed to be surveillance of mucosal/epithelial surfaces for foreign antigens not previously encountered, as these cells have the unique attribute of presenting potent antigen-specific inductive signals to naive T cells (20). Their further likely specialization for uptake of soluble protein antigens and viruses (35) suggests that they play a major role in the etiology of allergic and infectious diseases in the respiratory system, particularly in view of the fact that they constitute the sole source of class II MHC (Ia) expression within the epithelium lining the airways during the steady state (36).

It has been postulated further that the induction of T cell immunity by DC is a distinctly biphasic process. In the first phase, DC function as "sentinels" in peripheral tissues, internalizing and processing antigens, but paradoxically they are unable to effectively present the processed antigens to T cells (17–20). In the second phase, DC migrate to regional lymph nodes (RLN) and home to T cell zones, where they rapidly lose their capacity to process antigen and are transformed into potent APC. This functional maturation process can be mimicked by in vitro culture in appropriate media (17–20).

In the context of the respiratory tract, such a model provides a theoretically ideal mechanism for limiting local T cell responses at the fragile epithelial interface with the outside environment. In support of the operation of such a mechanism in the lung, the present study demonstrates that: (*a*) freshly isolated DC from this organ function relatively poorly in presentation of protein antigens to immune T cells, after brief antigen pulsing in vitro (Fig. 2 *B*) or in vivo (Fig. 2 *A*); (*b*) this functional inadequacy is not due to defective antigen uptake, and moreover is reversible, as demonstrated by the marked improvement in APC activity achieved by overnight preincubation of antigen-pulsed DC before titration into cultures of immune T cells (Fig. 2 *C*), suggesting that it involves step(s) in processing and/or presentation; and (*c*) the capacity of lung DC to sensitize naive T cells in a primary MLR demonstrates identical maturation in overnight culture (Fig. 3).

Although it is currently believed that the presence of processed peptide in Bjorkman's groove is necessary for recognition of allogeneic class II MHC in a one-way MLR (37), the finding that MLR-stimulating activity improves markedly during overnight culture in serum-free medium (Fig. 3 *B*) argues against upregulation of capacity to internalize and cleave proteins per se as the sole basis for the functional maturation of lung DC. Furthermore, previous studies on DC from other

mAb. Note the lack of staining in the epithelium (*E*). (*A–D*) ×730. (*E*) Electron micrograph of pulmonary alveolar macrophage (*M*) spread upon the type I epithelial lining of an alveolus in the region of a septal junction, with its characteristic electron-dense cytoplasmic inclusions (lysosomes, phagosomes, and residual bodies). Note the candidate dendritic cell (*dc*) in the interstitial tissue with irregularly shaped indented nucleus. (*c*) capillaries. ×4000. (*F*) Electron micrograph of two pulmonary alveolar macrophages (*M*) at a septal junction, separated from a candidate dendritic cell (*dc*) by the intervening type I alveolar epithelial cell and basal lamina (*arrowhead*). ×4000; inset, ×17,000.

sites have clearly established that capacity to carry out this aspect of processing is maximal at the "peripheral tissue" stage of their life cycle (20). We have previously observed that overnight culture of lung DC upregulates surface expression of Ia (28; see also Table 1), and the density of Ia on the surface of APC has been shown to correlate directly with their potency in vitro (38). However, previous studies indicating disparity between the kinetics of upregulation of Ia expression on peripheral DC and upregulation of APC activity (34, 39, 40) suggest that additional factors are involved such as surface expression of cell–cell interaction molecules.

The results of this study additionally provide evidence for a hitherto unrecognized level of regulation of DC functions, viz. active suppression of APC activity during their residence in peripheral tissues by (in the case of the lung) endogenous tissue macrophages.

This evidence takes two forms. First, we demonstrate that selective in vivo depletion of PAM leads to rapid upregulation of the capacity of lung DC to present antigen to both naive (Fig. 1) and primed T cells (Fig. 2, *A* and *B*), the degree of enhancement approximating that achieved by overnight preculture of isolated DC. Second, we demonstrate that the addition of PAM to the DC culture system across a semipermeable membrane, strongly inhibits maturation of their APC functions (Figs. 3 *C*, 4, and 5), and is also capable of inhibiting the activity of DC which were already functionally mature at the time of extraction (examples in MLR results of Fig. 5).

These observations provide an explanation for our earlier observations on rat lung DC (2, 6), and subsequent reports from other laboratories on DC from rat (5) and human lung (4, 7), which indicated that the presence of contaminating tissue macrophages (particularly contaminating PAM) prevented the in vitro demonstration of the APC activity of DC in lung tissue digests. However, the results obtained with DMDP-liposomes argue that this inhibitory process is not confined to the test tube, but also occurs in the whole animal.

Our data also provide a mechanism for the observed effects of PAM depletion on immune function in live animals. We have previously demonstrated that PAM-depleted mice and rats become hyperresponsive to antigenic stimulation in the lung and mount large local and systemic immune responses to low levels of inhaled antigens which do not elicit responses in intact animals (21, 22, 41). Thus, whereas this heightened responsiveness may be partly attributable to release of local T cells from the lymphocytostatic effects of PAM (16), it appears likely that upregulation of local APC functions also plays an important role.

In relation to the plausibility of such a control mechanism in vivo, it is valid to question whether diffusible signals from PAM are likely to effectively blanket the epithelial surfaces of the respiratory tract and hence the underling DC, particularly in view of the extremely large surface areas involved (up to 75 m^2 in the adult human). The general perception of PAM as a highly mobile population of phagocytic cells that constantly patrol the alveolar surface within the epithelial lining fluids, scavenging inhaled particulates, is largely artefactual. Recent studies on the histological and ultrastructural appearance of the alveolar septa after various fixation regimes (26, 27) indicate that the presence of apparently "free" rounded PAM in the lumen of the airspaces represents an aberration caused by the common practice of intratracheal fixation, which (like the process of bronchoalveolar lavage) dislodges cells from the alveolar surface and alters their shape and position. To obtain an accurate impression of the in vivo situation, it is necessary to carry out fixation through the vascular bed at controlled perfusion pressures, whilst maintaining the lungs in the inflated state. The application of this procedure to both rat and human lung reveals a very different distribution pattern, in which resident PAM are intimately associated with epithelial surfaces in both the terminal airspaces and the conducting airways (26, 27), and in the case of human lung, virtually 100% of the PAM are localized at or directly adjacent to alveolar septal junctional zones formed by adjoining septa (27). Electron microscope examination of perfused-fixed peripheral lung samples from the animals used in this study revealed an identical picture (Fig. 7, *E* and *F*), where the spatial separation between PAM and the interseptal connective tissues, and the majority of the DC are resident, can be as small as the width of a single type I alveolar epithelial cell (i.e., $\leqslant 0.2$ μm).

We have not as yet investigated the corresponding situation in the conducting airways in detail, but initial observations at the LM level (Fig. 7, *C* and *D*) indicate close association of intraepithelial DC with mature tissue macrophages in the underlying mucosa, and furthermore, these latter macrophages markedly inhibit in vitro APC activity, analogous to PAM (6). In addition, studies from other laboratories have demonstrated PAM tightly adherent to the airway epithelium on the lumenal surface (26), suggesting that airway intraepithelial DC may receive inhibitory signals from both directions.

The nature of the cytokine(s) that mediate these effects have not been rigorously defined. As noted in Materials and Methods we have tested a range of candidate mediators alone and in combination, but thus far can report consistent results only with respect to TNF-α and nitric oxide. As noted above, TNF-α alone displays weak activity in overnight cultures, but synergizes effectively with transmembrane PAM in inhibiting functional maturation of the DC. mRNA for TNF-α is constitutively produced in high levels by PAM (42), and inhalation of inflammatory stimuli leads to the rapid secretion of bioactive cytokine in vivo (43). This cytokine has also previously been demonstrated to maintain the viability of DC in vitro without promoting their functional maturation (44), and additionally has been demonstrated to stimulate migration of peripheral DC to RLN after in vivo administration (45), suggesting that it may modulate the expression of cell–cell interaction molecules on the DC surface. The striking effects observed with MMA in the culture system (Fig. 6) strongly suggest that nitric oxide plays a more direct role in the inhibitory process, and the synergy observed with TNF-α may indicate differing (but complementary) targets on the DC for these two effector molecules, or (as suggested in 46, 47) stimulation of nitric oxide secretion by TNF-α. It is interesting to note in this context that the earlier literature on direct

suppression of in vitro T cell proliferation by PAM concluded that a dual signal was central to the mechanism of suppression, one being derived from the PAM and the other from the T cells themselves (for review see reference 16), and more recent studies on nitric oxide-mediated suppression by PAM produced identical conclusions (33). It should be emphasized, however, that PAM-mediated suppression of DC functions proceeds in the absence of T cells.

A potential complicating factor in our DC maturation experiments, which will be addressed in future studies, is the presence of contaminating cell types that copurify with the DC, and may secrete cytokines during in vitro incubation. In particular, we have identified a source of GM-CSF in our cultures (data not shown), which is likely to be due to the presence of epithelial cells that are known to be capable of secreting this cytokine (48). GM-CSF has been demonstrated to promote survival and maturation of DC in culture (49) and may therefore modulate responsiveness to other cytokines, and follow-up experiments will be required accordingly, employing purer lung DC preparations.

We also plan to test the effects of inflammatory agents on the secretion of DC-modulatory mediators by PAM, particularly in view of the recent demonstration by Bowers et al. (50) of the presence of both inhibitory and stimulatory activities in conditioned medium from LPS- and silica-exposed peritoneal exudate macrophages.

In conclusion, our study has demonstrated that, analogous to epidermal Langerhans cells, DC from lung and airway tissues sequester incoming antigens in situ, but do not appear to be capable of efficiently presenting inductive signals to T cells until they undergo a subsequent maturation step. We have further demonstrated that in the lung, they are maintained in this functional state via the secreted products of adjacent macrophages, which include nitric oxide and TNF-α. Experiments with cell preparations from other tissues will be required to ascertain whether this is an intrinsic component of the immunobiology of the overall DC system, or whether it represents an adaptive response peculiar to respiratory tract epithelial microenvironments.

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