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Airway Epithelial Cells Regulate the Functional Phenotype of Locally Differentiating Dendritic Cells: Implications for the Pathogenesis of Infectious and Allergic Airway Disease

Angela Rate,* John W. Upham,*† Anthony Bosco,* Kathy L. McKenna,* and Patrick G. Holt2*

Atopic asthma pathogenesis is driven by the combined effects of airway inflammation generated during responses to viral infections and aeroallergens, and both these pathways are regulated by dendritic cells (DC) that differentiate locally from monocytic precursors. These DCs normally exhibit a sentinel phenotype characterized by active Ag sampling but attenuated presentation capability, which limits the intensity of local expression of adaptive immunity. How this tight control of airway DC functions is normally maintained, and why it breaks down in some atopics leading to immunopathological changes in airway tissues, is unknown. We postulated that signals from adjacent airway epithelial cells (AEC) contribute to regulation of local differentiation of DC. We tested this in a coculture model containing both cell types in a GM-CSF-IL-4-enriched cytokine milieu characteristic of the atopic asthmatic airway mucosa. We demonstrate that contact with AEC during DC differentiation up-regulates expression of the function-associated markers MHC class II, CD40, CD80, TLR3, and TLR4 on DCs with concomitant up-regulation of Ag uptake/processing. Moreover, the AEC-conditioned DCs displayed increased LPS responsiveness evidenced by higher production of IL-12, IL-6, IL-10, and TNF-α. The Th2 memory-activating properties of AEC-conditioned DCs were also selectively attenuated. Data from microarray and blocking experiments implicate AEC-derived type 1 IFNs and IL-6 in modulation of DC differentiation. Collectively, these findings suggest that resting AECs modulate local DC differentiation to optimize antimicrobial defenses in the airways and in the process down-modulate capacity for expression of potentially damaging Th2 immunity.


The defining feature of inhalant allergy is development of Th2-polarized memory to airborne allergens and ensuing production of allergen-specific IgE Ab. This form of T cell immunity plays an important role in the pathogenesis of atopic asthma, in which Th2 cytokine responses to inhaled aeroallergens contribute to the development of airway obstruction and hyperresponsiveness (1). However, although up to 50% of Western populations are sensitized to one or more aeroallergens, only a proportion of these develop clinically significant asthma (2), suggesting that additional factors are involved. Recent human studies have identified a key cofactor role for respiratory viral infections in atopic asthma pathogenesis. Airway inflammation resulting from viral infection synergizes with allergic inflammation in driving progression from intermittent to persistent asthma (3); moreover, interactions between host responses to these two environmental stimuli represent the most common triggers for acute severe asthma attacks requiring hospitalization (4, 5). These observations highlight the complexity of the interactions between genetic and environmental factors that dictate downstream disease expression.

One such complexity involves the capacity of the local tissue microenvironment to regulate the effector functions of the incoming cell populations that mediate local host immune surveillance mechanisms in the airways. These local regulatory mechanisms are believed to play a major role in asthma pathogenesis, and elucidation of how they operate may provide novel insight into the choice of targets for therapy and/or prevention of the disease.

Airway mucosal dendritic cells (DC)3 represent major candidates for such a regulatory function, given the central role they play in the initiation of adaptive immune responses to both viruses and aeroallergens by virtue of their Ag capture and presentation activities (6–9). In common with DCs from other peripheral tissues, resting DCs in the airway mucosa are specialized for sampling the local antigenic environment, including within the airway lumen (10), but they lack the capacity for efficient presentation to T-memory cells (11). The latter requires a series of maturation steps that involve down-regulation of endocytic functions and concomitant mobilization of MHC to the cell surface and costimulatory expression (12). However, in resting airway mucosal tissues, this sequential activation and maturation process is not normally completed until after the DCs migrate to draining lymph nodes, and it is thought that this compartmentalization of functions protects airway tissues from T cell-mediated damage that could ensue from continuously responding to ubiquitous nonpathogenic Ags that are normally present in ambient air (11). At the same time, resident DCs in the mucosa are required to maintain efficient surveillance
for incoming pathogens, functioning as both an early warning system for triggering first-line innate defenses via TLR-mediated cytokine/chemokine secretion, and as the source of processed Ag required for priming of pathogen-specific Th1-polarized adaptive immunity. This fine balance between retention of efficient pathogen surveillance capability within locally differentiating airway DC populations and concomitant attenuation of their T cell activation properties represents a critical control point in relation to susceptibility to respiratory inflammatory diseases such as asthma. It is currently unclear how this control is achieved during DC differentiation within the airway mucosa.

Airway DCs are distributed as contiguous networks both within and beneath the airway epithelium, and it has been shown that under steady-state conditions there is continuous and rapid turnover of these populations (13); this already dynamic process can be further accelerated in response to inflammatory stimuli including viruses and allergens (14–17). One important source of the precursors for these DC networks is circulating PBMCs that, in response to certain chemokines, can readily migrate into peripheral tissues in both steady-state and inflammatory conditions (18) and subsequently differentiate into DC under direction of mediators such as GM-CSF. A number of studies have identified mesenchymal cells as having important roles in regulating this DC differentiation process in solid tissue microenvironments (19, 20). In the conducting airways, the mesenchymal cells of principal interest are airway epithelial cells (AEC) that are situated in close proximity to local DC networks. In particular the intraepithelial DC population at the direct interface between the airway mucosa and the external environment is closely associated with the epithelial basement membrane and hence the adjacent basal AEC (11, 21). AECs express an array of regulatory mediators (as reviewed in Ref. 22) and hence are obvious candidates for influencing the local differentiation and maturation of monocytic precursor cells after they migrate into the airway mucosal microenvironment.

There is a range of precedents for this suggestion. For example, it has been demonstrated that epithelial cells in the gastric mucosa condition local DCs through the release of soluble mediators, resulting in a noninflammatory phenotype that is crucial for the maintenance of intestinal homeostasis (23). More recent studies of the lung microenvironment suggest that AECs may also condition fully differentiated DCs (24, 25). However, there is no information available that relates specifically to the situation prevailing in conducting airway tissues, i.e., where incoming monocytes recruited by inflammatory stimuli undergo maturation and maturation of monocytic precursor cells after they migrate into the airway mucosal microenvironment.

Materials and Methods

Subjects

Adult volunteers (age range, 21–65 years) were recruited into the study and underwent skin prick testing to house dust mite (HDM) allergen. Adult subjects classified as HDM atopic if their HDM-specific wheal size was ≥3 mm (range, 3–15 mm) were included in the study. Subjects refrained from taking antihistamines or inhaled corticosteroids for 24 h before venipuncture.

16HBE140 cell line

The 16HBE140 cell line was a gift from Dr. G. A. Stewart (University of Western Australia, West Perth, Australia). Under submerged culture conditions, 16HBE140 cells form flattened layers with a height of 1–5 cells exhibiting well-defined tight, adherens, and gap junctions and are morphologically similar to basal AECs in vivo (26). Additional properties of bronchial basal cells displayed by 16HBE140 include ICAM-1 expression (27), specific lectin-binding activity (28), and the absence of secretory component (29). As such they appear to be an acceptable surrogate for the basal AECs that are in direct contact with intraepithelial DCs in vivo. Mycoplasma-free cells (passages 15–45) were maintained in EMEM with Earle’s balanced salts and 2 mM l-glutamine (SAFC Biosciences) supplemented with 10,000 U/ml penicillin, 10 mg/ml streptomycin (Life Technologies), and 10% FCS (JRH Biosciences) and incubated at 37°C in a humidified atmosphere of 5% CO2. Cells were seeded at a density of 105 cells/cm2 in 24-well uncoated plates to achieve approximately 105/50 cell line/cm2. As such they appear to be an acceptable surrogate for the basal AECs that are in direct contact with intraepithelial DCs in vivo.

Generation of monocyte-derived DCs (MDDC) in cultures with 16HBE140 cells

Peripheral blood from adult volunteers was collected into an equal volume of RPMI 1640 (Life Technologies) containing preservative-free heparin (20 U/ml). PBMCs were isolated after centrifugation of the blood over a Lymphoprep density gradient (Axis-Shield). Some PBMCs were cryopreserved for later use, and previous studies from our laboratory (30) have shown that cellular immune responses are not distorted by this process. From the remaining PBMCs, CD14+ monocytes were isolated by positive selection using MACS beads (Miltenyi Biotech) according to the manufacturer’s instructions. Monocytes were washed with medium and resuspended to 105 cells/ml in EMEM with Earle’s balanced salts and 2 mM l-glutamine (SAFC Biosciences) supplemented with 10,000 U/ml penicillin, 10 mg/ml streptomycin (Life Technologies), 5% FCS, recombinant human IL-4 (500 U/ml; ProSpec-Tany; Technogene), and recombinant human GM-CSF (1000 U/ml; ProSpec-Tany; Technogene), and reconstituent human IL-4 (500 U/ml; ProSpec-Tany; Technogene), and reconstituent human GM-CSF (1000 U/ml; Glaxo; 500–500 µl aliquots of the monocyte suspension (5 × 105 cells) were then added to all wells of an empty 24-well plate and a plate containing the s c m 16HBE cells for 5 days. This allowed the monocytes to take up position above and between AECs. Fresh medium with all supplements was added to all wells at day 2 or 3 of culture.

Cell sorting of MDDC by flow cytometry

Following a 5-day culture, MDDCs were removed from wells with and without AEC monolayers by consecutive washing with plain culture medium. Nonspecific binding of Abs to FcRs was controlled by preincubating MDDCs (5 × 105/50 µl/FACS tube; BD Biosciences) with human Ig (50 µg/ml; CSL). Cells were then incubated for 25 min at 4°C in the dark with optimized amounts of allopurinol-containing medium with CD11c (BD Biosciences). Following incubation, propidium iodide (PI; Sigma-Aldrich; for dead cell identification) was added to the final wash and cells were then resuspended in RPMI 1640 with 5% FCS. To achieve a relatively pure population of viable MDDC, CD11c+PI- cells were FACS sorted using a FACSAria flow cytometer (Becton Dickinson, USA; purity was routinely >97% for all experiments). MDDC generated in media alone were also subjected to immunostaining and sorting to control for any effects of these processes. 5 × 105 sorted MDDC were lysed in 500 µl of Trizol reagent (Life Technologies) and stored at −80°C before RNA extraction.

Phenotypic analysis of MDDC by flow cytometry

After sorting, MDDC were washed in PBS supplemented with 0.1% sodium azide and 1% normal human serum. Nonspecific binding of Abs to Fc receptors was controlled by preincubating MDDC (5 × 104 MDDC and 50 µl per FACS tube; BD Biosciences) with human Ig (50 µg/ml; CSL). Cells were then incubated for 25 min at 4°C in the dark with optimized amounts of fluorochrome-conjugated mAbs against HLA-DR, CD40, CD80, CD86, CD11c, CD1a, and CD14 (BD Biosciences). After incubation, cells were washed and fixed in 1% paraformaldehyde and analyzed within 24 h on a
normal individuals. The extract contains the two major allergens, Der p 1 and Der p 2, but does not contain some of the minor lipid soluble allergens found in Dermatophagoides spp. (B. Hales and W. Thomas, unpublished observation). Although the HDM extract contains trace amounts of endotoxin, removal of endotoxin from the allergen preparation (Acrodisc chromatography unit; Pall Corp.) has no effect on IL-5 and IL-13 synthesis by HDM-stimulated PBMCs (B. Hales, unpublished observation).

**Coculture of MDDCs and autologous PBMC**

Autologous PBMC were isolated from peripheral blood by density gradient separation as previously described. PBMCs (2 × 10^6 cells/well) were cultured in RPMI 1640 and 5% FCS in medium alone or with autologous MDDC (2 × 10^5 cells/well; 1:10 ratio). Cells were cultured without stimulation or with HDM (10 μg/ml). All cultures were performed in duplicate in round-bottom 96-well plates for 48 h at 37°C with 5% CO₂. Following culture, supernatants were collected and stored at −20°C. Cell pellets were stored in RNA later (100 μl/well; Ambion) at −20°C before RNA extraction.

**Quantitative real-time PCR**

Total RNA was isolated from cell pellets using TRIZol (Invitrogen Life Technologies) followed by the RNeasy minikit (Qiagen), according to the manufacturer’s directions. Reverse transcription was performed using the Omniscript kit (Qiagen) according to the manufacturer’s protocol with oligo(dT) and Superasin (Geneworks). Primer sequences were obtained from a database (http://pga.mgh.harvard.edu/primerbank; see Ref. 34), designed in-house using Primer Express software (Applied Biosystems) or purchased from Qiagen. Reverse-transcribed RNA samples were diluted 5-fold, and expression of target mRNA was quantified by real-time PCR using QuantiTect SYBR Green Master Mix (Qiagen) according to the manufacturer’s protocol. The RNA samples were of high purity.

**Microarray methodologies**

Total RNA was extracted using TRizol (Invitrogen) followed by RNeasy (Qiagen) according to the Qiagen protocol. The RNA samples were of high purity.
quality as assessed on the Bioanalyzer (Agilent Technologies). Total RNA samples (1 μg) were labeled with the one-cycle labeling kit (Affymetrix) and hybridized to U133 Plus 2.0 microarrays (Affymetrix) as previously described (35). Microarray data were analyzed in the R environment for statistical computing (www.r-project.org/) using the pseudoknot local motif algorithm (default model) for data preprocessing (36). The data were transformed to the log2 scale, and differentially expressed genes were identified using the moderated t test (37) with false discovery rate control for multiple hypothesis testing (38). The differentially expressed genes (false discovery rate cutoff, <0.01) were ranked based on their fold change values and were screened for membership of known biological pathways catalogued in the Molecular Signatures Database (Subramanian et al.; Ref. 39). The Gene Expression Omnibus accession number for these data is GSE12773; it can be viewed at http://www.ncbi.nlm.nih.gov/geo. Selected genes were validated by quantitative real-time PCR in an independent sample set according to the methodology outlined above.

Blocking of type 1 IFN, IL-6, and IL-10 signaling in cocultures

Neutralization of type 1 IFN was achieved by addition of purified B18R protein (E-bioscience) to the cultures at days 0 and 3. This protein has been shown to bind to the surface of cells and act as a decoy receptor for type 1 IFN, preventing both paracrine and autocrine signal transduction by these cytokines into the target cell and importantly has high affinity for all human type 1 IFN subtypes (40–42). In our hands, a 0.05-μg/ml portion of the protein administered at initiation and halfway through coculture provided an ~80% reduction in type 1 IFN signaling into the cells as measured at day 5 (data not shown). Blocking Abs for IL-6 and IL-10 and corresponding matched IgG1 and IgG2 isotype controls were purchased from BD Biosciences and added at days 0 and 3 at 2.5 μg/ml.

Statistical analyses

Group data were expressed as means ± SE of the mean. Data were analyzed using Wilcoxon signed-rank test for paired data unless otherwise stated. All analyses were performed with SPSS version 11 for Macintosh (SPSS).

Results

AECs modulate the surface phenotype and cytokine profiles of DCs differentiated from moncytic precursors

We hypothesized that AECs from healthy individuals could contribute to immune homeostasis in the lung via modulation of DC differentiation and maturation from monocyte precursors. To examine this, we used the well-described in vitro culture system involving the cytokine-driven differentiation of monocytes into DCs in coculture with cells from the 16HBE airway epithelial line. Monocytes were introduced into semiconfluent 16HBE monolayers to allow the monocytes to take up position above and between AECs. We subsequently evaluated the phenotype and function of the MDDCs arising in these cultures and compared the results with those of monocytes that differentiated into DCs in the absence of AECs (Fig. 1). As expected, monocytes cultured on their own with IL-4 and GM-CSF significantly up-regulated their expression of HLA-DR and the costimulatory molecules CD40, CD80, and HLA-DR, whereas the expression of CD86, CD11c, DC-SIGN, and CD1a was not significantly different from the control MDDC. In addition, the AEC-conditioned MDDCs retained significantly higher levels of CD14 on their surface. These experiments were replicated with a second normal human bronchial cell line, BEAS-2B, and the surface phenotype of the resulting AEC-conditioned MDDCs was comparable with that seen with 16HBE (data not shown); subsequent experiments focused on the latter.

In follow-up experiments, it was demonstrated that the ability of AEC to modulate the MDDC phenotype was contact dependent, given that supernatants derived from resting AEC cultures had no effect on cytokine-driven differentiation of monocytes into MDDCs (data not shown). Moreover, dead AECs had no effect on cytokine-driven differentiation of monocytes into MDDCs (data not shown). Still, supernatants derived from resting AEC cultures had no effect on cytokine-driven differentiation of monocytes into MDDCs (data not shown). Moreover, dead AECs had no effect on cytokine-driven differentiation of monocytes into MDDCs (data not shown).

We next examined whether the presence of AECs during monocyte-to-DC differentiation was accompanied by changes in cytokine synthesis upon exposure to innate stimuli. As expected, LPS activation of MDDCs following their purification by flow cytometric sorting and exposed to LPS (10 ng/ml) for 21 h (C) or poly(IC) (10 μg/ml) for 24 h. Cell-free supernatants from control MDDC (CTRL) or AEC-MDDC (A) cultures were collected and assayed for IL-12p70, IL-10, IL-6, and TNF-α with time-resolved fluorometry. Data from eight independent experiments are presented as delta mean values ± SEM. * p < 0.05 compared with control (CTRL) MDDCs; ** p < 0.01 compared with control MDDC; *** p < 0.001 compared with control MDDCs. Before stimulation, total RNA was isolated for real-time PCR analysis from sorted control MDDC and AEC-MDDC. Data from 12 independent experiments are presented as mean ± SEM baseline expression of the TLR3 (D) and TLR4 (E) target genes normalized to the stable housekeeping gene EF1A1 for control MDDCs (CTRL) and AEC-MDDCs (A). * p < 0.05 compared with control MDDC; ** p < 0.01 compared with control MDDC. F, TLR4 protein expression was measured by flow cytometric analysis of sorted control MDDCs (CTRL) and AEC-MDDCs (A) before stimulation and normalized to levels present on autologous monocyte precursor cells which served as internal controls. Data from eight independent experiments are presented as mean MFI ± SEM. *** p < 0.001 compared with monocytes.
Supernatants were collected after 48 h and IFN-γ was supplemented with MDDCs and expressed as mean values ± SEM. *p < 0.05 compared with control MDDCs; **p < 0.01 compared with control MDDCs; ***p < 0.001 compared with control MDDCs.

AECs enhance Ag uptake and processing capacity of DC differentiated from monocytc precursors

To investigate whether AECs altered Ag uptake by DCs, we analyzed mannos receptor-mediated endocytosis of FITC-dextran. At all points throughout the time course of the uptake assay, AEC-conditioned MDDCs exhibited significantly higher levels of fluorescence than the control MDDCs, indicating increased levels of FITC-dextran uptake (Fig. 3A).

We next sought to determine whether the enhanced endocytic activity in AEC-conditioned MDDCs was paralleled by changes in Ag-processing activity. We used the self-quenched marker DQ-OVA, which consists of naturally mannosylated OVA extensively labeled with the fluorochrome BODIPY, to visualize Ag presentation. Control MDDCs exposed to DQ-OVA showed a time-dependent increase in fluorescence, corresponding to intracellular processing of this model Ag (Fig. 3B). In comparison, the mean fluorescence levels in MDDCs exposed to AECs during differentiation were significantly higher at all time points measured, indicating that conditioning of MDDC by the AEC markedly enhanced their ability to process internalized Ag.

AEC conditioning of monocyte-derived DC has downstream effects on the T cell memory response

We finally sought to identify whether these AEC-induced changes in the Ag-processing properties of MDDC influenced their capacity to subsequently present Ag-specific activation signals to T-memory cells. Our model system used PBMC from atopic subjects sensitized to HDM allergen. Stimulation of whole PBMC cultures with HDM resulted in modest Th2-polarized recall responses characterized by production of significant levels of IL-5, IL-9, and IL-13 triggered by endogenous APC within the PBMC population (Fig. 4A). Concomitant supplementation of these cultures with low numbers of MDDC (equivalent to 10% of PBMC cultures), either control or AEC-conditioned, resulted in markedly enhanced recall responses characterized by elevated levels of both Th2 and Th1 (IFN-γ) cytokine production. However, the Th2 cytokine component was consistently attenuated in the cultures containing AEC-MDDC relative to that triggered by control MDDCs. This attenuation of the Th2 dominance of the recall response was reflected in the elevated ratios of IFN-γ to the three Th2-associated cytokines measured (Fig. 4B).

To elucidate the mechanisms underlying the modification of downstream T-memory responses to HDM presentation by AEC-MDDCs, we examined the levels of transcripts of polarizing cytokine genes in the pellets of both MDDC populations by quantitative real-time PCR. It was observed that the AEC-MDDCs expressed significantly higher levels of IL-12p35 and IL-12p40 as compared with control MDDCs.
Molecular Signatures Database (39). Fig. 6 membership of known biological pathways cataloged in the pressed relative to control MDDC were initially ranked by fold replicate paired sets of cells. Genes that were differentially ex-
croarray in control MDDCs vs AEC-MDDCs, using three rep-
AEC conditioning of MDDCs, we performed a preliminary 
Gene expression profiling of maturing MDDCs 

Fig. 5. Measurement of mRNA transcripts for IL-12p35, IL-
12p40, IL-10, and IL-23p19 in MDDC. A, After flow cytometric sorting at
day 5, levels of IL-12p35, IL-12p40, IL-10, and IL-23p19 were measured in the unstimulated cell pellets by quantitative real-time PCR in control MDDCs (□) and AEC-MDDCs (■). Data from 12 independent experiments are presented as mean values ± SEM of the ratio of the target gene normalized to the stable housekeeping gene EEF1A1. *** p < 0.001 compared with control MDDC. B, Ratios of IL-12p35:IL-12p40 and IL-12p35: 

IL-10 were calculated for each experiment and expressed as mean values ± SEM for control MDDCs (□) and AEC-MDDCs (■). * p < 0.05 compared with control MDDC; *** p < 0.001 compared with control MDDC.

well as IL-10; however, there were no differences in the levels of the IL-23 subunit p19 (Fig. 5A). In addition, MDDCs differentiated in the presence of AECs demonstrated significantly higher IL-
12p40, IL-10, and IL-23p19 in MDDC.

Genotype expression profiling of maturing MDDCs
To gain some insight into the potential mechanisms involved in AEC conditioning of MDDCs, we performed a preliminary study comparing gene expression patterns by Affymetrix microarray in control MDDCs vs AEC-MDDCs, using three replicate paired sets of cells. Genes that were differentially expressed relative to control MDDC were initially ranked by fold change, and the top 50 genes (differentially expressed in the range of 10- to 100-fold in AEC-MDDCs) were screened for membership of known biological pathways cataloged in the Molecular Signatures Database (39). Fig. 6A illustrates the data for the top 15 most differentially expressed genes in AEC-MDDCs, of which 10 (Fig. 6A, closed symbols) are in the type 1 IFN-inducible pathway (43–45). Moreover, further analysis of the data set confirmed the up-regulated expression of ≥40 IFN-inducible genes in the AEC-MDDC population (data not shown). Furthermore, upon quantitative PCR examination of the AEC pellets recovered from the cocultures, levels of type 1 IFN mRNA expression were elevated compared with resting AECs and AECs stimulated with IL-4 and GM-CSF (Fig. 6C).

Examination of the cell pellets of the control MDDC and AEC-MDDC revealed increased levels of mRNA transcript coding for the two subunits of the type 1 IFN receptor in the latter population (Fig. 6D). Finally, a number of genes identified as up-regulated on the microarrays were flagged by pathway analyses as type 1 IFN sensitive, in particular MxA, which is the most prominent signature gene defining the type 1 IFN signaling pathway (43, 45). MxA gene transcription induced rapidly in the cocultures of monocytes and AEC (Fig. 6E) but was absent from monocytes cultured for 5 days with IL-4 and GM-CSF and in cultures of AEC in medium alone (data not shown). IL-4 and GM-CSF induced some MxA mRNA expression in AEC cultures without monocytes, albeit at much lower copy numbers and considerably slower relative to the AEC-monocyte cocultures. In addition to MxA, a large panel of other type 1 IFN-sensitive genes was identified as up-regulated on the AEC-MDDC microarrays (data not shown), and a subset of these genes was also validated by quantitative real-time PCR in an independent set of samples, including IRF7, STAT1, and OAS (data not shown). Collectively, these data implicate type 1 IFNs produced by AEC as potential candidates for the MDDC-conditioning role in this coculture model.

Fig. 6. Gene expression profiling studies. A, Following flow cy-
tometric sorting at day 5, total RNA from three paired control (CTRL) 
MDDC and AEC-MDDC populations was extracted and analyzed on 

microarrays. Genes differentially expressed in AEC-MDDCs relative to respective control MDDCs were identified using a moderated t test and were further ranked based on their fold change values. A subset of these genes representing the top 15 highest ranking genes up-regulated in AEC-MDDCs are illustrated for each pair. ○, IFN-inducible genes (43, 44); □, non-IFN-inducible genes. Gene abbreviations: IFI27, IFN-α-inducible protein 27; CCL8, chemokine ligand 8; RSAD2, radical S-adenosylmethionine domain containing 2; IFN-induced transmembrane protein 1; NEXN, nexilin; APOBEC3A, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3A; IFI71, IFN-induced protein with tetratricopeptide repeats 1; IFI44L, IFN-induced protein 44-like; IFI72, IFN-induced protein with tetratricopeptide repeats 2; BST1/ BST2, histone cluster 1, H2bg; NNMT, nicotinamide N-methyltransferase; KIAA0101, hypothetical protein LOC9768; SERPING1, serpin peptidase inhibitor, clade G (C1 inhibitor); CNN3, calponin 3, acidic. B, RNA extracted from sorted AEC following 5-day culture with 
or without IL-4-GM-CSF and/or monocytes was analyzed by quantita-
tive real-time PCR for levels of IFNAR1 and IFNAR2 transcripts (C), which were normalized to the housekeeping gene EEF1A1. Results are presented for six independent experiments and expressed as mean ± SEM. *, p < 0.05 compared with AEC alone. D, RNA extracted from control MDDCs (□) and AEC-MDDCs (■) following 5-day cultures was analyzed by quantitative real-time PCR for levels of IFNAR1 and IFNAR2 transcripts and MxA transcripts (E). Results are presented for eight independent experiments and are expressed as mean ± SEM. ***, p < 0.001 compared with control MDDC.
Blocking of type 1 IFN signaling during MDDC differentiation

To investigate the potential functional significance of the type 1 IFN signature in AEC-MDDCs, we used a commercially available purified protein, B18R, which blocks type 1 IFN signaling into target cells by acting as a decoy receptor (40–42). This protein exhibits high specificity and affinity for all known subtypes of the type 1 IFN family and thus circumvents the lack of a commercially available blocking Ab to the type 1 IFN receptor. We achieved an 80% reduction in the mRNA expression of gene transcripts downstream of the IFNAR complex when applied to cultures at days 0 and 3 at 0.05 μg/ml (data not shown).

Initial experiments focused on the effects of blocking type 1 IFNs on the surface markers CD14, CD40, CD80, and HLA-DR identified in Fig. 1 as differentially expressed on AEC-MDDCs. The presence of the B18R protein during the 5-day differentiation period resulted in significant attenuation of the effects of AEC on CD80 and HLA-DR expression during MDDC differentiation; however, AEC modulation of expression levels of CD14 and CD40 was not altered (Fig. 7A). Furthermore, blocking of type 1 IFN signaling during MDDC differentiation attenuated the AEC-mediated boosting of TLR3 and TLR4 expression (Fig. 7B) and downstream responses to LPS and poly(IC) (Fig. 7C). Finally, the Thi skewing activity of AEC-MDDCs relative to control MDDCs in T-memory cell activation assays was partially reversed if the AEC-MDDCs had been differentiated in the presence of the B18R protein (Fig. 7D). Taken together, these data are consistent with a role for type 1 IFN in the modification of MDDC surface phenotype and function in the context of innate and acquired immune responses in the airways.

IL-6 and IL-10 as other potential modifiers of maturing MDDCs

On the basis of the data obtained in the type 1 IFN blocking experiments showing incomplete reversal of phenotype and function of MDDC conditioning, we hypothesized that additional mediators are involved in the process. To address this issue, we re-examined the gene set analysis of the microarray data and identified additional signaling pathways that were differentially expressed in the two MDDC groups (data not shown). Of these, IL-6 and IL-10 pathogens were flagged as being significantly up-regulated in the AEC-MDDCs; thus, we elected to further examine their role.

We confirmed the presence of IL-6 in the cocultures by examining mRNA transcript levels and secreted protein concentrations in supernatants during the 5 days. Analogous to MxA, IL-6 copy number increased over time in the monocyte-AEC cocultures but
MDDCs (f). Data from eight independent experiments are presented as delta mean values of anti-IL-6 blocking Ab, surface marker expression was measured, and MFI values of control MDDCs (B) were normalized to monocyte levels. Data from 12 independent experiments are presented as mean fold change ± SEM. **, p < 0.01 compared with control MDDCs; ###, p < 0.001 compared with control MDDCs + anti-IL-6; ##, p < 0.01 compared with control MDDCs + anti-IL-6. B, Following 5-day differentiation of control and AEC-conditioned MDDCs with or without addition of anti-IL-6 Ab, MDDCs were exposed to LPS (1 ng/ml) or polyIC (10 µg/ml) for 24 h, and cytokine concentrations in cell-free supernatants were measured for control MDDCs (■) or AEC-MDDCs (■). Data from eight independent experiments are presented as delta mean values ± SEM. **, p < 0.01 compared with control MDDCs; #, p < 0.05 compared with control MDDCs + anti-IL-6; ##, p < 0.01 compared with control MDDCs + anti-IL-6. C, Autologous PBMCs were cocultured in medium alone or with each MDDC population generated with or without anti-IL-6 Ab addition in the presence or absence of HDM (10 µg/ml). Cell-free supernatants were collected after 48 h, and T cell cytokine concentrations were measured for PBMCs alone (■), PBMCs and control MDDCs (■), and PBMCs and AEC-MDDCs (■). Data from eight independent experiments are presented as delta mean values ± SEM. **, p < 0.01 compared with control MDDCs; ##, p < 0.001 compared with control MDDCs; #, p < 0.05 compared with PBMCs alone; ###, p < 0.01 compared with PBMCs alone; ##, p < 0.01 compared with PBMCs and control MDDCs; ##, p < 0.01 compared with PBMCs and control MDDCs + anti-IL-6. Matched isotype control Ab had no effect on surface marker expression in any condition (data not shown in the interest of brevity). Inhib., Inhibition.

not in those of MDDCs differentiated alone, and this was accompanied by a parallel elevation of IL-6 protein concentration in supernatants (data not shown). Following neutralization of soluble IL-6 by specific blocking Ab for the duration of culture, we again examined CD14, CD40, CD80 and HLA-DR to identify any changes in their expression levels on the MDDCs. In contrast to type 1 IFN inhibition, blocking IL-6 signaling had no effect on the boosted surface levels of CD80 or HLA-DR on the AEC-MDDCs but led to a significant reduction in CD14 and CD40 expression (Fig. 8A). We next sought to determine the consequences to the functional capacity of AEC-MDDC when IL-6 signaling was blocked during differentiation. Inclusion of anti-IL-6 Ab in the monocyte-AEC cocultures did not affect subsequent TLR responses of AEC-MDDCs (Fig. 8B). In contrast, the lack of IL-6 signaling during AEC-MDDC generation attenuated the Th1-skewing effects of these cells in Ag presentation assays similarly to that observed above with type 1 IFN blocking; however, statistical significance was not achieved in this case (Fig. 8C).

IL-10 was also examined as a potential MDDC modifier. Whereas IL-10 mRNA levels were not different in the two cultures containing differentiating monocytes, examination of supernatants revealed a measurable but decreasing concentration of IL-10 protein in coculture supernatants that was absent from control MDDC cultures (data not shown), thus confirming the microarray data and gene set analysis. Examination of day 5 MDDC and AEC-MDDC generated in the presence IL-10-neutralizing Ab showed no differences in the levels of candidate markers CD14, CD40, CD80, and HLA-DR compared with cultures without IL-10 blocking or those with matched isotype control Abs (data not shown). Thus, this cytokine appears to have no significant role in AEC conditioning of the MDDC surface phenotype.

Discussion

The pathogenesis of atopic asthma crucially depends on the function of DC populations that control local triggering of allergen-specific Th memory cells and also orchestrates innate and adaptive immune defense against viral infections. This study addressed the hypothesis that signals from AECs adjacent to airway mucosal DCs contribute toward regulation of their functional phenotype.

Rapid influx of DC precursors into the airway mucosa is a hallmark of host responses to the two principal environmental agents implicated in driving atopic asthma pathogenesis, notably virus and aeroallergen, and the available evidence points to monocytes as the major source of these precursors. In atopic asthmatics, a characteristic feature of the cytokine milieu of the airway mucosa is the presence of GM-CSF and IL-4 from a variety of cell types...
(46–49). We developed the present in vitro model to test the hypothesis that AEC may modulate DC differentiation in this cytokine-rich environment, in particular modifying their immune surveillance and T cell activation properties.

Our results show that the presence of AEC during MDDC generation resulted in a modified surface marker expression profile, indicating differences in differentiation and activation status. Levels of de novo CD1a expression on the surface of control MDDCs and AEC-MDDCs were comparable, a result that on its own would indicate that both cell types had progressed along the DC differentiation pathway (50). In addition, staining for MHC class II and costimulatory molecules revealed enhanced expression of these molecules on AEC-MDDCs which in the conventional view of DC maturation would suggest that these cells were phenotypically more mature than their control counterparts (51). In contrast, there was a significantly higher level of expression of CD14 retained on the surface of the MDDCs when differentiated in the presence of AECs compared with control MDDCs in which there was virtually no detectable CD14 protein. Our results are consistent with low levels of CD14 and moderate to high levels of MHC class II and costimulatory molecules observed on the surface of myeloid DCs obtained from surgically resected human lung specimens (52). This result would suggest that the differentiation/maturation of CD14+ monocytes into CD14− MDDCs was delayed by the presence of AEC (Fig. 1). This notion of attenuated differentiation is supported by the observation of higher levels of Ag uptake and processing by the AEC-MDDC (Fig. 3), a function that has been shown to be inversely correlated with the maturation status of DCs (51). This dichotomy of phenotype and function has been recently documented in an in vivo study of respiratory tract DCs from human subjects which showed that whereas DC from atopic asthmatic airways displayed higher levels of maturation markers, they were more adept at endocytosis than healthy controls (53). Their data and ours collectively suggest that regulation of phenotypic maturation and Ag uptake can be uncoupled in the airways and that AEC may play a role in the differential regulation of the two processes.

The AEC-MDDCs also showed a propensity for enhanced responses to activation with the innate stimuli LPS and poly(IC) relative to control MDDCs. Further investigation revealed increased levels of the genes TLR3 and TLR4 encoding receptors for these ligands on MDDCs (Fig. 2, D and E). This result, taken together with the observation of retention of CD14 on the surface of the AEC-conditioned MDDC (a molecule known to enhance TLR3- and TLR4-mediated signaling; see Refs. 54–56), provides a possible mechanism for the increased sensitivity of these cells to both innate stimuli. Another possibility is modulation of the TLR response at the intracellular signaling level as has been observed in other studies (57). Interestingly, a number of downstream TLR-signaling gene transcripts were flagged in the microarrays as being significantly increased at baseline in the AEC-MDDC compared with control MDDCs including MyD88, NFKB2, TRAM, and IFN regulatory factor 8 (data not shown), but further investigation of these differences was beyond the scope of this study. Priming with IFN-γ before the addition of LPS resulted in a further boosting in cytokine responses (particularly IL-12) by the AEC-MDDC that was not mirrored by the control MDDC population. This was not associated with a difference in IFN-γ receptor gene expression in the MDDC (data not shown) and accordingly may involve control at the level of receptor translocation to the cell surface or downstream signaling.

In addition to up-regulation of microbial surveillance mechanisms, the AEC-MDDC displayed enhanced Ag uptake and processing capability, and increased capacity for mobilization of MHC class II to the cell surface, together with elevated levels of CD40 and CD80 (Figs. 1 and 3). These changes are likely to increase capacity to deliver pathogen-specific priming signals to the T cell system in the regional lymph nodes and might also influence local T cell recall responses to incoming allerger. In this context, IL-5, IL-13, and IL-9 responses of atopic T cells to allergen presentation by AEC-MDDCs were selectively diminished relative to those in T cells stimulated with control MDDCs, indicating a potential bias directed against local expression of Th2 immunity.

There are a number of possibilities that could account for this difference in T cell responses to allergen presentation. For example, higher levels of peptide processing by AEC-MDDCs and enhanced MHC class II expression on their surface may result in higher effective concentrations of allergen presented to the T cells at the MHC-TCR synapse, resulting in preferential triggering of Th1 cytokine production. This suggestion is consistent with results from several previous human studies showing that low levels of antigenic stimulation in vivo or in vitro selectively drive Th2-dominated recall responses, while higher stimulation tends to preferentially trigger Th1 cytokine production (58, 59).

An alternative possibility is the shift in the balance of expression of CD80 and CD86 on the AEC-MDDC surface toward CD80 (Fig. 1). The interaction of these B7 family costimulator molecules on the surface of APC with the CD28 molecule on T cells has been shown to play a critical role in the MHC class II-restricted presentation of peptide and accompanying activation of responder T cells (60). CD86 is constitutively expressed on APCs and rapidly up-regulated upon maturation and interaction with CD28, whereas CD80 expression is inducible over a longer period and is more stable than the former (61). Despite exhibiting similar binding affinity to the CD28 ligand, CD80 and CD86 exhibit different biochemical characteristics that can result in different T cell functional outcomes (62). While there is not complete consensus in the literature, it is the generally held view that CD80 favors Th1 responses whereas CD86 promotes the expansion of a Th2 population and enhanced IL-4 production (63), and the shift toward CD80 dominance observed in AEC-conditioned MDDCs may thus contribute toward their Th1-stimulatory properties. Circumstantial in vivo evidence also favors this hypothesis including Langerhans cells expressing higher CD86 levels in Th2-mediated vernal keratoconjunctivitis (64); and in the lungs of patients with Th1-mediated sarcoidosis, there is an increased expression of CD80 but not CD86 (65). We additionally examined expression of other potential contributors, notably OX40 ligand (66) and Jagged/Delta Notch family ligands (67) but did not detect significant differences between MDDCs and AEC-MDDCs (data not shown).

An additional factor that could contribute to the Th1-trophic phenotype of AEC-MDDCs is their enhanced IL-12 production. As noted previously, mRNA transcript levels of the IL-12 subunits p35 and p40 were expressed at significantly higher levels in AEC-MDDCs relative to control MDDCs and the p35:p40 ratio was also significantly increased (Fig. 5). In the context of IL-12 function, both genes must be expressed coordinately in the same cells to produce the biologically active heterodimer; however, p35 has been determined to be the rate-limiting factor (68). In the case of the AEC-modified MDDC, we speculate that the higher p35:p40 ratio might result in increased expression of functional IL-12 protein, thus enhancing the capacity of these cells to prime for Th1 immunity (69–71). This may in parallel conserve the Th1 component of locally triggered allergen-specific memory T cell responses. In this context, it is pertinent to note earlier reports that IL-12 signaling from a source such as the DC is required to stabilize the IFN-γ transcriptional machinery during the generation of effector T cells from their previously primed memory precursors, thus effectively maintaining a Th1-polarized population (72, 73). In our system, we did not observe a consistent boosting of the T cell-derived IFN-γ by AEC-MDDCs, as
has been reported in studies on restimulation of Th2 memory cells in the presence of excess IL-12 (74). However, the cytokine phenotype of typical human atopics is most commonly Th0 (i.e., mixed Th1-Th2) with an already high IFN-γ component (33, 75); as observed in Fig. 4, this was preserved in AEC-MDDC cultures in the face of diminished output of Th2 cytokines.

Additionally, analysis of the p19 subunit of IL-12 that combines with the p40 molecule to form functional IL-23 revealed no differences between the two MDDC populations. IL-23 has been shown to also play an important role in supporting IFN-γ responses between the two MDDC populations. IL-23 was also noted that IL-10 transcripts were enriched in the AEC-MDDC. The potential role of IL-10 in this context remains unclear, but in a murine in vivo model of allergic airways inflammation, endogenous IL-10 production by DCs was found to contribute to a reduction in both Th2 cytokine production and goblet cell hyperplasia (77).

Our study overall provides support for a direct role for AECs in the regulation of local DC differentiation and maturation, which is in line with previous less detailed reports of AEC-DC interactions within the airways. In particular, it has recently been reported that the presence of AEC enhances the responsiveness of mature MDDC to bacterial and particulate Ags (24, 25). Additionally, another study has demonstrated that while supernatants derived from activated AEC drive partial differentiation of monocytes into DC, there was no such effect from AEC supernatants collected in the absence of exogenous stimuli (78). In accord with this latter study, we also found that the addition of supernatants from resting AECs did not modify MDDC differentiation (data not shown), suggesting that this regulatory phenomenon requires cell-to-cell contact. It is possible, however, that the concentrations of AEC-derived soluble mediators at the synapse between AEC and monocytes in direct physical contact can be sufficiently high to mediate the effects observed, but we were unable to directly test this.

AEC produce a broad range of immunoregulatory molecules (11, 79), and it is possible that a variety of their products may contribute to the changes in MDDC functions observed here. As an initial step toward elucidation of this process, we have taken a genomics approach, seeking to identify gene expression signatures in the MDDC that might be specifically associated with the presence of AECs during differentiation. Gene profiling using Affymetrix microarrays demonstrated a prominent type 1 IFN signature in the AEC-conditioned MDDCs. Consistent with this finding, parallel PCR analysis of the AECs cocultured with MDDCs demonstrated up-regulated levels of IFN-α2 and IFN-β mRNA compared with resting AECs or AEC cultured with IL-4-GM-CSF alone. It is well documented that type 1 IFNs form a crucial component of the antiviral defense system in the lung (as reviewed in Ref. 80) and can also direct the induction of adaptive immune responses via promotion of DC differentiation and activation (81).

AECs represent a significant source of type 1 IFN production in the lung during viral infection (82) and also at baseline (83) and a deficiency in production by AECs has been associated with increased risk for exacerbation of allergic asthma (84). In addition, studies using IFN-β knockout mice have demonstrated that in the absence of this type 1 IFN, there is an increased expansion of CD4+Th2 cells and release of their associated cytokines IL-4, IL-5, and IL-13 as well as a marked increase in IgE-producing cells (85). Moreover, administration of rIFN-α into sensitized animals prevented the aeroallergen-induced influx of eosinophils and Ag-induced CD4+ T cells in the airway (86) accompanying IL-5 production (87).

From our results, it appears that the expression of type 1 IFNs by AEC is for the most part contingent upon the contact of these cells with monocytes, as there was little or no detectable signal when AEC were grown in isolation (data not shown); the mechanism for their contact-dependent induction is as yet unknown. Blocking of type 1 IFN signaling in this system by addition of a global type 1 IFN inhibitor partially reversed the boosting of CD80 and MHC class II and cytokine responses to TLR ligands by AEC-MDDCs, presumably due in part to the down-modulation of the enhanced levels of TLR3 and TLR4 (Fig. 7, A–C). In addition, blocking type 1 IFN attenuated the Th1-skewing activity of AEC-MDDCs during Ag presentation (Fig. 7D). However, the absence of complete reversal of AEC conditioning when type 1 IFN signaling is blocked suggests that other AEC-derived mediators may contribute to this regulatory process.

IL-6 is another pleiotropic cytokine that can have substantial effects on immune cell function, and as noted in the results section, we detected a clear IL-6 signature in the AEC-MDDC array data. The possibility of IL-6 as an additional mediator of AEC-driven MDDC modification in our model was particularly interesting in the context of the results of the previous report upon which our differentiation coculture model was based (20). Differentiation of monocytes with IL-4 and GM-CSF in the presence of fibroblasts or epithelial cells gave rise to cells that were morphologically and functionally representative of macrophages, a process that was driven by the contact-dependent expression of IL-6 by the stromal cells. Thus, their studies and those of others (88, 89) point to IL-6 as representing potentially significant control factor in APC maturation.

In the current model, we detected IL-6 protein in the supernatants of the differentiation cocultures, the presence of which was contingent on the contact of monocytes and AECs. However, in contrast to the previous reported studies, we did not see convincing evidence of a switch to a macrophage program of differentiation. Specifically, although the AEC-conditioned day 5 cells retained some expression of CD14, which is characteristic of the macrophage phenotype (although also revealed to be representative of ex vivo human lung myeloid DC; see Ref. 52), they did not display typical macrophage vacuolar morphology and demonstrated efficient capacity to stimulate allogeneic T cells in an Ag presentation assay, a function generally lacking in the macrophage population. Other studies have revealed that an array of factors can interfere with IL-6 signaling including IL-1β, LPS, TNF-α, CD40L, and TGF-β1 (88, 90). Here we identified the presence of TNF-α in the supernatants of the monocyte-AEC cocultures (data not shown). In contrast, there is also evidence to suggest that type 1 IFN signaling via its receptor complex can enhance IL-6 signaling in target cells (91). Restrictions on the starting number of freshly isolated human monocytes precluded our ability to examine any additive effects of the simultaneous blocking of IL-6 and type 1 IFN or other mediators in the differentiation cultures. As a result, we may only presume given the data we have that there is a complex interplay of multiple factors existing in the interface of the AEC and differentiating monocytes that are responsible for the modification of the MDDC phenotype.

In conclusion, our results suggest that immunological homeostasis within the airway mucosa is regulated to a significant extent by secretion of factors including type 1 IFNs and IL-6 by resting AECs, resulting in downstream effects on the local differentiation of incoming monocytic precursors that replenish the rapidly turning over populations of airway mucosal DCs. The scenario of particular interest in this study is the airway mucosal microenvironment in atopy, which is enriched in cytokines such as GM-CSF and IL-4 and in which the maintenance of immunological homeostasis requires that local mucosal DC achieve tight control of potentially damaging Th2 immunity to aeroallergens while maintaining capacity for rapid mobilization of innate and Th1-polarized adaptive
immune defenses against pathogens. Our data suggest that low-level constitutive production of type 1 IFNs by AECs at baseline is central to maintaining local DCs in the ideal functional phenotype to achieve this balance. It is of interest to note in this context the recent report that disturbance of this milieu via a viral infection that escapes initial innate clearance mechanisms and as a consequence triggers high level production of type 1 IFN by AECs can result in the obverse, notably marked enrichment of local Th2 immunity via superinduction of high-affinity IgE receptor (FceRIa) on airway DCs (92). Up-regulation of this receptor does not occur in AEC-MDDCs in this system (data not shown) despite the presence of a type 1 IFN signature in the MDDC (Fig. 6A), which presumably indicates that the levels of type 1 IFN stimulation provided by the AECs are below the critical threshold required for FceRIa induction. Additionally, experimental models examining the effects of oral type 1 IFN on resistance to viral infection (93) and resistance to allergen-driven late apoptotic asthmatic responses (94) have reported biphasic type 1 IFN dose-response curves with maximal protection at low as opposed to high dosages.

We acknowledge that the present findings are based solely on the use of a bronchial epithelial cell line, and accordingly it will be important that this study is subsequently reproduced in a model using primary AECs. Ideally, follow-up studies should also involve the parallel use of primary AEC samples from subjects with vs without atopic asthma, particularly in view of recent findings that impaired production of type 1 IFN by AECs may be an important component of the high-asthma-risk phenotype (84).

Disclosures

The authors have no financial conflict of interest.

References


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