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**Supplementary Material**

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Severe asthma exacerbations in children requiring hospitalization are typically associated with viral infection and occur almost exclusively among atotics, but the significance of these comorbidities is unknown. We hypothesized that underlying interactions between immunoinflammatory pathways related to responses to aeroallergen and virus are involved, and that evidence of these interactions is detectable in circulating cells during exacerbations. To address this hypothesis we used a genomics-based approach involving profiling of PBMC subpopulations collected during exacerbation vs convalescence by microarray and flow cytometry. We demonstrate that circulating T cells manifest the postactivated “exhausted” phenotype during exacerbations, whereas monocyte/dendritic cell populations display up-regulated CCR2 expression accompanied by phenotypic changes that have strong potential for enhancing local inflammation after their recruitment to the atopic lung. Notably, up-regulation of FcεR1, which is known to markedly amplify capacity for allergen uptake/presentation to Th2 effector cells via IgE-mediated allergen capture, and secondarily programming of IL-4/IL-13-dependent IL-13Rα2 alternatively activated macrophages that have been demonstrated in experimental settings to be a potent source of autocrine IL-13 production. We additionally show that this disease-associated activation profile can be reproduced in vitro by cytokine exposure of atopic monocytes, and furthermore that IFN-γ can exert both positive and negative roles in the process. Our findings suggest that respiratory viral infection in atopic children may initiate an atopy-dependent cascade that amplifies and sustains airway inflammation initiated by innate antiviral immunity via harnessing underlying atopy-associated mechanisms. These interactions may account for the unique susceptibility of atotics to severe viral-induced asthma exacerbations. The Journal of Immunology, 2009, 183: 2793–2800.

Our current understanding of the cellular and molecular mechanisms responsible for triggering severe asthma attacks is derived largely from studies in murine models. This approach has identified cytokine-secreting Th2 cells in the airway mucosa as central in precipitating the symptoms characteristic of severe asthma exacerbations (1–3), together with a major triggering role for airway mucosal dendritic cells (DC) (4, 5). The animal models have proven ideal for elucidation of the individual inflammatory pathways triggered by aeroallergen challenge. However, these models cannot per se provide a picture of how these pathways interact in the corresponding human disease process, and resolution of this question requires additional studies on relevant human tissues collected during active expression of symptoms.

Studies focusing on the lesional site in human asthma are limited by constraints related to tissue access, and they are usually restricted to sampling during periods when asthma is stable. This is particularly the case in the pediatric age groups in which the disease is most frequent. Despite this limitation, the presence of Th2-associated biomarkers has been confirmed as a characteristic feature of the human asthmatic airway (1, 2), but beyond that qualitative conclusion little is known regarding precisely how Th2 immunity functions in the human airway to drive acute asthma symptoms. Understanding the pathogenesis of the human disease is also complicated by the evidence indicating that while Th2-associated atopy is a major risk factor for asthma, only a small proportion of humans sensitized to aeroallergens develop significant airway symptoms (6). This contrasts with the reproducible respiratory response profiles of sensitized/aerosol-challenged animals, and suggests that additional cofactors are operative in the human disease that are not accounted for in current experimental models.

The least understood phenotype in human asthma is the most severe form of the disease exemplified by acute severe exacerbations requiring hospitalization. These occur most frequently among children, in particular children who are sensitized to aeroallergens (7). An important clue to the nature of the underlying trigger mechanism(s) is the consistent observation that at the time of hospitalization most affected children also carry respiratory viral infections, and similar observations have been made with respect to severely exacerbating adult asthmatics (8–12). This suggests that
inflammation arising from host antiviral defense may interact in some way with underlying atopic inflammation to drive cumulative airway tissue damage above the critical threshold necessary to precipitate severe asthma attacks.

The aim of the current study was to seek evidence of interactions between host antiviral immunity and atopy in children hospitalized for acute severe asthma and to elucidate underlying pathways involved. We have devised an approach based on comparative analysis of circulating cells during acute asthma exacerbation vs subsequent convalescence. The rationale for this approach is two-fold. First, there is a well-established lung/bone marrow “axis” that operates during airway inflammation to replenish monocyted cell populations that are central to lung defense against pathogens (13); also, there are more recent precedents relating to activation of bone marrow eosinophil precursors in response to signals from inflamed airway mucosa (14). Second, it is recognized that migratory monocyted cells are preprogrammed during precursor stages via cytokine/chemokine signals generated at peripheral challenge sites, thus modifying their functions to optimize capacity to subsequently respond to the agents triggering the inductive signals (15). We accordingly reasoned that profiling gene expression in cells released into the blood during acute exacerbations may reveal information on the nature of the effector mechanisms that are activated during these events, as well as clues to the nature of the molecular stimuli responsible for their activation.

Employing this approach we first confirm that children hospitalized for acute severe asthma exacerbations are typically virally infected and are also severely atopic. Moreover, we show for the first time that during acute exacerbations they display marked up-regulation of Th2-associated functions in circulating monocytes/DC, including strongly enhanced surface expression of FcεRⅠα together with a concomitant gene expression signature indicative of the IL-4/IL-13-dependent “alternatively activated” phenotype. The latter has recently been identified among lung monocytes in an experimental murine parainfluenza model, and their activation was ascribed to signals from local invariant NKT cells (16). Our present findings demonstrate the operation in humans of a viral-induced pathway potentially leading to a comparable pathological endpoint, but utilizing instead endogenous IgE-mediated mechanisms that are operative in sensitized atopics.

Materials and Methods
Subjects and clinical assessments

The study population comprised a group of 67 children presenting with acute asthma exacerbation to Emergency at Princess Margaret Hospital, Perth. The institutional Ethics Committee approved the study, and parents provided written consent. The severity of the exacerbations was determined by a 15-point scoring system (17). Peripheral blood was collected during acute exacerbation and on average 6.5 h after glucocorticoid treatment (inhaled salbutamol and intravenous hydrocortisone) at 20-min intervals for first hour, and oral prednisolone 1 mg/kg) and at convalescence ≥6 wk later.

Postnasal aspirates were taken within 2 h of blood collection. The aspirates were tested for a panel of viral pathogens that account for ~90% of acute respiratory illness in local infants (18). Respiratory syncytial virus, adenovirus, influenza A and B, and parainfluenza types 1, 2, or 3 were either detected by 1) direct fluorescent Ab testing with virus-specific mAbs (Meridian Bioscience and Dako) or 2) isolation in cell culture (cell lines include LLC-MK, A549, Hep-2, Vero, and MRC-5) and identification with the virus-specific monoclonal antisera, 48 h after inoculation. Human rhinovirus (19, 20) and coronavirus 229E and OC43 (21) were detected by previously described RT-PCR studies, and human metapneumovirus detection utilized in-house primers designed to target nuc and matrix genes. For aspirates with sufficient sample remaining for retesting (the majority), respiratory viruses were detected by Respiratory MultiCode-PLx assay (22) and human rhinovirus was identified with a molecular typing assay (23).

Atonic status was defined by total serum IgE equal to or exceeding age-specific cut-offs (24) and either mite-specific or cat dander-specific IgE ≥0.35 kU/L, or skin prick test of wheal size ≥3 mm to at least one of eight common allergens. The overall study group was from the high end of the atopic spectrum (total IgE, 568.1 ± 84.7 kU/L).

Initial microarray screen

An initial microarray screen was performed comparing PBMC-derived RNA from 25 subjects bled at exacerbation and after convalescence. Standardized procedures were used for acquisition and analysis of microarray data employing Affymetrix U133 Plus 2.0 Gene Chips as described in our recent publication (25). Briefly, equal amounts of total RNA were extracted from freshly isolated PBMC of each patient using TRIzol (Invitrogen) and an RNAeasy kit (Qiagen). RNA samples from a total of 25 subjects were divided into five different pools, with each pool containing equal amounts of RNA from 5 subjects, either at acute exacerbation or convalescent stage. This pooling strategy using independent RNA pools was employed to minimize the effect of biological variation between different individuals in producing outliers and bias in the results (26, 27). The pooled RNA samples were labeled and hybridized to Affymetrix U133 Plus 2.0 Gene Chips, according to the manufacturer’s instructions as described previously (25). Microarray data were preprocessed in R software (www.r-project.org) employing the probe-level model algorithm (28). The microarray data were of high quality, as demonstrated by the scaling factor (5.62 ± 0.37), background (51.32 ± 2.58), percentage present (47.18 ± 0.51), and values of the RNA degradation controls: GAPDH (1.19 ± 0.02) and β-actin (1.55 ± 0.14) (mean ± SEM). Differentially expressed genes were identified using a moderated t test (29), with statistical significance reached at a false discovery rate-adjusted q value of <0.05 to account for multiple testing (27, 30). Statistically significant gene expression subsets and biological pathways were generated by gene set enrichment analysis (false discovery rate-adjusted q value of <0.05, www.broad.mit.edu/gsea) (51) and ingenuity pathways analysis (right-tailed Fisher’s exact p < 0.05; www.ingenuity.com).

Primary microarray data

The microarray data are available in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/projects/geo/) under accession no. 16032.

Validation of microarray data by quantitative RT-PCR (qRT-PCR)

Genes of interest selected via microarray screening were validated in qRT-PCR analyses employing individual paired exacerbation/convalescence samples from the subjects utilized for the initial screen, plus an independent set of 25 paired samples from a second cohort of children collected under the same conditions, employing standardized methodology (25) as follows. RNA was extracted from PBMC using TRIzol (Invitrogen) and RNAeasy kit (Qiagen). Total RNA was reverse-transcribed using an Omniscript kit (Qiagen), according to the manufacturer’s instructions, to generate cDNA, which was quantified using QuantiTect SYBR Green (Qiagen) on the ABI Prism 7900HT (Applied Biosystems). PCR primers were purchased from Qiagen (QuantiTect primer assays) or designed using Primer Express software (Applied Biosystems). Relative standard curves were obtained from serially diluted PCR products, and data were normalized to the housekeeping gene UBED2.

Flow cytometry and cell sorting

PBMC were phenotyped employing mAbs and corresponding isotype-matched control Abs. Patient PBMC were incubated for 30 min on ice with at least one of the following mAbs (obtained from BD Biosciences unless otherwise stated): anti-CD3, CD4, CD8, CD25, CD69, CD127 (Immunotech), CD14, and FcεRⅠ (eBioscience). Plasmacytoid DC (pDC) and myeloid DC (mDC) subsets were detected with the four-color Dengitric Value Bundle kit (BD Biosciences) and distinguished by the phenotypes CD123+LinHLA-DR+ and CD11c+LinHLA-DR+, respectively. Corresponding isotype-matched control mAbs were used. Cells were analyzed on a four-color LSR II flow cytometer (BD Biosciences). For each subset preparation, PBMC were stained with mAb cocktails and sorted into monocytes, DC, and NK, T, and B cells to at least 90% purity using a FACSAria flow cytometer (BD Biosciences).
Statistical analysis

SPSS 13 software was used to perform nonparametric Wilcoxon signed-rank test (for paired data), Mann-Whitney U test (for unpaired data), and Spearman’s rho correlation. Analytical procedures relevant to microarray screening are detailed in the relevant section above.

Results

Characteristics of study subjects

The study population comprising 67 children hospitalized for acute asthma is detailed in Table I. The study design involved screening of PBMC from an initial group of 25 children (cohort 1) followed by validation in a second group (n = 25; cohort 2), with inclusion of samples from an additional 17 subjects for follow-up studies on cell separation and asthma severity.

Gene expression profiling

An initial microarray screen was performed on RNA from 25 paired PBMC samples collected from children at admission plus follow-up samples collected at convalescence (cohort 1). The gene list identified as differentially expressed at exacerbation was further evaluated to identify immunological/inflammatory pathways statistically significantly enriched for the differentially expressed genes, and subsequent analyses focused on members of these pathways. We prioritized the differentially expressed genes based on known relevant biological pathways and/or immune-related/inflammatory roles from PubMed (www.ncbi.nlm.nih.gov). The resulting broad categories of interest derived from these genes included: 6) innate immunity, 5) complement/coagulation cascade, and 6) inflammation.

We focused primarily on genes that were differentially up-regulated during acute exacerbations for the core of the study. Fifty-two such genes were selected from the microarray profile for more detailed studies (supplemental Fig. 1, Table II, and supplemental Table I). We confirmed that expression of these genes was not significantly distorted by in vitro cell handling by parallel qRT-PCR analysis of fresh vs frozen samples (supplemental Table II). We also collected information for each patient on the timing of bleeding relative to final (oral) steroid administration, and subsequently tested for associations between expression of individual genes and the time lapse from steroid administration to PBMC collection, and observed relationships for only ∼10% of the genes selected below (supplemental Table III); moreover, only a small minority of the gene panel have previously been identified as “steroid inducible” in published studies (supplemental Table III). In addition to this panel we noted a smaller series of genes associated with T effector functions, including IFNG and LTA, which were uniformly down-regulated in these subjects (supplemental Fig. 2); these genes were not studied further.

PCR validation of differentially expressed genes

Initial confirmation of these findings was sought by qRT-PCR analysis of the samples employed for microarray screening (cohort 1 in Table II). Reconfirmation was provided via paired samples from an independent group of 25 severe asthmatics collected under identical conditions (cohort 2 in Table II).

Gene expression in relation to exacerbation severity

We next focused on the up-regulated genes and examined the relationship between the magnitude of differential gene expression during acute exacerbations and exacerbation severity, utilizing PCR data from a total of 61 subjects including cohorts 1 and 2. Within this combined population (right column of Table II), expression of 35 of the 46 differentially expressed genes showed significant correlation with disease intensity.

Localization of up-regulated genes in PBMC subpopulations during acute exacerbations

PBMC from an additional set of 10 subjects were sorted into monocytes, DC, and NK, T, and B cells for RNA extraction and qRT-PCR analysis, and relative gene expression levels within the individual cell populations during acute exacerbation are shown in Fig. 1. Up-regulated genes localized almost exclusively to the innate immune system, in particular monocytes/DC, which accounted for 44 of 46 genes tested. The specific activity of a subset of these genes was reassayed by qRT-PCR in the purified CD14+ monocytes comparing exacerbation and convalescence, which confirmed the pattern of exacerbation-associated up-regulation seen in unfraccionated PBMC (FceRIy, 2.1-fold up-regulation, p = 0.017; CCR2, 66.5-fold, p = 0.005; TLR2, 14.1-fold, p = 0.005; TLR4, 21.4-fold, p = 0.005; TLR7, 21.1-fold, p = 0.005; additionally, MRC1, 8.2-fold, p = 0.008; CD1D, 1.6-fold, p = 0.017 (detailed below)). Comparable up-regulation was observed in the purified DC samples (not shown).

Analysis of exacerbation-associated changes by flow cytometry

Follow-up flow cytometric studies focused primarily on monocytes/DC that displayed consistent evidence of activation at exacerbation and convalescence, which was accompanied by a 3-fold increase in expression of the high-affinity IgE receptor FceRIα among this population (Fig. 2B). Parallel increases were also seen in proportions of both pDC and mDC in PBMC (Fig. 2C) and accompanying up-regulation of expression of FceRIα (Fig. 2, D and E). Fig. 2, F and G, shows results from analyses of IgE levels in paired serum samples collected at exacerbation vs convalescence. Allergen-specific and total IgE titers were significantly higher during exacerbation relative to convalescence.

The data shown in Table II and supplemental Fig. 2B suggest down-regulation of some genes in the adaptive immune system during acute exacerbation. Of note in this context, employing the same samples used for the monocyte/DC analyses above, we noted a decline in the relative frequency of T cells in PBMC during acute disease (CD3+ T cells 53.3 ± 2.4% at convalescent stage vs

### Table I. Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cohort 1</th>
<th>Cohort 2</th>
<th>Whole Population*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. subjects</td>
<td>25</td>
<td>25</td>
<td>67</td>
</tr>
<tr>
<td>Age (2.2–15.6 years)</td>
<td>7.3 ± 0.7</td>
<td>6.7 ± 0.6</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>Male/Female</td>
<td>15/10</td>
<td>13/12</td>
<td>40/27</td>
</tr>
<tr>
<td>% Atopic</td>
<td>95.0</td>
<td>96.0</td>
<td>96.8</td>
</tr>
<tr>
<td>% Virally infected</td>
<td>85.0</td>
<td>82.4</td>
<td>80.0</td>
</tr>
<tr>
<td>Severity score* (5–15)</td>
<td>11.6 ± 0.2</td>
<td>11.3 ± 0.4</td>
<td>10.3 ± 0.3</td>
</tr>
</tbody>
</table>

* Ninety-six percent of the subjects had a previous diagnosis of asthma.

† National Institutes of Health severity scoring system.

‡ Rhinovirus was isolated in 95.0% of these virally infected subjects; 16.3% had other viruses (enterovirus, bocavirus, influenza, parainfluenza, human metapneumovirus, respiratory syncytial virus, and coronavirus). There were 10 dual infections and 2 triple infections, with HRV present in all of these cases.

The online version of this article contains supplemental material.
Table II. qRT-PCR validation of microarray data

<table>
<thead>
<tr>
<th>Pathway/Function</th>
<th>Gene symbol</th>
<th>Folda</th>
<th>q-value</th>
<th>Microarray Data</th>
<th>qRT-PCR Data: Cohort 1b</th>
<th>p-value</th>
<th>Microarray Data</th>
<th>qRT-PCR Data: Cohort 2b</th>
<th>p-value</th>
<th>Combined Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement and coagulation cascade</td>
<td>C3AR1 (A23B)</td>
<td>5.7</td>
<td>0.002</td>
<td>90.6 ± 12.3</td>
<td>956.6 ± 137.4</td>
<td>0.001</td>
<td>250.1 ± 42.1</td>
<td>1,323.2 ± 214.3</td>
<td>0.000</td>
<td>0.429 ± 0.001</td>
</tr>
<tr>
<td>Inflammation-associated</td>
<td>MS4A4A (CD20L1)</td>
<td>10.1</td>
<td>0.005</td>
<td>220.4 ± 78.1</td>
<td>1,588.7 ± 247.5</td>
<td>0.001</td>
<td>525.1 ± 117.3</td>
<td>3,560.5 ± 497.1</td>
<td>0.000</td>
<td>0.390 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MX1 (NL19)</td>
<td>4.1</td>
<td>0.004</td>
<td>438.5 ± 64.7</td>
<td>1,022.1 ± 248.5</td>
<td>0.001</td>
<td>742.7 ± 227.6</td>
<td>1,534.2 ± 191.1</td>
<td>0.000</td>
<td>0.295 ± 0.039</td>
</tr>
<tr>
<td></td>
<td>CALD1 (CDM)</td>
<td>3.6</td>
<td>0.006</td>
<td>35.1 ± 4.5</td>
<td>195.8 ± 55.3</td>
<td>0.000</td>
<td>31.5 ± 8.2</td>
<td>75.9 ± 9.4</td>
<td>0.001</td>
<td>0.514 ± 0.000</td>
</tr>
<tr>
<td></td>
<td>ADAMTS2 (NPI)</td>
<td>3.8</td>
<td>0.002</td>
<td>0.1 ± 0.0</td>
<td>58.5 ± 14.2</td>
<td>0.000</td>
<td>0.5 ± 0.5</td>
<td>79.4 ± 22.0</td>
<td>0.000</td>
<td>0.514 ± 0.000</td>
</tr>
</tbody>
</table>

4 RNA was extracted from PBMC collected during acute exacerbation (Ac) versus convalescence (Cv) and reverse transcribed prior to qRT-PCR assay. qRT-PCR data are presented as gene expression levels normalized to housekeeping gene UBE2D2.
5 Fold change (Ac/Cv) in the microarray data indicate higher gene expression level at Ac compared to Cv stage.
6 q-values were calculated using the false discovery rate (FDR)-adjusted q-values (Cv vs Ac) obtained by moderated t test (see Materials and Methods).
7 Values of p (Cv vs Ac) were obtained with Wilcoxon test.
8 Correlation between fold change (Ac/Cv) data and disease severity in 61 subjects by Spearman’s rho.
9 Value of p = 0.05—0.10. —. Data not significant at p = 0.05 cutoff.

38.6 ± 2.7% at acute stage; p = 0.008). In contrast, as shown in supplemental Fig. 2A, the expression of activation markers CD25 and CD69 on the circulating population was significantly elevated in both the CD4+ and CD8+ T cell populations during acute asthma. Furthermore, the relative frequency of regulatory T cells (CD4+CD25+CD127lo) was significantly higher during acute asthma compared with subsequent convalescence (supplemental Fig. 2B).

Bioinformatics of acute exacerbation gene signatures

Initial examination of the list of validated genes in Table II indicated the presence of type 1 IFN-sensitive and Th2 cytokine-sensitive genes in the exacerbation signature. More detailed analyses revealed a broader range of activated genes downstream of type I IFN (archetype, MX1; supplemental Table IV) and in particular downstream of IL-4/IL-13 (archetype, MRC1; supplemental Table V), with the latter being associated with the alternatively activated macrophage/monocyte (AAM) pathway.

Reproduction of major elements of the exacerbation-associated signature ex vivo

In the experiments in Fig. 3, circulating mononuclear cells from astatics were cultured overnight in the presence of the three key cytokines predicted above as central to development of the
exacerbation-associated gene expression signature. Cells were incubated in medium alone or medium supplemented with IFN-α, IL-4, IL-13, or these cytokines in combination, followed by analysis of expression of key exacerbation-associated biomarkers by qRT-PCR and/or flow cytometry. The Th2 cytokines, alone or in combination, up-regulated FcγRIIa, MRC1, CD1D, and CCR2. In contrast, type 1 IFN up-regulated MX1 (data not shown) plus FcγRI and strongly inhibited the stimulatory effects of the Th2 cytokines.

Discussion

Genome-wide expression profiling of paired PBMC samples from children collected during acute asthma exacerbations vs during subsequent convalescence revealed a broad-ranging differential expression signature associated with acute severe disease. As detailed in Table II, initial bioinformatics analyses identified a series of immunoinflammatory pathways that were significantly enriched for genes that were part of this expression profile, and subsequent studies localized the bulk of the activation signature to cells of the innate immune system. Of note was the finding that one of the most highly up-regulated genes at acute exacerbation was CCR2 (Table II and text), which is known to play a central role in trafficking of DC and monocytes to the lung during airway inflammation (32, 33), and the parallel finding of a 3- to 4-fold expansion of the circulating monocyte population at this time (Fig. 2).

This contrasts sharply with the marked reduction in effector gene expression in the circulating T cell compartment (supplemental Fig. 2), mirrored by a relative decline in T cell numbers in the blood. Of note was the accompanying enhanced expression of the CD25 and CD69 activation markers on the circulating T cell population during acute exacerbation (supplemental Fig. 2, A and B), which may be explicable in part by a relative increase in numbers of circulating regulatory T cells at this time (supplemental Fig. 2C). This circulating population may also contain postactivated and functionally “exhausted” effector memory T cells (36, 37).

The key question relates to the nature of the underlying molecular mechanisms that mediate susceptibility of the innate immune system to the asthmatic effects of respiratory infections. An important clue is provided by the observation that >95% of the subjects in this relatively rare subgroup of children who experienced severe virus-associated exacerbations were atopic, a finding consistent with many previous studies (2, 8, 9, 11, 12, 38). Several features of the atopic phenotype may contribute toward susceptibility in this context. For example, the efficiency of antiviral defense may be partially compromised in atopics. Consistent with this suggestion, one prospective study has demonstrated that atopic asthmatics manifest increased susceptibility to the spread of rhinovirus from upper to lower respiratory tract and subsequently display more severe and persistent symptoms relative to nonatopics (8). This may involve interference with T cell-mediated viral

![FIGURE 1. Cellular source of PBMC gene expression during acute exacerbation. Genes up-regulated at acute compared with convalescent stage are shown. qRT-PCR data presented are relative gene expression intensities during acute exacerbation within different cell types, from top to bottom of each bar graph: monocytes, DC, NK cells, T cells, and B cells (details of phenotypes in Materials and Methods). For each gene, expression levels normalized to housekeeping gene UBE2D2 were determined in each cell type and then expressed on a relative scale of 0–1. Data are expressed as means ± SEM of 10 subjects.](image)

![FIGURE 2. Higher FcγRI expression and IgE titers during acute exacerbation. PBMC collected at convalescent (Cv) or acute exacerbation (Ac) stage were stained with Abs to identify monocytes, total DC, or the pDC and mDC subsets, and surface FcγRI expression (details of Abs used in Materials and Methods). The frequency of monocytes (Mo) is presented as percentage of PBMC (A) and FcγRI expression as percentage of Mo (B), pDC or mDC frequency is presented as percentage of PBMC (C). FcγRIIIa expression on pDC or mDC is presented as either percentage of PBMC (D) or percentage of respective pDC or mDC populations (E). Total (F) and house dust mite (HDM)-specific (G) IgE titers (kU/L) were measured in sera of acute asthma patients collected at Cv and Ac stages. Data are expressed as means ± SEM of 6 subjects (A–E) or 54 subjects (F and G) and statistically analyzed by Wilcoxon test.](image)
clearance via the intrinsic Th1/Th2 imbalance in atopics (39) and/or intrinsic (40) or cytokine-driven acquired deficiencies (41) in airway epithelial innate defense mechanisms.

An additional indication of the underlying effects of atopy on innate immune functions is the constitutive expression of FcεRIα on circulating monocytes/DC in atopics, which correlates quantitatively with their levels of serum IgE (42, 43). These circulating populations contain the immediate precursors for the rapidly cycling airway intraepithelial DC network (44–46), and FcεRIα expression on these cells has been observed in bronchial biopsies from atopics (47). Expression of this receptor on monocytes/DC can have several functional consequences, including enhanced proinflammatory mediator production at challenge sites via an indirect mechanism involving a second cell type (44–46), and FcεRIα-regulated FcεRI-bearing monocytes/DC (Fig. 2, A–E) during acute exacerbations, with concomitant up-regulation of the recently been demonstrated to strongly up-regulate FcεRIα protein expression with concomitant up-regulation of the recently been demonstrated to strongly up-regulate FcεRIα protein expression on circulating monocytes/DC via flow cytometry. Statistically significantly different to treatment 1 (*, p < 0.05) or treatment 6 less than treatment 5 (#, p < 0.01–0.05) by Wilcoxon test.

It was therefore striking to note the ~10-fold increase in frequency of circulating FcεRIα-bearing monocytes/DC (Fig. 2, A–E) during acute exacerbations, with concomitant up-regulation of the gene encoding the FcεRIα chain (Table II), which stabilizes the FcεRIα chain on the cell surface (50). This infras transmission from the infected airway mucosa of an activation signal to monocytes/DC in the blood and/or bone marrow. A strong candidate is FcεRIα from the infected airway mucosa of an activation signal to monocytes/DC in the blood and/or bone marrow. A strong candidate is FcεRIα on the cell surface (50). This implies transmission of a stimulatory signal to the infected airway mucosa of an activation signal to monocytes/DC in the blood and/or bone marrow. A strong candidate is FcεRIα on the cell surface (50).

An additional finding of note was the presence of a prominent FcεRIα-regulated FcεRI-bearing monocytes/DC via an indirect mechanism involving a second cell type (51). We were not able to sample lung DC in exacerbating children, but analysis of their monocytoid precursors in blood revealed up-regulation of a series of type 1 IFN-sensitive genes (supplemental Table IV), indicating that bioactive levels of this cytokine or secondary inducers stimulated by it reach relevant bone marrow-derived precursors. Moreover, we were able to reproduce the predicted effects of type 1 IFN exposure on FcεRIα expression on atopic monocytes in vitro (Fig. 3). It is noteworthy that DC turn over extremely rapidly in the airway mucosa during infection (45, 52) and are continuously replenished by incoming precursors, and it is thus plausible that type 1 IFN may contribute toward up-regulation of FcεRIα on these DC during the infection cycle via this mechanism.

In the mouse parainfluenza model cross-linking of FcεRIα triggers secretion of chemokines selective for Th2 memory cells (51), and the authors suggested viral-specific IgE for this role. However, in sensitized human atopics a more immediately available trigger would be their preexisting IgE to ubiquitous indoor allergens. In these subjects an additional consequence of up-regulated FcεRIα expression on airway DC would be enhancement of their allergen uptake/presentation (APC) functions (49), employing IgE-loaded FcεRI for optimal allergen trapping. The potential for Th2-mediated inflammatory damage in this microenvironment is normally limited by maintenance of low baseline APC activity in resident airway DC (53), and up-regulation of APC function in airway DC via this mechanism has the potential to markedly amplify ongoing responses to allergens in virally infected sensitized children, adding further to their airway inflammation. In this regard, the higher IgE titers during exacerbation (Fig. 2, F and G) may indicate Th2 cytokine-driven stimulation of ongoing IgE production during the acute phase.

An additional finding of note was the presence of a prominent gene signature characteristic of the IL-4/IL-13-dependent AAM pathway originally identified in antiparasite responses (15). Notably, examination of the exacerbation-associated expression profiles revealed a comprehensive list of 52 AAM signature genes, including mannoseR and IL-13R (supplemental Table V). This IL-4/IL-13-dependent gene signature is found in vivo only in the context of acute exacerbation and is not evident in resting PBMC samples from atopic children (not shown) and can be readily reproduced via short-term culture of their cells with these Th2 cytokines.

**FIGURE 3.** Cytokine modulation of expression signatures on blood mononuclear cells from atopic children. PBMC from six atopic children were cultured overnight in the presence of cytokine(s) as illustrated. A, Expression of nominated genes (as specific mRNA) was determined by qRT-PCR. B, FcεRIα protein expression was determined on CD14+ monocytes by flow cytometry. Statistically significantly different to treatment 1 (*, p < 0.05) or treatment 6 less than treatment 5 (#, p < 0.01–0.05) by Wilcoxon test.
Migration of IL-13Rα2 AAM into the lungs of children during severe asthma has yet to be formally demonstrated, however they have recently been identified in asthma adults (16). We also acknowledge that the present findings do not preclude parallel contributions from activated invariant NKT cells, which have been suggested to contribute to lung AAM programming/activation in both human (50) and experimental asthma (16). Indeed, we have observed up-regulation of expression of the invariant NKT activation protein CD1d in the exacerbation-associated samples (not shown), but have not detected consistent exacerbation-associated changes in the circulating invariant NKT population. However, the pathway described herein, that is, infection-associated harnessing and enhancement of preexisting allergen-specific Th2 effector mechanisms within atopics, provides a discrete and more direct route to the same pathological endpoint.

A further finding of interest from this study was the apparently dualistic effects of type 1 IFN in this cascade in atopics. As noted in Fig. 3, type 1 IFN exposure in vitro can reproduce the FcεRI component of the asthma exacerbation-associated expression signature, which in other settings has been associated with indirect enhancement of surface expression of FcεRIα protein on human and murine DC (50, 51). However, as shown in Fig. 3, it additionally inhibits the direct stimulatory effects of the Th2 cytokines IL-4 and IL-13 on FcεRIα gene transcription. Thus, type 1 IFN may play a role in both the initiation and termination of this viral-induced inflammatory cascade in atopics, and the balance between these competing processes may be an important determinant of disease severity in atopic asthmatics.

In conclusion, this model provides a plausible explanation for epidemiological findings that severe asthma exacerbations triggered by virus infection are much more frequent in atopics than in nonatopics (2, 8, 9, 11, 12, 38), and that over a longer time scale risk for inflammation-driven progression to persistent asthma is highest in children experiencing these insults in combination (2, 56). In relationship to future human research in this area, our findings provide ethical justification for extending these invasive studies to a broader range of relevant clinical phenotypes, in particular to children who experience wheezing attacks in the apparent absence of viral infection. They also highlight the need for development of more complex animal models of atopic asthma focusing on interactions between multiple environmental triggers of the type operative in severe human disease, as opposed to the prevailing experimental approach involving allergen exposure in isolation, which may be only relevant to expression of mild forms of the disease.

Note added in proof. A recent publication from Semper et al. (2003. J. Allergy Clin. Immunol. 112: 411) demonstrated that FcεRIα expression is also up-regulated in epidermal Langerhans cells in patients during active asthma relative to that during remission, reflecting a similar mechanism to that reported here.

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Disclosures
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References


