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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 FcεRI, 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.

O ur 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 (DCs) (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 monocyte 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 monocyte cell precursors 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 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, which may be relevant in asthmatic bronchitis.

The findings are of interest both in isolation and in the context of an emerging body of information. First, the role of eosinophils in asthma and other chronic inflammatory disorders continues to receive attention, with the activity of these cells being increasingly recognized as being driven by Th2 cytokines (17). Thus, it is of interest that a putative “Th2-activated” phenotype was identified in circulating monocytes/DC and that this may be accompanied by activated eosinophils (18). Second, the finding that Th2-associated features are associated with the monocyte/DC subset in acute exacerbations of asthma is of importance in the context of the Th2/Th1 paradigm. The observation that this phenotype is also observed in circulating monocytes/DC during convalescence is in line with studies suggesting an elevated frequency of this subset in circulation during healthy states (19). The findings are also of relevance in the context of Th2 priming and Th2 bias in asthma, with the potential for these events to precipitate severe asthma attacks.

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 ipratropium bromide at standard doses) from 67 children presenting with acute asthma exacerbation at Princess Margaret Hospital (PMH) during the study period (14). Second, it is recognized that migratory monocyte cell precursors 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.

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Spearman’s rho correlation. Analytical procedures relevant to microarray screening are detailed in the relevant section above.

Characteristics of study subjects

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 detailed in Table II comprise genes associated with 1) arachidonic acid/prostaglandin metabolism, 2) leucocyte migration, 3) innate immunity, 4) adaptive 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 (detailed below). Comparable up-regulation was observed in the purified CD14+ monocytes comparing exacerbation and convalescence, which confirmed the pattern of exacerbation-associated up-regulation seen in unfractonated PBMC (FceRIγ, 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; MRC1, 8.2-fold, p = 0.005; TLR7, 21.1-fold, p = 0.005; TLR8, 21.4-fold, p = 0.005; TLR7, 21.1-fold, p = 0.005; 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 (Fig. 2, A–E). The largest quantitative changes were observed in monocytes, which expanded 3- to 4-fold during acute exacerbation to >20% of PBMC (Fig. 2A). This was accompanied by a 3-fold increase in expression of the high-affinity IgE receptor FceRIa 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 FceRIa (Fig. 2D 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 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 relationship 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 showed a decline in the relative frequency of T cells in PBMC during acute exacerbation to >20% of PBMC (Fig. 2A). This was accompanied by a 3-fold increase in expression of the high-affinity IgE receptor FceRIa 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 FceRIa (Fig. 2D 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.

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Table II. qRT-PCR validation of microarray dataa

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<td>Complement and coagulation cascade</td>
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Data: a RNA was extracted from PBMC collected during acute exacerbation (Ac) versus convalescent (Cv) and reverse transcribed prior to qRT-PCR assay. qRT-PCR data are presented as gene expression levels normalized to housekeeping gene UBE2D2. b Data are expressed as mean ± SEM of 25 subjects (cohort 1 or 2). c Official and alternative gene symbols, the latter in parentheses; see supplemental Table I for more details. d False discovery rate (FDR)-adjusted q-values (Cv vs Ac) obtained by moderated t test (see Methods and Materials). e Values of p (Cv vs Ac) were obtained by Wilcoxon test. f Correlation between fold change (Ac/Cv) data and disease severity in 61 subjects by Spearman’s rho. g Value of p = 0.05—0.10. —, Data not significant at p = 0.05 cutoff. h 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+CD127−) was significantly higher during acute asthma compared with subsequent convalescence (supplemental Fig. 2B).
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 FcεRI, MRC1, CD1D, and CCR2. In contrast, type 1 IFN up-regulated MX1 (data not shown) plus FcεRIγ 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 asthmogenic 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

![Image](https://example.com/image1.png)

**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](https://example.com/image2.png)

**FIGURE 2.** Higher FcεRI 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 FcεRI receptors (details of Abs used in Materials and Methods). The frequency of monocytes (Mo) is presented as percentage of PBMC (A) and FcεRI expression as percentage of Mo (B), pDC or mDC frequency is presented as percentage of PBMC (C). FcεRI 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.
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 atopic asthmatics (47). Expression of this receptor on monocytes/DC can have several functional consequences, including enhanced proinflammatory mediator production at challenge sites via NF-κB-dependent mechanisms and interference with development of sterilizing immunity (reviewed in Ref. 44), and also increased capacity to stimulate allergen-specific Th2 cells (49).

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 FcεRIγ-chain (Table II), which stabilizes the FcεRIα-chain on the cell surface (50). This transmits transmission from the infected airway mucosa of an activation signal to monocytes/DC in the blood and/or bone marrow. A strong candidate production is the infected airway epithelium and/or other cell populations of type 1 IFN, which in parainfluenza-infected mice has been observed to be produced by bronchial biopsies from atopic asthmatics (47). Expression of this receptor on monocytes/DC can have several functional consequences, including enhanced proinflammatory mediator production at challenge sites via NF-κB-dependent mechanisms and interference with development of sterilizing immunity (reviewed in Ref. 44), and also increased capacity to stimulate allergen-specific Th2 cells (49).

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.
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 asthmatic 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 changes in the circulating invariant NKT population. A further finding of interest from this study was the apparently dualistic effects of type 1 IFN in this cascade in atopics. As noted above, we may stimulate AAM programming distal to the lung in immature precursor populations.

Disclosures
The authors have no financial conflicts of interest.

References

Note added in proof. A recent publication from Semper et al. (2003. J. Allergy Clin. Immunol. 112: 411) demonstrated that FcεR1α 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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