

Interactions between Innate Antiviral and Atopic Immunoinflammatory Pathways Precipitate and Sustain Asthma Exacerbations in Children¹

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Severe asthma exacerbations in children requiring hospitalization are typically associated with viral infection and occur almost exclusively among atopics, 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-13R⁺ 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-α 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 atopics to severe viral-induced asthma exacerbations. *The Journal of Immunology*, 2009, 183: 0000–0000.

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)³ (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

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³ Abbreviations used in this paper: DC, dendritic cell; AAC, activated macrophage/monocyte; mDC, myeloid DC; pDC, plasmacytoid DC; qRT-PCR, quantitative RT-PCR.

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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 monocytoid 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 monocytoid 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 Fc ϵ RI α 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 ipratropium bromide 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 $\sim 90\%$ 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-PCRs, 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).

Atopic 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 atopy 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 detailed in our recent publication (25). Briefly, equal amounts of total RNA were extracted from ex vivo PBMC of each patient using TRIzol (Invitrogen) and an RNeasy 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 ± 2.54), 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 $\pm$ 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) (31) and ingenuity pathways analysis (right-tailed Fisher's exact *p* of <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 RNeasy 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 UBE2D2.

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 Fc ϵ RI α (eBioscience). Plasmacytoid DC (pDC) and myeloid DC (mDC) subsets were detected with the four-color Dendritic Value Bundle kit (BD Biosciences) and distinguished by the phenotypes CD123 $^{+}$ Lin $^{-}$ HLA-DR $^{+}$ and CD11c $^{+}$ Lin $^{-}$ HLA-DR $^{+}$, respectively. Corresponding isotype-matched control mAbs were used. Cells were analyzed using a six-color LSR II flow cytometer (BD Biosciences). For PBMC 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).

Table I. Characteristics of the study population

Characteristics	Cohort 1	Cohort 2	Whole Population ^a
No. subjects	25	25	67
Age (2.2–15.6 years) (mean ± SEM)	7.3 ± 0.7	6.7 ± 0.6	7.3 ± 0.4
Male/female	15/10	13/12	40/27
% Atopic	95.0	96.0	96.8
% Virally infected	85.0	82.4	80.0 ^c
Severity score ^b (5–15) (mean ± SEM)	11.6 ± 0.2	11.3 ± 0.4	10.3 ± 0.3

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

^b National Institutes of Health severity scoring system.

^c 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.

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 detailed in Table II comprise genes associated with 1) arachidonic acid/prostaglandin metabolism, 2) leukocyte 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 for more detailed studies (supplemental Fig. 1, Table II, and supplemental Table I).⁴ 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 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 confirmed the pattern of exacerbation-associated up-regulation seen in unfractionated PBMC (FcεRIγ, 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 (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 FcεRIα 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 FcεRIα (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 $53.3 \pm 2.4\%$ at convalescent stage vs

⁴ The online version of this article contains supplemental material.

Table II. *qRT-PCR validation of microarray data^a*

Pathway/Function	Microarray Data		qRT-PCR Data: Cohort 1 ^b			qRT-PCR Data: Cohort 2 ^b			qRT-PCR Data: Combined Population		
	Gene symbol ^c	Fold ^d	q ^e	Cv	Ac	p ^f	Cv	Ac	p ^f	R ^g	p ^g
Arachidonic acid/prostagandin											
FPRL1 (LXA4R)	4.5	0.004	101.6 ± 11.6	614.7 ± 70.2	0.000	334.8 ± 87.9	699.5 ± 127.5	0.000	0.514	0.000	
LTB4R (BLT1)	1.4	0.026	233.3 ± 22.6	404.1 ± 43.4	0.001	199.3 ± 12.9	317.2 ± 25.1	0.000	0.272	0.043	
ALOX15B	3.3	0.004	2.4 ± 0.4	141.8 ± 33.8	0.000	10.4 ± 6.3	235.6 ± 49.1	0.000	0.274	0.033	
ALOX5 (5-LO)	1.7	0.003	802.0 ± 60.0	1,779.3 ± 167.7	0.000	1,371.7 ± 115.9	2,706.3 ± 220.6	0.000	0.482	0.000	
PTGER2 (EP2)	1.5	0.019	1,434.3 ± 162.9	2,815.3 ± 235.0	0.000	894.0 ± 94.9	1,837.4 ± 148.6	0.000	0.393	0.002	
Leukocyte migration											
PECAM1 (CD31)	1.6	0.017	865.2 ± 125.5	1,841.2 ± 215.7	0.001	758.3 ± 108.5	1,332.3 ± 148.5	0.002	0.435	0.001	
CCR2 (MCP-1-R)	5.5	0.015	150.0 ± 31.7	1,092.1 ± 211.6	0.000	527.9 ± 126.3	1,680.7 ± 349.6	0.003	0.260	0.043	
CCR1 (CD191)	2.8	0.005	166.7 ± 29.9	612.4 ± 112.9	0.000			— ^h	0.397	0.002	
IL8RB (CXCR2)	2.3	0.036	19.3 ± 8.1	82.4 ± 28.4	0.002	14.7 ± 3.8	33.0 ± 4.7	0.017	0.283	0.032	
CXCL5 (ENA-78)	3.2	0.004	73.6 ± 11.2	298.7 ± 106.2	0.002	117.1 ± 31.0	195.9 ± 40.6	0.003			
CXCL1 (GRO1)	1.7	0.010	63.9 ± 10.8	187.1 ± 57.1	0.014			— ^h	0.331	0.011	
PF4V1 (CXCL4V1)	4.0	0.008	68.0 ± 8.4	338.0 ± 104.4	0.000	85.8 ± 28.8	189.9 ± 30.6	0.006			
Innate immunity											
TLR2	2.9	0.002	19,613.8 ± 2,027.6	6,5504.6 ± 5,738.1	0.000	20,787.8 ± 2,197.7	58,622.2 ± 5,618.2	0.000	0.514	0.000	
TLR4	3.6	0.005	2,247.8 ± 345.0	12,118.0 ± 1,618.6	0.000	3,280.1 ± 621.9	11,542.7 ± 1,616.5	0.000	0.429	0.001	
TLR7	2.4	0.043	5,335.5 ± 459.4	11,881.7 ± 1,447.7	0.002			— ^h	0.414	0.003	
TLR8	3.7	0.002	60.6 ± 15.5	313.8 ± 60.5	0.000	80.6 ± 21.1	307.4 ± 75.6	0.000	0.289	0.025	
CD14	2.3	0.004	6,388.3 ± 1,146.7	23,294.1 ± 4,433.7	0.000	7,196.5 ± 1,037.8	16,980.5 ± 3,169.9	0.001	0.474	0.000	
KIR2DL4 (CD158D)	1.8	0.036	44.9 ± 8.0	125.0 ± 32.8	0.001	118.0 ± 45.9	207.2 ± 59.0	0.001	0.277	0.035	
MX1 (MXA)	1.8	0.043	6,449.8 ± 1,271.2	15,277.1 ± 3,258.9	0.002	3,183.3 ± 471.2	5,861.0 ± 749.1	0.005	0.371	0.004	
MNDA (PYHIN3)	3.4	0.021	1,799.1 ± 550.9	5,478.5 ± 1,141.5	0.002	1,014.4 ± 255.2	2,519.8 ± 447.9	0.012	0.302	0.021	
FLT3 (CD135)	3.0	0.002	31.9 ± 3.6	254.9 ± 42.3	0.000	57.7 ± 8.3	345.9 ± 62.5	0.000	0.265	0.039	
CLEC4D (CLEC6)	3.3	0.000	84.8 ± 12.0	328.2 ± 31.5	0.000	81.1 ± 14.3	339.6 ± 46.4	0.000	0.448	0.000	
IL1RAP (IL1R3)	1.4	0.030	59.7 ± 5.6	94.3 ± 12.4	0.014	48.3 ± 3.1	103.7 ± 9.7	0.000	0.355	0.006	
CASP1 (ICE)	2.2	0.043	1,264.0 ± 231.4	2,617.1 ± 390.8	0.000	624.1 ± 101.9	1,234.1 ± 135.2	0.003	0.325	0.013	
FSTL1 (FRP)	3.9	0.002	9.2 ± 1.2	93.6 ± 26.7	0.000	19.7 ± 5.9	59.0 ± 10.1	0.001			
THBS1 (TSP1)	3.7	0.001	2,012.9 ± 384.3	10,108.3 ± 1,748.3	0.000	3,193.1 ± 664.0	11,153.8 ± 1,501.9	0.000	0.270	0.035	
CARD12 (CLAN)	3.5	0.006	25.6 ± 10.5	216.3 ± 43.8	0.000	79.4 ± 28.4	291.0 ± 81.2	0.008			
TNFAIP6 (TSG6)	2.2	0.009	68.7 ± 16.2	441.7 ± 89.3	0.000	54.3 ± 8.9	300.8 ± 60.7	0.000	0.355	0.006	
VSIG4 (A39IG)	9.2	0.000	39.8 ± 7.5	1,170.6 ± 219.6	0.000	49.9 ± 10.6	1,680.8 ± 651.9	0.000			
ADORA3 (A3AR)	4.9	0.002	144.4 ± 12.6	333.5 ± 58.7	0.001	871.2 ± 376.3	1,034.9 ± 416.8	0.037	0.341	0.007	
HBD (δ globin)	5.5	0.003	75.7 ± 7.0	387.4 ± 132.2	0.001	160.7 ± 51.7	562.0 ± 212.4	0.016	0.326	0.010	
SPARC (ON)	3.2	0.003	519.4 ± 75.0	2,604.6 ± 501.1	0.000	5,10.9 ± 140.0	1,209.3 ± 146.9	0.003			
Adaptive immunity											
FCER1G	1.8	0.009	6,674.5 ± 1,182.1	12,893.5 ± 2,046.1	0.001	2,752.3 ± 347.3	5,652.8 ± 582.9	0.000	0.401	0.002	
AREG (SDGF)	5.0	0.005	648.8 ± 87.5	3,115.2 ± 662.2	0.000	520.1 ± 135.8	2,104.6 ± 422.5	0.000	0.338	0.008	
TNFSF4 (OX40L)	3.6	0.010	5.4 ± 0.5	10.0 ± 1.2	0.001	9.0 ± 1.0	14.6 ± 1.2	0.006			
IL13RA1 (CD213A1)	2.2	0.007	369.5 ± 38.2	916.5 ± 89.4	0.000	251.2 ± 23.2	690.7 ± 69.9	0.000	0.371	0.004	
IL10 (CSIF)	1.8	0.002	203.4 ± 30.4	837.9 ± 157.0	0.000	163.7 ± 31.2	575.8 ± 65.5	0.000	0.414	0.001	
IL18 (IGIF; IL-1G)	1.7	0.006	601.2 ± 77.6	1,205.3 ± 145.7	0.000	245.5 ± 25.2	569.8 ± 65.9	0.000	0.292	0.026	
EGR1 (AT225)	2.5	0.043	850.8 ± 124.0	3,077.4 ± 516.8	0.000	1,105.7 ± 321.4	2,774.6 ± 533.9	0.000	0.477	0.000	
INDO (IDO)	1.3	0.047	120.7 ± 42.0	643.3 ± 190.8	0.001	186.4 ± 45.3	360.3 ± 53.3	0.019			
Complement and coagulation cascade											
C3AR1 (AZ3B)	5.7	0.002	90.6 ± 12.3	956.6 ± 137.4	0.000	250.1 ± 42.1	1,323.2 ± 214.3	0.000	0.429	0.001	
THBD (CD141; BDCA-3)	1.9	0.033	1,129.1 ± 172.3	1,984.0 ± 301.1	0.009	1,028.2 ± 254.2	1,428.7 ± 281.3	0.037	0.348	0.007	
Inflammation-associated											
MS4A4A (CD20L1)	10.1	0.005	220.4 ± 78.1	1,588.7 ± 247.5	0.000	525.1 ± 117.3	3,560.5 ± 497.1	0.000	0.390	0.002	
MYL9 (LC20)	4.1	0.004	438.5 ± 64.7	2,252.8 ± 495.5	0.000	742.2 ± 227.6	1,533.4 ± 191.1	0.002			
CALD1 (CDM)	3.6	0.006	35.1 ± 4.5	195.8 ± 55.3	0.000	31.5 ± 8.2	75.9 ± 9.4	0.001			
ADAMTS2 (NPI)	3.8	0.002	0.1 ± 0.0	58.5 ± 14.2	0.000	0.5 ± 0.5	79.4 ± 22.0	0.000			

^a 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.

^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 Positive fold change values (Ac/Cv) in the microarray data indicate higher gene expression level at Ac compared to Cv stage.

^e False discovery rate (FDR)-adjusted *q* values (Cv vs Ac) obtained by moderated *t* test (see *Materials and Methods*).

^f Values of *p* (Cv vs Ac) were obtained by Wilcoxon test.

^g Correlation between fold change (Ac/Cv) data and disease severity in 61 subjects by Spearman's rho.

^h 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⁺CD127^{low}) was significantly higher during acute asthma compared with subsequent convalescence (supplemental Fig. 2B).

Bioinformatics of acute exacerbation gene signatures

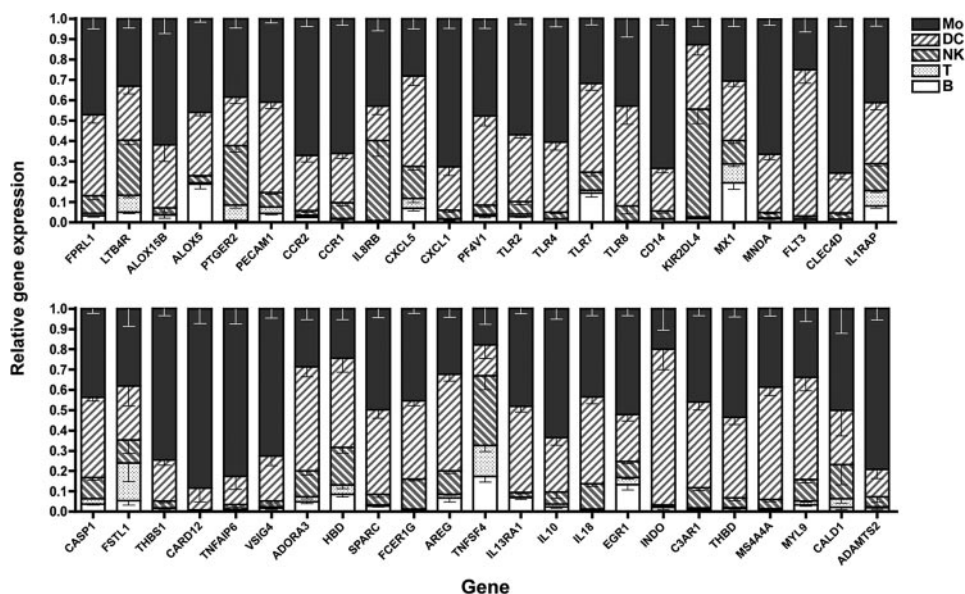
Initial examination of the list of validated genes in Table II indicated the presence of type 1 IFN-sensitive and Th2 cytokine-sen-

sitive genes in the exacerbation signature. More detailed analyses revealed a broader range of activated genes downstream of type 1 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 atopics were cultured overnight in the presence of the three key cytokines predicted above as central to development of the

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 $\pm$ SEM of 10 subjects.



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 Fc ϵ RI α , MRC1, CD1D, and CCR2. In contrast, type 1 IFN up-regulated MX1 (data not shown) plus Fc ϵ RI γ 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

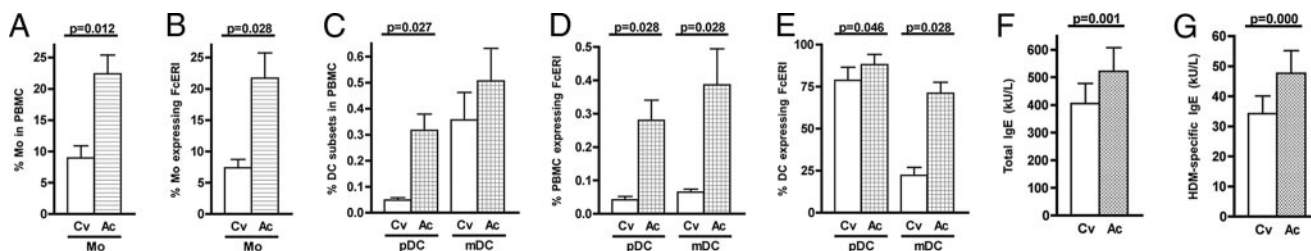


FIGURE 2. Higher Fc ϵ RI expression and IgE titers during acute exacerbation. PBMC collected at convalescent (Cv) or acute exacerbation (Ac) stage were stained with Abs to identify monocytes, total DC, or the pDC and mDC subsets, and surface Fc ϵ RI α receptors (details of Abs used in *Materials and Methods*). The frequency of monocytes (Mo) is presented as percentage of PBMC (A) and Fc ϵ RI α expression as percentage of Mo (B). pDC or mDC frequency is presented as percentage of PBMC (C). Fc ϵ RI α 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 $\pm$ SEM of 6 subjects (A–E) or 54 subjects (F and G) and statistically analyzed by Wilcoxon test.

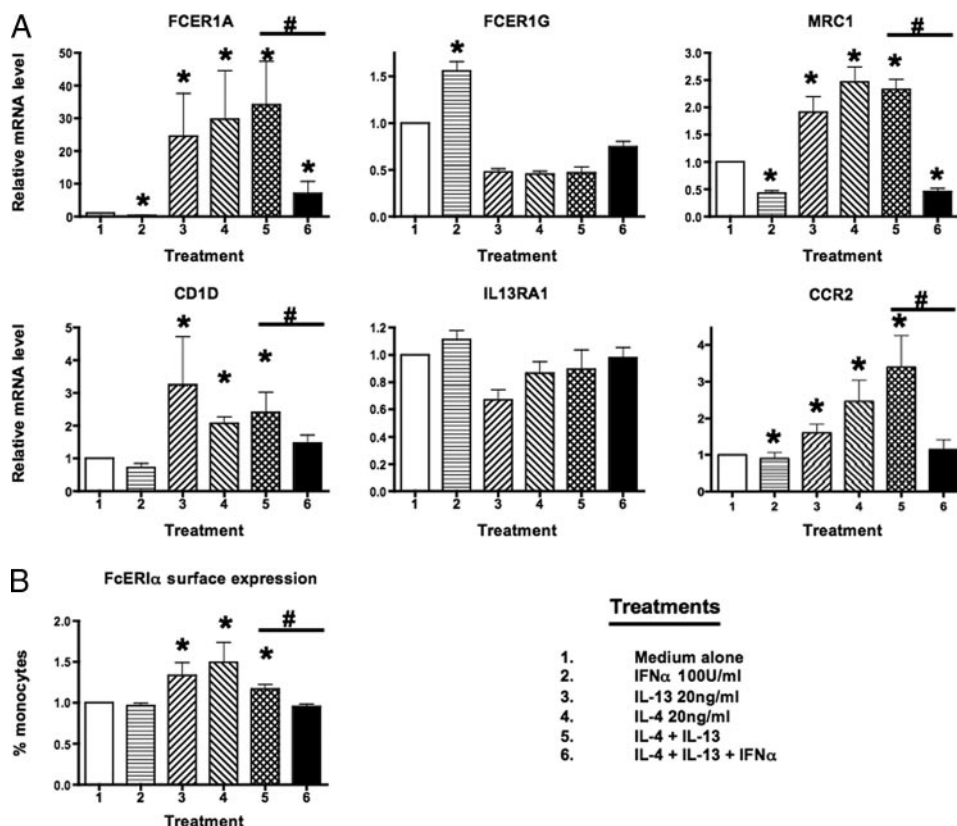


FIGURE 3. Cytokine modulation of expression signatures on blood mononuclear cells from atopic children. PBMC from six atopic children were cultured overnight in the presence of cytokine(s) as illustrated. **A**, Expression of nominated genes (as specific mRNA) was determined by qRT-PCR. **B**, FcεR1α 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.

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 infers transmission from the infected airway mucosa of an activation signal to monocytes/DC in the blood and/or bone marrow. A strong candidate is production by the infected airway epithelium and/or other cell populations of type 1 IFN, which in parainfluenza-infected mice has recently been demonstrated to strongly up-regulate FcεRIα on lung 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 monocytoic 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 FcεRIγ 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 FcεR1 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 aeroallergens 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

(Fig. 3). We suggest therefore that in atopic children a strong burst of IL-4/IL-13 generated locally during acute exacerbation, possibly stemming from allergen-specific Th2 responses enhanced via FcεR1-mediated allergen processing/presentation as suggested above, may stimulate AAM programming distal to the lung in immature precursor populations.

Migration of IL-13R⁺ 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 and enhancement of preexisting aeroallergen-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 FcεRIγ 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 aeroallergen 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

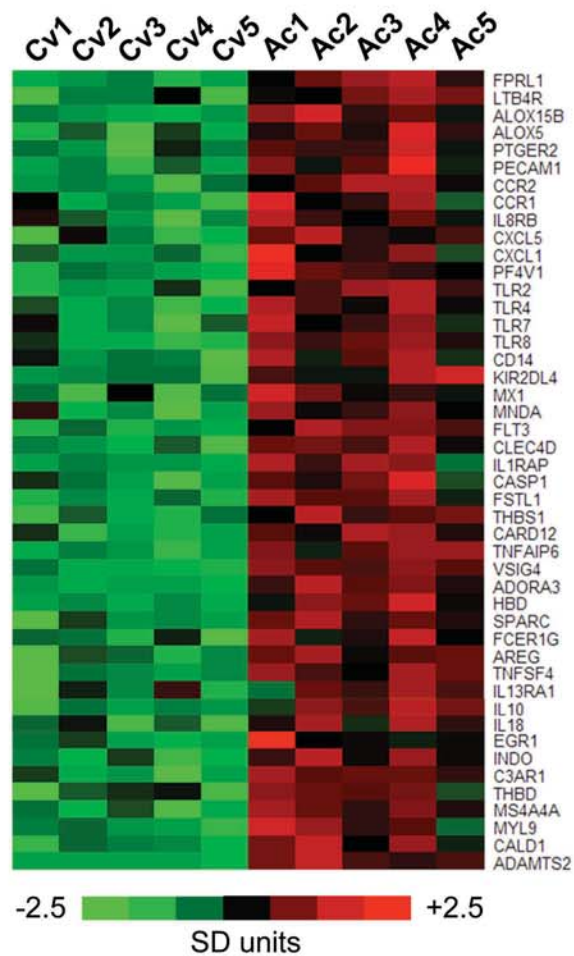
The authors have no financial conflicts of interest.

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Supplementary Figure 1:
Affymetrix microarray profiles of pooled convalescent (CV) vs Acute (Ac) RNA samples



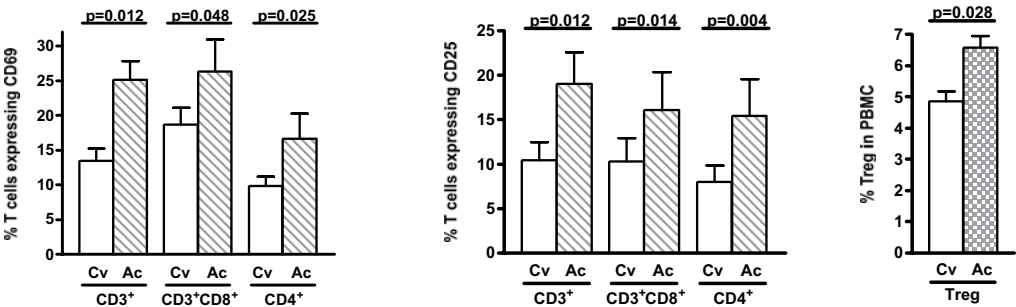
Supplementary Figure 2. Analysis of T cell-associated gene expression during acute exacerbation

a. qRT-PCR validation of downregulated T cell-associated genes^a

Gene symbol ^b	Pathway/ Function	Microarray data		qRT-PCR data: Cohort 1			qRT-PCR data: Cohort 2		
		Fold ^c	q ^d	Cv	Ac	p ^e	Cv	Ac	p ^e
IFNG	Adaptive	-3.0	0.024	515.6 ± 85.3	188.9 ± 28.6	0.001	261.6 ± 45.5	110.0 ± 18.5	0.007
IRF1 (MAR)	immunity	-1.5	0.011	3556.0 ± 294.9	2685.7 ± 269.8	0.022	2516.6 ± 177.5	1657.4 ± 192.7	0.002
LTA (TNFB)		-1.5	0.017	161.4 ± 15.3	100.3 ± 7.1	0.001	168.2 ± 42.5	150.6 ± 53.4	0.008
STAT4		-1.4	0.018	353.3 ± 48.8	286.0 ± 31.3	0.019	315.4 ± 19.7	250.0 ± 17.8	0.014
IL2RB (CD122)		-1.8	0.018	1710.7 ± 268.0	909.7 ± 155.5	0.011	1275.3 ± 145.0	875.5 ± 64.5	0.009
CXCR3 (IP10-R)		-1.4	0.040			^f	340.3 ± 51.0	251.8 ± 40.4	0.009

^a qRT-PCR data are presented as gene expression levels normalized to housekeeping gene UBE2D2. Data are expressed as mean ± SEM of 25 subjects (Cohort 1 or 2).
^b Official and alternative gene symbols, the latter in parentheses.
^c Negative fold change values (Ac/Cv) in the microarray data indicates lower gene expression level at acute (Ac) compared to convalescent (Cv) stage.
^d False discovery rate (FDR)-adjusted q values (Cv vs Ac) obtained by moderated T test (see Methods).
^e p values (Cv vs Ac) obtained by Wilcoxon Test ("-" designates data not significant at p=0.05 cutoff).
^f p=0.05-0.10

b. Upregulation of activation markers CD25 and CD69, and regulatory T (Treg) cells during acute exacerbation.



Peripheral blood mononuclear cells (PBMC) obtained during acute exacerbation (Ac) or convalescence (Cv) were stained with antibodies against CD69 (left panel), CD25 (centre panel), or CD4/CD25/CD127 (right panel) for flow cytometric analysis. The cells were also co-stained with anti-CD3, CD8, CD4 and CD127 antibodies as required. CD69 (left panel) or CD25 (centre panel) expression is presented as % of the respective CD3⁺, CD8⁺ or CD4⁺ T cell population, and the proportion of CD4⁺CD25⁺CD127^{low} regulatory T (Treg) cells (right panel) as % of PBMC. Data are expressed as mean ± SEM of 18 subjects (left and centre panels) or 10 subjects (right panel), and statistical analysis was performed by Wilcoxon test.

Supplementary Table 1. Differentially expressed genes (acute exacerbation vs convalescence) are enriched in biological pathway(s)

Gene symbol ^a	Pathways/Functions	Gene name
FPRL1 (LXA4R; ALXR)	Arachidonic acid/ prostaglandin	formyl peptide receptor-like 1
LTB4R (BLT1)		leukotriene B4 receptor
ALOX15B		arachidonate 15-lipoxygenase, type B
ALOX5 (5-LO)		arachidonate 5-lipoxygenase
PTGER2 (EP2)		prostaglandin E receptor 2 (subtype EP2), 53kDa
PECAM1 (CD31)	Leukocyte migration	platelet/endothelial cell adhesion molecule (CD31 antigen)
CCR2 (MCP-1-R)		chemokine (C-C motif) receptor 2
CCR1 (CD191)		chemokine (C-C motif) receptor 1
IL8RB (CXCR2; CD182)		interleukin 8 receptor, beta
CXCL5 (ENA-78; SCYB5)		chemokine (C-X-C motif) ligand 5
CXCL1 (GRO1; MGSA)		chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
PF4V1 (CXCL4V1)		platelet factor 4 variant 1
TLR2	Innate immunity	toll-like receptor 2
TLR4		toll-like receptor 4
TLR7		toll-like receptor 7
TLR8		toll-like receptor 8
CD14		CD14 molecule
KIR2DL4 (CD158D)		killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4
MX1 (MXA)		myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
MNDA (PYHIN3)		myeloid cell nuclear differentiation antigen
FLT3 (CD135)		fms-related tyrosine kinase 3
CLEC4D (CLEC6; MCL)		C-type lectin domain family 4, member D
IL1RAP (IL1R3)		interleukin 1 receptor accessory protein
CASP1 (ICE; IL1BC)		caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
FSTL1 (FRP; FSL1)		folliculin-like 1
THBS1 (TSP1)		thrombospondin 1
CARD12 (CLAN; IPAF)		caspase recruitment domain family, member 12
TNFAIP6 (TSG6)		tumor necrosis factor, alpha-induced protein 6
VSIG4 (Z39IG)		V-set and immunoglobulin domain containing 4
ADORA3 (A3AR; AD026)		adenosine A3 receptor
HBD (delta globin)		hemoglobin, delta
SPARC (ON; BM-40)		secreted protein, acidic, cysteine-rich (osteonectin)
FCER1G	Adaptive immunity	Fc fragment of IgE, high affinity 1, receptor for; gamma polypeptide
AREG (SDGF; CRDGF)		Amphiregulin (schwannoma-derived growth factor)
TNFSF4 (OX40L)		tumor necrosis factor (ligand) superfamily, member 4
IL13RA1 (CD213A1)		interleukin 13 receptor, alpha 1
IL10 (CSIF)		interleukin 10
IL18 (IGIF; IL-1G)		interleukin 18 (interferon-gamma-inducing factor)
EGR1 (AT225; KROX-24)		early growth response 1
INDO (IDO)	Complement & Coagulation Cascade	indoleamine-pyrrole 2,3 dioxygenase
C3AR1 (AZ3B)		complement component 3a receptor 1
THBD (CD141; BDCA-3)	Inflammation-associated	thrombomodulin
MS4A4A (CD20L1)		membrane-spanning 4-domains, subfamily A, member 4
MYL9 (LC20)		myosin, light polypeptide 9, regulatory
CALD1 (CDM)		caldesmon 1
ADAMTS2 (NPI; PCINP)		ADAM metalloproteinase with thrombospondin type 1 motif, 2

^a Official and alternative gene symbols, the latter in parentheses.

Supplementary Table 2. qRT-PCR validation using either frozen or fresh PBMC samples^a

Gene symbol ^b	Frozen PBMC ^c					Fresh PBMC ^c						
	Cv		Ac			Cv		Ac				
FPRL1 (LXA4R; ALXR)	165.2	±	33.2	896.1	±	106.3	140.1	±	29.6	941.4	±	132.2
LTB4R (BLT1)	98.7	±	58.0	369.8	±	161.9	114.0	±	13.4	388.8	±	77.0
ALOX15B	1.7	±	0.7	92.7	±	50.4	2.0	±	0.5	104.9	±	55.1
ALOX5 (5-LO)	501.6	±	71.1	980.0	±	145.8	563.0	±	62.4	1210.7	±	130.5
PTGER2 (EP2)	1007.4	±	391.4	1861.2	±	560.9	1157.0	±	444.5	2258.2	±	553.9
PECAM1 (CD31)	559.2	±	390.3	1953.8	±	825.6	639.2	±	355.7	1868.9	±	621.6
CCR2 (MCP-1-R)	236.7	±	41.1	752.2	±	166.4	221.5	±	51.1	800.2	±	182.6
CCR1 (CD191)	119.0	±	80.4	719.1	±	328.9	116.6	±	68.2	849.5	±	397.3
IL8RB (CXCR2; CD182)	51.3	±	33.1	209.2	±	112.0	20.5	±	5.6	89.5	±	4.6
CXCL5 (ENA-78; SCYB5)	29.6	±	11.9	564.0	±	410.4	35.2	±	12.9	385.1	±	211.1
CXCL1 (GRO1; MGSA)	9.8	±	3.2	29.6	±	10.6	10.3	±	3.0	36.4	±	10.0
PF4V1 (CXCL4V1)	47.7	±	12.8	527.2	±	308.3	46.6	±	13.6	486.2	±	266.7
TLR2	15499.7	±	1604.0	46560.1	±	6531.7	15919.3	±	1395.9	47402.4	±	6685.7
TLR4	2825.8	±	333.7	9740.3	±	981.5	2645.1	±	336.1	9776.4	±	992.5
TLR7	6893.8	±	721.7	15082.4	±	2258.8	6703.2	±	687.0	15604.9	±	2349.3
TLR8	38.6	±	12.6	207.4	±	101.6	37.4	±	11.2	224.8	±	97.6
CD14	2161.7	±	1619.5	19178.7	±	7690.3	3027.6	±	1163.5	21164.4	±	8283.7
KIR2DL4 (CD158D)	35.5	±	22.7	107.7	±	60.6	50.6	±	29.0	145.0	±	70.9
MX1 (MXA)	9139.2	±	1474.0	28490.7	±	11670.6	8080.1	±	1205.3	18241.2	±	5660.6
MNDA (PYHIN3)	5839.6	±	1009.7	11459.2	±	3166.9	3171.2	±	476.8	8829.9	±	540.4
FLT3 (CD135)	22.5	±	7.1	119.7	±	63.5	25.2	±	6.3	127.3	±	68.3
CLEC4D (CLEC6; MCL)	106.6	±	26.4	310.2	±	23.3	103.7	±	23.4	317.1	±	15.0
IL1RAP (IL1R3)	24.2	±	12.3	70.5	±	46.4	33.8	±	7.4	87.0	±	35.0
CASP1 (ICE; IL1BC)	2759.0	±	451.7	4943.7	±	436.1	1760.6	±	249.1	3410.2	±	631.2
FSTL1 (FRP; FSL1)	3.5	±	0.8	62.8	±	39.6	3.5	±	0.7	55.6	±	31.6
THBS1 (TSP1)	1330.6	±	869.8	4594.2	±	2128.2	1198.6	±	769.1	5175.8	±	2359.6
CARD12 (CLAN; IPAF)	30.7	±	14.7	151.8	±	55.1	30.4	±	15.9	163.8	±	59.9
TNFAIP6 (TSG6)	194.9	±	22.0	1124.1	±	129.9	193.8	±	25.5	1004.2	±	231.7
VSIG4 (A39IG)	73.4	±	29.7	1389.7	±	572.7	54.0	±	23.3	1201.1	±	449.3
ADORA3 (A3AR; AD026)	150.7	±	21.1	252.9	±	87.1	149.7	±	19.9	261.1	±	85.9
HBD (delta globin)	67.1	±	14.5	134.5	±	66.9	61.6	±	16.7	134.8	±	56.7
SPARC (ON; BM-40)	160.2	±	39.9	1664.7	±	789.2	178.0	±	34.8	1604.9	±	676.7
FCER1G	12943.8	±	2960.0	23188.5	±	5109.6	8173.5	±	2309.2	18604.9	±	3252.6
AREG (SDGF; CRDGF)	368.7	±	218.8	3107.9	±	2077.5	392.9	±	237.6	3194.9	±	2127.5
TNFSF4 (OX40L)	4.0	±	0.6	6.2	±	1.5	3.8	±	0.4	8.5	±	1.2
IL13RA1 (CD213A1)	366.9	±	41.4	1062.1	±	230.2	388.8	±	52.8	1051.2	±	167.7
IL10 (CSIF)	43.7	±	24.3	461.3	±	162.8	58.4	±	19.4	518.6	±	144.3
IL18 (IGIF; IL-1G)	928.6	±	217.1	1626.9	±	360.4	768.6	±	141.0	1614.2	±	369.0
EGR1 (AT225; KROX-24)	601.8	±	198.9	5214.9	±	1482.9	652.8	±	222.9	6160.1	±	2117.8
INDO (IDO)	83.9	±	54.9	1019.1	±	799.2	46.8	±	23.7	474.0	±	246.3
C3AR1 (AZ3B)	102.1	±	17.1	738.8	±	187.5	100.2	±	17.2	755.2	±	184.8
THBD (CD141; BDCA-3)	198.9	±	60.3	894.7	±	247.7	196.4	±	58.6	912.4	±	239.9
MS4A4A (CD20L1)	273.3	±	54.9	2477.4	±	833.8	212.9	±	50.2	2479.6	±	834.4
MYL9 (LC20)	195.2	±	55.5	2143.6	±	1162.3	208.9	±	61.7	2392.1	±	1195.9
CALD1 (CDM)	18.0	±	7.3	315.3	±	218.1	16.2	±	6.9	207.9	±	90.4
ADAMTS2 (NPI; PCINP)	0.0	±	0.0	31.3	±	20.9	0.1	±	0.1	34.3	±	17.9

^a qRT-PCR data are presented as gene expression levels normalised to house-keeping gene UBE2D2. Data are expressed as mean ± SEM of 5 subjects.

^b Official and alternative gene symbols, the latter in parentheses.

^c Comparison of gene expression in frozen versus fresh PBMC, at either convalescent (Cv) or acute (Ac) stage, generated non-significant p values (p>0.05) by Mann-Whitney U test.

In order to test whether cryobanking cells prior to analysis affected expression levels of genes in the panel identified in the study, RNA was extracted from 10 replicate samples of PBMC either immediately after collection or post cryobanking. Subsequent qRT-PCR analyses confirmed that none of the relevant gene expression was affected by PBMC cryopreservation, with no statistically significant differences found between expression levels in cryopreserved and fresh PBMC samples.

Supplementary Table 3. Gene expression in relation to sensitiveness to glucocorticoid (GC)^a

Gene symbol ^b	Correlation with time since GC treatment		GC-inducible (published) ^d
	R	p value ^c	
FPRL1 (LXA4R; ALXR)	-	-	1
LTB4R (BLT1)	-	-	
ALOX15B ⁶	-0.657	0.000	
ALOX5 (5-LO)	-	-	
PTGER2 (EP2)	-	-	
PECAM1 (CD31)	-	-	
CCR2 (MCP-1-R)	-	-	
CCR1 (CD191)	-	-	
IL8RB (CXCR2; CD182)	-	-	
CXCL5 (ENA-78; SCYB5)	-	-	
CXCL1 (GRO1; MGS A)	-	-	
PF4V1 (CXCL4V1)	-	-	
TLR2	-	-	1
TLR4	-	-	
TLR7	-	-	
TLR8	-	-	
CD14	-	-	
KIR2DL4 (CD158D)	-	-	
MX1 (MXA)	-	-	
MNDA (PYHIN3)	-	-	
FLT3 (CD135) ⁶	-0.467	0.003	
CLEC4D (CLEC6; MCL)	-	-	
IL1RAP (IL1R3)	-	-	
CASP1 (ICE; IL1BC)	-	-	
FSTL1 (FRP; FSL1)	-	-	
THBS1 (TSP1)	-	-	
CARD12 (CLAN; IPAF)	-	-	
TNFAIP6 (TSG6)	-	-	
VSIG4 (A39IG) ⁶	-	-	
ADORA3 (A3AR; AD026) ⁶	-0.555	0.000	
HBD (delta globin)	-0.467	0.003	
SPARC (ON; BM-40)	-	-	
FCER1G	-	-	1
AREG (SDGF; CRDGF) ⁶	-	-	
TNFSF4 (OX40L)	-0.451	0.005	
IL13RA1 (CD213A1)	-	-	
IL10 (CSIF)	-	-	
IL18 (IGIF; IL-1G)	-	-	
EGR1 (AT225; KROX-24)	-	-	
INDO (IDO)	-	-	
C3AR1 (AZ3B)	-	-	
THBD (CD141; BDCA-3)	-	-	
MS4A4A (CD20L1)	-	-	1
MYL9 (LC20)	-	-	1,5
CALD1 (CDM)	-	-	
ADAMTS2 (NPI; PCINP)	-	-	

^a qRT-PCR data of 61 subjects, normalised to house-keeping gene UBE2D2.

^b Official and alternative gene symbols, the latter in parentheses.

^c Correlation between gene expression at acute stage and time between GC treatment and PBMC collection by Spearman's rho ("-" designates data not significant at p=0.05 cutoff).

^d References cited are source references naming relevant genes as part of glucocorticoid-induced alternatively activated macrophage (AAM) gene expression signature.

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2. Yesner LM, Huh HY, Pearce SF, Silverstein RL. Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler Thromb Vasc Biol* 1996;16:1019-25.
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6. In a follow-up study PBMC samples from 10 volunteers were cultured for 6 hours with 1µm Dexamethasone, and expression of all genes in the panel assessed by qRT-PCR. Group mean expression of the indicated genes displayed unequivocal upregulation as defined by ≥ 4 fold above background control and no overlap between respective 95% confidence intervals.

Supplementary Table 4. Upregulated genes detected by microarray in acute PBMC samples which are part of the Type-1 interferon (IFN)-inducible gene signature

Gene symbol ^a	Gene name	Fold change Ac/Cv ^b	q value (Cv vs Ac) ^c	References ^d
HSPA1A	heat shock 70kDa protein 1A	1.9	0.049	1
IFITM3	interferon induced transmembrane protein 3 (1-8U)	1.6	0.044	2
ISG20	interferon stimulated exonuclease gene 20kDa	1.6	0.017	2
MX1	myxovirus (influenza virus) resistance 1	1.8	0.043	1,2,3,4,5
PLSCR1	phospholipid scramblase 1	1.9	0.021	1,3,5
RIN2	Ras and Rab interactor 2	2.0	0.007	1
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.1	0.039	3
TRIM22	tripartite motif-containing 22	1.4	0.020	4

^a Official human gene symbols, listed alphabetically. In bold are genes that are part of qRT-PCR validation in **Table 2**.

^b Fold change Ac/Cv values from microarray data.

^c False discovery rate (FDR)-adjusted q values obtained by moderated T test (see Methods).

^d References cited are source references naming relevant genes as part of the Type-1 IFN-inducible gene expression signature.

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4. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* 1998;95:15623-8.
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Supplementary Table 5. Upregulated genes detected by microarray in acute PBMC samples which are part of the expression signature of IL-4/IL-13-induced alternatively activated macrophages (AAM)

Gene symbol ^a	Gene name	Fold change Ac/Cv ^b	q value (Cv vs Ac) ^c	References ^d
ALOX12	arachidonate 12-lipoxygenase	3.1	0.003	1
ALOX15B	arachidonate 15-lipoxygenase, type B	3.3	0.004	1,2,3,4
BLVRA	biliverdin reductase A	1.5	0.014	3
CA2	carbonic anhydrase II	2.6	0.010	2
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	2.2	0.043	3
CD14	CD14 molecule	2.3	0.004	2
CD163	CD163 molecule	6.7	0.001	1,3
CD36	CD36 molecule (thrombospondin receptor)	2.3	0.019	2
CD86	CD86 molecule	1.7	0.031	1
CD9	CD9 molecule	2.1	0.020	2
CLEC4A	C-type lectin domain family 4, member A	1.6	0.021	2,3
CLEC7A	C-type lectin domain family 7, member A	2.0	0.007	2,5
EVI2B	ecotropic viral integration site 2B	1.3	0.018	2
F13A1	coagulation factor XIII, A1 polypeptide	3.3	0.002	2,3,4,6
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	1.7	0.009	1
FGD2	FYVE, RhoGEF and PH domain containing 2	2.1	0.008	3
FGL2	fibrinogen-like 2	2.2	0.018	2
FKBP1A	FK506 binding protein 1A, 12kDa	1.4	0.012	3
G6PD	glucose-6-phosphate dehydrogenase	1.6	0.015	3
GAS7	growth arrest-specific 7	1.7	0.007	2,3
GLIPR1	GLI pathogenesis-related 1 (glioma)	1.6	0.014	2
GSN	gelsolin (amyloidosis, Finnish type)	1.5	0.013	3
HNMT	histamine N-methyltransferase	2.0	0.004	2
HOMER2	homer homolog 2 (Drosophila)	1.4	0.041	2,3
HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	2.0	0.048	2
IL10	interleukin 10	1.8	0.002	1
IL13RA1	interleukin 13 receptor, alpha 1	2.2	0.007	1,7,8
IL1R2	interleukin 1 receptor, type II	42.7	0.000	3
LIPA	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	1.3	0.029	2,3
LTA4H	leukotriene A4 hydrolase	1.4	0.036	2
MAOA	monoamine oxidase A	1.5	0.038	3,4
MRC1 ^e	mannose receptor, C type 1	7.6 ± 2.4	0.008	1,2,3
MS4A4A	membrane-spanning 4-domains, subfamily A, member 4	10.1	0.005	2,3,4
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	2.4	0.019	2
MSR1	macrophage scavenger receptor 1	1.7	0.016	2
NFE2	nuclear factor (erythroid-derived 2), 45kDa	2.5	0.002	3
P2RY13	purinergic receptor P2Y, G-protein coupled, 13	3.2	0.004	2
P2RY14	purinergic receptor P2Y, G-protein coupled, 14	2.3	0.010	2
PDGFC	platelet derived growth factor C	2.1	0.002	2,3
PTGS1	prostaglandin-endoperoxide synthase 1	2.7	0.002	2
PYGL	phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	1.9	0.006	2
QPCT	glutamyl-peptide cyclotransferase (glutamyl cyclase)	1.9	0.017	2
RNASE6	ribonuclease, RNase A family, k6	2.7	0.002	2
SASH1	SAM and SH3 domain containing 1	1.6	0.039	2
SLA	Src-like-adaptor	2.2	0.016	3
SOCS1	suppressor of cytokine signaling 1	1.6	0.012	3
SORT1	sortilin 1	1.8	0.005	3
SPINT1	serine peptidase inhibitor, Kunitz type 1	1.4	0.038	3
TBXAS1	thromboxane A synthase 1 (platelet, cytochrome P450, family 5, subfamily A)	2.1	0.006	2
TLR5	toll-like receptor 5	2.3	0.029	2
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 (TRAIL)	2.6	0.049	2
TNFSF13	tumor necrosis factor (ligand) superfamily, member 13 (APRIL)	1.8	0.009	2

^a Official human gene symbols, listed alphabetically. In bold are genes that are part of qRT-PCR validation in **Table 2**.

^b Fold change Ac/Cv values from microarray data.

^c False discovery rate (FDR)-adjusted q values obtained by moderated T test (see Methods).

^d References cited are source references naming relevant genes as part of AAM gene expression signature.

^e The AAM signature gene MRC1 was not differentially expressed in the PBMC microarray data, but subsequent qRT-PCR analysis of sorted monocytes from **Fig. 1** demonstrated strong MRC1 upregulation at Ac compared to Cv stage (data shown as mean ± SEM).

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