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Functional Genetic Variation in *NFKBIA* and Susceptibility to Childhood Asthma, Bronchiolitis, and Bronchopulmonary Dysplasia

Salman Ali,*,[1] Aaron F. Hirschfeld,*,[1] Matthew L. Mayer,† Edgardo S. Fortuno, III,* Nathan Corbett,* Maia Kaplan,* Shirley Wang,* Julia Schneiderman,* Christopher D. Fjell,† Jin Yan,‡ Loubna Akhabir,‡ Farzian Aminuddin,‡ Nico Marr,* Thierry Lacaze-Masmonteil,§ Richard G. Hegele,¶ Allan Becker,‖ Moira Chan-Yeung,# Robert E. W. Hancock,† Tobias R. Kollmann,* Denise Daley,‡ Andrew J. Sandford,‡ Pascal M. Lavoie,* and Stuart E. Turvey*

Respiratory diseases are the most frequent chronic illnesses in babies and children. Although a vigorous innate immune system is critical for maintaining lung health, a balanced response is essential to minimize damaging inflammation. We investigated the functional and clinical impact of human genetic variants in the promoter of *NFKBIA*, which encodes IκBα, the major negative regulator of NF-κB. In this study, we quantified the functional impact of *NFKBIA* promoter polymorphisms (rs3138053, rs2233406, and rs2233409) on promoter-driven protein expression, allele-specific and total *NFKBIA* mRNA expression, IκBα protein expression, and TLR responsiveness; mapped innate immune regulatory networks active during respiratory syncytial virus infection, asthma, and bronchopulmonary dysplasia; and genotyped and analyzed independent cohorts of children with respiratory syncytial virus infection, asthma, and bronchopulmonary dysplasia. Genetic variants in the promoter of *NFKBIA* influenced *NFKBIA* gene expression, IκBα protein expression, and TLR-mediated inflammatory responses. Using a systems biology approach, we demonstrated that *NFKBIA*/IκBα is a central hub in transcriptional responses of prevalent childhood lung diseases, including respiratory syncytial virus infection, asthma, and bronchopulmonary dysplasia. Finally, by examining independent pediatric lung disease cohorts, we established that this immunologically relevant genetic variation in the promoter of *NFKBIA* is associated with differential susceptibility to severe bronchiolitis following infection with respiratory syncytial virus, airway hyperresponsiveness, and severe bronchopulmonary dysplasia. These data highlight the importance of negative innate immune regulators, such as *NFKBIA*, in pediatric lung disease and begin to unravel common aspects in the genetic predisposition to bronchopulmonary dysplasia, bronchiolitis, and childhood asthma. ***The Journal of Immunology*****, 2013, 190: 000–000.**

The most common chronic illnesses in children and babies are those of the respiratory system (1). Many of these serious childhood lung diseases are caused by infection and inflammation, including asthma, viral bronchiolitis, and bronchopulmonary dysplasia (BPD). The innate immune system plays a vital role in protecting our lungs but in many ways can be considered a double-edged sword that must be tightly regulated to minimize lung-damaging inflammation. Recent years have witnessed an explosion of interest in the innate immune system; nevertheless, most attention has focused on the elements involved in activation of the system, whereas negative regulators and modulators of innate immunity have received much less scrutiny

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BPD, bronchopulmonary dysplasia; CI, confidence interval; C&W, Children's and Women's Health Centre of British Columbia; LD, linkage disequilibrium; NCBI, National Center for Biotechnology Information; PIV, parainfluenza virus; RSV, respiratory syncytial virus; SNP, single nucleotide polymorphism.

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(2). We sought to address this knowledge gap by examining the role of a critical negative modulator of the innate immune system in some of the most prevalent pediatric infectious and inflammatory lung diseases.

NF-κB is a family of protein transcription factors that can orchestrate many inflammatory processes. IκBα, encoded by *NFKBIA*, is an important inhibitor of NF-κB activity (3). In the resting state, IκBα sequesters NF-κB in the cytoplasm. In response to specific stimuli, IκBα is ubiquitinated and degraded, allowing NF-κB to migrate to the nucleus where it can bind to response elements on proinflammatory genes and initiate their transcription. In vivo observations confirm the critical immunomodulatory role of IκBα in both mice (4) and humans (5).

In light of the vital role played by IκBα in regulating inflammation, proliferation, and apoptosis, it is not surprising that genetic variation in the promoter region of *NFKBIA* has been linked to alterations in susceptibility to infectious and inflammatory diseases, as well as a variety of cancers (6) (Table I). We hypothesized that these promoter variants in *NFKBIA* would have functional consequences, altering the "tuning" of immune responsiveness.

The objective of this study was to determine the functional impact of specific variants in the promoter of *NFKBIA* that appear to influence susceptibility to infectious and inflammatory diseases. To ensure clinical relevance, we built on our in vitro–derived mechanistic insights by examining how functional variants in *NFKBIA* alter in vivo susceptibility to childhood diseases with an inflammatory component in their pathogenesis: asthma, respiratory syncytial virus (RSV), bronchiolitis, and BPD. Although each of these clinical phenotypes has its own complex etiology, a central contribution of the innate immune system and NF-κB signaling is common to all (7–13). Greater understanding of the genetic control of the NF-κB pathway is particularly important given the growing interest in the identification of biomarkers that predict the risk for disease and the development of novel therapies that modulate NF-κB activity (14).

Materials and Methods

Analysis of NFKBIA promoter structure and population variation

Genetic variation and linkage disequilibrium (LD) patterns were examined using data from the HapMap consortium (http://www.hapmap.org) and Programs for Genomic Applications (http://www.nhlbi.nih.gov/resources/geneticsgenomics/programs/pga.htm). The Gene Regulation (http://www.gene-regulation.com/index2) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) bioinformatic tools were used to analyze the *NFKBIA* promoter for putative transcription factor binding sites.

NFKBIA promoter functional assay

Primers were designed to amplify a 1068-bp region, 13 bp upstream of the *NFKBIA* transcriptional start site, for cloning into a firefly luciferase reporter vector (Table II). The fragment was amplified with high-fidelity Phusion DNA polymerase (New England Biolabs), inserted into a pCRII-Blunt-TOPO vector (Life Technologies), and cloned into a pGL4.14 promoter-less luciferase-expressing vector (Promega). The pGL4.14-*NFKBIA* promoter constructs were sequenced to confirm genotype and orientation. CHO-K1 cells were grown in F-12K medium (HyClone) and cotransfected using a 4D Nucleofector (Lonza) with 200 ng plasmid and 8 ng pGL4.74 HSV-*Renilla* vector/2×10^5 cells. The cells were left to recover for 24 h and then were stimulated with 100 ng/ml IL-1β for 24 h. The stimulation was followed by a Dual Luciferase Assay (Promega) on an Infinite M200 luminometer (Tecan). Firefly luciferase luminescence was normalized to *Renilla* luminescence and reported as fold change.

Single nucleotide polymorphism genotyping

The *NFKBIA* single nucleotide polymorphisms (SNPs) rs2233406, rs3138053, and rs2233409 were genotyped using commercially available TaqMan assays (C_73867_10, C_73866_10, and C_15945891_10; Life Technologies). A custom assay was designed for SNP rs1050851 (Table II). SNPs were deemed acceptable for analysis if they had call rates > 95%, frequencies did not deviate from Hardy–Weinberg equilibrium (p value > 0.05), and no Mendelian errors were observed in the available complete trios. Genotype call rates were: RSV cohort (rs2233406 = 98%, rs2233409 = 97%), BPD cohort (rs2233406 = 99%; rs2233409 = 99%), and asthma cohort (rs2233406 = 92.7%, rs2233409 = 96.6%). Consequently, rs2233406 was not included in analysis of the asthma cohort.

Allele-specific gene expression

Blood samples were obtained with approval of the University of British Columbia Clinical Research Ethics Board (C04-0534). PBMCs were isolated by density-gradient centrifugation as previously described (15), suspended in RPMI 1640 medium containing 10% FCS (HyClone), and seeded into a 24-well plate (BD Biosciences) before stimulation with LPS (100 ng/ml; *Escherichia coli* 0111:B4; InvivoGen) or live *Streptococcus pneumoniae* (serotype 14). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 3 h poststimulus before harvesting mRNA with an additional DNAse treatment. Reverse transcription was achieved using the SuperScriptVILO cDNA synthesis kit (Life Technologies).

Modifying the technique described by Zhu et al. (16), we quantified allele-specific gene expression by measuring the expression of alleles of synonymous coding SNP rs1050851. This SNP is in LD ($r^2 > +0.82$) with the promoter SNPs (rs3138053, rs2233406, and rs2233409), acting as a "tag" for differentiating between the major (ACC) and minor (GTT) allele transcripts of *NFKBIA* (Fig. 1). When one rs1050851 allele was overexpressed relative to the other, the probe's fluorescence signal crossed the predetermined threshold earlier, generating a ΔCt value. Because PCR efficiency for the two alleles differs slightly, a correction was made by subtracting the ΔCt derived from heterozygous control genomic DNA (1:1 allele ratio) from the observed ΔCt derived from the cDNA sample. This corrected measure, designated ΔCt′, enabled calculation of an accurate allele-expression ratio.

NFKBIA gene and IκBα protein expression

Primers were designed (Table II), and expression of *NFKBIA* was calculated relative to *ACTB* by SYBR GreenER chemistry (Life Technologies). A 7300 Real Time PCR System (Applied Biosystems) was used under standard cycling conditions, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ Livak method (17). All quantitative PCR experiments were performed in triplicate. Statistical analysis was performed using the nonparametric Mann–Whitney test. To evaluate IκBα expression, PBMCs were stimulated with LPS (100 ng/ml; *E. coli* 0111:B4; InvivoGen) over 24 h, and lysates were analyzed by standard Western blotting protocols and probed for IκBα and β-actin (#9246, 4967; Cell Signaling). Band densitometry was calculated with an Odyssey Infrared Imaging System (LI-COR).

Quantifying innate immune responsiveness

Innate immune responsiveness was quantified using published techniques (18–20). Umbilical cord blood was obtained from healthy, full-term infants delivered by elective Caesarian section before the onset of labor. Neonatal cord blood mononuclear cells, isolated by density-gradient centrifugation, were stimulated at 37°C with a panel of TLR ligands at optimized concentrations: *E. coli* 0111:B4 LPS (TLR4), 3M-003 (an imidazoquinoline; TLR7/8), PAM_3CSK_4 (TLR2/1), 3M-002 (TLR8), and CpG type A (TLR9). Supernatants were analyzed for cytokine secretion by ELISA after 18 h of stimulation. Statistical comparisons were made using two-way ANOVA with the Bonferroni posttest.

Gene-expression microarray data processing and network analysis

Public microarray datasets were obtained from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) using GEOquery (21), and transcriptional data subsets of interest were identified (Supplemental Table I). The *NFKBIA* promoter haplotype of these datasets was not known. Associated calculations were performed using the Bioconductor project in the R statistical language, with microarray values normalized using quantile normalization (22). Differential expression of gene probes was calculated using the limma package with empirical Bayes methods (23), and adjusted p values were calculated using the Benjamini–Hochberg method (24). Differential gene expression between affected and control treatment groups was defined as gene fold changes ≥1.5 or ≤−1.5, with an associated adjusted p value ≤ 0.05. Network analysis was carried out using a previously published approach (25), using InnateDB (26). For each dataset, two networks were generated containing interactions between protein products of differentially expressed genes and interactions between the genes and their tran-

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Table I. Summary of published genetic-association studies examining *NFKBIA* promoter variants

Disease (Reference)	Reference SNP ID:	Position from Transcriptional Start Site −894 rs3138053 (A/G)	−839 rs2233406 (C/T)	−310 rs2233409 (C/T)	Association	*p* Value	Odds Ratio (95% CI)	Cases (*n*)	Controls (*n*)
Trachoma (38)		G	T	—	Protection	0.046	nc	199	194
Invasive pneumococcal disease (37)		G	T	—	Protection	10^{-4}	0.57 (0.43–0.76)	260	762
RSV in premature infants (57)		—	—	T	Protection	0.026	0.61	470	1008
Acute respiratory distress syndrome (58)		G	T	C	Risk	0.02	1.66 (1.06–2.53)	382	828
Graves' disease (59)		—	T	T	Risk	0.03	1.73 (1.02–2.94)	481	455
Rheumatoid arthritis (60)		—	T	—	Risk	0.01	1.8 (1.1–2.8)	140	115
Sarcoidosis (61)		G	T	T	Risk	0.01	nc	205	201
Myeloma (62)		G	—	C	Risk	0.006	2.29 (2.10–2.49)	157	196
Hepatocellular carcinoma (63)		G	T	—	Risk	0.002	3.142 (1.44–6.84)	202	482

—, SNP was not evaluated in referenced genetic-association study; nc, odds ratio not calculated in the original publication.

scription factors. Network analysis was carried out by merging these two networks with a first-order interaction network for *NFKBIA* into a single larger network using Cytoscape (2.8.0 for Windows) (27). Statistically significant subnetworks were identified within the larger interaction network using the jActiveModule plugin for Cytoscape, with significance defined as a Z-score > 3.0, consistent with well-established protocols (28). Network graphs were visualized using the software plugin Cerebral (29) to appreciate network directionality within the cell. Hub degree (the number of protein-level interactions with other members of the network) was determined for each gene "node" in the network using the software plugin cytoHubba for Cytoscape (30).

Description of patient cohorts, phenotype definition, and statistical analysis

RSV bronchiolitis. As previously published (31), DNA samples were obtained from nasopharyngeal wash samples from children (n = 352) suspected of having a respiratory viral infection visiting the emergency room at the Children's and Women's Health Centre of British Columbia, (C&W) (Vancouver, BC, Canada) or its affiliated institutions and from umbilical cord blood of healthy term neonates born in the same institution (population control; n = 296). The primary diagnosis of RSV infection on nasopharyngeal wash was by direct immunofluorescence assay (Chemicon). RSV-positive children were further classified as severe if they required admission to the hospital (n = 125). Case and control groups were analyzed with a dominant genetic model using the Fisher exact test. In all cohorts, the genotype frequencies did not deviate from Hardy–Weinberg equilibrium. Demographic data are shown in Table III.

Asthma. Data from the Canadian Asthma Primary Prevention Study have been published (32). Briefly, this was a prospective study that assessed the effectiveness of a multifaceted intervention program designed to prevent the development of asthma and other atopic disorders in 549 high-risk children who had a family history of allergies (33). The mothers of these children were recruited during the second and third trimester of pregnancy, and the children were assessed by a pediatric allergist for the presence of allergic phenotypes at three time points (12 and 24 mo and 7 y of life). A total of 545 families was initially recruited into the study, and 380 families were available for analysis by the end of 7 y of follow-up (34). In this study, we focused on the objective measure of airway hyperresponsiveness (AHR) at 7 y, which was defined as provocative concentration of methacholine chloride inducing a 20% decrease in forced expiratory volume in 1 s < 3.2 mg/ml. To monitor early life viral exposure, nasal swabs were obtained from children at 2 wk and 4, 8, and 12 mo of age, and these samples were analyzed by RT-PCR testing for parainfluenza virus (PIV), RSV, and picornavirus (rhinovirus/enterovirus) (35). Associations between SNPs and the clinical phenotype were tested using the software program STATA v11.0. Main effects were also tested, and viral exposure was classified by viral subtype as any positive PCR for that virus during the first 12 mo of life. Odds ratios were calculated using an additive genetic model in a logistic regression, with viral exposure as the predictor and AHR as the dependent variable. Interactions between viral exposures and SNPs were tested with the inclusion of an interaction term (SNP*Viral exposure) in the logistic regression, with adjustment for both recruitment center and intervention status. For SNPs and interaction terms with p values ≤ 0.05, a permutation procedure was applied in which the case and

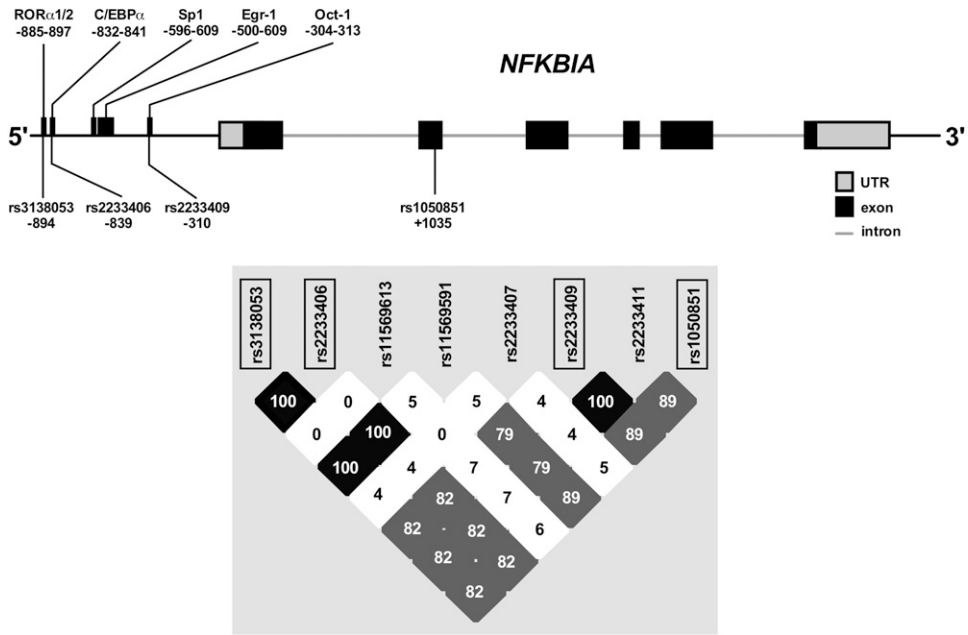

FIGURE 1. Analysis of *NFKBIA* promoter LD and transcription factor binding sites. LD plot of the *NFKBIA* promoter for the European population (Programs for Genomic Applications European panel). Variant information was downloaded from the genome variation server and uploaded into Haploview 3.1. Pairwise LD is shown as measured by r^2 and expressed as a percentile. To assess the potential functional impact of *NFKBIA* promoter variants, we used bioinformatic tools to identify putative transcription factor binding sites.

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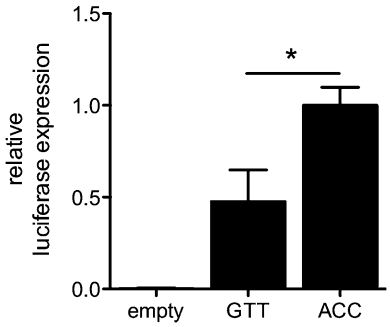

FIGURE 2. *NFKBIA* promoter variants demonstrate differences in promoter-driven luciferase production in a reporter system. CHO-K1 cells were cotransfected with either a promoter-less (empty) luciferase-expressing vector or with the same vector containing the *NFKBIA* promoter (ACC or GTT haplotype), plus a constitutively expressed *Renilla* luciferase plasmid. Data are expressed as firefly/*Renilla* luminescence normalized to the ACC ratio ($n = 3$). Statistical comparisons to ACC were made by ANOVA with the Tukey posttest. $*p < 0.05$.

control values were randomly shuffled 10,000 times, establishing an empirical p value.

Bronchopulmonary dysplasia. A total of 178 healthy white (biparental self-declared ethnicity) term-born (born at >37 wk gestation) and 156 white preterm (born at ≤30 wk gestation) neonates was prospectively recruited at birth or following admission to the neonatal intensive care unit of the C&W or the Royal Alexandra Hospital, Edmonton. Approximately 67 and 55% of all eligible premature neonates meeting the study criteria were enrolled at C&W or Royal Alexandra Hospital, respectively. BPD was defined as a chronic requirement for supplemental oxygen at 36 wk of postmenstrual age or at the time of discharge home, whichever came first. BPD was further graded by severity using criteria adapted from the National Institute of Child Health and Human Development (13), as described elsewhere (36). The Fisher exact test was used to determine significance of differences in genotype frequency between term and preterm infants using a dominant genetic model. Preterm infants with BPD grades of moderate to severe were grouped together and compared with preterm infants without BPD on the basis of the hypothesis that adverse and beneficial genetic variants would be enriched, respectively, in these two groups of disease severity. Multivariate regression analysis was performed, using gestational age, birth weight, blood culture-positive infections, and gender as covariables. Regression models were also adjusted for twins, using a covariable defining each singleton and twin pair. To minimize the confounding risk of population stratification, analysis was limited to infants for whom both parents reported their ethnicity as white.

Results

NFKBIA promoter structure and population variation

Genetic variation in *NFKBIA* has been examined in a variety of human diseases (Table I). What is most striking from these data is that three promoter variants (rs3138053, rs2233406, and rs2233409) have been repeatedly associated with many human diseases. Intriguingly, the same genetic variants that associate with protection from infectious disease are associated with increased risk for inflammatory conditions. Nevertheless, the functional impact of these variants has not been studied at a mechanistic level.

The promoter structure of *NFKBIA* and the LD pattern in the region were examined. Two promoter variants (rs2233406 and rs3138053) are in LD ($r^2 = +1$), and analysis of the European population revealed a four-variant haplotype consisting of rs2233409 in strong LD ($r^2 = +0.82$) with rs3138053 and rs2233406, as well as a fourth variant rs11569591 (an 8-bp insertion/deletion structural variant) that displayed LD ($r^2 = +1$) with rs2233406 and rs3138053 (Fig. 1). To model the potential functional impact of *NFKBIA* promoter variants, we identified putative transcription factor binding sites. Most notably, rs3138053 lies within a binding site for RORα1/2, and rs2233409 lies in the putative binding site of Oct-1. The polymorphisms of interest are also contained within putative binding sites for C/EBPα, SP1, and Egr-1 (Fig. 1). We hypothesized that the *NFKBIA* haplotype comprising the GTT minor promoter variants (in rs3138053, rs2233406, and rs2233409) would be associated with reductions in both allele-specific and total *NFKBIA* gene expression and decreased IκBα protein expression, resulting in altered innate immune function.

Modeling the functional impact of NFKBIA promoter variation

The region from −13 to −1081 bp from the transcriptional start site of *NFKBIA* was cloned into a promoter-less luciferase vector and expressed in CHO-K1 cells. This approach allowed us to directly compare the activity of the *NFKBIA* promoter containing the ACC common promoter variants (ACC-luc) with an otherwise identical construct containing the GTT minor promoter variants (GTT-luc). ACC-luc–transfected cells expressed double the amount of luciferase compared with the GTT-luc–transfected cells (Fig. 2).

NFKBIA promoter variants are associated with significant alterations in allele-specific gene expression, total gene expression, and IκBα protein expression

To validate our in vitro findings suggesting that the GTT promoter was less active than the ACC variant, we obtained fresh blood samples from healthy humans with different *NFKBIA* promoter haplotypes. Differential expression of *NFKBIA* alleles was quantified by an allele-specific expression assay. A synonymous SNP in the transcript (rs1050851) (Table II) was used as a marker to

Table II. PCR primer and probe sequences

Gene	NCBI Accession	Forward (5′→3′)	Reverse (5′→3′)	Region	Product (bp)	Temp (°C)
Primer sequences for promoter cloning						
NFKBIA	NG_007571.1	GGGCGGGCAGGATGGGACTA	CGCGGCGCCCTATAAACGCT	g.3905-4972	1068	60
Primer sequences for gene expression						
ACTB	NM_001101.3	GTTGCGTTACACCCTTTCTT	ACCTTCACCGTTCCAGTTT	c.*16-162	147	60
NFKBIA	NM_020529	TCAACAGAGTTACCTACCAGGGT	TCCTCTGTGAACTCCGTGAACTCT	c.728-902	175	60
Primer and probe sequences for custom SNP assay						
NFKBIA rs1050851	Primers	AAGTGATCCGCCAGGTGAAG	GCTGCAGGTTGTTCTGGAAGT	c.275-334	60	60
		Probe (VIC)	Probe (FAM)			
	Probes	ACCTGGC<u>C</u>TTCCTCA	ACCTGGC<u>T</u>TTCCTCA			

Underlined nucleotide represents SNP in the probe sequence.
Temp, Temperature.

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distinguish between the transcripts derived from each allele. To establish the accuracy of the system, various ratios of *NFKBIA* rs1050851 C and T alleles were prepared by mixing genomic DNAs homozygous for each SNP. Allele ratios were calculated from the ΔCt′ values. There was a strong correlation ($r^2 > +0.96$) between known and measured allele ratios, confirming the sensitivity of this experimental strategy (Fig. 3A).

The allele ratios of transcripts driven by the promoter haplotypes were quantified in PBMCs from ACC/GTT heterozygotes at baseline and after stimulation with both LPS and live *S. pneumoniae* (serotype 14). These stimuli were selected because genetic variation in the promoter of *NFKBIA* has been associated with altered susceptibility to both invasive pneumococcal infection (37) and Gram-negative organisms (38). In all conditions we detected significant allelic imbalance: specifically, the heterozygote allele ratio in RNA (cDNA) differed from the corresponding 1:1 ratio in genomic DNA (Fig. 3B). Mimicking our observation using the luciferase reporter system, in all conditions the transcript associated with the ACC haplotype was expressed at ~25% higher levels than the transcript associated with the GTT haplotype.

We determined that changes in relative allelic transcript abundance led to overall changes in *NFKBIA* mRNA levels. Individuals homozygous for the ACC haplotype had a 1.5-fold greater ex-

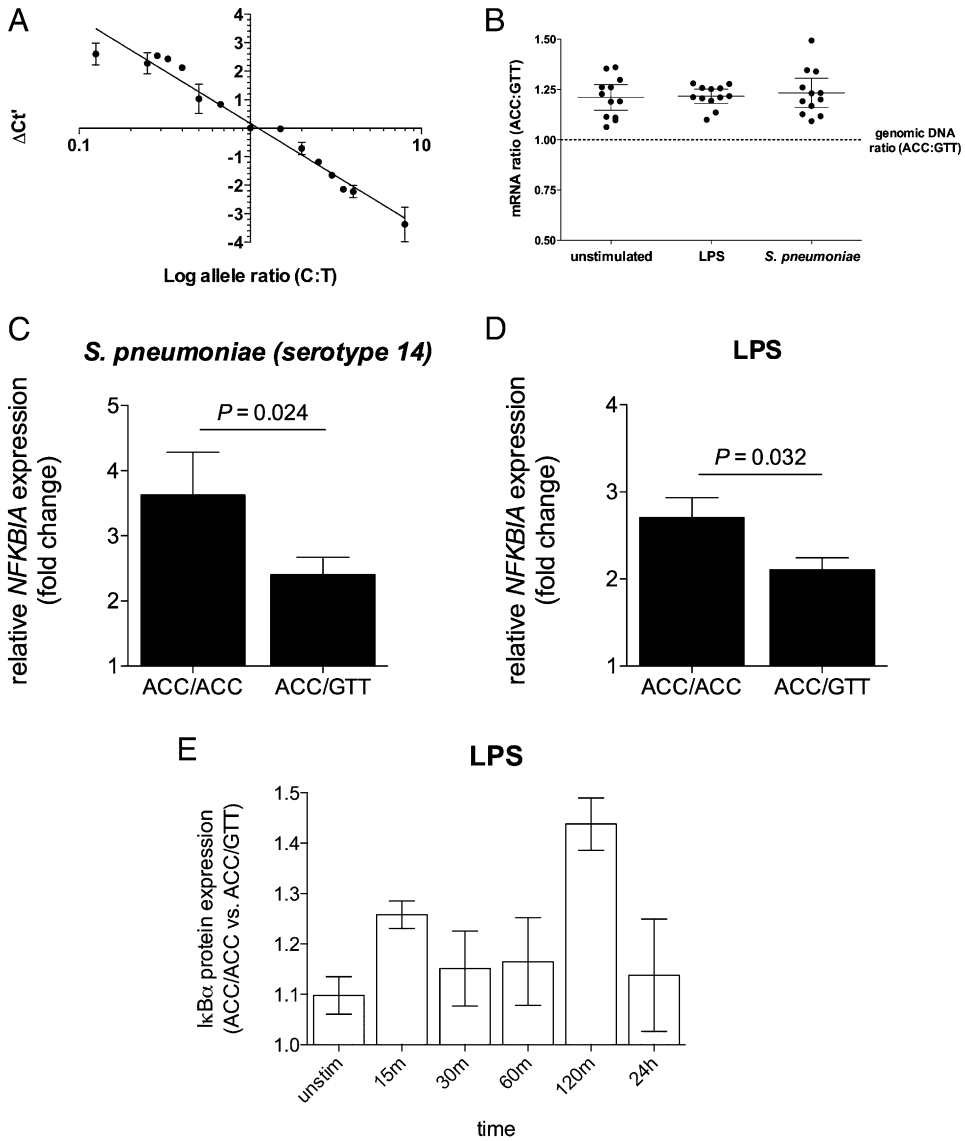

FIGURE 3. *NFKBIA* promoter variants are associated with significant alterations in allele-specific gene expression, total gene expression, and IκBα protein expression. (**A**) Allele-specific PCR was used to quantify the differential expression of *NFKBIA* alleles. Allelic imbalance was modeled by mixing different ratios of genomic DNA homozygous for the C or T alleles of rs1050851. There was a strong correlation ($r^2 = +0.96$) between the measured (as determined by ΔCt′) and known allele ratios, allowing us to use this assay to quantify allele ratios in experimental samples. (**B**) Allele-specific transcript levels in primary cells from white subjects ($n = 12$) who were confirmed to be heterozygous for both the promoter variants (ACC/GTT) and the "tag" SNP within the coding region (rs1050851, C/T). Allele ratios were quantified in PBMCs at baseline and after 3 h of stimulation with both live *S. pneumoniae* (serotype 14) and LPS. Values represent means with 95% CI. (**C** and **D**) PBMCs of individuals homozygous (ACC/ACC) or heterozygous (ACC/GTT) for the *NFKBIA* promoter SNPs were stimulated with 100 ng/ml of LPS and *S. pneumoniae* (serotype 14) for 3 h. Relative *NFKBIA* expression ($2^{-\Delta\Delta Ct}$ method) was measured in PBMC cDNA by quantitative PCR. Values represent mean ± SEM of homozygotes ($n = 11$) and heterozygotes ($n = 13$). Statistical analysis was performed using the nonparametric Mann–Whitney test. (**E**) IκBα protein expression was determined by Western blot in PBMCs stimulated with LPS. Values represent mean fold change (ACC/ACC versus ACC/GTT) with 95% CIs ($n = 4$).

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pression of *NFKBIA* compared with individuals who were heterozygous for the promoter variants following stimulation with *S. pneumoniae* ($p = 0.024$) and a 1.4-fold greater expression following stimulation with LPS ($p = 0.032$) (Fig. 3C, 3D).

Finally, we confirmed these differences at the protein level. IκBα protein expression at baseline and following LPS stimulation was significantly higher in individuals homozygous for the ACC haplotype of *NFKBIA* compared with individuals who were heterozygous for the promoter variants ($p < 0.05$, Fig. 3E). Because individuals homozygous for the GTT haplotype represent only 3% of the population, we were unable to analyze this rare population.

IκBα-dependent innate immune responses vary significantly among individuals with different NFKBIA promoter variants

Having demonstrated that the haplotype comprising the GTT minor promoter variants was associated with reductions in *NFKBIA* gene expression and IκBα protein expression, we continued to examine the functional impact of *NFKBIA* promoter variants on innate immune responsiveness. Cord blood mononuclear cells from ACC/GTT heterozygous neonates produced significantly more TNF-α than homozygous neonates following activation of TLRs (Fig. 4A–D). To determine whether the heightened TLR responsiveness associated with the *NFKBIA* GTT haplotype was specific to IκBα-dependent signaling pathways, rather than a more global process affecting inflammatory responses, we also measured IFN-α production following stimulation of mononuclear cells from the same individuals with CpG type A (a TLR9 agonist). TLR9 uses an IκBα-independent pathway to produce IFN-α (39, 40), and the response to CpG type A was not influenced by the *NFKBIA* promoter haplotype (Fig. 4E).

Systems biology validation of NFKBIA as a candidate gene in asthma, RSV infection, and BPD

To validate *NFKBIA* as a candidate gene in the pathogenesis of asthma, RSV bronchiolitis, and BPD, we used an in silico systems biology approach to map the major innate immune regulatory networks active in each condition using public microarray datasets for patients infected with RSV [GSE17156 (41)], patients with asthma [GSE15823 (42)], and at-risk premature infants who developed BPD [GSE8586 (43)] (Supplemental Table I). Network analysis of the cellular transcriptional responses revealed statistically significant subnetworks containing *NFKBIA* as a node (Fig. 5, Supplemental Figs. 1–3). *NFKBIA* was situated as a prominent mid-to-large–sized hub within each of the three networks (Fig. 5), establishing *NFKBIA* as a biologically plausible candidate gene that may be associated with different outcomes in RSV infection, asthma, and BPD.

Functional NFKBIA promoter variants are associated with differences in susceptibility to AHR, RSV bronchiolitis, and BPD

Informed by the pattern of LD (Fig. 1), we conducted case-control analysis to determine whether rs2233409 and rs2233406, the two functionally active genotypes that are not in complete LD, are differentially represented among subjects at risk for each childhood lung disease. The minor rs2233406 allele was associated with an increased risk for severe RSV bronchiolitis requiring hospitalization (OR = 1.83; 95% confidence interval [CI] = 1.20–2.80, $p = 0.005$) (Tables III, IV). In analyzing the asthma cohort, we focused on quantification of AHR by methacholine challenge, because this test is reliable, not influenced by variations in symptom perception or diagnostic trends, and closely related to the underlying pathophysiology of asthma. There was no significant

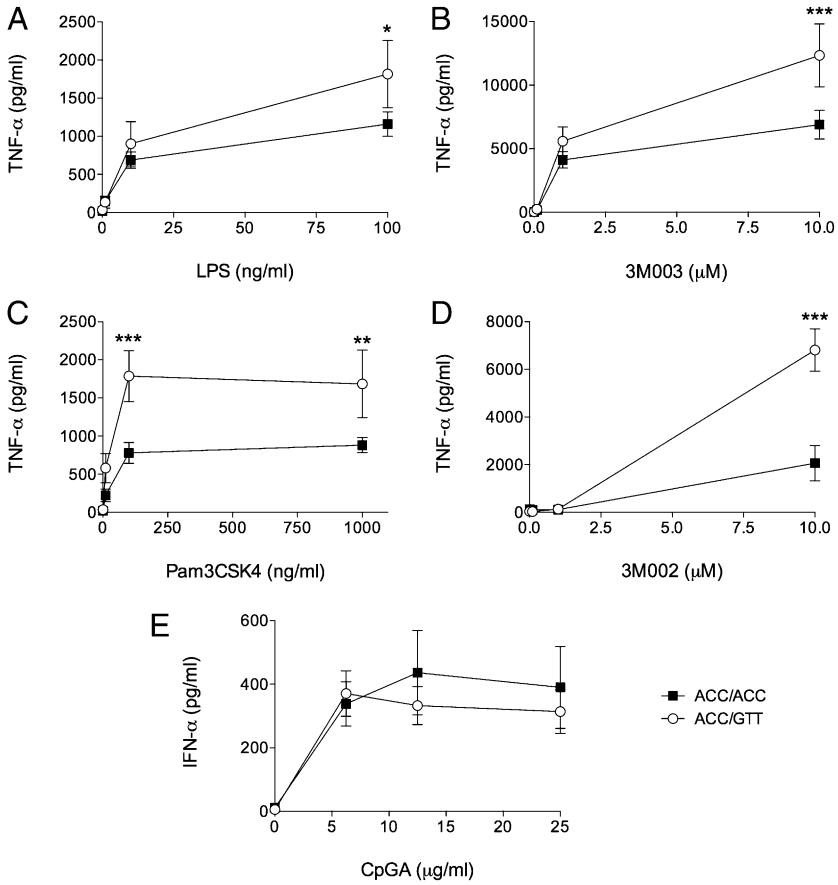

FIGURE 4. IκBα-dependent innate immune responses are significantly modulated by *NFKBIA* promoter variants. (**A**–**D**) Production of TNF-α by mononuclear cells stimulated with IκBα-dependent TLR ligands. (**E**) Production of IFN-α by the same mononuclear cells stimulated with CpG type A triggering IκBα-independent TLR9 pathways. Values represent mean ± SEM of heterozygotes (n = 5–7) and homozygotes (n = 5–10). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two-way ANOVA with the Bonferroni posttest.

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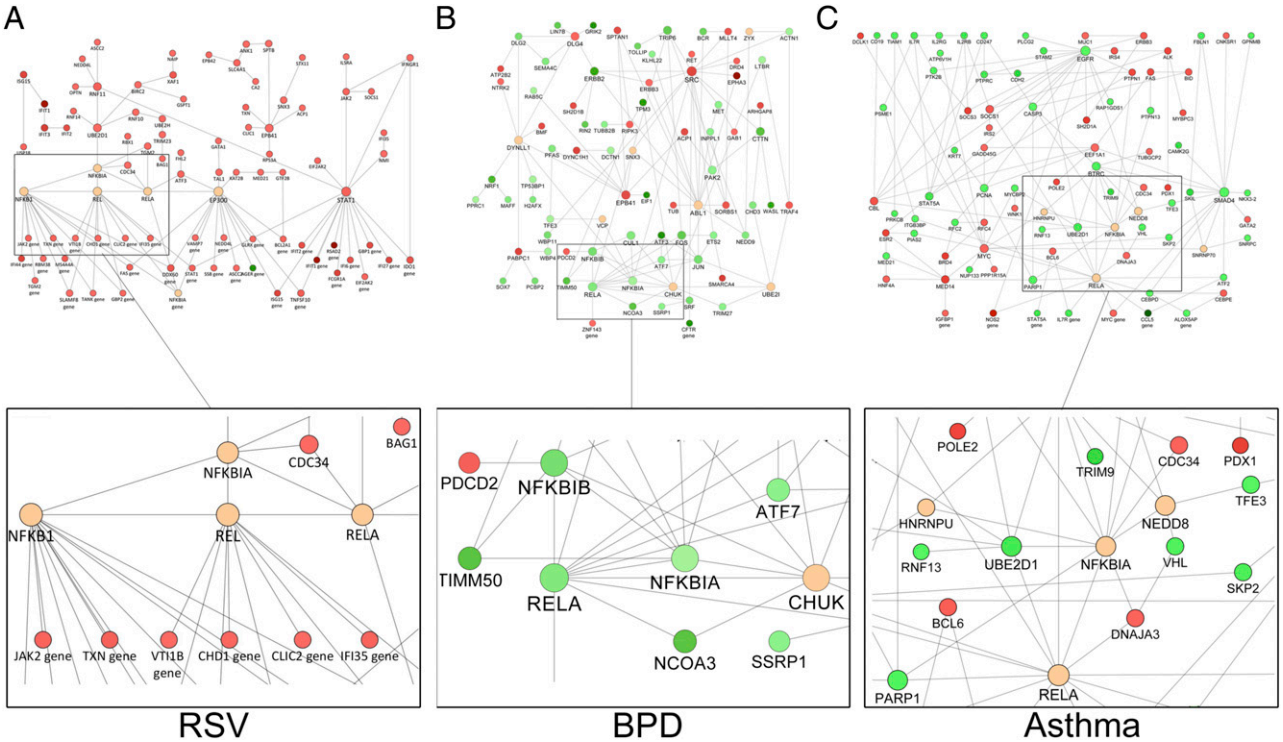

FIGURE 5. Network analysis reveals that *NFKBIA* is a key component of cellular transcriptional signatures during RSV infection (**A**), BPD (**B**), and asthma (**C**). Networks generated for RSV infection, BPD, or asthma were analyzed using jActive to determine the most statistically significant subnetworks within the broader networks. The highlighted regions demonstrate that *NFKBIA* is a statistically significant hub for each of the three disease datasets (shown in full detail as Supplemental Figs. 1–3), as determined by its interconnectivity within the subnetwork. Data used to generate these networks were available to the public (described in Supplemental Table I) and obtained through NCBI Gene Expression Omnibus.

association when AHR was considered in isolation (rs2233409: OR = 1.49, 95% CI = 0.95–2.34, $p = 0.081$). However, when early childhood viral exposures were included in the analysis, significant associations were revealed. Specifically, the minor allele at position rs2233409 was associated with AHR in children with PCR-documented RSV infection (OR = 2.55, 95% CI = 1.26–5.17, $p = 0.009$) or PIV infection (OR = 2.24, 95% CI = 1.12–4.48, $p = 0.023$) in the first 12 mo of life. Additionally, for RSV infection, there was evidence for interaction between rs2233409 and RSV increasing the risk for AHR ($p = 0.037$). There were no significant observations for picornaviruses (Table V). Genotyping of rs2233406 failed rigorous quality control and was not analyzed in the asthma cohort. Finally, the minor alleles at positions rs2233406 (OR = 0.13, 95% CI = 0.022–0.78, $p = 0.026$) and rs2233409 (OR = 8.28, 95% CI = 1.32–51.9, $p = 0.024$) were significantly associated with BPD severity but not prematurity ($p = 0.174$) in preterm infants (Table VI). Together, these data provide compelling evidence for a functional impact of *NFKBIA* promoter variants in influencing the outcome of infectious and inflammatory lung diseases in children.

Discussion

In this study we report three major novel findings: common genetic variants in the promoter of *NFKBIA* (rs3138053, rs2233406, and rs2233409) influence *NFKBIA* promoter function, gene and IκBα protein expression, and TLR-mediated inflammatory responses; *NFKBIA*/IκBα is a central hub in networked cellular transcriptional responses in RSV infection, asthma, and BPD; and immunologically relevant genetic variation in the promoter of *NFKBIA* is associated with differential susceptibility to severe RSV bronchiolitis, AHR, and severe BPD. We consider these associations particularly compelling given our functional immunological data combined with literature evidence from in vitro animal and human primary immunodeficiency studies that all support a vital role for IκBα in regulating inflammatory responses (4, 5).

The multiple published associations linking promoter variants in *NFKBIA* to alterations in susceptibility to malignancy and infectious and inflammatory diseases (Table I) were the motivation for investigating the functional immunological impact of these polymorphisms. Considering both previously published associations and the pattern of LD within the genetic region (Fig. 1), we focused our experimental attention on three *NFKBIA* promoter polymorphisms that were in strong LD ($r^2 > +0.80$): rs3138053, rs2233406, and rs2233409. We confirmed experimentally that these variants are indeed associated with alterations in promoter-driven protein expression, allele-specific and total *NFKBIA* gene expression, and IκBα protein expression (Figs. 2, 3).

To assess the functional immunological impact of the *NFKBIA* promoter polymorphisms, we quantified TLR-mediated innate immune responsiveness (Fig. 4). By carefully selecting TLR ligands and cytokine read-outs, we were able to analyze the impact of *NFKBIA* promoter polymorphisms on both IκBα-dependent and -independent TLR signaling in the same responder cell population. Consistent with the current understanding of TLR signaling cascades, the functional *NFKBIA* promoter polymorphisms were associated with differences in TLR-triggered

Table III. Demographic data for RSV-association study

	Female Gender Frequency	Median Age
Population control	0.51	Newborn
Severe RSV	0.42	5 mo (1 wk–18 y)

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Table IV. Functional *NFKBIA* promoter polymorphisms are associated with susceptibility to severe RSV infection

	rs2233406						rs2233409					
	CC (*n*)	CT/TT (*n*)	MAF	Risk Allele	*p* Value	OR (95% CI)	CC (*n*)	CT/TT (*n*)	MAF	Risk Allele	*p* Value	OR (95% CI)
Population control	186	110	0.22	T	**0.005**	**1.83** (1.20–2.80)	199	97	0.18	—	0.367	1.24 (0.80–1.91)
Severe RSV	60	65	0.30				78	47	0.20			

Text in bold is statistically significant.
—, No risk-associated allele; MAF, minor allele frequency.

TNF-α secretion, which is known to be negatively regulated by IκBα (44). Importantly, by taking advantage of the fact that TLR9 signaling stimulates IFN-α secretion via an IκBα-independent pathway (39, 40), we demonstrated that *NFKBIA* promoter polymorphisms only influence IκBα-dependent responses rather than altering global inflammatory signaling. Intriguingly, our results are consistent with data from an entirely different study design examining measles vaccine immunity; the minor allele *NFKBIA* variants at rs3138053, rs2233409, and rs1050851 were all associated with increased measles-specific TNF-α and IFN-α secretion by PBMCs following in vitro restimulation (45).

By investigating the impact of *NFKBIA* promoter polymorphisms at multiple molecular levels, we are in a position to develop a biologically coherent model of how *NFKBIA* promoter polymorphisms may influence innate immune responsiveness. At the level of promoter function and gene transcription, the *NFKBIA* transcript driven by the common promoter haplotype (ACC) was expressed at ~25% higher levels than the transcript associated with the minor promoter haplotype (GTT) (Figs. 2, 3). Because of these allele-specific differences in mRNA expression, the stimulated monocytes of individuals homozygous for the haplotype comprising the common promoter variants (i.e., ACC/ACC) expressed *NFKBIA* mRNA and IκBα protein at ~1.5-fold higher levels than individuals who were heterozygous for the promoter variants (ACC/GTT) (Fig. 3). Because they expressed higher levels of the gene encoding the major negative regulator of NF-κB, the proinflammatory responses of monocytes from individuals homozygous for the common *NFKBIA* promoter haplotype (i.e., ACC/ACC) were decreased compared with cells from those with the GTT haplotype comprising the minor promoter variants (i.e., ACC/GTT) (Fig. 4).

The expression level of most genes is regulated by transcription factors that bind to DNA regulatory sequences situated upstream of the site at which transcription is initiated. Polymorphisms located in promoter regions usually affect gene transcription by modifying the coordinated action of multiple regulatory proteins through complex protein–DNA and protein–protein interactions, thus influencing the kinetics and/or specificity of the transcription process. This explanation is consistent with the decrease in *NFKBIA* transcription that we observed to be associated with the minor promoter variants.

Increasing evidence supports the concept that a balanced NF-κB–driven inflammatory response determines the outcome following infection. Greatly diminished NF-κB responsiveness, as occurs in rare human primary immunodeficiencies caused by genetic mutations affecting NF-κB activation, results in recurrent, severe infections. At the other end of the spectrum, excessive inflammation can also be very harmful, with clinical data suggesting a pathologic role for NF-κB in sepsis and multiple-organ failure (46, 47). Similarly, our data indicate that *NFKBIA* promoter variants associated with increased TLR-mediated inflammatory responses are associated with severe RSV bronchiolitis and AHR in children with positive nasal swabs for RSV or PIV in the first year of life (Table V).

Innate immune hyperresponsiveness to RSV appears to play an important role in the pathogenesis of severe bronchiolitis (48). Our observations linking hyperinflammatory *NFKBIA* polymorphisms and severe RSV bronchiolitis are consistent with the published association between increased susceptibility to RSV-induced bronchiolitis and genetic variants mediating increased IL-8 transcription (49). In addition to causing acute bronchiolitis, RSV has strong epidemiological links to asthma and AHR. Children who experience severe RSV-induced bronchiolitis are at increased risk for the development of recurrent wheeze and asthma in later childhood (50). However, this association is bidirectional, because an asthmatic disposition and early wheezing also increase the risk for severe lower respiratory tract infections and RSV hospitalization (51). The bidirectional nature of this association indicates that severe RSV bronchiolitis and asthma may share a common genetic predisposition and/or environmental exposure. Our data suggest that genetic variants in the *NFKBIA* promoter associated with enhanced innate immune responsiveness may be one common genetic component that increases the risk for both severe RSV infection and AHR.

BPD is a serious chronic inflammatory lung disease frequently observed in premature infants (52). Children suffering from BPD have a dramatically increased risk for severe RSV infection, because up to 50% of preterm infants with BPD require hospitalization due to RSV in the first year of life (53, 54). Recently, our group (36) and other investigators (55) confirmed the strong contribution of complex polygenic influence on BPD susceptibility. In the current study, we found that functional genetic variation in the promoter of *NFKBIA* is associated with differential susceptibility to severe BPD but not premature birth (Table VI). However, the *NFKBIA* promoter variants that we investigated do not appear to contribute to the increased risk for severe RSV in-

Table V. Functional *NFKBIA* promoter polymorphisms are associated with AHR

					Main Effect of SNP			Main Effect of Virus		Interaction		
SNP	Major	Minor	MAF	Virus	OR (95% CI)	*p* Value	PP Value	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value	PP Value
rs2233409	C	T	0.209	RSV	2.55 (1.26–5.17)	**0.009**	**0.005**	1.14 (0.60–2.17)	0.692	0.37 (0.15–0.94)	**0.037**	**0.030**
				PIV	2.24 (1.12–4.48)	**0.023**	**0.016**	1.38 (0.71–2.68)	0.337	0.47 (0.19–1.17)	0.106	N/A
				Picornavirus	1.62 (0.73–3.58)	0.235	N/A	1.08 (0.55–2.12)	0.830	0.88 (0.33–2.31)	0.793	N/A

Text in bold is statistically significant.
MAF, minor allele frequency; PP, permutation *p* value.

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Table VI. Functional *NFKBIA* promoter polymorphisms are associated with susceptibility to moderate and severe BPD but not with prematurity

	rs2233406						rs2233409					
	CC (*n*)	CT/TT (*n*)	MAF	Risk Allele	*p* Value	OR (95% CI)	CC (*n*)	CT/TT (*n*)	MAF	Risk Allele	*p* Value	OR (95% CI)
Term neonates	98	80	0.27	—	0.507	0.85 (0.55–1.32)	105	73	0.23	—	0.174	0.72 (0.46–1.12)
Preterm neonates	92	64	0.24				104	52	0.19			
Preterm with no BPD	20	15	0.29	C	**0.026**	0.13 (0.022–0.78)	20	15	0.27	T	**0.024**	8.28 (1.32–51.9)
Moderate and severe BPD	47	29	0.22				58	18	0.13			

Text in bold is statistically significant.
—, No risk-associated allele; MAF, minor allele frequency.

fection experienced by premature infants with BPD, because the common allele at rs2233406 (i.e., C) was associated with an increased risk for severe BPD but was protective in the RSV cohort.

Infectious diseases are arguably one of the most influential selective pressures on the course of human evolution and have given rise to strong selective pressure for polymorphisms that decrease susceptibility to pathogens, regardless of their impact on the overall fitness of the host. For example, a polymorphism in the promoter of *TNF* confers both resistance to infection with tuberculosis and susceptibility to systemic lupus erythematosus, rheumatoid arthritis, and primary Sjögren's syndrome (56). Consistent with this paradigm, the *NFKBIA* promoter SNPs that we demonstrate to be associated with inflammatory lung diseases in children were reported to decrease susceptibility to pathogens (Table I), including *S. pneumoniae* (37), a leading infectious cause of death worldwide. Despite their potentially advantageous nature in the context of infection, SNPs that increase innate immune responsiveness may ultimately increase population level prevalence of other diseases with an inflammatory component, adding support to the concept of innate immunity as a double-edged sword.

Our functional immunological data and system biology analyses highlight the importance of *NFKBIA* in relevant signaling pathways and provide a biologically coherent model of how *NFKBIA* promoter polymorphisms may impact innate immune signaling in these childhood lung diseases. It is important to recognize that the genetic variants we are investigating are SNPs that are common in healthy populations, and they are not disabling mutations. Although the functional immunological changes we report are small in magnitude when compared with the changes found in humans with disabling genetic mutations, they are likely to be biologically relevant. Our data demonstrate that genetic variants in the promoter of *NFKBIA* influence *NFKBIA* gene expression, IκBα protein expression, and TLR-mediated inflammatory responses. When these functional immunological data are considered in the context of the multiple genetic association studies linking *NFKBIA* promoter polymorphisms with human disease (Tables I, III–VI), the weight of evidence indicates a biologically significant impact of these common *NFKBIA* promoter polymorphisms. However, our results must be interpreted with some caution given that the associations between the *NFKBIA* polymorphisms and the clinical phenotypes were of a modest magnitude. Ultimately, the genetic validity of these associations will only be firmly established through future replication studies in additional human cohorts.

In conclusion, we elucidated the functional immunological impact of common genetic variants in the promoter of *NFKBIA* that have been repeatedly associated with differences in susceptibility to cancer and infectious and inflammatory diseases. We built on these mechanistic in vitro insights to generate novel in vivo data showing that these functional variants in *NFKBIA* alter susceptibility to childhood AHR, RSV bronchiolitis, and BPD. These results provide new insights into the pathogenesis of childhood infectious and inflammatory lung diseases; strengthen our collective understanding of the importance of inhibitors of innate immunity, such as *NFKBIA*; and begin to unravel common aspects in the genetic predisposition to BPD, severe RSV bronchiolitis, and childhood asthma.

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ONLINE SUPPLEMENTAL MATERIAL

Functional genetic variation in *NFKBIA* and susceptibility to childhood asthma, bronchiolitis and bronchopulmonary dysplasia

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Running title: Functional genetic variation in NFKBIA and susceptibility to disease.

‡‡ Aaron Hirschfeld and Salman Ali made an equal contribution.

FIGURE LEGEND

Supplementary Figure S1: Network analysis visualization of PBMC transcriptional responses to infection with RSV.

Network analysis carried out on genes differentially expressed in PBMC during *in vivo* experimental infection of volunteers with RSV. This statistically significant subnetwork contains 89 gene nodes and 104 unique protein level interactions, with *NFKBIA* positioned as mid-sized hub with 6 unique interactions. Color of nodes is proportional to their relative fold-change in infected versus control groups, while the size of the node reflects its interconnectivity (hub degree) within the network. Data set derived from GEO accession GSE17156.

Supplementary Figure S2: Systems biology visualization of immune pathways activated in BPD.

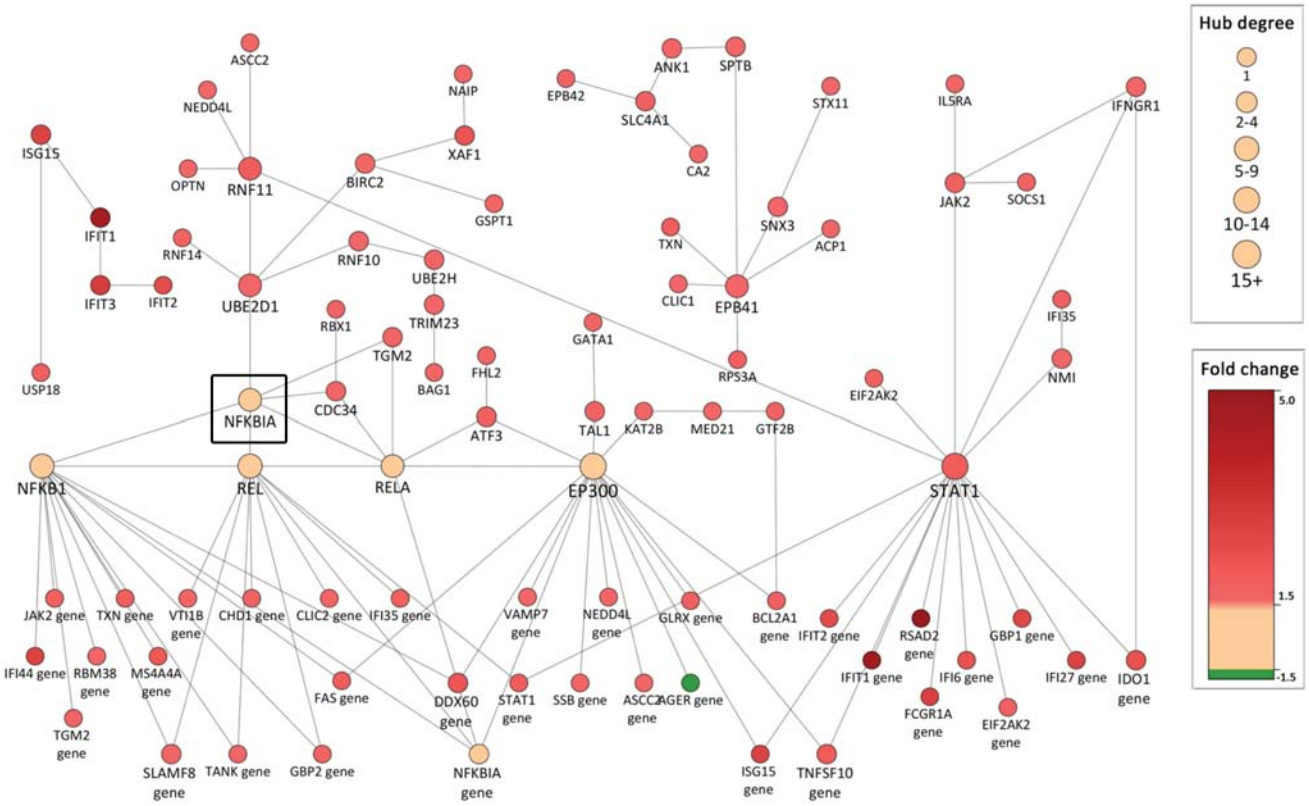
Network analysis of genes differentially expressed in cord blood of preterm neonates who developed BPD. This statistically significant subnetwork contains 87 gene nodes and 130 unique protein level interactions, with a downregulated *NFKBIA* positioned as mid-sized hub with 7 unique interactions. Color of nodes is proportional to their relative fold-change in biopsies from healthy patients, while the size of the node reflects its interconnectivity (hub degree) within the network. Data set derived from GEO accession 17916252.

Supplementary Figure S3: Network analysis visualization of transcriptional responses unique to the airways of asthmatic patients.

Network analysis of genes differentially expressed in lung biopsies of asthmatic patients. This statistically significant subnetwork contains 89 gene nodes and 146 unique protein level interactions, with *NFKBIA* positioned as large hub with 11 unique interactions. Color of nodes is proportional to their relative fold-change in biopsies from healthy patients, while the size of the node reflects its interconnectivity (hub degree) within the network. Data set derived from GEO accession 15038835.

Supplementary Table S1: Microarray datasets identified as containing suitable data subsets for network analysis of transcriptional responses and processed using MetaGEX.

GEO accession[1]	Associated publications [PMID]	Experimental description	Data subset used in analysis
GSE17156	19664979	Gene expression profiling of PBMCs from healthy volunteers (n = 60) at baseline, and again after intranasal challenge with human rhinovirus, respiratory syncytial virus (RSV), or influenza A. Blood was collected every 6-8 hours, and only the post-infection sample correlating with each patient's peak symptoms was analyzed.	Healthy volunteers challenged with intranasal RSV (n = 20), with comparisons between patient-matched pre- and post- infection data.
GSE8586	17916252	Umbilical cord blood was collected at birth from premature neonates (24-27 weeks gestational age, n = 54). Whole blood was sent for transcriptional profiling and neonates were followed until 36 weeks postmenstrual age.	From the patient sample (n=54), comparisons were carried out between those who developed BPD (n=20) and those who did not (n=34).
GSE15823	15038835	Comparison of transcriptional signatures from bronchial biopsies obtained from healthy adult subjects (n=4) or patients with allergic asthma, before (n=4) and after (n=4) initiation of inhaled corticosteroid therapy. Biopsies (7-11 per subject) were obtained from segmental bronchial carinae and lobar carinae throughout the lower, middle, and upper lung lobes, pooled, and analyzed.	Healthy adult subjects (n=4) compared to subjects with allergic asthma (n=4) before initiation of inhaled corticosteroid therapy.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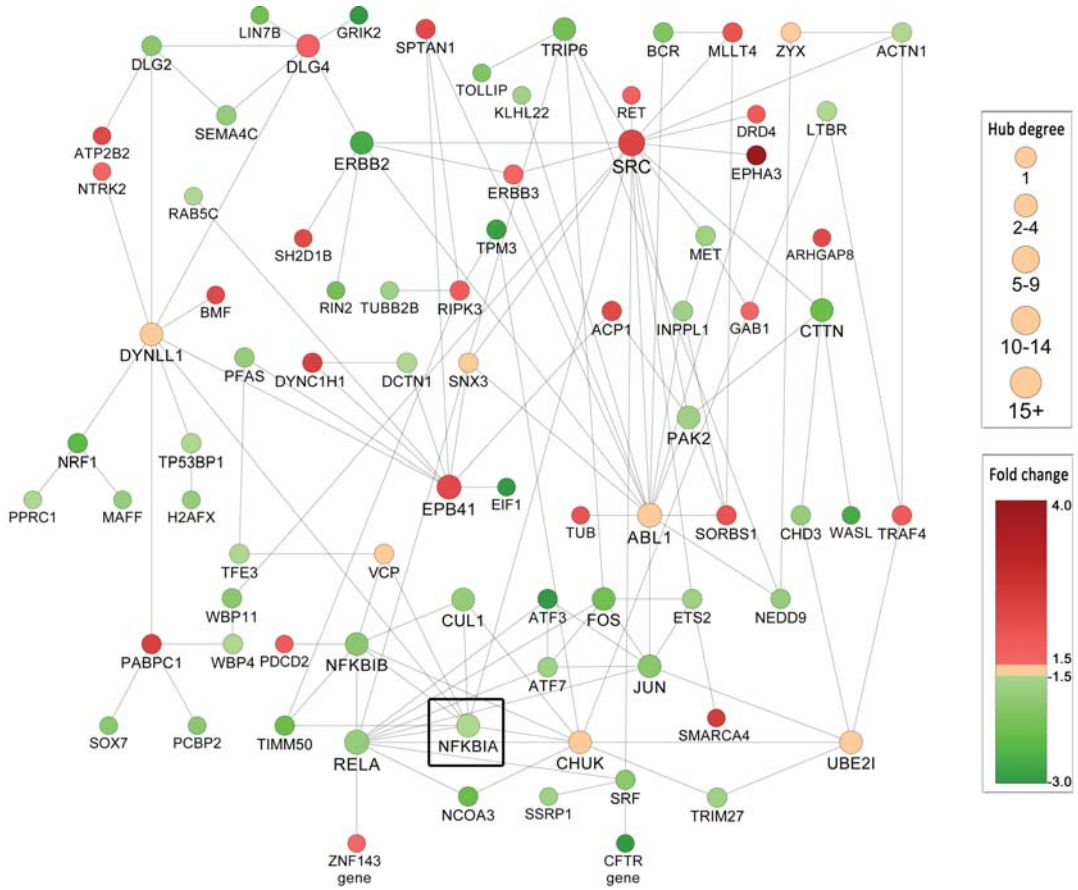
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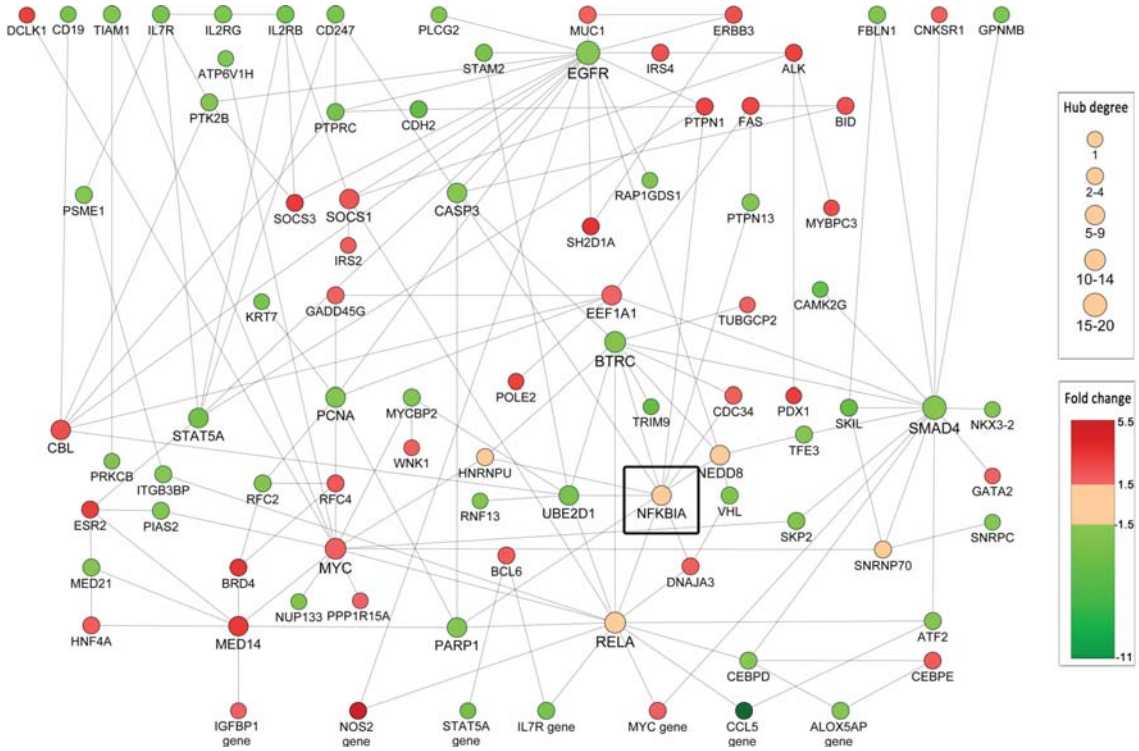
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