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


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

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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 IkBa, the major negative regulator of NF-kB. 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, IkBa 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, IkBa protein expression, and TLR-mediated inflammatory responses. Using a systems biology approach, we demonstrated that NFKBIA/IkBa 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: 3949–3958.

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.
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% CO₂ atmosphere for 3 h poststimulus before harvesting mRNA with an additional DNAase 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 (r² > 0.82) with the promoter SNPs (rs3138053, rs2233406, and rs2233409), acting as a “tag” for differentiating between the major (ACC) and minor (GTT) allele transcriptions 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.

### 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.ncbi.nlm.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 functional assay

Primers were designed (Table II), and expression of NFKBIA was calculated relative to ACTB by SYBR GreenER chemistry (Life Technologies).

#### 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), PAM3CSK4 (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 InNeteDB (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-
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.

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

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

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

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**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 l < 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 GEMATa 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 variable 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 viral exposure as the predictor variable. Significant interactions were defined as p values ≤ 0.05, a permutation procedure was applied in which the case and control groups were randomly permuted, and an interaction term was calculated for each permutation. The empirical p value was calculated as the number of permutations that produced a larger interaction term than the observed interaction term. This analysis was conducted using the R statistical software program (36)

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**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 r2 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.
control values were randomly shuffled 10,000 times, establishing an empirical p value.

**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 (r² = 1), and analysis of the European population revealed a four-variant haplotype consisting of rs2233409 in strong LD (r² = 0.82) with rs3138053 and rs2233406, as well as a fourth variant rs11569591 (an 8-bp insertion/deletion structural variant) that displayed LD (r² = 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 (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

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Accession</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
<th>Region</th>
<th>Product (bp)</th>
<th>Temp (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer sequences for promoter cloning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NFKBIA</strong></td>
<td>NG_007571.1</td>
<td>GGGCGGCGAGGATGGGACTA</td>
<td>GCGGCAGCCCTATGAAACGCT</td>
<td>g.3905-4972</td>
<td>1068</td>
<td>60</td>
</tr>
<tr>
<td>Primer sequences for gene expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACTB</strong></td>
<td>NM_001110.3</td>
<td>GTTCGGTTCACACCTCTCTT</td>
<td>ACCCTCACGGGTTCCAGTTT</td>
<td>c.*16-162</td>
<td>147</td>
<td>60</td>
</tr>
<tr>
<td><strong>NFKBIA</strong></td>
<td>NM_020529</td>
<td>TCAACAGGAGTACTGACCCAGGT</td>
<td>TCTCTGTAACCTCGCCTAAGCT</td>
<td>c.728-902</td>
<td>175</td>
<td>60</td>
</tr>
<tr>
<td>Primer and probe sequences for custom SNP assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NFKBIA rs1050851</strong></td>
<td>Primers</td>
<td>AAGTGATCCGCCAGGTGAAGGCTGCAGGTTGGAAGT</td>
<td>GCTGAGGCTGTTGTGAGAAGTTGACTGGACTA</td>
<td>c.275-334</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Probe (VIC)</td>
<td>ACCCTGCGGTCCCTCA</td>
<td>ACCCTGCGGGTCCCTCA</td>
<td>ACCCTGCGGTCCCTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe (FAM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Underlined nucleotide represents SNP in the probe sequence.

Temp, Temperature.
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 (r² > +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-
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. IKBa 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.

**IKBa-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 IKBa 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 IKBa-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 IKBa-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

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**FIGURE 4.** IKBa-dependent innate immune responses are significantly modulated by NFKBIA promoter variants. (A–D) Production of TNF-α by mononuclear cells stimulated with IKBa-dependent TLR ligands. (E) Production of IFN-α by the same mononuclear cells stimulated with CpG type A triggering IKBa-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.
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 IkBα protein expression, and TLR-mediated inflammatory responses; *NFKBIA*/IkBα 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 IkBα 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 IkBα 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 IkBα-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**

<table>
<thead>
<tr>
<th>Female Gender Frequency</th>
<th>Median Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population control 0.51</td>
<td>Newborn</td>
</tr>
<tr>
<td>Severe RSV 0.42</td>
<td>5 mo (1 wk–18 y)</td>
</tr>
</tbody>
</table>
The common promoter variants (i.e., ACC/ACC) expressed monocytes of individuals homozygous for the haplotype comprising with the minor promoter haplotype (GTT) (Figs. 2, 3). Because of the major negative regulator of NF-κB (43). Because they expressed higher levels of the gene encoding the minor promoter variants.

Increasing evidence supports the concept that a balanced NF-κB secretory activity is known to be negatively regulated by IkBα (44). Importantly, by taking advantage of the fact that TLR9 signaling stimulates IFN-α secretion via an IkBα-independent pathway (39, 40), we demonstrated that NFKBIA promoter polymorphisms only influence IkBα-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 IkBα 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 those from the GGT 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 did not appear to contribute to the increased risk for severe RSV infection.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Main Effect of SNP</th>
<th>Main Effect of Virus</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2233409</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>0.209</td>
<td></td>
</tr>
<tr>
<td>MAF</td>
<td>OR (95% CI)</td>
<td>p Value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>RSV</td>
<td>2.55 (1.26–5.17)</td>
<td>0.009</td>
<td>1.14 (0.60–2.17)</td>
</tr>
<tr>
<td>PIV</td>
<td>2.24 (1.12–4.48)</td>
<td>0.023</td>
<td>1.38 (0.71–2.68)</td>
</tr>
<tr>
<td>Picornavirus</td>
<td>1.62 (0.73–3.58)</td>
<td>0.235</td>
<td>1.08 (0.55–2.12)</td>
</tr>
</tbody>
</table>

Text in bold is statistically significant.

MAF, minor allele frequency; PP, permutation p value.
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.

Acknowledgments

We thank all subjects and their families who volunteered to participate in the patient cohorts.

Disclosures

The authors have no financial conflicts of interest.

References


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

<table>
<thead>
<tr>
<th>Allele</th>
<th>CC (n)</th>
<th>CT/TT (n)</th>
<th>MAF</th>
<th>Risk</th>
<th>p Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2233406</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>98</td>
<td>80</td>
<td>0.27</td>
<td></td>
<td>0.507</td>
<td>0.85 (0.55–1.32)</td>
</tr>
<tr>
<td>Preterm</td>
<td>92</td>
<td>64</td>
<td>0.24</td>
<td></td>
<td>1.052</td>
<td>0.39 (0.19–0.79)</td>
</tr>
<tr>
<td>Moderate and severe BPD</td>
<td>47</td>
<td>29</td>
<td>0.22</td>
<td>C</td>
<td>0.026</td>
<td>0.13 (0.022–0.78)</td>
</tr>
</tbody>
</table>

Text in bold is statistically significant.

—, No risk-associated allele; MAF, minor allele frequency.
functions in the innate immune system, especially RelA and cRel. The NF-kB family of transcription factors includes p50, RelA, and cRel, which are essential for regulating a wide range of biological processes, including inflammatory responses, cell survival, and tumor suppression.


directions involving MyD88 and TRIF pathways. The role of NF-kB in the activation of immune responses is evident in the expression of pro-inflammatory genes, such as TNF-α and IL-6. NF-kB activation is a critical event in the pathogenesis of many inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple myeloma.


to the expression of IL-2. It is suggested that TNF-α subtype 1 (TNF-α1) may be involved in the pathogenesis of rheumatoid arthritis and other inflammatory conditions.


to the development of chronic lung disease after premature birth. The study by Burg et al. (2007) provides evidence for the association between genetic polymorphisms and outcomes related to bronchopulmonary dysplasia.


to virus-induced type 1 IFN expression. The study by Corbett et al. (2007) highlights the role of NF-kB in subunit expression and its implications for viral infections.


to the regulation of NF-kB activity. The study by Li et al. (2006) underscores the importance of understanding the mechanisms that modulate NF-kB activation in different contexts.


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