Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

NKG2D gene variation and susceptibility to viral bronchiolitis in childhood

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BACKGROUND: Genetic factors associated with bronchiolitis are inadequately characterized. We therefore inspected a selected subpopulation of our previous genome-wide association study (GWAS) of bronchiolitis for overlap with known quantitative trait loci (QTLs) to identify susceptibility loci that potentially affect mRNA and protein levels.

METHODS: GWAS included a Finnish–Swedish case–control population ($n = 187$), matched for age and site. We integrated GWAS variants ($p < 10^{-4}$) with QTL data. We subsequently verified allele-specific expression of identified QTLs by flow cytometry. Association of the resulting candidate loci with bronchiolitis was tested in three additional cohorts from Finland and Denmark ($n = 1201$).

RESULTS: Bronchiolitis-susceptibility variant rs10772271 resided within QTLs previously associated with *NKG2D* (NK group 2, member D) mRNA and protein levels. Flow cytometric analysis confirmed the association with protein level in NK cells. The GWAS susceptibility allele (A) of rs10772271 (odds ratio [OR] = 2.34) corresponded with decreased NKG2D expression. The allele was nominally associated with bronchiolitis in one Finnish replicate (OR = 1.50), and the other showed directional consistency (OR = 1.43). No association was detected in Danish population

CONCLUSIONS: The bronchiolitis GWAS susceptibility allele was linked to decreased NKG2D expression in the QTL data and in our expression analysis. We propose that reduced *NKG2D* expression predisposes infants to severe bronchiolitis.

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INTRODUCTION

Bronchiolitis is a common lower respiratory infection (LRI) that affects infants and young children worldwide.[1] The most common etiological agent is respiratory syncytial virus (RSV), which infects almost every child by the age of 2 years.[2] Other viruses causing bronchiolitis include rhinovirus (RV), parainfluenza virus, and metapneumovirus.[1] The prevalence of different viruses varies by geographical location, and seasonal and annual variations occur. Although most infants undergo mild disease, up to 3% of infants of an age cohort suffer from bronchiolitis that requires hospital admission. Severe viral bronchiolitis in infancy is considered a risk factor for asthma.[3]

Young age, congenital heart disease, and prematurity are among the most important predisposing factors for severe bronchiolitis.[1] However, most children admitted to hospital with bronchiolitis were born full term without known risk factors. Neonatal bronchial hyperresponsiveness may explain such exaggerated response to RSV and other common respiratory viruses, like RV, whereas genetic susceptibility is another likely factor that could explain the variation in bronchiolitis phenotypes observed in otherwise healthy infants.[1, 4] Identifying infants with a high genetic risk of severe bronchiolitis could allow targeting the available treatments, such as passive immunization by monoclonal antibody Palivizumab.[5] Some infants with RV-induced wheezing respond to corticosteroids, and genetic markers are potential tools to help distinguish subgroups of children who could benefit from different treatments.[6] Prevention of severe virus-induced bronchiolitis can have significant short- and long-term benefits for respiratory health. In addition, characterization of variants contributing to bronchiolitis susceptibility will likely help achieve better understanding of the disease process and ultimately enable identification of new therapeutic strategies.

In previous hypothesis-driven studies, genes related to various aspects of the immune response were associated with variations in bronchiolitis susceptibility. Reported associations include polymorphisms in interleukins, toll-like receptors, surfactant genes, and *CDHR3* locus.[7–10] Some associations, including for variants in vitamin D Receptor (*VDR*), surfactant protein D (*SFTPD*), and toll-like receptor 4 (*TLR4*), were reproduced in further studies, and subsequent functional studies elucidated the molecular mechanisms for some of the reported associating loci.[2, 11] However, most of the reported associations have not been

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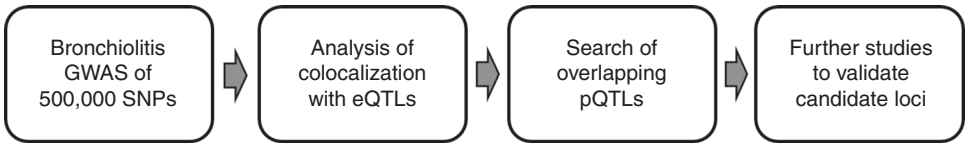


Fig. 1 Matched case–control population of 187 Finnish and Swedish infants genotyped with Illumina whole-genome genotyping array and tested for association with bronchiolitis. Variants with $p < 10^{-4}$ were assessed for overlap with known quantitative trait loci (QTLs). Expression QTLs (eQTLs) affecting variations in mRNA levels were screened from Genotype-Tissue Expression (GTEx) v6p data.[18] eQTLs harboring the defined GWAS variants were further examined for colocalization with protein QTLs (pQTLs) in the pGWAS server.[19] Variants with overlap to both types of QTLs were studied further. In subsequent analyses, protein expression was analyzed with flow cytometry to verify pQTL, and additional cohorts were tested to replicate the genetic association with bronchiolitis

replicated in subsequent studies, or the results between studies have varied. Thus, genetic factors playing a role in bronchiolitis susceptibility remain inadequately characterized.

Hypothesis-free genome-wide examinations facilitate the identification of loci associated with complex traits and generate hypotheses for ensuing studies.[12] Noncoding variants that affect the regulatory events of the genome appear to be the main drivers of complex traits.[13] More than 80% of genetic associations with common diseases occur outside of the protein-coding genome.[14] One possibility is that these variants affect disease risk by modifying gene expression. Therefore, integrating genome-wide association study (GWAS) signals and quantitative trait loci affecting gene expression (eQTLs) is one approach to interpreting noncoding disease-associated variants.

We recently conducted a GWAS of bronchiolitis in a population of 217 children with bronchiolitis and 778 controls, providing a hypothesis-free examination of bronchiolitis susceptibility.[15] We detected several loci that were suggestively associated ($p < 10^{-5}$ to 10^{-7}) with bronchiolitis. In the current study, our objective was to analyze an age-, site-, and gender-matched subset of the case–control population included in the GWAS by looking for colocalization with known eQTLs associated with mRNA levels. The GWAS subset comprised two cohorts with similar virus, age, and sex distributions, and the cohorts were genotyped on a same bead chip. To increase the likelihood of identifying functional bronchiolitis-susceptibility loci, we further inspected the identified colocalized GWAS–eQTL regions for overlap with QTLs associated with protein expression levels (pQTLs).

MATERIALS AND METHODS

Design

Figure 1 presents the outline of the study. The starting point of the study was a hypothesis-generating GWAS designed to discover variants associated with severe childhood bronchiolitis. Next, we analyzed GWAS signals with $p < 10^{-4}$ for overlap with known QTLs to identify functional bronchiolitis-associated variants that may affect gene or protein expression levels of nearby genes. eQTLs have been shown to be significantly enriched for loci detected through GWAS.[16] Functional criteria may be applied to identify trait-associated loci that do not reach genome-wide significance.[17] Thus, mining our GWAS data at a lower significance level when comparing it with overlapping functional elements such as QTLs could reveal additional genetic determinants of bronchiolitis susceptibility. Therefore, we chose variants with $p < 10^{-4}$ for QTL analysis. eQTLs associated with mRNA levels were screened from Genotype-Tissue Expression (GTEx) v6p data.[18] The primary tissues explored were whole blood, lung, and lymphocytes, due to biomarker potential or importance in bronchiolitis pathophysiology. Associations of loci with protein levels (pQTLs) were queried in the pGWAS server, which contains a GWAS of protein levels in blood plasma.[19] GWAS loci with overlap to QTLs associated with both gene and protein expression were studied further using flow cytometry to verify identified QTLs. Finally, we studied replication populations to validate genetic associations with bronchiolitis.

Study populations in genetic analyses

Altogether, the study cohorts comprised 358 bronchiolitis cases and 1030 controls, presented in Supplementary Table S1. Populations included a Finnish–Swedish GWAS ($n = 187$), two Finnish replication case sets ($n = 124$ and 101), and Danish replication samples ($n = 976$). Finnish replication cohorts were tested against the GWAS controls/Finnish samples from the 1000 Genomes Project data ($n = 99$).[20] Bronchiolitis was defined as an acute LRI with typical respiratory symptoms of bronchiolitis, including wheezing, labored breathing, chest retractions, and auscultative crackle.[21] RSV infection was tested by PCR of nasopharyngeal aspirates, antigen immunofluorescent or radio-immuno assays, or by serum antibody assays.

The GWAS population was collected in the areas of Kuopio, Finland, and Gothenburg, Sweden (93 cases and 94 controls). Study infants originated from prospective cohorts described previously.[22–24] Cases included in genetic analyses were hospitalized with doctor-diagnosed bronchiolitis, <24 months of age, full term, and of native Finnish or Swedish ethnicity. Healthy controls were collected and matched for age (±2 months), gender, and site. The Finnish replication samples were acquired from cohorts collected in Tampere ($n = 124$) and Turku ($n = 101$) regions.[3, 6, 25] All cases were healthy full-term infants diagnosed with bronchiolitis at <12 months of age. The study subjects from Denmark originated from two longitudinal birth cohorts (COPSAC2000 and COPSAC2010) containing 40 infants diagnosed with bronchiolitis at <12 months of age and 936 control children without bronchiolitis.[4, 26]

DNA extraction and genotyping

DNA was isolated following standard protocols.[10, 15] GWAS samples were genotyped in Technology Centre of the Institute for Molecular Medicine Finland (FIMM), University of Helsinki as described earlier.[15] Samples with >3% missing genotypes, and SNPs with a minor allele frequency (MAF) of <10%, a genotyping rate of <90%, or deviation from Hardy–Weinberg equilibrium (HWE; $p < 1 \times 10^{-3}$) were excluded. The SNPs rs10772271 and rs1049174 near *NKG2D* locus were genotyped in replication populations from Finland and Denmark. In the Finnish samples, genotypes of rs10772271 and rs1049174 were determined by Sanger sequencing and by RFLP genotyping with the DdeI restriction enzyme (New England Biolabs, Ipswich, MA), respectively. For the Danish cohorts, genotypes were acquired from Illumina Infinium HumanOmniExpressExome Bead chip. AROS Applied Biotechnology AS center, Aarhus, did the genotyping. Genotyping rate was >95% in all replicates and the cohorts were in HWE. Another variant of the *NKG2D* locus, rs1049174, was absent in the Finnish–Swedish GWAS data after quality control. The SNP genotypes were resolved by RFLP genotyping.

Flow cytometric analysis of NKG2D expression in NK cells

We performed flow cytometric analysis to verify the reported association between major alleles of rs10772271 and rs1049174, and lower levels of NKG2D protein expression in the relevant

Table 1. Overlap of bronchiolitis GWAS signals and significant eQTLs in data from the Genotype-Tissue Expression (GTEx) Project

Association test				eQTL information		
Chr: SNP	Gene[a]	A1	OR[b] (95% CI)	Gene	Effect size	Tissue
1: rs12730489	*WLS*	G	2.40 (1.58–3.65) †	*WLS*	–0.19†	Whole blood
10: rs303527	*LIPM*	G	2.94 (1.71–5.03) †	*ANKRD22*	0.43*	Skin—sun exposed
				LIPN	–0.42*	Lung
				LIPN	–0.31*	Whole blood
				ANKRD22	0.44**	Skin—not sun exposed
				ANKRD22	0.27†	Esophagus mucosa
12: rs10772271	*NKG2D*	A	2.34 (1.52–3.61) †	*NKG2E*	–0.25*	Whole blood
				NKG2D	–0.36**	Muscle, skeletal
				NKG2D	–0.34**	Adipose, subcutaneous
				NKG2C	0.33**	Cells—transformed fibroblasts
				NKG2C	0.37†	Stomach
				NKG2F	–0.30†	Adipose, subcutaneous

GWAS genome-wide association study, *eQTL* expression quantitative trait locus, *Chr* chromosome, *SNP* single-nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval
[a]Corresponding locus shown for variants within genes; the nearest characterized locus shown for intergenic SNPs (GRCh37/hg19 coordinates)
[b]OR for A1 allele in basic case–control association analysis (Chi-square allelic test)
*$p \le 10^{-8}$; **$p \le 10^{-6}$; †$p \le 10^{-4}$

target tissue. We isolated leukocytes from peripheral blood samples of 33 healthy Finnish volunteers as follows: red blood cells (RBCs) were sedimented with 3% dextran for 45 min; the nonsedimented fraction, which contains leukocytes, was collected for centrifugation; the residual RBCs were lysed with hypotonic NaCl solution. Leukocyte surface markers were identified using monoclonal mouse anti-human antibodies that are specified in Supplementary Table S2. Gating strategy for NK cells is presented in Supplementary Figure S1. Lymphocytes ($n = 250{,}000$/sample) were collected based on the lymphocyte gate and used for flow cytometric analysis with BD FACS Canto II (Becton, Dickinson and Company, Franklin Lakes, NJ). NKG2D expression was defined by mean fluorescence intensity (MFI) as a numerical value and assessed by genotypes of rs10772271 (AA, $n = 17$; AG, $n = 12$; GG, $n = 4$) and rs1049174 (CC, $n = 20$; CG, $n = 9$; GG, $n = 4$). Values were normalized by subtracting the MFI of the isotype control from the CD314-specific (NKG2D) MFI with FlowJo software (FlowJo LLC, Ashland, OR).

Statistical analyses

Genetic association tests were conducted using chi-square allelic test with PLINK 1.9.[27] Haplotype-level association tests were carried out with Haploview.[28] Pathway analysis was done with gene set analysis-SNP (GSA-SNP).[29] Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were queried. We tested normal distribution of MFI values from flow cytometry with Kolmogorov–Smirnov test, and assessed normality of standardized residuals with Shapiro–Wilk test and normal P-P plots using SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23. Armonk, NY: IBM Corp.). Associations between *NKG2D* genotypes and protein expression (i.e., MFI values) were analyzed by linear regression with PLINK 1.9. Box plots that represent NKG2D expression by genotypes of rs10772271 and rs1049174 were drawn with R.[30]

Ethics

The ethics committees of the participating centers approved the study. Informed consent was obtained from the participants or their guardians.

RESULTS

SNPs within eQTLs

The GWAS evaluated 511,132 variants for associations with bronchiolitis. The genomic inflation factor lambda was 1.01. We identified 36 SNPs with $p < 10^{-4}$ (Supplementary Table S3). To identify loci that may affect the expression of nearby genes, we screened these SNPs for overlap with GTEx eQTLs. Three SNPs were located within eQTLs in whole blood and lung tissue: the identified loci were associated with expression levels of genes encoding NKG2-E type II integral membrane protein (NKG2E), protein wntless homolog (WLS), and lipase member N (LIPN). We then explored whether these variants affect the expression of other genes in different GTEx tissues. Table 1 contains the association statistics and significant eQTL results for these loci. The eQTLs were associated with expression levels of *WLS, ANKRD22, LIPN*, and four genes of the *NKG2* locus (*NKG2E, NKG2D, NKG2C, and NKG2F*).

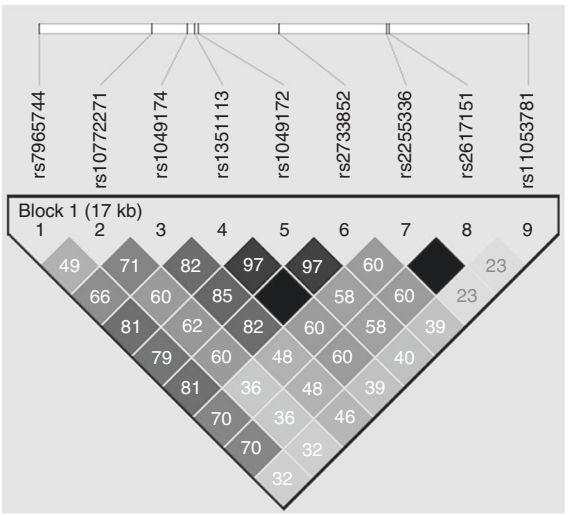
eQTL variants that overlap protein QTLs

Loci that harbored both significant GTEx eQTLs and previously defined association signals were further analyzed for colocalization with pQTLs in the pGWAS server. The NKG2D locus, encoded by *KLRK1* (killer cell lectin like receptor K1), overlapped with pQTL, eQTL, and GWAS signal. Variant rs10772271, among the eQTLs for genes in the NKG2 region, resided within a haploblock associated with NKG2D protein levels ($p = 7.79 \times 10^{-9}$, $\beta = -0.26$).

Association between *NKG2D* locus and bronchiolitis

NKG2D is an activating transmembrane receptor whose MHC class I-like ligands are expressed in response to stress, including viral infection.[31] When we investigated the genetic architecture of the *NKG2D* locus, we found that rs10772271 resided in the same haploblock as functional polymorphism rs1049174, located in the 3′ untranslated region of *NKG2D*. The correlation of rs10772271 with rs1049174 was 80% in the Finnish samples from the 1000 Genomes Project. Rs1049174 is an eQTL associated with *NKG2D* expression in multiple tissues and the variant also resides within the QTL associated with NKG2D protein levels ($p = 9.94 \times 10^{-12}$, $\beta = -0.33$). In the GWAS, the major allele (A) of rs10772271 was the bronchiolitis-susceptibility allele (odds ratio [OR] = 2.34, 95% CI:

a



b

Haplotypes

rs7965744	rs10772271	rs1049174	rs1351113	rs1049172	rs2733852	rs2255336	rs2617151	rs11053781	Frequency cases	Frequency controls	*p* value
A	A	C	G	A	A	G	G	A	0.565	0.391	8.0E-4
C	G	G	A	G	C	A	A	G	0.161	0.175	0.718
A	A	C	G	A	A	G	G	G	0.167	0.146	0.589
A	G	C	G	A	A	G	G	A	0.032	0.077	0.057
C	G	G	A	G	C	G	G	G	0.022	0.080	0.010
A	G	G	A	G	C	G	G	G	0.022	0.048	0.166
A	G	G	G	A	A	G	G	G	0.016	0.043	0.129
A	G	C	G	A	A	G	G	G	0.005	0.029	0.078

Fig. 2 Linkage disequilibrium (LD) plot for variants within *NKG2D* locus and haplotype-based association analysis. **a** LD plot spanning study variants rs10772271 and rs1049174 in the *NKG2D* locus was generated with Haploview using the genotyped Finnish–Swedish data. Pairwise correlation measures (R^2) shown in the plot. Correlation of SNPs rs10772271 and rs1049174 was 0.71 in Finnish–Swedish data. **b** The most common haplotype including major alleles of variants rs10772271 and rs1049174 showed an association with bronchiolitis ($p = 8 \times 10^{-4}$). This haplotype is consistent with a previously described LNK haplotype associated with low natural cytotoxic activity of NK cells[32]

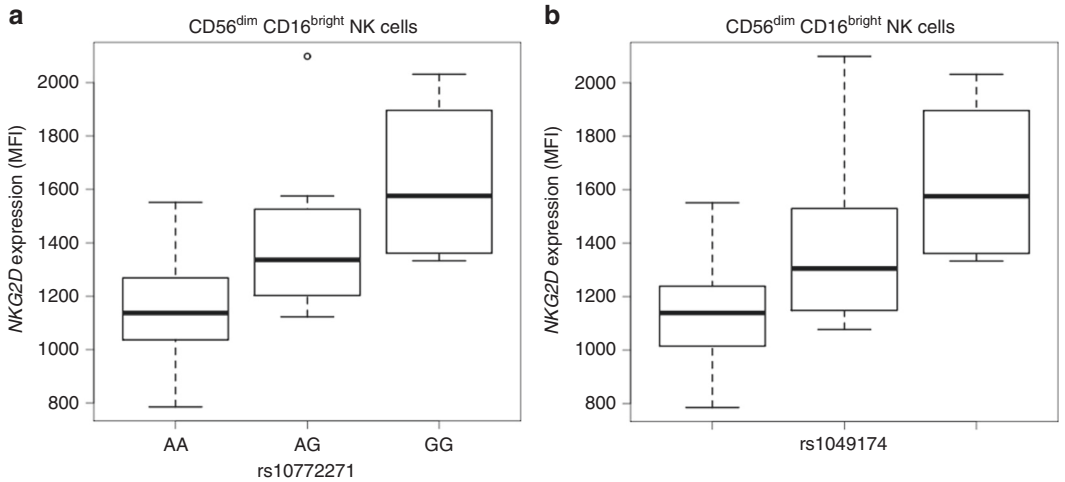


Fig. 3 NKG2D expression in cytotoxic NK cell population is affected by *NKG2D* genotypes. **a** NKG2D protein expression was measured as mean fluorescence intensity (MFI) in flow cytometry, and association with *NKG2D* variants was tested with linear regression. Variation of the data is presented as interquartile ranges (IQRs) around the median. Bronchiolitis-susceptibility allele (A) of rs10772271 was associated with decreased NKG2D expression ($p = 6.0 \times 10^{-4}$, β (standardized beta coefficient from linear regression) $= -0.57$). Medians for MFI values per genotype were 1138.80 (AA), 1304.90 (AG), and 1575.20 (GG). **b** Major allele of rs1049174 corresponded with decreased NKG2D .expression ($p = 4.3 \times 10^{-4}$, β (standardized beta coefficient from linear regression) $= -0.58$). MFI medians for each genotype were 1137.15 (CC), 1336.60 (CG), and 1575.20 (GG)

1.52–3.61, $p = 9.9 \times 10^{-5}$). Variant rs1049174 showed a milder association with bronchiolitis (OR = 1.8, 95% CI: 1.14–2.83, $p = 0.011$). However, the allele frequency difference between cases and controls was notable (>10%). The association was tested at the haplotype level; Fig. 2 shows a linkage disequilibrium (LD) plot with R^2 correlations and association statistics for different haplotypes spanning the variants under study. The most common haplotype, including the major alleles of the variants rs10772271 and rs1049174, showed association with bronchiolitis ($p = 8.0 \times 10^{-4}$). The haplotype was consistent with a low NK activity level (LNK) haplotype that has been associated with decreased NKG2D expression.[32] In association analyses restricted to RSV-positive or -negative bronchiolitis samples, the allele frequency differences between cases and controls were similar compared to the association test of the whole population.

Pathway analysis

We conducted pathway analysis to explore whether our GWAS data were connected to biological processes that would support the role of NKG2D signaling in bronchiolitis. One of the significant KEGG pathways involving NKG2D was hsa04650 (NK cell-mediated cytotoxicity; corrected $p = 0.0012$, false discovery rate = 0). Significant GO terms included immune response-activating cell surface receptor signaling pathway ($p = 0.0031$), positive regulation of NK cell-mediated immunity ($p = 0.0039$), and immune response-activating signal transduction ($p = 0.0081$).

Table 2. *NKG2D* allele frequency differences and association statistics in discovery data and replication cohorts

	rs10772271-A			rs1049174-C		
Population	Frequency, cases	Frequency, controls	OR (95% CI)	Frequency, cases	Frequency, controls	OR (95% CI)
GWAS Kuopio–Gothenburg	0.731	0.537	2.34 (1.52–3.61)**	0.769	0.649	1.80 (1.14–2.83)*
Finnish replication cohorts						
Tampere vs. GWAS controls	0.635	0.537	1.50 (1.00–2.24)*	0.662	0.649	1.06 (0.71–1.60)†
Tampere vs. 1000 G FINs	0.635	0.576	1.28 (0.86–1.91)†	0.662	0.616	1.22 (0.82–1.82)†
Turku vs GWAS controls	0.609	0.537	1.43 (0.90–2.01)†	0.654	0.649	1.02 (0.67–1.55)†
Turku vs 1000 G FINs	0.609	0.576	1.15 (0.77–1.71)†	0.654	0.616	1.18 (0.78–1.77)†
Danish replication cohorts						
$COPSAC_{2000/2010}$	0.563	0.610	0.82 (0.52–1.29)†	0.738	0.719	1.10 (0.66–1.83)†

*$p \le 10^{-4}$; **$p \le 0.05$; $^{\dagger}p$ = NS
OR odds ratio, *CI* confidence interval, *GWAS* genome-wide association study, *1000G FINs* Finnish samples from the 1000 Genomes Project

Association of *NKG2D* genotypes rs10772271 and rs1049174 with NKG2D expression

We performed flow cytometric experiments to study the reported association between *NKG2D* variants and NKG2D protein expression. NKG2D expression was analyzed in NK cells extracted from healthy individuals with different *NKG2D* genotypes. Distinct NK cell populations can be identified and further functionally subdivided by the expression of CD56 (an adhesion molecule) and CD16 (low-affinity Fc-receptor for IgG) surface markers. NK cells expressing less CD56 and more CD16 are mostly cytotoxic NK cells with a higher content of perforin and granzymes, whereas higher expression of CD56 combined with low or absent CD16 expression has been associated with immunoregulatory functions mediated by cytokines such as interferon-γ, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α.[33, 34] Three clear NK cell populations, $CD56^{bright}CD16^{-}$ and $CD56^{bright}CD16^{dim}$ (immunoregulatory), and $CD56^{dim}CD16^{bright}$(cytotoxic), were identified and analyzed for NKG2D expression. The results reinforced the association between *NKG2D* genotypes and NKG2D expression. Variants rs10772271 and rs1049174 were significantly associated with NKG2D expression in the primary cytotoxic $CD56^{dim}CD16^{bright}$ NK cell population (Fig. 3) and in two $CD56^{bright}$ populations representing immunoregulatory NK cells (Supplementary Figure S2).

Association between *NKG2D* locus and bronchiolitis in additional cohorts

We tested the association of the *NKG2D* genotypes with bronchiolitis in two additional Finnish cohorts and in one Danish cohort. Finnish cohorts originating from Tampere and Turku were tested against the GWAS control infants. The association for rs10772271 was nominally replicated in the Tampere cohort (OR = 1.50, 95% CI: 1.00–2.24, $p = 0.049$). In the Turku cohort, there was a similar but nonsignificant allele frequency difference (OR = 1.43). The cohorts were also tested against the Finnish samples from the 1000 Genomes Project. The allele frequency differences were not significant, but the effect showed directional consistency. This was also evident when the replication populations were pooled and tested against the GWAS controls (OR = 1.42, 95% CI: 1.00–2.01, $p = 0.05$). There was no association in the Danish replication population. Association statistics for all cohorts are shown in Table 2.

DISCUSSION

Recent clinical studies suggest that there are promising treatments of wheezing and bronchiolitis induced by common respiratory viruses.[5, 6] Targeting these treatments to appropriate subgroups of infants at high risk of severe LRI will likely promote short- and long-term respiratory outcomes of these individuals. Genetic studies aiming to resolve bronchiolitis-associated variants will likely, among clinical, immunological, and other studies, provide tools to distinguish such risk groups and further help understand disease mechanisms. In addition to more individualized treatment strategies, understanding the biological and genetic basis of bronchiolitis susceptibility may help develop new therapeutic options.

In the current study, we combined hypothesis-free and hypothesis-driven approaches by integrating bronchiolitis GWAS signals and the overlapping QTLs. This combined approach pinpointed the *NKG2D* locus, where the GWAS susceptibility locus coincided with QTLs associated with decreased NKG2D expression at the mRNA and protein levels. Thus, we hypothesize that reduced NKG2D expression predisposes infants to bronchiolitis and that the decrease in expression may be modulated by the *NKG2D* genotype.

NKG2D is an activating transmembrane receptor expressed by NK cells and subsets of T cells in humans.[31] NKG2D signaling is involved in cancer immunosurveillance, defense against viral infection, and autoimmune disease. Due to their pivotal roles in both adaptive and innate immune responses NKG2D and its ligands have been recognized as potential therapeutic targets.[31, 35] Moreover, many tumor cells and viruses have evolved means to escape detection by NKG2D, which suggests that promoting NKG2D-dependent activation could be beneficial.

A few studies have shown that RSV infection activates the immune system via the NKG2D receptor. Introduction of RSV infection to human dendritic cells in vitro caused upregulation of NKG2D ligand and led to NK cell proliferation and IFN-γ production.[36] Another study made similar observation in NK cells from the lungs of BALB/c mice.[37] However, in contrast with our study, it was suggested that high NKG2D expression and subsequent IFN-γ production is involved in lung immune injury during early stages of RSV infection. Another study with NKG2D-deficient mice observed a normal inflammatory response against RSV infection.[38] This is unsurprising, since the role of NKG2D seems to depend upon specific molecular environment at the time of infection. Further, different NKG2D ligands may activate parallel or opposite downstream pathways depending on the context.[39] It would be worthwhile to examine whether bronchiolitis due to RSV infection triggers the expression of specific ligands and to study the detailed course of the respective downstream signaling events.

Several viruses other than RSV may cause pediatric bronchiolitis. While it is possible that different viral infections activate the same pathways, it is probable that the engaged ligands differ. All populations included in our study represented overall bronchiolitis caused by any virus. A limitation was the different proportion of RSV-positive and -negative bronchiolitis cases between the study sets. For some of the RSV negative bronchiolitis cases, causal virus was unknown. This may in part be due to differences in virus detection methods before and after 90s. Nonetheless, when we restricted the association analyses to RSV-positive and -negative bronchiolitis populations within the Finnish cohorts, the direction of allele frequency differences for rs10772271 between cases and controls remained similar compared to the overall analysis. However, the sample sizes in the restricted analysis were too small to draw any firm conclusions on bronchiolitis subtypes. One limitation in the current study was the age heterogeneity of patient populations between the discovery population and the replication cohorts from different centers.

The bronchiolitis GWAS susceptibility variant rs10772271 resided in the same haploblock with a functional polymorphism rs1049174, with 70% pairwise correlation in our data. Rs1049174 has been recognized as an eQTL affecting NKG2D expression in several tissues, and recently the underlying molecular mechanism was elucidated. Rs1049174 alleles have differing affinity for microRNA mir-1245 that downregulates NKG2D expression.[40] In our flow cytometer experiments, the studied *NKG2D* genotypes affected NKG2D expression in both primary cytotoxic cells and in immunoregulatory (CD56bright) NK cells; this finding suggests that *NKG2D* genotypes may play a role in diverse downstream events. However, it should be acknowledged that the functions of NK cells display more heterogeneity than described by this dichotomy. Upon activation, NK cells expressing high levels of CD56 can become cytotoxic toward autologous activated CD4+ T cells, and NK cells expressing high levels of CD16 can be considered immunoregulatory, as they produce large quantities of chemokines and cytokines after activation.[34]

We did not detect genome-wide significant association signals, which was the major limitation in our GWAS. Therefore, we chose a higher *p* value threshold of 10^{-4} for functional characterizations of the GWAS variants. For *NKG2D* variant, the observed allele frequency differences between cases and controls were large. *NKG2D* was also involved in bronchiolitis-associated pathways and the association was seen in one of the replication populations. However, *NKG2D* variants were not associated with bronchiolitis in the two other replication sets with a smaller number of cases. Therefore, the association between *NKG2D* locus and bronchiolitis should be examined in a larger sample size. Other limitations in our study were differences in causal viruses and varying ages of patients among the study cohorts. In the next step, genetic associations should be studied in case–control cohorts with a more uniform clinical definition with regard to the upper age-limit of bronchiolitis. Also, associations between genetic variants and bronchiolitis should be studied in virus-specific cohorts to increase the power to detect an association, and to discover potential virus-dependent effects. Based on such virus-specific association analyses, relationship of specific causal viruses and NKG2D signaling could be studied in appropriate functional settings. There is reasonable evidence that the studied *NKG2D* genotypes influence *NKG2D* gene and protein expression, based on both previous studies and our current study. In the next step, NKG2D expression could be studied in samples collected from hospitalized bronchiolitis patients together with appropriate controls to test whether *NKG2D* genotypes affect NKG2D expression during the disease. Finally, studies of NKG2D expression using samples from bronchiolitis patients could be conducted to test whether NKG2D expression correlates with bronchiolitis severity.

In conclusion, our study addressed the potential importance of the *NKG2D* locus in bronchiolitis. Our results suggest that decreased NKG2D expression may predispose infants to bronchiolitis. These findings need to be confirmed in a larger sample size and further assessed in virus-specific bronchiolitis populations.

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ADDITIONAL INFORMATION

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