Gene-based analysis of regulatory variants identifies 4 putative novel asthma risk genes related to nucleotide synthesis and signaling

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Background: Hundreds of genetic variants are thought to contribute to variation in asthma risk by modulating gene expression. Methods that increase the power of genome-wide association studies (GWASs) to identify risk-associated variants are needed.

Objective: We sought to develop a method that aggregates the evidence for association with disease risk across expression quantitative trait loci (eQTLs) of a gene and use this approach to identify asthma risk genes.

Methods: We developed a gene-based test and software package called EUGENE that (1) is applicable to GWAS summary statistics; (2) considers both *cis*- and *trans*-eQTLs; (3) incorporates eQTLs identified in different tissues; and (4) uses simulations to account for multiple testing. We applied this approach to 2 published asthma GWASs (combined n = 46,044) and used mouse studies to provide initial functional insights into 2 genes with novel genetic associations.

Results: We tested the association between asthma and 17,190 genes that were found to have *cis*- and/or *trans*-eQTLs across 16 published eQTL studies. At an empirical FDR of 5%, 48 genes were associated with asthma risk. Of these, for 37, the association was driven by eQTLs located in established risk loci for allergic disease, including 6 genes not previously implicated in disease cause (eg, *LIMS1*, *TINF2*, and *SAFB*). The remaining 11 significant genes represent potential novel genetic associations with asthma. The association with 4 of these replicated in an independent GWAS: *B4GALT3*, *USMG5*, *P2RY13*, and *P2RY14*, which are genes involved in nucleotide synthesis or nucleotide-dependent cell activation. In mouse studies, P2ry13 and P2ry14—purinergic receptors activated by adenosine 5-diphosphate and UDP-sugars, respectively—were upregulated after allergen challenge, notably in airway epithelial cells, eosinophils, and neutrophils. Intranasal exposure with receptor agonists induced the release of IL-33 and subsequent eosinophil infiltration into the lungs.

Conclusion: We identified novel associations between asthma and eQTLs for 4 genes related to nucleotide synthesis/signaling and demonstrated the power of gene-based analyses of GWASs.

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Asthma is a highly polygenic disease with potentially hundreds or thousands of risk variants, with small effects contributing to variation in disease risk.[1] A small number of risk-associated variants has been identified through genome-wide association studies (GWASs), but the majority remain to be mapped. Identifying risk-associated variants is important because these could point to genes that were not previously suspected to be involved in disease pathophysiology[2,3] or that could represent drug targets with greater probability of clinical success.[4,5]

Several approaches have been proposed to increase the power of GWASs to identify variants with a modest but reproducible association with disease risk. These include larger sample sizes, analysis of more refined phenotypes,[6,7] multivariate association analysis of related phenotypes,[8] gene-based association analyses,[9,10] and association analyses restricted to functional

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Abbreviations used
ADP: Adenosine 5-diphosphate
AEC: Airway epithelial cell
ATP: Adenosine 5-triphosphate
BALF: Bronchoalveolar lavage fluid
eQTL: Expression quantitative trait locus
FDR: False discovery rate
GWAS: Genome-wide association study
HDM: House dust mite
LD: Linkage disequilibrium
SNP: Single nucleotide polymorphism
UDP: Uridine-diphosphoglucose (UDP-glucose)

variants, such as those that regulate gene expression levels.[11] The aim of this study was to develop a method that combined the 2 latter approaches and apply it to results from a published asthma GWAS to help identify new genes with expression associated with genotype and related to disease risk.

Specifically, we hypothesized that if the expression of a gene is causally related to asthma and gene expression is regulated by multiple independent expression quantitative trait loci (eQTLs), a gene-based approach that captures the aggregate signals from these eQTLs would be expected to improve power over the alternative approach of testing each variant individually. Recently, Gamazon et al[12] described a gene-based association method based on the same concept, called PrediXcan. Briefly, this approach includes 3 steps. First, for a given gene, eQTLs are identified from transcriptome data sets. Second, a model that can be used to predict gene expression levels based on the aggregate effect of those eQTLs is trained on a reference transcriptome data set. Third, this model is used to infer expression levels for a target GWAS data set that includes subjects genotyped for those eQTLs but for whom actual gene expression levels might not be available. The genetically inferred gene expression levels can then by tested for association with the phenotype of interest (eg, asthma).

As highlighted by Gamazon et al,[12] PrediXcan has several advantages over other gene-based tests, such as VEGAS.[9] However, in our view it has 1 major limitation: unlike VEGAS, it is not applicable to GWAS summary statistics, which are typically more readily available, and therefore can be applied to a larger sample size than available GWAS data sets with individual-level genetic data. The TWAS approach developed by Gusev et al[13] addresses this caveat but in its current release is applicable only to a relatively small number of genes (4,284 from 2 blood eQTL studies), *cis*- but not *trans*-acting eQTLs (eg, those located >1 Mb from the target gene), and to a single reference transcriptome data set at a time.

In this study we developed a gene-based association approach, called EUGENE, that combines the biological focus of PrediXcan and TWAS and the versatility of VEGAS. Our approach also considers eQTL evidence across different tissues and estimates empirical false discovery rates (FDRs) while accounting for the linkage disequilibrium (LD) between variants. We applied this new approach to a published asthma GWAS[14] to try to identify novel genes with a genetic component of gene expression associated with asthma risk. Finally, we investigated whether results from mouse models of experimental acute allergic asthma are consistent with a contribution of 2 selected genes to disease pathophysiology.

METHODS

EUGENE approach

The proposed gene-based approach is described in detail in the Methods section in this article's Online Repository at www.jacionline.org. Briefly, for a given gene, our approach includes 4 steps. First, we identify a set of variants that influence gene expression in any cell type or tissue relevant to the disease or trait of interest based on results from published eQTL studies. Including eQTLs identified in tissues not thought to be relevant for the disease of interest might improve power, but this is something we did not consider in our study. We include in this list eQTLs located in *cis* (≤1 Mb from the target gene) or *trans* (>1 Mb away or in a different chromosome). This list is then reduced to a subset of eQTLs with an LD r^2 value of less than 0.1; we refer to these as "independent eQTLs" for a given gene (see Fig E1 in this article's Online Repository at www.jacionline.org).

Second, we extract association results for these independent eQTLs from a disease or trait GWAS of interest and then calculate the gene-based statistic Q as the sum of the 1-df χ^2 values for the individual eQTLs. This represents the aggregate evidence for association in that GWAS across the independent eQTLs of that gene.

Third, we perform simulations using individual-level genetic data to estimate the statistical significance of Q while accounting for the residual LD between eQTLs.

Fourth, FDR thresholds are also estimated empirically to account for multiple testing. Simulations show that the type 1 error rate of EUGENE is close to the nominal expectation (see Table E1 in this article's Online Repository at www.jacionline.org). The software and input files required to run EUGENE are freely available at https://genepi.qimr.edu.au/staff/manuelF.

Application of EUGENE to published GWASs of asthma

We applied EUGENE to a published asthma GWAS[14] to illustrate the utility of the proposed approach. This GWAS included 6,685 patients with both asthma and hay fever and 14,091 asthma- and hay fever–free control subjects, all of European descent, tested for association with 4.9 million single nucleotide polymorphisms (SNPs) with a minor allele frequency of greater than 1%. In the original analysis of individual SNPs, 11 independent variants were found to be associated with disease risk at a genome-wide significance level of a P value of less than 3×10^{-8}. We used EUGENE to identify genes with an association with disease risk in the study by Ferreira et al[14] at an empirical FDR of 0.05 (corresponding to a P value threshold of 1.9×10^{-4}). At this FDR level, 5% of genes considered significant (ie, $P < 1.9 \times 10^{-4}$) are expected to be false-positive associations due to multiple testing.

To confirm putative novel associations, we then applied EUGENE to an independent asthma GWAS, the GABRIEL study,[15] for which summary statistics are publicly available. After excluding overlapping samples (the Busselton study), results from the GABRIEL study were based on 9,967 asthmatic patients and 15,301 control subjects.

Predicted direction of effect of gene expression on asthma risk

EUGENE can be used to identify a set of genes with expression levels determined by eQTLs and for which the eQTLs are collectively associated with disease risk. However, unlike PrediXcan[12] or TWAS,[13] EUGENE does not directly provide the predicted direction of effect of gene expression on disease risk. To understand whether a genetically determined increase in gene expression levels was predicted to increase or decrease disease risk, we compared the direction of effect of each eQTL on gene expression reported on the transcriptome GWAS with the effect on asthma risk reported in the Ferreira et al[14] asthma GWAS. Based on this information, for each eQTL, we report whether the allele associated with increased gene expression is associated with an increased or decreased asthma risk.

Functional studies in the mouse

We selected 2 putative novel asthma risk genes, *P2RY13* and *P2RY14*, for preliminary functional studies in the mouse. The criteria used to select these genes for functional follow-up were as follows: (1) significant gene-based association with asthma in the discovery GWAS at an empirical FDR of 5%; (2) the eQTLs that contributed to the significant gene-based association were not in LD ($r^2 < 0.1$) with established allergy risk variants (those with a $P < 5 \times 10^{-8}$ in published GWAS of asthma, hay fever, eczema and/or allergies); and (3) gene-based association replicated ($P < .05$) in an independent GWAS. Four genes satisfied all 3 criteria: *P2RY13*, *P2RY14*, *USMG5*, and *B4GALT3*. We prioritized the former 2 for follow-up because functional experiments were feasible with available tools/reagents (both are cell-surface receptors). We performed 2 sets of experiments, which are described in detail in the Methods section in this article's Online Repository and were performed in accordance with the Animal Care and Ethics Committees of the University of Queensland (Brisbane, Australia).

First, we used an established mouse model of acute allergic asthma[16] to identify cell types in the lung that express P2ry13 and P2ry14 in the context of allergen-induced airway inflammation. Two groups of wild-type C57BL/6 mice were anesthetized and sensitized intranasally with either saline solution (group 1) or 100 μg of house dust mite (HDM) extract (group 2) on day 0. Subsequently, mice were challenged with either saline (group 1) or 5 μg of HDM (group 2) at days 14, 15, 16, and 17 and killed 3 hours later. Total RNA was isolated from the left lung, and quantitative real-time PCR was performed to measure overall gene expression. Bronchoalveolar lavage fluid (BALF) was collected and cells were stained with anti-P2ry13 or anti-P2ry14 antibodies to identify individual cell types in the lung expressing P2ry13 and P2ry14. Cells were then stained with cell type–specific fluorescently labeled antibodies and enumerated with a BD LSR Fortessa cytometer (BD Biosciences, San Jose, Calif). Paraffin-embedded lung sections were prepared as previously described,[17] and probed with anti-P2ry13 or anti-P2ry14 antibodies to assess expression in airway epithelial cells (AECs). Photomicrographs were taken at × 400 and × 1000 magnification at room temperature and acquired with Olympus Image Analysis Software (Olympus, Center Valley, Pa).

We performed a second set of experiments to test the hypothesis that P2ry13 or P2ry14 receptor activation could influence the release of alarmins, such as IL-33, and contribute to airway inflammation. Naive mice were inoculated intranasally with saline, 10 nmol/L 2-methyl–adenosine 5-diphosphate (ADP; P2ry13 agonist), 10 nmol/L uridine-diphosphoglucose (UDP-glucose; P2ry14 agonist), or 10 nmol/L adenosine 5-triphosphate (ATP; agonist for all P2ry receptors, except P2ry6 and P2ry14), all in 50 μL. For comparison, 3 additional groups of mice were inoculated with 100 μg of HDM, 100 μg of cockroach extract *(Blattella germanica)*, or 25 μg of *Alternaria alternata* extract. Two hours after challenge, BALF was collected, as described above, and IL-33 levels were measured by means of ELISA. Seventy-two hours after challenge, BALF was again collected to obtain immune cell counts and stained for flow cytometry, as described above.

RESULTS

Application of EUGENE to results from a published asthma GWAS

We applied our proposed gene-based test of association to a published asthma GWAS,[14] including 6,685 cases and 14,091 control subjects to identify genes with eQTLs collectively associated with disease risk. We tested the association with 17,190 genes (Fig 1), which were found to have *cis*-eQTLs (n = 13,557), *trans*-eQTLs (n = 315), or both (n = 3,318) across 16 published eQTL studies representing 12 different cell types or tissues relevant to asthma (see Table E2 in this article's Online Repository at www.jacionline.org).

Of the 17,190 genes tested, 48 were associated with asthma at an empirical FDR of 0.05 (Fig 2 and Table I). Of these, 31 (65%) were located within 1 Mb (or on the MHC region) of established

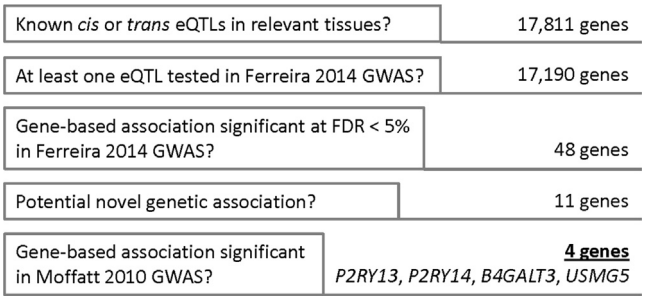

FIG 1. Outline of analytic procedure.

risk variants for allergic disease (highlighted with a "+" in Fig 2 and listed in Table E3 in this article's Online Repository at www.jacionline.org). For example, for thymic stromal lymphopoietin (*TSLP*; gene-based $P = 7 \times 10^{-6}$),[18] we identified 6 independent *cis*-eQTLs in 5 tissues, 4 of which were individually associated with asthma risk at a *P* value of less than .05 (see Table E4 in this article's Online Repository at www.jacionline.org). Multiple genes within the same risk locus had significant associations with asthma: 12 in the MHC,[15] 11 on 17q12,[2] 3 on 2q12,[15] and 2 on 16p13.[14] Some of these associations resulted from eQTLs being shared between neighboring genes, as observed for *ORMDL3*, *GSDMB*, and *ZPBP2* on 17q12 (see Table E5 in this article's Online Repository at www.jacionline.org) and for *CLEC16A* and *SOCS1* on 16p13 (r^2 between rs35441874 and rs7184491 [Table E3] = 0.64). eQTL sharing could arise, for example, if an underlying causal variant disrupts the activity of a regulatory element that controls the expression of multiple genes. However, that was not always the case; in the MHC region the individual *LTA* eQTL that was most strongly associated with asthma ($P = 2 \times 10^{-5}$) was in low LD ($r^2 \leq 0.02$) with the eQTLs for the other 11 significant MHC genes (see Table E6 in this article's Online Repository at www.jacionline.org). Similar results were observed for *NEU1*. Therefore, at least in the MHC region, the multiple significant associations observed were not entirely explained by eQTLs shared between genes.

On the other hand, 6 (12%; *LIMS1*, *AOAH*, *ZNF707*, *CLK3*, *SAFB*, and *TINF2*; "Δ" in Fig 2) of the 48 genes found to be significant at an FDR of 0.05 were not located in established risk loci for asthma, but the significant gene-based associations were (in most cases entirely) driven by *trans*-eQTLs located in the MHC region or near *ORMDL3* (see Table E7 in this article's Online Repository at www.jacionline.org). These include, for example, variant rs9268853, which is a *trans*-eQTL for *CLK3* ($P = 7 \times 10^{-17}$) in PBMCs,[19] *SAFB* ($P = 3 \times 10^{-6}$) in whole blood,[20] and *AOAH* in 3 tissues (best $P = 10^{-61}$,[19-21]; see Table E8 in this article's Online Repository at www.jacionline.org). This variant has also been found to be a *cis*-eQTL ($P < 5 \times 10^{-8}$) for *HLA-DQ* and *HLA-DR* genes across multiple tissues (data not shown). These results suggest that MHC and 17q12 variants might contribute to asthma risk not only by directly modulating the expression of nearby genes but also by indirectly influencing the expression of genes in different chromosomes (eg, through *cis*-mediation[22]).

Of potential greater interest, 11 (23%) of the 48 significant genes were located in potential novel asthma risk loci, and the gene-based associations were not driven by established allergy risk variants ("○" in Fig 2 and Table I). Because some of these

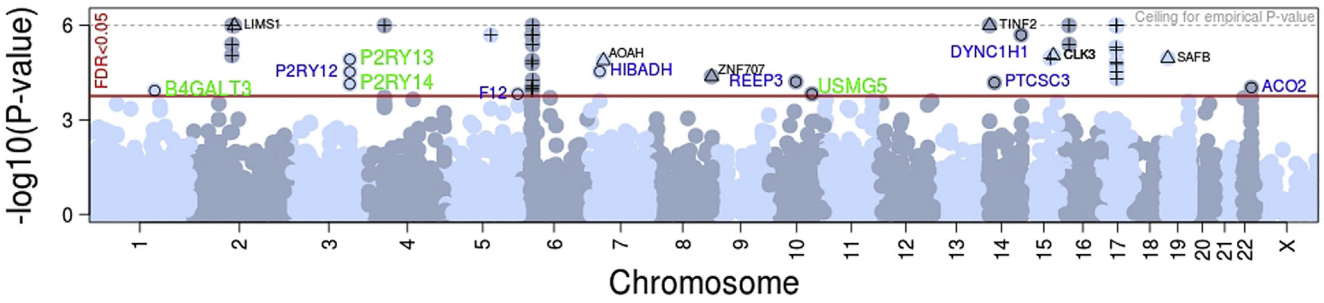

FIG 2. Summary of association results obtained for 17,190 genes by applying the proposed gene-based test of association to a published asthma GWAS.[14] The *red horizontal line* shows the *P* value threshold corresponding to an empirical FDR of 5% ($P = 1.9 \times 10^{-4}$). Forty-eight genes exceeded this threshold, including (1) 31 genes located in established risk loci for allergic disease (denoted by +; gene name shown in *black* font), (2) 6 genes located in new risk loci but with a gene-based association that was driven by *trans*-eQTLs located in the MHC or near *ORMDL3* (denoted by Δ), and (3) 11 genes with a gene-based association that was not driven by eQTLs located in established allergy risk loci (denoted by ○), including 4 (*green* font) for which the association replicated in an independent GWAS.[15] The *y axis* represents the $-\log_{10}$ value of the simulation-derived gene-based *P* value, which accounts for the residual LD between eQTLs of a given gene. The *P* value was based on up to 1 million simulations, and therefore it could not exceed a *P* value of 10^{-6} *(dashed gray line)*.

genes might represent false-positive findings, we studied their association with asthma in an independent GWAS.

Replication of the putative novel gene-based associations in an independent asthma GWAS

To confirm the putative novel associations, we applied EUGENE to an independent GWAS of asthma with publicly available summary statistics.[15] Based on results for 9,967 asthmatic patients and 15,301 control subjects, 4 of the 11 genes selected for replication had a significant gene-based association ($P < .05$ and see Table E9 in this article's Online Repository at www.jacionline.org) when simulations show that, on average, the expected number of genes significant at this threshold by chance alone given multiple testing was 0.53 (SD, 0.77).

We then explored whether the discovery and replication associations for those 4 genes (*P2RY13, P2RY14, B4GALT3* and *USMG5*) were consistent by comparing the direction of effect on disease risk for individual eQTLs. Overall, the direction of effect for most eQTLs of a given gene was the same between the 2 independent GWASs (see Table E10 in this article's Online Repository at www.jacionline.org). For example, of the 7 eQTLs for *USMG5* that were individually associated with asthma risk in either study, for 6, the allele that increased asthma risk was the same (or was on the same haplotype) in both studies; 1 eQTL was not tested in the replication GWAS, and therefore the direction of effect could not be compared. Therefore, the association between asthma risk and these 4 genes is generally consistent at the individual eQTL level between the 2 independent GWASs. Henceforth, we refer to these 4 genes with a reproducible gene-based association with asthma as putative novel asthma risk genes.

Contribution of *cis*- and *trans*-eQTLs to significant gene-based associations

For 3 (*P2RY13*, *P2RY14*, and *USMG5*) of the 4 putative novel risk genes, the gene-based association with asthma was entirely driven by *cis*-eQTLs. Most of these eQTLs were identified by using eQTL studies of whole-blood expression levels (see Table E11 in this article's Online Repository at www.jacionline.org). For the fourth gene, *B4GALT3*, 3 *cis*-eQTL (in neutrophils, blood, and fibroblasts) and 1 *trans*-eQTL (in blood) contributed to the association with asthma (see Table E11). The latter (rs1668873) was located 44-Mb away on chromosome 1 and was previously reported to associate with mean platelet volume and count.[23,24] This variant is also a *cis*-eQTL for *NUAK2*,[20] a nuclear transcription modulator that has been shown to induce expression of *B4GALT5*,[25] a galactosyltransferase related to *B4GALT3*.[26] Therefore these results suggest that both direct (*cis*-eQTLs) and indirect (through transcriptional modulators, such as *NUAK2*) genetic effects on *B4GALT3* expression can contribute to asthma risk.

Genetically predicted direction of effect of gene expression on asthma risk

To assess the direction of effect of gene expression on disease risk, we focused on the independent eQTLs for each gene that were individually associated with asthma in the discovery and/or replication GWASs. These variants had the greatest contribution to the significant gene-based tests. When we compared the direction of effect for each eQTL between asthma risk and expression levels, we found that the allele associated with increased gene expression was also associated with increased asthma risk for all independent eQTLs of *P2RY13* and *P2RY14* (see Table E11). The same pattern of results was observed for 6 of the 7 eQTLs of *USMG5*; for example, the rs1163073:C allele that was associated with asthma risk (odds ratio, 1.09; $P = .0005$) was associated with increased *USMG5* expression in 5 different cell types or tissues (neutrophils, lymphoblastoid cell lines, skin, PBMCs, and blood). These results suggest that in the tissues or cell types considered in our analysis, a genetically determined increase in gene expression for each of these 4 genes is associated with increased disease risk. For *B4GALT3*, there was no clear pattern across multiple eQTLs: of the 4 alleles associated with increased gene expression, 2 (in neutrophils and whole blood) were associated with increased and 2 (in fibroblasts and whole blood) with decreased disease risk. Such differences between eQTLs could arise, for example, if *B4GALT3* has opposing functional effects on different cell types relevant to

TABLE I. Forty-eight genes with a significant (FDR < 5%) association with asthma risk in the Ferreira et al[14] GWAS

Gene	Position	No. of eQTLs	No. of eQTLs tested	No. of eQTLs with $P < .05$	Best individual eQTL		EUGENE P value	Potential novel association*
					SNP	P value		
HLA-DQB1	6:32627244	78	26	15	rs1063355	1.8×10^{-13}	$<10^{-6}$	No
GSDMB	17:38060848	15	11	5	rs2952140	1.2×10^{-8}	$<10^{-6}$	No
LIMS1	2:109150857	15	14	4	rs1063355	1.8×10^{-13}	$<10^{-6}$	No†
TLR1	4:38792298	9	6	3	rs12233670	1.4×10^{-11}	$<10^{-6}$	No
ORMDL3	17:38077294	19	14	5	rs2952140	1.2×10^{-8}	$<10^{-6}$	No
IKZF3	17:37921198	9	7	3	rs7207600	4.5×10^{-7}	$<10^{-6}$	No
IL18RAP	2:103035149	18	16	6	rs13018263	5.0×10^{-6}	$<10^{-6}$	No
CLEC16A	16:11038345	4	4	2	rs35441874	2.9×10^{-8}	$<10^{-6}$	No
ZPBP2	17:38024417	2	2	1	rs9916765	1.9×10^{-9}	$<10^{-6}$	No
GRB7	17:37894180	2	1	1	rs14050	1.4×10^{-7}	$<10^{-6}$	No
TINF2	14:24708849	4	4	2	rs3135006	1.7×10^{-6}	$<10^{-6}$	No†
TAP2	6:32789610	48	36	11	rs2858312	1.9×10^{-5}	2.0×10^{-6}	No
TAP1	6:32812986	13	12	4	rs6928482	2.0×10^{-8}	2.0×10^{-6}	No
HSPA1B	6:31795512	13	13	6	rs13215091	4.7×10^{-4}	2.0×10^{-6}	No
TSLP	5:110405760	6	6	4	rs17132582	3.2×10^{-4}	2.0×10^{-6}	No
DYNC1H1	**14:102430865**	**6**	**5**	**2**	**rs4906262**	$\mathbf{1.1 \times 10^{-5}}$	$\mathbf{2.0 \times 10^{-6}}$	**Yes**
HLA-DRB1	6:32546546	97	46	18	rs3806156	1.4×10^{-4}	4.0×10^{-6}	No
IL18R1	2:102927989	11	11	5	rs6751967	3.2×10^{-6}	4.0×10^{-6}	No
SOCS1	16:11348262	7	6	5	rs7184491	3.5×10^{-6}	4.0×10^{-6}	No
CISD3	17:36886488	4	4	2	rs2941503	1.6×10^{-7}	5.0×10^{-6}	No
PGAP3	17:37827375	2	2	1	rs903502	1.5×10^{-6}	6.0×10^{-6}	No
IL1RL2	2:102803433	2	2	1	rs9646944	6.7×10^{-7}	9.0×10^{-6}	No
CLK3	15:74890841	2	2	1	rs9268853	1.8×10^{-6}	9.0×10^{-6}	No†
SMAD3	15:67356101	7	7	2	rs17293632	2.0×10^{-7}	1.1×10^{-5}	No
SAFB	19:5623046	2	2	1	rs9268853	1.8×10^{-6}	1.1×10^{-5}	No†
P2RY13	**3:151044100**	**8**	**8**	**5**	**rs9877416**	$\mathbf{1.2 \times 10^{-4}}$	$\mathbf{1.2 \times 10^{-5}}$	**Yes**
AOAH	7:36552456	26	21	3	rs9268853	1.8×10^{-6}	1.3×10^{-5}	No†
SLC44A4	6:31830969	2	2	1	rs9275141	1.1×10^{-6}	1.3×10^{-5}	No
STARD3	17:37793318	7	3	1	rs2941503	1.6×10^{-7}	1.5×10^{-5}	No
LTA	6:31539831	16	13	5	rs2442752	1.5×10^{-5}	1.6×10^{-5}	No
MED24	17:38175350	5	5	2	rs7502514	4.8×10^{-5}	1.7×10^{-5}	No
HIBADH	**7:27565061**	**14**	**12**	**6**	**rs6951856**	$\mathbf{9.6 \times 10^{-5}}$	$\mathbf{2.9 \times 10^{-5}}$	**Yes**
P2RY12	**3:151055168**	**6**	**6**	**4**	**rs17282940**	$\mathbf{7.0 \times 10^{-5}}$	$\mathbf{3.0 \times 10^{-5}}$	**Yes**
NR1D1	17:38249040	5	4	2	rs12150298	2.8×10^{-6}	3.0×10^{-5}	No
ZNF707	8:144766622	9	8	3	rs17609240	1.5×10^{-6}	4.2×10^{-5}	No†
TOP2A	17:38544768	1	1	1	rs2102928	4.1×10^{-5}	4.9×10^{-5}	No
HLA-DRB6	6:32520490	60	13	6	rs522254	6.3×10^{-4}	5.4×10^{-5}	No
REEP3	**10:65281123**	**6**	**4**	**2**	**rs7898489**	$\mathbf{9.1 \times 10^{-6}}$	$\mathbf{6.1 \times 10^{-5}}$	**Yes**
PTCSC3	**14:36605314**	**2**	**2**	**2**	**rs7148603**	$\mathbf{1.8 \times 10^{-4}}$	$\mathbf{6.5 \times 10^{-5}}$	**Yes**
P2RY14	**3:150929905**	**13**	**12**	**5**	**rs10513393**	$\mathbf{1.1 \times 10^{-4}}$	$\mathbf{7.2 \times 10^{-5}}$	**Yes**
HLA-DQA1	6:32595956	79	29	9	rs504594	1.7×10^{-5}	8.1×10^{-5}	No
ACO2	**22:41865129**	**3**	**3**	**2**	**rs960596**	$\mathbf{1.3 \times 10^{-4}}$	$\mathbf{9.4 \times 10^{-5}}$	**Yes**
HCP5	6:31368479	23	22	4	rs2071595	6.7×10^{-6}	9.6×10^{-5}	No
NEU1	6:31825436	8	8	5	rs9267901	9.1×10^{-4}	9.8×10^{-5}	No
MICB	6:31462658	41	30	9	rs9268764	3.3×10^{-5}	1.2×10^{-4}	No
B4GALT3	**1:161141100**	**10**	**9**	**3**	**rs1668873**	$\mathbf{1.5 \times 10^{-3}}$	$\mathbf{1.2 \times 10^{-4}}$	**Yes**
USMG5	**10:105148798**	**16**	**14**	**4**	**rs1163073**	$\mathbf{4.9 \times 10^{-4}}$	$\mathbf{1.5 \times 10^{-4}}$	**Yes**
F12	**5:176829141**	**3**	**3**	**2**	**rs4976765**	$\mathbf{1.7 \times 10^{-3}}$	$\mathbf{1.5 \times 10^{-4}}$	**Yes**

*Potential novel genetic associations with asthma (in boldface) are those for which the gene-based association was not driven by eQTLs located in known allergy risk loci. Known allergy loci are defined as those that contain a variant reported to be associated with asthma or other allergic diseases with a P value of less than 5×10^{-8} in published GWASs.
†Genes not located in an asthma risk locus but for which the gene-based association was driven by *trans*-eQTLs in LD with allergy risk variants (see Table E7 for more details).

asthma (eg, activation in one and inhibition in another). Further studies are required to test this possibility.

Functional studies in the mouse

The 4 putative novel asthma risk genes identified in our genetic association analysis are involved in nucleotide synthesis (*B4GALT3* and *USMG5*) and nucleotide-dependent cell activation (*P2RY13* and *P2RY14*). Based on this observation, we hypothesized that genetic dysregulation of nucleotide signaling contributes to asthma risk. In-depth functional experiments that comprehensively test this hypothesis were beyond the scope of this study. Nonetheless, we carried out 2 sets of experiments in the mouse to provide preliminary functional support for the involvement in allergic asthma for 2 of these 4 nucleotide-related genes: *P2RY13* and *P2RY14*. Both are cell-surface receptors with known agonists and therefore were well suited for functional studies.

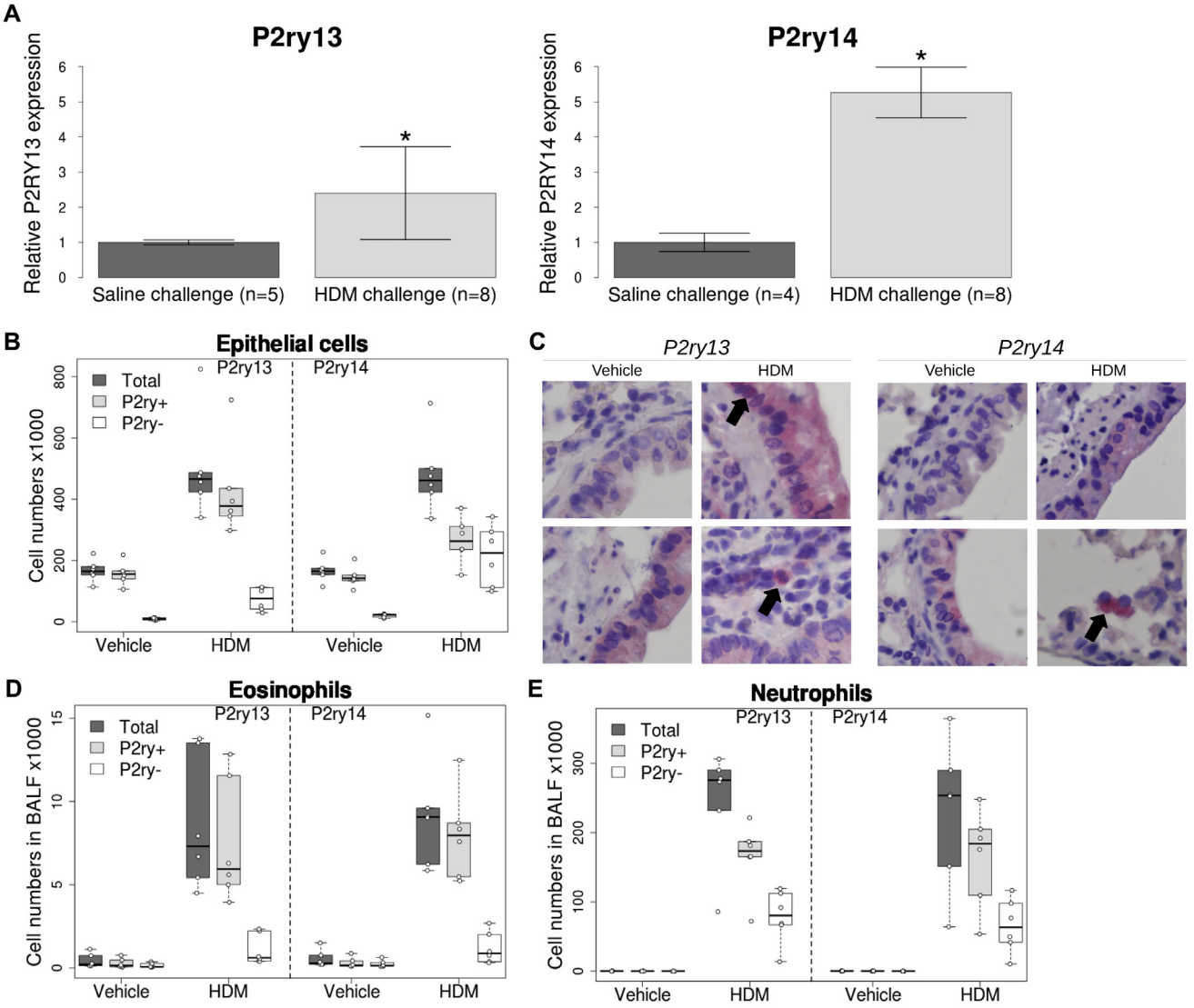

FIG 3. Expression levels of *P2ry13* and *P2ry14* in lungs of C57BL/6 mice sensitized and then challenged with a saline solution or an HDM extract. **A,** Overall gene expression in lung. Expression levels were normalized to *Hprt* and are expressed as fold change over the saline challenge group. Results show means ± SDs in each group. *Wilcoxon rank sum test: $P < .005$ when comparing HDM and saline groups. **B, D,** and **E,** Expression of P2ry13 and P2ry14 based on flow cytometric analysis in lung epithelial cells or eosinophils and neutrophils collected in BALF after saline or HDM challenge. **C,** Expression of P2ry13 and P2ry14 in lung sections of mice challenged with saline or HDM.

First, we used an established experimental model of acute allergic asthma[16] to study P2ry13 and P2ry14 expression in the lungs of C57BL/6 mice sensitized and subsequently challenged with HDM allergen. In this model, mice have granulocytic airway inflammation that has a predominant eosinophil contribution.[16] When considering overall lung expression, HDM challenge resulted in a significant increase in *P2ry13* and *P2ry14* expression relative to that seen in control mice challenged with a saline solution (Fig 3, *A*). To understand which lung cell types contributed to this increase in gene expression, we used flow cytometry to measure protein expression in AECs and major immune cell types collected through bronchoalveolar lavage. There was widespread expression of both receptors in AECs, both at baseline and after HDM challenge (Fig 3, *B* and *C*). Most eosinophils collected in BALF after HDM challenge stained positive for both receptors (Fig 3, *D*); expression in neutrophils was also high (Fig 3, *E*). Lymphocytes and dendritic cells had low expression of both receptors (see Fig E2 in this article's Online Repository at www.jacionline.org).

Second, given the high level of P2ry13 and P2ry14 expression observed in AECs at baseline (ie, in the absence of allergen challenge) and the previously reported proinflammatory effect of the respective agonists,[27,28] we postulated that receptor activation could promote airway inflammation by inducing alarmin release. To test this possibility, we collected BALF from naive mice 2 and 72 hours after intranasal challenge with saline, ADP (selective P2ry13 agonist), or UDP-glucose (selective P2ry14 agonist). At 2 hours after challenge, BALF levels of the alarmin IL-33 were significantly greater in mice exposed to the receptor agonists than in control mice (Fig 4, *A*). Of note, nucleotide-induced

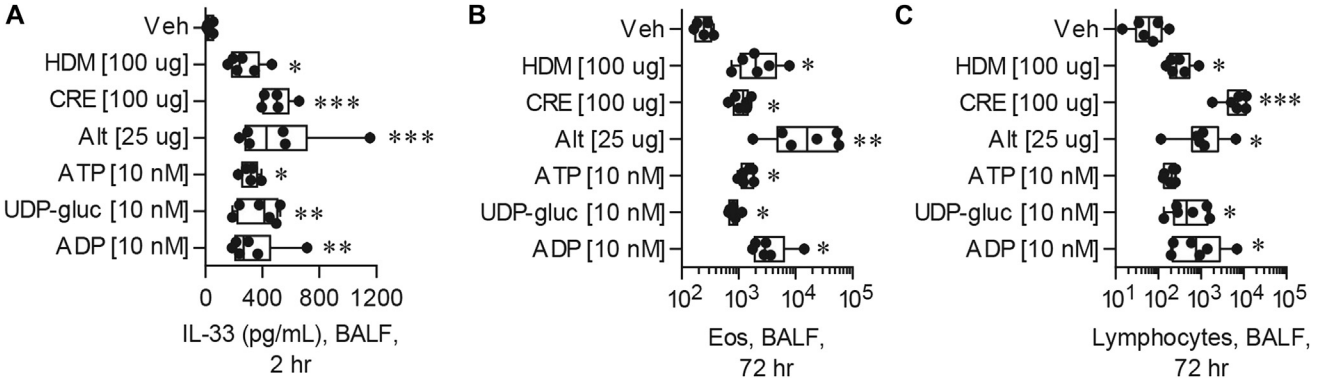

FIG 4. *In vivo* exposure to P2ry13 and P2ry14 receptor agonists in naive C57BL/6 mice. Mice were challenged with either vehicle, one of 3 allergens (HDM, cockroach allergen, or *Alternaria* species), or one of 3 nucleotides (ATP, UPD-glucose, ADP) and killed 2 and 72 hours after challenge. **A,** IL-33 expression in BALF collected 2 hours after challenge. **B** and **C,** Total numbers of eosinophils (Fig 4, *B*) and lymphocytes (Fig 4, *C*) recruited to the BALF at 72 hours after challenge based on flow cytometric analysis. *Alt*, *Alternaria* species; *CRE*, cockroach; *Veh*, vehicle. **P* < .05, ***P* < .01, and ****P* < .001.

IL-33 levels were comparable with allergen-induced IL-33 levels, indicating that both ADP and UDP-glucose are sufficient to induce IL-33 release. Furthermore, 72 hours after challenge, the number of BALF eosinophils and lymphocytes were significantly higher in agonist-treated mice (Fig 4, *B* and *C*), but this was not the case for neutrophils, dendritic cells, and monocytes (see Fig E3 in this article's Online Repository at www.jacionline.org). These results demonstrate that selective agonists of P2ry13 and P2ry14 can promote airway inflammation, even in the absence of allergen stimulation.

DISCUSSION

Dysregulation of gene expression is thought to be a common mechanism through which genetic variants can influence cellular function and, ultimately, variation in human traits and disease risk. This proposition is supported by the observation that eQTLs are more likely to be trait associated when compared to random variants,[18] and this was the motivation for the gene-based approach developed in this study.

EUGENE has some advantages when compared with other gene-based association approaches, of which we highlight 3: VEGAS,[9] PrediXcan,[12] and TWAS.[13] When compared with VEGAS, EUGENE avoids the requirement to use an arbitrary distance (same for all genes) from the known gene boundaries to define which SNPs to include in the gene-based analysis. If the distance is too small (eg, ±5 kb), then the contribution of important and more distantly located eQTLs might be missed, whereas a large distance (eg, ±1 Mb) could result in testing a large number of variants, many of which are likely to be unrelated to gene expression/function; in both cases the power to detect a significant gene-based association is reduced. Also, because the analysis in EUGENE is restricted to variants previously shown to influence gene expression, whether in *cis* or in *trans*, a significant gene-based trait association directly implies that genetically determined differences in gene expression contribute to trait variation. On the other hand, when compared with the recently described PrediXcan approach,[12] the main advantage of EUGENE is that it is applicable to GWAS summary statistics, which are typically easier to share than data sets with individual level genetic data. TWAS,[13] which is conceptually very similar to PrediXcan, is applicable to summary statistics, but its current release only includes weights for a relatively small number of genes obtained from 3 eQTL studies, although this is likely to increase in the future.

Another major difference when compared with both of these approaches is that with EUGENE, eQTLs identified in transcriptome studies of different cell types (and/or on cell stimulation) can be included in the same association analysis, all contributing with equal weight to the gene-based statistic. This might be important for traits or diseases for which multiple cell types or tissues are known to play a role in the underlying pathophysiology. In the PrediXcan and TWAS approaches, the weights assigned to different eQTLs in the model used to predict gene expression levels are based on the effect (eg, regression coefficient) of those variants on expression levels measured in a single reference transcriptome data set. The extent to which those weights remain appropriate (ie, yield good prediction) if the reference transcriptome and target data sets are very different (eg, in age composition) is unclear.

A disadvantage of EUGENE is that the direction of effect between gene expression and disease risk (or trait variation) is not directly inferred. To do so with the EUGENE approach, the effect of individual eQTLs that contribute to the gene-based test needs to be compared *ad hoc* between the transcriptome and trait GWAS. These eQTLs also provide a specific small group of variants to test in validation studies. Lastly, EUGENE (but not the other 3 approaches) estimates FDR thresholds empirically, taking into account the LD between eQTLs of the same or different genes. This is important to account for multiple testing.

When we applied EUGENE to a published GWAS of asthma, we identified 48 genes with a significant gene-based association at an FDR of 0.05, including 11 associations that were not driven by established genetic risk variants for allergic disease. For 4 of these genes (*B4GALT3*, *USMG5*, *P2RY13*, and *P2RY14*), the association was nominally significant in an independent asthma GWAS, and therefore we refer to these as putative novel asthma risk genes.

B4GALT3 encodes the widely expressed enzyme β-1,4-galactosyltransferase III, which catalyzes the transfer of galactose from UDP-galactose to *N*-acetylglucosamine to form *N*-acetyllactosamine and UDP.[26,29] How variation in *B4GALT3*

expression might contribute to asthma risk is unclear, but potential mechanisms include activation of β_1-integrin,[30] which is important in the initiation of T-cell inflammatory responses,[31] or by influencing extracellular release of UDP-galactose,[32] a P2RY14 agonist.

USMG5 encodes a small subunit of ATP synthase,[33] an enzyme responsible for ATP synthesis in the mitochondria. *USMG5* knockdown in HeLa cells causes loss of ATP synthase, resulting in lower ATP synthesis and slower cell growth.[34] In $CD4^+$ T cells, mitochondria produce the ATP that is rapidly released into the extracellular space on cell stimulation.[35] In turn, this ATP establishes an autocrine feedback through purinergic receptors that is essential for proper T-cell activation.[36] Given these observations, we speculate that genetically determined increased *USMG5* expression results in increased mitochondrial production of ATP, increased extracellular ATP release, and increased T-cell activation. In turn, this would translate into an increased risk of asthma. ATP synthase has also been detected at the surface of different cell types,[37] where it is thought to play different physiologic roles, such as high-density lipoprotein endocytosis in hepatocytes through *P2RY13* activation[38] and nonconventional T-cell activation.[39] Whether *USMG5* associates with membrane ATP synthase and therefore could potentially influence its ectopic roles remains to be determined.

P2RY13, also known as *GPR86* or *GPR94*, is a purinergic receptor highly expressed in the immune system, lung, and skin but also in the brain[40-42]; it displays a significant homology with the nearby *P2RY12* and *P2RY14* genes, sharing 48% and 45% amino acid identity.[40] P2RY13 is strongly activated by ADP,[40] a degradation product of ATP. Airway epithelial goblet cells are a major source of extracellular ADP, which is released as a co-cargo molecule from mucin-containing granules.[43] In turn, ADP has been shown to enhance antigen-induced degranulation in mast cells through a P2RY13-dependent mechanism.[27] ADP has also been reported to promote IL-6 release from keratinocytes,[42] inhibit TNF-α and IL-12 production by mature dendritic cells,[44] and promote chemotaxis of immature dendritic cells[45]; however, these studies did not specifically test whether the observed ADP effects were mediated by P2RY13. Results from our genetic association analyses indicate that a genetically determined increase in *P2RY13* expression increases asthma risk, which is consistent with the proinflammatory effect suggested for ADP and P2RY13 by these functional studies.

P2RY14, also known as *GPR105*, encodes a G protein–coupled receptor that is potently and selectively activated by UDP-sugars, especially UDP-glucose.[46] UDP-glucose is thought to be an extracellular proinflammatory mediator,[47] which is constitutively released by different cell types, including AECs.[48] Importantly, infection with respiratory syncytial virus or treatment with IL-13 significantly increases UDP-glucose release by AECs,[49] and this coincides with increased mucus secretion.[50] Known proinflammatory effects of UDP-glucose, acting through *P2RY14*, include inhibition of TLR9-dependent IFN-α production,[51] increased chemotaxis of neutrophils,[52] induction of mast cell degranulation,[53] production of IL-8,[28,54] and STAT3-dependent epidermal inflammation.[55] A small-molecule antagonist for P2RY14 was recently developed and shown to effectively block chemotaxis of freshly isolated human neutrophils.[56] Of note, plasma UDP-glucose levels are increased in mice fed a high-fat diet,[57] which raises the possibility that obesity might contribute to chronic P2RY14 activation and, in that way, increase asthma risk, severity, or both. Studies that investigate this possibility are underway.

When we studied the expression of both P2ry13 and P2ry14 in mice, we found that both genes were highly expressed in AECs, both at baseline and after allergen challenge. High expression was also observed in infiltrating eosinophils after challenge and, to a smaller extent, neutrophils and monocytes. High expression in AECs at baseline suggested that receptor activation could contribute to airway inflammation, even in the absence of allergen exposure. This was indeed what we observed when naive mice were challenged intranasally with either ADP or UDP-glucose: 72 hours after challenge, numbers of BALF eosinophils were significantly increased when compared with those in control mice. Interestingly, eosinophil influx into the airways was preceded by a significant increase in levels of the alarmin IL-33. The effect of both receptor agonists on IL-33 release was comparable in magnitude with that observed with allergens known to have a potent effect on IL-33 production, namely the fungus *Alternaria alternata*.[58] These results demonstrate that activation of P2ry13 and P2ry14, in addition to P2y2,[58] can strongly induce IL-33 release in mice. Interestingly, P2ry13 expression was observed in the nuclei of AECs after allergen challenge. Given that IL-33 is constitutively stored in the nuclei of AECs,[59] it is possible that intracellular activation of P2ry13 expressed on the nuclear membrane plays a role in allergen-induced IL-33 release.

In conclusion, our genetic findings establish an association between asthma risk and genes involved in nucleotide synthesis (*B4GALT3* and *USMG5*) and nucleotide-dependent cell activation (*P2RY13* and *P2RY14*). In mice, *in vivo* activation of P2ry13 and P2ry14 induced IL-33 release and subsequent eosinophilic airway infiltration. These observations suggest that genetic dysregulation of nucleotide signaling contributes to the risk of asthma (allergic and potentially also nonallergic) and other related conditions; studies that test this possibility are now warranted. Our results also show that reanalysis of published GWASs with a gene-based test that exclusively focuses on documented eQTLs has the potential to identify novel associations.

We thank all study participants, including customers of 23andMe who answered surveys, as well as the employees of 23andMe, who together made this research possible.

Key messages

- **In human subjects asthma risk is associated with genetically determined expression of 4 genes related to nucleotide synthesis (*B4GALT3* and *USMG5*) and nucleotide-dependent cell activation (*P2RY13* and *P2RY14*).**
- **In mice intranasal exposure to selective agonists for P2ry13 (ADP) or P2ry14 (UDP-glucose) induced IL-33 release and eosinophil infiltration into the lungs in the absence of allergen stimulation.**

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