Protection from asthma in a high-risk birth cohort by attenuated $P2X_7$ function

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Background: Viral illnesses are important factors in both asthma inception and exacerbations, and allergic sensitization in early life further enhances asthma risk through unclear mechanisms. Cellular damage caused by infection or allergen inhalation increases ATP levels in the airways with subsequent purinergic receptor activation. The purinergic receptor $P2X_7$ can enhance airway leukocyte recruitment to the airways, and $P2X_7$ knockout mice display a reduced asthma-like phenotype.
Objective: Based on the $P2X_7$ knockout mouse, we hypothesized that children with low $P2X_7$ function would have decreased rates of asthma.
Methods: We used a functional assay to determine $P2X_7$ pore-producing capacity in whole-blood samples in a birth cohort at high risk for asthma development. The $P2X_7$ assay was validated with known loss-of-function alleles in human subjects. $P2X_7$ pore status categorization was used to assess asthma and allergy status in the cohort.
Results: Attenuated $P2X_7$ function was associated with lower asthma rates at ages 6 and 8 years, and the greatest effects were observed in boys. Children with asthma at age 11 years who had low $P2X_7$ capacity had less severe disease in the previous year. Attenuated $P2X_7$ function was also associated with sensitization to fewer aeroallergens.
Conclusion: $P2X_7$ functional capacity is associated with asthma risk or disease severity, and these relationships appear to be age related. (J Allergy Clin Immunol 2012;130:496-502.)

Key words: *Asthma, allergy, children, $P2X_7$, ATP*

Sensitization to aeroallergens and the occurrence of virus-associated wheezing illnesses are early childhood events known to increase the risk of asthma, and the occurrence of either is thought to be due to a balance between environmental and host factors.[1,2] Our group previously reported a significantly increased risk of asthma at age 6 years with acute wheezing illnesses in the first 3 years of life associated with human rhinovirus (HRV),[3] and aeroallergen sensitization might contribute to the risk of more severe virus-induced wheezing illnesses and asthma.[4-6] The risk of HRV-induced wheeze might also depend on factors related to the virus,[7-9] and susceptible subjects can be identified based on attenuated antiviral defense mechanisms leading to compromised type I and III interferon production.[10-12] Unfortunately, less is known about the control of allergic sensitization and the diverse molecular patterns and innate immune receptors comprising recognition of aeroallergens.[13-16] Additionally, the transition from innate to adaptive immune responses is thought to be pivotal in the development of sensitization.[17-19] A recent example is the observation that chronic activation of dendritic cells (DCs) enhances the development of polysensitization to new aeroallergens.[20] Although these findings have provided new insights, determining additional characteristics of allergen-host interactions will further identify potential interventions important in asthmatic patients.

In this regard a growing body of evidence supports the function of nucleotides and nucleotide receptors in the regulation of innate to adaptive responses.[15,21,22] Injury and inflammation in the lung lead to cell damage and subsequent release of intracellular danger signals in the airways, including ATP,[23-25] a natural ligand for a family of purinergic receptors.[26] Granulocytic cell influx to the airways after allergen challenge is linked to ATP levels and is blunted when ATP is hydrolyzed or purinergic receptor antagonists are administered.[23,27] Specifically, an absence of $P2X_7$ leads to a lack of proinflammatory cytokine IL-1β release[28] and prevents contact dermatitis in a murine model.[29] A sensitization and exposure model of allergic asthma in $P2X_7$ knockout mice showed decreased airway reactivity and fewer immune cells

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Abbreviations used
COAST: Childhood Origins of Asthma
DC: Dendritic cell
HBS: HEPES-buffered saline
HRV: Human rhinovirus
LOF: Loss of function
MFI: Median fluorescence intensity
OR: Odds ratio
P2RX7: $P2X_7$ receptor gene

recruited to the lung after challenge.[30] The immunologic amplification loop involving extracellular ATP and $P2X_7$ has been implicated in a growing number of diseases in which the resulting pathology is determined by the site at which ATP is released, including at neuroreceptors and in the liver, vasculature, and the lung.[29-33]

Because $P2X_7$ contributes to responses from both allergens and pathogens, we sought to assess the association between $P2X_7$ function and the development of asthma in a birth cohort at high risk for asthma and allergy.[34] The gene encoding the $P2X_7$ receptor *(P2RX7)* is polymorphic, with nonsynonymous single nucleotide polymorphisms resulting in functional alterations.[35] These functional differences allow us to use a flow cytometric assay to assess whether a subject's *P2RX7* genotype confers normal or loss-of-function (LOF) potential for cell membrane pore formation.[36,37] Based on the *P2RX7* knockout mouse,[30] we hypothesized that low $P2X_7$ pore function would confer protection against asthma. We demonstrate that low functioning $P2X_7$, as measured in peripheral blood monocytes, is associated with reduced risk of childhood asthma and allergic sensitization.

METHODS

Study subjects

The participants were part of the Childhood Origins of Asthma (COAST) study, a previously described longitudinal study of a birth cohort that enrolled 289 children at high risk of asthma.[34] All children had at least 1 parent with a history of physician-diagnosed asthma, respiratory allergies, or both. All experiments were performed with approval of the Institutional Review Board and Human Subjects Committee at the University of Wisconsin–Madison; assent was obtained from the children, and informed consent was obtained from the children's parents.

Current asthma was defined at ages 6, 8, and 11 years, as described previously,[3] and asthma severity was assessed at age 11 years. Briefly, current asthma was diagnosed on the basis of the documented presence of 1 or more of the following in the previous year: (1) a physician's diagnosis of asthma; (2) use of albuterol for coughing or wheezing episodes (prescribed by a physician); (3) use of a daily controller medication; (4) step-up plan including use of albuterol or short-term use of inhaled corticosteroids during illnesses; and (5) use of prednisone for asthma exacerbation. Asthma severity was assessed at the 11-year visit based on the National Asthma Education and Prevention Program's Expert Panel Report 3 criteria. For children using long-term controller medications, severity was classified by the level of treatment required for control of asthma, whereas children not receiving controller therapy were classified based on symptoms.[38]

Wheezing respiratory tract illnesses in the first 3 years of life were previously defined by 1 or more of the following: (1) physician-diagnosed wheezing at an office visit; (2) an illness for which a child was prescribed short- or long-acting β-agonists, controller medications, or both; and (3) an illness given the following diagnoses: bronchiolitis, wheezing illness, reactive airway disease, asthma, and/or asthma exacerbation.[3]

$P2X_7$ pore assay

Peripheral blood samples in citrate tubes were obtained from COAST children during annual study visits at ages 10 and 11 years for whole-blood pore assays to assess $P2X_7$ function.[37] Briefly, 500 μL of room temperature blood was rinsed twice with HEPES-buffered saline (HBS; 130 mmol/L NaCl, 5 mmol/L KCl, 20 mmol/L HEPES, 0.1% BSA, and 10 mmol/L glucose [pH 7.4]) and incubated with CD14 conjugated to phycoerythrin (CD14-PE; BD Biosciences, San Diego, Calif) in HBS for 20 minutes. Samples were rinsed twice with potassium glutamate buffer (130 mmol/L potassium glutamate, 5 mmol/L KCl, 20 mmol/L HEPES, 0.1% BSA, and 10 mmol/L glucose [pH 7.4]) and incubated with 250 μmol/L 2′(3′)-*O*-(4-benzoylbenzoyl) ATP (BzATP; Sigma, St Louis, Mo) and 1 μmol/L YO-PRO-1 (Molecular Probes, Eugene, Ore) in potassium glutamate buffer for 20 minutes before addition of magnesium chloride and HBS washing. Viable $CD14^+$ cells identified by using propidium iodide exclusion were examined for YO-PRO-1 median fluorescence intensity (MFI) by using bead-adjusted (BD Calibrite Beads, BD Biosciences) and calibrated (RFP-30-5A; Spherotech, Lake Forest, Ill) flow cytometry on a FACSCaliber (BD Biosciences). Archived DNA from COAST participants was genotyped in the laboratory of Dr Carole Ober (University of Chicago, Chicago, Ill). An adult population previously genotyped for *P2RX7* and with $P2X_7$ pore function measurements was also used for comparison.[37] By using our previous methods,[39] 5 functionally validated *P2RX7* LOF alleles were used to genomically validate the threshold of whole-blood $P2X_7$ pore activity discriminating normal and attenuated function in both children and adults. A receiver operating characteristic curve was used to instruct the threshold between low and normal $P2X_7$ pore activity by maximizing sensitivity and specificity in identification of *P2RX7* LOF alleles.

Allergen-specific IgE measurement

Allergen-specific IgE levels were measured in plasma by using an automated fluoroenzyme immunoassay (Unicap 100; Pharmacia Diagnostics AB, Uppsala, Sweden). At ages 1, 3, 6, and 9 years, IgE levels were measured for 2 species of dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), *Alternaria alternata*, cat dander, and dog. At ages 6 and 9 years, IgE levels were additionally measured for ragweed, birch, timothy grass, and cockroach.[40] Test results were considered positive for values of 0.35 kU_A/L or greater.

Statistical analysis

The relationships between children's $P2X_7$ pore function MFI values measured on different days and obtained at different ages were examined by using the Pearson correlation coefficient. Logistic regression was used to examine the relationships of asthma and viral wheezing outcomes to pore status. Multivariate logistic regression models of asthma included pore status and either sex or HRV-induced wheeze and the interaction term as covariates. The χ^2 test for association was used to compare aeroallergen sensitization rates by pore status and early-life demographic and risk factors by pore status. Birth weight was compared by pore status with the Wilcoxon rank sum test. The number of aeroallergens sensitized was compared by pore status with generalized linear mixed-effects quasi-Poisson regression models. The χ^2 test for trend in proportions was used to test the association between asthma severity and pore status. $P2X_7$ pore function measured by MFI values of YO-PRO-1 uptake was normalized by using square root transformation for analysis. A 2-sided *P* value of less than .05 was considered statistically significant.

RESULTS

$P2X_7$ pore assay characteristics

At least 1 $P2X_7$ pore assay was performed on 172 children in COAST during annual visits at ages 10 and 11 years. Assay results were similar to those previously performed on adults, with an approximately square root normalized distribution (Fig 1, *A*). To validate the reproducibility of our standard methods, a subset of 48 samples had pore assays performed on more than 1 day after

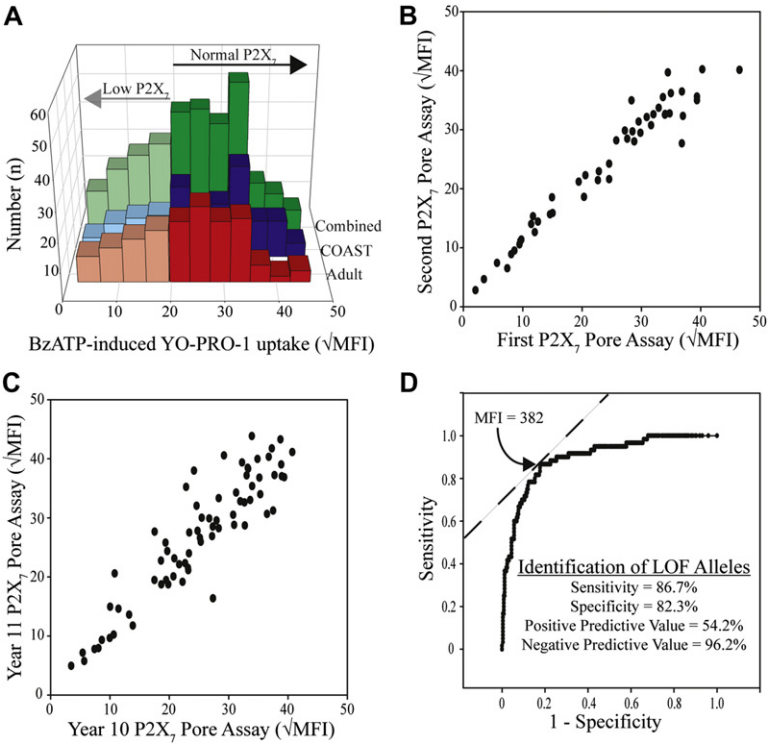

FIG 1. $P2X_7$ pore assay characteristics. **A,** $P2X_7$ pore assay distributions for COAST (n = 172) and adult (n = 156) populations are shown. *Lighter shading* indicates low $P2X_7$ function. **B** and **C,** $P2X_7$ pore assays are shown for the same sample at different days after phlebotomy ($r = 0.97$, $P < .001$; Fig 1, *B*) and at different subject ages ($r = 0.91$, $P < .001$; Fig 1, *C*). **D,** A receiver operating characteristic curve ($P < .001$, area under the curve = 0.90) is shown with characteristics for identifying *P2RX7* LOF alleles based on the optimized threshold MFI of 382, which is indicated as the *labeled point in the upper left corner*, maximizing both sensitivity and specificity.

phlebotomy, with an average daily coefficient of variation of 7% between the first and second day (Pearson $r = 0.97$, $P < .001$; Fig 1, *B*). Additionally, a subset of 71 children had pore assays performed at both the 10- and 11-year study visits, and the year-to-year reproducibility of the assay was also highly correlated (Pearson $r = 0.91$; $P < .001$; Fig 1, *C*). Overall, these results confirm the high reproducibility of our pore assay and independence from potential technical confounding factors.

Because the COAST $P2X_7$ pore assays were consistent with previous adult assays, we combined both for receiver operating characteristic analysis using 5 validated *P2RX7* LOF alleles to determine a threshold between subjects with low and normal $P2X_7$ function (Fig 1, *D*). From this analysis, a threshold MFI of 382 was identified and used to categorize all subjects with $P2X_7$ pore assays with either low or normal $P2X_7$ pore status, as indicated by the shading in Fig 1, *A*. The resulting performance properties of the assay in identifying LOF alleles for *P2RX7* are shown in Fig 1, *D* ($P < .001$, area under the curve = 0.90). Low $P2X_7$ pore capacity was observed in 28% of COAST participants, which is similar to rates seen in other populations.

$P2X_7$ pore status is independent of many demographic factors

To determine whether $P2X_7$ status was biased by risk factors at birth or in the first year of life, we examined the distribution of pore status across a number of risk factors for asthma. $P2X_7$ pore status was independent of birth and early-life characteristics (Table I).

TABLE I. $P2X_7$ function is independent of asthma risk factors

Birth and year 1 risk factors	$P2X_7$ pore function: Low (n = 48)	$P2X_7$ pore function: Normal (n = 124)	*P* value
Maternal asthma ever	37%	38%	.90
Paternal asthma ever	28%	34%	.49
Smoke exposure in year 1	27%	24%	.69
Day care attendance in year 1	50%	50%	1.00
Exclusive breast-feeding during first 6 mo	38%	35%	.73
Dog in home at birth	40%	33%	.42
Cat in home at birth	27%	32%	.51
Older siblings	54%	57%	.71
Active atopic dermatitis in year 1	30%	26%	.59
Birth month	—	—	.62
Birth weight ± SD (oz)	123 ± 16	125 ± 19	.96
Male sex	60%	56%	.64

Subjects' characteristics were stratified by $P2X_7$ function.

$P2X_7$ function, asthma inception, and disease severity

To determine the association between $P2X_7$ function and childhood asthma, we stratified the COAST cohort using $P2X_7$ pore status and determined the rates of asthma at ages 6, 8, and 11 years. Low $P2X_7$ pore status was associated with a decreased rate of asthma (Fig 2, *A*) at ages 6 (odds ratio [OR], 0.34; 95% CI, 0.15-0.79; $P = .01$) and 8 (OR, 0.42; 95% CI, 0.20-0.88; $P = .02$) years, but a significant association was not observed at

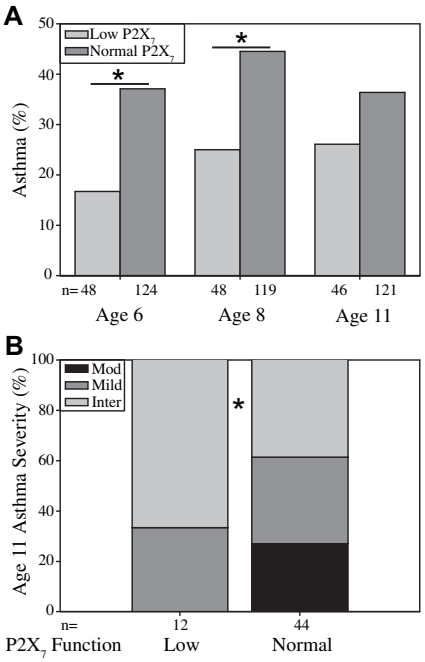

FIG 2. Low $P2X_7$ pore function is protective against asthma development. **A,** The rates of asthma at ages 6, 8, and 11 years are shown for low and normal $P2X_7$ status. **B,** Asthma severity at age 11 years is shown stratified by $P2X_7$ pore status. *Inter*, Intermittent; *Mod*, moderate. *$P < .05$.

age 11 years (OR, 0.62; 95% CI, 0.29-1.31; $P = .21$). To investigate whether there were any phenotypic effects in the children with asthma at age 11 years, we also stratified the severity of asthma using $P2X_7$ function. Asthmatic children with low $P2X_7$ pore function at age 11 years had evidence of less severe asthma compared with asthmatic children with normal $P2X_7$ pore function ($P = .03$; Fig 2, *B*). However, when examined at age 6 years, $P2X_7$ pore function was not associated with asthma severity ($P = .29$). Children with low pore function and asthma at age 11 years were also less likely to have used a step-up short-term plan, which is used for temporary loss of acceptable control with respiratory tract illnesses,[41] in the previous year (OR, 0.26; 95% CI, 0.07-1.00; $P = .04$).

$P2X_7$ function is associated with early-life wheezing with HRV

Because of $P2X_7$'s role in infections and airway reactivity[30,42] and our previous observation that wheezing illnesses associated with HRV in the first 3 years of life correspond to an increased rate of asthma,[3] we assessed the rates of wheezing in early life with or without virus detected based on $P2X_7$ function groups. Low $P2X_7$ pore status was not associated with preschool wheezing in general but was associated with decreased wheezing associated with HRV infections in the first 3 years of life (Table II).

Decreased asthma risk associated with attenuated $P2X_7$ function is varied by a history of HRV-induced wheezing and sex

To test whether reduced asthma risk in subjects with low $P2X_7$ function was only due to the association with decreased early-life HRV wheezing, we modeled the interaction of $P2X_7$ status and HRV-induced wheezing on asthma diagnosis by using logistic regression. The greatest risk for asthma was present in children who had the combination of normal $P2X_7$ function and a history of HRV-induced wheezing in the first 3 years of life (Fig 3, *A*), and this interaction was significant at ages 6 ($P = .03$) and 8 ($P = .01$) years.

Because of different rates of asthma in boys and girls at different ages,[43] we also examined whether there was an interaction between sex and $P2X_7$ status with regard to asthma risk using logistic regression. The protective effect of low $P2X_7$ pore status was more pronounced in boys than girls (Fig 3, *B*), and a significant interaction between sex and $P2X_7$ pore status was observed at ages 8 ($P = .02$) and 11 ($P = .03$) years. When only including boys in factoring asthma risk by $P2X_7$ pore status, protection against asthma in boys was significantly associated with low pore status at ages 6 (OR, 0.20; 95% CI, 0.06-0.64; $P = .004$), 8 (OR, 0.19; 95% CI, 0.06-0.54; $P = .001$), and 11 (OR, 0.30; 95% CI, 0.10-0.87; $P = .02$) years.

TABLE II. $P2X_7$ and early-life wheezing

Wheezing illnesses in first 3 y	$P2X_7$ pore function: Low (n = 48)	$P2X_7$ pore function: Normal (n = 124)	OR (95% CI), low pore function	*P* value
Any cause	44%	52%	0.71 (0.36-1.38)	.31
RSV detected	19%	31%	0.50 (0.22-1.14)	.10
HRV detected	19%	40%	0.35 (0.16-0.79)	.01

Wheezing illnesses in the first 3 years of life were compared between low and normal $P2X_7$ pore function groups.
RSV, Respiratory syncytial virus.

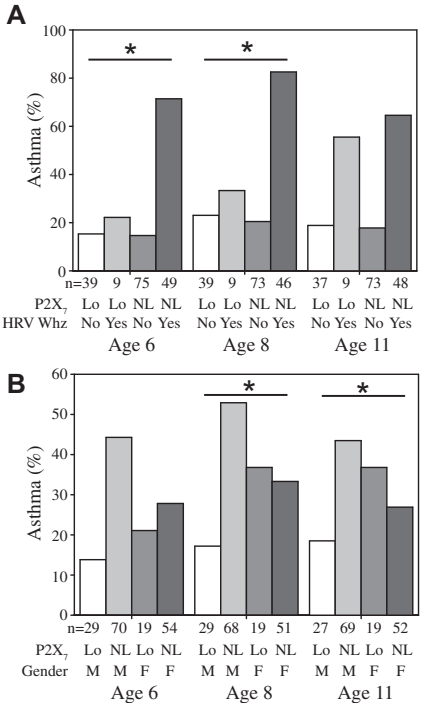

FIG 3. Interactions of $P2X_7$ with HRV-induced wheezing and sex. **A** and **B,** Both $P2X_7$ status and HRV-induced wheezing in the first 3 years of life (Fig 3, *A*) and $P2X_7$ status and sex (Fig 3, *B*) displayed an interactive effect on asthma risk. *$P < .05$ for logistic regression model interaction terms. *F*, Female; *HRV Whz*, wheezing with HRV during first 3 years of life; *Lo*, low $P2X_7$ function; *M*, male; *NL*, normal $P2X_7$ function.

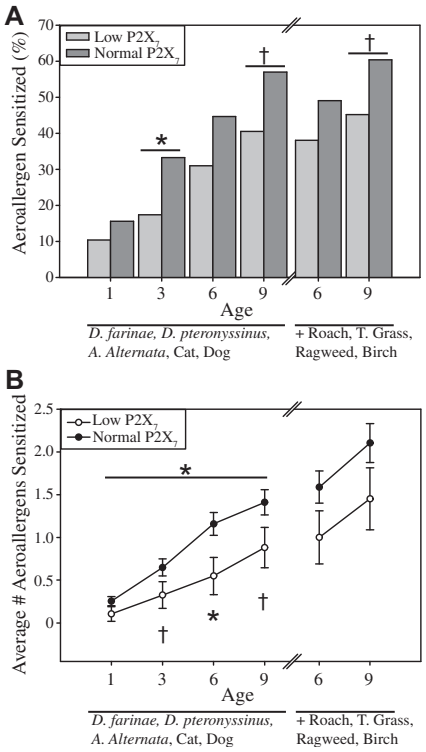

FIG 4. Aeroallergen sensitization status. **A,** The prevalence of children with positive sensitization to any aeroallergen measured is stratified by $P2X_7$ function. **B,** The average number of allergens to which each subject was sensitized was stratified by $P2X_7$ pore activity. For both panels, aeroallergens measured during all 4 ages are shown on the *left,* and inclusion of additional aeroallergens measured at ages 6 and 9 years is shown on the *right. Error bars* are the SEMs. *$P < .05$. †$P < .1$.

Allergic polysensitization and $P2X_7$ status

Effects of $P2X_7$ function on allergic sensitization rates have not previously been tested. In COAST, children with low $P2X_7$ function were less likely to be sensitized to common aeroallergens at age 3 years ($P = .04$), with a similar trend at age 9 years ($P = .07$; Fig 4, *A*). Because increased activation of DCs is reported to increase rates of polysensitization,[20] we also examined the rates of sensitization to aeroallergens as the average number of positive sensitizations per child. When 5 common aeroallergens were modeled by using mixed-effects quasi-Poisson regression, children with low $P2X_7$ function were sensitized to fewer aeroallergens across ages 1, 3, 6, and 9 years (mean fold change, 0.45; 95% CI, 0.22-0.91; $P = .03$; Fig 4, *B*). At individual ages, children with low $P2X_7$ function were sensitized to significantly fewer allergens at age 6 years ($P = .02$), and children with low $P2X_7$ function trended to be sensitized to fewer allergens at ages 3 ($P = .09$) and 9 ($P = .07$) years. When including additional aeroallergens measured only at ages 6 and 9 years, a similar trend was observed, but the model no longer remained significant across both years ($P = .14$).

DISCUSSION

This study adds to a growing body of research revolving around the role of nucleotides in airway disease. Similar to previous work in adults, we demonstrate good performance of a whole-blood $P2X_7$ function assay as a method to detect *P2RX7* LOF alleles (Fig 1). By using this robust assay, we have demonstrated that a lack of $P2X_7$ pore activity in high-risk children is associated with a reduced risk of asthma (Fig 2), as well as sensitization to fewer aeroallergens (Fig 4). However, the mechanisms underlying these observations are not clear. Discerning the role of $P2X_7$ activation by extracellular ATP in concert with secondary signals, including allergen exposure, viral infections, or both, might help determine how $P2X_7$ activity could modulate the risk of chronic conditions, such as asthma.

Previous studies indicate that the amount of extracellular ATP might be related to airway disease severity.[25] Rather than directly measuring ATP levels in the airway after injurious events, our study has the strength to study the potential for differential host responses to natural *in vivo* extracellular ATP fluctuations. Our results (Fig 1, *D*) recapitulate that considerably more contributes to $P2X_7$ pore function than validated *P2RX7* LOF alleles and illustrate the power of our functional approach to evaluate potential gene-environment interactions. The COAST population has already demonstrated gene-environment interactions, including between *IFNG* and sex,[44] which might be important to *in vivo* $P2X_7$ function because IFN-γ reportedly regulates $P2X_7$.[45] Although our current results are from prepubertal children, they display a varied risk of asthma by $P2X_7$ status based on sex (Fig 3, *B*). Whether the dynamics of this relationship change during and after puberty will be of great interest.

Our current results are in general agreement with findings from $P2X_7$ knockout mice, wherein low $P2X_7$ function is protective from asthma-like symptoms.[30] These $P2X_7$ knockout mice demonstrate decreased cell influx into the lung after allergen or smoke challenge,[30,46] and we have previously shown decreased neutrophil infiltration in the nose during an acute cold in adults with low $P2X_7$ function.[47] Although our current study might have been strengthened if $P2X_7$ pore assays could have been performed in early life before the earliest asthma evaluations, the high reproducibility and genetic basis of our results (Fig 1) indicate that assays should be similar at any age and mitigate these potential concerns.

Although low $P2X_7$ pore status protection against asthma in the current COAST cohort is consistent over multiple ages, these results seem counter to the inverse relationship between $P2X_7$ function and exacerbation risks in adults with a natural cold.[47] Differences in study populations and in the pathogenesis of asthma inception compared with exacerbations might help reconcile these findings. There are significant differences in study populations: the COAST population is comprised of high-risk children followed prospectively from birth, whereas the previous study enrolled symptomatic asthmatic adults during the peak cold season. In the children asthma was more common in boys, whereas in adults asthma was predominantly observed in women. It is possible that the overall lack of association at age 11 years between asthma and $P2X_7$ status might continue to change throughout puberty into adulthood and reflect the exacerbation risks observed in adults. Specifically, it is intriguing to note that a small percentage of children had both low $P2X_7$ status and a history of HRV-induced wheezing and that this group demonstrated the largest shift in rates of current asthma at different ages. Whether modification of $P2X_7$ function from nucleotide activation is sufficient to alter asthma outcomes in human subjects has yet to be measured.

How does $P2X_7$ influence asthma risk? Although $P2X_7$ is present in airway epithelial cells, the receptor is more highly

expressed and active in immune cells, including DCs.[48-50] Both nucleotides and nucleotide receptors, including $P2X_7$, affect DC function,[27,30,49-52] and loss of $P2X_7$ function, specifically from LOF alleles detected by using our pore assay, leads to a decrease in DC pore activity, as well as other $P2X_7$-dependent functional responses.[50,51] T-cell maturation, including regulatory T and T_H17 cell phenotypes, is modified by nucleotide activity on T cells and DCs, either directly or by engaging pathways associated with $P2X_7$, including the NLRP3 inflammasome or pannexin-1, and this suggests that functional $P2X_7$ activation might lead to a decrease in regulatory T-cell populations.[53-57] A DC-focused role of $P2X_7$ is supportive of an amplified response to infections or allergens when comingled with danger signals acting as adjuvants. Our study demonstrates a potential role for monitoring host responsiveness to immunomodulatory danger signals.

$P2X_7$ sits at a balance point in the immune system in response to allergic and infectious events. It is not clear whether a single episode of $P2X_7$ activation is sufficient to increase the risk of asthma or whether frequent stimulation is required. Moreover, $P2X_7$ function might not always be beneficial or harmful in the immune response; the role of $P2X_7$ might be different when comparing disease inception with active chronic conditions with superimposed acute events, such as exacerbations. As examples, influenza virus activation of the inflammasome has been linked to $P2X_7$ function,[58] whereas another report suggests $P2X_7$ might be necessary for some viruses to achieve cell entry.[59] Whether $P2X_7$ plays an active role in HRV infection or is secondary and solely responsive to cell injury could indicate when and where alterations of $P2X_7$ function are relevant. To study these relationships, the $P2X_7$ pore assay system described in this report is a useful tool to identify subjects at altered risk for disease and should be considered when further studying the role of danger signaling in disease pathogenesis.

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Key messages

- **$P2X_7$ functional capacity is easily assessed in children.**
- **Low $P2X_7$ function might decrease susceptibility to diseases, including asthma.**

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