THE EFFECTS OF FUNCTIONAL P2X$_7$ ON ASTHMA AND RHINOVIRUS INFECTION

By

David Matthew Manthei

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The dissertation is approved by the following members of the Final Oral Committee:

Loren C. Denlinger, Associate Professor, Medicine
James E. Gern, Professor, Pediatrics
Anna Huttenlocher, Professor, Pediatrics
Shigeki Miyamoto, Professor, Oncology
Deane F. Mosher, Professor, Biomolecular Chemistry

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David M. Manthei

Under the supervision of Loren C. Denlinger

University of Wisconsin-Madison

Asthma is a disease of the airways characterized by airflow limitations and shortness of breath. A history of wheezing in early childhood with human rhinovirus (HRV), the cause of the common cold, is strongly associated with asthma. The presence of allergic sensitization to multiple allergens has also been implicated in asthma inception. The interactions between an individual's genotype and these extrinsic exposures have helped frame the interdependency and variability of the causes of asthma.

Sudden worsening of asthma symptoms, or exacerbations, are triggered by viral infections or other environmental exposures, including allergens or smoke. However, the understanding of the mechanisms linking infection to exacerbations is incomplete and only some asthmatics will exacerbate from the same exposure or infection.

Both asthma inception and exacerbations demonstrate associations with HRV infections and subsequent inflammatory responses. During infection, cellular damage occurs, releasing intracellular contents of cells into the extracellular space. Adenosine triphosphate (ATP) is a prominent danger molecule that signals through a variety of purinergic receptors. Specific investigation of purinergic receptor $P2X_7$ has revealed associations with production of interleukin (IL)-1 family cytokines and control of various pathogens. These findings strengthen

the possibility of a role for $P2X_7$ in asthma inception and exacerbations by altering the host response during inflammation.

In the following chapters, I present studies on the role of $P2X_7$ in asthma inception and response to HRV infection. I utilize a functional assay identifying $P2X_7$ loss-of-function alleles to demonstrate a protective association between low $P2X_7$ function and asthma inception in childhood (CHAPTER TWO). The functional assay is well adapted to analysis of subjects in multi-center clinical trials (APPENDIX A). I then show that although responses to symptomatic viral infections are similar between asthmatic and non-asthmatic adults, elevated nasal cytokines among asthmatics – including those regulated by $P2X_7$ – are associated with exacerbations (CHAPTER THREE). Finally, I present evidence for functional $P2X_7$ in human respiratory epithelium (CHAPTER FOUR). The sum of this work strengthens the foundation of a role for $P2X_7$ in asthma and in response to infection and directs future studies on alternative cell populations including dendritic cells (CHAPTER FIVE).

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ABBREVIATIONS

ACQ – Juniper Asthma Control Questionnaire

ACRN – Asthma Clinical Research Network

APC – antigen presenting cell

ATP – adenosine-5'-triphosphate

AUC – area under the curve

BAL – bronchoalveolar lavage

BASALT – Best Adjustment Strategy for Asthma in the Long Term trial

BEC – bronchial epithelial cell

BSA – bovine serum albumin

BzATP – 2'(3')-*O*-(f-Benzoylbenzoyl)adenosine-5'-triphosphate

CD – cluster of differentiation

cDNA – complementary deoxyribonucleic acid

CI – confidence interval

COAST – Childhood Origins of ASThma

COPD – chronic obstructive pulmonary disease

CV – coefficient of variation

Da – Dalton

DAMP – danger associated molecular pattern

DAPI – 4',6-diamidino-2-phenylindole

DC – dendritic cell

DCC – data coordinating center

DIC – differential interface contrast

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

EPR-3 – expert panel report 3

ERK – extracellular signal-regulated kinase

FEIA – fluoroenzyme immunoassay

FeNO – fractional exhaled nitric oxide

FEV_1 – forced exhalation volume at 1 second

G-CSF – granulocyte colony-stimulating factor

G3PDH – glyceraldehyde 3-phosphate dehydrogenase

GERD – gastroesophageal reflux disease

GRB2 – growth factor receptor-bound protein 2

GWAS – genome-wide association study

h – hour

HBS – HEPES-buffered saline

HEK-293 – human embryonic kidney-293

HR – hazard ratio

HRV – human rhinovirus

IB – immunoblot

ICAM – intercellular adhesion molecule

ICS – inhaled corticosteroids

IFN – interferon

IgE – immunoglobulin E

IL – interleukin

IP-10 – interferon-γ-induced protein 10

IU – international unit

LABA – long-acting beta-agonist

LOF – loss-of-function

LPS – lipopolysaccharide

LTRA – leukotriene receptor antagonist

MAF – minor allele frequency

MIA – Macrolides In Asthma clinical trial

MCP – monocyte chemotactic protein

MFI – median fluorescence intensity

min – minute

MMP-9 – matrix metalloproteinase-9

mRNA – messenger ribonucleic acid

n – number

NAEPP – national asthma education and prevention program

NLF – nasal lavage fluid

NSAID – non-steroidal anti-inflammatory drug

oATP – oxidized adenosine triphosphate

OCS – oral corticosteroids

OR – odds ratio

PAMP – pathogen associated molecular pattern

PCR – polymerase chain reaction

PMN – polymorphonuclear neutrophil

Poly (I:C) – polyinosine-polycytidylic acid

RNA – ribonucleic acid

ROC – receiver operating characteristic

RSV – respiratory syncytial virus

RT-PCR – reverse transcription polymerase chain reaction

s – second

SABA – short-acting beta-agonist

SD – standard deviation

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP – single nucleotide polymorphism

TBS – tris-buffered saline

TBST – tris-buffered saline with 0.05% Tween

T_h – T helper cell

TLR – Toll-like receptor

TNF-α – tumor necrosis factor-α

TRAIL – TNF-related apoptosis-inducing ligand

T_{reg} – regulatory T cell

TSLP – thymic stromal lymphopoietin

URI – upper respiratory infection

VEGF – vascular endothelial growth factor

CHAPTER ONE

A literature review of asthma and the role for danger signaling

David M. Manthei

University of Wisconsin-Madison

OVERVIEW

Asthma is a disease of the airways characterized by episodic symptoms of shortness of breath, cough, and wheeze. Asthma features hyperresponsive smooth muscle surrounding airways that contract spasmodically causing airway narrowing, which is characteristically reversible with bronchodilating medications. Asthma is often associated with allergic disease and an expanding literature has linked early life wheezing and viral infections to inception. A general inflammatory state underlies much of the phenomena associated with asthma, including cellular influx and airway remodeling. Asthma exacerbations, sudden worsening of symptoms, are a burden on patients, caregivers, and the use of health care resources. The majority of exacerbations are preceded by a viral cold, although other causes include exposure to allergens and smoke.

Exposure to harmful infections or irritants, coupled with the underlying inflammation, creates an environment for increased cell damage and release of intracellular contents to extracellular space. These normally intracellular molecules act as danger signals by activating receptors and pathways to augment the immune response. Adenosine triphosphate (ATP) is a prototypical danger signal and acts through a class of extracellular receptors. Of the purinergic receptors responsive to ATP, $P2X_7$ is highly polymorphic with relatively common alleles conferring loss-of-function. By using a flow cytometric functional assay in peripheral blood to assess individual capacity for $P2X_7$ function, small numbers of individuals may be evaluated for functional and gene-by-environment studies to assess risk for disease-related outcomes. $P2X_7$ has been implicated in a variety of diseases in the lung, largely observed in mouse knockout models. This introduction provides the framework to analyze human $P2X_7$ function and interactions with viral infections related to asthma.

ASTHMA AND ITS MANIFESTATIONS

Overview of asthma

Asthma is a chronic disease of the airways characterized by symptoms of cough, wheeze, and shortness of breath.[1,2] Between 5 and 10% of adult and pediatric populations are estimated to suffer from asthma and its prevalence has increased in recent decades, particularly in Westernized societies, even though asthma may still be under-diagnosed.[3,4] Lung function is often summarized by means of spirometry when measuring airflow characteristics, and while most asthmatics are aware of symptoms, a minority do not perceive their airflow limitations appropriately.[5] The degree of airflow limitation may be variable over days and years, either spontaneously or in response to treatment. Hyperresponsiveness smooth muscle surrounding the airways is characteristic of asthma and contributes to airflow limitations.[6] Day-to-day variability in symptoms is typical, and underlying inflammation leading to airflow decrease by bronchospasm is a classic symptom of asthma, which is typically responsive to various bronchodilating agents. Asthma symptoms decrease quality of life, especially when improperly managed or not under control, with severe disease correlating to an even lower quality of life.[7-11]

Asthma status is often considered in two areas, or domains, of disease. The Impairment domain is evaluated as the degree of current symptoms and management while the Risk domain is assessed for the likelihood of future adverse events including exacerbations, or loss of asthma control.[12] Some of the most unpredictable and deleterious aspects of asthma are due to exacerbations and their associated high costs with health care usage and lost productivity.[13] Asthma exacerbations range from a mild loss of control that is difficult to distinguish from daily symptom variability to events severe enough to require medical intervention, including hospitalization.[14,15] A prior history of exacerbations is one of the biggest predictors of

exacerbations in the future, indicating there may be a high-risk subset of individuals.[16] Even in well-controlled asthma with minimal symptoms at baseline, it is unknown why some individuals exacerbate and others do not. There are relatively few measures used to predict exacerbations and a useful clinical biomarker is still being awaited.

In many ways, asthma reflects a symptomatic clinical syndrome with many causes rather than a single disease with a known, progressive course.[17] Anecdotal evidence and recent clustering analyses have revealed many phenotypes, or subgroups, of asthma. Some of these phenotypes relate to the underlying age of onset, contributions of co-morbid diseases, increases in particular cell populations, or individual responsiveness (or lack thereof) to different medications.[18-23] In the growing age of personalized medicine, advancing the methods to optimize therapy and management of asthma is a difficult but worthwhile goal.

Natural course of asthma

Before considering the underlying causes of asthma or appropriate interventions, it is helpful to examine what the natural course of asthma entails.[24,25] Just like disease symptoms, the incidence and course of asthma is variable and differs by both age and gender. In early life, a subset of children will wheeze, especially with illnesses, which is a hallmark for asthma later in childhood. A significant minority of these children will continue wheezing while most enter remission.[26] Yet other children with no history of wheeze will also go on to develop asthma, and children from both early wheezing and non-wheezing groups tend to have allergic disorders.

In childhood, boys have a higher prevalence of asthma than girls but throughout puberty many boys will enter remission while an increased incidence of asthma is observed in girls. This trend will continue into adulthood where asthma more often affects females.[3] The severity of

disease, as determined by lung function and adverse events, has been measured over the course of life. The greatest declines often occur in early life, and additional factors like smoking may also increase the deficit of lung function.[27,28] While individual components of disease appear to be reversible, if one has longstanding asthma some changes in the lung appear to be more deleterious and difficult to address. Lung function tends to decrease with the amount of time suffering from asthma, particularly if someone had asthma as a child and continues with disease into adulthood although there is great variability.[29-31]

Pathophysiology

The underlying pathology of asthma arises from generalized chronic inflammation with superimposed episodes of sporadic acute inflammation. This inflammation leads to textbook characteristics of the asthmatic lung: excess mucus production and edema in the airways, tissue remodeling such as angiogenesis and thickening and fibrosis of the underlying airway structures, and a characteristic hypertrophy of smooth muscle surrounding the smaller airways, or bronchioles.[24,32-34] The sum of these factors contributes to the decrease in airflow that is a hallmark of asthma.

Consistent with inflammation having a major role in asthma, many types of inflammatory cells are observed at increased levels in the airways. Lymphocytes, macrophages, eosinophils, neutrophils, mast cells, and dendritic cells have all been postulated to contribute to asthma responses in the airways.[35-39] In particular, one intensively studied cell is the eosinophil, present in increased numbers in most asthmatic individuals, although there are notable subsets without increased eosinophils.[40] In particular, the absolute and relative numbers of eosinophils and neutrophils in sputum has been correlated to disease severity, outcomes, and management

goals.[22,41] Many of these cells release factors that have a role in asthma by turning on pro-inflammatory pathways or recruiting other cells. It is likely that the mere presence of these cells is not sufficient for disease and that priming and activation by various factors, including paracrine and autocrine mechanisms, is necessary to induce the functional consequences and symptoms of disease.[42]

Many classes of factors released by cells associated with asthmatic disease have been measured. Mediators include a host of cytokines and chemokines, various classes of arachidonic acid-derived cysteinyl-leukotrienes, nitric oxide, reactive oxygen species, and antibodies such as immunoglobulin E (IgE), presenting opportune targets for drug development.[43,44] Relationships between these factors are often multidirectional. For example, IgE may bind to mast cells, causing degranulation and release of histamine and other active factors like eicosanoids, which can trigger constriction of the hyperresponsive smooth muscle around the airways.[45,46] Activation of macrophages resulting in production of chemokines attracts other cells to the airways, continuing the cellular inflammatory cascade. Some of these factors and cells are increased in the peripheral blood as well as in the lung, including eosinophils and IgE.[47] The natural question arises of whether these cells and factors have the same mechanisms systemically as well as locally in the lung. The answer may be important in the localization of disease, but also a factor in the constant milieu for inflammation and damage that is a part of the difficulty in treating asthma.

Pathogenesis and risks of asthma

Underlying mechanisms of asthma may be different depending on which phase of disease is considered. First, one may consider what causes disease inception. Second, what makes

disease worse, either acutely, such as for exacerbations, or long-term in regards to disease progression and persistence? An interaction between innate host factors, like gender and genetic background, and environmental factors, such as exposure to infections or allergens, appears to be important.[48]

Inception

Great effort has been put forth into finding underlying causes of asthma in hopes of preventing disease onset. Although there is a large hereditary component to asthma, simple Mendelian genetics do not apply to such a complex disorder.[49,50] Even with many genome-wide association studies (GWAS), a relative paucity of genes have been identified with an attributable risk for asthma of less than 50%.[51] Both rare alleles that are not routinely included in association studies as well as gene-by-environment interactions may have major roles in asthma, in part due to the heterogeneous nature of the disease.[52,53] Gene-by-environment interactions require both a predisposing, susceptible genotype as well as some environmental exposure, bridging the postulated arms of innate and extrinsic causes of asthma.[54-56]

Asthma is usually not a disease found in isolation and allergic diseases are highly co-morbid with asthma.[57] Atopy, broadly defined as elevated IgE in the blood, includes states of allergic sensitization and is commonly measured when determining categorization of risk for asthma; what is often considered "classic" asthma is in practice atopic asthma, although different groups have reported different rates.[58] These observations have played a large role in the hypothesis that asthma is a disease of imbalance between T-helper (T_h) cells, tipped in the balance to T_h2 production over T_h1 with a resultant lack of cell-mediated and increased humoral

immune responses including IgE production.[59,60] The balance of different parts of immune function is a recurring theme in the study of asthma.

Multiple cohorts of children have been studied to determine the course and origins of asthma.[61-64] Methods have been developed to predict the future risk of asthma, an example of which is the modified asthma predictive index.[65] While these predictive measures are typically sensitive, they have modest positive predictive measures, indicating that just because a child is at risk for asthma, it does not necessarily indicate he or she will develop disease. While not a risk for asthma, per se, gastroesophageal reflux disease (GERD) is also observed in a high proportion of asthmatics.[66] While there are many links regarding inception, particularly in children, there is less consensus regarding inception in adults.

Childhood risk factors

The role of viral infections and illnesses in asthmatic disease has gained prominence over the last two decades.[67] Early work postulated wheezing illnesses due to respiratory syncytial virus (RSV) infection led to subsequent development of asthma.[68,69] Recently, wheezing episodes with human rhinovirus (HRV), the cause of the common cold, have been implicated in subsequent development of asthma and decreased lung function.[70,71] Even with these findings providing a role for viral illnesses in asthma inception, risks due to allergic sensitization are also observed and show interactive risks.[72]

When determining risk of asthma due to sensitization, both the type and degree of sensitization may be important. Sensitization to aeroallergens appears to carry a stronger risk and sensitization to cockroach, dust mite, and *Alternaria* in particular are positively associated with asthma.[65,73-75] Additionally, sensitization to an increased number of allergens

(polysensitization) and the timing of early life sensitization are associated with development of asthma.[76,77] Similarly, allergic rhinitis, eczema, and peripheral blood eosinophilia are all associated with development of asthma.[24,78] Exposures to irritants, such as pollution and tobacco smoke, are also risks for asthma and thought to occur through overlapping and distinct pathways to other risks.[79,80]

As mentioned previously, parental history of asthma is a risk, and encompasses known and unknown genetic factors. Obesity has become an increasingly important trait associated with asthma.[81] Some of these hereditary risks are age-dependent, and perhaps order-dependent. The idea of the "hygiene hypothesis" has been popularized and implies that an increased exposure to bacteria and viruses in early life provides protection from disease later by building up an immune response.[82,83] However, multiple studies have shown that living on a farm or exposure to animals, and their protective effects, are dependent on individual genotypes.[84-87] The specific examples reinforce the concept that asthma risk is a combination of an individual's particular susceptibility as a genetic determinant, combined with personal exposures and environment.

Adult risk factors

Adult risk factors for asthma development are largely unknown, particularly regarding newly diagnosed asthma. However, low lung function as a child is a risk in general for adulthood asthma.[88,89] Many risk factors associated with childhood asthma are still observed, including smoking, atopy, and a history of airway hyperresponsiveness, with varying contributions to adult disease severity.[90-92] In contrast, some of asthma is related to life changes associated with adulthood, including occupational exposures to various materials causing

approximately 10% of adult cases.[93] Increases in asthma and symptoms have been noted during pregnancy and may be related to immune changes associated with pregnancy.[94] Some women experience altered symptoms and changes in lung function associated with the stage of the menstrual cycle with possible involvement of more general remodeling and angiogenic signals under hormonal control.[95,96] It is unclear how much of the increased prevalence of asthma in adult females is related to variations in hormones. While not observed frequently in children, adults may have a form of asthma associated with a sensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) that is thought to act by tipping the balance of prostaglandins and prostacyclins by inhibition of cox-1.[97] The differences in risks between adults and children may even be a part of the differences in observed phenotypes of disease.

Exacerbations

Exacerbations are a sudden worsening of asthma symptoms, or a loss of control, greater than day-to-day variability. Asthma exacerbations are linked to health care costs, hospitalizations, and mortality.[13,98] Exacerbations are difficult to study and define objectively.[15,99] Some definitions are symptom-based and partially anchored in medication history, while other methods use objective measures of lung function; a balanced approach has recently incorporated both aspects.[100] The mechanisms and phenomena underlying the cause of exacerbations including why some individuals are more susceptible are of debate, although they are thought to relate to level of function and underlying disease.[101]

From an epidemiological perspective, evidence has linked viral infections to exacerbations and hospitalizations, whether in children or adults.[102-104] Viral upper respiratory infections (URIs) often precede and are thought to contribute to asthma exacerbations. The

progression of viral infections from upper to lower airways and the host immune response to infection, including inflammation or neutrophil influx, may have roles in provoking exacerbations.[105-108] Increased neutrophils in the lower airways are associated with exacerbations, perhaps recruited in response to viral presence.[109] While viral URIs are linked to exacerbations, the underlying mechanisms are only partially known.[110]

Additional factors including tobacco smoke, smog, or allergen exposure may trigger exacerbations, perhaps by causing mast cell degranulation or initiating cell injury and inflammation.[24] Part of these exposures could cause injury of the innate barrier formed by respiratory epithelium, allowing another insult or infection access to more susceptible underlying tissue, and in some cases individuals may also be predisposed to an altered response to infections.[111] Children with viral infections also have longer duration of airway reactivity after infection.[112] Synergism between viral infections and allergic reactions have been observed, illustrating this cooperative effect.[113] Sputum eosinphils have also been associated with exacerbations, and treatment to reduce eosinophils is effective.[41,114] However, in general, there have been unsatisfactory results in predicting and preventing exacerbations.

Asthma therapy

Appropriate asthma therapy is usually determined after assessment for severity of disease, including both the Impairment and Risk domains. Asthma therapy is largely geared towards an anti-inflammatory or airway-relaxing effect, driven by individual patient symptoms, although studies have indicated a biomarker approach could be beneficial.[22,41,114] A guideline-based, step-wise approach is utilized to increase or decrease therapy depending on symptom control.[115] While there are questions over what ideal guidelines should be, adhering to current

guidelines for management reduces loss-of-control and increases quality of life; however, the majority of asthmatics worldwide are not receiving optimal therapy[116,117]

Short-term medications

Short-term therapy is often focused on treating the symptoms of asthma for rapid relief of constricted airways. Early in modern medicine, methods to relax the airways of asthmatics included epinephrine. While the choice of epinephrine is now considered risky, in particular due to its cardiac and vasculature altering properties, the underlying pharmacology is part of the choice for immediate relief today. Bronchodilators, particularly short-acting beta-agonists (SABAs) are used to open the airways by relaxing the smooth muscle around bronchioles.[118] SABAs are often used for daily control of symptoms, as needed, or for prophylactic management. Some individuals with a single nucleotide polymorphism (SNP) in the $beta_2$-adrenergic receptor gene have a worsening of symptoms when treated with SABAs, raising the concern of whether testing for these polymorphisms is justified before treatment is initiated.[119,120] Similar rationale for relaxing airway muscles exists for the use of anticholinergics, with an increasing number of reports indicating good efficacy.[121,122]

Long-term medications

The goal of long-term therapy is not only to control airway responsiveness and patency but also to prevent risk of exacerbations with progressive lung function decline.[123] Targeting specific pathways up-regulated with disease attempts to control or reverse underlying pathophysiologic changes. Various classes of medications have been evaluated for use when

measuring these endpoints, including sputum cell counts, as eosinophils are associated with airway remodeling.[124,125]

Corticosteroids form the bedrock of treatment for people with more than intermittent symptoms because of their anti-inflammatory effects.[126] While used for treatment of short-term changes in symptoms or exacerbations, the correct management is not always straightforward.[127,128] Maintenance therapy with steroids should be at the lowest effective dose. Inhaled corticosteroids (ICS) are often used first due to lower systemic dosing, while oral corticosteroids (OCS) are used in more severe disease. Part of the balance in steroid treatment is minimizing side effects like slowing growth and from sudden withdrawal of medication or the variable effects of steroids.[129] While slowing of growth with is even observed with ICS in children, height was not different between asthmatic children taking ICS and controls years following discontinuing ICS.[130] Different benefits take different lengths of time to occur after steroid dosing is initiated, ranging from hours for anti-inflammatory effects to weeks and months for symptom changes.[131,132]

With the success of SABAs as frontline medications came the desire for decreased dosing and long-lasting benefit of bronchodilating effect. Long-acting beta-agonists (LABAs) were chosen for all of those properties. However, there have been serious safety concerns with LABAs used as monotherapy, as was previously commonplace, warranting caution when used.[133] Yet when used in combination therapy, the addition of LABAs have shown benefit.[134] These differences underscore the importance in patient education in selection of appropriate medication.

Altered balances of arachidonic acid metabolism products are observed in asthma and drugs preventing metabolite generation or action are logical intervention points.[135] Leukotriene

receptor antagonists (LTRAs) and related 5-lipoxygenase inhibitors act to block receptor signaling or stop the arm of arachidonic acid metabolism, respectively. They are used with the goal of re-balancing the metabolites from pro- to anti-inflammatory eicosanoids. These lipid mediators are involved in numerous cell populations and some individuals appear to have drastic responses to treatment. Trials with LTRAs have shown increased benefit including a decrease in exacerbation rates.[136,137]

Other categories of treatment are often used when an individual is unresponsive to more common therapies. Mast cell stabilizers attempt to prevent mast cell degranulation and histamine release. They may have benefit in individuals with exercise-induced asthma where exertion is associated with bronchospasm.[138] Methylxanthines act via phosphodiesterase inhibition and adenosine receptor antagonism to promote smooth muscle relaxation and bronchodilation.[139] At lower doses, both anti-inflammatory and immunomodulatory effects are observed, but concern with off-effects has led to low utilization as a front-line medication.

A variety of molecules, including any altered cytokine during asthma, might be an appropriate target in treating disease symptoms.[43] Immudomodulators have been targeted to known factors associated with asthma and have the potential to change underlying disease processes. Two antibodies have been particularly noteworthy to date and both target the axis of allergic disease. Anti-IgE (omalizumab) has been associated with decreased hospitalizations and required dosage of ICS.[140,141] Anti-IL-5 (mepoluzimab), which attacks eosinophil generation and activation, has also been implicated in decreases of exacerbations and exacerbations.[124,142] This class of medication is typically expensive and has yet to be incorporated as cost-effective therapy. However, targeted treatment with subjects of specific risk or season of year may be a promising intervention and can have symptomatic benefits.[143]

In conclusion, many of these drugs empirically address the symptoms of asthma and even target alterations observed in disease, but may not address the underlying causes of these mediators. Additionally, there are specific concerns for risk with some medications and wide gaps in efficacy for others. Comparisons of ICS and LTRAs have demonstrated that ICS may be more effective overall, but that a substantial minority of individuals may respond preferentially to LTRAs.[144] In a similar regard, when stepping-up treatment in children with ICS, LTRA, or LABA, the LABA step-up was showed the greatest benefits overall, but yet again a heterogeneous preferential response was observed.[145] When one considers the heterogeneity of asthma to begin with, it quickly becomes clear that many asthmatics – even when on guideline-optimized therapy – may not be receiving the most appropriate care. The rest of this chapter will focus on the background and role for so-called Danger signaling, and how it relates to asthma.

EVALUATION OF DANGER SIGNALING IN THE AIRWAYS

Danger signals and airways

The understanding of immunology and how the host responds to foreign challenges has evolved greatly over the previous decades, including an increased understanding of interactions between innate and adaptive immune responses. A leap forward occurred when the idea of self and non-self recognition was postulated by Macfarlane Burnet.[146,147] Important ideas stemming from the basic theory have now come to include the recognition of foreign cells and pathogens being processed by antigen presenting cells (APCs) and pathogen associated molecular patterns (PAMPS) recognized by Toll-like receptors (TLRs) to initiate signaling for an immune cascade.[148] These pathways provided means for the immune system to respond appropriately to foreign substances.

However, the self-non-self theory did not provide for why low levels of microbes may not produce an immune response, why a cancer would be fought by the host immune system, or why autoimmune diseases would occur. Even before the identification of TLRs, Polly Matzinger helped frame the idea known as the Danger hypothesis, postulating the presence of some local signal produced by distressed, damaged cells to initiate immune responses.[149,150] These danger signals are thought to complement and act as an adjuvant to other immune-response pathways. Uric acid, cytochrome c, and a variety of nucleotides were eventually identified as intracellular contents acting as danger signals, collectively identified as danger associated molecular patterns (DAMPs). DAMPs help distinguish pathogens compared to normal flora, and if a response is required at interfaces with external environments.[151]

One can visualize many ways for extracellular presence of DAMPs in significant levels. Possible sources of increased cellular contents include active release by exocytosis, channel, receptor-mediated means, degranulation, and cytolysis. In the airways, many release pathways could converge during infections, allergen exposure, or irritant inhalation. Indirect measures have indicated increased intracellular contents in the airways during disease by measure of lactate dehydrogenase.[152] More recently, elevated ATP in the airways was reported following allergen exposure in mice and humans with subsequent leukocyte influx.[153] Increased ATP has been reported in bronchoalveolar lavage (BAL) fluid from chronic obstructive pulmonary disease (COPD) patients while similar and mixed results have been demonstrated in cystic fibrosis.[154-156] ATP is also increased with mucin production by airway epithelium.[157] Our laboratory has also observed a non-significant increase in ATP in BAL of asthmatics with more severe disease (Figure 1-1; unpublished observations). These collected works provide a

framework that DAMPS, and ATP in particular, act as a mechanistic link in the asthmatic response, as reviewed by Willart.[158]

Nucleotides and purinergic receptors

Early work identifying extracellular receptors to ATP was surprising considering that ATP is the essential energy source within a cell. ATP was not merely found as a bystander in extracellular space, but had potent actions on cells in experiments by Alan Drury.[159] Many paths exist for release of ATP including neurotransmission, degranulation, cell death, and both active and passive release.[160] Practically, high concentrations of ATP are found for short durations and in microenvironments with inflammation as ATP in the extracellular space is rapidly metabolized and hydrolyzed, the rates of which may be altered during inflammation.[161] Early work by Geoffrey Burnstock identified ATP as an actively released molecule at neurons with a functional role.[162,163] Investigations regarding ATP have grown to where it is now considered to be regulated and not merely a bystander, even when released during cell death.[164]

Multiple classes of receptors to nucleotides have been described and the term "purinergic" was introduced in 1972.[165] Purinergic receptors may have differing affinity to bases with varying numbers of phosphate groups, ranging from the P2 receptors to adenosine (P1) receptors. Adenosine receptor activity has been implicated in other disorders while P2 receptor activation has been reported in multiple cells including, relevant to the airways, mast cell mediator alterations, production of eosinophil-specific proteins, activation of alveolar macrophages, and neutrophil migration to sites of inflammation.[166-169] The P2 receptors, generally responding with the highest potency to ATP, are divided into distinct P2X and P2Y receptors signaling through different mechanisms and cellular expressions.[170]

P2Y receptors have been identified as 7-transmembrane-spanning G-protein coupled receptors with importance in both health and disease.[171] Various downstream effects are determined by which G-protein is coupled to the receptor. Pharmacologic study of different P2Y receptors shows a preferential response to different nucleotides, based both on the nucleoside base and number of phosphates.[172] P2Y receptors have a wide cellular distribution and function, including water efflux and cellular migration. Of note in cells found in airway disease, activation of $P2Y_{11}$ was found to inhibit apoptosis of neutrophils and P2Y2 is implicated in dendritic cell and eosinophil chemotaxis.[173,174]

P2X receptors ($P2X_{1-7}$) span the cell membrane twice, with both the N- and C-terminus in the cytoplasm. The receptors form trimeric complexes in the cell membrane, and both homo- and heterotrimers have been observed.[175] Unlike P2Y receptors, P2X receptors all respond preferentially to ATP compared to other naturally found nucleotides and each P2X receptor has a different pharmacologic property compared to other members. When P2X receptors are activated, an ion channel is opened through the trimeric complex space. Cellular distribution of P2X receptors is also varied and differentially inducible.[176]

There is homology between P2X receptors, but $P2X_7$ has many distinct properties. Compared to other P2X receptors, $P2X_7$ has a long intracellular C-terminus and was initially identified as purinergic receptor P2Z.[177] The intracellular signaling of $P2X_7$ includes activation of phospholipase C, calcium signaling, and anchoring to a variety of other cytoskeletal components.[176,178,179] $P2X_7$ is activated by higher concentrations of ATP compared to other P2X receptors. Aside from cationic currents, $P2X_7$ is also associated with dilation of a cell-membrane pore allowing passage of molecules up to 900 Da in size.[177] $P2X_7$ is also most highly expressed in cells of immune and neural lineages, and is involved with the processing and release of the

interleukin (IL)-1 family of cytokines (IL-1, IL-18, IL-33).[180,181] ATP is important in the release of IL-33 in response to either virus or allergen exposure.[182,183] Moreover, IL-1 has been reported to be required for the induction of the Th2 responses to aeroallergens and subsequent airway hyperresponsiveness.[184] Given the importance of the IL-1 family of cytokines in infectious and allergic outcomes, alterations to pathways involved with the IL-1 family, including $P2X_7$ function, may have functional consequences on asthmatic disease.

Variable $P2X_7$ host response from danger signals

The gene encoding $P2X_7$ (*P2RX7*) spans 13 exons at chromosome location 12q24 and expression may be regulated by inflammatory cytokines including interferon-γ. [185,186] *P2RX7* is highly polymorphic with hundreds of SNPs, many of which result in amino-acid alterations in $P2X_7$ protein.[187] Upon ATP exposure, these SNPs result in alterations to cell currents, membrane cell pore formation, and other intracellular signaling events including release of cytokines. $P2X_7$ gained prominence when one of these loss-of-function SNPs was associated with loss of control of tuberculosis.[188] The variability of outcomes due to single amino changes in a protein involved in response to DAMPs has been the basis of further work identifying loss-of-function alleles and their functional importance.

$P2X_7$ and airway disease

$P2X_7$ has been implicated in the host response to many infectious events, particularly intracellular pathogens. As noted above, a link between $P2X_7$ function and tuberculosis control has been identified. Furthermore, mycoplasma, chlamydia, and a host of other infections including influenza are at least partially modulated by $P2X_7$ activity.[189,190] Even when not

evaluating actively infectious agents, a bacterial wall component like endotoxin can have interactions with $P2X_7$ signaling pathways by downstream convergence on IL-1 processing, release, and other events leading to septic shock.[178,191] Notably, many of these infections and activities are associated with the lungs and airways, setting the stage for the potential of purinergic receptor involvement in airway disease set up by infectious events.

$P2X_7$ knockout mice have been developed and help to frame the role of $P2X_7$ *in vivo*.[192] These $P2X_7$ knockout mice undergo decreased ovalbumin-induced inflammation and airway hyperresponsiveness after sensitization and re-exposure.[193] While these are characteristics consistent with asthma, it is only reflective of an isolated allergic component of asthma and not the range of human disease. The knockouts are also protective in other models of lung disease including emphysema and fibrosis.[194,195] $P2X_7$ has been implicated in various immune such as contact dermatitis, fitting into a role for diseases with a skewed immune function.[196] The $P2X_7$ knockout is suggestive of a role in airway disease, but the background strain of the knockout is heterozygous for a known loss-of-function allele, warranting caution when interpreting results from the mouse model.[197]

Human studies with functional assessment of $P2X_7$

We have developed a functional assay to identify individuals with genotypes conferring a loss of $P2X_7$ function.[198,199] By using a simple peripheral blood sample and a flow cytometric assay to analyze pore formation, we determine low or normal functioning potential for $P2X_7$. By capitalizing on the unique properties of pore formation, this analysis provides a simple method to group individuals based on function instead of relying on rare allele frequencies or overlapping downstream readouts of activation such as transcription factor activation. The assay performs

well in identifying known loss-of-function alleles of *P2RX7*, but has a much lower positive predictive value (PPV), as will be shown in Chapter Two. The low PPV demonstrates the value of the functional approach in determining risk associated with genetic background since other alleles or factors are involved with losses of pore activity. This approach allows us to assess disease outcomes without the large numbers of subjects required for a strict genetic analysis, which is particularly useful for such a variable disease as asthma.

Work by our group has begun to complement the mouse-based observations by determining functional capability of $P2X_7$ in human subjects. Similar to the $P2X_7$ mouse asthma model, we have demonstrated a lack of neutrophil migration to the nose during infection in individuals with a lack of $P2X_7$ function and identified altered $P2X_7$ function as a risk for asthma exacerbations.[105] Our methods utilizing the pore assay lend themselves well to collective gene-by-environment analysis, as $P2X_7$ acts to amplify immune responses to the exposure of pathologic species or damaging particles relevant to the airways. The minimally invasive and robust nature of our assay also lends itself to utilization as a tool to test the responses in many populations.

SUMMARY OF BACKGROUND

Asthma is an inflammatory disease of the airways characterized by difficulty breathing. Asthma is associated with viral infections and allergy, both in disease inception and during asthma exacerbations. During inflammatory processes and infections, increased DAMPs are observed at active areas of disease. Evaluation of the role of DAMPS, and in particular ATP, in the response to infections has implicated a putative role for $P2X_7$ in asthma inception and exacerbation.

The following chapters will address the importance of inflammation related to asthma and precipitating events, evaluating the role for functional $P2X_7$. It is postulated that *P2RX7* genetic background influences the risk for future events related to infection and allergen exposure. The potential for $P2X_7$ functional capacity to influence the inception of asthma and allergy in childhood is first described (Chapter Two) and the feasibility to utilize the assay in a multicenter clinical trial is also reported (Appendix A). These findings are followed by studies examining what effecter molecules (Chapter Three) and potential cell populations (Chapter Four) are involved in altered predisposition to asthma and asthmatic events related to viral infections.

In evaluating the role of $P2X_7$ and infections on asthma, the following chapters will

i. Describe a correlation between loss of $P2X_7$ pore function and protection from development of asthma or severity of symptoms in a high-risk birth cohort, which are influenced by gender, history of wheezing with HRV, and age.

ii. Report the findings of nasal cytokines during a naturally acquired cold as biomarkers for subsequent asthma exacerbations.

iii. Demonstrate functional $P2X_7$ including pore activity in primary human respiratory epithelial cells, the modulation of which demonstrates small magnitude changes of HRV infection.

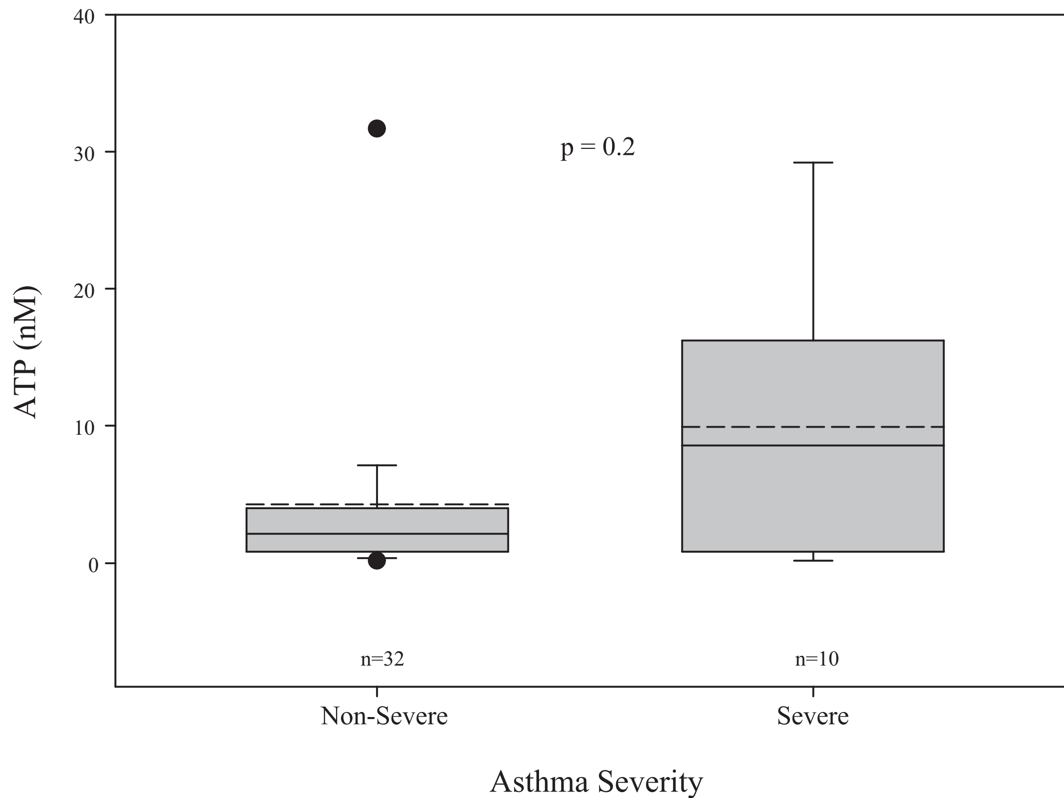

Figure 1-1. **ATP levels in asthmatic bronchoalveolar lavage (BAL) fluid**. BAL fluid from asthmatics was analyzed for ATP. Box plots indicate median (solid line), mean (dashed line), interquartile range (shaded region), and 95th percentile (contained by whiskers). A two-sided t-test on log-transformed measures was performed. (Following outlier analysis, a trend for a difference between groups was observed, p=0.08; additionally, asthmatics taking oral steroids compared to other asthmatics had higher ATP, p=0.04.)

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CHAPTER TWO

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

David M. Manthei[1], MS, Daniel J. Jackson[2], MD, Michael D. Evans[3], MS, Ronald E. Gangnon[3,4], PhD, Christopher J. Tisler[2], MT, James E. Gern[1,2], MD, Robert F. Lemanske[1,2], Jr, MD, and Loren C. Denlinger[1], MD/PhD

University of Wisconsin-Madison

Departments of Medicine[1], Pediatrics[2], Biostatistics & Medical Informatics[3], and Population Health Sciences[4]

ABSTRACT

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 due to infection or allergen inhalation increases ATP in the airways with subsequent purinergic receptor activation. The purinergic receptor $P2X_7$ may enhance airway leukocyte recruitment to the airways and $P2X_7$ knockout mice display a reduced asthma-like phenotype.

Objective: Based upon the $P2X_7$ knockout mouse, we hypothesized that children with low functioning $P2X_7$ would have decreased rates of asthma.

Methods: We utilized a functional assay to determine $P2X_7$ pore-producing capacity in whole blood samples in a birth cohort study at high risk for asthma development. The $P2X_7$ assay was validated with known loss-of-function alleles in humans. $P2X_7$ 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 and the greatest effects were observed in boys. Children with asthma at age 11 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.

INTRODUCTION

Sensitization to aeroallergens and the occurrence of virus-associated wheezing illnesses are early childhood events known to increase the risk of developing 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 may contribute to the risk of more severe virus-induced wheezing illnesses and asthma.[4-6] The risk of HRV wheeze may also depend on factors related to the virus,[7-9] and susceptible individuals may be identified by attenuated antiviral defense mechanisms leading to compromised Type I and III interferon (IFN) 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 developing sensitization.[17-19] A recent example is the observation that chronic activation of dendritic cells (DCs) enhances 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 asthma.

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 adenosine 5'-triphosphate (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 levels of ATP 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 pro-inflammatory cytokine interleukin (IL)-1β release[28] and prevents contact dermatitis in a mouse model.[29] A sensitization and exposure model of allergic asthma in $P2X_7$ knockout mice showed decreased airway reactivity and fewer immune cells 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 where the resulting pathology is determined by the site at which ATP is released including at neuroreceptors, in the liver, vasculature, and the lung.[29-33]

Since $P2X_7$ 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 non-synonymous single nucleotide polymorphisms (SNPs) resulting in functional alterations.[35] These functional differences allow us to utilize a flow cytometric assay to assess whether an individual's *P2RX7* genotype confers normal or loss-of-function (LOF) potential for cell membrane pore formation.[36,37] Based upon the *P2RX7* knockout mouse,[30] we hypothesized that low $P2X_7$ pore function would confer protection from 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 for development of asthma.[34] All children had at least 1 parent with a history of physician-diagnosed asthma and/or respiratory allergies. 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 documented presence of 1 or more of the following in the previous year: 1) physician 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-tern use of inhaled corticosteroids during illnesses; and 5) use of prednisone for asthma exacerbation(s). Asthma severity was assessed at the 11 year visit based on National Asthma Education and Prevention Program (NAEPP) Expert Panel Report 3 (EPR-3) criteria. For children using long-term controller medications, severity was classified by the level of treatment required for control of asthma, while children not on controller therapy were classified based upon 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 beta-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 room temperature blood was rinsed twice with HEPES-buffered

saline (HBS; 130 mM NaCl, 5 mM KCl, 20 mM HEPES, 0.1% bovine serum albumin, 10 mM glucose, pH 7.4) and incubated with CD14 conjugated to phycoerythrin (CD14-PE, BD Biosciences, San Diego, CA) in HBS for 20 minutes. Samples were rinsed twice with potassium glutamate buffer (130 mM potassium glutamate, 5 mM KCl, 20 mM HEPES, 0.1% bovine serum albumin, 10 mM glucose, pH 7.4) and incubated with 250 μM 2'(3')-*O*-(f-benzoylbenzoyl) adenosine-5'-triphosphate (BzATP; Sigma, St. Louis, MO) and 1 μM YO-PRO-1 (Molecular Probes, Eugene, OR) in potassium glutamate buffer for 20 min before addition of magnesium chloride and HBS washing. Viable CD14+ cells identified by propidium iodide exclusion were examined for YO-PRO-1 median fluorescence intensity (MFI) by bead-adjusted (BD Calibrite Beads; BD Biosciences) and calibrated (RFP-30-5A; Spherotech, Lake Forest, IL) flow cytometry on a FACSCaliber (BD Biosciences). Archived DNA from COAST participants were genotyped in the lab of Dr. Carole Ober, University of Chicago. An adult population previously genotyped for *P2RX7* and with $P2X_7$ pore function measurements was also used for comparison.[37] Using our previous methods,[39] five 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 (ROC) 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 was measured in plasma by automated fluoroenzyme immunoassay (FEIA; Unicap 100®; Pharmacia Diagnostics AB, Uppsala, Sweden). At ages 1, 3, 6, and 9, IgE was measured for 2 species of dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), *Alternaria alternata*, cat

dander, and dog. At ages 6 and 9, IgE was additionally measured for ragweed, birch, timothy grass, and cockroach.[40] Tests were considered positive for values ≥ 0.35 kU_A/L.

***Statistical analysis*:** The relationships between children's $P2X_7$ pore function MFI measured on different days and obtained at different ages were examined 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 gender or HRV 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 using the Wilcoxon rank-sum test. The number of aeroallergens sensitized was compared by pore status using 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 of YO-PRO-1 uptake was normalized by square root transformation for analysis. A 2-sided P value less than 0.05 was considered statistically significant.

RESULTS

***$P2X_7$ pore assay characteristics*:** At least one $P2X_7$ 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 (Figure 2-1A). To validate the reproducibility of our standard methods, a subset of 48 samples had pore assays performed on more than one day post-phlebotomy with an average daily coefficient of variation (CV) of 7% between the first and second day (Pearson r = 0.97, P <

0.001; Figure 2-1B). 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; $P < 0.001$; Figure 2-1C). 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 ROC analysis using 5 validated *P2RX7* LOF alleles to determine a threshold between individuals with low and normal functioning $P2X_7$ (Figure 2-1D). From this analysis, a threshold of 382 MFI was identified and used to categorize all individuals with $P2X_7$ pore assays with either low or normal $P2X_7$ pore status, indicated by the shading in Figure 2-1A. The resulting performance properties of the assay in identifying LOF alleles for *P2RX7* are shown in Figure 2-1D ($P < 0.001$, area under the curve (AUC) = 0.90). Low $P2X_7$ pore capacity was observed in 28% of COAST participants, similar to rates 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 2-I).

***$P2X_7$ function, asthma inception, and disease severity*:** To determine the association between $P2X_7$ function and childhood asthma, we stratified the COAST cohort by $P2X_7$ 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 (Figure 2-2A) at ages 6 (Odds Ratio (OR) 0.34, 95% Confidence Interval (CI) 0.15-0.79, $P = 0.01$) and 8 (OR 0.42, 95% CI 0.20-0.88, $P = 0.02$) years, but a

significant association was not observed at age 11 (OR 0.62, 95% CI 0.29-1.31, $P = 0.21$) years. To investigate whether there were any phenotypic effects in the children with asthma at age 11 years, we also stratified the severity of asthma by $P2X_7$ function. Low $P2X_7$ pore children with asthma at age 11 years had evidence of less severe asthma compared to normal $P2X_7$ pore children with asthma ($P = 0.03$; Figure 2B). However, when examined at age 6 years, $P2X_7$ pore function was not associated with asthma severity ($P = 0.29$). Children with low pore function and asthma at age 11 years were also less likely to have used a step-up short-term plan, 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, $P = 0.04$).

***$P2X_7$ function is associated with early life wheezing with HRV*:** Due to $P2X_7$'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 upon $P2X_7$ functional 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 2-II).

***Decreased asthma risk associated with attenuated $P2X_7$ function is varied by a history of HRV wheezing and gender*:** To test whether reduced asthma risk in low $P2X_7$ individuals was only due to the association with decreased early life HRV wheezing, we modeled the interaction of $P2X_7$ status and HRV wheezing on asthma diagnosis by 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 wheezing in the first 3 years of life (Figure 2-3A), and this interaction was significant at ages 6 (*P* = 0.03) and 8 (*P* = 0.01) years.

Due to different rates of asthma in boys and girls at different ages,[43] we also examined whether there was an interaction between gender and $P2X_7$ status with regard to asthma risk by logistic regression. The protective effect of low $P2X_7$ pore status was more pronounced in boys than girls (Figure 2-3B) and a significant interaction between gender and $P2X_7$ pore status was observed at ages 8 (*P* = 0.02) and 11 (*P* = 0.03) years. When only including boys in factoring asthma risk by $P2X_7$ pore status, protection from asthma in boys was significantly associated with low pore status at ages 6 (OR 0.20, 95% CI 0.06-0.64, *P* = 0.004), 8 (OR 0.19, 95% CI 0.06-0.54, *P* = 0.001), and 11 (OR 0.30, 95% CI 0.10-0.87, *P* = 0.02) years.

Decreased asthma risk associated with attenuated $P2X_7$ function is varied by a history of HRV wheezing and gender 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* = 0.04), with a similar trend at age 9 years (*P* = 0.07; Figure 2-4A). 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 mixed effects quasi-Poisson regression, low $P2X_7$ children were sensitized to fewer aeroallergens across ages 1, 3, 6, and 9 years (Figure 2-4B; mean fold change = 0.45, 95% CI 0.22-0.91, *P* = 0.03). At individual ages, low $P2X_7$ children were sensitized to significantly fewer allergens at age 6 years (*P* = 0.02) and low $P2X_7$ children trended to be sensitized to fewer allergens at ages 3 (*P* = 0.09) and 9 (*P* = 0.07) 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 ($P = 0.14$).

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 $P2X_7$ functional assay as a method to detect *P2RX7* LOF alleles (Figure 2-1). By utilizing 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 (Figure 2-2), as well as sensitization to fewer aeroallergens (Figure 2-4). 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 and/or viral infections may help determine how $P2X_7$ activity could modulate risk of chronic conditions such as asthma.

Previous studies indicate the amount of extracellular ATP may be related to airway disease severity.[25] Rather than directly measuring ATP 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 (Figure 2-1D) recapitulate that considerably more contributes to $P2X_7$ pore function than validated *P2RX7* LOF alleles and illustrates the power of our functional approach to evaluate potential gene-by-environment interactions. The COAST population has already demonstrated gene-by-environment interactions including between *IFNG* and sex[44] which may be important to *in vivo* $P2X_7$ function since IFN-γ reportedly regulates $P2X_7$.[45] Although our current results are from pre-pubertal children, they display a varied risk of

asthma by $P2X_7$ status based on gender (Figure 2-3B). 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 a decreased neutrophil infiltration in the nose during an acute cold in adults with low $P2X_7$ function.[47] Although our current study may 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 (Figure 2-1) indicates assays should be similar at any age and mitigate these potential concerns.

While low $P2X_7$ pore status protection from 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 to exacerbations may help reconcile these findings. There are significant differences in study populations: the COAST population is comprised of high risk children followed prospectively from birth, while the previous study enrolled symptomatic asthmatic adults during the peak cold season. In the children asthma was more common in boys, while in adults asthma was predominantly observed in women. It is possible the overall lack of association at age 11 years between asthma and $P2X_7$ status may 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 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 humans has yet to be measured.

How does $P2X_7$ influence asthma risk? While $P2X_7$ 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 $P2X_7$, impact DC function[27,30,49-52] and loss of $P2X_7$ function – specifically from LOF alleles detected by 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 T_{reg} and T_h17 phenotypes, is modified by nucleotide activity upon T cells and DCs, either directly or by engaging pathways associated with $P2X_7$ including the NLRP3 inflammasome or pannexin-1 and suggest that functional $P2X_7$ activation may lead to a decrease in T_{reg} populations.[53-57] A DC-focused role of $P2X_7$ is supportive of an amplified response to infections or allergens when co-mingled 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 may not always be beneficial or harmful in the immune response; the role of $P2X_7$ may be different when comparing disease inception to 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] while another report suggests $P2X_7$ may 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 individuals at altered risk for disease and should be considered when further studying the role of danger signaling in disease pathogenesis.

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Table 2-I. $P2X_7$ function is independent of asthma risk factors. Subject characteristics were stratified by $P2X_7$ function.

Birth and year 1 risk factors	**$P2X_7$ pore function**		***P* value**
	Low (n = 48)	**Normal** (n = 124)	
Maternal asthma ever	37%	38%	0.90
Paternal asthma ever	28%	34%	0.49
Smoke exposure in year 1	27%	24%	0.69
Day care attendance in year 1	50%	50%	1.00
Exclusive breastfeeding during first 6 months	38%	35%	0.73
Dog in home at birth	40%	33%	0.42
Cat in home at birth	27%	32%	0.51
Older siblings	54%	57%	0.71
Active atopic dermatitis in year 1	30%	26%	0.59
Birth month	--	--	0.62
Birth weight ± standard deviation (oz)	123 ± 16	125 ± 19	0.96
Male gender	60%	56%	0.64

Table 2-II. P2X$_7$ and early life wheezing. Wheezing illnesses in the first 3 years of life were compared between low and normal P2X$_7$ pore groups.

Wheezing illnesses in first 3 years	P2X$_7$ pore function		OR [95% CI] (Low Pore)	*P* value
	Low (n = 48)	Normal (n = 124)		
Any cause	44%	52%	0.71 [0.36-1.38]	0.31
RSV detected	19%	31%	0.50 [0.22-1.14]	0.10
HRV detected	19%	40%	0.35 [0.16-0.79]	0.01

RSV – respiratory syncytial virus

HRV – human rhinovirus

OR – odds ratio

95% CI – 95% confidence interval

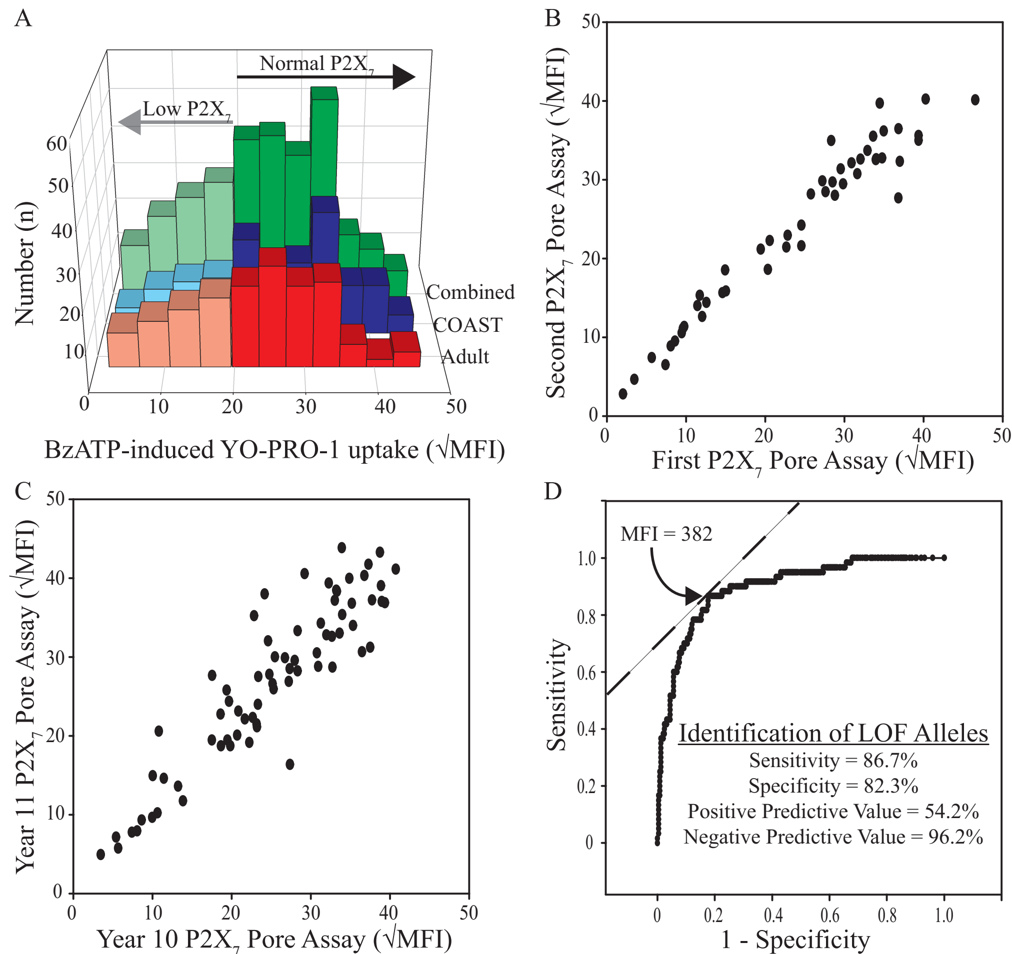

Figure 2-1. $P2X_7$ pore assay characteristics. A) $P2X_7$ pore assay distributions for COAST (n = 172) and adult populations (n = 156) are shown. Lighter shading indicates low $P2X_7$ function. $P2X_7$ pore assays are shown for B) the same sample at different days post-phlebotomy ($r = 0.97$, $P < 0.001$) and C) at different subject ages ($r = 0.91$, $P < 0.001$). D) A ROC curve ($P < 0.001$, AUC = 0.90) is shown with characteristics for identifying *P2RX7* LOF alleles based on the optimized threshold of 382 MFI, indicated as the labeled point in the upper left corner maximizing both sensitivity specificity.

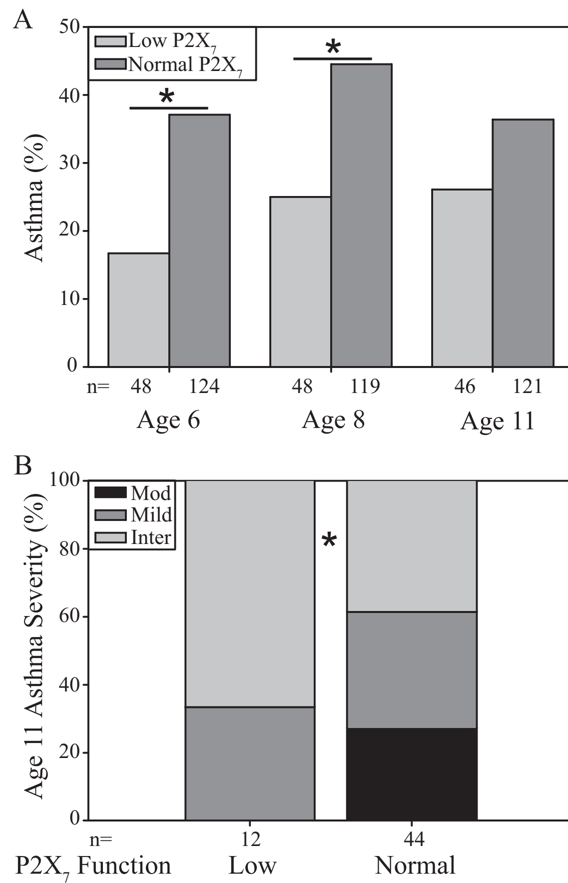

Figure 2-2. Low $P2X_7$ pore function is protective from asthma development. A) The rates of asthma at ages 6, 8, and 11 are shown for low and normal $P2X_7$ status. B) Asthma severity at age 11 is shown stratified by $P2X_7$ pore status. Inter = intermittent, Mod = moderate. n = number in each group. * indicates $P < 0.05$.

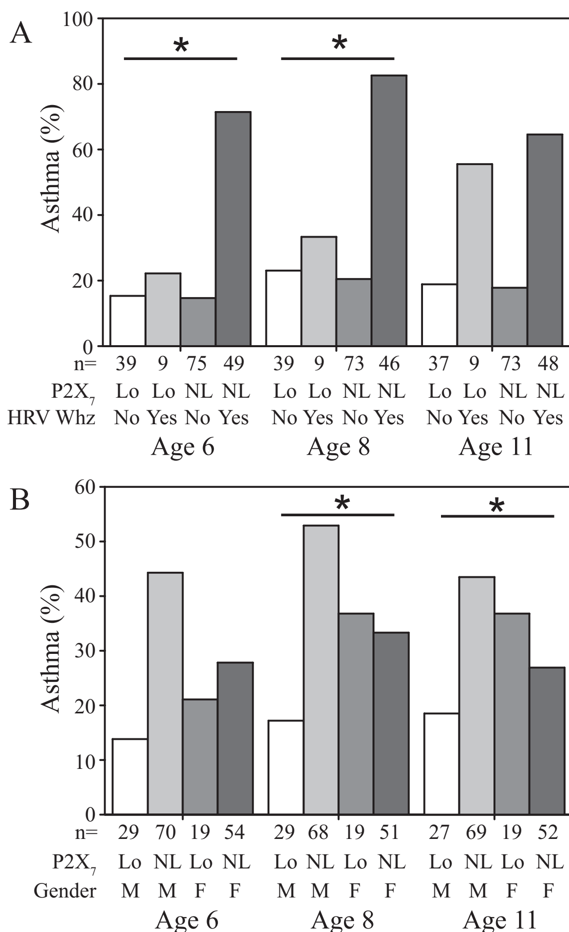

Figure 2-3. Interactions of $P2X_7$ with HRV wheezing and gender. Both A) $P2X_7$ status and HRV wheezing in the first 3 years of life and B) $P2X_7$ status and gender displayed an interactive effect on asthma risk. * indicates $P < 0.05$ for logistic regression model interaction terms. Lo = Low $P2X_7$ function, NL = Normal $P2X_7$ function, HRV Whz = Wheezing with HRV during first 3 years of life, M = male, F = female, n = number in each group.

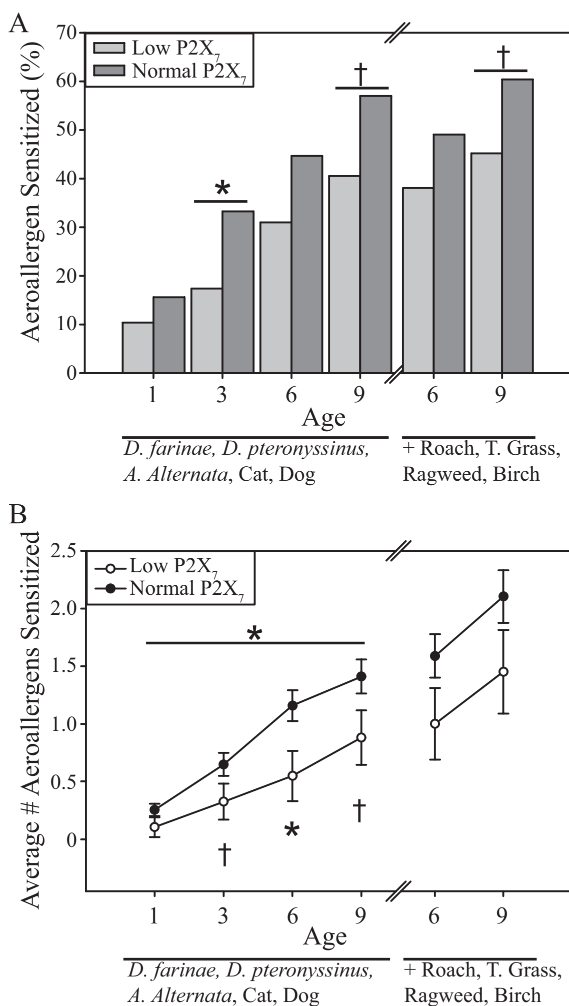

Figure 2-4. Aeroallergen sensitization status by $P2X_7$ function. A) The prevalence of children with positive sensitization to any aeroallergen measured is stratified by $P2X_7$ function. B) The average number of allergens each subject was sensitized to was stratified by $P2X_7$ pore activity. For both panels, aeroallergens measured during all 4 ages are shown on the left and inclusion of additional aeroallergens measured at ages 6 and 9 is shown on the right. Error bars are the standard error of the mean. * indicates $P < 0.05$, † indicates $P < 0.1$.

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CHAPTER THREE

Acute cytokine profiles during symptomatic colds predict asthma exacerbations

David M. Manthei, M.S., Elizabeth A. Schwantes, Sameer K. Mathur, M.D., Ph.D., Arturo G. Guadarrama, Elizabeth A. Kelly, Ph.D., Nizar N. Jarjour, M.D., Loren C. Denlinger, M.D., Ph.D.

University of Wisconsin-Madison

Department of Medicine

ABSTRACT

Background: Asthmatic and healthy individuals contract viral upper respiratory tract infections (URIs) at similar rates and suffer similar burdens, but asthmatic individuals may progress to more severe sequelae including exacerbations. Since asthma exacerbations contribute significantly to the health care burden, identifying unique features of upper respiratory infections continuing to exacerbations could lead to interventions preventing exacerbations. We therefore sought to analyze measurable cytokines in nasal lavage fluid (NLF) as a marker for future exacerbations.

Methods: 59 subjects with upper respiratory symptoms were used for analysis of cytokines in NLF during acute and resolution periods of natural colds. NLF samples corresponding to peak and trough nasal neutrophil levels were used for cytokine measures. Analyte protein levels were measured by ELISA and Luminex.

Results: Acute cytokine elevation with viral URIs was similar between asthmatic and non-asthmatic individuals. Elevated cytokines with viral URIs included IFN-γ, MCP-1, IL-10, TNF-α, VEGF, MMP-9, TRAIL, IL-1β, G-CSF, IL-8, and CXCL13. Acute levels of VEGF, TNF-α, and IL-1β were elevated in asthmatics who exacerbated compared to asthmatics without an exacerbation with a corresponding shorter time-to-exacerbation.

Conclusions: Cytokine generation in the nose in response to virus infection is similar between asthmatic and control individuals, indicating an appropriate response in asthmatics. However, asthmatics with higher levels of IL-1β, TNF-α, or VEGF may be at risk for exacerbation linked to a viral URI.

INTRODUCTION

Individuals with asthma acquire a similar number of viral upper respiratory tract infections (URIs) compared to healthy individuals and yet have an increased risk of lower respiratory symptoms during and following illness.[1,2] Asthma exacerbations contribute significantly to morbidity, mortality, and utilization of health care resources.[3] As upper respiratory symptoms often precede and are thought to contribute to asthma exacerbations,[4,5] determining and preventing cold-associated factors leading to exacerbations would provide a useful target for prevention. Multiple components, including both the initial host immune response to virus and the secondary reaction to those immune responses, may be involved in differences in cold severity and the pathogenesis of exacerbations in airway disease.[6]

Previous studies of viral colds depict similar features for both persons with asthma and otherwise healthy individuals. Cohabitation of asthmatic and non-asthmatic adults revealed no difference in symptoms from naturally transmitted URIs with similar infection rates and additional studies have demonstrated comparable upper respiratory symptoms regardless of asthma[1,7] while analogous results have been noted in pediatric populations.[8] Like naturally acquired colds, experimental inoculations with human rhinovirus (HRV) have demonstrated similar duration and severity of URIs irrespective of asthma status.[9,10] The progression of viral infections from upper to lower airways is implicated in the asthmatic response to colds and viral presence in lower airways may have a role in exacerbations.[11] The magnitude of HRV burden has been associated with disease severity, particularly in asthmatics,[12,13] although there is evidence to the contrary[14] and emerging work describes particular HRV groups contributing to more severe disease.[15] The host response has also been implicated in URI symptoms[16] while specific cells including airway epithelial cells from asthmatics have diminished innate immune

function in response to HRV *in vitro* by impaired production of interferons (IFNs)[17,18] and unclear relationships of IFN-λ to asthma exacerbations have been reported.[8,19] Although informative and provocative, these studies leave an incomplete picture of the host response to natural infections.

While the majority of viral URIs are caused by HRV and appropriately induce an antiviral response, whether a specific pathogen or host factor plays the defining role leading to asthma exacerbations is still an unresolved issue.[20] Airway neutrophils are associated with both viral infections and asthma exacerbations and many cytokines produced in inflammation play a role in cell recruitment to the airways.[21] Identification of differences in asthmatic host production of cytokines in the upper airway in response to infection has proved elusive to date.[22] Since we recently documented that both the peak and change in nasal neutrophils predict exacerbations,[23] we hypothesized that associated cytokine signatures in the upper airway at this time would also be predictive of virus-induced events inducing exacerbations.

In this study, we have characterized cytokine levels in nasal lavage fluid (NLF) of individuals with upper respiratory symptoms and following symptomatic recovery. We observe that there are not differences in induced cytokine production in response to viral URIs due to the presence or absence of underlying asthmatic disease. The differences in disease-specific responses may instead be in how the host responds to appropriately induced cytokines, including vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), as we report elevated levels in asthmatics with an asthma exacerbation. Measuring these or other markers may help identify asthmatics who would benefit from additional or more aggressive disease management when presenting with upper respiratory symptoms.

METHODS

Subject recruitment and characterization: Studies were performed with approval of the University of Wisconsin-Madison Health Sciences Institutional Review Board (approval H-2005-0070) and with subject written and informed consent. Adult subjects were recruited during peak URI seasons with symptomatic colds. Participants were characterized for asthma and asthma exacerbations as reported.[24,25]

Airway sample collection, cell differential, and pathogen determination: Samples were collected and processed as reported previously.[24]

Cytokine measurement: For each patient one acute NLF sample with the highest neutrophil count and one resolution sample with the lowest neutrophil count were selected for analysis. NLF was analyzed for immune factors in the following categories: anti-viral (IFN-α, -β, -γ, -λ1, TRAIL, IP-10), cell recruiting (G-CSF, TNF-α, MCP-1, MCP-3, IL-1β, IL-8), polarizing (IL-17, CXCL13, IL-10, TSLP, IL-13), and remodeling/other (VEGF, IL-33, MMP-9, fibronectin). For each subject, the acute and baseline samples were analyzed on the same plate in duplicate. IFN-α and -β were measured by ELISA using manufacturer suggestions (PBL Interferon Source, Piscataway, NJ). Additionally, fibronectin, IL-8, MMP-9, and IFN-λ1 were analyzed by ELISA with established methods[26] and the remaining analytes were measured by Luminex using manufacturer instructions (Millipore, Billerica, MA). To compare values from multiple plates, the limits of detection were determined as the upper 95% confidence interval of the mean calculated blank concentrations. For continuous measurements, samples below the limit of

detection were analyzed at the limit of detection. Upper limits were defined as the highest concentration standard and measures over the limit were set as the highest standard.

Data analysis: All cytokine levels and cell counts were log-transformed for analysis. Analytes were treated as a continuous variable if at least 25% of the samples had values above the limit of detection. Cytokines with less than 25% detection were analyzed as categorical variables. ANOVA, student's t-test, and χ^2 comparisons and Wilcoxan rank-sum and Kaplan-Meier tests were performed as appropriate with a 2-sided significance value of 0.05. In data reduction, Bonferroni correction for multiple comparisons was used. Statistical analyses were performed using JMP 9 (SAS, Cary, NC) and Sigmaplot 11 (SysStat Software, San Jose, CA).

RESULTS

Subject characteristics and sample analysis: 66 published subjects were enrolled in the study as previously described.[24] 59 subjects had adequate NLF samples. These included 13 non-asthmatics with an acutely detectable virus and 46 asthmatics, 35 of whom had an acutely detectable virus. Characteristics were not different between this and the published population over the entire study period for outcomes including detection of any virus, detection of HRV, and asthma exacerbation (p = 0.61, 0.84, 0.98, respectively). Asthmatics with upper respiratory symptoms but no acute virus detected had fewer peak neutrophils (0.86 ± 1.6 (million cells/mL; mean ± SD)) compared to either asthmatics with viral URIs (3.43 ± 6.46; p = 0.006) or non-asthmatics with viral URIs (5.00 ± 15.29; p = 0.03), but there was no difference at resolution (0.05 ± 0.07, 0.09 ± 0.13, 0.05 ± 0.08, respectively; p = 0.63). 22 subjects exacerbated during the study (48% of asthmatics) and 73% (n = 16) of subjects with exacerbations had acute NLF

samples analyzed for cytokines prior to the exacerbation. The cold day of acute NLF sample analysis was not different between the aforementioned groups (6 [3-16] (median cold day [interquartile range]); 3 [2-6]; 2 [2-5], respectively; ANOVA p = 0.13)

Cytokine detection: The detection limits for each cytokine and the proportion of subjects with detectable analyte are shown in Supplemental Table 3-I. Fibronectin and MMP-9 detection limits are shown as effective limits due to sample dilution. Acute IP-10 measures were greater than the upper limit of the Luminex standard curve in 66% of subjects and not included in analysis other than noted. For the remaining cytokines, less than 10% of samples were above the upper standard for any analyte. One plate of IFN-α was not included in analysis due to poor standard curve characteristics. TSLP was not detected in any sample and not included in further analysis.

***Cytokines are elevated with viral URIs*:** With 19 analytes remaining, comparisons between acute and baseline cytokines measures were Bonferroni corrected for multiple comparisons, requiring p = 0.0026 between acute and recovery levels for inclusion in continued analysis. For viral URIs, significantly elevated levels were observed for all continuously analyzed cytokines (Table 3-I). The proportion of detectable analyte was greater for both MCP-3 and IL-17 during viral URIs but did not maintain significance when correcting for multiple comparisons. Detection rates of IFN-α, -β, and -λ1 were not increased with acute viral colds compared to resolution, even when examining only non-asthmatic subjects (p = 0.61, 1.00, 1.00, respectively).

Similar methods were used to analyze the cytokine changes in asthmatics with cold symptoms but no detectable virus. We excluded fibronectin from further analysis because acute levels were also elevated compared to baseline in virus-negative colds ($p = 0.0037$). TRAIL, G-CSF, and MCP-1 were also increased at acute visits ($p = 0.02, 0.02, 0.02$, respectively), but did not meet the multiple comparisons criteria for exclusion.

Cytokines IL-1β and IFN-γ were also analyzed *post-hoc* as categorical variables due to detection rates of less than 50%. Categorical analysis demonstrated the same results as our continuous analyses. IP-10 was elevated 3.4 fold over baseline with viral URIs ($p < 0.0001$).

Elevated acute cytokines in viral URIs are not different with asthma: For virus-positive URIs, cytokines measured at acute visits were not significantly different between individuals with or without asthma for any analyte (Supplemental Table 3-II).

Elevated cytokines in asthmatics with subsequent exacerbations: We examined the remaining cytokines as candidates for altered levels in asthmatics based on the occurrence of an exacerbation in the first 3 weeks of the cold (Table 3-II). VEGF, TNF-α, and IL-1β were elevated in acute NLF in subjects with asthma who had an exacerbation compared to non-exacerbators.

Receiver operating characteristic (ROC) curve analysis for each of the 3 cytokines identifying exacerbations is shown in Figure 3-1. The threshold for VEGF was equal at 2 distinct values. The lower threshold for VEGF (105 pg/mL) and the threshold for IL-1β, corresponded all detectable samples for each cytokine.

A time-to-exacerbation analysis in asthmatics based on ROC thresholds from self-reported cold onset is shown in Figure 3-2, and was significant for elevated VEGF (either threshold $p = 0.03$) or TNF-α ($p = 0.04$) and a trend with IL-1β ($p = 0.07$). When determining time-to-exacerbation from the day of NLF cytokine measurements to exacerbation (and thereby excluding exacerbations prior to sample collection), both TNF-α and the upper VEGF threshold (157 pg/mL) remained significant ($p = 0.04$, $p = 0.03$, respectively) while IL-1β detection remained a trend for more rapid exacerbations ($p = 0.09$).

DISCUSSION

Our study reinforces the similarity of early host responses to viral URIs in both asthmatic and non-asthmatic individuals as we assessed numerous inflammatory mediators in the nasal passage during symptomatic upper respiratory illnesses. We did not observe a difference in acute induction of cytokines between subjects with viral URIs, whether having asthma or not, similar to previous work.[14] In noting similar inflammatory mediator levels between non-asthmatic and asthmatic individuals it appears those with asthma have an adequate host response to infection but perhaps a different response to appropriately induced mediators. Cytokines including VEGF and TNF-α and perhaps IL-1β may have more perilous downstream effects in asthmatics leading to a predisposition for asthma exacerbations.

A general increase in NLF cytokines during viral URIs has been reported in previous studies. Most of the analytes we measured were elevated or detectable more often during viral URIs (Table 3-II). We did not observe differences between acute and resolution samples or between subjects with and without asthma in Type I and III IFN, unlike previous reports,[8,17-19] although our detection rates for IFNs, ranging 6-21% for IFN-α, -β, and -λ, and 45% for IFN-γ,

were similar to those measured following inoculation with HRV16.[9] Similar to other inoculation models, when a viral URI was observed we detected increased IFN-γ, G-CSF, IL-8, and IL-1β,[27-30] all factors implicated in part of neutrophil recruitment, consistent with our NLF sample selection. Our viral URI cytokine measures further correspond with cytokine production by *in vitro* HRV infection with elevated IL-8, IL-1β, MCP-1, MMP-9, and VEGF.[31-36] Furthermore, we noted increased cytokines similar to those during natural viral infections in a cohort of year-long monitored subjects, including IL-1β, TNF-α, IL-8, MCP-1, and IFN-γ.[37] Similar cytokine production following infections by non-HRV viruses are also in agreement with our predominantly HRV viral exposure.[38,39] Viral URIs appear to induce similar responses in host NLF cytokine generation, unaltered by underlying asthmatic disease; however, we suggest IL-1β, VEGF, or TNF-α levels in NLF of subjects with asthma is preferentially elevated in individuals who will exacerbate.

We measured increased IL-1β with viral URIs consistent with previous studies[30] but observed no difference in IL-1β induction based upon asthma status, unlike other previous findings.[27,40] Yet we observed increased IL-1β levels in asthmatics who exacerbated (Table 3-II), with a trend for occurrence of exacerbations based on IL-1β detection. Possible mechanisms involving IL-1β changes leading to exacerbations are intriguing and include links to generation of neutrophil chemokines and response to HRV infection[41] and modulation by nucleotide receptor $P2X_7$.[23] Although our study was limited by relatively modest proportion samples with detectable IL-1β, these results indicate further evaluation in a larger population is warranted.

The observation of remodeling in airway disease has led to a body of work evaluating angiogenic factors in asthma.[42] VEGF is a powerful factor in angiogenesis and is induced by HRV in various cells.[36] VEGF measured in NLF of adults during an asthma exacerbation was

elevated with a protracted return to baseline levels.[43] In children VEGF was greater during acute asthma in NLF and sputum samples compared to baseline.[36,44] However, comparisons have not been made to distinguish differences during acute upper respiratory symptoms, of which only some asthmatics will go on to have an exacerbation. Our results indicate VEGF is an appropriate candidate as a marker for exacerbations and may help elucidate a potential subset of asthmatics more prone to exacerbation.

TNF-α, an established target in autoimmune diseases, has been studied as a potential mediator of asthma, particularly in severe refractory disease.[45] Subjects with severe asthma have increased soluble TNF-α in bronchiolar lavage fluid and membrane-bound TNF-α on peripheral blood mononuclear cells compared to control and mild-to-moderate asthmatics. In these severe asthmatics, etanercept improved symptoms, lung function, and airway hyper-responsiveness[46,47] although in complementary studies only small or absence of benefits were observed with etanercept treatment.[48,49] A study of severe asthmatics treated with another anti-TNF-α agent, golimumab, was discontinued early, reflecting concerns with anti-TNF-α side effects, and main study outcomes were not different between treatment groups. However, secondary analysis revealed a significant difference in exacerbation free days in a subgroup with >12% baseline FEV_1 reversibility.[50] Of consideration in these latter studies is the lack of measuring TNF-α, raising the question of whether optimal sub-groups were receiving therapy. Additionally, extended study periods might be appropriate for an exacerbation-focused endpoint. While our study included only mild asthmatics, and an acute cold produces elevated airway neutrophils, this provides evidence to bolster the prospect of TNF-α as a target in select subjects for prevention of exacerbations.

Overall, it appears that the presence of virus drives inflammation and NLF cytokine increases during acute colds regardless of asthmatic disease. How an individual with asthma and any other underlying disease factors interplay with these immune responses is an important consideration. Future studies should focus on how the host responds to increases in these identified factors.

ACKNOWLEDGEMENTS

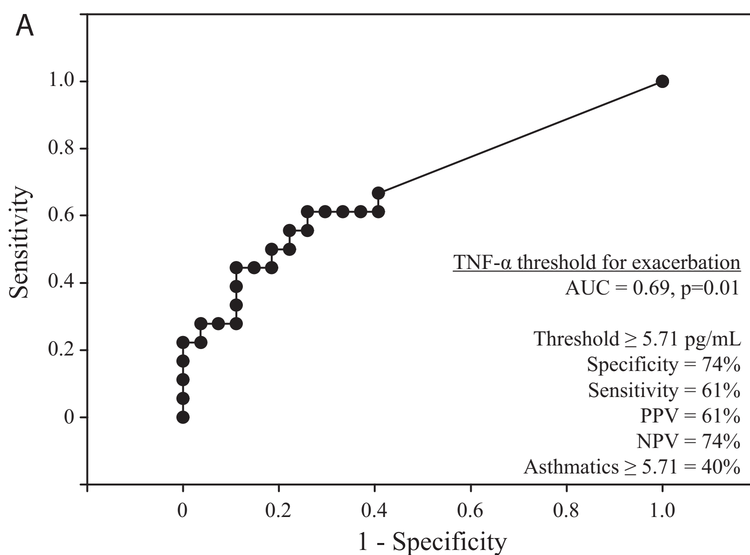
We would like to thank the subjects and coordinators, without whom the study would not be possible.

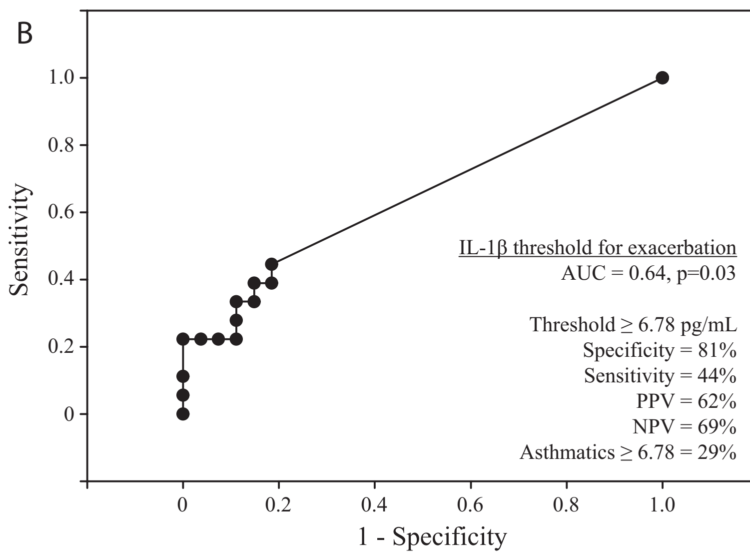
Table 3-I: Changes in cytokine concentrations in NLF during viral URIs. Correction for multiple comparisons established significance of a P value of 0.0026. For continuous variables values are mean ± standard deviation and for categorical variables the percent of subjects with detectable samples are shown. n=47, except IFN-α n=33.

		Cytokine	Acute	Resolution	P-value
Continuously analyzed	pg/mL	IFN-γ	64.5 ± 109	12 ± 0	<0.0001
		TRAIL	3288 ± 2827	418 ± 250	<0.0001
		G-CSF	2315 ± 2500	55.0 ± 83.5	<0.0001
		TNF-α	14.1 ± 30.6	1.5 ± 0.6	<0.0001
		IL-1β	23.6 ± 70.7	6.7 ± 0.5	0.001
		MCP-1	309 ± 367	48.9 ± 29.4	<0.0001
		IL-8	92.7 ± 59.8	46.1 ± 36.5	<0.0001
		IL-10	127 ± 225	5.8 ± 1.3	<0.0001
		CXCL13	82.0 ± 88.7	44.2 ± 105.6	<0.0001
		VEGF	230 ± 245	101 ± 18.9	<0.0001
	ng/mL	FN	15163 ± 10925	6253 ± 4626	<0.0001
		MMP-9	1096 ± 1233	2801 ± 544	<0.0001
Categorically analyzed	% Detectable	IFN-α	21	15	0.52
		IFN-β	17	15	0.78
		IFN-λ1	6	2	0.31
		MCP-3	21	4	0.01
		IL-13	6	4	0.65
		IL-17	21	2	0.004
		IL-33	6	0	0.08

Table 3-II: Cytokine levels of asthmatics with symptomatic upper respiratory symptoms. Comparison of candidate cytokines levels in acute NLF in asthmatics grouped by presence or absence of exacerbation in first 3 weeks of upper respiratory symptoms. Cytokine values are mean ± standard deviation, pg/mL, except for MMP-9, which are ng/mL. p-values are from log-transformed concentrations.

Cytokine	**Exacerbation** (n=18)	**No Exacerbation** (n=27)	**P-value**
IFN-γ	51.7 ± 107	50.4 ± 105	0.95
TRAIL	3321 ± 3245	2485 ± 2630	0.32
G-CSF	2861 ± 2619	1147 ± 1513	0.16
TNF-α	23.0 ± 47.9	4.5 ± 4.6	0.01
IL-1β	37.2 ± 110	7.9 ± 3.3	0.048
MCP-1	353 ± 509	197 ± 235	0.13
IL-8	81.1 ± 59.2	75.3 ± 52.7	0.72
IL-10	125 ± 318	87.8 ± 140	0.66
CXCL13	98.2 ± 107	66.6 ± 67.7	0.22
VEGF	251 ± 153	151 ± 84.5	0.006
MMP-9	1445 ± 1568	858 ± 1156	0.52


A
Sensitivity
1.0
0.8
0.6
0.4
0.2
0
TNF-α threshold for exacerbation
AUC = 0.69, p=0.01
Threshold ≥ 5.71 pg/mL
Specificity = 74%
Sensitivity = 61%
PPV = 61%
NPV = 74%
Asthmatics ≥ 5.71 = 40%
0 0.2 0.4 0.6 0.8 1.0
1 - Specificity


B
Sensitivity
1.0
0.8
0.6
0.4
0.2
0
IL-1β threshold for exacerbation
AUC = 0.64, p=0.03
Threshold ≥ 6.78 pg/mL
Specificity = 81%
Sensitivity = 44%
PPV = 62%
NPV = 69%
Asthmatics ≥ 6.78 = 29%
0 0.2 0.4 0.6 0.8 1.0
1 - Specificity

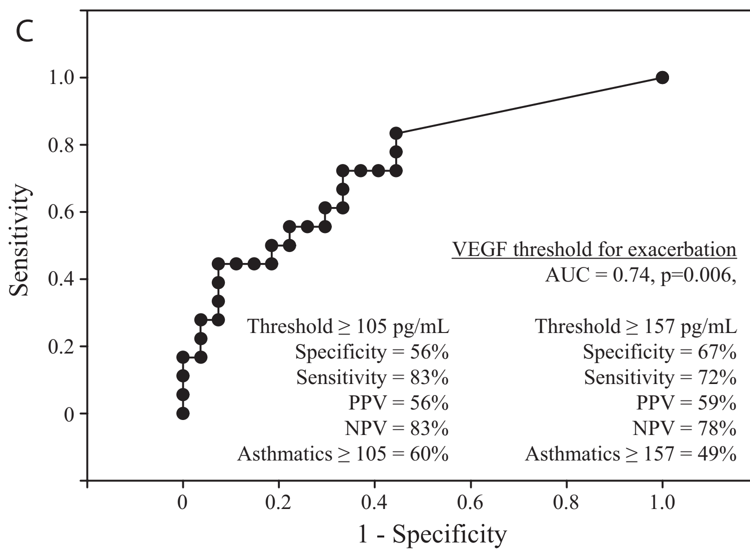

C
Sensitivity
1.0
0.8
0.6
0.4
0.2
0
VEGF threshold for exacerbation
AUC = 0.74, p=0.006,
Threshold ≥ 105 pg/mL
Specificity = 56%
Sensitivity = 83%
PPV = 56%
NPV = 83%
Asthmatics ≥ 105 = 60%
Threshold ≥ 157 pg/mL
Specificity = 67%
Sensitivity = 72%
PPV = 59%
NPV = 78%
Asthmatics ≥ 157 = 49%
0 0.2 0.4 0.6 0.8 1.0
1 - Specificity

Figure 3-1: Determination of acute cytokine thresholds for asthma exacerbations. Receiver operating characteristic curve analysis is shown for (A) TNF-α, (B) IL-1β, and (C) VEGF in the identification of asthmatics exacerbating in the first 3 weeks of the study. The threshold for each analyte is based on the described points chosen by Youden's J statistic. Performance characteristics for the identified thresholds and the proportion of asthmatics above the threshold are shown. AUC = area under the curve, PPV = positive predictive value, NPV =negative predictive value.

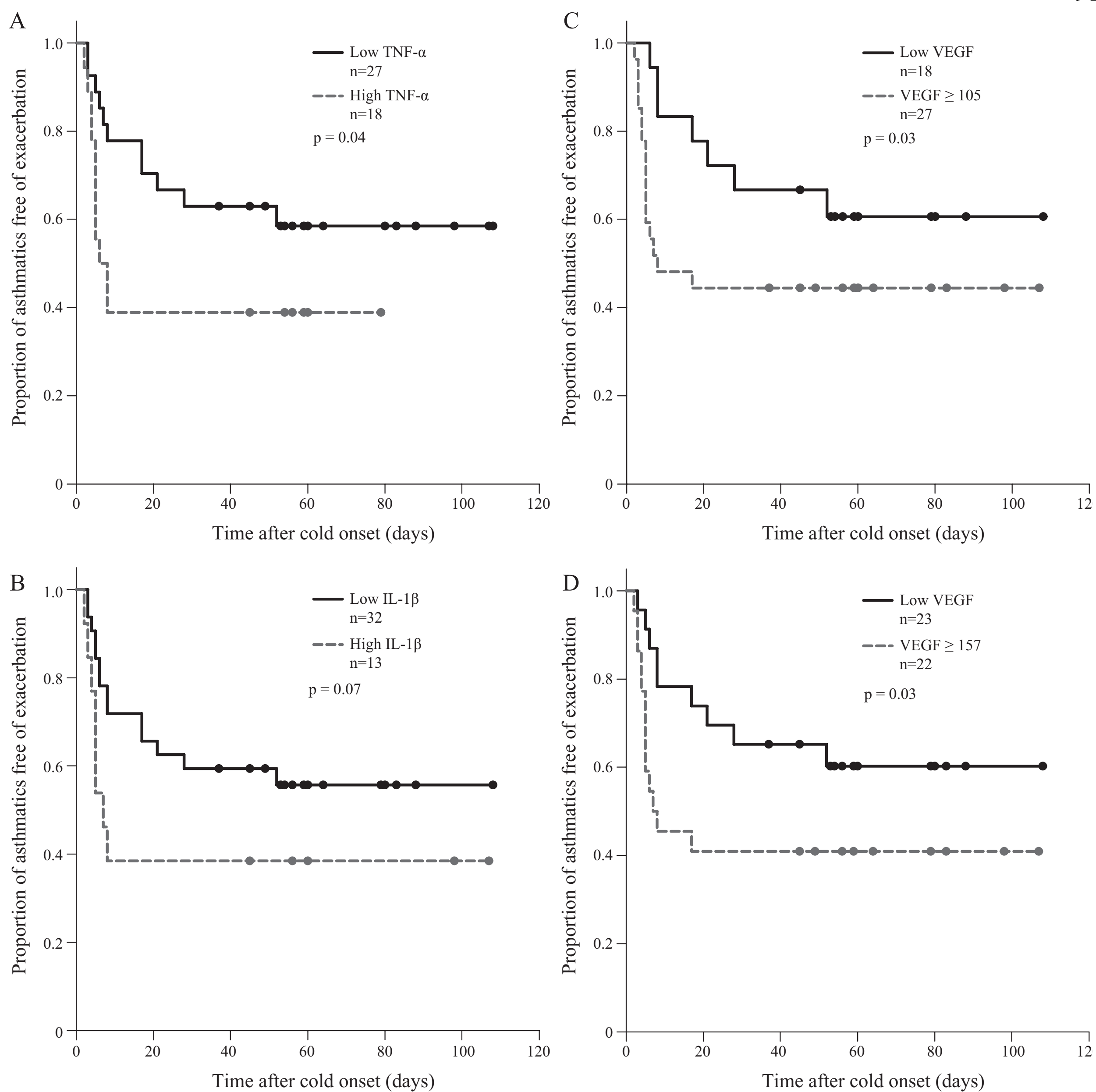


Figure 3-2: Time to exacerbation after cold symptoms. Asthmatic time-to-exacerbation is shown for (A) TNF-α, (B) IL-1β, and (C, D) VEGF based on the receiver operating characteristic thresholds for each analyte. The time is from the self-reported cold onset. Wilcoxan p-values for Kaplan-Meier analysis are displayed.

Supplemental Table 3-I: Detection rates and thresholds for each analyte measured.

Cytokine	**Acute** (% Detected)	**Resolution** (% Detected)	**Threshold**
IFN-α	17	19	8.5 pg/mL
IFN-β	16	17	66.5 pg/mL
IFN-γ	38	0	12 pg/mL
IFN-λ1	5	2	50 pg/mL
IP-10	100	98	50 pg/mL
TNFα	55	5	1.4 pg/mL
MCP-3	17	5	15.6 pg/mL
IL-1β	31	3	6.6 pg/mL
IL-10	66	7	5.6 pg/mL
IL-13	05	5	6.1 pg/mL
IL-17	17	2	3.3 pg/mL
IL-33	05	0	35 pg/mL
VEGF	62	31	92 pg/mL
TSLP	0	0	10.5 pg/mL
TRAIL	100	100	13.5 pg/mL
G-CSF	98	84	9 pg/mL
MCP-1	100	93	16.1 pg/mL
IL-8	100	98	6 pg/mL
CXCL13	97	84	3 pg/mL
Fibronectin	100	100	300 ng/mL
MMP-9	95	91	12 ng/mL

Supplemental Table 3-II: Comparison of acute cytokines by asthma status and viral detection. Values are mean ± standard deviation and p-values are from log-transformed measures.

		Cytokine	Asthma + Virus -	Asthma + Virus +	Non-asthma Virus +	p-value		
						ANOVA	t-test: Virus+	t-test: Asthma
Continuously analyzed	pg/mL	IFN-γ	14.5 ± 8.4	62.7 ± 118.3	69.2 ± 84.2	.07	.31	.08
		IP-10	4815 ± 3690	8423 ± 2816	9237 ± 2180	.0005	.49	.0003
		TRAIL	1337 ± 1691	3299 ± 3044	3259 ± 2273	.02	.38	.02
		G-CSF	562 ± 796	2244 ± 2322	2499 ± 3014	.004	.86	.001
		TNF-α	2.0 ± 1.3	15.1 ± 35.6	11.5 ± 9.6	.01	.60	.006
		IL-1β	7.7 ± 3.6	23.5 ± 80.1	23.8 ± 39.0	.34	.43	.32
		MCP-1	84.3 ± 61.6	316 ± 412	288 ± 219	.01	.54	.009
		IL-8	52.8 ± 53.9	85.6 ± 53.4	111.3 ± 73.1	.006	.22	.009
		IL-10	12.5 ± 22.1	131.9 ± 253.8	113.1 ± 130.2	.01	.73	.007
		CXCL13	86.4 ± 117.8	76.9 ± 74.7	95.3 ± 120.7	.92	.90	.74
		VEGF	143.9 ± 75.2	206.2 ± 135.3	291 ± 416.8	.32	.64	.21
	ng/mL	Fibronectin	14274 ± 8943	15941 ± 11500	13128 ± 9363	.89	.63	.91
		MMP-9	960 ± 1462	1136 ± 1328	992 ± 980	.33	.87	.14
Categorically analyzed	% Detectable	IFN-α	0	18	27	.29	.55	.20
		IFN-β	10	18	15	.84	.85	.56
		IFN-λ1	0	6	8	.67	.82	.41
		MCP-3	0	35	38	.20	.54	.08
		IL-13	0	9	0	.33	.27	.31
		IL-17	0	15	38	.04	.08	.18
		IL-33	0	9	0	.33	.27	.31

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CHAPTER FOUR

Rhinovirus-induced IL-1β release from bronchial epithelial cells is independent of functional $P2X_7$

Lei Shi[1,*], David M. Manthei[1,2,*], Arturo G. Guadarrama[1], Lisa Y. Lenertz[3], Loren C. Denlinger[1]

University of Wisconsin-Madison.

[1]Department of Medicine, [2]Cell & Molecular Biology Program, [3]Department of Biomolecular Chemistry

[*] Denotes equal contribution as co-first authors

ABSTRACT

Airway epithelial cell defenses to viral infections are often compromised in disease or injury. Danger molecules including ATP are released during infection and contribute to nucleotide receptor dependent inflammatory responses largely through $P2X_7$. Although respiratory epithelium has been shown to express a variety of nucleotide receptors, the functional contribution of $P2X_7$ to the epithelial cell inflammatory response is unclear. We utilized human donor bronchial epithelial cells (BECs) and primary brushed epithelium to explore responses upon nucleotide and Toll-like receptor stimulation. $P2X_7$ mRNA and protein were observed in unprimed BECs while inflammatory cytokine stimulation increased both mRNA and protein. Functional pore activity characteristic of $P2X_7$ was observed in BECs and IL-1β was rapidly released by BECs after TLR3 agonist Poly (I:C) priming followed by ATP administration, although no change was observed in IL-18 release. BECs produced more IL-1β after stimulation with Poly (I:C) than LPS, showing a different preferential response than monocytes. Additionally, blockade of nucleotide receptors with oATP significantly increased human rhinovirus (HRV) recovered 24 h after infection in BECs while BzATP treatment of brushed epithelial cells and respiratory cell lines non-significantly decreased HRV recovery. IL-1β release was detected after HRV infection in both BECs and brushed cells but BzATP did not significantly further increase IL-1β release. BEC processing of pro-IL-1β to the mature, cleaved 17 kDa form was confirmed by Western blotting. These results support the expression of functional $P2X_7$ in human lung epithelium, although its role in epithelial pathogen defense is likely independent of IL-1 family cytokine processing.

INTRODUCTION

Respiratory epithelium provides a complex physical barrier against foreign material and is the first site of exposure to pathogens. When infected, respiratory epithelium may serve as a primary site of replication. Once a cell detects an invasion, it may attempt to destroy the pathogen or signal for help to prevent other cells from succumbing to its fate.[1] Airway epithelial cells are sources of inflammatory cytokines including IL-1β and IL-18, as detailed in reviews[2-4] and sometimes generated by unclear mechanisms upon exposure to cigarette smoke[5] or other injury models including bleomycin.[6] Epithelium also respond to a host of other cytokines including tumor necrosis factor-α (TNF-α) and INF-γ present during both viral and gram-negative bacterial infections. Efficient release of the IL-1 family of cytokines involves synthesis induced by ligand activation of Toll-Like Receptors (TLRs) and $P2X_7$-regulated activation of caspase 1 leading to proteolytic cleavage.[7] Previous studies in rodent epithelial cells document mRNA and protein expression of $P2X_7$, as well as characteristic, non-desensitizing, ATP-induced cation flux, but have not addressed the ability of this receptor to form pores and concomitant capacity for IL-1 family processing.

The IL-1 family of cytokines is important in the pathology of asthma. Airway hyperresponsiveness was reported to be attenuated with IL-1 blockade[8,9] and severe asthmatics demonstrated increased IL-1β in the sputum compared to non-severe asthmatics.[10] IL-33 has been linked to Th2 cytokines and was reported to have increased expression in severe asthma.[11] Aditionally, IL-18 knockout mice demonstrated decreased inflammation and remodeling during ovalbumin challenge models.[12] Since multiple members of the IL-1 family have been shown to be modulated in asthmatic phenotypes, an upstream regulator of IL-1 processing is an attractive investigational tool and target.

ATP has emerged as a mediator of lung inflammation in animal models and clinical studies of patients with asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and idiopathic pulmonary fibrosis.[13-16] Extracellular ATP, acting as a danger signal, is released as a consequence of cell damage, stress, infection, allergen, tobacco, or platelet degranulation due to inflammation.[17] Despite rapid metabolism, ATP and ADP act on a family of nucleotide receptors expressed by epithelial cells and leukocytes to activate danger-signaling pathways as an adjunct to other cellular responses involved in both the innate and adaptive immune responses.[13,18]

Various P2 purinergic receptors have been implicated in the airway responses to ATP. Respiratory epithelial $P2Y_2$ (a heptohelical, G-protein coupled receptor) has been linked to water efflux and targeted as a potential therapeutic pathway in cystic fibrosis.[19] $P2Y_2$ has also been shown to regulate chemotaxis of resident dendritic cells.[20] In addition, a $P2X_7$-like non-selective cation channel activity was associated with epithelial cell ciliary beat frequency.[21] Genetic deficiency of $P2X_7$ in mice slows the development of ovalbumin-induced inflammation,[22] tobacco smoke triggered emphysema,[23] and bleomycin-induced lung fibrosis.[6] To balance these findings, we have shown in humans that attenuated $P2X_7$ pore function is a risk factor for virus-induced asthma exacerbations,[24] while others demonstrated a protective role in eliminating tuberculosis,[25] suggesting that this receptor is critical to infection control while also promoting inflammatory conditions. A heterogeneous population of cells is present in the airway both in health and disease. The relative contribution of resident epithelial cells compared to leukocytes in responses to nucleotides in the airway is unresolved.

Given the frequent exposure of bronchial epithelial cells (BECs) to TLR ligands and danger signals, we hypothesized that primary human respiratory epithelium express functional

$P2X_7$ and that such expression confers functional processing of IL-1 family cytokines. To begin evaluating the contribution of BEC $P2X_7$ function on the inflammatory response to viral TLR ligand activation or infection, the present study assesses the canonical $P2X_7$ functions in respiratory epithelial cells to extend previous findings regarding channel activity and IL-1 processing observed in cell lines. Herein we report the function of $P2X_7$ in BECs and a differential response to TLR agonists including effects upon viral infection.

METHODS AND MATERIALS

Cell cultures: Human cells were obtained with approval from the UW-Madison Institutional Review Board. Human primary BECs were isolated from residual tracheal/bronchial rings.[26] Subject BECs (B39, B45 and B47) were cultured in supplemented bronchial epithelial growth medium (BEGM; Cambrex, East Rutherford, NJ). Primary epithelial cells from asthma patients were obtained from brushings during bronchoscopy as described.[27] Additional details are in Supplemental Methods.

Fluorescent dye uptake: $5x10^4$ BECs/chamber on collagen-IV (Sigma, St. Louis, MO) coated chamber slides (Thermo Fisher Scientific, Pittsburgh, PA) were cultured in BEGM for 24 h, washed with Hepes-buffered saline (HBS; 130 mM NaCl, 5 mM KCl, 20 mM Hepes, 0.1% BSA, 10 mM glucose, pH 7.4) and stimulated with HBS containing 10 µM YO-PRO-1 (Invitrogen, Carlsbad, CA) with or without 300 µM 2'-3'-O-(4-benzoylbenzoyl) ATP (BzATP; Sigma) at 37°C for 20 min. Cells were washed 3 times with HBS before observation by fluorescent microscopy (Olympus, Minneapolis, MN).

Immunofluorescense: Cells were fixed and permeablized by standard methods before incubation with anti-P2X_7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Alexa donkey anti-rabbit antibody (Invitrogen) and DAPI (Invitrogen). Details are in Supplemental Methods.

Cell stimulation:

Polyinosine-polycytidylic acid (Poly (I:C)), LPS, ATP: 1.5x105 BECs/well were seeded on collagen-IV coated 12-well plates (Costar, Cambridge, MA), cultured overnight in BEGM, followed by 24 h in BEGM with vehicle, 3 µg/mL Poly (I:C) (Sigma) or 10 µg/mL LPS (Sigma). Cells were washed with HBS and stimulated 30 min with 3 mM ATP (Sigma) or vehicle in HBS.

IFN-γ/TNF-α: 2x10^5 BECs/well were seeded onto collagen-IV coated 6-well plates (Costar) and cultured 24 h in BEGM without hydrocortisone supplementation before stimulation with 10 ng/mL IFN-γ (Peprotech, Rocky Hill, NJ) and 5 ng/mL TNF-α (BD Pharmingen, San Diego, CA) for 48 h.

IL-1 receptor antagonist (IL-1RA) and cell-derived IL-1β: A549 cells were incubated for 24 h with media-diluted BEC supernatants and IL-1RA (Sigma). Details similar to previous studies[28] are in Supplemental Methods.

Protein determination:

ELISA: ELISAs were performed as described.[29]

Immunoblotting: Details are in Supplemental Methods.

***RNA isolation and PCR techniques**:* Standard methods were used and found in Supplemental Methods.

Human rhinovirus serotype 1a (HRV1a) infection: Purified and concentrated HRV1a was provided by Dr. W.M. Lee (UW-Madison). 1.5×10^5 BECs/well were seeded in 12-well plates for 24 h and pre-treated with 500 µM periodate oxidized ATP (oATP; Sigma) or vehicle in BEGM for 2 h at 37°C. After PBS washing, BECs were infected with 350 µL/well of HRV1a diluted in reduced hydrocortisone (10^{-8} M) BEGM to 10 PFU/cell with or without 100 µM BzATP, or incubated with media alone for 2 h at 34°C. After 3 PBS washes, 1 mL BEGM with or without 100 µM BzATP was added to each well and incubated 24 h at 34°C.

Statistics: Cytokines and HRV were normalized by natural log transformations for statistical analyses. Two-tailed t-, one way ANOVA, and repeated measures ANOVA tests were determined with SigmaPlot (Systat Software Inc., San Jose, CA) with a significance level of $p=0.05$. Pair-wise comparisons were Bonferroni corrected.

RESULTS

***Donor BECs exhibit modest BzATP-induced dye uptake**:* A characteristic of $P2X_7$ is its agonist-induced ability to form a non-selective cation pore in the plasma membrane. BzATP activates $P2X_7$ at lower concentrations than is required for activation of other purinergic receptors[30] and YO-PRO-1, a 629 Da intercalating fluorescent dye, will move through these open pores specific to $P2X_7$ activation. Initial BEC $P2X_7$ stimulation experiments utilizing a plate reader suggested slower and less robust YO-PRO-1 uptake than monocytic controls (not shown). This observation led us to adopt a method to observe cell dye uptake via fluorescent microscopy. After

stimulation with BzATP, monolayer BECs (B39, B45, B47) with intracellular YO-PRO-1 were counted and reported as a proportion of all cells. Representative images for B45 are shown in Figure 4-1A. The percentage of BzATP stimulated dye uptake was significantly increased in all BEC groups (Figure 4-1(B-D)) with an average fold increase of positive cells over vehicle of 2.2, 3.4, and 1.9 in B39 (n=4), B45 (n=6), and B47 (n=6) BECs, respectively.

$P2X_7$ is expressed in BECs and enhanced by inflammatory cytokine priming: $P2X_7$ mRNA was observed in all three BECs examined with a RT-PCR product the same size as that from human embryonic kidney (HEK)-293 cells transfected with human $P2X_7$ (Supplemental Figure 4-1A). The truncated $P2X_7$ splice variant ($P2X_{7\text{-}j}$) reported to act as a dominant negative[31] was not observed in any monolayer BECs (data not shown) using standard RT-PCR. As recent reports indicate that $P2X_7$ may form a large pore complex via association with the hemichannel protein, Pannexin-1,[32] analysis of *Pannexin-1* expression by RT-PCR also revealed a specific band in all three subject BECs (Supplemental Figure 4-1B).

BEC lysates were probed for $P2X_7$ by Western Blotting with positive controls including THP-1 cells differentiated for 2 days with 100 nM phorbol-12-myristate-13-acetate (PMA; Sigma) and stably transfected $P2X_7$ HEK-293 cells. A 72 kDa band was observed in both positive controls, consistent with the size of $P2X_7$, whereas bands were difficult to observe at 72 kDa in any BECs. Large amounts of cell lysate were required for detection of $P2X_7$ in BECs and brushed epithelial cells compared to other cell types (Figure 4-2(A,B)).

Previously, Humphreys, *et. al.,*[7] showed that TNF-α/IFN-γ were the most potent combination of pro-inflammatory cytokines tested to increase *P2RX7* expression in THP-1 cells. Monolayer BECs stimulated with TNF-α and IFN-γ for 48 h were used for quantitative RT-PCR

measures of genes related to $P2X_7$ pore function. Compared to unstimulated cells, incubation with combined TNF-α/IFN-γ synergistically increased expression of $P2X_7$ mRNA and more modestly increased Pannexin-1 mRNA (Table 4-I). The $P2X_{7\text{-}j}$ splice variant was not detected in any BEC, even with cytokine priming. BECs B39 (n=1) and B45 (n=2) were used to examine immunofluorescent localization of $P2X_7$; priming of these BECs with TNF-α and IFN-γ increased immunofluorescent signal for $P2X_7$. HEK-293 cells with $P2X_7$ and empty pcDNA3 vectors were used as controls. Representative images of BEC B45 are shown in Figure 4-2(C-F).

ATP promotes IL-1β release in Poly (I:C) primed BECs: Reports indicate $P2X_7$ receptors modulate early IL-1β, and to a lesser extent IL-18, processing and release in response to ATP in LPS-primed monocytes.[33] In fact, $P2X_7$ activation of caspase-1 has been reported to require TLR engagement.[34] Intestinal epithelial cells express IL-1β and display enhanced expression after LPS exposure.[35] However, it is not clear if ATP-induced release of IL-1β occurs in LPS-primed human BECs or whether other TLR receptors can also induce IL-1β release in BECs. To address these questions, either LPS or Poly (I:C) was utilized to prime monolayer B45 BECs for 24 h. Poly (I:C) priming induced the release of IL-1β, which was augmented by ATP treatment (n=4; Figure 4-3A). However, IL-18 did not display a similar increase in rapid release after Poly (I:C) priming (n=4; Figure 4-3B). Priming with Poly (I:C) in respiratory epithelium appears similar to LPS priming in THP-1 monocytes as separate systems to augment release of IL-1β upon ATP stimulation (Supplemental Figure 4-2A). That the methods of isolating BECs results in a cell population with greater than 99% purity[36] combined with the differential sensitivity of BECs to Poly (I:C) priming is enhanced compared to LPS priming are supportive and consistent with a non-leukocyte cell population releasing the IL-1β measured in these experiments.

HRV stimulated IL-1β release is largely independent of $P2X_7$, despite limited differences in HRV infection after nucleotide receptor modulation: BECs showed an increased pro-IL-1β after infection with HRV1a (Supplemental Figure 4-2B). BECs co-treated with BzATP during and after HRV infection demonstrated a small, non-significant decrease in HRV load at 24 h. However, a more robust, significant difference in HRV recovery was observed when comparing either vehicle or BzATP treated cells with oATP treated cells (n=6, Figure 4-4A). The magnitude of the difference was approximately a half log increase in recovered HRV1a with oATP treatment although the amount of virus associated with the BECs was not different at the end of the 2 hour infection period (data not shown). Brushed primary respiratory epithelial cells from bronchoscopy also displayed a trend to decreased HRV production with BzATP stimulation (n=5, Figure 4-4B).

We performed similar HRV infection experiments in human respiratory cell lines to confirm the observation that blocked $P2X_7$ function increased HRV recovery. Both A549 and BEAS-2B cell lines had undetectable $P2X_7$ when measured by quantitative RT-PCR leading us to generate stably transfected cells containing either vector control or $P2X_7$ constructs. HRV1a infection of these cell lines was consistent with the above results, demonstrating an increased level of HRV1a in the respiratory cells without $P2X_7$ at 24 h after infection (Figure 4-4(C,F)).

HRV infection increased IL-1β release 24 h after monolayer BEC infection with HRV1a. Co-treatment of BECs with BzATP during infection augmented the release of IL-1β from infected cells compared to BzATP treatment alone (n=6). The measured IL-1β and IL-18 in BEC supernatants are shown in Figure 4-4(D,G). While results are similar to the early release observed with Poly (I:C) priming, the HRV infection appears to drive cytokine generation in these experiments at 24 h. Epithelial cells obtained by bronchoscopic brushings (n=5) infected

with HRV1a showed similar trends in IL-1β production as that in BECs (Figure 4-4E). IL-1β was undetectable in A549 and BEAS-2B cell lines, with and without $P2X_7$, whether or not infected with HRV.

To confirm that the IL-1β detected by ELISA was not merely non-selective release of pro-IL-1β induced by TLR activation, Western blotting was performed on cell-free supernatants. Bands were detectable near the 17 kDa expected size with varying efficiency with two separate antibodies (Figure 4-5(A,B)) as well as bands consistent with pro-IL-1β in HRV infected sample supernatants. Lanes with detectable bands representing cleaved IL-1β corresponded to the conditions in Figure 4 showing an increased level of IL-1β measured by ELISA when compared to vehicle-treated cells. Additionally, when using HRV1a preparations at 20 times the amount used in any experiments, IL-1β was below the limit of detection measured by ELISA.

Culture with BEC supernatants demonstrate IL-1β induced release of IL-8: BEC IL-8 concentrations in the same supernatants as those in Figure 4D correlated to IL-1β measurements (natural log normalized, Pearson R=0.84, p=0.04). As further evidence for a functional role for released IL-1β, cell supernatants from Poly (I:C) primed and ATP treated BECs elicited a robust production of IL-8 from A549 cells similar to previous studies.[28] The IL-8 production was significantly decreased by IL-1RA in a dose-dependent manner (Figure 4-5C).

DISCUSSION

Whereas $P2X_7$ function in leukocytes is reasonably well established, its role in epithelial cells defenses is less understood. We provide additional evidence for $P2X_7$ function in human respiratory epithelium including pore activity, a property associated with active $P2X_7$. To our

knowledge, the incorporation of YO-PRO-1 into BECs by BzATP stimulation is the first report of $P2X_7$ pore function in primary human respiratory epithelium. Recent reports indicate Pannexin-1, in physical association with the $P2X_7$ receptor, is linked to both dye uptake and IL-1β release.[32,37,38] Our results confirm that primary BECs also express Pannexin-1 mRNA and is supported by our observation of pore activity in epithelial cells. Kim, *et. al.,*[39] previously reported that human nasal epithelial cells express $P2X_7$ mRNA, but did not show protein expression or pore activity. Our results reinforce those from animal models, where studies indicate airway epithelial cells express $P2X_7$ mRNA, protein and channel activity.[40-42] In addition, the ATP-initiated increase in rapid BEC IL-1 cytokine release was augmented by TLR3 engagement. Whether the detected IL-1β is immature or cleaved and provides adequate biological activity *in vivo* is often assumed in literature while others, including Riteau, *et. al.,*[6] have demonstrated processing capability in immortalized respiratory cells (BEAS-2B) under various stimuli. We demonstrate comparable results in primary respiratory cells as well as in response to viral infection and TLR agonists. While we cannot rule out some contribution of pro-IL-1β measured by ELISA, by Western analysis we have confirmed at least some portion of the IL-1β present is cleaved upon $P2X_7$ stimulation in human respiratory epithelium and have demonstrated that under these conditions the released IL-1β confers subsequent production and release of IL-8. In total, we have strengthened the evidence for the presence of functional $P2X_7$ in primary human respiratory cells.

HRV, a prominent respiratory pathogen, augmented the release of IL-1β in primary epithelial cells. Many different pathways, including non-classical routes,[43] for the maturation and release of IL-1 family of cytokines have been described and under what conditions $P2X_7$ mediates IL-1β release in respiratory epithelial cells is becoming more clear. However, our

results add to the debate of what cellular machinery and at which stage of processing IL-1β, may be released from respiratory epithelium under different conditions. LPS recognition requires the functional interaction of TLR4 with MD-2 and CD14.[44] Although airway epithelial cells express most TLRs,[45] TLR4 appears to have a limited role,[46] in agreement with our finding of less IL-1β induction from LPS compared to Poly (I:C), unlike monocytic lineage cells. Our early detection of IL-1β after Poly (I:C) stimulation in BECs is in contrast to experiments in peripheral blood mononuclear cells.[47] In addition, it is possible that pro-IL-1β may be cleaved after cytolysis. An intriguing possibility is whether individuals with known loss-of-function $P2X_7$ alleles would generate less active IL-1β during infection, although there was not a significant difference at 24 hours in our experiments with oATP treatment. Rapid release (30 min) of IL-1β in monocytes and macrophages and MMP-9 release in monocytes are all dependent upon $P2X_7$, while detection of IL-1β at later time points (24 h) may be observed with non-$P2X_7$ dependent pathways in monocytes, but not macrophages.[47-49] Additionally, in other experimental systems markers including IL-10 and TNF-α have shown differences upon LPS whole blood stimulation at both 6 h and 24 h secondary to differences in functional $P2X_7$.[50] Clearly the timing of assessment of mediator release, as well as assessment of cell population, may be critical to the discernment of $P2X_7$-dependent effects.

$P2X_7$ is a known modulator of intracellular infection control. In fact, $P2X_7$ function is prominently involved in the control of tuberculosis. IL-1β and $P2X_7$ activation are linked to neutrophil influx at areas of injury and inflammation[51] and we have previously demonstrated a correlation between $P2X_7$ pore activity and nasal neutrophil levels during naturally acquired colds.[24] In the experiments performed here, blockade of purine signaling during infection of BECs increased HRV1a levels, even with no leukocyte contribution to the experimental system.

These initial findings with HRV support our previous report that a decrease in $P2X_7$ function predisposes towards the risk of asthma exacerbations following natural HRV infections.[24] Given that inflammatory cytokines enhance expression of $P2X_7$ in BECs, it is possible that there may be a paracrine effect to limit spread of the infection. TLR priming also increases resistance to HRV infection and in asthmatics HRV infection is patchy, providing a manner for $P2X_7$ to act in a paracrine fashion to limiting viral spread. Extending the *in vitro* epithelial cell experiments to air-liquid interface differentiated cells would be a natural extension of our monolayer system to investigate these processes and what role $P2X_7$ plays in infectious resistance by differentiated epithelium.

When combined with recent reports,[6,22,52] a growing role for purinergic receptors in general and $P2X_7$ in particular within lung disease is taking shape. Our results support the presence and function of $P2X_7$ in human lung epithelia independent of leukocytes with an antiviral capacity including IL-1β processing and release. Finally, determining the full extent of $P2X_7$ function in primed and differentiated cells will illuminate the therapeutic potential of receptor modulation in disease states including asthma.

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Table 4-I. Inflammatory cytokines increase $P2X_7$ mRNA. BECs were treated with TNF-α/IFN-γ for 48 h before RNA isolation to examine expression changes of $P2X_7$ and Pannexin-1 mRNA. The fold change in expression is normalized to housekeeping gene *G3PDH* and unstimulated controls. Results are displayed as average fold change ± standard deviation. n=3 for each group.

BEC donor	$P2X_7$ Expression	Pannexin-1 Expression
B39	4.36 ± 2.22	1.78 ± 0.33
B45	7.82 ± 4.72	2.11 ± 0.29
B47	18.69 ± 13.62	2.10 ± 0.31

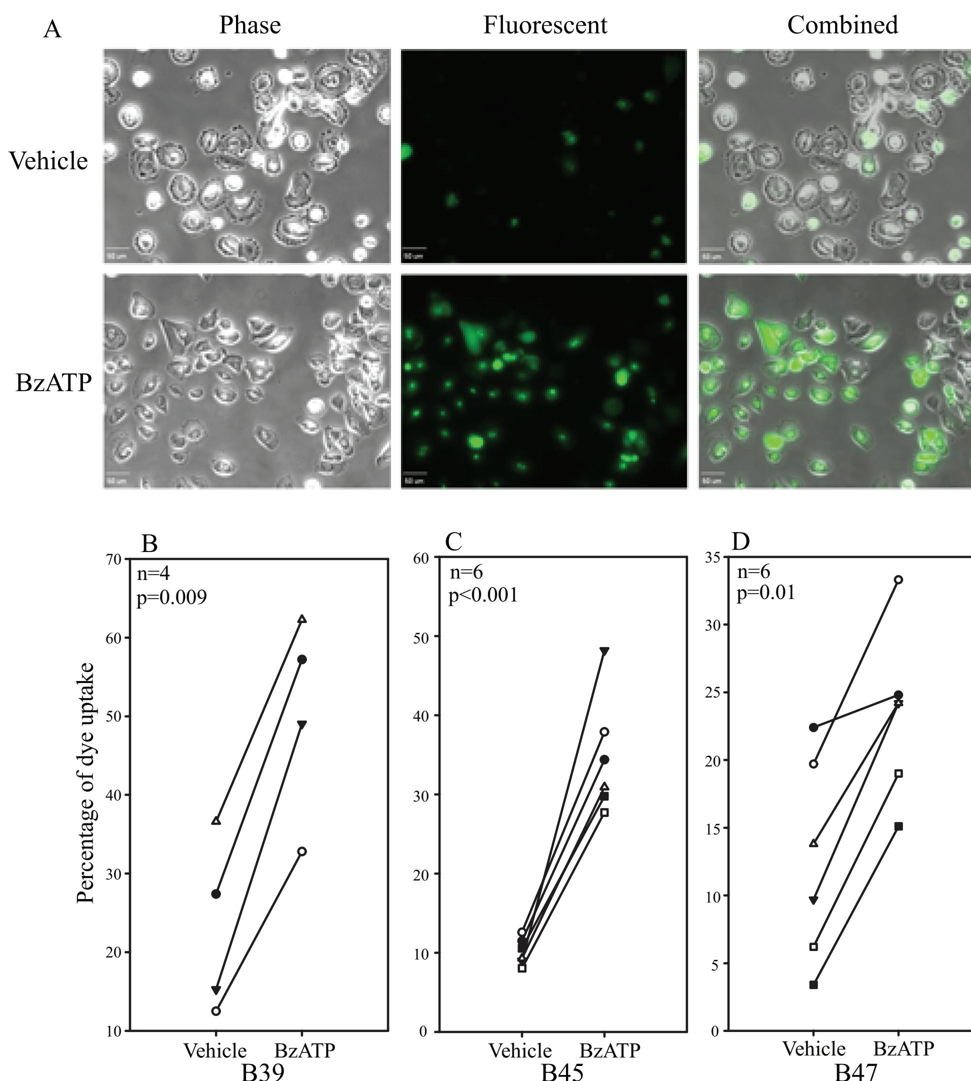


Figure 4-1. BECs have functional $P2X_7$ pore activity. Fluorescent dye YO-PRO-1 uptake in BECs after BzATP stimulation was observed by fluorescent microscopy and counted as a proportion of all cells. Representative images of BEC B45 are shown (A). The percentage of adherent BECs containing YO-PRO-1 was calculated and BzATP treated cells demonstrated a significant increase of uptake for all 3 BECs (B-D). Unpaired t-tests were performed within each group. Scale bar = 50 μm.

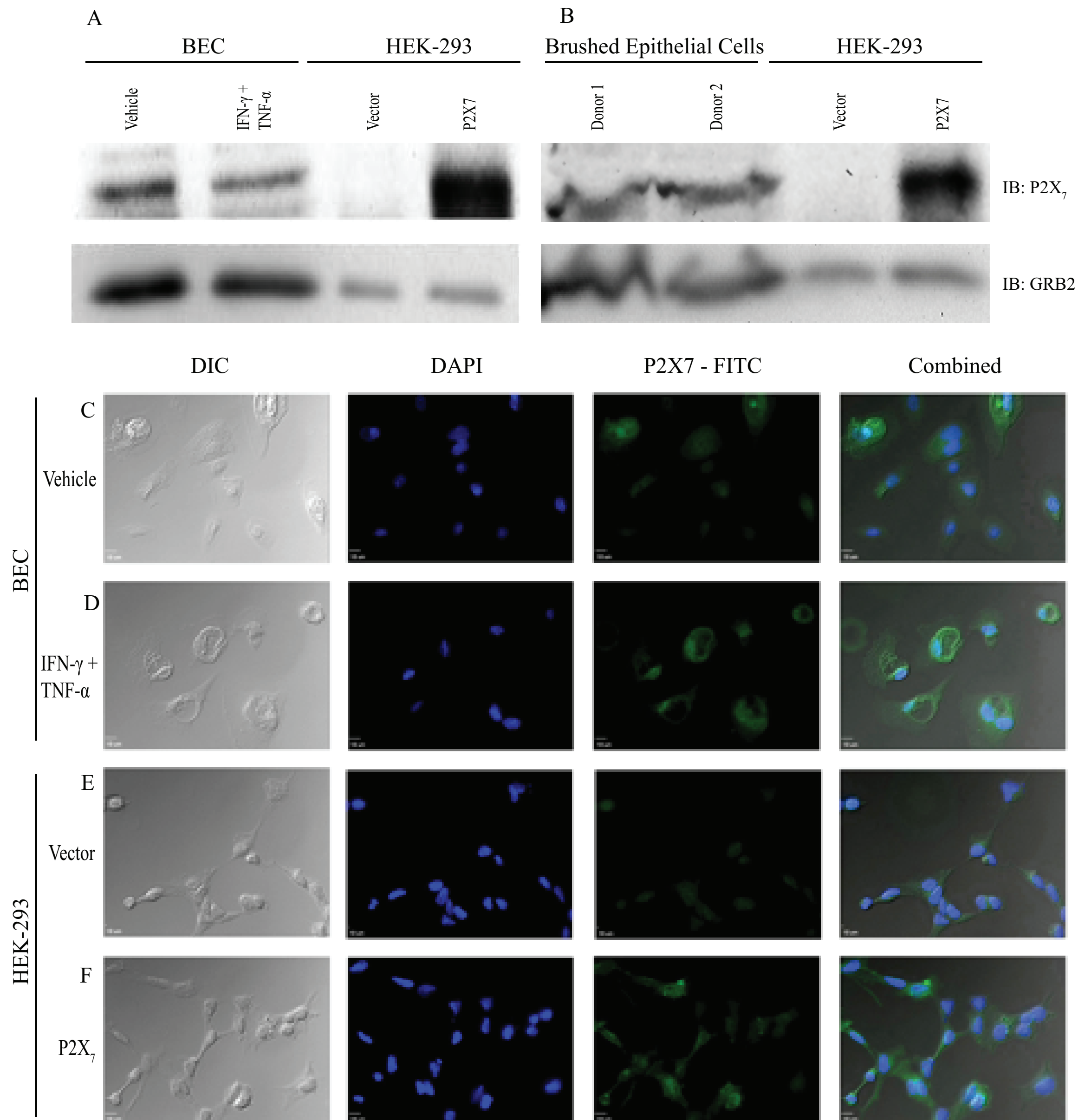


Figure 4-2. $P2X_7$ is detected in primary respiratory epithelial cells. BECs cultured with vehicle or TNF-α/IFN-γ for 24 h (A) and brushed epithelial cells from 2 donors (B) were probed for $P2X_7$ by Western blotting and indicate GRB2 as a loading control. HEK-293 cells with vector or P2X7 were used as controls. BECs treated with vehicle (C) or TNF-α/IFN-γ (D) for 48 h were examined for $P2X_7$ protein. DAPI and secondary antibody were co-incubated before

visualization. HEK-293 cells contain an empty vector (E) or express $P2X_7$ (F) for negative and positive controls, respectively. DIC and fluorescent signals are combined in the right panels. Scale bar = 10 μm.

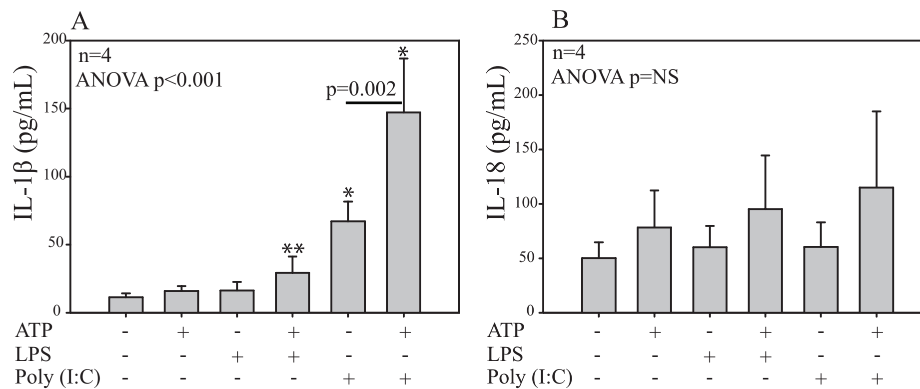


Figure 4-3. ATP increases IL-1β release from Poly (I:C) primed BECs. Supernatants from BEC B45 (n=4) primed with LPS or Poly (I:C) were collected after stimulation with ATP for 30 minutes and analyzed by ELISA. IL-1β levels were augmented by poly (I:C) priming, which was further increased with ATP treatment (A). IL-18 did not demonstrate the same pattern of release (B). The limit of detection for both cytokines was 12.5 pg/mL and data shown are mean + standard deviation. Pairwise comparisons were Bonferroni corrected. *p<0.05 compared to Vehicle, ATP, LPS, and ATP/LPS treated cells. **p<0.05 compared to Vehicle.

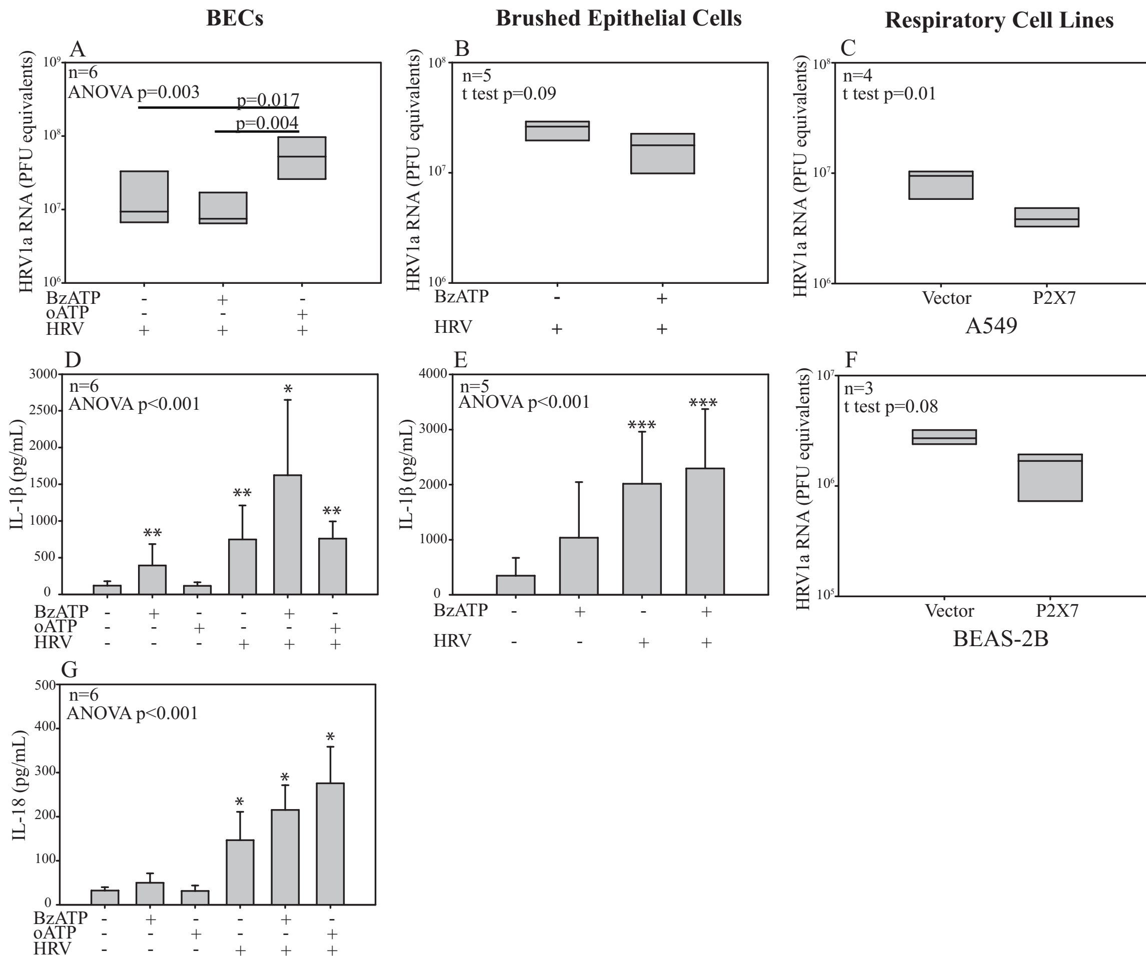


Figure 4-4. Nucleotide receptor blockade increases HRV recovery after infection while HRV infection increases IL-1 family cytokine release. Cellular HRV levels at 24 h was affected by nucleotide receptor modulators. oATP treatment of BECs increased HRV1a compared to both vehicle and BzATP groups (A) while brushed epithelial cells trended to a small decrease with BzATP treatment (B). Respiratory cell lines A549 (C) and BEAS-2B (F) transfected with either vector control or $P2X_7$ were also infected with HRV1a for 24 h. Supernatants of BECs or brushed epithelial cells treated with oATP and/or BzATP and incubated with HRV1a were analyzed for cytokine production. BEC IL-1β measured at 24 h (D) was

increased with HRV infection in all groups compared to vehicle or oATP treated. Epithelial cells from bronchoscopic brushings showed similar results with IL-1β (E) generating more IL-1β in the supernatant at 24 h compared to vehicle, but not BzATP-treated cells. BEC IL-18 measured at 24 h (G) was increased in all HRV groups. Box plots display median and interquartile range for (A-C) and (F) and bar graphs show mean + standard deviation for (D), (E), and (G). Pairwise comparisons are Bonferroni corrected. For cytokine measures *$p<0.05$ compared to Vehicle, BzATP, and oATP treated cells, **$p<0.05$ compared to Vehicle and oATP treated cells, ***$p<0.05$ compared to vehicle.

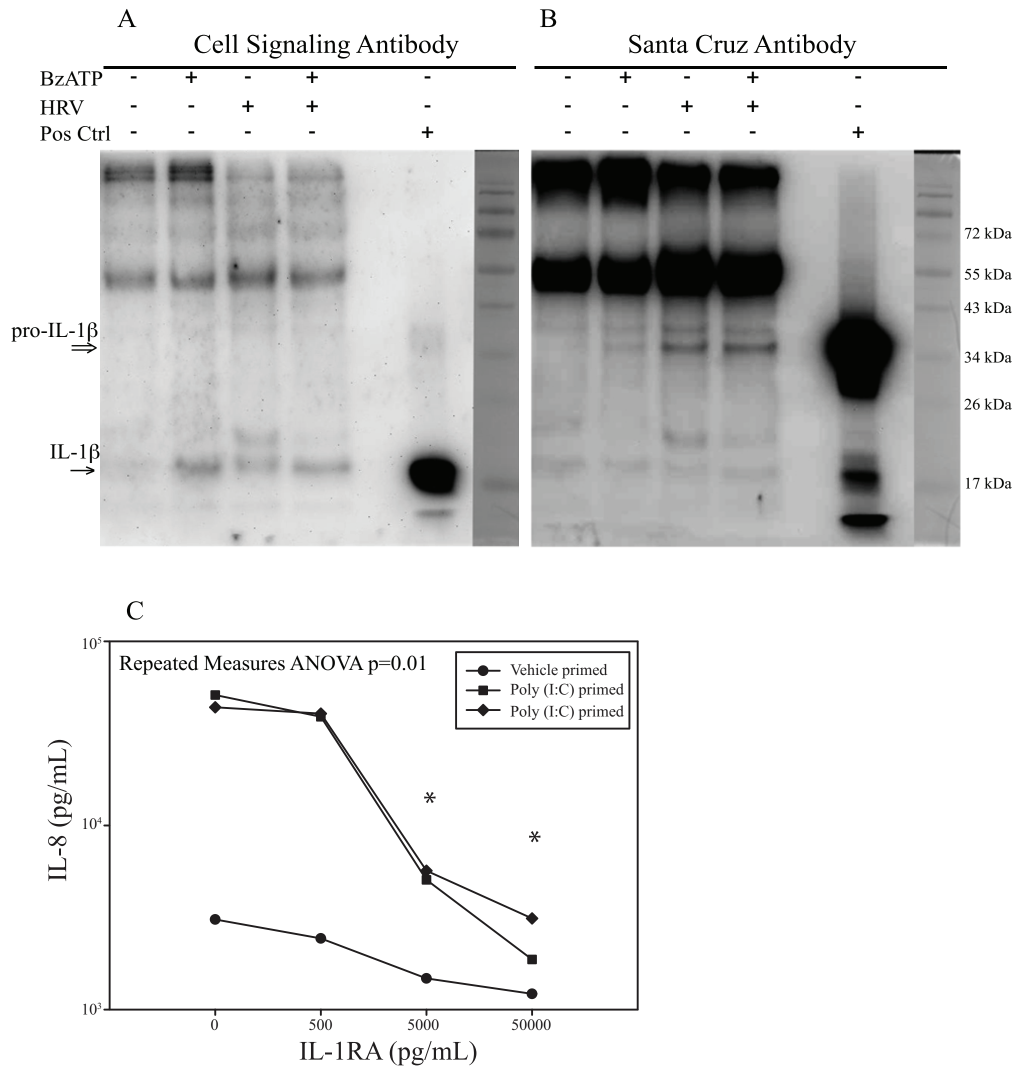


Figure 4-5. IL-1β released from BECs is cleaved and demonstrates functional activity. Western blotting of concentrated supernatants from BEC B45 infected for 24 h with HRV1a and/or stimulated with BzATP are shown with detectable bands similar in size to the positive control 17 kDa band from primed THP-1 cells and is representative of 3 experiments. Immunoblots with Cell Signaling (A) and Santa Cruz (B) antibodies display preferential detection of the cleaved and pro- forms of IL-1β, respectively. Positive control indicates LPS primed and ATP treated THP-1 cells. Molecular weight markers are shown on the right. (C)

Supernatants from BECs primed with Poly (I:C) or vehicle and treated with ATP were used to culture A549 cells for 24 h. Data points are the average of technical replicates. Increasing concentrations of IL-1RA were added and IL-8 was measured by ELISA. *$p<0.05$ compared to IL-8 release with no IL-1RA.

SUPPLEMENTAL METHODS

Cell Culture: Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 unless otherwise indicated. BECs were used at passage 4 or less. BEAS-2B, A549, and human embryonic kidney (HEK)-293 cells and THP-1 monocytes (ATCC, Manassas, VA) were cultured under standard conditions.

Transfection of respiratory cell lines: BEAS-2B and A549 cell lines (ATCC) were used to produce stably transfected cells with either pcDNA3 vector or $P2X_7$ by methods previously described with HEK-293 cells.[53]

Immunofluorescence: $5x10^4$ cells per well were plated on collagen-IV coated coverslips in 6-well plates and primed for 48 h with combined 10 ng/mL IFN-γ (Peprotech) and 5 ng/mL TNF-α (BD Pharmingen). Coverslips were rinsed with cold PBS and fixed with pre-warmed 4% paraformaldehyde for 10 min, then washed 3 times with Tris-buffered saline (TBS;10 mM Tris-HCl, ph 8.0, 150 mM NaCl) and permeablized with 0.1% Triton in TBS for 10 min. Cells were washed for 5 min twice with TBS and once with TBST (0.05% Tween 20 in TBS) before blocking with 1% BSA in TBST for 30 min. Cells were incubated in 1:200 anti-P2X7 antibody (Santa Cruz Biotechnology, CA) in blocking solution for 1 h at room temperature, washed for 5 min three times in TBST, then incubated in 1:10,000 Alexa donkey anti-rabbit secondary antibody (Invitrogen) and 1:10,000 DAPI (Invitrogen) for 1 h at room temperature in the dark. Cells were washed in TBST for 5 min three times before adding mounting media (Electro Microscopy Sciences, Hatfield, PA) and kept dark overnight before detection with a fluorescent

microscope (Olympus). Images were obtained and processed with SlideBook software (Intelligent Imagine Innovations, Denver, Co).

Immunoblotting: Samples were lysed in SDS sample buffer (10 mM Tris, 1 mM EDTA, 0.5 mM Na_3VO_4, 1 mM DTT, 1% SDS, 10% glycerol) and protein concentration for each sample was quantified using a bicinchoninic acid protein assay (Pierce/Thermo, Rockford, IL). Alternatively, 1 mL cell supernatants were concentrated using 3-kDa nominal molecular mass cutoff filters (Millipore, Billerica, MA). Equivalent amounts of protein for each sample (or volume for supernatants) were resolved on 12.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked in 5% milk in TBST and immunoblotting was performed according to manufacturer's protocols. Primary antibodies included $P2X_7$ (Santa Cruz Biotechnology), Pannexin-1 (abcam, Cambridge, MA), IL-1β (Santa Cruz Biotechnology; Cell Signaling, Danvers, MA), GRB2 (Santa Cruz Biotechnology), and total ERK1/2 (Millipore) and were incubated with 5% milk/TBST except the Cell Signaling IL-1β which was incubated with 5% BSA/TBST. Membranes were washed with TBST and incubated with secondary antibodies conjugated to HRP (Santa Cruz Biotechnology or Sigma) and immunoreactive bands were visualized using SuperSignal West chemiluminescent substrates (Pierce) and an Epichemi II darkroom (UVP, Upland, CA).

ELISA: Detection limits were 12.5 pg/mL for both IL-1β (eBioscience, San Diego, CA) and IL-18 (MBL International, Woburn, MA) mAb pairs.

IL-1 antagonism: Cell supernatants from vehicle or Poly (I:C) primed and ATP treated cells (Figure 4-3) were diluted 1:4 with media and used to incubate A549 cells for 24h, similar to methods used by Coulter, *et. al*.[28] IL-1RA was added at increasing concentrations to eliminate signaling due to IL-1R. Cell supernatants were measured for IL-8 by ELISA.

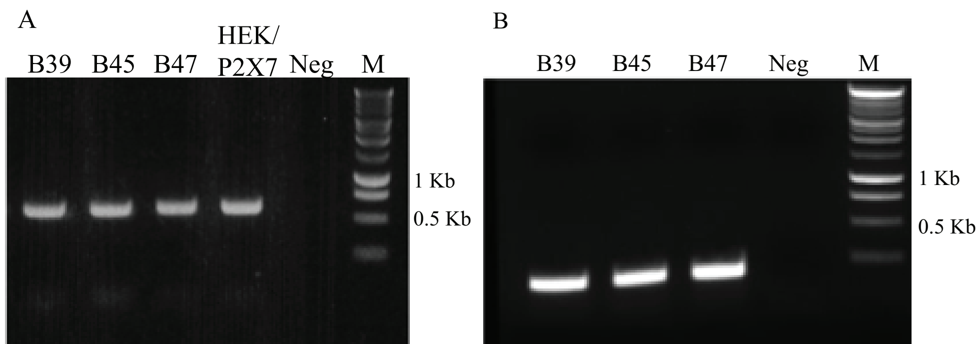
PCR: Total RNA was extracted using RNeasy mini kits (Qiagen, Valencia, CA) and treated with Turbo DNA-free (Ambion, Austin, TX) for 1 h at 37ºC and quantified by UV spectrophotometry (NanoDrop Inc, Rockland, DE). cDNA was generated from 1 µg of total RNA with iScript cDNA synthesis kit (Bio-Rad Laboratories, Richmond, VA). RT-PCR utilized AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and primers were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, CA) or from published literature and are shown in Supplemental Table 4-I. PCR products were separate by electrophoresis on a 1.5% agarose gel, stained by ethidium bromide, and visualized by UV transillumination. Quantitative PCR was performed in duplicate using an ABI 7500HT (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer specifications. Expression was normalized to housekeeping gene *G3PDH* and the fold change was calculated as the difference between the C_t values determined by the formulation $2^{-\Delta Ct}$.

Analysis of HRV RNA: Total RNA was extracted from infected cells with RNeasy mini kit (Qiagen) and reverse transcribed with iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed as described by Mosser and colleagues[54] and normalized to housekeeping gene *G3PDH* in each sample.

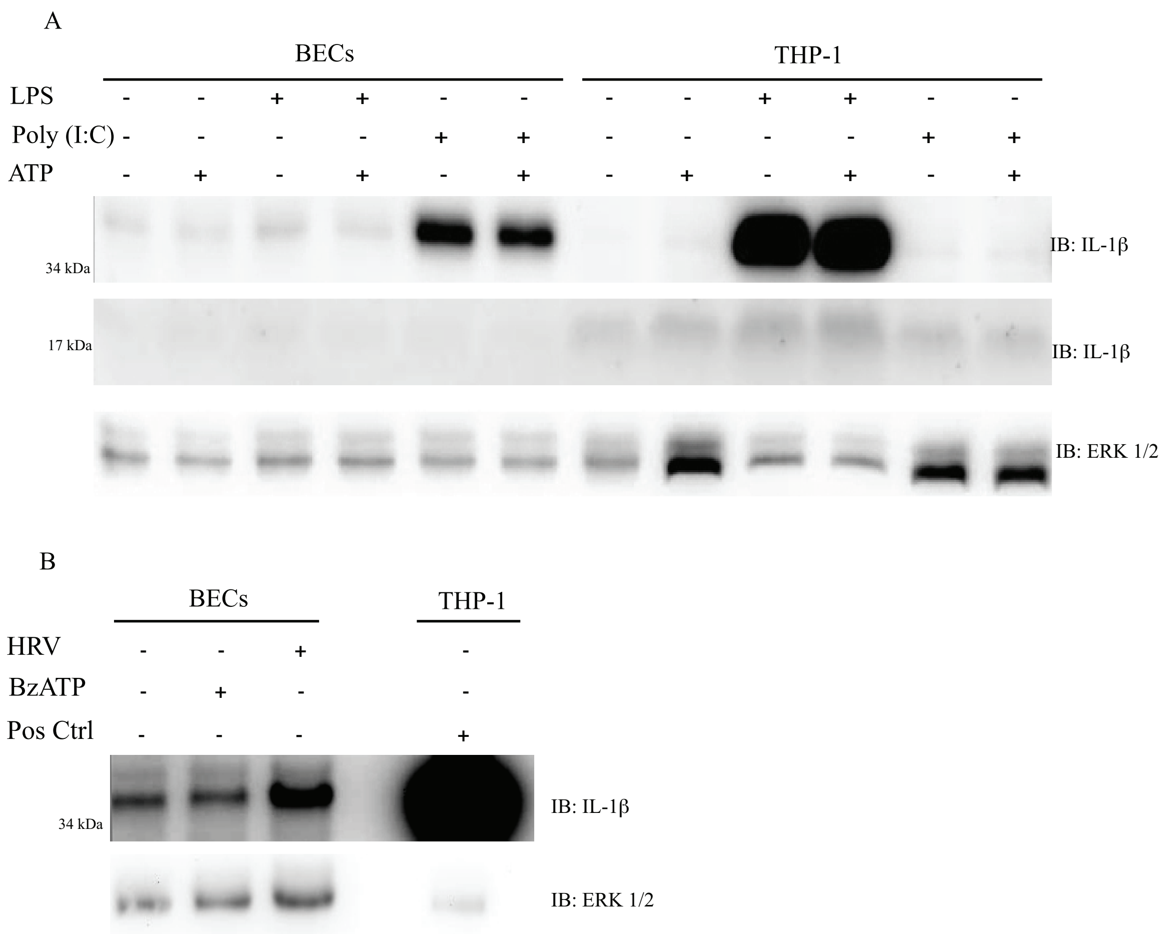
Supplemental Table 4-I: Primer sequences used for PCR.

Name or Accession Number	**Quantitative RT-PCR** (Forward and Reverse, 5' to 3')	**RT-PCR** (Forward and Reverse, 5' to 3')
$P2X_7$	ATACAGTTTCCGTCGCCTTG	GTCACTCGGATCCAGAGCATG
Y09561	AACGGATCCCGAAGACTTTT	CGGCACTGTTCAAGAGAGCA
$P2X_{7\text{-}j}$	TTTCAGATGTGGCAATTCAGATA	Same
From Feng[31]	AAGTAGGAGAGGGTTGAGCC	Same
Pannexin-1	AGCCTCTCCTCACTCTCAG	Same
NM_015368	GTCGGAATGGAACAAACAGC	Same
G3PDH	ATCACTGCCACCCAGAAGAC	
NM_002046	GCCATGCCAGTGAGCTTCCC	

G3PDH – glyceraldehyde-3-phosphate dehydrogenase



Supplemental Figure 4-1: Human primary BECs express $P2X_7$ and Pannexin-1 mRNA. cDNA generated from BEC mRNA was resolved on agarose gels. Bands for $P2X_7$ (A) and Pannexin-1 (B) were identified.



Supplemental Figure 4-2: BECs increase cellular pro-IL-1β upon TLR3 stimulation. (A) Cell lysates from cells primed for 24 h and treated with ATP for 30 min were used in Western blotting for IL-1β (Santa Cruz Biotechnology). Exposure times were 8 min for the main blot and 16 min for the long exposure showing the 17 kDa range. Total Erk 1/2 was used for a loading control. (B) Cell lysates from BECs infected with HRV for 24 h or treated with vehicle or BzATP for 24 h were used in Western blotting for IL-1β.

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CHAPTER FIVE

Summary, conclusions, and future directions

David M. Manthei

University of Wisconsin-Madison

Our laboratory has developed a non-invasive whole blood flow cytometric assay that determines the functional capacity of $P2X_7$. Using these methods, I have demonstrated an association between loss of $P2X_7$ function with a protection from asthma at ages 6 and 8 and with decreased asthma severity at age 11 in a high risk birth cohort (Chapter Two). The degree of aeroallergen polysensitization was also decreased in the low $P2X_7$ children, consistent with a less immunologically-primed environment during allergen exposures. The protection from asthma was more pronounced in boys, and future evaluation through puberty in both genders will help determine if the function of $P2X_7$ is differentially important at different stages of life.

Furthermore, we previously noted that low-functioning $P2X_7$ was a risk factor for exacerbations following a naturally acquired cold, although with a relatively small number of subjects. We have further complemented these studies with additional groups that, in contrast to our previous studies, were not recruited and focused during active cold seasons. These subjects also tended to exacerbate more with low $P2X_7$ function (Appendix A). Coupled with our data from children, it is intriguing to postulate that $P2X_7$ function may have different roles with different types of exposure or at different ages. For example, a general decrease in inflammatory responses following cell injury in childhood – due to low functioning $P2X_7$ – may provide protection from progression of events leading to asthma inception, including airway remodeling or development of polysensitization. Yet individuals in adulthood with established asthmatic disease could have a different course of events. In this case a lack of $P2X_7$ function might cause an incomplete response to, and control, of viral upper respiratory infections, allowing for development of increased viral load. Without an appropriately induced rapid inflammatory response after infection, the increased pathogen levels could lead to delayed and even increased

inflammation by other mechanisms, eventually leading to exacerbations. Further evaluation with multiple populations will help provide a basis to determine an underlying mechanism.

By measuring nasal cytokines during symptomatic colds, I undertook analysis to determine a signature of exacerbation-prone individuals (Chapter Three). While numbers were limited, candidate cytokines, including VEGF, TNF-α, and IL-1β were revealed, consistent with much of the existing literature for both roles in asthmatic disease in and response to HRV infection. Even further, $P2X_7$ has been implicated in varying degrees with the production of all three cytokines. Previous studies showed an association between low $P2X_7$ function and exacerbations, which might lead one to predict low cytokines would be found in individuals who exacerbated. However, our study demonstrated that increased levels of these cytokines were associated with more rapid asthma exacerbations. Similar to the observations noted above, these findings may be reconciled by positing that a decrease in $P2X_7$ is involved in decreased control of viral infections, allowing greater progression of infection and a delayed, secondary immune response even larger than would normally be appropriate. As the samples used in this study corresponded to peak nasal neutrophil influx, a variable number of days of symptoms before sample acquisition; however, there was not a difference in the average number of cold days prior to sample collection. Whether a subset of asthmatics is more responsive to increased inflammatory cytokines leading to the initiation of exacerbations certainly warrants further studies. Additionally, studies stratifying enrollment by $P2X_7$ function will be important in determining the role of $P2X_7$ in exacerbations and whether the mechanisms could be different than for inception.

Finally, with a postulated role for $P2X_7$ modulating viral infections, we examined the role of $P2X_7$ in respiratory epithelium because it is the primary site of viral replication (Chapter

Four). While reports exist of purinergic receptor activity in respiratory epithelium, the characteristics are inconsistent and the canonical property of pore formation has not been demonstrated in primary human respiratory epithelium. Because $P2X_7$ is engaged in IL-1 family cytokine processing and release, we determined the release of IL-1β as a secondary functional measure of $P2X_7$ activity. We demonstrated pore formation following BzATP stimulation as well as IL-1β processing and release after priming with TLR3 ligand and exposure to ATP. When infecting primary respiratory epithelial cells with HRV1a, we noted small numerical changes in HRV load with blockade or stimulation of $P2X_7$. Additionally, generation of IL-1β was driven by HRV infection, although uncertainties remain about the processing of the cytokine. Overall, while $P2X_7$ is present and functional in respiratory epithelium, its active role in a response to infection by epithelium is likely minimal. However, during damaging events, the epithelium is a prime repository of site of dangers signals for potential release. Future work will focus on cells of immune lineage present and recruited to the airways after release of ATP.

These studies support a role involving $P2X_7$ with inflammation during chronic conditions. When combined with recent work, dendritic cells have emerged as a likely candidate for functional responses to $P2X_7$ stimulation. In mouse models, dendritic cells have been shown to be responsive to ATP although the relative contributions of P2Y and P2X receptors are still under investigation and early reports indicate P2Y receptor involvement in cell chemotaxis. $P2X_7$ has been implicated in the dendritic cell role directing T-cell maturation, including a balance between T_{reg} and T_h populations. In fact, ATP may be involved in decreasing T_{reg} and increasing T_h17 populations based not only on dendritic cell function, but also by direct effects on the T-cells. Confirmatory experiments will be important, particularly with the potential for differential responses to nucleotide exposures.

We are well positioned to follow up many of the possibilities mentioned herein. Having further validated the whole blood $P2X_7$ pore assay in the identification of loss-of-function alleles, an extension of the assay in two ways would both be exciting and increase the potential for replication in other populations. First, measuring $P2X_7$ pore function in multiple cell populations simultaneously would allow us to observe other cells with pore activity, and whether this function is different among individuals. Second, the pore assay displays extremely stable characteristics, which may lend themselves to the development of measuring $P2X_7$ activity from frozen samples. If we are able to modify the assay to measure pore function in archived PBMCs, a host of other clinical populations would be available for a retrospective analysis.

From a clinical perspective, use of the whole blood $P2X_7$ pore assay as a screening tool to identify subsets of patients could be the most practical and useful focus, and it is feasible as utilized in Appendix A. As $P2X_7$ is implicated in conditions other than airway disease, a peripheral blood-based functional assay responds to recent calls for a method to screen for the potential for $P2X_7$ function. With the noted heterogeneity in asthmatic disease and responses to therapy, the pore assay could be used as a tool to stratify individuals into groups with differential effectiveness of medications and risks for exacerbations. If $P2X_7$ function is truly predictive of these differences, it would introduce a basis for personalization in patient management beneficial for both patients and providers.

APPENDIX A

$P2X_7$-regulated protection from exacerbations and loss of control is independent of asthma maintenance therapy

Loren C. Denlinger, MD, PhD[a], David M. Manthei, M.S., Max A. Seibold, PhD[b], Kwangmi Ahn, PhD[c], Eugene Bleecker, MD[g], Homer A. Boushey, MD[d], William J. Calhoun, MD[e], Mario Castro, MD[f], Vernon M. Chinchili, PhD[c], John .V. Fahy, MD[d], Greg A. Hawkins, PhD[g], Nicolina Icitovic, MS[c], Elliot Israel, MD[h], Nizar N. Jarjour, MD[a], Tonya King, PhD[c], Monica Kraft, MD[i], Stephen C. Lazarus, MD[d], Erik Lehman[c], Richard J. Martin, MD[b], Deborah A. Meyers, PhD[g], Stephen P. Peters, MD, PhD[g], Dagna Sheerar, BS[a], Lei Shi, PhD[a], E. Rand Sutherland, MD[b], Stanley J. Szefler, MD[b], Michael E. Wechsler, MD[h], Christine A. Sorkness, PharmD[a], Robert F. Lemanske, Jr., MD[a], and the NHLBI Asthma Clinical Research Network Investigators

University of Wisconsin School of Medicine & Public Health-Madison[a], National Jewish Health-Denver[b], Pennsylvania State University-Hershey[c], University of California at San Francisco[d], University of Texas Medical Branch at Galveston[e], Washington University-St. Louis[f], Wake Forest University Health Sciences-Winston-Salem[g], Brigham and Women's Hospital and Harvard Medical School-Boston[h], and Duke University Medical Center[i]

ABSTRACT

Rationale: The function of the $P2X_7$ nucleotide receptor protects against exacerbation in mild-intermittent asthmatics during viral illnesses, but the impact of disease severity and maintenance therapy has not been studied.

Objectives: To evaluate the association between $P2X_7$, asthma exacerbations and incomplete symptom control in a more diverse population.

Methods: A matched *P2RX7* genetic case-control was performed with samples from ACRN trial participants enrolled before July 2006, and $P2X_7$ pore activity was determined in whole blood samples as an ancillary study to two trials completed subsequently.

Measurements and main results: One hundred eighty-seven exacerbations were studied in 742 subjects, and the change in asthma symptom burden was studied in an additional 110 subjects during a trial of ICS dose optimization. African American carriers of the minor G allele of the rs2230911 loss-of-function SNP were more likely to have a history of prednisone use in the previous 12 months, with adjustment for ICS and LABA use (OR = 2.7, 95% CI 1.2 – 6.2, p = 0.018). Despite medium dose ICS, attenuated pore function predicted earlier exacerbations in incompletely controlled patients with moderate asthma (HR = 3.2, CI 1.1 – 9.3, p = 0.033). After establishing control with low dose ICS in patients with mild asthma, those with attenuated pore function had more asthma symptoms, rescue albuterol use and FEV_1 reversal ($p < 0.001$, 0.03 and 0.03 respectively) during the ICS adjustment phase.

Conclusions: $P2X_7$ pore function protects against exacerbations of asthma and loss of control, independent of baseline severity and the maintenance therapy.

INTRODUCTION

The release of ATP during cell injury is a danger signal to the airway inflammatory response.[1] Levels of ATP in the airway are elevated after allergen challenge in humans and mice, contributing to production of dendritic cell-derived cytokines important to efficient recruitment of eosinophils and lympocytes and the subsequent development of airway hyperresponsiveness to methacholine.[2] In addition, airway ATP levels are likely elevated during virus-induced asthma exacerbations in humans[3] and serve as an airway biomarker of neutrophilic inflammation during infection.[4] The nucleotide receptor, $P2X_7$, regulates this process. This receptor is a trimeric, non-selective cation channel expressed by leukocytes and epithelial cells that opens a larger pore upon full activation (size restriction 900 Da).[5,6] Data by Muller *et al*. in the ovalbumin-sensitized mouse model suggest that inhibition of $P2X_7$ partially attenuates dendritic cell-dependent influx of eosinophils and lymphocytes and the development of airway hyper-responsiveness and remodeling.[7] Consistent with a role for this receptor in compartmental recruitment of granulocytes, we have previously shown that $P2X_7$ pore function correlates with early recruitment of neutrophils in nasal lavage samples from asthmatic patients experiencing a viral induced cold.[8]

The gene for this receptor *(P2RX7)* localizes to chromosomal position 12q24,[9] a minor asthma locus containing multiple related genes.[10-16] *P2RX7* is highly polymorphic with over 800 single nucleotide polymorphisms.[17] At least ten loss-of-function alleles have been validated in recombinant expression systems;[18-22] however, the frequency of the minor alleles for most of these variants is rare (MAF < 3%) and contributes to relatively small blocks of linkage disequilibrium for this gene.[23,24] To circumvent these problems, we have developed a functional

screening assay for $P2X_7$ pore activity that detects the presence of five of the most common loss-of-function genotypes, and now permits analyses of multi-center studies.[25,26]

In patients with mild-intermittent asthma at baseline, attenuated $P2X_7$ pore function is associated with a 15-fold increased risk of exacerbations in the setting of a rhinovirus cold.[8] In accord with this, bronchial epithelial expression of $P2X_7$ confers a small protective effect in terms of limiting HRV replication.[27] The direction of these effects in our human studies are in some ways opposite to that predicted from the role of $P2X_7$ in regulating ovalbumin-induced airway hypersensitivity and inflammation in the mouse. Additionally, the ability of maintenance asthma therapy with inhaled corticosteroids (ICS) to reduce the risk of exacerbation has not been evaluated with stratification by $P2X_7$ function. Given this background, and because early *in vitro* experiments suggested that corticosteroids have minimal impact on $P2X_7$ pore activity, we predicted that normal $P2X_7$ function protects against asthma exacerbations in adults with established disease, independent of the asthma maintenance therapy.

METHODS

Human subjects participation: We studied three cohorts within the ACRN: i) a cross-section of participants enrolled in ACRN trials prior to July 2006, with mild-to-moderate severity at baseline and a diverse mix of maintenance therapy, ii) incompletely controlled patients despite medium-dose ICS during the run-in, and iii) well-controlled patients using low-dose ICS followed during an ICS-adjustment strategy trial. Details of these trials have been previously published.[28-30] Briefly, ACRN enrolled adults with physician-diagnosed asthma; harmonized phenotypic data and follow-up was available across the studies. Asthma exacerbations were defined as new administration of oral prednisone or other systemic corticosteroids. These

analyses were approved by both the ACRN Steering Committee and Data & Safety Monitoring Board. Sample collection was described in the trial consent form, approved by the local Internal Review Board of each clinical center and signed by each participant.

Measurement of P2X$_7$ pore activity in whole blood samples in multi-center trials**:** The methods of our pore assay in whole blood monocytes have been described, including refinements to accommodate loss of viability due to overnight shipping; non-viable cells were excluded after pore closure by the addition of propidium iodide.[23,25,26] The median fluorescence intensity (MFI) of YO-PRO-1 associated with viable CD14+ cells treated with the P2X7 ligand BzATP was used as the measure of pore activity, with a receiver operator curve defined threshold between low and normal pore activity of 382 MFI.[31] Pore function was independent of participant age, sex, ethnicity and ACRN Center (Supplemental Figure A-1).

Genotyping**:** DNA was harvested and stored at the Channing Laboratory (Boston, MA). Genotyping was performed using the Sequinom iPLEX platform. Five previously validated, non-synonymous loss-of-function *P2RX7* SNPs (rs28360447, rs28360457, rs2230911, rs3751143, rs1653624) were chosen in combination with five HapMap tagSNPs (rs656612, rs1653583, rs507085, rs1718119, rs2230912) to optimize gene coverage. Simulation of multiplex reactions was performed beforehand to maximize primer compatibility during the amplification step. The allele frequencies from all the SNPs were in Hardy-Weinberg equilibrium.

Statistical analysis**:** Phenotype data were managed at Data Coordinating Centers (DCC) of the ACRN with a database that does not include *P2RX7* genetic data. One author from the DCC (E.L.) remained blinded to the genetic data and performed matching of the cases with controls lacking the history of prednisone. Matching was performed on the basis of participant-reported ethnicity, sex and percent predicted FEV_1 at the screening visits. Given our focus on validated loss-of-function alleles for the ACRN analysis and the trimeric nature of the receptor that allows cooperative ligand binding,[32,33] the dominant model was chosen for the primary analysis. The Cochran-Mantel-Haenzel test was used for a matched case-control analysis of allele frequencies in the ACRN cross-sectional population. Multivariable logistic regression to model case-control status was adjusted for the match identifier. For the pore assay functional analysis, Kaplan-Meier models of the time to first exacerbation was performed using the log-rank test, and the time to multiple events was evaluated via a repeated measures proportional hazards regression model. The changes in secondary endpoints over the duration of these trials were evaluated by a repeated measures ANCOVA model with comparisons made between the low and normal pore groups. For all considerations, a p-value < 0.05 was considered significant without adjustment for multiple comparisons.

RESULTS

P2RX7 genetic association with exacerbations in a cross-section of ACRN participants at enrollment is independent of maintenance inhaled corticosteroids**:** From 1435 genotyped samples, we identified 170 case subjects who were randomized in ACRN trials, and also had a history of prednisone use in the 12 months prior to enrollment. Additionally, 481 controls were matched from this cohort on the basis of race, gender and FEV_1. The distribution of cases to

controls was as follows: 151 cases with three matched controls each, 9 cases with 2 matched controls each, and 10 cases with one matched control. Table 1 shows the distribution of ethnicity and the baseline phenotype variables. There was a numeric trend for the cases to have slightly lower percent-predicted FEV_1 values and slightly higher exhaled nitric oxide measurements. Methacholine responsiveness, sputum eosinophils and serum IgE values were not different, but the cases were more likely to be taking ICS and/or long acting beta2-agonists LABA prior to trial enrollment (Table A-1).

The association of exacerbation status with the minor allele frequencies of the ten genotyped SNPs is shown in Table A-2, with adjustment for the matching variable. The minor allele for rs2230911 was enriched in the cases compared to controls, an effect which is driven largely by African American participants. The presence of a minor allele for rs2230911 remained significantly associated with a history of prednisone use in African Americans using a multivariate logistic regression model with adjustment for both ICS and LABA use (OR = 2.70, 95% CI 1.18 – 6.17, p = 0.018).

Attenuated pore function and the time to exacerbation in symptomatic patients with moderately severe asthma on medium-dose ICS**:** Given that rs2330911 has been previously validated as a loss-of-function allele,[21,22] we implemented our pore assay to increase the sample size efficiency in a prospective trial. To assess whether ICS treatment mitigates against this $P2X_7$-dependent risk of exacerbation, we performed an ancillary study to an ACRN trial that targeted symptomatic patients with moderately severe disease who were felt to be at risk for exacerbation. Participants in this trial could not have had a cold within 6 weeks of enrollment, but were required to have incompletely controlled asthma symptoms despite medium dose ICS at

the end of the run-in period.[29] During the course of the trial, seventeen exacerbations occurred in fourteen participants who also had available pore assay data. Seven of 27 (25.9%) participants with low pore function had an exacerbation despite maintenance ICS, whereas this occurred in only 7 of 61 (11.5%) with normal pore activity. Figure A-1 shows a Kaplan-Meier analysis of the time to the first exacerbation, with a trend toward an earlier exacerbation time in the low pore group. Of note, four of these first-exacerbation events occurred on the day of the bronchoscopy; excluding these events results in a log-rank p-value of 0.027. Additionally, by allowing for multiple events including these procedure-associated prednisone bursts, a proportional hazards model demonstrates a hazard ratio of 3.2 (95% CI = 1.1, 9.3, $p = 0.033$) associating low pore activity with exacerbations. We also assessed the impact of pore function on the change in asthma control during the course of the trial in participants who were only receiving ICS as their maintenance therapy. Specifically, a logistic regression model showed an inverse relationship between continuous $P2X_7$ pore function and the likelihood of not having symptomatic improvement (change in ACQ score > 0.5) while on a fixed dose, medium strength ICS ($p = 0.049$). The direction of this effect suggests that patients with low pore activity are less likely than those with normal $P2X_7$ function to receive symptomatic improvement on ICS during the context of a clinical trial.

***Attenuated pore function and the return of asthma symptoms in a mild-to-moderate population during maintenance therapy adjustment*:** These genetic and functional data suggest that individuals with low pore activity might have a greater propensity to have asthma symptoms at baseline and/or be less responsive to ICS. To determine whether patients with asthma and low $P2X_7$ pore function are more likely to have asthma symptoms return after a period of control, we

performed an ancillary study to an ACRN trial that enrolled subjects with a mild-to-moderate asthma who could achieve good symptom control during the run-in period while taking a low-dose ICS as the only maintenance therapy.[30] Pore activity was assessed from 110 of the subjects in this trial at the time of randomization to three different ICS-adjustment strategies designed to guide decisions regarding step-up and step-down therapy in this population. Table A-3 shows the change in several variables related to asthma control during the 36 weeks of follow up in these participants, stratified by pore function. Neither group had a significant change in their ACQ score over the course of the trial. By contrast, the individuals with low pore activity experienced a small increase in their daily symptom scores over the course of the trial, that corresponded with an increase in rescue albuterol use and an increase in the bronchodilator-induced percent reversal of FEV_1; these changes were smaller in the normal pore group such that the comparison between low and normal status remained significant (Table A-3). Both groups had an increase in exhaled nitric oxide and had trends towards being more responsive to methacholine over the course of the trial, but these parameters did not differ according to $P2X_7$ pore status. Of note, the 30-day average exposure to ICS was not different between the two groups (mean ± standard deviation of the natural log of 30-day ICS dose; low pore, 7.42 ± 1.38, normal pore 7.25 ± 1.21, p = 0.488).

DISCUSSION

Our initial observations with mild-intermittent asthmatics are extended in several ways by the main findings of this analysis. First, adult African American carriers of a loss-of-function rs2230911 G allele (attenuates $P2X_7$ function in recombinant expression systems[21,22]) have an increased frequency of prednisone use in the past year, even after adjustment for ICS and LABA

use. Second, attenuated whole blood monocyte pore function was associated with a more rapid rate of exacerbation in a moderately severe population despite medium-dose ICS. In contrast to our previous work in mild asthmatics enrolled at the time of a cold experiencing a loss-of control, the present analysis focused on prednisone use, to be consistent with guideline definition of severe exacerbation. These events are likely representative of exacerbations from a diverse set of triggers, particularly in that cold symptoms were not required for study enrollment. Third, individuals with low pore activity have a higher asthma symptom burden than those with normal $P2X_7$ activity despite ICS in our prospective studies involving both mild and moderate populations. Whether $P2X_7$ function is a biomarker for ICS responsiveness is an intriguing possibility that will require further study.

These data also confirm that attenuated whole blood monocyte pore function is common in patients with asthma, representing 35% of the participants in the two trials combined. Despite robust receiver-operator curve derived performance characteristics of this assay for identifying samples with the five most common loss of function genotypes, the relatively low positive predictive value likely reflects the presence of samples with additional loss-of-function variants which could be identified by targeted sequence analysis, as we have done previously.[23] Given the abundance of SNPs for this gene and the presence of at least 10 loss-of-function alleles (most of which are rare), it is possible that the common *P2RX7* alleles have weaker effects than the rare variants. For example, the rs2230911 SNP with an effect in the ACRN cross-sectional cohort has been shown to be weaker in its influence on $P2X_7$ pore activity than other loss-of-function alleles.[23] As such, it is likely that replication efforts to test the *P2RX7* genetic association to exacerbation risk will yield other SNPs, an aspect of reproducibility that is more common for genes with rare functional variants.[34] Thus, the use of the whole blood monocyte $P2X_7$ pore

assay as an epidemiological tool significantly strengthens our findings. Deep sequencing of this gene in a larger study with uniform participant entry criteria and prednisone-dependent exacerbation data will likely define which *P2RX7* SNPs confer the most risk.

In addition to monocytes, the *P2RX7* gene is expressed by most leukocytes and by airway epithelial cells.[1] Dendritic cell production of chemokines has been implicated as a $P2X_7$-regulated mechanism for the recruitment of lymphocytes and eosinophils leading to development of airway hyperresponsiveness in the ovalbumin-sensitized mouse model.[7] The relative protection of the $P2X_7$ knock-out mouse from the development of allergic airway or atopic sensitization,[7,35] however, suggests that another cell type may be involved for the protective effects of this receptor during the inflammatory response to triggers capable of causing asthma exacerbations. In this regard, most exacerbations in children and adults with established asthma are driven by respiratory virus infections with neutrophilic inflammation,[36] and it is likely that the pathways involved in exacerbation are distinct from those driving sensitization and asthma development.[37-40] We have shown that the early recruitment of neutrophils to the upper airway directly correlates with $P2X_7$ function and is protective of exacerbation, potentially by limiting the spread of infection to the lung.[8] The recruitment of neutrophils to the lung in non-allergic models is also attenuated in the $P2X_7$-deficient mouse challenged with cigarette smoke or bleomycin.[41,42] Interestingly, $P2X_7$ is involved in the early steps of granulocyte extravasation before the activation of the Mac-1/ICAM-1 integrin receptors involved in neutrophil adherence to endothelial cells, such that the knock out mouse has diminished PMN adherence.[43] Additionally, amplification of Th1-type cytokine production is a primary role of $P2X_7$ (6), and individuals with attenuated Th1 responses during asthma exacerbations are more likely to have loss of lung function,[44] although the role of $P2X_7$ was not evaluated in the latter study. Other

possible $P2X_7$-dependent mechanisms that may be relevant during infectious triggers of acute asthmatic inflammation include differences in the rates of epithelial cell apoptosis and/or macrophage phagocytosis of spent inflammatory cells.[45-47] In this case, delayed epithelial cell apoptosis in individuals with attenuated pore function could provide an advantage to viral replication.[48,49] Similarly, inefficient $P2X_7$-dependent macrophage phagocytosis of apoptotic epithelial cells or neutrophils would likely prolong the inflammation in the lower airway, consistent with observations in patients with severe asthma.[50,51] In conclusion, $P2X_7$ function helps protect a diverse group of asthma patients from exacerbation and may be important for baseline symptom control while on maintenance ICS; the precise mechanism warrants further study.

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Table A-1. ACRN participant screening variables for the matched case-control analysis. Cases were defined as the acute use of prednisone in the 12 months prior to ACRN trial entry. Controls were matched by ethnicity, sex and percent predicted FEV_1. Spirometry data are reported as the means and standard deviations, whereas the median values and interquartile ranges are shown for the variables requiring log transformation prior to ANOVA. The percent of sputum eosinophils is also reported as the median and interquartile range with comparison by the Kruskal-Wallis test. Categorical use of any or no ICS, LABA, or leukotriene receptor antagonists (LTRA) was compared in the two groups by a χ^2 test.

	Cases	**Controls**	**p-value**
Total (N)	170	481	
Caucasian (N)	103	305	
African American (N)	40	110	
Hispanic (N)	17	45	
Asian/Pacific Islander (N)	7	17	
Other (N)	3	4	
% Female	67.6	67.2	
Age (years)	33 ± 10	32 ± 11	0.581
% Predicted FEV1	80.4 ± 14.6	82.7 ± 13.5	0.067
Methacholine PC20 (mg/mL)	0.8 (0.3, 2.1)	0.9 (0.4, 2.3)	0.359
FeNO (ppb, N = 151, 421)	15.8 (10.8, 25.8)	14.7 (9.3, 22.0)	0.062
% Sputum EOS (N = 67, 207)	0.3 (0, 1.7)	0.4 (0, 1.6)	0.987
IgE (IU/mL) (N = 52, 220)	156 (44, 512)	141 (48, 333)	0.288
ICS use (%)	88.7	65.4	< 0.001
LABA use (%)	50.3	26.7	< 0.001
LTRA use (%)	13.9	13.2	0.819

Table A-2. Loss-of-function *P2RX7* minor allele frequencies in matched ACRN cases and controls. The minor allele frequencies (MAF) are listed for the total population, as ethnicity was a strict matching variable for the case-control pair definitions. SNPs that have previously been validated as loss-of-function variants are designated by the asterisks.[18-24] Blocking on the match variable, the Cochran-Mantel-Haensel odds ratio and confidence intervals for the dominant model are presented with corresponding p – values.

***P2RX7* SNP (Minor Allele, Genotyping Call Rate)**	**MAF-Cases**	**MAF-Controls**	**Cochran-Mantel-Haensel OR (95% CI)**	**p - value**
rs656612 (C, 0.969)	0.348	0.390	0.76 (0.53-1.09)	0.121
rs1653583 (T, 0.975)	0.087	0.085	0.92 (0.56-1.50)	0.709
rs28360447 (A, 0.988) ***	0.003	0.013	0.23 (0.03-1.79)	0.105
rs507085 (G, 0.985)	0.276	0.290	0.85 (0.60-1.21)	0.329
rs28360457 (A, 0.982) ***	0.015	0.013	1.17 (0.41-3.38)	0.816
rs1718119 (T, 0.959)	0.352	0.393	0.74 (0.51-1.06)	0.139
rs2230911 (G, 0.978) ***	0.154	0.109	1.74 (1.16-2.60)	0.012
911-Caucasian only	0.078	0.089	1.02 (0.55-1.89)	1.000
911-African American only	0.262	0.126	3.44 (1.61-7.38)	< 0.001
911-Hispanic only	0.294	0.170	2.68 (0.88-8.49)	0.081
rs2230912 (G, 0.986)	0.132	0.131	0.96 (0.63-1.44)	0.791
rs3751143 (G, 0.972) ***	0.151	0.156	0.96 (0.65-1.42)	0.814
rs1653624 (A, 0.982) ***	0.018	0.013	1.42 (0.52-3.84)	0.416

Table A-3. Change in asthma control variables during ICS adjustment in patients with mild asthma stratified by pore function. Data reflect the change in each endpoint from randomization to the end of the treatment period, with the last column representing the differences between the low and normal pore groups. The p – values and 95% confidence intervals are reported from repeated measures ANCOVA models.

Change in Endpoints During ICS Adjustment	**Low Pore** (n = 40)	**Normal Pore** (n = 70)	**Low - Normal**
	Estimate (95% Confidence Interval); p-value		
ACQ Average	0.10 (-0.14, 0.35); 0.41	-0.07 (-0.26, 0.11); 0.44	0.18 (-0.13, 0.48); 0.26
Daily Symptom Diary Scores	0.07 (0.05, 0.09); <0.001	0.00 (-0.01, 0.02); 0.97	0.07 (0.05, 0.10); <0.001
Rescue Albuterol Use (puffs/day)	0.26 (0.13, 0.39); <0.001	0.09 (0.01, 0.18); 0.03	0.17 (0.01, 0.32); 0.03
FEV_1-Albuterol Reversal (4 puffs, %)	2.62 (0.75, 4.50); 0.007	0.10 (-1.28, 1.48); 0.89	2.52 (0.19, 4.85); 0.03
Natural log (FeNO, ppb)	0.40 (0.10, 0.70); 0.008	0.51 (0.29, 0.73); <0.001	-0.11 (-0.48, 0.26); 0.57
Log_2 (PC_{20}, mg/mL)	-0.61 (-1.24, 0.01); 0.05	-0.39 (-0.85, 0.07); 0.10	-0.23 (-1.00, 0.55); 0.56

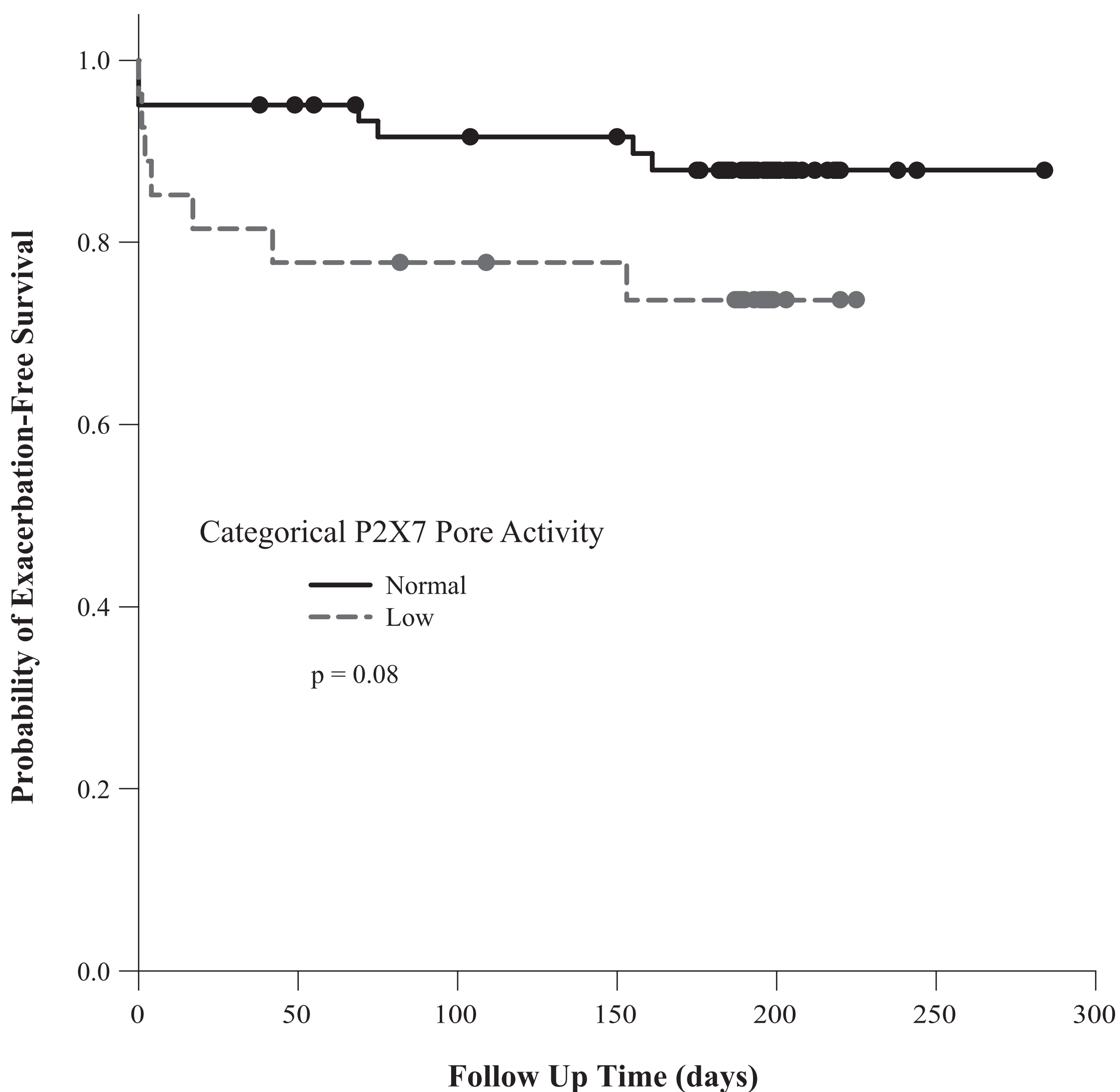
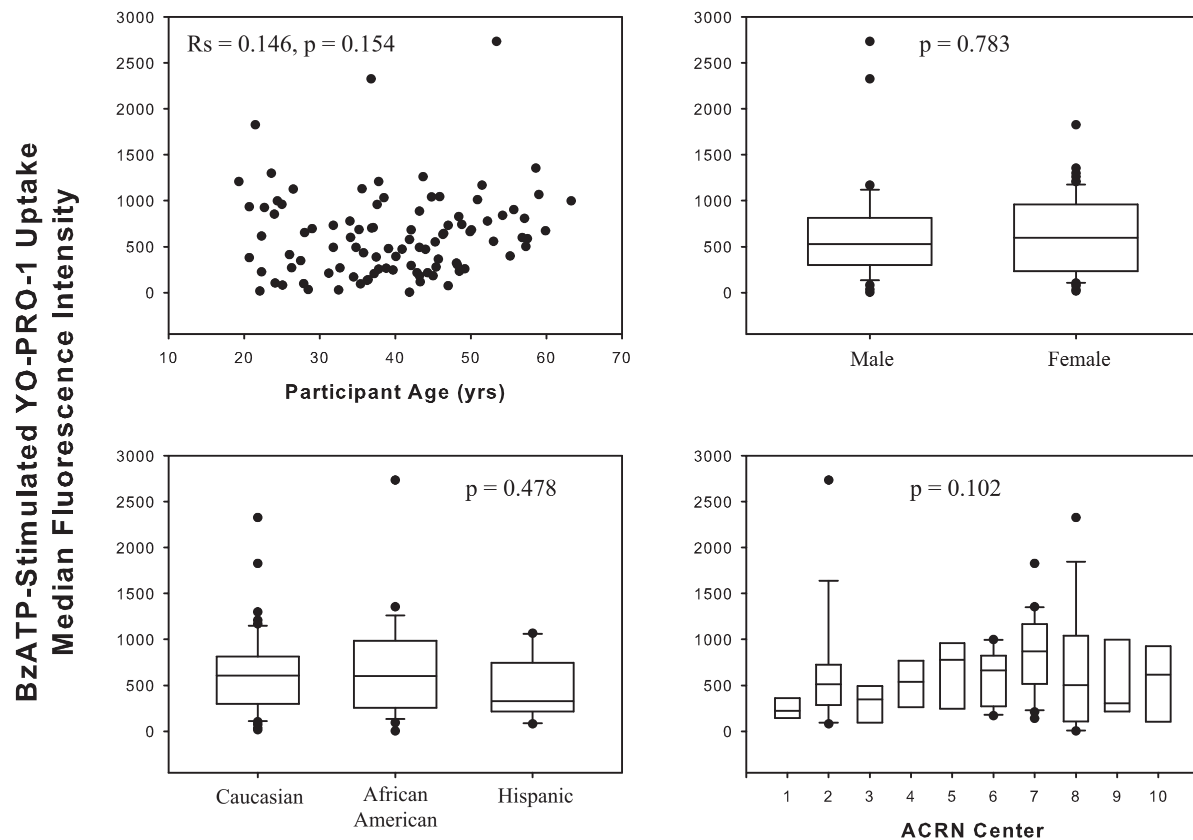


Figure A-1. Kaplan-Meier analysis of time to exacerbation in symptomatic patients with moderate asthma at baseline. The survival curves are shown separated by categories of $P2X_7$ pore function defined by the receiver-operator curve generated threshold. The solid and the dashed lines represent the normal and low pore groups respectively. Dots along these lines are the times of patient censure due to completing participation without an exacerbation. The comparison of event rates was performed with correction for multiple events in a repeated measures proportional hazards regression model.



Supplemental Figure A-1. Distribution of whole blood monocyte pore activity in participants of an ACRN trial. Data represent the amount of the indicator fluorescent dye that is taken up by $P2X_7$ ligand stimulation of monocytes in whole blood samples as a standardized measure of pore function.[26] Participant age, sex, ethnicity and ACRN Center are shown as stratifying variables with a corresponding Spearman correlation coefficient or ANOVA p – values. For reference, the threshold for low pore activity is a median fluorescence intensity less than 382 arbitrary units.

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