Attenuated $P2X_7$ Pore Function as a Risk Factor for Virus Induced Loss of Asthma Control

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ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Recruitment, Entry Criteria and Visit Structure

Paricipants were recruited from an established database and mass email prior to expected rhinovirus cold seasons (Spring and Fall) with instructions to call the study coordinators within 48 hr of a new upper respiratory tract infection. Inclusion criteria comprised the following: a) age of 18 to 45 years, b) physician diagnosis of asthma based on episodic symptoms of cough, wheeze and dyspnea established at least six months prior to enrollment, c) asthma medications consisting of as needed short acting β2 agonists, low dose inhaled corticosteroids (≤400 mcg beclomethasone/day or equivalent), or the combination product Advair® equivalent to low dose inhaled corticosteroids, d) database records of FEV1 ≥ 70% of predicted, e) prior history of FEV1 reversibility with β2 agonist ≥ 12% or methacholine PC20 <8 mg/ml, and f) skin test reactivity to at least one aeroallergen. Paricipants were not eligible to participate if they were a current smoker or had a history of ≥ 5 pack-yrs, were currently on immunotherapy, or had been enrolled in another clinical study within one month prior to the initial visit. Participants were asked to complete four visits within the first week of cold symptoms, followed by two visits when their cold symptoms returned to baseline at least six weeks later (Table E2).

Additional Phenotyping Methods

In addition to virus detection in airway samples by multiplex PCR, serum samples for *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* IgM, IgG and IgA detection were collected at one acute and one baseline, stored at -80°C, and performed at the end of the study using a ELISA kits according to manufacturers instructions (Savyon Diagnostics, Ashdod, Israel). Spirometry was performed at each visit and a

methacholine challenge was done on the visits corresponding to sputum induction; both of which were performed according to ATS guidelines.

The loss of asthma control is defined as follows. The convalescent baseline respiratory symptoms and medication use for each paricipant were calculated as the median values for the period of time beginning one week prior to the Visit 7 and extending through Visit 7A (Table E2). Similarly, the mean A.M. peak expiratory flow (PEF) for the same time period was used. The asthma symptom score combined the A.M. and P.M. values for chest tightness, wheeze, and shortness of breath, ranging from 0 to 18. Minor loss of control was defined as an increase in symptom score by at least 4 above the convalescent baseline value for two consecutive days, while not meeting the major criteria. A major loss of control included any of the following measures present on two consecutive days; a decline in PEF by ≥ 35%, an increase in rescue inhaler use above reference by at least 4 times per day, or greater than eight uses of rescue inhalers per day.

$P2X_7$ Pore Assay and Genotyping

Briefly, whole blood samples are stained with a phycoerythrin-labeled anti-CD14 antibody and then stimulated with buffer or 250 μM Bz-ATP in the presence of 1 μM YO-PRO-1. With recalled flow cytometer settings, monocytes were detected in the context of whole blood by a CD14+ threshold flow cytometric technique and the amount of fluorescent-dye uptake (YO-PRO-1) was quantified as the geometric mean. Nonviable cells were excluded from the analysis by gating out YO-PRO-1 positive cells in the buffer control, which conveniently have less intense CD14 expression. These gates are also applied to the Bz-ATP treated samples. Data are reported as the agonist-induced YO-PRO-1 uptake (fold change) by these cells. When pore assay data from an acute and

baseline visit were available from samples processed within 24 hrs, the average of these values was reported.

Genomic DNA was harvested using the PureGene kit and genotyping was performed by restriction fragment length polymorphism analysis of polymerase chain reaction products, as previously described (34). The RefSeq numbers for the genotyped *P2RX7* alleles are as follows: rs2393799, rs28360447 (cDNA—G474A, amino acid G150R), rs28360457 (cDNA—G946A, amino acid— R307Q), rs2230911 (cDNA—C1096G, amino acid—T357S), rs2230912, rs3751143 (cDNA—A1513C, amino acid E496A), and rs1653624 (cDNA--T1729A, amino acid— I568N).

Logistic regression was performed to determine how well $P2X_7$ activity corresponds with a binomial of the presence or absence of known loss of function genotypes. A receiver-operator curve (ROC) was constructed that determines the threshold of pore activity with the best predictive value for identifying samples with loss of function genotypes, while also excluding those with normal genotypes. The whole blood $P2X_7$ assay has previously been validated in samples from 200 healthy volunteers with robust identification of previously characterized and novel loss-of-function polymorphisms, with an area under the receiver-operator curve (AUC) of 0.927 (34). The AUC for identifying loss-of-function genotypes in this population is 0.870 ($p < 0.001$), incorporating an allele (*i.e.* G474A) we have previously proposed to confer attenuated function (34).

Site Directed Mutagenesis and Recombinant Line Characterization

To test whether the *P2RX7* 474 A allele is associated with attenuated function (confers an amino acid substitution G150R), we created a point mutant for recombinant expression in HEK293 cells. After confirming similar levels of mRNA and protein expression relative to the wild-type control (not shown), the function of these

recombinant cells was assessed for $P2X_7$ pore function by the agonist-induced uptake of YO-PRO-1 in a fluorimetric plate assay (Supplementary Figure 1). Briefly, a pcDNA3 expression vector containing a cloned human *P2RX7* cDNA associated with normal pore activity was subjected to point mutagenesis using the Quick Change Kit (Stratagene Inc., La Jolla, CA) to create the variant P2X7-G150R. HEK 293 cells were transfected by the Superfect liposomal method and stable populations were selected by G418 resistance. Expression of P2X7 mRNA and protein in unstimulated transfected HEK cell cultures was verified by RT-PCR and immunoblotting as previously described (E1). Transfected HEK cells were washed in HEPES-buffered saline (HBS; 130 mM NaCl, 5 mM KCl, 20 mM HEPES-pH 7.4, 0.1% BSA, and 10 mM glucose, components from Sigma-Aldrich, St. Louis MO), stimulated for 20 min at room temperature with various concentrations of 2'-3'-O-(4-benzoylbenzoyl)adenosine 5'triphosphate (BzATP, 0 or 1 to 300 μM) in the presence of 10 μM YO-PRO-1 (Invitrogen, Carlsbad, CA), equilibrated to 10 mM MgCl2, and washed again in HBS. YO-PRO-1 fluorescence was measured by a Synergy-HT fluoresent plate reader (BioTek Instruments, Inc., Winooski, VT) using 485 ± 20 nm excitation and 528 ± 20 emission filters. In this assay system, the YO-PRO-1 fluorescence was increased roughly four fold by Bz-ATP stimulation of HEK cells expressing the $P2X_7$ wild type control with an EC_{50} of < 1 μM. The control loss-of-function mutant, 496A, had a similar EC_{50} but a markedly reduced maximal response. By contrast, the 150R mutant exhibited no agonist stimulated increase in (Figure E1). In this regard, HEK 293 cells stably transfected with a recombinant expression vector containing this point variant have substantially reduced pore function (Figure E1). Collectively, the $P2X_7$ pore assay in whole blood has very good predictive power for identifying samples with known and novel variant alleles, and can be used as a continuous variable to investigate relationships between the $P2X_7$ receptor system and disease biomarkers or endpoints.

Regression Analysis of the Change in Asthma Symptoms and the Loss of Asthma Control

Least squares linear regression analysis was used to model normal-transformed univariate factors associated with the change in asthma symptoms during a naturally occurring cold. The methacholine PC_{20} and neutrophil counts required a log transformation, whereas pore activity data were normalized using the square root function. A preliminary multivariate model was built by the stepwise method, sequentially entering univariate factors with the next highest F-statistic until the statistical power was depleted. A generalized linear model of the change in asthma symptoms was then used to calculate multivariate coefficient estimates, confidence intervals, and corresponding statistics. In all cases, residual plots of the models had random distributions. For analysis of the loss of asthma control, any loss (minor or major) was categorized in a binomial variable described above. Logistic regression of the loss of control was performed with pore activity as the main dependent variable and the calculation of a crude odds ratio. Adjustment for the detection of virus and the pre-cold use of ICS was then included for an adjusted OR, receiver-operator analysis, and performance assessment of the model.

References

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P2RX7 Genotype						Validated LOF Genotypes		Validated + G474A	
G474A	G946A	C1096G	A1513C	T1729A	Pore Activity	Sens	1 - Spec	Sens	1 - Spec
HET	WT	WT	HET	WT	1.0	0	0.04	0.125	0
WT	WT	WT	HET	HET	1.8	0.167	0.04	0.25	0
WT	WT	WT	WT	WT	8.6	0.167	0.08	0.25	0.044
WT	WT	HET	HET	WT	8.9	0.333	0.08	0.375	0.044
WT	WT	HET	HET	WT	9.2	0.5	0.08	0.5	0.044
HET	WT	WT	HET	WT	20.1	0.5	0.12	0.625	0.044
WT	WT	WT	WT	HET	21.9	0.667	0.12	0.75	0.044
WT	WT	WT	HET	WT	24.9	0.667	0.16	0.75	0.087
WT	WT	WT	HET	WT	25.3	0.667	0.2	0.75	0.130
WT	WT	WT	WT	WT	29.2	0.667	0.24	0.75	0.174
WT	WT	HET	WT	WT	34.3	0.667	0.28	0.75	0.217
WT	WT	WT	HET	WT	38.5	0.667	0.32	0.75	0.261
WT	WT	WT	HET	WT	38.7	0.667	0.36	0.75	0.304
WT	WT	WT	HET	WT	39.1	0.667	0.4	0.75	0.348
WT	WT	WT	WT	HET	42.3	0.833	0.4	0.875	0.348
WT	WT	WT	HET	WT	47.3	0.833	0.44	0.875	0.391
WT	WT	WT	WT	WT	49.2	0.833	0.48	0.875	0.435
WT	WT	WT	HET	WT	51.0	0.833	0.52	0.875	0.478
WT	WT	WT	HET	WT	54.9	0.833	0.56	0.875	0.522
WT	HET	WT	WT	HET	55.9	1	0.56	1	0.522
WT	WT	WT	HET	WT	57.7	1	0.6	1	0.565
WT	WT	WT	WT	WT	58.3	1	0.64	1	0.609
WT	WT	WT	WT	WT	63.2	1	0.68	1	0.652
WT	WT	HET	WT	WT	65.4	1	0.72	1	0.696
WT	WT	WT	WT	WT	82.6	1	0.76	1	0.739
WT	WT	WT	WT	WT	98.8	1	0.8	1	0.783
WT	WT	WT	WT	WT	104.2	1	0.84	1	0.826
WT	WT	WT	WT	WT	108.8	1	0.88	1	0.87
WT	WT	WT	WT	WT	110.2	1	0.92	1	0.913
WT	WT	WT	WT	WT	122.9	1	0.96	1	0.956
WT	WT	WT	WT	WT	133.7	1	1	1	1

Table E1. Performance analysis of pore function to identify samples with loss of function genotypes. Genotypes and pore activities were assessed on all samples in the present study using procedures described in the Methods Section. Previously validated loss of function genotypes (1513 CC, 1729 TA, 946 GA, 1513 AC-1096 CG) are shaded (E2-E5). The 474 GA genotype has also been associated with low pore function in healthy paricipants ((34), please also refer to Figure E1 for functional data in a recombinant expression system). Calculations are shown for the sensitivities and

specificities at varying thresholds of pore function to identify loss of function genotypes using the validated alleles without and with G474A. Logisitc regression with a receiver-operator analysis has an area under the curve of 0.797 and 0.870 ($p = 0.012$ and < 0.001) respectively. The optimal threshold is < 22-fold of BzATP-induced YO-PRO-1 uptake, indicated by the highest Jaeger statistic (sensitivity – (1 – specificity). This threshold of pore activity corresponded with a YO-PRO-1 median fluorescence intensity of approximately 675 arbitrary fluorescence units in the BzATP-stimulated whole blood samples. The positive predictive values are 0.571 and 0.857 for the two models respectively. In both cases, the negative predictive value is 0.917.

	ACUTE INFECTION				RECOVERY			POST COLD BASELINE	
	Visit 1	Visit 1a	Visit 2*	Visit 3	(Visit 4)	(Visit 5)	(Visit 6)	Visit 7	Visit 7a*
		24-48 hours post V1	24-48 hours post V1a	7 days post V1	14 days post V1	21 days post V1	28 days post V1	6 weeks post V1	24 hours post V7
Medical & asthma history	X								
Issue diary cards (asthma and cold sx)& peak flow meters	X								
Cold Symptom Assessment	X								
Physical examination	(X)	(X)	X						X
Monitor peak flow rates		X	X	X	X	X	X	X	
Check diary cards & assess AE		X	X	X	X	X	X	X	
Nasal lavage	X	X	X	X	X	X	X	X	
Sputum induction		X		X	X	X	X	X	
Spirometry	X	X	X	X	X	X	X	X	X
Methacholine challenge		X		X				X	
Blood for P2X7 Pore Assay	(X)								X

Table E2. Visit structure and data collection. Patients with asthma were enrolled at the start of an upper respiratory tract infection according to the entry criteria in the text. Items enclosed by parentheses were optional.

PEAK FLOW AND SYMPTOM DIARY CARD Paricipant ID: ______________________

If your asthma worsens, please call ____________________________________ at (_________)______________________

Date and time of next study visit: ____

Score each asthma symptom as follows: 0=not present, 1=mild, 2=moderate, 3=severe.

Date (month/day/year)	Number of puffs of albuterol you used in the past 24 hours	Number of times you woke up at night and required albuterol	**Peak Exp. Flow Rate**	Chest Tightness	Wheeze	Cough	Shortness of Breath	Peak Expiratory Flow Rate	Chest Tightness	Wheeze	Cough	Shortness of Breath	Comments: • Medical problems • Medications taken (other than albuterol)
	AM	AM	AM	AM	AM	AM	AM	PM	PM	PM	PM	PM	
				Circle one **0 1 2 3**	**Circle one** **0 1 2 3**	**Circle one** **0 1 2 3**	**Circle one** **0 1 2 3**		**Circle one** **0 1 2 3**	**Circle one** **0 1 2 3**	**Circle one** **0 1 2 3**	**Circle one** **0 1 2 3**	
				0 1 2 3	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**		**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	
				0 1 2 3	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**		**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	
				0 1 2 3	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**		**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	
				0 1 2 3	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**		**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	**0 1 2 3**	

Table E3. Asthma symptom and peak flow diary used for this study. Shaded areas are nighttime values.

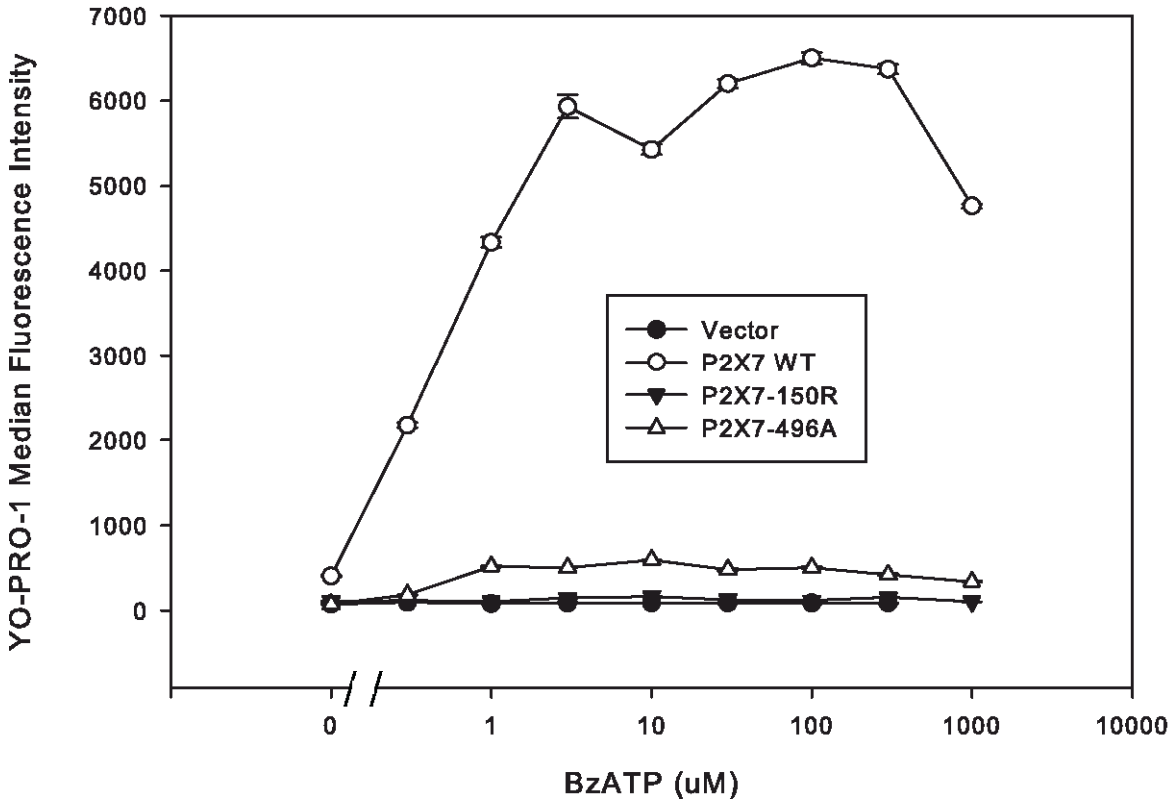
				Nasal Lavage Neutrophil Counts (10e6/mL)							
Pore Activity	Baseline ICS	RV detected	Loss of Control	Peak	V1	V1a	V2	V3	V4/7	V4a/7a	Average Baseline
1	No	No	Yes	1.709	1.709	0.093	0.048	0.136	0.043		0.043
1.8	No	No	Yes	0.029	0.023	0.017	0.029	0.009	0.004		0.004
8.6	No	No	No	0.406	0.380	0.08	0.068	0.046		0.406	0.406
8.9	No	No	Yes	0.198	0.029	0.198	0.023	0.183		0.045	0.045
9.2	No	Yes	Yes	0.071	0.007	0.035	0.071	0.009	0.059	0.047	0.053
20.1	No	No	Yes	1.367	0.722	0.3	1.367	0.175	0.319	0.107	0.213
21.9	No	No	Yes	3.609	3.609	0.535	1.1	2.962		0.175	0.175
24.9	Yes	Yes	Yes	0.192	0.095	0.192	0.067	0.108		0.029	0.029
25.3	No	Yes	Yes	0.107	0.069	0.027	0.107	0.04	0.008	0.009	0.009
29.2	No	No	No	0.038	0.007	0.004	0.01	0.002	0.017	0.038	0.028
34.3	No	No	Yes	0.268	0.268	0.019	0.069	0.04	0.166	0.07	0.118
38.5	No	No	No	1.563	0.088	0.027	0.463		1.563	0.096	0.83
38.7	No	Yes	Yes	3.964	0.028	0.463	0.072	3.964		0.004	0.004
39.1	No	Yes	No	4.468	4.468	0.281	0.089	0.029	0.043		0.043
42.3	No	No	No	59.555	2.074	59.555	30.353	1.205	0.115	0.04	0.077
47.3	No	Yes	Yes	24.857	24.857	0.356	0.992	0.261	0.111	0.086	0.099
49.2	Yes	Yes	Yes	0.023	0.001	0.023		0.009		0.001	0.001
51	No	Yes	No	19.829	19.829	16.13	1.431	1.359	0.505		0.505
55.9	No	No	Yes	0.238	0.004	0.003	0.02	0.238	0.036		0.036
57.7	No	Yes	No	0.353	0.010	0.049	0.162	0.211	0.098	0.353	0.225
58.3	No	Yes	No	22.501	0.156	22.501	4.16	4.17	0.518	16.422	8.47
63.2	No	Yes	Yes	9.843	2.880	9.843	0.666	7.425	0.01	0.97	0.49
65.4	Yes	Yes	Yes	3.809	3.809	0.045	0.039	0.139	0.344	0.318	0.331
82.6	No	Yes	No	29.49	0.212	0.278	27.244	29.49	0.013		0.013
104.2	No	Yes	Yes	1.141	0.166	1.141	0.029	0.187	0.099	0.107	0.103
108.8	No	No	No	2.092	1.717	2.092	0.568	0.089		0.068	0.068
122.9	No	No	No	9.898	5.099	9.898	0.408	0.223	1.569	9.054	5.311
133.7	No	No	No	14.054	2.216	12.259	14.054	0.549	0.32	0.971	0.645

Table E4. Predictors of loss of asthma control during an acute cold, and the nasal lavage neutrophil counts stratified by study visit. The 28 subjects with complete pore function and clinical data used for the models described in the Results section and Figure 3 are tabulated here. Pore activity reflects the fold of Bz-ATP induced YO-PRO-1 uptake in whole blood monocytes relative to the saline control. Prior to logistic regression analysis of the loss of asthma control, pore function data must be adjusted by the

square root transformation to achieve a normal distribution. Neutrophil counts in the nasal lavage fluid are also displayed according to study visit, however, these were not predictive of the loss of asthma control.

Figure E1. The $P2X_7$-150R variant (encoded by the 474A allele) was recombinantly expressed in HEK 293 cells along with controls with similar levels of mRNA and protein (not shown). Cells were stimulated with potassium glutamate containing varying concentrations of BzATP and 10 μM YO-PRO-1 for 20 min before the addition of 10 mM $MgCl_2$ to close the pore, followed by plate reader fluorescence detection. A representative of two experiments is shown with each data point reflective of the mean ± 50% of the range of duplicate measurements.

Figure E1


YO-PRO-1 Median Fluorescence Intensity
7000
6000
5000
4000
3000
2000
1000
0
0
1
10
100
1000
10000
BzATP (uM)
Vector
P2X7 WT
P2X7-150R
P2X7-496A