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MAJOR ARTICLE

Regulation of the Acute Sickness Response by the P2RX7 Receptor

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Background. The acute sickness response to infection is a stereotyped set of illness manifestations initiated by proinflammatory signals in the periphery but mediated centrally. *P2RX7* is a highly polymorphic gene encoding an ATP-gated cationic pore, widely expressed on immune cells and the brain, and regulating the NLRP3 inflammasome, as well as diverse neural functions.

Methods. Associations between *P2RX7* genotype, pore activity, and illness manifestations were examined in a cohort with acute viral and bacterial infections (n = 484). Genotyping of 12 *P2RX7* function-modifying single-nucleotide polymorphisms (SNPs) was used to identify haplotypes and diplotypes. Leucocyte pore activity was measured by uptake of the fluorescent dye, YO-PRO-1, and by ATP-induced interleukin-1β (IL-1β) release. Associations were sought with scores describing the symptom domains, or endophenotypes, derived from principal components analysis.

Results. Among the 12 SNPs, a 4-SNP haplotype block with 5 variants was found in 99.5% of the subjects. These haplotypes and diplotypes were closely associated with variations in pore activity and IL-1β production. Homozygous diplotypes were associated with overall illness severity as well as fatigue, pain, and mood disturbances.

Conclusions. *P2RX7* signaling plays a significant role in the acute sickness response to infection, likely acting in both the immune system and the brain.

Keywords. purinergic receptor; *P2RX7*; acute sickness response; genetics; disease association.

The acute sickness response to a wide spectrum of pathogens is a stereotyped set of clinical manifestations, including fever, fatigue, musculoskeletal pain, anorexia, disturbed mood, hypersomnia, and cognitive impairment [1, 2]. This response is highly conserved across species and is believed to represent a critical physiological adaptation that favors survival of the organism during infection. The response is initiated in the periphery by induction of proinflammatory cytokines acting on endothelial and epithelial cells of the blood-brain barrier and ultimately translated into central nervous system events via activation of resident microglial cells, secondary production of mediators, and ultimately altered neural transmissions, although the latter pathways are undefined [3, 4]. The response predominantly features subjectively reported symptoms such as pain and fatigue, but fever can be objectively recorded, and the associated biochemical changes in acute phase reactants such as C-reactive protein may be measured in the serum [5]. It is also clear in clinical practice that there is significant interindividual variability in the severity and pattern of illness manifestations during acute infections caused by the same pathogen [6]. This variability is partly attributable to host genetic determinants, including functional polymorphisms in proinflammatory cytokine genes [7, 8].

The P2X7 receptor (P2RX7) is a trimeric ligand-gated ion channel, which is widely expressed on cells of the immune system, as well as microglia, astrocytes, and neurons in the brain [9]. The ligand is extracellular adenosine 5′-triphosphate (ATP), which is a key damage-associated molecular pattern (DAMP) molecule when released from infected or injured cells. Ligation of ATP opens a cationic channel resulting in an influx of Ca^{2+} and Na^{+}, followed by larger pore formation, which may be evidenced by uptake of dyes such as YO-PRO-1 [10]. Multiple downstream events follow pore formation, including activation of numerous intracellular pathways related to inflammation, notably the NLRP3 inflammasome [11]. The gene (*P2RX7*) is highly polymorphic with numerous single-nucleotide polymorphisms (SNPs) identified (approximately 1500), including 3 recognized gain-of-function and 10 loss-of-function alleles [9]. Heterozygous combinations of individual SNPs and haplotypes generate complex and incompletely defined patterns of altered pore activity. These genetic variants have been associated with susceptibility to several infectious and inflammatory diseases,

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notably tuberculosis, as well as neurobehavioral phenomena relevant to the acute sickness response, including pain sensitivity and mood disturbance [9, 12].

The potential role of P2RX7 in the acute sickness response has not previously been studied.

METHODS

Subjects

Subjects in the Dubbo Infection Outcomes Study were originally recruited from primary care after presentation with an acute febrile illness and screening serology led to a provisional diagnosis of acute Epstein-Barr virus (EBV), Ross River virus (RRV), or Q fever infection [13]. Subsequently, acute and convalescent sera were tested for acute EBV, RRV, or Q fever infection to document IgG seroconversion or a 4-fold rise in antibody titer. Caucasian ancestry was designated on the country of birth of the parents of each participant. The study was approved by the relevant institutional review boards (approval numbers University of New South Wales HREC 04257 and 05091). All subjects provided written informed consent.

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the Wizard DNA kit (Promega). DNA was quantified using NanoDropR ND-1000 (BioLab), and the quality verified by agarose gel electrophoresis. Genotyping of SNPs was performed using Sequenom Mass ARRAY at the Australian Genome Research Facility. The 12 *P2RX7* SNPs included tag SNPs for previously reported haplotypes in Australian and European populations, and several SNPs associated with gain or loss of pore function [14]. Pairwise linkage disequilibrium (LD) was analyzed using the program Haploview (http://www.broad.mit.edu/mpg/haploview). In addition, a single *P2RX4* SNP was genotyped.

$P2X_7$ Pore Activity

PBMCs were separated from whole blood and stored under endotoxin-minimized conditions in aliquots, as described previously [7, 8]. The flow cytometric assay of pore activity was performed as previously described [10, 15]. Thawed PBMCs were costained with phycoerythrin-conjugated anti-CD14 Ab (BD Bioscience) and allophycocyanin-conjugated anti-CD3 Ab (BD Bioscience) were used to measure YO-PRO-1 dye (Molecular Probes) uptake into monocytes and lymphocytes. Flow cytometry was conducted on a FACSCalibur with CellQuest software (BD Bioscience). The data were analyzed as a batch using FlowJo (version 7.6.1) to apply consistent $CD14^+$ and $CD3^+$ gates to the complete dataset. The fold difference in geometric mean YO-PRO-1 fluorescence was used as the measure of pore activity by calculating fluorescence derived from $CD14^+$ or $CD3^+$ cells stimulated with benzoylbenzoyl-ATP (Bz-ATP; Sigma-Aldrich) relative to fluorescence from unstimulated cells. The coefficient of variation was 9.8% on samples from 4 subjects tested on 2 separate days (data not shown).

ATP-Induced IL-1β Release

Thawed PBMCs were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% (v/v) fetal bovine serum (JRH Biosciences) in the presence of 5 ng/mL lipopolysaccharide (LPS; from *Salmonella typhimurium*; Sigma-Aldrich, ATCC 7823). After 3 hours the supernatant was removed and cells were cultured for a further 30 minutes in media alone or in 125 μM Bz-ATP. The supernatants were harvested and stored at −80°C. Concentrations of interleukin-1β (IL-1β) were measured by enzyme-linked immunosorbent assay (ELISA; DUO Set, R & D Systems). The ratio of IL-1β production released from Bz-ATP treated cells to untreated cells was calculated.

Statistics

The distribution of genotype frequencies was tested for Hardy-Weinberg equilibrium using χ^2 tests. *P* values and regression coefficients were calculated using PLINK (http://zzz.bwh.harvard.edu/plink/) [16]. Linear regression analyses were applied to assess the association of the genotypes and pore activity with illness severity and endophenotypes (adjusted for age and sex, and infection type) using SPSS for Windows, version 25. Cytokine release and pore activity comparisons were undertaken using 2-tailed nonparametric tests in GraphPad Prism version 7.0. *P* values less than .05 were considered significant.

RESULTS

Subjects and Illness Characteristics

Clinical data, stored PBMCs, and genomic DNA from unambiguously Caucasian subjects (n = 484) enrolled in the Dubbo Infection Outcomes Study [13] were utilized, including 248 women (51%) with a mean age of 34 years (Table 1). The serologically documented acute infections included infection with: EBV, which causes infectious mononucleosis (n = 144; 29%); RRV, the mosquito-borne causative agent for epidemic polyarthritis in the Pacific region (n = 98; 20%); *Coxiella burnetii,* the intracellular bacterium that causes the zoonotic infection, Q fever (n = 84; 17%); as well as individuals with an acute febrile illness provisionally diagnosed with 1 of these 3 infections but not confirmed on acute and convalescent serological testing (n = 158; 33%). The symptom domains of the acute sickness response were characterized as described previously using principal components analysis of self-report symptom data to derive indices of overall illness severity, plus the key symptom domains or endophenotypes including fatigue, pain, neurocognitive difficulties, and mood disturbance [6]. The individual indices accounted for 39%–62% of the variance in the sample. Scores on the severity index were correlated with an independent measure of disability, the Brief Disability Questionnaire ($r = 0.46$, $P < .001$).

Table 1. Demographics, Infection Types, and Acute Sickness Response Characteristics

Characteristic	Infection Type				
	Epstein-Barr Virus (n = 144)	Ross River Virus (n = 98)	Q Fever (n = 84)	Unknown Acute Infection (n = 158)	Total (n = 484)
Age, y	22 (9)	41 (13)	40 (15)	35 (15)	34 (15)
Female, No. (%)	94 (65)	49 (50)	14 (17)	91 (58)	248 (51)
Acute sickness response characteristics, principal component					
Illness severity					
Upper tertile	1.07 (0.38)	1.17 (0.50)	1.12 (0.46)	1.36 (0.46)	1.20 (0.45)
Lower tertile	−1.10 (0.24)	−1.01 (0.28)	−1.02 (0.27)	−0.99 (0.32)	−1.04 (0.27)
Fatigue					
Upper tertile	1.16 (0.23)	1.04 (0.31)	1.05 (0.33)	1.37 (0.16)	1.23 (0.23)
Lower tertile	−1.02 (0.42)	−1.26 (0.37)	−1.02 (0.40)	−0.93 (0.52)	−1.01 (0.45)
Pain					
Upper tertile	0.73 (0.66)	1.59 (0.32)	1.20 (0.62)	1.38 (0.54)	1.26 (0.55)
Lower tertile	−1.05 (0.12)	−0.77 (0.32)	−1.02 (0.13)	−0.97 (0.18)	−0.99 (0.17)
Neurocognitive difficulties					
Upper tertile	1.24 (0.79)	1.33 (0.69)	1.32 (0.57)	1.55 (0.64)	1.45 (0.66)
Lower tertile	−0.85 (0.00)	−0.85 (0.00)	−0.85 (0.00)	−0.67 (0.26)	−0.85 (0.00)
Mood disturbance					
Upper tertile	1.21 (0.55)	1.13 (0.77)	1.09 (0.68)	1.38 (0.61)	1.25 (0.63)
Lower tertile	−0.95 (0.08)	−0.99 (0.01)	−0.96 (0.07)	−0.93 (0.10)	−0.95 (0.08)
Disability, Brief Disability Questionnaire					
Total score	9.57 (5.48)	10.05 (5.79)	11.24 (6.48)	10.32 (5.95)	10.19 (5.88)
Days out of role[a]	13 (9)	10 (10)	15 (11)	11 (10)	12 (10)

Data are mean (SD) except where indicated.

[a]Mean number of days out of role in the last month.

Genotyping

SNP genotyping was successful for 98.7% of samples, with an overall pass rate of 99.6% for all subjects. The minor allele frequency was greater than 0.01 for each SNP. Genotypes for all SNPs were in Hardy-Weinberg equilibrium (all $P > .7$). The previously reported 4 SNP linkage disequilibrium block was confirmed in the study population, defining 5 haplotypes previously designated as *P2RX7-1-5* (Figure 1A) [17].

P2RX7 Pore Activity

Stored PBMCs were available for n = 147 subjects, including samples from each infection subgroup. ATP-induced uptake of the fluorescent dye, YO-PRO-1, was examined in flow cytometry. As expected, pore activity data were normally distributed (Supplementary Figure 1), and tightly correlated between monocyte and lymphocyte subpopulations ($r = 0.93$; $P < .001$). The upper, mid, and lower tertiles of pore activity were labelled as high, normal, and low activity to allow examination of potential associations with *P2RX7* genotypes, intermediate phenotype (ie, pore activity), and illness endophenotypes.

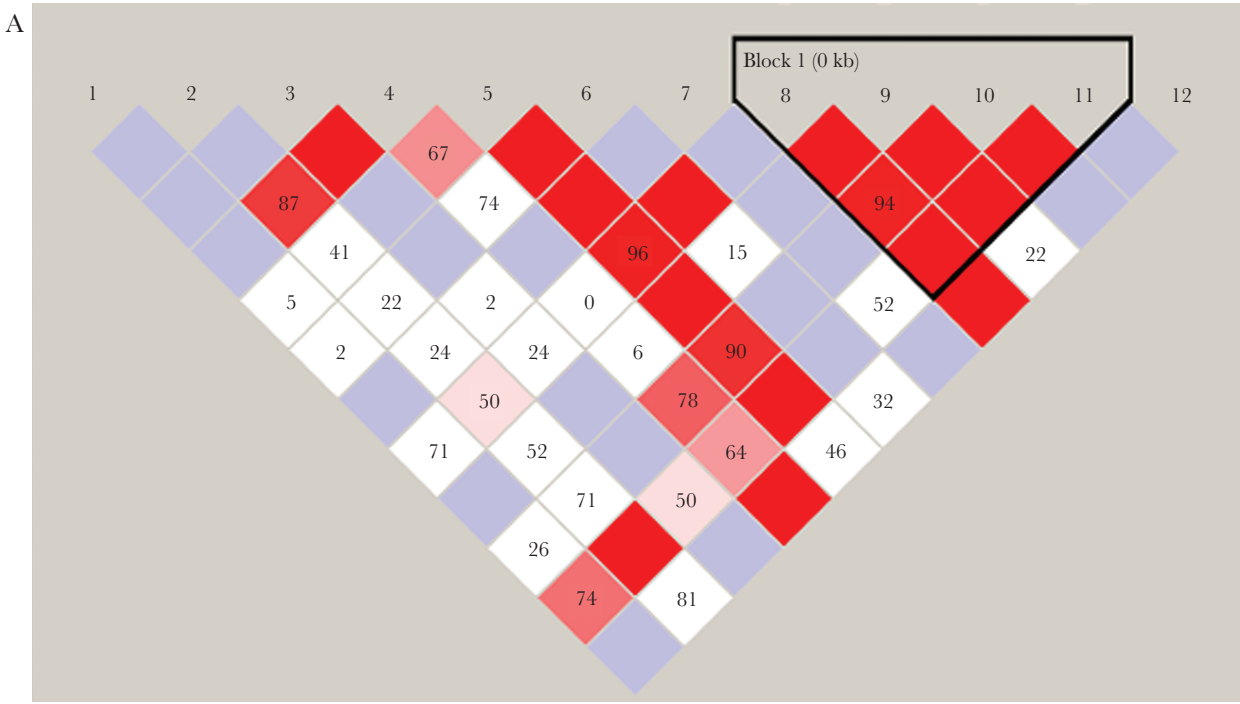
Associations Between P2RX7 Pore Activity and Genotype

The previous association of 2 haplotypes (*P2RX7-2* and *P2RX7-4*) with gain-of-function [17] was confirmed with the upper tertile of pore activity in individuals with homozygous diplotypes, and to low pore activity for the *P2RX7*-3 and -5 diplotypes, with the *P2RX7*-1 diplotype being the wild type with normal pore activity (Figure 1B). The pore activity measured by YO-PRO-1 uptake was confirmed in an assay measuring LPS-primed, ATP-induced activation and release of IL-1β from PBMCs (Supplementary Figure 2).

Associations Between *P2RX7* Genotype and Illness Endophenotypes

Individual *P2RX7* SNPs and heterozygous haplotypes were only weakly associated with overall severity and endophenotypes of the acute sickness response, reflecting the complex and often competing influences of the alleles (Supplementary Tables 1 and 2). The homozygous diplotypes were significantly associated with the clinical features (Figure 2), including overall severity, fatigue, and mood disturbance (all $P < .01$) as well as pain ($P < .05$), but not neurocognitive disturbance ($P = .1$). These associations were independent of age, sex, or infection type. Pore activity per se was not associated with the illness phenotypes ($P = .4$).

Given this finding and the observation that other purinergic receptors are known to contribute to pore activity in the assay, a *P2RX4* SNP (rs28360472, Tyr-315-Cys), which is in linkage with the *P2RX7* SNP rs28360447 (Gly-150-Arg) and has previously been associated with reduced phagocytosis [18], was also examined. The P2RX4 315-Cys containing haplotypes were associated with increased severity of fatigue,



Haplotype	8	9	10	11	Frequency
P2RX7-1 WT	G	C	A	T	0.34
P2RX7-2	A	C	A	T	0.222
P2RX7-3	G	C	A	G	0.205
P2RX7-4	A	C	G	T	0.154
P2RX7-5	G	G	A	T	0.074

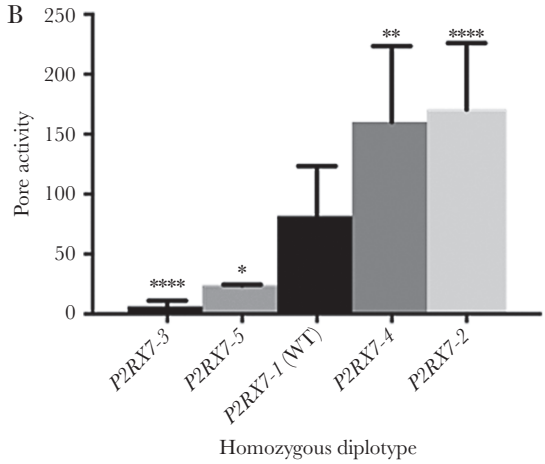


Figure 1. P2RX7 genotypes, haplotypes, and pore activity. *A*, Haploview analysis of pairwise linkage disequilibrium in the *P2RX7* gene using 12 marker SNPs. The colors represent the relative D′/LOD score: bright red, D′ = 1, LOD ≥ 2; blue, D′ = 1, LOD < 2; shades of pink, D′ < 1, LOD ≥ 2; and white, D′ < 1, LOD < 2. The D′ values indicate linkage disequilibrium between each pair of SNPs. The 4-SNP haplotype block represents 5 variants with their relative frequencies in the study population listed. *B*, The relationship between pore activity and P2RX7 genotype measured in 39 subjects with homozygous diplotypes. Pore activity was measured by YO-PRO-1 fluorescence in $CD14^+$ monocytes in lipopolysaccharide-primed peripheral blood mononuclear cells stimulated with benzoylbenzoyl-ATP relative to fluorescence from unstimulated cells. Each diplotype differed significantly from WT (Mann-Whitney tests). * $P < .05$, ** $P < .01$, **** $P < .001$. Abbreviations: LOD, logarithm of odds; P2RX7, purinergic receptor 7 gene; SNPs, single-nucleotide polymorphism; WT, wild type.

only in individuals with acute Q fever (β = .808, $P = .0380$; Supplementary Table 3).

DISCUSSION

In combination, these findings link genetic variations in *P2RX7* with variations in the severity and characteristics of the acute sickness response to infection. The P2X7 receptor has been linked to the pathogenesis of a wide range of infectious diseases, including with intracellular bacteria, viruses, parasites, and extracellular pathogens [19–21]. Polymorphisms in *P2RX7* have been associated with altered susceptibility, which may result from enhanced immunopathology (versus asymptomatic pathogen clearance or control), and also with a propensity to specific end-organ complications, such as the eye in acquired toxoplasmosis [22]. The data presented here extend these links to the universal phenomenon of the acute sickness response to infection, across diverse individual pathogens.

Taken together these data are consistent with the central role of the P2X7 receptor in induction of inflammation via activation of: the NLRP3 inflammasome leading to production of IL-1β and IL-18; the stress-activated protein kinase pathway, resulting in cellular apoptosis; and enhanced killing of pathogens via mitogen-activated protein kinase (MAPK) pathway activation. *P2RX7*-knockout mice show impaired induction of inflammation, cytokine release, and immune responses against many pathogens [20, 23, 24]. The findings reported here indicate that genetically determined regulation of P2RX7 pore function is an important contributor to symptom severity. These findings add to those in previous studies in this cohort, which revealed that functional polymorphisms in interferon-γ and IL-10 genes were associated with both severity and prolonged duration of the illness [8]. Further, production of IL-1β was correlated with the pattern and severity of symptoms of the acute sickness response [7]. As the heritability estimate in twin studies for LPS-stimulated production of IL-1β production is 86% [25], the findings here suggest that *P2RX7* genotype contributes to both IL-1β production and illness severity.

As the *P2RX7* gene is highly polymorphic with many gain and loss of function alleles [9], individuals with homozygous diplotypes offer the best opportunity to reliably investigate the functional consequences of these complex genetic variations. The previously identified 4 SNP haplotype block and frequencies in the Caucasian study population was confirmed, and also high and low pore activities comparable to those previously reported [17]. The 4 SNPs in this haplotype block are all nonsynonymous, and include: 2 that are known to confer loss of function (rs2230911, which encodes a Thr-357 to Ser mutation, and rs3751143, which encodes a Glu-496 to Ala mutation); rs2230912, which confers a Gln-460 to Arg mutation and has no significant functional effect in isolation; and rs1718119, which encodes an Ala-348 to Thr mutation and confers a gain-of-function effect. In addition, the rs2230912 Gln-460 to Arg allele in the $P2X_7$-4 variant was in linkage disequilibrium with additional minor alleles that were not part of the haplotype block, including 2 gain-of-function ectodomain SNPs, that is rs208294 His-155 to Tyr (D′ = 0.78) and rs7958311 His-270 to Arg (D′ = 0.90), confirming the previous report that this haplotype usually contains a total

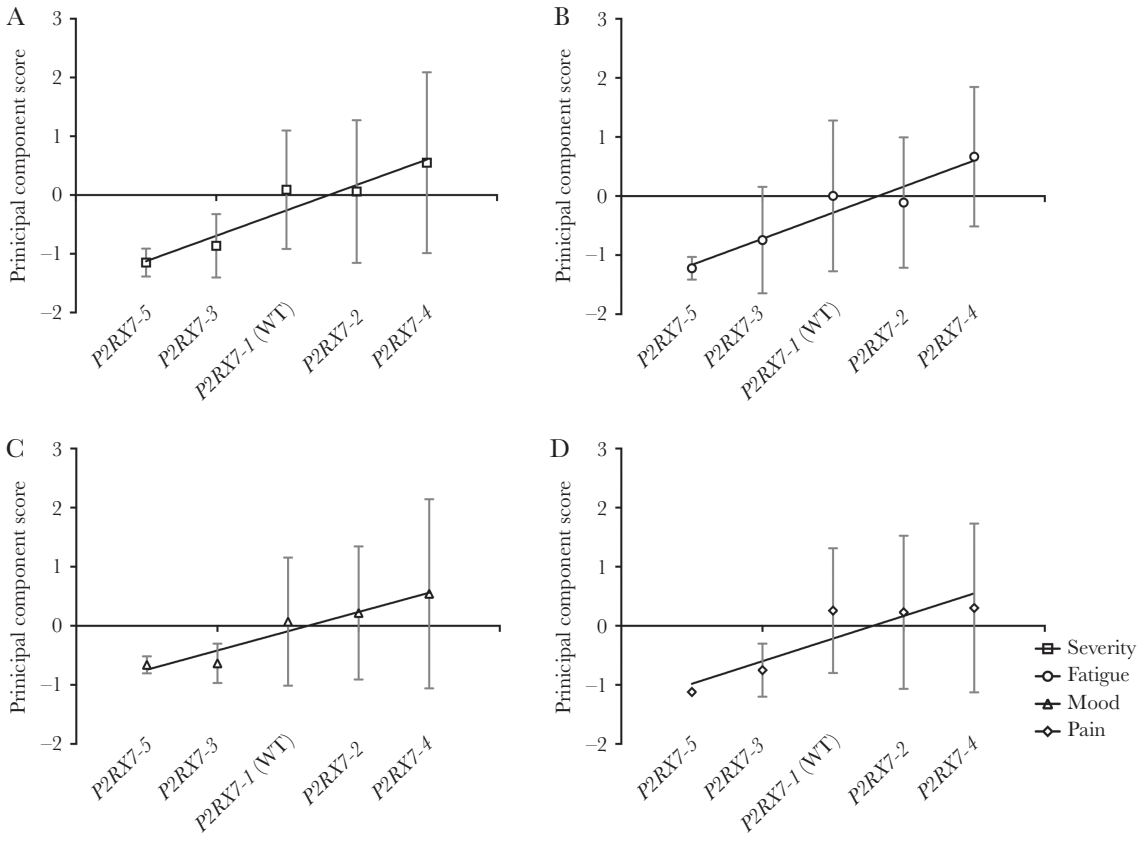


Figure 2. Homozygous *P2RX7* diplotypes and symptom scores. Linear relationships between homozygous *P2RX7* diplotypes associated with low pore activity (*P2RX7-5* and *P2RX7-3*), normal pore activity (*P2RX7-1* (WT)), or high pore activity (*P2RX7-2* and *P2RX7-4*) and illness characteristics of the acute sickness response measured by principal component scores in 39 subjects. Significant linear trends were evident in the relationship between symptom scores and overall illness severity (*A*; $P < .01$), as well as fatigue (*B*; $P < .01$), mood disturbance (*C*; $P < .01$), and pain (*D*; $P < .05$). There was no significant association with neurocognitive disturbance (not shown; $P = .14$). Abbreviations: *P2RX7*, purinergic receptor 7 gene; WT, wild type.

of 3 gain-of-function alleles [17]. Similarly, the rs1718119 Ala-348 to Thr mutation was also in strong linkage with the rs7958311 His-270 to Arg mutation, indicating that the *P2RX7-2* haplotype usually contains 2 gain-of-function alleles and confers increased receptor function (Figure 1B). In contrast, the loss-of-function SNP rs3751143 Glu-496 to Ala in the *P2RX7-3* variant was often coinherited with the 1 intronic SNP rs35933842 (D′ = 0.74), which is associated with reduced efficiency of mRNA splicing [26].

The findings here linking high-pore *P2RX7* genotypes with increased pain experienced during the acute sickness response are generally concordant with previous reports implicating the gain-of-function minor allele of rs7958311 (ie, Arg-270) with increased pain after mastectomy, or pain in association with osteoarthritis or experimental (cold pressor) testing [27, 28]. The 5 variants in the haplotype block were not included in these previous studies, although linkage between the minor allele of rs2230912 of *P2RX7-24* and rs7958311 was observed in the former study and was identified in the current cohort.

Genetic variations in *P2RX7* have also been implicated in susceptibility to major depression [29], with a meta-analysis confirming an association with the rs2230912 Gln-460 to Arg allele, which is associated with high pore activity [30]. Given this report, the finding of an association between the high pore activity diplotypes, *P2RX7-2* and *P2RX7-4*, and greater mood disturbance during acute infection suggests firstly that the association may be more generalizable than major depression per se, to include mood disturbance in other settings such as during infection, and secondly that further investigation of *P2RX7* haplotypes and pore activity in major depression is warranted.

The lack of a clear association between P2RX7 pore activity and illness phenotypes is consistent, firstly, with the fact that the leucocyte YO-PRO-1 assay provides an incomplete measure of the spectrum of P2RX7 biological activities, notably including phagocytic activity [31] and neurotransmitter release within the central nervous system [32]; and, secondly, that the assay lacks some specificity with recognized influences also from P2RX2, P2RX4, and P2RX5 [9]. The *P2RX4* SNP rs28360472 (Tyr-315-Cys), which is in linkage with the *P2RX7* SNP rs28360447 (Gly-150-Arg) and previously associated with reduced phagocytosis [18], was associated with increased severity of fatigue in individuals with acute Q fever. This finding is potentially consistent with the key role of phagocytosis in the host response to the causative intracellular bacterium, *C. burnetii*.

Taken together, the findings in this study suggest that the essentially universal human experience of an acute sickness response to infection may be regulated in part by the P2RX7 receptor, and therefore may be amenable to modulation by a combination of personalized medicine and therapeutics targeting the P2RX7 receptor.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. H. L. conducted the laboratory studies and genetic analyses. E. C. derived the symptom domain scores by principal components analysis. U. V. C. and A. R. L. led the design and conduct of the cohort study with the other DIOS study group members. J. M. and B. G. provided expert genetics advice. A. R. L. drafted the manuscript. All authors contributed to the final manuscript.

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