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Detection of Human P2X7 Nucleotide Receptor Polymorphisms by a Novel Monocyte Pore Assay Predictive of Alterations in Lipopolysaccharide-Induced Cytokine Production

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The nucleotide receptor P2X7 is expressed by most leukocytes and initiates signaling events that amplify numerous LPS responses. We tested the hypothesis that loss-of-function polymorphisms in the human P2X7 gene predispose to the production of an anti-inflammatory mediator balance. Accordingly, we developed a novel P2X7 pore assay in whole blood that magnifies the activity from wild-type alleles and preserves the gene dosage effect for the 1513 C polymorphism (AA, 69 ± 4; AC, 42 ± 4; and CC, 6 ± 1-fold stimulation). Thirty of 200 healthy individuals were identified as having low P2X7 pore activity. Seven low pore subjects were 1513 CC, 3 and 11 participants had the other known variants 946 GA and 1729 TA respectively; the remaining 9 volunteers likely have novel polymorphisms. Because platelets are a large source of extracellular ATP during inflammation, whole blood was treated ex vivo with Salmonella typhimurium LPS in the absence of exogenous nucleotides. LPS-stimulated whole blood from individuals in the low pore activity group generated reduced plasma levels of TNF-α (p = 0.036) and higher amounts of IL-10 (p < 0.001) relative to the high pore controls. This reduction in the TNF-α to IL-10 ratio persisted to at least 24 h and is further decreased by cotreatment with 2-methylthio-ATP. The ability of P2X7 polymorphisms to regulate the LPS-induced TNF-α to IL-10 ratio suggests that 15% of healthy adults may exhibit anti-inflammatory mediator responses during major infectious perturbations of the immune system, which can be predicted by P2X7 pore activity. The Journal of Immunology, 2005, 174: 4424–4431.

Despite rapid metabolism, endogenous adenine nucleotides are present at millimolar concentrations surrounding sites of inflammation, as a consequence of platelet degranulation and parenchymal cell death (1). In this, and in other contexts (e.g., neurotransmission and bronchial constriction), adenine nucleotide hormones interact with a family of receptors that are encoded by at least 17 distinct genes (2–4). Expression of this family of purinergic receptors is ubiquitous in many tissue types, and with multiple members found within a given cell type. P1 (adenosine) receptors bind nucleosides with one or no phosphate moieties, whereas P2 receptors bind diphosphate and/or triphosphate derivatives. P1 and P2Y members are heterotrimERIC G protein-coupled receptors with seven predicted transmembrane domains. By contrast, members of the P2X subfamily act as a ligand-gated, predominantly homotrimERIC, nonselective cation channels with each monomer containing two predicted membrane-spanning domains.

Unique features of the P2X7 nucleotide receptor suggest that it is a global thermistor of immune function (1). Most leukocytes express P2X7 (5), particularly in response to a variety of inflammatory stimuli (6), however, its localization to the cell surface is tightly regulated (7) by C-terminal lipid interaction motif resembling a conserved binding site for LPS (8–10). Upon nucleotide stimulation, this receptor regulates a variety of cell signaling mechanisms including calcium influx, increased activities of matrix metalloproteases, caspase-1, and stress-activated protein kinases, and nuclear localization and DNA binding capacity of the transcription factor NF-κB (11–15). In aggregate, this medley of signals contributes to monocyte and macrophage production of inflammatory mediators including TNF-α, IL-1β, and NO (12, 15–18). Similar signaling mechanisms may account for B cell transendothelial migration via the shedding of L-selectin and CD23 (19). Additionally, agonist stimulation for more than a few seconds causes a reversible expansion of the P2X7 selectivity filter, allowing for the passage of larger molecules (≥900 Da) (5). Independent from its channel function, P2X7 pore activity contributes to neutrophil superoxide formation (20), macrophage phagolysosomal maturation (21), and dendritic cell Ag presentation (22). Moreover, this receptor has a negative-feedback mechanism in that prolonged P2X7 stimulation results in leukocyte apoptosis (5), which can be aborted in the presence of additional inflammatory stimuli (23). Although most of the evidence to date has been pharmacological, the in vivo significance of these effects in the absence of exogenously administered nucleotides has recently been suggested by the attenuation of anti-collagen Ab-induced, LPS-promoted reactive arthritis in P2X7 knockout mice (24).
A number of species-specific P2X7 polymorphisms are known. Strains of mice have an allele conferring a P451L substitution (25), and a spontaneously derived subclone of RAW 264.7 macrophages has been shown to express a S342F change (8). In both, murine P2X7 pore activity is decreased. Additionally, five single nucleotide polymorphisms have been reported in the human P2X7 promoter, none of which alters receptor abundance or activity under the tested conditions (26). Finally, two polymorphisms have been identified in human P2X7-coding regions. The A1513C polymorphism encodes an E496A change resulting in defective pore formation (27), and the T1729A variant causes an I568N substitution that disrupts normal receptor trafficking (28).

Despite case control studies suggesting that the frequencies of two P2X7 polymorphisms are correlated with the incidence and the prognosis of familial chronic lymphocytic leukemia and tuberculosis (29–32), little is known regarding the effects of human P2X7 alleles on immune cell function. Because of established links between P2X7 function and the production of inflammatory mediators (12, 15–18), we tested the hypothesis that individuals with polymorphisms depressing P2X7 pore activity have lower levels of inflammatory mediators in response to challenge with LPS. To accomplish this objective, we devised a novel assay capable of rapidly segregating variant from common P2X7 phenotypes, thereby identifying 15% of a healthy adult population as having depressed monocyte P2X7 pore function. We present data herein to suggest that these individuals with reduced capacity for P2X7 pore formation are predisposed to an anti-inflammatory cytokine profile in the setting of immune system perturbation.

**Materials and Methods**

**Human subject participation**

Investigations were conducted with approval of the University of Wisconsin Institutional Review Board, and written informed consent was obtained from all the participants. Two hundred healthy (paid) volunteers between the ages of 18 and 50 were enrolled for the first phase of the study on 35 days over the course of a year with one to nine subjects enrolled per study-day. None had been hospitalized in the last year or used medicines on a daily basis. Ten milliliters of whole blood were obtained by routine phlebotomy from each participant, assigned an anonymized code number, and anticogulated with EDTA or citrate, respectively, for genetic and flow cytometric experiments.

Forty of these initial 200 subjects were recruited for a second cytokine phase of the study. In this phase, all 7 subjects with the P2X7 1513 CC genotype and low monocyte pore activity were enrolled, together with 4 randomly selected subjects from both the 1513 AA and AC groups (7 per group) with high (i.e., normal) pore activity. As discussed in Results, 23 phase 1 subjects exhibited low pore activity despite P2X7 1513 common AA or heterozygote AC genotypes. Of this latter group, 19 subjects were enrolled with 4 lost to follow up. Enrollement for the 40 subjects in the second phase was done on 7 different days with 3–7 subjects per day, and the investigators were blind to the scheduling details of any individual subject. Fifteen milliliters of whole blood were obtained from each participant at the return visit in either EDTA or citrate tubes for genetic, flow cytometric, and cytokine experiments. A second anonymized code was assigned to these samples such that the investigators performing the cytometric experiments. A second anonymized code was assigned to these samples such that the investigators performing the cytometric experiments.

**Determination of the P2X7 G946A, A1513C, and T1729A genotypes**

Genomic DNA was prepared from frozen whole blood samples using the Puregene DNA Isolation Kit (Gentra Systems). PCR primers for exon 13 of the human P2X7 gene were identical with those described by Gu et al. (27) (which amplifies a 356-bp product sufficient to incorporate both the 1513 and 1729 loci), and were synthesized by Integrated DNA Technologies. The final concentration of magnesium chloride was 1.5 mM and the annealing temperature was 58°C. The PCR product was digested with 2 U of the restriction endonuclease BseRI overnight at 37°C. The fragments were separated by gel electrophoresis in 1.5% agarose and observed by ethidium bromide staining. The P2X7 1513 C allele disrupts the BseRI palindromic sequence, thus the corresponding PCR fragment is not digested, producing three bands for the 1513 CC genotype (356, 256, and 100 bp) and one band for the CC individuals (356 bp). Because the latter result cannot be discerned from the uncut fragment, PCR products from subjects with the 1513 CC genotype were sequenced bidirectionally (University of Wisconsin Biotech Center). Additionally, the PCR products from respective P2X7 exons were sequenced for all subjects enrolled in the cytokine phase of the protocol to determine the G946A and T1729A genotypes.

**Monocyte P2X7 pore activity measured by flow cytometry of washed whole blood**

Monocytes were selected as the cell population to screen because of the greater variability in pore function noted between individuals participating in a small study with 45 healthy subjects (27). Aliquots of citrated whole blood (500 μl/aliquot) were washed twice in HEPES-buffered saline (130 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.4), 0.1% BSA, 10 mM glucose; components purchased at Sigma-Aldrich) and labeled at room temperature with 125 ng of an anti-human CD14 Ab conjugated to PE (BD Biosciences). After 20 min, the cells were washed twice in a potassium glutamate buffer (130 mM potassium glutamate, 5 mM KCl, 20 mM HEPES (pH 7.4), 0.1% BSA, 10 mM glucose; components from Sigma-Aldrich) to maximize the differences between high and low pore activities (33). In the absence of NaCl, cells were stimulated for 20 min with 0 or 250 μM 2′,3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (Bz-ATP) (Sigma-Aldrich) in the presence of 1 μM YO-PRO-1 (Molecular Probes). Samples were then adjusted to 10 mM magnesium chloride, washed in HEPES-buffered saline, and diluted to a volume of 2.5 μl in HEPES-buffered saline.

Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson) calibrated daily using standard fluorometric beads in conjuction with the CellQuest and CellQuestPro acquisition and analysis software (version 3.3 and 4.0; Becton Dickinson). Instrument settings (forward scatter, B00 mV; side scatter, 458 mV; FL-1, 410 mV; FL-2, 412 mV; acquisition threshold for FL-2, 324 mV; compensation, FL-2 − 32.6% FL-1) were derived before enrolling study subjects using purified blood monocytes (obtained from investigator L. C. Denlinger) that had been separated from the red cells with Ficoll-Hypaque (Sigma-Aldrich), and stained with a PE-conjugated anti-CD14 Ab in the presence and absence of YO-PRO-1. The PE signal is collected with a 585-nm filter with a 42-nm band pass (FL-2), whereas the YO-PRO-1 signal is collected with a 530-nm filter and a 30-nm band pass (FL-1). Using the results from the purified monocytes, the instrument is then set to trigger on the PE signal by adjusting the acquisition threshold above the background associated from unlabeled cells. Thus, data from all non-PE-labeled cells are not acquired, and pilot experiments with the isotype control Ab documented that this threshold was specific for CD14+ cells with forward and side scatter characteristics consistent with monocytes (unpublished data). Because the YO-PRO-1 signal is so intense, compensation was used to eliminate the YO-PRO-1 signal in the PE channel. These standard settings were then used without adjustment for the remainder of the investigation. Whereas this enhances reproducibility of our study, it also contributes to day-to-day assay variability. We therefore chose the Bz-ATP-induced fold stimulation of YO-PRO-1 uptake as a measurement of P2X7 pore activity in an attempt to account for these systematic factors, as well as minimize the potential variability from P2X7-independent sources of YO-PRO-1 uptake such as pinocytosis.

**Quantification of plasma cytokine levels after ex vivo stimulation of whole blood with LPS**

Aliquots of citrated whole blood (1 ml/aliquot) were stimulated with Hank’s standard PBS without calcium or magnesium in the presence and absence of Salmonella typhimurium LPS (LPS, 0.1 μg/ml; American Type Culture Collection strain 14028, List Biologicals, www.listlabs.com) for 6 or 24 h at 37°C with 5% CO2, in the presence or absence of 2-MeS-ATP, 2-methylothio-ATP (2-MeS-ATP; A. G. Scientific), a nucleotide previously known to reduce LPS-stimulated TNF-α levels in mice (34). Plasma samples were collected after centrifugation, aliquoted, and stored at −80°C. Sandwich ELISA quantification of TNF-α, IL-1β, and IL-10 levels in diluted plasma was done with the OpELIA reagents for 20 plates (BD Biosciences) according to standard methods. Data from a custom Search Light software (version 8.0; Imaging Research) for these cytokines were also compared. Standard curves were generated with the provided recombinant cytokines mixed with assay diluent and an identical dilution of unstimulated Monocytes

![Image](https://via.placeholder.com/150)
citrated plasma. Each plate contained one or more sets of plasma samples with known cytokine concentrations. All subject samples were run in duplicate on the same plate.

Statistical analysis

A $\chi^2$ goodness of fit test (35) was used to determine whether the 1513 C allele frequency was in accordance with the principles of the Hardy-Weinberg equilibrium (36). For flow cytometric experiments, data analysis was done as a batch using FlowJo software (version 4.3; Tree Star) to apply the same CD14$^+$ analysis gates to the entire study. Monocytes are known to take up fluorescent dyes by macropinocytic mechanisms (evidenced by comparing the fluorescence associated with unstimulated monocytes in the presence and absence of YO-PRO-1, data not shown), and this process likely has variability within a large sample independent from P2X$_7$ (37). Thus, to make the measurements of dye uptake more reflective of P2X$_7$ pore activity, a fold stimulation was calculated using the ratio of the geometric mean of YO-PRO-1 fluorescence from 10,000 CD14$^+$ cells treated with Bz-ATP relative to the geometric mean fluorescence derived from 10,000 CD14$^+$ cells treated with the vehicle control.

The ratios of these means were entered in to one-way ANOVA with three classes as determined by the A1513C genotype, followed by unpaired Student’s $t$ tests with correction for unequal variance. To determine the lowest fold stimulation of monocyte pore activity statistically different from the P2X$_7$ 1513 CC group, the SD of pore activity in this group was multiplied by 2.41 (the $t$ statistic for six degrees of freedom), and this product was added to the group mean. By this method, high P2X$_7$ pore activity was defined as $>$15-fold Bz-ATP-induced YO-PRO-1 uptake by CD14$^+$ cells, and low activity was established as $\leq$15-fold. Thus, any new subject with $>$15-fold Bz-ATP-induced pore activity has a 95% chance of being statistically different from the group of subjects with the 1513 CC genotype.

For the cytokine portion of the study, subject assignment to the low or high pore activity group was verified by replication of the phase I monocyte pore assay on the day of phase II re-enrollment. Although one individual in the high pore group and three subjects with low activity crossed over the assignment threshold defined above, the cytokine data were analyzed by the intention to treat method such that the initial group designations from phase I were applied for all of the data. Regarding the cytokine comparisons between groups, unpaired Student’s $t$ tests were again used with correction for unequal variance. Comparisons with and without 2-MeS-ATP cotreatment were performed with the paired Student’s $t$ test. All calculations were performed using Excel/Mac 2001, version SR1 (Microsoft) with a $p$-value of 0.05 adopted as the threshold for significance.

Results

Monocyte P2X$_7$ pore activity as a screen for individuals with P2X$_7$ genetic polymorphisms

To test the influence of various P2X$_7$ alleles on immune function, we established a rapid screening assay sensitive to the presence of known polymorphisms. Although P2X$_7$ is expressed in most leukocytes, monocytes exhibit the greatest variability in pore activity (27). Previous methods used to study the P2X$_7$ pore activity in primary cells include the lysis of erythrocytes, the isolation of whole blood leukocytes by gradient centrifugation, or the purification of lymph node T cells (25, 27). These techniques are too laborious for large phenotypic screens, and are confounded by the premature release of endogenous nucleotides as well as the potential for Percoll gradient-induced activation of monocytes by physical factors and/or contaminating LPS. By contrast, the labeling of whole blood with a CD14-specific Ab allowed for the use of a flow cytometry acquisition threshold technique to rapidly identify monocytes in these samples (Fig. 1), and dramatically reduced the potential for systematic variability associated with these isolation procedures. To maximize the differences in pore activity between the groups of subjects, we implemented a long treatment time (20 min) at ambient temperature with a medium dose of a selective P2X$_7$ agonist (250 $\mu$M Bz-ATP) in the absence of sodium chloride, followed by pore closure at the end of the assay upon adjustment to 10 mM MgCl$_2$ before washing (our unpublished data and Ref. 33). These conditions selectively allowed for robust monocyte uptake of the fluorescent dye YO-PRO-1 in samples from 1513 AA subjects, with little to no P2X$_7$-stimulated activity associated with the CC genotype (Fig. 1).

For all subjects, we measured the baseline fluorescence of CD14$^+$ cells in whole blood samples mixed with YO-PRO-1, and compared them to readings obtained after stimulation with 250 $\mu$M Bz-ATP. The basal YO-PRO-1 fluorescence associated with untreated CD14$^+$ cells in whole blood had a coefficient of variance of 0.40 over the course of the study, approximately half of which was due to day-to-day assay variability. We chose the Bz-ATP-induced fold stimulation of YO-PRO-1 uptake as a measurement of P2X$_7$ pore activity in an attempt to account for these systematic factors, as well as minimize the potential variability from P2X$_7$-independent sources of YO-PRO-1 uptake such as pinocytosis.

With this rapid whole blood assay, we phenotypically screened 200 healthy adults and correlated the results with the P2X$_7$ A1513C genotype, because gene dosage is known to predict pore activity measured by other methods (27). Sixty-nine AC heterozygous and seven CC homozygous individuals were identified, yielding a P2X$_7$ 1513 C allele frequency of 0.21 with a distribution in accordance with the Hardy-Weinberg equilibrium ($\chi^2 = 0.7, p > 0.5$) (36). Despite conditions that favor the identification of low responders, the rapid pore assay produced average fold stimulations of monocyte pore activity that were statistically distinct for each group according to the P2X$_7$ 1513 CC genotype (Fig. 2). Notably, all samples taken from subjects with the variant CC genotype had relatively low inducible P2X$_7$ pore activity (Fig. 2). An ANOVA demonstrated that there was significantly more pore activity associated with the CC genotype compared with the genotypes with $p < 0.001$. The three $t$ test comparisons between the groups were significantly different with $p < 0.001$ (Fig. 2). Thus, the washed whole blood monocyte pore assay correctly identified all individuals with the P2X$_7$ 1513 CC genotype, and preserved the gene dosage effect previously described for the C allele (27).
Frequency of depressed monocyte pore activity in a healthy adult population, identification of individuals with other P2X7 polymorphisms, and performance of the whole blood pore assay

Given the results of the P2X7, 1513 CC group, we defined low monocyte pore activity statistically as \( \leq 15 \)-fold induction of Bz-ATP stimulated uptake of YO-PRO-1 (please see Statistical analysis in Materials and Methods). Using this threshold, 23 additional subjects had low pore activity despite their 1513 AA \((n = 11)\) or AC \((n = 12)\) genotypes, after confirmation of the latter results by sequence analysis of the PCR products from P2X7 exon 13. This exon also contains a single nucleotide polymorphism (T1729A) that confers an amino acid substitution (I568N) influencing the cell surface localization of the receptor (28). Hence, 11 individuals in the low pore activity group were identified with the P2X7, 1729 TA (but none with 1729 AA) genotype, 9 of which were enrolled in the cytokine portion of our study (see below as well as Table I). This was in keeping with its previously observed low allele frequency (0.02; Ref. 28). The 1513 C and the 1729 A P2X7 polymorphisms segregated independently in our population; the 1729 A allele was equally present in individuals with the common 1513 AA and the heterozygotic AC genotypes \((n = 6 \text{ and } 5, \text{ respectively})\), and none of the 1513 CC subjects carried the 1729 A change. As three heterozygos subjects were identified with the recently described loss-of-function allele at position 946 (38), this left nine individuals who had low monocyte P2X7 pore activity despite the presence of the common 946 GG and 1729 TT genotypes, in conjunction with the absence of the variant 1513 CC genotype. These data suggest the presence of yet-to-be-discovered P2X7 alleles and/or distinct genetic loci affecting nucleotide-stimulated monocyte pore activity.

Although the basal YO-PRO-1 fluorescence obviously affects the calculated Bz-ATP-induced fold stimulation of dye uptake, these values did not differ between the high and low pore activity groups \((p = 0.62)\). Evaluation of the distribution of baseline data and replacement of outlier baseline data (those \(>\text{mean} \pm 2 \text{SDs}\)) with the group mean of unstimulated fluorescence showed that the calculation of fold stimulation resulted in only 1 of 200 subjects receiving an inappropriate pore activity group assignment. In sum, this whole blood pore assay accurately identified individuals with loss-of-function P2X7 alleles.

Cytokine production by LPS-stimulated whole blood

Previous pharmacological studies have linked P2X7 activity to the modulation of the levels of a variety of NF-kB-dependent inflammatory cytokines and mediators (14–16, 18), and the TNF-\(\alpha\) to IL-10 ratio in particular is important to the outcome of a variety of inflammatory disorders (39–42). Hence, we hypothesized that individuals with the 1513 CC genotype and/or low pore activity regardless of their P2X7 genotype would produce an anti-inflammatory cytokine profile in response to LPS. To test this hypothesis, we re-enrolled 40 of the initial 200 phase I subjects; 26 from the low pore group and 14 randomly selected controls with high activity and with equal representation of the 1513 AA and AC genotypes (Table I). The pore assay group assignments from phase I were reproducible for 36 of the 40 phase II subjects staying below or above the 15-fold stimulation cut-off and with collective intrasubject day-to-day coefficients of variance of 0.16 and 0.32 for the low and high groups, respectively. Three subjects with low pore activity in phase I had a 13, 45, and 57% increase in their phase II pore assay results, whereas one subject with high phase I pore activity had a 53% reduction on retesting such that the replicate result predicted the opposite group assignment in phase II. In all cases, the phase I group assignments were used for an intent-to-treat analysis of the cytokine data.

Whole blood samples were used to measure the cytokine responses in vitro after 6 or 24 h of stimulation with 0 or 100 ng/ml S. typhimurium LPS, where the former time point produces half-maximal responses in most donors for many cytokines (43–46). Additionally, LPS-stimulated platelets in whole blood are the source for abundant levels of endogenous adenine nucleotides (47) and the interaction between LPS-stimulated platelets and monocytes has been shown to augment the production of both TNF-\(\alpha\) and IL-1\(\beta\) (48). With respect to TNF-\(\alpha\), IL-10, and IL-1\(\beta\), the saline-treated samples contained undetectable plasma levels for all but one of the subjects, whereas LPS-treatment of whole blood produced a robust stimulation of these cytokines (Fig. 3). In comparing the results from the subjects with variant P2X7 function, the samples associated with low P2X7 monocyte pore activity had lower LPS-induced levels of TNF-\(\alpha\) relative to the high pore activity group (Fig. 3). This coincided with higher levels of IL-10 in the low pore group and no difference with respect to IL-1\(\beta\) (Fig. 3).

When the TNF-\(\alpha\) to IL-10 ratio was calculated on an individual subject basis, this measure for subjects with high pore activity was >3.2-fold on average than that of the low pore group, and this effect persists up to 24 h (Fig. 4). Thus, the monocyte pore assay

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**Table I. P2X7 genotype distribution for subjects enrolled in the LPS-induced cytokine study separated by pore activity**

<table>
<thead>
<tr>
<th>P2X7 Pore Activity</th>
<th>P2X7 A1513C Genotype</th>
<th>P2X7 T1729A Genotype</th>
<th>No. of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>AA</td>
<td>TT</td>
<td>7</td>
</tr>
<tr>
<td>High</td>
<td>AC</td>
<td>TT</td>
<td>7</td>
</tr>
<tr>
<td>Low</td>
<td>AA</td>
<td>TT</td>
<td>4</td>
</tr>
<tr>
<td>Low</td>
<td>AA</td>
<td>TA</td>
<td>5</td>
</tr>
<tr>
<td>Low</td>
<td>AC</td>
<td>TT</td>
<td>6</td>
</tr>
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<td>AC</td>
<td>TA</td>
<td>4</td>
</tr>
<tr>
<td>Low</td>
<td>CC</td>
<td>TT</td>
<td>7</td>
</tr>
</tbody>
</table>

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*Fourteen and 26 subjects were enrolled into Phase II of the study with high and low pore activity assignments and the genotypes were confirmed by sequence analysis of PCR products from exon 13. The latter group includes 19 of the 23 Phase I subjects with low pore activity despite the presence of at least one 1513 A allele. The genotypes of the four subjects with low pore activity lost to follow up are AA/TT, AA/TA, AC/TT, and AC/TA; these four subjects are not included in the table.*
predicted the P2X7 genotype, as well as the TNF-α to IL-10 ratio in response to whole blood treatment with LPS.

We also performed a cotreatment with 1 mM 2-MeS-ATP for 6 and 24 h (Fig. 5). Like many endogenous hormones such as epinephrine, 2-MeS-ATP exerts its effects via interactions with multiple receptors. This nucleotide has previously been shown to reduce LPS-stimulated murine TNF-α serum levels in vivo and to protect mice from endotoxic death (34). Consistent with these observations, 2-MeS-ATP cotreatment reduced the LPS-stimulated levels of TNF-α in whole blood samples at 6 h by 50 ± 7%, as well as at 24 h (Fig. 5), independent of P2X7 pore activity. By contrast, there was no effect of 2-MeS-ATP cotreatment on LPS-induced IL-10 levels at either time point (data not shown and Fig. 5). Because 2-MeS-ATP is an agonist for P2X7 (49), we reasoned that if small differences in IL-1β levels at 6 h between the high and low pore groups existed, cotreatment with this nucleotide would amplify them. However, 2-MeS-ATP cotreatment had no influence on IL-1β levels at 6 h (5 ± 10% change). There was an increase in production of this cytokine at 24 h, although again via a pore-independent mechanism (Fig. 5).

**Discussion**

The present investigation confirms the P2X7r 1513 C allele frequency in a large sample, and extends these results to include individuals from North America. Previous studies have documented a 1513 C allele frequency of 0.09 in Gambians (32), 0.12 in Australians (27), and 0.14 in Swedes (30), in comparison to our findings of 0.21 in the upper Midwest. The 1513 allele is more common than the P2X7r 1729 polymorphism, with an estimated 1729 A allele frequency of 0.02 in Australians (28) and at least 0.03 in our sample. Coupled with the five other human P2X7 promoter polymorphisms (26) and two murine structural variants, this genetic locus may be a region of greater variability than presently documented.

This is the largest study to date to evaluate the variability of P2X7 function in monocytes. In particular, we have developed a novel method for characterizing P2X7 pore function with several distinct advantages. The Ab labeling and flow cytometric threshold techniques allow for the functional assessment of monocytes (or other cell types) using 1 ml of whole blood, an aspect that has tremendous significance regarding the potential for future use in an unstable, critically ill patient population. With the existing method, results are available in 3 h from the time of phlebotomy, making possible the design of immunomodulatory clinical trials with prospective stratification of patient subsets. Moreover, the technique is readily adaptable for use in a clinical lab of an average community hospital, broadening its applicability compared with previous methods.

In addition, we demonstrate a subset of healthy subjects with discordance between their P2X7r 1513 genotypes and monocyte LPS-stimulated whole blood production of cytokines. Whole blood samples from phase II subjects were stimulated for 6 h at 37°C with 0 or 100 ng/ml *S. typhimurium* LPS, followed by centrifugation to harvest plasma. The control samples contained undetectable levels of TNF-α, IL-10, and IL-1β. Subject samples were quantified by sandwich ELISA in duplicate, and the data shown are the group means and SEs from 14 and 26 subjects in the high and low pore groups respectively. Similar results were also seen using a Search Light cytokine array.

**FIGURE 3.** LPS-stimulated whole blood production of cytokines. Whole blood samples from phase II subjects were stimulated for 6 h with 0 or 100 ng/ml *S. typhimurium* LPS, followed by centrifugation to harvest plasma. The control samples contained undetectable levels of TNF-α, IL-10, and IL-1β. Subject samples were quantified by sandwich ELISA in duplicate, and the data shown are the group means and SEs from 14 and 26 subjects in the high and low pore groups respectively. Similar results were also seen using a Search Light cytokine array.

**FIGURE 4.** Effect of variant P2X7r function on the TNF-α to IL-10 ratio. Whole blood samples from phase II subjects were stimulated for 6 or 24 h at 37°C with 0 or 100 ng/ml *S. typhimurium* LPS followed by centrifugation to harvest plasma. The control samples contained undetectable levels of these cytokines. As in Fig. 3, subject samples were quantified by sandwich ELISA in duplicate, and the data shown are the group means and SEs from 14 and 26 subjects in the high and low pore groups, respectively. The TNF-α to IL-10 ratio was calculated on an individual subject basis before deriving group means and SEs. Results from unpaired Student’s *t* tests with adjustment for unequal variance are also shown.

**FIGURE 5.** Tumor Necrosis Factor-α and Interleukin-10 production in response to 2-MeS-ATP cotreatment. Whole blood samples from phase II subjects were stimulated for 6 or 24 h at 37°C with 0 or 100 ng/ml *S. typhimurium* LPS followed by centrifugation to harvest plasma. The control samples contained undetectable levels of these cytokines. As in Fig. 3, subject samples were quantified by sandwich ELISA in duplicate, and the data shown are the group means and SEs from 14 and 26 subjects in the high and low pore groups respectively. Similar results were also seen using a Search Light cytokine array.

**FIGURE 6.** P2X7r Pore Activity.
pore activities. Twenty-three individuals in our sample had low pore activity despite the presence of at least one wild-type 1513 A allele (Fig. 2 and Table I). Three and 11 of these 23 were 946 GA and 1729 TA heterozygotes respectively, suggesting that at least 9 individuals in this study have yet to be disclosed polymorphisms affecting monocyte P2X7 pore activity. In combination with the subjects in this study, it is unlikely that defects in the P2X7 pore are associated with gross immunodeficiency, however, these alleles may contribute to the variability in the immune response when the system is under stress, such as during a major infection. A potential trade off might be enhanced microbial clearance at the expense of a higher incidence of autoimmune disorders and vice versa. Most candidate genes for these types of questions have multiple alleles, each with variable influence on protein function, inconsistent allele frequencies among distinct substrata of a given population, and unequal associations with clinical disease. Thus, functional tests, like the rapid monocyte pore assay, that are able to account for the influence of multiple alleles in linked pathways and to screen for polymorphisms at novel loci, are needed to assess the biological relevance of genetic variation in the pathogenesis of a given disease process.

Two P2X7 alleles have been associated with human disease. Gu et al. (27) identified the A1513C polymorphism associated with normal P2X7 protein expression levels and subcellular localization, but defective pore formation. Although the C allele has been correlated with resistance to ATP-induced apoptosis and with an increased incidence of familial chronic lymphocytic leukemia, an intriguing observation is that this subset of chronic lymphocytic leukemia patients has improved survival, perhaps via attenuated inflammatory mediator production (29–31). Additionally, Lammas and colleagues (32) found that the C allele of a P2X7 –762 promoter polymorphism was associated with a lower incidence of smear-positive pulmonary tuberculosis in a Gambian population. Although neither of these nor any other promoter polymorphisms appear to affect surface P2X7 expression (26), this receptor has been shown to have a large intracellular pool that promotes phagolysosomal maturation needed to facilitate killing of Mycobacteria tuberculosis (21, 50). Thus, if the –762 C allele is associated with enhanced mRNA and/or protein trafficking, these individuals may be better able to clear the initial infection such that they do not progress to active disease. Regardless of the mechanisms, in both of these association studies it is conceivable that an altered immune response might be associated with the clinical conditions.

Recently, Sluyter et al. (51) showed that LPS-primed monocytes from four subjects with the P2X7 1513 CC genotype release 75% less IL-1β after 30 min of ATP treatment relative to wild-type controls, however, this effect is no longer significant at 60 min. They demonstrate that this effect may extend to 2 h of ATP-treatment in LPS-primed whole blood (51). Given the transience of these results, it is not surprising that we did not detect a difference in IL-1β levels between the high and low pore groups after 6 h of whole blood stimulation with LPS and endogenous platelet-derived nucleotides (Fig. 3). Because LPS-treatment of monocytes causes the release of multiple forms of IL-1β (52), it is possible that when whole blood from subjects with low P2X7 pore activity is stimulated with LPS alone, less of what has been released in total is the active 17-kDa form but that this difference is not detectable by ELISA due to shared epitopes on both the pro and the active forms. However, in designing the current protocol with whole plasma samples, pilot experiments demonstrated that Western blotting with or without IL-1β immunoprecipitation for sample enrichment were semiquantitative at best with respect to analysis of the release of the 17-kDa active form of IL-1β (data not shown). Additionally, purification of monocytes was felt to be too cumbersome and variable for our study with 40 subjects. Therefore, to minimize sources of systematic variability in our large clinical study, we used whole blood assays for the detection of pore activity as well as the generation of LPS-induced cytokines. In this regard, LPS-stimulated platelets in whole blood are the source for abundant levels of endogenous adenosine nucleotides (47) and the interaction between LPS-stimulated platelets and monocytes has been shown to augment the production of both TNF-α and IL-1β (48), although the mechanism remains poorly characterized. Additionally, the use of whole blood and the inherent cotreatment with endogenous platelet-derived nucleotides avoids the controversial issue of whether purified myeloid lineage cells release ATP for autocrine stimulation (53, 54). Moreover, the P2X7 agonist, 2-MeS-ATP, failed to augment differences in LPS-stimulated...
IL-1β release between the high and low pore activity groups, suggesting that the contribution of this receptor to the postranslational processing of this cytokine is primarily at early time points.

This is the first study to demonstrate an influence of human P2X7 alleles on the levels of TNF-α and IL-10, an effect that is sustained for as long as 24 h. The opposite direction of the effects on these two cytokines likely reflects a relative lack of a systematic bias of our methods. The lower plasma levels of TNF-α in the LPS-treated whole blood samples from subjects with low pore activity is predictable from pharmacological studies with P2X7 agonists and antagonists (55). However, there previously has been little information regarding the control of IL-10 by P2X7. Although there is preliminary work showing that ATP-Y-S can synergize with LPS cotreatments to increase IL-10 steady-state mRNA and protein levels produced by human monocyte-derived dendritic cells, there was insufficient data to conclude a role for a particular purinergic receptor and this analog binds both P2X7, as well as P2Y11 (56). Similar preliminary data have also been observed with ATP-pretreatment of LPS-stimulated murine peritoneal macrophages (57). Because the P2X7 C-terminal domain has multiple motifs able to participate in channel-independent signaling (10), and because stimulation of P2X7 may also activate NF-κB-independent gene transcription via AP-1 and NFAT (58, 59), it will be of great interest to resolve the mechanism(s) by which individuals with attenuated P2X7 pore activity exhibit high LPS-induced production of IL-10. Alternatively, wild-type P2X7 activity may suppress IL-10 production, which is achieved in the absence of a functional allele. Another model is suggested by the regulation of IL-10 production by a P2Y receptor (56), wherein platelet-derived nucleotides interact with more than one receptor and this putative P2Y receptor might have elevated expression levels in the absence of functional P2X7. Finally, it is possible that defects in monocyte P2X7 pore activity serve a marker for other hyporesponsive loci in the TLR4 pathway, such that LPS-stimulated inflammation of whole blood results in TLR2-dependent IL-10 production (3). North, R. A. 2002. Molecular physiology of P2X receptors. Physiol. Rev. 82:1013.

Disclosures
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References


