A Thr^357 to Ser Polymorphism in Homozygous and Compound Heterozygous Subjects Causes Absent or Reduced P2X7 Function and Impairs ATP-induced Mycobacterial Killing by Macrophages

Received for publication, July 19, 2005, and in revised form, October 13, 2005 Published, JBC Papers in Press, November 1, 2005, DOI 10.1074/jbc.M507816200

Anne N. Shemon, Ronald Sluyter, Suran L. Fernando, Alison L. Clarke, Lan-Phuong Dao-Ung, Kristen K. Skarratt, Bernadette M. Saunders, Khai See Tan, Ben J. Gu, Stephen J. Fuller, Warwick J. Britton, Steven Petrou, and James S. Wiley

From the Department of Medicine, University of Sydney, Level 5, Spurrett Building, Nepean Hospital, Penrith, New South Wales 2750, the Centenary Institute of Cancer Medicine and Cell Biology, Camperdown, New South Wales 2042, the Discipline of Medicine, Central Clinical School, University of Sydney, Sydney, New South Wales 2006, and the Howard Florey Institute, University of Melbourne, Victoria 3010, Australia

The P2X7 receptor is a ligand-gated cation channel that is highly expressed on mononuclear leukocytes and that mediates ATP-induced apoptosis and killing of intracellular pathogens. There is a wide variation in P2X7 receptor function between subjects, explained in part by four loss-of-function polymorphisms (R307Q, E496A, I568N, and a 5′-intrinsic splice site polymorphism), as well as rare mutations. In this study, we report the allele frequencies of 11 non-synonymous P2X7 polymorphisms and describe a fifth loss-of-function polymorphism in the gene, T357S, which changes Thr^357 to Ser (T357S) with an allele frequency of 0.08 in the Caucasian population. P2X7 function was measured by ATP-induced ethidium^+ influx into peripheral blood lymphocytes and monocytes and, when compared with wild-type subjects, was reduced to 10–65% in heterozygotes, 1–18% in homozygotes, and 0–10% in compound heterozygotes carrying T357S and a second loss-of-function polymorphism. Overexpression of the T357S mutant P2X7 in either HEK-293 cells or Xenopus oocytes gave P2X7 function of ~50% that of wild-type constructs. Differentiation of monocytes to macrophages, which also up-regulates P2X7, restored P2X7 function to near normal in cells heterozygous for T357S and to a value 50–65% of wild-type in cells homozygous for T357S or compound heterozygous for T357S/E496A. However, macrophages from subjects that are compound heterozygous for either T357S/R307Q or T357S/stop codon had near-to-absent P2X7 function. These functional deficits induced by T357S were paralleled by impaired ATP-induced apoptosis and mycobacteria killing in macrophages from these subjects. Lymphocytes, monocytes, and macrophages from subjects homozygous for T357S or compound heterozygous for T357S and a second loss-of-function allele have reduced or absent P2X7 receptor function.

The P2X7 receptor and its genetic variants have been implicated in a variety of physiological and pathological processes, including the killing of intracellular pathogens by macrophages (1–4), inflammatory responses (5–9), and bone homeostasis (10). P2X7 receptors are highly expressed on cells of hemopoietic origin, including cells of the monocytic-macrophage series, dendritic cells, mast cells, and lymphoid cells of all subtypes, which collectively lead to the concept of P2X7 as a pro-inflammatory receptor (11). Activation of P2X7 opens a cation-selective channel allowing an influx of Ca^2+ and Na^+ and efflux of K^+ (12–16). Prolonged exposure to ATP induces a second permeability state (dilated channel or pore), which allows the influx of larger cations such as ethidium^+ (314 Da) or YO-PRO-1^2+ (375 Da) (12–14). It is currently unclear whether the two permeability states are an intrinsic property of the P2X7 receptor or whether the second permeability pathway depends on recruitment of an unidentified protein to the surface of the host cell (17). Activation of this receptor initiates a cascade of downstream signaling events such as the stimulation of phospholipase D (PLD) (18, 19) and the subsequent killing of mycobacteria and chlamydiae (1, 2, 20), processing and secretion of interleukin-1β and interleukin-18 (5, 7, 8), and the stimulation of membrane metalloproteases resulting in the shedding of CD23 and L-selectin (21–24). P2X7 activation also stimulates intracellular caspases and kinases, which eventually leads to cytolysis of lymphocytes (25), monocytes/macrophages (26, 27), and dendritic cells (28).

The P2X7 receptor has intracellular amino and carboxyl termini with two hydrophobic membrane-spanning domains, separated by a long glycosylated extracellular loop containing the proposed ATP-binding sites (29–32). The P2X7 receptor forms trimeric complexes of identical subunits (28). Differentiation of monocytes to macrophages, which also up-regulates P2X7, restored P2X7 function to near normal in cells heterozygous for T357S and to a value 50–65% of wild-type in cells homozygous for T357S or compound heterozygous for T357S/E496A. However, macrophages from subjects that are compound heterozygous for either T357S/R307Q or T357S/stop codon had near-to-absent P2X7 function. These functional deficits induced by T357S were paralleled by impaired ATP-induced apoptosis and mycobacteria killing in macrophages from these subjects. Lymphocytes, monocytes, and macrophages from subjects homozygous for T357S or compound heterozygous for T357S and a second loss-of-function allele have reduced or absent P2X7 receptor function.

This work was supported by the National Health and Medical Research Council of Australia, the Leukaemia Foundation of New South Wales, the Community Health Anti Tuberculosis Association, the Cecilia Kilkeary Foundation, the New South Wales Department of Health, a Faculty of Medicine Postgraduate Award (to A. N. S.) from the University of Sydney, a National Health and Medical Research Council of Australia Postgraduate Award (to S. L. F.) and a Sesqui Fellowship (to B. J. G.) from the University of Sydney. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 61-2-4734-3277; Fax: 61-2-4734-3432; E-mail:wileyj@medicine.usyd.edu.au.

2 The abbreviations used are: PLD, phospholipase D; IFN-γ, interferon-γ; GFP, green fluorescent protein; EGFP, enhanced GFP; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; BCG, bacille Calmette-Guerin.
A Polymorphism Impairs P2X7 Function

former affecting receptor function and the latter affecting receptor trafficking (35, 36). Work from our group has identified a third loss-of-function polymorphism in the ATP-binding site of the extracellular loop of the receptor that changes Arg307 to Gln (946G → T) so that ATP can no longer bind to the receptor (32). Two other genetic variations give loss-of-function of the P2RX7 gene. The first is an intronic polymorphism at the 5′-donor splice site at the exon 1-intron 1 boundary (151 + 1g → t), which results in a null allele in 1–2% of Caucasian subjects (37), whereas in one subject an exonic mutation (699C → T) introduces a stop codon in exon 7 of the P2RX7 gene (4). These polymorphisms and mutations, however, do not account for all observed cases of variation in P2X7 function in humans.

In this study we report a fifth polymorphism within the human P2RX7 gene. This polymorphism (1096C → G) changesThr357 to Ser (T357S) in a region of the carboxyl terminus that has been proposed as a β-arrestin-2 binding motif on the receptor (38). Freshly isolated lymphocytes and monocytes from subjects heterozygous for this polymorphism have ~50% P2X7 function compared with wild-type subjects, whereas lymphocytes and monocytes from subjects homozygous for T357S or compound heterozygous for T357S and a second loss-of-function polymorphism have near-to-absent P2X7 function.

EXPERIMENTAL PROCEDURES

Materials—ATP, RPMI 1640 media, bovine serum albumin, d-glucose, tryptone, yeast extract, kanamycin, Luria-Bertani agar powder, glycerol, 7-amino-actinomycin D, ethidium bromide, and salts were purchased from Sigma. TaqDNA polymerase, fetal calf serum, trypsin-EDTA free solution, mebro, and salts were purchased from Sigma. HEPES, recombinant human P2RX7 protein, and Opti-MEM I medium were from Invitrogen. Ficoll-Paque™ (density 1.077) and GFXTM PCR DNA and Gel Band Purification kit were purchased from Amersham Biosciences. Interferon-γ (IFN-γ) was from Roche Applied Science (Penzberg, Germany). DNA ladder (2-log) and NotI and BsmBI restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The QuikChange™ II site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The pEGFP-N1 vector was from BD Biosciences Clontech (Palo Alto, CA). The Mini-prep Plasmid and Wizard Genomic DNA Purification kit was from Qiagen (Valencia, CA). Phycoerythrin-conjugated Annexin V and propidium iodide were from BD Biosciences Clontech (San Jose, CA). Ficoll-Paque™ and GFXTM PCR DNA and Gel Band Purification kit were purchased from Amersham Biosciences. Interferon-γ (IFN-γ) was from Roche Applied Science (Penzberg, Germany). DNA ladder (2-log) and NotI and BsmBI restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The QuikChange™ II site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The pEGFP-N1 vector was from BD Biosciences Clontech (Palo Alto, CA). The Mini-prep Plasmid and Wizard Genomic DNA Extraction kit was from Promega (Madison, WI), and the HiSpeed™ plasmid purification kit was from Qiagen (Valencia, CA). Phycoerythrin-conjugated Annexin V and propidium iodide were from BD Biosciences.

Antibodies—Fluorescein isothiocyanate (FITC)- and phycocerythrin-conjugated anti-CD monoclonal antibodies (mAb) were from Dako (Carpentaria, CA). Murine anti-human P2X7 receptor mAb (clone L4) (39) was conjugated to FITC (Sigma) as described before (40) or to Alexa-647 (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol.

Selection of Subjects—Blood was collected from 841 adult subjects of whom 712 were analyzed at nucleotide position 1096. Of the 712 subjects 554 were Caucasian, 94 were Asian, 49 were Indian sub-continental, and 15 were of other ethnicity. Of the 554 Caucasian subjects, 218 were normal, whereas the remainder were patients with either chronic lymphocytic leukemia (n = 110), lymphoma (n = 47), other lymphoproliferative disorders (n = 26), various infectious (n = 52), or autoimmune (n = 26) diseases. The remainder of the Caucasian subjects (n = 75) had a range of disorders, including non-hematological cancers and allergies. Studies were conducted with informed, written consent and approval from local human ethics committees.

Preparation of Leukocytes—Peripheral venous blood from adult subjects was diluted 1:1 (v/v) with RPMI 1640 medium or phosphate-buffered saline. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll-Paque, washed once in RPMI 1640 medium or phosphate-buffered saline and resuspended in NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM d-glucose, and 1 mg/ml bovine serum albumin, pH 7.5) or RPMI 1640 medium containing 10% fetal calf serum, 2 mM l-glutamine, and 5 μg/ml gentamicin (complete RPMI 1640 medium). For the generation of macrophages, PBMCs suspended in complete RPMI 1640 medium were incubated for 2 h in plastic flasks and then gently washed to remove non-adherent cells. The adherent monocytes were differentiated to macrophages by culturing for 7 days at 37°C in 95% air/5% CO2 in complete RPMI 1640 medium. In the final 24 h of culture macrophages were activated by adding 100 units/ml IFN-γ.

DNA Extraction—Genomic DNA was extracted from peripheral whole blood as previously described (32). Plasmid DNA was extracted from XL1-Blue supercompetent cells transformed with wild-type or mutant P2X7 cDNA using either the Mini-prep Plasmid DNA Extraction or HiSpeed™ plasmid purification kits according to manufacturer’s protocols.

Single Nucleotide Polymorphism Detection—Twelve primers were used to amplify the 13 exons of the human P2RX7 gene from genomic DNA (GenBank™ accession number NT_009775.8), and the PCR products were sequenced as described (32). For detection of the 1096C → G polymorphism in a large number of samples, genomic DNA was analyzed using the high throughput TaqMan assay using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) at the SUPAMAC Facility (Royal Prince Alfred Hospital, Sydney, New South Wales, Australia) using the following primer and probe set (5′→3′): forward primer: GGGACGCAAGCAGTACTG; reverse primer: CAGCGCTGTGTGCATTCTC; Probe: VIC CTCATGACACTTACCT MGB NFQ; Probe: FAM TCATGACACTTACCT MGB NFQ. The identification of the 1096C → G polymorphism was verified by conventional sequencing.

Site-directed Mutagenesis—The C terminus of P2X7 was tagged with EGFP by sub-cloning the human P2X7 receptor into the Nhel/NotI cloning sites of the pEGFP-N1 vector as described previously (41). The single amino acid change at 1096C → G (Thr357 to Ser) was introduced using the QuikChange™ II site-directed mutagenesis kit with the wild-type human P2X7 in the pEGFP vector as the template. The point mutation was constructed using a pair of complimentary mutagenic primers described below consisting of a mutagenic codon flanked by sequences homologous to the wild-type strand of the template. After digestion by DpnI, intact mutation-containing DNA was transformed into XL1-Blue supercompetent cells. All mutations were confirmed by sequencing. Purified mutagenic oligonucleotides (PROLIGO, Sydney, New South Wales, Australia) were as follows (base changes are in boldface type and underlined): Thr357 to Ser forward GACCTTCTCATGACGTTAAGCT; Thr357 to Ser reverse: GAGTTTACTGGAGTAACTGTCGATGAGGAAGTC.
washed, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Surface P2X7 expression (FITC labeling) on subsets of PBMCs was measured by gating on CD19+ and CD3+ lymphocytes and on CD14+ monocytes. Dead cells were excluded using 7-amino-actinomycin D staining. For HEK-293 cells, EGFP expression was used to determine the total P2X7 expression, whereas surface P2X7 expression (Alexa-647 labeling) was measured by gating on EGFP-positive HEK-293 cells. Negative control values were subtracted for each subset to determine the mean channel of fluorescence intensity or the percentage of cells positive for surface P2X7 expression.

**Ethidium**^+** Influx Measurements**—ATP-induced ethidium^+ influx into PBMCs, macrophages, and EGFP-positive HEK-293 cells in KCl medium (150 mM KCl, 10 mM HEPES, 5 mM d-glucose, and 1 mg/ml bovine serum albumin, pH 7.5) was measured using time-resolved flow cytometry as described (32), with the FL-2 (ethidium^+) channel voltage reduced for macrophages as described (36).

**Bar**^2+** Influx Measurements**—ATP-induced Bar^2+ influx into PBMCs in KCl medium was measured using time-resolved flow cytometry as described (32).

**Electrophysiology**—Oocytes from adult female Xenopus laevis were surgically removed and prepared as outlined previously (42) and plated in 96-well plates. cDNA encoding wild-type P2X7-EGFP or mutant P2X7-EGFP (~0.7 μg/μl) was injected (20 nl) into the nuclei of Stage 5 or 6 oocytes using the Roboocyte Robot (Multi Channel Systems, Reutlingen, Germany) and stored at 18 °C for 2 days prior to experimentation. Oocytes were studied with the two-electrode voltage clamp technique using the Roboocyte Robot. Oocytes were impaled with two glass electrodes containing 1.5 M potassium acetate and 0.5 M KCl and held at a membrane potential of ~70 mV. Oocytes were continually perfused with a ND96 solution (96 mM NaCl, 2 mM KCl, 0.1 mM CaCl2, 5 mM HEPES, pH 7.5) using a Gilson 222 XL Liquid Handler and Gilson Minipulse 3 Peristaltic Pump (Gilson Inc., Middleton, WI). ATP (200 μM) dissolved in bath solution was applied to the oocytes for 20 s. Following ATP (200 μM) application, the oocytes were again perfused with bath solution for 2 min to allow for the full recovery of the current to baseline levels.

**Mycobacterial Killing and Macrophage Apoptosis**—IFN-γ activated macrophages were infected with **Mycobacterium bovis** bacille Calmette-Guerin (BCG)-GFP (43) for 4 h at a multiplicity of infection of 1:1 (day 0) then washed twice to remove extracellular bacteria. On day 2, cells were pulsed with 3 mM ATP in complete RPMI 1640 medium for 20 min, washed, and incubated overnight at 37 °C in 95% air/5% CO2. On day 3, half the wells were lysed with 0.1% Triton-X for 30 min to release the remaining mycobacteria. The remaining wells were stained for phycoerythrin and incubated at 37 °C for 3–4 weeks to determine the load of viable mycobacteria. The remaining wells were stained for propidium iodide and annexin V and propidium iodide to measure apoptosis as described (42). Oocytes were impaled with two glass electrodes containing 1.5 M potassium acetate and 0.5 M KCl and held at a membrane potential of ~70 mV.

**Presentation of Data and Statistics**—Data are presented as mean ± S.E. Differences between groups were compared using the two-tailed Student’s unpaired t test.

RESULTS

**A Single Nucleotide Polymorphism at Position 1096 of the P2RX7 Gene**—In addition to the known loss-of-function polymorphisms at nucleotide positions 155+1, 946, 1513, and 1729 in the human P2RX7 gene (32, 35–37), additional non-synonymous polymorphisms have been identified by our group at positions 253, 474, 835, 1068, 1069, and 1405 (Table 1). The polymorphisms at positions 489 and 1068 have been previously described (29, 44–46), whereas the other additional polymorphisms, with the exception of the 474G → A polymorphism, have all been reported previously.3 In addition, we have identified six synonymous polymorphisms at positions 488, 530, 558, 1448, 1628, and 1772, four of which have been reported on-line.2 The synonymous polymorphisms at positions 1448 and 1628 were found in linkage with the 1096G polymorphism reported in this study.

A number of subjects with low or absent P2X7 function carried the 1096C → G allele in exon 11 of the P2RX7 gene with an allele frequency of 0.10 (Table 1). A similar frequency was observed when only Caucasian subjects were included in the analysis. In 4 of 554 Caucasian subjects a homozygous nucleotide substitution (1096C → G) was found, whereas 83 subjects were heterozygous at this position. The values give an overall allele frequency of this single nucleotide polymorphism of 0.08 in the Caucasian population, and analysis shows the values are in Hardy-Weinberg equilibrium (expected homozygotes = 3.73). The four subjects homozygous for the 1096G polymorphism were wild-type for the 155+1, 946, 1513, and 1729 alleles. The 1096C → G substitution predicts a single amino acid change of threonine 357 to serine (T357S) in the carboxyl terminus of the P2X7 receptor. 17 of 542 Caucasian subjects (~3%) were compound heterozygotes for T357S as well as E496A, the latter being the most prevalent loss-of-function polymorphism with an allele frequency of 0.16 (47). Four of these compound heterozygotes were available for functional studies. In addition, two of the subjects homozygous for the T357S polymorphism were also heterozygous for other loss-of-function polymorphisms (R307Q and the 5′-intron splice site at nucleotide 151+1t, respectively), and another carried a stop codon mutation in exon 7 (nucleotide 699C → T); all of whom were available for functional studies.

Reduced P2X7 Function in Lymphocytes and Monocytes from Subjects with the Thr517 to Ser (T357S) Polymorphism—ATP-induced ethidium^+ influx was measured in monocytes and lymphocyte subsets pre-labeled with antibodies to enable gating as previously described (40). Subjects heterozygous for the T357S polymorphism had reduced ATP-induced

### Table 1: Polymorphisms within the P2RX7 gene which change an amino acid residue

| Base change | Amino acid change | n | Minor allele frequency |
|-------------|------------------|---|-----------------------|
| 151+1g → t | Null allele | 779 | 0.01 |
| 253T → C | Val354 to Ala | 37 | 0.07 |
| 474G → A | Gly150 to Arg | 41 | 0.05 |
| 489T → C | Tyr355 to His | 55 | 0.50 |
| 835G → A | Arg270 to His | 69 | 0.36 |
| 853G → A | Arg276 to His | 69 | 0.01 |
| 946G → A | Arg307 to Gin | 794 | 0.01 |
| 1068G → A | Ala348 to Thr | 172 | 0.29^a |
| 1096C → G | Thr357 to Ser | 712 | 0.10^a |
| 1405A → G | Gin460 to Arg | 465 | 0.16 |
| 1513A → C | Gln496 to Ala | 841 | 0.19^a |
| 1729T → A | Ile564 to Asn | 826 | 0.02 |

^a 1068 and 1096 single nucleotide polymorphism occur frequently together, therefore the allele frequency for 1068A is spuriously high, because it has only been determined as a by-product of sequencing to confirm heterozygotes at 1096.

^b Frequencies of the 1096G and the 1513C allele calculated for only the Caucasian cohort were 0.08 (n = 554) and 0.17 (n = 645), respectively.
A Polymorphism Impairs P2\textsubscript{X}\textsubscript{7} Function

TABLE 2

\begin{tabular}{|c|c|c|c|}
\hline
 & \textbf{B-cell} & \textbf{T-cell} & \textbf{Monocyte} \\
\hline
\textbf{T-cell} & & & \\
\textbf{Normals} & & & \\
\textbf{WT/T357S} & \text{328} \pm 24 & \text{205} \pm 251 & \text{13,082} \pm 8,705 \\
\textbf{T357S/T357S} & \text{216} \pm 305 & \text{17} \pm 23 & \text{4,078} \pm 2,506 \\
\textbf{Compound} & \text{3} \pm 46 & \text{4} \pm 5 & \text{86} \pm 44 \\
\textbf{heterozygotes} & \text{3} \pm 44 \pm 281 & \text{1,782} \pm 373 & \text{22,817} \pm 2,090 \\
\textbf{WT/WT} & & & \\
\hline
\textbf{CLL subjects} & & & \\
\textbf{WT/T357S} & \text{4,454} \pm 1,216 & \text{723} \pm 260 & \text{13,017} \pm 2,454 \\
\textbf{Compound} & \text{688} \pm 360 & \text{38} \pm 32 & \text{1,080} \pm 803 \\
\textbf{heterozygotes} & \text{49} \pm 528 & \text{1,536} \pm 285 & \text{20,161} \pm 1,452 \\
\textbf{WT/WT} & & & \\
\hline
\end{tabular}

\textsuperscript{a} Data are the mean ± S.E. \\
\textsuperscript{b} Subjects were compound heterozygotes for either T357S and the stop codon or T357S and R307Q or T357S and E496A. \\
\textsuperscript{c} Subjects were wild-type (WT) at amino acid 307, 357, 496, and 568, as well as the 5' intronic splice site. \\
\textsuperscript{d} Three subjects were compound heterozygotes for T357S and E496A, and one subject was compound heterozygous for T357S and the 5' intronic splice site.

Influx of ATP-induced ethidium\textsuperscript{+} compared with subjects wild-type for all known polymorphisms (Fig. 1 and Table 2). P2\textsubscript{X}\textsubscript{7} function in all PBMC subsets was even lower in subjects homozygous for the T357S polymorphism with values between 5 and 20% of wild-type (Table 2 and Fig. 1). The most severe loss-of-function was seen in all seven compound heterozygote subjects that were tested in whom P2\textsubscript{X}\textsubscript{7} function in all PBMC subsets was absent or reduced below 5% of wild-type (Table 2). The reduced function observed in PBMCs carrying the T357S polymorphism was not due to lack of surface P2\textsubscript{X}\textsubscript{7} expression as the receptor was present on PBMC subsets from these subjects (results not shown).

Reduced ATP-induced Ba\textsuperscript{2+} Influx in T-Lymphocytes from Subjects Homozygous for the Thr\textsuperscript{357} to Ser Polymorphism—The permeability of the P2\textsubscript{X}\textsubscript{7} channel/pore was studied by two-color flow cytometry in which the influx of Ba\textsuperscript{2+} was measured into T-lymphocytes identified by FITC-conjugated anti-CD3 mAb. Fig. 2 shows the ATP-induced uptake of Ba\textsuperscript{2+} into T-lymphocytes loaded with Fura-Red whose fluorescence emission measured by flow cytometry decreases on chelating this divalent cation as it enters the cell. The rate of Ba\textsuperscript{2+} uptake into T-lymphocytes from a subject who was homozygous for T357S was nearly absent compared with a wild type subject (Fig. 2). Similar results were obtained for T-lymphocytes as well as natural killer cells and B-lymphocytes from another homozygous subject (data not shown).

Reduced P2\textsubscript{X}\textsubscript{7} Function of Thr\textsuperscript{357} to Ser (T357S)-mutated P2\textsubscript{X}\textsubscript{7} in HEK-293 Cells—Transfection experiments confirmed that the T357S polymorphism reduced P2\textsubscript{X}\textsubscript{7} function. cDNA for wild-type P2\textsubscript{X}\textsubscript{7} receptor and a mutant carrying the 1096C

- intronic splice site.

- the P2\textsubscript{X}\textsubscript{7} channel/pore was studied by two-color flow cytometry in

- the transfected HEK-293 cells. Total P2\textsubscript{X}\textsubscript{7} expression was

- also shown.

\textbf{TABLE 2}

P2\textsubscript{X}\textsubscript{7} function in lymphocytes and monocytes from subjects with Thr\textsuperscript{357} to Ser polymorphism

P2\textsubscript{X}\textsubscript{7} function was measured as described in the legend of Fig. 1 and is expressed as mean arbitrary units over ethidium\textsuperscript{+} influx over 5 min. Ethidium\textsuperscript{+} influx in the absence of ATP was negligible.

\begin{tabular}{|c|c|c|c|}
\hline
 & \textbf{WT/T357S} & \textbf{T357S/T357S} & \textbf{T357S/E496A} \\
\hline
\textbf{Basal} & & & \\
\textbf{WT/WT} & & & \\
\textbf{WT/T357S} & & & \\
\textbf{T357S/T357S} & & & \\
\textbf{T357S/E496A} & & & \\
\hline
\end{tabular}

FIGURE 1. ATP-induced ethidium\textsuperscript{+} influx into lymphocytes and monocytes. PBMCs from normal subjects of various genotypes (WT/WT, wild-type; WT/T357S, T357S heterozygote; T357S/T357S, T357S homozygote; and T357S/E496A, compound heterozygote) were labeled with FITC-conjugated anti-CD mAb and sus-}
Reduced Function of Thr357 to Ser (T357S) Polymorphism—We have previously reported the absence of P2X7 function in macrophages from two subjects who were compound heterozygous for loss-of-function polymorphisms (32). Both were R307Q, a defective receptor for ATP binding, combined with either the trafficking defective I568N or the common E496A, respectively. Fig. 5B shows that macrophages from another two compound heterozygous subjects, each with the T357S polymorphism, had near to absent ATP-induced ethidium influx. In one subject the second allele coded for R307Q, whereas the other subject carried a mutation for a stop codon in exon 7 (nucleotide 699C → T). In contrast, the ATP-induced ethidium influx into macrophages from subjects heterozygous for both T357S and E496A was only about half that of wild-type macrophages (mean arbitrary units of ethidium influx of 20,910 ± 7,081, n = 3; Fig. 5B).

Reduced ATP-induced Apoptosis in Macrophages from Subjects with the Thr357 to Ser (T357S) Polymorphism—We have previously reported that ATP-induced apoptosis as well as killing of intracellular mycobacteria (BCG) is impaired in subjects carrying loss-of-function polymorphisms in the P2RX7 gene (3, 4). We studied seven subjects with the T357S polymorphism, including one homozygote and four compound heterozygotes for the ability of ATP to induce apoptosis of their macrophages and to kill intracellular mycobacteria. Adherent monocyte-derived macrophages from these subjects and from controls who were wild-type for all known loss-of-function polymorphisms were infected with BCG for 48 h and then pulsed with ATP for 20 min. Following removal of ATP and overnight incubation, the percentage of apoptotic macrophages was determined (Table 3). In wild-type individuals, the addition of ATP on average led to a 24% increase in apoptotic cells compared with unstimulated macrophages. ATP-induced apoptosis in macrophages either heterozygous or homozygous for the T357S polymorphism was reduced by ~43% and ~74%, respectively. This reduction
TABLE 3

ATP-induced apoptosis and BCG killing in macrophages derived from subjects with Thr^357 to Ser polymorphism

| Genotype                  | ATP-induced ethidium$^*$ uptake | ATP-induced apoptosis | ATP-induced bacterial killing |
|---------------------------|---------------------------------|-----------------------|-------------------------------|
| **Heterozygote**          | **Wild-type**                   | **Homozygote**        | **Compound heterozygote**    | **Wild-type** |
| WT/T357S                  | 25.185                          | 16.4                  | 84.5                          | 39,222 ± 2947         |
| WT/T357S                  | ND$^*$                          | 10.6                  | 83.4                          | 1.5                  |
| T357S/R307Q               | 1.874                           | 1.5                   | 16.8                          | 1.5                  |
| T357S/E496A               | 21,000                          | 3.0                   | 53.2                          | 2.0                  |
| T357S/E496A               | ND$^*$                          | 2.0                   | 43.8                          | 23.8 ± 1.6           |
| **Wild-type**             | **WT/WT**                       | **T357S/R307Q**       | **T357S/E496A**               | **39,222 ± 2947**    |
| ND$^*$                    | ND$^*$                          | 1.5                   | 16.8                          | 23.8 ± 1.6           |
| ND                        | ND$^*$                          | 2.0                   | 43.8                          | 10.8                |

$^*$ ND, not determined.

$^a$ Subjects were wild-type (WT) at amino acid 307, 357, 496, and 568, and the 5'-intronic splice site; data is the mean ± S.E. ($n = 7$).

**DISCUSSION**

The present data confirm the highly polymorphic structure of the P2RX7 gene and identify a fifth loss-of-function allele at position 1096 of cDNA. Within the coding region of the P2RX7 gene, we have identified 11 polymorphisms that change an amino acid residue, an average of one base change per 160 bp of cDNA. In addition, we have identified a 5'-intronic splice site polymorphism in 1–2% of Caucasian subjects with no identifiable product from the affected allele, presumably due to nonsense-mediated RNA decay. The present as well as previous studies from our group have identified five polymorphisms, including T357S, which lead to reduction or loss of P2X$_7$ function, whereas another three (at amino acids 155, 348, and 460) have a negligible effect on ATP-induced ethidium$^*$ influx in transfected HEK-293 cells (31). The effect of the amino acid 155 polymorphism is however controversial with a recent study proposing that the predicted amino acid change, His$^{155}$ to Tyr, confers a gain of function (46). In addition, we have found two exonic mutations in single individuals that abolish function of the mutant allele, one within the trafficking domain of the receptor (Arg$^{574}$ to His) and a second in exon 7 of the gene (699C → T in cDNA) that produces a premature stop codon (4). The splice site polymorphism as well as the uncommon R307Q and I568N polymorphisms confer complete loss of P2X$_7$ function in fresh PBMCs and have biological significance in heterozygous dosage (30, 33–34). In contrast, the two most common polymorphisms at residues 496 and 357 of the protein result in complete loss of P2X$_7$ function only when present in homozygous dosage or in combination with another loss-of-function polymorphism as a compound heterozygote (32, 35, 37) (Table 1). Because the T357S variation is present in ~11% of the population and E496A is found in over one quarter of Caucasian subjects (47) (Table 1), the combination of T357S/E496A is found in ~3% of subjects and is the single most common cause of near-to-absent P2X$_7$ function in fresh PBMCs. Collectively, when the prevalence of all five loss-of-function polymorphisms

FIGURE 5. ATP-induced ethidium$^*$ influx into macrophages. Monocyte-derived macrophages (activated with IFN-γ) from (A and B) wild-type (WT/WT) subjects; A T357S heterozygote (WT/T357S) or homozygote (T357S/T357S) subjects, or B, compound heterozygote subjects (as indicated) were labeled with FITC-conjugated anti-CD14 mAb and suspended in KCl medium at 37 °C. Ethidium$^*$ (25 μM) was added following 40 s later by the addition of 1 mM ATP (arrow). The mean channel of cell-associated fluorescence was measured at 5-s intervals by time-resolved flow cytometry. The voltage setting for ethidium$^*$ was reduced to gain the full scale of influx increase. Basal ethidium$^*$ influx measured in the absence of ATP is also shown.

in ATP-induced apoptosis was even greater in macrophages heterozygous for both T357S and one other loss-of-function allele, which on average was 92% lower than that of wild-type values.

**Reduced Mycobacterial Killing in Macrophages from Compound Heterozygotes**—In addition to measuring ATP-induced apoptosis of macrophages, the viability of engulfed mycobacteria within these cells was also determined following removal of ATP and overnight incubation (Table 3). Unlike ATP-induced apoptosis, the effect of the T357S polymorphism in either heterozygous or homozygous dosage on ATP-induced killing of mycobacteria was minimal with less than a 15% reduction in bacterial viability compared with wild-type values. However, ATP-induced mycobacterial killing was greater with less than a 15% reduction (from triplicate wells) in viable bacilli in ATP-pulsed cultures minus non-ATP-pulsed cultures.

4 B. J. Gu and J. S. Wiley, unpublished observations.
A Polymorphism Impairs P2X<sub>7</sub> Function

A major finding of this study is that the T357S substitution reduced P2X<sub>7</sub>-receptor function, although the effect of this polymorphism in heterozygous dosage was modest. P2X<sub>7</sub>-function, as measured by ethidium<sup>+</sup> influx in freshly isolated PBMCs, was reduced by 80% or was absent in subjects homozygous or compound heterozygous, respectively, for the T357S polymorphism (Fig. 1 and Table 2). Ba<sup>2+</sup> influx was also absent in freshly isolated PBMCs from homozygous subjects (Fig. 2). Results in the present and our previous studies (32, 35, 36) show that the loss-of-function imparted by deleterious polymorphisms are more severe in freshly isolated human mononuclear cells. Thus forced over-expression of the T357S-mutated P2X<sub>7</sub> in HEK-293 cells or Xenopus oocytes allows the impairment of P2X<sub>7</sub>-function to be partially reversed (Figs. 3 and 4). In both these heterologous systems the function of mutant P2X<sub>7</sub>, was ~50% of wild-type, which is a strikingly different value to the <1% of wild-type function observed in fresh human lymphocytes homozygous for the T357S polymorphism (compare Fig. 1, A and B, to Figs. 3 and 4). Our data also show that impaired P2X<sub>7</sub>-function in monocytes from a subject homozygous for T357S was partially corrected upon differentiation of monocytes to macrophages, a process that up-regulates P2X<sub>7</sub>-expression by at least one order of magnitude (36, 48, 49). We and others have previously shown that the ability of ATP to induce formation of ethidium<sup>+</sup>-permeable pores in cells that natively express P2X<sub>7</sub>, receptors is modulated by receptor subunit density at the cell surface (14, 49). Collectively, data from macrophages and both heterologous systems indicate that P2X<sub>7</sub>-function can be partially restored by increased expression of the T357S-mutated P2X<sub>7</sub>-receptor on the cell surface. Although the T357S P2X<sub>7</sub>-variant shows partial function at high receptor density, certain combinations of loss-of-function alleles lead to a complete loss of P2X<sub>7</sub>-function not only in fresh PBMCs but also in cultured macrophages. Thus Fig. 5 shows that P2X<sub>7</sub>-function was totally ablated in macrophages from a subject who was a compound heterozygote for both T357S and R307Q, as well as from the subject with T357S and a stop codon on the other allele. In contrast, P2X<sub>7</sub>-function in macrophages from subjects compound heterozygous for both T357S and E496A was on average 53% of that from wild-type subjects.

The threonine to serine polymorphism is at position 357 and lies in the carboxyl terminus several residues from the putative second transmembrane domain. Thr<sup>357</sup> forms part of a threonine-serine cluster (Thr<sup>357</sup> - Tyr<sup>358</sup> ), which has been proposed as a binding motif for β-arrestin-2 in a human endocervical carcinoma cell line and HEK-293 cells (38). Although threonine residues on P2X<sub>7</sub>, are known to be phosphorylated (38), and analysis of the human P2X<sub>7</sub>, receptor using the PhosBase version 2.0 program<sup>6</sup> revealed several threonine residues, including Thr<sup>357</sup> as potential phosphorylation sites, it is currently unknown if Thr<sup>357</sup> is actually phosphorylated. Serine, which is structurally similar to threonine, can also be phosphorylated. However, despite these similarities the substitution of serine for threonine at position 357 reduced ATP-induced currents in Xenopus oocytes by ~40% (Fig. 4) despite injecting equal amounts of cDNA, whereas this same substitution reduced ATP-induced ethidium<sup>+</sup> influx in HEK-293 cells by ~60% (Fig. 3) despite similar surface expression of P2X<sub>7</sub>, (data not shown). A similar reduction in ATP-induced ethidium<sup>+</sup> influx was also observed in monocyte-derived macrophages homozygous for the T357S polymorphism. This reduction in P2X<sub>7</sub>-function was even greater in fresh lymphocytes and monocytes. There are few reports describing the effect of threonine to serine substitutions on ion channel function. However, substitution of threonine in place of serine at residue 906 in the human Na<sub>1.4</sub> channel slowed entry into and recovery from the slow inactivation state resulting in a change-of-function (50). It remains to be determined if the effect of T357S on P2X<sub>7</sub>-function is due to changes in phosphorylation, altered interactions with β-arrestin-2, changes in free energy barriers impeding pore dilatation, or a modification in tertiary protein structure.

Activation of the P2X<sub>7</sub>-receptor on macrophages has been shown to lead to the subsequent apoptotic death of this cell type (26, 27). Our data (Table 3) confirm P2X<sub>7</sub>-induced apoptosis of human macrophages, because brief exposure to ATP gave a 24% increase in the percentage of apoptotic wild-type macrophages measured 16 h later. Loss-of-function polymorphisms in the P2X<sub>7</sub>-receptor reduced this value of ATP-induced apoptosis to half the wild-type value for T357S heterozygotes and to one-fourth of wild-type values for the single subject who was homozygous for T357S. Comparison of these values (Table 3) with the monocyte (Table 2) and macrophage P2X<sub>7</sub>-function (Table 3) for the same polymorphisms reveals a strong correlation between receptor activation and the downstream effects on apoptotic death of the cell.

Our current data are consistent with our previous studies (3, 4) demonstrating that loss-of-function polymorphisms lead not only to reduced ATP-induced apoptosis but also to impaired ATP-induced killing of an intracellular mycobacterium (BCG) by macrophages. However, unlike the R307Q, E496A, and I568N polymorphisms, which in heterozygous dosage impair killing of mycobacteria on average by 56% (4), the T357S polymorphism in heterozygous dosage had no significant effect, whereas homozygous dosage gave only modest (15%) reduction in killing. By contrast, killing of mycobacteria was markedly reduced in macrophages homozygous for T357S when combined with another loss-of-function polymorphism, collectively supporting the gene dosage effect observed with P2X<sub>7</sub>-loss-of-function polymorphisms (4). The relatively normal killing of BCG in T357S homozygous or homozygous macrophages despite impaired ATP-induced apoptosis in these same cells may somehow reflect the many steps required for killing of mycobacteria. Alternatively, the T357S polymorphism may not impair ATP-induced PLD stimulation, which is necessary for the killing of intracellular pathogens. In murine thymocytes, the Pro<sup>451</sup> to Leu polymorphism within the P2X<sub>7</sub>-receptor impairs ATP-induced cation fluxes, phosphatidylinerse exposure, and cell death but not ATP-induced PLD stimulation (51, 52). Moreover, transfection of HEK-293 cells with cDNA encoding this mutated murine P2X<sub>7</sub> gives values for ATP-induced YOP-PRO-1<sup>+</sup> uptake around half that for wild-type P2X<sub>7</sub>, (51). This difference is remarkably similar to our observations with ATP-induced ethidium<sup>+</sup> uptake into HEK-293 cells transfected with T357S-mutated human P2X<sub>7</sub>, cDNA (Fig. 2). Further studies are required to determine if T357S impairs ATP-induced PLD stimulation or other processes downstream of P2X<sub>7</sub>-activation.

Our results in Table 3 further define which combinations of loss-of-function polymorphisms completely ablate P2X<sub>7</sub>, function in cultured human macrophages. Compound heterozygotes with T357S together with either R307Q (the ATP-binding defect) or defects producing a null allele (nucleotides 151 +1t or 699t) gave absent P2X<sub>7</sub>-function (<1% of wild-type) and nearly absent (<5%) ATP-induced apoptosis and mycobacterial killing by macrophages (Table 3). Macrophages are central to the innate immune response, and complete abolition of P2X<sub>7</sub>, function may be a susceptibility factor in a range of intracellular infections, including tuberculosis, chlamydia, and toxoplasmosis. Our data confirm that the T357S polymorphism in homozygous dosage or in com-

---

<sup>5</sup> A. N. Shemon, R. Sleyter, and J. S. Wiley, unpublished observations.
<sup>6</sup> www.cbs.dtu.dk/services/NetPhos.
<sup>7</sup> A. N. Shemon, unpublished observations.
bination with another loss-of-function polymorphism has major effects on macrophage function and should be included in studies of susceptibility to infections by intracellular pathogens.

Acknowledgment—We gratefully acknowledge Jennifer G. Georgiou for additional genotyping.

REFERENCES

1. Fairbairn, I. P., Stober, C. B., Kumararatne, D. S., and Lammas, D. A. (2001) J. Immunol. 167, 3300–3307
2. Coutinho-Silva, R., Stahl, L., Raymond, M. N., Jungas, T., Verbeke, P., Burnstock, G., Marville, T., and Ojcius, D. M. (2003) Immunity 19, 403–412
3. Saunders, B. M., Fernando, S. L., Snyr, R., Britton, W. J., and Wiley, J. S. (2003) J. Immunol. 171, 5442–5446
4. Ferreira, D., Loo, M., Bauer, M. K. A., Vandenabeele, P., and Schulze-Dalwigk, S. (2003) J. Immunol. 171, 149–155
5. pkt, N., Freir, D., Bronte, V., Collavo, D., and Zanovello, P. (1989) J. Immunol. 143, 1955–1960
6. Ferrari, D., Los, M., Bauer, M. K. A., Vandenabeele, P., Wesselborg, S., and Schulze-Dalwigk, S. (1999) FEBS Lett. 447, 71–75
7. Humphreys, B. D., Rice, J., Kertesz, S. B., and Dubyak, G. R. (2000) J. Biol. Chem. 275, 26792–26798
8. Coutinho-Silva, R., Persechini, P. M., Bisaggio, R. D., Perfettini, J. L., Neto, A. C., Nakanoupolou, J. M., Moitta-Ly, I., Dauny-Varsat, A., and Ojcius, D. M. (1999) Am. J. Physiol. 276, C1139–C1147
9. Rassendren, F., Buell, G. N., Vinogino, C., Collno, G., North, R. A., and Surprenant, A. (1997) J. Biol. Chem. 272, 5482–5486
10. Kah, B. S. (2001) Nat. Rev. Neurosci. 2, 165–174
11. Worthington, R. A., Smart, M. L., Gu, B. J., Williams, D. A., Petrow, S., Wiley, J. S., and Barden, J. A. (2002) FEBS Lett. 512, 43–46
12. Gu, B. J., Shyter, R., Rassendren, F., Shemon, A. N., Dao-Ung, L. P., Fuller, S. J., Barden, J. A., Clarke, A. L., Petrow, S., and Wiley, J. S. (2004) J. Biol. Chem. 279, 31287–31295
13. Torres, G. E., Egan, T. M., and Voigt, M. (1999) J. Biol. Chem. 274, 6651–6659
14. Smart, M. L., Gu, B. J., Panchal, R. G., Wiley, J. S., Crompton, B., Williams, D. A., and Petrow, S. (2003) J. Biol. Chem. 278, 8853–8860
15. Gu, B. J., Zhang, W., Worthington, R. A., Shyter, R., Dao-Ung, P., Petrow, S., Barden, J. A., and Wiley, J. S. (2001) J. Biol. Chem. 277, 11136–11142
16. Wiley, J. S., Dao-Ung, L. P., Li, C., Shemon, A. N., Gu, B. J., Smart, M. L., Fuller, S. J., Barden, J. A., Petrow, S., and Shyter, R. (2003) J. Biol. Chem. 278, 17108–17113
17. Rassendren, F., Fuller, S. J., Shyter, R., Dao-Ung, L. P., Gu, B. J., and Wiley, J. S. (2005) FEBS Lett. 579, 2675–2678
18. Feng, Y. H., Wang, L., Wang, Q., Li, X., Zeng, R., and Gorodeski, G. (2005) Am. J. Physiol. 288, C1342–C1356
19. Buell, G., Chessell, I. P., Michel, A. D., Cogo, G., Salazano, M., Herren, S., Grestener, D., Grahames, C., Kaur, R., Kosovboil, M., and Humphrey, P. A. (1998) Blood 92, 3521–3528
20. Gu, B. J., Zhang, W. Y., Belland, L. J., Chessell, I. P., Buell, G. N., and Wiley, J. S. (2000) Am. J. Physiol. 279, C1189–C1197
21. Smart, M. L., Panchal, R. G., Bowser, D. N., Williams, D. A., and Petrow, S. (2002) Am. J. Physiol. 283, C77–C84
22. Buell, G., Ugur, M., Drummond, R. M., Singer, J. J., and V. Walsh Jr., J. (1997) FEBS Lett. 411, 339–345
23. Triccas, J. A., Berthet, F. X., Pellic, V., and Giqueul, B. (1999) Microbiology 145, 2923–2930
24. Buell, G., Talbot, F., Gos, A., Lorenz, J., Lai, E., Morris, M. A., and Antonarakis, S. E. (1998) Neuron Receptors 5, 347–354
25. Wiley, J. S., Gu, B. J., Zhang, W., Worthington, R. A., Dao-Ung, P., Shemon, A. N., Shyter, R., Liang, S., and Barden, J. A. (2001) Drug Develop. Res. 53, 72–76
26. Calvari, G., Falzoni, S., Forcch, S. L., Pellegrati, P., Balboni, A., Agostini, P., Cuneo, A., Castoldi, G., Baricci, O. R., and Di Virgilio, F. (2005) J. Immunol. 175, 82–89
27. Dao-Ung, L. P., Fuller, S. J., Shyter, R., Rassendren, K. K., Thunberg, U., Tobin, G., Byth, K., Ban, M., Rosenquist, R., Stewart, G. J., and Wiley, J. S. (2004) Br. J. Haemato. 125, 815–817
28. Hickman, S. E., el Khoury, J., Greenberg, S., Schieren, I., and Silverstein, S. C. (1994) Blood 84, 2452–2456
29. Gudipaty, L., Humphreys, B. D., Buell, G., and Dubyak, G. R. (2001) Am. J. Physiol. 280, C943–C953
30. Kuzmenkin, A., Jurkat-Rott, K., Lehmann-Horn, F., and Mitrovic, N. (2003) Pflüg. Arch. Eur. J. Physiol. 447, 71–77
31. Adirouch, S., Dux, C., Welge, V., Semon, M., Koch-Nolte, F., and Haag, F. (2002) J. Immunol. 169, 4108–4112
32. Le Stunff, H., Auger, R., Nakanoupolou, J. M., and Raymond, M. N. (2004) J. Biol. Chem. 279, 16918–16926