A Loss-of-Function Polymorphism in the Human P2X7 Receptor Abolishes ATP-Mediated Killing of Mycobacteria

Bernadette M. Saunders, Suran L. Fernando, Ronald Sluyter, Warwick J. Britton and James S. Wiley

*J Immunol* 2003; 171:5442-5446; doi: 10.4049/jimmunol.171.10.5442

http://www.jimmunol.org/content/171/10/5442

References

This article cites 29 articles, 13 of which you can access for free at: http://www.jimmunol.org/content/171/10/5442.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
A Loss-of-Function Polymorphism in the Human P2X7 Receptor Abolishes ATP-Mediated Killing of Mycobacteria

Bernadette M. Saunders, Suran L. Fernando, Ronald Slaysyer, Warwick J. Britton, and James S. Wiley

Protective immunity to mycobacterial infections requires activation of the antibacterial mechanisms of infected macrophages. It has previously been reported that ATP treatment of mycobacteria-infected macrophages induces apoptosis mediated via the P2X7 pathway and that this leads to the death of both the host cell and the internalized bacilli. We have recently identified a single nucleotide polymorphism in the P2X7 gene (1513A→C), with 1–2% prevalence in the homozygous state, which codes for a nonfunctional receptor. IFN-γ-primed, mycobacteria-infected macrophages from wild-type individuals were incubated with ATP and this induced apoptosis and reduced mycobacterial viability by 90%. Similar treatment of macrophages from individuals homozygous for the 1513C polymorphism failed to induce apoptosis and did not lead to mycobacterial killing via the P2X7-mediated pathway. These data demonstrate that a single nucleotide polymorphism in the P2X7 gene can allow survival of mycobacteria within infected host cells.


Copyright © 2003 by The American Association of Immunologists, Inc. 0022-1767/03/$02.00

Received for publication March 17, 2003. Accepted for publication September 9, 2003.

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 This work was supported by the National Health and Medical Research Council of Australia, the Community Health Anti Tuberculosis Association, the Cecilia Kilkeary Foundation, and the New South Wales Department of Health through its research infrastructure grant to the Centenary Institute of Cancer Medicine and Cell Biology. B.M.S. is a Rolf Edgar Lake Research Fellow of the Faculty of Medicine, University of Sydney.

2 Address correspondence and reprint requests to Dr. Bernadette M. Saunders, Centenary Institute of Cancer Medicine and Cell Biology, Royal Prince Alfred Hospital, Locked Bag No. 6, Newtown, 2042 NSW, Australia. E-mail address: b.saunders@centenary.usyd.edu.au

3 Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; PI, propidium iodide.
Materials and Methods

Subject recruitment

A total of 115 adult subjects of Caucasian background were screened for P2X7 genotype to identify three who were homozygous for the 1513A→C allele (10). Blood was taken with informed consent (and ethics approval) from the 18 subjects described in this study.

Human monocytes and monocyte-derived macrophage cultures

PBMC were separated on Ficoll-Paque PLUS (Amersham Pharmacia Bio-Tech, Uppsala, Sweden) and used to provide fresh monocytes. To generate monocyte-derived macrophages, PBMC were re-suspended at 1–2 × 10⁶ cells/ml in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated FCS (Life Technologies, Auckland New Zealand) and 2 mM glutamine (Sigma-Aldrich). Cells were incubated for 2 h and washed twice to remove non-adherent cells. Monocytes (adherent cells) were cultured for 6 days in complete medium. For ATP-induced ethidium uptake and P2X7 expression measurements, 6-day macrophages were cultured overnight with IFN-γ (100 IU/ml; Roche, Sydney, Australia) and propidium iodide (PI) to measure apoptosis as per the manufacturer protocol (BD Biosciences) and analyzed on a FACSCalibur flow cytometer.

Ethidium influx measurement by flow cytometry

PBMC or IFN-γ-activated macrophages, pre-labeled with FITC-conjugated anti-human CD14 mAb (DAKO, Carpinteria, CA), were re-suspended in 1 ml of HEPES-buffered KCl medium (10 mM HEPES (Life Technologies), 150 mM KCl, 5 mM d-glucose, and 1% BSA (Sigma-Aldrich), pH 7.5) at 37°C. All samples were stirred and temperature controlled at 37°C. Ethidium (25 μM; Sigma-Aldrich) was added, followed 40 s later by addition of 1 mM ATP (Sigma-Aldrich). Cells were analyzed at 1000 events on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The linear mean channel of fluorescence intensity for gated CD14⁺ cells over successive 5-s intervals was analyzed by WinMDI software version 2.7 (available at http://facs.scripps.edu/software.html) and plotted against time. Because of the increased P2X7 function on macrophages, data for macrophages were acquired at a reduced voltage setting for FL-2 (ethidium fluorescence) as described previously (19).

Immunofluorescent staining and flow cytometry

PBMC or IFN-γ-activated macrophages were labeled with FITC-conjugated anti-human P2X7 (20) or isotype control mAb (DAKO), PE-conjugated anti-human CD14 mAb (DAKO) and 7-amino actinomycin D (Sigma-Aldrich), or alone with PerCP-conjugated anti-human HLA-DR mAb (BD Biosciences) in the presence of 10% human AB serum for 20 min, washed, and analyzed using a FACSCalibur flow cytometer.

Mycobacterial killing and apoptosis

IFN-γ-activated macrophages were infected with BCG-green fluorescent protein strain (10), then washed twice to remove extracellular bacteria. On day 2, cells were pulsed with 3 mM ATP for 20 min, washed, and incubated overnight (7). On day 3, one-half of the wells were lysed with 0.1% Triton-X for 30 min to release viable bacilli. Serial dilutions of cell lysates were plated onto 7H11 agar and incubated at 37°C for 3–4 wk to determine the load of viable mycobacteria. The remaining wells were stained for PE-conjugated annexin V and propidium iodide (PI) to measure apoptosis as per the manufacturer’s protocol (BD Biosciences) and analyzed on a FACSCalibur flow cytometer.

DNA extraction, PCR, and DNA sequencing

Genomic DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). A primer pair within exon 13 of the P2X7 gene amplified a 376-bp product from genomic DNA. The forward primer was 5’-ACTCTTAGATCAGGGATAGCC-3’ and the reverse primer was 5’TACAGACGTGAGCCACGGT-3’. PCR amplification (25 cycles of denaturation at 95°C for 45 s, annealing at 56°C, and extension at 72°C for 1 min). PCR products were separated in 2% agarose gel and visualized by ethidium bromide staining. Amplified PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Piscataway, NJ) and sequenced using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Statistics

Ethidium uptake and P2X7 expression data were analyzed by the unpaired Students’ t test. Mycobacterial killing and apoptosis data were analyzed by the Mann-Whitney U test. Data were considered significant when p < 0.001.

Results

P2X7 expression and function in monocytes

The functional activity of the P2X7 receptor was investigated in three individuals homozygous for the 1513C single nucleotide polymorphism (SNP). Monocytes from these individuals were treated with ATP and ethidium uptake was measured (Fig. 1). Unlike wild-type individuals, all three homozygous subjects displayed no detectable uptake above basal levels. P2X7 function was quantitated by measurement of the arbitrary units of area under the ethidium uptake curve; this was 19393 ± 1803 (n = 16) for the wild-type monocytes compared with 73 ± 73 (n = 3, subjects tested at least twice) for monocytes from the 1513C homozygous subjects (p < 0.001). The surface expression of P2X7 on monocytes from wild-type individuals (37.5 ± 6.1, n = 11) was also higher than the P2X7 expression on monocytes from homozygous individuals (23.9 ± 11.2, n = 3); however, this difference did not reach significance (p = 0.29).

Reduced P2X7 expression and function on homozygote macrophages

ATP-induced ethidium uptake and P2X7 expression were also measured in IFN-γ-activated macrophages. ATP-induced ethidium uptake and P2X7 expression were 18- and 12-fold higher, respectively, in wild-type macrophages than homozygous macrophages (Table I). However, this single nucleotide polymorphism did not affect the expression of class II molecules because we saw similar levels of HLA-DR expression (Table I). Further BCG infection induced increased TNF secretion by all macrophages regardless of their P2X7 phenotype (Fig. 2). Although the level of TNF produced varied among individuals, no significant difference was seen in TNF induction between macrophages from wild-type homozygote individuals.

FIGURE 1. ATP induced ethidium uptake curve. PBMC (2 × 10⁶) from a wild-type individual (A) and an individual homozygous for the 1513A→C polymorphism (B) were labeled with anti-CD14-FITC conjugate and incubated in HEPES-buffered KCl at 37°C. Ethidium bromide (25 μM) was added and followed 40 s later by 1 mM ATP. The mean channel of cell-associated fluorescence intensity was measured at 5-s intervals. Data are representative of wild-type individuals and those with the 1513A→C polymorphism. Ethidium uptake in the absence (○) or presence of ATP (○).
A P2X7 mutation was determined by flow cytometry.

### Table 1. P2X7 Function and Expression, and MHC Class II Expression on Macrophages

<table>
<thead>
<tr>
<th>Exp.</th>
<th>P2X7 Function</th>
<th>P2X7 Expression</th>
<th>HLA-DR Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Homozygote</td>
<td>Wild-type</td>
</tr>
<tr>
<td>1</td>
<td>27,779</td>
<td>677</td>
<td>218.85</td>
</tr>
<tr>
<td>2</td>
<td>39,624</td>
<td>3,163</td>
<td>313.77</td>
</tr>
</tbody>
</table>

*IFN-γ-treated macrophages from wild-type individuals and individuals homozygous for the 1513A→C polymorphism were labeled with anti-CD14-FITC conjugated mAb and incubated in HEPES-buffered KCl at 37°C. Ethidium bromide (25 μM) was added, followed 40 s later by 1 mM ATP, and the mean channel of cell-associated fluorescence intensity was measured at 5 s intervals.

### Failure of ATP-induced killing in homozygous macrophages

It has previously been shown that ATP-induced apoptosis, but not H2O2-induced necrosis, of mycobacteria-infected macrophages leads to the death of the intracellular bacilli (22). We investigated whether the loss-of-function polymorphism at 1513 of the P2X7 gene affects the ability of phagocytic cells to kill mycobacteria via ATP-mediated apoptosis. Adherent monocyte-derived macrophages were infected with M. bovis BCG for 48 h and then pulsed with ATP for 20 min. Following overnight incubation, the percentage of apoptotic cells and the number of viable bacilli were determined. ATP treatment of BCG-infected macrophages from 1513C homozygote subjects failed to increase the number of apoptotic macrophages (~0.3–5.7%; Fig. 3), whereas in wild-type individuals ATP treatment of infected macrophages led to a 10-fold increase in the level of apoptosis (19.8–31.2%; Fig. 3). The level of necrosis (measured as PI+/annexin− macrophages) was equivalent in both groups, 0.29–5.25 for wild-type individuals and 0.11–3.01 for homozygous individuals. In wild-type macrophages, this increase in apoptosis was associated with a 90% reduction in mycobacterial load (Fig. 4). By contrast, the bacillary load in homozygous macrophages was unchanged after ATP treatment (Fig. 4).

### Discussion

These data demonstrate a complete loss of ATP responsiveness and a corresponding inability to kill mycobacteria via ATP in BCG-infected macrophages from individuals homozygous for the 1513C polymorphism.

There is increasing evidence that P2X7 plays an essential role in mycobacterial killing by cells of the monocyte/macrophage lineage. Extracellular ATP, added to infected human macrophages, has been shown to induce the apoptotic death of the host cell and its internalized mycobacteria (7, 23). This effect of ATP is known to be mediated by P2X7, as ATP-induced apoptosis and subsequent bacterial killing were inhibited by treatment of infected macrophages with the P2X7 antagonist KN62 (7, 24). Furthermore, macrophages from P2X7 gene knockout mice were unable to affect killing of mycobacteria by the addition of ATP (25).

The mechanism of P2X7-mediated killing involves a transient increase in cytosolic calcium which is necessary for the fusion of lysosomes with infected phagosomes and subsequent mycobacterial killing within the phagolysosome (23, 26). ATP-mediated killing of BCG within Nramp-susceptible, p47phox−/− and INOS−/− mice was unaffected, demonstrating that the pathway of ATP killing is independent of reactive oxygen intermediates, reactive nitrogen intermediates, and Nramp (25). Another downstream effect of P2X7 receptor activation is to stimulate the activity of phospholipase D, which is required for the killing of intracellular mycobacteria (8).

ATP-induced apoptosis and ATP-mediated killing of mycobacteria were essentially absent in macrophages from homozygous subjects despite ATP inducing a low level of ethidium uptake into...
These cells were previously observed to express the P2X7 receptor in the ATP-induced function of macrophages from homoyzgoites compared with wild-type individuals as measured by ethidium uptake (10). These differences in channel function were associated with marked differences in biological function, as measured by apoptosis and mycobacterial killing. ATP-induced apoptosis of macrophages is dependent on caspase activation (16), while ATP-induced killing of mycobacteria is dependent on caspase activation, IFN-γ-mediated apoptosis and the stimulation of cytolytic caspases with distinct roles in apoptotic and necrotic alterations of cell death. 

Our data suggest that loss-of-function polymorphisms in the P2X7 gene may confer a genetic susceptibility to tuberculosis. Recently, a significant association between the 1513A allele and susceptibility to TB was found for a single polymorphism (31), again consistent with our observations. We observed that the 1513A allele in homozygous dosage, or some other defect, results in the inability of ATP to mediate killing of intracellular mycobacteria. Recently, a second loss-of-function polymorphism (1513C) has been defined (19). This polymorphism is also in exon 13, which encodes the intracellular domain of P2X7. This prevents normal trafficking of the P2X7 receptor to the cell surface and reduces channel function. It will be of interest to determine whether this also results in impaired ATP-mediated killing of intracellular mycobacteria.

The overall impact of single nonfunctional polymorphisms, which affect macropage activity, on TB susceptibility in a population depends on the gene frequency for that allele in the population studied. In the study on a Gambian population (28), there was a lower frequency (7.6%) of expression of the 1513A→C polymorphism than the frequency of observed in this Australian Caucasian population (13.9%, n = 115) (Ref. 10 and R.S. and J.S.W., unpublished observations). Therefore, this polymorphism may confer significant susceptibility to TB in this Caucasian population.

References


