

Response heterogeneity of human macrophages to ATP is associated with P2X₇ receptor expression but not to polymorphisms in the *P2RX7* promoter¹

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Received 18 July 2002; revised 2 September 2002; accepted 2 September 2002

First published online 2 October 2002

Edited by Ned Mantei

Abstract A region 2 kb upstream of exon 1 of the P2X₇ gene was sequenced using DNA from nine healthy individuals who exhibited three different ATP response phenotypes (i.e. high, low and interferon gamma-inducible). Five single nucleotide polymorphisms were identified within the nine donor promoter sequences but none were associated with a specific ATP response phenotype. A P2X₇ loss of function polymorphism (1513 in exon 13) was also screened for within donor DNA but no response associations were identified. ATP response phenotype was positively associated with P2X₇ receptor expression, as assessed by flow cytometry, but not with any identified receptor or promoter gene polymorphisms.

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Key words: P2X₇; Promoter; Polymorphism; ATP; Cell lysis; 1513

1. Introduction

We have previously characterised a P2X₇-purinergic receptor-mediated pathway through which infected human macrophages can be signalled by exposure to extracellular ATP in vitro to kill both virulent and avirulent mycobacterial species rapidly [1–3]. This was shown to be a macrophage-mediated process, as ATP had no mycobactericidal activity within cell-free cultures. However, individual healthy cell donors were found to display marked heterogeneity in response to ATP in terms of the susceptibility of their macrophages to ATP-mediated cell death and killing of intracellular mycobacteria [2].

Only approximately 10% of individuals infected with *Mycobacterium tuberculosis* actually go on to develop clinical

tuberculosis (TB) [4]. A similar percentage of healthy donors were found to exhibit a low or non-ATP response phenotype [2]. Moreover, cells from non-ATP responder individuals also failed to kill intracellular mycobacteria following ATP stimulation whereas cells from ATP responders efficiently killed 50–70% of such bacteria within 6 h [2]. As multiple genetic influences are thought to affect human susceptibility to TB it was postulated that heterogeneity in ATP/P2X₇ responsiveness might prove to be an additional genetic factor influencing host immunity to infection with *M. tuberculosis*.

A single nucleotide polymorphism (SNP) at position 1513 in exon 13 of the P2X₇ gene was recently reported by Gu et al. to cause a total loss of receptor function when inherited as a homozygous mutation and resulted in a 50% reduction in receptor activity as a heterozygous mutation [5]. However this polymorphism was not found to account for all the non-ATP responsive donors identified in their study, suggesting that other factors may also be involved in regulating ATP response phenotype [5]. The 1513 SNP was also not reported to reduce P2X₇ receptor expression on affected cells [5]. This was of interest as our laboratory has observed that the ATP response phenotype of cells derived from healthy human donors is positively associated with P2X₇ receptor expression (Stober et al., submitted for publication). The heterogeneity in donor macrophage responsiveness to ATP is therefore predicted to be associated with genetic factors regulating P2X₇ expression rather than receptor function.

To investigate this hypothesis, a search was undertaken for polymorphisms in the putative promoter region of the P2X₇ gene 2 kb upstream of exon 1 of the published *P2RX7* sequence within the DNA of macrophages isolated from nine healthy cell donors. The donors were individuals who had previously been characterised by our laboratory as exhibiting either a high, low or interferon gamma (IFN γ)-inducible ATP responder phenotype [6].

2. Materials and methods

2.1. Cell donors

Nine healthy volunteers were recruited as blood donors to obtain 7-day cultured monocyte-derived macrophages, as previously described [2]. These nine individuals had been identified as exhibiting either a high responder phenotype (i.e. those exhibiting a high response to ATP with or without IFN γ pre-activation), a low responder phenotype (i.e. those showing little response to ATP in the presence or absence of IFN γ), or an IFN γ -inducible responder phenotype (i.e.

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¹ The sequences reported have been submitted to HGBASE and granted the following accession numbers: SNP000063001 –298 polymorphism; SNP000063002 –762 polymorphism; SNP000063003 –838 polymorphism; SNP000063004 –1140 polymorphism; SNP000063005 –1269 polymorphism.

Abbreviations: *P2RX7*, P2X₇ gene; SNP, single nucleotide polymorphism; FACS, flow cytometry staining; IFN γ , interferon gamma; TB, tuberculosis

those showing a positive response to ATP which was markedly increased on pre-activation with IFN γ [6]. The study cohort then comprised of three donors of each of the three described ATP response phenotypes.

2.2. ^{51}Cr Chromium release assay

^{51}Cr release assays were performed to confirm donor ATP response phenotype as described in [2].

2.3. Flow cytometry

Flow cytometry staining (FACS) was performed on 7-day cultured monocyte-derived macrophages from the nine cell donors examined. Aliquots of cells (5×10^5 – 1×10^6) were pelleted at $400 \times g$ for 4 min and labelled with either an IgG $_{2a}$ isotype control antibody (Dako A/S, Denmark) (final 1:100 dilution) or an anti-human P2X $_7$ receptor antibody L4 (Glaxo Wellcome, Stevenage, UK) (final 1:50 dilution) and incubated for 30 min. The cells were then washed twice at $400 \times g$ for 4 min in 1 ml of phosphate-buffered saline (PBS)+1% normal goat serum and then resuspended in a secondary antibody (anti-mouse Ig Fab' 2 FITC conjugate) (Amrad Operations, Australia) (final 1:100 dilution) and incubated for 30 min on ice in the dark. The cells were then washed one time with cold PBS+1% normal goat serum, and resuspended in 200 μl of FACS fixative (4% paraformaldehyde in PBS, pH 7.4), and stored at 2–8°C in the dark until analysed using a Becton Dickinson (FACS 440) flow cytometer.

2.4. DNA isolation

DNA was isolated from the blood of nine healthy volunteer donors using a Nucleon BACC2 kit (Tepnel). The DNA was then dissolved in sterile distilled water and used at a final concentration of 10 ng/ μl .

2.5. Polymerase chain reaction (PCR) amplification of products for sequencing

2.5.1. Promoter. The P2RX7 sequence was obtained from GenBank (accession number Y12851). The promoter region was assumed to be within a 2-kb sequence upstream of P2RX7 exon 1. This 2-kb region was split into five fragments for sequencing.

Fragment 1: forward (5'-CCT GCC TAT ATG CAA CTG AG-3'), reverse (5'-CAG GAA ACA GCT ATG ACC GAT ACA TGT CAA ATG TGT TC-3'); fragment 2: forward (5'-GCC TGG AAG ATT TAA GTC ATC-3'), reverse (5'-CAG GAA ACA GCT ATG ACC CTC TGA GTT ACT CTC CAC C-3'); fragment 3: forward (5'-ACC TAA CTT TGT TGG ACT GC-3'), reverse (5'-CAG GAA ACA GCT ATG ACC AGC CTT GCA AAT GTC ACA AG-3'); fragment 4: forward (5'-ACC TAA CTT TGT TGG ACT GC-3'), reverse (5'-CAG GAA ACA GCT ATG ACC ATC ACT GCA GCT GCA GCA GG-3'); fragment 5: forward (5'-GCAGGCCAGG-GAGTCA-3'), reverse (5'-ATCAGATTGTCAGGGGTAGG-3').

Primers were used at a final concentration of 200 nM. PCR conditions were as follows: fragments 1 and 2 contained 2.0 mM MgCl $_2$, KCl buffer (Applied Biosystems) (resulting in final concentrations of 10 mM Tris (pH 8.3) and 50 mM KCl), 0.025 U/ μl Amplitaq Gold (Perkin-Elmer), 0.2 mM dNTPs. Cycling conditions were 95°C for 14 min, 38 cycles of 94°C for 15 s, 56°C for 30 s and 72°C for 90 s followed by a final extension of 2 min at 72°C, using a PTC225 thermal cycler (MJ Research). Fragment 3 contained 2.5 mM MgCl $_2$, KCl buffer, 0.05 U/ml Amplitaq Gold (Perkin-Elmer), 0.02 mM dNTPs. Fragment 4 contained 1.5 mM MgCl $_2$, KCl buffer, 0.05 U/ml Amplitaq Gold (Perkin-Elmer), 0.2 mM dNTPs. Cycling conditions were 95°C for 14 min, 38 cycles of 94°C for 15 s, 56°C for 30 s and 72°C for 120 s followed by a final extension of 2 min at 72°C. The PCR product sizes generated were 467 bp, 445 bp, 395 bp, 460 bp and 859 bp respectively for each fragment. PCR products were separated by gel electrophoresis using a 1.5% agarose gel. The product was cut out from the gel over low intensity ultraviolet (UV) light and extracted using the Qiaquick gel extraction kit (Qiagen).

2.5.2. 1513. Forward primer: 5'-AGACCTGCGATGGACTT-CACAG-3'; reverse primer: 5'-GCCAGGTGGCGTAGCAC-3'.

The PCR mix contained 1.0 mM MgCl $_2$, KCl buffer, 200 nM of each primer, 0.025 U/ μl Amplitaq Gold (Perkin-Elmer), 0.2 mM dNTPs. Cycling conditions were 95°C for 14 min, 38 cycles of 94°C

for 15 s, 61°C for 30 s and 72°C for 60 s followed by a final extension of 2 min at 72°C, to generate a 379-bp product. The product was cut from the gel over low intensity UV light and extracted using the Qiaquick gel extraction kit (Qiagen).

2.6. Sequencing

Reverse strand for fragments 1–4 was sequenced using the dye primer cycle sequencing reaction M13 reverse kit (Applied Biosciences). Forward strands and the reverse strand for fragment 5 and the 1513 fragment were sequenced using the big dye terminator kit (Applied Biosciences). Template DNA and 3.2 pmol of the appropriate primer were used in the sequencing reaction. Thermal cycling conditions on a PTC225 thermal cycler (MJ Research) were 24 cycles of 1°C/s to 96°C, 96°C for 10 s; 1°C/s to 50°C, 50°C for 5 s; 1°C/s to 60°C, 60°C for 4 min then 10 min at 15°C. The sequencing reactions were ethanol precipitated using sodium acetate and resuspended in 2 μl of a 5:1 mix of formamide (Sigma) to ABI loading dye (Applied Biosystems). These were denatured at 95°C for 2 min and kept on ice before loading onto an ABI Prism 373. Analysis of the sequencing gel was carried out using ABI Prism sequencing analysis software (Applied Biosystems) to produce sequencing traces. These were aligned and analysed using the SeqMan programme of the Lasergene package (DNASTar).

3. Results

3.1. ATP response phenotype of the cell donors

The ATP response phenotype of 7-day cultured monocyte-derived macrophages isolated from nine cell donors utilised in this study were screened for their reactions to stimulation with 3 mM ATP+/-IFN γ (100 IU/ml) for cell lysis, and P2X $_7$ surface expression. Both the ^{51}Cr release assay (Fig. 1), and the flow cytometry results (Fig. 2) identified and confirmed that donors 2, 3 and 9 exhibited a low or non-ATP responder phenotype, donors 4, 5, and 8 exhibited a high ATP responder phenotype and donors 1, 6 and 7 exhibited an IFN γ -inducible phenotype.

3.2. Sequencing of the P2RX7 promoter region

Genomic DNA was isolated from the blood of the nine healthy cell donors. The promoter region 2 kb upstream of P2RX7 exon 1 was then sequenced from each of three low ATP responders (donors 2, 3 and 9), the three IFN γ -inducible

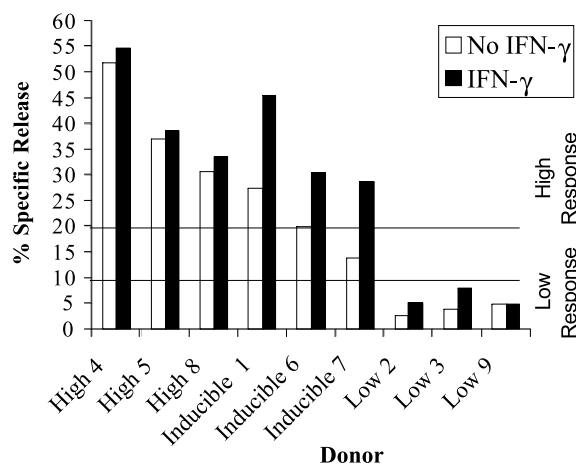


Fig. 1. Comparison of the cytotoxic ATP response phenotype of 7-day cultured monocyte-derived macrophages isolated from three high (donors 4, 5 and 8), three low (donors 2, 3 and 9) and three IFN γ -inducible responders (donors 1, 6, and 7) as assessed within a ^{51}Cr release cytotoxicity assay in the presence or absence of IFN γ (100 IU/ml).

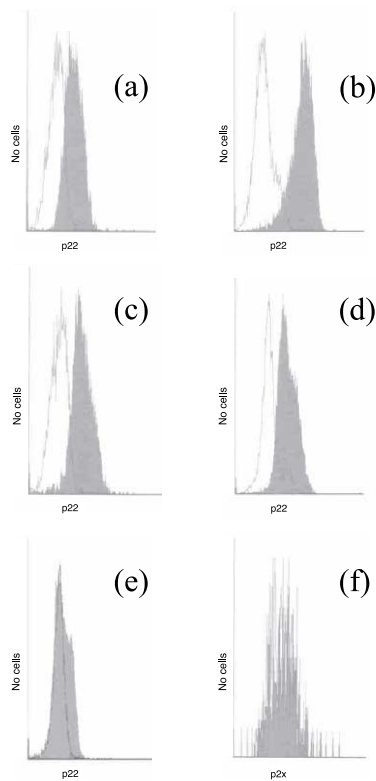


Fig. 2. Flow cytometry results depicting representative P2X₇ surface staining profiles of 7-day cultured monocyte-derived macrophages isolated from two high ATP responding donors (donors 4 and 5, a and b), two IFN γ -inducible responder donors (donors 1 and 6, c and d) and two low responder donors (donors 2 and 9, e and f). The isotype control staining pattern is represented by the open histogram, and the specific anti-P2X₇ staining is represented as the closed histogram.

responders (donors 1, 6, and 7) and the three high responders (donors 4, 5 and 8). Their nucleotide sequences were then compared. Five SNPs were observed in both strands of the putative 2-kb *P2RX7* promoter region within the nine donors examined (see Fig. 3). These were designated as follows:

3.2.1. –298 polymorphism. On aligning the promoter sequence of the nine cell donors an A/G polymorphism was observed at position –298 (Fig. 3a). Of the three low responders examined, donor 9 had an AA genotype and donors 2 and 3 exhibited GG genotypes. Of the three high responders examined, donor 8 had a GG genotype and donors 4 and 5 had A/G heterozygous genotypes. Of the three IFN γ -inducible responders examined, donor 6 had an A/G heterozygous genotype and donors 1 and 7 had a GG genotype. The AA genotype in donor 9 was therefore unique amongst the DNA samples examined.

3.2.2. –762 polymorphism. A T/C polymorphism was observed at position –762 (Fig. 3b). Of the three low responders, donor 9 was again unique within these samples in having a TT genotype while donor 2 had a T/C genotype and donor 3 had a CC genotype. All three high responder donors 4, 5 and 8 had a TC genotype while of the three IFN γ -inducible responders, donors 1 and 6 also exhibited a heterozygous T/C genotype and donor 7 a CC genotype.

3.2.3. –838 polymorphism. A C/A polymorphism was observed at position –838 (Fig. 3c). The low responding donors were again genotypically different, with donor 9 exhibiting an

AA genotype whereas donors 2 and 3 had a CC genotype. This latter genotype was also observed in donors 1, 4, 6, 7 and 8. The exception was the high responder donor 5, who exhibited a heterozygous AC genotype.

3.2.4. –1140 polymorphism. A T/C polymorphism was observed at position –1140 (Fig. 3d). The genotype of the low responder donor 9, was again distinct having a TT genotype while donors 2 and 3 were both CC genotypes. This genotype was also observed in the two high responder donors 4 and 8 and in the IFN γ -inducible responder donors 1, 6 and 7. Donor 5 was heterozygous at this site with a C/T genotype.

3.2.5. –1269 polymorphism. A T/G polymorphism was observed at position –1269 (Fig. 3e). Donor 9 expressed a GG genotype while the other two low responder donors 2 and 3 were both TT genotypes at this position. The latter genotype was also expressed by the two high responder donors 4 and 8 while the remaining donors, donors 1, 5, 7, had a heterozygous TG genotype.

Overall, none of the *P2XR7* promoter SNPs identified segregated completely with any specific ATP response phenotype.

3.2.6. 1513 polymorphism. The *P2RX7* gene from each of the nine cell donors was also sequenced to screen for the presence of the 1513 loss of function polymorphism as described previously [5]. This A/C mutation, which causes an amino acid change from glutamic acid to alanine, was found to be polymorphic in the nine donors examined (Fig. 3f). Donor 2 had a CC genotype, donors 1 and 3 were heterozygous and donors 4, 5, 6, 7, 8 and 9 were homozygous for the A allele. All three low responders exhibited different genotypes, indicating that the 1513 SNPs did not segregate with a non-ATP responsive phenotype as assessed in this study.

4. Discussion

Donor response heterogeneity to ATP was confirmed to be positively associated with macrophage P2X₇ expression suggesting that response phenotype may be genetically regulated by polymorphisms in the *P2RX7* promoter.

DNA from nine healthy cell donors, exhibiting three different ATP response phenotypes were then screened for polymorphisms in a 2-kb region upstream of *P2RX7* exon 1 and five SNPs were subsequently identified in this putative promoter region. However, none of the individual SNPs were found to be consistently associated with a specific donor ATP response phenotype. A recently described *P2RX7* functional polymorphism (1513) was also screened for within the DNA of the nine cell donors examined but this SNP was also not associated with any of the three defined ATP response phenotypes.

Our failure to find an association between the various SNPs identified in the *P2RX7* promoter and donor ATP response phenotype may be attributed to a number of factors. These include the possibility that the 2-kb sequence upstream of *P2RX7* exon 1 was not the actual receptor promoter. This hypothesis is supported by the fact that no characteristic promoter site markers, such as TATA boxes, were identified in this region. However, it may be that the promoter SNPs identified only subtly affect P2X₇ expression or alternatively, only affect it under certain physiological conditions that were not reproduced in this study.

No common polymorphisms were identified within this promoter sequence, with the exception of the genotypes from

	-323		-273	
Consensus	GAAAGGGGGGAAAAGGGAGAATTCTGAAAATGCCCATCCTCTGAACACCAT			(a)
Donor 1	-----			
Donor 2	-----			
Donor 3	-----			
Donor 4	-----R-----			
Donor 5	-----R-----			
Donor 6	-----R-----			
Donor 7	-----			
Donor 8	-----			
Donor 9	-----A-----			
	-787		-737	
Consensus	TGGTGTCCTCACTGAATAGGTCAACAAACCTAACTTTGTTGGACTGCCAC			(b)
Donor 1	-----Y-----			
Donor 2	-----Y-----			
Donor 3	-----			
Donor 4	-----Y-----			
Donor 5	-----Y-----			
Donor 6	-----Y-----			
Donor 7	-----			
Donor 8	-----Y-----			
Donor 9	-----T-----			
	-863		-813	
Consensus	ACTCCACTTTCCTCGGAATCCTATACCTAAATTGCTGTTTCCTTTGTTGG			(c)
Donor 1	-----			
Donor 2	-----			
Donor 3	-----			
Donor 4	-----			
Donor 5	-----M-----			
Donor 6	-----			
Donor 7	-----			
Donor 8	-----			
Donor 9	-----A-----			
	-1165		-1115	
Consensus	TAAATCAGAGACCTTCAGAACTTCGCTGTTTGAAATTACATGACTAAGA			(d)
Donor 1	-----			
Donor 2	-----			
Donor 3	-----			
Donor 4	-----			
Donor 5	-----Y-----			
Donor 6	-----			
Donor 7	-----			
Donor 8	-----			
Donor 9	-----T-----			
	-1294		-1244	
Consensus	ACTTAGCAGGCTGGAATAACAGAAATGGATCAAGCCAGCTGTAAAGATAAC			(e)
Donor 1	-----K-----			
Donor 2	-----			
Donor 3	-----			
Donor 4	-----			
Donor 5	-----K-----			
Donor 6	-----			
Donor 7	-----K-----			
Donor 8	-----			
Donor 9	-----G-----			
	1488		1538	
Consensus	cctgagagccacaggtgcctggaggAgctgtgctgccggaaaaagccggg			(f)
Donor 1	-----M-----			
Donor 2	-----C-----			
Donor 3	-----M-----			
Donor 4	-----			
Donor 5	-----			
Donor 6	-----			
Donor 7	-----			
Donor 8	-----			
Donor 9	-----			

Fig. 3. Polymorphisms were identified in the putative promoter region of the *P2RX7* at –298 (a), –762 (b), –838 (c), –1140 (d), –1269 (e) and the 1513 polymorphism of exon 13 of the *P2RX7* (f) in three high responding donors (4, 5, 8), three inducible donors (1, 6, 7) and three low responding donors (2, 3, 9). Key heterozygous alleles are represented using the IUB codes: R = A or G, Y = C or T, M = A or C, K = T or G, ‘–’ denotes the homozygous allele found in the consensus sequence.

donors 3 and 9. None appears to be associated with any of the three defined ATP response phenotypes (i.e. high, low and IFN γ -inducible responders) within the nine donors examined. However due to the small sample size, more individuals expressing the distinct genotypes of donors 3 and 9 need to be identified and assessed to determine whether these are more frequently associated with low expression of the P2X $_7$ receptor. This latter hypothesis would also suggest that it is the overall genotype or a combination of alleles that affects receptor expression rather than the influence of a single allele.

Support for a regulatory role for the promoter SNPs comes from the fact that we have found a positive association between one of the promoter SNPs (–762) and susceptibility to TB within a Gambian population [12]. Although no known binding motifs were found at or around the –762 SNP site, a search through the transfac database [7,8] indicated that three transcription factors CCAAT enhancer binding protein (C/EBP) [9], GATA binding factor 1 (GATA-1) [10] and delta crystalline enhancer binding protein EF-1 (δ EF-1) [11] could recognise a motif in this region. Indeed the –762 SNP was found to reside in this motif. We are currently characterising this region and investigating the effects of the –762 and the other promoter SNPs on P2X $_7$ function by cloning their regulatory sequences into reporter plasmids and transfecting them into both human and murine monocytic cell lines to look for differential transcript activity. It is hoped that this approach will help to identify the regulatory mechanisms underlying the heterogeneity in ATP responsiveness observed within human populations.

Acknowledgements: We thank Dr Ian Chessell and Dr Gary Buell for the gift of the anti-human P2X $_7$ monoclonal antibody. This study was supported by an EU RTD Grant IC18*CT970236, the United Bir-

mingham Hospitals Committee, Grant No. F08613 and the Wellcome Trust Grant No. 064013/Z/01/2. A.V.S.H. and S.J.C. are also supported by the Wellcome Trust. This work was presented in part at the Third International Symposium on Nucleotides and Nucleosides, Purines 2000, Biochemical, Pharmacological and Clinical Perspectives, 10th July, 2000, Madrid, Spain.

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