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ORIGINAL PAPER

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Expression of P2X receptors on immune cells in the rat liver during postnatal development

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Abstract Single and double-labeling immunofluorescence and RT-PCR expression of P2X receptor proteins and mRNAs were used in a study of the liver of postnatal rats. OX62 and ED1 were used as markers for dendritic and macrophage (Kupffer) cells respectively. The results showed that the $P2X_6$ receptor subunit was up-regulated by 15-fold on hepatic sinusoid cells during postnatal days P1 to P60. Subpopulations of Kupffer cells co-expressed $P2X_4$ and $P2X_6$ receptor subunits and dendritic cells co-expressed $P2X_4$ and $P2X_7$ receptor subunits. Lipopolysaccharide (endotoxin) injected into the peritoneal cavity led to increased expression of the $P2X_6$ receptor on Kupffer cells, suggesting that the $P2X_6$ receptor subunit may be up-regulated by endotoxin. This study presents the first evidence that P2X receptors are widely distributed in the rat liver immune system and that activation of Kupffer and dendritic cells in the rat liver might be regulated by extracellular ATP.

Keywords P2X receptors · Immune system · Development · Liver · Rat · Kupffer

Introduction

The liver macrophage (Kupffer cell) is the first line of defense against the microbes and toxins from the intestinal system. Although Kupffer cells themselves are highly phagocytic and contribute directly to the removal of bacteria, they also act to organize a proinflammatory response, resulting in the rapid recruitment of neutrophils to the liver (Gregory and Wing 2002). Within minutes after binding to the Toll family of receptors on macrophages, the bacterial endotoxin lipopolysaccharide (LPS) triggers activation of the three major mitogen-activated protein kinase pathways (Larsen and Henson 1983; Ulevitch and Tobias 1995; Sweet and Hume 1996; Means et al. 2000). These rapidly evoked signals modulate the expression of multiple inflammatory response genes via transcriptional and/or translational regulation. As a result, macrophages synthesize and release a variety of inflammatory mediators for several hours following the initial exposure to endotoxin. Many of these mediators act in an autocrine fashion via cell surface receptors to provide positive or negative feedback to the macrophage signaling cascades initiated by endotoxin (Larsen and Henson 1983; Sweet and Hume 1996). It has recently been proposed that endotoxin-activated macrophages also release nucleotides, such as ATP, to provide an additional pathway for autocrine or paracrine modulation of endotoxin-dependent responses (Murgia et al. 1993; Ferrari et al. 1997; Hu et al. 1998; Sperlagh et al. 1998; Balboa et al. 1999; Sikora et al. 1999). Both G protein-coupled (P2Y) and ionotropic (P2X) nucleotide receptors are expressed by macrophages (Dubyak and El-Moatassim 1993; Di Virgilio 1995; Ralevic and Burnstock 1998; Bowler et al. 2003). In normal liver, resident dendritic cells typically reside only around portal triads (Prickett et al. 1998; Sato et al. 1998) and, like dendritic cells in other peripheral sites, are able to efficiently capture, process, and transport antigens to regional lymphoid tissues.

There is no data available about the expression of P2X receptors on Kupffer cells and dendritic cells in the liver. Do Kupffer cells and dendritic cells in the liver have similar expression patterns of P2X receptors on immune cells in other organs such as brain and lungs? In the present study single- and double-labeling immunofluorescence

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and reverse transcription-polymerase chain reaction (RT-PCR) methods have been used to localize P2X receptors on Kupffer cells and dendritic cells in rat livers during postnatal development and in adults. Since we found that $P2X_6$ receptors on Kupffer cells were up-regulated during the postnatal days, we examined whether the bacterial endotoxin from the intestine up-regulates $P2X_6$ receptors on Kupffer cells by LPS injection into the peritoneal cavity.

Materials and methods

Tissue preparation

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with Home Office (UK) regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. All protocols were approved by the local animal ethics committee. Wistar rats of five different postnatal day (P)1, P7, P14, P30, P60 and embryological day 21 (E21) were used. Livers from four rats from each stage were used. Rats of postnatal days were killed by asphyxiation with CO_2, embryo rats were killed by immersion in ice water, and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. After perfusion livers were dissected out and refixed in 4% paraformaldehyde in 0. 1 mol/l phosphate buffer pH 7.4 overnight. The liver blocks were then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the blocks were rapidly frozen by immersion in isopentane at −70°C for 2 min. Sections (10 μm in thickness) were cut with a Leica (Heerbrugg, Switzerland) cryostat and thawed on the gelatin-coated slides.

Injection of LPS

Lipopolysaccharide (Sigma Chemical Co., Poole, UK) was dissolved in saline (NaCl 0.9%) at a concentration of 1 mg/ml. Three rats were given a single intraperitoneal injection of LPS in a volume of 1 ml/kg. The rats were killed 24 h later by asphyxiation with CO_2 and perfused as previously described.

Immunohistochemistry

Immunohistochemistry for P2X receptors were performed by using rabbit polyclonal antibodies against a unique peptide sequence of P2X receptor subtypes provided by Roche Palo Alto (CA, USA) (Oglesby et al. 1999). The immunogens used for production of polyclonal P2X antibodies were synthetic peptides corresponding to the carboxyl terminal of the cloned rat P2X receptors, covalently linked to keyhole limpet hemocyanin. The polyclonal antibodies were raised by multiple, monthly injections of New Zealand rabbits with the corresponding peptides (prepared by Research Genetics, Huntsville, AL, USA). The specificities of the antisera, verified by immunoblotting with membrane preparation from CHO K1 cells expressing the cloned P2X receptors was reported and no cross-reactivity was observed among the P2X receptor antisera (Oglesby et al. 1999).

The following protocol was used for immunocytochemistry. After being washed in PBS for 5 min three times the sections were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with P2X antibodies, diluted 1:300–500 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, the sections were incubated with Cy3 conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA, USA) at a dilution of 1:300 in PBS containing 1% NHS for 1 h. The sections for double-labeling immunostaining were incubated with the second primary antibodies of OX62 (mouse anti-rat, Serotec, Raleigh, NC, USA), a marker for dendritic cells (Brenan and Puklavec 1992), diluted 1:5, and ED1 (mouse anti rat, Chemicon International, Temecula, CA, USA), a marker for macrophages (Kupffer) cells (Dijkstra et al. 1985), diluted 1:400 in the antiserum dilution solution overnight at 4°C. Subsequently, the sections were incubated with FITC conjugated donkey-anti-mouse (Jackson) diluted 1:200 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3 × 10 min washes in PBS.

Double-labeling immunofluorescence with two rabbit polyclonal antibodies of P2X receptors

Simultaneous detection of two antigens by immunostaining usually requires primary antibodies from two different species. A novel double-labeling immunostaining method for immunodetection of two independent antigens using two antibodies from the same species of animals has been described (Teramoto et al. 1998). The principle of the method was that the first antigen is detected by the first primary antibody that is diluted so extensively that it cannot be detected with conventional methods; a highly sensitive tyramide signals amplification (TSA) system is used to identify this antibody; the second antigen is stained with the secondary primary antibody and detected by conventional immunostaining. The following protocol was modified from this protocol. Endogenous peroxidase was blocked by 1% H_2O_2 in PBS for 30 min. The sections were pre-incubated in 10% NHS, 0.2% Triton X-100 in PBS for 30 min, followed by incubation with $P2X_4$, $P2X_6$ and $P2X_7$ receptor antibodies, diluted 1:2,000 in antibody dilution solution overnight at 4°C. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in ExtrAvidin peroxidase

(Sigma) diluted 1:1000 in PBS for 30 min at room temperature. The immunoreactivity was visualized by the TSA Fluorescein system (NEL701, NEN, USA). After visualization the sections were incubated with a second different primary antibody of P2X diluted 1:300 in the antiserum dilution solution overnight at 4°C. Subsequently the sections were incubated with Cy3 conjugated Donkey-anti-rabbit (Jackson) diluted 1:300 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3 × 10 min washes in PBS.

Photomicroscopy and data analysis

Images of the immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged by using Adobe-Photoshop 5.0. The focal plane on the microscope was not adjusted whilst determining whether a particular cell colocalised both antigens. Only cells that demonstrated the same morphology, orientation and position when viewed under the two different filters (in the same focal plane) for the detection of Cy3 and FITC were deemed to colocalise. The fields chosen for cell counting were different as the distribution patterns of these P2X receptor immunoreactive (ir) cells were different. For $P2X_4$ receptor-ir cells the fields were randomly chosen, but for $P2X_6$ and $P2X_7$ receptor-ir cells the centers of the fields for cell counting were always the triad. Quantitative analysis for the single and double staining was performed as follows: five random fields (each area was 0.62 mm^2) at 200-fold magnification for one section of the liver were chosen and the number of positive cells was counted and expressed as the positive cell numbers/mm^2. Five fields for each of five sections from each of four rats were used for each marker. The mean number of positive cells/mm^2 from each rat was calculated ± standard error of the mean (n = number of rats). Statistical significance of P2X receptor subtype expression during postnatal development was tested by a one-way analysis of variance (ANOVA) followed by a post hoc (Bonferroni's) test and the effect of LPS on P2X receptor subtype expression was tested by an unpaired t test using GraphPad Prism (GraphPad software, Inc., San Diego, CA, USA). A probability of $P < 0.05$ was considered significant for each test.

Control experiments

Control experiments were carried out with P2X antisera-preabsorbed with P2X peptides at a concentration of 25 μg/ml. These peptides were synthesized by Roche Bioscience, Palo Alto. No staining was observed in those specimens incubated with the antibody solutions re-absorbed with P2X peptides, an example is shown for $P2X_6$ receptors in Fig. 4f.

Reverse transcriptase-polymerase chain reaction

Three adult Wistar rats (250–300 g) were used for RT-PCR. Three copies of total RNA were extracted from livers using the SV Total RNA Isolation System (Promega). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech). Reverse transcription was performed using the Molofney murine leukemia virus reverse transcriptase. Primer sequences for $P2X_1$ to $P2X_7$ (Shibuya et al. 1999) were used for amplification reactions. The sequences of the primers for P2X receptors were as follows: $P2X_1$, sense (S) GAAGTGTG ATCTGGACTGGCACGT, antisense (AS): GCGTCA AGTCCGGATCTCGACTAA, accession number for genebank (AN) X80447; $P2X_2$, (S) GAATCAGAGTGC AACCCCAA, (AS) TCACAGGCCATCTA-CTTGAG, (AN) U14414; $P2X_3$, (S) TGGCGTTCTGGGTATTAA GATCGG, (AS) CAGTGGCCTGGTCACTGGCGA, (AN) X90651; $P2X_4$, (S) GAGGCATCATGGGTATC AGATCAAG, (AS) GAGCGGGGTGGAAATGTAA CTTTAG, (AN) X87763; $P2X_5$, (S) GCCGAAAGCT TCACCATTTCCATAA, (AS) CCTACGGCA-TCCG CTTTGATGTGATAG, (AN) X92069; $P2X_6$, (S) AAA-GACTGGTCAGTG-TGTGGCGTTC, (AS) TGCCT-GCCCAGTGACAAGAATGTCAA, (AN) X92070; and $P2X_7$, (S) GTGCCATTCTGACCAGGGTTGTAT AAA, (AS) GCCACCTCTGT-AAAGTTCTCTCCGA TT, (AN) X95882. Reverse transcriptions and cDNA amplifications for P2X receptors were carried out with a thermal cycler (Hybaid, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK). Every sample was further treated with Amplification Grade DNase I (Sigma) to remove any residual DNA present that could generate false-positive results. Briefly, 1 μg of total RNA was reverse transcribed using the pd(T)12–18 as the first-strand primer at 42°C for 30 min and the enzyme was denatured at 95°C for 5 min. The sequence specific primers were then added to the reaction mixtures, and the PCR cycling parameters were 95°C for 45 s, 58°C for 1 min, 72°C for 1 min for 35 cycles, followed by a further stage of 10-min extension at 72°C. The resulting PCR products were resolved in a 1.5% agarose gel containing ethidium bromide and observed under ultraviolet illumination. RT-PCR results for the livers were confirmed by repetition with three separate RNA samples. Control experiments were conducted by denaturing the reverse transcriptase (95°C for 10 min) before the RT-PCR reaction. These experiments demonstrated that, on denaturation of the reverse transcriptase, no P2X receptor cDNAs could be detected.

Results

The majority of the cells (78–83%) with ED1-ir (a macrophage marker) were also labeled with $P2X_4$ receptor-ir (Fig. 1a) at all developmental stages examined. However,

only 11–13% of the cells with ED1-ir also expressed $P2X_7$ receptors (Fig. 1e). The percentage of cells with both ED1-ir and $P2X_7$ receptor-ir or $P2X_4$ receptor-ir did not change significantly at any age, but the percentage of cells with both ED1-ir and $P2X_6$ receptor-ir changed significantly with age, from zero at embryo day 21 to 87% at postnatal day 60 in the triad areas (Fig. 1c). The number of cells and the percentages of cells showing double-labeling with P2X receptors and ED1-ir at the different developmental stages are summarized in Table 1.

OX62 is believed to be a dendritic cell marker and most of the OX62-ir cells were found in the interlobular tissues. About 53–61% of the cells labeled by the OX62 antibody at all stages were also found to be immunoreactive for $P2X_4$ receptors (Fig. 1b). About 54—69% of the cells that were OX62-ir at all stages were also immunoreactive for $P2X_7$ receptors (Fig. 1f). None of the cells with

Table 1 The number of cells (with percentages underneath) with ED1-ir and $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir at different postnatal days (cell number/mm^2)

Stages	ED1+		ED1+		ED1+	
	Total	$P2X_4$ +	Total	$P2X_6$+	Total	$P2X_7$ +
P1	53±6	43 ± 6	56 ± 4	5 ± 2	61 ± 5	8 ± 3
%		81 ± 11		9 ± 4		13 ± 5
P7	57 ± 7	47 ± 7	60 ± 6	24 ± 5	57 ± 6	6 ± 3
%		82 ± 12		40 ± 8		11 ± 5
P14	55 ± 6	43 ± 6	65 ± 5	45 ± 6	62 ± 8	8 ± 5
%		78 ± 11		75 ± 9		13 ± 8
P30	61 ± 7	48 ± 5	63 ± 7	53 ± 8	67 ± 9	8 ± 6
%		79 ± 8		84 ± 13		12 ± 9
P60	58 ± 5	48 ± 6	69 ± 8	60 ± 5	71 ± 6	8 ± 4
%		83 ± 10		87 ± 7		11 ± 8

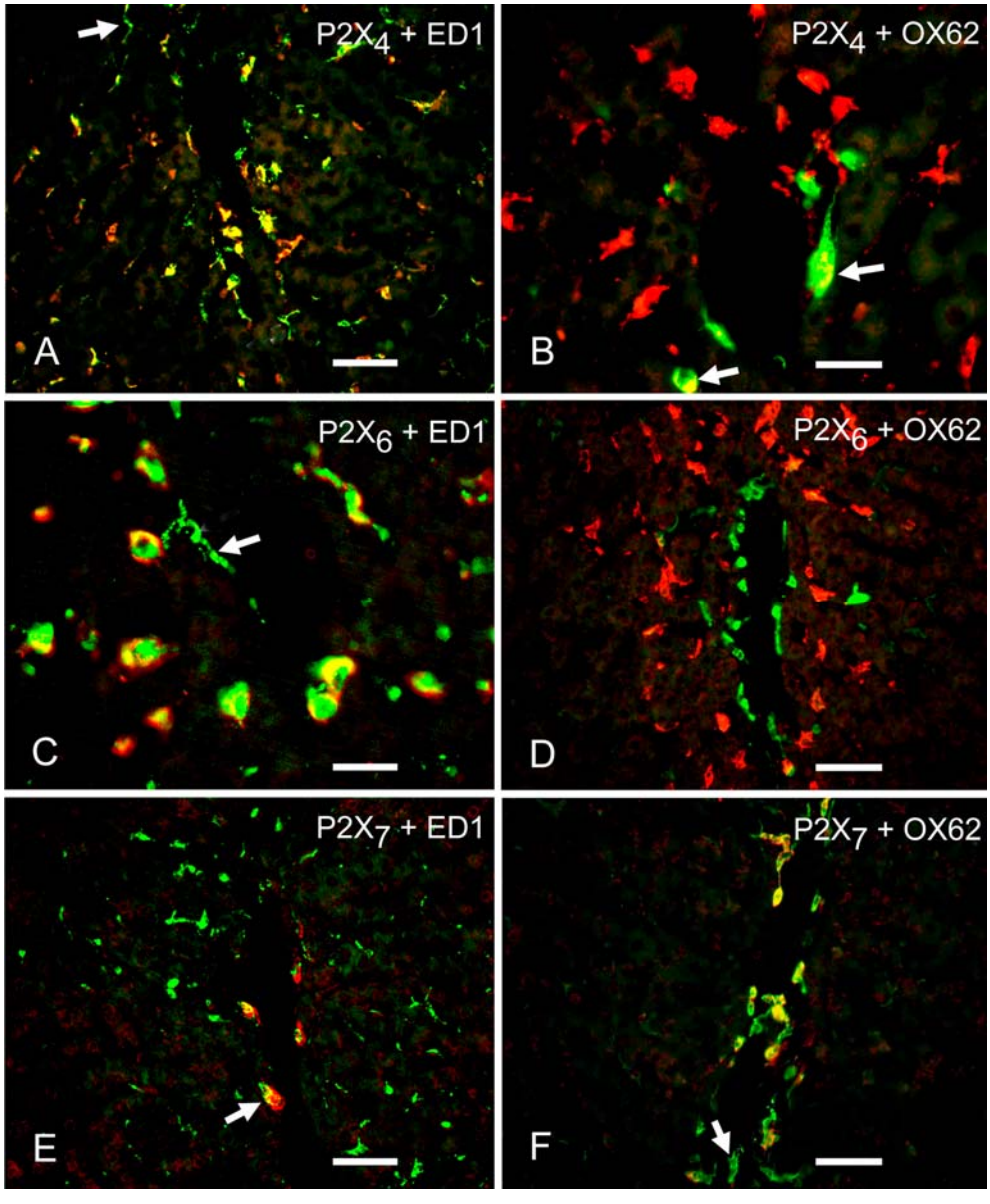

Fig. 1 Co-existence of $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir with ED1 (marker for Kupffer cells) and OX62 (marker for dendritic cells) in the rat liver at P60 developmental stage. **a** $P2X_4$ receptor-ir cells coexist with ED1 (*yellow*) in a subpopulation of cells. Note that some cells (*green*) show ED1-ir only (*arrow*), while other cells (*red*) show $P2X_4$ receptor-ir only. **b** $P2X_4$ receptor-ir cells coexist with OX62 in a subpopulation of cells (*yellow*; *arrow*). Note that many cells express the $P2X_4$ receptor only (*red*). **c** $P2X_6$ receptor-ir cells coexist with ED1 in a subpopulation of cells (*yellow*). Note that some cells express ED1 only (*green*; *arrow*). **d** $P2X_6$ receptor-ir (*red*) shows no co-expression with OX62 (*green*). **e** $P2X_7$ receptor-ir cells coexist with ED1-ir in a subpopulation of cells (*yellow*; *arrow*). Note that many cells express ED1-ir only (*green*) and a few cells only express $P2X_7$ receptors (*red*). **f** $P2X_7$ receptor-ir cells coexist with OX62 (*yellow*) in a subpopulation of cells. Note that some cells expressed OX62-ir only (*green*; *arrow*). Scale bars in **a**, **d**, **e**, **f** = 60 μm, in **b**, **c** = 30 μm

OX62-ir were also labeled by the $P2X_6$ receptor antibody at any developmental stage (Fig. 1d). The number of cells and the percentages of cells showing double-labeling for P2X receptors and OX62 at the different developmental stages are summarized in Table 2.

Among the seven P2X receptor subtypes ($P2X_1$–$P2X_7$) only $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir was found in Kupffer and dendritic cells in the liver at postnatal day 1(P1). The number of $P2X_4$ receptor-ir cells was greater than that with $P2X_6$ and $P2X_7$ receptor-ir, only a few scattered cells with $P2X_6$ and $P2X_7$ receptor-ir were seen at this stage (Fig. 2a, d, g). At E21 there were no $P2X_6$ receptor-ir cells to be found in the liver (Fig. 2d). From P1 to P30 the number of $P2X_6$ receptor-ir cells increased gradually (Fig. 2d, e, f), but the number of the other two P2X receptor-ir cells did not change significantly (Fig. 2a–c, g–i). The distribution patterns of the cells that were positively stained with $P2X_4$, $P2X_6$ and $P2X_7$ receptors were different in the liver. The cells with $P2X_4$ receptor-ir were found in the whole hepatic sinusoids and the triad of interlobular connective tissues (Fig. 2a–c). The cells with $P2X_6$ receptor-ir were only found in the hepatic sinusoids around the interlobular blood vessels (Fig. 2d–f). $P2X_7$ receptors were only found in the area around blood vessels of the triads (Fig. 2g–i). The number of the positive cells with $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir at different stages is summarized in Fig. 3. No staining was seen for the other four P2X receptors, $P2X_1$, $P2X_2$, $P2X_3$ or $P2X_5$ (Fig. 4g).

The coexpression studies between $P2X_4$, $P2X_6$ and $P2X_7$ receptor subunits in Kupffer and dendritic cells showed that most of the cells with $P2X_6$ receptor-ir (Fig. 4b) were also labeled for $P2X_4$ receptors (Fig. 4a, c), but not by the $P2X_7$ receptor antibody (Fig. 4d). Some of the cells with $P2X_4$ receptor-ir were associated with blood vessels and were found to express $P2X_7$ receptors although some cells with $P2X_7$ receptor-ir were not labeled by the $P2X_4$ receptor antibody (Fig. 4e). The percentage of double-labeling among P2X receptors is summarized in Table 3.

Table 2 The number of cells (with percentages underneath) with OX62+-ir and $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir at different postnatal days (cell number/mm^2)

Stages	OX62+		OX62+		OX62+	
	Total	$P2X_4$ +	Total	$P2X_6$+	Total	$P2X_7$ +
P1	14 ± 3	8 ± 3	12 ± 3	0	11 ± 3	6 ± 2
%		57 ± 21		0		55 ± 18
P7	12 ± 4	7 ± 4	11 ± 4	0	13 ± 5	7 ± 4
%		58 ± 33		0		54 ± 31
P14	15 ± 6	9 ± 5	13 ± 5	0	15 ± 4	9 ± 5
%		60 ± 33		0		60 ± 33
P30	14 ± 5	9 ± 4	16 ± 6	0	12 ± 5	7 ± 3
%		64 ± 29		0		58 ± 25
P60	13 ± 4	8 ± 3	13 ± 3	0	13 ± 6	9 ± 3
%		61 ± 23		0		69 ± 23

After i.p. injection of LPS (1 mg/kg) for 24 h, the number and staining density of the cells with $P2X_6$ receptor-ir increased greatly (Fig. 5c, d), while the number and staining density of the cells with $P2X_4$ and $P2X_7$ receptor-ir did not change significantly (Fig. 5a, b, e, f). The number and percentage of cells showing positive staining for $P2X_4$, $P2X_6$ and $P2X_7$ receptors are summarized in Fig. 6.

The result of RT-PCR confirmed that $P2X_4$, $P2X_6$ and $P2X_7$ receptors were expressed in the rat liver because a clear band was detected for these P2X receptor subtype mRNAs (Fig. 7).

Discussion

In this study the methods of single-labeling, double-labeling immunofluorescence and RT-PCR were used to study the expressions of P2X receptor proteins and mRNAs in the liver of the postnatal rat. The results showed that the $P2X_6$ receptor subunit was up-regulated during postnatal days 1 to 60 and by LPS; $P2X_4$ and $P2X_6$ receptor subunits were expressed and coexisted in the Kupffer cells perhaps as heteromultimers, and $P2X_4$ and $P2X_7$ receptor subunits were expressed and coexisted in the dendritic cells. This study has provided the first evidence that P2X receptors are widely distributed in the rat liver immune system, suggesting that there may be regulation of Kupffer cells and dendritic cells in the rat liver by extracellular ATP.

Kupffer cells in liver sinusoids are known to carry out a number of different functions, including removing bacteria and worn out erythrocytes from the blood, producing bile, storing glucose as glycogen, using amino acids to make plasma protein, playing a role in detoxification by converting ammonia in blood to urea and storing heavy metals such as iron and mercury and lipids. It will be interesting to find out if $P2X_4$ and/or $P2X_6$ receptors are involved in any of these functions.

In this study we found that $P2X_4$, $P2X_6$, but not $P2X_7$ receptor subunits were expressed in the Kupffer cells. The Kupffer cell is believed to be a macrophage belonging to the monocyte-macrophage system. The $P2X_7$ receptor subunit has been described in the monocyte–macrophage system of other tissues such as microglia (Collo et al. 1997; Ferrari et al. 1997; Visentin et al. 1999; James and Butt 2002; Boucsein et al. 2003), macrophage cell lines (Collo et al. 1997; Hu et al. 1998; Sanz et al. 1998; Beigi and Dubyak 2000) and osteoclasts (Gartland et al. 2001; Naemsch et al. 2001; Jorgensen et al. 2002). These data clearly showed that there is a tissue differential expression of P2X receptor subunits in the monocyte–macrophage system, which were further supported by the fact that only the $P2X_4$ receptor subunit among the seven members of P2X family was found in rat alveolar macrophages (Bowler et al. 2003). $P2X_4$ and $P2X_7$ receptor subunits were found to express in the dendritic cells at all stages we examined. This result is consistent

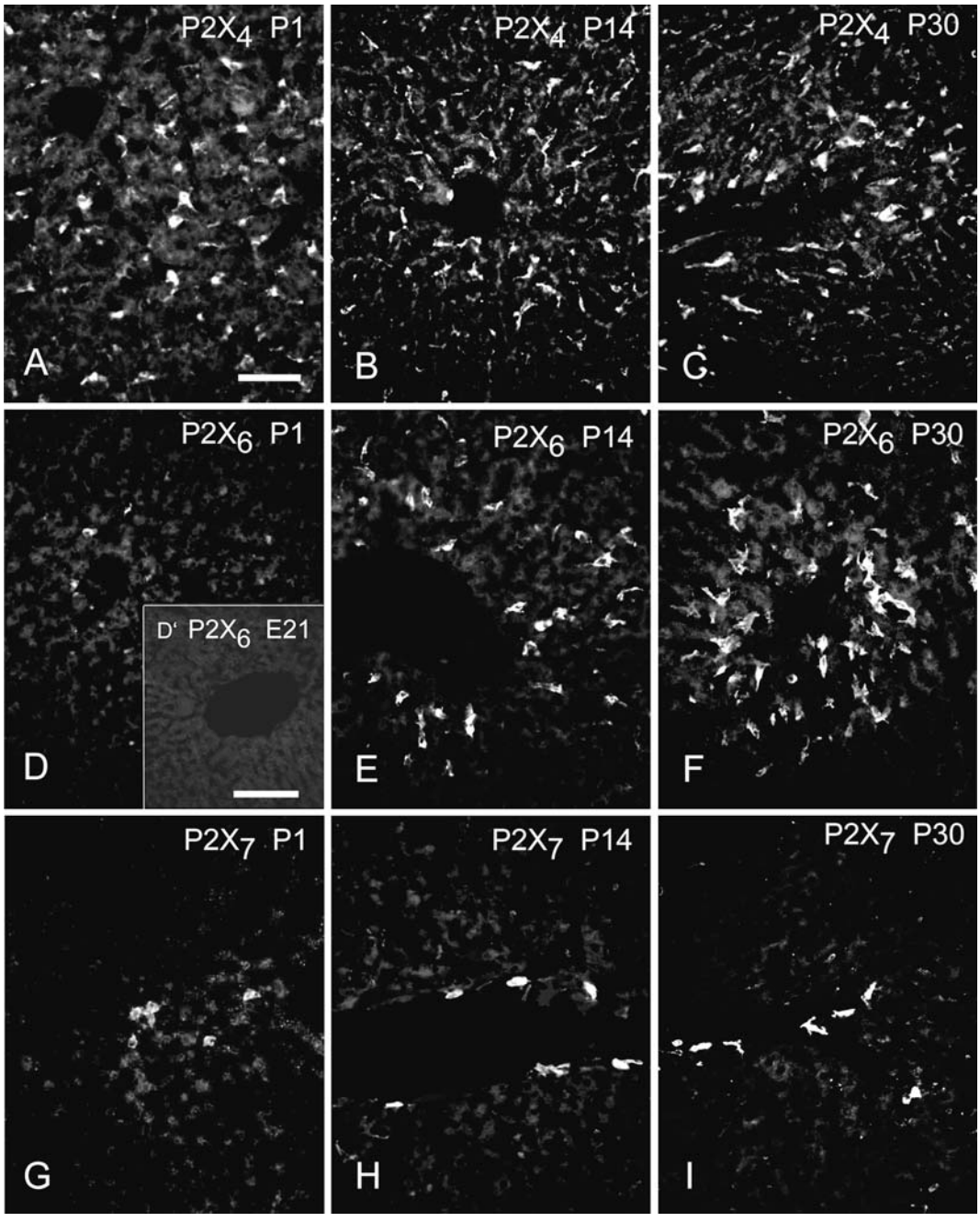

Fig. 2 Expression of $P2X_4$, $P2X_6$ and $P2X_7$ receptor subunits (in Kupffer and dendritic cells) in rat liver during the postnatal days from P1 to P30. **a** Expression of the $P2X_4$ receptor subunit at P1. **b** Expression of $P2X_4$ receptor subunit at P14 and **c** expression of $P2X_4$ receptor subunit at P30. **d** Expression of $P2X_6$ receptor subunit at P1. Note that there is no expression of $P2X_6$ receptor subunit at embryological stage E21 (**d′**, *box*). **e** Expression of $P2X_6$ receptor subunit at P14. **f** Expression of $P2X_6$ receptor subunit at P30. **g** Expression of $P2X_7$ receptor subunit at P1. **h** Expression of $P2X_7$ receptor subunit at P14. **i** Expression of $P2X_7$ receptor subunit at P30. Note that there is a marked increase in expression of $P2X_6$ receptors, but no change in $P2X_4$ or $P2X_7$ receptor expression during development. Scar bar **a**–**i** = 60 μm and **d′** = 120 μm

with previous reports about dendritic cells in other tissues although two other P2X receptor subunits ($P2X_1$ and $P2X_5$) were also reported in these cells (Berchtold et al. 1999; Coutinho-Silva et al. 1999; Mutini et al. 1999; Ferrari et al. 2000; Nihei et al. 2000; la Sala et al. 2001; Sluyter and Wiley 2002). In all those previous reports dendritic cell lines were used, in contrast to this study where rat liver tissue was used.

With double immunofluorescence staining, we found that $P2X_4$ and $P2X_6$ receptor subunits were co-expressed in the Kupffer cells around the triad and $P2X_4$ and $P2X_7$ receptors were co-expressed in some of the dendritic cells in the triad. It was accepted that $P2X_6$ receptor subunits could not form homomeric complexes but formed heteromeric assemblies with other subunits (Torres et al. 1999). Most of the Kupffer cells with $P2X_6$ receptor-ir

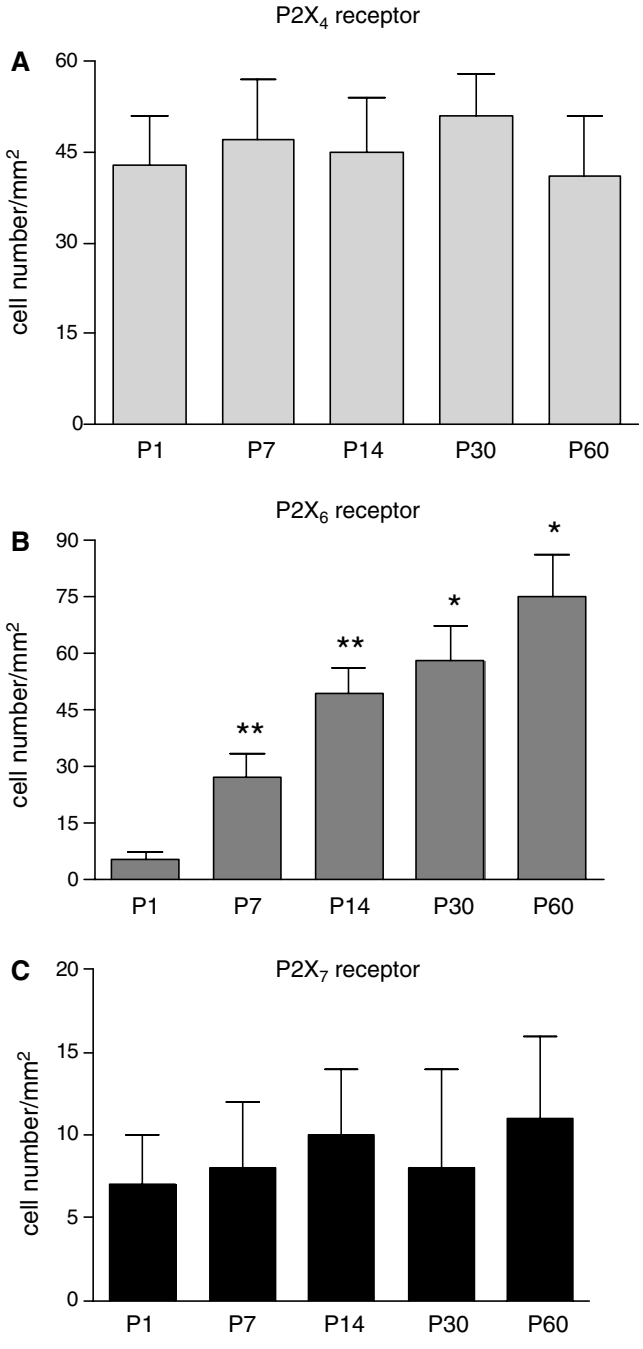

Fig. 3 Bar charts showing the number of cells expressing P2X receptor immunoreactivity at different postnatal ages (P1–P60). **a** $P2X_4$ receptors, **b** $P2X_6$ receptors and **c** $P2X_7$ receptors. All bars show mean ± standard error of the mean. Statistical analyses were by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$, $^{**}P < 0.01$

also expressed $P2X_4$ receptors, which suggested that the heteromeric complexes of $P2X_4$ and $P2X_6$ receptors are formed in these cells (see Le et al. 1998; Khakh et al. 1999). The present data implies that $P2X_6$ receptors may form homomeric complexes in the Kupffer cells of the triad since only $P2X_6$ receptors were up-regulated following LPS stimulation. Homomeric $P2X_6$ receptor channels do not naturally form (see North 2002) but a recent report has shown that $P2X_6$ receptors could form functional homomeric complexes after N-glycosidase F treatment (Jones et al. 2004).

The unique hepatic microenvironment is characterized by the presence of bacterial antigens and bacterial constituents, such as LPS, as well as mediators released by hepatic cells in response to those antigens. LPS and other products of bacterial breakdown in the gastrointestinal tract reach the liver with portal venous blood (Jacob et al. 1977; Lumsden et al. 1988). The concentration of LPS in portal venous blood has been shown to range between 10 pg and 1 ng/ml (Lumsden et al 1988).

The present study showed that the $P2X_6$ receptor subunit was not expressed in Kupffer cells at embryonic day 21. After birth, $P2X_6$ receptors were found, the expression of which increased from P1 to P30. Almost all the cells with $P2X_6$ receptor-ir were found in hepatic sinusoids that first came into contact with blood that comes from the intestine.

The number and the positive signal density of the cells with $P2X_6$ receptor-ir increased greatly after bacterial endotoxin LPS stimulation in vivo. These data suggest that the increase in expression of the $P2X_6$ receptor subunit could be triggered by intestinal bacteria or its toxin after birth. It has recently been proposed that endotoxin-activated macrophages also release nucleotides, such as ATP, provides an additional pathway for autocrine or paracrine modulation of endotoxin-dependent responses (Murgia et al. 1993; Ferrari et al. 1997; Hu et al. 1998; Sperlagh et al. 1998; Balboa et al. 1999; Sikora et al. 1999). In the rat liver these activated Kupffer cells could release ATP to modulate endotoxin-dependent responses via the heteromeric complexes of $P2X_4$ and $P2X_6$ receptors. It should be mentioned that conventional LPS preparations contain highly active lipoprotein contaminants (endotoxin proteins). LPS signals predominantly via the Toll-like receptor (TLR) 4, whereas endotoxin proteins signal via TLR2. Several TLR2-dependent responses of immunocytes to conventional LPS in vitro are triggered by endotoxin proteins and not by LPS itself (Steiner et al. 2005). In the present study it was not possible to say definitively whether LPS or endotoxin proteins were responsible for the up-regulation in $P2X_6$ receptors seen. It would be very interesting to study this issue in the future.

Reverse transcription-polymerase chain reaction results of this study further confirmed that P2X receptor subunit ($P2X_4$, $P2X_6$ and $P2X_7$) are expressed in the rat liver, although it is not clear from these results which hepatic cells the mRNAs for the receptor subtypes is originating, hepatocytes, immune cells, endothelial cell etc. Previous data has shown that P2X receptors are present on primary cultured hepatocytes of rat, guinea pig hepatocytes and rat bile duct epithelium (Zoetewij et al. 1996; Capiod 1998; Bo et al. 2003). In this study, P2X receptor subunits from $P2X_1$ to $P2X_7$ were not found in hepatocytes or bile duct epithelium although antibodies for these seven P2X receptor subunits have been used successfully in our Lab in previous studies (Xiang and Burnstock 2005a, b; Xiang et al. 1999).

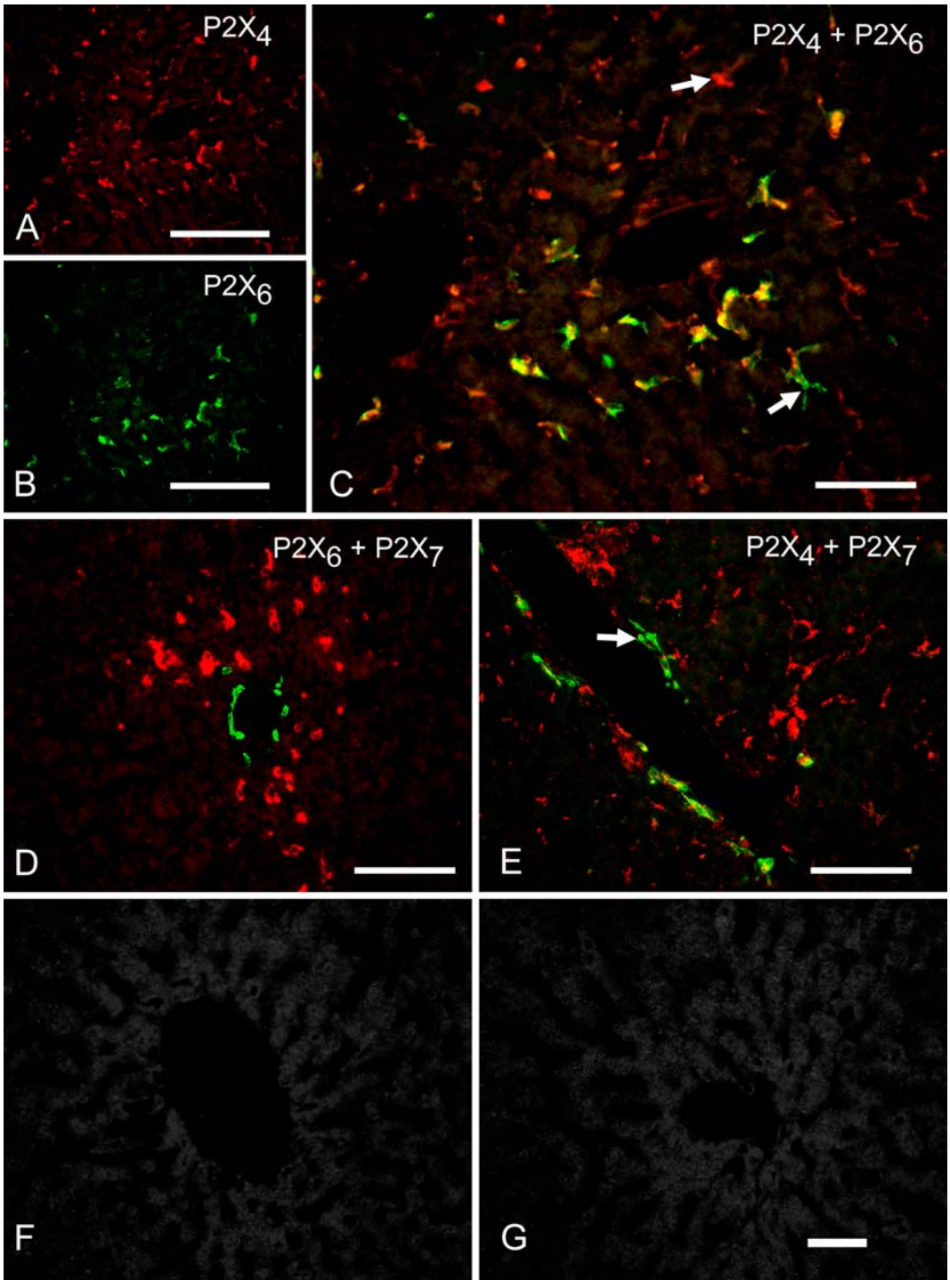

Fig. 4 Co-existence of $P2X_4$, $P2X_6$ and $P2X_7$ receptors in the rat liver at P60. **a** Expression of $P2X_4$ receptor-ir (*red*) in the rat liver at P60. **b** Expression of $P2X_6$ receptor-ir (*green*) in the rat liver of P60 from the same field as (**a**). **c** $P2X_4$ receptor-ir coexists with $P2X_6$ receptors in a subpopulation of cells (*yellow*). Note that many cells express $P2X_4$ receptor-ir only (*red*), others express $P2X_6$ receptor-ir only (*green*) (*white arrows*). **d** There is no co-expression of $P2X_6$ receptors (*green*) and $P2X_7$ receptors (*red*) in the rat liver of P60. **e** Coexpression of $P2X_4$ and $P2X_7$ receptors in a subpopulation of cells in the rat liver at P60 (*yellow*). Note that many cells express $P2X_4$ receptor-ir only (*red*) and a few express $P2X_7$ receptor-ir only (green) (*arrow*). **f** Control showing no staining for $P2X_6$ receptors when the primary antibody was preabsorbed with the corresponding peptide. **g** Negative staining for $P2X_2$ receptors in the rat liver at P60. Scale bars in **a** 65 μm; in **b**, **c** 150 μm; in **d**, **e** 100 μm; in **f**, **g** 60 μm

In summary, in the present study we used single-, double-labeling fluorescence immunohistochemistry and RT-PCR to study the expressions of P2X receptor proteins and mRNAs in the liver of the postnatal rat. We found that the $P2X_6$ receptor subunit was up-regulated during postnatal days from P1 to P30; Kupffer cells expressed

Table 3 The number of cells (with percentages underneath) with co-expression among $P2X_4$, $P2X_6$ and $P2X_7$ receptors in the liver at P60 (cell number/mm^2)

	$P2X_4$+			$P2X_6$+			$P2X_7$ +		
	Total	$P2X_6$+	$P2X_7$+	Total	$P2X_4$+	$P2X_7$+	Total	$P2X_4$+	$P2X_6$+
	46 ± 6	28 ± 6	8 ± 4	52 ± 8	48 ± 8	0	12 ± 4	8 ± 4	0
%		61 ± 13	17 ± 9		92 ± 15	0		67 ± 33	0

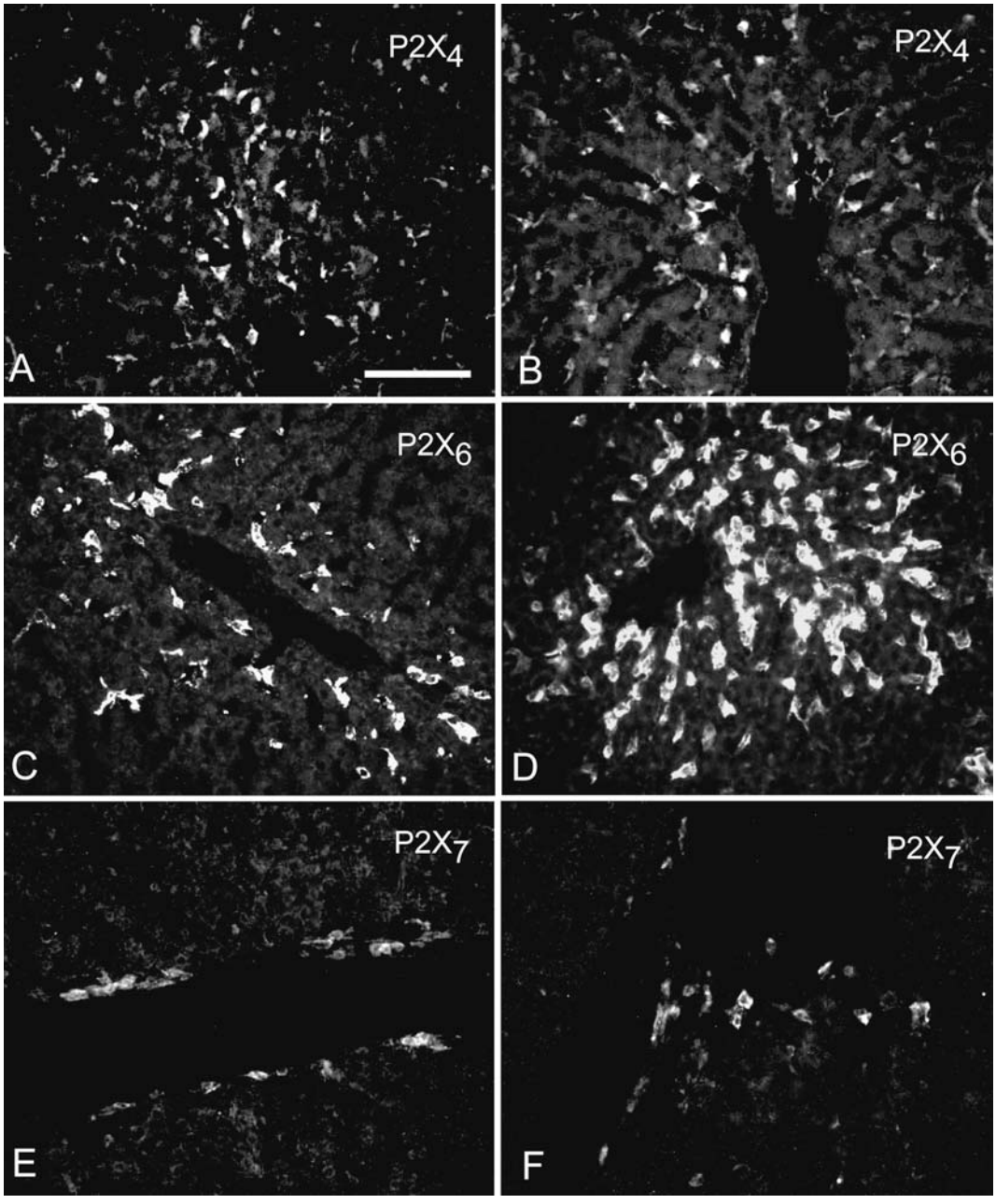

Fig. 5 The expression of $P2X_4$, $P2X_6$ and $P2X_7$ receptor subunits in Kupffer and dendritic cells in the adult rat liver in controls and after LPS-stimulation. **a** Expression of $P2X_4$ receptor in the normal liver. **b** Expression of $P2X_4$ receptors in the LPS-stimulated liver. Note that there is no significant change in the expression. **c** Expression of $P2X_6$ receptors in the normal liver. **d** Expression of $P2X_6$ receptor in the LPS-stimulated liver. Note that the number of the cells with $P2X_6$ receptor-ir is increased significantly. **e** Expression of $P2X_7$ receptors in the normal liver. **f** Expression of $P2X_7$ receptor in the LPS-stimulated liver. Note that there is no significant change in expression. Scale bar for all figures = 100 μm

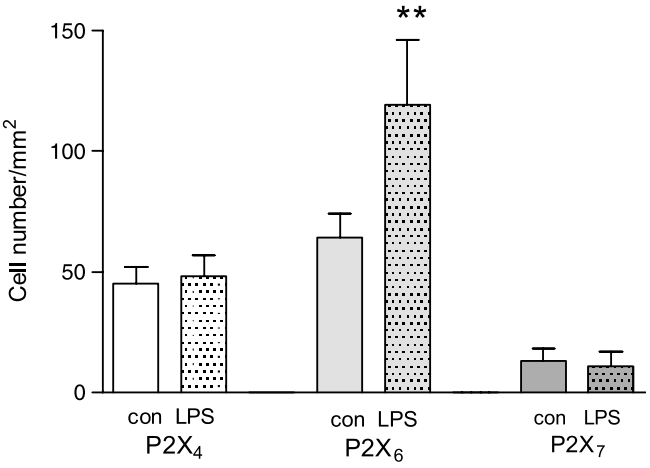

Fig. 6 Bar chart showing the number of cells with $P2X_4$, $P2X_6$ and $P2X_7$ receptor-ir in the liver in controls (con) and following LPS stimulation (cell number/mm^2). All bars show mean ± standard error of the mean. Statistical analyses were by unpaired *t* tests. $^{**}P < 0.01$

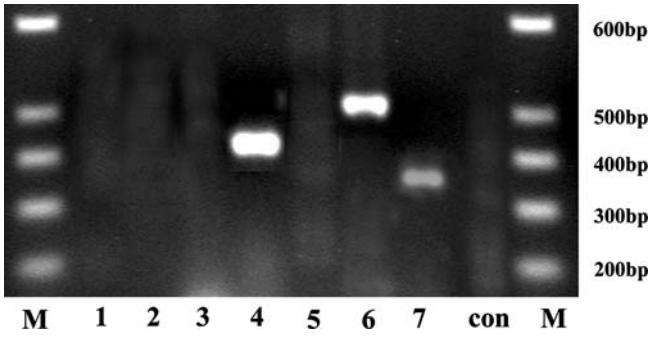

Fig. 7 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to investigate the expression of P2X receptor transcripts in the liver of adult rat. *Lane M* is DNA molecular weight marker; *lane 1–7* are the RT-PCR results with $P2X_1$–$P2X_7$ primers respectively, *lanes 4*, *6* and *7* show one band, respectively; *lane con* is the RT-PCR result of control group after the reverse transcriptase was denatured

$P2X_4$ and $P2X_6$ receptor subunits and dendritic cells expressed $P2X_4$ and $P2X_7$ receptor subunits; $P2X_4$ and $P2X_6$ receptors coexisted in Kupffer cells and $P2X_4$ and $P2X_7$ receptors coexisted in dendritic cells; the $P2X_6$ receptor subunit was substantially up-regulated by exposure of the animals to LPS, suggesting that they may be evoked by endotoxin. This study has provided the first evidence that P2X receptors are widely distributed in the rat liver immune system and suggests that they may be involved in the regulation of the activities of both Kupffer cells and dendritic cells in the rat liver by extracellular ATP.

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