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# Impairment of the splenic immune system in $P2X_2/P2X_3$ knockout mice

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## Abstract

The isolated spleens from male and female mice lacking $P2X_2$ and $P2X_3$ receptors ($P2X_2/P2X_3$ knockout (KO) mice) and those from wild-type (WT) mice were investigated by flow cytometry, immunohistochemistry and functionally by organ-bath pharmacology. The spleens from the $P2X_2/P2X_3$ KO mice weighed significantly more than the corresponding WT mice. Flow cytometry was used to isolate the mononuclear cells, which were then phenotyped. T-lymphocytes, B-lymphocytes and macrophages were identified and counted. It was found that the increase in size of the spleens from the KO animals corresponded to an increase in the numbers of mononuclear cells present and that all three cell types (T-lymphocytes, B-lymphocytes and macrophages) increased in much the same proportion as those from the WT animals. Immunohistochemical localisation of $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors revealed their presence on the spleen capsule and trabeculae. $P2X_1$ receptors were also present on blood vessels. There was no difference in the expression of these receptors between the WT and $P2X_2/P2X_3$ KO spleens. Functional studies revealed the presence of multiple P2 receptors inducing the contraction of the spleen capsule, from both WT and KO mice. There was no difference in the contractions induced by adenosine 5′-triphosphate (ATP), α,β-methylene ATP, 2-methylthio ADP or uridine triphosphate from WT and KO mice. It is concluded that mice lacking both $P2X_2$ and $P2X_3$ receptors have enlarged spleens and that this is correlated with an increase in the number of immune cells, perhaps as a consequence of a compromised immune system and chronic infection.


***Keywords:*** Spleen; P2 receptors

## Introduction

The spleen, in common with other immune organs such as the thymus, is innervated by sympathetic nerves, which release noradrenaline (NA; Elenkov and Vizi, 1991; Haskó et al., 1995a) and probably adenosine 5′-triphosphate (ATP) as a cotransmitter (Burnstock, 1990). Activation of $\alpha_1$-, $\alpha_2$- and β-adrenoceptors on immune cells regulate immunomodulatory functions such as the production of inflammatory mediators (nitric oxide and cytokines; Elenkov et al., 1995; Haskó

*Abbreviations:* ATP, adenosine 5′-triphosphate; α,β-meATP, α,β-methylene ATP; BSA, bovine serum albumin; BSS, buffered saline solution; DAB, 3, 3′-diaminobenzidine; IL-6, interleukin-6; KO, knock out; 2-MeSADP, 2-methylthio ADP; NA, noradrenaline; PBS, phosphate-buffered saline; NGS, normal goat serum; NHS, normal horse serum; UTP, uridine 5′-triphosphate; WT, wild-type

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et al., 1995b, 1998a, b) and most aspects of cellular and humoral immunity (Elenkov et al., 2000; Haskó and Szabó, 1998). P1 and P2 receptors are also abundantly expressed on immune cells (Burnstock, 2001).

The splenic capsule of the guinea pig, rat and mouse spleen is known to contract to NA via $\alpha_{1B}$-adrenoceptors (Eltze, 1994, 1996; Han et al., 1987), although the physiological significance of the capsular contraction is not clear. Agonists to other receptor types also initiate contraction of splenic capsular smooth muscle, for example, the rat spleen contracts in response to adenosine (Fozard and Milavec-Krizman, 1993) and the mouse spleen to bethanechol (Wong, 1990). *In vivo* infusions of both NA and $\alpha,\beta$-methylene ATP ($\alpha,\beta$-meATP) induced splenic capsular contraction of the pig as measured by an increase in venous blood flow (Lundberg et al., 1989), suggesting the presence of $P2X_1$ and/or $P2X_3$ receptors.

In the present study, the effect of P2 receptor agonists was examined on the splenic capsular smooth muscle of wild-type (WT) and mice that were genetically modified such that they lacked both $P2X_2$ and $P2X_3$ receptors. In addition to the functional study, the immunohistochemical expression of $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors in the mouse spleen was investigated.

## Materials and methods

### General procedures

Adult male and female mice that were lacking the gene for $P2X_2$ and $P2X_3$ receptors were generated on an inbred (C57Bl6) and an outbred (MF1) genetic background (Cockayne et al., 2000, 2002). These mice are referred to as $P2X_2/P2X_3$ knockout (KO) mice and are compared to WT mice.

All mice were killed by $CO_2$ asphyxiation and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering Schedule 1 procedures.

The spleen was dissected free and placed immediately in modified Krebs solution. The spleen was weighed and then prepared either for flow cytometry experiments, immunohistochemistry or functional studies.

### Preparation of splenocytes for flow cytometry experiments

Spleens were collected from the WT and $P2X_2/P2X_3$ KO mice as described above and cells were gently removed by mechanical dissociation and then re-suspended in buffered saline solution (BSS). The erythrocytes were removed and mononuclear cells enriched by centrifugation on a Ficoll density-gradient Histopaque 1083 (Sigma, St. Louis, MO, USA). The cells were counted and cell viability following this procedure was greater than 95% in all cases, as measured by Trypan blue exclusion. The splenocytes were adjusted to $10^6$ cells/sample, washed with BSS and incubated with BSS containing 5% normal goat serum (NGS), on ice, for 20 min. The cells were then incubated for 30 min with FITC-conjugated rat anti-mouse CD3 (1:100; PharMingen, UK), FITC-conjugated rat anti-mouse B220 (1:100; PharMingen, UK) or FITC-conjugated rat anti-mouse CD11b (1:100; PharMingen UK) to stain T-lymphocytes, B-lymphocytes and macrophages, respectively, diluted in BSS with bovine serum albumin (BSA). The negative controls for the isotypes used were: FITC rat $IgG_{2b}$ (PharMingen, UK) for CD3 and CD11b antibodies and FITC rat $IgG_{2a}$ (PharMingen, UK) for B220 antibodies control. The isotype controls were used at the same concentration as the antibody tested. Samples were then washed fixed in fresh 4% paraformaldehyde (Sigma) for 10 min on ice and then extensively washed in cold BSS. The cells were then re-suspended in phosphate-buffered saline (PBS) and analysed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA). Post-analysis was carried out using WinMDI (Multiple Document Interface Flow Cytometry Application, V2.8) software.

### Preparation of spleens for immunohistochemistry

#### Tissue handling

The spleens were removed, weighed and put in Hanks BSS solution, then embedded in OCT tissue compound (BDH Laboratory Supply, UK), progressively frozen in isopentane (pre-cooled in liquid nitrogen) and then stored in liquid nitrogen. Cryostat sections of the spleens were cut as a set of serial sections 10 μm thick. The sections were thaw-mounted on gelatine-coated slides and air-dried at room temperature. The slides were stored at −20 °C until use. Tissues were post-fixed for 2 min at room temperature in 4% formaldehyde (BDH) and 0.03% picric acid in PBS. Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% $H_2O_2$ for 25 min. Blocking of non-specific binding sites was achieved by pre-incubation with normal horse serum (NHS; Harlan Sera-Lab, UK) in PBS containing 0.05% thimerosal (Methiolate; Sigma) at room temperature for 20 min, as described by Llewellyn-Smith et al. (1993).

#### Immunostaining

An indirect immunohistochemical method with three-layer amplification was used. Antibodies against $P2X_1$ (Roche bioscience, Palo Alto, CA, USA), $P2Y_1$ and $P2Y_2$ receptors (Alomone Labs. Ltd., Jerusalem, Israel) raised in rabbit were allowed to react with biotinylated

donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, PA, USA) and detected with avidin-coupled horseradish peroxidase/nickel-intensified 3,3′-diaminobenzidine (DAB). Briefly, the sections were incubated overnight with the primary antibodies diluted to 5 and 2.5 μg/ml (determined as optimal by previous titration) with 10% NHS in PBS containing 0.05% Methiolate. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) diluted 1:500 in 1% NHS in PBS containing 0.05% Methiolate for 30 min, followed by incubation with extravidin–horseradish peroxidase (Sigma) diluted 1:1000 in PBS containing 0.05% Methiolate for 30 min. All the incubations were held at room temperature and separated by three 5-min washes in PBS. Finally, freshly prepared colour reaction mixture containing 0.5% DAB, 0.1 M sodium phosphate, 0.004% $NH_4Cl$, 0.2% glucose, 0.004% nickel ammonium sulphate and 0.1% glucose oxidase was applied to the section for 5–10 min. The sections were washed, dehydrated, cleared in xylene and mounted using Eukitt (BDH, Poole, UK). Control experiments were performed using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and thus confirm a specific immunoreaction. The sections were viewed using a Zeiss Axioplan (Germany) microscope coupled with a Leica DC 200 image acquisition system (Leica, Cambridge, UK). The figures were prepared using Adobe Photoshop 5.0.

### Preparation of spleens for functional studies

Male and female spleens were bisected longitudinally into two equal halves. Silk ligatures were applied to each end of the hemi-spleen; one end was attached to a rigid support and the other end to a FT03C force–displacement transducer. Each hemi-spleen was suspended in a 10 ml organ bath containing gassed (95% $O_2$/5% $CO_2$) modified Krebs solution of the following composition (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; $NaH_2PO_4$, 1.4; glucose, 7.7; and $CaCl_2$, 2.5; pH 7.3. Experiments were carried out at $37 \pm 1$ °C.

Mechanical activity was recorded using the software PowerLab Chart for Windows (version 4; ADInstruments, Australia). An initial load of 0.8 g was applied to each hemi-spleen, which was then allowed to equilibrate for not less than 45 min prior to the start of the experiment. The weight of each hemi-spleen was noted at the end of the experiment and contractile responses expressed as mg tension developed per mg hemi-spleen weight to compensate for the larger spleens of the $P2X_2$/$P2X_3$ KO mice.

Cumulative concentration–response curves were constructed for ATP (0.1 μM–1 mM), α,β-meATP (10 nM–0.1 mM), 2-methylthio ADP (2-MeSADP; 10 nM–0.1 mM), uridine triphosphate (UTP; 1 nM–0.1 mM) and adenosine (0.1 μM–1 Mm).

### Drugs used

Adenosine, ATP, α,β-meATP (sodium salt), 2-MeSADP and UTP were obtained from Sigma Chemical Co. (Poole, UK). Stock solutions of adenosine, ATP and UTP (all 0.1 M) were prepared in distilled water and aliquots and frozen. For each experiment aliquots were defrosted and dilutions made. All other agonists were prepared fresh for each experiment. The volume added to the organ bath to produce the final concentration was not in excess of 100 μl.

### Statistical analysis

Significant difference between populations of cells from the WT and $P2X_2$/$P2X_3$ KO animals were compared using an unpaired *t*-test. $P < 0.05$ was taken as significant.

Contractile responses to ATP, α,β-meATP, 2-MeSADP and UTP are expressed as mg tension developed per mg of spleen tissue ± s.e.m. (*n*). As none of the concentration–response curves reached a maximum response, it was not possible to calculate $pD_2$ values ($-\text{Log}\,EC_{50}$ concentration). Statistical significance between the WT and $P2X_2$/$P2X_3$ KO animals was tested by a two-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) followed by a *post hoc* test. A probability of $P < 0.05$ was considered significant.

## Results

### Spleen weights and cell numbers

The spleens from both the male and female $P2X_2$/$P2X_3$ KO animals were larger and weighed significantly more than the corresponding WT spleens (Fig. 1A and B). Following maceration of the spleens, the numbers of immune cells present were counted as described in the Materials and methods section. An equivalent rise in spleen mononuclear cell numbers in the $P2X_2$/$P2X_3$ KO animals was observed when compared with WT. The total number of mononuclear cells found in the spleens was significantly ($P < 0.05$) greater in the female $P2X_2$/$P2X_3$ KO animals compared to the WT, suggesting that the increase in spleen weight observed was due to an increase in the number of immune cells (Fig. 1C). In addition, phenotyping of mononuclear cells obtained from the spleens of the WT and KO animals was performed. We found that the

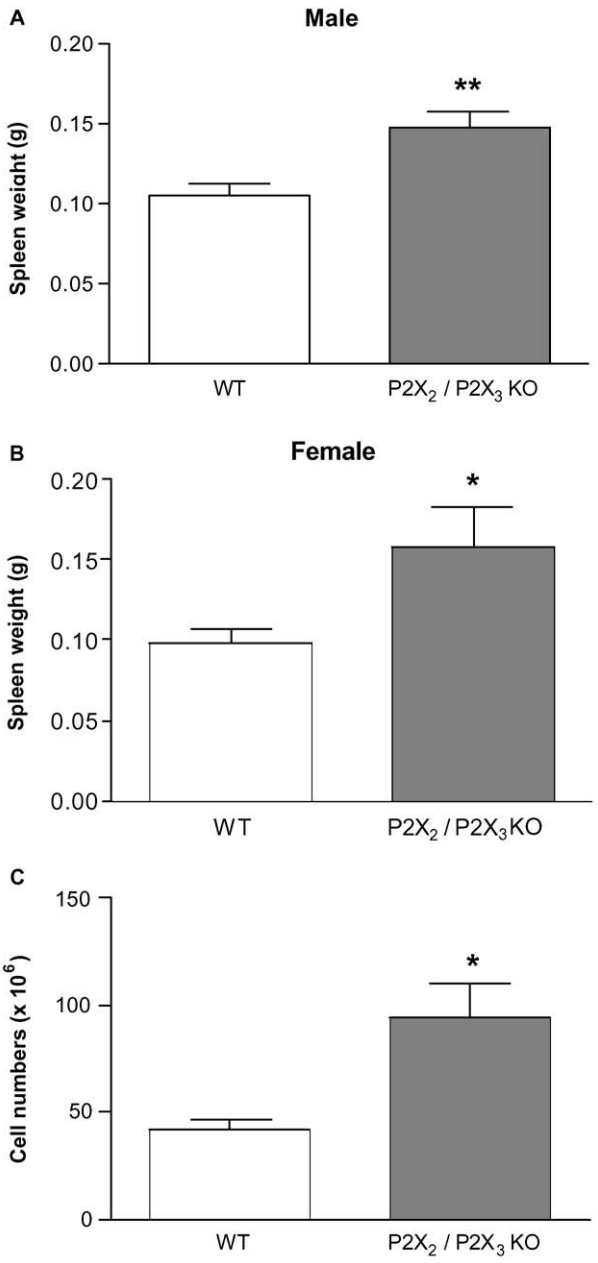


**Fig. 1.** Bar charts showing the weights and cell numbers of the spleens from the WT and $P2X_2/P2X_3$ KO mice. (A) Bar chart showing mean spleen weights from the male WT ($n = 9$) and $P2X_2/P2X_3$ KO ($n = 9$) mice. (B) Bar chart showing mean spleen weights from the female WT ($n = 10$) and $P2X_2/P2X_3$ KO ($n = 9$) mice. (C) Bar chart showing spleen cell numbers ($\times 10^{-6}$) from the WT ($n = 5$) and $P2X_2/P2X_3$ KO ($n = 5$) mice. All bars show mean $\pm$ s.e.m. Statistical significance was tested using an unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

proportion of $CD3^+$ cells (T-lymphocytes), $B220^+$ cells (B-lymphocytes) and $Mac1^+$ cells (macrophages) was the same for the spleens from the $P2X_2/P2X_3$ KO and WT mice (Table 1).

**Table 1.** Proportion of mononuclear cells (as a percentage) from the spleens of the WT and $P2X_2/P2X_3$ KO mice

| Percentage cells | WT | $P2X_2/P2X_3$ KO |
|---|---|---|
| T-lymphocytes ($CD3^+$ cells) | 21.0 $\pm$ 3.0 (5) | 26.4 $\pm$ 1.3 (6) |
| B-lymphocytes ($B220^+$ cells) | 61.6 $\pm$ 4.0 (5) | 61.9 $\pm$ 4.0 (6) |
| Macrophages ($Mac1^+$ cells) | 14.6 $\pm$ 0.4 (5) | 13.6 $\pm$ 1.0 (6) |

Note that there appears to be some increase in the percentage of T-lymphocytes in the KO mice, but this change was not statistically significant.

## Concentration–response curves

ATP, $\alpha,\beta$-meATP, 2-MeSADP and UTP all caused concentration-dependent contractions of the hemi-spleens from both the WT and $P2X_2/P2X_3$ KO animals. The concentration–response curves to ATP (Fig. 2A), $\alpha,\beta$-meATP (Fig. 2B), 2-MeSADP (Fig. 2C) and UTP (Fig. 2D) in the KO spleens were not significantly different from those obtained from the WT animals. Adenosine failed to contract the spleen capsule from either WT or KO animals.

## Immunohistochemistry

Consistent staining for $P2Y_1$ and $P2Y_2$ receptors was observed on the splenic muscle capsule and trabeculae from the WT mice (Fig. 3A and C). The spleens obtained from the $P2X_2/P2X_3$ KO mice also expressed both $P2Y_1$ and $P2Y_2$ receptors on their capsule and trabeculae (Fig. 3B and D). There was $P2X_1$ receptor expression on the spleen capsule and more clearly on the trabeculae of both the WT and $P2X_2/P2X_3$ KO mice (Fig. 3E and F). $P2X_1$ receptors were also observed staining smooth muscle of spleen blood vessels. There was no difference in the expression of $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors between the WT and KO animals. The staining was specific for all P2 receptors studied since pre-absorption of the primary antibody resulted in no observed immunoreactivity (Fig. 4A–D), with the exception of some non-specific background staining for the $P2X_1$ antibody (Fig. 4E and F).

# Discussion

This study has shown that spleens from mice lacking both $P2X_2$ and $P2X_3$ receptors have enlarged spleens, and that this enlargement can be correlated to an increase in the number of mononuclear cells (T-lymphocytes, B-lymphocytes and macrophages). A comparison in the actual numbers of specific immune cells between the WT and $P2X_2/P2X_3$ KO animals revealed that there was a number increase of

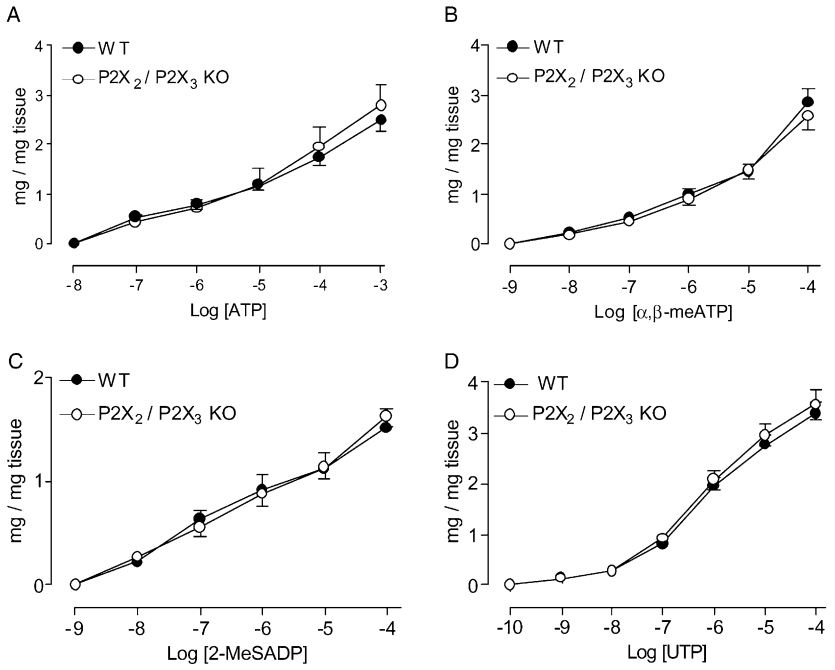


**Fig. 2.** Contraction of the splenic capsule induced by P2 receptor agonists on the WT and $P2X_2/P2X_3$ KO mice. (A) Concentration–response curves for ATP on the WT ($n = 8$) and $P2X_2/P2X_3$ KO mice ($n = 9$). (B) Concentration–response curves for α,β-meATP on the WT ($n = 9$) and $P2X_2/P2X_3$ KO mice ($n = 8$). (C) Concentration–response curves for 2-MeSADP on the WT ($n = 8$) and $P2X_2/P2X_3$ KO mice ($n = 8$). (D) Concentration–response curves for UTP on the WT ($n = 9$) and $P2X_2/P2X_3$ KO mice ($n = 9$). All data are expressed as mg tension developed per mg spleen tissue ± s.e.m. Significance was tested by a two-way ANOVA followed by a Tukey's *post hoc* test.

T-lymphocytes, B-lymphocytes and macrophages in the spleens from the $P2X_2/P2X_3$ KO animals compared to the WT animals, although the proportion of individual mononuclear cells did not alter between WT and KO.

The increase in absolute numbers of T- and B-lymphocytes and macrophages found in the $P2X_2/P2X_3$ KO spleens may suggest that mice lacking $P2X_2$ and $P2X_3$ receptors have altered mononuclear immune cell homing. Recently, a hypocellularity of the thymus and bone marrow has been observed in the $P2X_2/P2X_3$ KO animals (Cockayne et al., 2004). It should also be noted that a high percentage (approximately 90%) of the $P2X_2/P2X_3$ KO mice die prior to weaning due to acute pulmonary infections and multiple organ dysfunction (Cockayne et al., 2002, 2004); although the surviving mice appear healthy, there may be residual weaknesses and as there are fewer immune cells being produced by the thymus and bone marrow, the enlarged spleen observed in the KO animals may reflect an adaptive compensatory response. The enlarged spleens of the $P2X_2/P2X_3$ KO mice may also be as a consequence of chronic infection found to occur in these animals. It has been reported that mice expressing a truncated c-Rel protooncogene (encoding a member of the Rel/nuclear factor-κB family of transcriptional factors), which also have a compromised immune response, present histopathological alterations of haemopoietic tissues, including an enlarged spleen due to lymphoid hyperplasia and bone marrow hypoplasia (Carrasco et al., 1998). A health screen of the animals used in this study revealed that the double KO animals tested positive for *Pasteurella pneumotropica* and *Helicobacter hepaticus*. Although it is not currently known whether these mice have infected spleens, future studies may resolve this. Mice infected with *Staphylococcus epidermidis* commonly have enlarged spleens (Molnar et al., 1994) and in mice infected with *Ehrlichia risticii* thymic atrophy and splenomegaly have been described (Rikihisa et al., 1987). In HIV-1 transgenic mice and nude mice, the Gram-negative commensal bacterium *P. pneumotropica* was commonly present (Dickie et al., 1996). Other possibilities are that the enlarged spleen is due to an autoimmune response or to the development of amyloidosis (Abankwa and Ali-Khan, 1988; Hanayama et al., 2004).

The splenic capsule contains smooth muscle and elastic fibres that contribute to contraction of the whole spleen (Kai et al., 2000). Adrenergic and cholinergic agonists have been shown to induce contraction of the mouse splenic capsule, including bethanechol acting via muscarinic receptors (Wong, 1990) and NA acting via $\alpha_{1B}$ receptors (Eltze, 1996). ATP is an agonist at $P2X_1$, $P2Y_1$ or $P2Y_2$ receptors and ATP may be acting on any or all of these receptors. In addition, ATP rapidly

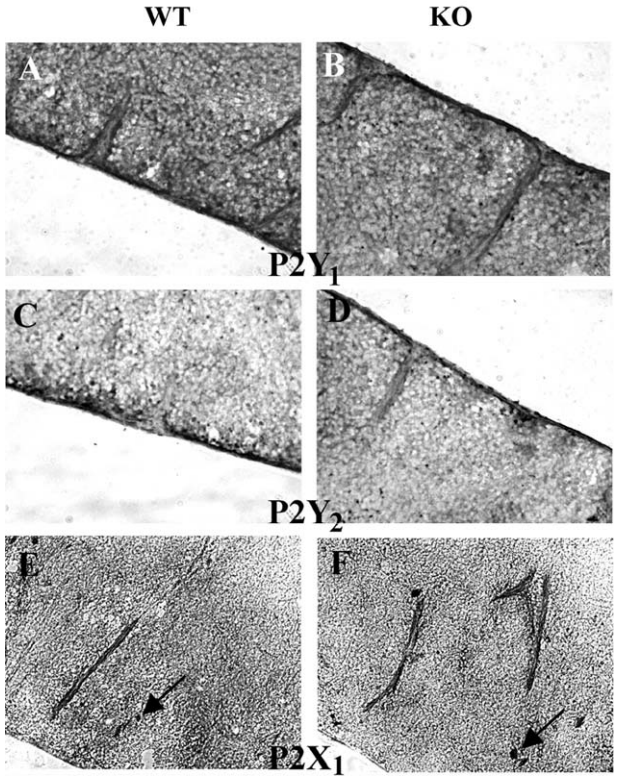


**Fig. 3.** $P2Y_1$, $P2Y_2$ and $P2X_1$ expression in the spleen capsule and trabeculae. (A, C and E) WT mice and (B, D and F) $P2X_2$/$P2X_3$ KO mice. (A, B) $P2Y_1$ immunostaining; (C, D) $P2Y_2$ immunostaining; and (E, F) $P2X_1$ immunostaining. Note in panels E and F in addition to capsule and trabeculae the immunoreactivity for $P2X_1$ on blood vessels (arrows). All figures 200 × .

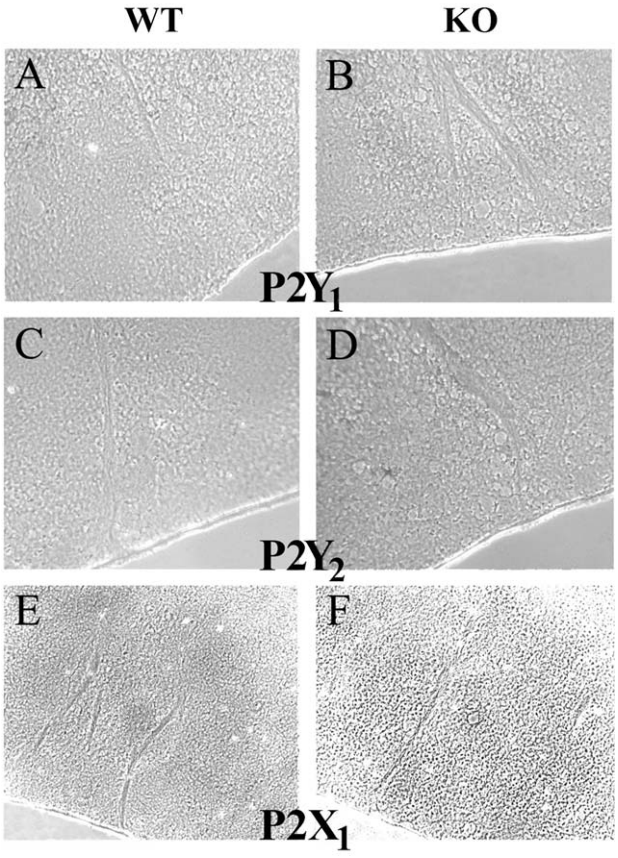


**Fig. 4.** Pre-absorption controls for $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors on the mouse spleen capsule and trabeculae. (A, C and E) WT mice and (B, D and F) $P2X_2$/$P2X_3$ KO mice. (A, B) $P2Y_1$ immunostaining; (C, D) $P2Y_2$ immunostaining; and (E, F) $P2X_1$ immunostaining. All figures 200 × . Note that there is some non-specific background staining for the $P2X_1$ antibody in panels E and F.

breaks down to adenosine due to the action of ecto-ATPases. The isolated spleen of the *rat* contracts in the presence of adenosine and several adenosine receptor agonists; the effect of selective agonists and antagonists identified the receptor as being of the $A_1$ subtype of P1 receptors (Fozard and Milavec-Krizman, 1993). The spleen capsule of the *mouse*, however, from both the WT and KO animals failed to contract to adenosine and thus a contractile adenosine receptor is discounted.

In order to characterise the P2 receptor subtype mediating the contractile response of ATP, more selective P2 agonists were used. The contraction of the spleen capsule induced by the purine and pyrimidine compounds suggests the presence of several subtypes of P2 receptors. The strong activity of 2-MeSADP and UTP suggests the presence of $P2Y_1$ and $P2Y_2$ receptors, respectively, and the presence of both of these receptors was confirmed by specific immunostaining. There was no difference in the contractions induced by 2-MeSADP and UTP or in the expression of $P2Y_1$ and $P2Y_2$ receptor following immunostaining between the WT and $P2X_2$/$P2X_3$ KO animals. The activity of $\alpha,\beta$-meATP suggests the presence of either $P2X_1$ or $P2X_3$ receptors. However, the continued activity of $\alpha,\beta$-meATP on the splenic capsule of mice lacking $P2X_3$ receptors would indicate that the receptor is of the $P2X_1$ subtype and this is further supported by the presence of immunoreactivity for $P2X_1$ receptors. Again, there was no difference in the response to $\alpha,\beta$-meATP between the WT and KO animals; thus, there was no compensatory change in $P2Y_1$, $P2Y_2$ or $P2X_1$ receptor activity of the spleen capsule as a result of the lack of $P2X_2$ or $P2X_3$ receptors.

In addition to positive staining for $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors on the spleen capsule, there was evidence for strong positive immunoreactivity to $P2Y_1$ and $P2X_1$ receptors and weak $P2Y_2$ immunoreactivity on cells within the body of the spleen. This has been reported previously. $P2Y_1$ and $P2X_1$ receptors have been identified which modulate interleukin-6 (IL-6) secretion, $P2X_1$ receptors mediate inhibition and $P2Y_1$ receptors mediate stimulation of IL-6 secretion (Straub et al., 2002). The presence of both $P2Y_1$ and $P2Y_2$ receptors has been reported for T-lymphocytes, whereas $P2Y_1$

receptors have been identified on B-lymphocytes and macrophages (Burnstock, 2001).

In the pig and dog, NA, ATP and neuropeptide Y were proposed as sympathetic cotransmitters, controlling spleen vascular tone and capsule contraction (Corder et al., 1987; Felten et al., 1985; Lundberg et al., 1989). It has been suggested that the central nervous system influences the responses of the immune system via the local interaction between nerves and immune cells in lymphoid organs (Straub et al., 1998) and the functional interplay of these nerves and immune cells in the spleen has been described in several reports (Elenkov et al., 2000; Madden, 2001; Straub et al., 2002).

In conclusion, this study has shown that the spleens from the $P2X_2/P2X_3$ KO animals are significantly enlarged with more immune cells than the WT's. The enlarged spleens may be the result of a compensatory change for a compromised immune system (hypocellularity of the thymus and bone marrow) in the double KO's and subsequent chronic infection. The presence of multiple P2 receptor subtypes mediating contraction of the spleen capsule has been demonstrated, although the activity of these receptors did not alter as a consequence of the loss of $P2X_2$ and $P2X_3$ receptors.

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