

## Changes in expression of P2X<sub>7</sub> receptors in NOD mouse pancreas during the development of diabetes

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(Submitted: 5 April 2006; accepted: 2 November 2006)

### Abstract

This study examined the expression of P2X<sub>7</sub> receptors in pancreatic islets of the non-obese diabetic (NOD) mouse model of human autoimmune insulin-dependent diabetes mellitus, to determine whether they are involved in islet cell destruction during early- and late-developing diabetes. Pancreatic cells containing glucagon ( $\alpha$ -cells), insulin ( $\beta$ -cells) and somatostatin ( $\delta$ -cells) were co-localized with P2X<sub>7</sub> receptors. We examined P2X<sub>7</sub> receptor expression in normal and diabetic spleens using flow cytometry. In non-diabetic NOD controls, P2X<sub>7</sub> receptors were expressed in glucagon-containing cells at the periphery of islets, being consistent with previous studies. In early NOD diabetes (12 weeks), there was migration of peripheral P2X<sub>7</sub> receptor positive, glucagon-containing cells into the center of islets. In late NOD diabetes (34 weeks), P2X<sub>7</sub> receptor- and glucagon-stained  $\alpha$ -cells were gone from islets. Migration of macrophages and dendritic cells into islets took place, but they lacked P2X<sub>7</sub> immunoreactivity. There was no significant difference in the percentage of splenic macrophages stained for P2X<sub>7</sub> receptors from control and diabetic spleens. In conclusion, in the development of early to late diabetes, there is a down-regulation of P2X<sub>7</sub> receptors on islet cells and a loss of  $\alpha$ - and  $\beta$ -cell populations. P2X<sub>7</sub> receptor signalling might be involved in  $\alpha$ -cell clearance from late diabetic islets.

**Keywords:** Diabetes, NOD, P2X<sub>7</sub> receptors, islet cells

### Introduction

Non-obese diabetic (NOD) mice, which spontaneously develop diabetes, have many features characteristic of human autoimmune insulin-dependent diabetes mellitus [1]. It is regarded as a good model for studying the pathogenesis of this autoimmune disease [2]. The disease in NOD mice is characterized by a very long pre-clinical period during which there is leukocyte infiltration in and around the islets, macrophages and dendritic cells being the first mononuclear cells to be detected inside the islets [3–5].

The P2X<sub>7</sub> receptor is a ligand-gated ion channel that is activated by extracellular ATP [6]. It is

expressed on many different cell types such as exfoliated epithelial cells [7,8], tumour cells [9], macrophages and dendritic cells [10–12]. The role of P2X<sub>7</sub> receptors is not completely understood but is associated with multinucleated cell formation on macrophages [13], elimination of intracellular parasites, such as mycobacteria [14,15] and Chlamydia [16,17] and release of mature interleukin-1 $\beta$  (IL-1 $\beta$ ) from macrophages and microglial cells [18,19]. The P2X<sub>7</sub> receptor also has the characteristic of mediating apoptosis in various cell types [11,16,20,21].

It has been proposed that damage to pancreatic islets in diabetes may involve IL-1 $\beta$  secretion [22,23] and apoptosis [23–27].

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In the present study, we investigated whether P2X<sub>7</sub> receptors were involved in cell destruction within islets from the NOD model of diabetes. The expression of P2X<sub>7</sub> receptors in diabetic islets and pancreas of early- and late-developed diabetes in NOD mice was investigated by immunohistochemistry. In addition, using flow cytometry analysis, we also examined the expression of P2X<sub>7</sub> receptors in spleens from normal and diabetic animals.

## Materials and methods

### *Animals*

The St Bartholomew's Hospital NOD colony (NOD/Ba) was established in 1987. There is a stable cumulative diabetes incidence of 55% in females and 15% in males [28]. Breeding, maintenance and killing of the animals used in this study followed the 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. Pancreatic and spleen tissue was taken from three groups of female NOD mice (5–7 week old pre-diabetic; 12, 14 and 21 week old diabetic and 29–34 week old diabetic), and two groups of males NOD mice (19 and 28 week old pre-diabetic, and 29 week old diabetic) and from 8 to 10 week old mature adult male Balb/c mice. The animals were kept in a constant 12/12 h light–dark cycle with free access to food and water. The diabetic animals were kept for two weeks as diabetics before culling, enabling the confirmation of disease but before the disease became too severe. The diabetes was confirmed by both a urinary glucose of more than 2% and a blood glucose of over 11 mmol.

### *Immunohistochemistry*

*Tissue handling.* The pancreas and spleen tissues were removed, put in Hanks balanced salt solution (HBSS), embedded in OCT tissue compound (VWR International, Poole, UK), progressively frozen in isopentane (pre-cooled in liquid nitrogen) and then stored in liquid nitrogen for later use. Cryostat sections were cut as sets of serial section 10  $\mu$ M thick. The sections were thaw-mounted on gelatine-coat slides. The slides were stored at  $-20^{\circ}\text{C}$  until use. Tissues were post-fixed for 2 min at room temperature in 4% formaldehyde (VWR) and 0.03% picric acid in phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by incubation for 10 min (pancreas) and 25 min (spleen) with 0.3% H<sub>2</sub>O<sub>2</sub> prepared in 50% methanol. Blocking of non-specific binding sites was achieved by preincubation with normal horse serum (NHS; Harlan Sera-Lab, UK) in

PBS containing 0.05% Merthiolate (Sigma Chemical Co., Poole, UK) at room temperature for 20 min. The establishment of insulinitis was evaluated by staining the samples with haematoxylin–eosin for histological examinations.

*Immunostaining.* An indirect immunohistochemical and immunofluorescent method with two layers of antibodies was used. Antibodies for P2X<sub>7</sub> receptors from rabbit were allowed to react with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and detected with avidin-coupled horseradish-peroxidase/nickel-intensified 3,3'-diaminobenzidine (DAB) or with either Oregon green or avidin-coupled Texas Red (Sigma). The P2X<sub>7</sub> antibodies were obtained from Roche Bioscience (Palo Alto, CA, USA). P2X<sub>7</sub> receptor antibodies were raised in rabbits against a specific 15 amino acid residue at the carboxy-terminus of the P2X<sub>7</sub> receptor molecule [29]. The sections were incubated overnight with the primary antibodies diluted to 5 and 2.5  $\mu\text{g/ml}$  with 10% NHS in PBS containing 0.05% Merthiolate. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson) diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 30 min, followed by incubation with ExtrAvidin-horseradish peroxidase (Sigma) diluted 1:1000 in PBS containing 0.05% Merthiolate for 30 min. All incubations were carried out at room temperature and separated by three 5 min washes in PBS. Finally, a freshly prepared colour reaction mixture containing 0.5% DAB, 0.1 M sodium phosphate, 0.004% NH<sub>4</sub>Cl, 0.2% glucose, 0.04% nickel ammonium sulphate and 0.1% glucose oxidase was applied to the sections for 5–10 min. Sections were then washed, dehydrated, cleared in xylene and mounted using Eukitt (VWR). Control experiments were performed using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and thus confirm a specific immunoreaction. In a series of experiments where a fluorescent marker was used, sections were incubated for 1 h with either streptavidin-conjugated Oregon green or Texas Red (Sigma) both at a concentration of 1:100. These experiments were performed using a modified version of the protocol of Ref. [30] (omitting 0.02% of a saturated solution of picric acid, inactivation of endogenous peroxidase, and the Ni-DAB reaction steps).

For anti-insulin, anti-glucagon and anti-somatostatin staining, a modified version of the protocol for P2X receptors was used. The primary antibodies used were guinea pig anti-insulin (Inestar Stillwater, Minn., USA) at a concentration of 1:1000 and 1:2000 and goat anti-glucagon and goat anti-somatostatin (Santa Cruz Biotechnology, CA, USA) both at a concentration of 1:200. In anti-insulin experiments, normal

goat serum (NGS) was used to block non-specific binding, instead of NHS. A goat anti-guinea pig biotinylated secondary antibody (Sigma) was diluted in 1% NGS and applied to slides for 30 min, followed by incubation with streptavidin-FITC for 45 min. Donkey anti-goat-FITC, at a concentration of 1:100, was used as a secondary antibody for anti-glucagon and anti-somatostatin staining. The microscopes used were a Zeiss Axioplan, (Zeiss, Oberkochen, Germany), and an Edge True-View 3D fluorescence microscope (Edge Scientific Instruments, Santa Monica, CA, USA). Images were prepared with Zeiss Axioplan microscope coupled with a Leica DC200 image acquisition system (Leica, Heerbrugg, Switzerland). The figures were prepared using the Adobe Photoshop 5.0 program. Prints were made with an Epson Stylus Photo 700 printer.

**Haematoxylin and eosin staining.** Haematoxylin and eosin (H + E) stain was used on some frozen sections. Sections were stained with Meyer's haematoxylin (Sigma) for 5 min, washed in tap water and stained in 1% eosin (Sigma) for 5 min. Sections were then dehydrated in ethanol, cleared in xylene and mounted using Eukitt.

#### *Preparation of splenocytes*

The spleens were collected from Balb/c and diabetic NOD mice. Splenocytes were gently removed by mechanical dissociation and re-suspended in HBSS. The erythrocytes were removed and the mononuclear cells enriched by centrifugation on a Ficoll density gradient Histopaque 1083 (Sigma). Cells viability was over 95% in all cases.

#### *Flow cytometry assays*

The splenocytes were adjusted to  $10^6$  cells/tube, washed twice with PBS and incubated with PBS containing 5% NGS, on ice, for 20 min. Cells were washed once in PBS and then incubated with FITC-conjugated rat anti-mouse CD3 (1:100; Serotec, UK), FITC-conjugated rat anti-mouse B220 (1:100; Serotec) or FITC-conjugated rat anti-mouse F4/80 ( $10 \mu\text{l}$  neat antibody/ $10^6$  cells; Serotec) for 30 min. The samples were washed and fixed in 4% paraformaldehyde (Sigma) for 10 min on ice and then washed in cold PBS. The rabbit anti-rat P2X<sub>7</sub> antibody was diluted in PBS containing 0.1% saponin and applied to the samples at a concentration of  $1 \mu\text{g}/10^6$  cells, overnight at 4°C. The samples were then washed three times in PBS and incubated with phycoerythrin (PE)-goat anti-rabbit monoclonal antibody (Caltag Lab. Burlingame, CA, USA) at a concentration of  $1 \mu\text{g}/10^6$  cells for 30 min. The cells were then washed three

times in PBS, re-suspended in PBS and analysed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA). Negative control experiments were performed by preabsorbing the P2X<sub>7</sub> antibody with its homologue peptide antigen and/or by omission of the primary P2X<sub>7</sub> antibody. Cells were initially gated by forward and side scatter and then by cell type-specific antibodies for macrophages (F4/80), B lymphocytes (B220) or T lymphocytes (CD3<sup>+</sup>). The data were post analysed using the program WinMDI (Multiple Document Interface Flow Cytometry Application, version 2.8).

## Results

### *The expression of P2X<sub>7</sub> receptors on the pancreas of NOD and Balb/c mice*

The expression of P2X<sub>7</sub> receptors of young (5–7 week) pre-diabetic animals was found to be located preferentially on the islets of Langerhans and staining formed an annular pattern (Figure 1(a)). A similar pattern of P2X<sub>7</sub> receptor staining was observed in islet from adult (28 week) non-diabetic animals (Figure 1(b)). In early-developed (12 week) diabetic animals, there was clear evidence for the infiltration of mononuclear cells in the islets of Langerhans (Figure 1(c)). There was also positive staining for P2X<sub>7</sub> receptors over the islets (Figure 1(d)). A similar expression pattern of staining for P2X<sub>7</sub> receptors was observed from 17 weeks diabetic animals and to a lesser extent from 21 week diabetic animals. The islet of Langerhans of late-developed diabetic animals (34 week) clearly demonstrated an infiltration of immune cells (Figure 1(e)), but there were no evidence for the expression of P2X<sub>7</sub> receptors (Figure 1(f)). The same pattern of immune cell infiltration and the absence of P2X<sub>7</sub> receptor positive cells was observed in islets of two of the 29 week old diabetic animals. The annular pattern of P2X<sub>7</sub> receptor staining was found in islets from the pancreas of adult Balb/c mice.

We then investigated which of the pancreatic cell types were positive for P2X<sub>7</sub> receptors. We performed double labelling for  $\alpha$ -cells,  $\beta$ -cells and  $\delta$ -cells with P2X<sub>7</sub> receptors from pre-diabetic mice (five week old). The insulin-positive cells ( $\beta$ -cells) made up the majority of the islet of Langerhans cells, but only a few  $\beta$ -cells were found to be positively stained for P2X<sub>7</sub> receptors (Figure 2(a)–(c)). The somatostatin-positive cells ( $\delta$ -cells) were found mainly around the central  $\beta$ -cells, but the majority of  $\delta$ -cells was negatively stained for P2X<sub>7</sub> receptors (Figure 2(d)–(f)). The glucagon-positive ( $\alpha$ -cells) cells were also found forming a ring around the islet and all of the  $\alpha$ -cells colocalized with P2X<sub>7</sub> receptor-positive cells (Figure 2(g)–(i)).

Double-labelling experiments on early 12 week old diabetic animals showed the presence of islet cells

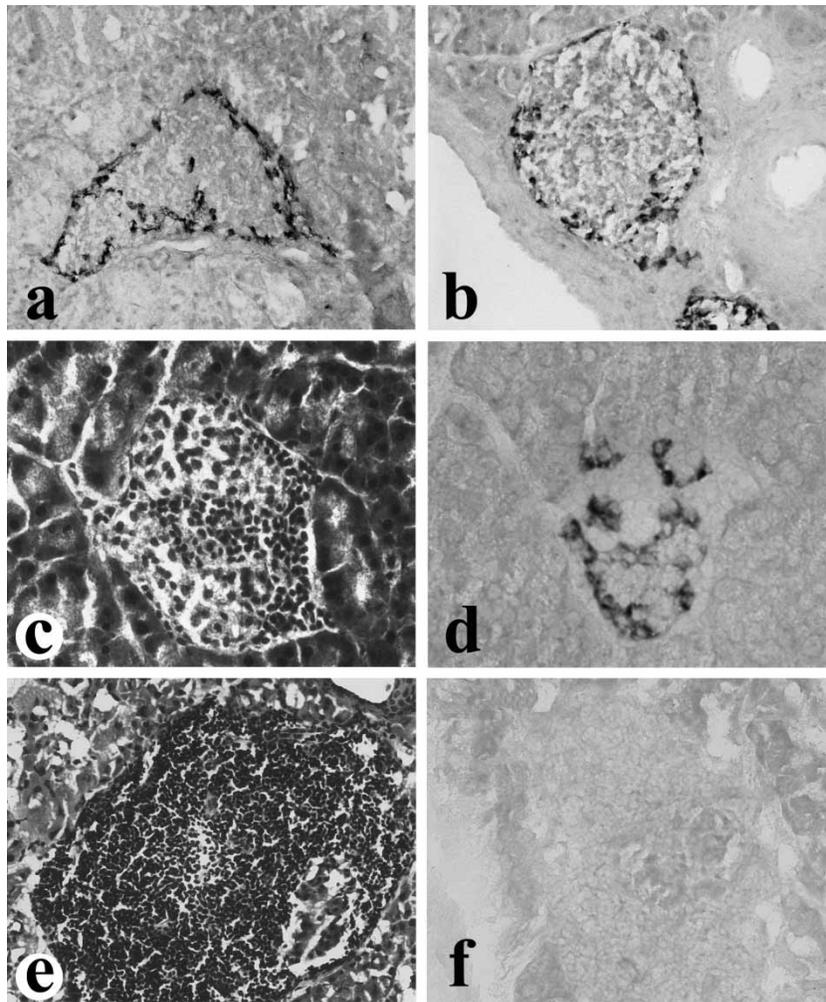


Figure 1. Expression of P2X<sub>7</sub> receptors in pancreatic islets of diabetic NOD mice. Serial cryostat sections showing (a) P2X<sub>7</sub> receptor staining in five week old and (b) 28 week old non-diabetic NOD mice. Note the disposition of P2X<sub>7</sub> receptor-positive cells in an annular formation around the islet. (c) Haematoxylin and eosin (H + E) and (d) P2X<sub>7</sub> immunostaining of early 12 week NOD diabetic mice. Note a clear cellular infiltration in (c) and a more diffuse immunostaining for P2X<sub>7</sub> receptors in (d). (e) H + E staining of late 34 week NOD diabetic mice. Note a strong (cellular) infiltration of macrophages and dendritic cells into the islet, (f) P2X<sub>7</sub> immunostaining with no reactivity for P2X<sub>7</sub> receptors. (a), (b), (e) and (f)  $\times 200$ ; (c), (d)  $\times 400$ .

positively stained cells for P2X<sub>7</sub> receptors (Figure 3(a)) and cells positively stained for glucagon ( $\alpha$ -cells) (Figure 3(b)). The majority of the glucagon staining was in core cells. Double labelling experiments on late diabetic animals showed the absence of cells positively stained for P2X<sub>7</sub> receptors (Figure 3(d) and (f)) and for glucagon (Figure 3(e)). Somatostatin-positive cells were found on the core of a few islet cells but there was no colocalization with P2X<sub>7</sub> receptors (Figure 3(g)). Based on the double labelling with P2X<sub>7</sub> receptors, the islet cells exhibiting the greatest expression of P2X<sub>7</sub> receptors were characterised as  $\alpha$ -cell in pre-diabetic and early diabetic NOD and Balb/c mice.

#### *The expression of P2X<sub>7</sub> receptors in the spleen*

The migration of macrophages and dendritic cells to pancreatic islets has been associated with the initiation of insulinitis and  $\beta$ -cell destruction [3,5,31]. In

addition, it has been suggested that  $\beta$ -cell antigens are transported by presentation to pancreatic lymph nodes [32]. We therefore examined the expression of P2X<sub>7</sub> receptors on macrophages from spleens from 14, 17, 22 and 29 week old diabetic animals and Balb/c animals, using flow cytometry analysis. (Figure 4(a) and (b)) illustrates the flow cytometry histograms of P2X<sub>7</sub> receptor expression in macrophages from spleens of Balb/c mice (Figure 4(a)) and early-developed diabetic NOD mice (Figure 4(b)). We found that  $80 \pm 7\%$  ( $n = 4$ ) and  $78 \pm 6\%$  ( $n = 3$ ) of F4/80 positive cells (spleen macrophages) expressed P2X<sub>7</sub> receptors from early-developed diabetic NOD and Balb/c mice, respectively. We performed immunohistochemical experiments on spleens from Balb/c, five week pre-diabetic and 34 week late diabetic NOD mice. We found positive immunostaining for P2X<sub>7</sub> receptors on cells from spleens from five week old pre-diabetic (Figure 4c) and adult Balb/c mice. The cells

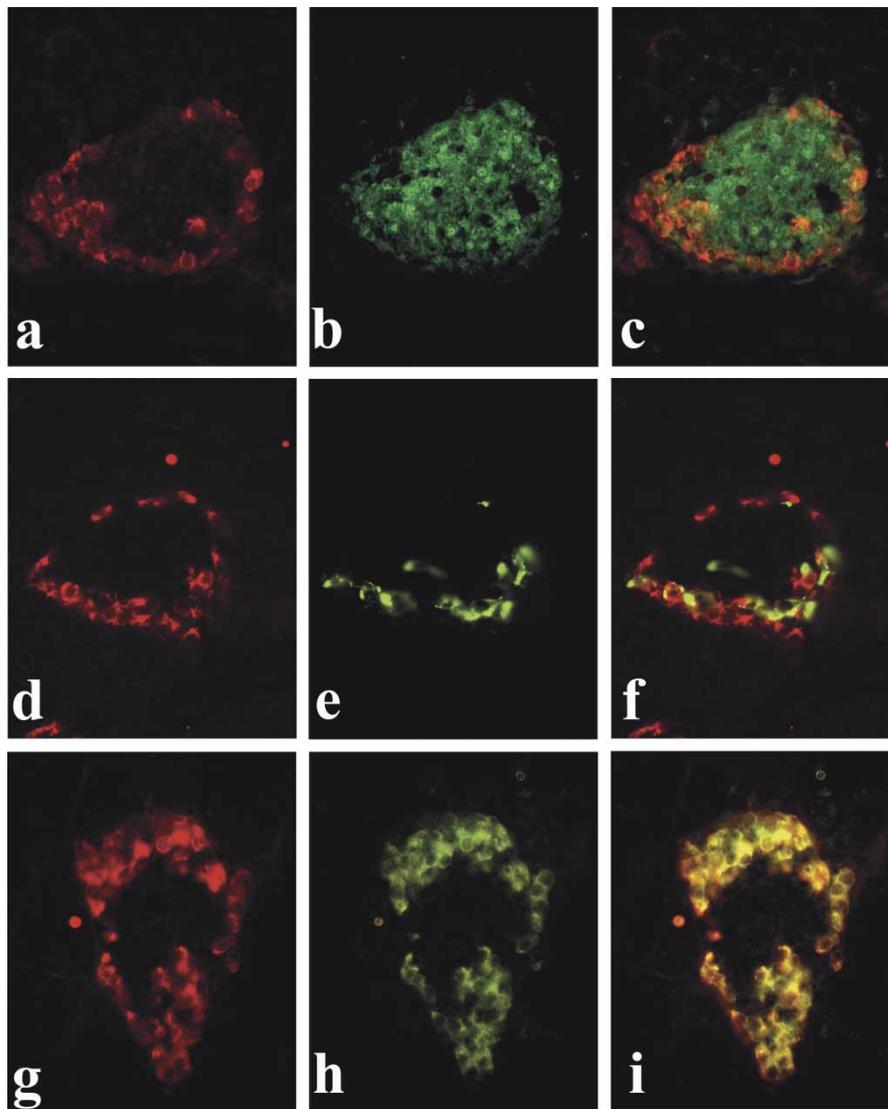


Figure 2. Islet of Langerhans double-labelled for P2X<sub>7</sub> receptors (a, d and g) and insulin (b) and (c), somatostatin (e) and (f) or glucagon (h) and (i) in five week old NOD diabetic mice. (a). P2X<sub>7</sub> receptor immunostaining (red) of islet cells. (b). Insulin immunostaining (green) showing  $\beta$ -cells. (c). Co-localization (yellow–orange) of P2X<sub>7</sub> receptors and insulin. Note very sparse co-localization. (d). P2X<sub>7</sub> receptor immunostaining (red). (e). Somatostatin immunostaining (green) showing  $\delta$ -cells. (f). Co-localization of P2X<sub>7</sub> receptors and somatostatin. Note that there are no double-labelled cells. (g). P2X<sub>7</sub> receptor immunostaining (red). (h). Glucagon immunostaining (green) showing  $\alpha$ -cells. (i). Co-localization (yellow–orange) of P2X<sub>7</sub> receptors and glucagon. Note that most of the glucagon-positive cells co-express P2X<sub>7</sub> receptors. (a)–(i)  $\times$  400.

positively stained for P2X<sub>7</sub> receptors were found on follicles and the germinal center (GC) of white pulp of the spleen, some of which presented dendritic morphological characteristics of macrophages and dendritic cells (Figure 4(c) insert). The cells labelled strongly in the marginal zone and within the light zone of the GC. Blood vessel in the GC strongly stained with the P2X<sub>7</sub> receptors antibody. We observed a similar pattern of P2X<sub>7</sub> receptor staining from spleen from 12 to 34 week old diabetic NOD animals (Figure 4(d)).

## Discussion

In this study, pancreatic islet cells from NOD mice show a change in expression of P2X<sub>7</sub> receptors and

glucagon-expressing ( $\alpha$ -cells) cells during development from early to late diabetes. In pre-diabetic mice, we found that  $\alpha$ -cells, showing positive staining for P2X<sub>7</sub> receptors, were distributed around the pancreatic islets forming a ring, consistent with that described in normal pancreas of adult rat and mice [33]. However, in young diabetic animals we observed that  $\alpha$ -cells had migrated to the core of the islets. Such a pattern of reorganization of  $\alpha$ -cell distribution is well documented for diabetic NOD mice [34] and with streptozotocin-induced diabetic rats [35].

The expression of P2X<sub>7</sub> receptors differed between the diabetic and non-diabetic animals. In the pre-diabetic animals, P2X<sub>7</sub> receptors were expressed in an annular pattern around the

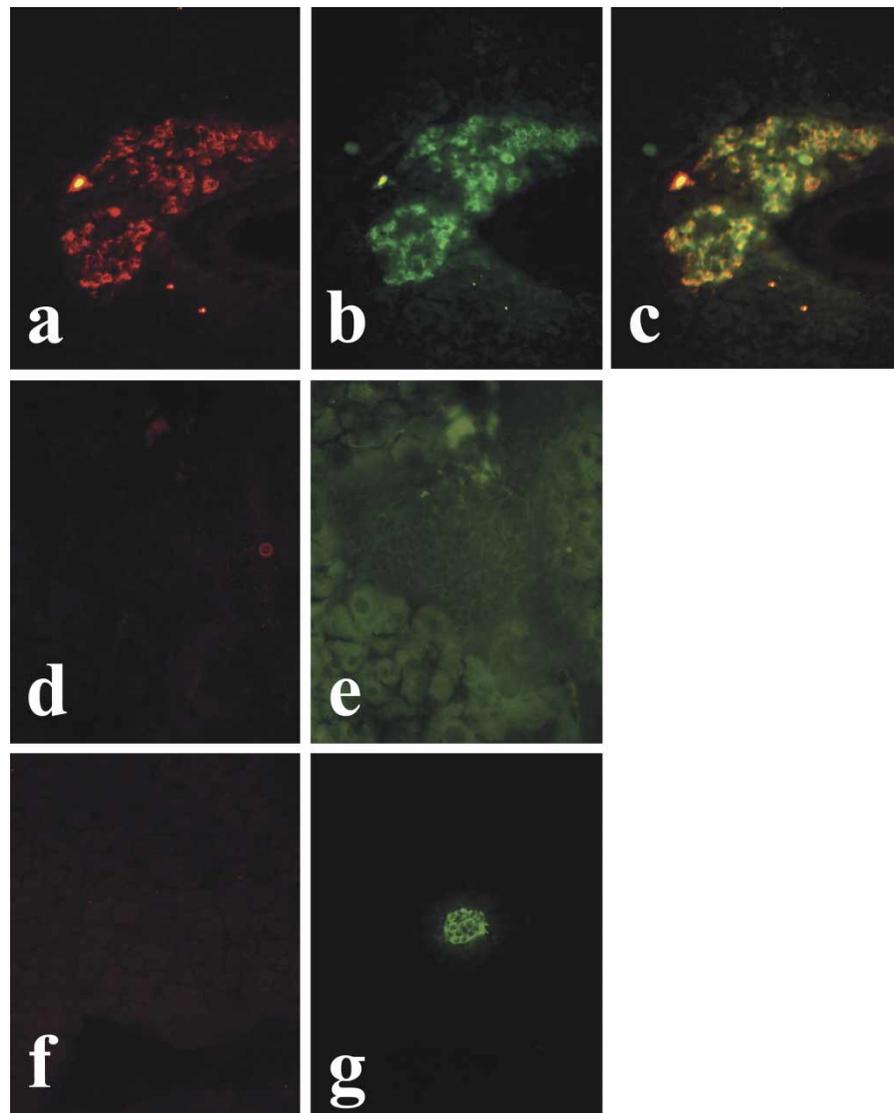


Figure 3. P2X<sub>7</sub> receptor expression in early (12 week) and late (34 week) NOD diabetic mice. (a–c) Immunostaining for P2X<sub>7</sub> receptors and glucagon in pancreas from 12 week diabetic mice. (a) P2X<sub>7</sub> receptor staining (red), (b) glucagon staining (green), (c) strong co-localization (yellow–orange) of P2X<sub>7</sub> receptors and glucagon. (d–e) Immunostaining for P2X<sub>7</sub> receptors and glucagon in pancreas from 34 week diabetic mice. Note that there is no immunoreactivity for P2X<sub>7</sub> receptors (d), or for glucagon (e). (f–g) Immunostaining for P2X<sub>7</sub> receptors and somatostatin in pancreas from 34 week diabetic mice. Note that there is no immunoreactivity for P2X<sub>7</sub> receptors (f) but there is immunostaining for somatostatin (green) on pancreatic islets (g). (a–g)  $\times 200$ .

pancreatic islet, whereas in the early-diabetic animals, the expression appeared throughout the islet. In contrast, in the late-developed diabetes, there was a lack of expression of P2X<sub>7</sub> receptors. An effect of age on diabetes has been suggested for streptozotocin-induced diabetes of CD-1 mice [36] and for the streptozotocin model of diabetes in rats [37]. In the rat model, it was shown that streptozotocin treatment of newborn rat pups induced cell death of both  $\alpha$  and  $\beta$ -cells and during the first three weeks of the treatment, the number of  $\alpha$  and  $\beta$ -cells decreased to the same extent. One explanation for the findings is that animals with a reduced number of  $\alpha$ -cells in islets of Langerhans may resist to a greater extent  $\beta$ -cells destruction, thus developing the disease only later.

The role of pro-inflammatory cytokines in intra-islet cell elimination is well known [25]. It was shown that the combination of IL-1 $\beta$  plus interferon (IFN)- $\gamma$  induced apoptosis in both rat  $\alpha$ -cells and  $\beta$ -cells [26]. It is possible that the pattern of intra-islet cytokine release from late-developed diabetic mice is different from that of early-developed diabetes. This appears to be the case for the time course of cytokine-secreting macrophages that accumulate between and surrounding the islet that occurs with aging and disease progression in the NOD mice model of diabetes [3]. Indeed, it has been established that macrophages are an absolute requirement for the development of type I diabetes in animal models [3,5,38].

It is well known that macrophages and dendritic cells strongly express P2X<sub>7</sub> receptors [11,39–41]. An

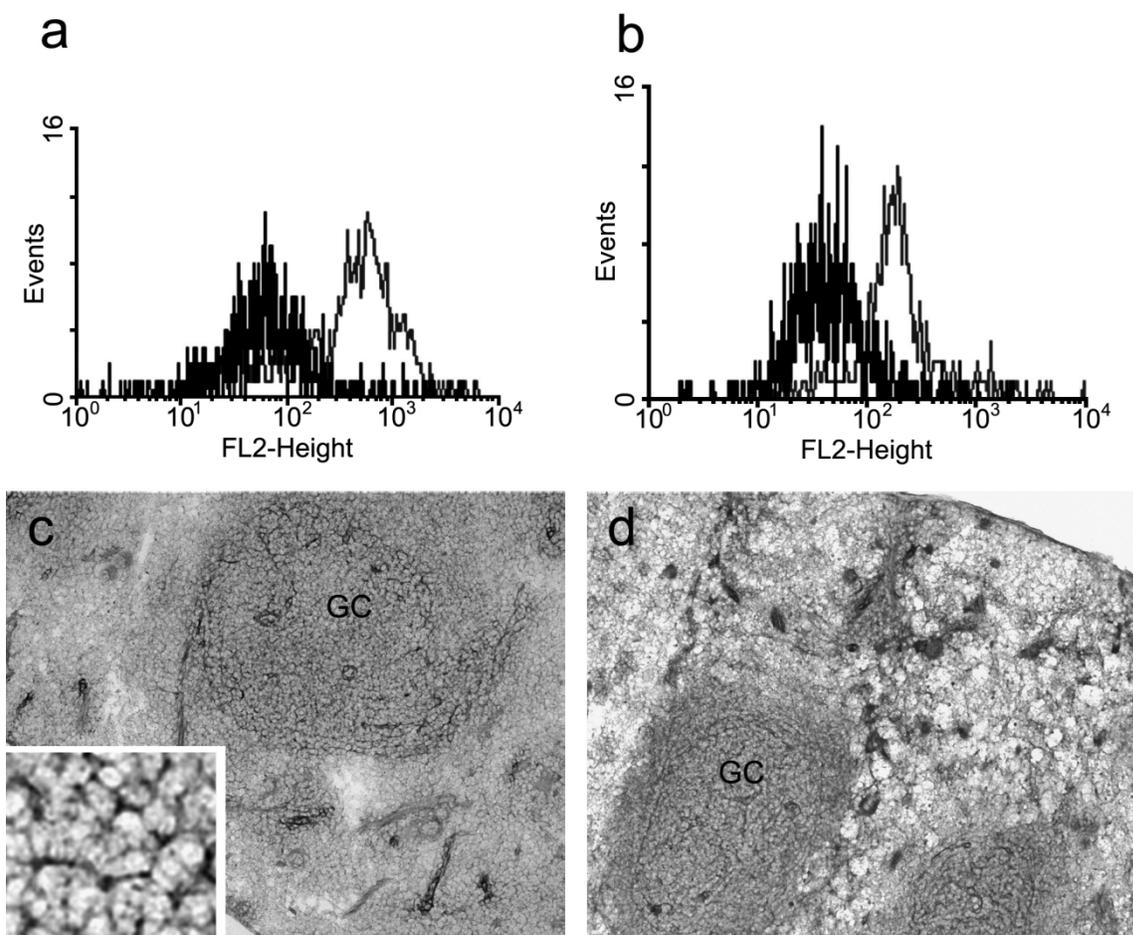


Figure 4. Detection of P2X<sub>7</sub> receptors by flow cytometry and immunohistochemistry of spleen cells. (a–b) Fluorescence histograms of P2X<sub>7</sub> receptor expression on F4/80-positive cells (macrophages) of spleen mononuclear cells from adult Balb/c mice (a) and 12 week old NOD diabetic mice (b). Cells were initially gated by forward and side scatter and then by cell type-specific antibodies for macrophages (F4/80), T-lymphocytes (CD3<sup>+</sup>) and B-lymphocytes (B220<sup>+</sup>). (a) and (b) are representative traces of spleen macrophages from three Balb/c animals in (a) and four early diabetic NOD mice in (b). The thin traces represent P2X<sub>7</sub> expression and dark traces the negative peptide control. Note that for both animals the majority of cells are stained. (c–d) Expression of P2X<sub>7</sub> receptors in NOD mice spleen by immunohistochemistry. (c) P2X<sub>7</sub> receptor expression on spleen from five week old non-diabetic animal. (d) P2X<sub>7</sub> receptor expression on spleen from 34 week old diabetic animal. Note the clear immunostaining for P2X<sub>7</sub> receptors on GC of white zone of spleen in both animals. Insert in (c) shows a higher magnification of positive cells in the GC detailing the dendritic morphology. (c) and (d) × 200.

unexpected finding from the immunostaining data was that, in addition to there being clear insulinitis with mononuclear cell infiltration from late diabetic animals, there were no P2X<sub>7</sub> receptor-positively stained cells on infiltrating mononuclear cells. In contrast, our results from the immunostaining and flow cytometry studies have shown that spleen macrophages from Balb/c, five week old pre-diabetic and early 12 week old diabetic and late 34 week old diabetic mice expressed P2X<sub>7</sub> receptors. A down-regulation of P2X<sub>7</sub> receptors on macrophages has been recently described for the pancreas from streptozotocin-diabetic rats [35]. In both NOD and streptozotocin diabetic models, the down-regulation of P2X<sub>7</sub> receptors on macrophages could have a functional significance long-term. Sustained activation of P2X<sub>7</sub> receptors can lead to cell death of macrophages and dendritic cells [11,16]. Indeed

necrotic and apoptotic cells are observed in diabetic and IL-1-treated islets [25,26]. ATP can be released by  $\beta$ -cells [42] and modulate insulin release [43,44]. It would therefore be advantageous for macrophage survival if P2X<sub>7</sub> receptor down-regulation occurred during infiltration of sites where extracellular ATP is present. If this is the case, the P2X<sub>7</sub> receptor would not be involved in IL-1 $\beta$  release in this disease model.

Recently, it has been proposed that, compared with normal lymphocytes, NOD lymphocytes are highly sensitive to P2X<sub>7</sub> receptor-stimulated apoptosis and the shedding of CD62L (L-selectin) and MHC class I [45]. It was speculated that defective clearance of neonatal apoptotic pancreatic  $\beta$ -cells by NOD mice might result in sufficient ATP release to stimulate P2X<sub>7</sub> receptors and consequently the secretion of IL-1 $\beta$  from macrophages, CD62L shedding and extravasation of T-cells. It was proposed that subsequent

T-cell-mediated killing of pancreatic  $\beta$ -cells and the consequent release of ATP might then exacerbate the P2X<sub>7</sub>-dependent inflammatory cascade. The findings from the study might also explain the results from this study relating to the differences in the spleen from the early-developed diabetic animals (where only  $\beta$ -cells are absent) from that of the late-developed diabetic animals (where  $\beta$ -cells and P2X<sub>7</sub>-expressing  $\alpha$ -cells are absent). It is known that macrophage phagocytosis is impaired in NOD mice [46]. It is possible that during late-developed diabetes in mice, there are high concentrations of ATP released by dying  $\beta$ -cells that are not removed by macrophages. ATP released by dying cells can be cytotoxic for  $\alpha$ -cells expressing P2X<sub>7</sub> receptors. In order to clarify the role of purinergic receptors on pancreatic islet cells during the development of diabetes, further investigations would need to be carried out.

In conclusion, this study has shown that in early-developed diabetes in the NOD model in mice, expression of P2X<sub>7</sub> receptors changes from an annular expression around the pancreatic islets (as found in pre-diabetic animals) to a more diffuse pattern, but that in late-developed diabetes, P2X<sub>7</sub> receptors are absent. The co-expression of P2X<sub>7</sub> receptors with cells containing glucagon, insulin and somatostatin varies between early- and late-developed diabetes.

### Acknowledgements

Authors wish to thank Dr Doris Rosenthal for help with the Discussion and Dr Gillian E. Knight for editorial assistance with the manuscript. This work was supported by funds from the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and the Wellcome Trust. Dr Coutinho-Silva was a Wellcome Trust fellow, number 062754/Z00Z.

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