

Fig. S1. Differentiation and IL-1 β secretion of monocytes, and analysis of cell-associated TG2 in macrophages.

(A) P2X7R mediated IL-1 β release. Untreated or LPS treated THP-1 cells were ATP stimulated in PSS for 30min as indicated, followed by analysis of cell free supernatants for secreted IL-1 β by capture ELISA (mean \pm s.e.m., nd=not detectable; 2 independent experiments).

(B) Morphological changes confirm cell differentiation. Phase contrast images of THP-1 cells analysed in Fig. 1A are shown. THP-1 cells were differentiated for indicated time with 50ng/ml TPA and stimulated with 100ng/ml LPS as indicated. Bar=50 μ m.

(C) Detection of cell surface-associated TG2. *In vitro* differentiated primary human macrophages were stimulated with BzATP for 10min, followed by biotinylation of cell surface proteins prior to cell extraction. Biotinylated proteins were isolated from extracts on a NeutrAvidin-agarose matrix, and both unbound (cytoplasm) and bound (cell surface) proteins analysed for TG2 by Western blotting alongside the respective cell supernatants from stimulation (medium). The biotin-active ester was omitted from a parallel sample as indicated to corroborate specificity of the cell-surface protein isolation procedure. Note, only free but not cell-associated extracellular TG2 was cleaved, and cation chelation with EDTA during stimulation did not prevent TG2 processing indicating that it is not a metalloproteinase-mediated event.

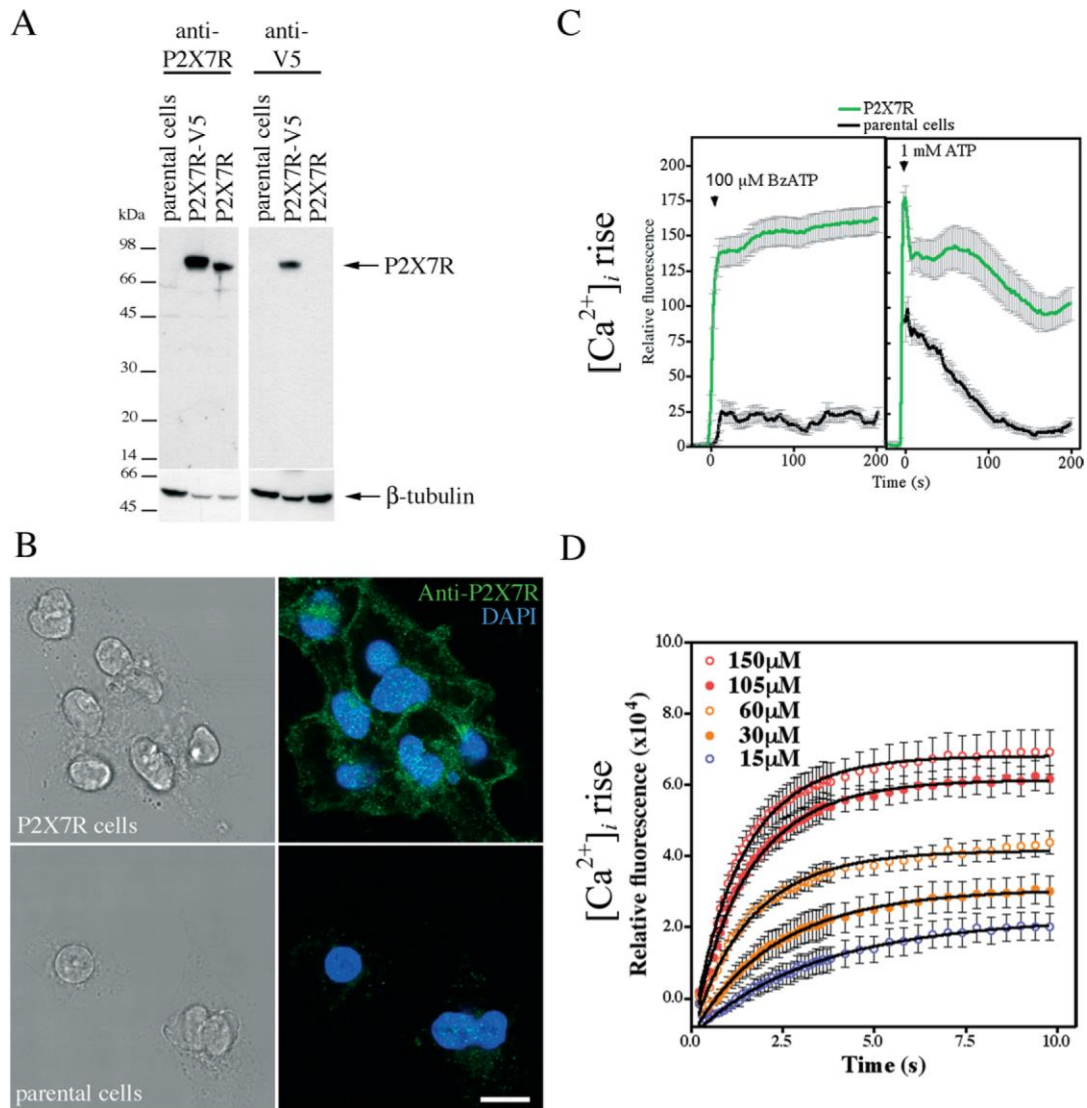


Fig. S2. Characterization of cells expressing P2X7R as a transgene.

(A) Expression of P2X7R in HEK293 cells. Cells stably expressing wild-type or V5-tagged P2X7R were extracted and proteins (25 μg/lane) separated by SDS-PAGE under reducing conditions, followed by Western blotting using antibodies to P2X7R, to V5, or to β-tubulin as a loading control.

(B) Immunolocalization of P2X7R. P2X7R and parental cells were fixed, permeabilized and stained with anti-P2X7R antibodies recognizing the intracellular C-terminal domain as well as DAPI to highlight nuclei. Fluorescence images (right) representing an optical section acquired by confocal microscopy are shown alongside phase contrast images (left). Bar=10 μ m.

(C) Intracellular Ca²⁺ response in P2X7R and parental cells. Cells were loaded with Ca²⁺-indicator Fluo-4-AM in OptiMEM for 20min prior to stimulation with ATP or BzATP as indicated. Fluorescence change in individual cells was monitored by confocal microscopy (λ_{Ex} =488nm, λ_{Em} =500-535nm) and is given as mean \pm s.e.m. (n=12) of a representative experiment. Note, ATP elicits P2Y receptor responses whereas BzATP is P2X receptor specific. Therefore, parental cells are not responsive to BzATP but respond to ATP stimulation with transient oscillations in [Ca²⁺]_i which is likely due to activation of P2Y1R or P2Y2R that are constitutively expressed in these cells (Schachter et al., 1997).

(D) Changes in intracellular free Ca²⁺ concentration in response to different concentrations of agonist in P2X7R cells. Fluo-4-AM loaded cells were stimulated with various concentrations of BzATP ranging from 15-300 μ M. For clarity, only selected concentrations of a representative experiment are shown. Data reflects the mean fluorescence (λ_{Ex} =485-12nm, λ_{Em} =520-10nm) of the cell layer from 8 wells after normalization for well-specific baseline fluorescence and subtraction of data from unstimulated control cells. For analysis, the initial 10s following stimulation were considered and data was fitted using second order kinetics as outlined in Materials and Methods. Note, BzATP concentrations <10 μ M were unable to elicit [Ca²⁺]_i changes.

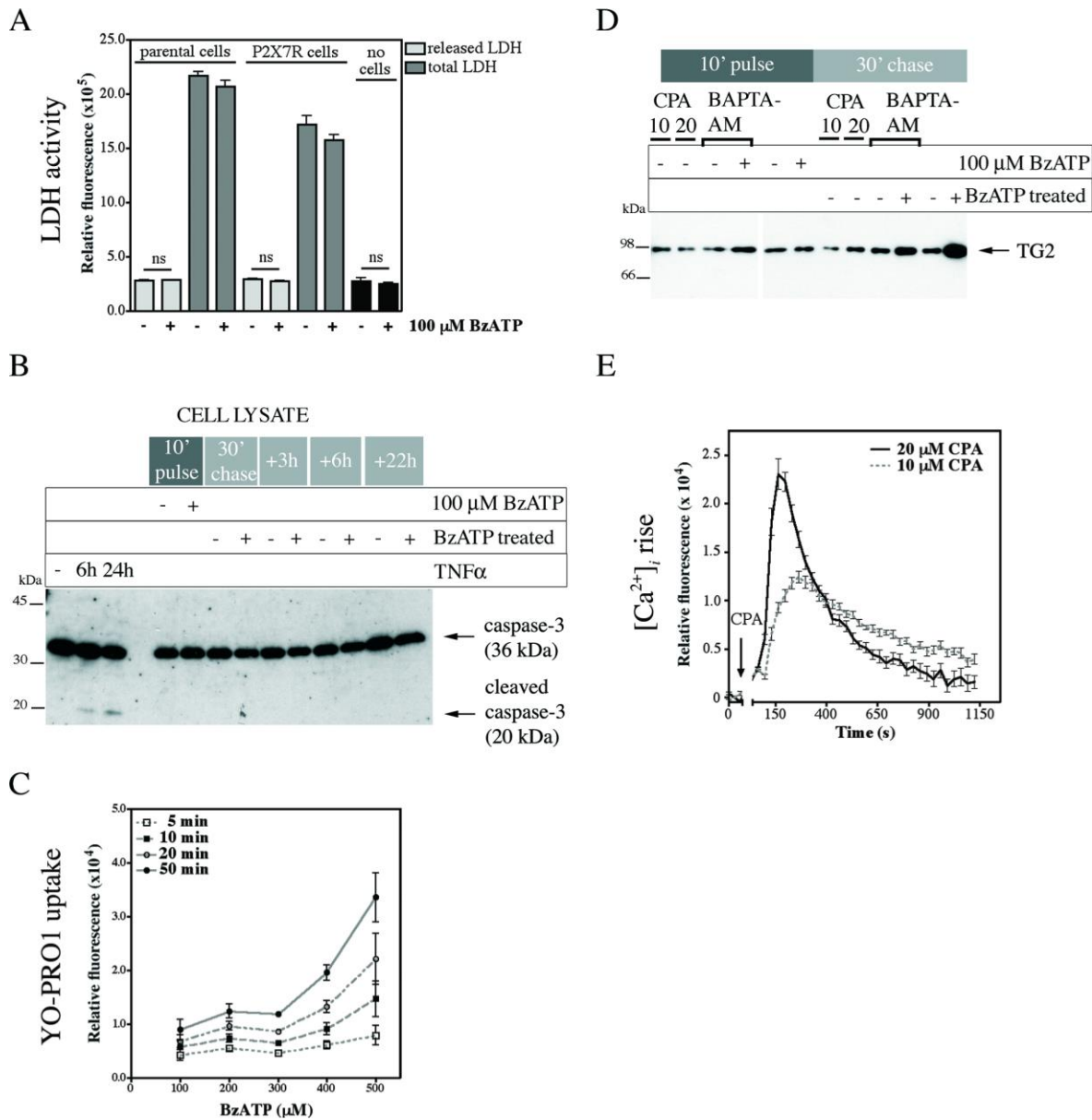


Fig. S3. P2X7R-mediated TG2 release is not due to compromised cell integrity or induction of cell death, and requires events beyond Ca²⁺ signaling.

(A) P2X7R activation is not compromising cell integrity. HEK293 P2X7R or parental cells were stimulated with BzATP for 10min as indicated, and then conditioned media assayed for LDH with (total LDH) or without (released LDH) prior cell lysis by measuring NADH-dependent resorufin fluorescence ($\lambda_{Ex}=540-10nm$, $\lambda_{Em}=590-10nm$) (mean \pm s.e.m., n=3; ns=not significant).

(B) P2X7R activation is not inducing apoptosis. P2X7R cells were BzATP stimulated and chased as before, and subsequently cultured in serum containing medium for up to 22h. Alternatively, apoptosis was induced with 20ng/ml TNF α for indicated time. Cell extracts were analysed by Western blotting for cleaved caspase-3.

(C) P2X7R-dependent “membrane pore” activity is characterized by two states induced at different agonist concentrations. P2X7R cells were stimulated with various concentrations of BzATP in PSS

containing YO-PRO1, and fluorescence changes over time upon continuous agonist stimulation monitored as outlined in Fig. 5C. Results are given as mean fluorescence \pm s.e.m. at different time points, and show that the cell response to different agonist concentrations displays a biphasic profile. This highlights firstly that for BzATP \leq 300 μ M, altering BzATP concentration relative to Ca²⁺ concentration does not alter dye uptake in line with changes in free nucleotide concentration and secondly, that the cellular response $>$ 300 μ M BzATP involves an additional mechanistic component with slower kinetics.

(D) Rise in intracellular Ca²⁺ concentration alone is not sufficient to induce TG2 secretion but is a necessary component. P2X7R cells expressing TG2 were treated with 10 or 20 μ M CPA in Ca²⁺-free medium to release Ca²⁺ from intracellular stores. Alternatively, cells were washed with Ca²⁺-free medium, pre-treated with 10 μ M cell permeable Ca²⁺-chelator BAPTA-AM for 10min where indicated, and then BzATP stimulated for 10min as indicated. Cells were then chased for 30min in BzATP/CPA-free medium. Conditioned media were analyzed by Western blotting for TG2.

(E) Changes in [Ca²⁺]_i mediated by CPA. Change in fluorescence in Fluo-4-AM loaded cells in response to CPA relative to control was monitored (mean \pm s.e.m. of 8 replicate wells).

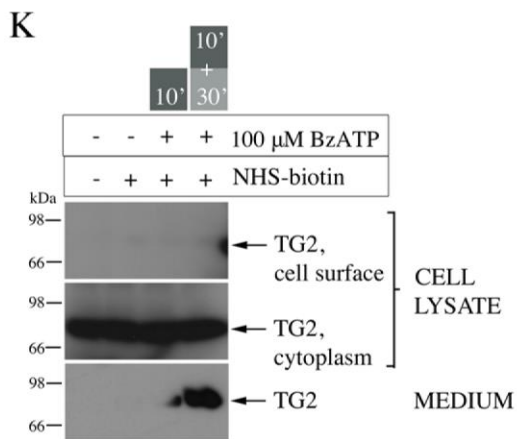
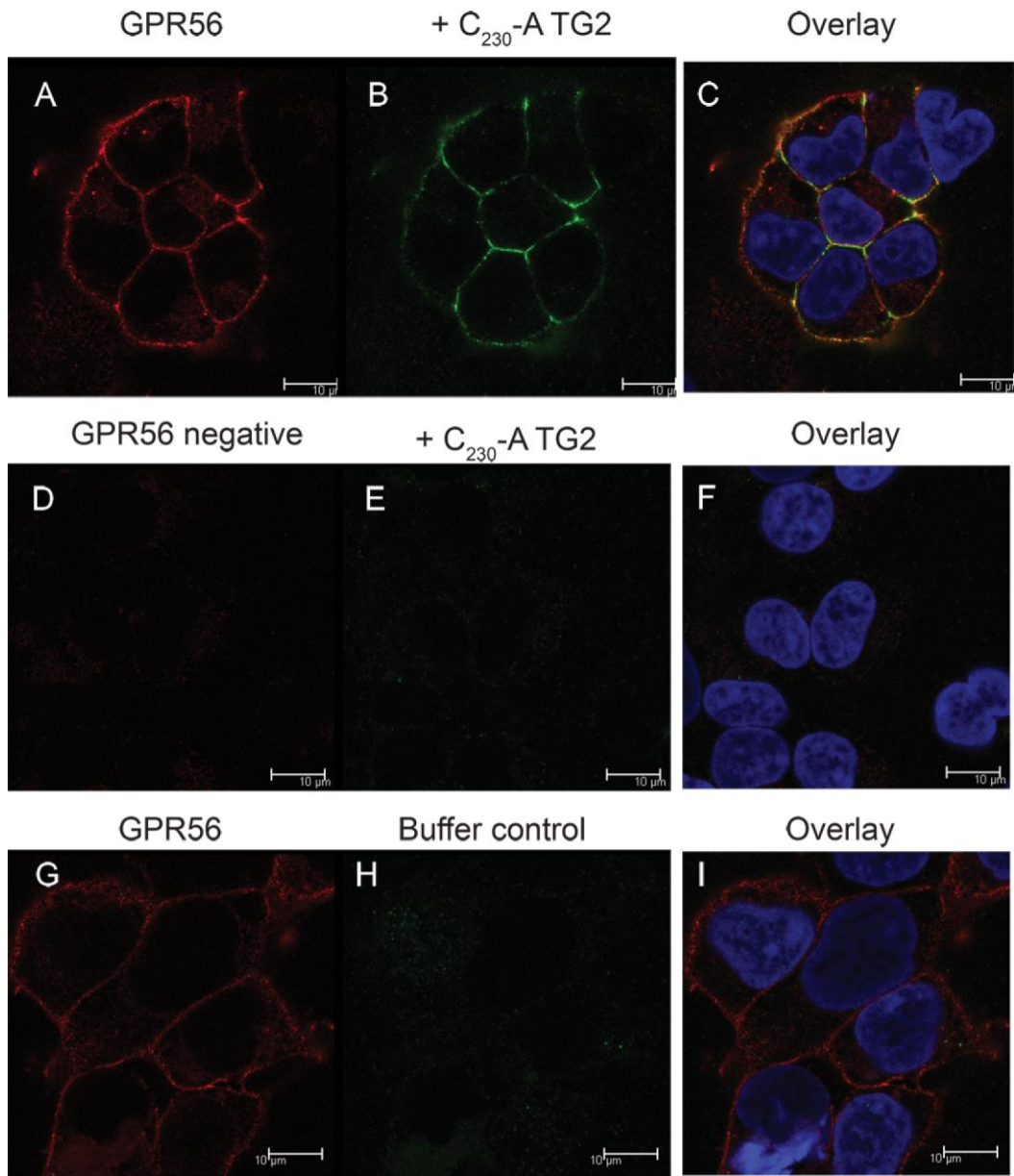


Fig. S4. TG2 is not sequestered to cell surface in HEK293 cells.

(A-I) HEK293 flp-in cells stably expressing the G-coupled receptor GPR56 under control of a tetracycline repressor sensitive promoter (T-REx system) were seeded onto poly-L-lysine coated coverslips and receptor expression induced with doxycycline for 48h (A-C, G-I) prior to treatment with 20µg/ml of the oxidation resistant TG2 C²³⁰A mutant (A-C, D-F) or control buffer (G-I) for various times. Cells were fixed, permeabilised and stained with antibodies to the *N*-terminus of GPR56 (red) or to TG2 (green). Data shown reflects 5s after stimulation with TG2. In GPR56 negative cells, no cell surface association of TG2 was observed (E,F), and this remained unchanged even after prolonged incubation for up to 30min. In contrast, expression of one of the receptors, GPR56, known to interact with TG2 lead to rapid cell surface association of TG2 (B,C), and subsequent internalisation.

(K) To further confirm the absence of extracellular cell- or matrix-associated TG2, HEK293 P2X7R cells expressing wildtype TG2 were BzATP stimulated as before, and the cell layer biotinylated as in Fig. S1C either immediately after 10min of stimulation or following a 30min chase without agonist. Extracted unmodified proteins (cytoplasm) or proteins carrying biotin (cell-surface/matrix) were analysed for TG2 by Western blotting alongside media collected after pulse and chase, respectively. Virtually none of the externalized TG2 was associated with the cell layer in HEK293 cells in contrast to macrophages (compare to Fig. S1C) which are known to harbor active enzyme at the cell surface (Toth et al., 2009).

Table S1. Oligonucleotides used to generate expression constructs. Mutated nucleotides are shown in bold face.

cDNA Construct	Forward primer	Reverse primer
P2X7R	5' – TTAGGTACCTTCACCATGCCGGCC TGCTGC	5' – TTTCTCGAGTCAGTAAGGACTCTTGAAGCC ACTGTA
P2X7R-V5	see P2X7R	5' – TTTCTCGAGGTAAGGACTCTTGAAGCCACT GTA CTG
P2X7R variant B	see P2X7R	5' – TTCTCGAGTTAGTCACTTCCTTCTCCAAAC CATTTTCCTAAAGCATGGAAAAGAGA
P2X7R P⁴⁵¹L	5' – GACACACCC C TGATTCCTGGAC	5' –CAGGAAT C AGGGGTGTGTCATGG
P2X7R A³⁴⁸T	5' –GTCTGGCC A CTGTGTTTCATCG	5' –GAACACAG T GGCCAGACCGAAG

Table S2. Expression constructs for GFP-tagged human TG2 based on pRc/CMV vector^a

Fusion protein	Sequence
TG2-GFP	atg... gcc-cccaagctt-atg... aaa-gcggccgctcgagcatgcatctag ¹ M -hTG2- ⁶⁸⁷ A - P K L - ¹ M -GFP- ²³⁸ K - A A A R A C I
GFP-TG2	atg... aaa-gcggccgctcgagcagatcccgcggcc-atg... gcctaa ¹ M -GFP- ²³⁸ K - A A A R A D P A A - ¹ M -hTG2- ⁶⁸⁷ A

a; pRc/CMV-GFP vector was a kind gift of Dr Olmstedt, University of Rochester, New York , USA (Olson et al., 1995).