Supplementary Material

Pharmacological inhibition of platelet-tumor cell cross-talk prevents platelet-induced overexpression of cyclooxygenase-2 in HT29 human colon carcinoma cells

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Molecular Pharmacology

Supplementary Methods

Western Blot Analysis

Cells were lysed in Triton 1% with 1mM of PMSF. For experiments regarding nuclear and cytoplasmic protein fractionation, NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL USA) was used, according to the manufacturer's protocols. Proteins were loaded onto 4-9% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes (GE Healthcare, Milan, Italy) and incubated with anti-COX-2, anti-TP, anti-EP1, anti-EP2(Cayman Chemical), anti-PCNA, anti-p21WAF1/CIP1, anti-cyclinB1, anti-cyclinD1, anti-galectin-3, anti-galectin-4, anti-ODC, anti-bcl-2, anti-EP4 and anti-Lamin A/C (Santa Cruz Biothecnology, Santa Cruz, CA, USA), anti-GPVI (4C9, kindly provided by Dr. Münch, CorImmun GmbH, Munich, Germany).
Quantification of optical density (OD) of different specific bands was calculated using laser densitometry (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to the OD of β-actin.

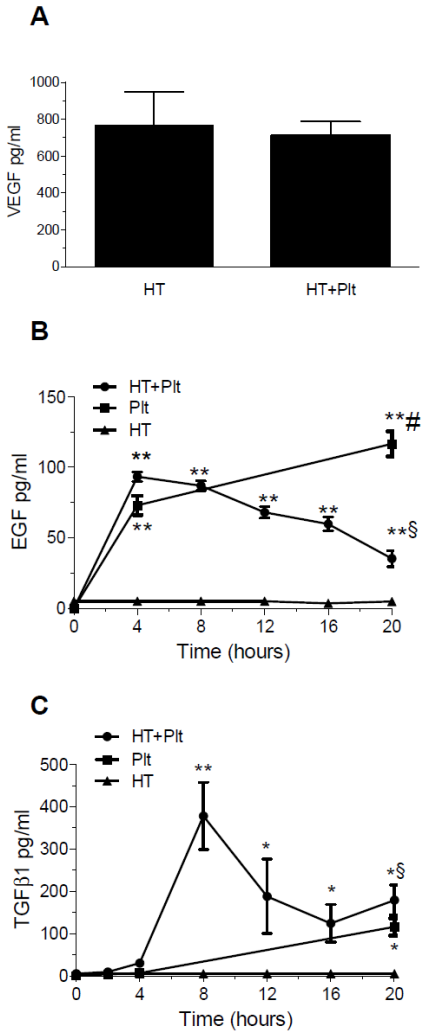
Immunofluorescence

For studies involving COX-2 and COX-1, the cells were blocked with 3 % filtered donkey serum (Sigma-Aldrich) diluted in PBS for 30min at room temperature. Endogenous COX-2 and COX-1 were detected with an anti-COX-2 polyclonal antibody (C-20; 1:100, Santa Cruz) and anti-COX-1 polyclonal antibody (1:100, Cayman Chemical) diluted in the blocking serum. The cells were incubated with the anti-COX-2 and anti-COX-1 overnight at 4°C, washed, and then incubated with FITC conjugated to donkey anti-goat IgG (1:1000, Santa Cruz) for COX-2 and with IRDye 680LT conjugated to donkey anti-rabbit IgG (1:1000, LI-COR Bioscience, USA) for COX-1, 1 h at room temperature.

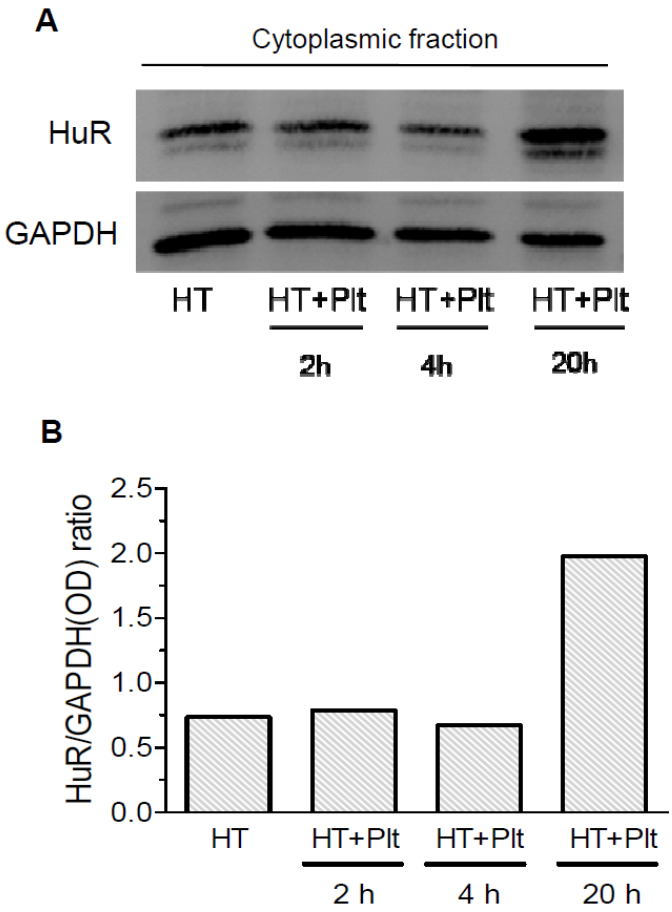
For studies involving HuR and GAPDH, the cells were blocked with 3% filtered goat serum (Sigma-Aldrich) diluted in PBS for 30min at room temperature. Cells were incubated overnight at 4°C with an anti-HuR monoclonal antibody (3A2; 1:250, Santa Cruz) and with anti-GAPDH

monoclonal antibody (1:100, Cell Signalling, Boston, MA, USA). The cells were washed and incubated with an anti-mouse secondary antibody conjugated to FITC (1:1000, Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) for HuR and with an anti-rabbit secondary antibody conjugated to Texas Red (1:1000, Jackson ImmunoResearch Laboratories) 1 h at room temperature. DAPI staining were obtained using VECTASHIELD hard-set mounting medium with DAPI (Vector Laboratories, CA, USA). Confocal images were obtained using a confocal Laser Scanning Microscope system Leica TCS SP5. The calculation of ratio between pixel summ of cytoplasmic and nuclear HuR staining was performed using LAS AF software, version 2.2.1.

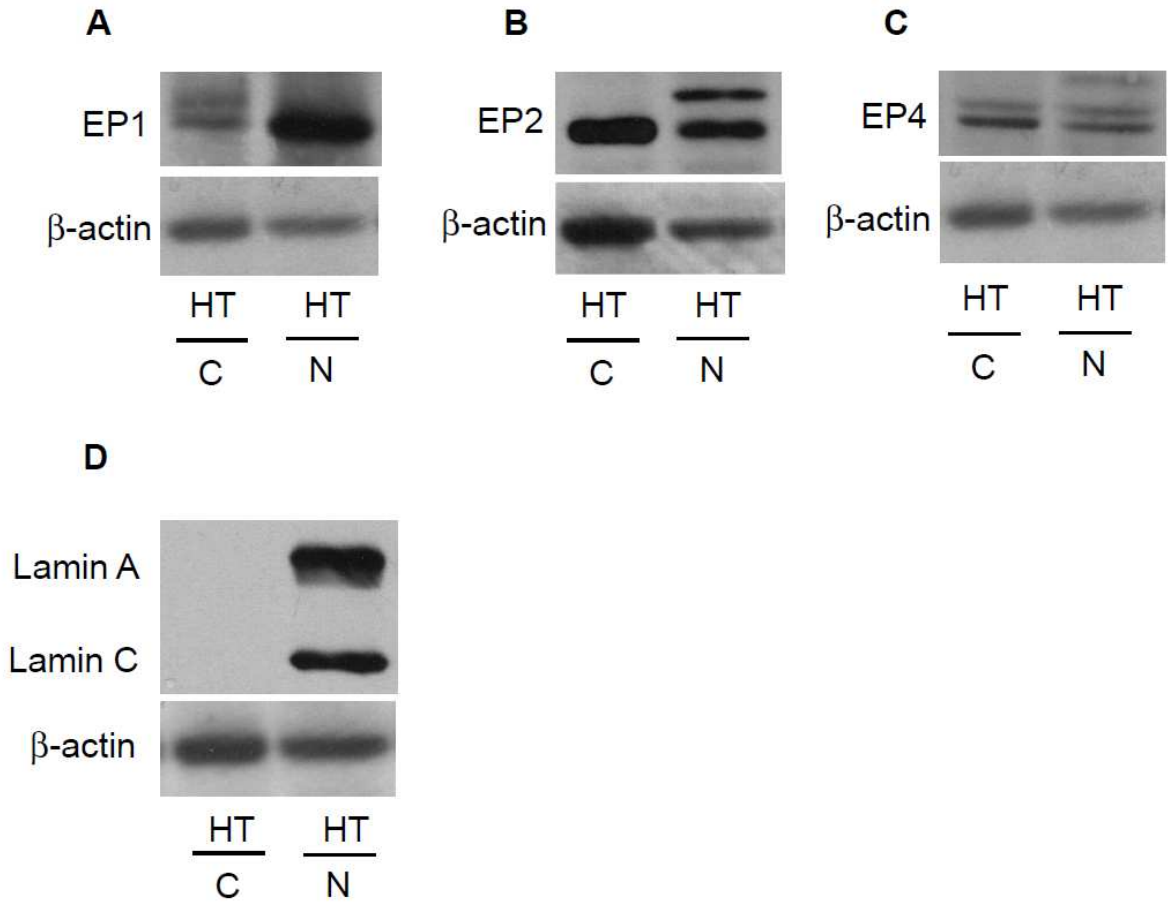
Supplementary Figures



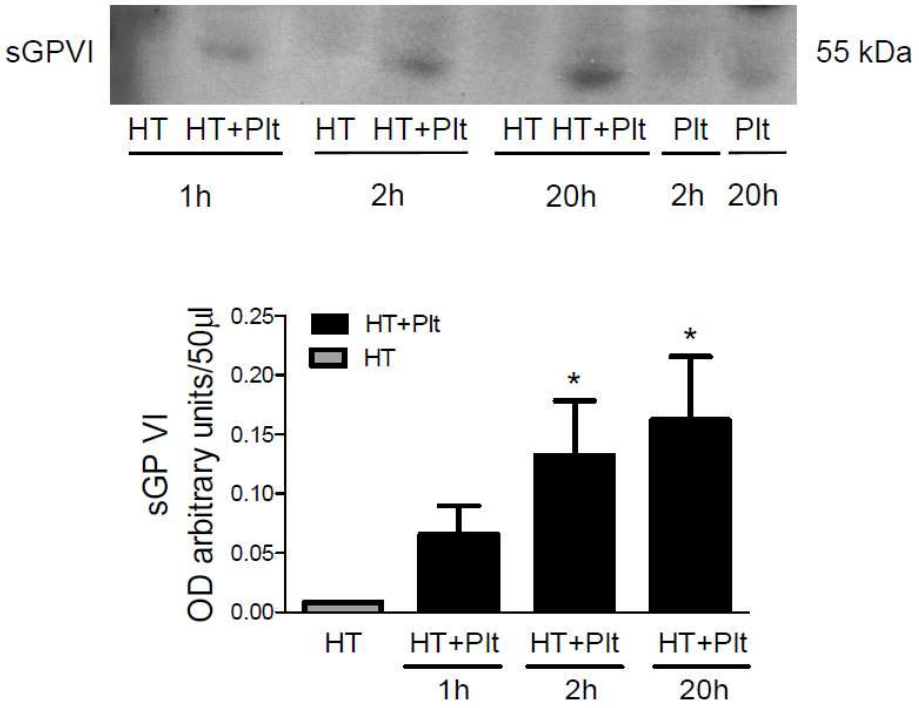
Supplementary Figure 1. Generation of VEGF, EGF and TGFβ1 in platelet–HT29 cells co–cultures. HT29 cells($1x10^6$)(HT) or isolated unstimulated platelets($100x10^6$)(Plt) were cultured alone or co-cultured (HT+Plt) up to 20h. The levels of VEFG (A) were measured in HT29 cells cultured alone (HT) or co–cultured with platelets (HT+Plt) for 20h. Values are reported as mean±SEM (n=4), pg/ml. The release of EGF (B) and TGFβ1 (C) were assessed in the culture medium at different time points. Values are reported as mean±SEM (n=3), pg/ml. (B) **P<0.05 vs HT at each time; §P<0.05 vs Plt(20h) and HT+Plt(4h); #P<0.01 vs Plt(4h). (C) *P<0.05 and **P<0.01 vs HT at each time; §P<0.05 vs HT(8h).



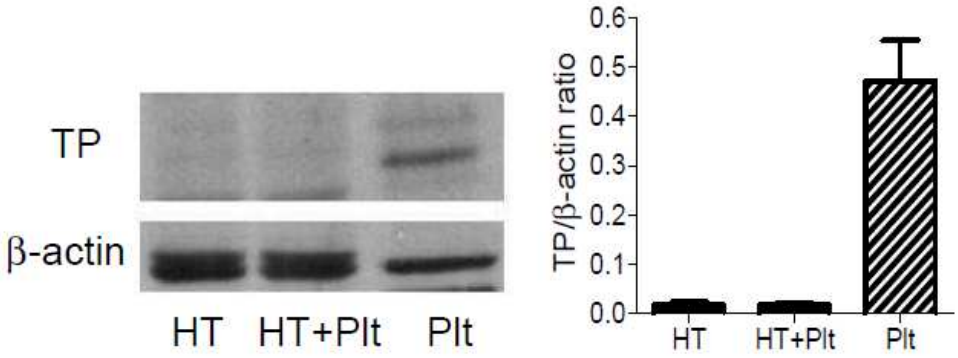
Supplementary Figure 2. Time–course of the cytoplasmatic accumulation of HuR in HT29 cells co–cultured with platelets. (A) Immunoblot of HuR protein levels was assessed by Western blot in the cytoplamic fraction of HT29 cells ($1x10^6$cells) cultured alone (HT) or with platelets up to 20h and normalized to GAPDH protein expression(B). Quantification of optical density (OD) of different specific bands was calculated using laser densitometry (B). Data are reported as mean of two separate experiment, as ratio between optical density (OD) values of HuR immunoreactive bands to that of GAPDH bands(B).



Supplementary Figure 3. Protein expression of PGE_2 receptor subtypes EP1 (A), EP2 (B), and EP4 (C) in the cytoplasmic and nuclear fraction of HT29 cells. (A-C) EP protein expression was assessed by Western blot in HT–29 cells ($1x10^6$ cells) cultured alone (HT) for 20h, both in the cytoplasmic (C) and nuclear (N) fraction. Quantification of optical density (OD) of different specific bands was calculated using laser densitometry and normalized to the OD of β–actin and values were reported as mean±SEM (n=3). (D) Lamin A and C, which are essential scaffolding components of the nuclear envelope, expression was detected in the same samples by Western blot technique.



Supplementary Figure 4. **Time–dependent release of soluble (s) GPVI in the medium of HT29 cells co-cultured with platelets**. Levels of sGPVI in the conditioned culture medium (50 µl) of HT29 cells ($1x10^6$ cells) (HT), platelets ($100x10^6$) (Plt) or their co–cultures (HT+Plt) up to 20h. The levels of sGPVI were analyzed by Western Blot. Quantification of optical density (OD) of different specific bands was calculated using laser densitometry. Data are reported as mean±SEM (n=3). *P<0.05 vs HT.



Supplementary Figure 5. Protein expression of TXA_2 receptor (TP) in HT29 cells, in platelets and in HT29–platelet co–cultures. TP protein expression was assesed by Western blot in HT29 cells ($1x10^6$cells) cultured alone (HT), in platelets ($100x10^6$) (Plt) and in HT29 cells co–cultured for 20h with platelets (HT+Plt). Quantification of optical density (OD) of different specific bands was calculated using laser densitometry and normalized to the OD of β-actin. Data are reported as mean±SEM from 3–5 experiments.