

Inhibition of spleen tyrosine kinase as treatment of postoperative ileus

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Abbreviations:

POI – postoperative ileus
SYK – spleen tyrosine kinase
GI – gastrointestinal
IM – intestinal manipulation
GC – geometric center
L – laparotomy
GSK143 – GSK compound143
SP – substance P
TNP – trinitrophenyl
DOX – doxantrazole
PMC – peritoneal mast cell
SCF – Stem cell factor
PBS – phosphate buffered saline
i.p. – intraperitoneal
FITC – fluorescein isothiocyanate
4-MUG – 4-methylumbelliferyl glucosaminide
HPF – high power field
DMSO – dimethyl sulphoxide

ABSTRACT

Objective Intestinal inflammation resulting from manipulation-induced mast cell activation is a crucial mechanism in the pathophysiology of postoperative ileus (POI). Recently it has been shown that spleen tyrosine kinase (Syk) is involved in mast cell degranulation. Therefore, we have evaluated the effect of the Syk-inhibitor GSKcompound143 (GSK143) as potential treatment to shorten POI.

Design In vivo: In a mouse model of POI, the effect of the Syk inhibitor (GSK143) was evaluated on gastrointestinal transit, muscular inflammation and cytokine production.

In vitro: The effect of GSK143 and doxantrazole were evaluated on cultured peritoneal mast cells and bone marrow derived macrophages.

Results In vivo: Intestinal manipulation resulted in a delay of gastrointestinal transit at t=24h (Geometric Center (GC): 4.4 ± 0.3). Doxantrazole and GSK143 significantly increased gastrointestinal transit (GC doxantrazole (10 mg/kg): 7.2 ± 0.7 , GSK143 (1mg/Kg): 7.6 ± 0.6), reduced inflammation and prevented recruitment of immune cells in the intestinal muscularis.

In vitro: In peritoneal mast cells, SP (0-90 μ M) and trinitrophenyl (0-4 μ g/mL) induced a concentration-dependent release of β -hexosaminidase. Pretreatment with doxantrazole and GSK143 (0.03-10 μ M) concentration-dependently blocked SP and trinitrophenyl induced β -hexosaminidase release. In addition, GSK143 was able to reduce cytokine expression in endotoxin treated bone marrow derived macrophages in a concentration dependent manner.

Conclusion The Syk-inhibitor GSK143 reduces macrophage activation and mast cell degranulation in vitro. In addition, it inhibits manipulation-induced intestinal muscular inflammation and restores intestinal transit in mice. These findings suggest that Syk inhibition may be a new tool to shorten POI.

SUMMARY BOX

What is already known about this subject?

- Postoperative ileus is mediated by intestinal inflammation resulting from manipulation-induced mast cell and macrophages activation.
- Spleen tyrosine kinase (Syk) is one of the critical tyrosine kinases involved in mast cell degranulation and macrophage activation.
- GSK143 is a potent and highly selective Syk inhibitor with efficacy in a range of inflammatory models.

What are the new findings?

- GSK143 significantly reduces the inflammatory response to intestinal manipulation thereby preventing POI.
- The Syk inhibitor GSK143 inhibits SP and TNP induced peritoneal mast cell degranulation.
- The Syk inhibitor GSK143 reduces cytokine expression in LPS treated macrophages.

How might it impact on clinical practice in the foreseeable future?

- These data indicate that GSK143 inhibits mast cell degranulation and macrophage activation, and prevents the inflammatory cascade leading to POI.
- This suggests that Syk can be a potential target for intestinal inflammatory diseases and may be a new tool to shorten POI.

INTRODUCTION

Postoperative ileus (POI) is characterized by generalized hypomotility of the gastrointestinal tract after abdominal surgery and leads to increased morbidity and prolonged hospitalization. Recent evidence shows that POI is mediated by infiltration of leukocytes in the intestinal muscle layer in response to surgical handling of the gut.(1-3) The importance of this inflammatory response in POI is underscored by the beneficial effect of pharmacological interventions blocking the influx of leukocytes.(1;2;4) We previously demonstrated that mast cells are involved in this inflammatory response: leukocyte recruitment was reduced in mast cell deficient mice whereas the mast cell stabilizers ketotifen and doxantrazole reduced muscular inflammation and shortened POI.(5) Based on these results, we performed a pilot study with ketotifen (4 or 12 mg for 6 days) in patients undergoing abdominal surgery.(6) Although gastric emptying was significantly improved by ketotifen, this compound has central side effects such as sleepiness and dizziness and has anticholinergic properties potentially counteracting the beneficial effect of mast cell stabilization on gastrointestinal motility.(7-9) A potential alternative approach to stabilize mast cells is blockade of intracellular spleen tyrosine kinase (Syk). Syk is one of the critical tyrosine kinases involved in mast cell degranulation induced by IgE crosslinking. Crosslinking of the FcεRI receptor causes phosphorylation of Syk subsequently activating intracellular pro-inflammatory pathways.(10) Therefore, Syk inhibitors suppress the signaling cascades that normally lead to degranulation of mast cells.(11) In addition to mast cells, activation of macrophages residing in the muscularis externa has been correlated with the intestinal inflammatory response resulting in POI.(12) Interestingly, Syk is also playing a crucial role in macrophage activation.(13-15) Indeed, inhibition of Syk signalling resulted in inhibition of the NF-kappaB (NF-κB) pathway and reduced cytokine production in lipopolysaccharide (LPS)-treated macrophages. Therefore, we hypothesized that modulation of the Syk pathway may be a potential new therapeutic strategy for POI.

GSK143 is a potent and highly selective Syk inhibitor with efficacy in a range of inflammatory models.(16) The aim of this study was to investigate the effect of GSK143 in preventing manipulation-induced intestinal inflammation thereby shortening POI.

MATERIAL AND METHODS

Animals

Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with standard mouse chow and water *ad libitum*; 20°C–22°C, 55% humidity). Ten to twelve weeks old wild type C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) and the Animal Experiments Committee of the Medical Faculty of the Catholic University of Leuven (Leuven, Belgium).

Primary culture of peritoneal mast cells

Peritoneal cells were harvested by abdominal lavage with phosphate buffered saline (PBS) in C57NL/BL6 mice. After centrifugation, peritoneal cells were resuspended in peritoneal mast cell (PMC) culture medium (RPMI 1640 (GIBCO) containing 10% fetal calf serum, 1% penicillin/streptomycin and 6% bone marrow mast cell supplement (containing 20% MEM non-essential amino acids, 1% L-glutamine, 0.22% Sodium Pyruvate, 0.005% β -mercapto-ethanol). Stem cell factor (SCF) 100 ng/mL was added to achieve enrichment of PMC. Cells were incubated in a 5% CO₂ humidified atmosphere at 37°C in 75 cm² tissue culture flasks for a minimum of 3 weeks. PMC cultured for 4-7 weeks were used for the in vitro experiments. Fc ϵ RI and CD117 expression was assessed by direct immunofluorescence. Cells were harvested and washed using ice-cold staining buffer (PBS supplemented with 0.5% BSA, 0.3mM EDTA and 0.01% NaN₃). Next, cells were incubated at 4°C with FITC-labeled anti-mouse CD117 (c-kit; 1:150) and PE labelled Fc ϵ RI- α (1:800) or corresponding isotype control and subsequently washed with staining buffer. Fluorescence was analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) equipped with CellQuest software.

Activation of peritoneal mast cells

Cells were collected and centrifuged for 5 minutes at 370 g and resuspended in Tyrode's buffer supplemented with 0.1% BSA at a density of 1×10^6 cells/mL. Cells (50 μ L/well; 5×10^4 cells/well) were

seeded in 96-well plates and activated by SP or TNP-Ovalbumin. To this end, cells were incubated with Tyrode's buffer at 37 °C for 30 minutes and challenged with Tyrode's buffer (control), SP (0-90 µM) or TNP-Ovalbumin (0-4 µg/mL) at 37°C for 10 or 30 minutes. Cells challenged with TNP-Ovalbumin were incubated overnight with mouse IgE anti-TNP (0.5 µg/µL) in PMC culture medium at a density of 2x10⁶ cells/mL. To stop this reaction, plates were centrifuged at 390 g at 4 °C for 5 minutes and supernatant was collected. In a separate series of experiments, the effect of GSK143 and doxantrazole on SP and IgE crosslinking (TNP-Ovalbumin induced) mediated mast cell activation was evaluated. Cells were pretreated with 10 µL Tyrode's buffer (placebo), GSK143 (0.03-10 µM) or the classic mast cell stabilizer doxantrazole (227 µM) at 37°C for 30 minutes. Subsequently, cells were activated with 50 µL SP (90 µM) or TNP (40 ng/mL) at 37°C for 10 minutes.

Primary Bone Marrow-Derived Macrophages

Bone marrow cells were isolated from C57NL/BL6 mice. One hundred and fifty thousand bone marrow cells were plated in 10 cm plates in 2 ml of BM-medium (DMEM supplemented with 20% low-endotoxin fetal bovine serum, 30% L929-cell conditioned medium, 1% L-glutamine, 1% Pen/Strep, 0.5% Na Pyruvate, 0.1% β-mercaptoethanol) and cultured for 10 days (17). At day 10, macrophages were incubated with different concentration of GSK143 or with doxantrazole as shown in figure 4 for 15 minutes before stimulation with LPS (100ng/ml, *E. coli* 055:B5) for 2 h. To assess purity at day 10, cells were harvested and washed using ice-cold staining buffer (PBS supplemented with 0.5% BSA, 0.3mM EDTA and 0.01% NaN₃). Next, cells were incubated at 4 °C with Pe-Cy7-labeled anti-mouse CD11b (1:200) and APC labelled F4/80 (1:100) or corresponding isotype control for 40 minutes and subsequently washed with staining buffer. Fluorescence was analyzed by flow cytometry using a FACSCanto (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo (version 4.6.2, Treestar).

Measurement of degranulation of peritoneal mast cells

To quantify mast cell activation, we measured the release of β-hexosaminidase in the supernatant. Supernatant was incubated during 2 h with a 4-methylumbelliferyl glucosaminide (4-

MUG) substrate solution (3.79 mg MUG/mL DMSO) in 0.1 M citrate buffer (pH 4.5) at 37°C in a 5% CO₂ humidified atmosphere. The reaction was stopped by adding 0.2 M glycine buffer (pH 10.7). Fluorescence was measured using a multiwell plate reader at an emission wavelength (λ) of 360 nm and excitation wavelength (λ) of 460 nm. The percentage of degranulation was calculated as follows: $((a - b)/(t - b)) \times 100$, where a is the amount of β -hexosaminidase released from stimulated cells, b is the amount released from unstimulated cells (basal release by cells incubated with Tyrode's buffer only), and t is total cellular β -hexosaminidase cellular content, determined by total lysis of cells by 1% Triton X-100.

Reagents

RPMI 1640 (containing 10% fetal calf serum, 1% penicillin/streptomycin and 6% bone marrow mast cell supplement (containing 20% MEM non-essential amino acids, 1% L-glutamine, 0.22% Sodium Pyruvate, 0.005% β -mercapto-ethanol)) was purchased from GIBCO. Tyrode's buffer (5.6 mM glucose, 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, pH 7.2) supplemented with bovine serum albumin (BSA) was used as stimulation medium. Recombinant Mouse SCF/C-KIT Ligand (1.0 mg Recombinant Mouse SCF in 1.8 mL 40 mM Tris Buffer) was purchased from Invitrogen. Doxanzole (a kind gift of Agnès François, Institut Gustave Roussy, Villejuif, France) was dissolved in dimethyl sulphoxide (DMSO). GSK143 (kindly provided by GlaxoSmithKline, Gunnels Wood Road, Stevenage, United Kingdom) was dissolved in DMSO at 10 mM and serial dilutions were prepared in DMSO. The final concentration of DMSO in cell suspensions was 0.5% and did not evoke more β -hexosaminidase release compared to basal release by cells incubated with Tyrode's buffer only. Mouse IgE anti-TNP was obtained from BD Pharmingen. SP was purchased from Sigma-Aldrich and TNP-Ovalbumin from Biosearch Technologies. Lipopolysaccharide from *Escherichia coli* 055:B5 was obtained from Sigma-Aldrich.

Surgical procedure

Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg/kg) and Xylazine (Rompun 10 mg/kg). Anesthetized mice underwent a laparotomy alone or a laparotomy followed by small intestinal manipulation (IM).(18) Surgery was performed under sterile

conditions and performed as follows: a midline abdominal incision was made, and the peritoneum was opened over the linea alba. The small bowel was carefully externalized and layered on a moist gauze pad. Contact with or stretch on stomach or colon was strictly avoided. The small intestine was manipulated from the cecum to the distal duodenum and back for a total of three times, using a sterile moist cotton applicator attached to a device enabling the application of a constant pressure with 9 grams of weight. After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6-0 silk). After closure, mice were allowed to recover for 3 h in a heated (32°C) recovery cage without administration of analgesic agents as these can interact with intestinal motility and the postoperative inflammatory process.

Drugs administration

Mice received doxantrazole (10 mg/kg in 5% NaHCO₃, pH 7.4) 16 h and 1 h before and 1 h after IM by i.p. injection. The other groups of animals received orally GSK143 (0.1 - 10 mg/kg in 0.5% methylcellulose solution in water) or vehicle (0.5% methylcellulose solution in water) also at 3 time points; 1.5 h before, and 1.5 and 6 h after IM (n= 5-8 per group). In another group of mice the effect of a single oral administration of GSK143 (1, 3 or 10 mg/kg in 0.5% methylcellulose solution in water) was given 1.5 h before intestinal manipulation. The researcher performing the operations was blinded for the type of pharmacological treatment.

Gastrointestinal transit measurements

Gastrointestinal function 24 h postoperatively was determined in vivo by measurement of gastrointestinal transit of liquid non-absorbable fluorescein labeled dextran (FITC-dextran). Ten microliters of FITC-dextran (70,000 Da; Invitrogen, Paisley, UK) dissolved in 0.9% saline (6,25 mg/mL) were administered via oral gavage. Ninety minutes later, animals were killed by cervical dislocation and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), the cecum, and colon (3 segments of equal length) were collected and the amount of FITC in each bowel segment was determined in duplicate using a fluorimeter (Synergy HT, BioTek Instruments Inc., VT, USA) with a excitation wavelength (λ) of 485 nm and emission wavelength (λ) of 528 nm. The distribution of the fluorescent dextran along the

gastrointestinal tract was determined by calculating the geometric center (GC): Σ (percent of total fluorescent signal in each segment \times the segment number)/100 for quantitative statistical comparison among experimental groups.(19)

Whole mount preparation and histochemistry

To quantify the degree of inflammation in whole mounts of the intestinal muscularis, segment number 7 of the small bowel was cut open, fecal content was washed, and segments were fixed with 100% ethanol for 10 minutes, transferred to ice cold modified Krebs solution and pinned flat in a glass-dish. Mucosa and submucosa were removed and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hucker-Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 minutes. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields (HPF, 668.4 μm \times 891.2 μm) was counted and the average was calculated. Tissue sections were coded so that the researchers were unaware of the surgical and pharmacological treatment of the specimens when the number of MPO positive cells was determined.

Cytokine measurements

For cytokine measurements, 2 jejunal muscularis segments (segments 4 and 5) were added to 500 μL lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl_2 , 2 mM CaCl_2 , 1% Triton X-100, pepstatin A, leupeptin and aprotinin (all 20 ng/mL; pH 7.4), homogenized, and incubated at 4°C for 30 minutes. Homogenates were centrifuged at 1500 g at 4°C for 15 minutes and supernatants were stored at -20°C until assays were performed. Ccl2, IL-6, IL-1 β and TNF- α in supernatants were analyzed by mouse ELISA (R&D Systems, Abingdon, England) according to the manufacturer's instructions.

RNA extraction and inflammatory gene expression

Total RNA was extracted from bone marrow derived macrophages stimulated with LPS (100ng/ml, *E. coli* 055:B5) for 2 h. RNA extraction was performed using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. Quantitative real-time transcription polymerase chain reactions (RT-PCR) were performed with the

10

LightCycler 480 SYBR Green I Master (Roche) on the Light Cycler 480, (Roche). Results were quantified using the $2^{-\Delta\Delta CT}$ method (20). The expression levels of the genes of interest were normalized to the expression levels of the reference gene *rpl32*. PCR experiments were performed in triplicate. Primer sequences used are listed in supplementary table 1.

Cell isolation from the intestinal muscularis for flow cytometry

24 h after the surgery, muscularis externa from the small intestine was isolated and enzymatically digested in MEM α medium (Lonza) containing 100 μ g/ml of Penicillin, 100 μ g/ml of Streptomycin, 50 μ M beta-mercaptoethanol, 5% FCS, 5mg/ml protease type I (Sigma-Aldrich), 20 mg/ml collagenase type II (Sigma-Aldrich) and 5U/ml DNase I for 15 minutes at 37 °C. Cell suspensions were filtered through a nylon mesh. Before staining, cells were pre-incubated with an anti-FcR antibody (clone 24G2; BD Biosciences). Cells were then stained with the following antibodies: CD45.2-FITC (104, BD Biosciences), CD11b-PeCy7 (M1/70, BD Biosciences), F4/80-APC (BM8, eBioscience), Ly6G-PerCPy5.5 (IA8, BD Biosciences), Ly6C-PE (AL-21, BD Biosciences) and analyzed by flow cytometry using a FACSCanto (BD Biosciences). Analysis was performed using FlowJo (version 4.6.2, Treestar). Immune cells were gated for their surface expression of CD45.2. Subsequently monocytes were identified for their surface expression of CD45.2, CD11b, F4/80 and Ly6C, while neutrophils were identified as CD45.2, CD11b and Ly6G positive but F4/80 negative cells (21).

Blood quantification of GSK143

GSK143 blood concentration levels were assessed in mice treated with a single oral dose of GSK143 (prepared in 0.5% Methylcellulose) at 1, 3 and 10 mg/kg or vehicle alone. Blood samples were taken at 1.5 h post dose (prior to surgery) and at 6 h and 25.5 h post dose. The blood concentration levels were determined by reverse phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at the laboratories of the Quantitative Pharmacology group at GlaxoSmithKline, Stevenage, United Kingdom. In brief, 40 μ L of the diluted samples (blood:water, 1:1, v/v) were assayed against matrix matched calibration standards prepared over the range 1 to 2000 ng/mL. Samples and calibration standards were extracted by protein precipitation with 200 μ L of

acetonitrile containing 50 ng/mL of a GSK proprietary internal standard and centrifuged at 3600 *g*. All prepared samples and standards were analysed on a LC gradient using a Phenomenex Kinetex C18, 2.6 μm , 50 x 2.1 mm column. GSK143 concentration data was generated and processed using Analyst 1.4.2 software.

Statistical analysis

The data on muscularis cytokine production were not normally distributed. The Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney U test was used to identify the statistical differences within the cohort. All other data were statistically analyzed by use of one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison test (for in vitro data) or Bonferroni’s multiple comparison test (for in vivo data). A probability level of P less than 0.05 was considered statistically significant and results are shown as means \pm SEM. Graph Pad Prism version 5.01 software was used to perform statistical analysis and create graphs.

RESULTS

GSK143 prevented manipulation-induced influx of leukocytes and improved postoperative gastrointestinal transit

Intestinal manipulation (IM) resulted in an impairment of gastrointestinal transit shown by a reduction in the geometrical center (GC) (laparotomy + placebo GC = 9.5 ± 0.3 , IM + placebo GC = 4.4 ± 0.3 , $P < 0.05$, $n = 5$ and 7 respectively). Treatment with doxantrazole (10mg/kg) or GSK143 (0.1-10mg/kg) did not affect intestinal transit in mice that underwent laparotomy (supplementary fig. 1). In contrast, IM-induced delay in intestinal transit was significantly prevented in mice treated with three doses of doxantrazole (10 mg/kg) (fig. 1: GC: 7.2 ± 0.7 , $n=6$) or GSK143 (1–3 mg/kg) (1 mg/kg): 7.6 ± 0.6 , $n=8$; 3 mg/kg 7.3 ± 0.5 , $n=8$). On the contrary GSK143 three doses of 10 mg/kg did not significantly protect against the development of POI (GC: 6.1 ± 0.6 ; GSK143: $n = 9$). In the same line, a single dose of 3 mg/kg of GSK143 administered 1.5 h before surgery was able to significantly improve intestinal transit (GC: 6.83 ± 0.6 , $n = 8$) and to reduce MPO-positive cell recruitment in the muscularis externa 24 h after IM (supplementary fig. 2).

Intestinal manipulation, but not laparotomy resulted in an influx of MPO-positive inflammatory cells in the muscle layer of the small intestine 24 h after surgery (fig. 2: 257 ± 45 MPO⁺ cells/HPF). As shown in figure 2, both doxantrazole (10 mg/kg) and GSK143 (1-10 mg/kg) significantly reduced the influx of MPO positive cells in a dose dependent manner.

GSK143 reduced manipulation-induced cytokine production in the intestinal muscularis

Intestinal manipulation of the small intestine markedly increased IL-1 β , CCL-2 and IL-6 protein expression, but not that of TNF- α , in the intestinal muscularis compared to laparotomy alone (data not shown). Interestingly, GSK143 (1 mg/kg) administered three times significantly reduced the levels of IL-1 β and Ccl2 compared to placebo treated mice. IL-6 production was reduced by both treatments but this effect was not statistically significant (fig. 3A).

As Syk inhibition resulted in a reduced number of MPO-positive cells and a lower amount of cytokine secretion in the muscularis layer of manipulated mice, we addressed whether GSK143 may also affect recruitment of specific subsets of inflammatory cells. As shown in figure 3B, treatment with GSK143 (1mg/kg) administered three times resulted in a significant reduction of immune cell recruitment in the muscularis externa. Syk inhibition affected recruitment of both neutrophils and monocytes suggesting a potent and broad anti-inflammatory effect of this treatment (fig. 3B).

GSK143 inhibited SP and TNP induced peritoneal mast cell degranulation and LPS-induced activation in macrophages

To define the concentration range of GSK143 to be tested in vitro, blood samples were collected from mice treated with a single dose of 1, 3 or 10 mg/kg GSK143 (Supplementary figure 3). Based on these data, the effect of GSK143 in the concentration range of 0.03-10 μ M GSK143 on isolated mast cells and macrophages was further studied.

Freshly isolated peritoneal cells from C57NL/BL6 mice were cultured for 4-7 weeks yielding a > 94% (Fc ϵ RI+, CD117+) pure PMC population resulting from an expansion of differentiated PMC in the presence of 100 ng/mL SCF (supplementary fig. 4A). Basal release of β -hexosaminidase by PMC incubated without stimulus (control) was below 7% of total cellular β -hexosaminidase content. Stimulation with SP (0-90 μ M) or TNP (0-4 μ g/mL) for 10 minutes resulted in a concentration-dependent response of β -hexosaminidase release with maximal release $54.7 \pm 2.6\%$ and $92.6 \pm 2.2\%$ of total cellular β -hexosaminidase content for SP and TNP respectively (supplementary fig. 5). Stimulation with SP or TNP for 30 minutes showed similar results (supplementary fig. 5).

Subsequently, the effect of GSK143, vehicle or doxantrazole was studied on PMC stimulated with 90 μ M SP or 0.04 μ g/mL TNP. Pretreatment with doxantrazole (227 μ M) significantly reduced SP induced β -hexosaminidase release from $43.5 \pm 1.0\%$ of total cellular content to $2.9 \pm 0.2\%$. β -hexosaminidase release by SP stimulated PMC was also inhibited significantly by $\geq 0.3 \mu$ M GSK143, but to a lesser extent than TNP-induced mast cell activation. (fig. 4A). The TNP induced β -hexosaminidase release was $70.3 \pm 4.3\%$ of total cellular content. Pretreatment with doxantrazole (227 μ M) and

GSK143 ($\geq 0.3 \mu\text{M}$) significantly reduced this release to a maximum of 2.8 and 8.7% respectively (fig. 4B).

Cultured bone marrow derived macrophages (purity > 95% (CD11b, F4/80; supplementary fig. 4B) from C57NL/BL6 mice were pretreated with GSK143 (0.1-10 μM) or doxanzole (227 μM) for 30 minutes prior LPS stimulation (100 ng/ml). Two hours after stimulation, macrophages were harvested and cytokine expression was assessed by quantitative PCR. GSK143 significantly reduced expression of cytokines such as IL-6, TNF- α , IL-1 β and CCL2 (fig. 5). Interestingly, pretreatment with doxanzole (227 μM) did not affect macrophage activation.

DISCUSSION

Postoperative ileus is mediated by intestinal inflammation resulting from manipulation-induced mast cell and macrophage activation. In the present study, we investigated the anti-inflammatory effect of a new Syk inhibitor and its ability to reduce POI. The Syk inhibitor GSK143 significantly reduced the inflammatory response to intestinal manipulation thereby preventing POI. In addition, we demonstrated that GSK143 inhibited both FcεRI and SP mediated degranulation of PMC and endotoxin-induced macrophage activation. Taken together, these data strongly suggest that Syk inhibition may represent a new therapeutic approach for POI.

Syk is required for FcεRI signalling in mast cells and activates intracellular signaling cascades involved in the transcription and translation of inflammatory mediators.(22;23) Syk inhibitors suppress the signaling cascades that normally lead to degranulation of mast cells.(11;22-24) In animal models Syk inhibitors have successfully prevented mast cell mediated inflammatory diseases such as rheumatoid arthritis and allergic rhinitis. (25;26) Moreover, Syk has been reported to play a crucial role in macrophage activation.(13-15) As both mast cells and macrophages have been implicated in the pathophysiology of POI, the present study was designed to establish whether blockade of intracellular Syk could represent an alternative approach to inhibit immune cell activation evoked by intestinal manipulation and thus represent a new tool to shorten POI.

At first, we evaluated the effect of GSK143 on primary cultured PMC. We have chosen to study this subset of mast cells as they functionally resemble connective tissue mast cells,(27) the subpopulation of mast cells most likely involved in POI.(1;27) In addition to IgE crosslinking, mast cells can also be activated by SP released by visceral afferent nerves with subsequent activation of inflammatory cells, a mechanism referred to as neurogenic inflammation.(5;27;27;28) In our in vitro experiments, we indeed showed concentration-dependent activation of PMC by SP and IgE crosslinking. In addition, we demonstrated that GSK143 significantly blocked the FcεRI and SP mediated degranulation of PMC. Of note, the concentrations of GSK143 inhibiting the activation of mast cells and macrophages in vitro were in the range of serum levels obtained following single

administration of the compound. Interestingly, the inhibitory effect of GSK143 was more potent for β -hexosaminidase release induced by IgE crosslinking compared with SP. This finding suggests that the signaling pathways involved in SP-induced mast cell activation are less dependent on spleen tyrosine phosphorylation compared to the Fc ϵ RI induced degranulation. Several lines of evidence indicate that SP can stimulate mast cells not only via its NK1 receptor, but also by NK1 receptor-independent pathways. Notably, at high concentrations SP induces mast cell degranulation by receptor-independent pathways,(29) which is mediated by G protein(s), protein kinase C, calcium, and phospholipase C.(29-35) These data would suggest that in addition to NK1 receptor mediated activation of Syk, other mechanisms may be involved explaining why GSK143 markedly inhibited Fc ϵ RI mediated and, to a lesser degree at higher drug concentrations, SP mediated degranulation. Alternatively, if SP-induced mast cell activation does not involve Syk, our data would indicate that GSK143 interacts with different intracellular signaling pathways. Potential mechanism could be competitive inhibition at the SP receptor level by membrane incorporation of GSK143 resulting in the competition of SP binding to the cell membrane surface proteoglycans.(29;36;37) Secondly, GSK143 may interfere with intracellular inositol triphosphate and subsequent increase intracellular calcium ions ultimately inhibiting degranulation.(38;39) An in-depth analysis of these signaling pathways was beyond the scope of this article, and further study is needed to fully understand the mechanisms underlying Syk-inhibition of SP induced mast cell degranulation.

Syk inhibition has been proposed as an important therapeutic strategy for the treatment of mast cell mediated upper airway diseases, such as allergic rhinitis.(40) Furthermore, Weinblatt et al. detected mast cells and Syk expression in the synovium of rheumatoid arthritis patients and demonstrated a significant clinical improvement after treatment with an oral Syk inhibitor.(41) As a consequence, inhibition of Syk has received increasing attention as new therapeutic approach for a variety of disorders. Previously, we have shown in mice that mast cells are important players in triggering the local intestinal inflammatory response leading to POI.(42-44) In patients undergoing surgery, even gentle inspection of the intestine at the very beginning of the surgical procedure triggers the release of mast cell mediators.(43) In accordance with these data, recent in vitro work

demonstrated that Syk-deficient mast cells fail to release mast cell mediators.(10) In the study reported here, GSK143 significantly reduced the upregulation of pro-inflammatory cytokines in the intestinal muscularis 24 h after intestinal manipulation. Moreover, GSK143 and the mast cell stabilizer doxantrazole attenuated the leukocytic influx and in addition improved gastrointestinal transit. This confirms the beneficial effect of Syk inhibition on the postoperative inflammatory phase within the intestine.(24)

Syk has been recently reported to also represent a key player in the regulation of the NF- κ B pathway in LPS-treated macrophages. Indeed, inhibition of Syk signalling in rat alveolar as well as in peritoneal macrophages and in the monocytic cell line THP-1 resulted in reduction of pro-inflammatory cytokine secretion.(14) In the present study, we evaluated the effect of GSK143 on cultured macrophages treated with endotoxin. In line with previous reports, we showed that GSK143 reduced the expression of IL-6, TNF- α , IL-1 β and CCL2 in the intestinal muscularis from intestinal manipulated mice. As macrophages are key players in orchestrating the leukocytic influx in the manipulated intestine (12), these data suggest that interaction of GSK143 with macrophages in the intestinal muscularis also contributes to the beneficial effect of GSK143 reducing muscular inflammation. Similar findings were previously reported by Moore *et al.* as treatment of mice with the inhibitor of protein tyrosine kinase tyrphostin reduced inflammatory influx and upregulation of pro-inflammatory cytokines, and significantly inhibited activation of NF- κ B.(3) Taken together, our in vitro data indicate that GSK143 may inhibit at the same time mast cells and macrophages exerting a broad anti-inflammatory effect in vivo. It should be emphasized though that Syk also affects the adaptive immune system.(45) Hence, we cannot exclude that the beneficial effects of GSK143 on POI reported here may extend beyond its interaction with mast cells and macrophages.(24)

To date, the exact triggers activating mast cells and macrophages during abdominal surgery are still unclear but besides neuropeptides (such as SP, VIP and CGRP) and specific antigens (via IgE crosslinking)(28) a variety of stimuli, including bacterial components and several physical stimuli could be involved. Physical stimuli may be perioperative temperature changes, or the inevitable surgical induced tissue damage that may activate mast cells via the local release of Damage Associated

Molecular Pattern Molecules, IL-1, reactive oxygen species and complement fragments such as C3a and C5a.(46) Interestingly, Pamuk *et al.* recently investigated the ability of a Syk inhibitor to protect mice against mesenteric ischemia-reperfusion induced injury and found that both local and remote lung injury were reduced with a significant reduction of leukocytic infiltration,(47) suggesting the use of Syk inhibitors in the suppression of tissue damage evoked inflammatory response. The effect of GSK143 on resident macrophages or peritoneal mast cells should be further evaluated by studying the levels of Syk phosphorylation. Nonetheless, our data indicate that GSK143 inhibits mast cell and macrophage activation, and consequently prevents the inflammatory cascade leading to POI.(12;42-44)

Clinical studies have shown that Syk inhibitors are efficient in allergy,(48) immune thrombocytopenic purpura,(49) B-cell lineage malignancies and autoimmune diseases like rheumatoid arthritis.(41;50) Importantly, these compounds were mostly well tolerated,(48) and not associated with serious side effects.(41;47;49-51) Syk inhibition in patients with rheumatoid arthritis for 6 months was reported to be associated with the development of elevated blood pressure, mild neutropenia and gastrointestinal adverse effects such as gastritis and nausea.(41) Although these side effects may develop after prolonged use of Syk inhibitors, it is suspected that even milder or no side effects will develop in postoperative patients who require shorter treatment. This is in line with our experimental model where one single dose of GSK143 prior surgery was able to prevent delay in gastrointestinal transit and to significantly reduce intestinal inflammation.

In conclusion, our study showed that GSK143 inhibited degranulation of peritoneal mast cells and reduced the expression of inflammatory cytokines by macrophage. In addition, Syk inhibition improved intestinal inflammation and intestinal transit, suggesting that GSK143 can be a useful tool to treat POI.

Figure Legends

Fig. 1: GSK143 prevented manipulation-induced delayed gastrointestinal transit.

(A) Effect of three times administration of placebo (vehicle of GSK143; black bar), GSK143 (0.1 mg/kg - 10 mg/kg; grey bars) or DOX (10 mg/kg; diagonally striped bar) on gastrointestinal transit 24 h after intestinal manipulation. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was significantly increased in the doxantrazole treated group, and GSK143 treated mice at a concentration of 1 and 3 mg/kg. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; * $P < 0.05$ for GSK143 or DOX versus Placebo; ** $P < 0.001$ for GSK143 versus Placebo. Data are expressed as mean \pm SEM. Placebo: $n = 7$, GSK143: $n = 5-8$ per group, DOX: $n = 6$.

Fig. 2: GSK143 reduced manipulation-induced recruitment of myeloperoxidase positive cells in the muscularis externa.

(A) Representative images of myeloperoxidase (MPO) positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation for the different experimental groups. Effect of three times administration of placebo, GSK143 (0.1 mg/kg - 10 mg/kg) or DOX (10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Scale bar is 100 μm .

(B) Effect of three times administration of placebo, GSK143 (0.1 mg/kg - 10 mg/kg) or DOX (10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; * $P < 0.05$, *** $P < 0.001$ for GSK143 or DOX versus Placebo. Dots represent individual mice.

Fig. 3: GSK143 reduced manipulation-induced production of inflammatory cytokines and recruitment of immune cells in the muscularis externa. (A) Cytokine production in the muscle layer of the small

intestine 24 h after intestinal manipulation and treatment three times with placebo (black bars), GSK143 (1 mg/kg; grey bars) or doxanztrazole (DOX 10 mg/kg; diagonally striped bars). IL-1 β and CCL-2 levels were significantly ameliorated by GSK143. The Kruskal-Wallis test was performed to identify statistical differences between the groups. The Mann-Whitney U test was used to compare Placebo versus GSK143 and Placebo versus DOX. Data are expressed as mean \pm SEM. * P < 0.05 (IL-1 β : P = 0.0140; CCL-2: P = 0.0401), GSK143 versus Placebo. Placebo: n = 7, GSK143: n = 8 per group, DOX: n = 6.

(B) Immune cell recruitment in the muscle layer of the small intestine 24 h after intestinal manipulation and treatment three times with placebo or GSK143 (1 mg/kg). Cells were isolated from enzymatically digested intestinal muscularis and assessed by flow cytometry. Absolute number of CD45 positive immune cells, Ly6G & CD11b positive neutrophils and F4/80 & CD11b positive monocytes were significantly reduced by GSK143 treatment. The Mann-Whitney U test was used to compare Placebo versus GSK143. * P < 0.05 and ** P < 0.01, GSK143 versus Placebo. Dots represent individual mice.

Fig. 4: GSK143 inhibited substance P (SP) and TNP induced degranulation in a concentration dependent manner. Mast cells were incubated with Tyrode's buffer (placebo, black bars), GSK143 (0.03 μ M-10 μ M; grey bars) or doxanztrazole (DOX (227 μ M); diagonally striped bar), followed by stimulation for 10 minutes with (A) 90 μ M SP or (B) 0.04 μ g/mL TNP. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to vehicle, one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. Data are expressed as mean \pm SEM of at least 3 independent experiments.

Fig. 5: GSK143 reduced cytokine expression in macrophages in a concentration dependent manner. Bone marrow derived macrophages were incubated in cultured medium alone (placebo, black bar) or with GSK143 (0.1 μ M-10 μ M; grey bars) or doxanztrazole (DOX (227 μ M, diagonally striped bars). Thirty minutes later macrophages were stimulated for 2 h with LPS (100 ng/ml) and cells were harvested for mRNA expression analysis of IL-6 (A), TNF- α (B), IL-1 β (C), and CCL-2 (D) . *** P < 0.001 GSK143

compared to vehicle, one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. Data are expressed as mean \pm SEM of at least 3 independent experiments.

Supplementary figure legends.

Supplementary fig. 1: Intestinal transit is not affected by GSK143 and doxantrazole treatment in mice that underwent laparotomy.

A. Effect of three times administration of placebo (black bar), GSK143 (0.1 mg/kg - 10 mg/kg; grey bars) or DOX (10 mg/kg; diagonally striped bar) on gastrointestinal transit 24 h after laparotomy. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was not significantly altered in doxantrazole and GSK143 treated mice. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Data are expressed as mean \pm SEM. Placebo: n = 5, GSK143: n = 5 per group, DOX: n = 5.

Supplementary fig. 2: Single dose of GSK143 prevented manipulation-induced delayed gastrointestinal transit and recruitment of inflammatory cells.

(A) Single dose treatment of GSK143 (1- 10 mg/kg; grey bars) or placebo 1.5 h before surgery on gastrointestinal transit 24 h after IM. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was significantly increased in the GSK143 treated mice at a concentration of 3 mg/kg. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; **P < 0.001 for GSK143 versus Placebo. Data are expressed as mean \pm SEM. Placebo: n = 7, GSK143: n = 7 per group.

(B) Effect of placebo, or single dose of GSK143 (1 mg/kg - 10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; * P < 0.05, **P < 0.01 for GSK143 versus Placebo. Dots represent individual mice.

Supplementary fig. 3: Blood concentration levels of GSK143 after single oral dose treatment. GSK143 blood concentration levels were assessed in mice treated with a single oral dose of GSK143 (prepared in 0.5 % Methylcellulose) at 1, 3 and 10 mg/kg or vehicle alone. Blood samples were taken at 1.5 h (prior to surgery) and at 6 h and 25.5 h post dose. The blood concentration levels were determined by

reverse phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). (A) Graph presenting the blood concentration levels obtained after a single oral dose of GSK143. (B) Data (mean \pm sd) from each group of animals surgically treated during this study at 1.5, 6 and 25.5 h post-dose.

Supplementary fig. 4: FACS plot of cultured mouse peritoneal mast cells and bone marrow derived macrophages. (A) Fc ϵ RI and CD117 expression was assessed by flow cytometry in cultured mouse peritoneal mast cells. Only more than 94% pure PMC (Fc ϵ RI+, CD117+) populations were used for the experiments. Data are representative of 3 experiments. (B) CD11b and F4/80 expression was assessed by flow cytometry mouse bone marrow derived macrophages (BMDMs) after 10 days of culture. Only more than 94% pure BMDMs (CD11b+, F4/80+) populations were used for the experiments. Data are representative of 3 independent experiments.

Supplementary fig. 5: Substance P and TNP induced concentration-dependent peritoneal mast cell degranulation. Mast cells were incubated with (A, C) substance P; 0-90 μ M and (B, D) trinitrophenyl (TNP); 0-4 μ g/mL. The β -hexosaminidase release was significantly increased by SP at a concentration of \geq 30 μ M and by TNP at a concentration of \geq 0.004 μ g/mL. Cells incubated with vehicle (Control) reflect the basal release. *** P < 0.001 compared to control, one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. Data are expressed as mean \pm SEM of at least 3 independent experiments.

Supplementary table S1. Primer sequences used for qRT-PCR.

Gene	Sense	Antisense
<i>Rpl32</i>	5'-AAGCGAAACTGGCGGAAAC-3'	5'-TAACCGATGTTGGGCATCAG-3'
<i>Tnfa</i>	5'- TCTTCTCATTCTGCTTGTGG-3'	5'- CACTTGGTGGTTTGCTACGA-3'
<i>Il18</i>	5'-GACCTTCCAGGATGAGGACA-3'	5'-TCCATTGAGGTGGAGAGCTT-3'
<i>Il6</i>	5'-CCATAGCTACCTGGAGTACATG-3'	5'-TGGAATTGGGGTAGGAAGGAC-3'
<i>Ccl2</i>	5'-CACGTGTTGGCTCAGCCAGATGC-3'	5'-CCTTCTGGGGTCAGCACAGACC-3'

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A

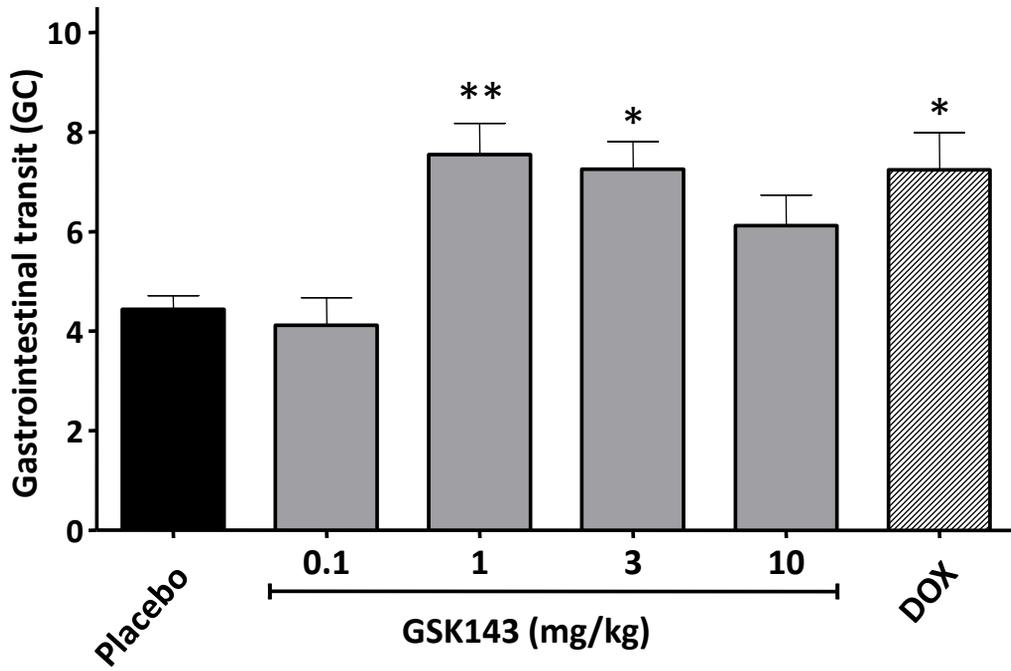
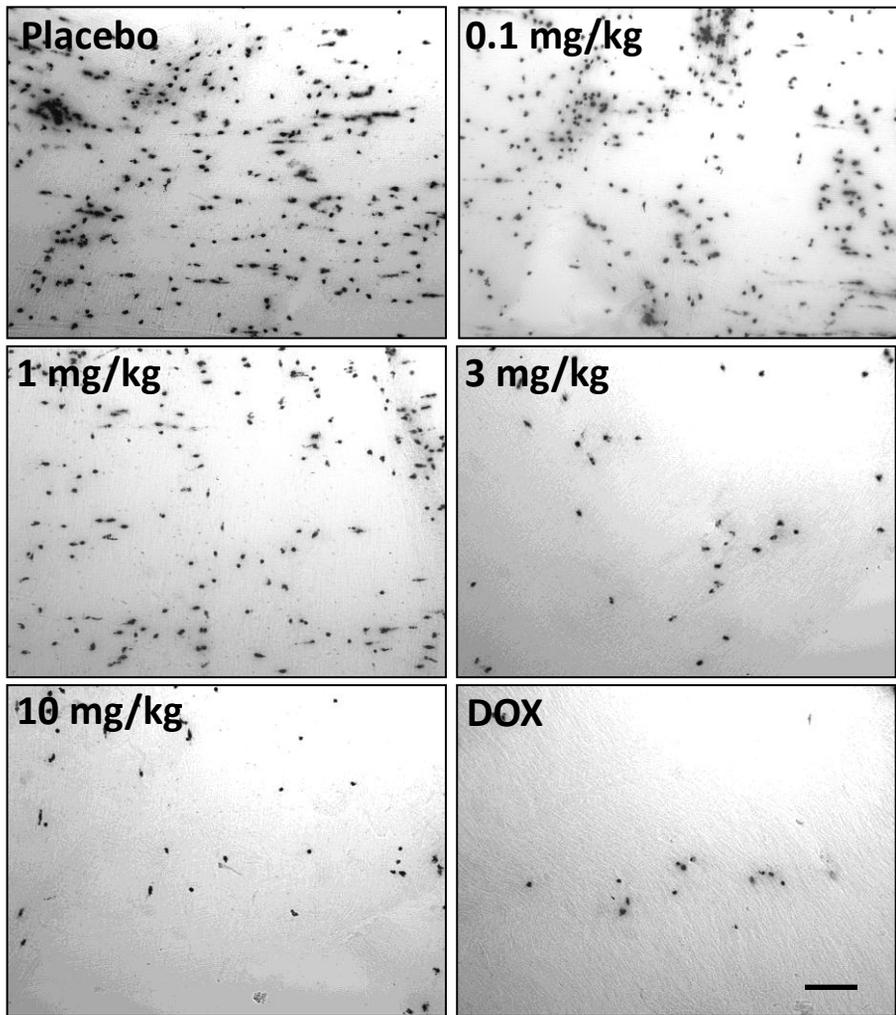


Figure 1

A



B

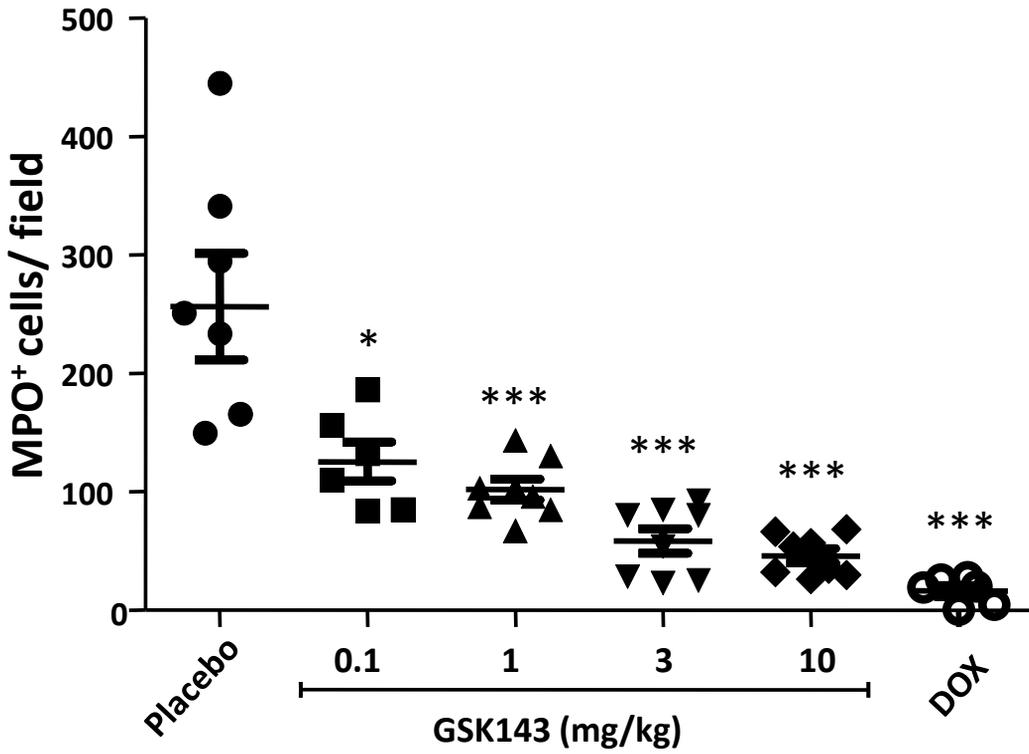


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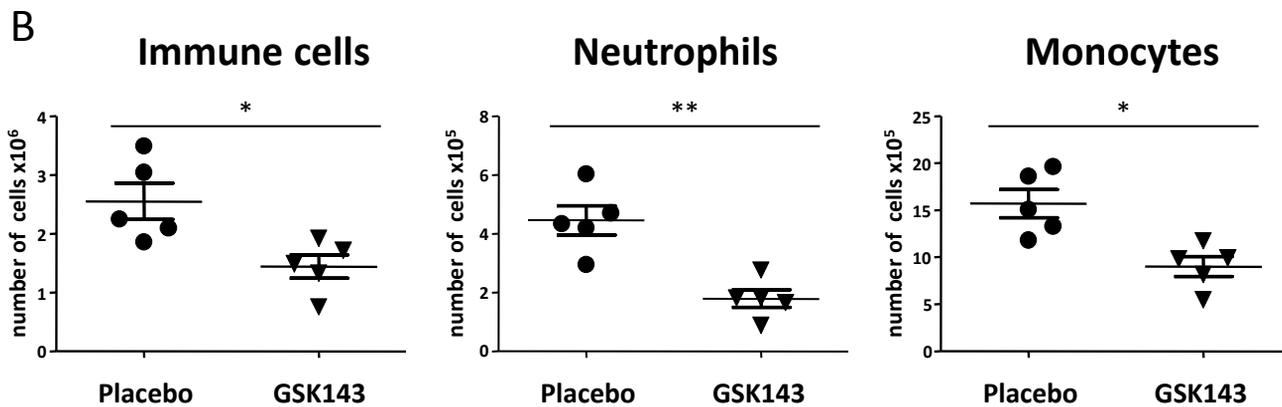
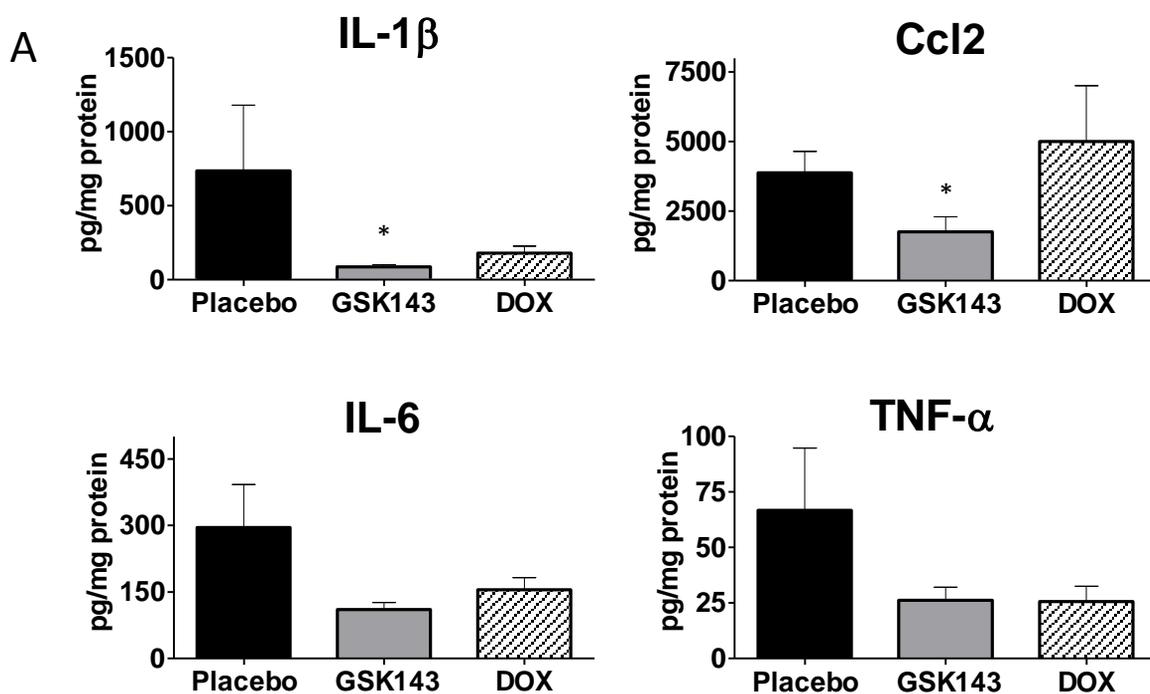


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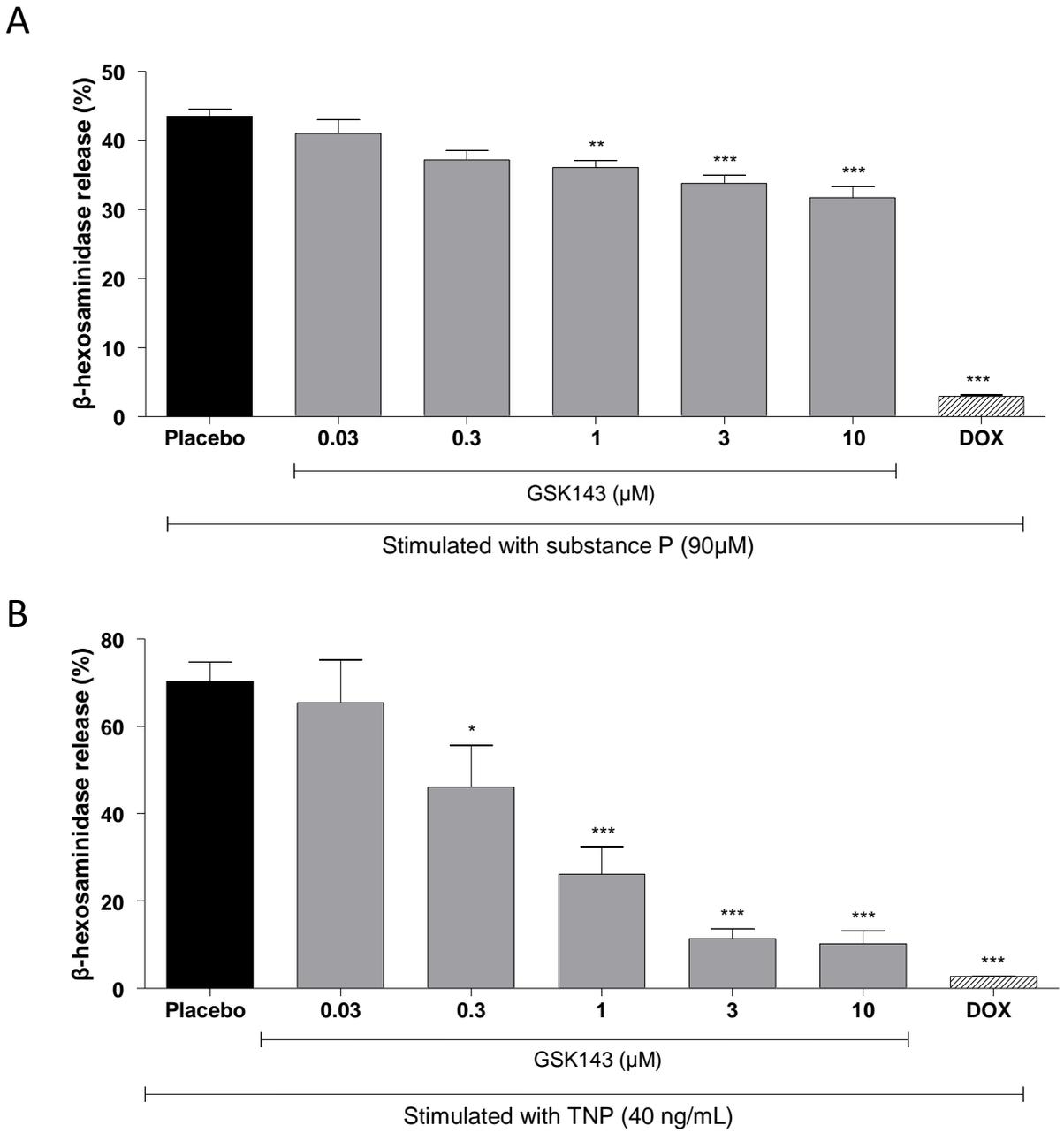


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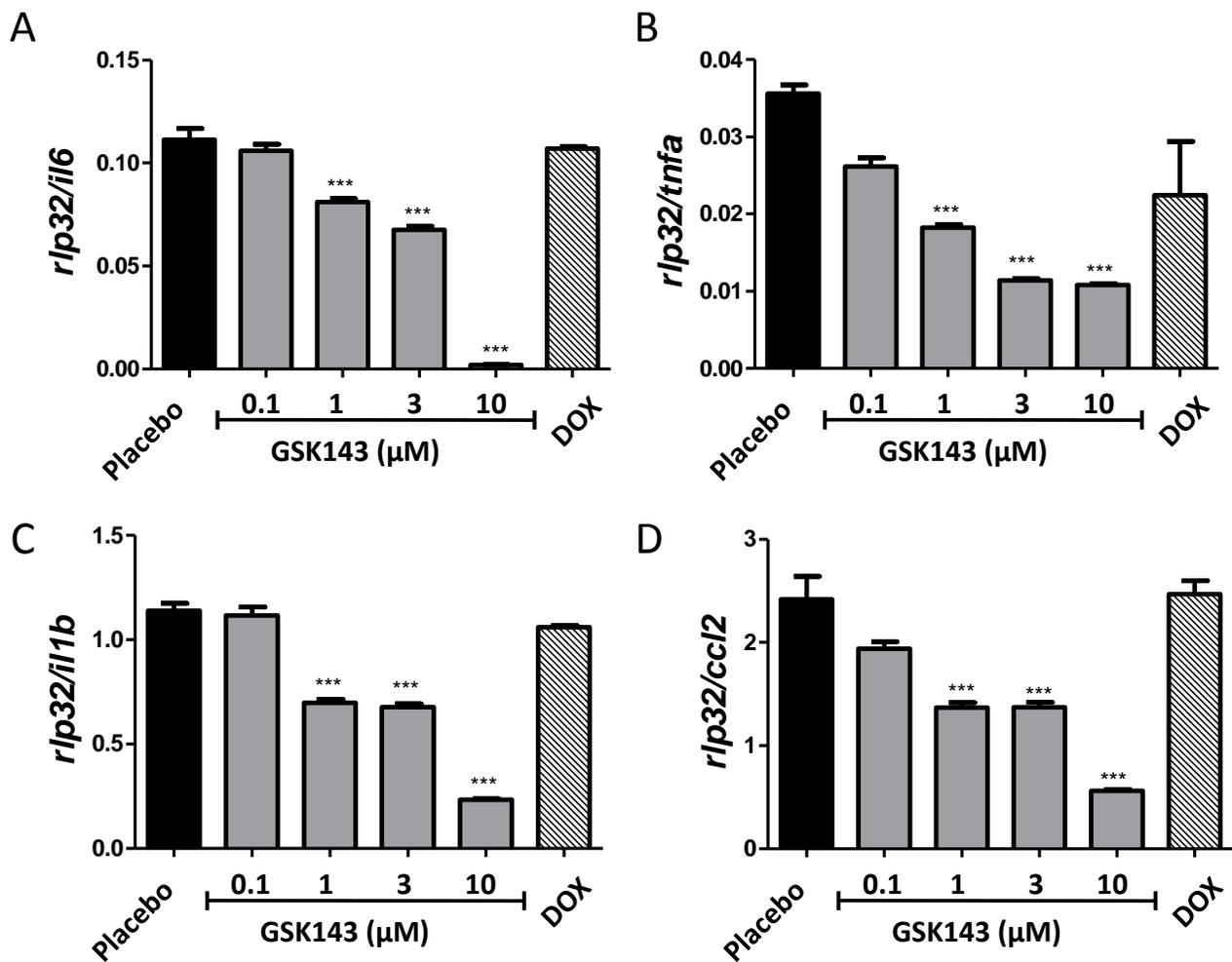
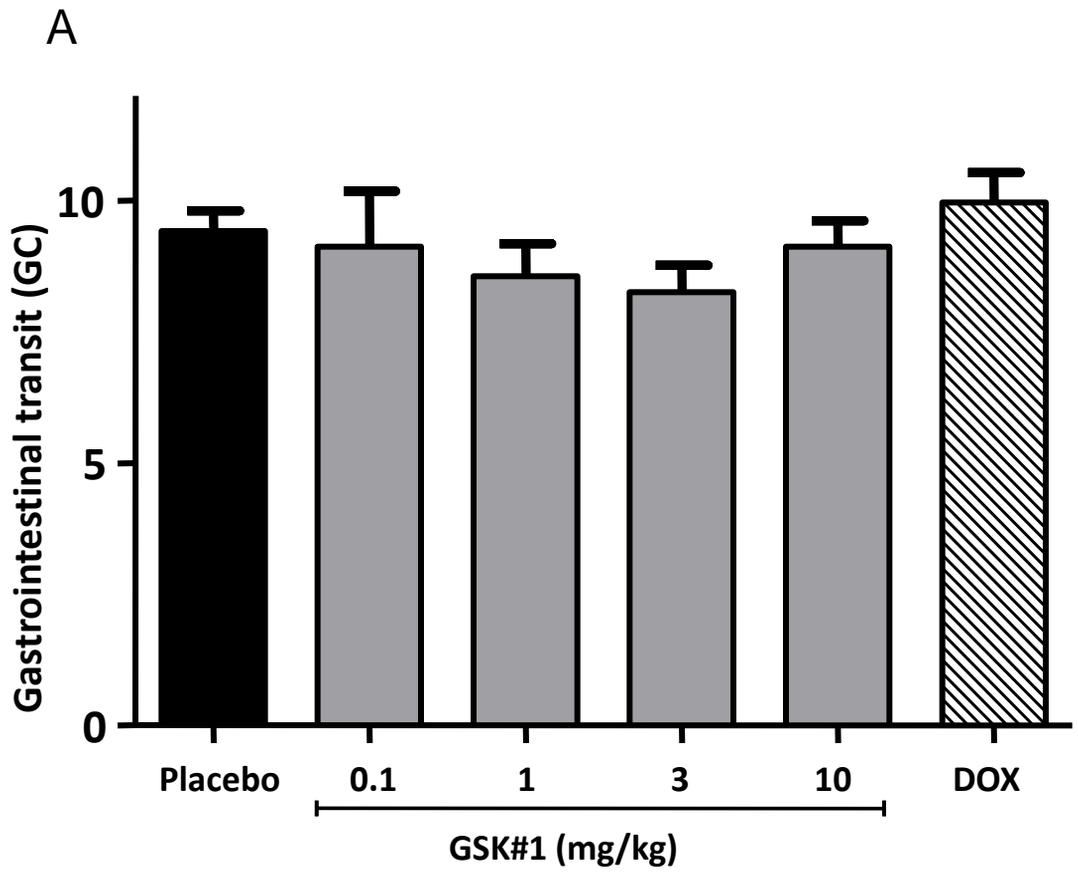
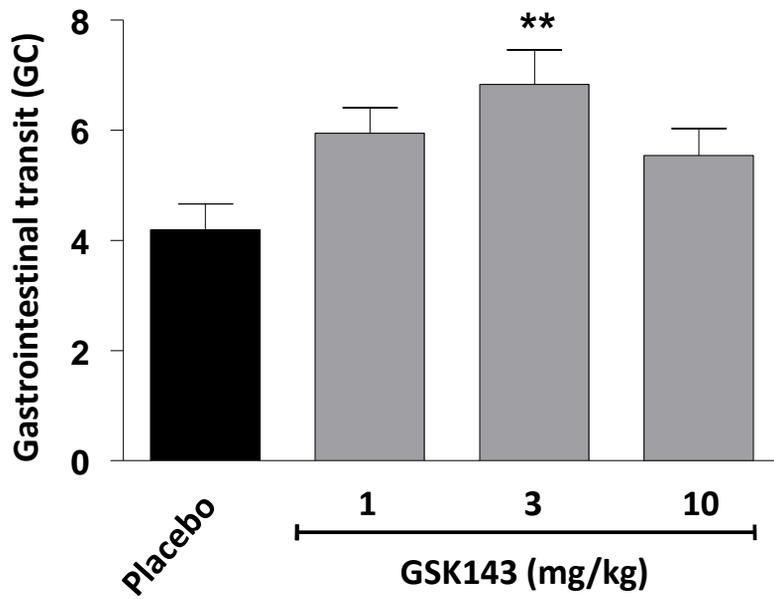


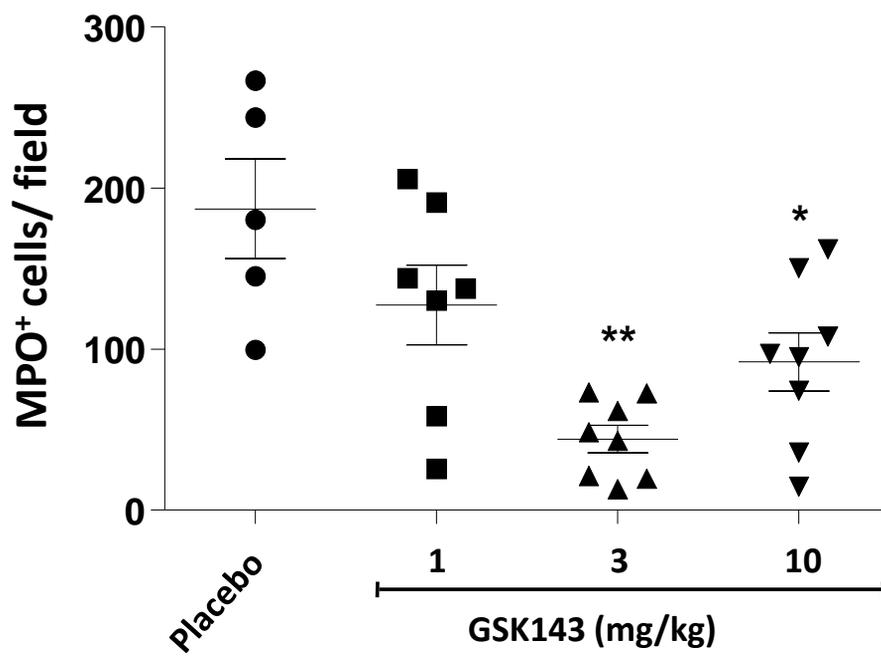
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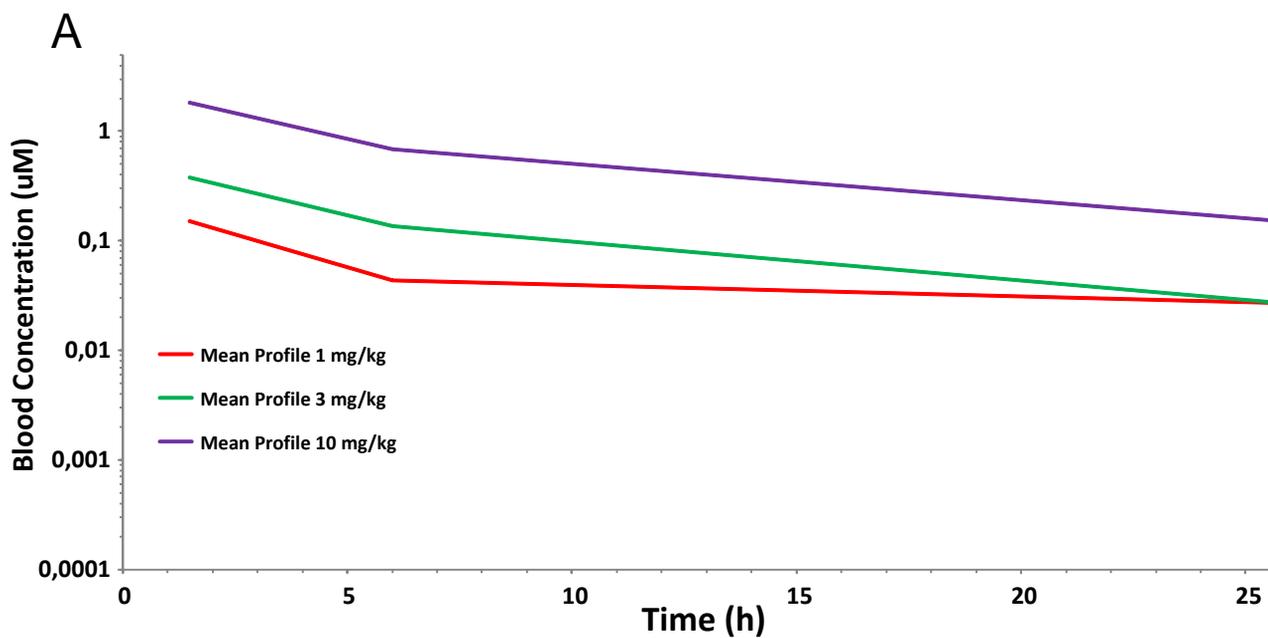
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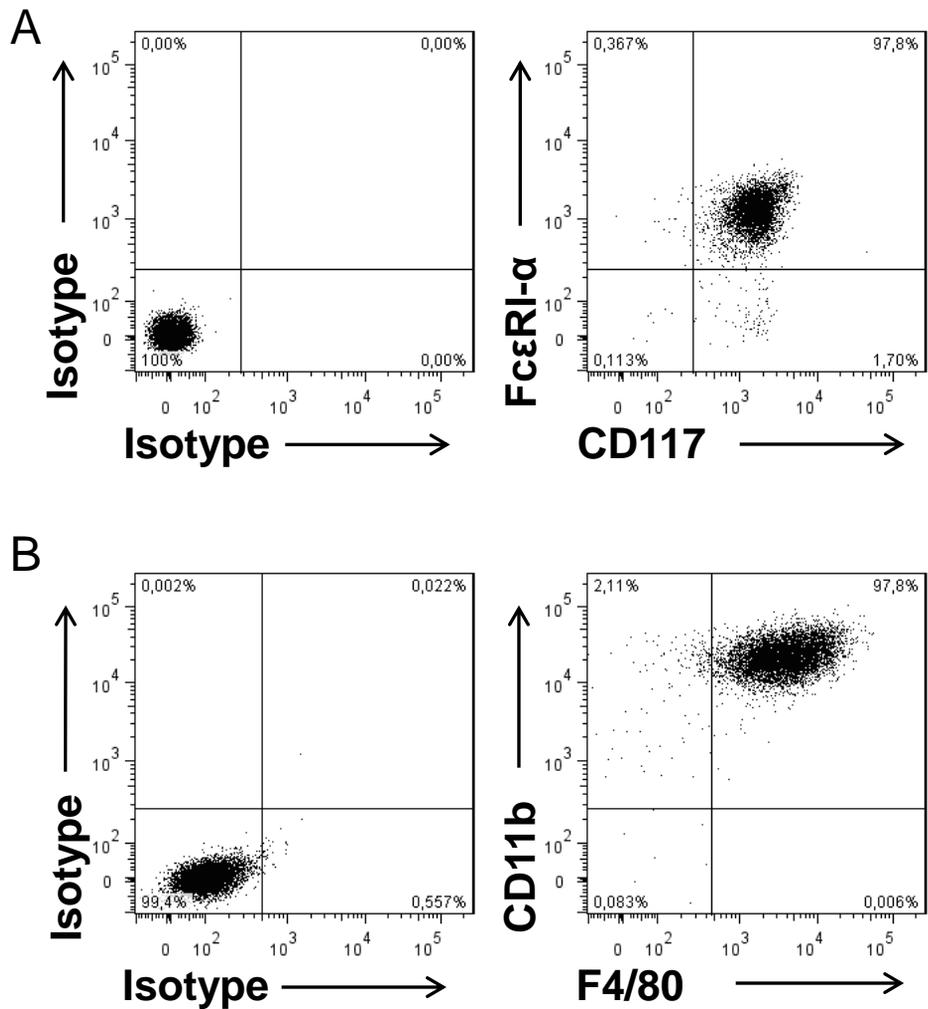
Supplementary figure 2



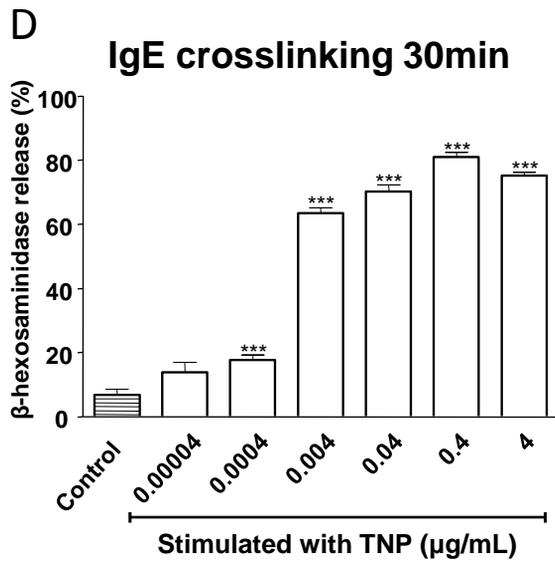
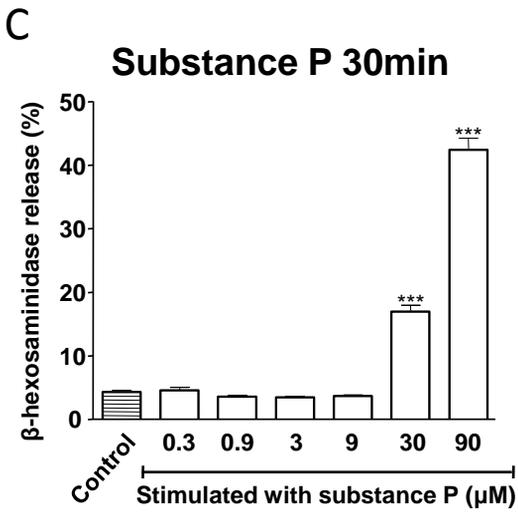
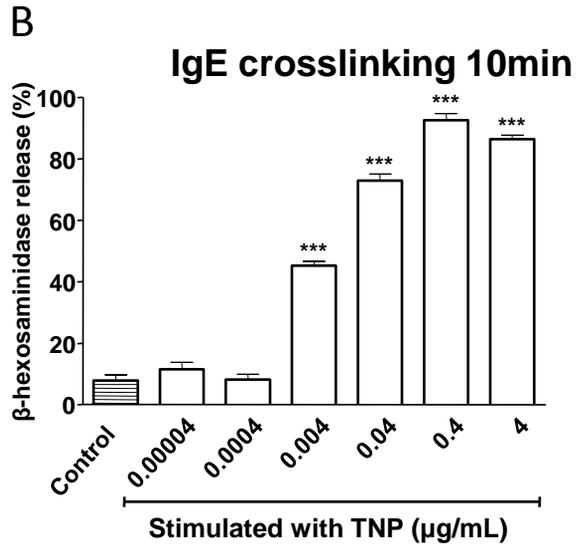
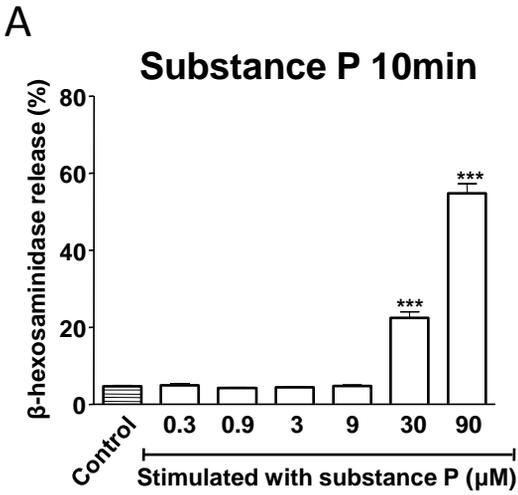
B

GSK143 Blood Concentration (uM)			
	Dose (mg/kg)		
Time (h)	1	3	10
1.5	0.15 ± 0.05	0.38 ± 0.24	1.84 ± 1.03
6	0.04 ± 0.02	0.14 ± 0.03	0.68 ± 0.44
25.5	0.03 ± 0.06	0.03 ± 0.01	0.15 ± 0.10

Supplementary figure 3



Supplementary figure 4



Supplementary figure 5