SUPPLEMENTAL MATERIALS

SOLUBLE GUANYLATE CYCLASE AGONISTS INHIBIT EXPRESSION AND PROCOAGULANT ACTIVITY OF TISSUE FACTOR

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METHODS

Cell preparation and experimental protocols

Blood sampling was performed according to the study protocol approved by the Regional Committee for Medical Research Ethics. Informed consent was obtained from six healthy volunteers of both sexes (age 24-59 yrs). Venous blood was collected using plastic syringes and 19-G needles into sterile polystyrene tubes (BD Biosciences Pharmingen, Franklin Lakes, NJ) containing heparin (Sigma-Aldrich, Munich, Germany) at a final concentration of 10 U/ml.

We utilized a whole blood system to test the potential of the soluble guanylate cyclase (sGC) agonists, BAY 41-2272 and BAY 58-2667, to attenuate tissue factor (TF) activation in monocytes stimulated with bacterial lipopolysaccharide (LPS). Sampled blood was exposed to BAY 41-2272 (1, 10, 100, or 200 μM) or BAY 58-2667 (1, 10, 50, or 100 μM) for 10 or 45 min prior to the LPS challenge. Thereafter, 1-ml aliquots of whole blood were stimulated by adding 5 ng/ml of LPS (strain 026:B6; Difco Laboratories, Detroit, MI) for 2 hrs in a rotary incubator (180 rpm) at 37^{o}C. The LPS stimulation was terminated by adding disodium ethylenediamine tetraacetic acid (EDTA; Merck, Darmstadt, Germany) at a final concentration of 5 mM. Blood was then diluted by adding one volume of sterile normal saline, carefully layered over a Lymphoprep solution (Axis-Shield, Oslo, Norway), and subjected to density centrifugation at 415 *g* for 15 min. Mononuclear cells (MNC) were washed once, counted on a Sysmex K1000 (TOA

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Medical Electronics, Kobe, Japan), pelleted, and kept frozen at –20°C until analyzed for TF procoagulant activity and protein levels.

We also studied the efficacy of both sGC agonists to inhibit TF activation in human vascular endothelium in a model of resting or tumor necrosis factor-α (TNF-α)-stimulated human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA). HUVEC were grown in a RPMI full growth medium supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, Munich, Germany) in a humidified 5% CO_2/air incubator. Forty-eight hrs after plating, the cells were starved from FCS overnight and exposed to 0.1, 1, 10, 50, or 100 μM of BAY 41-2272 or BAY 58-2667. Two hrs after exposure, HUVEC were stimulated with TNF-α (Sigma-Aldrich, Munich, Germany) at a concentration of 10 ng/ml for 4 hrs. At the end of the stimulation, the cells were harvested for further analysis of TF procoagulant activity and protein levels.

sGCα1 RNA interference

We investigated whether the effects of BAY 41-2272 and BAY 58-2667 on TF were dependent on the presence of functional sGC by performing experiments in human monocytes or HUVEC with knocked-down expression of the α1 subunit of sGC (sGCα1). Three million Lymphoprep-isolated monocytes were nucleofected with 3 μg of small interfering RNA (siRNA) with the sense sequence 5'-AUAUGUUACGAGGAAGAUG-3' using a Nucleofector II device (Lonza Cologne, Cologne, Germany) according to the manufacturer's instructions. Control monocytes were nucleofected with scrambled control siRNA. Following electrical pulse, 500 μl of the CO_2- and temperature-equilibrated nucleofection medium was immediately added to neutralize the nucleofection solution. The cells were seeded into 12-well plates and kept in a water-jacketed cell culture incubator for further use. For assessment of nucleofection efficiency in the sGCα1-silencing experiments, 1 μg of the green fluorescent protein (GFP)-encoding plasmid pGFP-C1 was used. Thereafter, monocytes were reintroduced to whole blood samples, which were previously depleted for MNC [1], and the highest effective concentrations of BAY 41-2272 or BAY

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58-2667 preceding the LPS stimulation were applied as described above. Similarly, three million HUVEC were nucleofected with siRNA, plated and treated as described above after a recovery period. The quality of the anti-TF antibody used in this study was controlled in a TF knock-down experiment, in which HUVEC were nucleofected with TF-specific siRNA with the sense sequence 5′-GCGCUUCAGGCACUACAAA-3′. Forty eight hrs after nucleofection, the cells were stimulated with TNF-α, harvested, and kept frozen for further western blotting analysis.

All reagents and materials were screened for contamination by LPS with a CoaTest according to the manufacturer's instuctions (Haemochrom Diagnostica, Frederiksberg, Denmark).

Western blotting

Pellets of isolated monocytes or harvested HUVEC were lysed in an ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were briefly sonicated and centrifuged at 10,000 *g* for 15 min at 4^{o}C. Supernatants were mixed with a SDS sample buffer, electrophoresed on 10% polyacrylamide gels and electroblotted onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked in 5% skimmed milk and probed with a mouse anti-human TF monoclonal antibody at a 1:1,000 dilution (clone TF9 10H10; Calbiochem, San Diego, CA) and a HRP-conjugated goat anti-mouse antibody at a 1:2,000 dilution (BD Biosciences Pharmingen, Franklin Lakes, NJ). Detection and quantification of immunopositive bands were performed using a LumiImager F1 and LumiAnalyst software (Boehringer Mannheim, Mannheim, Germany). Stripping of membranes was done by incubation in 0.2 M NaOH for 10 min before washing and re-blocking with 5% skimmed milk. Protein loading was determined by probing the membranes with a rabbit anti-human β-actin polyclonal antibody at a 1:2,000 dilution (Calbiochem, San Diego, CA) and a HRP-conjugated goat anti-rabbit antibody at a 1:2,000 dilution (BD Biosciences Pharmingen,

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Franklin Lakes, NJ). The efficacy of sGCα1 silencing was ascertained by immunoblotting lysates of monocytes or HUVEC with a monoclonal anti-sGCα1 antibody (Abcam, Cambridge, MA), and the expression of TF protein was used as a readout in the sGCα1-knock-down experiments. Levels of IκBα in monocytes and HUVEC were measured by using a mouse anti-human IκBα antibody (Cell Signaling Technology, Danvers, MA).

In-Cell Western assay

We utilized the In-Cell Western assay to characterize total and surface TF levels in monocytes. This assay allows comparison of levels of the antigen of interest in their cellular context in plated fixed cells [2]. Following treatments, monocytes were prepared as described above, plated for 30 min on a 96-well plate in RPMI medium, fixed with 4% paraformaldehyde, permeabilized with methanol to evaluate total (intracellular and surface) TF or left intact to evaluate only surface TF, blocked with 3% goat serum, and immunostained with anti-TF (Calbiochem, San Diego, CA) and anti-glyceraldehydephosphate dehydrogenase (GAPDH; Sigma-Aldrich, Munich, Germany) antibodies. The signal from the mouse anti-human TF monoclonal antibody was detected with IRDye800CW-conjugated goat anti-mouse polyclonal antibodies (1.25 μg/ml; Rockland Immunochemicals, Rockland, PA). The signal from the rabbit anti-GAPDH polyclonal antibody was detected with Alexa680-conjugated goat anti-rabbit polyclonal antibodies (1 μg/ml; Invitrogen, Carlsbad, CA). After washing, images of Alexa680 and IRDye800CW fluorescence were obtained on 700-nm and 800-nm channels of an Odyssey infrared imager (LI-COR Biosciences, Bad Homburg, Germany). For statistical analysis, integrated intensities of fluorescence in wells were processed using software provided with the imaging station.

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TF procoagulant activity

TF was measured in the intact cells and in frozen/thawed preparations of monocytes using a two-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as previously described [3].

Flow cytometry

Following completion of treatments, HUVEC were harvested, fixed with 4% paraformaldehyde, blocked with 3% goat serum and immunostained with an Alexa-conjugated (Alexa-488 Protein Labeling Kit; Invitrogen, Carlsbad, CA) mouse anti-human TF monoclonal antibody (Calbiochem, San Diego, CA) at a concentration of 10 μg/ml for 45 min on ice. Simultest γ2a/γ1 (BD Biosciences Pharmingen, Franklin Lakes, NJ) was used as an isotype control. After immunostaining, cells were washed once, re-suspended in PBS with 0.1% BSA (Sigma-Aldrich, Munich, Germany) and kept on ice until further analysis.

Flow cytometry was performed using a FACSCalibur flow cytometer calibrated with Calibrate™ beads (BD Biosciences, Franklin Lakes, NJ). Background fluorescence for isotype control was set at less than 2% of the stained cells. The fluorescence intensities of at least 5,000 events were recorded. Data acquisition and analysis were done using a CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Real time RT-PCR

Total RNA was isolated from cell pellets using an RNeasy Plus Mini Kit (Qiagen Norge, Oslo, Norway) followed by complementary DNA (cDNA) synthesis with High-capacity cDNA Reverse Transcription Kit. Real-time PCR was performed on a 7900HT Fast Real-Time PCR System using TaqMan Fast Universal PCR MasterMix. Tissue factor mRNA expression was analyzed by using forward primer 5'-CCCCAGAGTTCACACCTTACCT-3', a reverse primer 5'-CACTTTTGTTCCCACCTGTTCA-3', and a probe 6-FAM-5'-

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AGACAAACCTCGGACAGCCAACAATTCA-3'-BHQ-1. For assessment of an irrelevant housekeeping gene, we analysed an expression of cyclophilin using forward 5'-GTACTATTAGCCATGGTCAACCCC-3' and a reverse 5'-CAGTCAAAGGAGACGCGGCC-3' primer, and a probe 6-FAM-5'-AGACAAACCTCGGACAGCCAACAATTCA-3'-BHQ-1. The results were analyzed on a Sequence Detection System (v2.2.1). All reagents for reverse-transcriptase reaction and real-time PCR were purchased from Applied Biosystems (Foster City, CA).

Nuclear factor-kappa B (NF-κB) reporter gene assay

Five million MNC were isolated from fresh whole blood by the Lymphoprep density centrifugation and nucleofected with 3 μg of plasmid DNA. The pNF-κB-conA-LUC reporter plasmids (Clontech Laboratories, Mountain View, CA) feature a firefly luciferase gene driven by a promoter sensitive to NF-kB [4]. Nucleofection efficiency was monitored by using pCMV-bgal plasmid constitutively expressing β-galactosidase. The Amaxa Nucleofector II device was used together with a Human Monocyte Nucleofector kit according to the manufacturer's instructions for plasmid DNA delivery (Lonza Gologne, Cologne, Germany). Similarly, pNF-κB-conA-LUC and pCMV-bgal plasmids were nucleofected into HUVEC using the Amaxa Nucleofector II together with a HUVEC nucleofector kit (Lonza Gologne, Cologne, Germany).

After nucleofection, the cells were seeded into 24-well plates and kept in a 5% CO2/air incubator at 37°C. Following a 6-hr stabilization period, the cells were treated with the highest effective concentrations of BAY 41-2772 or BAY 58-2667 for 45 min prior to stimulation with LPS or for 2 hrs prior to stimulation with TNF-α. Thereafter, the cells were harvested into the lysis buffer. Luciferase and β-galactosidase activities were assessed by a dual light reporter gene assay kit (Tropix; Promega, Madison, WI) on a Lumoskan RS microplate reader (Labsystems Oy, Vantaa, Finland).

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Measurements of intracellular cGMP concentrations

Concentrations of cGMP in lysates of MNC and HUVEC were quantified using a cGMP enzyme-immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Briefly, following various treatments, one million cells were harvested and centrifuged at 100 *g* for 10 min at 4°C. The pellets were re-suspended in 250 µl of a lysis reagent and shaken for 10 min at room temperature. Following centrifugation at 1,000 *g* for 3 min at 4°C to remove the debris, cell lysates were used for the cGMP assay. Intracellular cGMP content was expressed in femtomoles per one million cells.

Assessment of cell viability

Viability of MNC and HUVEC after various treatments was assayed by flow cytometry analysis of propidium iodide (PI) uptake by cells as previously described [5]. Briefly, upon completion of treatments one million MNC or HUVEC were washed once in ice-cold PBS and transferred to 1 ml of PBS containing freshly dissolved PI at final concentration of 20 µg/ml. Cell were incubated for 20 min at 37^{o} C, then washed once in PBS, and the PI-positive population was analyzed using the FACSCalibur cytometer (BD Biosciences, Franklin Lakes, NJ). Cells were gated from the debris using a forward scatter channel against a side scatter channel acquisition, and fluorescence of PI was recorded on the FL2 channel. A sample of cells, not stained with PI, was used to establish a PI-negative cell population. Data acquisition and analysis were done using the CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Data analysis

Each experiment was performed at least four times. Samples, where applicable, were assayed in triplicates. Data are expressed as mean ± SEM. The treatment effects were tested by ANOVA

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followed by a Holm-Sidak *post hoc* test (SigmaStat 3.0; Systat Software, Richmond, CA). Probability values <0.05 were considered statistically significant.

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. BAY 41-2272 inhibits the LPS-induced surface and total TF expression in human monocytes. Plots illustrate dose-dependent reductions in surface (**A** and **C**) and total (**B** and **D**) TF protein expression in the LPS-stimulated monocytes after pretreatment with BAY 41-2272 for 10 or 45 min. AU, arbitrary units. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure II. BAY 58-2667 inhibits the LPS-induced surface and total TF expression in human monocytes. Plots illustrate dose-dependent reductions in surface (**A** and **C**) and total (**B** and **D**) TF protein expression in the LPS-stimulated monocytes after pretreatment with BAY 58-2667 for 10 or 45 min. AU, arbitrary units. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure III. BAY 41-2272 and BAY 58-2667 inhibit the LPS-induced TF mRNA expression in human monocytes. Plots illustrate dose-dependent reductions in the LPS-induced TF mRNA levels when whole blood aliquots were pre-treated with BAY 41-2272 (**A** and **C**) or BAY 58-2667 (**B** and **D**) for 10 or 45 min prior to LPS. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure IV. BAY 41-2272 inhibits TF expression and functional activity in resting human endothelial cells. TF-Alexa488 mean fluorescence intensities (MFI) (**A**), populations of TF-presenting cells (**B, C**), TF protein expression (**D**), and TF procoagulant activity (**E**) in resting HUVEC treated with BAY 41-2272 for 6 hrs. Plot on the panel D represents changes in the TF band densities relative to non-treated cells. WB, western blotting. Data are mean ± SEM. * P < 0.05 vs. non-treated cells.

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Supplemental Figure V. BAY 58-2667 inhibits TF expression and functional activity in resting human endothelial cells. TF-Alexa488 mean fluorescence intensities (MFI) (**A)**, populations of TF-presenting cells (**B**, **C**), TF protein expression (**D**), and TF procoagulant activity (**E**) in resting HUVEC treated with BAY 58-2667 for 6 hrs. Plot on the panel D represents changes in the TF band densities relative to non-treated cells. WB, western blotting. Data are mean ± SEM. * $P < 0.05$ vs. the untreated cells.

Supplemental Figure VI. Intracellular cGMP concentrations in monocytes and HUVEC. Stimulation of monocytes (**A**) and HUVEC (**B**) nucleofected with control siRNA with LPS and TNF-α, respectively, increased intracellular cGMP levels. Pretreatment of control monocytes and HUVEC with BAY 41-2772 or BAY 58-2667 prior to stimulation with LPS or TNF-α further increased intracellular cGMP concentrations. However, no increase in intracellular cGMP levels occurred in cells nucleofected with siRNA against sGCα1. * $P < 0.05$ vs. resting cells; † $P < 0.05$ vs. the LPS- or TNF-α-stimulated cells.

Supplemental Figure VII. Fractions of apoptotic cells. Rates of apoptosis in monocytes (**A-D**) and HUVEC (**E, F**) following different treatments including BAY 41-2272, BAY 58-2667, LPS or TNF-α.

Supplemental Figure VIII. Specificity of the anti-TF antibody. Specificity of the anti-TF antibody was confirmed by the loss of TF immunopositive band in a sample of HUVEC transfected with anti-TF siRNA. Plot represents changes in the TF band density relative to cells nucleofected with control siRNA. WB, western blotting.

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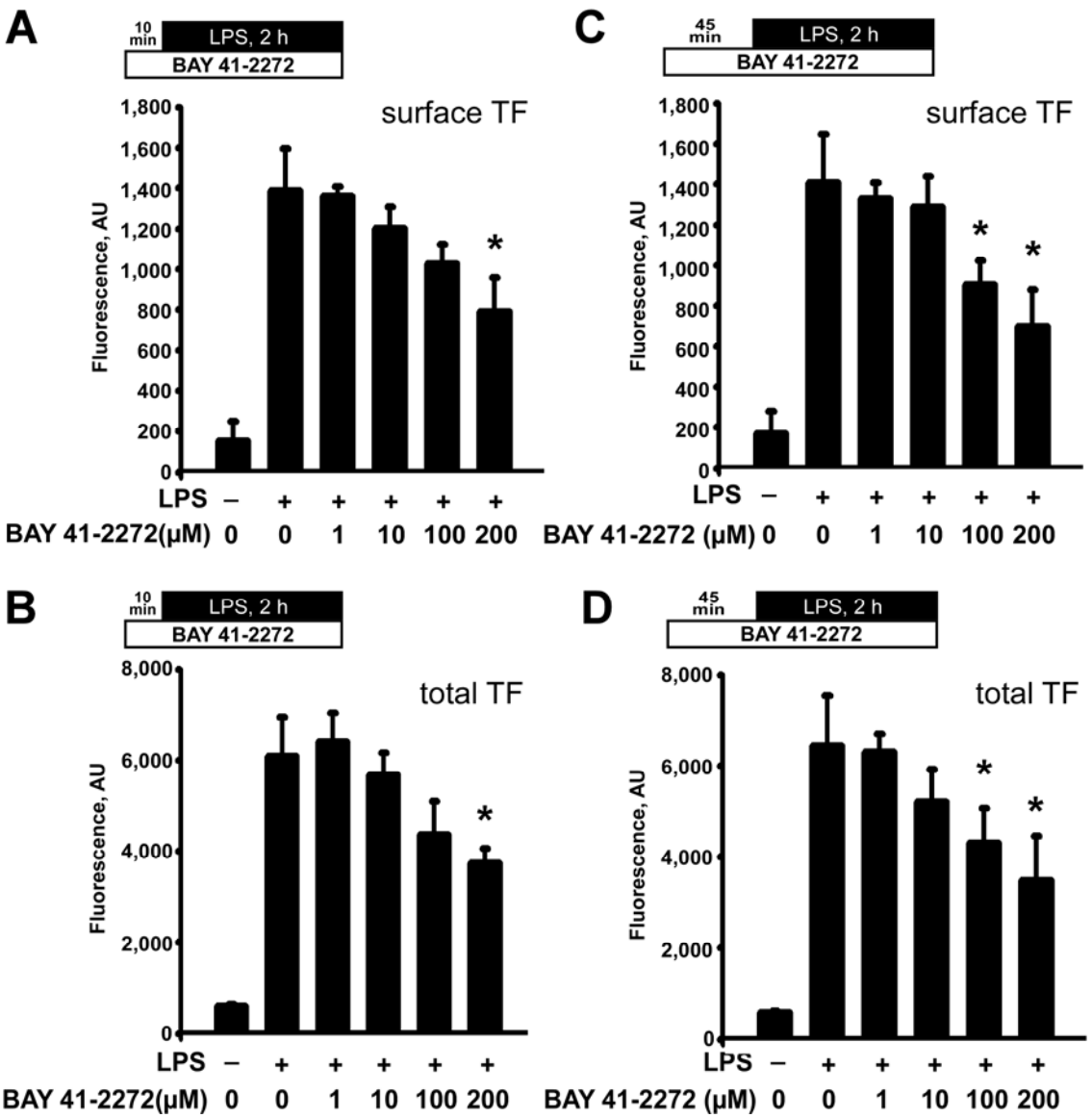

Supplemental Figure I.

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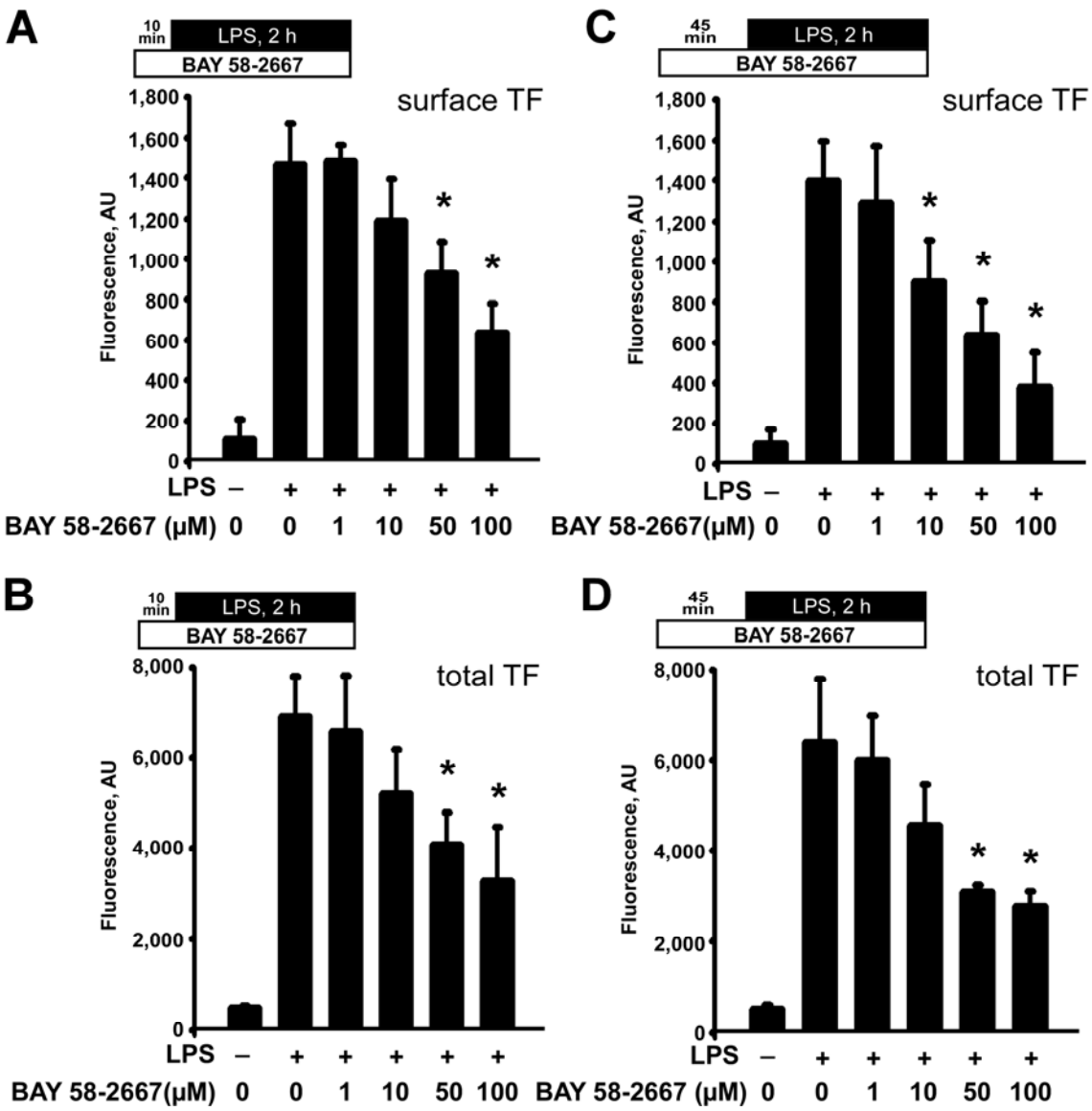

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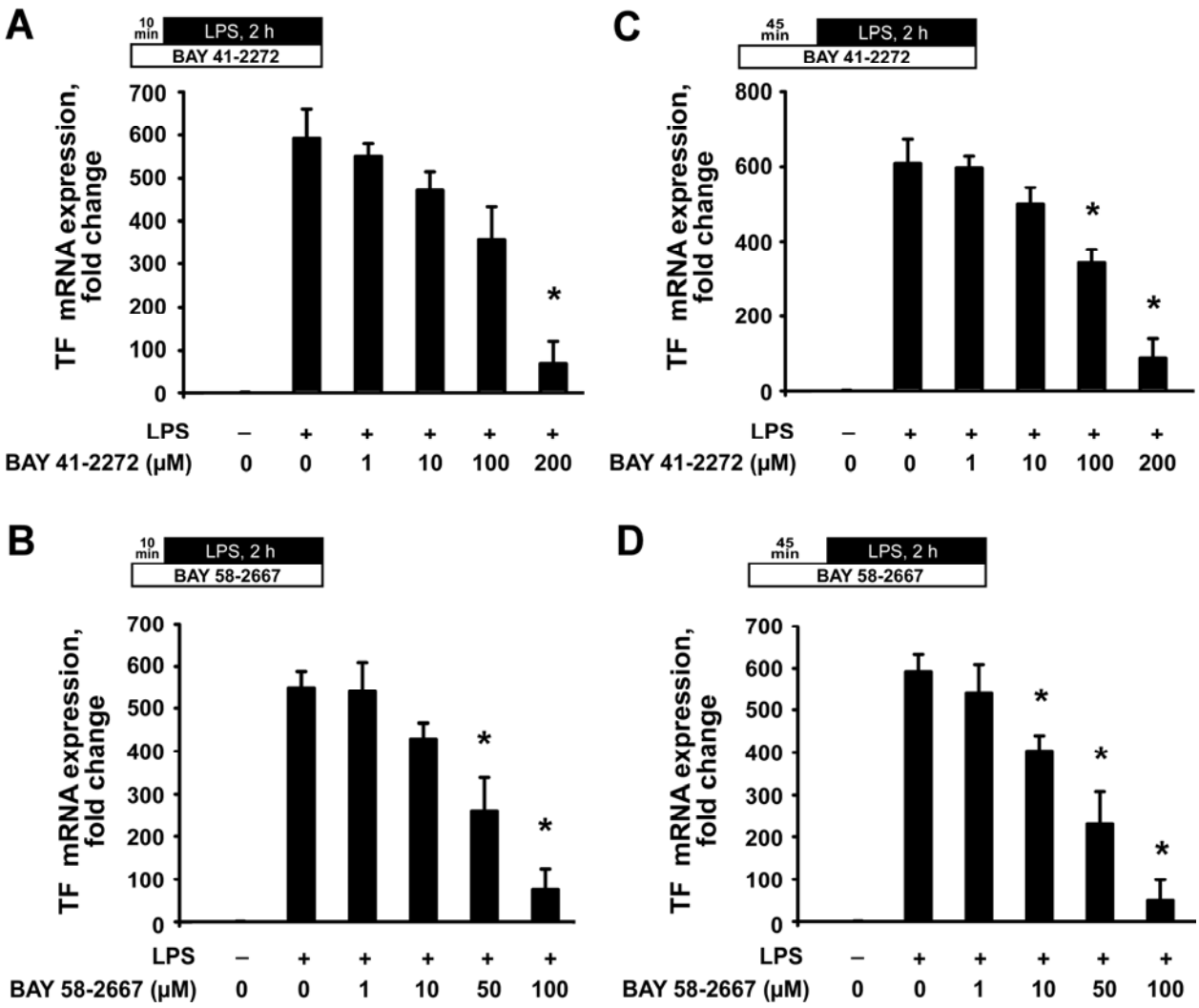

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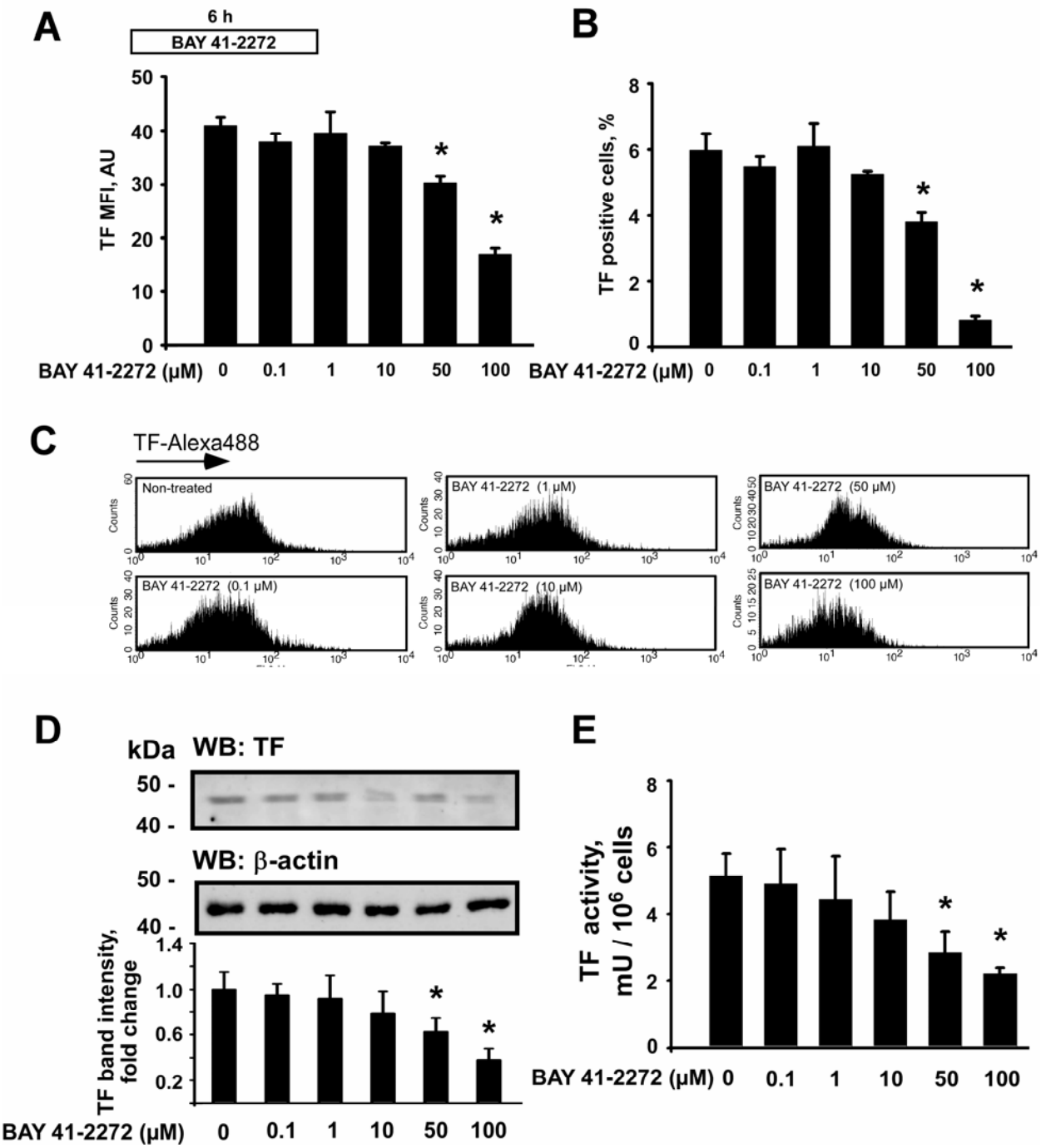

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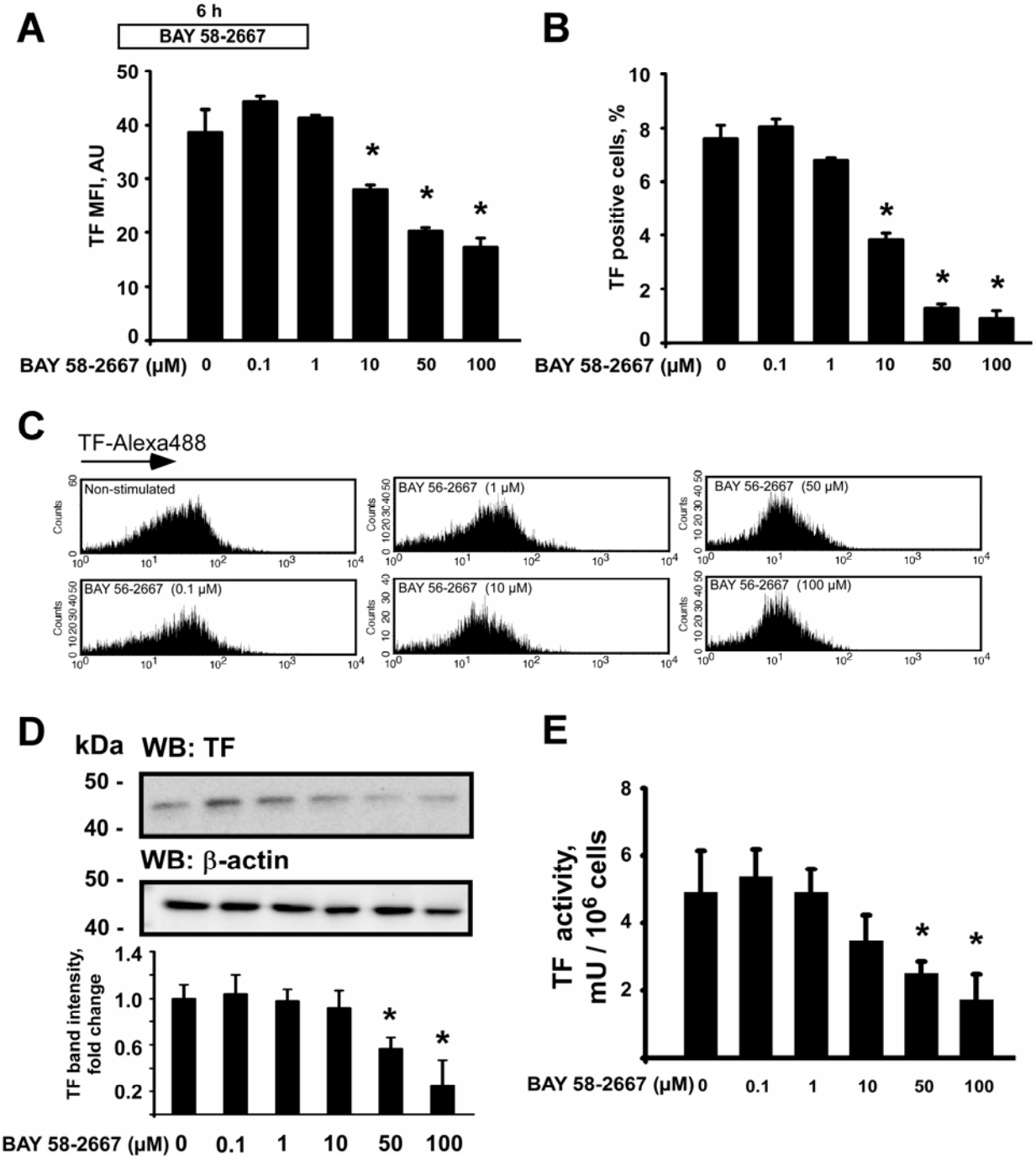

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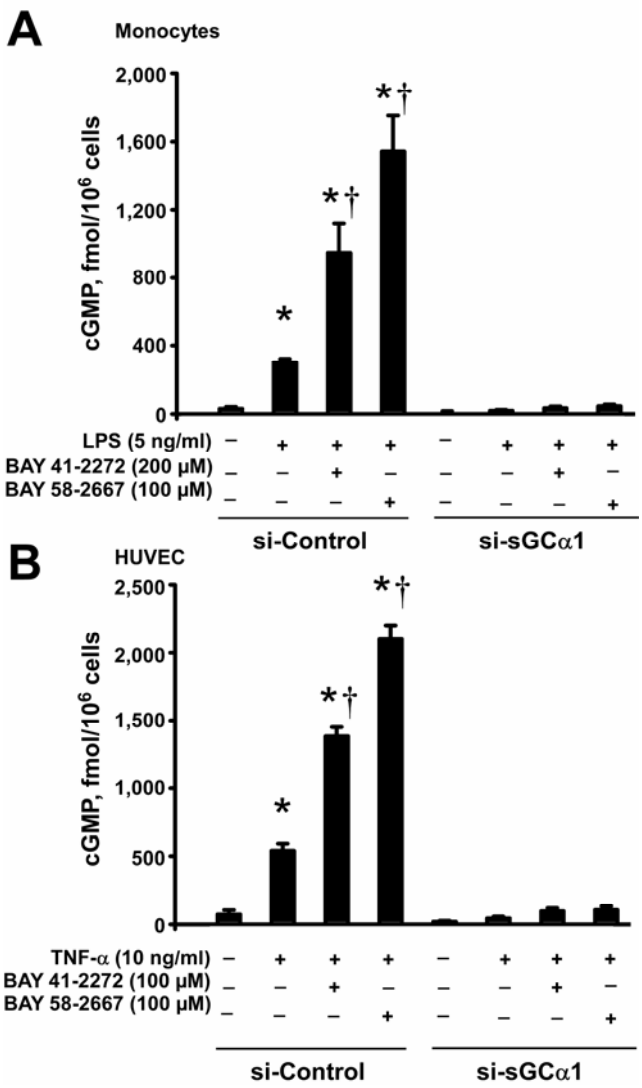

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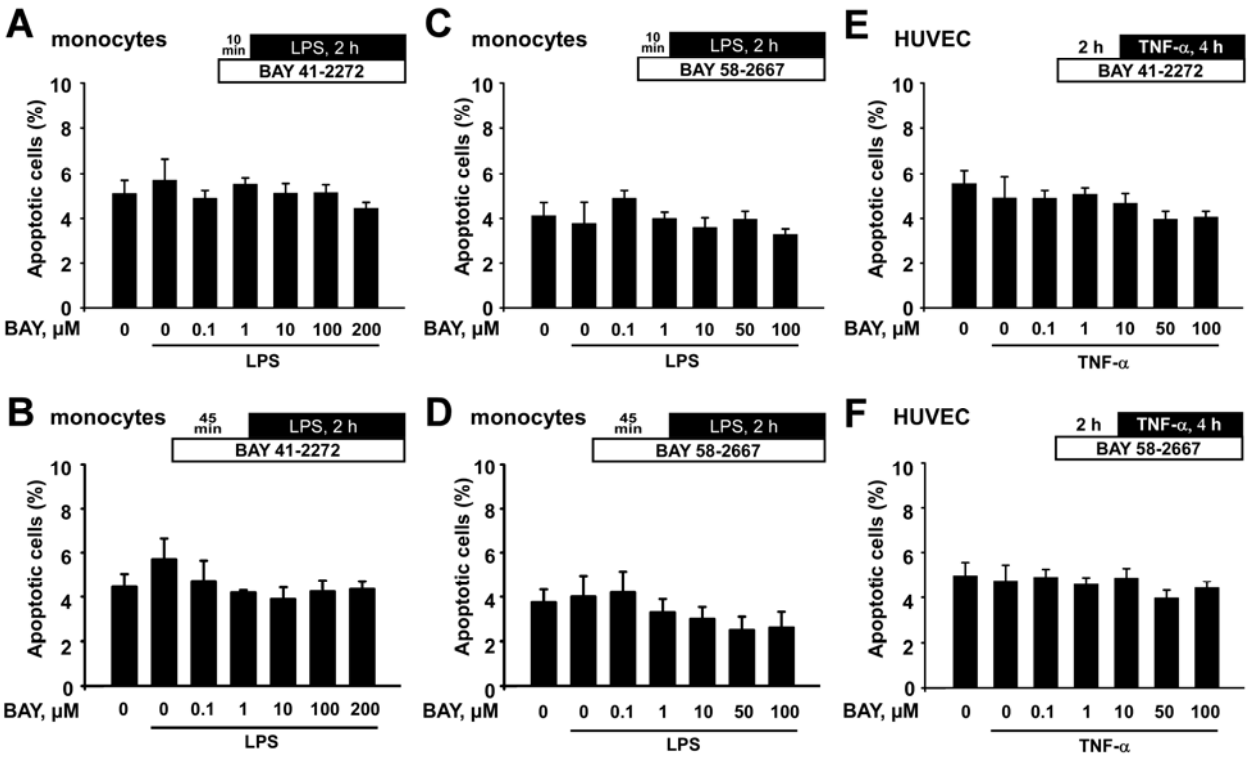

Supplemental Figure VII.

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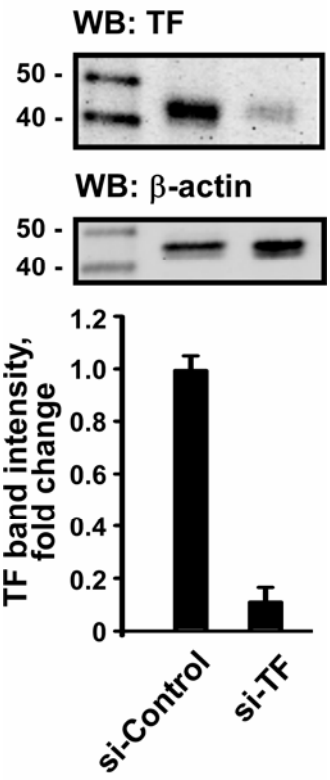

Supplemental Figure VIII.

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