

## Supplementary material

# **The adhesion G protein-coupled receptor GPR97/*ADGRG3* is expressed in human granulocytes and triggers antimicrobial effector functions**

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## SUPPLEMENTARY MATERIALS AND METHODS

### **Antibody neutralization assay**

GFP-positive HEK-293T cells transfected with pReceiver–GPR97–GFP (GeneCopoeia, MD, USA) were sorted and used for subsequent analysis. Cells were blocked for 1 h in cold blocking buffer (1% BSA, 5% normal goat serum in PBS), followed by incubation with GPR97-A mAb (5 µg/ml) with or without mFc or GPR97–mFc (20 µg/ml) at 4°C for 1 h. Cells were then washed extensively and incubated for 1 h with Alexa Fluor 647-conjugated goat anti-mouse IgG (2 µg/ml; Thermo Fisher) in blocking buffer. Cells were washed again extensively and subjected to analysis by flow cytometry.

### **Adhesion assay**

Isolated PMNs ( $1 \times 10^6$  cells/ml) were suspended in RPMI and incubated with immobilized control IgG1, G97-A mAb (10 µg/ml), or PMA (2 nM; Sigma-Aldrich) at 37°C for 1 h. Following gentle washing with PBS, cells were incubated with WST-1 (Roche) at 37°C incubator for 30 min. The levels of adherent cells were measured by the absorbance at OD<sub>440</sub>.

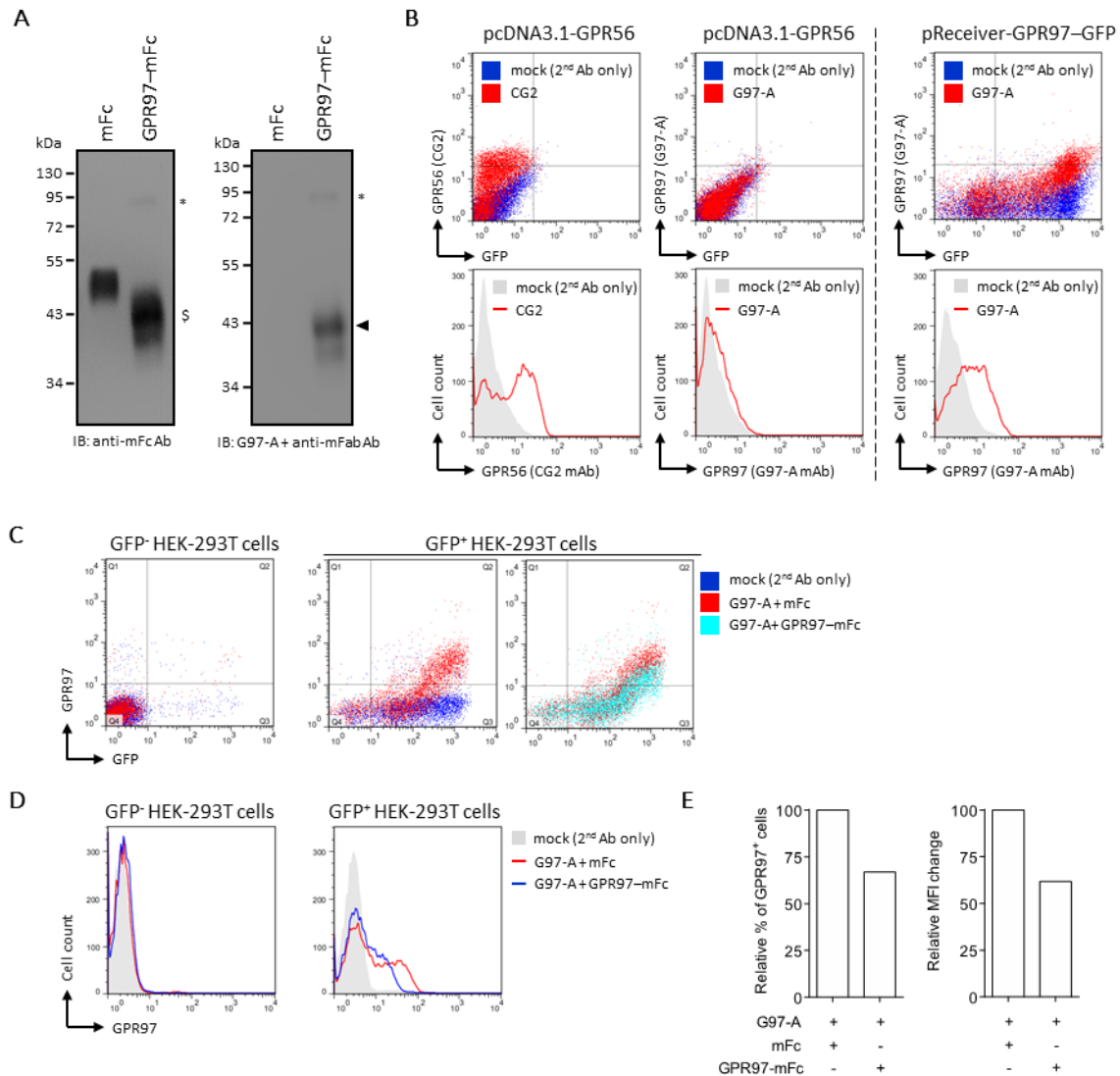
### **Apoptosis assay**

Isolated PMNs ( $2 \times 10^6$  cells/ml) suspended in RPMI were incubated with immobilized control IgG1, G97-A mAb (10 µg/ml), or LPS (200 ng/ml) for 20 h. Cell apoptosis was analyzed using the Annexin V-FITC Apoptosis Kit (Biovision, Milpitas, CA, USA) and flow cytometry.

### **Receptor internalization assay**

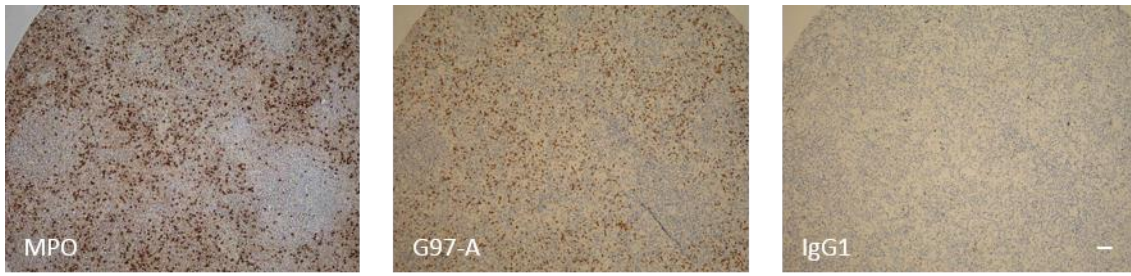
Isolated PMNs were suspended in ice-cold PBS buffer supplemented with 1% BSA and 5% normal goat serum. Cells were then incubated with Alexa Fluor 647-labeled G97-A mAb (Invitrogen) at 4°C for 1 h. Following extensive washing, cell samples were transferred to 37°C for 10 min, 30 min, and 60 min, respectively. When necessary, cells were treated with dynasore (50 µM; Sigma-Aldrich) or cytochalasin D (1 µM; Sigma-Aldrich) to stop receptor internalization. The level of receptor internalization was detected by flow cytometry.

## FIGURES

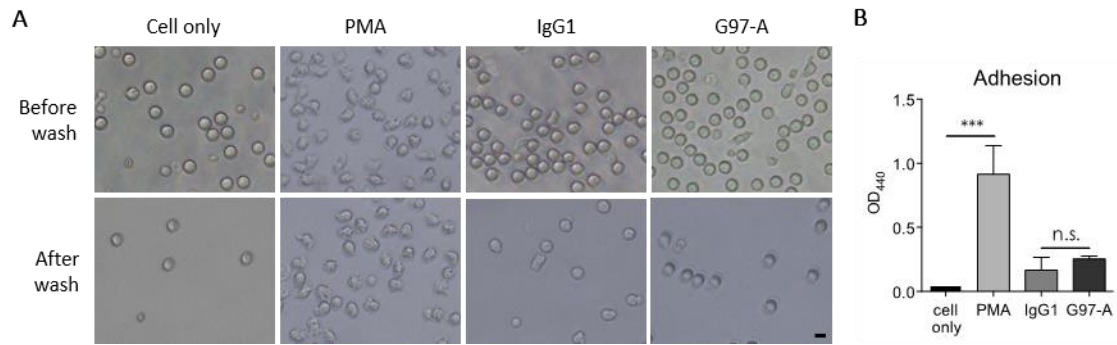


**Figure S1. Determination of the specificity of G97-A mAb.** (A) Western blot analysis of the GPR97-ECD-mFc protein using Abs specific to mFc (left panel) and GPR97-ECD (right panel). For the detection of mFc (left panel), anti-mouse IgG (Fc specific)-peroxidase was used, whereas the GPR97-ECD (right panel) was detected using G97-A as first Ab, followed by anti-mouse IgG (Fab specific)-peroxidase. The molecular weight of GPR97-NTF is ~43 kDa (indicated by arrowhead), very similar to the size of mFc fragment (indicated by the dollar sign). A minor fraction of uncleaved GPR97-ECD-mFc was expressed as a ~80-kDa band and detected by both Abs (indicated by asterisk). The soluble mFc was included as a control and appeared slightly larger than the cleaved mFc fragment of the GPR97-ECD-mFc protein due

to additional sequences encoded by pSecTag2A-mFc expressional construct, which contain multiple cloning sites and an additional biotinylation sequence. **(B)** Flow-cytometric analysis of HEK-293T cells transfected with pcDNA3.1-GPR56 and pReceiver-GPR97-GFP with mAb directed against GPR56 (CG2) and GPR97 (G97-A). **(C)** Flow-cytometric analysis of HEK-293T cells transfected with pReceiver-GPR97-GFP. GFP<sup>-</sup> and GFP<sup>+</sup> cell populations were sorted by flow cytometry and stained for surface GPR97 expression using G97-A mAb. To demonstrate specificity, G97-A mAb was pre-incubated with a 2-fold concentration of soluble mFc or GPR97-mFc before flow cytometry. **(D)** Flow-cytometric analysis of GPR97 surface levels in sorted GFP<sup>-</sup> and GFP<sup>+</sup> GPR97-expressing HEK-293T cells using G97-A mAb. Cells were pre-incubated with a 2-fold concentration of soluble mFc or GPR97-mFc before flow cytometry. **(E)** Quantification of the flow cytometry plots shown in panel D.

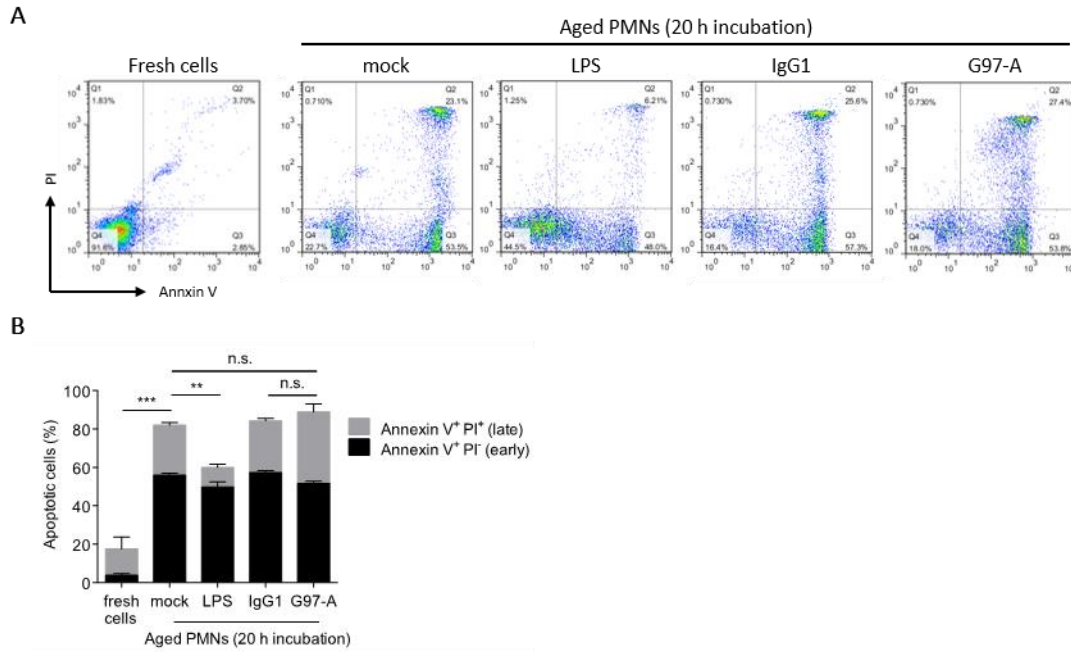


**Figure S2. Specific GPR97 expression in tissue-infiltrating PMNs.** The specific reactivity of the G97-A mAb on tissue-infiltrating neutrophils was examined on formalin-fixed and paraffin-embedded human spleen tissue sections by comparing the staining patterns obtained with anti-MPO, G97-A, and control IgG1 mAb as indicated. Scale bar: 50  $\mu$ m.

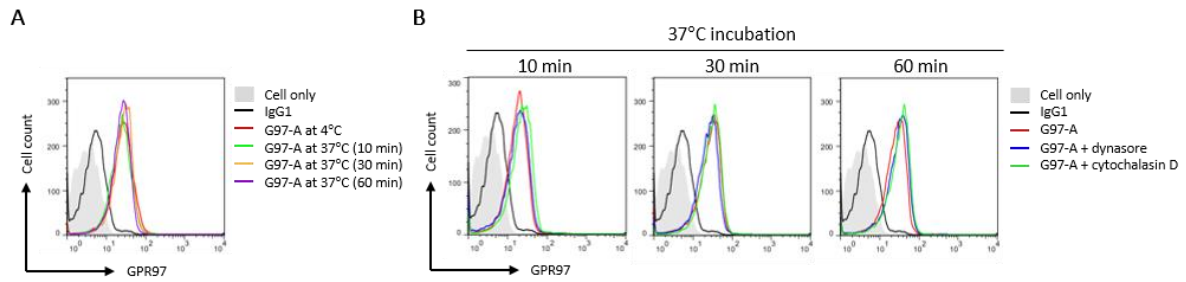


**Figure S3. GPR97 activation does not alter the morphology and adhesion of neutrophils.**

(A) Purified human neutrophils were cultured in the absence or presence of PMA (2 nM) or on plates pre-coated with control IgG1 or G97-A mAb (10 µg/ml) for 1 h before gentle washing and microscopic analysis of cell adhesion. Cell morphology of neutrophils before and after washing is shown. Scale bar: 10 µm. (B) Quantitative comparison of cells adhesion at the indicated conditions. Data are means ± SEM of 3 independent experiments. \*\*\*  $p < 0.001$ ; ns, non-significant.



**Figure S4. GPR97 activation does not affect neutrophil apoptosis.** (A) Cell apoptosis of fresh and aged PMNs (cultured at 37°C incubator for 20 h) was detected using flow-cytometric analysis of cells stained with Annexin V and propidium iodide (PI). (B) Quantitative comparison of cells apoptosis at the indicated conditions. Data are means  $\pm$  SEM of 3 independent experiments. \*\*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; ns, non-significant.



**Figure S5. GPR97 mAb binding does not induce GPR97 receptor internalization.** (A) Flow cytometric analysis of freshly isolated PMNs stained by Alexa Fluor 647-labeled control IgG1 or G97-A mAb (5  $\mu$ g/ml) and incubated at 4°C or shifted from 4°C to 37°C for 10, 30, and 60 min as indicated. (B) PMNs were treated without or with dynasore (50  $\mu$ M) or cytochalasin D (1  $\mu$ M) before staining with Alexa Fluor 647-labeled G97-A mAb (5  $\mu$ g/ml). Cells were stained at 4°C then shifted to 37°C. Cell surface GPR97 levels were measured by flow cytometry at different time points as indicated.