Supplementary Material

Antithrombin Together with NETs Inhibitor Protected Against Postoperative Adhesion Formation in Mice

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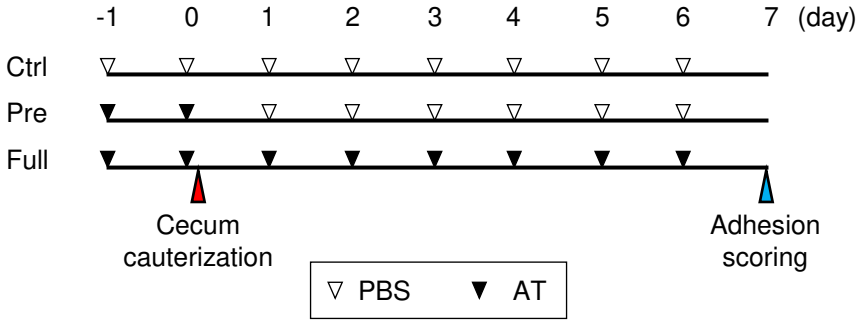
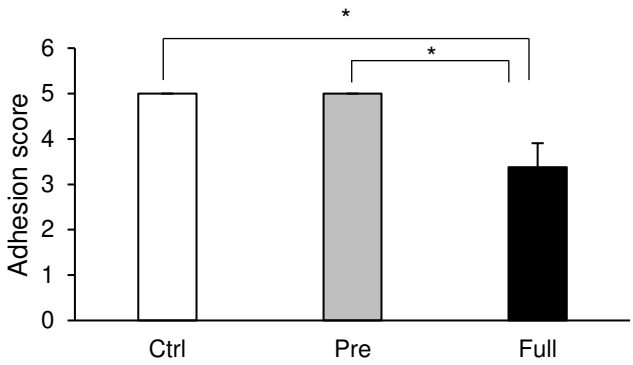
Supple. Fig. 1.

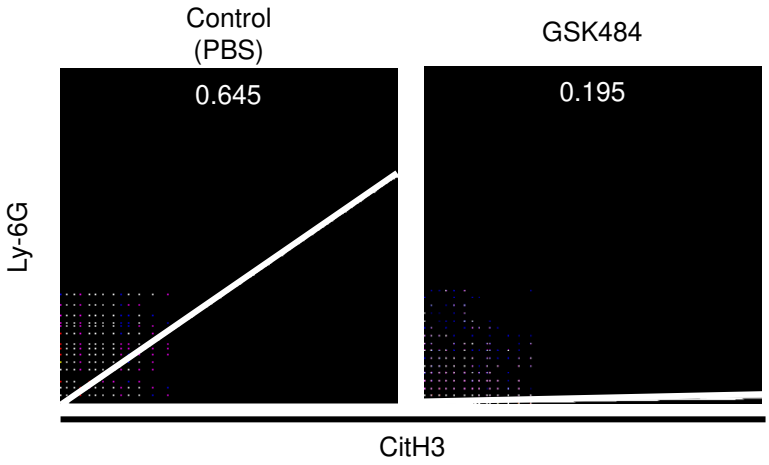

-1
0
1
2
3
4
5
6
7
(day)
Ctrl
Pre
Full
Cecum cauterization
Adhesion scoring
PBS
AT


*
*
Adhesion score
0
1
2
3
4
5
6
Ctrl
Pre
Full

Supplementary Fig. 1 Preadministration of antithrombin does not affect inhibiting adhesion formation.

Mice were administrated with phosphate-buffered saline (PBS; Ctrl) or antithrombin (AT) (10 U/mL) 1 day and 1 h before cecum cauterization (Pre) or AT (10 U/mL) 1 day and 1 h before cecum cauterization and after once a day for 5 days (Full). Adhesion scores were evaluated at day 7 post-operation. Eleven mice were used for each experimental group. Data are presented as mean ± SEM. Tukey's test. *$p < 0.05$ indicated statistically significant differences.

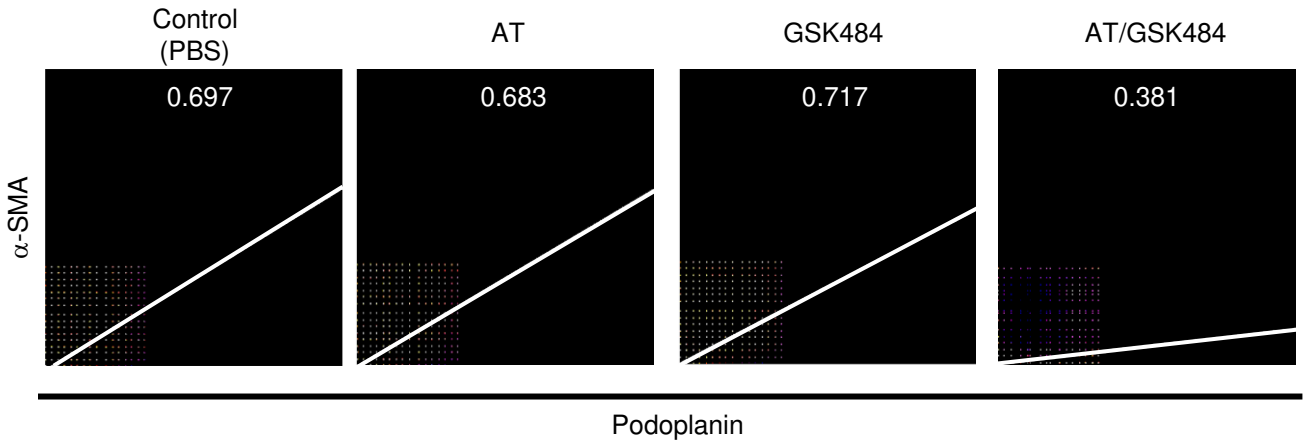
Supple. Fig. 2.



Supplementary Fig. 2 PAD4 inhibitor inhibits histone H3 citrullination in neutrophils in the adhesion bands.

Phosphate-buffered saline (PBS) (left panel) or GSK484 (80 μg/mouse) (right panel) was administrated intraperitoneally 24 h and 1 h before cecum cauterization. Intestinal specimens around the injured serosa were sampled at 24 h after cecum cauterization. Sample slices were stained with immunofluorescence for Ly-6G (red) and citrullinated histone 3 (CitH3) (green) (Fig. 3B). To analyze the neutrophils undergoing NETs, we measured the co-localization between Ly-6G and CitH3 intensities by ImageJ Fiji (SciJava software ecosystem's open source software project). Briefly, Red (Ly-6G) and green (CitH3) fluorophore intensities in the individual pixels were measured, and correlation coefficient between the two fluorescence intensities was estimated using Spearman rank correlation (SRC). In adhesion band of control mice, neutrophils were positively corelated with CitH3 (SRC: 0.645). In contrast, neutrophils in the GSK484-treated specimen were little correlated with CitH3 (SRC: 0.195). These data coincide with Ly-6G and CitH3 merged images (Fig. 3B).

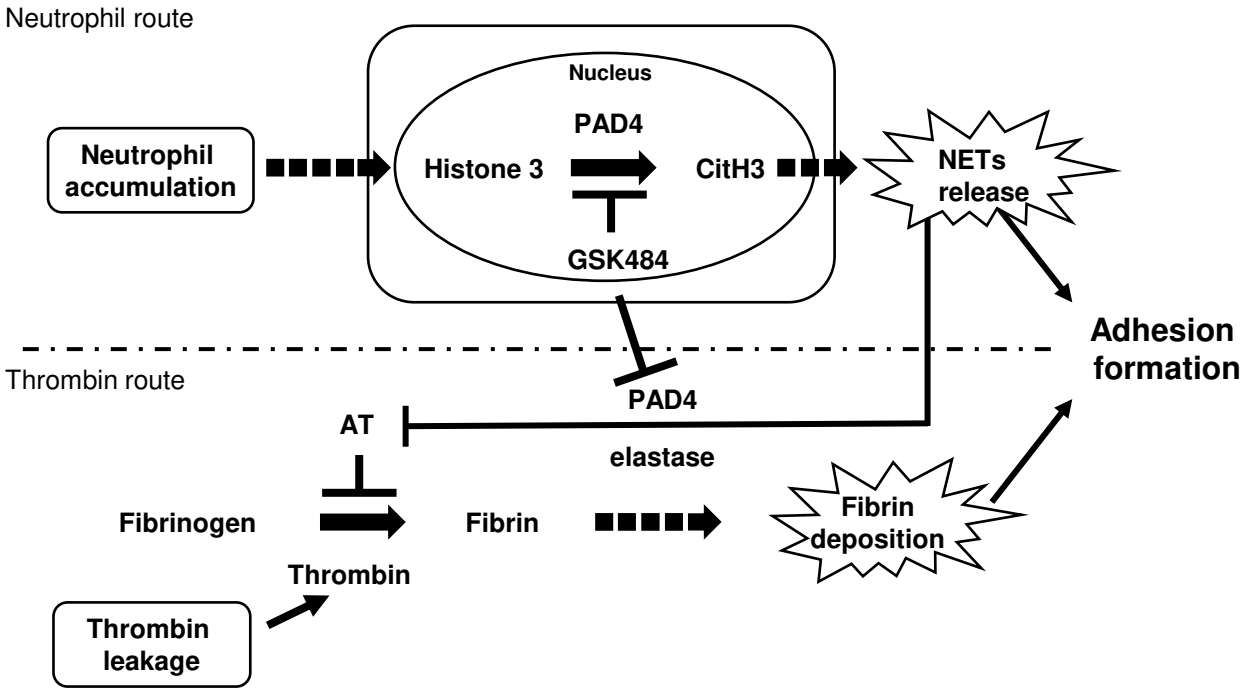
Supple. Fig. 3.


Control
(PBS)
AT
GSK484
AT/GSK484
0.697
0.683
0.717
0.381
α-SMA
Podoplanin

Supplementary Fig. 3 AT in combination with GSK484 attenuates myofibroblast accumulation.

Phosphate-buffered saline (PBS), AT, GSK484, or AT together with GSK484 (AT/GSK84) were administered into the mice that underwent cecum cauterization according to the experimental protocol as shown above. Seven days after cecum cauterization, immunofluorescence analysis was performed onto the adhesion bands (Fig. 4B). To analyze myofibroblast derived from mesothelial cells, we measured co-localization between α-SMA (red) and podoplanin (green) fluorescence intensities by ImageJ Fiji. α-SMA and podoplanin fluorophore intensities in the individual pixels were measured, and the relationship between the two fluorophores were estimated using Spearman rank correlation (SRC). In control, AT-, GSK484-, or AT/GSK484-treated mice, correlation between α-SMA and podoplanin was 0.697, 0.683, 0.717 or 0.381, respectively. This implicate that single treatment with AT or GSK484 did not profoundly prevent the mesothelial cell-derived myofibroblast accumulation as found in control mice, whereas combination treatment with the two reagents appeared to protect against it.

Supple. Fig. 4.


Neutrophil route
Nucleus
PAD4
Neutrophil accumulation
Histone 3
CitH3
NETs release
GSK484
Adhesion formation
Thrombin route
PAD4
AT
elastase
Fibrinogen
Fibrin
Fibrin deposition
Thrombin
Thrombin leakage

Supplementary Fig. 4 Proposed model for the role of thrombin and NETs in the induction of postoperative adhesion formation

Upon abdominal surgery, Thrombin route: leakage of thrombin converts fibrinogen into fibrin (lower half). Neutrophil route: neutrophil accumulation and following NETs formation (upper half). Thrombin route can inhibit by AT. Although AT cannot inhibit neutrophil route. PAD4 inhibitor (GSK484) can inhibit neutrophil route but cannot thrombin route. GSK484 can also inhibit citrullination of AT by PAD4 which released from NETs. Blockage of postoperative adhesion formation might need both thrombin and neutrophil routes inhibition by AT and GSK484, respectively.