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Original Paper

Blockade of Tumor Necrosis Factor by Etanercept Prevents Postoperative Adhesion Formation in Mice

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Key Words
Postoperative adhesions • Murine cecum cauterization model • Etanercept • Tumor necrosis factor • Neutrophil extracellular trap formation

Abstract
Background/Aims: Although adhesion formation is a frequent adverse event following intraperitoneal surgery, efficient prophylactic interventions have not yet been established. We recently reported that blockade of interleukin (IL)-6 prevented postoperative adhesion after cecum cauterization. Intriguingly, this intervention dampened tumor necrosis factor (TNF) induction in the injured serosa. Herein, we addressed whether TNF might be a key target and, if so, how TNF blockade rescued adhesion formation. ***Methods:*** Mice were administered an anti-TNF biologic (etanercept) on days -2 and -1 before and upon cecal cauterization. The adhesion scores were evaluated at day 7 postoperatively. Histological alterations were examined by immunochemistry/immunofluorescence studies. We incubated human neutrophils and mesothelial cell line cells with recombinant TNF in the presence of etanercept and measured transcript levels of cytokines and chemokines by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). ***Results:*** Etanercept rescued mice from adhesion formation, accompanied by a robust reduction of neutrophilia in the injured serosa. Immunofluorescence revealed a substantial formation of neutrophil extracellular traps (NETs) with the potential to induce tissue damage and profibrotic responses. In contrast, the etanercept-treated mice lacked NET formation. In addition, etanercept inhibited TNF-induced IL-6, TNF, and neutrophil-recruiting chemokines in neutrophils and mesothelial cells, a major cellular source of myofibroblasts in the adhesion band. ***Conclusion:*** Prophylactic administration of etanercept might be a potential strategy for preventing postoperative adhesion formation.

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Introduction

Postoperative adhesion formation frequently occurs after abdominal surgeries and can cause adverse effects including chronic abdominal pain, infertility in women, small bowel obstruction, and obstacles to re-operation [1-4]. To address these complications, additional treatments with possible high morbidities and substantial costs might be needed. To date, a few preventive medications for postoperative adhesions have been established. Several types of barrier sheets can prevent the physical contact of surgically injured abdominal organs with neighboring intact abdominal organs and abdominal wall [5, 6]. Although these instruments can cover the injured peritoneum of abdominal wall and that of the organs, adhesion formation occurs on not only between these injured peritoneum, but also occurs between multiple surgical serosa such as intestine and intestine, and liver and intestines. Laparoscopic surgery was reported to reduce the incidence of adhesion-related readmissions [7]. However, the complications by adhesion-related readmissions still remain to be a big problem [7]. Further approach is needed to reduce the incidence of adhesion-related postsurgical complications.

We previously observed that mice undergoing cecum cauterization or partial hepatectomy developed adhesion formation around the injured cecum and the surgical surface of the remnant liver, respectively, within 1 week [8, 9]. Using these two types of animal models, we demonstrated that fibrin deposition and rapid induction of IFN-γ are commonly required for the development of these adhesion formations. Upon cecum cauterization, invariant natural killer T (*i*NKT) cells immediately migrated into the injured serosa and produced interferon (IFN)-γ, which, in turn, promoted hypercoagulation by inducing potent anti-fibrinolytic factor and plasminogen activator inhibitor 1 (PAI-1), eventually leading to adhesion formation. Additionally, *Ifng*$^{-/-}$ mice and *i*NKT-deficient mice both were equally free from adhesion formation, concomitant with abrogated induction of PAI-1. Moreover, blockade of PAI-1 by the administration of neutralizing anti-PAI-1 antibodies prevented adhesion formation in wild-type mice. Notably, the importance of the IFN-γ-PAI-1 axis was also demonstrated for adhesion formation following partial hepatectomy [9]. Thus, the prompt induction of IFN-γ might initiate adhesion formation. Very recently, we found that transcripts of interleukin-6 (*Il6*) were immediately induced after cauterization and verified that the administration of antibody (Ab) against receptor (R) for IL-6 alleviated adhesion formation [10]. Intriguingly, mice receiving the IL-6R monoclonal Ab (mAb) showed a substantial reduction in tumor necrosis factor (TNF) expression. This prompted us to propose the hypothesis that neutralization of endogenous TNF via etanercept will prevent postoperative adhesion formation.

Etanercept, a recombinant fusion protein of human type 2 receptor for TNF (TNFR2) and Fc of human immunoglobulin G was approved for the treatment of human chronic inflammatory diseases and autoimmune diseases, including rheumatoid arthritis (RA), psoriasis, and ankylosing spondylitis [11, 12]. Etanercept competitively inhibits the binding of TNFα to TNFR1 and TNFR2 [11, 13]. Clinical trials have demonstrated the efficacy and safety of etanercept in these diseases, particularly RA [12]. Etanercept can protect against mouse models of chronic inflammatory diseases such as arthritis, gout, and glucocorticoid-insensitive asthma [14-16]. Here, to test the above hypothesis we investigated whether neutralizing endogenous TNF by etanercept pretreatment protects against the adhesion formation and, if this is the case, we investigated how etanercept prevents it.

Materials and Methods

Reagents

Recombinant (r) human (h) TNF (catalog number 210-TA/CF) was purchased from R&D. We purchased a fusion protein containing a ligand-binding fragment of human TNF receptor (R)-2, named etanercept, from Pfizer Inc., anti-Ly6G mAb (551459) from BD Biosciences, and anti-Histone H3 (citrulline R2 + R8 +R17) antibody-ChIP Grade (ab5103) from Abcam.

Mice

Previously we performed cecum cauterization in both male and female mice, and found no differences in cecum cauterization-induced adhesion formation in terms of clinical score and histological changes between male and female mice [7, 9, 10]. In this study we used female mice. We purchased BALB/c female mice (8–10 weeks of age) from Japan SLC, Inc. All animal experiments were performed under specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care Committee, Hyogo College of Medicine (approval protocol number: 17-019).

Mouse model of surgical adhesion formation

We previously described the mouse cecum-cauterization model [7, 10]. Briefly, we anesthetized mice with isoflurane followed by a midline abdominal incision and cauterization of the cecum isolated using the coagulation mode of bipolar forceps (MERA MS-1500; output dial 3; 5 W, 500kHz, 100 Ω Senko Medical Instrument Manufacturing CO, Japan) with a pinch width of 0.3 mm for 2 s. We closed the incision in two layers with silk sutures. At the indicated time points post-operation, we sampled injured ceca or adhesion tissues. At 168 h after operation, we scored adhesion formation from 0 to 5 according to the following scoring system: 0, no adhesion; 1, one thin filmy adhesion; 2, more than one thin adhesion; 3, thick adhesion with focal point; 4, thick adhesion with plantar attachment or more than one thick adhesion with focal point; and 5, very thick vascularized adhesion or more than one plantar adhesion.

Tissue staining

Hematoxylin and eosin (HE) and Sirius red staining were performed as described elsewhere [10].

Immunohistochemistry

We performed immunohistochemical analysis as described previously [10]. Briefly, we fixed tissue specimens in IHC Zinc Fixative (BD Pharmingen™) and embedded them in paraffin wax before sectioning. For immunohistochemical analysis, we preincubated the sections with a serum-free protein block (Agilent Technologies) for 30 min at room temperature. The primary antibodies were added to each slide and then secondary Ab staining was performed using Histofine Simple Stain Mouse MAX-PO (Nichirei Biosciences Inc.) according to the manufacturer's instructions. The staining was visualized under a Nikon Eclipse Ni-U microscope (Nikon).

Immunofluorescence staining with confocal microscopy

Immunofluorescence analysis was performed as previously described [10]. Briefly, tissue samples were fixed in IHC Zinc Fixative and embedded in paraffin wax. To detect fibrosis, the tissue slices were incubated with antibodies for α-SMA (ab124964, Abcam) and podoplanin (ab11936, Abcam) or 4',6-diamidino-2-phenylindole (DAPI) to detect DNA. To detect neutrophil extracellular traps (NETs), the slices were incubated with antibodies against Ly6G (551459 BD Bioscience) and anti-citrullinated histone H3 mAbs as well as DAPI. The fluorescence was visualized under a confocal microscope (Zeiss LSM780.)

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

We performed RT-qPCR as previously described [10]. Total RNA was prepared from tissue or cell samples using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA), and reverse transcripts were synthesized using PrimeScriptTM RT Master Mix (Perfect Real Time) (Takara Bio, Inc.). Taqman Fast Advanced Master Mix, TaqMan Gene Expression Assays for *Il6* (Mm00446190_m1), *Tnf* (Mm00443258_m1), *Pai1* (Mm00435858_m1), *Cxcl2* (Mm00436450_m1), *IL6* (Hs00174131_m1), *TNF* (Hs00174128_m1), *CXCL2* (Hs00601975_m1) and *18S* (Hs99999901_s1) were purchased from Applied Biosystems. Real-time qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The results were analyzed using StepOne software v.2.0. The fold-change of each gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. According to this method, each threshold cycle (Ct) value was first normalized to the internal reference gene (18S ribosomal RNA) of the sample and then to the controls.

Statistical analyses

Data are presented as means ± SEM of either the results from each experimental group or triplicate samples. We performed statistical comparisons using Tukey tests. All statistical analyses were performed using free web software (www.gen-info.osaka-u.ac.jp/MEPHAS/tukey.html). $P < 0.05$ indicated statistically significant differences.

Results

Etanercept protected against postoperative adhesion formation

To investigate the role of TNF in the adhesion formation induced by cecum cauterization, we administered 100 or 50 μg/mouse of etanercept at -2 and -1 days before and on the day of cecum cauterization. Mice treated with 100 μg/mouse of etanercept exhibited a significantly reduced adhesion score compared to that of control mice treated with phosphate-buffered saline (PBS) at day 7 post-operation (Fig. 1A). Treatment with etanercept (50 μg/mouse) reduced the adhesion sore, but not significantly (Fig. 1A). We observed a very thick adhesion band connecting the injured serosa to the organs in the vicinity in PBS-treated control mice, with substantial vascularization (Fig. 1A). In contrast, we observed only a thin filmy adhesion in mice in the etanercept (100 μg) group (Fig. 1A). Consistent with

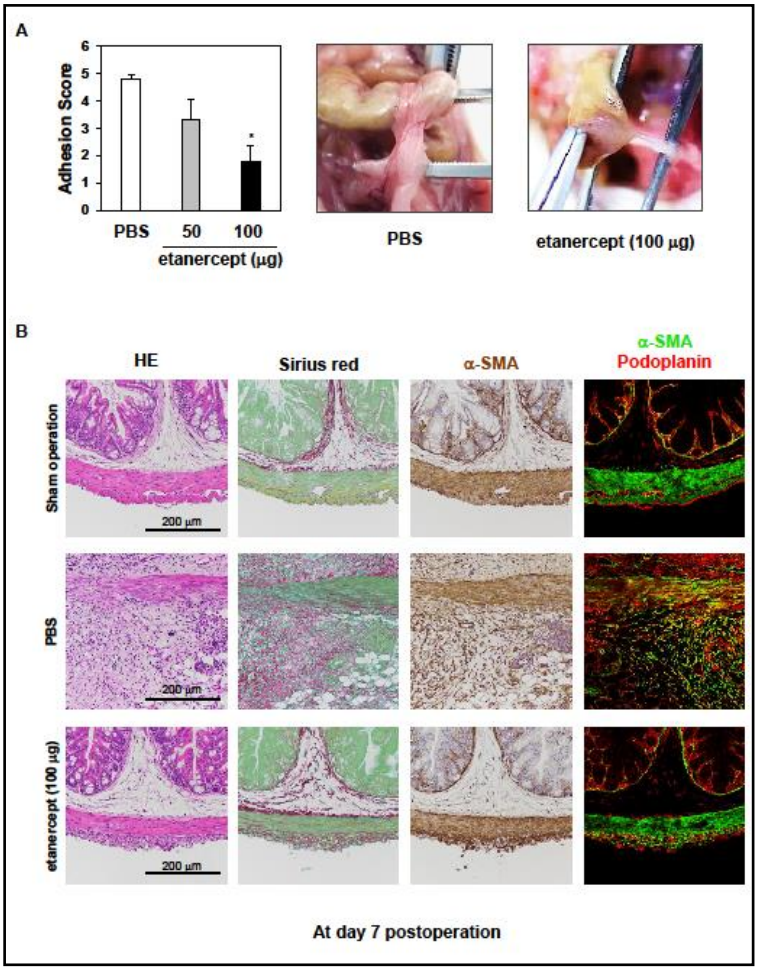

Fig. 1. Prophylactic administration of etanercept suppresses adhesion formation. (A) Phosphate-buffered saline (PBS), etanercept (50 μg/mouse), or etanercept (100 μg/mouse) was administered subcutaneously on days -2, -1, and the day of cecum cauterization. We evaluated the adhesion scores at day 7 post-operation. Data are presented as mean ± SEM. *p<0.05 indicated statistically significant differences. Representative photos of intraabdominal adhesion formations in the PBS (score 5) and etanercept (100 μg/mouse) (score 1) groups are shown. (B) Intestinal specimens around the injured serosa were sampled from mice treated with PBS or 100 mg/mouse etanercept on day 7 after cecum cauterization. We also sampled the cecum of mice on day 7 after sham operation. Sample slices were stained with hematoxylin and eosin (HE) (left panels) or Sirius red (second from left panels). Immunohistochemical staining for α-SMA was performed (second from right panels). Immunofluorescence analysis of α-SMA (green) and podoplanin (red) were performed (right panels). Representative photographs are shown. Three to five mice were used for each experimental group. We performed the experiments three times with similar results.

this, Sirius red staining revealed very thick fibrosis in the PBS-treated mice receiving cecum cauterization compared to mice that underwent sham operations (Fig. 1B). In contrast, only moderate changes positive for Sirius red were found in etanercept (100 μg/mouse)-treated mice (Fig. 1B). This was also the case for immunohistochemical analysis of α-SMA. Robust and only mild accumulation of α-SMA$^+$ cells were observed in the serosa of PBS- and etanercept-treated mice, respectively, (Fig. 1B). As previously reported, mesothelial cells are the major cellular source of myofibroblasts [10, 17]. α-SMA$^+$ cells co-stained with podoplanin, a cellular marker of mesothelial cells, were abundant and only scattered in the PBS- and etanercept-treated mice, respectively, (Fig. 1B). These results demonstrated that etanercept protected against adhesion formation following cecum cauterization. Etanercept impaired adhesion-associated molecule induction

To investigate the molecular mechanisms underlying the anti-adhesion effects of etanercept, we investigated the kinetics of mRNA expression of various molecules involved in adhesion formation after cecum cauterization by real-time qPCR. Consistent with our previous report [10], in the control group, *Tnf*α expression levels started to increase at 3 h (approximately 17-fold), continued to increase to 24 h (approximately 40-fold), and reduced by 72 h after cecum cauterization. In the etanercept group, however, *Tnf* induction was significantly reduced at 6 to 24 h compared to that in the control group (Fig. 2A), suggesting a positive circuit for *Tnf* induction in an autocrine/paracrine manner. Our previous study showed that *Il6* induction peaked at 3 h after cauterization [10]. Consistent with this, *Il6* expression was increased by approximately 850-fold at 3 h in the control group (Fig. 2). At 6 h and later, the levels remained around 200-fold (Fig. 2). However, *Il6* induction was significantly suppressed in the etanercept group at 3 h, suggesting the involvement of TNF signaling in *Il6* induction. However, no significant difference in *Il6* induction was observed between the control and etanercept groups thereafter (Fig. 2B). CXCL-2, a major chemokine for neutrophil recruitment in mice, was also aberrantly induced post-operation [10]. Expectedly, *Cxcl2* induction was observed in the control group, while that in the etanercept group appeared to be suppressed, although not significantly, compared to the control group (Fig. 2C). PAI-1, a potent coagulation factor that inhibits fibrinolysis, was induced after cecum cauterization in IFN-γ and IL-6 pathway-dependent manners [7, 10]. Consistent with our previous studies [7, 10], *Pai1* was induced in the control group postoperatively (Fig. 2D). Intriguingly, etanercept did not affect the early induction of *Pai1* (3 and 6 h) but did significantly reduce *Pai1* induction in the late phase (24 and 72 h) (Fig. 2D), suggesting that PAI-1 was induced in a manner independent of and dependent on TNF in the early and late phases, respectively.

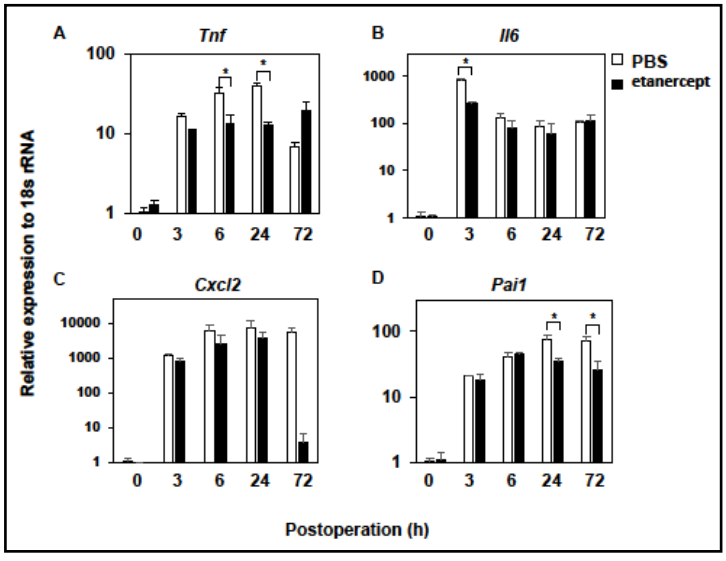

Fig. 2. Etanercept protects against the induction of mRNA expression of adhesion-associated molecules. At 0, 3, 6, 24, and 72 h after cecum cauterization, cecal specimens were sampled to measure the mRNA levels of tumor necrosis factor (Tnf) (A), interleukin-6 transcript (Il6) (B), chemokine (C-X-C motif) ligand 2 transcript (Cxcl2) (C), and plasminogen activator inhibitor-1transcript (Pai1) (D). Data are presented as mean ± SEM. *p<0.05 indicated statistically significant differences. Three mice were used for each experimental group. We performed the experiments three times or more with similar results.

Importance of TNF for neutrophil accumulation

Other studies have demonstrated that neutrophils are promptly and robustly recruited to the injured serosa and that *in vivo* depletion of neutrophils rescued mice from postoperative adhesion [10, 18], suggesting the importance of neutrophils in the development of adhesion formation. We found that etanercept treatment, similar to treatment with anti-IL-6R mAb [10], tended to reduce *Cxcl2* induction (Fig. 2C), prompting us to investigate whether etanercept inhibited neutrophilia. Immunohistochemical analysis of Ly-6G, a surface marker of neutrophils, showed consistent migration of neutrophils into the cauterized serosa in the control mice (Fig. 3A). We counted total $Ly6G^+$ cells in the serosa or adhesion indicated by the green dotted line in the control and experimental group (Fig. 3A, B), quantified the Ly6G-positive proportion to the area, and calculated the relative ratio of each proportion to that of tissue slices of the control group at 6 h (Fig. 3C). We found that etanercept inhibited neutrophil accumulation at 24 and 72 h (Fig. 3C). These results, together with those of our previous report [10], suggested the dependence of adhesion formation on serosal neutrophilia.

NETs may participate in adhesion formation

We next assessed whether neutrophils that migrated to the serosa were activated or in resting form. NETs released from activated neutrophils reportedly to serve as a potent host defense mechanism against pathogen microbes such as bacteria, fungi, and viruses [19, 20]. NETs are neutrophil-derived chromatin filaments decorated by cytotoxic molecules, including histones, proteases, and cytoplasmic and granular proteins [21]. After activation by microbes or microbial products, neutrophils release NETs, which trap and efficiently kill them the microbes by means of their web-like configuration. Recent intensive studies, however, unveiled their dark side: NETs also participate in various noninfectious diseases such as those related to autoimmunity and thrombosis [21, 22]. This allowed us to investigate whether the infiltrating neutrophils might be activated to form NETs. To detect NETs, we performed co-staining for Ly-6G, DNA, and citrullinated histone H3 (CitH3), which are essential for the generation of NETs by inducing nuclear decondensation, a key event for NET release [21, 23]. We found that the unique fibrous structures comprising CitH3 and DNA were generated around the neutrophilia at 24 h after cecum cauterization (Fig. 4A), suggesting that the migrated neutrophils might have released substantial NETs. In contrast to the PBS-treated mice, NETs were almost absent in the injured serosa of etanercept-treated mice (Fig. 4B). These results suggest that, upon cecum cauterization, neutrophils promptly migrate to the injured sites to generate NETs in a TNF-dependent manner.

Etanercept inhibited TNF action in human neutrophils and mesothelial cells

Mesothelial cells were the major cellular source of myofibroblasts in the adhesions (Fig. 1B). We investigated whether etanercept directly protected against TNF induction of the adhesion-associated molecules in mesothelial cells. Consistent with our previous report [10], human mesothelial cell line, MeT5A cells expressed higher levels of *IL6, CXCL2*, and *PAI1* in response to TNF (Fig. 5A). Pretreatment with etanercept suppressed the TNF induction of these molecules to the control levels (Fig. 5A). Consistent with our findings [10], human neutrophils showed increased *TNF*, *CXCL2*, and *IL6* levels (Fig. 5B). As was observed in MeT5A, etanercept completely prevented TNF induction of these molecules (Fig. 5B). Etanercept alone did not affect the expression levels of these molecules in either cell type (Fig. 5A, 5B).

The results of the present study suggested that TNF produced by both neutrophils and mesothelial cells promptly after cauterization might be a key trigger to initiate postoperative adhesion formation at the injured serosa. Etanercept may neutralize TNF to suppress the induction of adhesion formation-related molecules. Thus, prophylactic administration of etanercept could prevent postoperative adhesion formation, presumably by inhibiting inflammation, neutrophil accumulation, and NET formation (Fig. 5C).

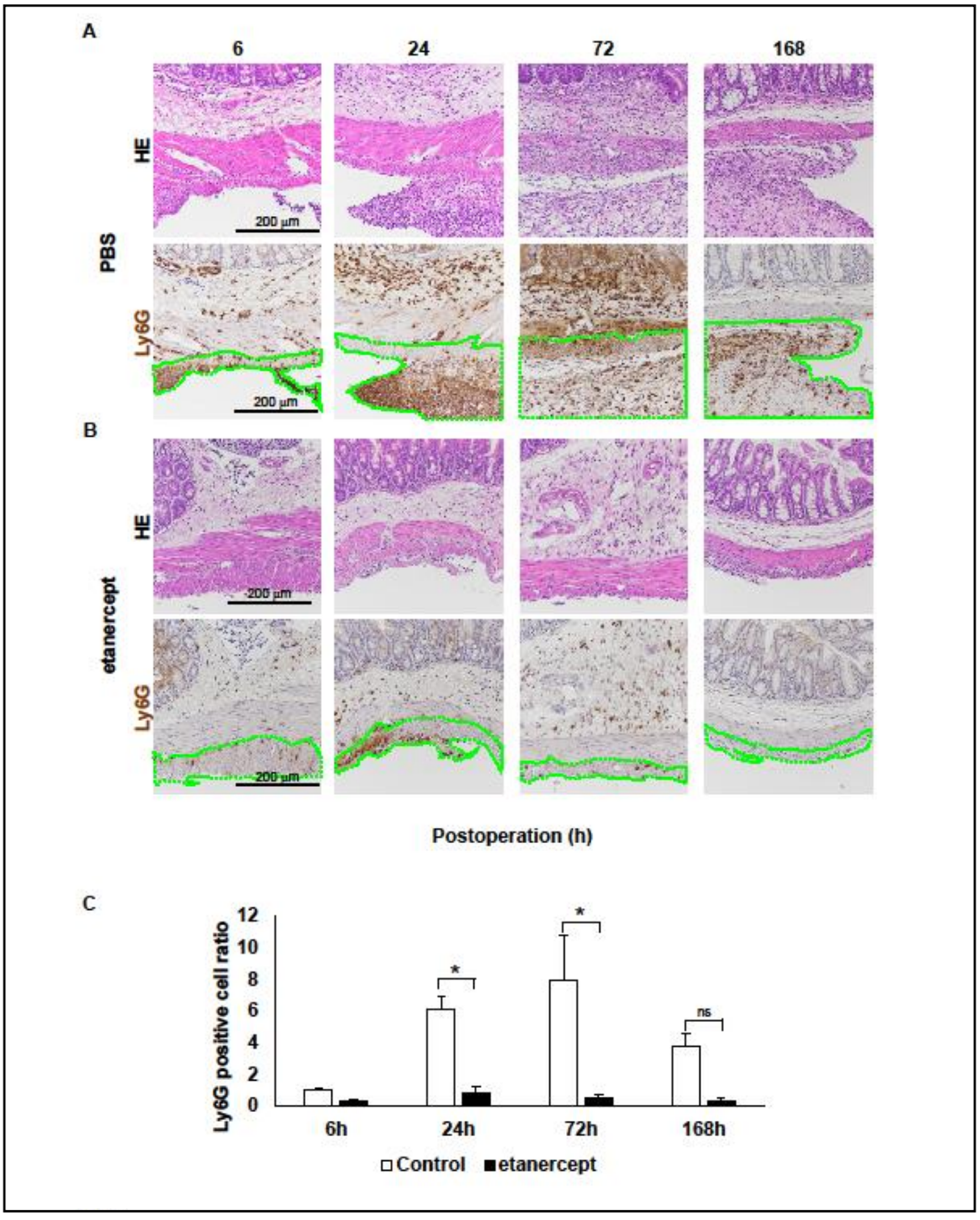

Fig. 3. Etanercept hampers neutrophil accumulation at injured serosa. Mice were administered phosphate-buffered saline (PBS) (A) or etanercept (100 μg/moues) (B) and the cecal lesions were sampled at the indicated time points after cecum cauterization for hematoxylin and eosin staining or immunohistochemistry for Ly6G. (C) We counted total $Ly6G^+$ cells in the serosa or adhesion indicated by the green dotted line (A, B), quantified the Ly6G-positive proportion to the area, and calculated the relative ratio of each proportion to that of tissue slices of the control group at 6 h. Data are given as means ± SEM. *$p<0.05$ indicated statistically significant differences. ns, not significant. Three mice were used for each experimental group. We performed the experiments three times with similar results.

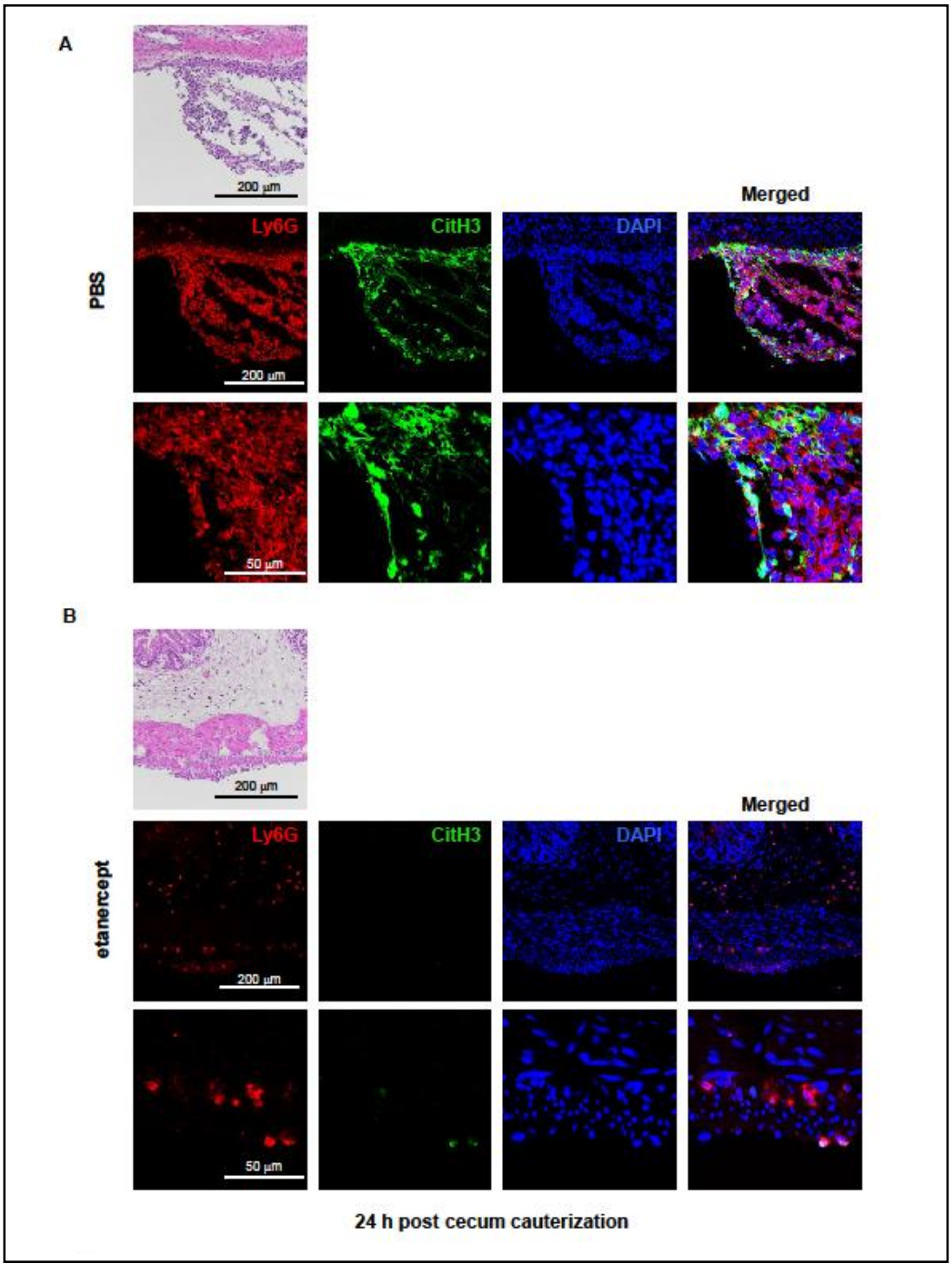

Fig. 4. Etanercept prevents neutrophil extracellular trap (NET) formation in injured serosa. Mice were administered phosphate-buffered saline (PBS) (A) or etanercept (100 mg/mouse) (B). The cecal lesions were sampled at 24 h after cecum cauterization for HE staining or immunofluorescence for Ly6G (red), citrullinated histone 3 (CitH3) (green), and DAPI (blue). Photographs with low (upper panels) and high (lower panels) magnifications are shown. Three mice were used for each experimental group. We performed the experiments three times with similar results.

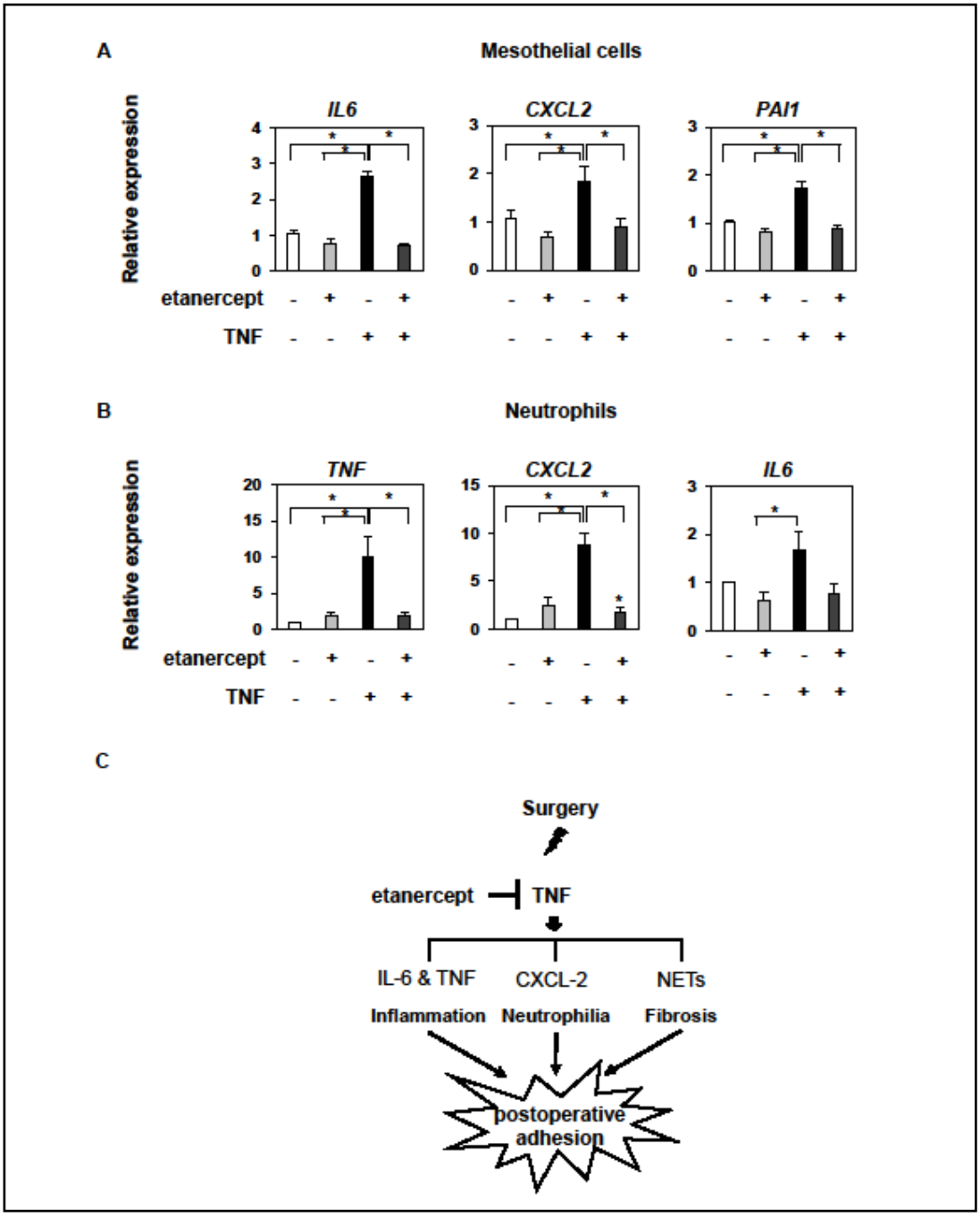

Fig. 5. Etanercept inhibits tumor necrosis factor (TNF) action on mesothelial cells and neutrophils. (A, B) Human mesothelial cell line, MeT5A cells (A) or primary human neutrophils (B) were exposed to 10 ng/mL TNF for 21 h with or without 3 h-etanercept pretreatment. Cells were harvested for quantification of mRNA levels of IL-6 (A, B), CXCL-2 (A, B), PAI-1 (A), and TNF (B). Data are presented as mean ± SEM. *p<0.05 indicated statistically significant differences. (C) Proposed model for the role of TNF in the induction of postoperative adhesion: upon abdominal surgery, the injured serosa is exposed to TNF in the early phase. TNF triggers various adhesion formation-related events such as IL-6 and CXCL-2 induction and neutrophil extracellular trap (NET) formation. Prophylactic administration of etanercept might prevent postoperative adhesion formation by inhibiting these events.

Discussion

In this study, we found that TNF blockade by etanercept protected against adhesion formation following cecum cauterization in mice, concomitant with a reduction in the induction of proinflammatory cytokines such as *Il6* and *Tnf*, pro-fibrotic and anti-fibrinolytic protein *Pai1*, and neutrophil accumulation with somewhat impaired induction of *Cxcl2*. A similar protective efficacy was also induced by treatment with anti-IL-6R mAb [10]. We previously reported that blockade of the IL-6-mediated signal pathway prevented adhesion formation with reduced induction of *Tnf* and neutrophilia in the injured serosa. IL-6 can induce *IL6* and *TNF* expression. TNF can also induce *IL6* and *TNF* [10]. This bidirectional positive circuit between these two proinflammatory cytokines and unidirectional positive circuits of the individual cytokines might account for the phenomena by which IL-6 and TNF appeared to equally contribute to adhesion formation.

Many biologics targeting TNF, including anti-TNF mAbs and etanercept, have been clinically used to treat chronic inflammation and autoimmune diseases with substantial efficacy [12]. TNF is a soluble protein (sTNF) and transmembrane protein (mTNF), both of which have equivalent biological actions [24]. TNF-targeting biologics, anti-TNF mAbs, and etanercept have neutralizing activity against both mTNF and sTNF. Furthermore, both types of biologics can equally exert complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) against mTNF-expressing cells [12]. However, mAb types of biologics but not etanercept can additionally activate reverse signaling in mTNF-expressing cells, eventually leading to the apoptotic cell death of mTNF-expressing cells [13, 24]. This difference in anti-TNF action mode between the two types of biologics might be attributed to their distinct clinical efficacy against Crohn's disease; etanercept has no efficacy, whereas mAbs show substantial efficacy [24-26]. It remains to be elucidated whether etanercept protected against adhesion formation by neutralizing sTNF and mTNF, complement-dependent cytotoxicity against mTNF-expressing cells, ADCC against mTNF-expressing cells, and/or induction of reverse signaling in mTNF-expressing cells in mice.

Our results showed that treatment with etanercept protected against NET release in injured serosa. TNF activates neutrophils to release NETs [27, 28]. By immunoblotting analysis, Neeli et al. reported that human peripheral blood neutrophils citrullinated histone H3 in response to TNF [27]. Wang et al. also verified this TNF-induced citrullinated histone H3 by immunofluorescence analysis [28]. Interestingly, peripheral blood and synovial neutrophils of patients with rheumatoid arthritis (RA) spontaneously released NETs according to endogenous TNF [29]. These reports indicate that TNF can induce NET formation. As shown here, etanercept could hamper serosal neutrophilia after cecum cauterization. Therefore, etanercept might directly protect against NET formation by inhibiting neutrophil decondensation through abrogation of histone H3 citrullination and presumably indirectly via suppression of neutrophil accumulation.

It has been well documented that NETs potently trigger or promote proinflammatory responses in various diseases [20, 21]. Recently, NETs were reported to induce fibrotic alterations [30]. Chrysanthopoulous et al. reported that NETs induced fibroblast differentiation to myofibroblasts [31]. In response to NETs, primary human lung fibroblasts expressed higher levels of *ACTA2* (transcript of αSMA) and increased collagen production *in vitro*. Fibrosis-related agents, including cigarette smoke extract and bleomycin, could activate human neutrophils to release NETs [31]. Thus, NETs may be involved in tissue fibrosis. Furthermore, a recent report by Martinoid et al. demonstrated that NETs contributed to organ fibrosis, particularly age-related cardiac and pulmonary fibrosis in mice [32]. During aging, organ function declines, presumably due to the replacement of parenchymal cells with age-related fibrosis [32]. The authors found that aged wild-type mice but not young mice, developed cardiac and pulmonary fibrosis accompanied by robust NETs in these organs. In contrast, PAD4-deficient mice were resistant to age-related fibrosis. Together with our present study, these findings suggest that NETs might become a new target for tissue fibrosis.

The present study revealed prophylactic effects of etanercept on the postoperative adhesion. However, this study contains several limitations that would have been broken through for clinical trials. First, the injury used in this mouse model is cauterization. However, actual surgical procedures generate other types of injuries, such as an abrasion. Therefore, we need to test protective roles of etanercept in adhesion formation induced by other types of intraperitoneal damages. Second, it is unclear whether etanercept protects against adhesion formation upon surgery in human. Third, single or double etanercept treatments might render the patients immunocompromized to be susceptible to microbial infection. It is well established that multiple treatments with chronic inflammatory diseases with etanercept potentially activate endogenous microbial infection, such as *Mycobacterial tuberculosis* [33]. Beyond these limitations, this study might still provide a milestone to generate the prophylactic maneuver against adhesion formation after intraperitoneal surgery.

Conclusion

The prophylactic administration of etanercept might be a potential strategy to prevent clinical postoperative adhesion formation.

Acknowledgements

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Author contributions

J.F., M.S, K.I., and H.T. conceptualized the study; M.S., K.I., K.M., M.J., and J.F. performed experiments; M.S., K.I, H.T., and J.F. analyzed the data; M.S., K.I, H.T., and J.F. wrote the manuscript; and all authors reviewed and approved the final version of the manuscript.

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Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the Institutional Animal Care Committee, Hyogo College of Medicine (approval protocol number: 17-019).

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Holmdahl L: Making and covering of surgical footprints. Lancet 1999;353:1456-1457.
2 Cheong YC, Laird SM, Li TC, Shelton JB, Ledger WL, Cooke ID: Peritoneal healing and adhesion formation/reformation. Hum Reprod Update 2001;7:556-566.
3 Vrijland WW, Jeekel J, van Geldorp HJ, Swank DJ, Bonjer HJ: Abdominal adhesions: intestinal obstruction, pain, and infertility. Surg Endosc 2003;17:1017-1022.

4 ten Broek RP, Issa Y, van Santbrink EJ, Bouvy ND, Kruitwagen RF, Jeekel J, Bakkum EA, Rovers MM, van Goor H: Burden of adhesions in abdominal and pelvic surgery: systematic review and met-analysis. BMJ 2013;347:f5588.
5 Lin LX, Yuan F, Zhang HH, Liao NN, Luo JW, Sun YL: Evaluation of surgical anti-adhesion products to reduce postsurgical intra-abdominal adhesion formation in a rat model. PLoS One 2017;12:e0172088.
6 Ward BC, Panitch A: Abdominal adhesions: current and novel therapies. J Surg Res 2011;165:91-111.
7 Kosaka H, Yoshimoto T, Fujimoto J, Nakanishi K: Interferon-gamma is a therapeutic target molecule for prevention of postoperative adhesion formation. Nat Med 2008;14:437-441.
8 Krielen P, Stommel MWJ, Pargmae P, Bouvy ND, Bakkum EA, Ellis H, Parker MC, Griffiths EA, van Goor H, Ten Broek RPG: Adhesion-related readmissions after open and laparoscopic surgery: a retrospective cohort study (SCAR update). Lancet 2020;395:33-41.
9 Ohashi K, Yoshimoto T, Kosaka H, Hirano T, Iimuro Y, Nakanishi K, Fujimoto J: Interferon γ and plasminogen activator inhibitor 1 regulate adhesion formation after partial hepatectomy. Br J Surg 2014;101:398-407.
10 Uyama N, Tsutsui H, Wu S, Yasuda K, Hatano E, Qin XY, Kojima S, Fujimoto J: Anti-interleukin-6 receptor antibody treatment ameliorates postoperative adhesion formation. Sci Rep 2019;9:17558.
11 Scallon B, Cai A, Solowski N, Rosenberg A, Song XY, Shealy D, Wagner C: Binding and functional comparisons of two types of tumor necrosis factor antagonists. J Pharmacol Exp Ther 2002;301:418-426.
12 Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP: Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol Ther 2008;117:244-279.
13 Mitoma H, Horiuchi T, Tsukamoto H, Ueda N: Molecular mechanisms of action of anti-TNF-α agents - Comparison among therapeutic TNF-α antagonists. Cytokine 2018;101:56-63.
14 Belmellat N, Semerano L, Segueni N, Damotte D, Decker P, Ryffel B, Quesniaux V, Boissier MC, Assier E: Tumor Necrosis Factor-Alpha Targeting Can Protect against Arthritis with Low Sensitization to Infection. Front Immunol 2017;8:1533.
15 Amaral FA, Bastos LF, Oliveira TH, Dias AC, Oliveira VL, Tavares LD, Costa VV, Galvão I, Soriani FM, Szymkowski DE, Ryffel B, Souza DG, Teixeira MM: Transmembrane TNF-α is sufficient for articular inflammation and hypernociception in a mouse model of gout. Eur J Immunol 2016;46:204-211.
16 Dejager L, Dendoncker K, Eggermont M, Souffriau J, Van Hauwermeiren F, Willart M, Van Wonterghem E, Naessens T, Ballegeer M, Vandevyver S, Hammad H, Lambrecht B, De Bosscher K, Grooten J, Libert C: Neutralizing TNFα restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation. Mucosal Immunol 2015;8:1212-1225.
17 Tsai JM, Sinha R, Seita J, Fernhoff N, Christ S, Koopmans T, Krampitz GW, McKenna KM, Xing L, Sandholzer M, Sales JH, Shoham M, McCracken M, Joubert LM, Gordon SR, Poux N, Wernig G, Norton JA, Weissman IL, Rinkevich Y: Surgical adhesions in mice are derived from mesothelial cells and can be targeted by antibodies against mesothelial markers. Sci Transl Med 2018;10:eaan6735.
18 Kanamaru R, Ohzawa H, Miyato H, Matsumoto S, Haruta H, Kurashina K, Saito S, Hosoya Y, Yamaguchi H, Yamashita H, Seto Y, Lefor AK, Sata N, Kitayama J: Low density neutrophils (LDN) in postoperative abdominal cavity assist the peritoneal recurrence through the production of neutrophil extracellular traps (NETs). Sci Rep 2018;8:632.
19 Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. Science 2004;303:1532-1535.
20 Papayannopoulos V: Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol 2018;18:134-147.
21 Jorch SK, Kubes P: An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med 2017;23:279-287.
22 Németh T, Sperandio M, Mócsai A: Neutrophils as emerging therapeutic targets. Nature Reviews Drug Discovery 2020;19:253-275.
23 Lewis HD, Liddle J, Coote JE, Atkinson SJ, Barker MD, Bax BD, Bicker KL, Bingham RP, Campbell M, Chen YH, Chung CW, Craggs PD, Davis RP, Eberhard D, Joberty G, Lind KE, Locke K, Maller C, Martinod K, Patten C, et al.: Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. Nat Chem Biol 2015;11:189-191.
24 Eissner G, Kolch W, Scheurich P: Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. Cytokine Growth Factor Rev 2004;15:353-366.

25 Lügering A, Schmidt M, Lügering N, Pauels HG, Domschke W, Kucharzik T: Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 2001;121:1145-1157.
26 ten Hove T, van Montfrans C, Peppelenbosch MP, van Deventer SJ: Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50:206-211.
27 Neeli I, Khan SN, Radic M: Histone deimination as a response to inflammatory stimuli in neutrophils. J Immunol 2008;180:1895-1902.
28 Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, Hayama R, Leonelli L, Han H, Grigoryev SA, Allis CD, Coonrod SA: Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 2009;184:205-213.
29 Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V, Thompson P, Chen P, Fox DA, Pennathur S, Kaplan MJ: NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med 2013;5:178ra140.
30 Dimmeler S, Zeiher AM: Netting Insights into Fibrosis. N Engl J Med 2017;376:1475-1477.
31 Chrysanthopoulou A, Mitroulis I, Apostolidou E, Arelaki S, Mikroulis D, Konstantinidis T, Sivridis E, Koffa M, Giatromanolaki A, Boumpas DT, Ritis K, Kambas K: Neutrophil extracellular traps promote differentiation and function of fibroblasts. J Pathol 2014;233:294-307.
32 Martinod K, Witsch T, Erpenbeck L, Savchenko A, Hayashi H, Cherpokova D, Gallant M, Mauler M, Cifuni SM, Wagner DD: Peptidylarginine deiminase 4 promotes age-related organ fibrosis. J Exp Med 2017;214:439-458.
33 Baddley JW, Cantini F, Goletti D, Gomez-Reino JJ, Mylonakis E, San-Juan R, Fernandez-Ruiz M, Torre-Cisneros J: ESCMID Study Group for Infections in Compromised Hosts (ESGICH) Consensus Document on the safety of targeted and biological therapies: an infectious diseases perspective (Soluble immune effector molecules [I]: anti-tumor necrosis factor-alpha agents). Clin Microbiol Infect 2018;24:S10-S20.