Neutrophil extracellular traps orchestrate formation of peritoneal adhesions

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**Article**

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Abstract

Peritoneal adhesions are a poorly understood but highly prevalent condition that can lead to intestinal obstruction, pelvic pain, and infertility. While there is consensus that stress-induced inflammation triggers peritoneal adhesions, the process of their formation remained elusive to date. Herein, we show that neutrophil extracellular traps (NETs) serve as essential scaffold for adhesion formation and that DNases interfere with this process. Thus, peritoneal adhesions in murine models and in humans showed that these lesions are largely based on extracellular DNA derived from neutrophils. Furthermore, treatment with DNASE1 or a DNASE1L3 analog significantly reduced or even prevented peritoneal adhesions in experimental models. These data not only suggest that NET formation plays an essential role in peritoneal adhesions but also show that therapeutic application of DNases can prevent the formation of peritoneal adhesions.

Introduction

Peritoneal adhesions, a common consequence of serosal repair after abdominal interventions, represent a major burden for clinicians and surgeons alike. In fact, the formation of adhesions has been shown to occur as often as 93-100% of patients following abdominal surgery and can cause serious complications such as intestinal obstruction, pelvic pain, and infertility. As a result, the quality of life of millions of patients throughout the world is affected by peritoneal adhesions. These lesions are also associated with considerable costs of roughly two billion US dollar per year in the US. Their actual burden in the medical setting is highlighted by a Scottish survey performed over a 10-year period. They suggested that 5.5% of all hospital re-admissions can directly be attributed to the formation of adhesions. Lastly, the formation of adhesions has not only been linked to a reduced quality of life and significant health care costs, but also mortality rates of 6-15%.

To date, no molecular therapies exist that interfere with the formation of peritoneal adhesions. Instead, therapeutic interventions to impede the formation of adhesions are limited to bioabsorbable films, placed on surgical wounds to prevent adhesions of the peritoneum and the viscera. A number of barrier agents with various features are also available. However, these measures are complicated and time consuming. Furthermore, a recently published large Cochrane review, including 19 randomized controlled trials on the prevention of adhesion after gynecological surgery, did not reveal conclusive evidence of the effectiveness of such approaches. Taken together, a treatment preventing peritoneal adhesions represents an unmet medical need.

Despite the clinical impact of adhesions, the pathomechanism of their formation is poorly understood. Peritoneal healing is a highly complex process involving hemostasis, inflammation, angiogenesis, formation of granulation tissue, deposition of extracellular matrix (ECM), and tissue remodeling. However, there is evidence that the most important element of peritoneal healing and the formation of adhesions after peritoneal injury during surgery is inflammation. After a peritoneal injury, the innate immune system reacts within hours through a plethora of mechanisms that are endowed with both pro...
and anti-inflammatory activities. These remain activated for several days and create a delicate balance which, if disrupted, can tip over from protecting the host from microbes to mediating hyperinflammation, inhibiting healing and increasing mortality.

Neutrophils, the predominant leukocytes of acute inflammatory reactions, are immediately recruited into injured areas and remain there for about two days. They are (1) the most abundant immune cells in the human circulation, (2) regarded as the first line of defense of the innate immune system, and (3) the main leukocyte subset involved in the early phases of wound healing. In response to infection and/or injury, neutrophils form NETs, which consist of high molecular weight double-stranded DNA filaments that build robust scaffolds. These are decorated with histones and cytotoxic proteins, such as myeloperoxidase (MPO) and neutrophil elastase (NE), accounting for 70% and 20% of all proteins of NETs, respectively. Neutrophils release NETs by multiple mechanisms: (1) NETosis, a programmed cell death pathway, (2) non-lytic discharge of parts or their entire nucleus, and (3) mitochondrial DNA release, providing an additional DNA source for NET formation.

NETs are “double-edged swords” as they regulate homeostatic and pathological inflammation. During infection NETs exhibit antimicrobial functions, trap and kill extracellular pathogens in blood and tissue. However, NETs also form during sterile inflammation. NETs stimulate platelet adhesion and coagulation and the proteolytic activity of aggregated NETs traps histones and contributes to the resolution of inflammation.

While the function of NETs is essential in combating infection and inflammatory responses, a spatial and temporal inappropriate production of NETs can have detrimental effects. In fact, it has been shown that NETs contribute to the pathology of several inflammatory conditions, such as autoimmune diseases, wound healing, sepsis, and ischemia reperfusion injury. A common denominator of these disorders is the involvement of NETs as mediators of thrombosis and hyperinflammation and of the occlusion of vessels and ducts.

Based on response-to-stress and inflammation as potential triggers for peritoneal adhesions we hypothesized that NETs play an important role in the formation of such adhesions. Furthermore, we considered that therapeutic application of DNases that cleave NETs may represent a new treatment option for peritoneal adhesions.

**Results**

**Laparotomy-induced adhesions in Dnase1- and Dnase1L3-KO mice**

First, we examined the role of two endogenous DNases; DNASE1 and DNASE1L3 in abdominal adhesions. It is known that deficiency of the former contributes to lupus, whereas the latter is associated with scleroderma and autoimmunity. Mice deficient in DNASE1 or DNASE1L3 insufficiently metabolize extracellular DNA and nuclear remnants. We studied these knockout mice with the same
protocol for the formation of adhesions which included laparotomy, coagulation and two sutures. We observed that the knockout of *Dnase1* or *Dnase1l3* augmented formation of peritoneal adhesions (Figure 1A,B,E). This finding underscored the importance of DNases in the regulation of abdominal adhesions. The most distinctive phenotype with respect to the formation of adhesions were *Dnase1l3*-KO mice, indicating that DNASE1L3 is of paramount importance to prevent the formation of adhesions. The peritoneal thickness was also increased in these DNase mutant mice with more pronounced results in *Dnase1l3*-KO than *Dnase1*-KO mice (Figure1C,D).

Upon closer examination, local and systemic DNase treatment not only affected the formation of adhesions, but also influenced various aspects of peritoneal wound healing, as seen by an altered histology of the affected area (Figure 2A-H). Compared to controls, the *Dnase1l3*-KO mice displayed a significantly higher collagen type I to type III ratio (Figure 2F,I) three weeks after injury. This adds further evidence to the role of DNASE1L3 in fibrosis. *Dnase1l3*-KO mice developed dense, parallel collagen fibers at the site of peritoneal injury (Figure 2G,I). Other factors like fibrin or small muscle actin were not affected by the DNase treatment (Figure 2A,D,E,H). It appears that DNase1l3 has an important role in collagen regulation.

**Neutrophil- and NET-markers during laparotomy-induced adhesions formation**

As DNases cleave NETs, we tested the influence of reduced NETs degradation on peritoneal NET formation after abdominal injury. While the absence of DNases did not affect the total amount of Ly6G positive neutrophils (Supplement 2A,G), *Dnase1l3*-KO mice showed significantly higher levels NETs formation, as measured by staining for myeloperoxidase (MPO), neutrophil elastase (NE) (Supplement 2B,C,F,H,J) and citrullinated histone H3 (citH3) (Supplement 2D,I,J). Moreover, control mice demonstrated very high levels of DNases (especially DNASE1L3), which supports the hypothesis that DNases have a crucial role in the formation of peritoneal adhesions (Supplement 2J). When measured NE activity in the surgical sites, we observed a robust NE activity (Supplement 3A). Importantly, this activity was resistant to both the pharmacological (Sivelestat) as well as the endogenous inhibitor a1-antitrypsin (a1-AT) (Supplement 3B,C). It appears that neutrophil recruitment was similar in WT and KO mice in the context of adhesion formation.

**Laparotomy-induced adhesions in WT mice-treatment with DNases**

We next aimed to identify the procedure that most effectively reduces and/or prevents the formation of peritoneal adhesions. Recombinant DNASE1 and/or a DNASE1L3 analog were applied topically during the surgery and also systemically 24h and 48h afterwards. The DNASE1L3 analog significantly reduced adhesions at day 21 post-surgery; evaluated with the Leach and Nair adhesion scores (Figure 3A,B)). Severity of adhesion was reflected by peritoneal thickness, which was significantly reduced by treatment with DNASE1 or DNASE1L3 analog compared to controls (Figure 3C-E).

**Anastomosis, deserositation, and thermal injury-driven adhesion formation; treatment with Domase alfa**
Next, we evaluated the effects of DNASE1 on wound healing in three typical clinical settings: (1) intestinal anastomosis (2), deserosation as a consequence of abdominal surgery, and (3) thermal injury. Assessing the effects of DNase on “beneficial” wound healing is of importance to surgeons, as DNases could potentially facilitate the spread of bacteria or delay wound healing. However, our results showed that mortality rates amongst animals treated with DNASE1 undergoing deserosation, intestinal anastomosis, and thermal injury were not elevated in comparison to controls; i.e. animals treated with inactivated DNASE1 (failure of intestinal anastomosis: DNASE1 0.0% vs. controls 10.0%, p>0.05; deserosation: Dornase alfa 22.2% vs. controls 29.3%, p>0.05; heat exposure: DNASE1 0.0% vs. controls 0.0%, p>0.05). No case of wound infection or incisional hernia was found. In contrast, peritoneal adhesions were again significantly reduced in mice treated with DNASE1 (Figure 4A-C). These findings clearly demonstrate that application of DNases reduced the formation of adhesions without negatively affecting wound healing.

**Topical treatment with DNASE1 changes gene expression in peritoneal cells**

DNASE1 treatment ameliorated immune response and reduced leucocyte activation. mRNA analysis of murine peritoneal cavity cells showed that 2875 of the 3718 differentially expressed genes were downregulated after Dornase alfa treatment (Supplement 4A). The GO terms regulation of leukocyte activation (Supplement 4B) and activation of immune response (Supplement 4C) were significantly enriched in the DNASE1 treated peritoneum. The top 20 most significantly up and downregulated genes were related to leukocyte adhesion (Supplement 4B), activation of the immune response (Supplement 4C), NET-associated genes (Supplement 4D) and genes involved in nucleosome assembly (Supplement 4E) 34. The 5 most upregulated genes were Il31ra (IL31 receptor alfa), 493341K16Rik (K16RIK), Bpifa2 (BPI Fold Containing Family A Member 2), Masp2 (Mannan-binding lectin-serine-protease 2) and Bpifbb1 (BPI Fold Containing Family B Member 1) The 5 most downregulated genes were Dmbt1 (Deleted In Malignant Brain Tumors 1), Epcam (Epithelial cell adhesion molecule), Wfdc2 (WAP four-disulfide core domain protein 2 or human epididymis protein 4), Aqp5 (Aquaporin 5), Clca3a2 (Chloride channel accessory 3A2). These are characterized by the gene ontology GO:0002694 (regulation of leukocyte activation), GO:0002791 (regulation of peptide secretion), GO:0002253 (activation of immune response), GO:0019221 (cytokine-mediated signaling pathway) and GO:0002443 (leukocyte mediated immunity). We conclude that the immune system plays a key role in the formation of the adhesions.

**Role of DNASE1L3 in healing of thermal wounds in mice**

To further elucidate the role of DNASE1L3 in wound healing, a second repair model based on thermal injury was employed. Animals with the Dnase1l3-KO had similar wound closure time (Figure 5B) but significantly worse scar appearance as measured by the Yeong scale (Figure 5A, 4C). Treatment with a DNASE1L3 analog significantly improved scarring and resulted in a significantly faster wound closure (Figure 5A-C). Similar to the adhesion model, the Dnase1l3-KO animals had a significantly higher collagen type I to type III ratio (Figure 5D) and a more parallel alignment of collagen (Figure 5E,F). Particularly the last finding is indicative of immature scars 35. The absence of DNASE1L3 did neither significantly affect neutrophil concentrations nor NETs formation. However, there was a trend towards higher Ly6G positive
neutrophils and higher expression of NE and citH3 (Figure 5G-J). Treatment with a DNASE1L3 analog significantly reduced neutrophil activation and NETs formation (Figure 5H,I).

**Human adhesions contain neutrophil-borne proteins and extracellular, NET-like DNA.**

To find out whether NETs are also components of human peritoneal adhesions, we analyzed human surgery material. As shown in representative images (Figure 6 and Supplement 5), the NET-associated proteins NE and MPO were expressed in the human peritoneal adhesions (Figure 6A). Additionally, the expression of DNASE1L3, especially at the margins of the adhesion, was detected (Figure 6D). The isotype controls (Figure 6B, 6E) were negative for the fluorescence signal, confirming the specificity of the signal obtained for NE, MPO and DNASE1L3. Figure 6C shows a Hematoxylin & Eosin (HE) staining of the same biopsy. Enlarged details of the surgical biopsy material are depicted in Supplement 5 with additional positive immunofluorescence signals for the expression of the hallmark NET marker citH3. In a longitudinal section of a human adhesion the canonical NET markers MPO, citrullinated histone H3 and DNA were detected in addition to fibrin (Supplement 6).

**Discussion**

Our findings suggest that NETs play a pivotal role in the formation of peritoneal adhesions in mice and humans. We support previous research, which suggests that the pathogenesis of the formation of adhesions is based on a combination of inflammation, coagulation, and fibrinolysis.

It is established that the inflammatory phase precedes wound healing. However, there are also controversial reports: (1) Wounds in areas with inherently lower levels of macrophages, neutrophils, and T cell infiltration, such as oral wounds, heal instantly with marginal inflammation and scar formation, (2) neutrophil depletion in mice accelerates the re-epithelialization rate of uninfected diabetic wounds, (3) in the wounds of diabetic mice elevated levels of citrullinated histone H3 (citH3) were found and healing was delayed; (4) wound healing was accelerated in peptidylarginine deiminase 4 (Pad14)-KO mice which have very limited NETs formation when compared with WT mice, and (5) DNASE1 accelerated healing of incisional wounds in diabetic mice.

In peritoneal as well as thermal wound healing, NETs appear to have similar disadvantageous effects. This is supported by our findings that at sites of peritoneal injury NETs were found and DNases were expressed in both humans and mice. This finding suggests that NETs and DNases regulate the formation of adhesions. A loss-of-function single nucleotide polymorphism in Dnase1l3 is associated with systemic sclerosis, a rare condition that is characterized by hardening of the skin and tissue due to excessive accumulation of collagen. We show that Dnase1l3-deficient mice formed massive deposits of collagen in the skin and peritoneum upon injury. Further, topical application of a recombinant DNASE1L3 analog reduced scarring and facilitated healing. Collectively, the data suggest that DNASE1L3 is critical for tissue regeneration.
Assessment of peritoneal adhesions revealed widespread abundance of NETs characterized by extracellular DNA and neutrophil elastase activity. NETs may likely represent scaffolds for peritoneal adhesions. Thus, injury leads to recruitment of neutrophils which increase steadily at inflammatory foci, where they form NETs. When NETs reach high local densities, they aggregate and form structures that span over several centimeters, that can easily bridge two intestinal loops. Since DNA builds the backbone of NETs, they are robust, sticky, highly flexible and elastic. If they are not cleaved by DNases in time, they go through a certain degree of maturation, activate platelets and fibrinogen and are decorated with high molecular weight fibrin. The latter physically strengthen these structures by providing attachment points for fibroblasts, endothelial cells and, smooth muscle cells and immune cells, which eventually organize and develop into mature adhesions.

The study's results are promising and DNase therapy to prevent the formation of adhesions is ready to be evaluated in a clinical study. As our data shows, DNases do not appear to negatively affect wound healing. Indeed, our results suggest an enhanced physiological wound healing process after treatment with DNases, particularly with the DNASE1L3 analog. The main effect appears to be the reduction of NETs-associated inflammation as confirmed by the RNA seq data. We observed that DNases application or KO-Models reduced and increased the formation of adhesions, respectively; in both models wound healing was preserved. It did not enhance the incidence of hemia formation or anastomotic insufficiency.

DNases are an elegant option to prevent the formation of adhesions; they are cost effective and can metabolize extracellular DNA in vitro. DNases are already being used for inhalation in patients with cystic fibrosis. So far, no serious adverse effects have been recorded with the application of DNases. Furthermore, our data also did not show any adverse effects of DNase treatment on wound healing. In summary, we have shown that the formation of peritoneal adhesions is driven by NETs, and can be counteracted by DNase treatment.

Declarations

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**Conflict of Interest:** M. B., M. H., and T. A. F. are stakeholders of and/or receive research funding from Neutrolis. The lead compound of Neutrolis was not used in this study. Apart from that, the authors have no conflicts of interest relevant to this article to disclose.

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Methods

Study design:

The study was approved by the Hamburg State Administration for animal research (73/15, 63/16). A total of 143 six–week-old mice were utilized for the experimental model and all environmental parameters within the animal facility complied with the German guide for the care and use of laboratory animals (Animal Welfare Act). All animals including the genetic knockouts (\textit{Dnase1-KO} and \textit{Dnase1l3-KO}) used to examine the role of DNases in the process of the formation of adhesions and wound healing had the same genetic background (C57BL/6). The \textit{Dnase1-KO} and the \textit{Dnase1l3-KO} mice were generated as described earlier\textsuperscript{13,44,45}. We obtained the WT mice from Jackson Laboratory and employed littermates for all treatment groups.

Additionally, peritoneal samples of nine children who underwent a second laparotomy within two weeks after their primary laparotomy were included in the study (Department of Pediatric Surgery of the University Medical Center Hamburg-Eppendorf from 2017 to 2019) and analyzed histologically. Samples were obtained only from cases with non-infectious conditions that lead to re-operative surgery. Anonymized tissue collection was in accordance with the guidelines of the medical research ethics committee of Hamburg (Ethik-Kommission der Ärztekammer Hamburg, PV5489) and with the 1964 Helsinki declaration and its later amendments. We obtained written informed consent from the legal representatives.

Human sample collection

Peritoneal tissue samples from the maximum of the adhesion were collected at the time of secondary relaparotomy surgery. Patients with infectious conditions were excluded from this current study. We stained and analyzed the samples as described above for murine samples.

Tissue sampling

After blood collection, morphologic analysis was performed and captured using a 4K/12-megapixel camera. Next, the scar was dissected and evenly distributed into test tubes containing Bouin solution.

Generation of \textit{Dnase1} knockout mice

\textit{Dnase1} mutant mice were generated by CRISPR/Cas9 mediated mutagenesis in JM8A3 embryonic stem (ES) cells from C57BL/6N origin 44. In brief, ES cells were transfected with pX458 (obtained from Addgene, Watertown, MA, in which the \textit{Dnase1} specific gRNA-sequence 5´ TGACATCGCTGTTATCCAAG 3´ was inserted 45. GFP-expressing ES cells were sorted and mutations in individual ES cell clones were analyzed by sequencing the amplicon generated by primers flanking the target sequence in exon 3. One clone showing a 65 bp deletion from intron 2 into exon 3 was selected for blastocyst injections,
generation of chimeric mice and further breeding with C57BL/6N mice. The deletion does not allow splicing into exon 3, and potential alternative splicing into exons 4, 5 or 6 containing the active sites for DNASE1 enzymatic activity lead to frameshift mutations and premature stop codons.

Animal Procedures

We first determined the time course of extracellular DNA formation in the course of NET formation after injury (laparotomy) in wild-type mice (see methods section). We visualized extracellular DNA using SYTOX orange and observed a peak 72 hours after the induction of adhesions (not shown). We observed the cumulative maximum of adhesions at 21 days using the Leach and Nair adhesion score (not shown). Therefore, we used both day 3 as well as day 21 post injury to assess the effects of DNases on NET formation and abdominal adhesions (Supplement 1A).

Mice were randomized into groups of equal size. For better standardization, a single surgeon performed all operations. Anesthesia was induced with 5% isoflurane (Baxter, Unterschleißheim, Germany) and maintained with 2.5% isoflurane gas delivered through a facemask. Preoperative antisepsis was performed with iodopovidone and all mice received 0.02 mg/kg bodyweight (BW) buprenorphine (Reckitt Benckiser, Mannheim, Germany) subcutaneously, 30 min preoperatively for analgesia.

The study involved two models: (1) adhesion model to assess the effect and implications of NETs and DNase treatment on the formation peritoneal adhesion, (2) secondary intention wound healing model to evaluate DNases in wound healing after a thermal injury.

Model 1: Adhesion formation

Adhesions were induced using a bipolar electrocoagulation method 52: Standardized lesions were inflicted on an area measuring 0.5 cm × 1.5 cm by sweeping the bipolar electrocoagulation forceps over the abdominal peritoneum for 2 seconds. The current was delivered using the following settings: Bipolar Soft, Effect 4, 40 Watts. The defects were subsequently closed using two interrupted sutures (6/0 Vicryl, Ethicon, Norderstedt, Germany) to induce an ischemic field around the traumatized area. The sutures were placed equidistantly (5 mm) along the defect and 1 mm from the wound's edge.

To establish the most effective therapy, several treatment combinations using recombinant human DNASE1 (Dornase alfa, Roche, Mannheim, Germany) with a dosage of 10 mg/kg BW, as well as NTR-10, a recombinant human DNASE1L3 analog provided by Neutrolis, Cambridge, MA, USA, with a dosage of 1 mg/kg BW were tested. The control groups received a vehicle. Ultimately, a sham group without (1) the intervention, other than the laparotomy, and without (2) treatment was included.

To determine the effects of DNases on wound healing, three typical clinical scenarios were reproduced: (1) Deserositation – induced by rubbing a mini-prep on the wall of the small intestine, (2) Intestinal anastomosis - performed with a 8x0 Vicryl continuous suture after dissection of a small segment of the small intestine, and (3) Thermal injury - induced by heat exposure on the intestine using a red lamp with a distance of 1 meter for 10 minutes.
Model 2: thermal injury

Thermal injuries, serving as a model for secondary intention wound healing, were induced as described previously. In short, a 1.5cm x 1.5cm large burn injury was induced on the neck of the animals. At the two timepoints (72 hours or 21 days), animals were euthanized after anesthesia using isoflurane as described above. Re-laparotomy was conducted and assessment of the formation of adhesions or dermal scaring was performed. Ultimately, resection of the lesion took place prior to all animals being euthanized via decapitation.

Assessment of the adhesions

All adhesions were evaluated immediately after re-laparotomy. Macroscopic grading of the formation of adhesions was assessed by two independent surgeons, blinded to the animal groups and blinded to each other, using the Leach grade, as well as the Nair grade. The Leach grade was originally designed to score adhesions of the uterine horn and was thus modified for this study to evaluate peritoneal adhesions. The Leach score consists of three factors: (1) severity of adhesions (0=no adhesion, 1=filmy avascular, 2=vascular or opaque, 3=cohesive attachment), (2) degree of adhesions (0=no adhesion, 1=adhesion separable with gentle traction, 2=adhesion separable with moderate traction, 3=requiring sharp dissection), and (3) extent of adhesions (0=no adhesion, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%).

The Nair score consists of two factors: (1) macroscopic adhesions (0=no adhesion, 1=single band of adhesion between viscera to abdominal wall, 2=two bands between viscera to abdominal wall, 3=more than two bands to abdominal wall), and (2) microscopic adhesions (0=no fibrosis, 1=fibrosis with thin collagen bundle, 2=tissue with wider and less vascularized collagen fibrosis, 3=tissue with thick collagen bundle).

Assessment of the scars

Burn scars were evaluated before euthanasia using the modified Yeong scale, by two surgeons, blinded for the treatment groups. This 3-item wound evaluation scale was specifically developed for thermal injuries assessing the scar surface appearance, height and color mismatch from 1 (best) to 4 (worst) for each item, resulting in a total score ranging from 1 (best scar) to 12 (worst scar).

Microscopic grading

All specimens were evaluated histologically. In our burn model the scars were marked with blue dye for better microscopic evaluation and standardization. All specimen were then washed in phosphate buffered saline (PBS) and fixed in 10% buffered formalin before being embedded in paraffin and cut into 3µm thick sections, slides were then stained using hematoxylin and eosin (HE) and examined by two researchers who were blinded to the groups in light microscopy, using a magnification of ×4 and ×10. Assessment of wound healing (epithelialization) was carried out in a standardized manner and expressed as a percentage of the whole wounded area. The unhealed wound was measured as the distance...
between both edges of the wound and the total wound diameter as the distance between the wound edges.

**Immunohistochemistry (HE, Ly6g, Collagen I/III, SMA, Fibrin)**

Hematoxylin and Eosin (HE) and Lymphocyte Antigen 6 Complex Locus G6D (1A8-Ly6G) staining was performed with a standardized staining procedure. Collagen fibers were stained using Pico Sirius red (ab150681, Abcam, Cambridge, UK), using polarized light microscopy was used to differentiate collagen I from III. An antibody for smooth muscle actin (SMA, ab5694, Abcam, Cambridge, UK) was applied to the samples, serving as a marker for myofibroblast, which induce wound contraction. Fibrin deposition was determined using a fibrinogen antibody (ab58207, Abcam, Cambridge, UK). Subsequently, the stained samples were incubated according to manufacturer’s instructions. In accordance with each antibody examined, an appropriate isotype control antibody was used as a negative control. All samples were scored semi-quantitatively using following score: (I) None 0: – no signs of tissue staining; (II) Little 1 – small amount of tissue staining; (III) Medium 2 – medium amount of tissue staining; (III) Strong 3 – strong amount of tissue staining. The assessment of collagen alignment was scored based on the orientation of the bundles (0= diffuse with bundles in 90° angle to 4=parallel).

**Immunofluorescence Staining (MPO, NE, citH3, anti-DNA, fibrin)**

3μm-paraffin tissue sections underwent a deparaffinization and rehydration process followed by immunofluorescence staining for myeloperoxidase (MPO), neutrophil elastase (NE), citrullinated histone 3 (citH3), DNA and fibrin. Antigen retrieval was assessed by incubating the sample slides with Target Retrieval Solution pH 6 (Dako, Santa Clara, USA) in a 97°C water bath for 10 min following a cooling step of 30 min. After rinsing the sections twice for three min with a solution of tri-buffered saline and polysorbate 20 (Tween 20) (TBST), blocking of the probes was performed with a Donkey Block (BioGenex, Fremont, USA) for 30 min at room temperature (RT). Tissue specimens were further incubated with either isotype- or antigen-specific-antibodies at 4°C: Goat anti-mouse MPO- (AF 3667, R&D Systems, Minneapolis, USA) diluted 1:20, rabbit anti-mouse NE- (AB68672, Abcam, Cambridge, UK) diluted 1:200, rabbit anti-mouse citH3- (AB219406, Abcam, Cambridge, UK) diluted 1:100, mouse anti-DNA- (CBL186, Merck KGaA, Darmstadt, Germany) diluted 1:100 and rabbit anti-human fibrinogen alpha chain-antibodies (AB92572, Abcam, Cambridge, UK) diluted 1:100. Twelve hours later, sections were rinsed 3x5 min with TBST and subsequently incubated 1:200 with AF647- or Cy3 at RT for 30 min (Jackson ImmunoResearch Europe Ltd., Cambridge, UK). After a 3x 5 min rinsing-step with PBS, nuclei were counterstained by incubating probes with DAPI for 5 min at RT. Finally, slides were rinsed 5 min with H2O, and mounted with Fluoromount-G (Southern Biotech, Birmingham, USA). Isotype control antibodies were used as a negative control (MPO = AB-108-C, R&D Systems, Minneapolis, USA; NE = AB37415, citH3 = AB172730, Abcam, Cambridge, UK). Images were taken employing an Aperio VERSA 8 Slide Scanner (Leica Biosystems, Wetzlar, Germany) and processed with Aperio ImageScope Version 12.3.3.5048 (Leica Biosystems) and Adobe Photoshop CC Version 19.1.8 (Adobe Inc, Mountain View, CA, USA).
Activity of neutrophil elastase

Material from the surgical site of all groups of mice (n=5/group) was washed twice in PBS (ThermoFisher Scientific, 14190250). Then the fluorogenic substrate MeOSuc-AAPV-AMC (Santa Cruz Biotechnology, sc-201163) was added to a final concentration of 100 µM into 48-well plates. Neutrophil elastase from human leukocytes (Sigma-Aldrich, St. Louis, MO, USA, E8140) was used to quantify the activity. To assess the inhibitory effect of the pharmacological and endogenous inhibitors, Sivelestat (final concentration 400 µM, Sigma-Aldrich, S7198) and α1-antitrypsin (final concentration 1 mM, Sigma-Aldrich, A9024) were added to the surgical material, respectively. Fluorescent readings at 37°C were collected on a TECAN Infinite 200 Pro (Tecan, Männedorf, Switzerland) using the filter set (excitation 360nm, emission 465nm) at 20 min intervals. To evaluate the inhibitory potential of Sivelestat and α1-AT we compared the gain of fluorescence two hours before and two hours after addition of the inhibitors.

RNA Sequencing

Sequencing was performed at NovoGene, Peking, China. Treated samples were measured in quadruplets while control samples were measured in triplets and between 36 and 43 M paired-end sequence reads of length 150bp were obtained per replicate. Sequence data have been submitted to the European Nucleotide Archive (ENA). They are publicly available under accession PRJEB40510. Fastp (v0.20.1) was used to remove sequences of sequencing adapters and low quality (Phred quality score below 20) sequences from the 3’-end of the sequence reads 56. Thereafter, reads were aligned to the human reference assembly (GRCh38.98) using STAR (v2.7.5c) 57. Differential expression was assessed with DESeq2 58. Genes were considered differentially expressed when the absolute log2FC was 1 or higher and the FDR was 0.1 or lower. WebGestalt (v.2017) was employed for over-representation analysis (ORA) of gene ontology (GO) terms and Reactome pathways 59.

Statistics

All data were analyzed using SPSS Statistics 26 (IBM, NY, USA) and GraphPad Prism 9 (GraphPad, CA, USA). A pre-power study calculation was performed using G*Power 3.1. The power was deducted from previous trials regarding inflammation and NET formation 18. Differences between groups were calculated using mixed-effect model with Geisser-Greenhouse correction as well as Dunnett’s multiple comparison test. Data is presented as mean ± standard deviation (SD). The level of significance was set at 0.05.

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**Figures**
NETs precipitate the formation of adhesions. (A, B) Animals with targeted deletion of Dnase1 and even more Dnase1l3 displayed significantly higher Leach and Nair adhesion scores than controls. (C) Adhesions are reflected by significantly increased thickness of the parietal peritoneum in the area of the peritoneal injury. (D) Representative HE images of DNases knockout mice compared to controls and
shams. Data shown as mean±SD. Statistics: ANOVA with Dunnett’s correction or Kruskal-Wallis test with Dunn’s correction.

**Figure 2**

DNASE1L3-deficiency affects wound healing by inducing intensive collagen deposition. (A,E) DNases appear not to affect SMA in short- and long-term which is a marker of wound contractility. (B,C, F,G) Collagen 1:3 ratio and collagen alignment are used to access wound maturation. In Dnase1l3 knockout
mice very high levels of collagen I and III were to be found. Even after three weeks, the collagen fibrils remained aligned in parallel. This pattern is often associated with an immature wound and fibrosis. (D,F) Fibrin remained the same in all groups. Data shown as mean±SD. Statistics: Kruskal-Wallis test with Dunn's correction.

Figure 3

Topical therapy with DNASE1L3 analog reduces the formation of adhesions. (A-C) The Leach and Near score were used to access the formation of adhesions at day 21. The most effective treatment option was NTR-10, a DNASE1L3 analog. (D,E) The thickness of the parietal peritoneum was significantly reduced in mice that received topical DNase treatment when compared to controls. (E) Representative images of the peritoneal thickness. Data shown as mean±SD. Statistics: ANOVA with Dunnett’s correction or Kruskal-Wallis test with Dunn’s correction.
Figure 4

DNASE1 reduces the formation of adhesions but maintains wound healing. To test various typical clinical scenarios, mice were subjected to anastomosis of the small intestine (A), deserositation (B) and thermal injury (C). Treatment with DNASE1 (Dornase alfa; Dor-a) prevented the formation of adhesions in all three scenarios almost completely. Additionally, it did not affect the rate of incision hernia, suture insufficiency,
peritonitis or mortality but rather improved it. Data shown as mean±SD. Statistics: Kruskal-Wallis test with Dunn’s correction.

Figure 5
A C day 7

In a murine model of wound healing after thermal injury therapy with DNASE1L3 analog reduces scarring and fibrosis. (A) Assessment with the scar score showed significant worse scare appearance in affected mice compared to controls and mice treated with NTR-10, a DNASE1L3 analog. (B) Mice with targeted
deletion of Dnase1l3 did not affect wound closure. Topical NTR-10 significantly accelerated wound closure. (C) Representative images of the wounds. (D-F) Collagen 1:3 ratio and alignment were affected similarly as in the adhesion model. The intense collagen depositions in the skin displayed parallel alignment. (G-J) Neutrophils and NETs formation. Data shown as mean±SD. Statistics: Kruskal-Wallis test with Dunn's correction.

Figure 6

Human adhesions show NETs and DNASE1L3 deposition. (A) Paraffin section of a human adhesion shows expression of the characteristic NET proteins myeloperoxidase (MPO, red) and neutrophil elastase (NE, green). (B) Isotype control for MPO and NE from (A). (C) HE staining of the same human adhesion. (D) Human adhesion also shows expression of DNASE1L3 (D1l3; red). (E) Isotype control for the staining of DNASE1L3 in (D). (A,B,D,E) DNA was counterstained using DAPI. The size bar represents 100 µm.

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