PART II

The role of coagulation and oxidative stress in the pathophysiology of burns
Neutrophil extracellular traps coincide with a pro-coagulant status of microcirculatory endothelium in burn wounds

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ABSTRACT

Background: Burn-induced tissue loss is partly related to secondary expansion of necrosis into vital dermis neighboring the initial burn injury. An important factor herein is the severe loss of perfusion of the burn wound, probably caused by microvascular damage induced by the intense local inflammatory responses as well as burn-induced hypercoagulation. We hypothesize that the formation of neutrophilic extracellular traps (NETs) play an important role in this. The purpose of this study was to investigate post-burn intravascular thrombosis, NETs formation and the coagulant state in the microvasculature of burns in both animal models and patients.

Methods: We used two in vivo burn wound models: rats and pigs. In rats, the entire wound was excised at day 14 post-burn and in pigs burn wound biopsies were collected at different time points up to 60 days post-burn. To confirm the data in patients, eschar from the burn wound was obtained from burn wound patients at different time points after wounding. The number of intravascular thrombi, the presence of intravascular NETs and the number of tissue factor (TF) positive blood vessels in the burn wound was determined.

Results: In rats, a significant increase in intravascular thrombi and TF expression was observed 14 days post-burn, that in majority coincided with NETs. In pigs, a significant increase in intravascular thrombi and TF expression was found over time up to 60 days post-burn, that in majority coincided with NETs too. Also in eschar of burn wound patients, a significant increase in intravascular thrombi was noted, that in majority coincided with NETs, already 0.5 days post-burn and remained elevated up to 46 days post-burn.

Conclusion(s): This study shows the presence of NETosis in microcirculatory thrombosis of burn wounds and a switch in the microcirculatory endothelium toward a pro-coagulant phenotype.
INTRODUCTION

Patients suffering from burn injuries endure lifelong consequences, due to the loss of vital tissue and subsequent defective healing resulting in extensive scarring. The severity of these consequences is determined by multiple factors, including the depth of the wound and the percentage of body surface affected [1]. A significant proportion of the tissue loss can be caused by secondary expansion of necrosis into the surrounding vital dermis neighboring the initial burn injury, leading to an increased burn depth and area [2]. As such, partial thickness burns often convert into deeper or full-thickness burns, a process called “burn wound conversion” [3]. A potentially important contributing factor is the severe loss of perfusion in the center and the border zone (i.e., zone of stasis) of the burn wound [2], as a result of microvascular damage. This loss of perfusion may delay clearance of dead tissue and leads to ischemia, which in turn result in delayed wound healing, wound expansion, and excessive scarring. This damage is probably instigated by a combination of both the intense local inflammatory responses and the burn-induced hypercoagulation [2,3]. However, the exact mechanisms underlying this burn-induced microangiopathy and thrombosis are not fully understood yet.

Essential mediators for microvascular damage in the zone of stasis are thought to be neutrophils [4]. Neutrophils are the first inflammatory cells known to infiltrate the wound after burn trauma and are shown to persist for weeks post-burn [5]. Neutrophils are important in the antibacterial defense. However, their activation also results in the production of proinflammatory cytokines and reactive oxygen species (ROS) which may damage vital skin and microvasculature and enhance ischemic tissue necrosis. Indeed, neutrophil depletion resulted in significantly reduced dermal vascular permeability in a rat burn wound model [4].

In 2004 Brinkmann et al. introduced a novel defense strategy of neutrophils, whereby so-called neutrophilic extracellular traps (NETs) are formed. NETs are assembled through an active, generally suicidal process called NETosis, whereby neutrophils eject net-like structures consisting of DNA and histones, and a number of different antimicrobial and proinflammatory granular proteins, including myeloperoxidase (MPO), elastase and cathepsin G [6,7]. NETs are essential in the innate immune response against microbial infection as they trap and neutralize microbial pathogens [6]. However, NETosis also occurs independent of infection and was recently identified as a possible link between sterile inflammation and thrombosis [8]. The question remains whether formation of NETs is a consequence of the hypercoagulative state present within the burn wound, or whether the NETs set the stage for the increased thrombus formation. However, the externalized nucleosomes in conjunction with elastase and cathepsin G can initiate coagulation, through activation of platelets as well as the intrinsic (“contact activation pathway”) and extrinsic (tissue factor (TF) pathway) coagulation cascades [9,10]. Moreover, Martinod et al. described that the extracellular release of DNA
and protein components of NETs, such as histones and serine proteases, contributes to coagulation and platelet aggregation, causing eventually thrombus formation [10]. Furthermore, expression of TF in NETs was suggested as a novel mechanism for the induction of thrombosis and inflammation in vasculitis [11].

Since its discovery, NETosis was shown to be an important mediator of tissue damage in various pathologies. NETs have been found in tissues and the circulation in major trauma, shock, and sepsis [12]. However, knowledge regarding a possible role for NETs in burn injury, is scarce.

Increased levels of circulating cell free DNA/NETs were measured in patients after burn trauma [13,14], indicating their putative relevance in human burn victims also. Although, the link with thrombosis is still unknown.

We hypothesize that after burn trauma, burn-induced microvascular damage and intravascular thrombosis occur and that NETosis is involved in these phenomena. Therefore, the aim of the present study was to investigate post-burn intravascular thrombosis, NETs formation and presence of pro-coagulant TF in the microvasculature of burned skin in time after burn trauma in both animal models and patients.

MATERIALS AND METHODS

All procedures were executed in agreement with the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam.

In vivo rat burn wound model

Twelve-week-old female Wistar rats (n = 13) were acclimatized for 1 week prior to the experiment. During the whole study, the rats were individually housed with ad libitum access to food and water. Seven rats were anesthetized using 2.5% isoflurane and received Temgesic (buprenorphine, 0.05 mg/kg, subcutaneous) as an analgesic. Subsequently, a dorsal full-thickness burn wound (4x2 cm) was created on a shaved part of the skin (just below the shoulders) by resting a copper stamp heated to 100°C for 15 seconds on the skin. The wounds were covered with nonadhesive bandage. Six rats did not receive a burn wound and served as controls. After 14 days, the rats were terminated. From the rats who received the burn wounds, the entire wound as well as healthy skin from the hind leg (internal control) was excised. From the control rats, dorsal skin was excised. The skin samples were immediately transferred to formalin for fixation and subsequently embedded in paraffin. Tissue from these rats was used in a previous study [15].
In vivo pig burn wound model

In order to study the different parameters in time, we used tissue samples from a porcine experiment [16].

Three-month-old female Yorkshire pigs (n = 7), weighing approximately 30 kg at arrival, were acclimatized for 1 week prior to the experiment. During the whole study, the pigs were individually housed and fed twice daily, with ad libitum access to water. The pigs were divided in two groups. Four pigs did not receive burn wounds and served as controls.

The sedation, anesthesia and analgesia procedures used in the three pigs that received burn wounds were described in detail in our previous study.16 Briefly, one day prior to burn, the pigs received a transdermal Fentanyl 35 mg/hour patch of the posterior pinna (which was replaced at day 2) and oral administration of meloxicam 0.4 mg/kg (for a period of 3–5 days) for analgesia.

The next day the pigs were sedated with a combination of ketamine 10 mg/kg, midazolam 0.5 mg/kg and atropine 0.5–1.0 mg by intramuscular (i.m.) injection.

Before wound infliction, full anesthesia was induced using propofol 100–150 mg via intravenous (i.v.) injection, and analgesia fentanyl 200 mg, which was maintained by i.v. injection of propofol 10 mg/kg/hour and fentanyl 6.5 mg/kg/hour. For postoperative pain relieve meloxicam 0.4 mg/kg was administered i.m. 15 minutes prior to surgery.

Anesthesia during the biopsy and bandage-change procedures was induced by intramuscular injection of zolazepam and tiletamine 6 mg/kg and xylazine 1.5 mg/kg, while analgesia was induced by meloxicam, 0.4 mg/kg.

The burn wound procedures used in the three pigs we previously described in detail.16 Briefly, eight (four wounds on each flank) dorsal full-thickness burns of 4x4 cm burned area (6.1.5% total body surface area [TBSA]) were created using a heated copper device (170°C) during 20 seconds. The wounds were covered with wound dressing sterile gauzes, which were kept in place by adhesive bandage and elastic stockings.

From the burn wounds biopsies (4 mm diameter, containing the complete dermis and part of the subcutis in depth) from two different wounds per time point (one from each flank) were collected at day 3, 6, 9, 14, 21, 30, 40, and 50 post-burn. At 60 days post-burn, all seven pigs were terminated with euthasol 100–200 mg/kg. From the experimental burned pigs, the entire wounds were excised as well as skin samples from remote nonburned areas from the back (internal control). From the control pigs corresponding dorsal skin samples were excised. All skin samples were fixed in formalin and embedded in paraffin.
Burn wound patients

The investigation conforms to the principles of the declaration of Helsinki.

Eschar from the burn wounds from 37 burn wound patients who were admitted to the Burn Center of the Red Cross Hospital in Beverwijk, the Netherlands were obtained on medical indication. Control skin biopsies, containing the epidermis and dermis were taken from seven nonburn wound patients undergoing abdominoplasty (table 1).

All tissues were fixed in formalin and embedded in paraffin.

(Immuno)histochemistry

For immunohistochemistry, sections were dewaxed and rehydrated in xylene and ethanol (100%) followed by incubation in a methanol/H$_2$O$_2$ solution for 30 minutes to block endogenous peroxidases. Antigen retrieval was performed by either boiling slides in a citrate pH 6.0 (antihuman CD31, anti-human CD61, and anti-rat/pig/human H3-Citrulline) or a TRIS-EDTA pH 9.0 (ant-rat/pig CD31 and anti-rat/pig/human TF) solution in a microwave for 10 minutes. Thereafter, the slides were incubated with either rabbit anti-rat CD31 (1:400), anti-pig CD31 (1:100) (Bioss, Woburn, MA), or mouse anti-human CD31 (1:40, Dako, Glostrup, Denmark) to visualize thrombi. In order to confirm intravascular thrombi found with CD31, mouse anti-human CD61 (1:50, Dako) was used. Rabbit anti-rat H3-Citrulline (1:100), anti-pig H3-Citrulline (1:100), or anti-human H3-Citrulline (1:100) (Abcam, Cambridge, United Kingdom) were used to visualize NETs. Rabbit anti-rat TF (1:250), anti-pig TF (1:100), or anti-human TF (1:100) (Biorbyt, Cambridge, UK) were used to visualize TF. Slides were incubated for 1 hour at room temperature.

Subsequently the slides were incubated with undiluted goat anti-mouse/rabbit EnVision (Dako, Denmark) for 30 minutes at room temperature. Positive staining was visualized using 3,30-diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated, and covered. As a control, the same staining procedure was used, but instead of the primary monoclonal or polyclonal antibody, 1% bovine serum albumin/phosphate buffered saline solution was used. These slides were found to be negative (data not shown).

Immunohistochemical analysis/the scoring system

After immunohistochemistry, the tissue slides were evaluated by light microscopy.

(1) CD31: Thrombi were visualized with CD31+ thrombocyte aggregations inside the lumen of blood vessels (figure 1A) and confirmed with CD61 staining (figure 1B). The number of intravascular thrombi per tissue slide was quantified by counting the number of blood vessels containing...
thrombi, divided by the total surface of the tissue, which resulted in the number of intravascular thrombi per square millimeter (mm²).

The counting was performed manually using a light microscope, while for determining the total surface of the tissues, the tissue slides were scanned with Pathscan Enabler IV (Meyer Instruments, Houston, TX) and measured with the QuickPhoto software (windows version 3.0, Promica, Prague, Czech Republic).

(2) H3-citrulline: The presence of NETs in intravascular thrombi was evaluated by using serial slides of: CD31 staining to visualize thrombi, MPO staining to visualize neutrophils and H3-citrulline staining to visualize cell free DNA (H3 Histone) [17] (figure 1C).

(3) TF: In order to visualize the pro-coagulant blood vessels in the tissues TF was used (figure 1D). The number of TF+ blood vessels per tissue slide was quantified by counting the number of blood vessels showing TF expression on their endothelium, divided by the total surface of the tissue, which resulted in the number of TF+ blood vessels per square millimeter (mm²).

Statistical analysis

Statistical analyses were performed with SPSS (Windows version 20.0, IBM corp., Armonk, NY). To avoid the normality assumption, data were compared using the nonparametric Kruskal-Wallis test. The Dunn’s multiplicity correction was applied to the resulting p-values in order to control the type I error. Dunn’s multiple testing corrected p-values_0.05 were considered significant.

Results are described as: median; first quartile (Q1) to third quartile (Q3) and mean (“+”).
RESULTS

NETs within intravascular thrombi and increased TF-positive blood vessels at 14 days post-burn, in burned rats

First the presence of intravascular thrombi, visualized as CD31+ thrombocyte aggregations, was analyzed in the burn wound and remote healthy skin obtained 14 days post-burn and in healthy skin from noninjured control rats.

In healthy skin from control rats no thrombi were observed (not shown). In the burn wound however, a significant increase was found in the number of thrombi positive vessels per mm² compared to control rats (burn: 0.6/mm², control: 0/mm²; p < 0.001) (figure 2A). Interestingly, an increase in the number of intravascular thrombi was noted also in remote healthy skin from injured rats, albeit nonsignificant (0.2/mm²). The observed thrombi showed no signs of morphological organization, indicative of recent thrombi formation.

Next, the presence of NETs in intra-microvascular thrombi were evaluated. NETs were observed in the majority of intra-microvascular thrombi of the burn wound. An example is shown in figure 2B. NETs were also observed in intra-microvascular thrombi in the remote healthy skin of rats who received burn wounds (not shown).

Finally, to analyze a putative pro-coagulant state of the microvasculature, TF expression in the microvasculature was quantified as the number of TF-positive blood vessels/mm². In control skin, no TF-positive blood vessels were observed. Conversely, the number of TF-positive blood vessels per mm² in burn wounds (0.2/mm²) as well as in internal control skin (0.1/mm²) was increased significantly compared to control skin (p < 0.05) (figure 2C).

Figure 1. Examples of an intravascular thrombus, NETs and TF-positive vessels. An intravascular thrombus visualized with CD31 (A) and CD61 (B), NETs (C), and TF-positive vessels (D).
Thus, thrombi were observed in the microvasculature of the burn wound and of remote healthy skin in rats 14 days post-burn. In the burn wound, these thrombi in majority colocalized with NETs. The occurrence of intramicrovascular thrombosis in time after burns and the presence of NETs herein was then assessed in a pig burn wound model.
Figure 2. Intravascular thrombi, NETs, and TF-positive blood vessels at 14 days post-burn, in burned skin of rats.
The number of intravascular thrombi per square millimeter (A), an example of NETs within intravascular thrombi (B) and (C) the number of TF-positive blood vessels per square millimeter in the skin of control (C), internal control (IC) and burned rats, 14 days post-burn (C). Median ("line"); first quartile (Q1) to third quartile (Q3) and mean ("+").
*p ≤ 0.05. **p ≤ 0.01.

Post-burn intravascular thrombi, NETs and TF-positive blood vessels up to 60 days post-burn, in a porcine burn wound model

Intra-microvascular thrombi formation, the presence of NETs as well as microvascular TF expression were analyzed in the burn wound 3, 6, 9, 14, 21, 30, 40, 50, and 60 days post-burn and in remote healthy skin in experimental burn wound pigs and in healthy control pigs.

In the skin of healthy control pigs, no intramicrovascular thrombi were observed. In the burn wounds of experimental pigs, a nonsignificant increase in the thrombi was noted between 3 and 6 days post-burn (0.5/mm²) (figure 3A). The numbers of thrombi positive vessels increased further after 9–14 days (1.6/mm²; p < 0.01), 21–30 days (1.0/mm²) and remained increased up to 40–60 days (1.6/mm²; p < 0.01). Interestingly, as observed in the rats, a nonsignificant increase in the number of intramicrovascular thrombi was observed in the remote healthy skin of experimental pigs at 60 days post-burn (0.4/mm²) (figure 3B). Moreover, at all time points up to 60 days post-burn, observed thrombi showed no signs of morphological organization, indicative of recent thrombi formation.

Next, the presence of NETs in intra-microvascular thrombi were evaluated. As in the rats NETs were observed in the majority of intra-microvascular thrombi of the burn wound. An example is shown in figure 3C. The NETs were present in the thrombi at all time points up to 60 days post-burn. NETs were also observed in intramicrovascular thrombi in the remote healthy skin of experimental pigs (not shown).
Together with the thrombi and NETs, there was a concomitant increase in the number of TF-positive blood vessels in the burn wounds (figure 3D). In the skin of healthy control pigs, no TF-positive blood vessels were observed. In burn wounds, a nonsignificant increase in the number of TF-positive blood vessels was noted at 3–6 days (0.5/mm²) and 9–14 days (1.0/mm²), which increased further and significantly after 21–30 days (2.8/mm²; p < 0.01) and 40–60 days (3.4/mm²; p < 0.01). A nonsignificant increase in TF-positive blood vessels were also observed in the remote healthy skin of affected pigs (0.8/mm²) (figure 3E). Thus, microvascular thrombi were formed in the burn wound and in remote healthy skin in pigs and this persists for at least 60 days after burn injury. These thrombi in majority colocalized with the presence of NETs. We then evaluated if these findings can be extrapolated to burn patients.
Figure 3. Post-burn intravascular thrombi, NETs and TF-positive blood vessels up to 60 days post-burn, in burned pigs. The number of intravascular thrombi per square millimeter (A and B), an example of NETs within intravascular thrombi (C) and the number of TF-positive blood vessels per square millimeter (D and E) in the skin of control (C) and burned pigs, 3–60 days post-burn. Median ("line"); first quartile (Q1) to third quartile (Q3) and mean ("+"). **p ≤ 0.01.

Post-burn intravascular thrombi as well as NETs up to 46 days post-burn, in burned human skin

As in the porcine burn wound model, intravascular thrombi were found in skin samples obtained from burn wound patients, while in skin samples of healthy controls no intravascular thrombi were found (not shown). The number of thrombi per mm² was significantly increased in burn wound tissues/biopsies of 0.5–2 days (6.2/mm²), 3–6 days (4.9/mm²), 9–14 days (2.6/mm²), and slightly (not significantly) increased at 21–46 days post-burn (0.8/mm²) in comparison with healthy control skin (p < 0.05) (figure 4A). It is noteworthy that in comparison with the pig model, intravascular thrombi in patients were found earlier after burn. Moreover, the average number of thrombi/mm² in patients was higher (approximately threefold) than in the pigs. As in the pigs, the thrombi found in wound of patients showed no signs of morphological organization at all time points up to 46 days post-burn, indicative of recent thrombi formation.

As in the rats and pigs, NETs were observed in the majority of intra-microvascular thrombi of the burn wound of patients. An example is shown in figure 4B. The NETs were present in the thrombi at all time points up to 21–46 days post-burn.

Lastly, we found a slight increase in the number of TF-positive blood vessels per mm² after 0.5–2 days (0.8/mm²) and 3–6 days post-burn (1.1/mm²) in comparison with control skin (0.1/mm²), although this was statistically not significant (figure 4C). Thus, the microvascular thrombosis NETs and TF expression that were found in the animal models are also found in burn wound patients.
A

Humans

![Graph showing thrombi per mm²](image)

Days post-burn

B

![Image of tissue sample with an arrow](image)

C

Humans

![Graph showing TF positive blood vessels per mm²](image)

Days post-burn
DISCUSSION

An important factor in secondary expansion of necrosis into vital dermis is severe loss of perfusion, likely caused by microvascular damage and burn-induced hypercoagulation [2,3,19]. However, the mechanisms underlying this burn-induced microangiopathy and thrombosis are poorly understood. In this study, we showed long term presence of microcirculatory thrombi in the burn wounds of rats, pigs, and humans that in majority colocalized with NET formation. We also demonstrated a switch in the microcirculatory endothelium toward a pro-coagulant phenotype.

Previous preclinical studies have shown that microvascular thrombosis and hypercoagulation contribute significantly to wound expansion after burns and are therefore biologically relevant [2,4,20-22]. The results of the present study confirm that thrombosis occurs in the microvasculature of burn wounds early after burns, in both patients (0.5-day post-burn) and in a pig burn wound model (3 days post-burn). Additionally, the thrombi formation coincides with the presence of NETs. Therefore, this points to a role of NETosis in burn-induced secondary thrombosis and wound progression. The microvascular thrombosis and NETosis we observed 14 days after burn trauma in rats were also observed in a prolonged episode up to 60 days in the pig burn wound model and in patients, suggesting that microvascular thrombosis and NETosis may play a pathogenic role in burn wound for an extended period of time. In line with this, we and others have observed previously that large numbers of neutrophils remain present in the burn wound up to weeks after trauma [5,16].

Neutrophils are important in the antibacterial defense. However, their activation also results in the production of proinflammatory cytokines and ROS which may damage skin and microvascular endothelial cells and enhance ischemic tissue necrosis. Ravage et al. found that neutrophil depletion resulted in significantly reduced dermal vascular permeability [4]. We now show the presence of NETs in thrombi in the microvasculature of burn wounds in rats, pigs, and patients. This could point to a role for neutrophils in the instigation of thrombosis in the microcirculation of the burn wound as well. Indeed, NETosis has been identified as a possible link between (sterile) inflammation and thrombosis [8]. NETs formation has also been linked to thrombotic events in other pathologies, e.g., stroke and acute coronary syndrome [23]. NETs have been shown to initiate coagulation directly through the externalized nucleosomes in conjunction with elastase and cathepsin G can initiate coagulation that in turn can activate platelets as well as the intrinsic and extrinsic coagulation cascades.
Alternatively, NET formation may result in thrombus formation indirectly through the induction of injury to the endothelium of (micro)vessels, in particular via the toxicity of the released histones. Yang et al. showed that extracellular histones induce TF expression in vascular endothelial cells [24]. In our study, we found increased expression of TF in the microvasculature of burn wounds, that supports this hypothesis. This increased TF expression further supports the pro-coagulant state of the microvasculature in burn wounds [25]. Moreover, activated endothelial cells were shown to induce NETs formation, which in turn triggered endothelial cell damage and death [26]. As such, NETs can induce collateral damage in inflamed tissues, as was shown in animal models of heart and brain infarctions [27]. NETosis was first described as an antimicrobial defense mechanism [6]. Although in all of the analyzed burn wounds there was no macroscopic evidence of infection we cannot exclude the possibility that (part of) the NETosis may have been triggered by microbes and/or microbial material present in the burn wounds.

Interestingly, it has recently been shown that NETosis impaired the healing of excisional skin wounds, particularly in diabetes, in which neutrophils are more susceptible to NETosis. The prevention of NETosis (using peptidylarginine deiminase 4 (PAD4)-knockout mice (padi4−/−)), without interfering with other neutrophil functions, resulted in improved wound healing. In addition, increased levels of circulating cell free DNA (cfDNA)/NETs were measured in patients after burn trauma [13,14], indicating their putative relevance also in human burn victims. Serum cfDNA/NETs levels were found to predict inflammatory second hit and sepsis in trauma patients [29] and mortality in burn victims [13], although their relation with thrombosis, NETs formation, secondary necrosis and healing locally in the burn wound were not investigated. Nevertheless, our findings in the present study indicate the possible involvement of NETosis in the microvascular thrombosis in the burn wound. Furthermore, impaired NETs degradation was shown to intensify inflammation via complement activation in systemic lupus erythematous patients [30] and to associate with acute thrombotic microangiopathies [31]. Here we found in pigs and burn wound patients, that there seems to be a prolonged thrombi formation after burn also, up to 60 days and more than 21 days post-burn respectively. Moreover, at all time points observed thrombi showed no signs of morphological organization, visualized via myofibroblasts infiltration, indicative of recent thrombi formation.

However, a remarkable finding was the presence of intravascular thrombi and TF-positive blood vessels in remote nonburned skin of both rats and pigs (internal controls). Even though, this was not significantly more than in skin of nonburned healthy rats and pigs, this systemic effect was caused even with a TBSA burned of 1.5%. Conversely, we found in humans that higher percentages of TBSA burned coincided with relatively higher numbers of intravascular thrombi. Moreover, these findings are in line with systemic hypercoagulation, which was observed in burns, and might be involved in post-burn multiple
organ failure [32,33]. Furthermore, several studies support the hypothesis claiming a central role for the release of NETs by neutrophilic granulocytes within thrombus formation [8,12,34,35]. The question remains whether formation of NETs is a consequence of the hypercoagulative state present within the burn wound, or whether the NETs set the stage for the increased thrombus formation. However, besides immunohistochemical stainings our analyses are based on morphology as well, therefore the observations reported in this study serve as a strong indication of the presence of NETs within intravascular thrombi. Remarkably, we found a difference in thrombus formation in time, in pigs and in humans. In pigs, thrombus formation seems to occur later, in comparison to humans. We agree that based on data obtained from the present study, we cannot exclude that the infiltration of neutrophils and NETosis is a consequence rather than the primary initiator of post-burn intravascular thrombus formation. However, the ejection of DNA into the extracellular milieu creates an extreme pro-coagulant environment [8–10] and it is known that the ejected histones are toxic to the vascular wall [36]. This at least suggests that even if the formation of intravascular NETs does not primarily initiate thrombosis, though their pro-coagulant and toxic effects, they exacerbate thrombosis. However, to prove this in burn wounds more research is necessary.

In conclusion, in the present study we showed for the first time evidence for the presence of NETosis in the microvascular thrombosis in the burn wound and a clear switch in the microcirculatory endothelium toward a pro-coagulant phenotype. This, in a rat and pig burn wound model in time up to 60 days post-burn, as well as in wound of burn wound patients, already from 0.5 days post-burn up to 46 days post-burn.

REFERENCES


