The local and systemic inflammatory response in a pig burn wound model
with a pivotal role for complement

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ABSTRACT

Background: In patients with burns, a massive inflammatory response is induced which negatively affects the healing process of the burn wound and additionally exerts systemic effects. An important factor herein is the complement system. Here we analyzed the effects of burns on complement and inflammatory cells both locally and systemically after burn in time in a pig burn wound model.

Methods: In burned pigs, burn wound biopsies and blood were collected up to 60 days after burn. Complement in blood as well as complement and inflammatory cells in the burn wound and several organs were determined.

Results: In the blood, C3 was significantly increased after 9 to 60 days, whereas C4 after 21 to 30 days after burn. In the burn wound, C3 levels were significantly increased after 9 days and C4 after 3 days, whereafter both declined after 21 and 9 days, respectively. Neutrophils, macrophages, and lymphocytes were significantly increased in the burn wound after 3 days, all declined after 21 days after burn. In the heart, at 60 days after burn, an increase of neutrophils and macrophages was observed, mainly in the right atrium. In contrast to the heart, the inflammatory cell infiltrates in the lungs, liver, and kidney of burned pigs were lower than in control pigs.

Conclusion(s): In pigs, following burn there is a prolonged increase in complement levels both in the burn wound and the blood and increased inflammatory cell infiltrate in the burn wound and the heart. However, complement levels in the burn wound and in the blood seem not to be correlated in time.
INTRODUCTION

Severe burns can result in fatal complications including infection, respiratory failure, shock, and severe psychological distress due to long-term hospitalization, scarring, and deformity [1]. Severe burn injury is characterized by an extensive and complex inflammatory response, in which the acute phase response (APR) is playing an important role. It is activated immediately after the trauma and coincides with a strong increase of specific proteins produced by the liver, the so-called acute phase proteins (APPs). These secreted APPs play important roles in the restoration of hemostasis, antibacterial defense, and the regulation of the inflammatory response. Activation of the complement system plays a central role in the post-burn APR. The complement system is composed of several proteins that interact in three different enzymatic cascades: the classical, lectin, and alternative pathways. All three pathways converge at the point of C3, making it the common component of the complement system, while C4 is the component of the classical and lectin pathways. The final step in the complement cascade is the formation of the membrane attack complex (MAC) (i.e. C5b-9), which triggers the lysis of targeted host cells or pathogens [2–5]. Activated complement induces capillary vasodilatation and leakage as well as the influx of neutrophilic granulocytes and lymphocytes in the wound [6–9]. Subsequently, macrophages invade the burn wound. These cells not only phagocytose cell debris but also stimulate fibroblast and keratinocyte migration, thereby initiating the formation of granulation tissue and tissue repair [10].

However, although the inflammatory response initially is indispensable for proper wound healing, in burn victims it can be overactivated and persist for up to months after the initial trauma [11–13]. As such it can contribute to secondary wound expansion and excessive scarring, and can exerts systemic effects, including the heart [8,14–17], that can result in secondary organ failure and hence can be life threatening. Therefore, this sustained or exaggerated inflammatory response may be an attractive target for therapeutic intervention. Indeed, administration of C1-esterase inhibitor (C1inh), an inhibitor of the complement system, in the blood immediately after burn, did protect the dermal microcirculation during the APR in burned pigs [18]. Moreover, we found that daily administration of C1inh for 14 days intravenously after burn reduced the amount of granulation tissue and macrophage infiltration in the burn wound and enhanced its reepithelialization and also reduced the burn-induced macrophage infiltration in the heart [19]. Lastly, in burned pigs systemic application of an anti-C5a antibody or of C1inh reduced cardiac dysfunction, as measured by monitoring cardiac hemodynamics (eg, electrocardiography, heart rate, and blood pressure) [20,21]. These studies indeed show that therapeutic inhibition of the complement system after burns may be a viable treatment option to reduce both the local and systemic effects of excessive post-burn inflammation.
However, the pathophysiology of the burn wound is still not fully understood, including the role of inflammatory mediators herein [2–5]. In animal models and in humans, the post-burn complement blood or tissue levels were shown to be elevated up to months after burn injury [5,22–24]. To optimize therapeutic strategies targeting the complement system, a more detailed knowledge regarding complement blood levels and their relation with the complement levels and the cellular inflammatory response locally in the burn wound as well as systemically in the heart is warranted. Therefore, the aim of the present study was to determine the effects of burn wounds on complement levels/inflammatory cells in the burn wound (local effect), and also in the blood, the heart, the lungs, the liver, and the kidney (systemic effect) in a pig burn wound model in time.

**MATERIALS AND METHODS**

**In Vivo Pig Burn Wound Model**

All procedures were executed in agreement with the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam. Three-month-old female Yorkshire pigs (n = 10), weighing approximately 30 kg at arrival, were used in the study. During the whole study, the pigs were individually housed and fed twice daily, with ad libitum access to water. After arrival, the pigs were acclimatized for 1 week prior to the experiment. Six pigs were exposed to burns, while four healthy pigs served as controls and were terminated after 1 week acclimatization and blood sample collection. After termination, skin samples and the hearts were collected too.

**Sedation/Anesthesia/Analgesia**

One day prior to burn induction, the six “burn” pigs received a transdermal fentanyl 35 μg/h patch on the posterior pinna and an oral administration of meloxicam 0.4 mg/kg for analgesia. The next day, 20 minutes before the start of all procedures, the pigs were sedated with a combination of ketamine 10 mg/kg, midazolam 0.5 mg/kg, and atropine 0.5 to 1.0 mg by intramuscular injection. Before the creation of full-thickness burn wounds, full anesthesia was induced using propofol 100 to 150 mg via intravenous injection, and analgesia fentanyl 200 μg. During procedure, the anesthesia was maintained by intravenous injection of Propofol 10 mg/kg/h and fentanyl 6.5 μg/kg/h. Artificial respiration was applied with 45 to 50% O2. Vital functions such as CO2 expiration concentration, blood gas values, heart rate, and temperature were monitored during the entire procedure. For postoperative pain relief, meloxicam 0.4 mg/kg was administered intramuscularly 15 minutes prior to the surgery. From day 1, meloxicam 0.4 mg/kg was orally administered once a day for a period of 3 to 5 days and the transdermal fentanyl patch was replaced at day 2.
Anesthesia during the biopsy and bandage-change procedures was induced by intramuscular injection of Zoletil 100 (Virbac Nederland, Barneveld, The Netherlands) (zolazepam and tiletamine) 6 mg/kg and xylazine 1.5 mg/kg. Analgesic meloxicam 0.4 mg/kg was administered intramuscularly.

**Burn Wound Infliction**
The burn wound procedures used in these pigs were previously (slightly modified) described by Middelkoop et al. [25] and Hoekstra et al. [26].

Prior to burn wound infliction, blood samples (40 ml) were collected (from the jugular vein) in serum tubes (day 0 control serum). Eight (four wounds on each flank) dorsal full-thickness burns of 4 × 4 cm burned area (±1.5% TBSA) were created on the shaved part of the skin by application of a heated copper device (170°C) during 20 seconds. The wounds were covered with Allevyn wound dressing (Smith & Nephew, Hull, UK) and sterile gauzes, which was kept in place by adhesive bandage (Curafix Lohman & Rauscher, Neuwied, Germany) and elastic stockings (Tubigrip [Mediq Medeco/Klinion, Oud-Beijerland, The Netherlands]). All procedures were performed under proper anesthesia and pain relief as described above.

Blood samples (40 ml) via the jugular vein and skin biopsies (4 mm diameter, depth until the whole dermis and partly the subcutis) from two different wounds (one from each flank) were collected at day 3, 6, 9, 14, 21, 30, 40, 50, and 60 after burn.

At 60 days after burn, all six pigs were terminated with Euthasol 100 to 200 mg/kg (AST Farma B.V., Oudewater, The Netherlands), whereafter the entire wound was excised. Furthermore, the left atrium, left ventricle, right atrium, right ventricle, the lungs, the liver, and the kidney were also collected. All blood samples were centrifuged (1530g, 10 minutes, 21°C), whereafter serum samples were stored at −80°C. All skin, heart, lung, liver, and kidney tissues were fixed in formalin and embedded in paraffin.

**Macroscopic Wound Analyses**
Digital pictures of the wounds were taken at 3, 6, 9, 14, 21, 30, 40, 50, and 60 days after burn. The pictures were visually evaluated, whereby the color, shape, debridement, and closure of the wound were determined.

**Immunohistochemistry**
For immunohistochemical analysis, 4-μm sections were dewaxed in xylene and dehydrated in ethanol (100%) followed by incubation in a methanol/H₂O₂ 0.3% solution to block endogenous peroxidases activity. Antigen retrieval was performed by either boiling slides in a citrate pH 6.0 (MPO and CD45) or
a Tris-EDTA pH 9.0 (MAC-387 and C3) solution in a microwave for 10 minutes (no antigen retrieval was needed for C4). Next, the sections were incubated with 1:1500 polyclonal rabbit anti-human/pig myeloperoxidase (MPO) (DAKO, Glostrup, Denmark), 1:100 monoclonal mouse anti-pig CD45 (Serotec, Kidlington, UK), 1:200 monoclonal mouse anti-pig macrophage (MAC 387) (Acris, Herford, Germany), 1:100 polyclonal rabbit anti-pig complement C3 (Bioss, Woburn, MA), or 1:50 monoclonal mouse anti-pig complement C4 (Acris, Herford, Germany) for 1 hour at room temperature. The sections were then incubated with undiluted goat anti-mouse/rabbit envision (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Staining was visualized using 3,3-diaminobenzidine (DAB, 0.1 mg/ml, 0.02% H_2O_2) (Dako, Glostrup, Denmark). The sections were counterstained with hematoxylin, dehydrated, and covered. As a control, the same procedure was used, but instead of the primary monoclonal or polyclonal antibody, normal antibody diluent (Immunologic, Duiven, The Netherlands) was used. All controls yielded negative results (data not shown).

**Immunohistochemical Analysis**

The infiltration of neutrophilic granulocytes (MPO) and macrophages (MAC 387) was quantified manually (microscopically) as the number of cells per tissue section, whereafter the surface area of the tissue section was measured using QuickPHOTO MICRO software (windows version 3.0, PROMICRA, Prague, Czech Republic), to determine the number of cells per square millimeter surface area. The infiltration of lymphocytes (CD45) and the presence of complement (C3 and C4) were digitally quantified as the percentage of the surface area of the tissue slide positive for these stainings using QuickPHOTO MICRO software.

To ensure analysis consistency, in burn wound samples of 60 days after burn, for all markers only the area of the burn wound (not the healthy skin bordering the burn wound) was analyzed, similar to the burn wound biopsy samples of other time points, to analyze consistently.

**Serological Analyses**

For the quantitative determination of pig complement C3 and C4 protein concentration in serum samples, a sandwich enzyme-linked immunosorbent assay (ELISA) were used (Pig Complement C3 ELISA Kit, CUSABIO, Wuhan, China; Porcine C4 [Complement Component 4] ELISA Kit, Elabscience, Wuhan, China). Serum samples and standards were analyzed according to the manufacturer’s instructions. Data were linearized by standard curve, and regression analysis was used to determine sample complement C3 and C4 concentrations in microgram per milliliter.

**Statistical Analysis**
Statistics were performed on the R statistical computing platform [27]. Data were compared using the Kruskal–Wallis test. The Hochberg multiplicity correction was applied to the resulting $p$ values to control the type I error. Hochberg multiple testing corrected $p$ values $\leq 0.1$ were considered significant. To identify the contrasts between data giving rise to the significance of the Kruskal–Wallis test, the test was followed by a nonparametric multiple comparison procedure implemented in the “kruskalmc” function available through the R-packages “pgirmess,” [28] which was described in detail by Siegel and Castellan [29]. Results are described as: median (first quartile [Q1]–third quartile [Q3]).

RESULTS

Macroscopical Wound Characterization

Macroscopically, we found differences in the color, shape/contraction, and closure of the burn wounds in time (0–60 days after burn) (figure 1). At day 0, the burn wounds had a yellow/white color with a red border. At 3 days after burn, the (full-thickness) burn wounds appeared lighter in color, with a more prominent red edge (visible are the three biopsy sites in the center of the burn). At 14 days after burn, the necrotic tissue was rejected, and the burn wounds were red, wet, and a little contracted. At 30 days after burn, the burn wounds were still open, looked red, but were less wet and were more contracted. Finally, at 60 days after burn, the burn wounds were completely closed and showed contraction.

Figure 1. A burn wound at different time points after burn (0–60 days after burn). Pictures of a burn wound at day 0 (D0), 3 (D3), 14 (D14), 30 (D30), and 60 (D60) after burn. The three holes (arrows) at day 3 are the result of the biopsy collection. Wound contraction at day 60 is depicted with arrows.

Complement Levels in the Blood and the Burn Wound in Time Following Burn
In the blood, complement C3 and C4 levels were analyzed to determine the post-burn circulating complement levels in time (0–60 days after burn). At day 0 (before burn wound infliction), complement C3 and C4 levels (µg/ml), expressed in terms as median (Q1–Q3), were 1034 (961–1158) and 56 (48–62), respectively (uninterrupted lines in figure 2A, B). C3 levels in the blood showed a slow increasing trend in time (figure 2A). At 3 to 6 days after burn, C3 levels were elevated (1475 [1366–1479]), which persisted up to 60 days after burn to significantly elevated levels: 2199 (1938–2479) (corrected $p \leq 0.05$). In contrast, complement C4 levels remained around basal levels up to 14 days after burn (67 [63–84]) (figure 2B). At 21 to 30 days after burn, the C4 levels were significantly increased to 116 (108–139) (corrected $p \leq 0.01$) and then declined again after 40 to 60 days following burn (81 [74–86]).

In nonburned control skin, complement C3 and C4 were virtually absent (dotted lines in figure 2A, B). At 3 to 6 days after burn, the percentage of complement C3 positivity in the burn wound was not significantly different compared with nonburned control skin. However, at 9 to 14 days after burn, C3 positivity in the burn wound peaked, and diffuse staining mainly in the dermis showed significantly increased C3 positivity, compared with nonburned control skin (0.005 [0.002–0.007]) (corrected $p \leq 0.1$). Thereafter, C3 levels declined in time (not significant), but were still significantly elevated compared with controls at 60 days after burn (0.001 [0.001–0.002]) (corrected $p \leq 0.1$). In contrast to C3, at 3 to 6 days after burn the percentage of the complement C4-positive area (also diffuse in the dermis) peaked and was significantly increased, compared with control skin (0.0004 [0.0001–0.0005]) (corrected $p \leq 0.1$). Thereafter, C4 levels decreased in time (not significant), but were still significantly elevated in comparison with control skin 60 days after burn (0.00003 [0.00002–0.00004]) (corrected $p \leq 0.1$).
Figure 2. Analysis of complement levels in the blood and the skin following burn. Complement C3 (A) and C4 (B) in the blood (μg/ml) and in the skin (%/mm²) at day 0 (C) and at 3–6, 9–14, 21–30, and 40–60 days after burn. */# = corrected p ≤ 0.1 in the blood / skin. **/# = corrected p ≤ 0.05 in the blood / skin. ***/### = corrected p ≤ 0.01 in the blood / skin.

Inflammatory Cell Infiltrate in the Skin
We next analyzed the post-burn inflammatory cell infiltrate in the skin (figure 3A). In nonburned control skin, the MPO-positive neutrophils, MAC 387-positive macrophages and the percentage of CD45-positive lymphocytes per surface area (mm²) were virtually absent. In the burn wound, the amount of infiltrate of all three inflammatory cell types was significantly increased at 3 to 6 days (neutrophils: 3 [1–27]; macrophages: 13 [6–59]; lymphocytes: 0.0005 [0.0003–0.002]), peaked at 9 to 14 days (neutrophils: 37 [15–47]; macrophages: 55 [39–84]; lymphocytes: 0.006 [0.003–0.01]), and then declined in time until 60 days after burn. However, the inflammatory cell infiltrate at 60 days after burn was still significantly increased in comparison with nonburned control skin (neutrophils: 0.4 [0.2–0.6]; macrophages: 5 [4–15]; lymphocytes: 0.001 [0.0006–0.001]) (corrected p ≤ 0.1).

Complement and Inflammatory Cell Infiltrate in the Heart, the Lungs, the Liver, and the Kidney

Next, we analyzed the inflammatory cell infiltrate in the heart, at 60 days after burn (figure 3B–D). In both healthy heart tissue and heart tissue from burned pigs, no complement C3 and C4 were found (data not shown). In healthy control heart tissue, no or low numbers of MPO-positive neutrophils and MAC-387-positive macrophages were found in both atria and ventricles (figure 3B, C). In the heart collected from burned pigs, a slightly increased, although not significant, number of neutrophils was found only in the right atrium (RA). Similarly, macrophages showed also a slight, although statistically significant, increase especially in the RA, but also in the left and right ventricles (LV and RV) of burned pigs (RA: 0.6 [0.1–0.7]; LV: 0.07 [0.02–0.2]; RV: 0.08 [0.06–0.1]) (corrected p ≤ 0.05). Remarkably, we found, albeit minor, CD45-positive lymphocytes in healthy control heart tissue (figure 3D). The percentage of CD45-positive lymphocytes in both atria and ventricles of burned pigs was not significantly different compared with nonburned healthy pigs.

Next, we analyzed the inflammatory cell infiltrate in the lungs, liver, and kidney at 60 days after burn (figure 4A–C). In the lungs, liver, and kidney collected from burned pigs, the numbers of neutrophils and macrophages per surface area (mm²) were remarkably lower than in control pigs, by a factor of 2 to 9, which was significantly in the liver (p < 0.05) (figure 4A, B). Similarly, the percentage of CD45-positive lymphocytes in the lungs, liver, and kidney of burned pigs was lower than in control pigs, which was significantly in the kidney (p < 0.05) (figure 4C).
Figure 3. Analysis of inflammatory cell infiltrate in the skin and the heart following burn. The number of neutrophils, macrophages, and the percentage of lymphocytes per surface area (mm²) in the skin (A) at day 0 (C) and at 3–6, 9–14, 21–30, and 40–60 and in left atrium (LA), left ventricle (LV), right atrium (RA), and right ventricle (RV) of the heart (B–D) at day 60 after burn. */#/$/ = corrected $p \leq 0.1$ for neutrophils / macrophages / lymphocytes. **/#/$$ = corrected $p \leq 0.05$ for neutrophils / macrophages / lymphocytes. ***/#/#/$$$/ = corrected $p \leq 0.01$ for neutrophils / macrophages / lymphocytes.
A

**Lung**

![Box plot showing neutrophils in Lung](image)

- **C**
  - n = 4
- **Burn**
  - n = 5

**Liver**

![Box plot showing neutrophils in Liver](image)

- **C**
  - n = 4
- **Burn**
  - n = 5

**Kidney**

![Box plot showing neutrophils in Kidney](image)

- **C**
  - n = 4
- **Burn**
  - n = 5
B

Lung

Liver

Kidney

Macrophages in Lung (#/mm²)

Macrophages in Liver (#/mm²)

Macrophages in Kidney (#/mm²)

C  n = 4  Burn  n = 5

C  n = 4  Burn  n = 5

C  n = 4  Burn  n = 5
Lymphocytes in Lung (% CD45 positive area / mm²)

Lung

n = 4  n = 5

Lymphocytes in Liver (% CD45 positive area / mm²)

Liver

n = 4  n = 5

Lymphocytes in Kidney (% CD45 positive area / mm²)

Kidney

n = 4  n = 5
DISCUSSION

In burn wound patients, a massive inflammatory response occurs that not only negatively affects the local healing of the wound but could also exert negative systemic effects in other organs, including the heart [8,14–17]. The APR, particularly the complement system, is suggested as an important factor in this response [2–5]. In this study, we show in a pig burn wound model burn-induced increased and prolonged complement levels both in the blood and in the burn wound, as well as an increased inflammatory cell infiltrate in the burn wound and in the heart up to 60 days after burn. However, there seems to be no correlation between post-burn complement levels in the burn and the blood in time. While complement C3 levels in the burn wound do correlate with inflammatory cell infiltrate and a role for C4 in the cellular inflammatory response in the wound cannot be excluded as its levels in the wound are increased prior to C3 and inflammatory cell infiltration. Remarkably, in contrast to the heart, the inflammatory cell infiltrates in the lungs, liver, and kidney of burned pigs were lower than in control pigs. Further research is necessary to explain this phenomenon.

A severe burn wound induces a local inflammatory response that is important in the process of wound healing. It causes clearance of cellular debris and protects the wound against microbial agents [30]. In normal wound healing, this inflammatory response is resolved in a few weeks [31]. However, in burn wounds a prolonged local acute inflammatory reaction has been described [10]. This can have detrimental effects for the wound healing such as delayed wound closure, excessive wound contraction, and severe scarring, as we see also to a certain extent in our pig burn wound model (figure 1). Several studies assessed the inflammatory response after burns, both in animals and in human. These former studies have shown that complement blood or tissue levels were elevated up to months after burn injury [5,22–24]. However, a possible relation between complement blood and tissue levels, the levels of different complement factors in time, and the relation with inflammation in the burn wound and heart have not been studied until now. The complement system includes a large family of proteins. In the current study, we analyzed complement C3 and C4. C3 is the common component of all three complement pathways, while C4 is the component of the classical and mannann-binding lectin (MBL) pathways [2–5].

First of all, there seems to be a difference in post-burn complement C3 and C4 levels in blood and in the burn wound (figure 2). In the blood, we found prolonged elevated levels of complement C3 up to 60 days after burn implicating a persistent systemic acute inflammation. In contrast to C3, complement C4 in blood was increased much later and persists shorter, while in the burn wound, complement C4 was increased earlier (3–6 days after burn) in comparison with complement C3. This might be an indication
of an earlier activation of the classical and/or lectin pathway of the complement system locally in the skin (not systemic), as C4 is a component of these pathways. Previous studies have already shown that MBL plays an important role in the first-line host defense against infectious agents after burn injury. MBL initiates the lectin complement pathway and act as an opsonin [32,33], supporting the idea that (at least) the lectin complement pathway is activated after burn. The activation of the complement system by the classical, alternative, or lectin pathway results, namely, in the cleavage of C3 and C4 in i.a. C3a and C4a [10,34]. In addition, complement C4 was increased early in the burn wound, in contrast to its blood levels. This could implicate that C4 is also produced locally in the burn wound. It is already known that complement can be produced locally i.a. endothelial cells [34]. In addition, complement could also leak from the blood into the wound, explaining the high levels of C4 and C3 in the burn wound. Furthermore, the inflammatory response in the burn wound seemed to persist shorter than the systemic response [35,36]. Although it is prolonged when compared with “nonburn” wound healing [10], at 21 to 30 days after burn, complement levels (coinciding with inflammatory cell infiltrates) in the burn wound declined again toward basal levels, which is in contrast to the systemic response in the blood and in contrast with human [24]. The findings in the present study indicate that there is no correlation between complement blood and burn wound levels.

An important feature of local complement activation is its chemotactic recruitment of inflammatory cells to sites of injury [37,38]. In accordance with this, the amount of inflammatory cell infiltrate in the burn wound seemed to correlate with the complement C3 levels in the burn wound, as they show a similar trend in time following burn (figures 2A and 3A). In addition, a role for C4 in the cellular inflammatory response in the wound cannot be excluded as its levels in the wound are increased prior to C3 and inflammatory cell infiltration (figure 2B).

Severe burn trauma has also been associated with several cardiodepressive effects. Post-burn reduced heart function most likely is the result of a variety of different causes, both physiological and inflammatory. Post-burn fluid loss through increased vascular leakage and edema can lead to intravascular hypovolemia and hypovolemic cardiac shock. Both in humans and several animal models, severe burn injury has been shown to result in reduced cardiac function and increased cardiac inflammation [21,39–48]. Although the exact role of complement in relation with cardiac dysfunction has not been described yet, Hoesel et al. [20] showed that there was a significant increase in cardiomyocyte expression of C5a receptor within 24 hours after burn injury. This coincided with a significant decrease in left ventricular pressures and cardiomyocyte sarcomere contractility. An in vivo blockade of C5a attenuated burn-induced cardiac dysfunction. In a rat burn wound model, an increase in plasma and cardiomyocyte levels of proinflammatory cytokines (interleukin-1β, tumor necrosis factor-α, and interleukin-6) and related loss of contractile function were shown [46,49,50].
Furthermore, we previously showed in a rat burn wound model that burn wound infliction resulted in inflammation in the heart at 14 days after burn. In addition, daily systemic administration of C1inh during 14 days after burn reduced macrophage infiltration and facilitated the transition to the anti-inflammatory subtype of macrophages, in both atria and ventricles of the heart [19]. In the present study, in a model closer to humans, we show, in line with previous studies, post-burn systemic infiltration of inflammatory cells (i.e. neutrophils and macrophages) in the heart. Although this was not in high numbers and, therefore, may not directly lead to dysfunction, it was caused even with a TBSA burned of 1.5% and to be present still at 60 days after burn.

The significance of the presence of neutrophils and macrophages in the heart still remains unclear. It has to be noted that we did not perform heart function measurements during our study and therefore cannot relate it directly to dysfunction, although intramyocardial macrophages f.i. have been related to chronic heart failure [51]. Previous studies by our group suggest that the presence of inflammatory cells in the heart could be the result of cardiomyocyte death [52,53]; however, we did not see this in the present study. In addition, intramyocardial macrophages may secrete cardiac-depressing proinflammatory cytokines, such as interleukin-1β, tumor necrosis factor-α, and interleukin-6, that have been shown to be involved in burn-induced contractile dysfunction in rats [46,50].

Furthermore, we did not find a significant difference in the infiltration of lymphocytes in the heart of burned pigs, in comparison with healthy pigs. This could be explained by the fact that we created burn wounds with a relatively low TBSA of 1.5%. Remarkably, we found (limited) infiltration of lymphocytes in healthy control heart tissue too; therefore, stress-induced infiltration of lymphocytes in the heart during the study cannot be excluded. A limitation of the study is that we were not able to analyze the heart at an earlier time point because this would mean that we had to terminate the animals earlier.

Remarkably, we found that the number of neutrophils and macrophages and the percentage of lymphocytes per surface area (mm²) in the lungs, the liver, and the kidney were lower in burned pigs than in control pigs. This in contrast to the skin and the heart and the findings of Liu et al. [54]. Liu et al. showed increased numbers of neutrophils and macrophages in the heart, lungs, liver, and kidney after severe burns in rats. However, they used a rat burn wound model with a TBSA of 30 to 50% burned and performed their analyses during the first 48 hours after burn, while we used a pig burn wound model with a TBSA of 1.5% burned and analyzed the inflammatory cell infiltrate in the lungs, the liver, and the kidney at 60 days after burn.

In conclusion, in the present study we showed in pigs, with a TBSA of 1.5% burned, a prolonged increase in complement levels both in burn wound and blood following burn, which coincided with increased inflammatory cell infiltrate in the burn wound. However, complement levels in the burn wound (local) and in the blood (systemic) following burn seemed not to be correlated in time. In the burn wound, the
overall inflammatory response seemed to persist relatively shorter, indicating that even after the local inflammation response subsides, the systemic response may proceed that may in turn cause several systemic negative effects in other organs. Moreover, systemic effects were found (although moderate) even with small burns with a TBSA of 1.5% burned, which is important for the clinic (f.i. for therapeutic intervention). In addition, the amount of inflammatory cell infiltrate in the burn wound seemed to correlate with the complement C3 levels and a role for C4 in the cellular inflammatory response in the wound cannot be excluded as its levels in the wound are increased prior to C3 and inflammatory cell infiltration. Also the increased number of inflammatory cells in the heart, still at 60 days after burn, is most likely caused by the elevated systemic inflammation.

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