SHORT COMMUNICATION

Human Streptococcal Necrotizing Fasciitis Histopathology Mirrored in a Murine Model

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Streptococcal necrotizing fasciitis (NF) causes high morbidity and mortality despite state-of-the-art therapy. Low incidence and rapid disease progression, necessitating immediate initiation of therapy, have proven challenging aspects for setting up prospective randomized trials. This has resulted in little therapeutic progress over the past decade. The validation of reliable murine NF models to study both pathogenesis and optimized therapeutic regimens of streptococcal NF are thus essential. In this study, we characterized a murine NF model and compared the pathology with an in-depth tissue analysis of streptococcal NF in patients. We found that the streptococcal murine NF model closely reflected all histologic characteristics encountered in human streptococcal NF. This murine NF model helps understanding of human NF pathology better in a time-dependent manner and will allow studying novel therapeutic options in the future. (*Am J Pathol* 2018, 188: 1517–1523; <https://doi.org/10.1016/j.ajpath.2018.03.009>)

Necrotizing fasciitis (NF) is a rapidly progressing, often fatal infection and mostly is caused by group A *Streptococcus* (GAS or *Streptococcus pyogenes*).¹ NF morbidity and mortality remain high despite prompt antibiotic, intravenous immunoglobulin, and intensive care therapy coupled to aggressive surgical debridement.² Aiming to improve therapeutic efficiency, randomized prospective clinical trials were initiated. Low overall incidence of NF and urgency for therapy initiation, however, jeopardized recruitment and caused premature stop of clinical trials.³ Current treatment regimens are based on observational clinical studies⁴ and some experimental work in murine NF models.^{5,6} However, GAS is not a murine pathogen, and no detailed comparison and validation of the histologic presentation of human and murine NF have been performed so far. Although human NF histopathology has been extensively described,^{1,7–9} this has been lacking for murine models. In addition, histology of murine NF models has so far not been compared with human NF histology. Human NF is characterized by rapidly progressing necrosis along the deep dermis, superficial fascia, and adipose tissue,^{1,7–9} whereas dermis and epidermis are spared until a later stage.⁷ Neutrophils and fibrin deposition

along fascia and deep soft tissue can be observed,^{1,7–9} together with abundant bacteria.^{1,7,9} Adjacent muscle fibers may be affected, whereas deep muscle is usually spared.^{7,8} Microvascular thrombosis and secondary inflammatory changes in neighboring vessels are common, and tissue hemorrhage is sometimes observed. The presence of intracellular GAS inside neutrophils and macrophages^{5,10} as well as the presence of GAS biofilm-like structures¹¹ in NF tissue was recently described in a clinical observational study.

To elucidate NF pathogenesis and to optimize NF therapy, validation of a reliable mouse model is crucial. We thus assessed the histology of streptococcal NF over time in a murine NF model and compared it with the histology of patients with streptococcal NF.

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Table 1 Characteristics of Patients Included in This Study

Patient	Sex	Age, years	Risk factors	<i>emm</i> -type	Site of infection	Time symptoms to biopsy, hours	Outcome
CI 13	Female	84	Diabetes	M1	Cervical	24	Died
CI 15	Male	47	Diabetes, concurrent influenza B infection	M12	Cervical, mediastinal	15	Cured
CI 16	Female	63	None	M3	Right foot/lower leg	72	Cured
CI 406	Male	40	None	M12	Right leg	28	Cured
CI 48*	Male	42	None	M1	Right chest	54	Cured
CRF 28	Female	30	None	M89	Left lower leg	36	Cured
CRF 47	Male	64	Alcohol abuse	NA	Left lower arm	25	Cured
CRF 8	Female	37	None	M1	Left trunk	27	Cured
CRF 9	Male	70	None	M28	Left arm	24	Cured

Overview of patient characteristics including gas *emm*-type and time until surgery after onset of symptoms.

*Histology from patient CI 48 is presented in [Figure 1](#).

Materials and Methods

Patient Characteristics

Patient samples and data were collected in accordance to the declaration of Helsinki and approved by the Canton's Ethics Committee (Kantonale Ethikkommission Zurich, Switzerland, KEK-ZH-Nr. BASEC 2016-00145). NF histologic samples of nine patients treated at the University Hospital Zurich between January 1, 2006, and December 31, 2013, were retrospectively

included in this study; surgery and tissue collection occurred up to 72 hours after onset of clinical symptoms. All patients received a β -lactam antibiotic, eight of nine patients received clindamycin, and seven of nine patients received intravenous immunoglobulin. GAS was isolated in all patients and typed according to the recommendation of the Centers for Disease Control and Prevention (<https://www2a.cdc.gov/ncidod/biotech/strepblast.asp>, last accessed July 2013); the following *emm*-types were identified: M1 (3 of 9), M12 (2 of 9), M3 (1 of 9), M28 (1 of 9), M89 (1 of 9), and not

Figure 1 Patient histology 72 hours after onset of symptoms. **A:** Overview of hematoxylin & eosin (H&E)-stained biopsy (**left panel**) with fibrin deposits indicated by **arrow** (**right panel**). **B:** H&E-stained biopsy with fibrin, platelets, and erythrocytes mixed thrombus. **C:** Representative overview of Brown Brenn (BB)-stained biopsy (**left panel**); intracellular group A *Streptococcus* (GAS) in BB-stained biopsy indicated by **arrows** (**right panel**). **D:** Fluorescence *in situ* hybridization of patient biopsy showing an overview (**top row**) and the **boxed area** shows a section with single cocci as well as chains by using specific probes that detect bacteria (EUB338-Cy3; EPI Filter, Chroma 41007A) or specifically streptococci (STR405-FAM; EPI Filter, Olympus U-MNIBA). **Boxed areas** are shown at higher magnification in the **bottom row**. DAPI added as control for nuclear and bacterial DNA (Filter, Olympus U-MWUA). Images are representative of one patient infected with an M1-serotype marked with an asterisk in [Table 1](#). Scale bars: 100 μ m (**A** and **C**, **left panels**); 10 μ m (**A** and **C**, **right panels**, and **B** and **D**).

Table 2 Summary of Histologic Characteristics of Necrotizing Fasciitis in Patients and Mice

Variable	Mice				Patients
	6—8 hours after infection (<i>n</i> = 5) (GAS M1T1 5448)	24 hours after infection (<i>n</i> = 6) (GAS M1T1 5448)	72 hours after infection (<i>n</i> = 16)		≤72 hours after onset of symptoms (<i>n</i> = 9)
			GAS M1T1 5448 (<i>n</i> = 6)	GAS clinical isolates CI416 (<i>n</i> = 5), CI529 (<i>n</i> = 5)	
Epidermis and superficial dermis	Intact in all biopsies	Intact in 3 of 6 or partially necrotic in 3 of 6 biopsies	Intact in 1 of 6 or completely necrotic in 5 of 6 biopsies	Intact in 7 of 10, partially necrotic in 2 of 10, or completely necrotic in 1 of 10 biopsies	Not sampled in 7 of 9, intact in 1 of 9, or partially necrotic in 1 of 9 biopsies
Deep soft tissue/fascia inflammatory infiltration	Discrete (2 of 5) or moderate (3 of 5) inflammatory infiltration	Discrete (4 of 6) or moderate (2 of 6) inflammatory infiltration	Discrete (1 of 6), moderate (4 of 6), or severe (1 of 6) inflammatory infiltration	Discrete (6 of 10), moderate (2 of 10), or severe (2 of 10) inflammatory infiltration	Discrete (1 of 9), moderate (5 of 9), or severe (3 of 9) inflammatory infiltration
Deep soft tissue/fascia necrosis	Present in 1 of 5 biopsies	Present in 5 of 6 biopsies	Present in all biopsies	Present in 8 of 10 biopsies	Present in all biopsies
Muscle tissue	Present in 3 of 5 biopsies	Present in 4 of 6 biopsies	Superficial damage of muscle tissue in all cases, deep muscle vital in 4 of 6 biopsies	Present in 6 of 10 biopsies	Present in 3 of 9 biopsies
Fibrin deposition in fascia/deep soft tissue	Present in 3 of 5 patients	Present in all biopsies	Present in 5 of 6 patients	Present in all biopsies	Present in all biopsies
Thrombosis	Not found in any patient	Present in 1 of 6 biopsies	Present in 1 of 6 biopsies	Present in all biopsies	Present in all biopsies
Tissue hemorrhage	Minimal present in 2 of 5 biopsies	Minimal present in 4 of 6 or present in 1 of 6 biopsies	Present in 2 of 6 biopsies	Present in 9 of 10 biopsies	Present in all biopsies (unknown whether surgically induced or not)
Bacterial aggregates	Present in 4 of 5 biopsies	Present in 5 of 6 biopsies	Present in 5 of 6 biopsies	Present in 5 of 10 biopsies	Present in 5 of 9 biopsies
Intracellular bacteria	Present in all biopsies	Present in all biopsies	Present in 5 of 6 biopsies	Present in 8 of 10 biopsies	Present in 4 of 9 biopsies

Histologic characteristics of necrotizing fasciitis (NF) are summarized for a total of nine patients and 27 mice. Group A *Streptococcus* (GAS) M1T1 5448 NF skin samples were harvested after 6 to 8 hours (5 mice), 24 hours (6 mice), or 72 hours (6 mice). GAS clinical isolates CI416 (5 mice) or CI529 (5 mice) NF skin samples were harvested after 72 hours.

determined (1 of 9). Multiple and extensive surgical debridement were performed in all patients. NF was cured in eight of nine patients, one patient died shortly after diagnosis.

Murine Infection Model

The clinical GAS isolates M1T1 5448,¹² CI416, and CI529⁵ were mixed 1:1 with sterile cytotoxic beads (Sigma-Aldrich, Buchs, Switzerland) and 3×10^7 colony forming units in 100 μ L were injected subcutaneously into the flanks of 7- to 9-week-old female C57Bl/6 wild-type mice (Janvier Labs, Le Genest-Saint-Isle, France).⁵ Mice were sacrificed 6 or 8 hours after infection (five mice in total), as well as 24 hours (six mice) and 72 hours after infection (16 mice), and skin samples were collected for histology. This work, conducted under the protocol ZH251/14, was approved by the

Institutional Animal Care and Use Committee of the University of Zurich.

Histology

Patient and mouse tissues were fixed in 4% buffered formalin and paraffin-embedded or snap-frozen (Leica, Muttens, Switzerland). Sections (2 μ m) were stained with hematoxylin and eosin and Brown Brenn. Whole-slide scanning and photomicrography were performed with a NanoZoomer 2.0-HT digital slide Scanner (Hamamatsu, Houston, TX). Histology slides were independently assessed by two scientists, two medical doctors, and an experienced board-certified pathologist with special interest in infectious disease pathology. The concordance rate was high, and discordant cases were re-analyzed together by

Figure 2 Mouse histology over time. **A–C:** Skin of mice infected with group A *Streptococcus* (GAS) M1T1 5448 were harvested and analyzed at the indicated time points. **A:** Representative overviews of mouse skin 6 to 8 hours after infection stained with hematoxylin and eosin (H&E) (**top left panel**) or Brown Brenn (BB) (**top right panel**). Vascular congestion and stasis (H&E; **bottom left panel**), microthrombus (H&E; **bottom middle panel**), and intracellular bacteria (BB; **bottom right panel**) are indicated by arrows. **B:** Representative overviews of mouse skin 24 hours after infection stained with H&E (**top left panel**) or BB (**top right panel**). Inflammatory cells (H&E; **bottom left panel**) localized between necrotic muscle fibers (arrows) and necrotic adipose tissue (dashed arrow). Intracellular bacteria in BB staining is indicated by arrows (**bottom right panel**). **C:** Representative overviews of mouse skin 72 hours after infection, stained with H&E (**top left panel**) showing pronounced cellular infiltration along the fascia (short arrow), necrotic epidermis, and subcutis (long arrows). Representative overview of BB-stained skin (**top right panel**) depicting bacterial infiltration along the muscle fascia (arrow). Inflammatory infiltrates along muscle fascia (H&E; **bottom left panel**), thrombus (arrow, **bottom middle panel**), and intracellular bacteria indicated by arrows (BB; **bottom right panel**). **D:** Fluorescence *in situ* hybridization of mouse histology showing an overview (**top row**), and the boxed areas show a section with single cocci as well as chains (arrows) with the use of specific probes detecting bacteria (EUB338-Cy3; EPI Filter, Chroma 41007A) or specifically streptococci (STR405-FAM, EPI Filter, Olympus U-MNIBA). Boxed areas are shown at higher magnification in the bottom row. DAPI added as control for nuclear and bacterial DNA (Filter, Olympus U-MWUA). Images showing histology of representative mice for each time point. $n = 2$ mice (**A**); $n = 1$ mouse (**B–D**). Scale bars: 100 μm (**A**, **B**, and **C**, top row); 20 μm (**A**, **B**, and **C**, bottom row); 10 μm (**D**).

using a multihead microscope. Fluorescence *in situ* hybridization (FISH) was performed as previously described.¹³ DAPI was detected with a blue Filter, Olympus U-MWUA, EUB338-Cy3 with an EPI Filter, Chroma 41007A, and STR405-FAM with an EPI Filter Olympus U-MNIBA (Olympus, Tokyo, Japan).

The inflammatory infiltrate predominantly consisted of neutrophils and macrophages, whereas eosinophils, lymphocytes, and plasma cells were exceedingly rare. The biopsies were thus evaluated according to the following parameters: 0 (none), + (discrete), ++ (moderate), or +++ (severe). Zero was defined as no neutrophilic infiltration, + as minimal neutrophilic infiltration without visible necrosis, ++ as predominant neutrophilic infiltration without

necrosis, and +++ as predominant neutrophilic infiltration with necrosis.

Results

Histologic Characteristics of Human Streptococcal NF

Patient characteristics, including site of infection, age, and sex, are given in Table 1. All patient tissues showed marked necrosis and variable degrees of inflammatory infiltrates (Figure 1A). Fibrin deposits (Figure 1A) and thrombosis (Figure 1B) were detected, and large amounts of bacteria presented both as bacterial aggregates and intracellularly (Figure 1C). Brown Brenn stain and FISH staining revealed

Figure 3 Mouse histology 72 hours after infection with group A *Streptococcus* (GAS) clinical isolates. **A and B:** Necrotizing fasciitis histology of mice infected with GAS clinical isolates CI416 (**A**) or CI 529 (**B**) for 72 hours. Representative overview of hematoxylin and eosin stain (**left panels, A and B**) or Brown Brenn (BB) (**right panels, A and B**). **Insets:** Depict intracellular bacteria (BB; **arrows**). Images showing histology of representative mice infected with either clinical isolate. $n = 2$ mice. Scale bars: 100 μm (**A and B, main images**); 20 μm (**A and B, insets**).

distinct streptococcal chains at the site of infection (Figure 1, C and D). The defined parameters used to characterize NF in mouse and humans^{1,7–9} are summarized in Table 2.

Characteristics and Progression of GAS NF during 72 Hours in a Murine NF Model

Six to 8 hours after infection with GAS M1T1 5448 a dense inflammatory infiltration, fibrin deposits, vascular stasis, and small thrombi formations were present (Figure 2A and Table 1). Abundant bacteria and intracellular bacteria were found (Figure 2A). Necrosis was present only in one sample (Figure 2A).

Further progression of the infection was observed in the histology of mice sacrificed 24 (Figure 2B) and 72 (Figures 2C and 3) hours after infection. Over time, extensive necrosis developed (Figures 2, B and C, and 3, and Table 2), inflammatory infiltrates further increased, and both intracellular and abundant bacterial aggregates (Figure 2, B and C) were found, reflecting histologic characteristics

typically found in patients (Figure 1 and Table 2). These bacterial aggregates colocalized with extracellular polysaccharides (Supplemental Figure S1). In addition, single bacterial cocci and small chains were found by FISH analysis (Figure 2D). Infection with GAS M1T1 5448¹² and GAS M1 clinical isolate CI416 and CI529⁵ resulted in comparable histologic characteristics after 72 hours (Figures 2C and 3 and Table 2), including inflammatory infiltrates, thrombosis, and presence of bacteria.

Discussion

The typical histologic presentation of human streptococcal NF was reflected in the murine NF model, in particular 24 and 72 hours after infection and irrespective of the GAS strain used. The murine NF model serves as a valuable tool for in-depth study of NF pathophysiology and will allow assessing and further developing current treatment options. This is essential because prospective trials are lacking and difficult to implement, whereas NF morbidity and mortality

remain high despite aggressive antibiotic treatment and surgical intervention.²

The GAS serotype M1 is most often isolated from patients with NF^{14–16} as was the case in one-third of our patients. To test for potential differences between M1 clinical isolates, the histology of murine NF caused by three distinct GAS isolates, MIT1 5448,¹² CI416, and CI529,⁵ was analyzed and compared. Thereby, reflecting clinics where no relevant differences in clinical presentation of the various GAS strains are observed, also histology revealed no isolate-specific differences.

This murine NF model allowed assessing NF progression over time providing hints on how the disease evolves. At the earlier time points, the epidermis was still unaffected despite the presence of abundant bacteria and dense inflammatory infiltrate found along the fascia, representing the spreading of the infection. Rarely small areas of necrosis were found in the dermis. Vascular stasis, occasional thrombosis, secondary inflammatory changes in neighboring vessels, and extravascular fibrin deposits were present and increased over time as was the case for inflammatory infiltrates and necrosis. All these findings are consistent with the histologic description of NF in patients,^{1,7–9} further underlining the suitability of this murine NF model to study human streptococcal NF.

Although GAS has been considered an extracellular bacterium, it is able to survive intracellularly after phagocytosis by professional phagocytes.^{10,17,18} Intracellular bacteria were detected 6 hours after infection in mice. In addition, intracellular bacteria were found in the patients' tissue. The potential pathophysiologic importance of intracellular GAS remains to be further studied in particular because β -lactams, used for treating NF patients, lack intracellular activity. In addition, bacterial aggregates resembling biofilms were observed in histologic samples from mouse and humans, as previously shown.^{11,19,20}

At our hospital, as is the case for many other centers, histologic samples are, in contrast to microbiology samples, rarely taken from NF patients for further analysis. Thus, in >50% of the patients with NF no histology was available. For this comparative analysis only patients in whom surgery was performed within 72 hours of symptom onset were included, resulting in a total number of nine NF patients available within the 8-year period (2006 to 2013).

The low number and the long collection period of cases available for analysis is a limitation of our study. However, NF histologic characteristics in humans have been described previously and are consistent with our findings.^{1,7,8}

Biopsies obtained from patients are usually heterogeneous regarding time of first surgery, localization of the infection, and patient characteristics, including age, comorbidities, site of infected area, and clinical outcome. Depending on the extent and localization of the infection, the tissue debridement and thus the resulting histologic specimens vary. Typically, not all tissue layers are always present for histologic assessment, as was the case in our NF

patients. The surgeon typically removes macroscopically altered tissue, which may present a sampling bias. As an additional diagnostic tool, FISH staining was added, confirming the presence of streptococci, as single cocci and chains, in both mouse and humans.

Conclusions

Despite the mentioned variables and limitations, the histologic findings of human streptococcal NF were well reflected in the murine NF model, indicating the robustness of this model. This will help studying the pathophysiology and therapeutic options of the rapidly progressive disease in more detail.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.03.009>.

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