Defects in neutrophil granule mobilization and bactericidal activity in Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations

Xi Wen Zhao1,*, Roel Gazendam1,* , Agata Drewniak1,* , Michel van Houdt1, Anton T.J. Tool1, John L. van Hamme1, Iwan Kustiawan2, Alexander B. Meijer3,4, Hans Janssen5, David G. Russell6, Lisette van de Corput7, Kiki Tesselaar7, Jaap J. Boelens8, Ingrid Kuhnle9, Jutte Van Der Werff Ten Bosch10, Taco W. Kuijpers1,11, Timo K. van den Berg1.

1Department of Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
2Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
3Department of Plasma Proteins, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
4Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands.
5Division of Cell Biology, Dutch Cancer Institute, Amsterdam, The Netherlands
6Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States
7Department of Medical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
8Department of Immunology/Hematology and BMT, University Medical Center Utrecht, Utrecht, The Netherlands
9University Medicine Göttingen, Department of Pediatrics, Göttingen, Germany
10Department of Pediatrics, Universitair Ziekenhuis Brussel, Brussels, Belgium
11Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

* These authors contributed equally to this work

Running title: bacterial killing defects in FHL-5 neutrophils

Corresponding author:
Timo K. van den Berg, PhD
Sanquin Research and Landsteiner Laboratory, Academic Medical Center
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands
Phone: +31205123317
Fax: +31205123310
Email: t.k.vandenberg@sanquin.nl
Key Point:

- Neutrophils of FHL-5 patients with munc18-2/STXBP2 mutations have impaired granule fusion and bacterial killing

Abstract:

Familial hemophagocytic lymphohistiocytosis (FHL) is caused by genetic defects in cytotoxic granule components or their fusion machinery, leading to impaired Natural Killer (NK) cell and/or T lymphocyte (CTL) degranulation and/or cytotoxicity. This may accumulate into a life-threatening condition known as macrophage activation syndrome. STXBP2, also known as MUNC18-2, has recently been identified as the disease-causing gene in FHL5. A role for STXBP2 in neutrophils, and for neutrophils in FHL in general, has not been documented thus far. Here, we report that FHL5 neutrophils have a profound defect in granule mobilization, resulting in inadequate bacterial killing, in particular of Gram-negative Escherichia coli, but not of Staphylococcus aureus, which rather depends on intact NADPH oxidase activity. This impairment of bacterial killing may contribute to the apparent susceptibility to gastrointestinal inflammation in FHL5 patients.
Introduction

Rare mutations (incidence: ~1-4 cases/million depending on ethnicity) in four different genes, including PRF1 (encoding perforin-1) in FHL2, UNC13D (encoding Munc13-4) in FHL3, STX11 (encoding syntaxin 11) in FHL4, and, more recently, also STXBP2 (also known as MUNC18-2), encoding the granule-associated docking protein syntaxin binding protein 2 (STXBP2), in FHL5, have been identified as disease-causing in FHL patients. FHL-5 accounts for an estimated 10% of all FHL cases. STXBP2 is important for regulating intracellular granule trafficking and docking at the plasma membrane. Previous reports have documented the role of STXBP2 in NK, CTL and platelet degranulation in FHL type 5 patients (FHL-5). Some of the clinical features of FHL5, such as the increased susceptibility to gastrointestinal bacterial infection, have remained unexplained and it therefore seems possible that STXBP2 plays a role in other leukocytes, such as neutrophils. Indeed, STXBP2 has been reported to localize to the different types of granules in human neutrophils. Neutrophil granules contain an array of anti-microbial constituents and proteases that will be secreted and/or released into the phagosome upon mobilization and this process contributes to microbial killing. Although there is some evidence for an involvement of STXBP2 in neutrophil granule function, compelling evidence for a direct role has not been documented. Here, we demonstrate that STXBP2 is involved in neutrophil granule mobilization and bacterial killing using neutrophils from three genetically-defined FHL5 patients.
Materials and methods

FHL5 patients, control subjects and neutrophil isolation

Heparinized blood was collected, after informed consent and according to the declaration of Helsinki 1964, from three unrelated FHL-5 patients (supplementary table 1) and healthy controls, which included umbilical cord blood and blood from healthy adults. Granulocytes were isolated by density gradient centrifugation with isotonic Percoll (Pharmacia, Uppsala, Sweden) and erythrocyte lysis, as described before. Granulocytes were washed and resuspended in Hepes-buffered saline solution (HBSS containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human serum albumin, pH 7.4). The purity of granulocytes always exceeded 95%. The study has been approved by local institute Review Board.

Degranulation assays

Neutrophil degranulation was examined as described before. Briefly, neutrophils (2×10⁶/ml) were incubated in Hepes-buffered saline solution (Hepes-buffer) at 37°C in a shaking water bath before adding the (priming) agents PAF (1 μM, Sigma, 5 minutes) or cytochalasin B (5 μg/ml, Sigma, 5 minutes) and were subsequently stimulated with fMLP (1 μM, Sigma, 15 minutes). After stimulation, cells were put on ice, washed with Hepes-buffer once, and subsequently stained with antibodies against neutrophil-granule markers: CD63-PE (IgG1, 435); CD66b-FITC (IgG1, CLB-B13.9). Data are expressed as mean fluorescence intensities (MFI). The cells were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD). The release of elastase and lactoferrin was
evaluated using ELISA kits (HyCult Biotechnology) according to the manufacturer’s instructions.

**Bacterial killing**

Granulocyte bactericidal activity was determined using, *Escherichia coli*, strain ML-35, and *Staphylococcus aureus*, strain 502A. Bacterial survival was measured by assaying bacterial colony formation as previously described\(^{10}\).

**Statistics**

Statistical significance was determined where possible using Students t-test, or otherwise by Grubb’s outlier test; \( p \leq 0.05 \) was considered to be significant.
Results and discussion

In order to characterize a role for STXBP2 in neutrophil function and a possible contribution of neutrophils to FHL5, we analyzed three unrelated patients with defined mutations in STXBP-2 (Suppl. Table 1). The absence of STXBP2 protein in neutrophils of patients A and C was confirmed by Western blot analysis, whereas patient B carrying a homozygous exon 15 splice site mutation, which is known to be associated with residual NK function and a milder clinical phenotype11, did express substantial levels of a STXBP2 protein (Suppl.Fig.1A). As reported before12, this splice site mutation yields a variant STXBP2 protein (Suppl.Fig.1B), most likely with a partially impaired function. In line with this, and also with previous findings11, a complete (patients A and C) or partial (patient B) absence of NK cell-mediated degranulation and cytotoxicity towards target cells was observed (Suppl.Fig.2). The exocytosis of the different types of neutrophil granules6, induced by either cytochalasin-B/fMLP or PAF/fMLP, was evaluated by monitoring secretion of the granule components elastase (azurophilic granules) or lactoferrin (LAF) (specific granules) and the exposure of cell surface CD63 (azurophilic granules) or CD66b (secretory, tertiary and specific granules) (Fig.1). This revealed a pronounced defect in the release of both types of vesicles in FHL5 neutrophils of all three patients. Consistently, the cytoB/fMLP-stimulated release of extracellular proteolytic activity, analyzed by DQ-BSA proteolysis assay, which primarily measures neutrophil-derived serine proteases, appeared completely absent in FHL5 neutrophils, although the total cellular content of the proteases was not different (Suppl.Fig.3). Immuno-EM analysis of the tertiary, specific and azurophilic granules, using respectively gelatinase, lactoferrin and MPO as markers, showed a normal granule appearances and frequencies
(Suppl.Fig.4), thereby excluding defects in granule biosynthesis. Measurement of intraphagosomal serine protease activity suggested that mobilization of neutrophil granules to phagosomes was also substantially impaired in FHL-5 neutrophils (Suppl.Fig.5), although it was certainly not completely absent as also shown by the presence of both specific and azurophilic granule components in *E.coli*-containing phagosomes (Suppl.Fig.6). Collectively, these findings suggested, for the first time, that STXBP2 is required for neutrophil granule exocytosis, and likewise also, at least in part, for granule mobilization to the phagosome.

Neutrophils are essential for controlling bacterial and fungal infections. There are two mechanisms that primarily contribute to neutrophil-mediated killing of microbes: (a) the NADPH oxidase, which upon assembly and activation in the phagosomal- and/or plasma-membrane produces toxic reactive oxygen species, and (b) proteolytic killing, which is assumed to require the fusion of the protease-loaded azurophilic granules with phagosomal- and/or plasma-membrane. Although gene targeting experiments in the mouse have demonstrated that both mechanisms may act independently or in concert to achieve full destructive power, the relative contribution of each activity depends on the nature of the microbe as well.13-15

However, in spite of abundant information on neutrophil microbial killing in a human context with respect to the role of the NADPH oxidase, an activity that is defective in chronic granulomatous disease (CGD), much less is known about the contribution of granule mobilization in human neutrophils. Clearly, the availability of FHL5 neutrophils with a granule mobilization defect allowed us to explore this directly. Neutrophil-mediated killing of *S. aureus* and *E. coli* was evaluated. Figure 2 shows that
the *S. aureus* killing was virtually unaffected in FHL5 neutrophils, but the killing of *E. coli* was substantially impaired in cells from all patients investigated. Of relevance, the phagocytosis of the bacteria was apparently not significantly affected (Suppl.Fig.7). In line with a complementary role for the NADPH oxidase in this context, *S. aureus* killing was more or less abolished in cells treated with the NADPH oxidase inhibitor diphenylene iodonium (DPI), while the killing of *E. coli* was only partially impaired (Suppl.Fig.8). Similar abnormalities in killing have been observed with neutrophils from CGD patients\(^1\) (and data not shown). It should be noted that the production of reactive oxygen species by the NADPH oxidase in response to particulate stimuli, such as unopsonized or opsonized zymosan (Suppl.Fig.9A), or soluble stimuli, such as PAF/fMLP (not shown), was not impaired in FHL5 neutrophils. However, the intra-phagosomal oxidation of DHR-coated *E.coli* appeared slightly delayed in FHL-5 neutrophils (Suppl.Fig.9B). Because DHR oxidation requires MPO activity\(^1\) this is consistent with the impaired mobilization of granules to phagosomes noted above. Collectively, these findings suggest that, while *S. aureus* killing depends primarily on the NADPH oxidase, both the oxidase as well as granule mobilization-dependent mechanisms, either to the plasma membrane and/or to the phagosomal membrane, play a role in *E. coli* killing. Finally, chemotaxis of FHL5 neutrophils was apparently normal (Suppl. Fig.10).

Taken together, to our knowledge these findings provide the first direct demonstration of a requirement for STXBP2 in particular, and granule mobilization in general, in the killing of bacteria by human neutrophils. This defect in bacterial killing may potentially also explain some of the unexplained symptoms, such as the increased
susceptibility to gastrointestinal inflammation, which has previously been reported in FHL5 patients.

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Authorship contribution

XWZ, RG, AD, MvH, JvH, AT, HJ, ABM, LvdC, KT performed experiments and analyzed results; DGR, JJB, IK, JVDWTB provided reagents, patient material and/or advice; TWK and TvdB designed experiments; TvdB wrote the paper.
Reference List


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Figure Legends

**Figure 1.** Defective granule mobilization by FHL5 neutrophils. Degranulation of neutrophils was examined in one day old healthy adult (n=11) and fresh cord blood (n=6) controls, and the FHL-5 patient A (fresh), B and C (both one day old) by monitoring the release of (A) lactoferrin (LAF, specific granules) and surface exposure of (B) CD66b (secretory, tertiary and specific granules) upon stimulation with PAF/fMLF, and the release of (C) elastase (azurophilic granules) and surface exposure of (D) CD63 (azurophilic granules) upon stimulation with cytoB/fMLF, respectively. Concentrations (ng/ml) of released factors, or the mean fluorescence intensities (MFI) of surface markers for stimulated (white, blue, green, red) and unstimulated (black parts of the bars) cells are shown. The values shown are averages ± SEM, from at least two independent experiments, each performed in triplo, with the exception of patient A, which was evaluated once; *, p < 0.05; n.s., non-significant; Grubb’s outlier test. Note that granule release is essentially absent in all three FHL-5 patients.

**Figure 2.** Impaired bacterial killing by FHL5 neutrophils. Killing of *S. aureus* (A) and *E. coli* (B) was assessed in cord blood (control A; n=6) and normal (control B/C; n=10) controls and the FHL-5 patients as described in the Materials and Methods section (see also the legend of Fig. 1). Remaining viable bacteria were quantified as colony forming units (CFU) and expressed as % of CFU at t=0. For the control groups the values shown are averages ± SEM, and for the patients the average of two measurements from two (patient A/B) or three (patient C) independent experiments are shown; *, p < 0.05;
Grubbs’ outlier test (patient A/B) or Students-t-test (patient C). Note that the killing of *S. aureus* is virtually normal, while *E. coli* killing is significantly impaired for all three FHL-5 patients.
Figure 1

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Figure 2

**E. coli**

**S. aureus**

- controls A (n=6)
- controls B/C (n=6)
- patient A
- patient B
- patient C

* p ≤ 0.05
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