Original Investigation

The synthetic antimicrobial peptide LTX21 induces inflammatory responses in a human whole blood model and a murine peritoneum model

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Abstract

The global spread of antimicrobial resistance and the increasing number of immune-compromised patients are major challenges in modern medicine. Targeting bacterial virulence or the human host immune system to increase host defence are important strategies in the search for novel antimicrobial drugs. We investigated the inflammatory response of the synthetic short antimicrobial peptide LTX21 in two model systems: a human whole blood *ex vivo* model and in a murine *in vivo* peritoneum model - both reflecting early innate immune response.

In the whole blood model, LTX21 increased secretion of a range of different cytokines, decreased the level of tumour necrosis factor (TNF), and activated the complement system. In a haemolysis assay, we found 2.5% haemolysis at a LTX21 concentration of 500 mg/L.

In the murine model, increased influx of white blood cells (WBC) and polymorphonuclear neutrophils (PMN) in the murine peritoneal cavity was observed after treatment with LTX21. In addition, LTX21 increased monocyte chemoattractant protein-1 (MCP-1).

In conclusion, LTX21 affected the inflammatory response; the increase in cytokine secretion, complement activation and WBC influx indicates an activated inflammatory response. The present results indicate impact of LTX21 on host-pathogen interplay. Whether this will also affect the course of infection has to be investigated.

Keywords: LTX21, cationic peptides, human whole blood model, murine model

Introduction

The global spread of antimicrobial resistance and the increasing number of immune-compromised patients are major challenges in modern medicine (1). The search for novel antimicrobial drugs now includes agents targeting bacterial virulence or host defence immunity (2). Cationic host defence peptides (HDPs) are a diverse class of molecules, acting as a first-line defence against microbial threats (3, 4). Their modes of action are not completely resolved but a proposed central mechanism is bacterial membrane disruption affecting both dormant and dividing bacteria (5). However, studies indicate that the lytic effect may be less important under physiologically relevant conditions (6, 7). A potential important function of HDP s are immune-modulating effects enhancing protective immunity and suppressing inflammation (8, 9). During infection, HDPs may reduce levels of pro-inflammatory cytokines, modulate the expression of chemokines, enhance recruitment and activation of immune cells and stimulate angiogenesis and wound healing (3, 6, 10). Hence, they have potential as new antimicrobial, immune-modulating drugs. Strategies to improve HDP's function and reduce toxicity include design, modification and synthesis of peptide sequences with improved pharmacokinetic, antimicrobial or immune modulating properties (3, 11).

We have previously reported that short synthetic antimicrobial peptides (SAMPs) have a superior *in vitro* killing of biofilm-embedded Coagulase negative staphylococci (CoNS) compared to Vancomycin (12). In a follow-up study, we compared the efficacy of Vancomycin versus a SAMP (LTX21) in a murine peritonitis CoNS-infection model (13). Eradication of CoNS biofilms was not achieved, but LTX21 therapy reduced the bacterial load similar to Vancomycin. Moreover, we found modulation of the immune response among mice treated with LTX21, which was not seen in mice treated with Vancomycin or placebo (13).

The aim of the present study was to investigate potential immune modulating properties of LTX21 on the innate immune response in two different model systems; a human whole blood *ex vivo* model

reflecting an early innate immune response and a murine peritoneal *in vivo* model, reflecting both an early and a late innate immune response.

Materials and methods

LTX21

LTX21 (Lytix Biopharma, Norway) is a tetra-peptide (arginine-tri-tert-butyl-tryptophan-arginine), derived from bovine lactoferricin (5). The two arginine moieties provide cationic properties and the modified tryptophan provides the lipophilic bulk. LTX21 powder was dissolved in NaCl to final concentrations between 25 mg/L and 1000 mg/L for the different *ex vivo* and *in vivo* experiments.

Human whole blood *ex vivo* model

This model has been described earlier (14, 15). Briefly, for each experiment, approximately 25 ml fresh blood from each of the six healthy donors was used. The Regional Committee for Medical Research Ethics approved the collection of blood from healthy adults (2011/2020/REK nord). Informed written consent was obtained from the blood donors before the experiments were started. The anticoagulant lepirudin (Refludan®, Hoechst, Sanofi-Aventis, Frankfurt am Main, Germany) was added to the collected blood at a concentration of 50 µg/ml and the experiments were performed in six parallels, with different donors each time. The effect of LTX21 on innate immunity components in human whole blood was compared to placebo (0.9% NaCl) and assessed at two different concentrations, 50 mg/L and 500 mg/L which is 10 and 100 times the minimal inhibitory concentrations (MIC) of LTX21 against *Staphylococcus epidermidis* (13) . All experiments were started within 30 min after blood collection and LTX21 was added immediately before incubation. Samples were added to silicone tubing (MediPlast, Malmø, Sweden,) which was circularized and

incubated rotating at 37 °C for 30 or 180 min. After incubation the samples were centrifuged and plasma was collected and stored at -80°C until further analyses. We performed two different readouts of these experiments. First, we determined complement activation in plasma after 30 min incubation. Second, we determined cytokine concentrations in plasma after 180 min incubation.

Murine peritoneum *in vivo* model

Forty-six female BALB/c mice (Taconic M&B A/S Ry, Ejby, Denmark), aged 12 weeks were used. There were eight mice per cage and all mice had access to water and food ad libitum. The animal studies were performed in accordance to the ARRIVE guidelines and the European Convention and Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation. The animal experiments were approved by the National Animal Ethics Committee, Denmark.

All animals received intraperitoneal injections (400 µl) of LTX21 500 mg/L or placebo (0.9% NaCl) every 24 hours for up to three days. The LTX21 dosage was 10 mg/kg, based on previous toxicology studies, a pilot treatment study and *in vitro* MIC studies (data not shown) and a previous *in vivo* study comparing the effects of LTX21 versus vancomycin (13). In order to study early immunomodulatory effects of LTX21, mice were sacrificed three hours after the first intraperitoneal administration of LTX21 (n=10) or placebo (n=10) on day one. In order to study late immunomodulatory effects mice were sacrificed three hours after the third intraperitoneal administration of LTX21 (n=10) or placebo (n=10) on day three. One group of mice without any interventions (n=6) were included as normal controls and sacrificed on day one.

Blood sampling and peritoneal lavage were performed after the mice were deeply sedated with a subcutaneous injection of 0.1 ml pentobarbital 200 mg/ml (KVL, Copenhagen, Denmark). Blood was drawn by cardiac puncture and transferred to tubes with added heparin (LEO Pharma A/S, Ballerup, Denmark) for further analyses. Peritoneal lavage was performed by injecting 3 ml phosphate-buffered saline (PBS) into the peritoneal cavity, followed by gentle massage of the

abdomen before withdrawing peritoneal lavage fluid (PLF). Mice were euthanized by removal of the heart whilst in deep sedation. By the end of all experiments 100 µl of PLF was spread on blood agar plates to check bacterial growth due to possible perforation of the intestine during the intraperitoneal injections, which could influence inflammatory markers.

Enzyme immune assays for cytokines.

From the human whole blood assay, plasma from samples incubated for 180 minutes were analysed to quantify the level of 27 different cytokines (Table 1) using Bioplex cytokine assays (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. From the murine *in vivo* model, five cytokines (TNF-α, IL-1β, MIP-2 MCP-1 and GM-CSF) were quantified both in plasma and PLF by using Luminex® Assays (R&D systems, Inc., Minneapolis, MN, USA), according to the manufacturer's description. The selection of cytokines quantified in the murine *in vivo* model, was based on the findings from the human whole blood model and previously published work of a similar *in vivo* murine study (13).

Enzyme immune assays for complement activation products

In the human whole blood *ex vivo* model central complement activation products C5a, C4bc, C3bBbP, C3bc and TCC were quantified by enzyme-linked immunosorbent assay as previously described, in plasma from samples incubated for 30 minutes (16, 17).

Cellular response and flow cytometry

We measured the total white blood cell (WBC) count and polymorphonuclear neutrophils (PMNs) in murine blood and PLF in response to LTX21 or NaCl, as described previously (13) Briefly, the total WBC count was determined by staining with allophycocyanin-conjugated monoclonal rat anti-mouse CD45 antibody (Becton Dickinson, Franklin Lakes, NJ, USA). Fractions of PMNs were estimated by staining with allophycocyanin-conjugated rat anti-Ly-6G antibody (Becton Dickinson, Franklin Lakes, NJ, USA), fluorescein isothiocyanate-conjugated rat anti-mouse F4/80 antibody (BioLegend, San Diego, CA, USA), and peridinin chlorophyll protein complex-conjugated monoclonal rat anti-mouse CD45 antibody (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were fixed with 10% FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed on a FACSCanto (Beckton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15-mW argon-ion laser tuned at 488 nm and a red diode laser at 635 nm for excitation. Light scatter and logarithmically amplified fluorescence parameters for >10000 events were recorded in list mode after gating on forward light scatter and fluorescence for CD45 staining to excluded debris, cell aggregates and bacteria. The instrument was calibrated with CST beads (Becton Dickinson, Franklin Lakes, NJ, USA).

Haemolytic activity

As an indicator of cytotoxicity, the haemolytic activity of LTX21 was determined in human plasma and NaCl, as previously described (17). Briefly, human erythrocytes from fresh whole blood were prepared by centrifugation at 194 x g (Eppendorf, Hamburg, Germany) and washed three times with preheated NaCl before being diluted to 10% in either physiological NaCl or pooled human plasma. The erythrocytes were incubated for 1 hour at 37°C with LTX21 at concentrations ranging from 25 to 1000 mg/L. The samples were centrifuged at 344 x g (Eppendorf, Hamburg, Germany) for 5 min before absorbance of the supernatant was measured at 405 nm in a microplate reader (Versamax®, Molecular Devices, San Jose, CA, USA). Positive controls were human erythrocytes treated with 10% Triton (Teknova, Hollister, CA, USA) and the negative control was physiological NaCl. The experiments were repeated three times with different blood donors in two parallel experiments. A haemolysis ≤ 2.5% does not seem to cause a clinically relevant influence on release of potassium and

other intracellular substances (18), whereas higher values will increasingly become clinically significant and potentially dangerous for the host (19).

Statistical analyses

Data were analyzed using IBM-SPSS version 25 (IBM, Armonk NY, USA) statistical software. The non-parametric Mann-Whitney U-test was used to compare two groups. We used a Bonferroni correction when testing the cytokines and complement at two different LTX21 concentrations, and for these analyses a p value < 0.025 was considered statistically significant. For all other analyses a p value < 0.05 was considered statistically significant.

Results

Clinical appearance of the mice

All mice were in good clinical condition before each treatment. The group treated with NaCl remained clinically unchanged after treatment. In mice treated with LTX21, a change in behaviour and appearance was noticed during the first 20 min after administration, the mice became less active, had ruffled fur and huddled closely together. Within one hour post administration, their activity level was restored and their clinical appearance was the same as the placebo treated and control mice.

Bacterial growth in murine peritoneal lavage fluid (PLF)

PLF from one of the placebo treated mice had $1{,}1 \times 10^3$ CFU/ml indicating a possible bacterial contamination from intestinal perforation. Data obtained from this mouse was excluded from all analyses. In five other placebo mice 10 CFU/ml was observed, this was not considered significant contamination or intestinal perforation that would affect the results of the study. This is confirmed by

200 the significant increase found in several inflammatory markers for the LTX21 treated mice compared
201 to placebo mice, described below.

202

203 **Cellular responses in murine peritoneal lavage fluid (PLF)**

204 We found significantly higher total levels of WBCs in PLF among mice treated with LTX21 compared
205 to placebo, both on day one ($p=0.023$) and day three ($p=0.002$) (Figure 1a). A significant increase in
206 the WBCs was observed from day one to day three ($p=0.019$) in the LTX21 group. Similarly, there
207 were significantly higher levels of PMNs in PLF among mice treated with LTX21 compared to
208 placebo, both on day one ($p<0.001$) and day three ($p<0.001$) (Figure 1b).

209

210 **Cellular responses in murine blood**

211 There were higher levels of PMNs in mice treated with LTX21 compared to placebo both on day one
212 ($p=0.01$) and on day three ($p<0.001$) (Figure 2b). No significant differences in WBCs were found
213 between the LTX21 and placebo, neither on day one nor three (Figure 2a). Both WBC ($p= 0.015$) and
214 PMN ($p= 0,009$) levels decreased in the placebo group from day one to three, while only a significant
215 fall in WBC level was observed in the LTX21 group (Figure 2b).

216

217 **Cytokine secretion in murine plasma and PLF**

218 We detected increased levels of the monocyte chemoattractant protein-1 (MCP-1) in both plasma (p
219 <0.001) and PLF ($p <0.001$) among mice treated with LTX21 compared to placebo on day one (Figure
220 3a and b). On day three we only found significantly higher levels of MCP-1 in the PLF ($p = 0.011$),
221 but not in plasma, among mice treated with LTX21 (Figure 3a and b). There was a significant decrease
222 in MCP-1 levels from day one to three in both PLF ($p= <0.001$) and plasma ($p <0.001$). The other

cytokines measured in plasma from mice treated with LTX21 or placebo showed no significant biological relevant differences.

Cytokine secretion in human plasma

A significant increase of cytokine secretion in plasma from blood exposed to LTX21was observed compared to blood exposed to NaCl. In Table 1 we present cytokines showing significant change compared to the control after LTX21 exposure. LTX21 induced cytokine secretion in a dose-dependent manner for several cytokines. In contrast, we observed a significant decrease in TNF-α secretion in response to LTX21.

Complement activation in human whole blood

In the *ex vivo* human whole blood model, we observed significant complement activation as measured for C5a and TCC in response to LTX21 at a concentration of 500 mg/L (Table 2). The lower LTX21 concentration (50 mg/L) did not significantly activate the complement system.

Haemolytic effects of LTX21 in plasma

LTX21 induced haemolysis at concentrations ≥100 mg/L, while concentrations ≥500 mg/L resulted in very high haemolysis (Figure 4) when erythrocytes where diluted in NaCl. Haemolysis was markedly reduced when erythrocytes where diluted in physiological plasma solution. Under this latter condition only low haemolysis (2.5%) at the highest LTX21 concentration (1000 mg/L) was observed.

Discussion

Our results show that the response to LTX21-in the human whole blood model was dominated by induced cytokine secretion and an activation of the complement system. In the murine peritoneal model, we found significant increases in WBC counts, particularly PMNs as a response to LTX21.

The immune response during an infection is largely mediated by cytokines e.g. by attracting WBCs to the site of infection and thus augmenting the immune response. We observed a significant increase in several pro- and anti-inflammatory cytokines as a response to LTX21 in the *ex vivo* human whole blood model. However, not all cytokines increased in a dose-dependent manner, and some, albeit statistically significant, increased only modestly with uncertain biological significance.

A clear dose-dependent increase was observed for the chemokine MCP-1, eotaxin and VEGF which all play a role in angiogenesis (20). The only cytokine displaying reduced levels, was TNF, which is an important pro-inflammatory cytokine in the acute phase response of innate immunity (21). Increase in chemokines and reduced levels of TNF secretion has previously been shown in response to synthetic cationic peptides (22, 23). In the murine model, MCP-1 was increased in response to LTX21, a response also described previously for other synthetic HDPs (22). MCP-1 is primarily secreted by monocytes, macrophages and dendritic cells and recruits more of these cells to the site of inflammation (24, 25). Resident peritoneal macrophages are known to be an important source of chemokines for leukocyte recruitment during peritoneal infections and were probably one of the cell types that increased MCP-1 production in response to LTX21 (26-28). A significant increase in activation of the terminal complement pathway was observed for LTX21 in the *ex vivo* whole blood model. This supports the theory that both natural HDPs and synthetic peptides may activate the host immune defence. However, they seem to cause a more modest inflammatory response in contrast to the strong and often inappropriate pro-inflammatory response observed after stimulation with Gram negative cell wall components (lipopolysaccharides) (29). Our findings are also in line with recent data reporting complement activation in response to synthetic cationic peptides (30).

In the murine model increased influx of WBCs, and in particular PMNs, to the peritoneal cavity was found, indicating that LTX21 induces a biologically significant inflammatory response. Other studies

have shown that HDPs induce chemotaxis of WBC/PMNs through stimulation of chemokine secretion in addition to suppression of potentially harmful inflammation (22, 23, 31, 32). We could not detect any increased levels of cytokines attracting PMNs. Other studies have shown that some HDPs are chemotactic themselves and induce WBC/PMN influx without the production of cytokines (33). The observed influx of immune cells in response to LTX21 and other lactoferricin-derivates has to our knowledge not previously been studied, and calls for further investigation of the mechanisms. We did not measure complement products in the murine model. We can therefore not rule out that complement-mediated recruitment of PMNs could be an explanation for our finding. However, a pilot study (data not published) indicate that LTX21 does not significantly activate complement in the animal model setup used in this study. Another limitation with our study is that we did not check specifically for obligate anaerobes when plating the peritoneal fluid, since anaerobe bacteria make up a significant proportion of the gut microbiome they could theoretically explain some of the inflammatory response observed in the placebo group.

Mammalian cytotoxicity differs between different HDPs. We tested the cytotoxicity of LTX21 in a haemolysis assay with human erythrocytes in human plasma. The amphipathic structure of LTX21, with its hydrophobic and cationic residue is an important hallmark of antimicrobial peptides, mimicking the mammalian membranes. There is a well-known correlation between increased hydrophobicity and increased lysis of erythrocytes (34, 35). In our study, haemolysis was low even at the highest LTX21 concentration (1000 mg/L) when we used a physiologically relevant media. This reflects how complex biological environments and possible peptide binding to plasma proteins may reduce the potential of HDP's toxicity to mammalian cells compared to *in vitro* findings using assays with NaCl (36, 37).

We have used the synthetic peptide LTX21, structurally closely related to LTX109 (Lytixar™), a drug already evaluated in human clinical trials (38). LTX109 is a promising drug for nasal decontamination of methicillin-resistant *Staphylococcus aureus* and treatment of Gram-positive skin infections (38). The antimicrobial and antifungal effects of LTX109 have been extensively investigated (12, 39, 40). However, this is the first study where the innate immune response to the

298 closely related peptide LTX21 is examined both under *ex vivo* and *in vivo* conditions. The immune-
299 modulatory effects of synthetic HDPs have not been explored to the same extent as their antimicrobial
300 properties. Prophylactic treatment with protease-labile L-amino acid peptides may reduce both local
301 and systemic infections in mice acting as an immune defence regulator (23). Our observations are in
302 agreement with these findings, indicating that further studies should be repeated in an *in vivo* model,
303 assessing both the early and the late inflammatory response in addition to further mechanistic studies
304 assessing the potential of LTX21 in regulating proinflammatory cytokines, and alleviating
305 inflammation.

Conclusion

LTX21 induced cytokine secretion and activated the complement system in the human whole blood model and induced influx of WBC and PMNs in a murine peritoneal model. The most pronounced effect; increase in chemokine secretion and PMN influx, points towards similar functions as those previously described for other HDPs. The PMN influx to the treatment site in the *in vivo* model may enhance the bacterial killing. LTX21 induced innate immune responses, which may augment the inflammatory response during an infection. Additionally, the reduced TNF secretion may even prevent some of the tissue damage mediated by this cytokine during severe immune responses. Further *in vivo* experiments are necessary to study how LTX21 may affect the course of an infection alone or in combination with traditional antibiotics.

Abbreviations

HDP Host defence peptides

PMN Polymorphonuclear neutrophils

CoNS Coagulase negative staphylococci

CFU Colony-forming units

FACS Fluorescence-activated cell sorting

ELISA Enzyme-linked immunosorbent assay

WBC White blood cells

PLF Peritoneal lavage fluid

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Consent for publication

All authors have approved this version of the publication.

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Table 1. LTX21 induced cytokine responses in an *ex vivo* human whole blood model after 3 h incubation. Interval data are presented as median (IQR). Concentrations of the cytokines are given in pg/ml. Of the 27 cytokines studied, this table includes those (n=13) with significant differences between the groups.

Cytokin (pg/ml)	Control[1]	LTX21 50mg/L	LTX21 500mg/L	P-value[2] Control vs 50mg/L	P-value[2] Control vs 500mg/L
IL2	0 (0-3.25)	6.5 (5-7.25)	12 (12-13)	0.002	0.002
IL6	3.5 (2.5-4.0)	5.5 (3.75-6)	14 (9.75-14)	0.041	0.002
IL10	15 (13-17)	23 (21-30)	32 (22-59)	0.009	0.002
IL13	5 (4.5-7.25)	6.5 (4.75-7.25)	14 (9.75-25)	0.132	0.002
IL15	0 (0)	0 (0)	7.5 (3-12)	1.0	0.015
Eotaxin	39 (32-48)	57 (41-72)	92 (75-110)	0.093	0.002
GMCSF	0 (0)	29 (8.5-36)	85 (62-100)	0.002	0.002
IP10	301 (225-394)	525 (379-725)	869 (606-1314)	0.026	0.004
MCP1	7 (6-12)	15.5 (14-24)	40 (30-45)	0.004	0.004
MCP1α	3 (1.5-4.5)	6.5 (5-8.5)	9.5 (8.75-13)	0.009	0.004
PDGFBB	906 (710-1026)	2472 (2066-2914)	3189 (2090-3724)	0.002	0.002
TNF	51 (43-63)	48 (48-53)	29 (24-44)	1.0	0.002
VEGF	18 (17-25)	41 (30-57)	102 (85-133)	0.009	0.002

[1]Untreated control: human blood and PBS.

[2]p value is determined by Mann-Whitney U-test. Bonferroni correction was done and a ρ value <0.025 was considered statistically significant.

Table 2. Complement activation by LTX21 in an *ex vivo* human whole blood model after 30 min incubation. Interval data are presented as median (IQR)

Complement activation product	Control[1]	LTX21 50mg/L	LTX21 500 mg/L	P-value[2] Control vs 50 mg/L	P-value[2] Control vs 500 mg/L
C4bc (CAU/ml)	31 (16-49)	28 (16-48)	59 (32-180)	0.937	0.093
C3bBbP (CAU/ml)	585 (464-879)	509 (398-650)	548 (320-994)	0.485	0.937
C3bc (CAU/ml)	55 (41-62)	46 (36-61)	81 (55-121)	0.589	0.132
C5a (ng/ml)	46 (42-57)	45 (29-56)	191 (153-249)	0.699	0.002
TCC (CAU/ml)	25 (16-35)	24 (16-37)	52 (42-110)	1	0.004

[1] Human blood and PBS

[2]p-value determined by Mann-Whitney U-test with a Bonferroni correction test; a p-value <0.025 was considered statistically significant

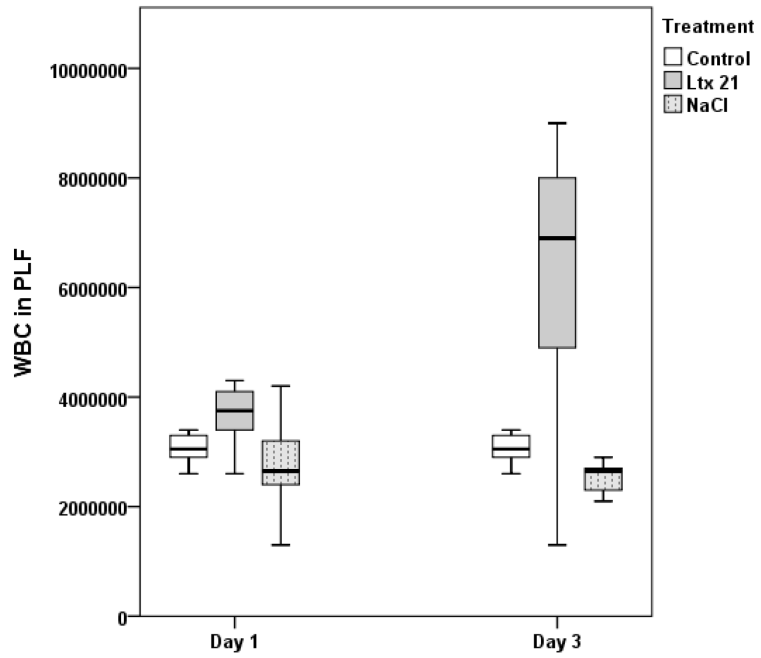


Fig. 1 a: Levels of white blood cell count (WBC) in peritoneal lavage fluid (PLF) after treatment with LTX21 or NaCl.

The number of WBC were estimated in PLF from mice treated with either LTX21 or NaCl once (sacrificed on day 1) or three times with 24 h intervals (sacrificed on day 3). Controls were six mice sacrificed on day 1 without any intervention or treatment.

*p < 0.05, **p < 0.01, ***p < 0.001; differences within one day (solid lines)

*p < 0.05, **p < 0.01, ***p < 0.001; differences between days (dotted lines). With comparison between days; comparison of the same substance on two different days.

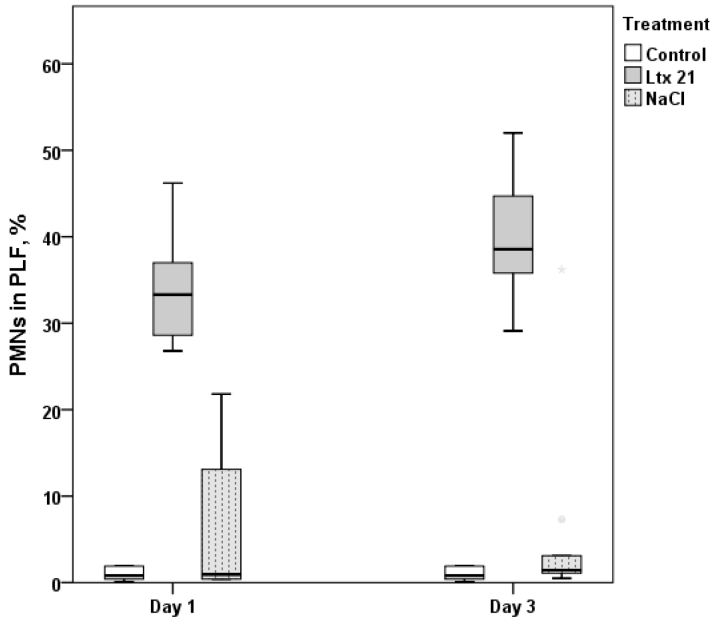


Fig. 1 b: Amount of polymorphonuclear neutrophils (PMNs) in peritoneal lavage fluid (PLF) after treatment with LTX21 or NaCl.

The amount of PMNs were estimated in PLF from mice treated with either LTX21 or NaCl once (sacrificed on day 1) or three times with 24 h intervals (sacrificed on day 3). Controls were six mice sacrificed on day 1 without any intervention or treatment.

*p < 0.05, **p < 0.01, ***p < 0.001; differences within one day (indicated by solid lines)

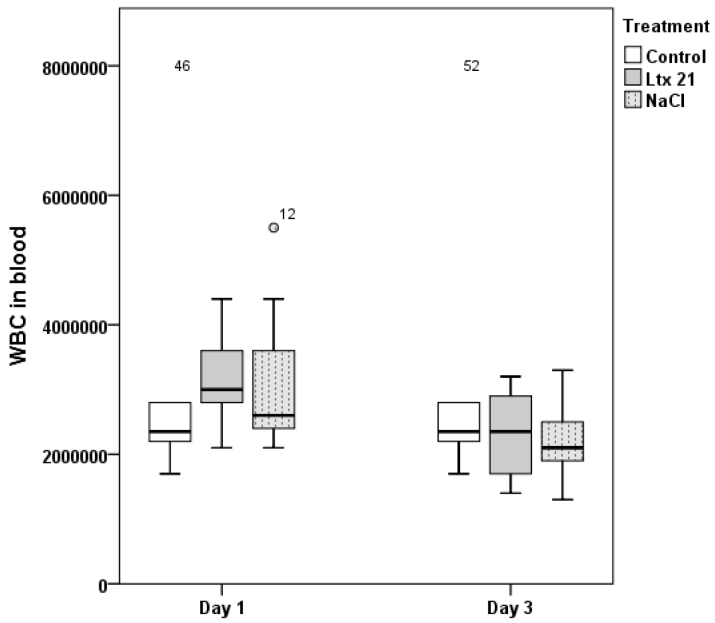
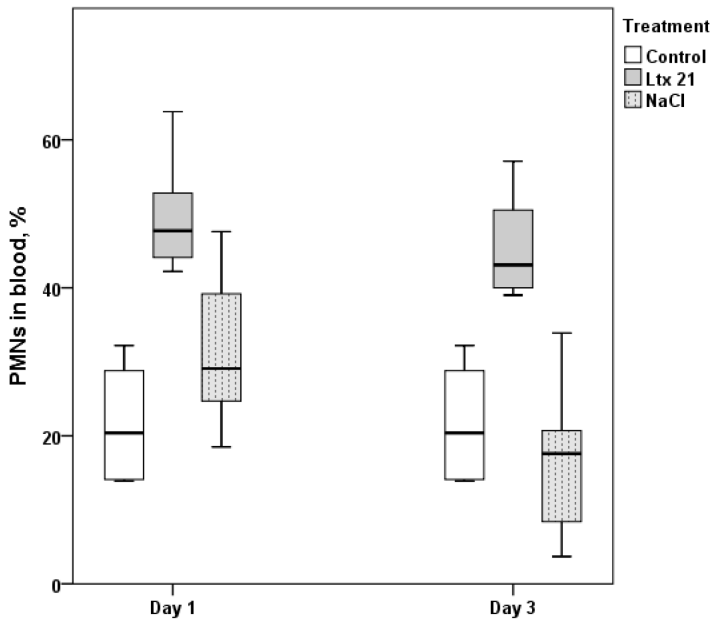


Fig. 2 a: Levels of white blood cell count (WBC) in murine blood after treatment with LTX21 or NaCl. The number of WBC were estimated in blood from mice treated with either LTX21 or NaCl mice (sacrificed on day 1) or three times with 24 h intervals (sacrificed on day 3). Controls were six mice sacrificed on day 1 without any intervention or treatment.

*p < 0.05, **p < 0.01, ***p < 0.001; differences between days (dotted lines). With comparison between days; comparison of the same substance on two different days.



504

505 **Fig. 2 b:** Amount of polymorphonuclear neutrophils (PMNs) in murine blood after treatment with
506 LTX21 or NaCl. The amount of PMNs were estimated in blood from mice treated with either LTX21
507 or NaCl mice (sacrificed on day 1) or three times with 24 h intervals (sacrificed on day 3). Controls
508 were six mice sacrificed on day 1 without any intervention or treatment.

509 *p < 0.05, **p < 0.01, ***p < 0.001; differences within one day (solid lines)

510 *p < 0.05, **p < 0.01, ***p < 0.001; differences between days (dotted lines). With comparison
511 between days; comparison of the same treatment on two different days.

512

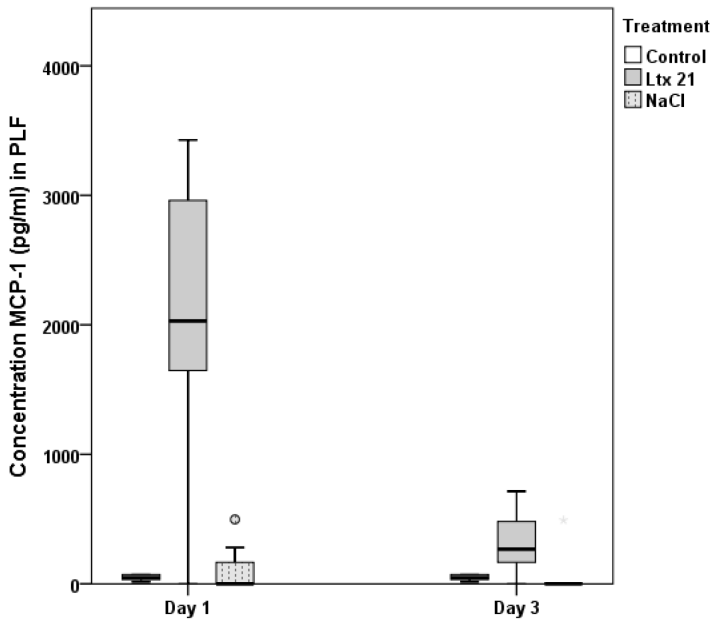


Fig. 3a: Level of Monocyte chemoattractant protein-1 (MCP-1) in peritoneal lavage fluid (PLF) after treatment with LTX21 or NaCl.

The level of MCP-1 was estimated in PLF from mice treated with either LTX21 or NaCl once (sacrificed on day 1) or three times with 24h intervals (sacrificed on day 3). Controls were six mice sacrificed on day 1 without any intervention or treatment.

*p < 0.05, **p < 0.01, ***p < 0.001; differences within one day (solid lines)

*p < 0.05, **p < 0.01, ***p < 0.001; differences between days (dotted lines). With comparison between days; comparison of the same substance on two different days.

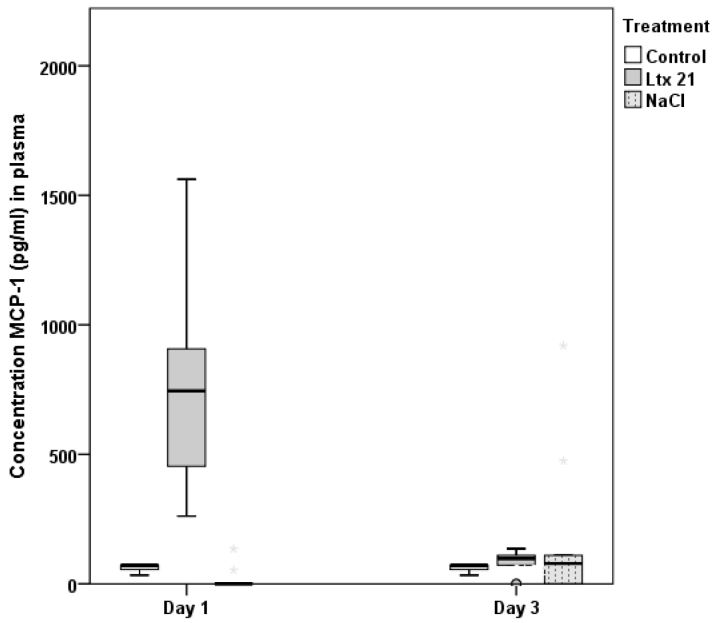
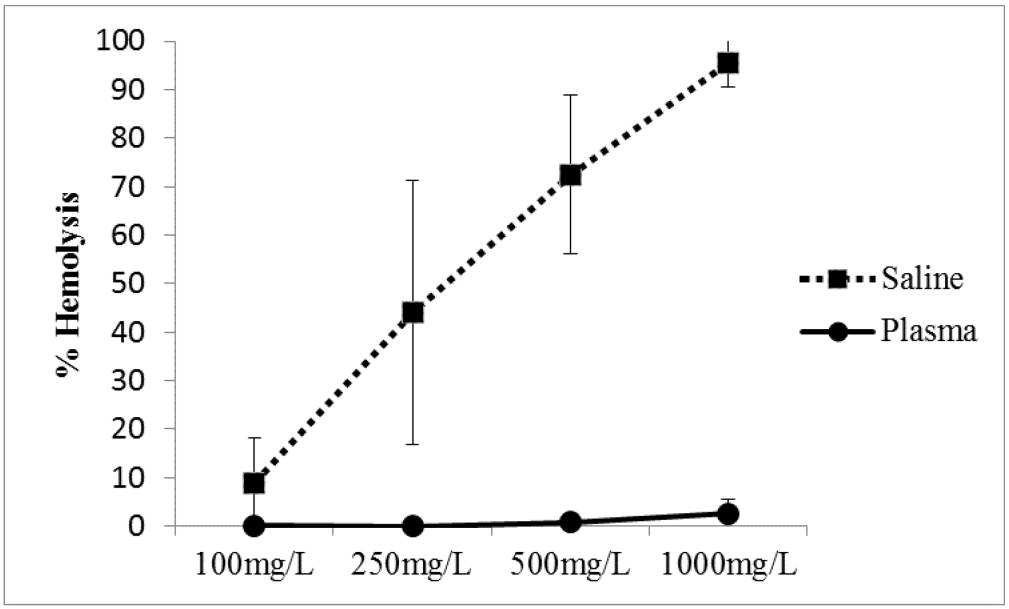


Fig. 3b: Level of Monocyte chemoattractant protein-1 (MCP-1) in plasma after treatment with LTX21 or NaCl.

The level of MCP-1 was estimated in blood from mice treated with either LTX21 or NaCl once (sacrificed on day 1) or three times with 24h intervals (sacrificed on day 3). Controls were six mice sacrificed on day 1 without any intervention or treatment.

*p < 0.05, **p < 0.01, ***p < 0.001; differences within one day (solid lines)

*p < 0.05, **p < 0.01, ***p < 0.001; differences between days (dotted lines). With comparison between days; comparison of the same substance on two different days.



532

533 **Fig. 4:** Haemolytic activity of LTX21 on human erythrocytes. Haemolytic activity of LTX21 was
534 determined in NaCl and plasma. Each line represents the average of two parallel samples from 3
535 donors. Error bars display the standard error of mean.

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