# Recombinant Human Tissue Non-Specific Alkaline Phosphatase Successfully Counteracts Lipopolysaccharide Induced Sepsis in Mice

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## Summary

Sepsis is a life threatening condition that arises when the body's response to an infection injures its own tissues and organs. Sepsis can lead to shock, multiple organ failure and death especially if not recognized early and treated promptly. Molecular mechanisms underlying the systemic inflammatory response syndrome associated with sepsis are still not completely defined and most therapies developed to target the acute inflammatory component of the disease are insufficient. In this study we investigated a possibility of combating sepsis in a mouse model by intravenous treatment with recombinant human tissue non-specific alkaline phosphatase (rhTNAP) derived from transgenic rabbit milk. We induced sepsis in mice by intraperitoneal injection of LPS and three hours later treated experimental group of mice by intravenous injection with rhTNAP derived from transgenic rabbits. Such treatment was proved to be physiologically effective in this model, as administration of recombinant rhTNAP successfully combated the decrease in body temperature and resulted in increased survival of mice (80 % vs. 30 % in a control group). In a control experiment, also the administration of bovine intestinal alkaline phosphatase by intravenous injection proved to be effective in increasing survival of mice treated with LPS. Altogether, present work demonstrates the redeeming effect of the recombinant tissue non-specific AP derived from milk of genetically modified rabbits in combating sepsis induced by LPS.

## Key words

Tissue non-specific alkaline phosphatase • Lipopolysaccharides • Sepsis • Mice • Survival

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## Introduction

Sepsis is defined by an overwhelming systemic inflammatory response syndrome to pathogens or pathogen associated molecular patterns. The most severe form of sepsis can result in septic shock characterized by multiple organ failure. Unfortunately, treatment strategies are limited and a number of promising attempts to combat this medical condition have failed in clinical trials (Riedemann *et al.* 2003, Angus and van der Poll 2013). Although thanks to intensive campaign the mortality in the last years in severe septic patients decreased in some countries to 30 % (Levy *et al.* 2010), the availability of effective therapy is of critical importance in septic patients. Septic shock can be induced experimentally in response to endotoxin lipopolysaccharide (LPS), an essential component of the outer membrane of Gram negative bacteria, resulting in an uncontrolled release of pro-inflammatory mediators (Heine *et al.* 2001, Uhrin *et al.* 2013).

In our study, we investigated a possibility of combating sepsis in a LPS-induced murine model of sepsis, by treatment of mice with recombinant human tissue non-specific alkaline phosphatase (rhTNAP) derived from milk of transgenic rabbits previously generated in our laboratory (Bodrogi *et al.* 2006).

Tissue-nonspecific alkaline phosphatase (TNAP) is a member of a family of alkaline phosphatases (APs) consisting of at least three additional, distinct but related alkaline phosphatases: intestinal, placental and placental-like (ALPPL2). TNAP is expressed in a variety of tissues and cells including bone, cartilage, liver and kidney (Hoshi *et al.* 1997). In humans, genetic deficiency of TNAP results in hypophosphatasia, a disorder of bone mineralization (Henthorn *et al.* 1992). Inactivation of TNAP in knockout mice causes growth impairment and development of epileptic seizures due to defective metabolism of vitamin $B_6$ (Waymire *et al.* 1995, Narisawa *et al.* 1997).

Members of the group of APs were shown to exhibit a detoxifying effect on LPS through its dephosphorylation *in vitro* (Poelstra *et al.* 1997a,b) and *in vivo* (Koyama *et al.* 2002). Subsequently, usage of AP derived from calf intestine or human placenta was demonstrated to be beneficial in sepsis models attenuating LPS toxicity (Bentala *et al.* 2002, Beumer *et al.* 2003, Verweij *et al.* 2004, Su *et al.* 2006). In our study we show now the redeeming effect of administration of the rhTNAP in combating sepsis in a LPS-induced model of sepsis in mice.

## Methods

*Partial purification of rhTNAP and endotoxin determination*

Rabbit milk samples containing rhTNAP were obtained from homozygous transgenic females (Bodrogi *et al.* 2006). Control samples were derived from milk of non-transgenic females. Briefly, animals were separated over night from their pups, i.p. injected with oxytocin (2 IU, Kela N.V., Belgium) and milked. After centrifugation of fresh milk samples for 20 min at 800 g and 4 °C, the upper fat fraction was collected and resuspended in milliQ water. To extract rhTNAP from milk fat globule membranes, a single phase extraction method previously elaborated for obtaining of AP was used (Ahn and Snow 1993). Briefly, n-butanol in a final concentration of 10 % (v/v) was used for the extraction of fat globule membranes binding in the case of transgenic milk the rhTNAP. The samples were kept shaking for 30 min at room temperature and centrifuged for 90 min at 4 °C and 100,000 g. Water phase containing the rhTNAP fraction was collected, aliquoted, freeze-dried and re-dissolved in sterile 0.9 % (w/v) NaCl. This procedure yielded rhTNAP at concentration of 7.8-11.7 U per 1 ml

of transgenic rabbit milk. Some aliquots of milk extracts were also re-dissolved in water and used for testing for contamination with endotoxin, using Limulus Amoebocyte Lysate (LAL) Assay (Endotoxin Detection Service, Hyglos GmbH, Regensburg, Germany).

*SDS-PAGE and determination of AP activity*

Milk extracts derived from transgenic and non-transgenic rabbits were examined on 10 % gels on their residual protein content and AP activity. Protein content was estimated using SDS-PAGE gels and Coomassie Brillant Blue-R (PageBlue, Vilnius, Lithuania) and silver staining (Sigma, St. Louis, MO). AP activity in protein extracts was determined by 10 % native-PAGE gels by staining with a mixture of 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Western Blue Stabilized Substrate for Alkaline Phosphatase, Promega, Madison WI, USA).

*Animals and experimental design*

Two months old male Balb/c mice (obtained from Harlan, Wobe Ltd., Hungary) were subjected to LPS-induced model of sepsis. The animals were divided into two groups consisting of 10 mice (designated as LPS+rhTNAP and as LPS+control group). Both groups were treated by i.p. injection of LPS (*Escherichia coli* 0111:B4, L5293 Sigma, St. Louis MO, USA, 15 µg/g body weight, dissolved in 200 µl of 0.9 % NaCl). Three hours later animals in a group LPS+rhTNAP received i.v. injected 9 units of rhTNAP, dissolved in 500 µl of 0.9 % (w/v) NaCl. Mice in the LPS+control group were treated by injecting of milk extract dissolved in 500 µl of 0.9 % (w/v) NaCl derived from non-transgenic rabbit milk and lacking rhTNAP activity. Body temperature of mice was measured by rectal thermometry immediately before the LPS injection, and at time points 3 h and 21 h later. At these time points, 50 µl of blood were collected by orbital punction into tubes without anticoagulants to determine AP activity. Survival of mice was monitored for at least up to 7 days.

In a subsequent experiment three groups of two months old Balb/c male mice consisting of 10 mice per group were studied. Mice were treated with i.p. LPS injection followed by i.v. injection of 9 units of rhTNAP, or of 9 units of bovine intestinal alkaline phosphatase (bIAP, Alloksys Life Sciences B.V., Bunnik, The Netherlands, dissolved in 500 µl of 0.9 % (w/v) NaCl) or of control milk fraction. Also in this experiment survival of mice was monitored for up to 7 days.
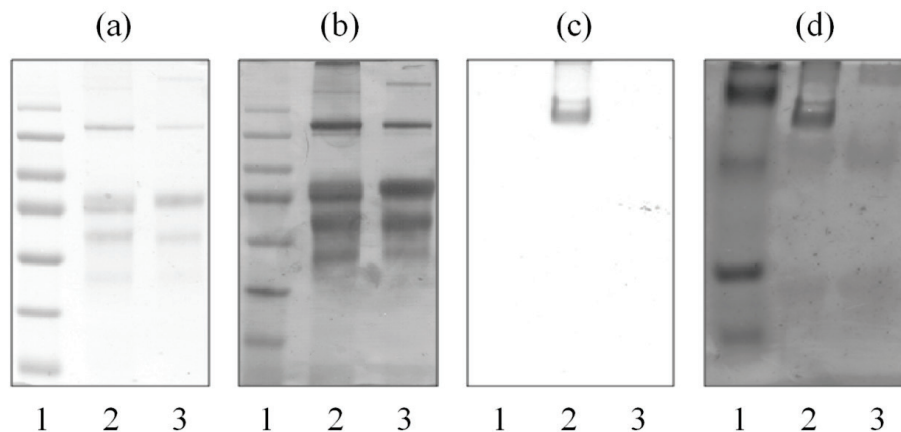
**Fig. 1.** Gel electrophoresis of protein extracts derived from transgenic and non-transgenic rabbit milk. SDS-PAGE with (**a**), Coomassie staining and (**b**), silver staining to determine residual total protein content. (**c**), Native-PAGE and BCIP/NBT treatment to confirm AP activity in protein extracts derived from transgenic rabbit milk. (**d**), Coomassie staining after native-PAGE and BCIP/NBT treatment to analyse residual protein content. Lane 1: molecular weight markers, from top to bottom: (**a** and **b**), 170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 35 kDa, 25 kDa; (**c** and **d**), 240 kDa, 160 kDa, 67 kDa, 45 kDa, 25 kDa. Lane 2: rhTNAP extract from transgenic rabbit milk. Lane 3: AP extract from control rabbit milk

All animal experimental protocols complied with the European Guidelines on Laboratory Animal Care and were performed upon the approval of the Directorate for Animal Care Committee (Reg. number: 22.1/1911/2007).

*Determination of AP activity*

The AP activity of the partially purified protein fractions and of the serum samples was determined with a *p*-nitrophenol-phosphate (pNPP) chromogenic based method (Cyboron and Wuthier 1981). Here, the colorless pNPP was hydrolyzed by AP at room temperature and at pH 9.6 forming yellow free *p*-nitrophenol. The change in optical density at 405 nm per unit time was indicative of AP activity, where one unit of activity is defined as 1 µmol of substrate hydrolyzed per min using the molar absorption coefficient ($\varepsilon$) of 1.87 cm$^2$/µmole of *p*-nitrophenol at 405 nm. Volume activity (U/ml) was determined by measuring the change in absorbance per min over the linear portion of the curve and using this value in the following calculation:

$$\text{Volume activity U / ml} = \frac{(\Delta E_{405\,nm} / \min) \times V_t}{V_s \times \varepsilon} \times \text{dilution factor}$$

where $V_t$ is the final volume of reaction mix (ml), and $V_s$ is the sample volume (ml).

*Statistical analysis*

Values are expressed as mean ± standard deviation. Data were analyzed by GraphPad Prism 5 software. For survival analysis we performed log rank tests with Kaplan-Meier curves. Criteria for significance for all experiments were p<0.05.

**Results**

*Characterization of protein extracts derived from transgenic and non-transgenic rabbit milk by gel electrophoresis, determination of AP activity and of endotoxin content*

We examined protein content and AP activity in milk extracts derived from milk of transgenic and control non-transgenic rabbits in SDS-PAGE gels by Coomassie (Fig. 1a) and silver staining (Fig. 1b). Subsequently, we proved the AP activity in milk extracts derived from transgenic rabbits, contrary to those derived from control milk samples, by treatment of gels with a mixture of BCIP and NBT (Fig. 1c) and estimated residual protein by Coomassie staining (Fig. 1d).

We further quantified the AP activity in samples derived from milk of transgenic rabbits by spectrophotometry using chromogenic substrate. Based on the outcome of these analyses, we diluted samples to achieve the AP activity of 9 U per 500 µl. Control samples derived from milk of non-transgenic rabbits were diluted in a similar way under the consideration of the residual protein content (data not shown).

As the contamination of samples derived from milk with excessive endotoxin LPS could influence the outcome of the *in vivo* model of sepsis, we determined endotoxin levels by LAL assay. Indeed, levels of endotoxin in these extracts were very low (7.1 pg and

5.3 pg in 500 μl volume used for i.v. injection, respectively). Altogether, we can conclude that the biological effects reported in our study are attributed to the enzymatic activity of the rhTNAP protein and not to relatively minor contamination of probes with endotoxin.

*Pilot experiment to determine the effective amount of rhTNAP and the optimal time of administration*

We treated Balb/c mice (3 mice per group) with 15 μg/g LPS (i.p.), followed by the i.v. injection of the milk extracts containing 3, 6 and 9 units of rhTNAP at 1, 2, 3 and 4 h after LPS injection. Based on the outcome of this pilot experiment (data not shown), we selected usage of 9 units of rhTNAP for treatment of mice, 3 h after the LPS injection. As described below, next we determined AP activity levels in serum of mice upon such treatment and monitored body temperature and mice survival.

*AP activity in blood serum*

Intravenous injection of 9 units of rhTNAP resulted in relatively high levels of AP activity detected in the serum of blood samples taken 5 min after administration of rhTNAP (Fig. 2). Since the injection of the recombinant protein through the tail vein is a delicate procedure (and not reproducible with 100 % accuracy), the levels of AP in their serum ranged between 1.38 to 5.11 U/ml. At 21 h after administration, AP activity levels in serum decreased in all animals to the background levels (Fig. 2), consistent with the observed plasma elimination curves of calf intestinal phosphatase determined in a previous study (Beumer *et al.* 2003).
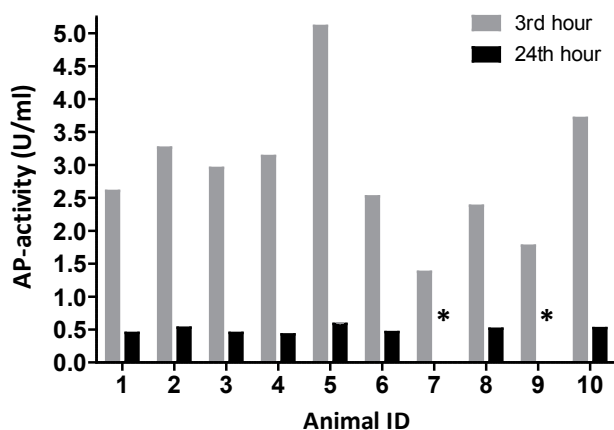


**Fig. 2.** Alkaline phosphatase activity in blood serum upon rhTNAP injection. Mice were i.p. injected with LPS and 3 h later treated by i.v. injection of rhTNAP or control fraction. AP activity was measured in serum derived from blood obtained 5 min after rhTNAP administration and 21 h later. Animals labeled with asterisk died within 10 to 24 h upon LPS administration.
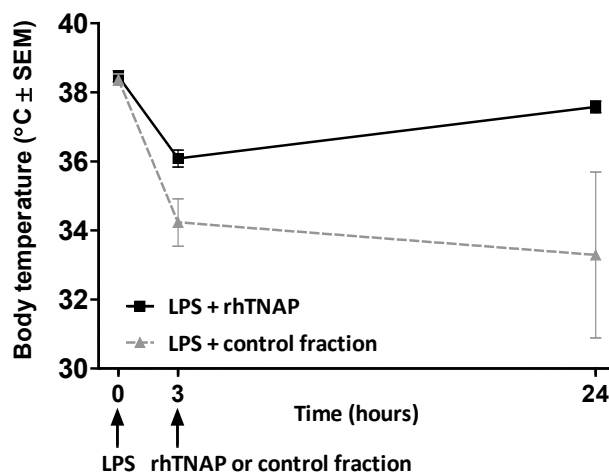


**Fig. 3.** Effect of LPS and rhTNAP on body temperature in mice. Mice were i.p. injected with LPS and 3 h later treated by i.v. injection of rhTNAP or control fraction. rhTNAP treatment normalized body temperature of mice ($p<0.05$).

*Body temperature*

LPS injection in control group of mice, similarly as in the interleukin beta-1 deficient mouse model (Kozak *et al.* 1995) and in *E. coli* induced sepsis (Beumer *et al.* 2003), decreased body temperature of mice. Importantly, however, the temperature in a group of mice treated with rhTNAP reached at 24 h after LPS injection, upon an initial decrease, normal levels indicating beneficial effect of treatment of the animals with rhTNAP (Fig. 3).
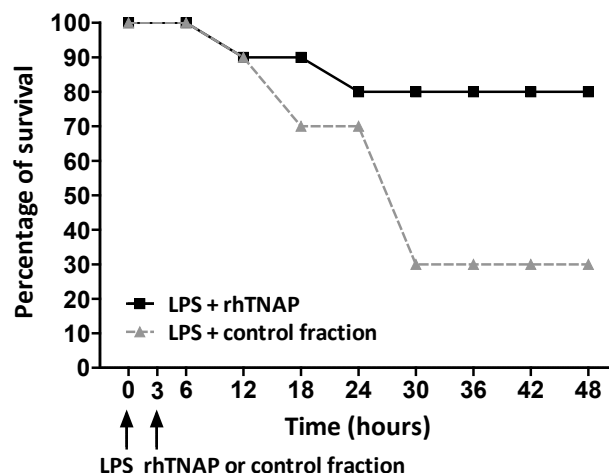


**Fig. 4.** Survival of mice within 48 h after LPS injection and upon rhTNAP administration. Mice were i.p. injected with LPS and 3 h later treated by i.v. injection of rhTNAP or control fraction. rhTNAP treatment significantly ($p<0.05$) increased survival of mice.

*Increased survival of animals treated with rhTNAP*

The above treatment of mice with 9 units of rhTNAP applied 3 h upon LPS injection dramatically

increased survival of mice (Fig. 4), as only 2 of 10 animals died in this group, contrary to a control group of mice treated with a control fraction derived from non-transgenic rabbits (7 out of 10 mice died within 10-30 h after LPS administration). Indeed, the two animals in LPS+ rhTNAP group that died had the lowest AP activity levels in their blood upon rhTNAP injection (mice with ID 7 and 9, and levels l.38 and 1.77 U/ml, respectively, Fig. 2), further pointing to the protective effect of circulating AP regarding the survival. Mice that did not die within 48 h permanently survived the experiment, as revealed by monitoring for at least 7 following days.
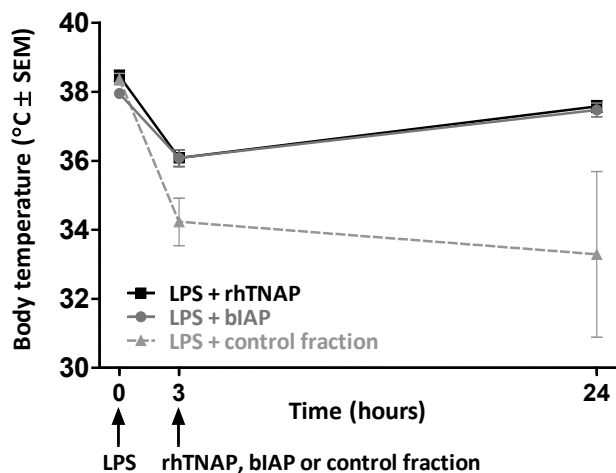


**Fig. 6.** Survival of mice within 48 h after LPS injection and upon rhTNAP or bIAP administration. Mice were i.p. injected with LPS, followed by i.v. injection with rhTNAP, bIAP or control fraction 3 h later. Both, rhTNAP and bIAP treatment significantly ($p<0.05$) increased survival of mice.

## Discussion

A number of strategies have been explored to treat life-threatening complications of sepsis in patients (Lam *et al.* 2013). Detoxification of LPS itself represents a promising strategy to this problem (Schulte *et al.* 2013). It was Poelstra *et al.* (1997a,b) who first suggested the role of APs in the protection against endotoxin insult typical for Gram negative bacteria by showing that LPS is a substrate for AP. In their subsequent study the authors demonstrated the capability of human placental AP to detoxify LPS, which was achieved by dephosphorylation of its lipid A moiety (Bentala *et al.* 2002). When mice received human placental AP immediately after the LPS challenge, the survival rate was 100 % compared to 57 % in a control group (Bentala *et al.* 2002). Beneficial effect of using human placental AP was also shown in sepsis model in mice treated with *E. coli* (Verweij *et al.* 2004), and of intestinal AP in a sepsis model induced by feces injection in sheep (Su *et al.* 2006). Usage of bovine and calf intestinal alkaline phosphatase was shown to milder inflammatory response in a murine cecal ligation and puncture (CLP) model of polymicrobial sepsis, lowering the levels of inflammatory cytokines TNF-alpha, interleukin-6 and macrophage chemoattractant protein 1 and of lung inflammation and liver damage (van Veen *et al.* 2005, Ebrahimi *et al.* 2011). Lately, intestinal alkaline phosphatase was shown to prevent the systemic inflammatory response associated with necrotizing enterocolitis in rats (Riggle *et al.* 2013). The clinical success using calf intestinal AP was demonstrated by



**Fig. 5.** Effect of LPS and rhTNAP or bIAP on body temperature in mice. Mice were i.p. injected with LPS and 3 h later treated by i.v. injection of rhTNAP, bIAP or control fraction. Both, rhTNAP, and bIAP treatment normalized body temperature of mice ($p<0.05$).

*Increased survival of animals treated with bovine intestinal alkaline phosphatase (bIAP)*

To underline that the beneficial effects of rhTNAP in combating of LPS-induced decreased body temperature and mortality are indeed due to AP activity, in additional control experiment we treated 10 mice, 3 h after LPS injection, with 9 units of bIAP, in parallel with 10 mice treated with 9 units of rhTNAP and 10 animals treated with control extracts derived from non-transgenic rabbit milk. Indeed, both types of APs, the bIAP and rhTNAP, similarly restored body temperature to normal levels within 21 h (Fig. 5). Both type of treatment also increases survival, as in a group of mice treated with bIAP and rhTNAP 3 and 2 mice died, respectively, compared to 7 mice in a control group (Fig. 6). Altogether, these results demonstrate the beneficial effects of treatment with APs in a LPS induced model of sepsis.
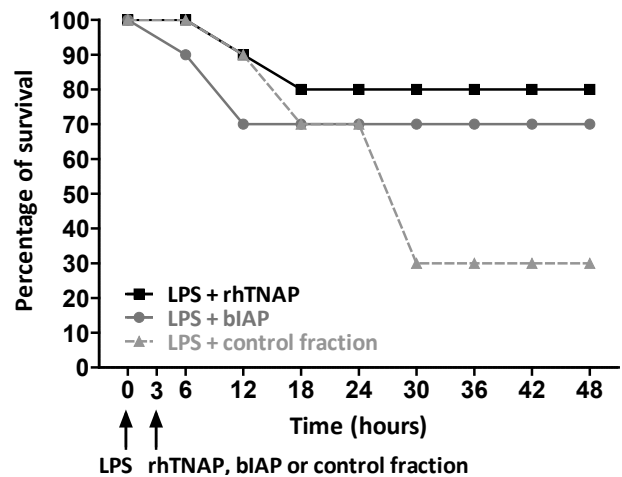
ongoing clinical trials targeting sepsis in human renal patients, leading to the improvement of their renal function (Heemskerk *et al.* 2009, Pickkers *et al.* 2012, Peters *et al.* 2013, 2014) and in cardiac surgery patients (Kats *et al.* 2012). Besides the ability of alkaline phosphatase to reduce inflammation through dephosphorylation detoxifying LPS, alkaline phosphatase was shown to convert adenosine triphosphate, released during cellular stress caused by inflammation and hypoxia, into adenosine with anti-inflammatory and tissue-protective effects, thus eliminating its detrimental effects (Peters *et al.* 2014).

In our study we investigated the potentially therapeutic *in vivo* effect of the rhTNAP derived from milk of transgenic rabbits (Bodrogi *et al.* 2006). We received a protein fraction with high activity of rhTNAP and used it for treatment of mice in a LPS-induced model of sepsis. Three hours after LPS administration, mice received by i.v. injection 9 units of rhTNAP which was efficiently taken up by the peripheral blood system, as revealed by the high AP activity in the serum of treated mice. Although the AP activity at 21 h later decreased to background levels, consistent with rather high AP turnover observed in a model of sepsis induced by *E. coli* infection (Beumer *et al.* 2003), such treatment was also in our model of LPS-induced sepsis efficient. It is important to note that the lower temperature determined in case of control-treated animals was most probably caused by the fact that the measurement of the temperature of these

animals was in fact delayed, due to the spent handling time needed for the intravenous injection of the experimental group with TNAP or with bovine AP. Crucially though, the treatment of the animals (either with TNAP or with bovine AP) contrary to the control situation reverted the decrease in body temperature and significantly increased survival of mice, an effect that was not seen in control-treated animals. Our study also shows that treatment of mice with i.p. injected rhTNAP fraction was effective even after 3 h of LPS administration.

In summary, our work demonstrates beneficial effects of administration of rhTNAP in a LPS induced model of sepsis. The milk of the transgenic rabbits generated in our laboratory could be the source for large scale production of biologically active rhTNAP supplementing other mammalian phosphatases currently used to treat several inflammatory conditions in patients.

## Conflict of Interest
There is no conflict of interest.

## Acknowledgements

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