Shock, Publish Ahead of Print

DOI : 10.1097/SHK.0000000000001252

Overexpression of Gilz protects mice against lethal septic peritonitis

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Short Report

Running Title: Gilz protects against sepsis

Conflict of interest: The authors have declared that no conflict of interest exists

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Abstract

Sepsis in humans and experimental animals is characterized by an acute inflammatory response. Glucocorticoids (GCs) are widely used for the treatment of many inflammatory disorders, yet their effectiveness in sepsis is debatable. One of the major anti-inflammatory proteins induced by GCs is GILZ (Glucocorticoid-Induced Leucine Zipper, coded by the *TSC22D3* gene). We found that *TSC22D3* mRNA expression is downregulated in white blood cells of human sepsis patients. Interestingly, transgenic Gilz overexpressing mice (Gilz-tg) showed better survival rates in the cecal ligation and puncture (CLP) model of mouse sepsis. To our surprise, Gilz had only mild anti-inflammatory effects in this model, since the systemic pro-inflammatory response was not significantly reduced in Gilz-tg mice compared to control mice. During CLP, we observed reduced bacterial counts in blood of Gilz-tg mice compared to control mice. We found increased expression of *Tsc22d3 mRNA* specifically in peritoneal exudate cells in the CLP model, as well as increased capacity for bacterial phagocytosis of $CD45^{+}$ Gilz-tg cells compared to $CD45^{+}$ Gilz-wt cells. Hence, we believe that the protective effects of GILZ in the CLP model can be linked to a more efficient phagocytosis.

Key words: ***sepsis, therapy, steroids, phagocytosis***

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Introduction

Sepsis is a major cause of mortality among hospitalized patients. It is the leading cause of death among patients admitted to intensive care units (ICUs). Sepsis is triggered by microbial invasion of normally sterile tissues or body fluids and is the clinical manifestation of the dysregulated systemic activation of the innate immune system (1). It has long been believed that the early phase of sepsis is typically characterized by a profound pro-inflammatory response. In many cases and over several days, the hyper-inflammatory response resolves, and patients enters a phase of immune suppression (2). However, a recent study has shown that the immediate transcriptional response in trauma patients includes both upregulation of the innate immune response and downregulation of the adaptive response which translates into the phenotypic changes of the pro-inflammatory and immunosuppressive syndrome simultaneously (3). Altogether, these and other findings suggest a more relevant paradigm of early systemic inflammatory response and immunosuppression and emphasize the importance of the very early post-injury period (1). Despite advances in supportive care, the mortality rate of sepsis remains high and after decades of intensive research, no new therapies targeting the immuno-inflammatory response have been proven to be beneficial in sepsis patients (2). To gain more insights into the complex pathophysiology of sepsis and possible therapeutic strategies, several animal models have been developed (4). The cecal ligation and puncture (CLP) model is considered as a golden standard for studying sepsis (5). Compared to other models, CLP provides a good representation of the complexity of human sepsis by causing hypothermia, tachypnea, tachycardia and hypotension (4, 6).

Glucocorticoids (GCs) are widely used for the treatment of a plethora of inflammatory disorders such as asthma, rheumatoid arthritis and psoriasis. They have potent anti-inflammatory

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actions but due to their metabolic functions, they also lead to adverse effects such as osteoporosis and diabetes mellitus, especially after long application (7). In sepsis, the use of GCs has been controversial for several decades. Despite recent results of a successful clinical trial, it still remains unclear which patients will benefit from GC treatment (8, 9). Careful consideration of each patient might be a workable approach, since GC resistance appears to be a very common phenomenon in sepsis (10). GC treatment has been shown to improve survival in mouse endotoxemia models by reducing the inflammatory response (11). However, in the CLP mouse model, which reflects the pathophysiology of human sepsis more accurately and which involves invasion of microbes from the cecum in the body, conflicting results have been observed. Van den Berg *et al.* found that in contrast to high doses, low doses of dexamethasone, a synthetic GC, were able to improve survival in the CLP model (12). Additionally, Osuchowski *et al.* found that stratifying mice predicted to die based on inflammatory parameters increased the survival benefit of GCs in this subpopulation (13). Greater understanding of the anti-inflammatory actions of GCs, as well as the mechanisms underlying GC resistance, may enable the development of therapies that circumvent GC resistance, or that mimic their mild immunomodulatory actions.

After binding of GCs to the glucocorticoid receptor (GR), GR translocates to the nucleus where it can modulate gene transcription. As a homo-dimer, GR can activate transcription in a process called transactivation while as a monomer, GR inhibits gene expression by tethering to other transcription factors such as NF-κB and AP-1 (14). *Tsc22d3*, a gene encoding the GC-induced leucine zipper (GILZ) protein, is one of the genes most sensitive to transcriptional activation by GCs/GR and believed to be one of the most important anti-inflammatory mediators induced by GCs (15). *Tsc22d3* expression is thus considered to be a biomarker for GC effectiveness. Induction or administration of GILZ protein could be a valid therapeutic strategy

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in patients suffering from inflammatory disorders or in patients where GCs are ineffective (16). Functionally, GILZ negatively regulates Ras signaling, suppresses pro-inflammatory cytokines and modulates T cell activation (17). At least some of the anti-inflammatory activities of GCs are thought to be mediated by the ability of GILZ to inhibit NF-κB, the master regulator of inflammatory responses (14). GILZ physically binds the p65 subunit of NF-κB and inhibits nuclear translocation (18). Different reports using GILZ overexpressing mice or recombinant GILZ protein support the protective effect of GILZ in models of acute inflammation, including lipopolysaccharide (LPS)-induced shock (19, 20).

In this study, based on the low GILZ levels in both mouse endotoxemia and human sepsis, we investigated the role of GILZ in sepsis, using Gilz overexpressing transgenic mice (Gilz-tg) (21) and the CLP mouse model (5) which induces polymicrobial sepsis. We found that Gilz overexpression results in decreased CLP-induced bacteremia and increased survival rates and suggest a mechanism involving enhanced phagocytosis by $CD45^+$ peritoneal cells.

Material and Methods

Human study protocol. The protocol for this clinical pilot study was approved by the ethics committee of the University Hospital of Ghent (registration number B670201419579). In total 13 patients were enrolled, meeting the criteria for severe sepsis or septic shock defined at the consensus conference of 2001 (22). Fresh human blood was collected from these patients within 24 hours after admission to the ICU. As a control, fresh blood was collected from 15 healthy volunteers at ambulatory centers. Inclusion and exclusion criteria for this study are depicted in **Table 1**. All patients provided informed consents before study related procedures were performed in compliance with the guidelines of the MedImmune institutional review board. Blood was collected in EDTA-coated vials to determine whole blood RNA expression.

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Mice. C57BL/6 wildtype mice were purchased from Janvier and the generation of Gilz-tg and Gilz-wt mice was previously described in Carceller *et al*. (21). Mice were kept in individually ventilated cages under a 12 h dark/light cycle in a specific pathogen free (SPF) animal facility and received food and water *ad libitum*. All mice were used at 8-12 weeks of age. Exclusively male mice were used for CLP. All the animal protocols were approved by the ethical committee of the Faculty of Sciences of Ghent University.

RT-qPCR analysis. For RNA isolation from human blood samples, the NucleoSpin® RNA blood kit (Macherey-Nagel) was used and the manufacturer's guidelines were followed. The InviTrap® Spin Universal RNA Mini Kit (Invitek) was used for RNA isolation from mouse blood. Liver tissue samples were collected in RNA later (Qiagen) and RNA was isolated according to the RNeasy Mini kit (Qiagen) protocol. RNA concentration was measured with Nanodrop 1000 (Thermo Scientific) and 300-1000 ng RNA was used to prepare cDNA with Superscript II (Invitrogen). RT-qPCR was performed using the Roche Light cycler 480 system (Applied Biosystems). Using Genorm, the best two performing housekeeping genes were determined and used in further analysis. Results are given as relative expression values normalized to geometric means of the housekeeping genes. Primers used for RT-qPCR analysis are listed in **Table 2**.

Cecal ligation and puncture (CLP)-induced septic peritonitis. Mice were subjected to CLP in order to induce polymicrobial septic shock, as described by a published standard operating procedure (SOP) (5). Briefly, mice were anesthetized by isoflurane inhalation and a one-centimeter incision was made in the abdomen after which the cecum was exposed and 75% ligated. This was followed by making two punctures in the cecum with a 21-gauge needle. During the procedure, some cecal content is pushed out using sterile forceps. The abdominal

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musculature and skin were closed with simple running sutures and metallic clips, respectively. In the SOP, animals are resuscitated by a single injection of 1000 µl saline post-operative. However, in this study we decided to reduce the amount of fluids in order to minimize their effect on pro-inflammatory cytokine production and subsequent mice survival (5). Therefore, we resuscitate the mice by intraperitoneal injecton of an antibiotic cocktail containing ceftriaxone (25 mg/kg; Sigma-Aldrich NV) and metronidazole (12.5 mg/kg; Sigma-Aldrich NV) 10 h and 24 h after CLP onset. In both cases a volume of 100 µl is injected, meaning that the mice received 200 µl of fluids during the first day of CLP. With this CLP model and using this 21-gauge needle, 80% to 100% lethality is generally obtained in wildtype B6 mice in our hands (23), which is very similar as is published with the SOP (5). For experiments aimed to isolate blood and organ samples, sham operated mice of which the cecum was exposed but not ligated or punctured, were used and are indicated as 0 h or sham.

Cytokine Measurement. Serum samples were assayed for IL-6 using Luminex technology (Bio-Rad) in accordance with the manufacturer's protocol.

Determination of bacterial load in blood. Blood was taken via cardiac puncture after sedation of the mice with a ketamine/xylazine solution and collected in EDTA coated tubes. Serial dilutions of blood were prepared in sterile PBS for plating on brain-heart-infusion agar plates. Plates were incubated at 37°C overnight. Viable counts of bacteria were expressed as colony-forming units (CFU) per ml blood. Blood composition was analyzed by Hemavet (Scientific Inc. Oxford).

Phagocytosis of E. coli bioparticles by peritoneal cells. Peritoneal exudate (PE) cells were isolated from sham operated mice using ice-cold 30% sucrose solution in PBS. Sham mice, and not CLP mice were used because the latter condition is thought to induce phagocytosis before the

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phagocytosis assay is initiated and thus to interfere with the basal phagocytic phenotype of the peritoneal cells. *E. coli* bioparticles (Invitrogen) labeled with pHrodo fluorescent reagent were resuspended at 1 mg/ml. Bioparticles were added to the PE cells at a ratio of 1×10^6 cells/ml, as per manufacturer's instructions. All cells were incubated with bioparticles for 30 min at either 4°C (negative control) or 37°C and then processed using a FACS system (LSRFortessa 4 laser; BD Biosciences) and analyzed using FlowJo software (Tree Star) to determine the level of fluorescence. % of PE-positive cells amongst $CD45^+$ cells are shown in the figure. Cell suspensions were stained at 4°C in the dark with the following antibodies: Life/death (APCCy7-efluor780) and anti-CD45 (BV510).

Statistical analyses. Survival curves (Kaplan-Meyer plots) were compared by a Logrank test, and final outcomes were compared by a $\chi 2$ test on the number of mice that died or survived. Data were expressed as means ± SEM. The statistical significance of differences between groups was evaluated with Student's t-tests, one-way ANOVA or two-way ANOVA tests with 95% confidence intervals. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$ and ns = not significant.

Results

***Tsc22d3* expression is downregulated in human and mouse sepsis**

Increasing evidence *in vitro* as well as *in vivo*, shows that downregulation of *Tsc22d3*/Gilz expression in response to inflammation is a recurrent phenomenon. For example, in epithelial cells, endothelial cells and macrophages, inflammatory cytokines have been reported to reduce GILZ expression. Therefore, it has been suggested that there is a negative correlation between GILZ expression and the inflammatory status and that a low expression of GILZ may

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reflect an ongoing inflammatory response (24, 25). To investigate the expression of *TSC22D3* in human sepsis, fresh blood of 15 healthy volunteers and 13 severe sepsis patients isolated within 24 hours after admission to the ICU (inclusion and exclusion criteria, see Table 1 in Materials and Methods) was isolated, and mRNA was prepared. In each qPCR reaction, the same amount of cDNA of was used correcting for the amount of white blood cells (WBCs) in the different groups and in between sepsis patients. Compared to healthy controls, the *TSC22D3* mRNA expression levels, measured by RT-qPCR were significantly downregulated in WBCs of sepsis patients (**Figure 1A**). In mice, the expression of *Tsc22d3* in WBCs was determined two hours after the CLP sepsis procedure and found to be significantly downregluated in CLP-operated mice compared to sham-operated control mice (**Figure 1B**). Interestingly, downregulation of *Tsc22d3* does not only occur in the blood, but also in the liver of mice subjected to CLP. Already after two hours, the levels are significantly reduced and the effect is even more pronounced after six hours (**Figure 1C**).

Gilz overexpression protects against CLP-induced peritonitis by reducing bacterial counts

To investigate the role of Gilz in murine sepsis induced by CLP, Gilz transgenic mice which overexpress mouse Gilz ubiquitously (in all cell types), were used. The generation of these mice has been described elsewhere (21). In brief, the mouse *Tsc22d3* cDNA, preceded by a loxP flanked stop cassette, was inserted in the mouse genome under control of the ROSA26 promoter. These mice were crossed with Nestin-Cre mice expressing cre recombinase ubiquitously and leading to strong expression of Gilz in all tissues investigated (21). When Gilz-tg mice and Gilz-wt mice were subjected to CLP and the *Tsc22d3* levels were measured in liver, we found higher levels in Gilz-tg mice compared to Gilz-wt mice, as expected. Gilz-wt mice also showed a significant downregulation of *Tsc22d3* after CLP as shown before, but there was a non-

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significant effect in Gilz-tg mice by CLP **(Figure 2A)**. Interestingly, Gilz-tg mice were found to be significantly less sensitive to CLP-induced lethality than the Gilz-wt animals (**Figure 2B**). During the acute inflammatory phase 6, 12 and 24 h after CLP onset, no major differences between Gilz-tg and Gilz-wt mice were observed on serum IL-6 levels, which is believed to be a predictive factor of mortality in experimental sepsis and in septic patients (26) (**Figure 2C**). Additionally, the bacterial load in blood was quantified 6 and 12 h after CLP. No significant difference between the two genotypes could be observed at the earliest time point, but at the 12 h time point, Gilz-tg mice had significantly reduced bacterial counts in their blood compared to Gilz-wt mice (**Figure 2D**). It is important to note that the amount of administered fluids in this study is lower than generally used in CLP protocols. For this reason, we cannot fully exclude the fact that Gilz overexpression might have an effect on hypovolemic shock in our model. However, based on the reduced bacterial counts in the blood of Gilz-tg mice, we believe that overexpression of Gilz might increase the host's ability to clear bacteria or prevent spreading into circulation.

Gilz expression in peritoneal cells is related to phagocytosis activity

It has been described that *Tsc22d3* expression in macrophages is downregulated upon toll-like receptor (TLR) or TNF stimulation (24, 25). In contrast, when we studied *Tsc22d3* mRNA levels in peritoneal exudate (PE) cells 6 h after CLP, we found increased expression in both Gilz-wt and Gilz-tg mice, compared to naive mice. As expected, *Tsc22d3* expression levels were significantly higher in Gilz-tg mice compared to Gilz-wt mice (**Figure 3A**). The elevated levels of *Tsc22d3* in leukocytes recruited to the peritoneal cavity of Gilz-tg mice might be important in the protection against CLP-induced lethality. The major function of leukocytes recruited to the peritoneal cavity is killing of bacteria via phagocytosis. Chiswick *et al.* have

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shown that early phagocytic impairment (within 6 h after CLP) ultimately leads to uncontrolled microbial growth and death (27). During CLP, WBCs migrate to the site of infection, and so the numbers of WBCs in circulation decline, while the amounts of WBCs in the peritoneal cavity increase. When comparing Gilz-tg and Gilz-wt mice, we found no differences in total number of WBCs measured in blood (**Figure 3B**) or in the peritoneum (**Figure 3C**) 6 and 12 h after CLP. In order to study the bacterial phagocytosis capacities of Gilz-tg and Gilz-wt leukocytes, PE cells were isolated after sham operation and phagocytosis assays were performed using pHrodo-labeled *E. coli* bioparticles. pHrodo is a fluorescent dye that increases in fluorescence as the pH of its environment becomes more acidic in the phagosome. Peritoneal leukocytes ($CD45^+$) derived from Gilz-tg mice displayed a significantly higher phagocytic capacity than Gilz-wt cells, correlating with the difference in bacterial counts between Gilz-tg and Gilz-wt mice and thus also their enhanced survival during CLP (**Figure 3D**).

Discussion

Despite improvements in the diagnosis, treatment and management of sepsis and septic shock over the last decades, sepsis remains the leading cause of ICU mortality (1). Gene expression studies in blood of sepsis patients have suggested that the pathogen recognition and inflammatory signaling pathways dominate sepsis. This profiling has also been used to identify diagnostic and prognostic gene signatures as well a novel therapeutic targets (28). Still, the current treatments of sepsis patients remain focused on supportive measures including fluid resuscitation, infection control and antibiotic treatment.

In light of the conflicting data reported with GCs in sepsis, we initiated our study. Despite the fact that some studies find protective effects of GCs on final survival in sepsis, a pattern of sepsis being a GC-resistant inflammatory condition emerges (8–10). Anti-inflammatory proteins

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that are normally induced by GCs and/or mediate GC's anti-inflammatory activities might be interesting therapeutic alternatives. One such a protein is Gilz.

To explore the role of Gilz in sepsis or septic shock, we conducted a study in which blood samples were collected from sepsis patients. We compared the *TSC22D3* expression in patients to healthy controls and detected a downregulation of *TSC22D3* in blood cells. Also in the mouse CLP sepsis model, *Tsc22d3* expression levels were downregulated both in blood cells, and in the liver. We also found that genetic overexpression of Gilz significantly increased survival in the CLP model. Although Gilz is a potent anti-inflammatory molecule, no significant difference in systemic inflammation characterized by elevated IL-6 cytokine expression was observed between the two genotypes. Interestingly, these data suggest that mice are able to survive CLP even with high systemic IL-6 values, probably because GILZ protects either downstream of IL-6 signaling or via another pathway, which is dominant over IL-6.

Next to their obvious harmful effects, pro-inflammatory cytokines are important in the activation of host defense mechanisms against invading pathogens by facilitating immune cell recruitment and activation (29). Hence, one might argue that a lack of inhibition of inflammation in the CLP model by Gilz overexpression might be a positive feature. Indeed, Van den berg *et al*. described that low doses of dexamethasone were associated with a mild, nonsignificant suppression of CLP-induced inflammation, and with improved survival (12). Finally, it was described that in sepsis, early suppression of the innate immune system results in excessive bacterial replication (30). Next to this, several studies have shown that Gilz can also increase anti-inflammatory pathways such as TGFβ signaling (21). This suggests that the suppressive effect of Gilz on cytokine expression might be disease or context specific.

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Even though Gilz overexpression did not affect WBC recruitment, reduced bacterial counts in circulation were detected. Interestingly, 6 h after the CLP onset, *Tsc22d3* expression levels were increased in peritoneal exudate cells of both Gilz-wt and Gilz-tg mice (with higher levels in the latter). These data suggest that already at early time points, the peritoneal exudate cells exhibit functions related to resolution of inflammation. Phagocytic capacity of peritoneal cells was assessed by incubating peritoneal cells derived from sham operated mice with pHrodo red labeled *E. coli* bioparticles conjugate. An enhanced bacterial killing capacity of CD45+ peritoneal exudate cells of Gilz-tg mice was observed in comparison to Gilz-wt cells. These data could explain the reduced bacterial dissemination and reduced lethality of Gilz-tg mice in the CLP model. These results are in accordance with the study of Vago *et al.* which shows that an increase of Gilz expression in M2 macrophages might contribute to their enhanced efferocytic capacities during resolution of inflammation (20). Although the effect of Gilz overexpression on the phagocytosis capacity of CD45+ cells provides an interesting hint to a possible mechanism, a more detailed investigation in different cell subpopulation is still warranted.

Based on our data, we believe that administration of Gilz could be a valid therapeutic strategy in sepsis. Gilz is a relatively small protein. The canonical protein consists of only 117 amino acids. Several exciting biological activities of Gilz have been described, a.o. binding and inhibition of NF-κB, c-jun and c-fos, decrease of antigen presentation, increase of IL-10 production by dendritic cells, and stimulation of regulatory T cell differentiation via facilitating TGF-β pathways (leading to transcriptional induction of Foxp3) and increase in phagocytosis (this study) (31). Unfortunately, the therapeutic application of Gilz as a protein is not straightforward. Gilz is a cytoplasmic protein, which also functions in the nucleus of cells, and no cell membrane receptor for Gilz is known, nor has an extracellular activity for Gilz been

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reported. Several groups have described therapeutic effects with recombinant Gilz. The group of Carlo Riccardi, who discovered Gilz, generated a fusion protein of Gilz with a membrane permeable hydrophobic protein sequence, which shuttles Gilz through the cell membrane. The protein had therapeutic effects in a Th1-mediated model of colitis (32). Optimization of delivery strategies of recombinant Gilz in the future may lead to a breakthrough in the management of sepsis. The increase in expression of Gilz in the Gilz-tg mice described here is moderate (about double, Fig. 3A) and yet sufficient to confer very strong protection in CLP sepsis. The key to success here, is probably that the transgenic *Tsc22d3* gene is not subjected to inflammation-induced decrease of *Tsc22d3* mRNA (Figure 2A).

Gilz is induced by GCs and mediates some of their anti-inflammatory effects (33). The high induction can be explained by the multiple GR binding sites discovered in the promoter of the *Tsc22d3* gene (34). During inflammatory stimulation *Tsc22d3* mRNA expression levels abruptly decline (e.g. in the blood and livers of CLP mice in our study, in the liver of TNF treated mice (35) or in LPS-stimulated macrophages *in vitro* (24)). This might be explained by an acute lack of GR induction of the expression of *Tsc22d3*, but Hoppstädter *et al* found that the effects are rather based on *Tsc22d3* mRNA lability which is induced by inflammatory cytokines (24). As a result, inflammation stimulates the acute decline of this major anti-inflammatory molecule. It is likely that the decline in *Tsc22d3* expression observed in sepsis patients and mice contributes to the inflammatory pathways that develop in these mice and even sepsis patients.

Interestingly, when studying the *Tsc22d3* expression in peritoneal cells after CLP, we found that *Tsc22d3* mRNA expression increases. Whether this is a reflection of GC actions is not known at this point. The lack of decline in *Tsc22d3* expression is fascinating if one considers the strong pro-inflammatory environment of the peritoneal cavity after puncture of the cecum.

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Perhaps decrease in *Tsc22d3* expression by cytokines and TLR ligands, such as LPS, is overruled by the high presence of live intact bacteria in the peritoneal cavity. These might stimulate *Tsc22d3* gene expression, which is clearly linked with more active phagocytosis.

In conclusion, the expression of the Gilz coding gene *Tsc22d3* sharply declines in sepsis white blood cells but increases in peritoneal phagocytes where it promotes phagocytosis and potentially forms the basis of Gilz-mediated protection against sepsis by reducing bacterial dissemination.

Acknowledgements

The authors wish to thank Joke Vanden Berghe and the staff of the animal care facilities for animal care. We acknowledge Dr. Sriram Balusu together with the medical staff of the University Hospital of Ghent for their assistance in collecting human blood samples of sepsis patients.

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References

1. Hotchkiss RS, Moldawer LL, Opal, SM, Reinhart K, Turnbulle IR, Vincent JL. Sepsis and septic shock. *Nat Rev Dis Prim* doi:10.1038/nrdp.2016.45, 2016.

2. Seeley EJ, Bernard GR. Therapeutic Targets in Sepsis: Past, Present, and Future. *Clin Chest Med* 37(2):181–189, 2016.

3. Tompkins RG. Genomics of injury: The Glue Grant experience. *Journal of Trauma and Acute Care Surgery* 2015.

4. Zanotti-Cavazzoni SL, Goldfarb RD. Animal models of sepsis. *Crit Care Clin* 25(4):703, 2009.

5. Toscano MG, Ganea D, Gamero AMC. Cecal ligation puncture procedure. *J Vis Exp* 51: 2860, 2011.

6. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends Microbiol* 19(4):198–208, 2011.

7. Vandewalle J, Luypaert A, De Bosscher K, Libert C. Therapeutic Mechanisms of Glucocorticoids. *Trends Endocrinol Metab* 29(1):42–54, 2018.

8. Suffredini AF. A Role for Hydrocortisone Therapy in Septic Shock? *N Engl J Med* 378(9):860–861, 2018.

9. Venkatesh B, Finfer S, Cohen J, Rajbhandari D, Arabi Y, Bellome R, Billot L, Correa M, Glass P, Harward M et al. Adjunctive Glucocorticoid Therapy in Patients with Septic Shock. *N Engl J Med* 378(9):797-808, 2018.

10. Dendoncker K, Libert C. Glucocorticoid resistance as a major drive in sepsis pathology. *Cytokine Growth Factor Rev* 35:85-9, 2017.

11. Wang X, Nelin LD, Kuhlman JR, Meng X, Welty SE, Liu Y. The role of MAP kinase phosphatase-1 in the protective mechanism of dexamethasone against endotoxemia. *Life Sci* 83(19–20):671–680, 2008.

12. van den Berg JW, van der Zee M, de Bruin RW, van Holten-Neelen C, Bastiaans J, Nagtzaam NM, Ijzermans JN, Benner R, Dik WA. Mild versus strong anti-inflammatory therapy during early sepsis in mice: a matter of life and death. *Crit Care Med* 39(6):1275–1281, 2011.

13. Osuchowski MF, Connett J, Welch K, Granger J, Remick DG. Stratification is the key: Inflammatory biomarkers accurately direct immunomodulatory therapy in experimental sepsis. *Crit Care Med* 37(5):1567–1573, 2009.

14. Hübner S, Dejager L, Libert C, Tuckermann J. The glucocorticoid receptor in inflammatory processes: transrepression is not enough. *Biol Chem* 396(11):1223–31, 2015.

15. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, Cannarile L, Migliorati G, Riccardi C. A new dexamethasone-induced gene of the leucine zipper family protects T

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lymphocytes from TCR/CD3-activated cell death. *Immunity* 7(6):803–812, 1997.

16. Hoppstädter J, Kiemer AKC. Glucocorticoid-induced leucine zipper (GILZ) in immuno suppression: master regulator or bystander? *Oncotarget* 6(36):38446–38457, 2015.

17. Ayroldi E, Zollo O, Macchiarulo A, Di Marco B, Marchetti C, Riccardi C. Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Mol Cell Biol* 22(22):7929–7941, 2002.

18. Di Marco B, Massetti M, Bruscoli S, Macchiarulo A, Di Virgilio R, Velardi E, Donato V, Migliorati G, Riccardi C. Glucocorticoid-induced leucine zipper (GILZ)/NF-kappaB interaction: role of GILZ homo-dimerization and C-terminal domain. *Nucleic Acids Res* 35(2):517–528, 2007.

19. Pinheiro I, Dejager L, Petta I, Vandevyver S, Puimege L, Mahieu T, Ballegeer M, Van Hauwermeiren F, Riccardi C, Vuylsteke M, Libert C. LPS resistance of SPRET/Ei mice is mediated by Gilz, encoded by the Tsc22d3 gene on the X chromosome. *EMBO Mol Med* 5(3):456–470, 2013.

20. Vago JP, Tavares LP, Garcia CC, Lima KM, Perucci LO, Vieira EL, Nogueira CR, Soriani FM, Martins JO, Silva PM et al. The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *J Immunol* 194(10):4940–4950, 2015.

21. Carceller E, Ballegeer M, Deckers J, Riccardi C, Bruscoli S, Hochepied T, Libert C, Pérez Paloma. Overexpression of Glucocorticoid-induced Leucine Zipper (GILZ) increases susceptibility to Imiquimod-induced psoriasis and involves cutaneous activation of TGF-β1. *Sci Rep* 6:38825, 2016.

22. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsey G. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 31:1250–1256, 2003.

23. Dejager L, Vandevyver S, Ballegeer M, Van Wonterghem E, An LL, Riggs J, Kolbeck R, Libert C. Pharmacological inhibition of type I interferon signaling protects mice against lethal sepsis. *J Infect Dis* 209(6):960-70,2014.

24. Hoppstädter J, Diesel B, Eifler LK, Schmid T, Brüne B, Kiemer AK. Glucocorticoid-induced leucine zipper is downregulated in human alveolar macrophages upon Toll-like receptor activation. *Eur J Immunol* 42(5):1282–1293, 2012.

25. Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, Zuraw BL. The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *J Allergy Clin Immunol* 119(1):115–122, 2007.

26. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: Interleukin-6 measured 6 H after the initiation of sepsis predicts mortality over 3 days. *Shock* 17(6):463-467, 2002.

27. Chiswick EL, Mella JR, Bernardo J, Remick DGC. Acute-Phase Deaths from Murine Polymicrobial Sepsis Are Characterized by Innate Immune Suppression Rather Than Exhaustion. *J Immunol* 195(8):3793–3802, 2015.

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28. Maslove DM, Wong HR. Gene expression profiling in sepsis: Timing, tissue, and translational considerations. *Trends Mol Med* 20(4):204–213, 2014.

29. Netea MG, Van Der Meer JWM, Van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: Not enough, or too much of a good thing? *Trends Immunol* 24(5):254–258, 2003.

30. Cao L, Hudson CA, Lawrence DA. Immune changes during acute cold/restraint stress-induced inhibition of host resistance to Listeria. *Toxicol Sci* 74(2):325–334, 2003.

31. Beaulieu E, Morand EF. Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nat Rev Rheumatol* 7(6):340–348, 2011.

32. Cannarile L, Cuzzocrea S, Santucci L, Agostini M, Mazzon E, Esposito E, Muia C, Coppo M, Di Paola R, Riccardi C. Glucocorticoid-Induced Leucine Zipper Is Protective in Th1-Mediated Models of Colitis. *Gastroenterology* 136(2):530–541, 2009.

33. Ronchetti S, Migliorati G, Riccardi C. GILZ as a Mediator of the Anti-Inflammatory Effects of Glucocorticoids. *Front Endocrinol.* 6:170, 2015.

34. Thormann V, Rothkegel MC, Schöpflin R, Glaser LV, Djuric P, Li N, Chung HR, Schwahn K, Vingron M, Meijsing SH. Genomic dissection of enhancers uncovers principles of combinatorial regulation and cell type-specific wiring of enhancer–promoter contacts. *Nucleic Acids Res* 46(6):3258, 2018.

35. Van Bogaert T, Vandevyver S, Dejager L, Van Hauwermeiren F, Pinheiro I, Petta I, Engblom D, Kleyman A, Schütz G, Tuckermann J et al. Tumor necrosis factor inhibits glucocorticoid receptor function in mice: a strong signal toward lethal shock. *J Biol Chem* 286(30):26555–26567, 2011.

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Figure legends

Figure 1: *TSC22D3/Tsc22d3* gene expression is downregulated in septic conditions. (**A**) Human blood samples were collected from healthy volunteers (n=15, white circles) and severe septic or septic shock patients within 24 h of admission to the ICU (n=13, black circles). Total blood mRNA was isolated and *TSC22D3* expression levels were analyzed via RT-qPCR. (**B**) Mouse blood samples were collected from sham operated (n=14, white circles) and CLP operated C57BL/6J mice (n= 9, black circles) 2 h after the procedure (combined data of three independent experiments). Total blood mRNA was isolated and *Tsc22d3* expression levels were analyzed via RT-qPCR. (**C**) Livers were collected from C57BL/6J mice 2 h and 6 h after CLP surgery (n=6-12; combined data of two independent experiments). Liver mRNA was isolated and *Tsc22d3* expression levels were analyzed via RT-qPCR. Data are represented as mean ± SEM. Asterisks refer to significant difference between the relevant bar and sham operated mice (0 h). p-values were calculated using Student's t-tests (A & B) or one-way ANOVA test (C). **** $p < 0.0001$, *** $p < 0.001$, * $p \leq 0.05$ and ns = not significant.

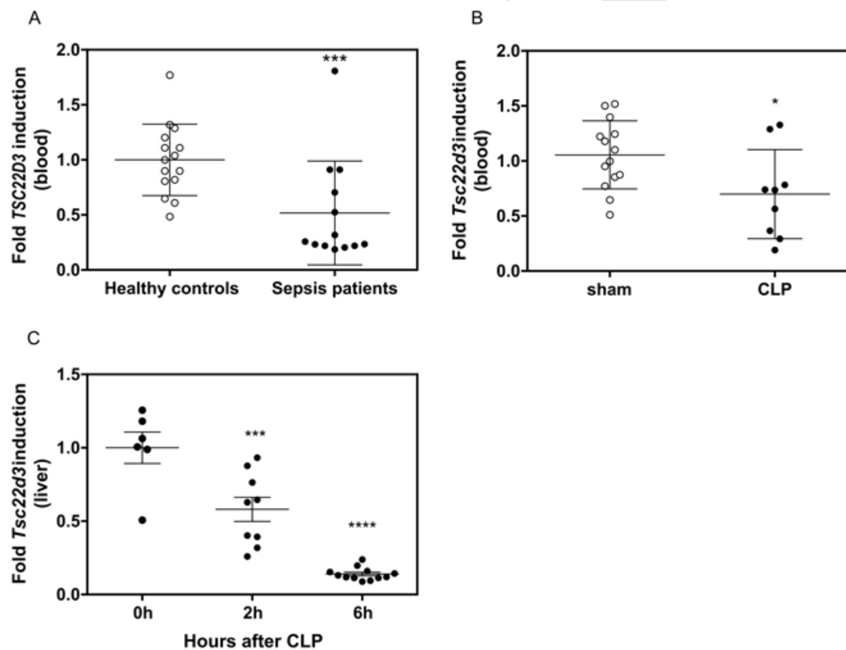

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Figure 2: Gilz overexpression protects against CLP-induced peritonitis by reducing the blood bacterial load. (**A**) Male Gilz-wt (white boxes) and Gilz tg (black boxes) mice were subjected to CLP. Liver samples were isolated after 0 h and 6 h and mRNA was prepared (n=5; representative data of three independent experiments). *Tsc22d3* expression levels were analyzed via qPCR and are shown as mean ± SEM. p-values were calculated via two-way ANOVA tests. (**B**) Male Gilz-wt (n=14, white boxes) and Gilz-tg (n= 18, black boxes) mice were subjected to CLP and survival was monitored for 7 days, after which no further deaths occurred (combined data of three independent experiments). Survival curve was analyzed with a Logrank test. ** $p < 0.01$. (**C**) Kinetics (0, 6, 12 and 24 h) of IL-6 protein levels in serum of Gilz-wt and Gilz-tg mice subjected to CLP (n=10-14; combined data of three independent experiments). (**D**) Kinetics (0, 6 and 12 h) of bacterial counts in blood of Gilz-wt and Gilz-tg mice subjected to CLP. Data are represented as colony-forming units (CFU) per ml blood (n=18; combined data of three independent experiments). p-values were calculated using two-way ANOVA tests. ** $p \leq 0.01$ and ns = not significant.

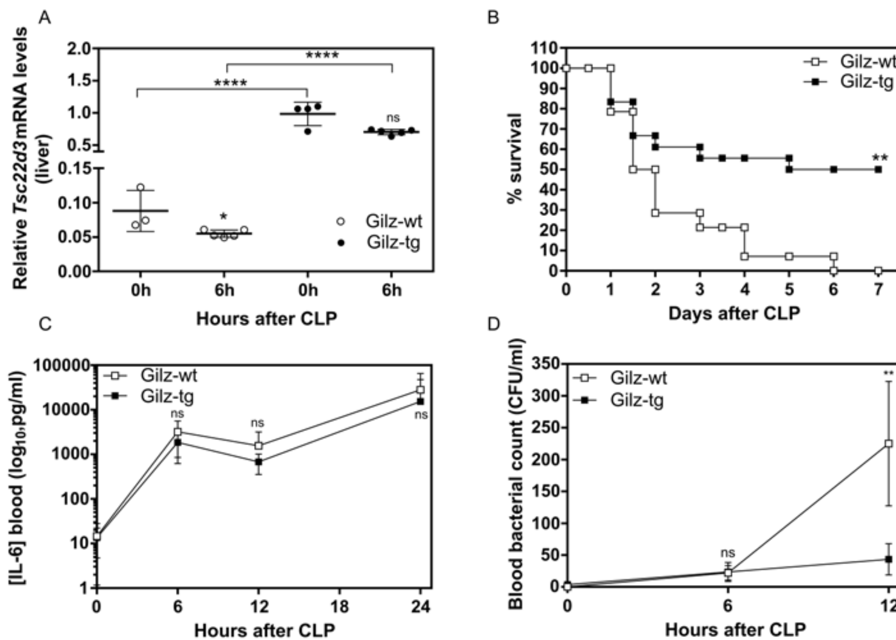

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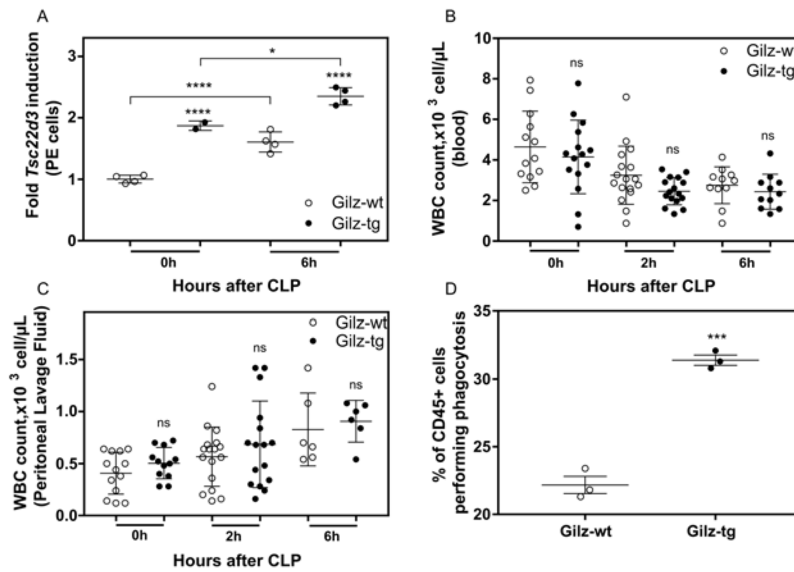
Figure 3: ***Tsc22d3*** **expression is upregulated in peritoneal exudate cells after CLP and is important for phagocytic activity** (**A**) Male Gilz-wt (white boxes) and Gilz-tg (black boxes) mice were subjected to CLP. PE cells were isolated after 0 and 6 h and mRNA was prepared. *Tsc22d3* expression levels were analyzed via RT-qPCR (n=4; one experiment). (**B-C**) Kinetics (0, 6, and 12 h) of WBC count in blood (**B**) and peritoneal lavage (**C**) of Gilz-wt and Gilz-tg mice subjected to CLP (n=10-14; combined data of three independent experiments). Data are represented as x10³ cells per µl. (**D**) Peritoneal exudate cells derived from sham operated mice were incubated with pHrodo-labeled bioparticles for 30 minutes and analyzed with FACS to measure the phagocytosis capacity. % of CD45+ cells which are positive is shown (n=3, one experiment). Data are shown as mean ± SEM. p-values were calculated using two-way ANOVA tests (A and C-D) or Student's t-test (B). **** $p < 0.0001$, *** $p < 0.001$, * $p \leq 0.05$ and ns = not significant.



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INCLUSION criteria
Severe sepsis
male or female
age > 18 years
two or more of following criteria of SIRS hyper- (> 38°C) or hypothermia (< 36°C) heart rhythm > 90/min respiratory rate > 20/min leukocytosis (> 12000/mm³) or leukopenia (< 4000/mm³)
suspected or present source of infection
one or more of following criteria of severe sepsis elevated lactate levels (> 12 mg/dl) despite adequate fluid resuscitation urinary output < 0.5 ml/kg/h during > 2 h acute lung injury with PaO_2/FiO_2 < 250 in the absence of pneumonia acute lung injury with PaO_2/FiO_2 < 200 in the presence of pneumonia thrombocytopenia (< 100000/µl) coagulopathy (international normalized ratio > 1.5)
Septic shock
criteria severe sepsis in combination with one of the following: persistent hypotension despite adequate fluid resuscitation systolic pressure < 90 mmHg OR reduction of > 40 mmHg compared to baseline need for vasopressors despite adequate fluid resuscitation
EXCLUSION criteria
age < 18 years
use of immunosuppressive medications or glucocorticoids
HIV positive
hematological malignancies
liver cirrhosis
chronic kidney insufficiency

Table 1: Inclusion and exclusion criteria clinical study protocol

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Gene	Forward primer (5'-3')	Reverse primer (3'-5')
CYCLOPHILIN	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC
PPIA	TCCTGGCATCTTGTCCATG	CCATCCAACCACTCAGTCTTG
TSC22D3	GGAGATCCTGAAGGAGCAGA	TTCAGGGCTCAGACAGGACT
Rpl	CCTGCTGCTCTCAAGGTT	TGGTTGTCACTGCCTCGTACTT
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Ubc	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACA
Tsc22d3	CCAGTGTGCTCCAGAAAGTGTAAG	AGAAGGCTCATTTGGCTCAATCTC

Table 2: Primer sequences used for RT-qPCR analysis.

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