Die Bedeutung des angeborenen Immunsystems und der endothelialen Entzündungsreaktion im septischen Schock

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*Geteilte Erstautorenschaft*
1. Inhaltsverzeichnis

Abkürzungsverzeichnis ........................................................................................................... 1

2. Einleitung ............................................................................................................................... 1

1. Inhaltsverzeichnis ................................................................................................................ 1

Abkürzungsverzeichnis ........................................................................................................... 2

2. Einleitung ............................................................................................................................... 3

Sepsis: Definition, Inzidenz und Mortalität ........................................................................ 3
Toll-like-Rezeptoren als Bestandteil des angeborenen Immunsystems .................................. 6
Einfluss von Stickstoffmonoxid auf den totalen peripheren Widerstand in der Sepsis ............. 7
Das Cullin-RING-Ligase System und seine Bedeutung für die Transkriptionsfaktoren NF-kB und HIF ......................................................... 9

3. Ergebnisteil ............................................................................................................................ 14

Ehrentraut, S., Frede, S., Stapel, H., Mengden, T., Grohe, C., Fandrey, J., Meyer, R., and Baumgarten, G. (2007). Antagonism of lipopolysaccharide-induced blood pressure attenuation and vascular contractility. *Arterioscler Thromb Vasc Biol* 27, 2170–2176........................................ 14

Ehrentraut, S.*, Lohner, R., Schwederski, M., Ehrentraut, H., Boehm, O., Noga, S., Langhoff, P., Baumgarten, G., Meyer, R., and Knuefermann, P. (2011). In vivo Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. *Shock* 36, 613–620......................................................... 22

Ehrentraut, S.F., Dorr, A., Ehrentraut, H., Lohner, R., Lee, S.H., Hoeft, A., Baumgarten, G., Knuefermann, P., Boehm, O., and Meyer, R. (2012). Vascular dysfunction following polymicrobial sepsis: role of pattern recognition receptors. *PLoS One* 7, e44531................................................................. 32

Ehrentraut, S.F., Kominsky, D.J., Glover, L.E., Campbell, E.L., Kelly, C.J., Bowers, B.E., Bayless, A.J., and Colgan, S.P. (2013). Central role for endothelial human deneddylase-1/SENP8 in fine-tuning the vascular inflammatory response. *J Immunol* 190, 392–400......................................................... 44

Ehrentraut, S.F.*, Curtis, V.F., Wang, R.X., Saeedi, B.J., Ehrentraut, H., Onyiah, J.C., Kelly, C.J., Campbell, E.L., Glover, L.E., Kominsky, D.J., et al. (2016). Perturbation of neddylation-dependent NF-kappaB responses in the intestinal epithelium drives apoptosis and inhibits resolution of mucosal inflammation. *Mol Biol Cell* ....................................................................... 57

4. Diskussion .............................................................................................................................. 65

5. Zusammenfassung ................................................................................................................ 69

6. Überlappung durch geteilte Autorenschaften ................................................................ 71

7. Bibliographie ......................................................................................................................... 72

8. Danksagung .......................................................................................................................... 81

9. Wissenschaftlicher Lebenslauf .......................................................................................... 82

10. Erklärung ............................................................................................................................. 84
| Abkürzung | Deutscher Begriff |
|-----------|------------------|
| CD        | Cluster of differentiation |
| CO        | Cardiac output |
| Cul       | Cullin |
| DAMP      | Danger associated molecular pattern |
| DNA       | Desoxyribonukleinsäure |
| EF        | Ejection Fraction |
| FIH       | Factor inhibiting HIF |
| HDAC      | Histone deacetylase |
| HIF       | Hypoxie-induzierter Faktor |
| HMEC      | Humane mikrovaskuläre Endothelzellen |
| HMGB-1    | High mobility group Box 1 |
| HZV       | Herzzeitvolumen |
| IKK       | Inhibitorische kappaB-Kinasen |
| IL        | Interleukin |
| iNOS      | Induzierbare NO-Synthetase |
| L-NAME    | N-Nitro-L-arginine-methylester |
| L-NMMA    | N(omega)-Monomethyl-L-Arginine Acetate |
| LPS       | Lipopolysaccharid |
| LTA       | Lipoteichonsäure |
| MAD       | Mittlerer arterieller Blutdruck |
| NAE       | NEDD8-aktivierendes Enzym |
| NEDD8     | Nukleär precursor cell expressed, developmentally downregulated |
| NF-κB     | Nukleärer Faktor kappa B |
| NO        | Stickstoffmonoxid |
| ODN       | Oligodeoxyneukleotid |
| PAMP      | Pathogen associated molecular pattern |
| PCR       | Polymerasekettenreaktion |
| PHD       | Prolylhydroxylasen |
| pVHL      | von Hippel-Lindau Protein |
| ROS       | Reactive oxygen Species |
| SCF       | Skp-Cullin-F-Box |
| SENP8     | Sentrin specific protease 8 |
| SIRS      | Systemic inflammatory response syndrome |
| SMT       | S-Methyl-Isothiurea |
| SOFA      | Sequential organ failure assessment |
| SV        | Schlagvolumen |
| SW        | Schlagarbeit |
| TLR       | Toll-like-Rezeptor |
| TNF       | Tumornekrosefaktor |
| TPR       | total peripherer Gefäßwiderstand |
| ZNS       | zentrales Nervensystem |
| ZVD       | Zentraler Venendruck |
2. Einleitung

Sepsis: Definition, Inzidenz und Mortalität

Die Sepsis wurde 1989 als überschießende, systemische Entzündungsreaktion auf eine (vermutete) Infektion, mit schweren Veränderungen der physiologischen und biochemischen Reaktion des Körpers, beschrieben (Balk and Bone, 1989).

Der lebensbedrohliche Charakter der Sepsis zeigt sich in der hohen Inzidenz gepaart mit einer außerordentlich hohen Mortalität. Die Zahlen hinsichtlich der Häufigkeit zeigen gewisse Schwankungen im internationalen Vergleich, da eine genaue Quantifizierung aufgrund der verschiedenen nationalen Kodiersysteme schwierig bzw. nicht wirklich erfassbar ist. In den USA schwankt die Inzidenz zwischen 81 Fällen / 100.000 Einwohner und 1031 Fällen / 100.000 Einwohnern, je nach verwendeter Kodiermethodik (Angus et al., 2001; Dombrovskiy et al., 2007; Gaieski et al., 2013; Wang et al., 2007). Für Deutschland wurden jüngst neue Zahlen basierend auf den ICD-10-GM-kodierten Krankenhausentlasszahlen veröffentlicht. Demnach liegt die Inzidenz der Sepsis in Deutschland bei 158 Fällen / 100.000 Einwohnern (Fleischmann et al., 2016). Es handelt sich somit weiter um eine häufige Erkrankung.

Auch hinsichtlich der Krankenhausmortalität gibt es keine einheitlichen, allgemeinverbindlichen Zahlen. In den USA schwanken die Angaben zur Mortalität der Sepsis zwischen 14,7% (Wang et al., 2007) und 29,9% (Dombrovskiy et al., 2007). Die Mortalität in Deutschland ist vergleichbar hoch. So zeigen aktuelle Daten aus dem Jenaer Sepsis Register eine intensivstationäre Sterblichkeit von 24% und eine Krankenhaussterblichkeit von 45% (Schmidt et al., 2020). Noch deutlicher wird die gesamtgesellschaftliche Bedeutung, wenn man die langfristige Mortalität der Sepsis nach dem Akutkrankenhausaufenthalt betrachtet. So betrug die beobachtete Sepsissterblichkeit sechs Monate nach Diagnosestellung bereits 59% und erhöhte sich im Beobachtungszeitraum von 4 Jahren auf 74% (Schmidt et al., 2020).

Neben der hohen Mortalität ergeben sich auch weiter gesundheitsökonomische Aspekte aufgrund der Sepsis. So kam eine Metaanalyse, beruhend auf 37 Einzelarbeiten, zu dem Ergebnis, dass die Behandlungskosten der Sepsis im Durchschnitt $32.421 betragen (Arefian et al., 2017). Die Sepsis ist somit eines der Krankheitsbilder mit den höchsten Gesamtkosten und stellt daher auch eine sozioökonomische Herausforderung für Gesundheitssysteme dar (Danna, 2018).
Erste Definitionsversuche des komplexen Geschehens erfolgten 1991 im Rahmen einer Konsensusdefinition und grenzten erstmals das sog. systemisch-inflammatorische Syndrom (systemic inflammatory response syndrome, SIRS, Kombination von zwei oder mehr Faktoren aus Kasten 1) von der sog. schweren Sepsis ab (Bone et al., 1992). Dies stellte die sog. Sepsis-1 Definition dar, welche 2001 durch die Sepsis-2 Definition abgelöst wurde (Levy et al., 2003).

Letztere Sepsisdefinition umfasst das SIRS in Kombination mit nachweisbarem Organversagen aufgrund einer vorhandenen oder vermuteten Infektion. Grundlage der Erfassung eines Organversagens bildete der sog. Sequential Organ Failure Assessment Score, SOFA (Vincent et al., 1996). Dieser erfasst sechs Organsystem (Lunge, Kreislauf, Niere, Thrombozyten, Leber und ZNS) und teilt sie je nach Höhe der Funktionseinschränkung in jeweils fünf Kategorien mit Punktwerten von 0 (keine Einschränkung) bis 4 (Organversagen) ein (Tabelle 1). Das Vorliegen von mehr als zwei Punkten zeigt ein relevantes Organversagen an, welches das SIRS von der schweren Sepsis abgrenzt.

**Kasten 1: Initialdefinition des SIRS**

| Parameter | 1 Punkt | 2 Punkte | 3 Punkte | 4 Punkte |
|-----------|---------|---------|---------|---------|
| Atmung    | PaO₂/FIO₂ < 400 mmHg | PaO₂/FIO₂ < 300 mmHg | PaO₂/FIO₂ < 200 mmHg | PaO₂/FIO₂ < 100 mmHg |
| Herzfrequenz | >90/min | >90/min | >90/min | >90/min |
| Atemfrequenz | >20/min | >20/min | >20/min | >20/min |
| Laktat | >4 mmol/l | >4 mmol/l | >4 mmol/l | >4 mmol/l |
| Temperatur | >38°C | >38°C | >38°C | >38°C |
| Herzfrequenz | >90/min | >90/min | >90/min | >90/min |
| Atemfrequenz | >20/min | >20/min | >20/min | >20/min |
| Leukozytenzahl | >12 000/mm³ | >12 000/mm³ | >12 000/mm³ | >12 000/mm³ |
| Hämoglobinkonzentration | <100 g/l | <100 g/l | <100 g/l | <100 g/l |
| Gerinnung | Thrombozyten < 150.000 /μl | Thrombozyten < 100.000 /μl | Thrombozyten < 50.000 /μl | Thrombozyten < 30.000 /μl |
| Niere | Kreatinin > 1.9 mg/dl | Kreatinin > 3.0 mg/dl | Kreatinin > 4.9 mg/dl | Kreatinin > 7.0 mg/dl |

Nach Bone et al., 1992

**Tabelle 1: Einzelmitems des SOFA Score nach Vincent et al. 1996**

Darüber hinaus wurde noch der sog. septische Schock, als Kombination aus schwerer Sepsis mit anhaltender Hypotension trotz adäquatem Flüssigkeitsersatz eingeführt (Bone et al., 1992).
Diese Definition hatte für nahezu 25 Jahre Bestand und wurde 2016 durch die sog. Sepsis-3 Kriterien angepasst (Singer et al., 2016). Grundlage der Anpassung war die Einsicht, dass die ursprüngliche Definition den „lebensbedrohlichen Charakter“, resultierend aus einer fehlregulierten bzw. überschießenden Immunantwort, der Sepsis nicht hinreichend berücksichtigte. Darüber hinaus ist das Auftreten eines SIRS durchaus auch im Rahmen anderer schwerer Geschehnisse, wie z.B. nach Trauma oder ausgedehnte Operationen, ohne das Vorliegen einer Infektion häufig. Die Anpassung vereinfachte die Diagnosestellung der Sepsis dahingehend, dass die Begrifflichkeit des SIRS und die schwere Sepsis weggefallen sind. Es wird nunmehr nur noch zwischen Sepsis (SIRS + Organversagen) und septischen Schock (Sepsis mit therapierefraktärer Hypotonie) unterschieden (Singer et al., 2016).

Die therapierefraktäre Hypotonie ergibt sich aus einer unzureichenden Blutdruckregulation. Hierbei sind sowohl die Herzleistung im Sinne des Herzzeitvolumens (HZV) als auch der totale periphere Widerstand (TPR) von Bedeutung. Beides geht entsprechend folgender Formel in die Genese des mittleren arteriellen Blutdrucks ein:

\[ TPR = \frac{MAD - ZVD}{HZV} \]

bzw.

\[ MAD = TPR \times HZV + ZVD \]

Daraus ergibt sich, dass sowohl eine negative Veränderung des Herzzeitvolumens als auch ein Verlust des TPR zu einem verringerten mittleren arteriellen Blutdruck (MAP) führen. Die diesen Effekten zugrundeliegenden molekularen bzw. pathophysiologischen Effekte in der Sepsis werden im weiteren Teil der Einleitung behandelt bzw. sind Bestandteil des Ergebnisteils.

Trotz vielfacher Bestrebungen, seit nunmehr über 30 Jahren, bleibt die septische Immunreaktion weiterhin ein unverstandenes hochkomplexes Krankheitsbild. Die hier vorgestellten grundlagenwissenschaftlichen Arbeiten beleuchten die verschiedenen Einflüsse des angeborenen Immunsystems für die septische Herzkreislaufregulation. Darüber hinaus wird auf neue Signaltransduktionswege für die Entstehung und Weiterleitung der Entzündungskaskaden im Endothel und Epithel eingegangen. Die folgenden Abschnitte behandeln den diesbezüglichen Kenntnisstand in der Zeit von 2007 bis 2016.
Toll-like-Rezeptoren als Bestandteil des angeborenen Immunsystems
Das angeborene Immunsystem stellt die sog. „erste Verteidigungslinie“ des Organismus gegen eine potentielle Schädigung dar (Hato and Dagher, 2015). Es kann exogene, d.h. von außen auf den Organismus einwirkende Pathogene erkennen und eine direkte Immunantwort auslösen. Grundlage der Erkennung ist die Bindung von entwicklungsgeschichtlich hochkonservierten, mikrobiellen, pathogenassoziierten Mustern, sogenannter PAMPs (pathogen associated molecular patterns) wie Flagellin oder bakterieller Zellwandbestandteile wie Lipopolysacchariden (LPS) (Takeda and Akira, 2015). Darüber hinaus können auch wirtseigene Noxen, z.B. infolge von Zellschädigung freigesetzt werden. In diesem Zusammenhang spricht man von sog. DAMPs (danger associated molecular patterns) wie z.B. dem, aus hämatopoetischen Zellen freigesetzten, HMGB-1 (high mobility group Box 1) (Gardella et al., 2002). Eine für die Erkennung dieser PAMPs/DAMPs wichtige Gruppe von Rezeptoren ist die Gruppe der sog. Toll-like-Rezeptoren (TLRs). Ursprünglich erstmals 1980 durch ihre Rolle in der Embryonalentwicklung von Drosophila melanogaster beschrieben, konnte bald gezeigt werden, dass die Rezeptorfamilie auf für die Abwehr von Pilzinfektionen relevant ist (Lemaitre et al., 1996). Nachfolgend wurde Mitte der 1990er Jahre der Toll-like-Rezeptor im Menschen und seine Bedeutung für die Immunantwort nachgewiesen (Medzhitov et al., 1997). Inzwischen konnten 13 verschiedene Varianten, von denen die TLR1-10 im Menschen vorkommen, nachgewiesen werden. Diese Rezeptoren binden verschiedene, in Tabelle 2 aufgeführte, Liganden.

| Toll like Rezeptor | Ligand (ohne Anspruch auf Vollständigkeit) |
|-------------------|-------------------------------------------|
| 1                 | Triacyl lipopeptide                        |
| 2                 | Peptidoglykan, Lipoteichonsäure, Zymosan, Glykolipide |
| 3                 | Doppelsträngige DNA                        |
| 4                 | Lipopolysaccharide, Taxol, verschiedene endogene Noxen wie Hitzeschockproteine, Hyaluronsäure) |
| 5                 | Flagellin                                  |
| 6                 | Diacyl lipopeptide                         |
| 7                 | Einzelsträngige DNA                        |
| 8                 | Einzelsträngige DNA                        |
Nach Bindung erfolgt eine komplexe Signaltransduktionskette, die den Transkriptionsfaktor Nukleärer Faktor kappa B (NF-κB) aktiviert, welcher wiederum in der Regulation verschiedener proentzündlicher Zytokine beteiligt ist (Lim and Staudt, 2013).

Die Bedeutung von TLRs, insbesondere der TLR2, TLR4 und TLR9, für die kardiale Funktion konnte in verschiedenen Arbeiten von Baumgarten und Knüfermann dargestellt werden (Knuefermann et al., 2002a). So konnten diese zeigen, dass es in Abhängigkeit von zu einer vermehrten Zytokinsekretion, einer verminderten Kontraktilität von Kardiomyozyten, aber auch veränderten Infarktgrößen nach Ischämie/Reperfusion kommt (Baumgarten et al., 2001, 2006b, 2006a). Zudem konnte dargestellt werden, dass diese Prozesse auch unter Beteiligung des Ko-Receptors CD (Cluster of differentiation)-14 bzw. TLR2 vermittelt werden (Knuefermann et al., 2002b, 2004). Des Weiteren zeigte sich, dass auch die Bindung von bakterieller DNA über den TLR9 negative Auswirkungen auf die kardiale Kontraktilität und die Ausbildung eines Lungenversagens hat (Knuefermann et al., 2007, 2008). Aufgrund der Beobachtung, dass die verminderte Kontraktilität, zumindest teilweise, von der Freisetzung von Stickstoffmonoxid (NO) abhängt, stellt sich die Frage der Auswirkungen von NO auf den totalen peripheren Widerstand, der zweiten wichtigen Stellgröße der Hämodynamik.

Einfluss von Stickstoffmonoxid auf den totalen peripheren Widerstand in der Sepsis

Bei einer bakteriellen Infektion werden Virulenzfaktoren freigesetzt und das Immunsystem reagiert mit der Bildung von entzündlichen Zytokinen (Baumgarten et al., 2001), was zu einer Senkung des totalen peripheren Widerstand des Kreislaufsystems führt. Dies wird unter anderem durch einen Anstieg der Herzfrequenz und des Herzzeitvolumens kompensiert. Im Rahmen des septischen Schocks kann

| Tabelle 2 Toll-Like-Rezeptoren und ihre Liganden, modifiziert nach Takeda und Akira 2015 |
|-----------------------------------------------|
| 9 | unmethylierte (CpG) DNA |
| 10 | HIV-1, andere Liganden vermittelt durch Dimerisierung mit anderen TLRs |
| 11 | Profilin |
| 12 | Profilin |
| 13 | Bakterielle ribosomale RNA |
das Herzzeitvolumen den Verlust des peripheren Widerstand nicht mehr ausgleichen, was zu einem drastischen Abfall des Blutdrucks und einer gestörten Organ- und Mikrozirkulation führt (Spronk et al., 2004).

Bedingt durch die septische Systemantwort kommt es, neben der Sekretion von proentzündlichen Zytokinen wie TNFalpha auch zur Generierung und Freisetzung unterschiedlicher anderer Mediatoren wie Stickstoffmonoxid und Adrenomedullin (ADM). Die Wirkung von NO und ADM ist breit gefächert und hat auch relevante Auswirkungen auf die Regulation des TPR (Evans et al., 1993; Parratt, 1997; Takeuchi et al., 2000; Vallance and Moncada, 1993). Es ist bekannt, dass das NO-Synthase-Isoenzym iNOS (induzierbare NOS oder Typ II NOS) im murinen Herzmuskel nach LPS-Stimulation hochreguliert wird (Baumgarten et al., 2006a) und dass vaskuläres NO aus iNOS für die vasomotorische Dysfunktion während des septischen Schocks relevant ist (Gunnnett et al., 1998). Frühe Studien zur Verwendung von nichtselektiven NO Synthase-Inhibitoren wie N-Nitro-L-arginine-methylester (L-NAME) oder N(omega)-Monomethyl-L-Arginine Acetate (L-NMMA) haben gezeigt, dass die Hemmung der NO-Genese die Sepsis-asoziierte Hypotonie verhindern kann, wenngleich L-NMMA eine verminderte Herzleistung zur Folge haben kann (Petros et al., 1991, 1994). Daher sind selektive Hemmstoffe von iNOS möglicherweise besser geeignet sein, den Verlust des TPR zu hemmen, ohne die schädlichen Auswirkungen der totalen NO-Hemmung durch L-NAME, die in Tierversuchen und klinischen Studien beobachtet wurden (Fukatsu et al., 1995; Kim et al., 2003; Minnard et al., 1994; Robertson et al., 1994).

Neben dem Verlust des totalen peripheren Widerstands ist eine Einschränkung der Herzleistung („Myokardiale Depression“) eine häufige und potenziell tödliche Komplikation des septischen Schocks. Parillo et al. wiesen bereits 1990 nach, dass etwa 40 % der Patienten mit Sepsis sowohl eine links- als auch eine rechtsventrikuläre Dysfunktion entwickeln (Parrillo et al., 1990). Dies stellt sich sowohl durch eine Dilatation der Herzkammern, als auch durch eine verringerte Auswurfleistung dar. Dies wiederum hat erhebliche Auswirkungen auf die sepsis-asoziierte Mortalität. Außerdem steigt bei Patienten, die eine kardiovaskuläre Beeinträchtigung entwickeln, die Sterblichkeit von 20 % auf 70-90 %, was einen Zusammenhang zwischen der Herzfunktion und dem Überleben während einer Sepsis zeigt (Natanson et al., 1986). Neben NO zeigt sich, dass Tumor-Nekrose-Faktor (TNF)-α, Interleukin (IL)-1β, und IL-6 entscheidend an der mit dem septischen Schock assoziierten Myokarddepression
beteiligt sind. Die Freisetzung dieser Mediatoren und ihr Einfluss auf das Myokard konnte in mehreren Tiermodellen infolge einer Stimulation mit gram-negativen Bakterien oder deren Zellwandbestandteilen gezeigt werden (Baumgarten et al., 2006b, 2006a; Cain et al., 1999; Panas et al., 1998).

Das Cullin-RING-Ligase System und seine Bedeutung für die Transkriptionsfaktoren NF-kB und HIF

Eines der Hauptmerkmale der Sepsis ist der Verlust der Endothelfunktion und die anschließende Aktivierung des Immunsystems (Paulus et al., 2011; Shapiro et al., 2010). Der NF-κB-Signalweg hat sich als einer der wichtigsten Signalwege für die intrazelluläre proinflammatorische Signalgebung etabliert (Baker et al., 2011; Barnes and Karin, 1997; Hayden and Ghosh, 2008). Darüber hinaus gibt es Hinweise, dass NF-κB den Spiegel des Hypoxie-induzierbaren Faktors (HIF) (Cockman et al., 2000; Semenza and Wang, 1992), eines Transkriptionsfaktors für die Sauerstoffhomöostase, beeinflussen kann und umgekehrt (Belaiba et al., 2007; Walmsley et al., 2005). NF-κB wird durch Inhibitorische kappaB-Kinasen (IKK) reguliert, und seine Aktivierung hängt vom Abbau der IKK durch das 26S-Proteasom ab (Hacker and Karin, 2006). Auch HIF wird posttranslational stark reguliert. Unter normoxischen Bedingungen wird HIF durch sog. Prolylhydroxylasen (PHD) hydroxyliert, anschließend ubiquitiniert und schließlich durch das Proteasom abgebaut (Semenza, 2007, 2007). Hypoxie ist häufig an Entzündungsherden nachweisbar (Colgan and Taylor, 2010). Unter solchen Bedingungen wird der Hypoxie-induzierbare Faktor stabilisiert, wandert in den Zellkern und verstärkt die Transkription einer Reihe von potentiell entzündungshemmenden Genen (Grenz et al., 2012). Die Ubiquitinierung der IκB-Kinase und der Alpha-Untereinheit von HIF-1 und HIF-2 wird durch Cullin-Proteine vermittelt, die, um aktiv zu sein, eine Konjugation mit dem kleinen ubiquitinähnlichen Modifikator NEDD8 (Neural precursor cell expressed, developmentally downregulated) benötigen (Kumar et al., 1993). Die Konjugation von NEDD8 an seine Zielproteine ist ein mehrstufiger Prozess (Siehe Abbildung 1 und 2).
Abbildung 1: Regulierung des hypoxie-induzierbaren Faktors durch Hydroxylierung. Unter normoxischen Bedingungen wird die HIFa-Untereinheit hydroxyliert durch Prolylhydroxylasen 1-3 (PHDs) oder den HIF-Inhibitor-Faktor (FIH) hydroxyliert. Hydroxyliertes HIF wird durch das von Hippel-Lindau-Protein (pVHL) erkannt, das in seinem aktivierte Zustand eine neddylierte Cullin-2-Untereinheit enthält. Dies führt zur Ubiquitinierung der HIF-1a-Untereinheit und deren Abbau durch das Proteasom. Im Rahmen von Entzündungsprozessen kann der lokale Sauerstoffgehalt aufgrund des erhöhten Stoffwechsels und durch die von den Neutrophilen erzeugten ROS begrenzt sein. Diese hypoxische Umgebung macht die PHDs impotent, ebenso wie eine pharmakologische Hemmung mit DMOG, wodurch die Ubiquitinierung von HIFa durch pVHL verhindert wird. Nicht ubiquitiertes HIFa transloziert in den Zellkern, bindet an die β-Untereinheit und das Heterodimer fungiert als Transkriptionsfaktor für eine Reihe von Genen, darunter Adrenomedullin (ADM). Darüber hinaus hemmt HIFa die durch die Fas-associated-death-domain (FADD) induzierte Apoptose und verbessert dadurch die Barrierefunktion des Darms. Die autokrine Freisetzung von ADM kann durch die ADM-Rezeptor-vermittelte Deneddylierung von Cul2 als negativer Rückkopplungsmechanismus dienen (Ehrentraut and Colgan, 2012).

Zunächst wird der NEDD8-Vorläufer an einem carboxyterminalen Glycinrest gespalten und an die UBA3-APPB1 E1-Ligase konjugiert, die auch als NEDD8-aktivierendes Enzym (NAE) bezeichnet wird (Huang et al., 2004; Mendoza et al., 2003; Wada et al., 1998). Der reife NEDD8-Anteil wird auf seine E2-Ligase UBC12 (Ubiquitin-konvertierendes Enzym) (Liakopoulos et al., 1999) übertragen und dann an seine E3-Ligase, ein Cullin-Protein, gebunden, wodurch es aktiviert wird (Jones et al., 2008; Parry and Estelle, 2004). Die Konjugation von NEDD8 mit Cul-1 ist notwendig, um den Vorläufer der NF-κB p50-Untereinheit p105 über das Skp-Cul1-FboxβTrCP (SCFβTrCP) zu verarbeiten (Amir et al., 2002). Amir et al. schließen aus ihren Ergebnissen, dass die NEDDylierung von Cullin-Proteinen für die induzierte Prozessierung von p105 und die NF-κB-Aktivität wesentlich ist (Amir et al., 2002). Neben NF-κB wirkt sich die Cullin-Neddylierung auch auf den Transkriptionsfaktor.
Hypoxie-induzierbarer Faktor aus. Die Alpha-Untereinheit von HIF (HIFα) wird unter normoxischen Bedingungen durch Prolyl-Hydroxylasen hydroxyliert (Semenza, 2010). Diese Hydroxylierung erleichtert die Erkennung und Bindung von HIFα durch das Von-Hippel-Lindau-Protein (pVHL), das Teil eines E3-Ligase-Komplexes ist, der Cullin-2 enthält (Sufan and Ohh, 2006), wodurch der proteasomale Abbau von hydroxyliertem HIF vermittelt wird (Semenza, 2010). In seiner nicht hydroxylierten Form wandert HIFα in den Zellkern, bindet an die konstitutiv exprimierte Untereinheit HIF1β und reguliert auf transkriptionelle Weise eine Vielzahl von Genen, die für die Entzündungsreaktion wichtig sind (Giatromanolaki et al., 2003; Mariani et al., 2009).

Eine schematische Darstellung der beteiligten Prozesse ist Abbildung 1 zu entnehmen.

Abbildung 1: Schematische Darstellung der Hydroxylierung und Verarbeitung von HIF-α. Die Alpha-Untereinheit von HIF (HIFα) wird unter normoxischen Bedingungen durch Prolyl-Hydroxylasen hydroxyliert (Semenza, 2010). Diese Hydroxylierung erleichtert die Erkennung und Bindung von HIFα durch das Von-Hippel-Lindau-Protein (pVHL), das Teil eines E3-Ligase-Komplexes ist, der Cullin-2 enthält (Sufan and Ohh, 2006), wodurch der proteasomale Abbau von hydroxyliertem HIF vermittelt wird (Semenza, 2010). In seiner nicht hydroxylierten Form wandert HIFα in den Zellkern, bindet an die konstitutiv exprimierte Untereinheit HIF1β und reguliert auf transkriptionelle Weise eine Vielzahl von Genen, die für die Entzündungsreaktion wichtig sind (Giatromanolaki et al., 2003; Mariani et al., 2009).

Abbildung 2: Die Erkennung pro-inflammatorischer Stimuli, das Vorhandensein von mikrobiellen Zellwandbestandteilen, sezerniertem TNFα oder verschiedenen anderen Zytokinen durch ihre jeweiligen Rezeptoren löst eine erste pro-inflammatorische Reaktion der IEC aus. Der NFκB-Signalweg wird durch die Phosphorylierung von IkB durch die Ik-Kinasen α und β aktiviert. Diese Phosphorylierung ermöglicht seine Erkennung durch den neddylierten Skp-Cullin-F-Box (SCF)-Komplex, Polyubiquitinierung und anschließenden proteasomalen Abbau. Die Neddylierung von Cullinen wird durch einen Multienzym-Prozess erreicht, bei dem eine NEDD8-Komponente an das Zielprotein konjugiert wird. Um konjugiert werden zu können, muss NEDD8 durch die Isopeptidase SENP8 von seiner Pro-Form zur reifen Form verarbeitet werden. Das gleiche Protein entfernt zusätzlich zum COP9-Signalosom NEDD8 von Cullinen. Neddylierte Cullins werden in den SCF-Komplex integriert und aktivieren diesen, um die vom SCF-Komplex vermittelte Ubiquitinierung zu ermöglichen. Das NFκB-Heterodimer kann dann in den Zellkern wandern und an die Promotorregionen verschiedener entzündungsfördernder und entzündungshemmender Zytokine, einschließlich TNFα, IL-1β und Interferon-γ, binden. Dies kann die Entzündung weiter fördern, z. B. durch TNFα-induzierte Apoptose über den FADD-Weg, und den Abbau der Barriere, aber auch den eingehenden Stimulus durch Induktion von NUB1 über den Interferonrezeptor aufheben. Zu den entzündungshemmenden Mechanismen gehören die Induktion der Deneddylierung von Cullin-Proteinen durch Adenosin und die ROS-vermittelte Hemmung der E2-Ligase NEDD8/UBC12. (Ehrentraut and Colgan, 2012)
In letzter Zeit ist ein Interesse am Verständnis der Mechanismen entstanden, die die Cullin-Neddylierung regulieren, genauer gesagt den umgekehrten Prozess, die Deneddylierung. Es hat sich gezeigt, dass Adenosin, das durch hypoxische Vorkonditionierung erzeugt wird, die Deneddylierung von Cul-1 vermittelt. Dieser Prozess ist vom COP9-Signalosom abhängig und stellt einen potenziellen Schutzmechanismus gegen hypoxiebedingte proinflammatorische Stimuli dar (Khoury et al., 2007). Die Entdeckung von SENP8, einer Isopeptidase, die in der Lage ist, Cullin-Proteine direkt zu deneddylieren (Mendoza et al., 2003; Wu et al., 2003), lieferte neue Erkenntnisse darüber, wie die Cullin-Neddylierung reguliert werden könnte. SENP8 scheint in der Lage zu sein, übermäßig neddylierte Cullin-Proteine zu deneddylieren und bietet damit einen Spaltungsweg jenseits des COP9-Signalosoms (Cope and Deshaies, 2003; Lyapina et al., 2001). Frühere Untersuchungen (Khoury et al., 2007; Lennon et al., 1998; Schingnitz et al., 2010) zeigen, dass insbesondere Adenosin potentiell positive Einflüsse auf verschiedene entzündliche Prozesse ausübt.

Darüber hinaus gelang es die genauere Funktion der sog. humanen Deneddylase-1 (SENP8, sentrin specific protease 8) (Amir et al., 2002; Chan et al., 2008) zu bestimmen. Anders als ihr Name vermuten lässt, ermöglicht SENP8 die Neddylierung von Cullinproteinen und erlaubt darüber ihre Aktivierung. Eine schematische Darstellung der beteiligten Prozesse ist Abbildung 2 zu entnehmen. Aufbauend auf diesen Ergebnissen wurde ein pharmakologischer Inhibitor (MLN4924, Millennium Pharmaceuticals, Boston, (Brownell et al., 2010; Milhollen et al., 2010)) des SENP8-Signaltransduktionsweges erfolgreich im Tiermodell getestet und es konnte gezeigt werden, dass dies eine schützende Wirkung in akuten entzündlichen Krankheitsmodellen hat. Des Weiteren zeigte sich, dass der Wirkstoff MLN4924 stabilisierende Effekte für den entzündungshemmenden Transkriptionsfaktor HIF1α hat und sich dadurch für den Einsatz in entzündlichen Krankheitsbildern eignen kann.

Aus den obenstehenden Ausführungen ergeben sich folgende Fragestellungen:

- Haben TLR eine Bedeutung für die septische Vasoplegie?
- Gibt es Unterschiede hinsichtlich der vaskulären Reaktion in Abhängigkeit verschiedener TLR und ihrer Liganden?
- Welche Rolle spielt endothelial generiertes NO für die septische Vasoplegie?
- Inwieweit hat der SENP8/Cullin-RING-Ligase Signalweg eine Rolle für die Regulation der endo-/epithelialen Immunantwort?
3. Ergebnisteil

Ehrentraut, S., Frede, S., Stapel, H., Mengden, T., Grohe, C., Fandrey, J., Meyer, R., and Baumgarten, G. (2007). Antagonism of lipopolysaccharide-induced blood pressure attenuation and vascular contractility. Arterioscler Thromb Vasc Biol 27, 2170–2176.

Zielsetzung der Arbeit - Es sollte untersucht werden, ob die durch Lipopolysaccharid (LPS) induzierte Verringerung des totalen peripheren Widerstands vom Toll-like-Rezeptor (TLR)4-Signalgebung abhängt. Zusätzlich wurde untersucht, ob die septische Vasoplegie auf einer endothelialen NO-Synthase beruht und durch TLR4-Antagonisten modulierbar ist.

Methoden und Ergebnisse - C3H/HeN-Mäuse (Kontrolle), die einen funktionellen, und C3H/HeJ-Mäuse, die einen nicht funktionellen TLR4 exprimieren, wurden miteinander verglichen. LPS (20 mg/kg) wurde 6 Stunden vor den hämodynamischen Messungen i.p. injiziert. L-NAME und SMT, Inhibitoren der NO-Produktion, und Eritoran, ein TLR4-Antagonist, wurden auf ihre Auswirkungen auf die vaskuläre Kontraktilität getestet. Isolierte Aortenringe wurden 6 Stunden lang mit oder ohne LPS (1 µg/mL) oder mit der Kombination von LPS+Eritoran (2 µg/mL) inkubiert. Nachfolgend wurde ihre Phenylephrin-induzierte Kontraktilität mit einem Mulvany Myographen gemessen. Die Expression von Zytokinen im aortalen Gewebe wurde mittels real-time Polymerase-Kettenreaktion untersucht. Bei Kontrollmäusen führte LPS zu einer signifikanten Abnahme des Blutdrucks und einem Anstieg der Herzfrequenz, während Mäuse ohne funktionalen TLR4 davon nicht betroffen waren (siehe Figure 1, p. 2172, Ehrentraut et al. ATVB 2007). LPS induzierte einen Anstieg der Zytokinexpression und eine Depression der Gefäßkontraktilität nur bei Mäusen mit funktionalem TLR4, nicht aber bei Mäusen, in denen der Rezeptor funktionslos war. L-NAME und der, für die induzierbare NO-Synthase iNOS spezifische, Inhibitor S-Methyl-Isothiurea erhöhten die Kontraktilität der Gefäßringe und stellten die LPS-abhängige Depression der Kontraktilität wieder her. Der TLR4-Antagonist Eritoran verhinderte den LPS-induzierten Verlust der Kontraktilität (siehe Figure 2, p. 2173, Ehrentraut et al. ATVB 2007).

Schlussfolgerungen - LPS reguliert die Zytokinexpression im Gefäß über TLR4 und induziert eine Abschwächung der Kontraktilität der glatten Muskulatur. Dies beruht auf der endothelialen, induzierbaren NO-Synthase iNOS. Diese Funktionseinschränkung kann mittels Blockade des TLR4 aber auch durch Blockade der iNOS verhindert werden.
Antagonism of Lipopolysaccharide-Induced Blood Pressure Attenuation and Vascular Contractility

S. Ehrentraut, S. Frede, H. Stapel, T. Mengden, C. Grohé, J. Fandrey, R. Meyer, G. Baumgarten

Objective—Aim was to assess whether lipopolysaccharide (LPS)-induced decrease of total peripheral resistance depends on Toll-like receptor (TLR)4 signaling and whether it is sensitive to NO-synthase or TLR4 antagonists.

Methods and Results—C3H/HeN mice (control), expressing a functional, and C3H/HeJ mice, expressing a nonfunctional TLR4, were compared. LPS (20 mg/kg) was injected i.p. 6 hours before hemodynamic measurements. L-NAME and SMT, inhibitors of NO production, and Eritoran, a TLR4 antagonist, were tested for their impact on vascular contractility. Aortic rings were incubated for 6 hours with or without LPS (1 μg/mL), or with LPS + Eritoran (2 μg/mL) and their phenylephrine-induced contractility was measured using a myograph. The expression of cytokines in aortic tissue was examined by real-time polymerase chain reaction. In control mice LPS induced a significant decrease of blood pressure and an increase of heart rate, whereas C3H/HeJ remained unaffected. LPS induced an increase of cytokine expression and a depression of vascular contractility only in control mice but not in C3H/HeJ. L-NAME and SMT increased contractility in all rings and restored LPS-dependent depression of contractility. Eritoran prevented LPS-induced loss of contractility.

Conclusions—LPS upregulates cytokine expression via TLR4 and induces attenuation of smooth muscle contractility which can be effectively antagonized. (Arterioscler Thromb Vasc Biol. 2007;27:2170-2176.)

Key Words: blood pressure ■ sepsis ■ toll-like receptor 4 antagonism ■ vascular contractility

Sepsis and septic shock are responsible for about 1400 deaths per day on noncardiac intensive care units in the United States.1,2 During bacterial infection virulence factors are released and the immune system reacts by generating inflammatory cytokines3 leading to a lowered total peripheral resistance (TPR) of the circulatory system. This is compensated by an increase in heart rate and cardiac output. In septic shock cardiac output cannot balance the loss in peripheral resistance any longer resulting in a drastic drop in blood pressure and disturbed microcirculation.4 It has been shown that different members of the Toll-like receptor (TLR) family specifically bind different virulence factors from a wide variety of pathogens. Those virulence factors are very important for the pathogenesis of sepsis. Therefore, competitive inhibition of virulence factors at the Toll-like receptor level might be a potential therapeutic option to treat pathogen-induced sepsis and septic shock. LPS is an important virulence factor of Gram-negative bacteria, which binds to and signals via the TLR4/CD14 complex, thereby inducing inflammatory mediators.3,5

Recently it has been demonstrated that a synthetic Lipid-A-analog called Eritoran (E5564, Eisai, currently also used in different clinical trials for the treatment of sepsis) competitively antagonizes LPS at the TLR4/CD14 complex and inhibits the synthesis and induction of sepsis relevant mediators. In addition, Eritoran was able to prevent the negative inotropic effect of LPS on isolated cardiac myocytes.6,7 In addition nitric oxide (NO) and cytokines or adrenomedullin (ADM) appear to be increased during sepsis and play an important role in the regulation of total peripheral resistance (TPR).9–11 It is known that the NO synthase isoenzyme iNOS (inducible NOS or type II NOS) is upregulated in the murine myocardium after LPS stimulation9 and that vascular NO from iNOS is relevant for vasomotor dysfunction during septic shock.12 Early studies on the use of nonselective NO synthase inhibitors such as L-NAME or L-NMMA have shown that the inhibition of NO can prevent the sepsis associated hypotension, albeit L-NMMA produced a decreased cardiac output.13,14 Therefore selective inhibitors of iNOS may be more suitable to inhibit the loss of TPR and do so without the detrimental effects of total NO inhibition by L-NAME shown in animal15–18 and clinical studies.19

The purpose of this study was to examine the role of TLR4 and inflammatory cytokines, as target genes in the TLR4 signaling cascade, in a murine model of Gram-negative sepsis.
for the regulation of hemodynamic parameters and vascular contractility. The second aim was to elucidate whether TLR4 antagonism or iNOS inhibition may serve as therapeutic targets for the treatment of TPR loss during septic shock.

Materials and Methods

Animal Model

Twelve- to 14-week-old LPS responsive C3H/HeN (control) mice and LPS hyporesponsive C3H/HeJ mice of both genders were purchased from Charles River (Sulzfeld, Germany). C3H/HeJ mice carry a point mutation in the cytoplasmic region of TLR4, a responsive phenotype.20 Mice were housed in pathogen-free cages with free access to water and standard rodent chow. The animals were handled according to the principles of laboratory animal care (NIH publication No. 85-23, revised 1996), and animal procedures were approved by the local committee for animal care.

In Vivo Stimulation

Controls and C3H/HeJ mice were injected i.p. with NaCl or LPS (20 mg/kg bodyweight, #L2630, E. coli 0111:B4; Sigma-Aldrich Chemical) before hemodynamic recordings.

Hemodynamics

Baseline hemodynamic parameters were recorded in anesthetized mice (1% isoflurane, flow 1 l/min Forene, Abbott GmbH) under constant regulation of body temperature. A polyethylene catheter (PE 10) was inserted into the right carotid artery. Pressure signals were digitized using a Powerlab (AD, Instruments GmbH) and were continuously recorded using Chart for Windows (Version 4.2.3). After a 15-minute stabilization period baseline hemodynamic parameters were recorded for 15 minutes. Afterward the animals were euthanized to allow excision of the aorta thoracica.

Mulvany Myograph and Contractility Studies

Periadventitial fat was removed from the explanted abdominal aorta and the lower part was cut into 2-mm rings according to the method of Mulvany and Halpern.21 Aortic rings were mounted in a Mulvany Myograph and Contractility Studies (PE 10) was inserted into the right carotid artery. Pressure signals were digitized using Myodaq V2.01 and converted using Myodata software (Myonic Technology).

Stimulation With TLR4 Antagonist

Rings were kept in Dulbecco Modified Eagle Medium (DMEM) (37°C, 5% CO2, 95% oxygen) as (1) controls or coincubated with either (2) E.coli LPS (1 μg/mL) or (3) LPS (1 μg/mL) and lipid A antagonist Eritoran (2 μg/mL; EISAI; with Eritoran administered every 2 hours).22,23 Afterward concentration response curves to PE were recorded as described above. The Eritoran concentration was chosen according to previous in vitro experiments from our group on isolated cardiac myocytes.6 We tested a concentration of 1 μg/mL Eritoran and 1 μg/mL LPS, which was not able to fully prevent the cardiac depressive action of LPS. However, 2 μg/mL of Eritoran, exceeding the LPS concentration twice, were sufficient to block LPS associated cardiac depression. Therefore we applied the same concentration of Eritoran in our experiments on aortic rings.

Inhibition of NOS

Another set of PE concentration response curves was recorded after 20 minutes incubation of the isolated aortic rings with the nonspecific NOS-inhibitor N-Nitro-l-arginine-methylester (L-NAME, 100 μmol/L, Sigma #N5751) or the specific iNOS inhibitor S-Methylisothiourea (SMT, 100 μmol/L, Sigma #67730).24

Cytokine mRNA Isolation and Real-Time PCR

Animals were injected i.p. with 20 mg/kg BW LPS for 6 hour prior to isolation of the aortae. Aortae were flash frozen in liquid nitrogen (LN2) and conserved at −80°C. For RNA extractions the whole material was homogenized and RNA was isolated as described elsewhere.25 For initiation of cDNA-synthesis an Oligo-(d)-T 15- Primer for Avian Myeloblastosis Virus Reverse Transcriptase was used and 1 μg RNA transcribed to cDNA. To estimate the specific cDNA-amount a PCR for β-actin, as house-keeping gene, was performed. The resulting PCR-products were seperated using 1.5%-agarosegels. Primers for iNOS, tumor necrosis factor (TNF)α, interleukin (IL)-1, IL-6 and ADM were selected according to previous in vitro experiments from our group on isolated cardiac myocytes.6 Primers and amplicon lengths were defined as shown in the Table. The reaction was processed in a TaqMan PCR system (Applied Biosystems, Foster City, CA, USA) (Search parameters: ampliconsize 100 bp, annealing-temperature 60°C, GC-content 60%). The reaction mixtures were prepared using the Master SYBR Green PCR kit (Eurorgenetic, Liege, Belgium). Primer sequences and amplicon lengths were defined as shown in the Table.

Statistical Analysis

Numerical results are given as mean±SEM of n observations, where n is the number of tested animals. For analysis of numerical data, Student’s unpaired t test was used to compare means between groups. Probability values ≤0.05 (*) are indicated.

Results

Clinical Manifestations of Infection

Six hours postinjection, clinical manifestations of shock could be observed in LPS-injected controls. These included diarrhea and lethargy beginning 2 hours after injection.
C3H/HeJ mice remained unaffected by LPS injection. These findings are consistent with those reported by numerous groups and confirm the LPS hyporesponsive phenotype of the C3H/HeJ strain and the LPS responsiveness of the control strain.3,5,10

Hemodynamic Results
To demonstrate the fact that LPS induces hemodynamic instability characteristic of septic shock, endotoxin responsive control mice were injected i.p. with E.coli LPS (n=10) or with saline as control (n=10). In response to a 6-hour challenge of LPS 20 mg/kg bodyweight the mean arterial blood pressure (MAP) of control animals was significantly reduced by 29% compared with controls (ie, 65.72±5.27 mm Hg in LPS treated animals; 91.99±2.20 in controls, not shown). The reduction of MAP originated in a LPS-dependent decrease in both systolic (106.12±2.80 mm Hg in controls and 73.30±5.33 mm Hg in treated mice; Figure 1A) and diastolic blood pressure (80.80±2.3 mm Hg in controls and 58.46±5.26 mm Hg in treated mice Figure 1B). The heart rate of LPS-treated control animals was significantly increased by 120% (612.62±44.87 beats per minute [bpm] versus 512.46±18.38 bpm in control mice; Figure 1C). However, this increase in heart rate was not sufficient to compensate for a loss of blood pressure. In TLR4-deficient mice of the C3H/HeJ strain none of the above mentioned parameters was significantly reduced (Figure 1A–C).

Contractility Studies
In Vivo LPS Treatment
To clarify whether the described decrease in blood pressure is related to changes in vasocontractility we recorded concentration response curves of aortic rings. Six hours after LPS injection mice were euthanized and aortic rings prepared. LPS stimulation led to a significant attenuation of contraction force of aortic rings from control mice at PE concentrations of 10⁻⁷ mol/L to 10⁻⁵ mol/L (Figure 2A) whereas arterial contractility of C3H/HeJ mice remained unaffected (Figure 2B).

LPS Induced Hypocontractility Through NO
After treating aortic rings from LPS-injected control mice with the nonspecific NOS-inhibitor, L-NAME in vitro arterial contractility was restored to normal levels at all PE concentrations. Significant differences between L-NAME–treated control rings and L-NAME–treated rings from endotoxinemic animals could not be detected (Figure 2C). In C3H/HeJ mice 10⁻⁹ mol/L to 10⁻⁶ mol/L L-NAME significantly increased contractility compared with contractility of saline-treated animals of the same strain (Figure 2D). The specific iNOS inhibitor SMT was as efficient as L-NAME, leading to complete restoration of the CRC of rings from LPS-treated control animals (Figure 2E).

Ex Vivo LPS Treatment
To determine whether the LPS-induced vascular hypocontractility can be antagonised by the TLR4 antagonist Eritoran, we coincubated aortic rings from control mice with LPS or with LPS and Eritoran in DMEM for 6 hours. Afterward, arterial contractility in response to PE was recorded. LPS-dependent attenuation of vascular contractility was lower in ex vivo stimulated rings. This might be attributable to lower LPS concentrations during ex vivo stimulation compared with LPS concentrations used for in vivo stimulation. Coincubation of Eritoran and LPS completely prevented attenuation of arterial contractility in LPS-treated aortic rings from control mice in comparison to rings incubated only with LPS (Figure 2F).

Expression of Cytokines in the Murine Aorta
To determine which cytokines are increased in aortic tissue after LPS stimulation we quantified the mRNA amounts of IL-1β, IL-6, TNFα. In addition, iNOS and ADM were also monitored.

LPS application for 6 hours led to a significant increase of mRNA for the proinflammatory cytokines IL-1β, IL-6, TNFα in control mice, but not in LPS hyporesponsive C3H/HeJ mice.

LPS stimulation caused a pronounced induction of iNOS in control mice compared with saline-treated control mice. LPS did not induce a significant upregulation of iNOS in LPS hyporesponsive C3H/HeJ mice. Comparable results were detected for adrenomedullin, showing a significant increase
of ADM in LPS-treated control mice but not in LPS hyporesponsive C3H/HeJ mice (Figure 3).

**Discussion**

The aim of our study was to elucidate whether TLR4 is involved in the regulation of vascular contractility during sepsis. Furthermore we wanted to clarify whether TLR4 antagonism can serve as therapeutic target during sepsis.

We demonstrate that blood pressure and vascular contractility are attenuated during septic shock. These results are in accordance with previous findings from other groups, proving the functionality of our model. Additionally our data show that animals lacking a functional TLR4 are not developing LPS-dependent downregulation of circulatory function (Figure 1). A major new finding of this study is that LPS-induced vascular relaxation depends on TLR4 expressed in the vessel wall. Moreover we show for the first time that a vascular block of TLR4 or iNOS can prevent LPS-induced changes of vascular contractility. Thus Eritoran might serve as a new tool in the clinical treatment of septical hypotension.

LPS stimulation leads to an induction of several target genes of the TLR4 cascade, including different proinflammatory cytokines like IL1β, IL6, TNFα, and potent vasodilatators such as iNOS and ADM in aortic tissue. Other groups demonstrated that iNOS-deficient mice show an altered response to LPS stimulation, indicating an important role of iNOS for the generation of...
septic shock. We wanted to elucidate whether direct iNOS inhibition or antagonism of the TLR4 receptor are also able to block LPS-dependent vascular hypocontractility.

Our data show that isolated aortic rings taken from control animals treated with LPS developed a significantly reduced contractility (Figure 2A), which was comparable to rings incubated with LPS in vitro. This demonstrates that circulating immune cells are not necessary for LPS-induced vascular relaxation (Figure 2F). Because there are usually no immune cells like macrophages in the aortic wall, this response has to be induced by local cells, eg, smooth muscle cells or endothelial cells. Smooth muscle cells are known to express functional Toll Like Receptor 2,30 3,31 and 4.32,33 TLR4 has also been localized on endothelial cells.34,35 Therefore, both cell types are susceptible to TLR4-stimulation and thus LPS is able to induce cytokine as well as iNOS expression in both cell types. The importance of TLR4 is further underlined by the observation that rings from C3H/HeJ animals did not show a loss of contractility after LPS treatment (Figure 2B). The vascular relaxation seems to rely mainly on NO as it can be blocked by L-NAME. SMT was also able to prevent vascular relaxation completely, therefore iNOS appears to play a major role. It has been shown in previous studies that the use of nonspecific NO synthase inhibitors prevents hypotension during sepsis.36–39 In this study we confirmed this effect and show furthermore that arterial iNOS expression is upregulated after LPS treatment (Figure 3), and that nonselective and selective inhibition of iNOS completely restored vascular contractility (Figure 2C–D) in aortae from septic mice.

In addition to the regulation of iNOS we wanted to elucidate how other target genes of the TLR4 cascade are regulated after LPS treatment. We were specifically interested in the regulation of ADM as a potent vasodilator.40 It has been shown that inflammatory stimuli led to an increase of ADM expression and thus to increased angiogenesis and

Figure 3. Six hours of LPS stimulation induce an upregulation of cytokines in aortae from control mice, compared with aortae from saline treated controls. LPS hyporesponsive mice of the C3H/HeJ strain show no significant differences in cytokine and iNOS and adrenomedullin (ADM) mRNA levels after LPS injection (n=5 in all groups, *P<0.05, all values are mean±SEM).
vasodilation. Frede et al could show that HIF1α is upregulated in monocytes after LPS stimulation leading to increased ADM expression. We were for the first time able to demonstrate that ADM upregulation is TLR4-dependent in aortic tissue (Figure 3) and might thus play an important role for catecholamine resistant hypotension in sepsis. However, it remains unclear whether this upregulation of ADM is dependent on the upregulation of HIF1α in aortic tissue or induced by a different pathway.

The LPS-dependent upregulation of ADM should lead to pronounced vasodilation. There are supposed to be 2 different pathways leading to ADM-dependent vasodilation: (1) direct increase of cAMP and activation of eNOS, or (2) a combined pathway of cAMP-NP mediated vasorelaxation. Those pathways focus on ADMs influence on NO produced by the NOS isoform eNOS. However our data suggest an additional interaction between ADM and iNOS, because the inhibition of iNOS also completely restored contractility in aortic rings. We therefore assume that in our experimental surrounding ADM-dependent NO is produced mainly by iNOS, not eNOS. The upregulation of iNOS, ADM, and cytokines, after LPS stimulation, was not detected in C3H/HeJ mice (Figure 3).

The most intriguing result of our data are that LPS-induced vascular relaxation can be inhibited by using a competitive inhibitor of TLR4, Eritoran, a second generation analogue of the lipid A component of LPS, prevented the loss of vascular contractility and led to normal vascular contractions after phenylephrine administration (Figure 3F). Baumgarten et al have demonstrated that the use of Eritoran is beneficial to maintain cardiac function and myocyte contractility during endotoxemia. Our data demonstrate that Eritoran is able to protect arterial contractility during sepsis. Thus inhibition of TLR4 by Eritoran might serve as a novel therapeutic concept to prevent cardiac dysfunction and hypotension after endotoxemia. However, further investigations regarding the impact of Eritoran on iNOS regulation are necessary.

Our data provide evidence that LPS-induced attenuation of blood pressure is TLR4-dependent and that the use of selective and nonselective inhibitors of NO synthase can restore vascular contractility during Gram-negative sepsis. Furthermore, the presented data show that the TLR4 is an important target for pharmaceutical modulation of sepsis and that the TLR4 inhibitor Eritoran might improve treatment during sepsis. However, it is still unclear whether Eritoran prevents cytokine upregulation in vivo and LPS-dependent hypotension in vivo.

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Disclosures

None.

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Ehrentraut, S.*, Lohner, R., Schwederski, M., Ehrentraut, H., Boehm, O., Noga, S., Langhoff, P., Baumgarten, G., Meyer, R., and Knuefermann, P. (2011). In vivo Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. Shock 36, 613–620.

Zielsetzung der Arbeit - Sepsis und septischer Schock gehen häufig mit einer akuten kardiovaskulären Depression einher. LPS kann, vermittelt durch den Toll-like-Rezeptor 4 (TLR4) eine septische Organdysfunktion auslösen. Das Ziel dieser Studie war es, die Auswirkungen eines pharmakologischen TLR4-Antagonismus auf die LPS-induzierte kardiovaskuläre Depression in vivo zu untersuchen.

Methoden und Ergebnisse - Um eine gram-negative Sepsis auszulösen, wurden C3H/HeN-Mäuse intraperitoneal mit zwei mg/kg Körpergewicht LPS infiziert. Mit der Absicht, die LPS-Effekte zu antagonisieren, wurde der selektive TLR-Antagonist Eritoran in einer Dosis von vier mg/kg Körpergewicht intravenös verabreicht. Körpere Aktivität, peripherer Blutdruck und die Herzfrequenz wurden vor und nach der LPS- und Eritoran-Injektion aufgezeichnet. Darüber hinaus wurden intrakardiale hämodynamische Parameter (systolischer und diastolischer Blutdruck und Herzfrequenz) und die Bewegungsaktivität mit einem implantierten Kathetersystem telemetrisch erfasst (siehe Figure 1, p. 615, Ehrentraut et al., Shock 2011) In einer separaten Kohorte wurden zusätzlich noch weitere, detailliertere Parameter (end-systolischer Ventrikeldruck, enddiastolischer Ventrikeldruck, Herzfrequenz, Schlagvolumen (SV), Schlagarbeit (SW), Ejektionsfraktion (EF), HZV, maximale erste Ableitung der Änderung des systolischen Druckanstiegs nach der Zeit (dP/dtmax) und maximale erste Ableitung der Änderung des diastolischen Druckabfalls nach der Zeit (dP/dtmin) ermittelt (siehe Figure 2, p. 615, Ehrentraut et al., Shock 2011). Zwei bzw. sechs Stunden nach LPS-Stimulation und Eritoran-Behandlung wurden die Herzen und Aorten entnommen, und die Expression von TLR sowie von Entzündungsmediatoren wurde mit Hilfe der quantitativen Polymerase-Kettenreaktion (Reverse Transkription) und verschiedener Enzymimmunoassays gemessen. Lipopolysaccharid senkte den arteriellen Blutdruck im Laufe der Zeit deutlich. Die Verabreichung von Eritoran verhinderte teilweise die LPS-abhängige Senkung des Blutdrucks und konnte die Herzfunktion weitestgehend erhalten. Darüber hinaus erhöhte LPS die Expression von CD14 und TLR2 im Herz- und Aortengewebe. Im Aortengewebe schwächte Eritoran diesen Anstieg ab, während im Herzen keine signifikante Reduktion beobachtet wurde (siehe Figure 4, p. 615, Ehrentraut et al., Shock 2011). Darüber hinaus waren die mRNA-Spiegel der induzierbaren
Stickstoffmonoxid-Synthetase im Herz- und Aortengewebe sechs Stunden nach der LPS-Applikation signifikant erhöht. Dieser Effekt wurde durch die Anwesenheit von Eritoran verringert.

Schlussfolgerungen - Zusammenfassend lässt sich sagen, dass der positive Einfluss von Eritoran auf die kardiovaskuläre Funktion in vivo hauptsächlich auf der Verringerung der LPS-induzierten induzierbaren Stickstoffmonoxid-Synthetase iNOS-Expression sowie auf einer abgeschwächten Zytokin-Expression in der Gefäßwand zu beruhen scheint. Der erhaltene Gefäßtonus bewirkt zusätzlich eine Stabilisierung der Herzfunktion im Sinne einer erhaltenen Herzfrequenz.
IN VIVO TOLL-LIKE RECEPTOR 4 ANTAGONISM RESTORES CARDIAC FUNCTION DURING ENDOTOXEMIA

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ABSTRACT—Severe sepsis and septic shock are often accompanied by acute cardiovascular depression. Lipopolysaccharide (LPS) signaling via Toll-like receptor 4 (TLR4) can induce septic organ dysfunction. The aim of this study was to elucidate the in vivo impact of pharmacological TLR4 antagonism on LPS-induced cardiovascular depression using eritoran tetrasodium (E5564). To simulate sepsis, C3H/HeN mice were challenged i.p. with 2 mg/kg body weight LPS. With the intent to antagonize the LPS effects, eritoran was administered i.v. (4 mg/kg body weight). Physical activity, peripheral blood pressure, and heart frequency were recorded before and after LPS and eritoran injection. In addition, intracardiac hemodynamic parameters were analyzed with a pressure conductance catheter. After 2 and 6 h of LPS stimulation ± eritoran treatment, the hearts and aortae were harvested, and TLR as well as inflammatory mediator expression was measured using reverse transcription–quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. Lipopolysaccharide significantly decreased arterial blood pressure over time. Administration of eritoran partially prevented the LPS-dependent reduction in blood pressure and preserved cardiac function. In addition, LPS increased the expression of CD14 and TLR2 in cardiac and aortic tissue. In aortic tissue, eritoran attenuated this increase, whereas no significant reduction was observed in the heart. Furthermore, cardiac and aortic inducible nitric oxide synthetase mRNA levels were significantly increased 6 h after LPS application. This effect was reduced in the presence of eritoran. In summary, the beneficial influence of eritoran on cardiovascular function in vivo seems to rely mainly on reduction of LPS-induced inducible nitric oxide synthetase expression as well as on attenuated cytokine expression in the vascular wall.

KEYWORDS—TLR4 antagonism, sepsis, cardiac depression, endotoxemia, eritoran

INTRODUCTION

Sepsis and systemic inflammatory response syndrome are still among the major causes of death in noncardiac intensive care units (1), with sepsis-related mortality increasing despite more differentiated treatment options (2, 3). Multiorgan dysfunction, in particular cardiovascular depression, dramatically increases mortality of sepsis (4).

It is well known that different pathogen-associated molecular patterns, such as bacterial cell components, are able to induce sepsis by the activation of the innate immune system. Pattern recognition receptors (PRRs) are a key feature of the innate immune system. Among the PRRs, Toll-like receptors (TLRs) play an outstanding role for the recognition of exogenous and endogenous ligands, thereby activating a signaling cascade leading to the secretion of proinflammatory cytokines, reactive oxygen species, antimicrobial peptides, chemokines, and acute-phase proteins (5–7). Increased proinflammatory cytokines and vasodilatory mediators result in impaired microcirculation (8), diminished cardiac output (CO), and finally complete cardiovascular failure and death (9).

Important inducers of sepsis are lipopolysaccharide (LPS), cell wall components of gram-negative bacteria, which bind to the TLR4/MD2/CD14 complex (10), thereby inducing proinflammatory cytokines (11). Cytokine induction relies on specific activation of the innate immune system. However, previous studies indicate that cytokine expression via TLR activation is also directly detectable in the heart (6, 12) and aortae of septic animals (13). Activation of the TLR4 pathway causes cardiac dysfunction by depressing sarcomere shortening (14) and impairing vascular tone, resulting in blood pressure decline (13).

Efforts have previously focused on the TLR4/CD14 complex as a possible therapeutic target during endotoxin challenge. Previous studies provide evidence that the LPS analog, eritoran tetrasodium (E5564; ESI, Andover, Mass), successfully prevented LPS-induced cytokine production in human (15) and equine blood monocytes (16), as well as corneal inflammation (17). Furthermore, it has been shown that TLR4 antagonism dampens clinical signs of endotoxemia such as increased body temperature and tachycardia (18, 19). The first phase II clinical trial indicated a trend toward lower mortality in eritoran-treated septic patients at highest risk of mortality according to Acute Physiology and Chronic Health Evaluation II score, albeit unchanged serum levels of the proinflammatory cytokine interleukin 6 (IL-6) (18). Our group was able to show in vitro that LPS-induced cardiac depression and loss of vascular tone were prevented by the use of eritoran (13, 14). Because in vitro efficacy of a substance is often not directly transferable to the in vivo situation, the aim of this study was to test whether an in vivo application of eritoran successfully prevented LPS-induced hypotension and cardiac depression. Furthermore, this
study analyzed the influence of TLR4 antagonism on LPS-induced cytokine production in the murine heart and aorta.

**MATERIALS AND METHODS**

**Animal handling and care**

Ten- to 14-week-old LPS-responsive C57Bl/6J mice of both sexes were purchased from Charles River (Sulzfeld, Germany). Mice were housed in pathogen-free cages with free access to water and standard rodent chow. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85–23, revised 1996), and animal procedures were approved by the local committee for animal care.

**In vivo stimulation**

Eritoran (4 mg/kg body weight [BW]; ESI) or placebo (lactose formulation of eritoran tetradsodium provided by ESI) was injected into the tail vein 15 min before LPS or saline injection into the peritoneal cavity. Lipopolysaccharide was dosed 2 mg/kg BW (E. coli 0113:B5, Sigma-Aldrich Chemical, Munich, Germany). A total fluid volume of approximately 700 µL was applied to each mouse (30 mL/kg BW) by injection of eritoran/placebo and LPS/saline. Animals were further investigated after 2 or 6 h.

**Hemodynamic measurements**

Telemetry—To measure blood pressure, heart rate (HR), and physical activity following endotoxin challenge without cardiodepressive effects of anesthesia, telemetry devices were implanted as described in the studies of Kawada et al. (20) and Bek et al. (21). Briefly, mice were anesthetized using 2% isoflurane (Forene, flow 1 L/min; Abbott GmbH, Ludwigshafen, Germany). A TA11PA-C20 telemetry catheter (Data Sciences International, St Paul, Minn) was introduced into the right carotid artery, and the tip was placed within the aortic arch. The transducer unit was implanted s.c. in the right flank. Animals were treated s.c. with buprenorphine 0.065 mg/kg BW for postoperative analgesia. After a 5-day recovery period, mice received either LPS + placebo or LPS + eritoran as described above. Systolic and diastolic blood pressure, HR, and physical activity (movement detection displayed as counts per minute) were recorded for 5 min every 6 min. Analysis was performed using Dataquest A.R.T. software (Data Sciences International).

**Measurements of cardiac function**—Measurements of cardiac function were performed with a 1.4F pressure conductance catheter (SPR-839; Millar Instruments, Houston, Tex). We used the closed-chest approach, which is the most suitable model to obtain pressure volume data from mice suffering from septic heart failure (22). In brief, mice were anesthetized with 1% isoflurane through a nasal cone without invasive ventilation. Sufficient anesthesia was ascertained by unresponsiveness to tail pinch. After preparation of the right carotid artery, the catheter was inserted, and peripheral blood pressure data were collected. Before insertion, the catheter was calibrated using a standard cylindrical calibration with fresh heparinized blood, and parallel conductance was assessed by injection of a 10-L µL bolus of a hypertonic (10%) saline solution into the left jugular vein. After a short recovery period to allow signals to return to baseline levels, peripheral blood pressure was recorded for 10 min; then, the catheter was advanced through the aortic valve into the left ventricle. After a short period of rest, intracardiac pressure-volume signals were recorded. Because sepsis is capable of inducing hypothermia in rodents, which has significant impact on cardiac performance, we maintained body temperature at 36°C controlled by a feedback loop coupled to a heating device. Pressure-volume signals were digitized using a Powerlab (ADInstruments GmbH, Spechbach, Germany) and continuously recorded using Chart for Windows (version 5.5.5; ADInstruments). Pressure-volume loops were analyzed with the PVAN 3.6 software package (Millar Instruments). The obtained conductance signal itself was noncalibrated and contained parallel conductance signals from the surrounding tissue. The following parameters were derived: end-systolic ventricular pressure, end-diastolic ventricular pressure, HR, stroke volume (SV), stroke work (SW), ejection fraction (EF), CO, maximum first derivative of change in systolic pressure rise with respect to time (dP/dtmax), and maximum first derivative of change in diastolic pressure fall with respect to time (dP/dtmin).

**Cytokine mRNA isolation and real-time PCR**

Animals were challenged with LPS or LPS + eritoran as described above. For RNA extractions, the hearts or aortae were homogenized, and RNA was isolated using the TRIzol reagent (Gibco Laboratories, Grand Island, NY). The RNA was subsequently reverse transcribed using the high-capacity cDNA synthesis kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s protocol. Relative RT-PCR was performed using TaqMan Gene expression Master Mix (part 4369016; Applied Biosystems) with the primers previously described (24): GAPDH (Mm99999915_g1), IL-1β (Mm00443258_m1), IL-6 (Mm00443258_m1), TLR2 (Mm00121945_g1), TLR4 (Mm00445273_m1), inducible nitric oxide synthetase (iNOS) (Mm00440491_g1), and tumor necrosis factor-α (TNF-α) (Mm00443258_m1). The reaction was performed in a TaqMan PCR system (Applied Biosystems), and the results were analyzed by calculating the ratio of target accumulation over GAPDH accumulation.

**Cytokine protein isolation**

At 2 and 6 h after stimulation, hearts were flash frozen in liquid nitrogen (LN2) and conserved at −80°C. Cytosolic proteins were isolated using NE-PER kit (78878; ThermoScientific, Rockford, Ill) according to the manufacturer’s protocol. Enzyme-linked immunosorbent assay was performed using Quantikine Mouse TNF-α (MTA00), IL-1β (MLB00B), or IL-6 (M6000B) kits (all from R&D Systems, Abingdon, UK) according to the manufacturer’s protocol.

**Statistical analysis**

Numerical results are given as mean ± SEM of n observations, n being the number of tested animals, n ≥ 5 in all groups. For analysis of numerical data, Student’s unpaired t test was used to compare means between groups. Groups larger than two were analyzed using one-way analysis of variance with Newman-Keuls multiple-comparisons posttest. P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***). Statistics were calculated using Prism 4.03 (GraphPad Software Inc, San Diego, Calif).

**RESULTS**

**Clinical manifestations of endotoxemia**

At 6 h after injection of LPS, clinical symptoms of endotoxemia were inspected. These included lethargy and nasal discharge beginning 30 min to 2 h after injection of LPS and are concordant with previous findings (6, 12, 13). In animals treated with eritoran and LPS, clinical symptoms were less severe.

**Telemetric recording of arterial pressure and HR**

After implantation of the telemetry devices, mice (n = 4/group) were given a recovery period of 5 days. Blood pressure, HR, and physical activity were continuously monitored in conscious and moving animals after injection of LPS + placebo or LPS + eritoran. Lipopolysaccharide application induced a significant reduction in physical activity compared with untreated controls (−92% ± 17%). The presence of eritoran attenuated the decrease in physical activity (−54% ± 11%; P < 0.05). Lipopolysaccharide treatment induced a transient depression of blood pressure after about 30 min with a temporary recovery. After 2 h, a second phase of blood pressure attenuation began, subsequently resulting in a significant blood pressure fall after 6 h (systolic: LPS −42% ± 14% vs. LPS + eritoran −18% ± 4% compared with baseline blood pressure levels, P < 0.05). Eritoran partially ameliorated this decrease in diastolic blood pressure without reaching statistical significance (diastolic: LPS −42% ± 21% vs. LPS + eritoran −14% ± 2%; P = 0.07). Heart rate in LPS-treated animals was significantly reduced (−39% ± 13%; P < 0.01), whereas eritoran blocked this response. Representative curves of telemetry recordings are shown in Figure 1A.

**Arterial blood pressure in mild anesthesia**

The LPS-induced depression of systolic blood pressure was also detectable in animals investigated during anesthesia. At 6 h after LPS stimulation, mean peripheral systolic blood pressure was reduced by approximately 16% (71.29 ± 4.25 mmHg, n = 8, vs. 84.77 ± 2.66 mmHg in untreated controls, n = 7, P < 0.05; Fig. 1B). Pretreatment with eritoran prevented the loss
of systolic blood pressure after LPS challenge (83.77 ± 3.61 mmHg, n = 7; Fig. 1B). Parallel to the observed changes in systolic blood pressure, diastolic blood pressure was significantly lower after LPS challenge compared with untreated controls (43.68 ± 3.81 mmHg, n = 7, vs. 60.91 ± 3.22 mmHg, n = 8, *P < 0.01). Also in diastolic blood pressure, eritoran prevented the LPS-induced decline (59.13 ± 3.65 mmHg, n = 7), indicating that eritoran preserves hemodynamic function despite LPS challenge.

Hemodynamic changes observed in the peripheral bloodstream were complemented by those detected in the ventricle using a pressure conductance catheter. Intraventricular end-systolic blood pressure in controls amounted to 89.32 ± 3.31 mmHg (n = 7), which was significantly higher than in LPS-treated mice (69.96 ± 2.00 mmHg, n = 7, *P < 0.001). This cardiodepressive effect was partially inhibited by administration of eritoran as end-systolic blood pressure declined only by 12% to 78.85 ± 1.67 mmHg (n = 7), being significantly higher than in the LPS group (**P < 0.001) (Fig. 2A). End-diastolic ventricular pressure showed a trend toward decreased values after LPS challenge without reaching the level of significance (Fig. 2B). Cardiac contractility, measured as dP/dt max and dP/dt min, was also significantly impaired by LPS, and again eritoran prevented this reduction (Fig. 2, C and D; Table 1). Ejection fraction and CO were both significantly reduced after LPS injection compared with control, whereas addition of eritoran blocked LPS-mediated changes (Fig. 2, E and F). Lipopolysaccharide diminished SV as well as SW, and eritoran-treated animals showed better performance in both parameters (Fig. 2, G and H).

Cardiac TLR and cytokine mRNA expression

To determine the inflammatory response induced by LPS, mRNA gene expression of CD14, TLR2, TLR4, TNF-α, IL-1β, IL-6, and iNOS was measured by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) at 2 and 6 h after application. Hearts from animals treated with a single injection of placebo served as controls.

Lipopolysaccharide treatment increased CD14 and TLR2 mRNA expression at both time points (Fig. 3, A and B). Additional application of eritoran did not change the LPS-dependent increase except for CD14 at 6 h after stimulation, where CD14 mRNA was even higher in the LPS + eritoran group than in the LPS group. Compared with the increased mRNA expression of CD14 and TLR2 (>10-fold compared with controls), changes in TLR4 mRNA expression were minor (1.18-fold TLR4; Fig. 3C). Therefore, differences in this group are not further considered.

Fig. 1. Peripheral blood pressure. Original telemetric recordings (A) of HR and systolic and diastolic blood pressure before and after LPS challenge i.p. (marked by vertical dotted line). Thirty minutes after LPS challenge (left panel), blood pressure and HR dropped transiently with subsequent recovery. Starting at about 3 h, all parameters consecutively declined. Animals treated with LPS and eritoran (right panel) showed a diminished initial hemodynamic depression without further reduction. One representative recording of four in each group is plotted. Recording in mild anesthesia (B). Systolic (left panel) and diastolic peripheral blood (right panel) pressures were recorded 8 h after LPS stimulation (mean ± SEM, **P < 0.01, ***P < 0.001, n = 7).

Fig. 2. Intraventricular hemodynamic parameters. End-systolic pressure (A), end-diastolic pressure (B), dP/dt max (C), dP/dt min (D), EF (E), CO (F), SV (G), and SW (H) were recorded by an intraventricular pressure conductance catheter. At 6 h after LPS stimulation, all parameters except end-diastolic pressure were reduced, whereas this effect was less prominent in the presence of eritoran (mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, n ≥ 7).
In cardiac tissue, the mRNA levels of all investigated cytokines were significantly elevated 2 h after LPS stimulation (Fig. 4, A–C). The presence of eritoran had no suppressive effect on mRNA levels, and in fact, TNF-α and IL-6 mRNA expression rose even higher in the LPS + eritoran group. Four hours later, TNF-α, IL-1β, and IL-6 had fallen to a lower level but remained above control level but still above control. Expression did not differ between LPS- and LPS + eritoran-treated mice. At 2 h, iNOS mRNA expression was significantly elevated neither in the LPS nor in the LPS + eritoran group. Four hours later, iNOS mRNA expression was significantly upregulated in both groups. However, the incremental increase in iNOS mRNA was significantly reduced by the presence of eritoran (Fig. 4D).

Cardiac cytokine protein levels
To further confirm the influence of eritoran on cardiac cytokines, protein levels were monitored additionally. At 2 h after LPS injection, a significant increase in TNF-α, IL-1β, and IL-6 protein levels compared with the untreated control group was detected (Fig. 5A. The addition of eritoran significantly attenuated this increase in TNF-α protein levels (Fig. 5A). In contrast, the elevated IL-1β and IL-6 protein levels were independent of the presence of eritoran (Fig. 5, B and C). At 6 h after LPS injection, only the protein level of IL-1 remained slightly elevated compared with control (Fig. 5B).

Aortic TLR and cytokine mRNA levels
In accordance with the measurements in the heart, mRNA expression of the same PRRs and cytokines was investigated in aortic tissue. Here, LPS caused again a clear and highly significant increase in CD14 and TLR2 mRNA expression at 2 and 6 h after challenge (Fig. 6, A and B). At both time points, the presence of eritoran reduced the LPS-dependent upregulation of the two PRRs. However, the eritoran effect on CD14 2 h after LPS challenge did not reach the level of significance. Interestingly, eritoran was able to attenuate the increased expression of both PRRs dramatically 6 h after stimulation. Like in cardiac tissue, LPS challenge had only a minor influence on TLR4 expression (Fig. 6C).

After 2 h of LPS treatment, a significant increase in mRNA levels was observed for the three investigated cytokines in aortic tissue (Fig. 7, A–C). Eritoran limited this increase in all three cytokines; however, statistical significance was reached only for IL-6 (Fig. 7C). At 6 h after LPS application, TNF-α, IL-1β, and IL-6 mRNA levels remained significantly elevated above control. The presence of eritoran significantly suppressed the mRNA expression of all three cytokines down to control level.

Inducible nitric oxide synthetase mRNA expression remained unaffected after 2 h of stimulation. At 6 h, iNOS mRNA was significantly increased in the aortic tissue of LPS-treated mice. The presence of eritoran prevented this augmentation of iNOS mRNA (Fig. 7D).

DISCUSSION
The main result of this investigation is that prophylactic injection with the TLR4 antagonist, eritoran, in vivo significantly attenuates hemodynamic depression during endotoxemia.

![Fig. 3. Cardiac PRR mRNA levels.](image)

The expression of CD14 (A), TLR2 (B), and TLR4 (C) was measured by RT-qPCR at 2 and 6 h after stimulation. Levels of CD14 and TLR2 were significantly elevated after LPS treatment at both time points. In the LPS + eritoran group, receptor expression was not lower than in the LPS group. All treatments caused minor changes in the expression levels of TLR4 (mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, n ≥ 5).

**TABLE 1. Hemodynamic parameters 6 h after LPS or LPS + eritoran treatment**

| Parameter | Control | LPS | Control vs LPS | LPS + eritoran | LPS vs LPS + eritoran |
|-----------|---------|-----|----------------|---------------|----------------------|
| HR, beats/min | 569.1 ± 0.65 | 581.5 ± 2.0 | NS | 638.7 ± 1.1 | 9 | <0.01 |
| ESP, mmHg | 89.32 ± 0.01 | 69.96 ± 0.02 | <0.001 | 78.85 ± 1.6 | 7 | <0.05 |
| EF, % | 60.35 ± 0.01 | 43.72 ± 2.1 | <0.001 | 55.39 ± 2.1 | 9 | <0.001 |
| CO, μL/min | 6,902 ± 0.09 | 5,375 ± 2.7 | <0.05 | 7,087 ± 4.2 | 9 | <0.01 |
| dP/dt max, mmHg/s | 8,514 ± 0.08 | 5,780 ± 1.7 | <0.001 | 8,325 ± 5.7 | 9 | <0.001 |
| dP/dt min, mmHg/s | -8,059 ± 0.09 | -5,266 ± 2.9 | <0.05 | -7,964 ± 5.8 | 6 | NS |
| SV, μL | 12.15 ± 0.06 | 9.301 ± 0.4 | NS | 11.49 ± 0.5 | 8 | NS |
| SW, mmHg * μL | 903 ± 0.00 | 534 ± 2.9 | <0.001 | 730.3 ± 4.4 | 8 | <0.01 |

ESP indicates end-systolic pressure; dP/dt max, maximal first derivative of pressure rise; dP/dt min, maximal first derivative of pressure fall; NS, not statistically significant.
These findings expand previous in vitro results to the clinically more relevant living organism (13, 14).

In conscious C3H/HeN mice, LPS depressed blood pressure, HR, and individual activity, indicating that this in vivo model of endotoxemia resembles the clinical and hemodynamic changes during sepsis (4). Here, an initial and transient loss of blood pressure followed by a temporary recovery after 2 h of LPS was detected. Subsequently, blood pressure, HR, and physical activity decreased continuously (Fig. 1A). To our knowledge, this biphasic course has so far not been described in vivo. Furthermore, Baumgarten et al. (14) showed that LPS application depressed sarcomere shortening of isolated cardiac myocytes in a biphasic time course consisting of an initial depression within the first hour and a second fall after 5 h. Thus, the observed hemodynamic changes in vivo are in accordance with the detected time course of depression of sarcomere shortening in vitro. The HR recorded in our study after LPS challenge did not exceed the physiological values. Thus, the murine model of sepsis does not fully resemble a hyperdynamic state in clinical sepsis. Application of eritoran attenuated the response to LPS by preserving HR and blood pressure. In addition, the physical activity of eritoran-treated animals was less affected by LPS, indicating a better physical condition.

Intracardiac catheter recordings performed under anesthesia allowed a more detailed hemodynamic characterization. These recordings revealed that eritoran prevented or at least attenuated the LPS effects on cardiac function by significantly increasing HR, end-systolic pressure, SV, EF, and dP/dtmax as well as dP/dtmin. Thus, the observed increase in CO is based on both, an elevated HR and an elevated SV. A possible increase in venous return leading to higher preload might also contribute to the observed improvements in CO. One plausible explanation might be that eritoran decreased fluid leakage and thereby improved venous return in accordance to the increase in end-diastolic ventricular pressure. Interestingly, the LPS-induced blood pressure decrease seemed to be more pronounced in conscious animals with implanted sensors. A possible explanation for the observed differences might be linked to the fact that telemetric data can be obtained from severely compromised animals, which would not survive Millar catheterization. In vivo preservation of hemodynamic parameters due to eritoran treatment may be explained by an additive pharmacological effect on the heart and the vascular system. The effect of eritoran on the cardiovascular system has already been demonstrated on isolated cardiac myocytes as well as on isolated aortic rings; thus, the results presented here consequently expand previous in vitro findings to the in vivo situation (13, 14). With respect to the clinical situation of sepsis, cardiovascular function was also monitored in conscious animals, where eritoran clearly improved hemodynamic function.

We are well aware that, in the clinical setting, fluid resuscitation is an important component to maintain hemodynamic stability during sepsis. The importance of fluid resuscitation has also been demonstrated in a murine model of sepsis (26). However, the aim of this study was to investigate whether eritoran is able to antagonize cardiovascular depression during sepsis. Thus, a model with moderate fluid resuscitation was chosen to gain maximal cardiovascular response. Even under these circumstances, the beneficial effects of eritoran developed.

For a better understanding of the underlying mechanisms responsible for the observed improvements, we monitored PRR...
expression as well as cytokine and iNOS expression in cardiac and aortic tissue 2 and 6 h after LPS application. Toll-like receptor 2 and CD14 mRNA expression were upregulated by LPS in both tissues at both time points. A significant reduction of this LPS-dependent change was obtained by etororan in aortic but not in cardiac tissue. Lipopolysaccharide-dependent upregulation of TLR2 has already been described in epithelial (25) and endothelial cells (26) but so far not in the intact cardiovascular system. Lipopolysaccharide-dependent upregulation of CD14 in the heart has already been described (12, 27). Thus, upregulation of TLR2 and CD14 may be seen as a reaction to the inflammatory stimulus, which seems to be sensitive to etororan treatment in the vascular system. Recently, it has been shown that CD14 is the coreceptor not only of TLR4 but also of TLR7 and TLR9 (28). Thus, the upregulation of CD14 might be regarded as the organism’s attempt to sensitize a key regulatory molecule for inflammatory responses. Downregulation of CD14 by etororan in aortic tissue might lead to a lower susceptibility for pathogens and therefore explain the preserved vascular function during endotoxemia previously described by our group (13).

Comparable to CD14 and TLR2, LPS treatment caused a profound increase in TNF-α, IL-1β, and IL-6 mRNA levels in both tissues at both time points. In aortic tissue, etororan attenuated the LPS-dependent cytokine induction at both time points. However, in cardiac tissue, TNF-α and IL-6 mRNA expressions were induced to even higher levels in the presence of etororan 2 h after LPS application. This etororan-associated increase in cardiac cytokine mRNA expression was not resembled in the respective protein expressions. In particular, TNF-α protein level at 2 h was even significantly reduced in the presence of etororan. Interleukin 1β and IL-6 protein levels were not affected by a single dose of the TLR4 antagonist.

Four hours later, cardiac TNF-α and IL-6 protein levels had returned to baseline. Posttranscriptional or posttranslational processes may explain these differences between mRNA and protein expression. The present findings underline the data from the phase II trial, where etororan did not change the serum levels of IL-6, regardless of dose (18).

The highest proinflammatory cytokine levels were accompanied by an initial recovery of peripheral blood pressure 2 h after LPS treatment independently of etororan application (Fig. 1). During further progression of endotoxemia, cardiac and arterial iNOS expression increased, reaching a high level after 6 h, whereas cytokine levels have declined (Figs. 4 and 7). The time course of cytokine and iNOS expression indicates that iNOS upregulation may depend on prior cytokine expression. As the main decrease in arterial blood pressure starts after 2 h, increasing iNOS expression may be an important determinant of cardiovascular performance.

In summary, a differential effect of etororan on proinflammatory cytokine expression was observed in cardiac and vascular tissue. In the heart, etororan did not reduce proinflammatory cytokine expression consistently, whereas vascular cytokine mRNA expression was reduced at both time points by the use of etororan. Thus, the vascular cytokine expression seems to resemble the etororan effect on blood pressure.

Cardiac iNOS mRNA expression was not affected by LPS stimulation after 2 h but was significantly augmented after 6 h. Application of etororan significantly dampened this response. Beneficial influence of etororan on iNOS mRNA expression is even more distinct in aortic tissue, where iNOS mRNA expression remained at control levels. Interestingly, LPS-dependent upregulation of iNOS has recently been shown to be MyD88-dependent/TRIF-independent (29, 30). Thus, one might conclude that etororan inhibits at least the MyD88-dependent pathway.
It has been shown that high concentrations of nitric oxide (NO) impair cardiac function through activation of protein kinase G (31) and by Ca\(^{2+}\) desensitization of troponin I (32). Lower concentrations of NO appear to increase myocardial contractility by opening voltage-gated calcium channels (33) and may thus increase intracellular calcium (34, 35), allowing better contractility. Previous recordings of isolated cardiac myocytes revealed that LPS-induced attenuation of sarcomere shortening could be antagonized by the application of eritoren as well as by application of the iNOS blocker S-methyl-isothiourea (14). Accordingly, increased myocardial iNOS levels led to elevated blood plasma nitrite/nitrate levels, resulting in circulatory failure (30, 36). The here observed reduction of cardiac iNOS mRNA expression by eritoren may explain the observed improvement in cardiac contractility during endotoxemia.

As mentioned above, eritoren prevented LPS-induced iNOS expression in the blood vessel wall. This lowered iNOS expression might be the key issue in preserving vascular tone in vivo. It was previously demonstrated in vitro that eritoren was able to restore LPS-dependent loss of vascular contractility, thus maintaining blood pressure and circulation (13).

We were now able to transfer our previous in vitro results into an in vivo model of sepsis and prove for the first time the beneficial impact of TLR4 antagonism on cardiac failure and blood pressure loss. The reduction of iNOS expression by TLR4 antagonism appears to be one of the crucial points resulting in attenuated hemodynamic depression and improved activity after LPS challenge.

Because earlier attempts have failed to generally block NO production (37, 38) and neutralize the biological activity of TNF-α (4) during sepsis failed, different approaches have to be found to preserve cardiac function. Upstream inhibition of the pathways leading to increased NO and TNF-α might suit this purpose. Our findings now link the beneficial effects of eritoren as a prophylactic agent against experimental gram-negative sepsis in vivo to preserved cardiac function providing evidence for functional and possibly clinical relevance of TLR4 antagonism. Further tests with eritoren, especially in a setting where eritoren is administered following the endotoxic stimulus, are needed to better evaluate its clinical potential.

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Zielsetzung - Ziel war es, die spezifische Rolle von verschiedenen Musterererkennungsrezeptoren des angeborenen Immunsystems für vaskuläre Dysfunktion während einer polymikrobiellen Sepsis (Colon-Ascendens-Stent-Peritonitis, CASP) zu klären. Zudem sollte die pharmakologische Wirksamkeit eines TLR9 spezifischen Antagonisten für die Gefäßkontraktilität untersucht werden.

Methoden und Ergebnisse - Die vaskuläre Kontraktilität von C57BL/6-Mäusen (Wildtyp) und Mäusen, denen jeweils einer der Toll-like-Rezeptoren 2/4/9 (TLR2-Defizient, TLR4-Defizient, TLR9-Defizient) oder der Ko-Rezeptor CD14 (CD14-Defizient) fehlte wurde 18 Stunden nach CASP mittels Mulvany-Myographie am isolierten Aortenring gemessen. Zur Erfassung der Auswirkungen einer TLR9 Blockade auf die Gefäßkontraktilität wurden Wildtypmäuse mit 0,2mg/kg Körpergewicht des selektiven TLR9 Antagonisten H154-Oligodeoxyneukleotid (-ODN) injiziert. 24 Stunden später erfolgte die CASP Prozedur und erneute 18h später die Messung der Gefäßkontraktilität. Die mRNA-Expression von pro- TNFa, IL-1ß, IL-6) und dem anti-inflammatorischen Zytokin IL-10 sowie iNOS wurde mittels quantitativer RT-PCR bestimmt.

Wildtyp-Mäuse wiesen nach Auslösen einer polymikrobiellen Sepsis durch CASP einen signifikanten Verlust der vaskulären Kontraktilität auf. Dies wurde bei TLR2-defizienten-Mäusen verschlimmert, bei TLR4-defizienten-Tieren abgeschwächt und bei TLR9-defizienten- und CD14-defizienten-Tieren aufgehoben (siehe Figure 3, p.5, Ehrentraut et al., PLoS 2012). Die TNF-a-Expression wurde nach CASP bei Wildtyp- und TLR2-defizienten-Tieren signifikant erhöht, nicht jedoch bei Mäusen mit TLR4-, -9- oder CD14-Mangel. iNOS war nur im aortalen Gewebe von TLR2-defizienten-Tieren signifikant erhöht. Tiere ohne TLR2 wiesen zudem signifikant höhere Konzentrationen von TLR4, TLR9 und CD14 im Gefäßgewebe auf. Die Verabreichung von H154-ODN, einem TLR9-Antagonisten, schwächte die CASP-induzierte Zytokinfreisetzung und die vaskuläre Dysfunktion bei Wildtyp-Mäusen ab (siehe Figure 5, p.7, Ehrentraut et al., PLoS 2012).

Schlussfolgerungen - In unserem Modell spielen CD14 und TLR9 eine entscheidende Rolle für die Entwicklung der vaskulären Dysfunktion und können mit H154-ODN
wirksam bekämpft werden. TLR2-D-Tiere sind anfälliger für polymikrobielle Sepsis, vermutlich aufgrund der kompensatorischen Hochregulierung von TLR4, TLR9 und CD14.
Vascular Dysfunction following Polymicrobial Sepsis: Role of Pattern Recognition Receptors

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Abstract

Aims: Aim was to elucidate the specific role of pattern recognition receptors in vascular dysfunction during polymicrobial sepsis (colon ascendens stent peritonitis, CASP).

Methods and Results: Vascular contractility of C57BL/6 (wildtype) mice and mice deficient for Toll-like receptor 2/4/9 (TLR2-D, TLR4-D, TLR9-D) or CD14 (CD14-D) was measured 18 h following CASP. mRNA expression of pro- (Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-1β, IL-6) and anti-inflammatory cytokines (IL-10) and of vascular inducible NO-Synthase (iNOS) was determined using RT-qPCR. Wildtype mice exhibited a significant loss of vascular contractility after CASP. This was aggravated in TLR2-D mice, blunted in TLR4-D animals and abolished in TLR9-D and CD14-D animals. TNF-α expression was significantly up-regulated after CASP in wildtype and TLR2-D animals, but not in mice deficient for TLR4, -9 or CD14. iNOS was significantly up-regulated in TLR2-D animals only. TLR2-D animals showed significantly higher levels of TLR4, -9 and CD14. Application of H154-ODN, a TLR9 antagonist, attenuated CASP-induced cytokine release and vascular dysfunction in wildtype mice.

Conclusions: Within our model, CD14 and TLR9 play a decisive role for the development of vascular dysfunction and thus can be effectively antagonized using H154-ODN. TLR2-D animals are more prone to polymicrobial sepsis, presumably due to up-regulation of TLR4, 9 and CD14.

Introduction

The innate immune system detects and binds pathogen associated molecular patterns (PAMPs) [1] by pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs) [2]. The consecutive transcellular signaling initiates an inflammatory response and can culminate into the clinical picture of sepsis [3,4]. The high mortality of this disease is mostly due to an evolving multi organ dysfunction caused in part by malperfusion due to reduction of total peripheral resistance [5]. Different sepsis models have been established to broaden the understanding of the underlying molecular mechanisms. These approaches include stimulation with distinct TLR agonists, like the TLR4 ligand lipopolysaccharide (LPS) from Gram-negative bacteria [6] or the TLR2 agonist lipoteichoic acid (LTA) from Gram-positive bacteria [7]. It has been shown that LPS induces vascular dysfunction in mice; this can be attenuated using the TLR4 antagonist Eritoran in vitro and in vivo enhancing vascular function and cardiac output [8,9]. Vascular dysfunction is further aggravated by LPS-dependent impairment of cardiac contractility culminating in septic heart failure [10]. Furthermore, it has been shown, that the soluble pattern recognition receptor for LPS CD14 is necessary for recognizing low amounts of LPS [11] and CD14-deficient mice are nearly insensitive to Gram-negative septic shock [5]. However, clinical sepsis is often not caused by a mono-virulent infection but by a polymicrobial stimulus. This type of sepsis is much more complex although it appears to be mediated through TLRs as well [12]. A multibacterial setting of sepsis is likely to stimulate several TLRs simultaneously, due to the appearance of various cell wall components and bacterial DNA. Interestingly, different experimental approaches to induce sepsis appear to stimulate variable pathways for the induction of vascular dysfunction [13,14].

Contrary to experimental endotoxaemia, where loss of vascular function is associated with iNOS-dependent pathways [8,10], work by Wang and colleagues indicates that experimental polymicrobial sepsis, utilizing the caecal ligation and puncture (CLP) model, resulted in loss of vascular contractility independent of NO production [13]. Hence, there might be differences in the organism’s reaction to multibacterial stimuli in regard to which specific TLR is stimulated. There is evidence in the literature, that TLR9 is crucial for the induction of polymicrobial sepsis. Pillas et
al. showed that TLR9-deficient mice have lower serum proinflammatory cytokine levels and a higher bacterial clearance during polymicrobial sepsis resulting in improved survival [15]. Furthermore, it has been shown that TLR9 antagonism attenuates sepsis-induced kidney failure [16].

The aim of this study was to further elucidate the role of different TLRs for their specific impact on vascular contractility during polymicrobial sepsis. Furthermore, we were interested, whether pharmacological inhibition of the relevant receptor might be a suitable target for treatment of vascular dysfunction during polymicrobial sepsis.

Materials and Methods

Animal Handling and Care
12- to 14-week-old C57BL/6 (wild-type, WT) mice of both genders were purchased from Charles River (Sulzfeld, Germany). TLR2- (TLR2-D), TLR4- (TLR4-D), CD14- (CD14-D) and TLR9-deficient (TLR9-D) mice were kindly provided by Professor Shizuo Akira (Osaka University, Osaka, Japan) and backcrossed to a C57BL/6 background. Mice were housed in individually ventilated cages with free access to rodent chow and water with 12 hours automated light/dark cycles.

Ethics Statement
All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Treatment protocols were approved by the district government of Northrhine-Westphalia, here the “State office for protection of environment, nature and consumers” in Recklinghausen is the responsible agency. The approval number for this project is: LANUV 8.87-51.04.20.09.391.

Figure 1. Effects of CASP and expression of PRR expression in the murine vessel wall. PRR mRNA expression during control conditions and following CASP-surgery (TLR2 (A), TLR4 (B), TLR9 (C), CD14 (D)). TLR2-D mice showed significantly higher baseline levels of TLR4 (B), TLR9 (C) and CD14 (D) compared to the other groups. All PRRs appeared to be regulated after CASP; however, only TLR2 and CD14 mRNA expression reached the level of significance (A, D; *p<0.05; **p<0.01; ***p<0.001; n=5 animals in each group; mean ± SEM). doi:10.1371/journal.pone.0044531.g001
Colon Ascendens Stent Peritonitis (CASP)

The CASP-model was chosen to induce polymicrobial sepsis [17]. Mice were anaesthetized with 1.5vol% isoflurane (Forene, Abbot GmbH, Ludwigshafen, Germany) with an oxygen flow of 1 l/min using a nasal cone. Sufficient anesthesia was ascertained by non-responsiveness to a tail pinch. A small midline incision was performed to open the peritoneal cavity. The caecum was exposed under sterile conditions, followed by insertion of an 18 gauge stent into the colon ascendens, which was kept in place with 7/0 Ethilodon thread (Ethicon, Norderstedt, Germany). After ascertaining free passage of feces through the stent, to ensure a save intraluminal placement, the caecum was relocated into the peritoneal cavity and the abdomen was closed with Ethilodon 5/0 thread. Sham-operated animals underwent the same procedure (laparotomy and caecal exposure) without stent insertion. To prevent hypothermia during surgery, the animal was placed on a heating pad and, in addition, body temperature was controlled via a rectal probe regulating an infrared lamp.

All mice received a single subcutaneous injection of 0.1 mg/kg bodyweight buprenorphine (in approx. 500 µl sterile PBS) for analgesia and fluid resuscitation.

Bacterial Culture

At the beginning of experiments and 18 h after CASP the peritoneal cavity was flushed with 3 ml of a sterile phosphate buffer solution (PBS) and aspirated into a sterile syringe. Aliquots of a serial log dilution of this peritoneal lavage fluid were plated on Columbia blood agar and MacConkey agar to select for either gram-positive or Gram-negative bacteria. The cell culture dishes were incubated for 48 h at 37°C and the amount of colony forming units (CFU) was evaluated.

Injection of TLR9 Antagonist H154-thioate

The immunosuppressive oligodeoxynucleotide (ODN) H154-thioate (5′-GCTCAA CGTGTAGGGG-3′) acts specifically via TLR9 [18]. In our setting we injected 0.2 mg/kg BW (approx. 50 µg/mouse) into the tail vein 24 h prior to CASP surgery to mimic a prophylactic preoperative treatment option.

LTA Treatment Protocol

Purified Staphylococcus aureus lipoteichoic acid (LTA; 15 mg/ kg; InvivoGen, San Diego, USA) dissolved in sterile, endotoxin free LAL water (InvivoGen) was injected intraperitoneally (i.p.). Phosphate buffered saline injections were used as negative controls. 6 h after stimulation aortic ring contractility was measured as described below.

Mulvany Myograph and Contractility Studies

18 h after CASP procedure, mice were put under deep isoflurane anaesthesia (>2vol%, sufficient depth was ascertained by non-response to tail pinch) and sacrificed by decapitation to prevent structural stress to the aorta. Vessels were then prepared and measured as described previously [8]. Isometric contractions were digitized using a Powerlab (ADInstruments GmbH, Spechbach, Germany) and continuously recorded using Chart for Windows (Version 5.5.5, ADInstruments).

Cytokine mRNA Isolation and RT-qPCR

Tissue was harvested 18 h after CASP or sham surgery. The aortae were homogenized and RNA was isolated using the thiooctanate-phenol-chloroform method [19]. Afterwards, RNA was purified using the Micro-to-Midi Total RNA purification kit (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany; Part No. 4368814) according to the manufacturer’s protocol. Relative RT-PCR for Actin, IL-1, IL-6, IL-10, TNFα, iNOS, TLR-2, TLR-4, TLR-9, and CD14 was performed using TaqMan Gene expression Master Mix (Applied Biosystems, Part No. 4369016) with the primers previously described [8,20]. The reaction was processed in a TaqMan PCR system (Applied Biosystems), and the results were analyzed by calculating the ratio of target accumulation over GAPDH accumulation.

Statistical Analysis

Numerical results are given as means ± standard error of the mean (SEM) of n observations, n being the number of tested animals. For analysis of numerical data, Student’s unpaired t test or 1-Way ANOVA with Newman-Keuls multiple comparison post-hoc test were used where appropriate using Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA). Probability values <0.05 (*), <0.01 (**), <0.001 (***), and <0.001 (****) are indicated.

Results

Clinical Symptoms and Mortality Following CASP

18 h after CASP surgery, clinical manifestations of infection were observed. These findings included lethargy, nasal and ocular discharge and piloerection, beginning as early as 2 h after surgery, and were most prominent in TLR2-D mice and not observable in TLR4-D, TLR9-D or CD14-D mice. Sham operation did not induce any obvious clinical symptoms.

Intraperitoneal Bacteria Counts

To validate the polymicrobial sepsis model, bacterial cultures were prepared and evaluated regarding the amount of bacteria in peritoneal lavages.

We did not detect any bacteria in the peritoneal cavity of WT sham animals. CASP-surgery induced a significant increase in the amount of bacteria in the peritoneal cavity of CASP-WT leading to $3.044 \times 10^7$ ($\pm 1.5359 \times 10^7$, n = 10; p<0.05 vs. 0 h) Gram-negative and $1.224 \times 10^6$ ($\pm 3.40552 \times 10^5$, n = 5; p<0.05 vs. 0 h) Gram-positive CFUs after 18 h. This indicates liberation of feces into the abdominal cavity after CASP surgery. Furthermore, there were no significant differences in the amount of bacteria released to the peritoneal cavity of TLR2-D or TLR9-D animals (TLR-9D: gram-positive $3.900 \times 10^6 \pm 1.444 \times 10^6$, TLR-9D: gram-negative $2.678 \times 10^6 \pm 1.048 \times 10^6$, n = 5; TLR-2-D: gram-positive $2.8 \times 10^6 \pm 8.74 \times 10^5$, gram-negative $2.702 \times 10^7 \pm 1.343 \times 10^7$, n = 5).
Figure 3. Arterial contractility 18 hours after CASP. A A significant hypocontractility of aortic rings in CASP- WT animals, but not in sham-operated WT animals was observed at phenylephrine concentrations of $10^{-7}$ to $10^{-3}$ M. B TLR2-D mice exhibited a significantly lower maximal contractile response compared to WT animals (TLR2-D 19.07 ± 0.454 mN vs. WT 23.59 ± 0.872 mN; p < 0.05). C Significant reduction of vascular contractility following CASP was also observed in TLR4-D mice compared to controls (p < 0.05), but it was less severe than in WT animals. D, E CASP
TLR4, -9 and CD14 were Up-regulated in Aortae of TLR2-D Animals Previous to CASP Surgery

PRR-dependent signal strength depends in part on the amount of receptor expression. Therefore, mRNA expression of PRRs in the aortic wall was investigated in a first step. TLR2-D mice showed a significantly higher baseline expression of TLR4, TLR9 and CD14 (Fig. 1B-D; the ablated receptor was not monitored in the respective group). In contrast, the baseline expression of PRRs in the aorta was not significantly influenced by gene ablation of TLR4, -9 and CD14 (Fig. 1A-D). CASP surgery led to a significant elevation of TLR2 mRNA in TLR4-D animals (Fig. 1A) and a significant, 2.5-fold up-regulation of CD14 mRNA (Fig. 1D) in all groups. The observed significantly higher baseline expression of TLR4 mRNA in TLR2-D animals compared with all other groups remained unaffected by CASP surgery (Fig. 1B). But the high baseline expression of TLR9 mRNA in aortae of TLR2-D animals was blunted by 18 h of polymicrobial stimulation, as significant differences to WT animals could not be detected any more (Fig. 1C). The co-receptor CD14 was already expressed to the highest level before CASP surgery in TLR2-D sham animals. During polymicrobial sepsis, the amount of CD14 mRNA was significantly up-regulated in all genotypes. Again, CD14 mRNA expression reached the highest level in TLR2-D animals, but was not significantly higher than in the other TLR-deficient groups after CASP surgery (Fig 1D).

CASP-induced Cytokine Induction was Significantly Increased in TLR2-D Mice

To monitor vascular inflammation, we investigated the pro- and anti-inflammatory cytokine levels in the aortic wall. Baseline cytokine levels did not differ between genotypes (not shown). In WT mice CASP-surgery induced a significant increase in the mRNA-level of the pro-inflammatory cytokine TNF-α (Fig. 2A). None of the investigated pro-inflammatory cytokines was significantly up-regulated due to CASP in TLR4-D, TLR9-D or CD14-D mice (Fig. 2A-C).

In contrast, CASP caused a significantly higher expression of TNF-α, IL-1β and IL-6 mRNA in TLR2-D mice compared to all other groups (Fig. 2A-C). The anti-inflammatory cytokine IL-10 was up-regulated in WT, TLR2-D and CD14-D animals following CASP, albeit not significantly (Fig. 2D). The up-regulation of the inducible NO-synthase (iNOS; NOS2) can lead to a reduced vascular tone, therefore iNOS mRNA expression following CASP was checked. Interestingly, iNOS was significantly up-regulated only in TLR2-D animals compared to all other groups (Fig. 2E).

Polymicrobial Sepsis Induced a Profound Loss of Vascular Contractility

To determine vascular contractility during polymicrobial sepsis, we measured the vessel’s response to ascending doses of phenylephrine (PE) 18 h after CASP surgery. Sham-operated WT compared to untreated animals showed a non-significant reduction of vascular contractility of 17.86% (Fig. 3A, F). CASP surgery, in contrast to sham-surgery, induced a significant loss (45.8%) of vascular tone, resulting in decreased maximal response, and decreased hill-slope as well as increased EC50 (Fig. 3A, F). Parameters of all sigmoidal-concentration-response curves to PE are collected in Table S1.

TLR-deficiency Influences CASP-induced Vascular Dysfunction

Untreated TLR2-D mice showed a significantly reduced vascular contractility following α1-stimulation compared to mice from all other strains (Tab. 1; supplement). CASP induced a significant impairment of vascular contractile force with an overall loss of 70.12% in TLR2-D animals compared to their respective controls (Fig. 3B, F). This was the most severe reduction of contractility of all groups. TLR4-D mice showed only a slight albeit significant reduction of contractile force after 18 h following CASP (~26.49% vs. control, Fig. 3C, F).

In contrast, CASP-operated TLR9-D animals did not respond differently to PE compared to their respective controls (~12.83% vs. control, Fig. 3D, F). Our findings provide evidence that TLR9 might be a very important receptor for the induction of vascular dysfunction in polymicrobial sepsis, since TLR9-D mice preserved vascular contractility.

There were significant differences in the vascular contractile response of untreated CD14-D mice compared to the untreated WT, TLR2-D and TLR4-D animals, visible in a higher EC50 (Table S1). Thus, this mouse strain seems to react less sensitive to α1-agonist stimulation than all other groups. With respect to polymicrobial sepsis CD14-D mice reacted comparable to TLR9-D animals, i.e. vascular tone did not change significantly following CASP surgery (~13.39% vs. controls, Fig. 3D, E, F). Therefore, signaling through CD14 appears to be similarly crucial to mediate the polymicrobial stimulus as TLR9.
Since in TLR2-D mice vascular tone was already strongly reduced in the control group, we were interested in the effects of specific TLR2 stimulation with LTA in WT mice. Application of LTA lowered the EC50 of phenylephrine leading to a small but significant improvement of contractile function of the vessel wall in the intermediate concentration range of phenylephrine (Fig. 4). Maximal force remained unchanged (not shown).

**Figure 5. Prevention of CASP induced aortic cytokine and iNOS production following TLR9 antagonist treatment.**

A-E 24 h of exposure to the synthetic TLR9 antagonist H154-thioate did not induce mRNA expression of the investigated cytokines, but prevented the CASP-dependent rise in mRNA expression of inflammatory mediators. There was no observable influence of H154-thioate on iNOS expression. F H154-thioate treatment prior to CASP completely prevented the CASP-induced arterial hypocontractility observed in WT animals (F) (*p<0.05; **p<0.01; ***p<0.001; n=5 animals in each group; mean ± SEM).

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**Vascular Contractility After TLR2 Stimulation**

Since in TLR2-D mice vascular tone was already strongly reduced in the control group, we were interested in the effects of specific TLR2 stimulation with LTA in WT mice. Application of LTA lowered the EC50 of phenylephrine leading to a small but significant improvement of contractile function of the vessel wall in the intermediate concentration range of phenylephrine (Fig. 4). Maximal force remained unchanged (not shown).
Discussion

The aim of this study was to elucidate the impact of polymicrobial sepsis on vascular contractility and to assess the role of different Toll-like receptors in this setting. It is well known that specific TLR-stimulation increases the expression of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6. This is associated with the development of cardiac dysfunction [21]. However, this study demonstrates for the first time that TLR9-antagonism revealed to be decisive during polymicrobial sepsis as vascular tone of TLR9-D mice remained unaffected while that of WT, TLR2-D, and TLR4-D mice was significantly reduced. Furthermore, in mice lacking TLR9 the pro-inflammatory mediators TNF-α, IL-1β and IL-6 were not raised by CASP. The baseline expression of PRRs was not influenced by the gene ablation of TLR9 itself. To test the role of TLR9 in WT mice we injected these with the TLR9-specific antagonist H154-thioate. Increased expression of TLR2 seems to be meaningful as this receptor is important for Gram-positive signaling, CD14 has been described as soluble LPS receptor and TLR4-cocoreceptor [28]. This view is based on earlier findings by Haziot [29] and Knuefermann [5] showing that CD14-deficiency leads to LPS insensitivity. At the first glimpse the up-regulation of CD14 might be taken as a compensatory mechanism for the missing TLR4. However, CD14 has further functions, which are discussed in detail below.

TLR9 signaling revealed to be decisive during polymicrobial sepsis as vascular tone of TLR9-D mice remained unaffected while that of WT, TLR2-D, and TLR4-D mice was significantly reduced. Furthermore, in mice lacking TLR9 the pro-inflammatory mediators TNF-α, IL-1β and IL-6 were not raised by CASP. The baseline expression of PRRs was not influenced by the gene ablation of TLR9 itself. To test the role of TLR9 in WT mice we injected these with the TLR9-specific antagonist H154-thioate. TLR9-antagonism prevented the loss of vascular contractility and also diminished the expression of pro-inflammatory cytokines significantly. Our results complement the finding of Yasuda and colleagues, that TLR9-antagonism was suitable to reduce sepsis-induced kidney failure [16].

In TLR9-D animals other PRRs are expressed. Therefore, a complete block of the inflammatory signal should not be expected. Interestingly, TLR9-deficiency was able to protect completely against polymicrobial challenge underlining the crucial role of TLR9 in vascular signaling. Since TLR4-D mice express TLR9 they were only partially protected. Thus, the effects of single TLRs are not simply additive during polymicrobial sepsis. Taken together our data give for the first time evidence that TLR9 is a key element in the development of vascular dysfunction during polymicrobial sepsis and might be a pharmacological target. Similar to the TLR-9 gene ablation CD14-deficiency protected vascular contractility completely against polymicrobial challenge. The pro-inflammatory cytokines as well as PRRs were also regulated comparably to TLR9-D animals. Recently, it has been demonstrated, that CD14 acts also as co-receptor for other TLRs than TLR4, including TLR7 and −9 [30]. In our setting, this means that the absence of CD14 interrupts TLR9- as well as TLR4-dependent signaling, thus preventing inflammation-dependent loss of vascular tone. This is consistent with our observation that during CASP CD14 is up-regulated in all investigated TLR-D genotypes (Fig. 1D) and that CD14-deficiency is responsible for complete protection from loss of vascular contractile function (Fig. 3E, F).

In contrast to the beneficial effects of CD14- and TLR9-deficiency TLR2-D mice developed an even significantly lower vascular tone than WT animals after CASP (Tab. 1; supplement).
Under baseline conditions TLR2-deficiency is accompanied by compensatory up-regulation of all other examined PRRs. Additionally, baseline vascular contractility was reduced in these animals. During CASP, all pro-inflammatory cytokines as well as iNOS were elevated to a level significantly above all other genotypes. This may be due to the elevated PRRs. The overexpression of TLR4, -9 and CD14 in TLR2-D mice might obscure possible effects conveyed by TLR2. Thus, we tested TLR2-specific stimulation with purified LTA. Interestingly, this did not reduce the vascular tone, which supports the view that loss of vascular contractility detected in TLR2-D aortic rings is caused by overexpression of other PRRs. Interestingly, Ha et al. were not able to detect a reduction of vascular tone in their TLR2-deficient mice, which is in accordance with our findings in the blood vessel.

However, work by Zhou and colleagues suggest protective effects of TLR2 knockdown during polymicrobial sepsis. Their data on TLR2-deficient mice undergoing CLP exhibited lower decreases of mean arterial blood pressure, increased survival, limited increases in circulating and cardiac tissue cytokines and maintained left ventricular function compared to wildtype animals [32]. Their findings might be perceived as being contradictory to ours; however, they only observed cardiac function and not vessel function or vessel cytokines. Furthermore, their TLR2 deficient mice also developed lowered mean arterial blood pressure when subjected to CLP. This loss of blood pressure might be due to the observed loss of vascular contractility found here. Their observations on load dependent parameters were derived from Langendorff perfused hearts with constant preload, which is, as Zhou et al. point out “not achievable in an *in vivo* septic condition”.

It has been described, that TLR2-deficient mice are more prone to infections, in particular of *Streptococcus pneumoniae* [33] and *Staphylococcus aureus* [34]. Our own findings in combination with those by Ha and colleagues now give evidence that TLR2-deficiency is significant for polymicrobial infections as well, indicating a higher susceptibility for a wide variety of pathogen induced effects.

The experiments of this study demonstrated that TLR4-deficiency protected partly and TLR9- as well as CD14-deficiency protected completely from sepsis-induced vasopenia. Consequently, the significant up-regulation of TLR4, TLR9 and CD14 in TLR2-D mice may explain their severe reaction to polymicrobial stimuli. Our findings in regard to TLR2 and -4 seem to be in contrast to findings from Williams et al. who found that CLP, but also sham surgery, induces TLR2 and TLR4 expression in a time-dependent manner in the lung, liver and spleen [12]. These differences might be due to the experimental approach (CASP vs. CLP), different mouse strains and the diverse organ systems examined.

The observed higher expression of TLR4, TLR9 and CD14 in TLR2-D mice might be considered a non-physiological knockout phenomenon, but in our understanding it is of clinical relevance since there are several TLR2 polymorphisms in humans, leading to non-functional variants of TLR2 [35]. These patients show a decreased cellular response to lipopolysaccharides and TLR2 gene mutations might also predispose for bacterial infections [36]. In addition, TLR2 ligands might be protective against cardiac [31] and vascular dysfunction in polymicrobial sepsis. Thus, the absence of TLR2 might further aggravate septic cardiac dysfunction.

Another aspect influencing vascular contractility in sepsis is the Angl/Ang2/Tie-2 receptor axis. Differences in the expression of these factors/receptors have been shown to be important for sepsis induced mortality [37,38] and to alter blood vessel response to vasoconstrictors [39]. Further studies are needed to better understand the role of these factors in polymicrobial sepsis, specifically looking for alterations of their expression attributable to TLR knockout.

In summary, our study provides evidence that polymicrobial sepsis leads to vascular dysfunction, possibly independent of iNOS. It also demonstrates that the absence of TLR2 appears to lead to a compensatory increase in other PRRs in the blood vessel concomitant with an increased susceptibility to polymicrobial sepsis with severely impaired vascular tone. We show for the first time, that functional CD14 and TLR9 are crucial for reducing vascular function and mediating the polymicrobial stimulus and that TLR9 antagonism results in improved vascular tone and reduced pro-inflammatory cytokine levels in the vessel wall.

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**Supporting Information**

**Table S1** Analysis of aortic sigmoidal dose-response curves for Phenylephrine induced contractions.

(DoC)

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**Author Contributions**

Conceived and designed the experiments: SFE HE GB OB RM. Performed the experiments: SFE HE AD SHL RL. Analyzed the data: SFE HE AD SHL RL AH OB PK GB RM. Contributed reagents/materials/analysis tools: OB PK AH GB RM. Wrote the paper: SFE HE OB AH RM PK GB.
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Ehrentraut, S.F., Kominsky, D.J., Glover, L.E., Campbell, E.L., Kelly, C.J., Bowers, B.E., Bayless, A.J., and Colgan, S.P. (2013). Central role for endothelial human deneddylyase-1/SENP8 in fine-tuning the vascular inflammatory response. J Immunol 190, 392–400.

Zielsetzung - Ein tieferes Verständnis der Mechanismen, welche die Wirtsreaktionen auf eine Entzündung steuern, ist für die Entwicklung wirksamer Therapien von entscheidender Bedeutung. Ziel dieser Studie war es, die proximalsten Regulatoren der Cullin (Cul)-RING-Ligasen zu definieren, die eine zentrale Rolle bei der Stabilisierung der Transkriptionsfaktoren NF-kB und dem Hypoxie-induzierbaren Faktor spielen. In dieser Studie identifizieren wir zusätzlich die menschliche Deneddylyase-1 (SENP8) als einen wichtigen Regulator der Cul-Neddylierungsreaktion in vitro und in vivo.

Methoden und Ergebnisse - Mit menschlichen mikrovaskulären Endothelzellen (HMECs) untersuchten wir den Cul-Neddylierungsstatus, die NF-kB- und HIF1α-Stabilisierung sowie die Zytokin-Sekretion nach proentzündlicher Stimulation. HMECs mit intaktem Neddylierungsweg zeigten eine zeitabhängige Induktion der Cul-1-Neddylierung, der nukleären Translokation von NF-kB, der Stabilisierung von HIF1α und eine erhöhte NF-kB/HIF1α-Promotoraktivität als Reaktion auf LPS. Zur Untersuchung des Einflusses von SENP8 wurden, mittels lentiviralem knockdown, HMECs ohne ausreichende Expression von SENP8 generiert (siehe Figure 2, p. 393, Ehrentraut et al., J Immunol 2013). Diese sind nicht in der Lage, Cul-1 zu neddylieren und können daher weder NF-kB noch HIF1α aktivieren (siehe Figure 3, p. 394, Ehrentraut et al., J Immunol 2013). Die pharmakologische Beeinflussung der Neddylierung, durch das Adenosinmonophosphatanalogon MLN4924, führte zu einer signifikanten Aufhebung der NF-kB-Antwort, zur Induktion der HIF1α-Promotoraktivität und zur Verringerung der Sekretion von TNF-a-induzierten pro-inflammatorischen Zytokinen. MLN4924 stabilisierte den Transkriptionsfaktor HIF und hob pro-inflammatorische Reaktionen, inklusive des Verlustes der Barrierefunktion, auf (siehe Figure 5, p. 396, Ehrentraut et al., J Immunol 2013). Die Sekretion von entzündungshemmenden IL-10 in vivo nach LPS-Verabreichung blieb aufrechterhalten.

Schlussfolgerungen - Diese Studien identifizieren SENP8 als proximalen Regulator der Cul-Neddylierung und zeigen eine wichtige Rolle für SENP8 bei der Feinabstimmung der Entzündungsreaktion. Darüber hinaus bieten unsere Ergebnisse die Möglichkeit,
den Cullin-Signalweg im Rahmen von Entzündungsreaktionen therapeutisch anzusprechen.
Central Role for Endothelial Human Deneddylase-1/SENP8 in Fine-Tuning the Vascular Inflammatory Response

Stefan F. Ehrentraut, Douglas J. Kominsky, Louise E. Glover, Eric L. Campbell, Caleb J. Kelly, Brittelle E. Bowers, Amanda J. Bayless and Sean P. Colgan

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Central Role for Endothelial Human Deneddylase-1/SENP8 in Fine-Tuning the Vascular Inflammatory Response

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A deeper understanding of the mechanisms that control responses to inflammation is critical to the development of effective therapies. We sought to define the most proximal regulators of the Cullin (Cul)-RING ligases, which play a central role in the stabilization of NF-κB and hypoxia-inducible factor (HIF). In these studies, we identify the human deneddylase-1 (SENP8) as a key regulator of Cul neddylation status in vitro and in vivo. Using human microvascular endothelial cells (HMECs), we examined inflammatory responses to LPS or TNF-α by assessing Cul neddylation status, NF-κB and HIF-1α stabilization, and inflammatory cytokine secretion. HMECs with an intact neddylation pathway showed a time-dependent induction of Cul-1 neddylation, nuclear translocation of NF-κB, stabilization of HIF-1α, and increased NF-κB/HIF-α promoter activity in response to LPS. HMECs lacking SENP8 were unable to neddylate Cul-1 and subsequently were unable to activate NF-κB or HIF-1α. Pharmacological targeting of neddylation (MLN4924) significantly abrogated NF-κB responses, induced HIF-1α promoter activity, and reduced secretion of TNF-α-elicited proinflammatory cytokines. MLN4924 stabilized HIF and abrogated proinflammatory responses while maintaining anti-inflammatory IL-10 responses in vivo following LPS administration. These studies identify SENP8 as a proximal regulator of Cul neddylation and provide an important role for SENP8 in fine-tuning the inflammatory response. Moreover, our findings provide feasibility for therapeutic targeting of the Culs during inflammation. The Journal of Immunology, 2013, 190: 392–400.

Sepsis remains one of the leading causes of death in developed countries (1, 2). The underlying mechanisms of sepsis severity are incompletely understood, and therefore treatment options beyond those available today are needed. One of the hallmarks of sepsis is loss of endothelial function and subsequent activation of the immune system (3, 4). The NF-κB pathway has been well established as one of the key pathways for intracellular proinflammatory signaling (5–7). Furthermore, there is evidence that NF-κB can influence hypoxia-inducible factor (HIF), an oxygen homeostasis transcription factor, levels and vice versa (8, 9). NF-κB is regulated through IκB kinases and its activation relies on IκB kinase degradation through the 26S proteasome (10). HIF is also tightly regulated in a posttranslational fashion. Under normoxic conditions, HIF is hydroxylated by prolyl hydroxylases, followed by ubiquitination and ultimately proteasomal degradation (11). Hypoxia is often detectable at sites of inflammation (12). Under such conditions, HIF is stabilized, translocates to the nucleus, and enhances transcription of a number of anti-inflammatory genes (13).

The ubiquitination of IκB kinase and the α subunit of HIF-1 and HIF-2 is mediated through Culin (Cul) proteins, which, to be active, require conjugation to the small ubiquitin-like modifier (SUMO) conjugation of Cul-1 (14). The conjugation of neddylation (MLN4924) significantly abrogated NF-κB responses, induced HIF-1α promoter activity, and reduced secretion of TNF-α-elicited proinflammatory cytokines. MLN4924 stabilized HIF and abrogated proinflammatory responses while maintaining anti-inflammatory IL-10 responses in vivo following LPS administration. These studies identify SENP8 as a proximal regulator of Cul neddylation and provide an important role for SENP8 in fine-tuning the inflammatory response. Moreover, our findings provide feasibility for therapeutic targeting of the Culs during inflammation.

The Journal of Immunology, 2013, 190: 392–400.
an isopeptidase capable of directly deneddylating Cul proteins (17, 27), provides new insights into how Cul neddylation might be regulated. SENP8 appears to be able to deneddylate hyper-neddylated Cul proteins, thus offering a cleavage pathway beyond the COP9 signalosome (28, 29).

In this study, we define the role of SENP8-mediated deneddylation during inflammation using several approaches, including knockdown and overexpression of SENP8. Furthermore, we use the small molecule inhibitor of neddylation, MLN4924 (30), which has been shown to impact NF-κB inhibition via Cul-mediated IκBα degradation (31), to evaluate its benefits for modulating the inflammatory response following LPS or TNF-α stimulation in vitro and in vivo. We identify a previously unappreciated role for SENP8 as a central regulator of the inflammatory process. Furthermore, we show that under physiological conditions, SENP8 functions to make mature Nedd8 available for conjugation to Cul5, impacting on two transcription factors (i.e., NF-κB and HIF) important for balancing the inflammatory response.

Materials and Methods

Cell culture

Human microvascular endothelial cell (HMEC)-1 cells were a gift of Francisco Candal (Centers for Disease Control, Atlanta, GA). HMEC-1 cells were cultured in molecular, cellular, and developmental biology-131 medium supplemented with heat-inactivated FBS, penicillin, streptomycin, 1-glutamine, epidermal growth factor, and hydrocortisone, as described previously (32). Stable knockdown of SENP8 was performed using short hairpin RNA (shRNA) with the following sequences: 5′-CCGGGCCTAATCTTCATGAGCAGCTTGGTCTTGAGATGACTGTGTTTTTG-3′ (TRCN0000073338, referred to as clone 38 in figures) or 5′-CCGGGACTGTGGGATGTACGTGATACTCGAGTATCACGTACATCCCACAAGCTTTTTGT-3′ (TRCN0000073342, referred to as clone 42 in figures; Sigma-Aldrich, St. Louis, MO) introduced using lentiviral particles. Cells transfected with scrambled shRNA were used as control. Knockdown cells were kept in cell culture medium with a maintenance dose of 0.25 μg/ml puromycin. Transient overexpression of SENP8 was achieved by transfecting 1 μg FLAG-tagged SENP8 (Addgene plasmid 18066) (33) construct under a CMV promoter into control HMEC-1 cells. Cells were cultured at 37°C in an atmosphere of 95% air and 5% CO2 in a humidified incubator. Where indicated, HUVECs (Promocell, Heidelberg, Germany) were obtained and cultured according to the manufacturer’s recommendations.

Real-time PCR

To initially assess the level of SENP8 knockdown by lentiviral knockdown, SENP8 transcript levels were measured by relative real-time PCR. mRNA was isolated using the thiocyanate–phenol-chloroform method. Relative real-time PCR was then performed using Power SYBR Master Mix (Applied Biosystems, Carlsbad, CA) and the following primer sequences:
SENPs, sense, 5′-CAACACAGGTTGCTGAAG-3′, antisense, 5′-CGGGGTCCATCTTACTGGA-3′; IL-1β, sense, 5′-GCTGACACATGGGATAAAGCA-3′, antisense, 5′-TCTTCTACACAGAGGAGCAGCAG-3′; IL-6, sense, 5′-ATGCCAATAACACCCCCCTGAC-3′, antisense, 5′-GAGGTGCCCATGCTACATG-3′; IL-8, sense, 5′-AGCACTTCAAGGCGCTGG-3′, antisense, 5′-CATATTAATGTGTTGGGCCGACTTG-3′; TNF-α, sense, 5′-TCTTCTAGACACCTCAACC-3′, antisense, 5′-AGGGCCTAGTTTGAAATCTT-3′; ICAM, sense, 5′-TTTCTGACGGCGCAAACAGC-3′, antisense, 5′-AATGCAAACAGGACAAGAGG-3′; β-actin, sense, 5′-GAAGCTTCCAGGACAGGAGG-3′; antisense, 5′-AGACTGTGTTGGCGTACAG-3′.

Cell treatments

For Western blot experiments and luciferase assays the following treatment regimen was used. Six hours prior to harvesting, cells were treated with 10 μg/ml medium of \textit{Escherichia coli}–derived LPS (\textit{E. coli} 055:B5; List Biological Laboratories, Campbell, CA), LPS plus MLN4924 (0.33 μM; Millennium Pharmaceuticals, Boston, MA), TNF-α plus MLN4924, or vehicle (DMSO; Sigma-Aldrich) for 0.5, 1, 2, 4, and 6 h under normoxic conditions. Adenosine (Calbiochem) was used as a 30-min pretreatment at concentrations from 100 μM to 100 nM prior to LPS stimulation.

Western blotting

Cytosolic and nuclear proteins were isolated using the NE-PER Nuclear and Cytoplasmic Protein Extraction kit (Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Lowry assay. Proteins and Cytoplasmic Protein Extraction kit (Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Lowry assay. Proteins were resolved under reducing conditions on SDS-PAGE gels followed by transfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Protein (40 μg) was then used as input on a 10% SDS-PAGE gel, and Western blot analysis using murine anti-SENP8 (1:500; Abcam) was performed as described above.

Immunoprecipitation

Protein lysate (100 μg) was precipitated on protein A μMACS protein beads (Miltenyi Biotec, Auburn, CA) precoated with 2 μl rabbit anti-SENP8 (Abnova). Following incubation, protein beads were washed and protein was eluted according to the manufacturer’s protocol. Protein (40 μg) was then used as input on a 10% SDS-PAGE gel, and Western blot analysis using murine anti-SENP8 (1:500; Abcam) was performed as described above.

Immunofluorescence

Control and SENP8 knockdown cells were seeded on collagen-coated coverslips in a 24-well dish format. After 24 h growth, cells were fixed and stained with an anti-SENP8 primary Ab (1:100; Abnova) overnight, washed, and stained with secondary Ab (Alexa Fluor 555, donkey anti-rabbit, 1:500, Invitrogen). Cells were washed and counterstained with DAPI (1:25,000; Invitrogen). Images were recorded from coverslips using a Zeiss Axio Imager A.1 microscope with an AxioCam MRc 5 at ×20 magnification (Zeiss EC Plan-Neofluar ×20/0.5) utilizing Zeiss AxioVision software version 4.6.3. SENP8 expression was quantified by assessing the fluorometric SENP8/DAPI ratio.

**FIGURE 3.** Functional influence of SENP8 on LPS-induced activation of NF-κB and HIF. (A) Following LPS stimulation, control HMECs exhibit an increased translocation of the NF-κB subunits p50 and p65 to the nucleus. This effect is abrogated in cells lacking SENP8. (B) Cells transfected with a constitutive SENP8 overexpressing (OE) vector showed baseline NF-κB–luciferase levels comparable to LPS treated control cells transfected with empty vector (EV) only. (C) MLN4924 (structure homolog of AMP) significantly quenched LPS-induced NF-κB–luciferase response. (D) LPS induced HIF-1α stabilization in the nucleus of empty vector cells after 4 and 6 h stimulation. This effect is not observable in SENP8 knockdown cells. All numerical data are means ± SEM from three or more experiments. **p < 0.01, ***p < 0.001.
Luciferase assay of HIF and NF-κB promoter activity

The promoter constructs for p-NF-κB-luciferase and p-hypoxia response element (HRE)–luciferase (34) were described previously. Transfection of HMEC-1 cells was performed using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) in addition to 0.5 (HRE) or 1.5 μg (NF-κB) DNA, following overnight transfection, luciferase activity was measured after LPS for 6 (for NF-κB activity) or 24 h (for HRE response) using the luciferase reporter assay (Promega, Madison, WI). Observed firefly luciferase activity was normalized to the total protein amount of each sample determined by Lowry assay.

Endothelial permeability assay

FITC-dextran flux over an endothelial monolayer was measured as previously described by Lennon et al. (35).

Animal experiments

Twelve-week-old ΔODD mice (FVB background) of both genders received i.p. LPS doses of 100 μg/kg body weight. A respective control cohort received vehicle i.p. only. Six hours after treatment mice were sacrificed under tribromoethanol anesthesia and blood samples were collected for measuring systemic cytokine release. All animal experiments have been reviewed by the Institutional Animal Care and Use Committee and are in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Cytokine measurements in serum and HIF-luciferase in kidney tissue

Proinflammatory cytokines in murine blood and organ samples were harvested following i.p. 6 h treatment with either 100 μg/kg LPS, vehicle, 3 mg/kg MLN4924, or LPS plus MLN4924 (1 h MLN4924 pretreatment), snap frozen in liquid nitrogen, and stored at −80°C until further analysis. Serum was collected by centrifugation and organ proteins were isolated using a tissue homogenizer. Serum cytokines were measured using the murine inflammatory 7-Plex assay (Meso Scale Discovery, Gaithersburg, MD). Luciferase activity in the kidney was measured using the luciferase reporter assay (Promega).

Data analysis

All raw and calculated data are expressed as means ± SEM of n observations, with n being the number of biological replicates, and analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Changes in transcript and protein levels and changes in NF-κB and HIF promoter activity levels were compared using a Student unpaired t test or one-way ANOVA with Newman–Keuls post hoc test where appropriate. A p value < 0.05 was considered significant.

Results

Model to study Cul neddylation

Neddylation of Cul-1, an E3 ligase critical for the negative regulation of NF-κB through ubiquitination of IκB (36, 37), has been strongly implicated in inflammation (21). To establish a model of inflammation-induced Cul neddylation, we determined the ability of LPS to modulate endothelial Cul-1. As shown in Fig. 1A, LPS increased endothelial Cul-1 neddylation (reflected as an increase in Nedd8-associated band shift upward in the gel) in a time-dependent manner. Densitometry analysis of Nedd8/loading control ratios revealed a 1 ± 0.07-, 1.3 ± 0.54-, 2.7 ± 0.51-, 2.1 ± 0.52-, 1.7 ± 0.38-, and 3 ± 0.67-fold increase for 0, 0.5, 1, 2, 4, and 6 h stimulation, respectively (n = 3 experiments).

Our previous work showed that extracellular adenosine promotes the neddylation of Cul-1 (26). We therefore determined whether adenosine might influence LPS induced Cul-1 neddylation. As depicted in Fig. 1B, the addition of extracellular adenosine (range, 10 nM–100 μM) attenuated LPS-induced Cul-1 neddylation in a concentration-dependent manner with nearly complete loss of LPS-induced Cul-1 neddylation at concentrations >1 μM compared with untreated control. Densitometry analysis revealed a 41 ± 3.3, 75 ± 8.2, and 86 ± 6.7% decrease relative to LPS alone at 10 nM, 1 μM, and 100 μM adenosine, respectively (n = 3 experiments).

SENP8 as a critical regulator of Cul neddylation

Having profiled endothelial LPS-induced Cul-1 neddylation, we turned our attention to defining molecular regulation. Our previous studies provided a role for the COP9 signalosome in the neddylation of Cul-1 (26), but they revealed little with regard to inflammation-associated neddylation of the Culs. Moreover, our previous work implicated targets upstream of the COP9 signalosome in control of Cul neddylation. This turned our attention to SENP8, and in this study we demonstrate a role for isopeptidase in LPS-mediated Cul-1 neddylation. To demonstrate this point, we used lentiviral-mediated shRNA knockdown to generate stable endothelial cell lines with reduced SENP8 expression. Efficiency of gene silencing was determined by real-time PCR and indicated a >50% loss of transcript in cells transfected with clone 42 shRNA targeting SENP8 compared with nontarget control and a less efficient clone 38 (Fig. 2A). Analysis of SENP8 protein levels by a combination of immunoprecipitation and Western

![FIGURE 4. Influence of neddylation inhibition on HIF stabilization, HIF hydroxylation, Cul-1 neddylation, and HIF activity. (A) Pretreatment with MLN4924 increases HIF-1α protein in nuclear lysates of HMECs stimulated with LPS to higher levels than MLN4924 alone, indicative of synergistic effects of both compounds. LPS-induced Cul-1 neddylation was lost when cells were pretreated with MLN4924. (B) Treatment with MLN4924 stabilized HIF-1α in its hydroxylated form and allowed for its translocation to the nucleus, implicating effects of MLN4924 on Cul-2 neddylation. (C) Luciferase activity of HRE-luciferase following 24 h LPS stimulation mirrored protein results. All numerical data are means ± SEM from three or more experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
blotting revealed an ~85% loss of SENP8 in clone 42 knockdown cells compared with control and clone 38 (Fig. 2A, insert). Likewise, localization of SENP8 by immunofluorescence (Fig. 2B) revealed perinuclear cytoplasmic distribution of SENP8 and a significant loss in SENP8 clone 42 cells (SENP8/DAPI ratio in empty vector controls 2.5 ± 0.42 versus SENP8 knockdowns 1.3 ± 0.07; p < 0.03). The secondary Ab-only control showed no detectable staining for SENP8 (data not shown).

To further confirm altered neddylation status of Culs, we assessed the conjugation of Nedd8 to Cul by probing directly with anti-Nedd8 Ab. LPS increased the abundance of Nedd8 protein in empty vector cells but not in SENP8 knockdown cells (Fig. 2C). Nedd8 detected in this manner (i.e., directly) likely reflects Nedd8 conjugation to several Cul proteins (e.g., Cul-1 and Cul-2, which have different molecular masses), and thus the diffuse nature of the bands on the blots shown in Fig. 2C. These same cells, when treated with LPS in a similar fashion to control cells, showed a blunted neddylation response of nuclear Cul-1 protein, indicating that SENP8 is necessary for LPS-induced Cul-1 neddylation (Fig. 2D).

To define the influence of endothelial SENP8 on functional inflammatory responses, we evaluated the NF-κB response in control and SENP8 knockdown cells following LPS exposure. As shown in Fig. 3A, LPS induced a profound translocation of the p50 and p65 subunit of NF-κB from the cytoplasm to the nucleus. Within 30 min, control cells showed a robust increase in the nuclear fraction of p50 and p65, which persisted to time points beyond 4 h (Fig. 3A). Both p50 and p65 responses were markedly reduced in cells lacking SENP8 (Fig. 3A). In SENP8 knockdown cells, both the kinetics and the magnitude of the p50/p65 response, that is, the nuclear translocation of said heterodimer, was attenuated compared with those observed in control cells, indicating a central role for SENP8 for the immediate NF-κB response following LPS exposure.

Conversely, overexpression of SENP8 in HMEC-1 cells using a SENP8 plasmid on a heterologous promoter significantly increased NF-κB responses. Indeed, as shown in Fig. 3B, compared with empty plasmid transfection controls, overexpression of SENP8 increased basal NF-κB reporter plasmid activity by as much as 2.8 ± 0.5-fold (p < 0.01), approaching that of maximal LPS-induced NF-κB activity. LPS treatment of SENP8 overexpressing cells did not further increase NF-κB activity compared with empty plasmid controls, suggesting that a maximum level of NF-κB activity had been achieved with these conditions. Nearly identical results were observed in primary endothelial cell cultures (HUVECs), where overexpression of SENP8 increased baseline and LPS-induced TNF-α transcript (Supplemental Fig. 1). Thus, both loss- and gain-of-function studies strongly implicate SENP8 as a critical component to vascular endothelial NF-κB responses.

Influence of the neddylation inhibitor MLN4924 on NF-κB and HIF activity

Recently, a small molecule inhibitor (MLN4924) targeting Nedd8 conjugation to the Culs became commercially available. Given our findings that adenosine (via metabolism from AMP) deneddylates Cul-1 (26), it is interesting that MLN4924 is a structural AMP analog (Fig. 4C) that functions to inhibit Nedd8-activating enzyme and results in the deneddylation of Cul-1 and Cul-2 (30, 38). As shown in Fig. 3C, the NF-κB activity elicited by LPS was quenched to nearly control levels in HMEC-1 cells that had been pretreated with MLN4924 (p < 0.01). Similar results were...
obtained in cultured HUVECs, where MLN4924 abrogated LPS-induced Cul-1 neddylation and LPS-induced TNF-α transcript (Supplemental Fig. 1).

Given the importance of Cul-2 neddylation for pVHL activity and thus in the degradation of HIF protein (39), we next addressed the impact of MLN4924 on HIF function. As shown in Fig. 4, in stark contrast to the inhibition of NF-κB (Fig. 3D), MLN4924 stabilized HMEC-1 HIF-1α protein (Fig. 4A), and more specifically the hydroxylated isoform of HIF-1α (Fig. 4B). This observation was significantly enhanced by the presence of LPS, which is consistent with previous studies indicating that LPS stabilizes HIF activity (40, 41). Moreover, HIF activity (as measured using a hypoxia-response element reporter plasmid) was significantly increased by MLN4924 (p < 0.05). These findings indicate that the status of Cul-2 neddylation strongly influences HIF activity.

We next addressed whether Cul neddylation in vascular inflammatory responses was specific for LPS. For these purposes, we selected TNF-α–elicited cytokine/chemokine induction in the presence and absence of MLN4924 as primary endpoints. As shown in Fig. 5, TNF-α potently induced HMEC-1 IL-1β, IL-6, and IL-8 mRNA and protein (all p < 0.05 or p < 0.01). In each case, the neddylation inhibitor MLN4924 significantly inhibited mRNA induction (p < 0.01 or p < 0.05) as well as IL-1β and IL-6 protein induction (p < 0.01). Moreover, MLN4924 significantly inhibited TNF-α–elicited IFN-γ protein (83 ± 8% inhibition, p < 0.05) and IL-12p70 protein (76 ± 5% inhibition, p < 0.01).

**FIGURE 6.** Impact of MLN4924 on LPS-mediated HIF activity and cytokine production in vivo. ΔODD mice received vehicle, MLN4924 (3 mg/kg body weight), LPS (100 μg/kg body weight), or MLN4924 plus LPS i.p. for 6 h. Inflammatory cytokines were measured in serum and HIF-luciferase was measured in renal lysates. (A) LPS plus MLN4924 induced a significant increase of renal HIF cytokines. (B–E, G, and H) Mice pretreated with the neddylation inhibitor MLN4924 prior to LPS showed no significant upregulation of all proinflammatory serum cytokines while maintaining increased levels of the anti-inflammatory cytokine IL-10 (F). All values are means ± SEM with with four or more animals per group. *p < 0.05, **p < 0.01, ***p < 0.001.
Furthermore, MLN4924 inhibited TNF-α-mediated induction of ICAM-1 transcript (Fig. 5D; 79 ± 9% decrease, p < 0.05) and completely abrogated barrier dysfunction elicited by LPS (measured by FITC-dextran flux; see Fig. 5E). Such findings place Cul neddylation as a central regulator of the vascular inflammatory response and clearly implicate Cul deneddylation as a target to inhibit this phenotype.

Cul neddylation and LPS-induced inflammation in vivo

Reagents to define specific aspects of Nedd8/Cul conjugation pathways in vivo have been limiting owing to the embryonic lethality of the gene-targeted mouse lines (42). Thus, we elected to employ MLN4924, which is well tolerated for use in murine tumor models (30). Initially, we profiled whether MLN4924 influenced HIF expression in vivo. For these purposes, we used the HIF reporter ΔODD-luciferase mouse model in combination with LPS (100 μg/kg body weight) exposure in the presence and absence of MLN4924 pretreatment. As shown in Fig. 6A, the combination of LPS and MLN4924 synergistically increased HIF stabilization (reflected as luciferase activity) in kidneys from ΔODD-luciferase mice (p < 0.01). Further analysis of serum cytokines in these mice revealed prominent increases in proinflammatory cytokines and chemokines compared with vehicle-treated mice (Fig. 6B–H). LPS also induced the anti-inflammatory cytokine IL-10 (Fig. 6F). Administration of MLN4924 30 min prior to LPS significantly attenuated the induction of each of the proinflammatory cytokines, including IL-1β, IL-6, TNF-α, IL-12p70, and IFN-γ (Fig. 6B–H). Interestingly, mice that received MLN4924 and LPS showed a further, albeit not statistically significant, increase in the anti-inflammatory cytokine IL-10 (Fig. 6F). Taken together, these in vivo observations reveal a potent anti-inflammatory role for MLN4924. (Fig. 7)

Discussion

In productive inflammatory responses, a number of transcription factors, including NF-κB and HIF, are tightly regulated by posttranslational modifications that control the kinetics of expression via proteasomal degradation pathways (reviewed in Ref. 43). The polyubiquitination of components within these pathways (e.g., IkB and HIF) is mediated through a multiprotein E3 ligase complex, for which one of these components is a member of the Cul family of proteins (44). E3 ligase activity is controlled through its neddylation status, that is, the conjugation of a Nedd8 moiety to the Cul subunit (45). Regulation of Cul neddylation is achieved through the COP9 signalosome and/or the human deneddylyase SENP8 (27, 29). Work by Mendoza et al. (17) identified the isopeptidase SENP8 to primarily deneddylate Culs, thereby inactivating the E3 ligase.

Limited information is available regarding the direct role of Cul regulation in inflammation. Amir et al. (21) demonstrated that lack of functional E2 or E3 ligases results in reduced breakdown of the NF-κB precursor p105, thereby limiting the induced inflammatory response. Their work, however, focused on the E1 and E2 ligases of the neddylation pathway, whereas the present studies address the function of upstream targets of said ligases. To address the role of Cul neddylation in inflammation, we initially established a model using HMECs and LPS to evaluate the biochemical features of Cul-1 neddylation. This model demonstrated prominent Cul-1 neddylation that was inhibited by adenine, which we have previously shown to potently deneddylate the Culs (26).

Based on these observations and our previous work suggesting that targets upstream of the COP9 signalosome should control Cul neddylation (26), we targeted SENP8 as a central regulator for the proinflammatory phenotype. Loss- and gain-of-function studies strongly implicated SENP8 as a central regulator of the inflammatory response. Indeed, lentiviral-mediated shRNA knockdown of SENP8 resulted in a loss of the neddylated Cul-1 that was reversed by constitutive overexpression of SENP8 (33). These observations are likely due to the dual protease actions of SENP8. For instance, Wu et al. (27) have described SENP8 as capable of not only removing Nedd8 from target proteins, but also cleaving

**FIGURE 7.** Neddylation pathways influencing NF-κB and HIF-α. Left, NF-κB pathway. Proinflammatory stimuli, such as LPS, facilitate the phosphorylation of IκB and result in the recognition of p-IκB by the Cul-1-Nedd8-Skp-βTRCP complex, culminating in its polyubiquitination and proteasomal degradation. The rate-limiting step for this is the conjugation of Nedd8 to Cul-1. Neddylation is achieved through a multi-enzyme process wherein SENP8 enables cleavage of the Nedd8 precursor and promotes Nedd8 conjugation to the Culs. Loss of SENP8, or pharmacological inhibition of Nedd8 conjugation by MLN4924, prevents activation of Cul-1 and thus prevents liberation of NF-κB from IκB, resulting in quenched proinflammatory signaling. Right, HIF-α pathway. In contrast to NF-κB, HIF-α in its hydroxylated form is degraded by the proteasome after ubiquitination through the pVHL. pVHL in its activated form contains neddylated Cul-2, thereby controlling cellular HIF-α levels. Use of MLN4924 prevents Cul-2 neddylation and, as shown in this study, leads to higher levels of the hydroxylated HIF-α isoform.
the precursor of Nedd8 to its mature form. Immature Nedd8 is incapable of coupling to the E1 and E2 ligases, thus making it unavailable for conjugation to the E3 ligase. Thus, our observations in the SENP8 knockdown line are likely due to a lack of mature Nedd8 available for conjugation. The molecular mechanism of Cul-RING ligase activity relies on the conjugation of mature Nedd8 to the Cul-RING ligase.

We have shown in the past that adenosine actively deneddylates Cul-1 and results in an inhibition of NF-κB signaling (26). Likewise, we have recently demonstrated that adrenomedullin deneddylates Cul-2 (46), a critical component of the HIF-α E3 Skp-Cul-1-Fbox ubiquitin ligase (47). Cul-2 neddylation should result in diminished pVHL activity and subsequently an accumulation of HIF-1α protein, an effect we did not observe with SENP8 knockdown. It is therefore likely that permanent loss of SENP8 triggers adaptive pathways for HIF-1α control independent of pVHL. In contrast, MLN4924 increased HIF-1α levels and HIF activity, suggesting that short-term inhibition of neddylation influences both Cul-1 and Cul-2 activity. This was also indicated by our in vivo data revealing that HIF levels increase in the kidney in response to LPS and that MLN4924 synergistically increases this response. Important in this regard, HIF stabilization has been proven beneficial in a number of murine disease models (12), including the kidney (48). The underlying mechanisms for HIF-mediated renoprotection remain unresolved but HIF-mediated induction of the ectonucleotidase CD73 (49), a transmembrane protein critical for the generation of extracellular adenosine, has been strongly implicated. For example, Grenz et al. (50) showed that renal CD73 is upregulated by ischemic preconditioning and protective against ischemic injury. Additionally, work by Song et al. (51) demonstrated protective influences of pharmacological HIF induction in the kidney using dimethyloxalylglycine, a known HIF stabilizer. Likewise, constitutive activation of HIF dampens glomerulonephritis progression, providing further evidence for beneficial influences of HIF for renal injury (52). Our results extend these findings to a murine model of septic renal injury, emphasizing the importance of renal HIF stabilization during kidney injury and hinting at potential beneficial influences of MLN4924 in various nephranepathies.

Proinflammatory cytokine responses were quenched in cells and animals treated with the AMP analog MLN4924. Uptregulation of circulating cytokines is one of the hallmarks of sepsis and also serves as a surrogate predictor of disease outcome. For instance, significant evidence suggests that IL-6 serum levels >2000 pg/ml are associated with higher mortality in mice suffering from polymicrobial sepsis (53). These results from a murine model are supported by data from human neonates. Cernada et al. (54) showed that cord blood from septic neonates displayed higher IL-6 levels and that IL-6 served as a good predictor for early onset neonatal sepsis. Our current in vitro data following LPS stimulation and in vivo data from experimental endotoxemia, a model of Gram-negative sepsis, show significantly attenuated proinflammatory cytokine levels, including IL-6, with MLN4924 treatment, indicating some degree of therapeutic potential. The anti-inflammatory properties of MLN4924 are further exemplified by the observation that LPS-induced IL-10 is not influenced by MLN4924. At present, differences in the mechanisms of cytokine-specific regulation are not known.

We conclude that 1) SENP8 is necessary for inflammatory activation of NF-κB, 2) inflammation-induced stabilization of HIF in part relies on functional SENP8, and 3) pharmacological inhibition of SENP8 using the structural AMP analog MLN4924 might serve as a suitable therapeutic target for modulating inflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

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Supplemental data

Figure S1
Influence of neddylation pathways in primary human umbilical vein endothelial cells (HUVEC)

To validate our findings from immortalized human microvascular endothelial cells (HMEC-1), we repeated key experiments in HUVECs. A Cultured HUVECs were exposed to media containing 100ng/ml LPS in the presence or absence of MLN4924, a potent inhibitor of neddylation. LPS alone induced Cullin-1 neddylation over the timeframe of 2hrs, an effect unobserved when cells were also treated with MLN4924. B Downstream of NFκB activation, which relies on Cullin-1 neddylation, proinflammatory cytokines, e.g. TNFα, are transcriptionally upregulated. LPS induced a robust, time-dependent, TNFα response by itself. This response was quenched in MLN4924 treated cells. C In confirmation of the findings in HMEC-1 cells, overexpression (OE) of SENP8 in HUVECs resulted in higher baseline levels of TNFα, resembling those of LPS treated HUVECs transfected only with empty vector (EV). LPS did not further induce TNFα in SENP8 overexpressors, hinting at a ceiling effect of the NFκB response.

A

B

C
Ehrentraut, S.F.*, Curtis, V.F., Wang, R.X., Saeedi, B.J., Ehrentraut, H., Onyiah, J.C., Kelly, C.J., Campbell, E.L., Glover, L.E., Kominsky, D.J., et al. (2016). Perturbation of neddylation-dependent NF-kappaB responses in the intestinal epithelium drives apoptosis and inhibits resolution of mucosal inflammation. Mol Biol Cell.

Zielsetzung - Jüngste Arbeiten haben gezeigt, dass die Neddylierung (die Konjugation einer Nedd8-Komponente an Cullin-Proteine) eine zentrale Rolle bei der Feinabstimmung der NF-κB-Reaktion (über Cullin-1) spielt. In der vorliegenden Studie untersuchten wir den Beitrag der Neddylierung von Cullin-1 und der NF-κB-Signalübertragung zu Entzündungsreaktionen der kolonischen Schleimhaut in vitro und in vivo.

Methoden und Ergebnisse - Erste in-vitro-Studien mit kultivierten Darmepithelzellen ergaben, dass der Neddylierungsinhibitor MLN4924 die Deneddylierung von Cullin-1 deutlich fördert. Parallele Western Blot-, Luciferase-Reporter- und Gen-Target-Assays identifizierten MLN4924 als potenoten Inhibitor des intestinalen epithelialen NF-κB. Anschließende Experimente ergaben, dass MLN4924 die Apoptose von Epithelzellen wirksam auslöst, allerdings nur in Gegenwart zusätzlicher Entzündungsreize. Die In-vivo-Verabreichung von MLN4924 (3 mg/kg pro Tag) in einem TNBS-induzierten Kolitis-Modell verstärkte den Schweregrad der Erkrankung erheblich. Tatsächlich führte MLN4924 bei Mäusen mit Kolitis zu einer Verschlechterung der klinischen Werte und zu einer erhöhten Sterblichkeit in der Frühphase der Entzündungsreaktion. Die histologische Analyse des Dickdarms zeigte, dass die Hemmung der Neddylierung zu einer verstärkten Gewebeschädigung und einer signifikant erhöhten Apoptose der Schleimhaut führt, die durch TUNEL und gespaltene Caspase-3-Färbung bestimmt wurde und besonders im Epithel auftrat. Erweiterungen dieser Studien ergaben, dass die anhaltende Entzündung mit einem signifikanten Verlust der Expression von Deneddylase-1 (SENP8) verbunden ist (siehe Figure 4, p. 3691, Ehrentraut et al. Mol Biol Cell 2016). Zudem konnte gezeigt werden, dass es auch im menschlichen Gewebe, bei Patienten mit chronisch entzündlichen Darmerkrankungen (Morbus Crohn bzw. Chronisch ulzerative Colitis) zu einer vergleichbaren, signifikanten Herabregulation von SENP8 kommt (siehe Figure 5, p. 3692, Ehrentraut et al. Mol Biol Cell 2016).

Schlussfolgerung – Diese Ergebnisse zeigen, dass eine intakte Cullin-1-Neddylierung für das Abklingen einer akuten Entzündung von zentraler Bedeutung ist. Die relative Expression von SENP8 scheint auf der mRNA Ebene zeittabhängig im Rahmen von akuten und chronischen Entzündungsreaktionen herunterreguliert zu werden.
Perturbation of neddylation-dependent NF-κB responses in the intestinal epithelium drives apoptosis and inhibits resolution of mucosal inflammation

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**ABSTRACT** Recent work has revealed a central role for neddylation (the conjugation of a Nedd8 moiety to Cullin proteins) in the fine-tuning of the NF-κB response (via Cullin-1). In the present study, we investigated the contribution of Cullin-1 neddylation and NF-κB signaling to mucosal inflammatory responses in vitro and in vivo. Initial in vitro studies using cultured intestinal epithelial cells revealed that the neddylation inhibitor MLN4924 prominently induces the de neddylation of Cullin-1. Parallel Western blot, luciferase reporter, and gene target assays identified MLN4924 as a potent inhibitor of intestinal epithelial NF-κB. Subsequent studies revealed that MLN4924 potently induces epithelial apoptosis but only in the presence of additional inflammatory stimuli. In vivo administration of MLN4924 (3 mg/kg per day) in a TNBS-induced colitis model significantly accentuated disease severity. Indeed, MLN4924 resulted in worsened clinical scores and increased mortality early in the inflammatory response. Histologic analysis of the colon revealed that neddylation inhibition results in increased tissue damage and significantly increased mucosal apoptosis as determined by TUNEL and cleaved caspase-3 staining, which was particularly prominent within the epithelium. Extensions of these studies revealed that ongoing inflammation is associated with significant loss of deneddylase-1 (SENP8) expression. These studies reveal that intact Cullin-1 neddylation is central to resolution of acute inflammation.

**INTRODUCTION** Posttranslational protein modifications (PPMs) play an important role in the regulation of protein function, allowing for rapid responses to external stimuli (Song et al., 2010). One of these PPMs, neddylation—

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Abbreviations used: SENP8, sentrin-specific protease 8/deneddylase-1; TER, transepithelial electrical resistance.

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enzyme (NAE; also called UBA3-APPB1-E1-ligase; Wada et al., 1998; Mendoza et al., 2003; Huang et al., 2004). Subsequently the Nedd8 moiety is transferred to its E2-ligase (Jones et al., 2002) and then the target Cullin-E3-ligase protein complex, thereby activating it (Parry and Estelle, 2004). Free Nedd8 can be generated through cleavage of conjugated Nedd8 from the Cullin-E3-ligase, a process known as deneddylation. This process depends, at least in part, on the COP9 signalosome, can be positively influenced by commensal bacteria (Kumar et al., 2007; Jones et al., 2015) and extracellular adenosine (Khoury et al., 2007), and offers a potentially protective mechanism during inflammatory processes. In addition, loss of the isopeptidase sentrin-specific protease 8 (SENPS8)/deneddylylase-1 leads to a loss of neddylyatory function and an inability to activate NF-κB (Ehrentraut et al., 2013).

Studies of neddylation and Cullin pathways in vivo have been hampered by the embryonic lethality of gene-targeted mice (Tateishi et al., 2001). Recent advances in the development of small-molecule therapeutics identified the adenosine monophosphate (AMP) homologue MLN4924 (Soucy et al., 2009). This compound disrupts the Cullin-E3-RING-ligase complex neddylation by inhibiting NAE. Given our previous observations that adenosine deneddylates Cullin proteins during ongoing inflammation (Khoury et al., 2007), we investigated the effect of MLN4924 on inflammatory responses and demonstrated that administration of this compound inhibits acute lipopolysaccharide (LPS)-induced endotoxemic shock (Ehrentraut et al., 2013). Other studies showed that low-dose MLN4924 activated HIF through Cullin-2 (Angus, 2011; Curtis et al., 2015). Here we sought to investigate the contribution of protein neddylation to mucosal inflammation using cultured epithelial cells and experimental colitis in mice as model systems.

RESULTS

Cytokine-induced Cullin-1 neddylation is abrogated by MLN4924

In the present study, we examined how neddylation affects epithelial NF-κB responses and mucosal inflammation endpoints. As shown in Figure 1A, Caco-2 intestinal epithelial cell exposure to tumor necrosis factor-α (TNF-α; 10 ng/ml, 1 h) induced p65 nuclear translocation. Coincubation of cells with the NAE inhibitor MLN4924 inhibited both p65 nuclear translocation and Cullin-1 (Cul-1) neddylation in a concentration-dependent manner. At doses as low as 100 nM MLN4924, the neddylation fraction of Cul-1 was significantly decreased (p < 0.05). Parallel studies using NF-κB reporter assays revealed concentration-dependent inhibition of NF-κB activity with MLN4924, with >60% loss of activity at 3 μM MLN4924 (Figure 1B). When Caco-2 and T84 intestinal epithelial cells were exposed to the combination of MLN4924 (3 μM) and TNF-α/interleukin-1β (IL-1β; 10 ng/ml each), we observed a 50–70% decrease in the induction of the NF-κB target genes IL-8 and ICAM-1 (Figure 1C; p < 0.025). These results indicate that MLN4924 is a potent NF-κB inhibitor and that loss of Cul-1 neddylation significantly inhibits NF-κB target gene induction.

Epithelial barrier function and neddylation

One of the hallmarks of mucosal diseases, including inflammatory bowel disease (IBD), is epithelial barrier dysfunction (Koch and Nusrat, 2012), allowing for translocation of luminal contents into the serosa. Here we examined the influence of neddylation on epithelial barrier function in the presence and absence of inflammatory stimuli. Epithelial barrier function has been widely modeled in vitro through measurement of transepithelial electrical resistance (TER).

Figure 1: The neddylation inhibitor MLN4924 inhibits NF-κB signaling in intestinal epithelial cells. (A) Western blot of nuclear/cyttoplasmic fractionation of Caco-2 cells treated with TNF-α (10 ng/ml) and increasing concentrations of MLN4924 for 1 h leads to deneddylylation of Cul-1 and decreased p65 NF-κB in the nucleus, with actin and TATA-binding protein (TBP) as loading controls (n = 3). (B) Luciferase assay in Caco-2 cells transfected with an NF-κB luciferase reporter plasmid and treated with TNF-α and IL-1β (10 ng/ml each) and increasing concentrations of MLN4924 leads to increased inhibition of NF-κB signaling (n = 3). (C) mRNA expression of NF-κB target genes 2 h after treatment with TNF-α and IL-1β (10 ng/ml each) is inhibited by pretreatment (30 min) with 3 μM MLN4924 treatment in the presence of (n = 3), *p < 0.05.

For these purposes, T84 intestinal epithelial cells were cultured on polycarbonate inserts and grown to confluence (>1000 Ω cm²). Cells were exposed to medium alone (control), cytokin (10 ng/ml each of TNF-α, IL-1β, and interferon-γ), MLN4924 (1 μM) alone, or the combination of cytokin and MLN4924. As shown in Figure 2A, exposure of confluent epithelia to MLN4924 alone did not influence baseline epithelial barrier compared with medium alone (p = 0.54), suggesting that neddylation per se is not necessary to maintain epithelial barrier function. Treatment of epithelia with cytokin led to a significant (p < 0.05) decrease in epithelial resistance over 24 h, indicative of a loss of tight junctional integrity (Figure 2A), which has been previously demonstrated (Brewer et al., 2003). This cytokin-dependent decrease in barrier was markedly enhanced in combination with MLN4924 (Figure 2A; p < 0.01) by analysis of variance (ANOVA), indicated by an earlier and more severe reduction in TER measurements.

TER measurements reflect changes in electrical conductivity from both transcellular and paracellular paths. To verify whether the observed changes were attributable to the paracellular path (i.e., tight junction permeability), we performed paracellular flux assays using 3-kDa fluorescein isothiocyanate (FITC)-dextran as a tracer. As shown in Figure 2B, similar to TER measurements, MLN4924 alone did not increase paracellular flux compared with control (p = 0.10), whereas cytokin treatment increased paracellular flux by a small (~10%) but significant amount (p < 0.05). The combination of MLN4924 and cytokin, however, increased transepithelial flux by nearly 50-fold (p < 0.01) compared with other treatment groups, clearly indicating that the loss of neddylation in combination with inflammatory stimuli results in a marked loss of tight junction integrity.

Previous studies showed that the disruption of epithelial junctions in response to inflammatory cytokines is related at least in part...
to an increase in apoptosis through the activation of caspase-3 (Nava et al., 2010). Once activated, caspase-3 initiates the nonreversible apoptotic cascade leading to DNA fragmentation and ultimately cell death (Porter and Janicke, 1999). To determine whether increased apoptosis was responsible for our observed neddylation-dependent loss of barrier, we characterized the influence of the combination of cytokine activation and MLN4924 on caspase-3 activity. As depicted in Figure 2C, MLN4924 alone did not significantly influence caspase-3 activity (p = 0.12). Although cytokinex alone trended toward increased caspase-3, the combination of neddylation inhibition and cytokinex increased caspase-3 activity by nearly ninefold compared with control and MLN4924 alone (p < 0.01). To define the functional significance of this observation, we used a general caspase inhibitor peptide to block caspase-3 activity. As shown in Figure 3A, the decrease in TER caused by cytokinex led to increased apoptosis through the activation of caspase-3 (Porter and Janicke, 1999). To determine whether the apoptotic response seen with MLN4924 treatment with cytokinex contributed to reduced barrier function and partially recapitulates the response of MLN4924 treatment with cytokinex.

**FIGURE 2:** The combination of proinflammatory cytokines with the neddylation inhibitor MLN4924 leads to increased barrier disruption. (A) TER of T84 cells on Transwell inserts during a 24-h time course with control, cytokinex (10 ng/μl each of TNF-α, IL-1β, and IFN-γ), 1 μM MLN4924, or cytokinex plus MLN4924 (n = 3). (B) FITC-flux assay of T84 cells on Transwell inserts after a 24-h time course with control, cytokinex, 1 μM MLN4924, or cytokinex plus MLN4924 (n = 3). (C) Luciferase assay detecting activation of caspase-3/7 of T84 cells treated with control, cytokinex, 1 μM MLN4924, or cytokinex plus MLN4924 for 24 h; *p < 0.05.

**Neddylation and intestinal inflammation in vivo**

Having defined the importance of neddylation for epithelial barrier integrity in vitro, we extended these results to define the relative importance of neddylation in colonic inflammation in vivo, using a murine 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis model. This is a model characterized by disruption of the epithelial barrier in vivo (Karhausen et al., 2004). After TNBS instillation, body weight was monitored twice per day. In accordance with previous observations, TNBS treatment led to increased mortality (33% after 3 d, n = 9, compared with 0% death in control group, n = 5, treated with ethanol only; Figure 4A). The earliest time point of animal death was 2 d into the trial period. Treatment with MLN4924 alone (3 mg/kg per day) did not alter this ratio, and all of the animals survived the 3-d trial period (n = 5). Daily subcutaneous injections of MLN4924 combined with TNBS significantly increased mortality (60% of animals by day 3.5, n = 10, p < 0.025 compared with vehicle treatment; Figure 4A).

Colon shortening, a hallmark feature of murine colitis (Karhausen et al., 2004), was not different between vehicle and MLN4924-alone exposures (Figure 4B). Colitis induced with TNBS showed a nonsignificant trend toward shorter colons at the time of killing, which was consistent with previous data for this model (Robinson et al., 2008). However, animals receiving repetitive doses of MLN4924 along with the induction of colitis showed a significant reduction of colon length compared with their littermates receiving only TNBS (Figure 4B).

TNBS colitis has been shown to lead to apoptotic cell death (Crespo et al., 2012; Hjerpe et al., 2012). TNBS-induced inflammatory response was characterized by a loss of crypt architecture and infiltration of large numbers of inflammatory cells with mucosal and submucosal injury (Figure 4C). Hematoxylin and eosin staining. MLN4924 treatment alone resulted in no observable change to colonic architecture compared with vehicle control. The combined use of MLN4924 and TNBS, however, significantly increased tissue destruction, leading to total loss of crypt structure, massive inflammatory cell infiltration, and transmural mucosal denudation (Figure 4C, bottom left). As a result, the tissue injury index in mice receiving MLN4924 with TNBS was significantly increased compared with those receiving TNBS alone (Figure 4F).
DISCUSSION

Posttranslational modifications of signaling proteins are critical to productive inflammatory responses and resolution of disease (Ehrentraut and Colgan, 2012). NF-κB is the quintessential signaling hub during acute inflammation, and its regulation is fine-tuned by multiple posttranslational modifications, including neddylation (Amir et al., 2002). Cullin proteins, as components of ubiquitin E3 ligases, are neddylated for the polyubiquitination of effectors (e.g., IκB). This neddylation response is regulated, in part, by the deneddylation of cullins by the deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activity. An intact deneddylase SENP8 as a mechanism to control E3 ligase activ

In addition, colonic tissue of animals treated with only MLN4924 showed no increase in the amount of fragmented DNA (a sign of apoptotic cell death) compared with vehicle-treated controls (Figure 4D, bottom). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, top right and left. TNBS-induced colitis increased the detectable amount of fragmented DNA, indicative of some degree of cell death. This was increased in the colonic epithelium of animals receiving both TNBS and MLN4924 (Figure 4D, bottom). The activation of caspase-3 is another keystone along the apoptotic cell death pathway. In our study, MLN4924 alone did not alter the amounts of detectable caspase-3 in the colonic epithelium. The induction of cell death via TNBS colitis increased the activation of caspase-3 (Figure 4E, bottom left). Again, this proapoptotic response was significantly enhanced in animals receiving both MLN4924 and TNBS, as reflected by cleaved caspase-3 (Figure 4E, bottom right). A loss of barrier function and cell breakdown is accompanied by the detection of colonic contents (e.g., bacteria) through the innate immune system. One of the major signaling pathways, the Toll-like receptor pathway, elicits the induction of proinflammatory cytokines such as IL-1β and IL-6. The induction of these cytokines was significantly increased in the colonic tissue of animals undergoing TNBS colitis while concomitantly being dosed with MLN4924 (approximately sixfold compared with normalized control, p < 0.01; Figure 4G).

We previously showed that human deneddylase-1 (SENP8) is crucial for enabling NF-κB-mediated inflammation (Ehrentraut et al., 2013). Whether SENP8 itself is regulated during chronic inflammation is unknown. For this purpose, we investigated the expression of SENP8 in our model systems (cultured epithelia, murine tissue), as well as human tissue from healthy and IBD subjects. As shown in Figure 5, there was a striking similarity between the three models that revealed a correlation between disease and the loss of SENP8 mRNA expression. Exposure of T84 cells to cytokin (24 h) resulted in a nearly 70% decrease in SENP8 mRNA expression (p < 0.01; Figure 5A). Epithelial isolates from animals undergoing TNBS colitis at days 1 and 3 after induction were examined and compared with ethanol-only controls. TNBS-colitic animals tended to express less SENP8 mRNA transcript at day 1 (n = 3 per group, p = 0.07; Figure 5B) and day 3 after colitis induction (p < 0.05, n = 5 per group; Figure 5B). The same response was observed in human tissue samples from patients with active IBD. Regardless of the type of colitis (i.e., ulcerative colitis vs. Crohn’s disease), mRNA levels of SENP8 were significantly lower than in samples from healthy individuals (p < 0.001; four nonactive controls, 19 individual samples per disease cohort; Figure 5C). Collectively these results suggest that intact neddylation is disease protective and that loss of SENP8 expression correlates with the development of mucosal inflammatory disease in both mice and humans.
In intestinal epithelia, MLN4924 more potently inhibits Cul-2 than Cul-1. Our previous studies, in fact, revealed EC_{50} \approx 5 \text{nM} for MLN4924 actions on Cul-2 and HIF stabilization (Curtis et al., 2015). In these studies, lower concentrations of MLN4924 (0.1 mg/kg, compared with 3 mg/kg used in the present studies) activated HIF in vivo and were protective for dextran sulfate sodium (DSS) colitis at multiple levels. There are distinct differences in our findings here, that is, aggravation of inflammatory tissue damage after MLN4924 plus TNBS-induced inflammatory disease and previously reported findings from our group in DSS colitis. These differences may reflect the different pathogenic mechanisms of both colitis models. DSS colitis is believed to occur independent of adaptive immune cells, whereas TNBS colitis is directly T-cell dependent (Neurath et al., 1996). MLN4924 was initially discovered for the treatment of NF-kB-dependent B-cell lymphoma (Milhollen et al., 2010). Hence use of MLN4924 in a disease model dependent on adaptive immune cells might explain the observed differences. Together these findings suggest that both in vitro and in vivo, Cul-2 responses are significantly more sensitive to MLN4924.

Given the central role of NF-kB in inflammation, it is not surprising that numerous studies have revealed that inhibition of NF-kB is antiinflammatory (Kanarek and Ben-Neriah, 2012). The intestinal mucosa—specifically, epithelial cells—appears to be somewhat unique in this regard (Karrasch and Jobin, 2008). For example, genetic deletion of NF-kB components (e.g., IkKb) within the intestinal epithelium results in significantly exacerbated pathogen-induced intestinal inflammation (Zaph et al., 2007). These studies revealed increased apoptotic responses with the loss of NF-kB signaling, resulting in a loss of epithelial barrier and ultimately septicemia. Our studies here indicate similar results with targeting neddylation in vivo and that as a preapoptotic sensitizer, the combination of deneddylation

the NF-kB pathway profoundly enhanced the inflammatory response. Other model systems show similar actions of MLN4924. For example, studies of T-cell activation revealed that whereas MLN4924 alone does not activate T-cells, neddylation inhibition significantly lowers the threshold for anti-CD3–stimulated cytokine production (Friend et al., 2013). Other studies in T-cells show that MLN4924 may have neddylation targets beyond the Cullins, including proteins in the Ras/Erk pathway and other adaptor proteins such as Sht (Jin et al., 2013). Godbersen et al. (2015) also showed that targeting neddylation with MLN4924 abrogates NF-kB activation in leukemic B-cells and, in the process, regulates a diverse set of target genes. Thus the “priming” activity elicited by MLN4924 within the mucosa likely represents a complex response to neddylation targets beyond that of Cullins.

In the presence of inflammatory stimuli significantly enhances the inflammatory response. It is noteworthy that throughout these studies, the inhibition of neddylation using MLN4924 had little to no detectable influence on basal epithelial function (i.e., in the absence of additional inflammatory stimuli). Barrier function, for example, was not changed by the inhibition of neddylation responses, even at relatively high concentrations of MLN4924. At multiple levels, these studies revealed a primed inflammatory response that correlated with the loss of neddylation and diminished NF-kB activity in vivo. Direct inhibition of NF-kB via Bay 11-7085 in conjunction with inflammatory stimuli partially recapitulated the reduction in TER measurements and thus barrier function observed with MLN4924.

A central component of the active deneddylation response is SEPN8, an isopeptidase capable of directly deneddylylating Cullin
proteins (Mendoza et al., 2003; Wu et al., 2003), offering a cleavage pathway beyond the COP9 signalosome (Lyapina et al., 2001; Cope and Deshaies, 2003). It was shown, for example, that knockdown of SENP8 prevented LPS-induced NF-κB activation and systemic cytokine release (Ehrentraut et al., 2013). We extended these results to define the expression of SENP8 in murine and human colitis. Across each model tested, including human IBD tissue, inflammation was associated with a loss of SENP8 expression. Such observations suggest that down-regulation of SENP8 in murine/human colitis serves as a compensatory mechanism to quench the ongoing inflammatory response. Mechanisms of such regulation await further studies.

In conclusion, we demonstrate the contribution of epithelial NF-κB and Cul-1 neddylation to inflammatory responses in the intestinal mucosa. In particular, these studies identify MLN4924 as an inhibitor of Cul-1 neddylation, leading to loss of NF-κB signaling. These findings support a role for Cul-1 deneddylation as an apoptotic presensitizer during inflammation, which in turn enhances intestinal inflammatory responses.

MATERIALS AND METHODS

Cell culture

Human T84 and Caco-2 intestinal epithelial cells were cultured in 95% air with 5% CO₂ at 37°C in DMEM and DMEM:F12, respectively, supplemented with 10% calf serum (Fisher Scientific, Waltham, MA) and penicillin/streptomycin (100 U/ml, 100 μg/ml; Invitrogen, Carlsbad, CA).

Transcriptional analysis

TRizol reagent (Invitrogen) was used to isolate RNA from Caco-2 or T84 cells. cDNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR analysis was performed using SYBR Green (Applied Biosystems, Carlsbad, CA). Western blot analysis

The NE-PER extraction kit was used to prepare nuclear and cytoplasmic lysates from Caco-2 cells per the manufacturer’s instructions (Thermo Scientific, Waltham, MA). Western blotting of these lysates was performed using Cul-1 Rb polyclonal antibody (pAb; Invitrogen), p65 Rb pAb (Cell Signaling, Danvers, MA), TATA-binding protein (TATA-BP) Ms monoclonal antibody (mAb; Abcam, Cambridge, United Kingdom), and human β-actin Rb pAb (Abcam). Each experiment was performed in triplicate.

Luciferase assays

An NF-κB luciferase reporter plasmid (500 ng; Ehrentraut et al., 2013) and a Renilla reporter plasmid (1 ng) were transfected into subconfluent Caco-2 cells per the manufacturer’s instructions (Thermo Scientific, Waltham, MA). Western blotting of these lysates was performed using Cul-1 Rb polyclonal antibody (pAb; Invitrogen), p65 Rb pAb (Cell Signaling, Danvers, MA), TATA-binding protein (TATA-BP) Ms monoclonal antibody (mAb; Abcam, Cambridge, United Kingdom), and human β-actin Rb pAb (Abcam). Each experiment was performed in triplicate.

Barrier integrity, permeability assays, and apoptosis assays

T84 cells were plated on 0.33-cm², 0.4-μm permeable polyester inserts (Corning, Corning, NY). TER was measured using the EVOM2 voltohmmeter (World Precision Instruments, Sarasota, FL) to monitor
barrier formation after treatment of confluent T84 monolayers with MLN4924 (1 μM; Millennium Pharmaceuticals, Cambridge, MA), cytomiix (10 ng/μl each of TNF-α, IL-1β, and IFN-γ; eBioscience, San Diego, CA), and Bay 11-7085 (30 μM; Tocris Bioscience, Minneapolis, MN).

Paracelllular permeability was assayed using FITC-dextran flux assay described previously (Furuta et al., 2001) on T84 monolayers with MLN4924 (1 μM), cytomiix, or a combination of both MLN4924 and cytomiix.

To detect caspase-3/7 activity, the Caspase-Glo 3/7 Assay (Promega) was used according to the manufacturer’s instructions. Briefly, 10,000 T84 intestinal epithelial cells per well were plated in 96-well plates and treated with 1 μM MLN4924, cytomiix, 30 μM Bay 11-7085, or a combination of either MLN4924 or Bay 11-7085 with cytomiix for 24 h. After 24 h, 100 μl of the Caspase-Glo 3/7 reagent was added to each well, and the luminescence was measured using the GloMax-Multi plate reader (Promega).

To inhibit caspase activation, a general caspase inhibitor peptide (Z-VDAD-FMK; 30 μM) or a caspase-3 inhibitor peptide (Z-DEVAD-FMK; 30 μM) was given as a 30-min pretreatment to T84 cells plated on permeable polyester inserts before treatment with cytomiix plus MLN4924 (1 μM; BD PharMingen, San Diego, CA) or cytomiix plus Bay 11-7085 (30 μM). A negative control peptide (Z-FA-FMK; 30 μM) was used on additional inserts (BD PharMingen).

Animals

Wild-type C57Bl/6 mice (Mus musculus) were obtained from Jackson Laboratories (Bar Harbor, ME). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado. TNBS colitis was induced as previously described (Furuta et al., 2002). Animals were administered either MLN4924 (3 mg/kg) or cycloedxin (Sigma-Aldrich, St. Louis, MO) via subcutaneous (s.c.) injection at day 1 of TNBS exposure, and this was continued daily. The groups were as follows: ethanol plus MLN4924 (n = 5), and TNBS plus cycloedxin (n = 9), and TNBS plus cycloedxin (n = 10).

Histology and immunofluorescence

Histological examination was performed on samples of the distal colon from each group; samples were fixed in 10% Formalin before staining with hematoxylin and eosin. Slides used for immunofluorescence were first blocked with 5% normal goat serum (NGS). An In Situ Cell Death Detection kit (Sigma-Aldrich) was used to visualize apoptotic cell death by TUNEL staining. For cleaved caspase-3 staining, Rb mAb was used (Cell Signaling) at a 1:400 dilution. Secondary antibody was Alexa Fluor 488 goat anti-rabbit (Invitrogen), used at 1:500 dilution in 5% NGS. Immunolabeling was visualized with an Axioscam MR c5 attached to an AxioImager A1 microscope (Zeiss, Oberkothen, Germany). Where indicated, series of confocal fluorescence images were obtained using a Zeiss Axiovert 200 M laser-scanning confocal/multiphoton-excitation fluorescence microscope with a Meta spectral detection system (Zeiss NLO 510 with META; Zeiss, Thornwood, NY). Representative hematoxylin and eosin and immunofluorescence sections are presented.

Histology

Histological examination was performed on three samples of the distal colon. Samples were fixed in 10% Formalin before staining with hematoxylin and eosin. All histological quantitation was performed in a blinded manner, using a previously described scoring system (Dieleman et al., 1998). The three independent parameters measured were severity of inflammation (0–3: none, slight, moderate, severe), extent of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (n = 1: 0–25%; n2: 26–50%; n3: 51–75%; n4: 76–100%), and all numbers were summed. Maximum possible score was 40.

Human tissue

Deidentified human intestinal tissue cDNA was obtained from the TissueScan Cronh’s/Colitis array (OnGene Technologies, Rockville, MD). Complete patient/sample characteristics can be accessed from the supplier’s website (www.origene.com).

Statistical analysis

Data are expressed as mean values ± SEM. Data were analyzed with Student’s t test between two groups or ANOVA coupled with post hoc Bonferroni test for multiple pairwise comparisons. p < 0.05 was considered to be statistically significant.

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4. Diskussion

Die oben aufgeführten Arbeiten beschäftigen sich inhaltlich mit der Frage, ob und inwieweit die septische Kreislauffdysregulation auf einer vaskulären TLR Antwort beruht. Diesbezüglich konnte durch unsere Untersuchungen erstmals dargestellt werden, dass die funktionale Expression von TLR4 für eine sepsisinduzierte Vasoplegie notwendig ist (Ehrentraut et al., 2007). Überdies zeigte sich, dass auch eine kompetitive Inhibition des TLR4 Signalweges am Endothel zu einer erhaltenen Kontraktilität führt. Diese Beobachtung wurde in mehreren Arbeiten bestätigt. Die Beobachtung, dass die induzierbare NO Synthase iNOS oder NOS II eine relevanten Bedeutung hat, wurde jüngst nochmals durch Trevelin et al. (Trevelin et al., 2021) untersucht. Während unsere Arbeit die Rolle des Gefäßes isoliert, d.h. ohne Einfluss zirkulierender Zellen des adaptiven Immunsystems betrachteten, zeigten Trevelin et al., dass eine septische Vasoplegie nicht von aus Leukozyten freigesetztmem NO abhängig ist. Zudem konnte dargestellt werden, dass es, vermittelt durch aus dem Endothel freigesetztem NO zu einer verminderten TLR4 Expression und einer verringerten NF-κB Antwort kommt (Trevelin et al., 2021). Weitere auf unserer Beobachtung basierender Studien zeigten, dass es, neben der NO vermittelten Vasoplegie, auch zu einer verminderten Katecholaminsensitivität kommt. Dies zeigte sich durch eine verringerte Ansprechbarkeit des A1-Adrenorezeptor nach LPS Gabe. Diese ist durch eine rasche Internalisierung des TLR4 durch Endozytose bedingt und führt nachfolgend zu einer verringerten Calciumfreisetzung in glatten Muskelzellen (Mazor et al., 2021). Neben den kardiovaskulären Auswirkungen des endothelialen TLR4 konnten Untersuchungen von Manouchehrian et al. darstellen, dass es durch eine TLR4 vermittelte Herzfrequenzsteigerung, wie von uns gezeigt, auch zu einer statistisch signifikanten Abnahme der perivaskulären Verteilung von Liquor nach LPS Stimulation kommt (Manouchehrian et al., 2021). Zudem zeigte sich auch eine Auswirkung des TLR4 auf die glattmuskuläre Funktion des Darms in einem Ileusmodell (Buchholz et al., 2015).

Unsere weiteren Untersuchungen zur TLR4-vermittelten Kreislauffdysregulation untersuchten die Möglichkeit einer in vivo Inhibiton des TLR4 und deren Auswirkungen auf die Kreislauffunktion (Ehrentraut et al., 2011b). So konnten wir zeigen, dass eine kompetitive Inhibition des TLR4 auch in vivo zu einer erhaltenen Herzfunktion führt und die LPS induzierte Hypotension verhindert (Ehrentraut et al., 2011b). Diese
Beobachtungen konnten in einer Vielzahl weiterer Studien bestätigt und erweitert werden. So konnte gezeigt werden, dass Eritoran nachweislich die Herzhypertrophie in einem Mausmodell der Aortenverengung durch Hemmung einer TLR4-vermittelten Entzündungsreaktion reduziert (Ehrentraut et al., 2011a). Neben den Auswirkungen auf die kardiale Funktion zeigte sich auch ein Effekt von Eritoran auf Ischämie/Reperfusionsschäden der Leber. McDonald et al. zeigten, dass die durch eine HMGB-1/TLR4 Interaktion vermittelten Leberschäden durch Blockade mit Eritoran abgeschwächt werden können (McDonald et al., 2015). Neben den positiven Effekten auf einen akuten Schaden, wurde durch die Arbeit von Hsieh et al. dargestellt, dass Eritoran eine chronische Leberentzündung und Fibrose durch Hemmung des TLR4-Signalwegs bei Mäusen mit chronischen Leberschäden verringert (Hsieh et al., 2021). Die Erkenntnis, dass diese TLR4 vermittelte Reaktion durch den kompetitiven Inhibitor Eritoran (E5564) blockierbar ist, führte zu einer Reihe weiterer Untersuchungen und klinischen Erprobung (Opal et al., 2013; Tidswell et al., 2010). Letztlich konnte ein positiver Einfluss auf die 28d-Mortalität von Sepsispatienten aber nicht nachgewiesen werden (Opal et al., 2013).

Wir untersuchten überdies die Bedeutung von TLR2 und TLR9 in einem polymikrobiellen Sepsismodell. Es zeigte sich, dass ein Verlust des TLR2 zu einer deutlich erhöhten Vasoplegie in der polymikrobiellen Sepsis einhergeht, vermutlich aufgrund der Hochregulierung von TLR4, TLR9 und CD14 (Ehrentraut et al., 2012). Die ursprüngliche Annahme, dass ein Verlust von TLR2, durch Verlust der Möglichkeit auf Zellwandbestandteile gram-positiver Bakterien zu reagieren, positive Effekte hat, konnte von uns nicht gezeigt werden. Dies erklärt sich möglicherweise durch Verlust pro-angiogener Effekte und der Tatsache, dass TLR2 von Bedeutung für regenerative Prozesse wie Wundheilung ist (Saber et al., 2011; Wagner et al., 2013; West et al., 2010).

Wir konnten zudem zeigen, dass TLR9 ein Schlüsselmediator für die Induktion einer vaskulären Dysfunktion bei polymikrobieller Sepsis ist (Ehrentraut et al., 2012). Diese Erkenntnis konnte in weiteren Studien bestätigt werden. So konnte durch Lohner et al., im Colon-Ascendens-Stent-Peritonitis-induzierten Sepsis-Modell, dargestellt werden, dass TLR9-defiziente Mäuse im Vergleich zu ihren WT-Pendants eine signifikante Verringerung der Herzentzündung und eine anhaltende Herzfunktion zeigten (Lohner et al., 2013). Die wesentliche Bedeutung von TLR9 in der polymikrobiellen Sepsis mit einer gesteigerten NF-κB Aktivität und folgend einem Anstieg pro-inflammatorischer
Zytokine im Herzen, konnte bereits gezeigt werden (Knuefermann et al., 2008). Unsere Ergebnisse der TLR9-Inhibition im Gefäßbett wurden aber auch in anderen Organsystemen dargestellt. Die Hemmung von TLR9 durch das suppressive ODN H154-Thioat führte zu einer signifikanten Verbesserung der Herzentzündung, zum Erhalt der Herzfunktion und zur Verbesserung der Überlebensrate (Boehm et al., 2013). Auch bei steriler, d.h. nicht infektiöser Inflammation, zeigt sich die Rolle von TLR9 für die Entzündungsreaktion. So konnte die Arbeit von Markowski et al. schlüssig darstellen, dass es eine Stimulation von TLR9 vor Ischämie/Reperfusion des Herzens positive Effekt im Sinne präkonditionierender Effektes hat (Markowski et al., 2013). Zur weiteren Abklärung der septischen Signaltransduktion verlagerte sich der Untersuchungsschwerpunkt auf intrazelluläre Aspekte. So konnten wir erstmals zeigen, dass die humane Deneddylase-1 von relevanter Bedeutung für die sepsis-assoziierte NF-κB Aktivierung ist (Ehrentraut et al., 2013). Ein Verlust, bzw. die pharmakologische Blockade, dieses Signalweges führt zu einer Aufhebung der LPS Antwort und begünstigt die Stabilisation des vermeintlich anti-inflammatorischen Transkriptionsfaktors HIF1α. Dies zeigt sich sowohl in einer verminderten transkriptionalen und translationalen Zytokinexpression, als auch in einer erhaltenen Zellbarrierefunktion. Basierend auf unseren Arbeiten konnten in anderen, entzündungsassozierten Modellen bzw. Krankheiten dargelegt werden. So zeigte sich in Arbeiten von Zhang et al., dass ein intakter Neddylierungsmechanismus notwendig für die Herpes-simplex Virus induzierte Interferon-β-Antwort ist (Zhang et al., 2016). Die von uns gemachten Beobachtungen wurden durch die Arbeit von Asara et al. um den Aspekt der Artherosklerose erweitert. Diese zeigen, dass die Inhibition des Neddylierungsweges potentiell protektiv gegenüber der Ausbildung von artherosklerotischen Läsionen ist (Asara et al., 2017). Arbeiten zur endothelialen Dysfunktion unterstreichen und erweiterten dies nochmals. Auch darin konnte nachgewiesen werden, dass eine Histone-deacetylase 2 und 6 (HDAC2 bzw. -6) relevant für die Endothelfunktion sind, und ihre Auswirkung auf dem SENP8-anhängigen Neddylierungsmechanismus beruhen (Nomura et al., 2021; Pandey et al., 2015). Neben der endothelialen Bedeutung, konnte auch eine wesentliche Rolle für die kardiale Funktion, den kardialen Ischämie/Reperfusionsschaden und die Genese der Lungenfibrose gezeigt werden (Deng et al., 2017; Kandala et al., 2014; Li et al., 2020; Liu et al., 2019; Zhang et al., 2021).
Die oben genannten Untersuchungen fokussieren sich größtenteils auf die Bedeutung des Neddylierungsmechanismus im Endothel. Aufgrund der enormen Bedeutung des Transkriptionsfaktors NF-κB für die septische Inflammation, stellten wir uns die Frage, ob und in wieweit eine Beeinflussung des SENP8-Transduktionsweges, und damit mittelbar auch der NF-κB Antwort, Auswirkungen in anderen Zelltypen und Inflamationsmodellen hat. Dafür beleuchteten wir die Auswirkungen einer pharmakologischen Inhibition der Neddylierung in Darmepithelzellen und in einem T-Zell abhängigen Kolitismodell (Tri-Nitro-Benzoesäure, TNBS-Kolitis) (Louis et al., 2008; Neurath et al., 1996). Während in einem nicht-T-Zell-abhängigen Kolitismodell (Natriums-Dextransulfat, DSS-Kolitis) die Neddylierungsinhibition positiv erscheint (Curtis et al., 2014), zeigen die T-Zell-abhängigen Untersuchungen ein anderes Bild. Die Inhibition von SENP9 führt in der TNBS Kolitis zu einer verstärkten Inflammation und erhöhten Apoptoserate. Dies liegt gegebenenfalls in der besonderen Bedeutung des NF-κB Signalwegs im intestinalen Epithel begründet. So zeigten Zaph et al., dass der Verlust des NF-κB-Signals letztlich in einer herabgesetzten epithelialen Barrierefunktion und auch zu einer erhöhten Vulnerabilität gegenüber septischen Stimuli führt (Zaph et al., 2007). Unsere Studien deuten auf ähnliche Ergebnisse mit der gezielten Neddylierungsinhibition in vivo hin und zeigen, dass die Kombination von Deneddylierung als präapoptotischer Sensibilisator in Gegenwart von inflammatorischen Stimuli die Entzündungsreaktion deutlich verstärkt. Wir haben diese Ergebnisse erweitert, um die Expression von SENP8 bei Colitis in der Maus und beim Menschen zu bestimmen. In allen getesteten Modellen, einschließlich Biopsiematerial von Patienten mit chronisch entzündlichen Darmerkrankungen, war die Entzündung mit einem Verlust der SENP8-Expression verbunden. Diese Beobachtungen deuten darauf hin, dass die Herabregulierung von SENP8 bei Colitis bei Mäusen und Menschen als Ausgleichsmechanismus dient, um die laufende Entzündungsreaktion zu unterdrücken. Die Mechanismen einer solchen Regulierung müssen noch weiter untersucht werden. Dies wird durch Beobachtungen von Wang et al. bestätigt. Diese postulieren, dass negative Effekte einer Neddylierungsinhibition in einer gestörten Zellzyklusregulation begründet sind (Wang et al., 2020).
5. Zusammenfassung

Die Sepsis bleibt, auch nach Dekaden intensiver Forschung, ein bislang unzureichend verstandenes, hochkomplexes Krankheitsbild mit hoher Mortalität. Wichtige Teilkomponenten des septischen Geschehens ist die Unfähigkeit des Wirtsorganismus eine adäquate, also nichtüberschießende, Immunantwort herbeizuführen. Häufig kommt es zu einer fehlmessenen, überschießenden Antwort mit vielfachen, für den Organismus letztlich nachteiligen, Folgen. Dazu gehört unter anderem die septische Vasoplegie, d.h. die Unfähigkeit einer ausreichenden Gefäßkontraktion zur Erhaltung eines ausreichenden totalen peripheren Widerstands und damit eines ausreichenden arteriellen Blutdrucks. Die erste hier vorgelegte Arbeit befasst sich mit der Bedeutung des TLR 4 für die septische Vasoplegie. Die Ergebnisse konnten erstmals zeigen, dass ein funktioneller TLR4 im Gefäßsystem notwendig für die Weiterleitung des septischen Stimulus ist, und letztlich NO-vermittelt zur Vasoplegie führt. Eine pharmakologische Blockade dieses Signalweges verhindert die septische Vasoplegie und ist in der Lage auch im Tier die septische Hypotension zu vermeiden, unter anderem durch eine Stabilisierung der Herzfrequenz und einer verminderten Ausschüttung proinflammatorischer Botenstoffe.

Auch andere Mustererkennungsrezeptoren neben TLR4 spielen eine besondere Rolle. So konnte durch uns dargestellt werden, dass es im Rahmen einer TLR2-Defizienz zu einer relevanten Überexpression anderer PRR kommt und sich daraus möglicherweise eine nachteilige Vasodilation in der polymikrobiellen Sepsis bei TLR2-defizienten Tieren erklärt.

Die letzten beiden Arbeiten beschäftigen sich mit der Rolle sog. NEDDylierungsprozesse für das Entstehen einer septischen Reizantwort. Wir konnten zeigen, dass es ein funktionales Vorliegen der sog. Humanen Deneddylase-1/SENP8 notwendige Voraussetzung für eine NF-κB-vermittelte endetheliale Entzündungsreaktion ist. Darüber hinaus konnten wir belegen, dass eine pharmakologische Inhibition des Neddylierungsmechanismus zu einer vermindertem LPS-vermittelten Ausschüttung pro-entzündlicher Zytokine führt.

Dieser Signalweg zeigte sich auch in anderen Zellmodellen, so z.B. in Darmepithelzellen, von besonderer Bedeutung. Auch dort kann eine Inhibition der Neddylierung positive Effekte haben, sofern es sich um eine nicht-T-Zell-vermittelte Reaktion handelt. Im Rahmen eher chronischer Geschehnisse, wie der T-Zell-vermittelten TNBS-Colitis zeigte sich hingegen ein nachteiliger Effekt der
Neddylierungshemmung. Dies liegt am ehesten an einer fehlerhaften septischen Apoptoseregulation. Ergänzend zeigte sich die Bedeutung von SENP8 auch in Proben von Patienten mit chronischen Darmerkrankungen, a.e. kompensatorisch, heruntergeregelt ist.

Die vorgelegten Arbeiten erweitern das pathophysiologische und pathobiochemische Verständnis der septischen Entzündungsreaktion, insbesondere in Hinblick auf die Bedeutung verschiedener Toll-like Rezeptoren und des Neddylierungsmechanismus.
6. Überlappung durch geteilte Autorenschaften

Die vorliegende Habilitationsschrift hat fünf publizierte Originalarbeiten zur Grundlage. Zwei der Arbeiten habe ich als Erstautor (Ehrentraut et al., 2011b, 2016) publiziert. Eine Arbeit habe ich mit Herrn Ralph Lohner als geteilter Erstautor zusammen veröffentlicht. Herr Lohner war medizinischer Doktorand in unserer Gruppe dessen überdurchschnittliches Engagement mit einer geteilten Erstautorenschaft gewürdigt wurde. Frau Dr. Valerie Curtis hat Teile der Experimente aus meiner amerikanischen Post-Doktorenzeit komplettiert und dadurch die Fertigstellung des Manuskriptes ermöglicht. Entsprechend ihres Engagements wurde die Autorenschaft geteilt.

Eine Überlappung mit anderen Habilitationsschriften ist nicht gegeben. Die 2007 in ATVB publizierte Arbeit stellt die publizierten Ergebnisse meiner Dissertation dar. Sie wird hier als mitaufgeführt, da sie den Grundstein des wissenschaftlichen Oeuvres darstellt und aus meiner Sicht in die Gesamtheit des Themas miteinbezogen gehört. Die von der Fakultät geforderte Mindestanzahl von vier Erst-/Letztautorenschaften wird auch ohne diese Arbeit erfüllt.
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Preise
- 1. Preis „Wissenschaftlicher Vortragswettbewerb der Deutschen Gesellschaft für Anästhesiologie und Intensivmedizin: Inflammatorische Hypoxie reprimiert die humane Deneddylose-1(SEN P8)“ Deutscher Anästhesie Kongress 2013, Nürnberg
- 2. Preis „Grundlagenforschung“: „Die Bedeutung der Toll-Like-Rezeptoren für die vaskuläre Kontraktilität nach CASP“ Hauptstadtkongress für Anästhesie und Intensivmedizin 2010, Berlin.

Stipendien
- Nachwuchsgruppenförderung der BONFOR-Kommission der medizinischen Fakultät der Universität Bonn von November 2017 bis November 2018
- DFG Forschungsstipendium „Adenosine-mediated HIF-1α and NFκB regulation during hypoxia and inflammation“

Drittmittelanträge
- COVID DataNET.NRW – Frühzeitige Vorhersage des Krankheitsverlaufs bei COVID-19 auf Basis Künstlicher Intelligenz
10. Erklärung
Hiermit bestätige ich, dass ich die Richtlinien zur guten wissenschaftlichen Praxis der Universität Bonn, laut Habilitations-Ordnung, zur Kenntnis genommen habe und ich versichere, dass ich sie beim Verfassen der Habilitationsschrift beachtet habe. Insbesondere versichere ich, dass ich alle in der Habilitationsschrift benutzten Quellen und Hilfsmittel angegeben habe.

Alfter, den 24.11.2021

(Hier bitte Ihre Unterschrift einfügen)

Dr. med. Stefan Felix Ehrentraut