Aus dem
Universitätsklinikum Düsseldorf
Klinik für Unfall- und Handchirurgie
Klinikdirektor: Univ.-Prof. Dr. med. Joachim Windolf

NEUE ASPEKTE DER DYSREGULIERTEN APOPTOSE UND HYPERAKTIVITÄT IN NEUTROPHILEN GRANULOZYTEN NACH SCHWEREM TRAUMA

-RELEVANZ FÜR DIE POSTTRAUMATISCHE IMMUNANTWORT-

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Dr. rer. nat. Adnana Nicoleta Paunel-Görgülü
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Dekan: Univ.-Prof. Dr. med. Joachim Windolf

Gutachter: 1. Prof. Dr. rer. nat. Victoria Kolb-Bachofen

2. Prof. Dr. med. Nikolas Stöcklein

Meiner Familie

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Abkürzungen

ARDS	Acute Resopiratory Distress Syndrome
CARS	Compensatory anti-inflammatory Response Syndrome
FADD	Fas-associated death domain
Fas	Fas Rezeptor
sFas	löslicher (soluble) Fas Rezeptor
FasL	Fas Ligand
GM-CSF	Granulozyten-Makrophagen-Kolonie-stimulierender Faktor
IL	Interleukin
ISS	Injury Severity Score
MAP	mittlerer arterieller Druck
MODS	Multiorgandysfunktionssyndrom
MPO	Myeloperoxidase
MOV	Multiorganversagen
PMN	neutrophile Granulozyten
PMNE	PMN Elastase
ROS	reaktive Sauerstoffspezies
SIRS	Systemisches Inflammatorisches Response-Syndrome
SOFA	Sequential Organ Failure Assessment
STS	Staurosporin

1. Darstellung des Habilitationsthemas

1.1 Einleitung

Durch das bessere Verständnis der Pathophysiologie des posttraumatischen systemischen inflammatorischen Response-Syndrome (SIRS) und des nachfolgenden Organversagens in jüngster Vergangenheit hat man die Schlüsselrolle polymorphkerniger neutrophiler Granulozyten (PMN) bei den Gewebeverletzungen und den daraus resultierenden Organdysfunktionen bereits erkannt. Nach einem schweren Trauma kommt es über zum Teil unerforschte Signalwege zu einer Hemmung der spontanen Apoptose und dadurch zu einer Verlängerung der PMN-Lebensdauer. Die Anzahl der PMN korreliert dabei mit der zunehmenden Freisetzung toxischer reaktiver Sauerstoffspezies (ROS) und Proteasen und den daraus resultierende Gewebeschädigungen.

Die vorliegende Arbeit hat es sich zum Ziel gemacht, die molekularen Mechanismen, die der PMN-Apoptoseresistenz zugrunde liegen, sowie die Bedeutung der reduzierten Apoptose für die posttraumatische Immunantwort näher zu charakterisieren. Ferner sollte die Überwindung der Apoptoseresistenz über alternative molekulare Signalwege geprüft werden. Die daraus resultierenden Erkenntnisse sollen zum besseren Verständnis antiapoptotischer Signalwege unter pathologischen Bedingungen beitragen.

1.1.1 Rolle neutrophiler Granulozyten für die posttraumatische Immunreaktion

Trauma gehört zu den häufigsten Todesursachen in der Bevölkerung der unter 50jährigen in der westlichen Welt. Die heute gültige Definition beschreibt ein Polytrauma als gleichzeitig entstandene Verletzungen verschiedener Körperregionen bzw. Organsystemen, von denen die Verletzung eines Organsystems oder mehrerer Organsysteme als lebensbedrohlich gilt (Tscherne, Regel et al. 1987). Die Patienten sterben als Folge ihrer schweren Verletzungen oder aufgrund einer zusätzlichen Organschädigung durch die Trauma-bedingten Immunreaktionen (Hietbrink, Koenderman et al. 2006). Über 5 % aller Patienten mit schwerem Trauma entwickeln ein multiples Organversagen (MOV), wobei die Funktion mehrerer Organe wie z. B. Lunge, Nieren, Leber und Herz nachträglich oder simultan versagt. Neben einer

genetischen Prädisposition der Patienten werden exogener Faktoren, wie das Trauma an sich („first hit“), sowie der anschließenden chirurgischen Intervention („second hit“) besondere Beachtung bei der klinischen Manifestation des Organversagens geschenkt (Rotstein 2003; Cobb and O'Keefe 2004). Die Organschädigung und das nachfolgende Organversagen sind Folgen einer Dysregulation des Immunsystems.

Es ist weitgehend anerkannt, dass die erste inflammatorische Immunreaktion auf das Trauma im Wesentlichen durch die PMN geprägt wird. So führt die Gewebeschädigung infolge eines Traumas unverzüglich zur Aktivierung der PMN, die als Zellen der unspezifischen Immunabwehr eine erste Barriere gegen eindringende Pathogene bilden (Hernandez, Grisham et al. 1987). Täglich werden bis zu 10^{11} PMN konstitutiv aus dem Knochenmark freigesetzt, und diese Zahl kann nach einem schweren Trauma um ein Vielfaches gesteigert werden (Christopher and Link 2007). Obwohl PMN eine essentielle Rolle für die angeborene Immunabwehr und Phagozytose von Mikroorganismen einnehmen, haben diese Zellen einen Janus-Charakter. Die posttraumatische Aktivierung dieser Zellen ist Teil einer systemischen Abwehrreaktion (Systemisches inflammatorisches Response-Syndrom, SIRS), welche mit einem erhöhten zytotoxischen Potential und verstärkter Bildung von ROS einhergeht. Die vermehrte Sekretion proteolytischer Enzyme wie Elastase und Myeloperoxidase (MPO) oder ROS kann wiederum zur Schädigung der Endothelzellschicht und somit zur Ödembildung und Gewebsschädigung beitragen (Ogura, Tanaka et al. 1999; Bhatia, Dent et al. 2006). Normalerweise haben PMN als ausdifferenzierte Zellen eine sehr kurze Lebensdauer von 8-20 h im Blutkreislauf und 1-4 Tagen in Geweben, da sie konstitutiv in die spontane Apoptose übergehen (Maianski, Maianski et al. 2004). Eine rezente Studie von Pillay *et al.* zeigt jedoch, dass unter *in vivo* Bedingungen die PMN-Lebensdauer in der Zirkulation mit 5,4 Tagen wesentlich länger zu sein scheint als ursprünglich postuliert (Pillay, den Braber et al. 2010). Unter Berücksichtigung der täglichen PMN-Freisetzungsrate aus dem Knochenmark sowie einer konstanten Zellzahl von 3×10^6 PMN pro ml Blut, wäre jedoch von einer beschränkten Lebensdauer von 5 h und nicht 5 Tagen auszugehen. Somit sind in zukünftigen Arbeiten sowohl die PMN-Mengen, die vom Knochenmark gebildet werden, als auch die Lebensspanne zirkulierender PMN im Hinblick auf ihre Gültigkeit zu prüfen. Ungeachtet dessen führen proinflammatorische Mediatoren (wie z. B. G-CSF, GM-CSF, IL-1, IL-2 und IL-6), die nach einer schweren

Verletzung sezerniert werden, zu einer signifikanten Verlängerung der PMN-Lebensdauer von bis zu 3 Wochen (Brach, deVos et al. 1992; Colotta, Re et al. 1992; Pericle, Liu et al. 1994; Biffl, Moore et al. 1995; Maianski, Maianski et al. 2004; Taneja, Parodo et al. 2004). Somit kann die Verzögerung bzw. Störung des Apoptoseprozesses als Folge eines schweren Traumas sowohl die Entwicklung einer SIRS als auch Gewebeschädigungen und MOV begünstigen. Die klinische Relevanz dieses Phänomens wird unterstrichen durch die Beobachtung, dass eine Korrelation zwischen erhöhter PMN-Aktivität und dem Vorkommen von SIRS und MOV besteht (Partrick, Moore et al. 1996). Es ist im Weiteren bekannt, dass die exzessive proinflammatorische Phase häufig von einer kompensatorischen antiinflammatorischen Phase, genannt CARS, gefolgt wird, welche u.a. durch eine verminderte HLA-DR Expression auf Monozyten, erhöhte T-Zell-Apoptose und T-Zellanergie gekennzeichnet ist (Cheadle, Pemberton et al. 1993; Bandyopadhyay, De et al. 2007; Frazier and Hall 2008). Während eine überschießende Inflammation Schock und Organschädigung vorantreibt, kann eine kompensatorische antiinflammatorische Gegenreaktion eine Immunsuppression und damit verbundenen Komplikationen, wie z. Bsp. die Entstehung einer Sepsis, positiv begünstigen. Aktuellste Untersuchungen belegen, dass Anzeichen für SIRS und CARS unmittelbar nach einem schweren Trauma zeitgleich und über mindestens 96 Stunden auftreten können (Kasten, Goetzman et al. 2010). Während die Rolle hyperaktivierter PMN bei der Verschiebung dieses Gleichgewichts Richtung Immunsuppression derzeit noch völlig ungeklärt ist, bleibt deren Bedeutung für das frühe posttraumatische Organversagen im Rahmen einer SIRS unumstritten.

1.1.2 Bedeutung neutrophiler Granulozyten für die Pathologie der Sepsis

Die häufigste Todesursache in der späten Phase nach einem Trauma ist das Sepsis-bedingte MOV, welches ursprünglich auf infektiöse Komplikationen im zeitlichen Verlauf nach Trauma und der damit verbundenen Fehlfunktion mehrerer Organsysteme zurückzuführen ist. Trotz intensivster Bemühungen die Sepsis durch adäquate Antibiotika- und Volumentherapie sowie Unterstützung der Organfunktionen einzudämmen, liegen die geschätzte jährliche Inzidenz in den Vereinigten Staaten bei 700.000 Fällen und die aktuelle Mortalitätsrate mit 35 % auf einem hohen Niveau.

Die heute allgemein anerkannte Definition beschreibt die Sepsis als eine systemische Inflammation (SIRS) als Antwort auf eine Infektion (Levy, Fink et al. 2003). Die initialen Stimuli sind häufig bakterielle Bestandteile, die zu einer übermäßigen Ausschüttung proinflammatorischer Mediatoren aus diversen Zellen des Immunsystems führen. Diese proinfalmmatorische Phase kann, ähnlich wie nach einem schweren Trauma, von einer kompensatorischen antiinflammatorischen Antwort begleitet werden, die mit einer schlechten Prognose und Anzeichen der Immunsuppression assoziiert ist (Hack, Aarden et al. 1997). Bei der Pathologie der Sepsis nehmen PMN, die systemisch aktiviert werden, eine wichtige Stellung ein. So konnte in Patienten mit akuter respiratorischer Insuffizienz (ARDS), eine im Verlauf der Sepsis häufig auftretende Schädigung der Lunge, ein deutlicher Zusammenhang zwischen Ausmaß der PMN-Infiltration, Verschlechterung der Lungenfunktion und hohen Konzentrationen an PMN-stämmigen proteolytischen Enzymen beobachtet werden (Windsor, Mullen et al. 1993). Mehrere Arbeiten konnten belegen, dass die PMN-Apoptose in Patienten mit SIRS, Sepsis, ARDS und solchen mit hohem Risiko ein Organversagen zu entwickeln, deutlich reduziert ist (Biffl, Moore et al. 1999; Wagner, Pioch et al. 2000; Martins, Kallas et al. 2003; Paunel-Görgülü, Zörnig et al. 2009). Dieser Effekt ist häufig auf zirkulierende Faktoren wie Lipopolysaccharide, Lipoteichonsäure und proinflammatorische Zytokine zurückzuführen. Die verlängerte Lebensspanne von gewebsinfiltrierten PMN erhöht die Wahrscheinlichkeit für extrazelluläre Schäden bedingt durch eine überschüssige unkontrollierte Freisetzung von ROS und Proteasen. Ferner wird das Überleben von PMN in Geweben durch lokale antiapoptotischen Faktoren begünstigt. So konnten Lesur *et al.* zeigen, dass die niedrige PMN-Apoptoserate in den Lungenalveolen von ARDS-Patienten im engen Zusammenhang mit den IL-2 Konzentrationen in der bronchoalveolären Flüssigkeit steht (Lesur, Kokis et al. 2000). Die genauen Mechanismen der reduzierten Apoptose in Sepsis und ARDS sind derzeit nicht vollständig verstanden. Vorangehende Studien gehen davon aus, dass u. a. eine erhöhte Expression des Transkriptionsfaktors NF-κB, eine Verminderung der Caspasenaktivitäten, eine Modulation von Mcl-1, PBEF (pre-B cell colony-enhancing factor) sowie von p38 MAPK Signalwegen in den antiapoptotischen Prozessen in PMN involviert sind (Sheth, Friel et al. 2001; Jia, Li et al. 2004; Taneja, Parodo et al. 2004; Paunel-Görgülü, Zörnig et al. 2009). Durch die Beobachtung einer inversen Korrelation zwischen PMN-Apoptoserate und Schweregrad der Sepsis wird die Relevanz der

PMN-Apoptose für die Pathogenese der Sepsis deutlich. In diesem Zusammenhang wurde in einer prospektiven Studie gezeigt, dass PMN aus Patienten mit Sepsis-induzierter ARDS und der höchsten Mortalitätsrate die niedrigste PMN-Apoptoserate aufweisen, verglichen mit der Apoptose in Patienten mit unkomplizierter Sepsis (Fialkow, Fochesatto Filho et al. 2006). Obwohl PMN essentielle Effektorzellen zur Beseitigung eingedrungener Pathogene sind, führt deren Hyperaktivierung im Rahmen einer Sepsis unvermeidlich zur kollateralen Schädigung von Organfunktionen. Ein besseres Verständnis der zugrunde liegenden molekularen Mechanismen würde die Entwicklung zielgerichteter Therapien zur Eindämmung inflammatorischer Prozesse vorantreiben.

1.1.3 Regulation der Apoptose in neutrophilen Granulozyten

PMN spielen eine essentielle Rolle bei der angeborenen Immunantwort. Die Einleitung der Apoptose wenige Stunden nach ihrer Freisetzung aus dem Knochenmark ist jedoch entscheidend bei der Aufrechterhaltung der Homöostase und Abklingen der Inflammation. Daher wird die Verlängerung der PMN-Lebensdauer als ein kritischer Faktor hinsichtlich Schweregrad und Dauer der Infektion bzw. Inflammation angesehen.

Bei der Regulation der Apoptose in PMN nehmen Mitglieder der Bcl-2-Familie eine essentielle Rolle ein. Diese Proteine zeichnen sich durch den Besitz von bis zu vier konservierte Bcl-2-Homologiedomänen (BH-Domänen) aus und werden je nach Funktion in pro- und antiapoptotische Faktoren unterteilt. Bei den proapoptotischen Faktoren unterscheidet man im Weiteren zwischen Mitgliedern mit mehreren BH-Domänen (wie z. Bsp. Bax und Bak) und solchen, die nur die BH3-Domäne besitzen (z. Bsp. Bid und Bim). Letztere können erst nach Interaktion mit den Mitgliedern, die mehr als eine BH-Domäne aufweisen, ihre proapoptotische Wirkung entfalten (Desagher, Osen-Sand et al. 1999; Wei, Lindsten et al. 2000; Cheng, Wei et al. 2001; Wei, Zong et al. 2001; Zong, Lindsten et al. 2001). PMN exprimieren eine ganze Reihe proapoptotischer Proteine der Bcl-2-Familie, einschließlich Bax, Bad, Bak, Bid und Bik (Moulding, Akgul et al. 2001). Die wichtigsten antiapoptotischen Faktoren, die bislang in PMN nachgewiesen wurden, sind Mcl-1 (Myeloid cell leukemia-1) und Bfl-1/A1 (= A1). Konditionell erzeugte *mcl-1* Knockout-Mäuse, bei denen das *mcl-1* Gen spezifisch in Makrophagen und PMN ausgeschaltet wurde,

weisen eine normale Makrophagenpopulation und gleichzeitig eine 3-fach erhöhte PMN-Apoptoserate im Vergleich zu den Kontrolltieren auf (Dzhagalov, St John et al. 2007). Ferner führte die Beobachtung, dass PMN aus *a1* -/- Mäusen eine erhöhte spontane Apoptose aufweisen, zu dem Schluss, dass A1 ebenfalls an der Regulation der PMN-Lebensdauer beteiligt ist (Kotani, Avallone et al. 2003). Die mRNAs von Mcl-1 und A1 (und Mcl-1-Protein) haben eine sehr kurze Halbwertszeit von 2-3 h, was im Allgemeinen eine schnelle Regulation der PMN-Lebensdauer sowie -Aktivität erlaubt (Dzhagalov, St John et al. 2007). Die Expression beider Faktoren kann durch Zytokine, wie GM-CSF und TNF-α, verstärkt werden (Cross, Moots et al. 2008). Aus der Literatur ist bekannt, dass die zellulären Mengen des Mcl-1 Proteins mit dem PMN-Überleben korrelieren, d.h. hohe Mcl-1 Proteinlevel erlauben der Zelle zu überleben, wohingegen eine Abnahme an Mcl-1 die Apoptose einleitet.

Die Entscheidung zwischen Zellüberleben und Apoptose wird primär durch das intrazelluläre Verhältnis von pro- und antiapoptotischen Proteinen sowie deren Lokalisation, Konformation und Phosphorylierungsstatus bestimmt (Gross, McDonnell et al. 1999). Beim intrinsischen Apoptose-Signalweg, der im Verlauf der spontanen Apoptose überwiegt, spielt das Mitochondrium die zentrale Rolle. Pro-apoptotische Bid und Bax Proteine translozieren zur depolarisierten Mitochondrienmembran, führen zu einer erhöhten Permeabilität der äußeren Mitochondrienmembran und somit zur Freisetzung proapoptischer Faktoren wie Cytochrom c (Fossati, Moulding et al. 2003; Maianski, Geissler et al. 2004; Maianski, Roos et al. 2004). Dieses bindet an Apaf-1 und pro-Caspase 9, um das Apoptosom zu bilden, welches essentiell für die Caspase-9-Aktivierung sowie nachfolgender Caspasen ist (Budihardjo, Oliver et al. 1999; Yuan and Yankner 2000). Gleichzeitig wird eine Abnahme der intrazellulären Mcl-1 Proteinmengen beobachtet, so dass Bax aus dem heterodimeren Mcl-1:Bax-Komplex entlassen und zu den Mitochondrien versetzt wird (Moulding, Quayle et al. 1998). Trotz zahlreicher kontroverser Berichte, die die Interaktion von A1 mit proapoptotischen Faktoren wie Bax, Bak, Bid und truncated Bid (tBid) beschreiben, ist der Mechanismus der A1-vermittelten antiapoptotischen Wirkung zurzeit ungeklärt (Holmgreen, Huang et al. 1999; Zhang, Cowan-Jacob et al. 2000; Werner, de Vries et al. 2002; Simmons, Fan et al. 2008).

Die Apoptose kann zusätzlich über den extrinsischen bzw. Rezeptor-vermittelten Signalweg eingeleitet werden. Das Fas (CD95; APO-1)/Fas Ligand (FasL; CD95L)-System gehört zu den wichtigsten zellulären Signalwegen zur

Apoptoseeinleitung in einer Vielzahl von Geweben und spielt eine wichtige Rolle bei der PMN-Apoptoseregulation und Herunterregulation der PMN-Aktivität (Cinatl, Blaheta et al. 2000). Der Fas-Rezeptor gehört zur Tumor-Nekrose-Faktor-/Nervenwachstumsfaktor (TNF/NGF)-Rezeptor-Familie und wird auf vielen Zelltypen exprimiert (Nagata 1994; Nagata and Golstein 1995). Die Ligandenbindung löst eine Trimerisierung und somit Aktivierung der Rezeptoren aus. Eine Aktivierung des Fas-Rezeptors kann durch FasL, sFasL (die lösliche Form des Liganden) sowie durch Kreuzvernetzung mit anti-Fas IgM Antikörpern erfolgen, wodurch eine Kaskade von proteolytischen Reaktionen ausgelöst wird, die schließlich in die Zellapoptose einmündet (Suda, Takahashi et al. 1993; Owen-Schaub, Angelo et al. 1995; Watson, Rotstein et al. 1997). Hierbei wird eine sogenannte Caspasen-Kaskade eingeleitet, in der die jeweils folgende Caspase durch proteolytische Spaltung aktiviert wird. Eine zentrale Funktion bei der apoptotischen Signaltransduktion nimmt die Caspase-8 ein, die zum DISC-Komplex (death-inducing signaling complex) gehört und sich über das Adapterprotein FADD (Fas-associated death domain) an den Fas-Rezeptor anlagert. Die durch Autoprozessierung aktivierte Caspase-8 setzt nun die Caspasen-Kaskade in Gang, indem es distal fungierende Caspasen wie Caspase-3 und -7 aktiviert (Muzio, Chinnaiyan et al. 1996; Ashkenazi and Dixit 1998). Obwohl beide apoptotische Signalwege, der intrinsische sowie der extrinsische, unabhängig voneinander agieren können, bildet das Bid-Protein den molekulare Link zwischen diesen beiden Signalwegen. Bid wird als Folge der Fas-Aktivierung durch die aktive Caspase-8 gespalten. Das gespaltene Protein tBid transloziert zu den Mitochondrien und induziert die Oligomerisierung von Bax und/oder Bak (Li, Zhu et al. 1998; Eskes, Desagher et al. 2000; Wei, Lindsten et al. 2000). Es wurde bereits gezeigt, dass sowohl Mcl-1 als auch A1 als Bindungspartner von tBid fungieren können, wodurch die tBid-induzierte Cytochrom c Freisetzung inhibiert werden kann (Simmons, Fan et al. 2008).

Während der spontanen Apoptose in PMN werden beide Signalwege, d. h. sowohl der Mitochondrien-abhängige als auch der Rezeptor-vermittelte Weg, aktiviert (Daigle and Simon 2001). Eine Reihe von Studien mit peripheren PMN konnte im Weiteren belegen, dass proinflammatorische Mediatoren aus dem Serum bzw. Plasma von Patienten mit inflammatorischen Erkrankungen zu einer ausgeprägten Hemmung beider Signalwege führen (Chitnis, Dickerson et al. 1996; Jimenez, Watson et al. 1997; Asensi, Valle et al. 2004; Taneja, Parodo et al. 2004; Paunel-

Görgülü, Zörnig et al. 2009). Diese proinflammtorischen Zytokine, wie GM-CSF, entfalten ihre anti-apoptotische Wirkung durch Aktivierung von MAPK- und PI3K/Akt-Signalkaskaden, so dass diesen Signalwegen eine Schlüsselrolle bei der Regulation der PMN-Apoptose sowie des Zellüberlebens zugesprochen wird (Klein, Rane et al. 2000; Klein, Buridi et al. 2001). In PMN konnte gezeigt werden, dass sowohl der ERK- als auch der PI3K/Akt-Signalweg durch anti-apoptotische Faktoren wie Lipopolysaccharide und rekombinantes GM-CSF aktiviert werden, was zu einer Erhöhung der Mcl-1-Proteinstabilität führt (Derouet, Thomas et al. 2004). Die ERK-vermittelte Proteinphosphorylierung resultiert in einem verminderten Abbau durch das Proteasom (Domina, Vrana et al. 2004), während Akt die GSK-3-Aktivität und dadurch die Phosphorylierung sowie die nachfolgende Mcl-1 Ubiquitinylierung hemmt (Maurer, Charvet et al. 2006). Sowohl ERK- als auch PI3K/Akt-Inhibitoren haben *in vitro* zu einer verstärkten Apoptose in PMN geführt (Hirata, Kotani et al. 2008).

1.2 Eigene Arbeiten und Diskussion

1. Arbeit: Paunel-Görgülü A., Zörnig M., Lögters T., Altrichter J., Rabenhorst U., Cinatl J., Windolf J., Scholz M.: Mcl-1-mediated impairment of the intrinsic apoptosis pathway in circulating neutrophil from critically ill patients can be overcome by Fas stimulation. *J Immunol* 2009, 183:6198-206.

Nach einem schweren Trauma kommt es bereits innerhalb weniger Stunden zu einer deutlichen Verringerung der Apoptoserate in zirkulierenden PMN, die mit einer systemischen Hyperaktivierung dieser Zellen einhergeht. Damit verbunden ist eine vermehrte Freisetzung proteolytischer Enzyme und ROS, begleitet von Schädigungen der Endothelzellschicht und des umliegenden Gewebes. Schwerverletzte Patienten versterben zumeist in Folge ihrer schweren Verletzungen, häufig jedoch auch bedingt durch die unkontrollierten Immunreaktionen und den damit assoziierten Organdysfunktionen. In der vorliegenden Arbeit wurden die molekularen Signalwege der verzögerten PMN-Apoptose nach schwerem Trauma erforscht und alternative Signalwege zur Überwindung dieser Apoptoseresistenz vorgeschlagen. Unsere Untersuchungen machten erstmals deutlich, dass die Trauma-bedingte verlängerte Lebensdauer von PMN auf einer Inhibition des Mitochondrien-abhängigen intrinsischen Apoptose-Signalweges beruht.

Die anfänglichen Versuche haben ergeben, dass die Behandlung von PMN aus gesunden Probanden mit dem Serum polytraumatisierter Patienten (ISS > 16; Tag 1 nach Trauma) eine signifikante Hemmung der PMN-Apoptose zur Folge hat, verglichen mit der Apoptoserate in Zellen, die in Anwesenheit von autologem Serum kultiviert wurden. Dies deutete darauf hin, dass die beobachtete Hemmung der PMN-Apoptose in polytraumatisierten Patienten auf Serumfaktoren zurückzuführen ist. Interessanterweise korrelierte diese mit einer deutlichen Erhöhung der Mcl-1 Proteinlevel in diesen Zellen. Mcl-1 ist ein antiapoptotisches Mitglied der Bcl-2-Familie, dessen Expression, wie gezeigt werden konnte, in PMN nach einem Trauma stark zunimmt. Durch die Interaktion mit proapoptotischen Faktoren vermag Mcl-1 deren Translokation zu den Mitochondrien zu unterbinden und auf diese Weise die Aktivierung des Mitochondrien-abhängigen intrinsischen Signalweges zu hemmen. Der intrinsischer Weg wird bekannterweise durch das intrazelluläre Verhältnis von

pro- und antiapoptotischen Proteinen, wie Mcl-1, bestimmt. Um zu prüfen ob Mcl-1 tatsächlich den mitochondrialen Signalweg in PMN aus polytraumatisierten Patienten hemmt, wurden frisch isolierte Zellen aus gesunden Probanden und Patienten mit Staurosporin (STS), einem Breitspektrum-Kinase-Inhibitor, der den intrinsischen Apoptosesignalweg zu induzieren vermag, behandelt. Tatsächlich führte diese Behandlung in Kontroll-PMN zu einer deutlichen Abnahme der intrazellulären Mcl-1 Level, verbunden mit einem Verlust des mitochondrialen Membranpotentials und zunehmender Aktivität der Caspase-9. Im Gegensatz dazu konnte nach STS-Zugabe in Patienten-PMN sowie in PMN, die mit Patientenserum vorbehandelt wurden, eine erhöhte Stabilität des Mcl-1 Proteins und eine geringere Apoptoserate ausgemacht werden, verglichen mit Zellen aus gesunden Probanden. Folglich wurde in den Patientenzellen eine Mcl-1-vermittelte Inhibition der intrinsischen Apoptose gezeigt.
Zusätzlich konnte als wichtiger Mediator der PMN-Apoptoseresistenz nach Trauma das proinflammatorische Zytokin GM-CSF identifiziert werden. Wie im Rahmen dieser Arbeit gezeigt, steigt die GM-CSF-Konzentration im Serum polytraumatisierter Patienten bereits wenige Stunden nach dem Unfall an und bleibt bis mindestens Tag 11 nach Trauma deutlich erhöht. Nachfolgende Untersuchungen haben ergeben, dass die Expression des wichtigsten antiapoptotischen Proteins in PMN, nämlich Mcl-1, einer positiven Regulation durch das Zytokin GM-CSF unterliegt. Eine Hemmung der biologischen Aktivität von GM-CSF im Patientenserum durch neutralisierende anti-GM-CSF Antikörper führte zu einer starken Abnahme der Mcl-1 Proteinlevel und gleichzeitig zu einer signifikanten Erhöhung der spontanen sowie der STS-induzierten Apoptose. Aktuellere eigene Untersuchungen mit rekombinantem GM-CSF machten jedoch deutlich, dass dieses allein keine STS-Resistenz induzieren kann. Somit wird deutlich, dass GM-CSF im Zusammenspiel mit mindestens einem weiteren zirkulierenden Faktor die intrinsische Apoptoseresistenz in PMN nach Trauma zu vermitteln vermag (unveröffentlichte Daten). Die Identifikation dieses Faktors bzw. dieser Faktoren ist Gegenstand zukünftiger Forschungsarbeiten.

Es ist darüber hinaus bekannt, dass neben dem intrinsischen Signalweg die Apoptose auch über den extrinsischen Weg, so z. Bsp. über Aktivierung des Oberflächen-Todesrezeptors Fas (CD95), initiiert werden kann. Die Ligandenbindung löst eine Trimerisierung und somit Aktivierung des Rezeptors aus. Aus der Literatur ist bekannt, dass die Aktivierung von Fas durch FasL, sFasL sowie durch

Kreuzvernetzung mit anti-Fas IgM Antikörpern erfolgen kann, wodurch eine Kaskade von proteolytischen Reaktionen ausgelöst wird, die schließlich in die Zellapoptose einmündet. Dieser alternative Apoptose-Signalweg wurde im Rahmen weiterer Versuchsreihen näher untersucht. Anhand von Gen- und Proteinexpressionsanalysen konnten wir bestätigen, dass PMN zum einen sowohl den Fas-Rezeptor als auch dessen Liganden (FasL) exprimieren und zusätzlich zeigen, dass die Expression beider Proteine im zeitlichen Verlauf nach Trauma (bis mindestens Tag 11) unverändert bleibt. Diese Ergebnisse sprechen dafür, dass der Fas-Rezeptor auf PMN ein potentielles therapeutisches Target zur Überwindung der Apoptoseresistenz darstellen könnte. Anderseits wurde bereits anhand mehrerer Kleintierstudien demonstriert, dass eine systemische Applikation agonistischer anti-Fas Antikörper mit einer unkontrollierten Apoptose in verschiedenen Zelltypen und massiver Organschädigung, wie z. Bsp. der Lunge und Leber, assoziiert ist (Ogasawara, Watanabe-Fukunaga et al. 1993). Folglich stellt die Immobilisierung von agonistischen anti-Fas Antikörpern (Klon CH-11) auf einer biokompatiblen Matrix (Polyurethanschaum) eine sinnvolle Alternative dar um die toxischen Effekte löslicher Antikörper zu umgehen. Dieser therapeutische Ansatz, der ausschließlich in Form einer extrakorporalen Immuntherapie Verwendung finden könnte, wurde im Weiteren geprüft.

Anhand von nachfolgenden Experimenten konnte zum ersten mal gezeigt werden, dass aktivierte PMN aus dem Blut polytraumatisierter Patienten nach *ex vivo* Aktivierung von Fas mittels immobilisierter anti-Fas Antikörper (Klon CH-11) in die Apoptose geleitet werden. Gleichzeitig wurde ein zunehmender Verlust des mitochondrialen Membranpotentials beobachtet, was darauf hindeutet, dass es sich bei PMN um sogenannte Typ II Zellen handelt, bei denen die Aktivierung des extrinsischen Signalweges von de Mitochondrien abhängt. Hier führt die schwache Aktivierung der Caspase-8 zur Spaltung des proapoptotischen Faktors Bid und zur Bildung von tBid, welches zu den Mitochondrien transloziert um dort die Freisetzung proapoptotischer Faktoren einzuleiten. In diesem Zusammenhang konnte eine unabhängige Studie belegen, dass PMN aus *bid* -/- Mäusen eine verringerte Sensitivität gegenüber Fas-induzierter Apoptose aufweisen (Geering, Gurzeler et al. 2011). Im Gegensatz dazu kommt es in Typ I Zellen zu einer sehr starken Aktivierung der Caspase-8 mit direkter Spaltung und dadurch Aktivierung der Caspase-3 (Hao and Mak 2010). In unseren Versuchen zeigten wir zusätzlich eine sichtbare Abnahme

von Mcl-1 nach Fas-Aktivierung die, ähnlich wie die Fas-vermittelte Apoptose, Caspasen-abhängig verläuft.

2. Arbeit: Paunel-Görgülü A., Lögters T., Flohé S., Cinatl J., Altrichter J., Windolf J., Scholz M.: Stimulation of Fas signaling down-regulates activity of neutrophils from major trauma patients with SIRS. *Immunobiology* 2011, 216:334-42.

Die PMN-Hyperaktivierung und deren erhöhtes zytotoxische Potential im Verlauf einer posttraumatischen SIRS sind Anzeichen einer unbändigen inflammatorischen Reaktion, die sowohl mit Schädigung des Wirtsgewebes als auch Organversagen assoziiert ist. Proinflammatorische Zytokine leiten die Rekrutierung von PMN ein und fördern die Phagozytoseaktivität sowie die Produktion von ROS (oxidative burst) (Botha, Moore et al. 1995). Obwohl wir in der vorangegangenen Studie bereits zeigen konnten, dass hyperaktivierte PMN durch den direkten Kontakt mit einem immobilisierten anti-Fas Antikörper in die Apoptose geleitet werden, war der Effekt auf die PMN-Aktivität bis zum damaligen Zeitpunkt noch völlig unklar. Die Frage, ob die Aktivierung von Fas auf geprimte, d. h. vorstimulierte, PMN zu einer unverzüglichen Inaktivierung der Zellen führt und in welchem Ausmaß diese stattfindet, sollte in dieser zweiten Arbeit geklärt werden.

In dieser prospektiven Studie wurden 23 polytraumatisierte Patienten sowie ein vergleichbares Kollektiv an gesunden Probanden eingeschlossen. In Übereinstimmung mit unseren früheren Daten zeigten am Tag 1 nach Trauma isolierte Patienten-PMN eine geringere Caspase-3-Aktivität im Vergleich zu Kontroll-PMN, was mit der verminderten Apoptoserate in diesen Zellen übereinstimmt. Kennzeichnend für die akuten inflammatorischen Bedingungen nach Trauma konnte im Blut polytraumatisierter Patienten eine signifikant erhöhte PMN-Anzahl sowie erhöhte Level an MPO und IL-8 nachgewiesen werden. Die *ex vivo* Stimulation der Zellen mit immobilisierten agonistischen anti-Fas Antikörpern (Klon CH-11) führte im Weiteren zu einer ausgeprägten Steigerung der Caspase-8-, -3/-7-, und -9-Aktivität, während die Hemmung der Caspasenaktivitäten durch Zugabe des Breitband-Inhibitors BocD-*fmk* die vollständige Aufhebung dieses Effektes zur Folge hatte. Um den Einfluss der Fas-Aktivierung auf die Zellaktivität zu untersuchen, wurden frisch

isolierte PMN aus gesunden Probanden und polytraumatisierten Patienten (Tag 1 nach Trauma) für 1 h und für 4 h mit den immobilisierten agonistischen Antikörpern inkubiert und anschließend die chemotaktische Aktivität, oxidative burst und Phagozytosefähigkeit der Zellen analysiert. Wir konnten zeigen, dass die CH-11-Stimulation von ruhenden PMN aus gesunden Probanden bereits nach 1 h zu einer markanten Verminderung der PMN-Aktivität führt. So fiel die Wanderung der PMN entlang eines IL-8-Gradienten, die Produktion von ROS nach PMA-Stimulation als auch die Phagozytosefähigkeit FITC-markierter *E. coli* Bakterien deutlich schwächer aus im Vergleich zur Kontrollgruppe ohne Antikörper. Im Gegensatz dazu konnte in geprimten Patienten-PMN erst nach einem längeren Zeitraum (4 h) ein vergleichbarer, hemmender Effekt auf die Zellaktivität beobachtet werden. Diese Daten weisen darauf hin, dass die Inaktivierung von geprimten PMN verzögert stattzufinden scheint. Tatsächlich entdeckte die Arbeitsgruppe von Kotone-Miyahara *et al.*, einen Mechanismus des GM-CSF-abhängigen verzögerten Fas-Signalweges (Kotone-Miyahara, Yamashita et al. 2004). Die Autoren beschreiben in ihrer Arbeit eine durch rekombinantes GM-CSF vermittelte Verzögerung der FADD-Rekrutierung zum Fas-Rezeptor, welche die Bildung des DISC-Komplexes negativ beeinflusst. Diese Annahme steht im Einklang mit unseren eigenen Ergebnissen, die eine signifikante Erhöhung von GM-CSF im Serum nach Trauma gezeigt haben (1. Arbeit). Somit könnte das GM-CSF aus dem Serum tatsächlich zu einer Beeinträchtigung der Fas-mediierten Effekte führen. Allerdings konnte die erwähnte Studie, als auch unsere eigenen Daten belegen, dass diese verminderte Fas-Sensitivität durch eine verstärkte Stimulation des Fas-Rezeptors wieder aufgehoben werden kann. Interessanterweise stellten wir im Laufe weiterer Untersuchungen fest, dass die Fas-mediierte Hemmung der chemotaktischen Aktivität von PMN unabhängig von der Aktivität von Caspasen abläuft und somit unabhängig vom Apoptoseprozess. Im Gegensatz dazu ließ sich die Inhibition der ROS-Produktion und der Phagozytose durch Zugabe von BocD-*fmk* vollständig unterbinden. Diese neuen Erkenntnisse lenkten den Blick auf erst kürzlich beschriebene Apoptose-unabhängige Signalwege, die durch Fas geleitet werden. In diesem Zusammenhang ist bereits bekannt, dass die Adapterproteine FADD, c-FLIP und Caspase-8 die Signale, die durch den membranständigen Fas-Rezeptor vermittelt werden, auf den MAPK- und NF-κB-Signalweg übertragen (Peter, Budd et al. 2007). Letztere sind in Mechanismen des zytoskelettalen Rearrangements involviert und beeinflussen somit

Zellmigration, -adhäsion und Chemotaxis (Johnson and Lapadat 2002; Huang, Chen et al. 2001). Diese Erkenntnisse führten zu der Annahme, dass die verminderte chemotaktische Aktivität von PMN nach Fas-Stimulation auf einer Beeinträchtigung der zytoskelettalen Komponenten basiert. Dieser Weg scheint parallel jedoch unabhängig von Apoptose-einleitenden Signalübertragungskaskaden abzulaufen. Folglich wurde in dieser Arbeit gezeigt, dass die Aktivierung von Fas nicht nur den apoptotischen Prozess in inflammatorischen PMN einleitet, sondern zusätzlich eine schnelle Herunterregulierung der zellulären Aktivität zur Folge hat.

Vor diesem Hintergrund weisen unsere Ergebnisse auf neue Möglichkeiten zur Entwicklung therapeutischer Verfahren auf der Basis von extrakorporalen Immuntherapien hin. So wäre die Verwendung immobilisierter biofunktioneller Effektormoleküle zur Limitierung der posttraumatischen SIRS-Reaktion und der damit verbundenen hohen Mortalitätsrate durchaus im Bereich des Denkbaren. Die Relevanz dieses innovativen Therapieansatzes für den Einsatz am Patienten sollte daher in einer nachfolgenden Studie am Großtiermodell getestet werden.

3. Arbeit: Lögters T., Altrichter J., Paunel-Görgülü A., Sager M., Witte I., Ott A., Sadek, S., Baltes J., Bitu-Moreno J., Schek A., Müller W., Jeri T., Windolf J., Scholz M.: Extracorporeal immune therapy with immobilized agonistic anti-Fas antibodies leads to transient reduction of circulating neutrophil numbers and limits tissue damage after haemorrhagic shock/resuscitation in a porcine model. *J Inflamm (Lond)* 2010, 7:18.

Der hämorrhagische Schock zählt mitunter zu den häufigsten Todesursachen von Patienten, die ein schweres Trauma erleiden. Pathophysiologisch ist das posthämorrhagische Organversagen mit einer verstärkten Rekrutierung von PMN assoziiert, die ins Gewebe einwandern und dort verstärkt zytotoxische Substanzen sezernieren, was zu Störungen der jeweiligen Organfunktionen führen kann. In den vorangegangenen Studien (1. und 2. Arbeit) konnten wir anhand von *ex vivo* Versuchen zeigen, dass immobilisierte agonistische anti-Fas Antikörper zu einer raschen und deutlichen Verminderung der PMN-Aktivität führen und zusätzlich Apoptose in den Zellen einleiten. Diese vorläufigen Ergebnisse dienten als Basis für

die Etablierung eines hämorrhagischen Schockmodels am Schwein, anhand dessen geprüft werden sollte, ob das Targeting von Fas auf PMN die posthämorrhagische Entstehung von SIRS sowie MODS zu limitieren vermag. Für die prospektive experimentelle Studie wurden 24 Münchener Miniaturschweine eingesetzt, die einem hämorhhagischen Schock über 30 min bei einem MAP von 35 ± 5 mmHg und weitere 15 min bei einem MAP von 40 ± 5 mmHg unterzogen wurden. Zur regulierten Schockeinleitung sowie -erhaltung wurde ein Sheldon-Katheter in die linke Vena jugularis externa eingesetzt. Mit Beginn der Reperfusionsphase wurde eine Versuchsgruppe (n = 12) einer extrakorporalen Immuntherapie mit immobilisierten anti-Fas Antikörpern unterzogen. Eine weitere Gruppe (n = 12) ohne Immuntherapie diente als Vergleichsgruppe. Hierfür erfolgte der Einbau eines Gehäuses, welches einen Polyurethanschaum, beschichtet mit anti-Fas Antikörpern, beinhaltete, über zwei Schenkeln des Sheldon-Katheters in den Blutkreislauf. Die extrakorporale Flussgeschwindigkeit lag bei 300 ml / min über einen Zeitraum von 180 min. Vorangegangene Untersuchungen unter Anwendung eines Gehäuses ohne anti-Fas Antikörper Beschichtung konnten unspezifische Effekte, etwa durch den künstlichen extrakorporalen Kreislauf, weitgehend ausschließen. Nach einer Nachbeobachtungszeit von 48 h und 72 h in jeder Gruppe wurden die Tiere euthanasiert und nachfolgende histologische als auch molekularbiologische Untersuchungen angeschlossen. In Übereinstimmung mit unseren *ex vivo* Experimenten konnte durch den Einsatz der extrakorporalen Immuntherapie eine deutliche Minderung der Anzahl an zirkulierenden PMN im Verlauf der Reanimationsphase beobachtet werden sowie eine sichtbare Hemmung der PMN-Infiltration in verschiedenen Organen, wie Herz, Leber, Niere und Darm im Vergleich zur Schockgruppe ohne Immuntherapie. Dieser Unterschied war im Lungengewebe am deutlichsten ausgeprägt. Untersuchungen der Hämoxygenase-1 (HO-1)-Expression, als Marker für die Schock-induzierte Hypoxie, zeigten eine deutliche Reduktion der HO-1 Gen- und Proteinexpression in der Lunge, im Darm sowie in der Leber der Tiere mit reduzierter PMN-Anzahl, möglicherweise als Folge der limitierten PMN-Infiltration. Ferner war die Apoptose im Darm- sowie Lungengewebe nach Einsatz immobilisierter anti-Fas Antikörper signifikant reduziert. Des Weiteren führte die *ex vivo* Aktivierung der PMN-Apoptose zu einer nachweisbaren Verbesserung der Lungen-, Herz- und Nierenfunktion. Interessanterweise konnte in Tieren mit extrakorporaler Immuntherapie eine deutliche Abnahme der zirkulierenden

Monozyten, aber keine Änderungen hinsichtlich der Anzahl an Lymphozyten, gemessen werden. Obwohl im Rahmen dieser *in vivo* Studie gezeigt werden konnte, dass der therapeutische Einsatz immobilisierter anti-Fas Antikörper zur Limitierung Inflammations-bedingter Kollateralschäden prinzipiell möglich ist, sind die erzielten Ergebnisse im Allgemeinen kritisch zu betrachten. Zwar führt die PMN-Hyperaktivierung nachweislich zu einem erhöhten Risiko für zusätzliche Gewebeschäden nach einem Trauma, jedoch kann das Auftreten posttraumatischer Komplikationen, wie z. Bsp. Sepsis, als Folge einer PMN-Inaktivierung sowie einer Verminderung der Monozytenanzahl, wie hier beobachtet, nicht ausgeschlossen werden. Weitere alternative, PMN-spezifische Wege zur Aufhebung der PMN-Apoptoseresistenz wären in Zukunft erforderlich um diesen therapeutischen Einsatz zu prüfen.

4. Arbeit: Paunel-Görgülü A., Flohé S., Scholz M., Windolf J., Lögters T.: Increased serum soluble Fas after major trauma is associated with delayed neutrophil apoptosis and development of sepsis. *Crit Care* 2011, 15:R20.

Die Überwindung der intrinsischen Apoptoseresistenz durch Aktivierung des Fas-Rezeptors mittels immobilisierter, agonistischer anti-Fas Antikörpern in PMN nach Trauma ist durch unsere Arbeiten erstmals gezeigt worden. Interessanterweise scheint diese extrinsische Apoptose-Einleitung *in vivo* nach einem schweren Trauma nur bedingt eine Rolle zu spielen. So bleibt die Apoptoserate von Fas-exprimierenden PMN trotz der ubiquitären Expression des FasL, wie z.Bsp. auf PMN und Endothelzellen, deutlich reduziert und die Anzahl zirkulierender PMN erhöht. Es stellte sich daher die wichtige Frage nach den regulatorischen Mechanismen, die unter inflammatorischen Bedingungen nach einem Trauma die Fas-Aktivierung unterbinden. Dazu konnten wir im Rahmen der aktuellen Studie zeigen, dass das Serum schwerverletzter Patienten bereits am Tag 1 nach Trauma erhöhte Level an lösliches Rezeptor Fas (sFas) enthält, welcher durch Bindung an den natürlichen Liganden FasL die Fas / FasL-Wechselwirkung antagonisiert. Im Gegensatz dazu konnten keine Unterschiede hinsichtlich der Level an löslichem Fas Liganden (sFasL) gemessen werden. Diese Beobachtungen führten zunächst zu der Hypothese, dass

sFas durch Bindung an FasL die *in vivo* Aktivierung von Fas auf PMN unterbindet. Die Tatsache, dass die höchsten sFas-Serumspiegel innerhalb der ersten 9 Tage nach dem Trauma ausschließlich in Patienten mit posttraumatischer Sepsisentstehung gemessen wurden, stellte sich für uns als ein weiterer wichtiger Befund heraus. Somit scheint die antagonistische Wirkung von sFas in Patienten mit posttraumatischer Sepsisentstehung von besonderer Relevanz zu sein, wohingegen die reduzierte Apoptose in PMN aus Patienten mit unkompliziertem Verlauf offensichtlich anderen Regulationsmechanismen unterliegt. Die Sepsisgruppe (n = 18) wies signifikant erhöhte sFas-Konzentrationen zum einen am Tag 1 nach dem Trauma auf im Vergleich zur Kontrollgruppe (n = 17) und zusätzlich am Tag 5 und Tag 9, verglichen mit den Leveln gemessen im Serum gesunder Probanden sowie in Patienten mit unkomplizierten Verlauf (n = 29). Dies führte zu der Fragestellung, ob sFas tatsächlich *in vivo* die PMN-Apoptose zu beeinflussen vermag. Im Weiteren sollte der Zusammenhang zwischen sFas und PMN-Apoptose sowie Verlauf bzw. Outcome der polytraumatisierten Patienten eingehend geprüft werden.

Tatsächlich fanden wir heraus, dass die PMN-Apoptoserate in der Sepsisgruppe bereits ab Tag 1 bis einschließlich Tag 10 nach Trauma lediglich geringfügig reduziert war, verglichen mit der nicht Sepsisgruppe. Auch konnten keine Unterschiede in der Leukozytenzahl zwischen den beiden Gruppen ausgemacht werden. Diese Befunde schienen auf dem ersten Blick unserer Hypothese zu widersprechen. Die Ergebnisse lassen sich jedoch durch die Tatsache erklären, dass aktivierte PMN in der Regel schnell zum geschädigten oder entzündeten Gewebe rekrutiert werden und somit nur für eine recht kurze Zeit in der Zirkulation verweilen. Somit wird nur eine eingeschränkte Population an PMN methodisch erfasst. Zusätzlich stellt die geringe Anzahl an septischen Patienten eine grundsätzliche Limitierung unserer Studie dar.

Daher wurde im Rahmen anschließender Experimente geprüft, ob das sFas aus dem Serum septischer Patienten die Fas-vermittelte PMN-Apoptose *in vitro* zu hemmen vermag. Wir konnten zeigen, dass sowohl Serum sFas als auch rekombinantes sFas in einer konzentrationsabhängigen Weise die anti-Fas Antikörper (CH-11)-induzierte Apoptose in PMN durch Blocken der Antigenbindungsstelle unterbindet. Dieses Ergebnis deutete darauf hin, dass die PMN-Apoptose *in vivo* tatsächlich auf erhöhte sFas-Level zurückzuführen ist. Bereits in einer früheren Arbeit konnten wir zeigen, dass die PMN-Apoptose zusätzlich einer

strengen Regulation durch verschiedene proinflammatorische Zytokine, wie GM-CSF, IL-8 und IL-6 unterliegt, die ihrerseits über Modulation antiapoptotischer Bcl-2 Faktoren eine intrinsische Apoptoseresistenz in den Zellen auslösen (1. Arbeit). Somit werden aus unserer Sicht sowohl die Inflammation als auch die progressive Organdysfunktion, die bei Sepsis beobachtet werden, durch die antiapoptotische Aktivität von extrinsischen Faktoren, wie sFas, alleine und/oder in Kombination mit der Hemmung des Mitochondrien-abhängigen Signalweges in PMN gefördert. Die sFas-Level in Patienten mit septischem Schock lagen am Tag 5 und Tag 9 nach Trauma tendenziell höher, verglichen mit den Werten gemessen im Serum von Patienten mit einfacher sowie schwerer Sepsis (unveröffentlichte Daten). Ein Zusammenhang zwischen sFas und dem Schweregrad von Sepsis (De Freitas, Fernandez-Somoza et al. 2004), maligner Erkrankungen (Mitani, Nishioka et al. 2003), Autoimmunerkrankungen (Cheng, Zhou et al. 1994), ARDS (Lee, Choi et al. 2008) oder postchirurgischer Komplikationen (Iwase, Kondo et al. 2006) wurde bereits im Rahmen früherer Studien beschrieben. Tatsächlich zeigt unsere Arbeit erstmals eine positive Korrelation zwischen sFas und der Leukozytenanzahl im Blut sowie dem PMN-Aktivitätsmarker Elastase und den Organdysfunktion-Scores „Sequential Organ Failure Assessment“ (SOFA) und „Multiple Organ Dysfunction Score“ (MODS), welche in septischen Patienten am stärksten ausgeprägt war. Dabei konnte die stärkste Korrelation zwischen sFas und Leukozytenzahl, PMNE, dem etablierten Sepsismarker IL-6 und den Organdysfunktion-Scores am Tag 5 nach Trauma ermittelt werden und somit zu einem Zeitpunkt, an dem häufig erste klinische Zeichen für eine Sepsis in Erscheinung treten.

In unserem Patientenkollektiv lag bei lediglich 7 der insgesamt 18 Patienten am Tag 5 nach dem Trauma die Diagnose einer Sepsis vor. Da ferner die sFas-Level bereits am Tag 1 nach Trauma eine positive Korrelation mit den SOFA-Scores in der Patientengruppe mit posttraumatischer Sepsisentstehung aufwies, stellt sFas einen potentiellen prädiktiven Marker für die Entstehung der posttraumatischen Sepsis dar. Es ist bislang jedoch nicht eindeutig geklärt, in welchem Kontext die sFas-vermittelten Effekte auf PMN mit der Entstehung der Sepsis stehen. Vielmehr scheint die zusätzliche Hemmung des extrinsischen Signalweges die PMN-Hyperaktivität und das Auftreten von Organdysfunktionen anzutreiben. In welchem Ausmaß sFas bzw. hyperaktivierte PMN zur Entstehung der bekannten Sepsis-assoziierten T-Zellanergie beitragen, sollte im Rahmen zukünftiger Studien näher untersucht

werden. Denkbar wäre in diesem Zusammenhang eine sFas-abhängige Modulation der adaptiven Immunantwort durch Auslösung eines phänotypischen und funktionellen Wechselns in PMN. So ist erst kürzlich eine durch PMN-eigene Arginase oder ROS ausgelöste Hemmung der T-Lymphozytenproliferation beschrieben worden (Munder, Schneider et al. 2006; Kusmartsev, Su et al. 2008).

Zusammenfassend konnten wir in dieser Arbeit die bedeutende Rolle von sFas als Mediator der PMN-Apoptoseresistenz belegen. Die starken Korrelationen mit dem Sepsismarker IL-6, PMNE und den Organdysfunktion-Scores unterstreichen zusätzlich dessen prognostischer Wert sowie klinische Relevanz für die Entstehung einer posttraumatischen Sepsis und MOF.

5. Arbeit: Paunel-Görgülü A., Kirichevska T., Lögters T., Windolf J., Flohé S.: Molecular mechanisms underlying delayed apoptosis in neutrophils from multiple trauma patients with and without sepsis. *Mol Med* 2012, 18:325-35.

Wie bereits ausführlich dargestellt, zeigen unsere bisherigen Untersuchungen erstmals einen Zusammenhang zwischen dysregulierter PMN-Apoptose und Outcome der Patienten nach einem schweren Trauma. Die Verlängerung der PMN-Lebensdauer, assoziiert mit einer zellulären Hyperaktivität, beruht zum einen auf einer Inhibition des Mitochondrien-abhängigen Signalweges (1. und 2. Arbeit). Zum anderen konnte in der vorangegangenen Studie eine zusätzliche Regulation durch extrinsische Faktoren gezeigt werden, welche im engen Kontext mit der posttraumatischen Sepsisentstehung, als auch Organdysfunktion steht (4. Arbeit). Diese Ergebnisse führten zu der Vermutung, dass bei der Regulation der PMN-Apoptose im Verlauf einer Sepsis extrinsische Signalwege zunehmend an Bedeutung gewinnen. Im Gegensatz dazu scheint das PMN-Überleben in Patienten mit unkompliziertem Heilungsverlauf eher auf einer hemmenden Regulation des intrinsischen Signalweges zu beruhen. Im Rahmen dieser Arbeit sollten vertiefende molekularbiologische Untersuchungen dazu beitragen, die aufgestellte Hypothese näher zu prüfen. Zu diesem Zweck wurden in der prospektiven Studie 12 Patienten mit posttraumatischer Sepsisentstehung innerhalb der ersten 10 Tage nach dem Trauma und 12 Patienten mit unkompliziertem Verlauf eingeschlossen. In

Übereinstimmung mit den bislang erzielten Daten konnten keine signifikanten Unterschiede in der PMN-Apoptoserate zwischen den beiden Patientengruppen am Tag 5 und Tag 10 nach Trauma gefunden werden. Diese Beobachtung liegt zum einen darin begründet, dass lediglich die Apoptose von zirkulierenden PMN erfasst wird, und zum anderen, dass die Anzahl der Patienten eingeschränkt sowie die Heterogenität des Patientenkollektivs recht groß ist. Die Bedeutung der PMN-Apoptose für den klinischen Verlauf nach Trauma wird durch die gefundene inverse Korrelation zwischen PMN-Apoptose und den Organdysfunktion-Scores SOFA ($p = 0,059$) und MODS ($p = 0,04$) am Tag 10 nach Trauma deutlich untermauert. Anhand von *gene array*-Analysen fanden wir heraus, dass, von den insgesamt untersuchten 94 Apoptose-relevanten Genen, zahlreiche Gene nach einem Trauma unterschiedlich reguliert werden im Vergleich zu der Expression in PMN aus gesunden Probanden. Dabei konnte kein eindeutiger Trend im Hinblick auf die Expression einer bestimmten Gengruppe festgestellt werden. Vielmehr machten wir die Beobachtung, dass zahlreiche pro- und antiapoptotische Gene hochreguliert werden, wie z. Bsp. *Mcl-1*, *A1*, *Dad1*, jedoch auch *Bax*, *Bad*, *Bid* und *Caspase-3*. Daneben wurde eine Hochregulation der mRNA-Expression von Todesrezeptoren gefunden, während Gene, die für Rezeptor-assoziierte Adapterproteine kodieren, deutlich runterreguliert waren. Die Ergebnisse aus unseren früheren Arbeiten gaben uns zum Anlass, unseren Forschungsfokus auf die Untersuchung der Expression von Bcl-2 Mitgliedern zu lenken. So belegen eigene (1. Arbeit) sowie weitere aktuelle Studien die essentielle Rolle von Mcl-1 bei der Regulation der PMN-Apoptose (Moulding, Quayle et al. 1998; Akgul, Moulding et al. 2001; Derouet, Thomas et al. 2004). In diesem Zusammenhang wurde die Genexpression der antiapoptotischen Faktoren *Mcl-1* und *A1* sowie des proapoptotischen Faktors *Bax* am Tag 1, Tag 5 und Tag 10 nach Trauma in beiden Patientengruppen untersucht. Zusätzlich erfolgte die Analyse der *Defender against cell death* (*Dad1)* Expression, einem Faktor, dessen Funktion in PMN derzeit völlig unklar ist, der jedoch auf mRNA-Ebene über den gesamten Zeitraum eine deutlich Hochregulation im Vergleich zur der Expression in Kontrollzellen gezeigt hatte. Dad1 ist ein integrales ER-Membranprotein, das für die N-Glykosylierung verantwortlich ist. Es ist bereits gezeigt worden, dass Dad1 mit Mcl-1 interagiert, wohingegen der Verlust dieses Proteins einen Anstieg der Apoptoserate zur Folge hat (Makishima, Yoshimi et al. 2000). Obwohl wir keine Unterschiede hinsichtlich des Expressionsmusters zwischen

septischen und nicht septischen Patienten finden konnten, lag der sichtbare Expressionspeak für das *Mcl-1* und *A1* Gen am Tag 1 nach Trauma. Von vielen Faktoren ist jedoch bekannt, dass sie posttranslational reguliert werden, u. a. Mcl-1, so dass die Ergebnisse der Genanalyse in der Regel keine Aussage über die tatsächliche Proteinexpression erlauben. Tatsächlich fanden wir eine deutliche Hochregulation von Mcl-1 und A1 in den polytraumatisierten Patienten im Vergleich zu Kontroll-PMN. In Übereinstimmung mit der gefundenen reduzierten PMN-Apoptoserate kam es bereits ab Tag 1 nach Trauma zu einer Reduktion der intrazellulären Bax-Proteinleveln. Im Gegensatz dazu konnte für Dad1 keine Änderung der Expressionsrate gefunden werden. Interessanterweise zeigten Patienten mit posttraumatischer Sepsisentstehung eine deutliche Abnahme der Mcl-1 Proteinexpression am Tag 5 nach Trauma und eine signifikante Herunterregulation von A1 am Tag 5 sowie von Mcl-1 und A1 am Tag 10 nach Trauma, verglichen mit nicht septischen Patienten. Dagegen zeigte sich in der Sepsisgruppe ein deutlicher Anstieg von Dad1 am Tag 10 nach Trauma. Die Frage, ob Dad1 in der Hemmung der ER-Stress-induzierten Apoptose oder eher in der Modulation der Mcl-1 Aktivität involviert ist, muss jedoch in zukünftigen Arbeiten beantwortet werden.

Diese Ergebnisse deuten in ihrer Gesamtheit auf einen Switch des Apoptose-Kontrollmechanismus in PMN in Abhängigkeit vom Patienten-Outcome. Somit spiegelt sich die von uns ursprünglich postulierte Hypothese in den aktuellen Daten wider. Sollten Mcl-1 und A1 tatsächlich den Mitochondrien-abhängigen Signalweg regulieren, so müsste eine Abnahme dieser Proteine in den Zellen mit einer zumindest teilweisen Aufhebung der intrinsischen Apoptoseresistenz verbunden sein. Durch *knock-down* Experimente mittels siRNA konnten wir bestätigen, dass eine Abnahme von Mcl-1 mit einem zunehmenden Verlust des mitochondrialen Membranpotentials sowie einem Anstieg der Apoptose nach STS-Behandlung verbunden ist. Ähnliche Ergebnisse konnten auch im Rahmen aktueller Forschung mit A1 erzielt werden, wodurch die essentielle Funktion dieser Faktoren bei der Regulation der intrinsischen Apoptose als erwiesen gilt (unveröffentlichte Daten). Es konnte sowohl in einer früheren (1. Arbeit) als auch in der aktuellen Arbeit gezeigt werden, dass die intrinsische Apoptoseresistenz auf Serumfaktoren zurückzuführen ist. So ist die Behandlung von PMN aus gesunden Spendern mit dem Serum polytraumatisierter Patienten (Tag 1 nach Trauma) völlig ausreichend um in diesen Zellen eine intrinsische Apoptoseresistenz auszulösen. Diese Beobachtung legt die

Vermutung nahe, dass die verminderte Mcl-1- und A1-Expression in PMN aus septischen Patienten unter anderem mit dem veränderten Zytokinmuster unter pathologischen Bedingungen zusammenhängt. Wir konnten nun erstmals zeigen, dass die Behandlung von PMN mit dem Serum septischer Patienten (Tag 1, Tag 5 und Tag 10) nach Trauma mit einer deutlichen Zunahme der mitochondrialen Membrandepolarisation nach STS-Behandlung verbunden ist, verglichen mit dem Serum nicht septischer Patienten. Dass dieser Effekt größtenteils mit den Seren, die am Tag 10 und nicht am Tag 5 gesammelt wurden, zu beobachten war, lässt sich durch die Tatsache erklären, dass am Tag 5 nach Trauma lediglich 5 der insgesamt 12 Patienten in der Sepsisgruppe klinische Symptome einer Sepsis aufwiesen. Somit wären generell die stärksten Effekte zu einem recht späten Zeitpunkt, d. h. am Tag 10 nach Trauma, zu erwarten. Die gefundene verminderte intrinsische Apoptoseresistenz in PMN septischer Patienten erklärte zu diesem Zeitpunkt aber nicht die verminderte Apoptose in diesen Zellen. Da im Serum von septischen Patienten bereits in einer früheren Arbeit (4. Arbeit) erhöhte Level an sFas gemessen wurden, erschien die Möglichkeit für einen verminderten extrinsischen Signalweg in den PMN dieser Patienten als sehr wahrscheinlich. Dass dies tatsächlich der Fall ist, konnten wir anhand einer reduzierten Caspase-8-Aktivität in diesen Zellen zeigen. Dieses Ergebnis wird zusätzlich durch die verminderte Bid-Spaltung und des daraus resultierenden erhöhten Bid/tBid-Verhältnisses gestützt. Zusammenfassend konnten unsere Ergebnisse zeigen, dass es während einer Sepsis zu einer Änderung des Expressionsmusters apoptotischer Faktoren in PMN kommt. Während in der frühen Phase nach einem Trauma die Apoptose überwiegend einer Regulation durch antiapoptotische Bcl-2 Mitgliedern unterliegt, scheint die Korrelation zwischen PMN-Überleben und Mcl-1 Expression im weiteren Verlauf immer mehr an Geltung zu verlieren. So kommt es in septischen Patienten trotz verminderter Mcl-1 Expression zu einer ausgeprägten Hemmung der Apoptoserate. Unsere Daten machen erstmals deutlich, dass diese Hemmung auf einer Störung des extrinsischen Signalweges beruht, dessen Ursprung sich z. T. auf erhöhte Level an zirkulierendem sFas zurückführen lässt. Die Ergebnisse dieser Arbeit bilden aus heutiger Sicht einen wichtigen weiteren therapeutischen Ansatzpunkt zur Prävention der PMN-vermittelten Schädigung des Wirtsgewebes im Rahmen einer Sepsis. Daraus abgeleitete Interventionsstrategien zur Blockade intrinsischer und extrinsischer Signalwege könnten somit in Zukunft Einzug in den klinischen Alltag finden.

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1.4 Zusammenfassung

Nach einem schweren Trauma kommt es häufig zu einer Hyperaktivierung neutrophiler Granulozyten (PMN), gefolgt von einer systemischen Abwehrreaktion und multiplem Organversagen. Obwohl bislang bekannt war, dass PMN nach Trauma eine verlängerte Lebensdauer aufgrund eines gestörten programmierten Zelltods aufweisen, sind die regulatorischen Mechanismen bislang weitgehend ungeklärt geblieben. Gegenstand der vorliegenden Habilitationsschrift war die Aufklärung der molekularen Zusammenhänge zwischen posttraumatischer Inflammation, Apoptose-regulierender Faktoren und Apoptoseresistenz in PMN nach schwerem Trauma. Ferner sollte die Bedeutung der PMN-Apoptose für die Entstehung posttraumatischer Komplikationen, wie Sepsis, näher untersucht werden.

Die vorliegende Arbeit zeigt, dass die Trauma-bedingte verlängerte Lebensdauer von PMN auf einer Inhibition des Mitochondrien-abhängigen intrinsischen Apoptose-Signalweges beruht. Allein die Behandlung von PMN gesunder Probanden mit dem Serum polytraumatisierter Patienten war völlig ausreichend um eine signifikante Hemmung der PMN-Apoptose zu erzielen. Interessanterweise korrelierte diese mit einer deutlichen Erhöhung der Proteinlevel des antiapoptotischen Bcl-2 Mitgliedes Mcl-1 in diesen Zellen. Zusätzlich konnte gezeigt werden, dass die Mcl-1 Expression in PMN nach einem Trauma generell stark zunimmt. Durch die Bindung des Mcl-1 Proteins an proapoptotische Faktoren wird deren Translokation zu den Mitochondrien unterbunden und der darauf folgende Verlust des mitochondrialen Membranpotentials gehemmt. Letzteres ist ausschlaggebend für die Freisetzung proapoptotischer Faktoren aus dem Mitochondrien-Intermembranraum und somit für die Einleitung der Mitochondrien-abhängigen intrinsischen Apoptose. Zusätzlich konnte als wichtiger Mediator der PMN-Apoptoseresistenz nach Trauma das proinflammatorische Zytokin Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF) identifiziert werden. Wie in der vorliegenden Arbeit gezeigt, steigt die GM-CSF-Konzentration im Serum polytraumatisierter Patienten bereits wenige Stunden nach dem Unfall an und bleibt bis mindestens Tag 10 nach Trauma deutlich erhöht. Nachfolgende Untersuchungen haben ergeben, dass die Expression des Mcl-1 Proteins in PMN einer positiven Regulation durch das Zytokin GM-CSF unterliegt. Eine Hemmung der biologischen Aktivität von GM-CSF im Patientenserum

durch neutralisierende Antikörper führte zu einer deutlichen Abnahme der Mcl-1 Proteinlevel und gleichzeitig zu einer signifikanten Erhöhung der intrinsischen Apoptose in PMN.

Es ist im Weiteren bekannt, dass neben dem intrinsischen Signalweg die Apoptose auch über den extrinsischen Weg, so z. Bsp. über Aktivierung des Oberflächen-Todesrezeptors Fas, eingeleitet werden kann. Mehrere Kleintierstudien haben in der Vergangenheit demonstriert, dass eine systemische Applikation agonistischer anti-Fas Antikörper mit einer unkontrollierten Apoptose in verschiedenen Zelltypen und massiver Organschädigung, wie z. Bsp. der Lunge und Leber, assoziiert ist. Folglich stellt die Immobilisierung von anti-Fas Antikörpern auf einer biokompatiblen Matrix eine sinnvolle Alternative dar um die toxischen Effekte löslicher Antikörper zu umgehen. Vor dem Hintergrund dieses therapeutischen Ansatzes konnte erstmals gezeigt werden, dass aktivierte PMN aus dem Blut polytraumatisierter Patienten nach *ex vivo* Aktivierung von Fas mittels immobilisierter agonistischer anti-Fas Antikörper bereits nach kürzester Zeit eine deutliche Reduktion der Phagozytose, oxidative burst sowie Chemotaxis aufweisen und schließlich in die Apoptose geleitet werden. Interessanterweise sind die beobachteten Fas-mediierten Effekte auf die Zellaktivität zum Teil auf einer nachweislichen Aktivierung Apoptose-unabhängiger Signalkaskaden zurückzuführen. Somit lassen sich sowohl die PMN-Hyperaktivität als auch die Mitochondrien-abhängige Apoptoseresistenz durch Aktivierung des Fas-Rezeptors überwinden. Anhand eines etablierten Großtiermodells wurde ferner eine sichtbare Minderung PMN-mediierter Organschäden nach hämorrhagischen Schock und Einsatz immobilisierten anti-Fas Antikörpern in Form einer extrakorporalen Immuntherapie nachgewiesen. Jedoch scheint diese extrinsische Apoptose-Einleitung durch Aktivierung des Fas-Rezeptors auf PMN *in vivo* nach einem schweren Trauma nur bedingt eine Rolle zu spielen.

In weiteren Folgestudien konnte gezeigt werden, dass das Serum schwerverletzter Patienten bereits am Tag 1 nach Trauma erhöhte Level an lösliches Rezeptor Fas (sFas) enthält, welcher durch Bindung an den natürlichen Liganden FasL die Fas / FasL-Wechselwirkung antagonisiert. Tatsächlich wurde in dieser Arbeit eine Korrelation zwischen sFas und der Leukozytenanzahl im Blut sowie dem PMN-Aktivitätsmarker Elastase und den Organdysfunktion-Scores gezeigt. Von besonderer Relevanz scheint die antagonistische Wirkung von sFas in Patienten mit

posttraumatischer Sepsisentstehung zu sein. Diese Patienten weisen bereits am Tag 1 nach dem Trauma bis einschließlich Tag 9 die höchsten sFas-Serumspiegel auf. Demnach konnte sFas als zusätzlicher Mediator der PMN-Apoptoseresistenz und potentieller prädiktiver Marker für die Entstehung der posttraumatischen Sepsis identifiziert werden. Obwohl die PMN-Apoptoserate in Patienten mit posttraumatischer Sepsisentstehung innerhalb der ersten 10 Tage nach dem Trauma deutlich reduziert ist, konnte in diesen Zellen eine signifikant verminderte Expression der antiapoptotischen Bcl-2 Proteine Mcl-1 und A1 im Vergleich zu den Expressionsraten in PMN aus Patienten mit unkompliziertem Verlauf nachgewiesen werden. In Übereinstimmung mit der reduzierten Mcl-1 Expressionsrate zeigten diese Zellen eine erhöhte Sensitivität gegenüber Substanzen, die den Mitochondrien-abhängigen intrinsischen Signalweg aktivieren. Zudem lies sich in PMN mit posttraumatischer Sepsisentstehung eine reduzierte Caspase-8-Aktivität und eine damit assoziierte verminderte Bid-Spaltung nachweisen. Diese Befunde belegen, dass die verminderte Apoptose in PMN in Sepsis auf einer Hemmung des extrinsischen, Rezeptor-vermittelten Signalweges beruht. Die Bedeutung dieser Ergebnisse für die Pathophysiologie nach schwerem Trauma wird durch die gefundene Korrelation zwischen PMN-Apoptoserate und Organdysfunktion-Scores und somit das Patienten-Outcome unterstrichen.

Zusammenfassend tragen die Erkenntnisse aus dieser Arbeit zum bessern Allgemeinverständnis der molekularen Pathophysiologie von inflammatorischen Erkrankungen bei. Zudem liefern unsere vorgestellten Daten eine potentielle Basis für die Entwicklung neuer therapeutischer Konzepte zur Prävention einer Neutrophilen-Hyperaktivität im Rahmen einer systemischen Inflammation.

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4 Lebenslauf

Persönliche Daten

Name:	Dr. rer. nat. Adnana Nicoleta Paunel-Görgülü (geb. Paunel)
Anschrift:	Kolberger Str. 5, 46149 Oberhausen
Geburtsdatum:	geboren am 01.12.1977 in Jassy (Rumänien)
Staatsangehörigkeit:	deutsch
Familienstand:	verheiratet, 1 Kind (geb. 22.07.2004)

Beruflicher Werdegang

seit 05/2011	Projektleiterin, gefördert durch die DFG an der Klinik für Unfall- und Handchirurgie, Universitätsklinikum Düsseldorf (Klinikdirektor: Univ.-Prof. Dr. J. Windolf)
05/2007-04/2011	Wissenschaftliche Angestellte (Postdoc) an der Klinik für Unfall- und Handchirurgie, Universitätsklinikum Düsseldorf (Klinikdirektor: Univ.-Prof. Dr. J. Windolf)
02/2006-04/2007	Wissenschaftliche Angestellte (Postdoc) am Institut für Anatomie, Abteilung Neuroanatomie, Universitätsklinikum Essen (Prof. Dr. N. Dünker)
12/2002-01/2006	Wissenschaftliche Angestellte (Doktorandin) am Institut für Molekulare Medizin, Abteilung für Immunbiologie, Heinrich-Heine-Universität Düsseldorf (Prof. Dr. V. Kolb-Bachofen)

Hochschulbildung

04/2006	Erlangung der Promotion „Biologische Relevanz der nicht-enzymatischen Stickstoffmonoxid-Generierung in der menschlichen Haut“ Abschluss: Dr. rer. nat. (magna cum laude)
10/1997-11/2002	Studium der Biologie Heinrich-Heine-Universität, Düsseldorf Diplomarbeit: „Charakterisierung von *imap*-Genen aus Maus und Mensch“ Abschluss: Diplom-Biologin (sehr gut)

Schulbildung

1990-1997	Elsa-Brändström Gymnasium, Oberhausen Abschluss: Abitur
1988-1990	Lyzeum, Jassy (Rumänien)
1984-1988	Grundschule, Jassy (Rumänien)

Mitgliedschaften

Sektion für Grundlagenforschung der DGOU

Eingeworbene Drittmittel

seit Mai 2011	Einzelförderung durch die Deutsche Forschungsgemeinschaft zum Projekt: Molekulare Aufklärung anti-apoptotischer Signalwege in neutrophilen Granulozyten nach schwerem Trauma (Mai 2011 – April 2013)

seit August 2009	Anschubförderung der Forschungskommission der Medizinischen Fakultät des Universitätsklinikums Düsseldorf zum Vorhaben: Molekulare Aufklärung der Apoptoseresistenz in Neutrophilen von Patienten mit schwerem Trauma (Antragsteller: Prof. Dr. Sascha Flohé, Dr. Adnana Paunel-Görgülü; August 2009 – Februar 2013)

5 Originalarbeiten zum Habilitationsthema

Mcl-1-Mediated Impairment of the Intrinsic Apoptosis Pathway in Circulating Neutrophils from Critically Ill Patients Can Be Overcome by Fas Stimulation[1]

Adnana Paunel-Görgülü,* Martin Zörnig,† Tim Lögters,* Jens Altrichter,* Uta Rabenhorst,† Jindrich Cinatl,‡ Joachim Windolf,* and Martin Scholz[2]*

The systemic inflammatory response syndrome and subsequent organ failure are mainly driven by activated neutrophils with prolonged life span, which is believed to be due to apoptosis resistance. However, detailed underlying mechanisms leading to neutrophil apoptosis resistance are largely unknown, and possible therapeutic options to overcome this resistance do not exist. Here we report that activated neutrophils from severely injured patients exhibit cell death resistance due to impaired activation of the intrinsic apoptosis pathway, as evidenced by limited staurosporine-induced mitochondrial membrane depolarization and decreased caspase-9 activity. Moreover, we found that these neutrophils express high levels of antiapoptotic Mcl-1 and low levels of proapoptotic Bax protein. Mcl-1 up-regulation was dependent on elevated concentrations of GM-CSF in patient serum. Accordingly, increased Mcl-1 protein stability and GM-CSF serum concentrations were shown to correlate with staurosporine-induced apoptosis resistance. However, cross-linking of neutrophil Fas by immobilized agonistic anti-Fas IgM resulted in caspase-dependent mitochondrial membrane depolarization and apoptosis induction. In conclusion, the observed impairment of the intrinsic pathway and the resulting apoptosis resistance may be overcome by immobilized agonistic anti-Fas IgM. Targeting of neutrophil Fas by immobilized agonistic effector molecules may represent a new therapeutic tool to limit neutrophil hyperactivation and its sequelae in patients with severe immune disorders. *The Journal of Immunology,* **2009, 183: 6198–6206.**

Critically ill intensive care patients die as a direct consequence of their severe injuries, or by the additional damage caused by subsequent deregulated immune reactions resulting in multiple organ dysfunction syndrome (MODS)[3] (1). Tissue damage leads to an immediate neutrophil activation and increased cytotoxicity yielding in endothelial dysfunction through the secretion of proteolytic enzymes such as elastase or reactive oxygen species, followed by edema and tissue destruction (2, 3). Normally, neutrophils spontaneously undergo apoptosis and their life span is limited to 8–20 h in circulation and 1–4 days in tissue. However, after severe injury, the neutrophil life span is significantly prolonged and can last up to 3 wk (4, 5). Thus, deregulated apoptosis, such as resistance to proapoptotic signals in neutrophils of trauma patients, may contribute to the development of systemic inflammatory response syndrome (SIRS) associated with tissue destruction and MODS (6–8).

From a therapeutic point of view, the transient prevention of posttrauma neutrophil hyperactivation is an important challenge. In this regard, a better understanding of posttrauma neutrophil apoptosis regulation is a prerequisite for the development of neutrophil-directed therapies.

Fas (CD95)/Fas ligand (FasL, CD95L) signaling has emerged as an important cellular pathway regulating the induction of apoptosis in a wide variety of tissues and plays a critical role for counter-regulation of neutrophil activity (9). The Fas receptor is a member of the TNFR family and is widely expressed on cell surfaces. Activation of this receptor by its ligand FasL or a cross-linking Ab (anti-Fas IgM mAb) results in receptor oligomerization and apoptosis induction (10). The execution of apoptosis occurs by activation of cysteine proteases, called caspases. Activated Fas forms the death-inducing signaling complex, which contains the FasR, the adapter protein Fas-associated death domain protein, and multiple procaspase-8 molecules, resulting in caspase-8 activation by autoprocessing. The initiator caspase-8 triggers a caspase cascade that activates downstream effector caspases such as caspase-3 and caspase-7 (11, 12).

Liles et al. (13) have shown that inhibition of the Fas receptor results in a partial inhibition of neutrophil apoptosis. Furthermore, different expression of proteins of the Bcl-2 family as well as caspase-mediated activation of protein kinase Cδ have been suggested to modulate neutrophil cell death (14, 15). The Bcl-2 family can be divided into antiapoptotic (such as Bcl-2, Bcl-x_L, Mcl-1, and A1/Bfl-1) and proapoptotic proteins (such as Bak, Bax, Bad, and Bid). The ratio of pro- and antiapoptotic molecules and their localization, conformation, and phosphorylation status can affect the function of these proteins and the decision between apoptosis and survival (16). Pro- and antiapoptotic proteins are associated

*Department of Traumatology and Hand Surgery, Heinrich-Heine University, Duesseldorf, Germany; †Georg Speyer Haus, Frankfurt am Main, Germany; and ‡Institute for Medical Virology, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

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[2] Address correspondence and reprint requests to Prof. Dr. Martin Scholz, Department of Traumatology and Hand Surgery, University Hospital Düsseldorf, Moorenstr. 5, D-40225 Düsseldorf, Germany. E-mail address: martin.scholz@uni-duesseldorf.de

[3] Abbreviations used in this paper: MODS, multiple organ dysfunction syndrome; ICU, intensive care unit; PI, propidium iodide; PU, polyurethane; SIRS, systemic inflammatory response syndrome; TBS-T, Tris-buffered saline containing 0.1% Tween 20.

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with the mitochondrial membrane where they compete to regulate the exit of proapoptotic factors such as cytochrome *c*. Once released into the cytosol, cytochrome *c* associates with Apaf-1 and procaspase-9 to form the apoptosome which activates caspase-3 by proteolytic cleavage (17).

In severely injured patients, elevated levels of proinflammatory cytokines such as GM-CSF are known to extend the life span of mature neutrophils by impairing Fas-mediated apoptosis and to augment neutrophil activity (13, 18). Additionally, it has been shown that GM-CSF up-regulates Mcl-1 in neutrophils in vitro (19, 20). To overcome this neutrophil apoptosis resistance, activation of the extrinsic apoptosis pathway by specific neutrophil Fas cross-linking remains an attractive strategy to transiently decrease neutrophil activity (21). To avoid the systemic application of agonistic Fas effector molecules resulting in nonspecific side effects, an extracorporeal immune therapy with agonistic anti-Fas IgM Abs immobilized on biocompatible carriers has been proposed to selectively target neutrophils within the circulating blood (22, 23).

In this study, we provide, for the first time, evidence for impaired intrinsic apoptosis sensitivity in neutrophils from severely injured patients. We show that apoptosis resistance is due to increased Mcl-1 protein level and stability and can be overcome by ex vivo cross-linking of the Fas receptor with immobilized anti-Fas Ab.

Materials and Methods

Patients

Study approval was obtained from the local ethics committee of the University of Duesseldorf (Duesseldorf, Germany). Twenty-six multiply injured patients who were admitted to the trauma center with an injury severity score >16 and intensive care unit (ICU) stay >3 days were included in this study. Informed consent was obtained from the patient's legal representative if the patient lacked consciousness. Exclusion criteria were death of the patient on the day of admission or within the first 2 days in ICU, ICU stay of <3 days, and withdrawal of patient consent. Immediately after admission (day 0) and every 24 h over a 12-day period, venous blood samples were collected.

Isolation and culture conditions of human neutrophils

Heparinized blood (10 ml) was collected from healthy volunteers and daily from patients after severe trauma over a 12-day period. Neutrophils were isolated by discontinuous density gradient centrifugation on Percoll (Biochrom) as previously described (24). After removal of PBMCs, RBCs were lysed using isotonic ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) at 4°C for 10 min. The purity and viability of neutrophils were >95% as examined by flow cytometry analysis and trypan blue exclusion, respectively. If not mentioned otherwise, neutrophils from patients at time A after trauma (days 0–2) were used in all experiments.

Freshly isolated neutrophils were resuspended in RPMI 1640 containing 2 mM glutamine (Biochrom), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (RPMI 1640 medium; Invitrogen), and 1% autologous serum to a final concentration of 1×10^6 cells/ml.

A neutrophil suspension of 400 μl was incubated with immobilized agonistic anti-Fas IgM (clone CH-11; MBL), on open porous polyurethane (PU) foam (1 cm^3) as a biocompatible carrier. For control, neutrophils were incubated with PU foams alone, without Ab. PU foam carriers with or without anti-Fas loading (4 μg/ml; clone CH-11) were provided by Leukocare. Foams were placed in cryotubes, and incubation with neutrophils was performed in a rocking system at room temperature for 1 and 4 h, respectively. Subsequently, cells were immediately frozen at −80°C or cultured overnight at 37°C in a humidified atmosphere containing 5% CO_2. For Western blot analysis, freshly isolated neutrophils were resuspended in RPMI 1640 supplemented with 1% or 10% autologous/pooled patient serum or 10% FCS (PAA Laboratories) to a final concentration of 2.5×10^6 cells/ml.

Effect of patient serum on neutrophil apoptosis

Neutrophils isolated from healthy volunteers were incubated overnight in RPMI 1640 supplemented with 1% or 10% autologous serum and patient serum (pool of three patients, time A), respectively. In some experiments anti-human GM-CSF Ab (R&D Systems) was additionally used. The 50% neutralization dose for this Ab was determined to be ~0.3–0.5 μg/ml in the presence of 0.5 ng/ml recombinant human GM-CSF. To neutralize the biological activity of GM-CSF in human serum, anti-human GM-CSF Ab was first incubated with the serum on ice for 30 min before addition to the culture medium.

Flow cytometric analysis of Fas and FasL expression

Neutrophils were obtained over time (time A, days 0–2; time B, days 3–6; time C, days 7–11) from multiple trauma patients and from healthy volunteers. Isolated neutrophils were resuspended in PBS supplemented with 5% normal goat serum (Dako) to inhibit nonspecific binding of Abs. Cells (1×10^6) were incubated with mouse anti-human Fas IgM (clone CH-11; MBL) and mouse anti-human FasL IgG (BD Biosciences) for 20 min on ice. Mouse IgG1 and mouse IgM to *Aspergillus niger* glucose oxidase (Dako) were used as negative controls. After a washing with PBS, cells were incubated with a FITC-conjugated goat anti-mouse IgM or IgG, respectively (both from Dianova) for a further 20 min on ice while protected from light. Cells were washed twice with PBS and analyzed by flow cytometry on a FACScan instrument using CellQuest software (BD Biosciences).

Quantification of neutrophil apoptosis

Apoptosis of neutrophils was evaluated either immediately after neutrophil isolation or after 18 h of culture. The proportion of neutrophils that display a hypodiploid DNA peak, i.e., apoptotic cells, was determined by the protocol described by Nicoletti et al. (25). Briefly, cell pellets were suspended in 300 μl of hypotonic solution (0.1% sodium citrate plus 0.1% Triton X-100) containing 50 μg/ml propidium iodide (PI; Sigma-Aldrich) and incubated for at least 3 h at 4°C. The red fluorescence of PI was measured by FACScan cytometer (BD Biosciences). A minimum of 10,000 events was counted per sample. Results are represented as the percentage of hypodiploid DNA (sub-G1) corresponding to fragmented DNA characteristic for apoptotic cells.

Detection of mitochondrial membrane depolarization

The change of mitochondrial membrane potential was examined by staining the cells with a mitochondrial membrane potential-dependent lipophilic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Sigma-Aldrich). In brief, neutrophils were stained by addition of 3 μM JC-1 for 20 min at 37°C. Thereafter, cells were harvested by centrifugation (450 × g for 5 min), washed twice in PBS, and immediately analyzed by flow cytometry (FACScan). The excitation wavelength was 488 nm, and the emission wavelengths were 530 nm (FL1 channel) for green fluorescence and 590 nm (FL2 channel) for red fluorescence. In cells with intact mitochondrial membrane potential, the dye concentrates in mitochondrial matrix and forms red fluorescence JC-1 aggregates. By contrast, in cells with disrupted mitochondrial membrane potential, the dye is dispersed throughout the cytoplasm, where it fluoresces green in its monomeric form. Results are expressed as the relative number of cells with high levels of green fluorescence (FL1), indicating mitochondrial membrane depolarization in the cells.

Determination of caspase-9 activity

Neutrophils incubated with staurosporine were harvested by centrifugation, and cell pellets were stored at −80°C for further investigation. Caspase-9 activity was measured by using the Caspase-Glo 9 Assay (Promega) according to the instructions of the manufacturer with some modifications. Briefly, cells were resuspended in PBS, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS followed by cell sonication. The protein concentration of the cell lysates was determined by using the Dc Protein Assay (Bio-Rad). Same protein concentrations were used for the determination of caspase activity.

GM-CSF detection by ELISA

Blood (10 ml) was collected from healthy volunteers and daily from patients after severe trauma over a 10- to 12-day period. Sera were harvested by centrifugation and stored at −80°C until further processing. A commercially available ELISA kit (Quantikine HS Human GM-CSF; R&D Systems) recognizing recombinant and natural GM-CSF was used for quantification of GM-CSF according to the manufacturer's instructions (minimal detectable level <0.26 pg/ml).

RT-PCR analysis

Total RNA from neutrophils was extracted using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. One microgram of

total RNA was reverse transcribed using oligo(dT)$_{15}$ primer, using Omniscript Reverse Transcriptase (Qiagen) and following the manufacturer's instructions. PCR was conducted using published gene-specific primer sequences for *Fas* (26), *FasL* (27), and *Mcl-1* and *Bax* (28). Primers for *GAPDH* (29) were used to control the quality of the cDNA samples. cDNA (2.5 μl) was amplified using *Taq*PCR Core Kit (Qiagen), and products were separated on 1.8% agarose gel and visualized under UV after SYBR Gold (Invitrogen) staining.

For real-time PCR, total RNA was isolated from cells using High Pure RNA Isolation Kit and transcribed into cDNA with Transcriptor First Strand cDNA Synthesis Kit (both Roche). Real-time PCR analysis was performed on a LightCycler 480 using RealTime Ready Human Apoptosis Panel 384 (Roche).

Western blot analysis

Neutrophils were resuspended in PBS, 1% Nonidet P-40, 0.5 mM sodium deoxycholate, 0.1% SDS supplemented with the complete protease inhibitor mixture (Roche). Samples were sonicated and centrifuged at 8000 $\times$ *g* for 10 min, and protein concentration was quantified using the Dc Protein Assay kit (Bio-Rad).

Protein (30–50 μg/sample) was separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were saturated in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% w/v nonfat dry milk for 60 min at room temperature and then immuno-labeled with monoclonal mouse anti-human Mcl-1 (BD Biosciences) and polyclonal rabbit anti-human Bax Ab (Cell Signaling) in TBS-T and 1% w/v nonfat dry milk or 5% BSA. After the blots were washed in TBS-T, the membranes were incubated for 60 min at room temperature with the goat anti-mouse or anti-rabbit HRP-conjugated secondary Ab (both Dako) in TBS-T and washed as described above. Bands were visualized by the ECL method. Equal loading of gels was confirmed both by Ponceau S staining of membranes and by reincubation of the filters with a polyclonal Ab for β-actin (Santa Cruz Biotechnology). The amount of specific protein was quantified by densitometry (Quantity One; Bio-Rad).

Statistics

All data are presented as mean $\pm$ SEM. If not mentioned otherwise, statistical analyses were performed by using one-way ANOVA, followed by the Newman-Keuls test. Alternatively, Student's *t* test was performed (GraphPad Prism Program, version 5; GraphPad Software). Values of $p < 0.05$ were considered statistically significant.

Results

Changes in gene and protein expression in neutrophils after major trauma

Activated neutrophils exhibit attenuated apoptosis, which is suggested to be the major reason for prolonged autodestructive effector functions (3, 30). To determine the apoptotic status of neutrophils after severe trauma, we quantified DNA fragmentation, as a hallmark of apoptosis, in freshly isolated neutrophils and found up to 7-fold reduced apoptotic rate in neutrophils from multiple trauma patients (time A, days 0–2) compared with control cells. Apoptosis was significantly diminished for at least 1 wk after severe trauma (Fig. 1).

We next studied whether delayed apoptosis of neutrophils is associated with changes in the expression of proapoptotic and antiapoptotic regulator molecules (Fig. 2). Indeed, screening of 372 different apoptosis-relevant gene transcripts by means of the human apoptosis panel revealed different patterns between neutrophils of patients and of healthy donors. In Fig. 2*A*, the gene expression for selected major molecules representing proapoptotic (Bax, Bad, Fas receptor) and antiapoptotic (Mcl-1) regulator functions are shown. This gross examination of cDNA revealed first evidence that multiple trauma did not alter the mRNA levels for proapoptotic Bax or Bad in neutrophils. However, substantially increased levels of mRNA for the Fas receptor, which promotes activation of the extrinsic apoptotic pathway (12), and for Mcl-1, an antiapoptotic protein that has been implicated in neutrophil survival (20), was detected. Because of the potential key roles of Mcl-1 and Bax expression in the regulation of apoptosis-signaling

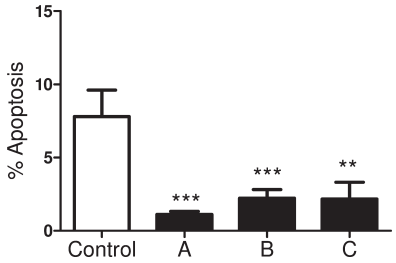

FIGURE 1. Disturbed spontaneous apoptosis in trauma neutrophils. Neutrophil apoptosis rate was determined after isolation of cells from healthy volunteers (control) and from patients at times A (days 0–2), B (days 3–6), and C (days 7–11) after trauma by propidium iodide staining and flow cytometry.

pathways in neutrophils, we chose the systematic analysis of these molecules in terms of mRNA and protein expression by semiquantitative PCR (Fig. 2*B*) and Western blot (Fig. 2*C*). Data are shown from experiments conducted with neutrophil samples from up to 13 healthy controls and 11 patients, and they confirm the results obtained by the real-time PCR analysis.

Overall, Mcl-1 gene expression was increased 2-fold in neutrophils after trauma, whereas Bax gene expression remained unchanged (Fig. 2*B*). Nevertheless, as two previous reports showed a depletion of Bax protein in neutrophils with delayed apoptosis (30, 31), we studied Bax protein expression in neutrophils after trauma and found a significant 2.3-fold reduction in Bax levels compared with control cells. In contrast, Mcl-1 protein expression in patients was 2.5-fold increased relative to neutrophils isolated from healthy volunteers (Fig. 2*C*).

These findings clearly demonstrate that neutrophils of patients after multiple trauma exhibit increased amounts of Mcl-1 and decreased amounts of Bax protein, both likely to be associated with prolonged neutrophil survival and unappreciated activity.

Effects of serum from severely injured patients and GM-CSF on neutrophil apoptosis

As previously noted, GM-CSF significantly reduces neutrophil apoptosis by increasing Mcl-1 protein levels (19, 20). To investigate whether GM-CSF levels are increased after trauma, we measured GM-CSF concentration in serum of healthy volunteers and severely injured patients over time by quantitative ELISA (Fig. 3). GM-CSF levels were significantly elevated and highest at time A (days 0–2) after trauma compared with concentrations determined in serum of healthy volunteers. Conversely, no significant increase in GM-CSF concentrations could be measured at time B (days 3–6) and time C (days 7–11) after trauma.

We therefore hypothesized, that elevated concentrations of GM-CSF in the serum of patients might be responsible for the attenuation of neutrophil apoptosis. When neutrophils isolated from healthy volunteers were incubated in medium supplemented with 1% or 10% pooled patient serum (time A), we found neutrophil apoptosis to be strongly down-regulated compared with apoptosis measured in cells cultured in medium containing autologous serum (Fig. 4*A*). The addition of neutralizing anti-GM-CSF Ab to 1% or 10% autologous serum did not significantly increase apoptosis of cultured cells (Fig. 4*B*). In contrast, when anti-GM-CSF Ab was added to pooled patient serum, a significant raise of apoptosis could be measured, indicating that elevated levels of GM-SCF measured in the serum of patients indeed lead to the impairment of neutrophil spontaneous apoptosis (Fig. 4*C*).

The antiapoptotic property of patient serum was found to correlate with marked increased Mcl-1 protein expression in neutrophils cultured in medium supplemented with 1% and 10% serum.

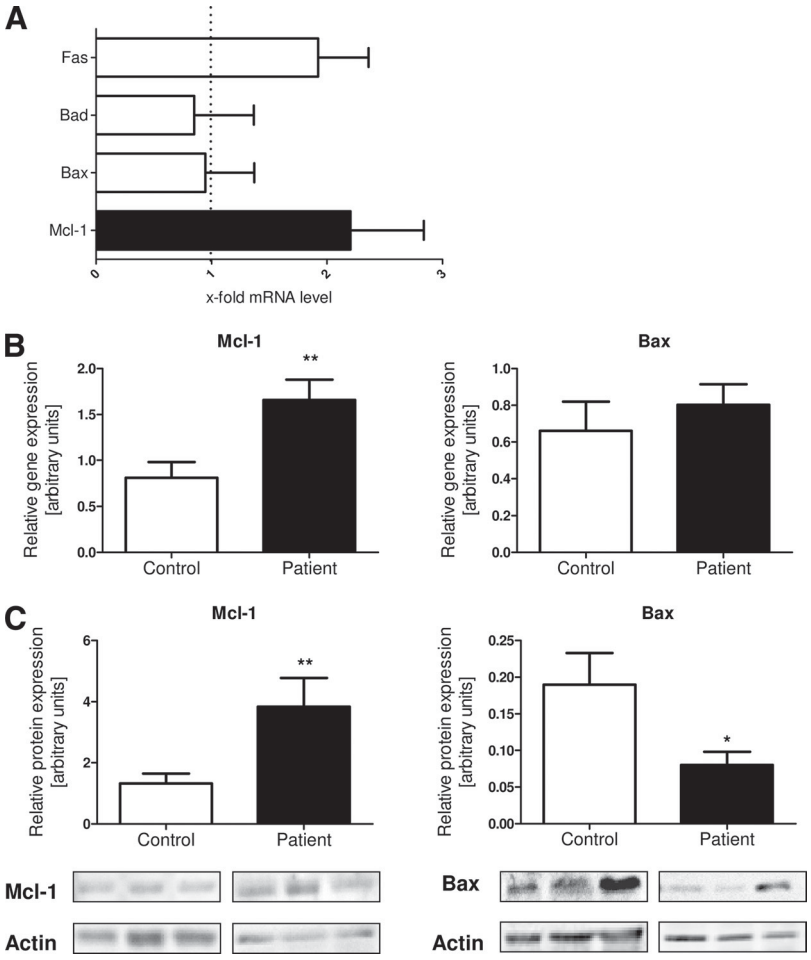

FIGURE 2. Expression of Fas and Bcl-2 family members. *A*, Quantitative real-time PCR evaluation of gene expression in control and patient neutrophils. Gene expression changes in patients are represented as fold increase as compared with controls (– – – –). The expression of four genes is depicted. Data were obtained from three healthy controls and three patients. *B*, *Mcl-1* ($n \geq 8$) and *Bax* ($n \geq 8$) gene expression in neutrophils isolated from healthy volunteers (control) and patients analyzed by semiquantitative PCR. Amplification levels were related to the housekeeping gene GAPDH. **, $p < 0.01$ vs control (Student's *t* test). *C*, Western blot analysis showing Mcl-1 ($n \geq 11$) and Bax ($n \geq 7$) expression in neutrophils isolated from healthy volunteers (control) and patients. Representative blots of three controls and patients are depicted. The normalized values for Mcl-1 and Bax, obtained by densitometric analysis, are reported as arbitrary units. *, $p < 0.05$ vs Control (Student's *t* test).

However, this increase in Mcl-1 protein levels was abolished in the presence of neutralizing anti-GM-CSF Abs (Fig. 4*D*). Taken together, GM-CSF was identified to be a key factor in maintaining neutrophil viability after multiple trauma by the up-regulation of prosurvival molecules such as Mcl-1.

The intrinsic pathway of apoptosis is impaired in trauma neutrophils

We hypothesized that reduced spontaneous apoptosis seen in neutrophils isolated from trauma patients is due to increased levels of Mcl-1 protein, which is known to prevent proapoptotic factors such as Bax from triggering mitochondrial membrane depolarization. To explore whether the intrinsic pathway of apoptosis is impaired in activated neutrophils, we used staurosporine to induce apoptosis in cells isolated from healthy volunteers and trauma patients (Fig. 5). Fig. 5*A* shows that incubation of cells with staurosporine induces a significant concentration-dependent increase in mitochondrial membrane depolarization in control cells, but not in cells from trauma patients or cells pre-incubated with pooled patient serum. The loss of mitochondrial membrane potential in controls was associated with increased apoptosis (Fig. 5*B*). Conversely, staurosporine-induced apoptosis increase in patient neutrophils and in control cells preincubated with patient serum was less prominent. To verify whether changes initiate apoptosis by caspase activation, we further measured caspase-9 activity after staurosporine challenge. In general, caspase-9 is activated through the formation of the apoptosome involving procaspase-9, Apaf-1 and cytochrome *c* (32). Activation occurs in the cytosol following the translocation of mitochondrial cytochrome *c*, a process that is facilitated by mitochondrial membrane depolarization (33). Consistent with our findings concerning mitochondrial membrane depolarization, patient neutrophils showed decreased caspase-9 activity after staurosporine treatment compared with the control (Fig. 5*C*).

As depicted in Fig. 5*D*, the level of the antiapoptotic protein Mcl-1 declined in control neutrophils undergoing apoptosis after treatment with 0.2 μM staurosporine, whereas in patient cells and in neutrophils incubated in the presence of patient serum, this decline was partially prevented. These data again indicate that GM-CSF is responsible for reduced apoptosis and constant levels of Mcl-1, presumably by delaying Mcl-1 protein turnover. To verify this assumption, we again performed GM-CSF blocking studies (Fig. 6). When control cells were preincubated with pooled patient

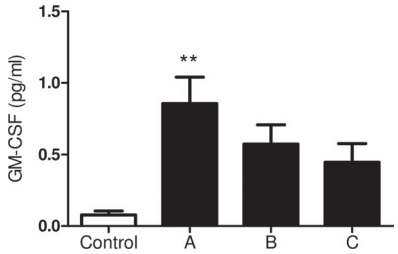

FIGURE 3. GM-CSF concentrations in serum. Sera of multiply injured patients ($n = 22$) were analyzed using a commercially available GM-CSF ELISA kit at time A (days 0–2), time B (days 3–6), and time C (days 7–11) after trauma and compared with healthy volunteers ($n = 8$). GM-CSF concentrations at time A (days 0–2) after trauma were significantly increased compared with GM-CSF levels in controls. **, $p < 0.01$ vs control.

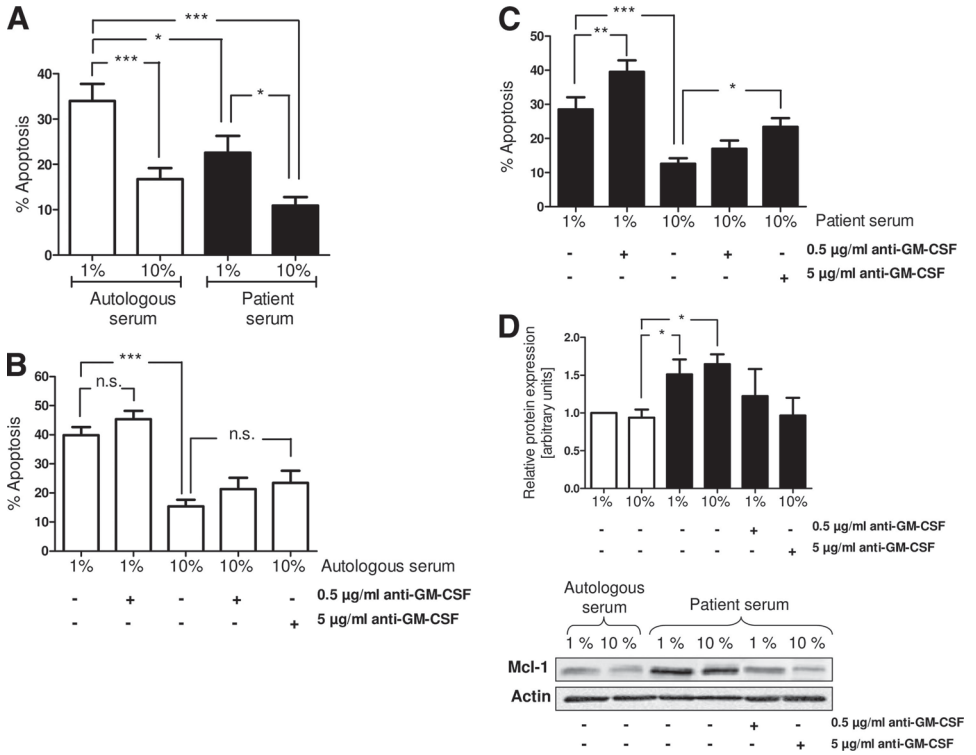

FIGURE 4. Effect of patient serum on neutrophil apoptosis and Mcl-1 protein expression. *A*, Neutrophils isolated from healthy volunteers were incubated for 18 h in medium containing 1% or 10% autologous serum or serum pooled from three severely injured patients (time A, days 0–2), respectively. Neutrophil apoptosis was determined by PI staining. *, $p < 0.05$; ***, $p < 0.001$. $n = 13$. In some experiments, serum was preincubated with 0.5 or 5 μg/ml anti-human GM-CSF Ab which was shown to neutralize the biological activity of human GM-CSF. Culture medium of neutrophils (10^6/ml) isolated from healthy volunteers was supplemented with autologous serum (*B*) or pooled patient serum (*C*) with or without Ab, and cells were incubated for 18 h before determination of DNA fragmentation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $n = 8$. *D*, Neutrophils (2.5×10^6/ml) from healthy donors were incubated overnight with medium supplemented with 1% or 10% autologous serum (□) or pooled patient serum (time A, ■). In addition, cells were incubated with 1% patient serum containing 0.5 μg/ml anti-GM-CSF Ab or 10% patient serum with 5 μg/ml anti-GM-CSF Ab, respectively. Mcl-1 protein expression was verified by Western blot. Blots were analyzed by densitometry and normalized to β-actin. Relative protein expression in cells incubated in medium supplemented with 1% autologous serum was set as 1. *, $p < 0.05$. $n \geq 4$.

serum supplemented with neutralizing anti-GM-CSF Ab, we observed significantly increased mitochondrial membrane depolarization (Fig. 6*A*) as well as apoptosis (Fig. 6*B*) following staurosporine treatment when compared with cells treated in the absence of anti-GM-CSF Ab. Altogether, these data confirm that apoptosis resistance in patient neutrophils after induction of the mitochondria-dependent intrinsic pathway is mediated by high levels of Mcl-1 protein, which was found to be stabilized in a GM-CSF-dependent way.

Expression of Fas and FasL on neutrophils

Neutrophil apoptosis may be accelerated by many extracellular stimuli and is often mediated by cell surface death receptors such as Fas. Neutrophil death in vitro can be partially blocked by Fas/FasL pathway antagonists, as previously reported (13, 34).

To determine whether Fas/FasL mRNA and protein are expressed by neutrophils from severely injured patients, we analyzed their expression by semiquantitative PCR and flow cytometric analyses (Fig. 7). As a control, mRNA and protein expression in neutrophils from healthy donors was examined. *Fas* mRNA was always detectable in neutrophils from trauma patients over a 12-day time period and showed a significant expression peak at time B (days 3–6) after trauma (Fig. 7*A*). Similarly, *FasL* mRNA expression was also significantly up-regulated in neutrophils from trauma patients (time A; Fig. 7*B*). On the contrary, no changes in Fas and FasL protein expression on the cell surface could be detected in cells from healthy volunteers and patients (Fig. 7*C*), supporting the assumption that prolonged neutrophil survival after trauma is not due to down-regulation of Fas receptor or FasL.

Immobilized agonistic anti-Fas IgM Abs induce apoptosis in trauma neutrophils

Despite controversial publications regarding the Fas sensitivity of neutrophils under inflammatory conditions in the past (7, 8, 34), the Fas receptor is an attractive target for therapeutic immune regulation (35). However, experimental systemic application of agonistic anti-Fas Abs has toxic effects, e.g., in liver and lung (36). Therefore, immobilized agonistic anti-Fas IgM (clone CH-11) on solid biocompatible PU as a carrier has been used in this study (22, 23).

To test whether immobilized agonistic anti-Fas IgM may overcome apoptosis resistance, isolated cells from patients and healthy controls were stimulated with mAb CH-11 coupled to a biocompatible PU carrier for 1 and 4 h, respectively. After an additional 18 h of cell culture, the apoptosis rate was determined by PI staining. As depicted in Fig. 8*A* (*top*), no significant difference in apoptosis was observed between control cells incubated with PU alone (−CH-11) or with Ab-loaded PU (+CH-11) for 1 h. Stimulation of patient neutrophils with immobilized CH-11 for 1 h induced an increase in the relative amount of fragmented DNA (Fig. 8*A*, *bottom*). Apoptosis induction was significantly increased after 4 h of incubation with immobilized CH-11 (Fig. 8, *A* and *B*). Prolonged Fas stimulation therefore seems to determine the extent of apoptosis. As shown in Fig. 8*C*, neutrophils from both controls and patients undergo mitochondrial membrane depolarization following

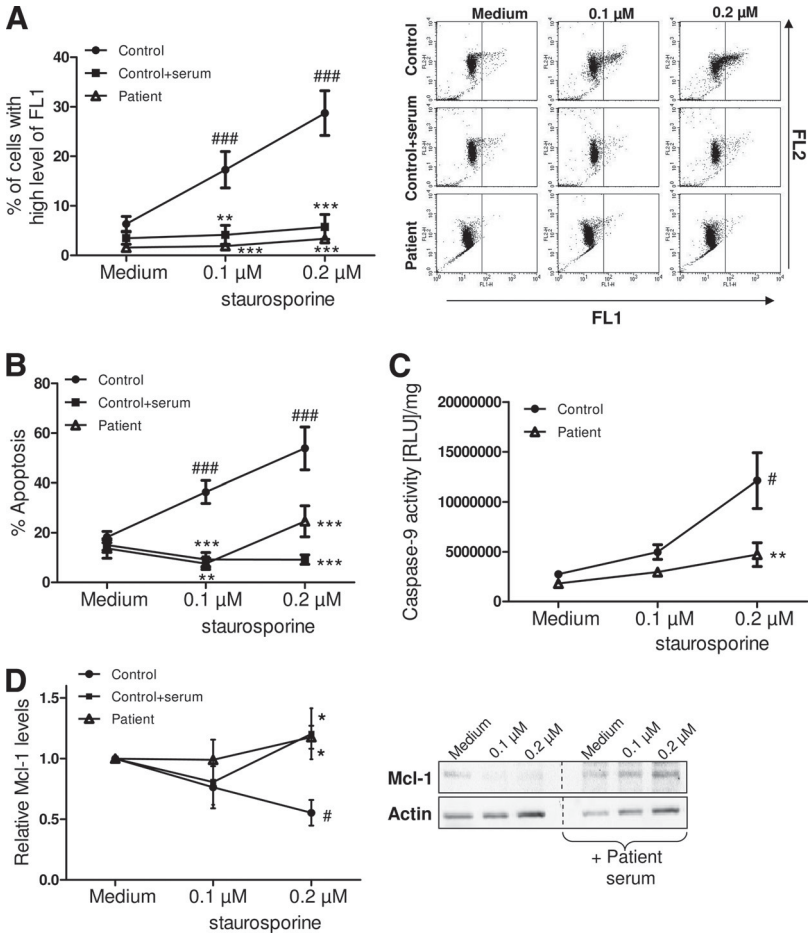

FIGURE 5. The intrinsic apoptosis pathway is impaired in patient neutrophils. *A*, Freshly isolated neutrophils from healthy volunteers (control) and from patients were incubated in the presence of 0.1 or 0.2 μM staurosporine for 4 h. In parallel experiments, control cells were preincubated with 10% pooled patient serum (time A) (control + serum) for 1 h before staurosporine treatment. After 4 h of incubation, mitochondrial membrane depolarization was quantified by JC-1 staining (*left*). Dot-plots of one representative experiment for control, patient, and control + serum are depicted. (*right*). Dot-plots show the increase in neutrophil numbers with high levels of green (FL1) fluorescence. **, $p < 0.01$; ***, $p < 0.001$ vs medium. $n \geq 5$. *B*, Freshly isolated neutrophils were incubated in the presence of staurosporine for 18 h before being assessed for apoptosis. **, $p < 0.01$; ***, $p < 0.001$ vs medium. $n \geq 6$. *C*, Caspase-9 activity in control and patient neutrophils after staurosporine treatment. *, $p < 0.05$ vs Medium; ##, $p < 0.01$ vs control; $n = 3$. *D*, Mcl-1 protein expression analyzed by Western blot after incubation of cells with staurosporine for 4 h. β-Actin was used as a loading control to normalize densitometry values. Relative protein expression in cells incubated in medium was set as 1. Western blot of one representative experiment (control and control + serum) is shown. *, $p < 0.05$ vs control; #, $p < 0.05$ vs medium. $n \geq 5$. FL2, red fluorescence.

Fas activation. This loss of mitochondrial membrane potential has been found to correlate with an increase in caspases activity (data not shown). However, when cells were preincubated with the broad-range caspase inhibitor Boc-aspartyl(OMe)fluoromethylketone, this increase was abrogated, which indicated that the activity of caspases is essential for Fas-mediated mitochondrial membrane depolarization. In accordance with previously published work, our experiments indicate caspase-dependent intracellular Mcl-1 degradation after activation of the FasR (Fig. 8*D*) (37).

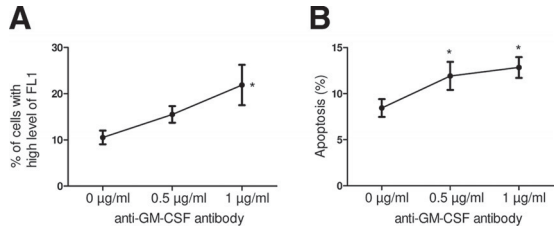

FIGURE 6. The impairment of the intrinsic apoptosis pathway depends on GM-CSF. *A*, Freshly isolated neutrophils from healthy volunteers (control) were preincubated with 1% pooled patient serum supplemented with 0, 0.5, or 1 μg/ml neutralizing anti-GM-CSF Ab for 1 h before treatment with 0.2 μM staurosporine. After 4 h of incubation mitochondrial membrane depolarization was quantified by JC-1 staining. *, $p < 0.05$ vs control sample (0 μg/ml). $n = 9$. *B*, Neutrophils from healthy volunteers (control) were cultured in the presence of pooled patient serum, supplemented with neutralizing anti-GM-CSF Ab and 0.2 μM staurosporine for 18 h before quantification of apoptosis. *, $p < 0.05$; **, $p < 0.01$ vs 0 μg/ml. $n = 9$. FL1, green fluorescence.

Overall, our results demonstrate that apoptosis resistance in neutrophils from multiple trauma patients may be overcome by stimulation of the FasR and the downstream extrinsic signaling pathway.

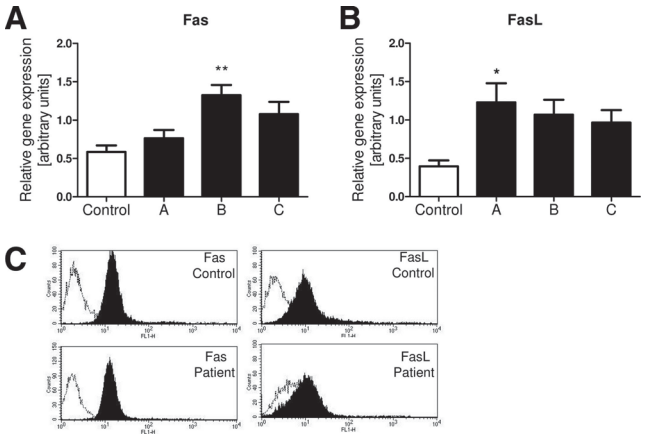

FIGURE 7. Analysis of Fas and FasL expression. Relative *Fas* (*A*; $n \geq 7$) and *FasL* (*B*; $n \geq 7$) gene expression in neutrophils isolated from healthy donors (control) and patients at time A (days 0–2), time B (days 3–6), and time C (days 7–11) and analyzed by semiquantitative PCR. **, $p < 0.01$; *, $p < 0.05$ vs control. *C*, Indirect immunofluorescence staining and flow cytometry were performed to detect cell surface Fas and FasL expression on freshly isolated neutrophils from healthy volunteers and patients (time A, days 0–2; time B, days 3–6; time C, days 7–11). Representative expression of Fas and FasL on neutrophils from one healthy person (control) and patient in the form of a fluorescence histogram (FL1-H) overlay depicting specific staining (solid) vs IgM and IgG isotype control (open) is shown.

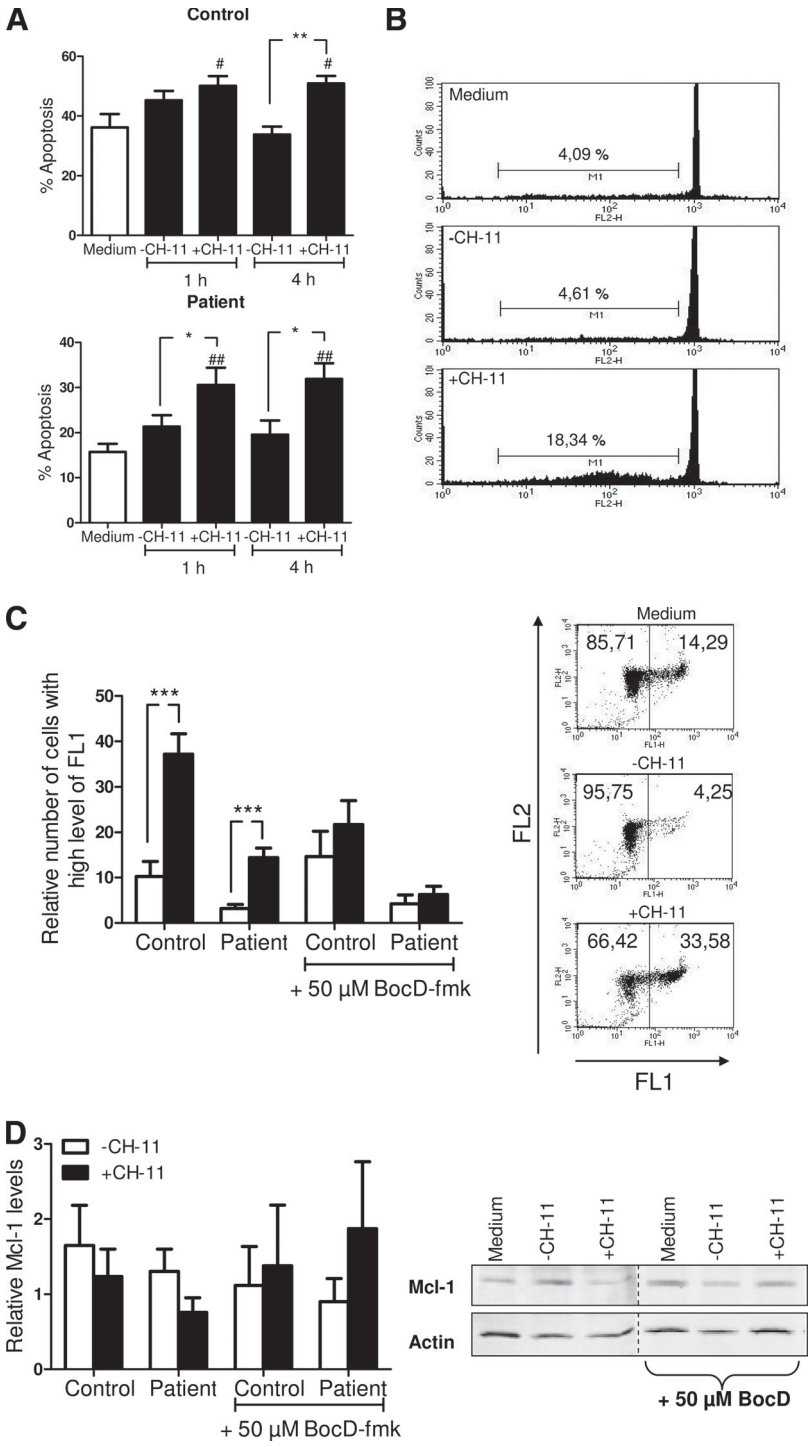

FIGURE 8. Effects of immobilized CH-11 Ab on neutrophil apoptosis. *A*, Freshly isolated neutrophils (10^6/ml) were incubated with unloaded PU foam carriers (−CH-11) and CH-11-loaded foams (+CH-11) for the times indicated. Apoptosis was quantified after a further 18 h in culture. *, $p < 0.05$; **, $p < 0.01$. $n = 9$. *B*, Representative DNA fluorescence histogram (FL) of patient cells incubated with medium only, PU foams alone (−CH-11), and CH-11-loaded foams (+CH-11) for 4 h. After further incubation for 18 h, apoptosis was determined by PI staining. *C*, Isolated neutrophils were incubated with unloaded PU foam carriers (−CH-11) and CH-11-loaded foams (+CH-11) for 4 h. In a parallel experiment, cells were preincubated with the caspase inhibitor 50 μM Boc-aspartyl(OMe)-fluoromethylketone (BocD-fmk). Mitochondrial membrane depolarization was measured by JC-1 staining after 4 h of CH-11 incubation. Results represent mean values of eight controls and five patients. Dot-plots of one representative control person are depicted. ***, $p < 0.001$ (Student's *t* test). $n \geq 3$. *D*, Western blot analysis of Mcl-1 protein expression normalized to β-Actin in cells pretreated with or without 50 μM Boc-aspartyl(OMe)-fluoromethylketone after 4 h of CH-11 incubation. Relative protein expression in cells incubated in medium was set as 1 (not shown). Western blot of one representative experiment (control) is depicted. $n \geq 4$.

Discussion

Extended neutrophil survival has been implicated in a variety of inflammatory diseases, and has been described in septic patients with burns, traumatic injuries, and pneumonia (6, 38–40). Deregulation of neutrophil apoptosis can result in the progression of posttraumatic SIRS and MODS, and prolonged neutrophil viability may continue to exacerbate inflammation because of the release of proteases, reactive oxygen species, and proinflammatory mediators (41, 42). Therefore, for the development of therapeutic strategies to limit unwanted outcomes of neutrophil activation a better understanding of neutrophil apoptosis resistance in critically ill patients is required. In this study, we demonstrate for the first time that apoptosis resistance in neutrophils isolated from severely injured patients is due to impaired activation of the intrinsic apoptosis pathway and may be overcome by Fas cross-linking with immobilized agonistic anti-Fas Abs.

Because changes in apoptosis during inflammatory conditions may correlate with different expression of apoptotic proteins (15, 19, 20), the expression of a broad range of genes involved in neutrophil apoptosis was examined. Although numerous genes were found to be deregulated in severely injured patients compared with controls, the antiapoptotic *Mcl-1* was one of the most prominent persistently up-regulated genes. It is well recognized that *Mcl-1* gene expression may be triggered by agents that delay neutrophil

apoptosis, e.g., cytokines and many signaling pathways including MEK/ERK, p38 MAPK, PI3K/Akt, and JAK/STAT3 (18–20, 43, 44). Mcl-1 is a short-lived protein with a half-life of <3 h because of its PEST (proline, glutamate, serine and threonine) motifs, which are targets for ubiquitination and which mark the protein for degradation by the proteasome (20). Because Mcl-1 has a rapid turnover, it is an ideal protein for apoptosis regulation. Indeed, cellular levels of Mcl-1 in human neutrophils closely correlate with their survival kinetics (45). Mcl-1 function may also be regulated by posttranslational events, such as phosphorylation, that leads to Mcl-1 inactivation (46).

However, contrary to the findings by Ertel et al. (6), we now provide data demonstrating that increased serum concentrations of GM-CSF measured in patients early after trauma (days 0–2) strongly correlate with increased intracellular Mcl-1 levels and a reduced apoptosis rate of neutrophils. Mcl-1 protein levels declined over time after trauma, thus showing a strong correlation with the GM-CSF concentrations measured in patient serum. Because neutrophil spontaneous apoptosis was significantly reduced until at least day 11 after trauma, we concluded that Mcl-1 might not be the only antiapoptotic factor involved in the regulation of neutrophil apoptosis but nevertheless inhibits apoptosis efficiently at higher GM-CSF serum concentrations as determined at days 0–2 after trauma (unpublished results). Although our blocking experiments clearly confirmed the Mcl-1-dependend antiapoptotic role of GM-CSF, these results also indicate that other yet undefined serum factors may also partly contribute to neutrophil apoptosis resistance. For instance, besides GM-CSF, factors such as TNF-α, IL-1β, IL-6, IL-8, IFN-γ, and G-CSF have been shown to prolong neutrophil lifespan (47). In addition, synergistic effects of GM-CSF and G-CSF or IL-8, respectively, have been already described (48, 49).

A critical finding of our study is the identification of limited mitochondria disruption and caspase-9 activation following activation of the intrinsic apoptosis pathway by staurosporine in patient neutrophils. Mitochondria lose their membrane potential due to the opening of permeability transition pores. Membrane depolarization may be prevented by antiapoptotic Bcl-2 members such as Mcl-1. Conversely, a reduced intracellular Mcl-1 level during neutrophil death leads to Bax release from the Mcl-1-Bax complex and a subsequent translocation to the mitochondrial membrane (45).

In this study, we clearly demonstrate that patient neutrophils which overexpress Mcl-1 protein are resistant to membrane depolarization in response to staurosporine. Although the amount of Mcl-1 protein declined in staurosporine-treated cells from healthy donors, we found stable Mcl-1 protein levels in patient neutrophils and in control neutrophils which were preincubated with pooled patient serum. These experiments indicate that the increase in Mcl-1 protein level and stability detected in patients 48 hours after major trauma is mediated by serum factors.

We therefore assume that a significant increase in the Mcl-1 level in response to GM-CSF and possibly other proinflammatory agents prevents cytochrome *c* exit and caspase-9 activation and therefore the initiation of apoptosis by maintaining the mitochondrial transmembrane potential. Our hypothesis is supported by findings by Derouet et al. (43), who showed enhanced neutrophil survival and Mcl-1 stability after incubation of cells with recombinant GM-CSF. Moreover, our experiments reveal that GM-CSF in the serum of patients as well as increased amounts of the Mcl-1 protein are responsible for the maintenance of the mitochondrial membrane potential in staurosporine-treated neutrophils. Therefore, this study provides an important insight into the mechanisms yielding neutrophil apoptosis resistance and thus contributing to the development of SIRS. Nonetheless, additional studies are needed to address the question of whether the ability of GM-CSF to inhibit neutrophil apoptosis is exclusively mediated by Mcl-1.

Presently, it was unknown whether this intrinsic apoptosis resistance in neutrophils may be overcome by ex vivo stimulation of death receptors and thus by activation of extrinsic apoptosis pathways. In general, neutrophils are highly susceptible to rapid apoptosis after Fas stimulation, and blocking of the Fas/FasL pathway partially inhibits neutrophil death in vitro (13, 34, 50).

In this study, we demonstrate for the first time that immobilized agonistic anti-Fas Abs induce ex vivo apoptosis in neutrophils from severely injured patients. In general, similar results were also obtained in control experiments when the same Abs were used in soluble form (data not shown). The proapoptotic effect of immobilized agonistic anti-Fas IgM was more pronounced after 4 h of incubation in neutrophils from both controls and patients, when compared with the apoptotic values after 1 h of incubation. This suggests that the effect of immobilized agonistic anti-Fas IgM overcoming the apoptosis resistance is time dependent. Recently, caspase-dependent Mcl-1 degradation during sodium salicylate- and TNF-α-induced neutrophil apoptosis have been described (51, 52). Here, we found that Fas-induced mitochondrial disruption is accompanied by increased Mcl-1 turnover which depends on caspase activity.

In summary, our data demonstrate for the first time that the intrinsic apoptosis pathway in neutrophils from severely injured patients is blocked at least in part by elevated Mcl-1 levels induced by GM-CSF. However, the cells remain sensitive toward activation of the extrinsic pathway. Taken together, our findings presented herein are important for the design of better therapeutic approaches on the basis of biofunctional medical devices to prevent and control neutrophil activity during aberrant inflammatory conditions such as SIRS.

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Disclosures

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Stimulation of Fas signaling down-regulates activity of neutrophils from major trauma patients with SIRS

Adnana Paunel-Görgülü [a], Tim Lögters [a], Sascha Flohé [a], Jindrich Cinatl [b], Jens Altrichter [a], Joachim Windolf [a], Martin Scholz [a,*]

[a] Department of Trauma and Hand Surgery, Heinrich-Heine University, Düsseldorf, Germany
[b] Institute for Medical Virology, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

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ABSTRACT

Posttrauma apoptosis resistance of neutrophils (PMN) is related to overshooting immune responses, systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF). Recently, we have shown that the apoptosis resistance in circulating PMN from severely injured patients which is known to be mediated by high serum levels of pro-inflammatory cytokines can be overcome by the activation of Fas death receptor. Here, we aimed to study whether stimulation of surface Fas leads to the inactivation of hyperactivated PMN from critically ill patients with SIRS. PMN from 23 multiple trauma patients (mean injury severity score (ISS) 34 ± 1.9) were isolated at day 1 after admission to the trauma center. PMN from 17 volunteer blood donors served as controls. Neutrophil activity has been determined after *ex vivo* short (1 h) and long-term (4 h) stimulation of freshly isolated PMN with immobilized agonistic anti-Fas antibodies. We found neutrophil chemotactic migration in response to IL-8, phagocytosis and oxidative burst to be significantly inhibited in control cells already after short-term (1 h) Fas stimulation. In contrast, inactivation of trauma PMN by agonistic anti-Fas antibodies was found to be efficient only after long-term (4 h) incubation of cells with agonistic antibodies. Thus, in trauma PMN down-regulation of neutrophil activity seems to be delayed when compared to cells isolated from healthy controls, suggesting impaired susceptibility for Fas stimulation in these cells. Interestingly, whereas Fas-mediated inhibition of phagocytosis and oxidative burst could be prevented by the broad range caspase inhibitor t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone (BocD-fmk), the chemotactic activity in response to IL-8 was unaffected. In conclusion, we demonstrate that stimulation of neutrophil Fas does not only initiate apoptosis but also induces inhibition of neutrophil functions, partially by non-apoptotic signaling.

Introduction

Polymorphonuclear leukocytes (PMN) play a major role in the first line of defence and their amount increases rapidly during inflammation. Normally, the neutrophil half life in the circulation is limited to 8–10 h because aged PMN undergo spontaneous apoptosis in the absence of cytokines or other inflammatory agents, followed by their removal by macrophages (Edwards, 1994; Savill et al., 1998). However, in acute inflammation and after trauma, the spontaneous neutrophil apoptotic pathway is delayed by the action of local inflammatory mediators and PMN numbers within tissues can be extremely high. The extended neutrophil survival within tissues may result in persistent inflammation and tissue damage by secretion of cytotoxic molecules, such as reactive oxygen species (ROS) and proteases (Edwards, 1994; Edwards and Hallett, 1997). Excessive systemic PMN activation as seen after trauma is part of a systemic inflammatory response syndrome (SIRS). It is widely accepted that the increased cytotoxic potential of PMN is a sign of an uncontrolled inflammatory reaction, which causes damage to tissues and leads to early multiple organ failure (MOF) (Botha et al., 1995). In this context, it has been shown in experimental models that blocking or depletion of PMN results in a reduction of organ failure in the early phase after trauma (Fabian et al., 1994). Neutrophil death by apoptosis and safe removal

Abbreviations: AIS, abbreviated injury scale; ANOVA, one-way analysis of variance; BE, base excess; BocD-fmk, t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone; DHR, dihydrorhodamine; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell scan; FasL, Fas ligand; FADD, Fas-associated death domain protein; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; c-FLIP, cellular FLICE-inhibitory protein; GM-CSF, granulocyte macrophage-colony stimulating factor; ICU, intensive care unit; ISS, injury severity score; MFI, mean fluorescence intensity; MAPK, mitogen-activated protein kinase; MOF, multiple organ failure; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes, neutrophils; PMNE, neutrophil elastase; PU, polyurethane; RFU, relative fluorescence units; RLU, relative luminescence units; ROS, reactive oxygen species; SEM, standard error of the mean; SOFA, sequential organ failure assessment; SIRS, systemic inflammatory response syndrome.

* Corresponding author. Tel.: +49 211 81 04406; fax: +49 211 81 04902.
E-mail address: martin.scholz@uni-duesseldorf.de (M. Scholz).

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by phagocytic cells thus helps to limit tissue damage during inflammation.

The Fas–FasL system represents a major pathway for the induction of apoptosis in cells and tissues (Peter and Krammer, 1998). Activation of the death receptor Fas induces the recruitment and activation of caspase-8, which in turns initiates apoptosis by the activation of downstream caspases, such as caspase-3 (Kischkel et al., 1995; Boatright et al., 2003; Pop et al., 2007). As it is well established that human PMN express functional Fas on their surface, this molecule has emerged as a critical pathway for the induction of neutrophil apoptosis, and may be involved in the regulation of acute inflammation (Iwai et al., 1994; Marsik et al., 2003). Previous work has stated that activation of the Fas receptor by soluble agonistic anti-Fas antibody (clone CH-11) leads to a strong stimulation of neutrophil apoptosis *in vitro* (Himpe et al., 2008). However, since Fas is expressed on many cell types in different organs, systemic application of anti-Fas revealed to have severe toxic effects, e.g. in lung and liver (Hagimoto et al., 1997; Ogasawara et al., 1993). Therefore, to avoid systemic application of anti-Fas antibodies the concept of challenging circulating PMN with an agonistic anti-Fas IgM antibody that is immobilized on an open porous biocompatible polyurethane (PU) foam (Fas+ foam) has been proposed (Scholz et al., 2004). The goal of the present study was to investigate whether Fas targeting might also be a useful strategy for the inhibition of neutrophil activity. We further questioned if down-regulation of cellular activity might be linked to the well known apoptotic process triggered by Fas ligation or might occur by non-apoptotic signaling pathways.

Materials and methods

Study population

Twenty-three primarily admitted patients (17 males, 6 females), mean age: 45.9 ± 3.5 years (range 18–80) were enrolled in this prospective study. Study approval was obtained from the Ethics Review Board of the University of Duesseldorf, Germany. Patients with blunt or penetrating multiple injuries who were admitted to our Trauma Center Level I during a 17-month period (June 2007–October 2008) with an injury severity score (ISS) greater 16 were enrolled in this study. Exclusion criteria were death of the patient on day of admission and patients withdrawing consent. In addition, patients with known preexisting immunological disorders or systemic immunosuppressive medication were excluded. The severity of injury was assessed by the ISS, based on the abbreviated injury scale (AIS; Greenspan et al., 1985). SIRS and sepsis were defined using the criteria outlined in the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (Calandra and Cohen, 2005). Patients were determined as septic if they fulfilled criteria for SIRS and had a definite source of infection. SIRS was defined by two or more of the following criteria: temperature > 38 °C or < 36 °C; heart rate > 90 beats per minute; respiratory rate > 20 breaths per minute or arterial carbon dioxide tension ($PaCO_2$) < 32 mm Hg; and white blood cell count >12,000 cells/mm^3 or <4000 cells/mm^3, or with >10% immature (band) forms. Furthermore, in order to evaluate early organ dysfunction/failure, the sequential organ failure assessment (SOFA) score was determined (Marshall et al., 1995). Seventeen volunteers (10 males, 7 females) from the staff of the hospital aged between 26 and 49 years (mean age 33.1 ± 1.7) were used as controls. All were free of infection at the time of blood sampling.

Heparinized, citrated and serum blood samples (each 5–10 ml) were collected from healthy volunteers and from patients at day 1 (during the first 24 h, 8:00 to 9:00 a.m.) after admission to the trauma center. Heparinized blood was immediately used after collection for neutrophil isolation. In parallel, sera and plasma were harvested by centrifugation and stored at −80 °C until further processing.

Isolation and incubation of PMN

Human PMN were isolated by discontinuous density-gradient centrifugation on Percoll (Biochrom) as previously described (Maianski et al., 2002). Isolated PMN were suspended in RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% autologous serum at a final concentration of 1×10^6/ml. Purity and viability were routinely above 95% as assessed by flow cytometry.

A volume of 400 μl of freshly prepared neutrophil suspension (1×10^6 cells/ml) was incubated with PU foams (1 cm^3) that carry immobilized agonistic anti-Fas IgM (clone CH-11) or with PU foams alone, without antibody (Leukocare, Munich). Control cells (without PU foams) were incubated in parallel under same conditions. In some experiments PMN were pre-cultured in medium containing 100 μM of the broad-spectrum caspase inhibitor t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone (BocD-fmk; Calbiochem) for 30 min at 37 °C.

For quantification of neutrophil apoptosis cells were incubated overnight (18 h) in 24-well cell culture plates at 37 °C in a humidified atmosphere containing 5% CO_2.

Flow cytometric analysis of the surface expression of Fas and FasL

The expression of Fas and FasL on PMN was determined as previously described (Paunel-Görgülü et al., 2009). Briefly, cells (1×10^6/100 μl) were incubated with mouse anti-human Fas IgM (clone CH-11; MBL, Woburn, MA) or mouse anti-human FasL IgG (BD Pharmingen) for 20 min on ice. Mouse IgM or IgG1 to *Aspergillus niger* glucose oxidase (Dako) were used as negative controls. After washing with PBS cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or IgG (Dianova), respectively for further 20 min on ice protected from light. Finally, PMN were washed twice with PBS and analyzed by flow cytometry.

Neutrophil chemotaxis

Human neutrophil chemotaxis was measured in 12-well plates containing transwell inserts with 3 μm pore size (Transwell Permeable Supports, 3.0 μm Polycarbonate Membrane, Costar, Corning NY). Transwell inserts containing 100 μl (1×10^5) of cells were placed in wells containing RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS and 25 ng/ml of IL-8 (R&D Systems) as chemoattractant. After incubation of plates for 60 min at 37 °C, the transwell inserts were removed and fluorescent CountBright counting beads (Invitrogen) were added to samples to quantify absolute cell numbers by flow cytometry. Chemotaxis of Fas−/Fas+ foams-treated cells is expressed as relative percentage of cells migrated in response to IL-8.

Neutrophil oxidative burst

To determine the percentage of PMN that produce reactive oxidants with or without Fas stimulation, the Phagoburst™ kit (Orpegen Pharma) was used according to the manufacturer's instructions with some modifications. In brief, a suspension of 100 μl was suspended in RPMI medium supplemented with 10% autologous serum and incubated for 20 min with phorbol 12-myristate 13-acetate (PMA) as high stimulus or without stimulus at 37 °C. The burst activity was determined by flow cytometry as the conversion of dihydrorhodamine 123 (DHR-123) to green fluorescent rhodamine 123 (R-123) by oxidative burst products. Results

are presented as relative oxidative burst (= % ROS-producing cells treated with Fas+/Fas− foams/% ROS-producing control cells) and relative MFI (mean fluorescence intensity; =MFI of Fas+/Fas− -treated cells/MFI of control cells), representing the mean oxidative burst activity per cell.

Neutrophil phagocytic activity

The Phagotest™ kit (Orpegen Pharma) was used to measure phagocytosis of PMN according to the manufacturer's instructions with some modifications. Untreated control cells (1×10^5/100 μl) or PMN incubated with Fas−/Fas+ foams were suspended in RPMI medium containing 10% autologous serum and incubated with FITC-labeled *E. coli* bacteria for 10 min at 37 °C, whereas a negative control sample remained on ice. Phagocytosis was terminated by placing the samples on ice and adding a quenching solution. After two washing steps, lysis solution was added to remove erythrocytes and samples were further incubated at room temperature for 20 min. The percentage of phagocyting cells was determined by flow cytometry. Results are presented as relative phagocytosis (= % phagocyting cells treated with Fas+/Fas− foams/% phagocyting control cells).

Assessment of neutrophil apoptosis

After incubation of PMN with immobilized anti-Fas IgM (Leukocare, Munich), cells were cultured overnight before centrifugation at 600 × *g* for 5 min. Apoptosis was measured by flow cytometry as the percentage of cells with fragmented DNA as previously described (Nicoletti et al., 1991; Paunel-Görgülü et al., 2009). Results are represented as the percentage of hypodiploid DNA (sub-G1) corresponding to fragmented DNA characteristic for apoptotic cells.

Quantification of myeloperoxidase (MPO), neutrophil elastase (PMNE) and IL-8 by ELISA

Levels of MPO (Hoelzel Diagnostika, Cologne) were determined in the serum, PMNE (Milenia Biotec, Gießen) and IL-8 (Hoelzel Diagnostika, Cologne) in the plasma of healthy volunteers and trauma patients by ELISA according to the manufacturer's instructions.

Determination of caspase-8, -3/7 and -9 activity

Freshly isolated PMN and cells incubated with PU foam carriers with or without CH-11 antibodies (Fas+ and Fas− foams; Leukocare, Munich) were harvested by centrifugation and cell pellets were stored at −80 °C for further investigation. Caspase activities were measured by using Apo-ONE® Homogeneous Caspase-3/7 Assay, Caspase-Glo® 8 Assay and Caspase-Glo® 9 Assay (all Promega) according to the instructions of the manufacturer with some modifications. Briefly, cells were suspended in PBS, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS followed by cell sonication. After determination of protein concentration, same protein concentrations were used for the determination of caspase activity.

Statistical analyses

All data are presented as mean ± SEM. Statistical analyses were carried out using one-way analysis of variance (ANOVA) and Newman–Keuls *post hoc* test. To compare differences between two groups the Mann–Whitney *U* test for nonparametric data has been used (GraphPad Prism Program, GraphPad Software, San Diego, CA). Differences between data groups were considered to be statistically significant at $p < 0.05$.

Results

Injury pattern, initial blood values, outcome

The mean ISS of the study group was 34 ± 1.9 (mean ± SEM, range 17–50). Patients demographics, injury severity and pattern, as well as initial values for one-stage prothrombin time test, lactate, base excess (BE), and SOFA scores are shown in Table 1. Patients exhibited a prothrombin time of less than 70% (61.4 ± 4.5%) and a negative base excess of -4.8 ± 1.1 mM on admission. All patients met criteria of SIRS within the first day after trauma. Highest

Table 1
Demographics, injury pattern and severity, initial blood values, and clinical outcome.

Subject	Sex	Age, years	ISS	Injured body parts, AIS				ICU, days	Sepsis	Survival	Initial blood values			
				Skull	Chest	Abdomen	Extremity				Prothrombin lime. %	Lactat, mM	BE, mM	SOFA <24 h
1	F	50	36	2	4		4	15	N	Y	73	1.9	0.5	7
2	M	48	34	3	3	4	3	74	Y	Y	48	11.7	−16	12
3	F	58	36	4	4		2	21	N	Y	65	3.9	−9.9	10
4	M	60	41		4	4	3	95	Y	Y	17	9	−20.8	13
5	M	63	41	3	4	3	4	83	V	Y	35	5.5	−5.3	13
6	M	28	24	2	2		4	6	N	Y	70	4.2	−6.3	13
7	F	34	24	2		4	2	5	N	Y	93	3.4	−2	4
8	F	26	41	4		5		11	N	Y	74	5	−7.7	5
9	M	80	32		4		4	55	Y	N	10	3.1	−3.3	14
10	M	67	41	2	3	4	4	50	Y	Y	44	1.7	−3.2	13
11	F	24	50	5	4		3	28	N	Y	65	2.5	−6.5	15
12	M	38	50	4	5		3	19	N	Y	63	1.4	2	7
13	M	50	29	3	4		2	20	Y	Y	60	2.5	1.7	13
14	M	28	27	3	3		3	15	N	Y	69	3	−3.2	10
15	M	29	29	2	4		3	12	y	Y	47	1.7	0.9	12
16	M	29	29	2	3		4	10	N	Y	98	5.4	−5	6
17	M	77	48	4	4	4		6	N	Y	85	3.1	−2.9	12
18	F	18	32	4	4			11	N	Y	64	1.3	−1.9	5
19	M	55	17	1			4	3	N	Y	60	2.1	−0.8	11
20	M	41	36	4	2		4	40	N	Y	81	5.6	−7.1	14
21	M	46	24	2	4		2	3	N	Y	81	1	−1.3	9
22	M	52	20	4			2	5	H	N	62	7.2	−10.4	9
23	M	55	41	3	4		4	15	N	Y	48	2.8	−2.9	11

ISS: injury severity score; AIS: abbreviated injury scale; ICU: intensive care unit length of stay; BE: base excess; SOFA: sequential organ failure assessment score: <24 h; highest score within the first 24 h after trauma; F: female; M: male; Y: yes; N: no.

Table 2
Increased neutrophil numbers and elevated levels of myeloperoxidase (MPO), neutrophil elastase (PMNE), and IL-8 after severe trauma.

	Healthy donors	Day 1 after trauma
Leukocytes ($\times 10^6$/ml), mean ± SEM	6.3 ± 0.4 (n = 5)	8.7 ± 0.8 (n = 19)
Neutrophils (% leukocytes), mean ± SEM	58.1 ± 2.6 (n = 5)	80.1 ± 2.1 (n = 19)†
MPO (ng/ml), mean ± SEM	20.15 ± 8.9 (n = 11)	228.3 ± 73.9 (n = 18)*
PMNE (ng/ml), mean ± SEM	156.7 ± 13.5 (n = 6)	185.2 ± 28.3 (n = 18)
IL-8 (pg/ml), mean ± SEM	Below detection limit (n = 7)	40.2 ± 17.5 (n = 17)

* $p = 0.038$ vs. healthy donors.
† $p < 0.0001$ vs. healthy donors.

SOFA score within 24 h after admission was 10.3 ± 0.7 (range 4–15) points. From all patients 18 sustained a blunt and 5 a penetrating trauma. 7 patients developed sepsis during the first 10 days after trauma, 2 patients died posttraumatically as a consequence of MOF. The mean intensive care unit (ICU) stay was 26.2 ± 5.6 days (range 3–95 days).

Acute inflammatory conditions after severe trauma

Severely injured patients admitted to the ICU of the hospital showed significantly elevated neutrophil numbers ($p < 0.0001$ vs. controls) indicating an acute inflammatory condition during the first 24 h after trauma. Additionally, serum MPO, IL-8 and plasma PMNE levels in patients were markedly increased when compared to the levels determined in the serum and plasma (for PMNE) of healthy volunteers (Table 2).

Assessment of Fas/FasL expression on neutrophils after major trauma

Because increased neutrophil activity during SIRS might be associated with altered Fas/FasL expression at the cell surface, we first measured relative Fas and FasL protein expression on PMN isolated from trauma patients and healthy controls. While Fas protein expression was slightly up-regulated in patient PMN, no changes in FasL expression could be detected by flow cytometry. However, already early after major trauma the Fas/FasL protein expression ratio was significantly increased in patients compared to controls (Fig. 1A) indicating that the Fas receptor might represent a feasible target for the modulation of cellular activity. Furthermore, reduced caspase-3 activity could be determined in these cells (Fig. 1B) suggesting prolongation of neutrophil lifespan which is known to be a hallmark of neutrophil activation *in vivo*.

Fas-mediated apoptosis fully depends on the activity of caspases

Having shown in a previous report that Fas activation overcomes apoptosis resistance in PMN from trauma patients (Paunel-Görgülü et al., 2009), we investigated the effects of anti-Fas antibodies-coated PU foams (Fas+ foams) in comparison with control foams without antibodies (−CH-11; Fas− foams) on the activity of caspases. As depicted in Fig. 2A, Fas stimulation of both control and patient PMN for 4 h was associated with significant increase in relative caspase-8, -3/7 and -9 activities when compared to the levels determined in cells treated with control foams. Notably, increase in caspase-8 activity after challenge to anti-Fas antibodies was less pronounced in patient PMN compared to control cells (414.8 ± 67.1% in +CH-11 control vs. 144.4 ± 29.8% in +CH-11 patient).

Nevertheless, Fas-induced as well as spontaneous apoptosis (−CH-11) of control cells could be almost totally prevented by the broad range caspase inhibitor BocD-fmk (Fig. 2B). Similar results were obtained with cells isolated from patients (data not shown).

Inhibition of neutrophil activity by anti-Fas antibodies is mediated by apoptotic and non-apoptotic signaling

It has been previously described that stimulation of neutrophil Fas by agonistic antibodies not only induces apoptosis but additionally attenuates neutrophil adhesion to endothelial cells (Hendey et al., 2002; Greenstein et al., 2000). In order to investigate the regulation of neutrophil activity by targeting Fas we tested the effect of Fas+ foams (+CH-11) on freshly isolated PMN in the presence and absence of the caspase inhibitor BocD-fmk. For this, cells were incubated with Fas+ or control foams for two different time points, 1 and 4 h, respectively, which were previously found to be the most accurate times for the investigation of Fas-mediated effects in this experimental setup.

As depicted in Fig. 3A, treatment of control PMN with Fas+ foams for 1 h (short-term) before chemotaxis assay caused a significant decrease of spontaneous migration ($p < 0.05$) as well as IL-8-induced chemotaxis ($p < 0.001$). However, under the same conditions no significant alteration in chemotactic activity could be observed with PMN isolated from trauma patients. In contrast, long-term (4 h) stimulation by Fas+ foams led to a significant down-regulation of IL-8-induced cell migration in both, control cells ($p < 0.01$) and patient cells ($p < 0.01$), when compared with the chemotactic activity of PMN incubated with Fas− foams (Fig. 3B). Interestingly, caspase inhibition in control cells prior to the incubation with Fas+ foams for 4 h, did not influence Fas-induced reduction in chemotaxis in response to IL-8 suggesting caspase-independent signaling mediated by Fas ($p < 0.01$; Fig. 3C). Similarly, we found strong decrease in the relative number of phagocyting control PMN after 1 h ($p < 0.05$; Fig. 4A) and 4 h ($p < 0.05$; Fig. 4B) of Fas stimulation. While phagocytosis of patient PMN was only slightly down-regulated after 1 h of Fas+ incubation (Fig. 4A), number of cells taking up opsonized *E. coli* bacteria markedly decreased after long term treatment ($p < 0.05$; 4 h; Fig. 4B). In contrast to the findings for chemotaxis, the process of phagocytosis has been found to depend on caspase activity because inhibition of caspases efficiently prevented impairment of neutrophil phagocytic activity by Fas stimulation (Fig. 4C).

Consistent with these findings, challenge of control PMN by Fas+ foams reduced the percentage of oxidative burst positive cells which was accompanied by diminished amounts of ROS (Fig. 5A and B). Long-term Fas stimulation (4 h) of activated PMN from patients significantly reduced the relative amount of ROS-producing cells ($p < 0.05$; Fig. 5B). Additionally, no consistent alterations in oxidative burst could be detected in control cells after caspase inhibition and Fas stimulation for 4 h (Fig. 5C).

Discussion

Severe trauma is associated with the induction of systemic inflammation and subsequent activation of PMN. Aberrant neutrophil activity and prolonged neutrophil survival after major trauma contribute to the development of SIRS, tissue damage, MOF,

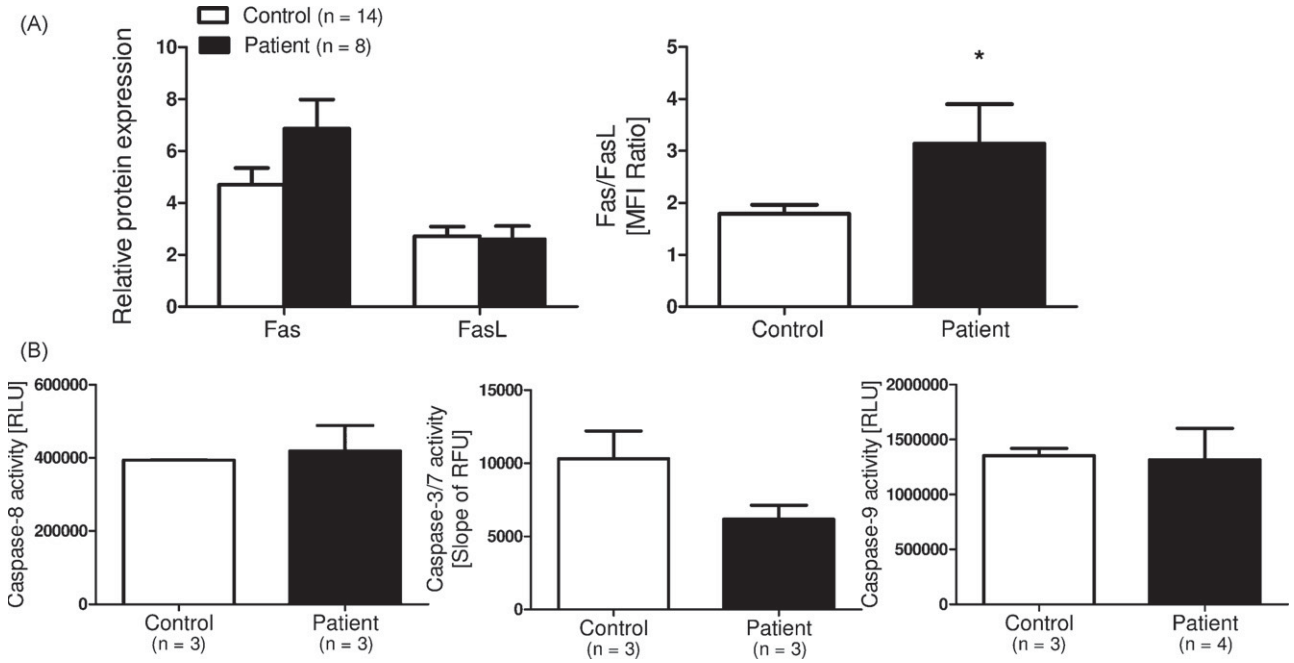

Fig. 1. Fas/FasL expression and caspase activities in trauma neutrophils. (A) Freshly isolated neutrophils (1×10^6) from healthy volunteers and trauma patients were labeled with mouse anti-human Fas or FasL antibody and analyzed by flow cytometry. As control, an isotype-matched IgM or IgG antibody was used. Relative protein expression was calculated as mean fluorescence intensity (MFI) of cells stained with anti-Fas or anti-FasL antibody divided by MFI of isotype control antibody-stained cells (*left panel*). The Fas/FasL ratio on neutrophils is shown for each control and trauma patient (*right panel*). $^*p < 0.05$ vs. Control. (B) Caspase-8, -3/7 and -9 activities were determined in neutrophils isolated from healthy controls and trauma patients. Results are expressed as relative luminescence units (RLU) for caspase-8 and -9 and as the slope of relative fluorescence units (RFU) for caspase-3/7.

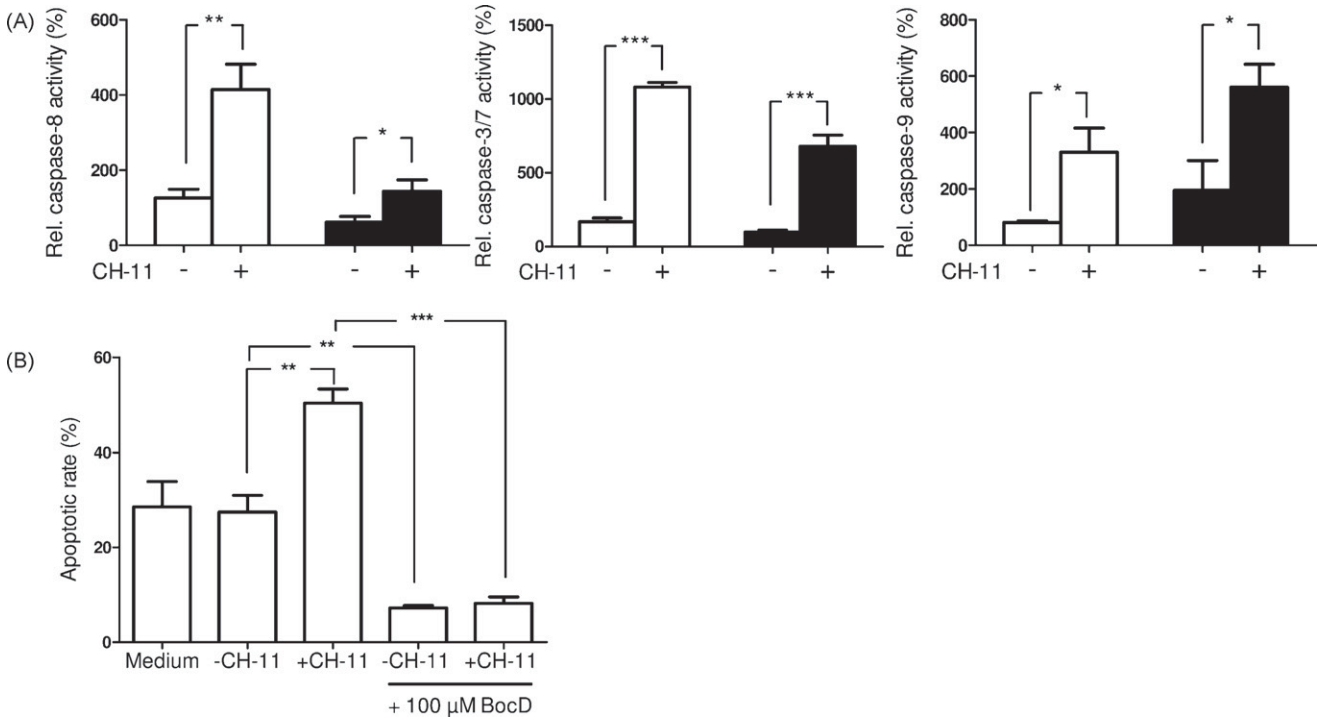

Fig. 2. Fas+ foams-induced apoptosis depends on the activity of caspases. (A) Freshly isolated PMN (1×10^6/ml) from healthy volunteers ($n = 3$, *white bars*) and trauma patients ($n = 3$, *black bars)* were incubated with Fas− (−CH-11) or Fas+ foams (+CH-11) for 4 h. Then, the activities of caspase-8, -3/7 and -9 were measured. Caspase activity in control cells incubated in medium was set as 100% (not shown). Data are expressed as relative percentage of caspase activity compared to control cells. (B) PMN from five healthy donors (1×10^6/ml) were pre-treated with the broad range caspase inhibitor BocD-fmk (100 μM) for 30 min before challenge to CH-11-coated or uncoated (+/−CH-11) foams for 4 h. Cells were subsequently transferred to culture plates and incubated for further 18 h at 37 °C. Apoptotic neutrophils with hypodiploid DNA content were quantified by propidium iodide staining and flow cytometry; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

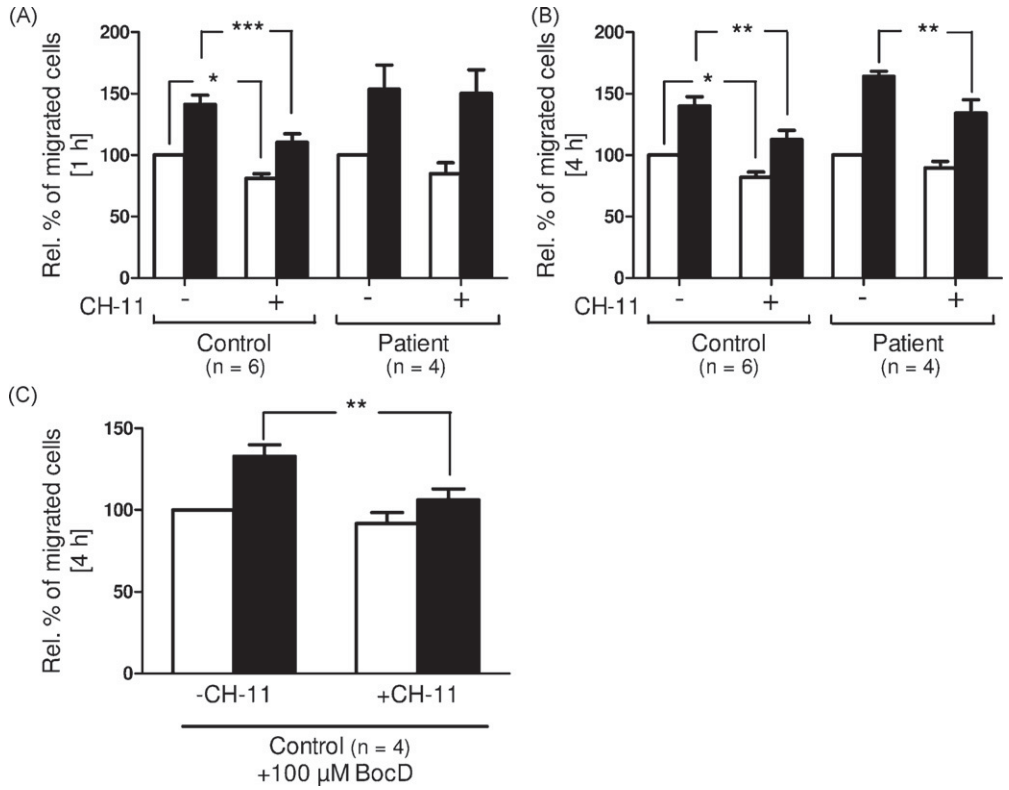

Fig. 3. Effect of time and caspases inhibition on Fas-mediated down-regulation of neutrophil chemotaxis. PMN (1×10^6/ml) isolated from healthy volunteers and trauma patients were incubated with uncoated Fas− foams (−CH-11) and Fas+ foams (+CH-11) for 1 h (A) and 4 h (B), respectively. In some experiments, control neutrophils were pre-treated with the caspase inhibitor BocD-fmk (100 μM) before challenge to Fas−/+ foams for 4 h (C). Directly after incubation with the foams, 1×10^5 cells were immediately transferred into transwell inserts and spontaneous neutrophil migration (*white bars*) as well as migration in response to IL-8 (*black bars*) were determined after 1 h of incubation at 37 °C. Numbers of spontaneously migrating cells incubated with Fas− foams (−CH-11, *white bars*) were set as 100%; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

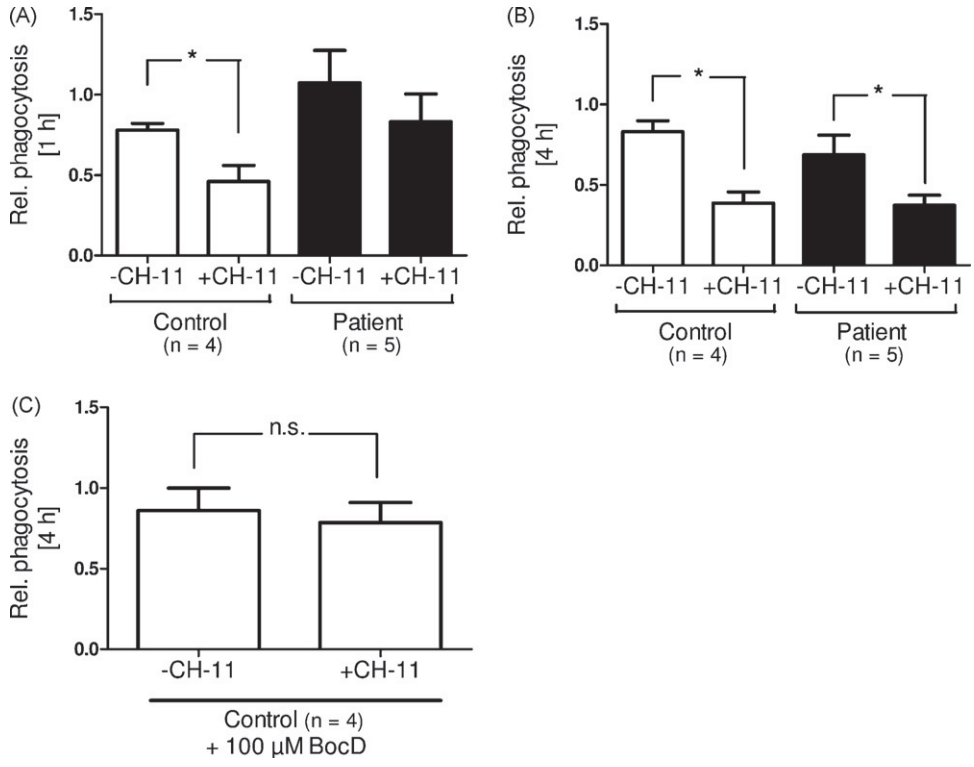

Fig. 4. Effect of time and caspases inhibition on Fas-mediated down-regulation of neutrophil phagocytosis. PMN (1×10^6/ml) isolated from healthy volunteers and trauma patients were incubated with uncoated Fas− foams (−CH-11) and Fas+ foams (+CH-11) for 1 h (A) and 4 h (B), respectively. In some experiments control cells were pre-treated with BocD-fmk (100 μM) and then incubated with Fas−/+ foams for 4 h (C). For phagocytosis assay neutrophils were stimulated with FITC-labeled opsonized *E. coli* bacteria at 37 °C. Fluorescence intensity of phagocytosed bacteria was determined by flow cytometrie. Relative phagocytosis was calculated as % of phagocyting Fas−/Fas+ -treated cells/% of phagocyting untreated control cells; $^*p < 0.05$. n.s. = not significant.

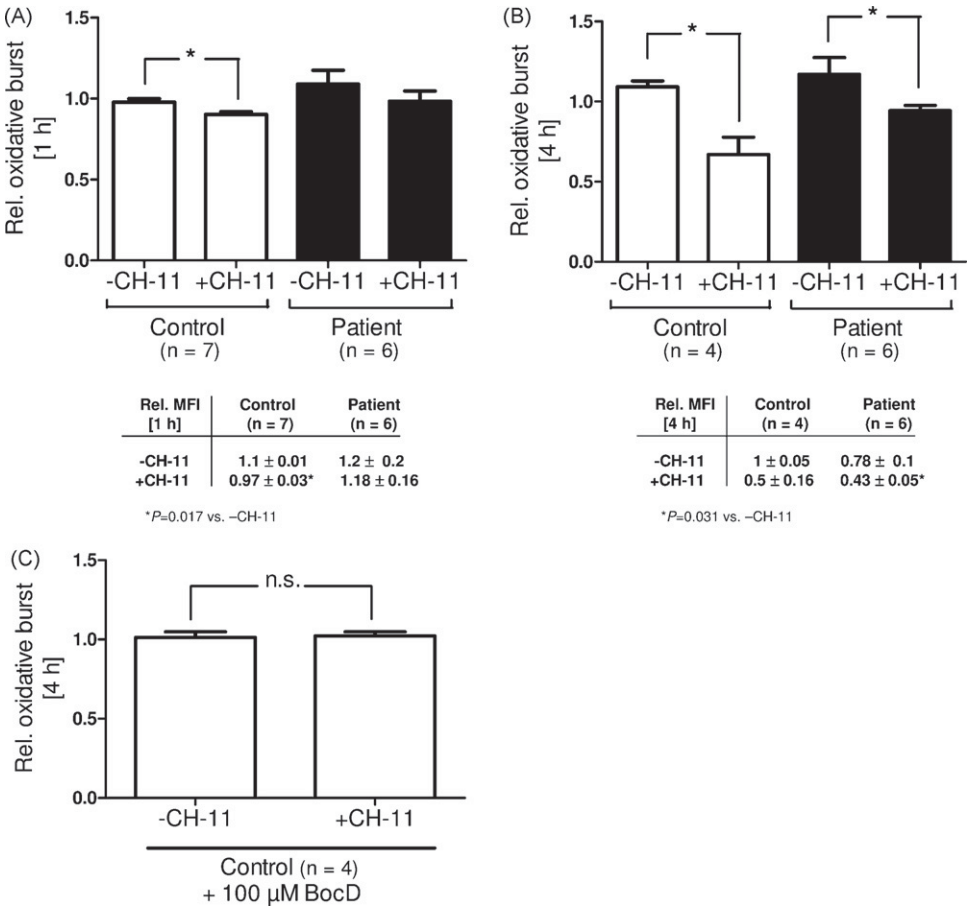

Fig. 5. Effect of time and caspases inhibition on Fas-mediated down-regulation of neutrophil oxidative burst. PMN (1×10^6/ml) isolated from healthy volunteers and trauma patients were incubated with uncoated Fas− foams (−CH-11) and Fas+ foams (+CH-11) for 1 h (A) and 4 h (B), respectively. Percentage of ROS-producing neutrophils was measured following maximal stimulation of PMN with PMA. Relative oxidative burst of Fas−/Fas+ foams-treated cells (as compared to untreated control cells) is depicted. In parallel, the amount of ROS per cell expressed as mean fluorescence intensity (MFI) was quantified. Relative MFI was calculated using the following equation: GeoMean value of Fas−/Fas+ -incubated cells/GeoMean value of control cells. *$p < 0.05$. (C) Control cells were pre-treated with 100 µM BocD-fmk before being incubated with Fas−/+ foams for 4 h. n.s. = not significant.

and sepsis (Jimenez et al., 1997; Muller Kobold et al., 2000). Besides triggering apoptosis, targeting of neutrophil Fas may rapidly impair neutrophil effector functions (Cinatl et al., 2000) and thus may prevent prolonged activation and protect from neutrophil-mediated tissue damage. Here, we studied whether the potentially pathogenic hyperactivity of PMN from severely injured patients with multiple trauma and SIRS might be attenuated by Fas stimulation with immobilized agonistic anti-Fas antibodies. We measured elevated levels of IL-8, MPO and PMNE in the serum/plasma of trauma patients, all of them being related to increased neutrophil activity and pathological effects in inflammation. However, neutrophil hyperactivity was found to be rapidly down-regulated by *ex vivo* challenge of cells to immobilized anti-Fas antibodies. We further demonstrate that in contrast to phagocytosis and neutrophil oxidative burst, inhibition of cell migration in response to IL-8 does not depend on caspase activity, and thus might represent an apoptosis-independent process triggered by Fas.

It is widely accepted, that activation of the Fas signaling pathway initiates extrinsic apoptosis in neutrophils *in vitro* and *in vivo* (Himpe et al., 2008; Kotani et al., 2003) by triggering downstream caspase cascades via activation of the initiator caspase-8, resulting in DNA fragmentation as a characteristic of apoptotic cell death (Ashkenazi and Dixit, 1998). However, there is increasing evidence that activation of Fas may also result in non-apoptotic responses like induction of cellular activation, proliferation, differentiation or migration (Peter et al., 2007). Although Chen et al. (2003) have demonstrated inactivation of neutrophils by tumor FasL in mice, it has not been clearly shown yet whether stimulation of extrinsic pathways, e.g. by Fas stimulation may also overcome neutrophil hyperactivity.

Because the functional expression of cell membrane Fas is the prerequisite for Fas-mediated signaling we determined surface expression of Fas and its ligand counterpart FasL on patient PMN in comparison with PMN from healthy donors. Our data implicate that circulating PMN express higher levels of Fas protein in the early posttraumatic phase than PMN isolated from healthy blood donors. Normally, increased Fas expression would lead to a higher rate of programmed cell death due to activation of the Fas/FasL apoptotic pathway. However, preliminary data from our group provide evidence for increased serum concentrations of soluble Fas early after multiple trauma (unpublished data), which is known to protect cells against autocrine/paracrine Fas activation by binding to membrane FasL (Liles et al., 1996). Therefore, despite increased Fas/FasL ratio on PMN, soluble Fas may functionally antagonize the Fas/FasL pathway and may further add to the anti-apoptotic and pro-inflammatory status of PMN after severe trauma. This apoptosis resistance might be overcome by stimulation with agonistic Fas molecules. Indeed, in the present study we showed that Fas molecules expressed on PMN after trauma are still functionally

active as demonstrated by Fas-mediated caspase-dependent apoptosis.

Whereas stimulation of Fas with agonistic anti-Fas antibodies for 1 h attenuated the activity of control cells efficiently, we found only slight down-regulation of phagocytosis and oxidative burst in patient PMN under same conditions. Challenge to Fas+ foams for 4 h significantly impaired IL-8-induced chemotaxis, phagocytic activity and PMA-stimulated oxidative burst in both, control and trauma PMN. However, in our patient cohort we did not find any age- or gender-related differences concerning the efficacy of neutrophil inactivation by Fas stimulation. Additionally, down-regulation of neutrophil activity after long term Fas stimulation did not display any correlation with the severity of patients' injuries, e.g. ISS. These results indicate that Fas-dependent signaling may be critical in the regulation of neutrophil effector functions after trauma. Further, the delayed inactivation found in patient PMN suggests that the Fas signaling pathway might be deregulated in these cells. It has been demonstrated previously that elevated levels of pro-inflammatory cytokines like granulocyte macrophage-colony stimulating factor (GM-CSF) in the blood of patients with SIRS, prolong both neutrophil life span and effector functions by mediating apoptosis resistance (Fanning et al., 1999; Hasegawa et al., 2003). In this context, Kotone-Miyahara et al. (2004) have described a GM-CSF-mediated delay of Fas-mediated apoptosis in PMN due to impaired recruitment of the Fas-associated death domain protein (FADD) to the Fas receptor. However, GM-CSF-mediated apoptosis resistance has been shown to be overcome by strong Fas stimulation. Thus, these results are in agreement with our present data showing delayed Fas-mediated inactivation of patient PMN when compared to control cells. Additionally, we have previously reported that GM-CSF not only activates neutrophil effector functions, but also prolongs the neutrophil life span by increasing the stability of the anti-apoptotic Mcl-1 protein (Paunel-Görgülü et al., 2009).

In the present study, *ex vivo* activation of Fas resulted in caspase-dependent neutrophil apoptosis which was completely abrogated in the presence of the broad range caspase inhibitor BocD-fmk. Notably, our data presented herein, demonstrate that in contrast to phagocytosis and the ability to produce ROS, inhibition of cell migration in response to IL-8 does not depend on caspase activity, and thus might represent an apoptosis-independent process triggered by Fas. Although little is known about the non-apoptotic signaling pathways activated by Fas, it is widely accepted that FADD, caspase-8 and c-FLIP connect the Fas receptor to MAPK and NF-κB signaling pathways (Peter et al., 2007) which are involved in cytoskeletal rearrangement mechanisms. Therefore, it is possible that impaired neutrophil adhesion and migration observed after Fas signaling stimulation (Cinatl et al., 2000) is due to changes in the cytoskeletal arrangement. Consequently, this would explain the herein observed time delayed responsiveness towards chemotactic signals such as IL-8 after challenging the cells with immobilized agonistic anti-Fas IgM.

In conclusion, our data show for the first time that Fas signaling not only promotes apoptosis in hyperactivated PMN from multiple trauma patients but also attenuates neutrophil effector functions. The results presented herein establish the feasibility of Fas targeting as an approach to limit posttraumatic SIRS. For instance, the treatment of critically ill patients with a medical device for extracorporeal immune therapy that contains immobilized agonistic anti-Fas molecules might be used to prevent early neutrophil hyperactivation and its sequelae. Although the apoptosis-related mechanisms triggered by immobilized anti-Fas antibodies have been extensively studied and clearly defined in a large patient cohort (Paunel-Görgülü et al., 2009), the small number of subjects might represent a major limitation of this study. Therefore, further study including a larger number of patients should be conducted to support the presented results.

Acknowledgments

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Extracorporeal immune therapy with immobilized agonistic anti-Fas antibodies leads to transient reduction of circulating neutrophil numbers and limits tissue damage after hemorrhagic shock/resuscitation in a porcine model

Tim T Lögters*[1,2], Jens Altrichter[1], Adnana Paunel-Görgülü[1], Martin Sager[1], Ingo Witte[1], Annina Ott[1], Sarah Sadek[1], Jessica Baltes[1], José Bitu-Moreno[3], Alberto Schek[1], Wolfram Müller[4], Teresa Jeri[1], Joachim Windolf[1] and Martin Scholz[1]

Abstract

Background: Hemorrhagic shock/resuscitation is associated with aberrant neutrophil activation and organ failure. This experimental porcine study was done to evaluate the effects of Fas-directed extracorporeal immune therapy with a leukocyte inhibition module (LIM) on hemodynamics, neutrophil tissue infiltration, and tissue damage after hemorrhagic shock/resuscitation.

Methods: In a prospective controlled double-armed animal trial 24 Munich Mini Pigs (30.3 ± 3.3 kg) were rapidly haemorrhaged to reach a mean arterial pressure (MAP) of 35 ± 5 mmHg, maintained hypotensive for 45 minutes, and then were resuscitated with Ringer' solution to baseline MAP. With beginning of resuscitation 12 pigs underwent extracorporeal immune therapy for 3 hours (LIM group) and 12 pigs were resuscitated according to standard medical care (SMC). Haemodynamics, haematologic, metabolic, and organ specific damage parameters were monitored. Neutrophil infiltration was analyzed histologically after 48 and 72 hours. Lipid peroxidation and apoptosis were specifically determined in lung, bowel, and liver.

Results: In the LIM group, neutrophil counts were reduced versus SMC during extracorporeal immune therapy. After 72 hours, the haemodynamic parameters MAP and cardiac output (CO) were significantly better in the LIM group. Histological analyses showed reduction of shock-related neutrophil tissue infiltration in the LIM group, especially in the lungs. Lower amounts of apoptotic cells and lipid peroxidation were found in organs after LIM treatment.

Conclusions: Transient Fas-directed extracorporeal immune therapy may protect from posthemorrhagic neutrophil tissue infiltration and tissue damage.

Background

Hemorrhagic shock is a leading cause of complications and death in combat casualties and civilian trauma [1]. It has been shown to cause systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and multiple organ failure (MOF) [2]. Despite intensive investigations, the pathophysiology of posthemorrhagic multiple organ failure remains incompletely understood. Recently, it has been reported that neutrophils recruited by mitochondrial products (formyl peptides and mitochondrial DNA) released from damaged tissues and cells are responsible for the inflammation seen in SIRS [3]. However, tissue infiltration with activated polymorphonuclear neutrophils is associated with collateral tissue damage elicited by excessive amounts of neutrophil-derived proteases and oxygen radicals which may affect all major organs and largely contribute to MODS [4-17].

* Correspondence: tim.loegters@med.uni-duesseldorf.de
[1] Department of Trauma and Hand Surgery, University Hospital, Düsseldorf, Germany
Full list of author information is available at the end of the article

One major reason for the collateral damage mediated by hyperactivated neutrophils is the prolonged neutrophil survival time in conjunction with resistance against apoptosis [18]. There is increasing evidence that prolonged neutrophil survival is due to reduced susceptibility to proapoptotic mediators as a result of proinflammatory cytokines [19] and cytokines [20]. Moreover, intracellular inhibitors of apoptosis proteins (IAPs) are important regulators of neutrophil survival time under inflammatory conditions [21]. Unfortunately, the role of modified neutrophil susceptibility against proapoptotic signaling in the posttraumatic/posthemorrhagic situation and its potential for therapeutic targeting is largely unknown.

Recently, we developed an extracorporeal immune therapy approach to inactivate circulating neutrophils by targeting neutrophil Fas [22-25]. It is known that adequate cross-linking of Fas (APO-1, CD95) on the neutrophil surface membrane stimulates proapoptotic signaling pathways [26,27] but probably may also lead to cellular changes independent from apoptosis [28]. In this regard, we could show earlier that neutrophils rapidly become inactive following contact with membrane bound FasL [29] or with immobilized agonistic anti-Fas IgM antibody [24]. Moreover, evidence has been obtained that the transient contact of technetium-labelled neutrophils with immobilized anti-Fas IgM leads to their rapid sequestration in the spleen [22]. This proposed mechanism might efficiently reduce the number of preapoptotic circulating neutrophils within the circulation. In addition, we recently showed that apoptosis resistance of hyperactivated neutrophils from patients with major trauma may be overcome by agonistic Fas stimulation [30] which may also lead to a shorter life time of activated circulating neutrophils.

This experimental study was done to find out whether neutrophil Fas-directed extracorporeal immune therapy may limit posthemorrhagic inflammation and MODS. Therefore, an extracorporeal mini circuit was developed for the use in a porcine hemorrhagic shock model. As the functional unit, a down-scaled adaptation of the anti-Fas containing leukocyte inhibition module (LIM) as it was used previously for the integration in heart-lung machines [24] was connected to the circuit. The module allows Fas specific inactivation of circulating neutrophils at a flow of 300 ml/min. At this flow neutrophils adhere to and roll over biofunctionally modified three dimensional polyurethane surfaces that carry covalently immobilized anti-Fas (anti-CD95) monoclonal IgM antibodies. Upon contact with the biofunctional surface, inactivated neutrophils rapidly lose their ability to adhere and to migrate towards chemotactic signals [12,29]. Consequently, neutrophils detach from the artificial surface and may be efficiently cleared from the blood probably by phagocytic engulfment [31] and degradation in the spleen [22].

To define whether this specific extracorporeal immune therapy is superior over standard medical care, one group of animals was hemorrhaged/resuscitated without any further treatment whereas the verum group underwent posthemorrhagic extracorporeal immune therapy with the mini-circuit.

Methods

Animals and groups

The animal experiments were performed according to the National Institutes of Health Guidelines for the use of experimental animals. This study was approved by the regional government of Düsseldorf and supervised by the animal health officer of the University of Düsseldorf. Twenty-four pigs (Munich mini pigs; 30.3 ± 3.3 kg) were allocated to 2 groups (each n = 12). All animals were fasted 24 hours before surgery and only received water ad libitum. For histological control samples five additional untreated healthy animals were sacrificed.

Premedication and anesthesia

The animals were premedicated with ketamine and azaperon. Pigs were anesthetized with analgosedation (Thiopental), relaxed, and intubated endotracheally. Ventilation was performed with Isoflurane (1%) and nitrous oxide:oxygen (3:1) mixture with a tidal volume adjusted to maintain $PaCO_2$ values between 36 and 44 Torr [4.8 and 5.9 kPa] and PaO_2 between 100 and 150 Torr [13.3 and 20 kPa].

Surgical preparation

All invasive procedures were accomplished using aseptic technique. Several catheters were inserted for hemodynamic monitoring, blood sampling and connection of the circuits for LIM. A median cut at the ventral neck was accomplished to allow insertion of a 5-Fr catheter into the left carotid artery for continuous arterial pressure monitoring. An 8-Fr Sheldon catheter was placed into the left external jugular vein. This catheter was used for controlled hemorrhage, extracorporeal circulation, and intermittent blood sampling. In addition an 8-Fr introducer sheath was placed into the right external jugular vein followed by a Swan-Ganz catheter (Edwards Lifesciences, Irvine, California, USA) insertion. After verifying proper calibration of arterial and Swan-Ganz-catheter all catheters were fixed subcutaneously.

Extracorporeal Fas-targeted immune therapy with the Leukocyte inhibition module (LIM)

The extracorporeal immune therapy circuit (Figure 1) consists of a Sheldon catheter, a tubing set, and a functional unit with a total volume of 70 ml housing an open

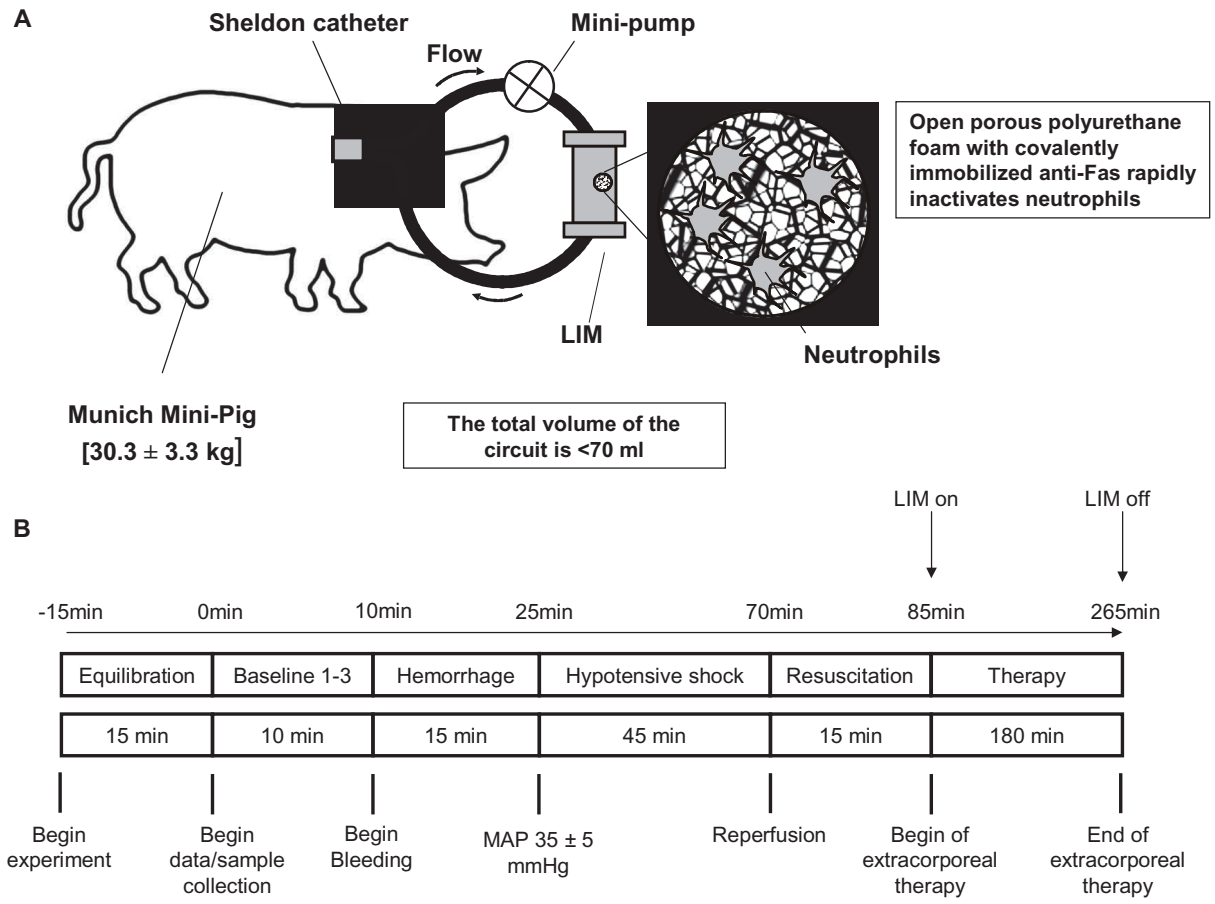

Figure 1 Scheme (A) of the Fas-directed extracorporeal immune therapy (LIM) in the porcine model and (B) schematic depiction of experimental procedures over time.

porous polyurethane foam with specific 3-dimensional characteristics that allows blood flow of 300 ml/min. The foam is coated with anti-Fas (CD95/APO-1) directed agonistic antibodies (clone CH11). The circuit was primed with 70 ml Ringer' solution. After anticoagulation by means of systemic administration of 200 IU/kg heparin (Liquemin; Roche, Grenzach-Wyhlen, Germany) the housing was connected with both lines to the Sheldon catheter (Fig. 1A). To rule out a possible bias, pigs undergoing hemorrhagic shock/resuscitation without extracorporeal immune therapy (standard medical care; SMC) received the same amounts of heparin.

Experimental protocol

All animals were allowed to equilibrate for 15 minutes before baseline measurements (time point 0; Figure 1B). After two additional baseline measurements within 10 minutes, each animal was hemorrhaged rapidly through the Sheldon catheter over 15 minutes in order to reach a mean arterial pressure (MAP) of 35 ± 5 mmHg. Average volume of withdrawn blood was 586 ± 22 ml (SMC: 555 ± 34 ml; LIM: 616 ± 26 ml, n.s.). All animals were kept hypotensive for the next 30 minutes at an MAP of 35 ± 5 mmHg and for further 15 minutes at 40 ± 5 mmHg.

Subsequently, resuscitation was carried out by transfusion of 961 ± 28 ml crystalloid (Ringer') solution back to about 90% of the baseline MAP level (SMC: 916 ± 50 ml; LIM: 1005 ± 18 ml, n.s.). Fifteen minutes after resuscitation extracorporeal circuits were connected to the Sheldon catheter and extracorporeal circulation was initiated with a flow rate of 300 ml/min (LIM group, n = 12). After 3 hours the circuit was flushed with Ringer's solution and disconnected. All animals were then allowed to recover and observed for 48 hours (n = 12, 6 of each group) or 72 hours (n = 12, 6 of each group). Then animals underwent anesthesia, intubation and ventilation again. Catheters were reconnected and after a steady-state stabilization period of 30 minutes hemodynamic parameters were examined for 15 minutes. Finally, pigs were sacrificed and autopsy was performed.

Hemodynamics

During anesthesia following hemodynamic variables were continuously measured with Swan-Ganz and arterial catheter: mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), central venous pressure (CVP), pulmonary capillary wedge pressure (PCWP), mean pulmonary arterial pressure (MPAP), and central venous oxygen saturation (svO2). Blood gas samples were collected every 10 minutes throughout the experimental procedure and measured with a blood gas analysis system (ABL800 Flex, Radiometer GmbH, Willich, Germany). From beginning of baseline measurements venous blood samples were collected at time points 10, 25, 70, 85, 95, 115, 145, 205, 265 minutes as well 12, 24, 48, 72 h after surgery and were analyzed with standardized methods of clinical chemistry. Red blood count, leukocyte count and differential, erythrocyte parameters and platelets were analyzed from EDTA blood (scil animal care company GmbH, Viernheim, Germany).

Histology and staining procedures

All animals included in this study as well as five healthy control animals without any treatment have been euthanised in order to harvest organs for histological evaluation. Tissue samples were fixed in 4% formaldehyde and embedded in paraffin according to standard procedures. Sections (5 μm) were stained with hematoxylin-eosin for pathological examination. In addition, chloracetatesterase staining was performed for specific detection and quantification of tissue infiltration by neutrophils. Neutrophils were counted in a blinded and standardized fashion by microscopy (Axiovert 40, Zeiss, Jena, Germany). Briefly, an ocular micrometer (x10) was used to count neutrophils in 10 different high power fields (HPF) of each section. Mean values from each organ and animal were allocated to predefined ranges of countings/0.09 mm^2 (0-5, 6-10, 11-20, 21-50, 51-100, 101-500).

Quantification of apoptotic cells in tissue sections by TUNEL - Assay

For histological evaluation of apoptotic cells in the porcine tissues, tissue samples of lung, liver, and bowel were frozen directly after removal in liquid nitrogen and stored at -80°C before further utilization. For Tdt-mediated dUTP Nick-End Labeling (TUNEL)-Assay, samples were first embedded in paraffin and 5 μm - sections were prepared according to standard protocols. All following steps were done according to instructions of DeadEnd™ Fluorometric TUNEL System kit (Promega GmbH, Mannheim, Germany). Microscopic examination of DAPI (4'-6-Diamidin-2'-phenylindol-dihydrochlorid) stained nuclei and apoptotic domains was carried out with a fluorescence microscope (Axioskop 40, Zeiss, Jena, Germany) in 400 fold magnification. Different visual fields were selected for each tissue type to count up to 1000 DAPI positive cells. The percentage of apoptotic cells was calculated as the number of TUNEL positive cells from all DAPI positive cells counted. As a positive control for the staining procedure some slides were incubated with DNase before TUNEL staining, resulting in 100% TUNEL positive cells in each field.

Polymerase chain reaction

Total RNA from tissue was extracted using TRI REAGENT (Sigma, Munich, Germany) according to the manufacturer's instructions. 10 μl of total RNA was reverse transcribed using oligo (dT) 15 primer (Sigma, Munich, Germany), employing Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) and following the manufacturer's instructions. PCR was carried out using gene specific primer sequences for heme oxygenase-1 (HO-1; pHO-1-R: 5'-CGTAGCGCTTGGTGGCCTGCG-3'; -F: 5'-CAGCCCAACAGCATGCCCCAG-3', Genosys-Sigma, Munich, Germany). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (hGAPDH-R: 5'-GAAGTCAGAGGAGACCACCA-3'; -F: 5'-CACCACCATGGAGAAGGCTG-3', Genosys-Sigma, Munich, Germany) were used as controls. 2.5 μl of cDNA were amplified using Taq PCR Core Kit (Qiagen, Hilden, Germany) and products were separated on 1.8% agarose gel and visualized under UV after Sybr Gold (Invitrogen, Karlsruhe, Germany) staining.

Western blot analysis

Tissue samples were suspended in RIPA buffer (1% Nonidet-P40 (NP40), 0.5 mM sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in PBS) supplemented with the Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Samples were sonicated and incubated at 4°C for 15 min. After centrifugation at 8,000 × g for 10 min and 4°C, protein concentration was assayed using the Dc Protein Assay kit from Bio-Rad. Protein (30 μg/sample) was separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were saturated in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% w/v nonfat dry milk (blocking buffer) for 60 min at room temperature and then incubated with mouse HO-1 monoclonal primary antibodies specific against pig HO-1 (Stressgen, Victoria, Canada) diluted in TBS containing 0.1% Tween-20 and 5% w/v nonfat dry milk. After three washes in TBS containing 0.1% Tween-20, the membranes were incubated for 60 min at room temperature with the horseradish peroxidase-labelled polyclonal goat anti-mouse secondary antibody for HO-1 (Dako Cytomation, Glostrup, Denmark), diluted 1:1,000 in TBS, 0.1% Tween-20 and washed as described above. Bands were visualized by the enhanced chemiluminescence method (SuperSignal West

pico Chemiluminescent Substrate, Pierce, Bonn, Germany). Equal loading of gels was confirmed both by Ponceau S staining of membranes and by re-incubation of the filters with a polyclonal antibody for beta-Actin (Santa Cruz, Heidelberg, Germany). The amount of specific protein was quantified by densitometry (Quantity One, Bio-Rad, Munich, Germany).

Lipid peroxidation assay

The determination of lipid peroxidation in tissue homogenates was done by quantification of thiobarbituric acid reactive substances (TBARS; Cayman Chemical Company, Ann Arbor, MI). Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde (MDA). The assay is based on the reaction of MDA with thiobarbituric acid (TBA) which is added to the sample. MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm (Victor 3, Perkin Elmer). Briefly, 25 mg of frozen tissue (-80°C) were mixed with RIPA buffer (1% Nonidet-P40 (NP40), 0.5 mM sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in PBS) with protease inhibitors (Complete Mini, Roche). The mixture was homogenized with a pestle and sonicated (Ultrasonic processor UP50H, Hielscher) for 15 seconds on ice. The tubes were then centrifuged at 1600 × g for 10 minutes at 4°C. The supernatant was used for protein concentration analysis (Dc Protein Assay, Biorad), standarized at 1 mg protein/ml solution and utilized for TBARS-assay immediately. The assay was done in duplicates in 96 well plates. Data were compared with standards provided by the manufacturer. The obtained MDA values were calculated using the formula provided by the manufacturer. The dynamic range of the kit is 0-50 μM MDA equivalents.

Statistical analysis

Statistical analysis was carried out using the SAS/Stat for Windows software (SAS Institute, Inc, Cary, NC, version 8) and SPSS (SPSS, Inc, Chicago, IL, version 15). Non-parametric tests of the raw data were used to analyze specific inter-group and over-time differences. Data was considered to be statistically significant at $p < 0.05$. Wilcoxon two-sample test was used for specific inter-group (LIM versus SMC groups) difference and Wilcoxon paired test for over time differences (time point versus start value).

Results

Effects of LIM on leukocyte counts

Time kinetics of leukocyte counts was determined throughout the entire experiments (Figure 2). As shown in Figure 2A, after beginning of resuscitation with LIM leukocyte counts were found to be depressed until the end of extracorporeal immune therapy in the LIM group compared with SMC. This was due to the depression of neutrophil numbers (Figure 2B) and monocyte numbers (Figure 2C), whereas lymphocyte numbers were not significantly modified (Figure 2D). Three hours after reperfusion, neutrophil counts increased in both groups. Furthermore, 72 hours after beginning of resuscitation neutrophil counts were significantly reduced in the LIM group compared to SMC ($p < 0.05$). However, 24 and 48 hours after beginning of resuscitation no intergroup differences were evident for neutrophil counts (data not shown).

Effects of LIM on hemodynamics

MAP in both groups was equivalent at baseline (SMC: 75.7 ± 2.57 mmHg; LIM: 75.2 ± 3.11 mmHg) and decreased in a similar pattern during hemorrhage (Figure 3). During resuscitation MAP reached 89% of the baseline levels. However, it was found to be significantly ($p < 0.05$) decreased in the post resuscitation period in both groups (Figure 3, Table 1). After 72 h MAP values were significantly higher in the LIM group compared with SMC ($p < 0.05$, Table 1). Heart rate (HR) for both groups was slightly different at baseline (SMC: 86.7 ± 3.41 beats/min; LIM: 96.2 ± 4.37 beats/min). As expected, HR increased during hemorrhage until begin of resuscitation (SMC: 128.6 ± 10.7; LIM: 164.9 ± 7.52 beats/min). HR remained increased during the post resuscitation period compared to baseline levels (data not shown). In contrast to the values for the SMC group, values for the LIM group were below baseline at 72 h (Table 1). Within the first 48 hours after resuscitation no significant improvement in hemodynamic variables (MAP, HR, CO, CVP, svO_2, PCWP, MPAP) was observed in the LIM group. However, after 72 hours MAP and CO were significantly ($p < 0.05$) higher in the LIM group compared to the SMC group (Table 1). SvO_2 was 63.1 ± 5.77% for the LIM group and 49.1 ± 3.7% for SMC ($p = 0.0625$).

Ischemia and tissue damage parameters

Transaminases (AST, ALT), creatine phosphokinase (CK), CK-MB, Troponin T, and lactate significantly ($p < 0.05$) increased over time in both groups (Table 2). In conjunction with the increase in lactate, base excess (BE) significantly decreased over time. At 24, 48, and 72 hours lactate values were slightly lower in the LIM group. After 72 hours lactate values were at pre shock level in both groups. CK values were significantly lower 72 hours after shock in the LIM-treated animals (1431 ± 305 U/l) compared with the SMC group (2337 ± 232 U/l).

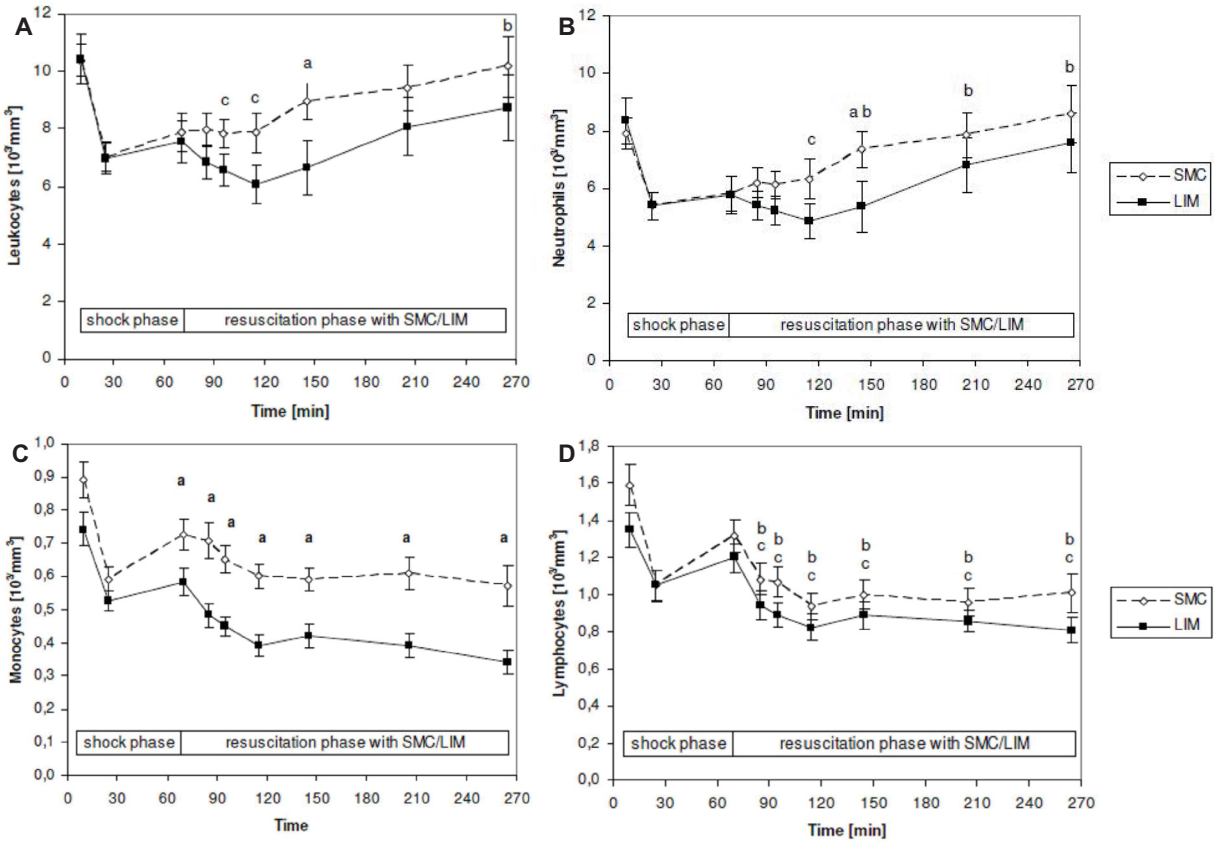

Figure 2 Time kinetics of leukocyte (A), neutrophil (B), monocytes (C), and lymphocytes (D) counts for the SMC group and LIM group; n = 12 per group. Mean ± SEM. [a] - Statistically significant ($p < 0.05$) between SMC and LIM group. [b] - Statistically significant ($p < 0.05$) difference compared with end of shock value (70 minutes) in SMC group. [c]-Statistically significant ($p < 0.05$) difference compared with end of shock value (70 minutes) in LIM group.

Neutrophil tissue infiltration

Representative tissue sections of lung, heart, liver, kidney, and bowel are depicted in Figure 4. Histopathological evaluation did not reveal tissue damage. However, counting of CHE positive cells/HPF revealed increase of neutrophil numbers in the tissues. All SMC animals exhibited neutrophil infiltration of the lungs versus control (SMC range: 101-500, n = 12; control range: 6-10, n = 5). Animals undergoing LIM treatment exhibited only a weak infiltration (11-20, n = 9; 21-50, n = 3). The LIM-mediated limitation of neutrophil infiltration was also found in heart (left ventricle), liver, kidneys (glomeruli), and bowel. However, the differences between SMC and LIM groups were less evident than in the lung.

HO-1 expression, lipid peroxidation, and apoptosis

HO-1 gene and protein expression as a counter-regulation mechanism of oxidative stress was found to be induced in bowels, lungs, and livers in animals that underwent hemorrhagic shock/resuscitation compared to control animals that did not undergo hemorrhagic shock (Figure 5). Both HO-1 gene (Figure 5A) and protein (Figure 5B) expression was lower in the LIM group as compared with SMC. In addition, MDA values that indicate lipid peroxidation and thus tissue damage were significantly lower in the bowels and slightly lower in the lungs of animals in the LIM group compared with the SMC group after shock (Figure 6A). Lipid peroxidation was not found in the livers of animals of either group when compared with control animals.

The putative contribution of apoptosis within bowels, lungs, and livers was studied by TUNEL staining. The numbers of TUNEL positive cells as the percentage from DAPI positive cells were calculated. Results are depicted as relative countings (Figure 6B) and qualitatively as microphotographs (Figure 6C). Apoptosis was lower in the lamina propria of the bowels ($p < 0.05$) and in the lungs (not significant) of animals in the LIM group compared with the SMC group. No Apoptosis was found by TUNEL staining in the liver.

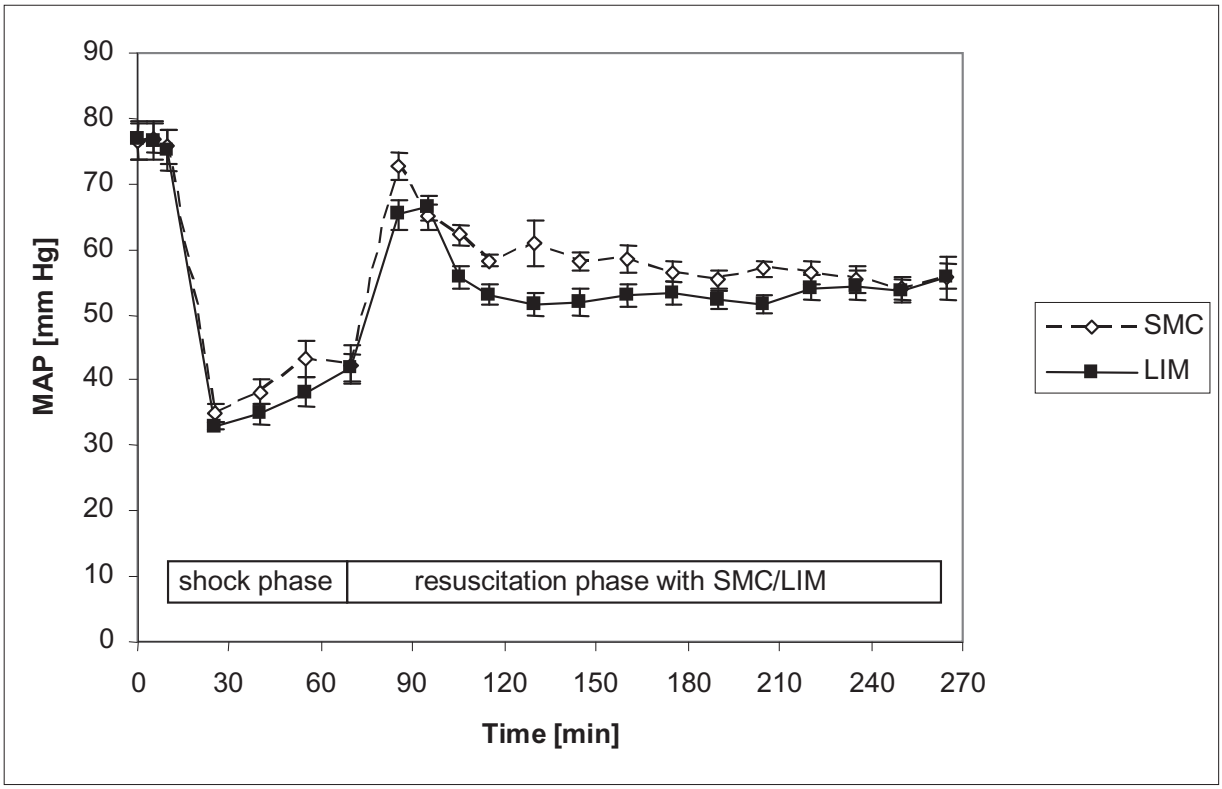

Figure 3 Time kinetics of mean arterial pressure (MAP) in the shock and resuscitation phase for the LIM group and the SMC group; n = 12 per group; Mean ± SEM. Mean arterial pressure is expressed as mmHg.

Discussion

In our porcine hemorrhagic shock/resuscitation model we observed impaired hemodynamics, neutrophil tissue infiltration, lipid peroxidation in the bowel, lung, and liver during an observation period of 72 hours Extracorporeal immune therapy targeting neutrophil Fas ameliorated shock-related pathophysiology. The ability of the mouse-anti-human agonistic anti-Fas IgM used in this study to induce porcine neutrophil apoptosis and to impair the effector functions was shown in earlier studies [22,25]. In previous experiments and in experiments that were done to establish this model, mini circuits without antibody coating were run to exclude effects mediated by the circuit itself. In these tests hemodynamics and leuko-

Table 1: Time kinetics of hemodynamic parameters

	0 h		48 h		72 h	
	SMC	**LIM**	**SMC**	**LIM**	**SMC**	**LIM**
MAP [mmHg]	75.7 ± 2.57	75.2 ± 3.11	44.9 ± 2.64[a]	40.3 ± 4.86[a]	43.8 ± 2.63[a]	52.9 ± 2.54[ab]
HR [beats/min]	86.7 ± 3.41	96.2 ± 4.37	91.9 ± 6.59	105.9 ± 6.63	95.6 ± 9.77	90.0 ± 5.00
CO [l/min]	3.0 ± 0.13	3.1 ± 0.12	2.3 ± 0.23	2.3 ± 0.30	2.2 ± 0.08[a]	3.1 ± 0.24[b]
CVP [mmHg]	3.3 ± 0.70	3.8 ± 0.55	1.1 ± 0.69	5.8 ± 2.19[b]	3.4 ± 1.70	4.8 ± 1.24
svO_2 [%]	86.9 ± 0.95	82.9 ± 2.69	56.0 ± 2.47[a]	57.7 ± 6.44[a]	49.1 ± 3.70[a]	63.1 ± 5.77
PCWP [mmHg]	7.3 ± 1.23	8.2 ± 0.57	3.8 ± 0.88	5.2 ± 0.89	5.9 ± 1.43	5.9 ± 1.12
MPAP [mmHg]	14.8 ± 1.22	17.8 ± 1.49	7.3 ± 1.01[a]	10.9 ± 1.10[ab]	12.2 ± 2.10[a]	13.7 ± 1.05[a]

MAP: mean arterial pressure, HR: heart rate; CO: cardiac output; CVP: central venous pressure; svO_2: central venous oxygen saturation; PCWP: pulmonary capillary wedge pressure; MPAP: mean pulmonary arterial pressure; n = 12 per group at time point 0; at 48 h and 72 h, n = 6; Mean ± SEM. *a*-statistically significant ($p < 0.05$) over-time; *b*-statistically significant ($p < 0.05$) between SMC and LIM group.

Table 2: Time kinetics of metabolic and organ specific parameters

	0 h		End shock		24 h		48 h		72 h	
	SMC	**LIM**	**SMC**	**LIM**	**SMC**	**LIM**	**SMC**	**LIM**	**SMC**	**LIM**
Lactate	3.3 ± 0.26	3.4 ± 0.38	3.4 ± 0.28	4.0 ± 0.43[a]	n.d.	n.d.	2.1 ± 0.39	2.4 ± 0.78	2.3 ± 0.22[a]	1.6 ± 0.31[a]
BE	3.1 ± 0.58	5.0 ± 0.57[b]	1.4 ± 0.91	1.4 ± 0.71[a]	n.d.	n.d.	4.3 ± 0.58	4.8 ± 1.59	5.2 ± 0.92	6.1 ± 1.05
Creatinine [1.1-1.8]	1.0 ± 0.03	0.9 ± 0.05[b]	1.0 ± 0.05	0.9 ± 0.06	1.2 ± 0.07[a]	1.2 ± 0.16	0.9 ± 0.09	1.1 ± 0.18	1.1 ± 0.06	0.8 ± 0.04[b]
AST [23-54]	56 ± 6.8	40 ± 2.8	37 ± 4[a]	31 ± 2.91	912 ± 193[a]	1853 ± 572[a]	378 ± 120[a]	854 ± 515[a]	62 ± 6.8	64 ± 9.7
ALT [50-90]	60 ± 5.68	51.1 ± 3.0	31 ± 3.3[a]	26 ± 1.28[a]	203 ± 25.3[a]	258 ± 33.8[a]	178 ± 19.2[a]	213 ± 30.0[a]	108 ± 5.84[a]	123 ± 16.5[a]
CK [251-810]	1643 ± 220	1183 ± 87	982 ± 134[a]	716 ± 56[a]	58420 ± 9767[a]	77653 ± 14960[a]	15851 ± 4185[a]	29439 ± 15529[a]	2338 ± 233	1431 ± 305[b]
CK-MB	180 ± 20	151 ± 6	95 ± 13[a]	97 ± 10[a]	767 ± 84[a]	969 ± 144[a]	294 ± 33	467 ± 152[a]	156 ± 12[a]	134 ± 31
Troponin T [< 0.05]	0.03 ± 0.01	0.02 ± 0.003	0.04 ± 0.01	0.04 ± 0.01[a]	0.08 ± 0.03	0.15 ± 0.05[a]	0.02 ± 0.004	0.04 ± 0.021	0.02 ± 0.006	0.01 ± 0.00

Reference Ranges in []. Lactate [mmol/l]; BE [mmol/l]: base excess; creatinine [mg/dl]; AST [U/l]: aspartate aminotransaminase; ALT [U/l]: alanine aminotransaminase; CK [U/l]: creatine phosphokinase; CK-MB [U/l]: „MB"-type isoenzyme of creatine phosphokinase; Troponin T [ng/ml]; n = 12 per group, 72 h n = 6; Mean ± SEM; *a*-statistically significant ($p < 0.05$) over-time; *b*-statistically significant ($p < 0.05$) between SMC and LIM group. n.d. = not determined.

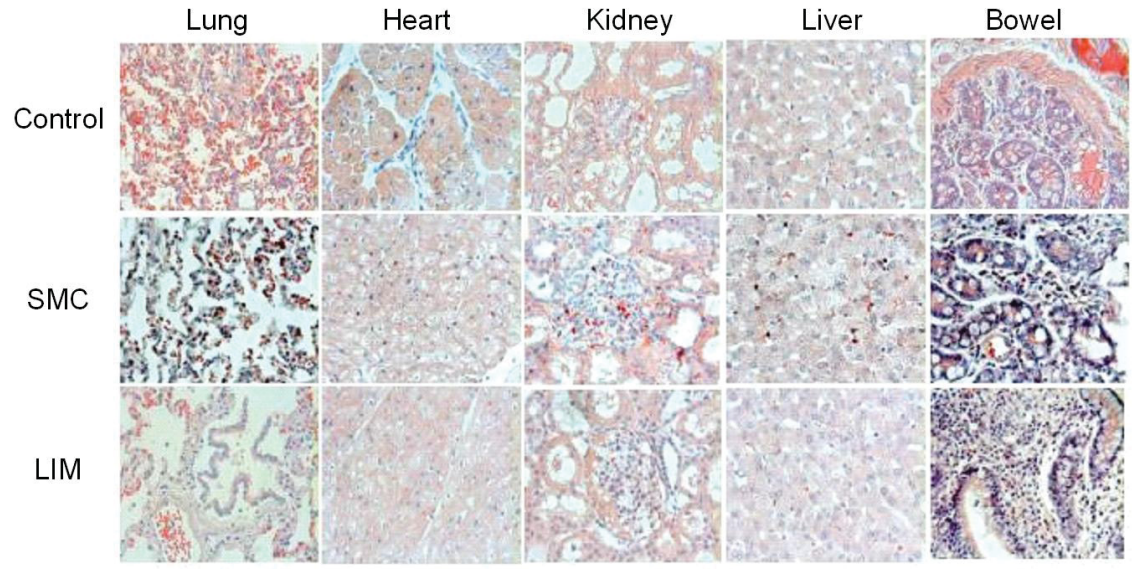

Figure 4 Chloracetatesterase staining of paraffin sections from heart, lung, liver, kidney, and bowel. Representative tissue samples for untreated healthy control pigs, pigs undergoing hemorrhage/resuscitation (SMC), and pigs undergoing hemorrhage/resuscitation with treatment (LIM). Except for control animals, organs were harvested 48 h after shock.

cyte counts were similar to the SMC group. However, in the current study we may not totally exclude LIM effects that are not dependent on Fas activation on neutrophils.

Our working hypothesis was that posthemorrhagic targeting of circulating neutrophil Fas may rapidly impair neutrophil effector functions and thus may prevent their prolonged hyperactivation and neutrophil-mediated tissue damage. We previously found that binding of neutrophils to membrane-bound but not soluble FasL inactivated neutrophils within minutes even before signs of apoptosis were detectable [29], leading us to the assumption that immobilized agonistic anti-Fas may be used to therapeutically limit hyperactivation of neutrophils. In addition, functionalized biocompatible surfaces with agonistic anti-Fas in extracorporeal immune therapy may be more suitable than systemic application of anti-Fas because the latter approach has been shown to have severe side effects such as liver toxicity and pulmonary fibrosis [32,33].

Therefore, in order to effectively inactivate neutrophils in an early phase of posthemorrhagic immune deregulation, an extracorporeal circuit with a neutrophil inhibition module (LIM) on the functional basis of immobilized agonistic anti-Fas IgM was used in a porcine hemorrhagic shock/resuscitation model. The proof of concept of such an approach had been previously shown in patients undergoing cardiac surgery [24,25].

In this study, the efficacy of LIM has been shown by the relative reduction of neutrophil counts during the treatment phase. Histopathological analyses of post hemorrhagic organs clearly revealed lower numbers of neutrophils within the pulmonary tissues and slightly less numbers in heart, liver, kidney and bowel in animals of the LIM group versus SMC. In addition, we found evidence of improved pulmonary, cardiac, and kidney function in the LIM group as indicated by partially higher svO_2, and better cardiac output, respectively. Moreover, CK values were lower in the LIM group, however, only after 72 hours. Due to high SEM values at 24 and 48 hours, the interpretation of these data has to be done carefully. Overall, the obtained evidence that posthemorrhagic hemodynamics and metabolism may be better in the LIM group versus SMC should be confirmed by future studies. In addition, the unexpected reduction of monocyte counts by LIM treatment requires further studies.

Although controversial reports exist regarding activation or inhibition of different cell types by Fas stimulation [34] we never observed increased activity upon challenging neutrophils ex vivo with immobilized agonistic Fas. One possible mechanistic explanation of our findings from this in vivo study may be that LIM treatment impairs the motility of circulating neutrophils which may partly result in the failure of neutrophils to transmigrate into tissues. Consequently, the well known neutrophil-

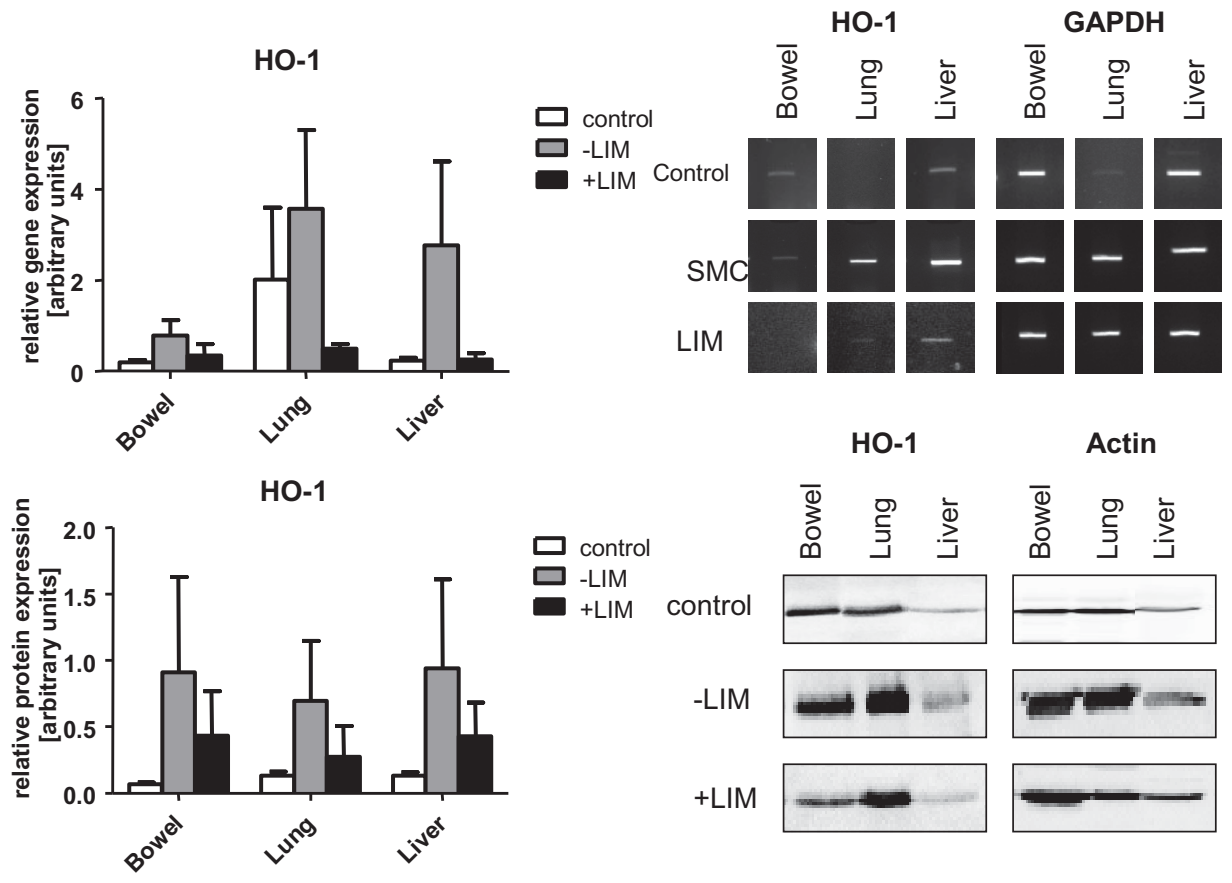

Figure 5 Heme oxygenase-1 (HO-1) gene expression (A), and HO-1 protein expression (B) in control (white bars), SMC (grey bars), and LIM (black bars) animals.

mediated disruption of the integrity of endothelial/epithelial layers, impairment of microcirculation, induction of oxidative stress with subsequent lipid peroxidation [35,36] might be limited by LIM. Indeed, neutrophil chemotactic activity has been shown previously to be reduced after LIM treatment [23]. It has been shown previously that blood cells made apoptotic by extracellular exposure to psoralen and UV light exerted anti-inflammatory effects in a graft-versus-host disease model [37]. It would be of interest to find out whether similar anti-inflammatory mechanisms may also exist upon Fas-mediated neutrophil apoptosis. Further evidence that apoptotic cells have anti-inflammatory and immunosuppressive effects when given systemically in a model of murine LPS-induced endotoxic shock has been reported [38].

Herein, shock/resuscitation-induced hemoxygenase-1 (HO-1) expression, probably as a consequence of posthemorrhagic oxidative stress [39,40], was clearly limited in the LIM group in lung, liver, and bowel, organs that frequently are impaired after trauma [41]. HO-1 is known to be induced by oxidative stress and has been shown by others to protect from hemorrhagic shock-induced tissue injury [39]. The finding that gene and protein expression of HO-1 was found to be lower in the LIM group may be a result of limited neutrophil infiltration and neutrophil-mediated oxidative stress.

Shock-induced lipid peroxidation was only observed in the bowels. However, there seems to be no direct correlation between the amount of lipid peroxidation and infiltrated neutrophils within the bowel since only low neutrophil numbers could be detected in the bowel after shock. In contrast, high numbers of apoptotic cells were found in the lamina propria of the bowel in the SMC but not in the LIM group suggesting that inhibition of peripheral inhibition of circulating neutrophils during posthemorrhagic inflammation may result in protection of the bowel. Similarly, shock-induced apoptosis in the lung tissue was also largely prevented by LIM. The underlying mechanisms remain to be defined. One possible explanation might be that LIM protects from the previously described no-reflow phenomenon associated with

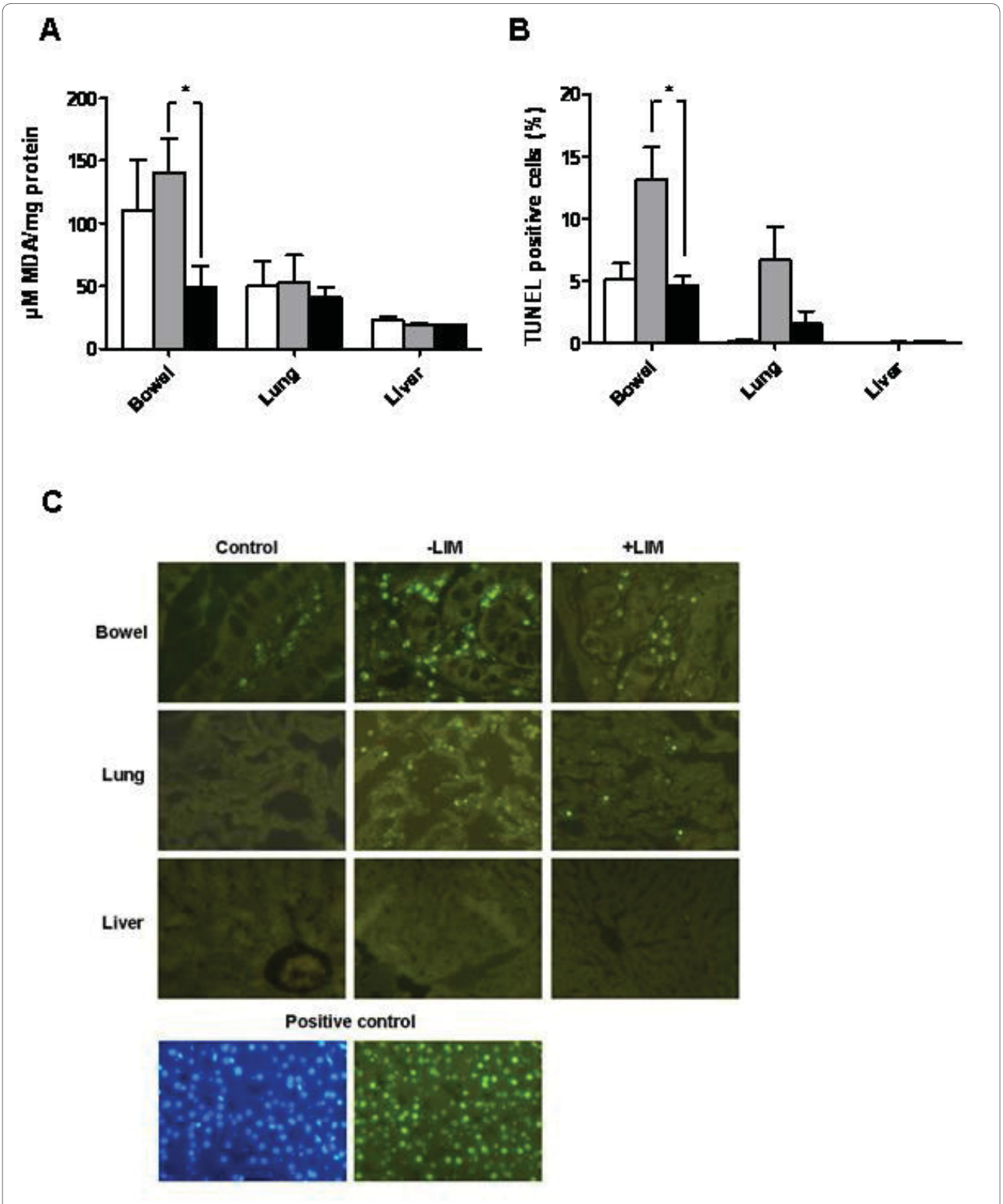

Figure 6 Lipid peroxidation (A) and apoptosis (B) in bowel, lung, and liver as determined by means of malondialdehyde (MDA) assay and Tdt-mediated dUTP Nick-End Labeling (TUNEL), respectively. Data is shown for control (white bars), SMC (grey bars), and LIM (black bars) animals. *Statistically significant ($p < 0.05$) difference. Positive controls indicate staining with 4'-6-Diamidin-2'-phenylindol-dihydrochlorid (DAPI; left) and TUNEL (right) after incubation of tissue with DNase.

neutrophils that are sequestered in the capillaries of the tissues, thus damaging the tissue in the absence of overt neutrophil tissue infiltration [41].

Conclusions

From our data we conclude that targeting of neutrophil Fas during the early posthemorrhagic or posttraumatic time period may ameliorate inflammation-mediated sequelae and thus may be of therapeutic benefit for trauma patients. Due to the small sample size the conclusions have to be made carefully. As usual for explorative studies that have the main objective in the identification of the best primary end point for subsequent confirmative studies, multiple testing of different parameters and time points had to be done, resulting in a reduction of the robustness of the tests performed. Nevertheless, the results obtained provide an interesting basis encouraging further evaluation.

However, the timing of neutrophil inhibition has to be critically considered since inhibition of neutrophil activation might impair anti bacterial phagocytic effects of neutrophils which are essential to prevent sepsis [42,43]. On the other hand, the early prevention of neutrophil-mediated disruption e.g. of the intestinal or pulmonary epithelium might in turn prevent bacterial dissemination and sepsis. Further studies investigating potential clinical benefits of neutrophil Fas-directed immune therapy in patients after hemorrhagic shock or severe trauma are encouraged.

Competing interests

JA and MS receive salary from and hold shares of LEUKOCARE. None of the other authors have anything to declare.

Authors' contributions

TL conducted the experiments and draft the manuscript. AP-G, MS, IW, AO, SS, JB, JB-M, AS participated in the experiments including surgical preparation and data collection. WM participated in the histological analysis. AP-G, JA, JW participated in the study design and revised the manuscript critically for important intellectual content. TJ was in charge of he statistical evaluation. MSch conceived of the study, and participated in its design and coordination and draft the manuscript. All authors read and approved the final manuscript.

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Author Details

[1]Department of Trauma and Hand Surgery, University Hospital, Düsseldorf, Germany, [2]Department of Thoracic and Cardiovascular Surgery, University Hospital, Frankfurt am Main, Germany, [3]Department of Vascular Surgery, Faculdade Medicina Marilia (FAMEMA), Marilia, Brasil and [4]Pathology Group Starnberg, Starnberg, Germany

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CRITICAL CARE

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Increased serum soluble Fas after major trauma is associated with delayed neutrophil apoptosis and development of sepsis

Adnana Paunel-Görgülü, Sascha Flohé, Martin Scholz*, Joachim Windolf, Tim Lögters

Abstract

Introduction: Deregulated apoptosis and overshooting neutrophil functions contribute to immune and organ dysfunction in sepsis and multiple organ failure (MOF). In the present study, we determined the role of soluble Fas (sFas) in the regulation of posttraumatic neutrophil extrinsic apoptosis and the development of sepsis.

Methods: Forty-seven major trauma patients, 18 with and 29 without sepsis development during the first 10 days after trauma, were enrolled in this prospective study. Seventeen healthy volunteers served as controls. Blood samples from severely injured patients were analyzed at day 1, day 5 and day 9 after major trauma. sFas levels, plasma levels of neutrophil elastase (PMNE) and levels of interleukin (IL)-6 were quantified by enzyme-linked immunosorbent assay and related to patients' Sequential Organ Failure Assessment (SOFA) score and Multiple Organ Dysfunction Score (MODS). Neutrophil apoptosis was determined by propidium iodide staining of fragmented DNA and flow cytometry. sFas-mediated effects on neutrophil apoptosis were investigated in cells cultured with agonistic anti-Fas antibodies in the presence of recombinant sFas, sFas-depleted serum or untreated serum from septic patients.

Results: Serum levels of sFas in patients who later developed sepsis were significantly increased at day 5 ($P < 0.01$) and day 9 ($P < 0.05$) after trauma compared with patients with uneventful recovery. Apoptosis of patient neutrophils was significantly decreased during the observation period compared with control cells. Moreover, Fas-mediated apoptosis of control neutrophils was efficiently inhibited by recombinant sFas and serum from septic patients. Depletion of sFas from septic patient sera diminished the antiapoptotic effects. In septic patients, sFas levels were positively correlated with SOFA at day 1 ($r = 0.7$, $P < 0.001$), day 5 ($r = 0.62$, $P < 0.01$) and day 9 ($r = 0.58$, $P < 0.01$) and with PMNE and leukocyte counts ($r = 0.49$, $P < 0.05$ for both) as well as MODS at day 5 ($r = 0.56$, $P < 0.01$) after trauma.

Conclusions: Increased sFas in patients with sepsis development impairs neutrophil extrinsic apoptosis and shows a positive correlation with the organ dysfunction scores and PMNE. Therefore, sFas might be a therapeutic target to prevent posttrauma hyperinflammation and sepsis.

Introduction

Major trauma is frequently associated with activation of polymorphonuclear neutrophils and systemic inflammation. Normally, the life span of neutrophils, which constitute an important line of innate host defense, is limited by apoptosis [1]. During inflammation, neutrophils rapidly migrate from the blood into solid tissues to protect organs from invading bacteria [2]. However, the life span of these neutrophils is prolonged, resulting in lung [3], liver [4] and kidney [5] injury. Further, neutrophil accumulation in the lung and distant organs represents a characteristic finding in patients dying of sepsis [6]. Neutrophils may cause tissue damage by the secretion of reactive oxygen species (ROS) and proteolytic enzymes, of which neutrophil elastase (PMNE) is the most abundant [7,8]. There is strong evidence for a direct correlation between impaired neutrophil apoptosis and overshooting inflammation [9].

* Correspondence: martin.scholz@uni-duesseldorf.de
Department of Trauma and Hand Surgery, University Hospital Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany

Apoptosis is tightly regulated and might be activated via membrane-bound "death" receptors, such as Fas (extrinsic pathway), or via the mitochondrion (intrinsic pathway). Fas/Fas ligand (FasL) signaling has emerged as an important cellular pathway regulating the induction of apoptosis in a wide variety of tissues as well as activated immune cells [10,11], thus playing a crucial role in the resolution of inflammatory responses [9]. The Fas receptor, also designated as CD95 or Apo-1, is a type I cell surface glycoprotein which belongs to the tumor necrosis factor (TNF) receptor superfamily of membrane receptors and has a broad distribution on various tissues [12]. The Fas molecule could occur as a cell surface receptor as well as a soluble protein. The soluble form of Fas (sFas) is derived either by alternative splicing from the membrane form or by proteolytic cleavage of membrane-bound receptors [13,14]. sFas seems to play an important role as a signaling molecule. It has been suggested that sFas modifies ligand concentration, downregulates membrane receptor numbers and specifically inhibits ligand-receptor association in the extracellular space, thus preventing the induction of apoptosis in Fas-bearing target cells. Furthermore, expression of sFas in mice leads to an autoimmune syndrome, and elevated levels of sFas have been found in some patients with autoimmune diseases [13]. FasL is a type II integral membrane protein which is more restricted and tightly regulated in its expression [12], and the procession by a matrix metalloproteinase results in protein cleavage and release of the extracellular domain [15]. The biologically active soluble form of FasL (sFasL) as well as agonistic anti-Fas antibodies are capable of inducing cytotoxicity, hepatocyte destruction and mortality in mice through the interaction with hepatocyte Fas [16,17] and might contribute to systemic tissue destruction during inflammation [18].

Neutrophils express both Fas and its endogenous ligand FasL on their surface, and therefore Fas-FasL interaction may represent a mechanism of autocrine/paracrine neutrophil death regulation [19]. Several previous studies have reported reduced Fas-mediated apoptosis in neutrophils obtained from humans with systemic inflammatory response syndrome (SIRS), burn injuries or surgical trauma [20,21], without elucidating the regulatory mechanisms of the disturbed apoptosis.

In the current study, we provide evidence for serum sFas-mediated inhibition of neutrophil apoptosis and have determined the prognostic value of sFas in post-traumatic sepsis.

Materials and methods

Patients

Forty-seven patients were enrolled in this prospective study. Study approval was obtained from the Ethics Review Board of the University of Düsseldorf (Düsseldorf, Germany). Patients with blunt or penetrating multiple injuries who were admitted to our Level I Trauma Center with an Injury Severity Score (ISS) >16, intensive care unit (ICU) stay >3 days and ages 18 years and older were enrolled in this study. Written, informed consent was obtained from all participants or their legal representatives if the patients were unconscious. Exclusion criteria were death of the patient on the day of admission or within the first 2 days on the ICU and withdrawal of patient consent. In addition, patients with known preexisting immunological disorders or systemic immunosuppressive medication were excluded. The severity of injury was assessed by using the ISS, which is based on the Abbreviated Injury Scale (AIS) [22], on admission to the emergency room. SIRS and sepsis were defined using the criteria outlined in 2005 by the International Sepsis Forum [23]. SIRS was considered to be present when patients' conditions fulfilled more than one SIRS criterion. Patients were determined as septic if they fulfilled criteria for SIRS and had a proven source of infection. To evaluate organ dysfunction and/or failure, the Sequential Organ Failure Assessment (SOFA) and Multiple Organ Dysfunction (MOD) scores [24] were determined. Severe sepsis referred to sepsis complicated by organ dysfunction. Organ dysfunction has been defined using the definition by the SOFA score with >2 points for at least one system (respiratory, coagulation, liver, cardiovascular, central nervous or renal system). Septic shock was defined as sepsis with acute persistent circulatory failure unexplained by other causes (>2 points in SOFA score for the cardiovascular system).

The patients included in this study did not receive low-dose hydrocortisone therapy as routine adjuvant treatment for septic shock. Seventeen healthy volunteers served as the control group.

Blood was collected from healthy volunteers and daily from patients from the day of admission until day 9. Heparinized blood was immediately used after collection for neutrophil isolation. In parallel, sera and plasma were harvested by centrifugation and stored at -80°C until further processing.

Quantification of sFas, sFasL, IL-6 and PMNE by ELISA

sFas (detection limit <47 pg/mL), sFasL (detection limit <12 pg/mL) (both evaluated by Hoelzel Diagnostika, Cologne, Germany) and interleukin (IL)-6 (detection limit <0.70 pg/mL) (evaluated by R&D Systems, Wiesbaden-Nordenstadt, Germany) were measured in serum and PMNE (detection limit 3 ng/mL) (evaluated by Milenia Biotec, Gießen, Germany) in plasma samples by using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Isolation of human neutrophils

Human neutrophils were isolated by discontinuous density gradient centrifugation using Percoll medium (Biochrom, Berlin, Germany) as previously described [25]. After hypotonic lysis to remove contaminating erythrocytes, cells were suspended in phosphate-buffered saline (PBS). Purity and viability were routinely >95% as assessed by forward and side scatter characteristics of FACScan (BD Biosciences, Heidelberg, Germany) and Trypan blue exclusion, respectively.

Immunoprecipitation of sFas from patient serum

The monoclonal anti-Fas antibody clone ZB4 (2 μg; Millipore, Schwalbach, Germany) was mixed with 40 μL of Protein G Plus/Protein A-Agarose beads (Calbiochem, Darmstadt, Germany) and incubated for 3 hours with gentle shaking. Then pooled serum from four septic patients was added and incubated for an additional 17hours at 4°C with gentle shaking. Bound immune complexes were spun down, and the supernatant was stored at -80°C until use.

Apoptosis assay

To neutralize the apoptotic activity of agonistic anti-Fas immunoglobulin (Ig) M antibody (clone CH-11; MBL, Woburn, MA, USA), antibodies (50 ng/mL) were first incubated with recombinant human sFas (R&D Systems, Wiesbaden-Nordenstadt, Germany) for 1 hour and then added to freshly isolated neutrophils (1×10^6/mL) from healthy controls. Cells were further cultured with anti-Fas antibodies in the presence of sFas for 18 hours in RPMI 1640 medium containing 2 mM glutamine (Biochrom, Berlin, Germany) and supplemented with 5% fetal calf serum (FCS) (PAA Laboratories, Coelbe, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere containing 5% CO_2 before being assessed for apoptosis.

Additionally, pooled patient serum and sera immunoprecipitated with ZB4 were used to block the activity of agonistic anti-Fas antibodies (clone CH-11; 200 ng/mL). After 1 hour of incubation, patient serum (10%) containing CH-11 antibodies was added to freshly isolated control neutrophils (1×10^6/mL). Cells were further cultured overnight in RPMI 1640 medium containing 2 mM glutamine (Biochrom, Berlin, Germany) and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere containing 5% CO_2.

Neutrophil apoptosis was measured by flow cytometry as the percentage of cells with fragmented DNA using the method described by Nicolleti *et al*. [26]. Briefly, cell suspensions of freshly isolated neutrophils or those incubated overnight were centrifuged at 450 × *g* for 5 minutes, and then cells were suspended in 300 μl of hypotonic fluorochrome solution (50 μg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100). Cell suspensions were stored in the dark at 4°C for at least 3 hours before they were analyzed by flow cytometry (BD Biosciences, Heidelberg, Germany). A minimum of 10,000 events were counted per sample. Results are represented as the percentage of hypodiploid DNA (sub-G1; percentage apoptosis) corresponding to fragmented DNA characteristics for apoptotic cells.

Statistical analyses

To evaluate differences between the study groups, a Kruskal-Wallis test with Dunn's *post hoc* test was performed. Correlation between numerical values was evaluated by using Spearman's rank-correlation coefficient (*r*). Nonparametric receiver operating characteristics (ROC) curves were generated in which the value for sensitivity (true positive rate) was plotted against the false-positive rate (1 - the value of specificity). Analyses were performed using GraphPad Prism software (version 5; GraphPad Software, San Diego, CA, USA). Comparison of ROC curves was performed with MedCalc software (version 11.1.1, MedCalc Software, Mariakerke, Belgium) using the method described by Delong *et al*. [27]. Data were considered to be statistically significant at $P < 0.05$.

Results

Demographics and initial blood values outcomes

The 47 patients (31 male, 16 female) enrolled in this study had a mean ISS of 32.9 ± 1.7 (range, 16 to 57). The patients' mean age was 45.9 ± 2.9 years (age range, 20 to 96 years). Among all patients, 18 (38.3%) developed sepsis within 6.1 ± 0.3 days (range, 4 to 9 days) after admission. Among the septic patients, nine patients met the criteria for severe sepsis and four patients met the criteria for septic shock. The infection site of sepsis and microbiological pathogens for each patient are given in Table 1. Five patients died posttraumatically after 30.7 ± 12.3 days (range, 16 to 55 days) as a consequence of multiple organ failure (MOF). The mean ICU stay was 18.1 ± 2.6 days (range, 3 to 74 days). The mean age of the 18 patients (3 female, 15 male) who subsequently developed sepsis (sepsis group) was 53.5 ± 4.6 (range, 20 to 78 years). The mean ISS in this patient group was 36.7 ± 2.8 (range, 16 to 50). Further patient characteristics as well as injury severity and outcomes are shown in Table 2.

Levels of sFas and sFasL in patients with or without sepsis after major trauma

Levels of sFas and sFasL were determined in the serum of healthy volunteers (control group) and patients within

Table 1 Infection site of sepsis and microbiological pathogens

Patient	Infection site	Pathogen	Evidence for sepsis, days after trauma
1	Pneumonia	*Klebsiella pneumoniae*	4
2	Pneumonia	*Klebsiella pneumoniae*	5
3	Pneumonia	*Pseudomonas aeruginosa*	8
4	Pneumonia	*Klebsiella pneumoniae, Pseudomonas aeruginosa*	5
5	Pneumonia	*Klebsiella pneumoniae, Enterococcus faecalis*	7
6	Pneumonia	*Escherichia coli*	6
7	Pneumonia	*Morganella morganii*	6
8	Pneumonia	*Haemophilus influenzae*	4
9	Pneumonia	*Klebsiella pneumoniae*	6
10	Peritonitis	*Enterococcus faecalis*	5
11	Pneumonia	*Escherichia coli*	7
12	Pneumonia	*Pseudomonas aeruginosa*	9
13	Pneumonia	*Staphylococcus aureus*	6
14	Pneumonia	*Staphylococcus aureus*	7
15	Pneumonia	*Klebsiella pneumoniae*	7
16	Pneumonia	*Klebsiella pneumoniae*	4
17	Surgical wound infection	*Enterococcus faecalis*	5
18	Pneumonia	*Enterobacter cloacae*	8

24 hours after admission (day 1), at day 5 and at day 9 after major trauma (Figure 1). Patients were divided in two groups: those who subsequently developed sepsis and those with uneventful recovery after major trauma.

Within the first day after admission, sFas values of patients who subsequently developed sepsis, but not the sFas values of those with uneventful outcomes (median, 101.6; interquartile range (IQR), 66.62 to 156.9), were significantly increased (median, 122.5; IQR, 92.84 to 230.7; $P < 0.05$) compared with the healthy control group (median, 70.29; IQR, 42.9 to 93.29) (Figure 1a). Furthermore, sFas levels in these patients remarkably increased within the next days and peaked at day 5 after trauma (median, 230; IQR, 145.2 to 291.2), whereas the values for patients without development of sepsis normalized at this time point (median, 86.46; IQR, 62.95 to 114.2). sFas values in the sepsis group remained enhanced until day 9 after trauma (median, 187.1; IQR, 80.22 to 297.4) compared with values in the nonsepsis group at the same time (median, 68.95; IQR, 52.44 to 128.8). Significant intergroup differences were detectable between patients with sepsis development and healthy volunteers at day 1 ($P < 0.05$), day 5 ($P < 0.001$) and day 9 ($P < 0.01$). Additionally, sFas levels increased significantly in sepsis patients at day 5 ($P < 0.01$) and day 9 ($P < 0.05$) compared with the nonsepsis patients.

In contrast, for both groups (with or without sepsis), sFasL values were on an equivalent level compared with that of healthy controls throughout the entire observation period ($P > 0.05$) (Figure 1b) and did not show any intergroup differences.

Table 2 Demographics, injury severity, and outcome among subsets of patients[a]

Parameter	All patients	Nonsepsis	Sepsis
Number, *n*	47	29	18
Age, yr (±SEM)	45.9 ± 2.9	41.1 ± 4.4	53.5 ± 4.6[b]
ISS (±SEM)	32.9 ± 1.7	30.5 ± 2.0	36.7 ± 2.8[b]
ICU, days (±SEM)	18.1 ± 2.6	13.2 ± 2.9	25.9 ± 4.3[b]
Sepsis, % (*n*)	38.3 (18)	0 (0)	100 (18)
Death, % (*n*)	10.6 (5)	0 (0)	27.8 (5)
Max SOFA day 1	9.2 ± 0.6	8.4 ± 0.9	10.6 ± 0.5
Max SOFA day 5	6.2 ± 0.6	4.4 ± 0.8	9.1 ± 0.7[b]
Max SOFA day 9	4 ± 0.6	2.0 ± 0.6	7.1 ± 1.0[b]

[a]ISS, injury severity score; ICU, intensive care unit length of stay; Max SOFA, maximal Sequential Organ Failure Assessment score; [b]$P < 0.05$ between sepsis and nonsepsis groups.

Prevention of neutrophil apoptosis by recombinant and serum sFas

It is well established that sFas may bind to membrane-bound FasL, thus blocking binding of the ligand to the Fas receptor and preventing apoptosis induction in the target cell. Therefore, we assumed that elevated serum levels of sFas may inhibit apoptosis in circulating neutrophils and promote prolonged cellular activity. Neutrophil apoptosis in both patient groups, those who developed sepsis subsequently and those with an uneventful recovery, was significantly reduced within the first day after trauma and continued to be reduced for the entire period until day 9 after trauma. The sepsis patients had a lower rate of neutrophil apoptosis at day 5 (median, 0.94; IQR, 0.6 to 1.67; vs. median, 2.08; IQR 0.64 to 3.36; in the nonsepsis group) and day 9 (median,

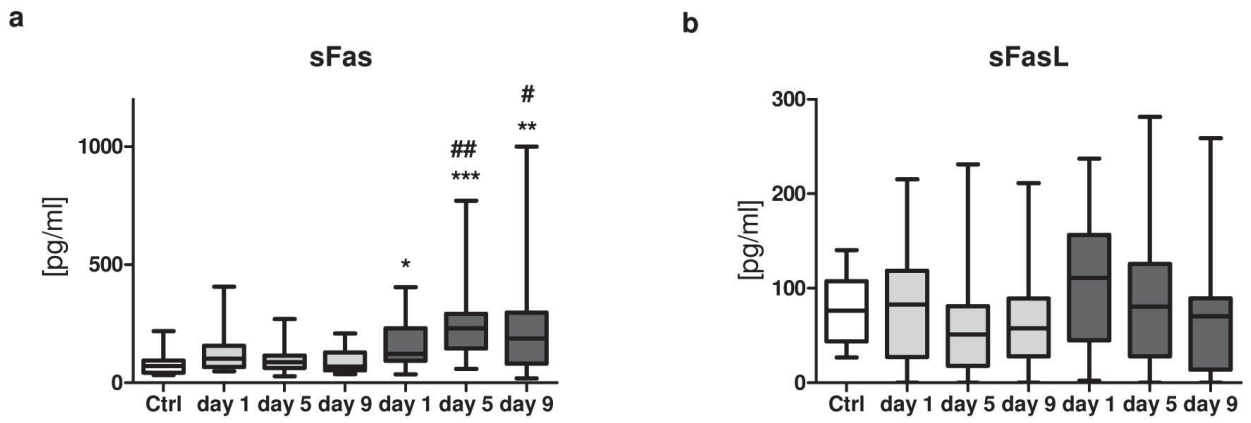


Figure 1 Kinetics of serum soluble Fas (sFas) and soluble Fas ligand (sFasL) after major trauma. **(a)** sFas levels in patients who developed sepsis during the first 10 days after trauma (n = 18, dark gray boxes) are significantly elevated when compared to the values determined in patients with uneventful recovery (n = 29, light gray boxes) and healthy volunteers (n = 17, white box). **(b)** No alterations in sFasL levels were observed between different groups. The horizontal line across the boxplots represents the median, and the lower and upper ends of the boxplots are the 25th and 75th percentiles, respectively. Whiskers indicate the minimum and maximum values, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 vs. nonsepsis group.

0.38; IQR, 0.28 to 0.94; vs. median, 0.47; IQR 0.26 to 2.5; in the nonsepsis group), although this difference did not reach the level of significance (Figure 2a).

We therefore speculated that sFas prevents the activation of Fas on trauma neutrophils, leading to strong inhibition of neutrophil extrinsic apoptosis in sepsis. To prove this hypothesis of sFas-mediated apoptosis inhibition, neutrophils from healthy donors were incubated with an agonistic anti-Fas antibody (CH-11) in the presence of serial dilutions of recombinant human sFas, which has been shown to inhibit FasL-induced apoptosis of Jurkat cells [13]. As depicted in Figure 2b, we found that sFas blocks apoptosis in a concentration-dependent manner.

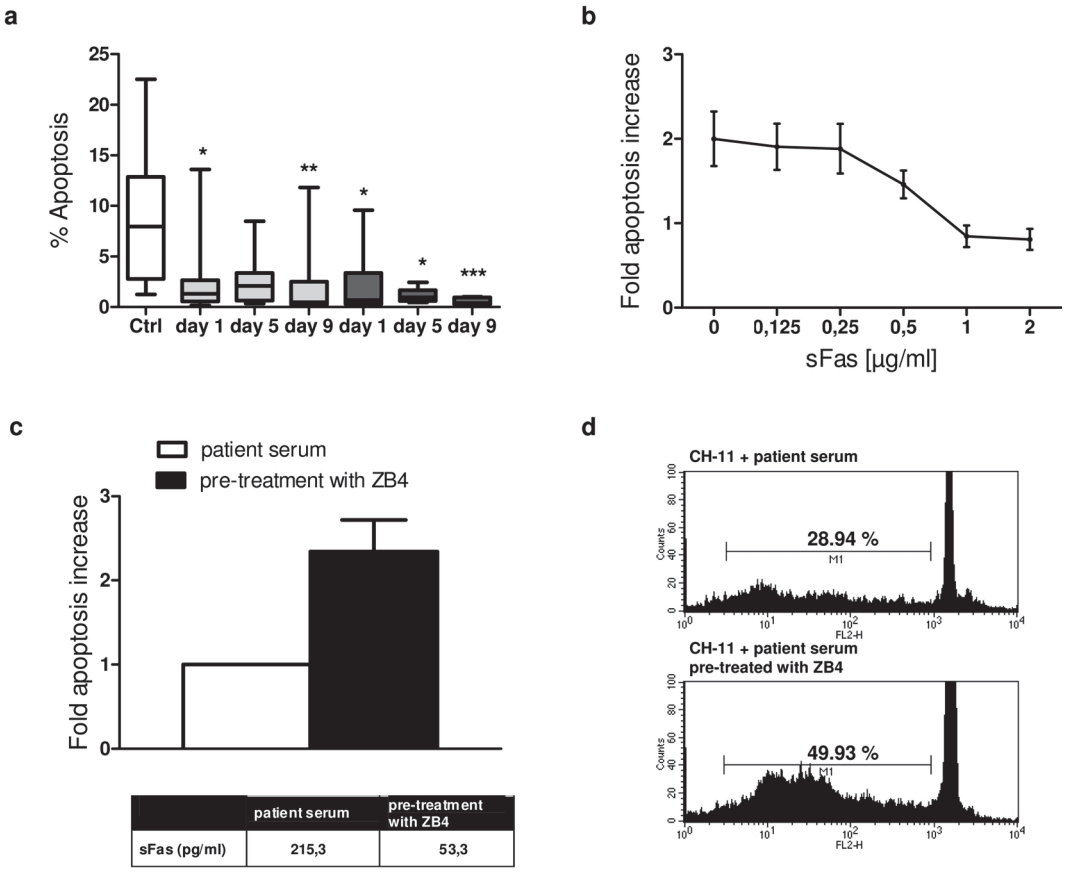
We further investigated whether sFas in the sera of patients with sepsis development might also inhibit CH-11-mediated neutrophil apoptosis. Patient serum contains a broad range of cytokines, especially high levels of granulocyte macrophage colony-stimulating factor (GM-CSF), which is known to reduce the neutrophil apoptosis rate during inflammation by inhibiting the intrinsic apoptosis pathway [28]. Because serum containing high or moderate levels of sFas might also differ in the concentrations of the cytokines mentioned above, we pooled sera from four sepsis patients before immunoprecipitation of sFas by anti-Fas antibodies (ZB4). Then sera were further used to block the proapoptotic activity of CH-11 monoclonal antibodies. As depicted in Figures 2c and 2d, neutrophils incubated with agonistic CH-11 antibodies and sera from sepsis patients immunoprecipitated with ZB4 (low sFas levels) displayed a twofold increased apoptosis rate when compared with cells cultured in the presence of CH-11 antibodies and pooled serum samples (control; high sFas levels).

Increased levels of PMNE in patients with development of posttraumatic sepsis

As shown in Figure 3a, leukocyte counts were found to be significantly increased in septic patients at day 9 after trauma (median, 12.7; IQR, 9.4 to 17.75) compared with the number of leukocytes determined in the nonsepsis group at day 1 (median, 7.7; IQR 6.05 to 9.85; P < 0.01) and day 5 (median, 7; IQR, 6.3 to 10.7; P < 0.05). Neutrophil degranulation was further examined by assessing the levels of PMNE in patients' plasma (Figure 3b). PMNE showed peak levels at day 5 in patients who developed sepsis (median, 301.4; IQR, 217.5 to 474) compared with controls (median, 165.9; IQR, 123.1 to 184.4) and in patients with uneventful recovery (median, 162.8; IQR, 111.4 to 268.9; P < 0.05). Interestingly, PMNE values as well as leukocyte counts were found to correlate with serum sFas concentrations in the sepsis group at day 5 after trauma (r = 0.49; P < 0.05 for both).

Relation of serum sFas levels with IL-6, SOFA and MOD scores and its prognostic value in septic patients

IL-6 is a widely accepted inflammatory parameter in response to major trauma and sepsis. Therefore, IL-6 values in patient serum were determined and correlated to the sFas values. As depicted in Figure 3c, IL-6 values of both groups were elevated at day 1 compared with control values, but decreased simultaneously on the following days. Differences were significant at day 5 and day 9 between the sepsis group (day 5: median, 191.7; IQR, 57.37 to 282.2; day 9: median, 54.94; IQR 29.51 to 191.4) and the nonsepsis group (day 5: median, 41.82; range, 22.74 to 69.43; P < 0.05; day 9: median, 19; IQR, 4.11 to 26.75; P < 0.05). In all patients, IL-6 showed a



	patient serum	pre-treatment with ZB4
sFas (pg/ml)	215,3	53,3

Figure 2 Inhibition of neutrophil extrinsic apoptosis by sFas. **(a)** Reduced percentage of apoptotic neutrophils isolated from healthy controls (n = 15, white box), sepsis patients (n = 7, dark gray boxes) and nonsepsis patients (n = 13, light gray boxes) at day 1, day 5 and day 9 after trauma. Boxplots represent the median (heavy line in boxes) and the 25th and 75th percentiles (lower and upper lines of the box, respectively). Whiskers indicate the minimum and maximum values, respectively. *P < 0.05, **P <0.01, ***P < 0.001 vs. control group. **(b)** Neutrophils from healthy controls were incubated with 50 ng/mL anti-Fas antibody (CH-11) in the presence of serial dilutions of recombinant human soluble Fas (sFas) (range, 0 to 2 µg/mL) for 18 hours. Thereafter cells were lysed in hypotonic solution containing propidium iodide, and the percentage of apoptotic cells was determined by flow cytometry. Data (means ± SEM) from three independent experiments are presented. **(c)** Control neutrophils were incubated with 200 ng/mL agonistic anti-Fas antibodies (clone CH-11) and pooled serum from four sepsis patients immunoprecipitated by anti-Fas antibodies (clone ZB4) or not. After 18 hours of culture, apoptotic neutrophils with hypodiploid DNA content were quantified by propidium iodide staining and flow cytometry. Data (means ± SEM) from six independent experiments are depicted. **(d)** Representative histogram of CH-11-induced apoptosis in the presence of patient serum. Region M1 describes the percentage of hypodiploid DNA.

positive correlation with SOFA score at all time points as well as with the MOD score at days 5 and 9 (Table 3). Furthermore, a strong correlation was determined between IL-6 and sFas at day 5 (r = 0.42; P < 0.01) and day 9 (r = 0.4; P < 0.05), but not at day 1 after major trauma. No correlation between sFas and IL-6 values was found in patients with sepsis development (sepsis group).

To investigate the predictive potential of sFas for the development of sepsis after major trauma, sFas values were additionally correlated to SOFA and MOD scores (Table 3). Elevated sFas concentrations determined in patients with sepsis development after severe trauma strongly correlated with patients' SOFA scores from day 1 until day 9 after trauma. In this patient cohort, sFas values at day 5 were also significantly correlated to the MOD score and were positively associated with the development of multiple organ dysfunction (Table 3). However, sFas did not correlate with SOFA and MOD scores of patients with uneventful recovery.

ROC curves

To verify the prognostic potential of sFas in relation to the established prognostic marker IL-6 for sepsis development after major trauma, we established ROC curves for both parameters at each time point. Figure 4 shows ROC curves of sFas and IL-6 at day 1 and day 5 after

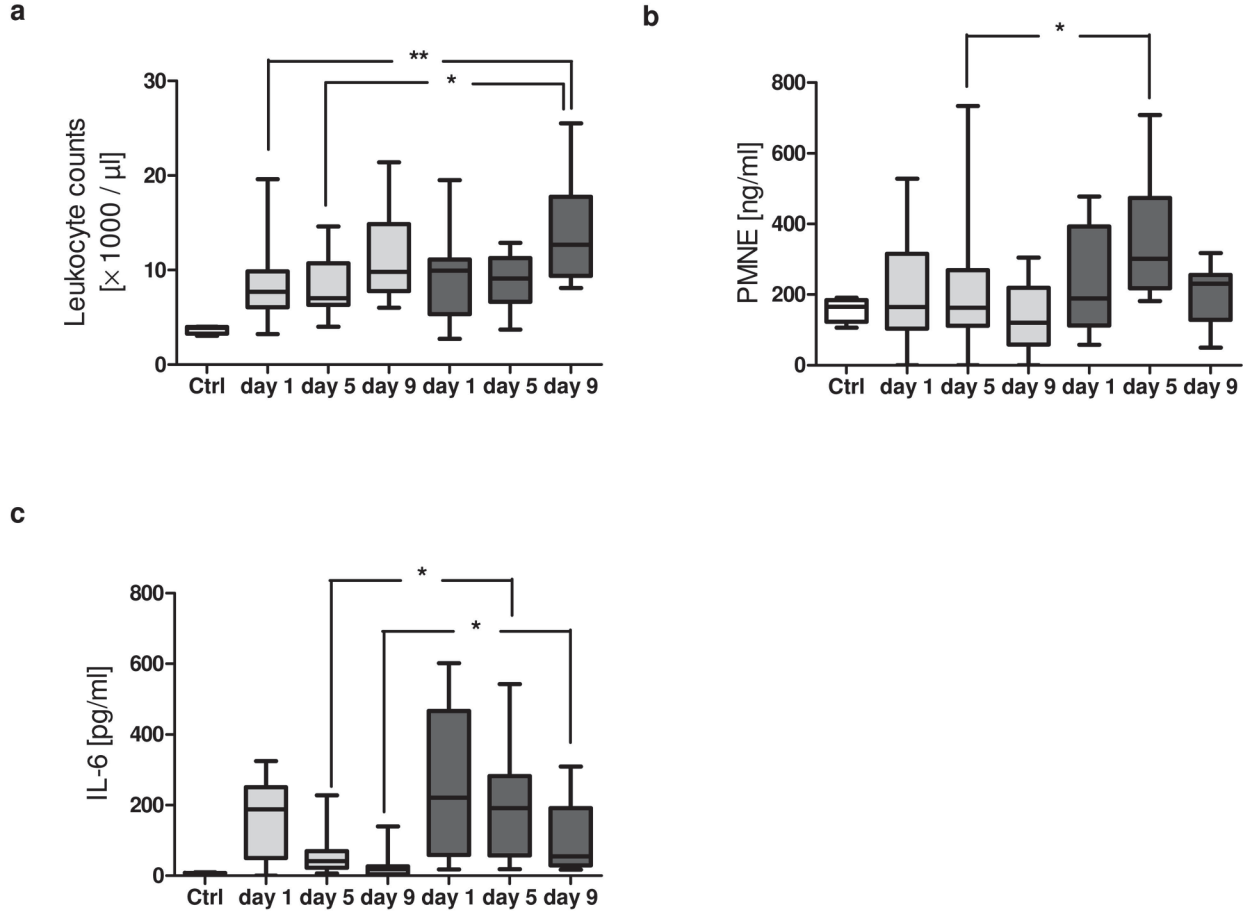


Figure 3 Leukocyte count, plasma levels of neutrophil elastase (PMNE) and IL-6 levels after major trauma. **(a)** Total leukocyte counts from controls (n = 4, white box), patients with sepsis development (n = 16, dark gray boxes) and patients with uneventful outcome (n = 29, light gray boxes). **(b)** PMNE in controls (n = 6, white box), the sepsis group (n = 16, dark gray boxes) and the nonsepsis group (n = 28, light gray boxes). **(c)** Serum IL-6 levels in trauma patients with sepsis (n = 18, dark gray boxes) and without sepsis (n = 27, light gray boxes). Boxplots represent the median (heavy line in boxes) and the 25th and 75th percentiles (lower and upper lines of the box, respectively). Whiskers indicate the minimum and maximum values, respectively. *P < 0.05, **P < 0.01.

Table 3 Correlations of sFas and IL-6 levels with the organ dysfunction scoring systems[a]

	SOFA			MODS		
Protein	**Day 1**	**Day 5**	**Day 9**	**Day 1**	**Day 5**	**Day 9**
sFas						
Nonsepsis	0.38^{NS}	0.25^{NS}	0.42^{NS}	0.35^{NS}	0.25^{NS}	0.29^{NS}
Sepsis	0.7^{d}	0.62^{c}	0.58^{c}	0.18^{NS}	0.56^{c}	0.13^{NS}
All	0.54^{d}	0.56^{d}	0.61^{d}	0.28^{NS}	0.44^{c}	0.37^{b}
IL-6						
Nonsepsis	0.55^{b}	0.57^{c}	0.21^{NS}	0.08^{NS}	0.15^{NS}	0.09^{NS}
Sepsis	0.46^{NS}	0.33^{NS}	0.59^{c}	0.35^{NS}	0.25^{NS}	0.71^{c}
All	0.35^{b}	0.54^{d}	0.57^{d}	0.2^{NS}	0.37^{b}	0.43^{c}

[a]SOFA, Sequential Organ Failure Assessment score; MODS, Multiple Organ Dysfunction Score; sFas, soluble Fas; IL-6, interleukin-6; *NS*, not significant; [b]P < 0.05; [c]P < 0.01; [d]P < 0.001.

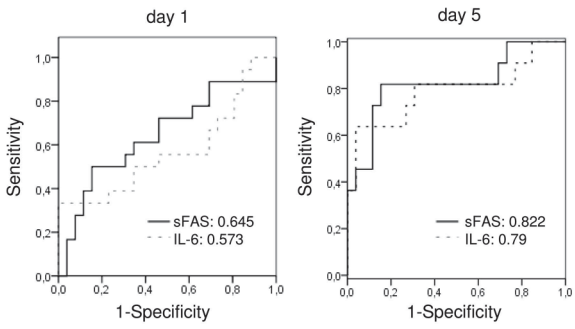


Figure 4 Receiver operating characteristic (ROC) curves using sFas and IL-6 as predictors of sepsis. The area under the curve (AUC) is given for each graph. On day 5, the seven patients who already had sepsis were excluded from the ROC curve analysis.

trauma. Pairwise comparison of the ROC curves displayed no statistical difference between the area under the curve (AUC) for the sFas and IL-6 values at the depicted time points (day 1, $P = 0.694$; day 5, $P = 0.911$).

Discussion

In this study, we have demonstrated that sFas, which has been found to be significantly elevated in the sera of trauma patients who subsequently developed sepsis, inhibits the activation of the Fas pathway and thus extrinsic apoptosis induction in neutrophils.

Neutrophil apoptosis is regulated by the expression of pro- and antiapoptotic factors and might be initiated by the activation of TNF family receptors such as Fas by naturally occurring ligands such as FasL. Many proinflammatory cytokines such as GM-CSF, IL-8 and IL-6 are known to prolong neutrophil survival [29]. Recent studies have shown that proinflammatory mediators activate both the extracellular signal-regulated kinase and phosphatidylinositol 3-kinase pathways [30,31] and might trigger upregulation of antiapoptotic factors such as Mcl-1 [32], thus promoting intrinsic apoptosis resistance in neutrophils [28].

The Fas/FasL system plays a key role in maintaining the homeostasis of the immune system. It is widely accepted that sFas can protect cells against Fas-mediated apoptosis by binding to FasL, thereby functionally antagonizing the Fas-FasL pathway [13]. Evidence has been reported for a relation between elevated sFas levels and severe illness [33-37], such as sepsis [36], malignant disease [37], autoimmune diseases [13] or acute respiratory distress syndrome [38], or after major surgery [39]. It has been suggested that sFas decreases neutrophil apoptosis in patients postoperatively [39].

In the present study, the sFas levels in patients who developed sepsis were found to be significantly elevated at day 1, day 5 and day 9 after major trauma compared with levels determined in the sera of healthy donors and at day 5 and day 9 compared with patients with uneventful recovery. Our *in vitro* experiments with recombinant sFas and sera from septic patients demonstrate the abrogation of CH-11-induced neutrophil apoptosis. We have clearly shown by immunoprecipitation that the antiapoptotic effects of patient serum were largely mediated by sFas. We therefore postulate that the antiapoptotic activity of sFas in combination with the previously reported impaired intrinsic apoptosis pathway in neutrophils after trauma might be an important factor in the ongoing inflammatory injury and progressive organ dysfunction seen in sepsis patients [40,41].

Indeed, serum sFas concentrations showed a strong positive correlation with SOFA and MOD scores, especially in those patients who developed sepsis. Additionally, sFas values in patients with septic shock tended to be higher at day 5 and day 9 after trauma compared with the sFas levels in patients suffering from sepsis and severe sepsis (data not shown). Thus, our data demonstrate that sFas levels correlate with patient prognosis and might be used as an additional prognostic sepsis marker already at day 1 after trauma when sepsis is clinically not apparent. Moreover, as an interesting new aspect, we found sFas levels in patients with sepsis development to be persistently increased even at day 5 and day 9 after trauma, thus showing an association with the reduced neutrophil apoptosis found at these time points. Surprisingly, no significant differences in peripheral circulating leukocyte numbers between both patients groups could be found. This finding might be explained by the fact that activated neutrophils become rapidly recruited to the injured tissue and thus cannot be further detected in the peripheral circulation.

These data show for the first time the role of sFas as a predictor for sepsis and the potential link to neutrophil activity and the pathophysiology of major trauma. However, the ISSs of the patients in our series ranged between 16 and 57. This heterogeneity between patients in terms of injury severity as well as the small number of patients included may present potential limitations of the current study.

In contrast to the work of Papathanassoglou *et al*. [33], here sFas strongly correlated with IL-6 levels in serum from trauma patients, except for day 1. Nevertheless, no association was found between IL-6 and sFas in patients with sepsis development. IL-6 levels did not specifically correlate with SOFA and MOD scores of the sepsis group, pointing to sFas as a marker for sepsis and clinical outcome.

The highest sFas serum concentrations as well as the best correlation with leukocyte counts, PMNE, IL-6 and MODS were found at day 5 after severe trauma. Interestingly, at this time point, sepsis frequently develops clinically [8]. Because it is known that sFas may also influence the adaptive T cell-mediated immunity [42,43], it may be speculated that sFas might contribute to T cell anergy and sepsis.

In this study, reduced neutrophil apoptosis has also been observed in patients who did not develop sepsis. This finding indicates that sFas-mediated effects on neutrophils contribute to the development of organ dysfunction due to prolonged neutrophil hyperactivity, but not directly to the development of sepsis. Moreover, it is likely that sFas might additionally promote a phenotypical and functional change in neutrophils, resulting in an indirect inhibition of T cell function, which is widely accepted to be associated with sepsis development [44,45]. In this context, impairment of T cell proliferation by soluble CD83 molecules, neutrophil-derived

arginase and ROS has been reported [46-48]. Nevertheless, the relationship between neutrophil hyperactivity and the extensive lymphocyte apoptosis seen in sepsis-related immunosuppression is currently incompletely understood and should be elucidated in future studies.

Conclusions

In summary, the present study demonstrates for the first time a role of serum sFas in the inhibition of neutrophil extrinsic apoptosis associated with increased levels of PMNE, a marker for systemic inflammation. Our results show a high correlation between sFas and patients' SOFA and MOD scores in sepsis and thus provide evidence for the clinical significance of the risk for the development of sepsis and MOF. Thus, sFas may represent a feasible target for new therapeutic strategies to limit neutrophil life span and hyperactivity.

Key messages

- Serum sFas levels have been shown to be significantly elevated in patients with sepsis development after major trauma compared with patients with uneventful recovery and healthy controls.
- Fas-mediated neutrophil apoptosis was efficiently inhibited by serum sFas from sepsis patients. Elevated sFas levels were associated with increased levels of PMNE, a marker for neutrophil activity.
- sFas showed a positive correlation with SOFA and MOD scores and sepsis development in severely injured patients.
- sFas may represent a feasible target for new therapeutic strategies to prevent neutrophil hyperactivity and sepsis.

Abbreviations

AIS: Abbreviated Injury Scale; ARDS: acute respiratory distress syndrome; AUC: area under curve; ERK: extracellular signal-regulated kinase; FasL: Fas ligand; FCS: fetal calf serum; GM-CSF: granulocyte macrophage colony-stimulating factor; ICU: intensive care unit; IL: interleukin; IQR: interquartile range; ISS: Injury Severity Score; MOD(S): Multiple Organ Dysfunction (Score); MOF: multiple organ failure; PI3K: phosphatidylinositol 3-kinase; PBS: phosphate-buffered saline; PMNE: neutrophil elastase; ROC: receiver operating characteristics; ROS: reactive oxygen species; SEM: standard error of the mean; sFas: soluble Fas; sFasL: soluble Fas ligand; SIRS: systemic inflammatory response syndrome; SOFA: Sequential Organ Failure Assessment; TNF: tumor necrosis factor.

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Authors' contributions

AP-G and SF conceived the study, analysed and interpreted data and drafted the manuscript. Experimental work was performed by AP-G. TL contributed to the acquisition and analysis of patient data as well as to the writing of the manuscript. MS and JW critically revised the manuscript for intellectual content and gave important advice. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Molecular Mechanisms Underlying Delayed Apoptosis in Neutrophils from Multiple Trauma Patients with and without Sepsis

Adnana Paunel-Görgülü, Tamara Kirichevska, Tim Lögters, Joachim Windolf, and Sascha Flohé

Department of Trauma and Hand Surgery, University Hospital Düsseldorf, Düsseldorf, Germany

Delayed neutrophil apoptosis and overshooting neutrophil activity contribute to organ dysfunction and subsequent organ failure in sepsis. Here, we investigated apoptotic signaling pathways that are involved in the inhibition of spontaneous apoptosis in neutrophils isolated from major trauma patients with uneventful outcome as well as in those with sepsis development. DNA fragmentation in peripheral blood neutrophils showed an inverse correlation with the organ dysfunction at d 10 after trauma in all patients, supporting the important role of neutrophil apoptosis regulation for patient's outcome. The expression of the antiapoptotic Bcl-2 protein members A1 and Mcl-1 were found to be diminished in the septic patients at d 5 and d 10 after trauma. This decrease was also linked to an impaired intrinsic apoptosis resistance, which has been previously shown to occur in neutrophils during systemic inflammation. In patients with sepsis development, delayed neutrophil apoptosis was found to be associated with a disturbed extrinsic pathway, as demonstrated by reduced caspase-8 activity and Bid truncation. Notably, the expression of Dad1 protein, which is involved in protein N-glycosylation, was significantly increased in septic patients at d 10 after trauma. Taken together, our data demonstrate that neutrophil apoptosis is regulated by both the intrinsic and extrinsic pathway, depending on patient's outcome. These findings might provide a molecular basis for new strategies targeting cell death pathways in apoptosis-resistant neutrophils during systemic inflammation.

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INTRODUCTION

Sepsis is a leading cause of death in intensive care unit patients, resulting in more than 200,000 deaths annually (1). Clinical and experimental observations have suggested that apoptosis may play a role in the pathogenesis of sepsis-associated multiple organ dysfunction syndrome (MODS) (2–5), but the mechanism by which MODS develops remains unclear. Neutrophils are terminally differentiated cells of the innate immune system playing an important role as a first line of defense against bacterial infections as well as in the modulation of the inflammatory response (6). Paradoxically, neutrophils also represent one of the main mediators of tissue injury in various human diseases, including sepsis (7,8). Under normal conditions neutrophil half-life in the circulation is limited to ~6–10 h, after which they undergo spontaneous apoptosis. However, during acute inflammation, neutrophil life span becomes significantly extended owing to the action of proinflammatory mediators and bacterial membrane components such as endotoxin (9). This prolonged neutrophil survival is associated with the accumulation of activated neutrophils contributing to an ongoing inflammation, and consecutive host tissue damage, with subsequent organ failure in critical ill patients. Profound inhibition of apoptosis has been already reported in neutrophils from patients with systemic inflammatory response syndrome (SIRS) (2,10), in sepsis (11–14), as well as after burn injuries (15) and acute respiratory distress syndrome (8,16,17). These observations suggest that the resolution of the inflammatory response is highly dependent from the induction of neutrophil programmed cell death.

During neutrophil spontaneous apoptosis both, mitochondria- and death receptor–mediated apoptotic signaling were shown to be activated (18). The intrinsic apoptotic pathway involves mitochondria, which release cytochrome c into the cytoplasm following outer membrane permeabilization by proapoptotic Bcl-2 family members. Cytochrome c then combines with caspase-9 and Apaf-1 to form the apoptosome, resulting in caspase-3 activation and apoptosis (19).

Address correspondence to *Tim Lögters, Department of Trauma and Hand Surgery, University Hospital Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany. Phone: +49-211-81 04411; Fax: +49-211-81 04902; E-mail: Tim.Loegters@med.uni-duesseldorf.de.*
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The Feinstein Institute for Medical Research North Shore LIJ

Human neutrophils express the proapoptotic proteins Bax, Bad, Bid, Bik and Bak and the antiapoptotic proteins Mcl-1, A1/Bfl-1 (A1) and Bcl-X_L (20). Mcl-1 and A1 have very short half-lives of 2-3 h whereas the half-lives of the proapoptotic proteins are relatively long (21). The cellular levels of Mcl-1 protein have been shown to closely correlate with neutrophil survival. Hence, neutrophil half-life may be predominantly governed by the cellular levels of the antiapoptotic factors (22,23).

In the extrinsic pathway, stimulation of death receptors such as Fas, TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 and TNF receptor-1 and -2 by their ligands, for example, FasL, TNF-α or TRAIL, allows the trimerization/reorganization of the death receptor on the cell surface and subsequent caspase-8 activation (24). Both, the extrinsic and intrinsic pathway may converge at the level of mitochondria.

Currently, the molecular mechanisms that underlie the delay of neutrophil apoptosis during acute inflammation and sepsis are not fully understood. Recently, we found evidence for an impaired extrinsic pathway in patients with posttraumatic sepsis development due to elevated serum levels of soluble Fas (sFas). Serum sFas has been also shown to inhibit neutrophil apoptosis *in vitro*. However, in this study, both trauma patients with and without sepsis development showed strong reduction of apoptosis in circulating neutrophils at least until d 10 after trauma (25).

Based on these previous findings, here we sought to characterize the impact of extrinsic and intrinsic pathways on the regulation of neutrophil apoptosis in patients with trauma-associated sepsis development, as well as in patients with uneventful outcome.

MATERIALS AND METHODS

Patients

Twenty-four patients were enrolled in this prospective study. Study approval was obtained from the Ethics Review Board of the University of Duesseldorf, Germany. Patients with blunt or penetrating multiple injuries who were admitted to our Level I Trauma Center with an Injury Severity Score (ISS) ≥16, intensive care unit (ICU) stay >3 d and aged 18 years or older were enrolled in this study. Written informed consent was obtained from all participants or their legal representatives if the patients lacked consciousness. Exclusion criteria were death of the patient on day of admission or within the first 2 d on ICU. In addition, patients with known preexisting immunological disorders or systemic immunosuppressive medication were excluded. The severity of injury was assessed by the ISS, based on the Abbreviated Injury Scale (AIS) (26). SIRS and sepsis were defined using the criteria outlined 2005 from the International Sepsis Forum (27). Patients were determined as septic if they fulfilled criteria for SIRS and had a proven source of infection. SIRS was defined by two or more of the following criteria: temperature >38°C or <36°C; heart rate >90 beats per min; respiratory rate >20 breaths per min or arterial carbon dioxide tension ($PaCO_2$) <32 mmHg; and white blood cell count >12,000 cells/mm^3 or <4000 cells/mm^3, or with >10% immature (band) forms. To evaluate organ dysfunction/failure, the Sequential Organ Failure Assessment (SOFA) and Multiple Organ Dysfunction (MOD) score were determined prospectively on a daily basis (28). Severe sepsis referred to sepsis complicated by organ dysfunction. Organ dysfunction has been defined using the definition by the SOFA score with >2 points for at least one system (respiratory, coagulation, liver, cardiovascular, central nervous or renal system). Septic shock was defined as sepsis with acute persistent circulatory failure unexplained by other causes (>2 points in SOFA score for cardiovascular system).

Blood was collected from healthy volunteers and routinely from patients from the day of admission until d 10. Heparinized blood was immediately used after collection for neutrophil isolation. In parallel, sera were harvested by centrifugation and stored at –80°C until further processing.

Isolation and Culture Conditions of Human Neutrophils

Human neutrophils were isolated by discontinuous density-gradient centrifugation on Percoll (Biochrom) as previously described (29). After hypotonic lysis to remove contaminating erythrocytes, cells were suspended in phosphate-buffered saline (PBS). Purity and viability were routinely >95% as assessed by forward and side scatter characteristics of FACScan (BD Biosciences) and trypan blue exclusion, respectively. Subsequently, cells were frozen at –80°C or suspended in RPMI 1640 containing 2 mmol/L glutamine (Biochrom) and 1% human serum, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

RNA Interference and Transfection

For siRNA experiments, freshly isolated neutrophils from patients at d 1 after admission were nucleofected with mcl-1 small interfering RNA (siRNA) (Qiagen) using Amaxa Nucleofector System (Lonza), according to the manufacturer's instructions (Human Monocyte Nucleofector Kit, Lonza) with some modifications. In brief, 5×10^6 neutrophils were suspended in 100 μL Nucleofector solution (Lonza) with 1.5 μg siRNA. The cells were electroporated and further cultured in RPMI 1640 medium containing 2 mmol/L glutamine (Biochrom) and 10% FCS (PAA Laboratories). Alexa488-conjugated control siRNA (Qiagen), used to detect intracellular siRNA by flow cytometry and to monitor transfection efficiency, showed that the fluorescent siRNA was taken up in >90% of the cells. After 24-h culture, 0.2 μmol/L staurosporine (Alexis) was added to the cells.

Analysis of mRNA Expression by RT-PCR and Quantitative PCR

Total RNA from neutrophils was extracted using TRI Reagent (Sigma) accord-

ing to the manufacturer's instructions. Contaminating DNA was removed by digestion with DNase (DNA-*free*, Ambion). 500 ng RNA were reverse transcribed to complementary DNA (cDNA) using oligo(dT)$_{15}$ primer, random primer, and Omniscript Reverse Transcriptase (Qiagen). Gene-specific primer pairs were designed by using the Primer Express® software (version 3.0; Applied Biosystems): *Mcl-1* forward: 5′-CAAGG CATGC TTCGG AAACT-3′; *Mcl-1* reverse: 5′-GATCA TCACT CGAGA CAACG ATTT-3′; *A1* forward: 5′-CTCAG CACAT TGCCT CAACA G-3′; *A1* reverse: 5′-GCCTG GTGGA GAGCA AAGTC-3′; *Bax* forward: 5′-TGGAG CTGCA GAGGA TGATT G-3′; *Bax* reverse: 5′-GAAGT TGCCG TCAGA AAACA T-3′; *Dad1* forward: 5′-GGCGT CGGTA GTGTC TGTCA-3′; *Dad1* reverse: 5′-CTGCG GAGTG GAGCT CAAG-3′; *18S* forward: 5′-CATGG TGACC ACGGG TGAC-3′; *18S* reverse: 5′-TTCCT TGGAT GTGGT AGCCG-3′. Relative gene expression levels were determined using SYBR Green (Applied Biosystems) incorporation following the manufacturer's recommended protocol with the following thermal cycling conditions: 95°C, 10 min (1 cycle); 95°C, 15 s, 60°C, 60 s (40 cycles); 4°C hold. All samples were run in triplicates (ABI Prism 7300, Applied Biosystems). Expression of each target gene was normalized to the *18S* RNA gene. Fold expression was calculated using the $2^{-\Delta\Delta C_T}$ method (30).

Human Apoptosis Quantitative Real-Time Polymerase Chain Reaction Array

To analyze the expression of apoptosis-related genes in neutrophils after major trauma, the Human Apoptosis 96 StellARay quantitative real-time polymerase chain (qPCR) array has been used according to the manufacturer's instructions (Lonza). Data were analyzed using Global Pattern Recognition (GPR) Analysis software (Bar Harbor Biotechnology), which normalizes the data of each analyzed gene to that of every other gene without dependence of single gene normalization (31).

Western Blotting

Isolated neutrophils were suspended in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfide), supplemented with complete protease inhibitor mixture (Roche) followed by cell sonication. Samples were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BioRad). Membranes were saturated in Tris-buffered saline supplemented with 0.1% Tween20 (TBST) and 5% w/v nonfat dry milk for 1 h and then immunolabeled with monoclonal anti-human Mcl-1 (BD Biosciences), polyclonal anti-human Bid and anti-human A1/Bfl-1 (both Cell Signaling), or anti-human Dad1 antibody (Imgenex), respectively. Blots were then washed three times with Tris-buffered saline + 0.1% Tween 20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako) for 1 h at room temperature. Bands were visualized using SuperSignal West Pico detection kit (Thermo Fisher Scientific). Equal loading was confirmed by PonceauS staining and reincubation of membranes with a monoclonal antibody for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Imgenex).

Quantification of Neutrophil Apoptosis

Neutrophil apoptosis was measured by using the Cell Death Detection Enzyme-Linked Immunosorbent Assay (ELISA) Plus Kit (Roche), which quantitatively detects mono- and oligonucleosomes in the cytoplasmatic fraction. Briefly, neutrophil pellets were suspended in lysis buffer and DNA fragments were detected according to the manufacturer's instruction. Apoptosis was normalized to total double-stranded (ds) DNA, which has been quantified by PicoGreen (Invitrogen) staining. In addition, neutrophil apoptosis was measured by propidium iodide staining and flow cytometry as previously described (10). Nuclei to the left of the G1 peak (sub-G1) containing hypodiploid DNA, corresponding to fragmented DNA, were considered apoptotic.

Detection of Mitochondrial Membrane Depolarization

To measure the mitochondrial membrane potential, neutrophils were stained with the lipophilic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Sigma Aldrich, Taufkirchen, Germany) as previously described (10). Then, cells immediately analyzed by flow cytometry. The excitation wavelength was 488 nm and the emission wavelengths 530 nm (FL1) for green fluorescence and 590 nm (FL2) for red fluorescence. In intact cells with a high negative mitochondrial membrane potential, JC-1 forms aggregates that emit a red fluorescence (590 nm). In mitochondria with low membrane potential (depolarized), the dye forms monomers in the cytosol that emit a green fluorescence (530 nm). Results are expressed as the relative number of cells with high levels of green fluorescence (FL1), indicating mitochondrial membrane depolarization in these cells.

Determination of Caspase-8 Activity

Freshly isolated neutrophils were harvested by centrifugation and cell pellets were stored by –80°C for further processing. The activity of caspase-8 has been measured by using Caspase-Glo 8 Assay (Promega) according to manufacturer's instructions.

Statistical Analyses

Data are presented as box plots representing the median (heavy line in boxes) and the 25th and 75th percentiles. Whiskers indicate the minimum and maximum values, respectively. To evaluate differences between several groups, a nonparametric Kruskal-Wallis test with Dunn *post hoc* test was performed. To compare differences between two groups, the Mann-Whitney *U* test has been used. For normally distributed data, the mean ± SEM is depicted. Statistical analysis was performed by using

one-way analysis of variance (ANOVA), followed by the Newman-Keuls test. Correlation between numerical values was evaluated by Spearman rank correlation coefficient (ρ). Analyses were performed with GraphPad Prism Program (version 5, GraphPad Software, San Diego, CA, USA). Data were considered to be statistically significant at $P < 0.05$.

RESULTS

Demographics, Initial Blood Values Outcome

The 24 patients (15 male, 9 female) enrolled in this study had an ISS of 46.7 ± 3.1 (mean ± SEM, range 16–75). The mean age was 41.7 ± 3.8 years (range 19–78 years). All patients fulfilled at least two of criteria of SIRS on admission to the ICU and on d 1 after trauma. From all patients, 12 developed sepsis within 6.1 ± 0.4 d (range 4–8 d) after trauma. Among the septic patients, one patient had uncomplicated sepsis, seven patients met the criteria for severe sepsis and four patients met the criteria for septic shock. The infection site of sepsis and microbiological pathogens for each patient are given in Table 1. The most frequent focus for sepsis was a pulmonary infection. Patients with posttraumatic sepsis development were apparently older and more severely injured than those without sepsis. Further, septic patients had a longer ICU stay as well as higher SOFA and MOD scores. Three septic patients died 30.7 ± 12.3 d (range 16–55 d) after trauma as a consequence of multiple organ failure. Further patient characteristics as well as injury severity, SOFA as well as MOD score values and outcome are shown in Table 2.

Table 1. Infection site of sepsis and microbiological pathogens.

Subject	Infection site	Pathogen	Evidence for sepsis, d
1	Pneumoniae	*Staphylococcus aureus*	5
2	Pneumoniae	*Escherichia coli*	7
3	Pneumoniae	*Klebsiella pneumoniae*	5
4	Peritonitis	*Enteroccocus faecium*	8
5	Pneumoniae	*Enterobacter cloacae*	4
6	Pneumoniae	*Escherichia coli*	7
7	Pneumoniae	*Staphylococcus aureus*	6
8	Pneumoniae	*Staphylococcus aureus*	7
9	Pneumoniae	*Escherichia coli*	7
10	Pneumoniae	*Klebsiella pneumoniae*	4
11	Pneumoniae	n.d.	8
12	Soft tissue	*Enterococcus faecalis*	5

n.d., Not detected; d, d after trauma.

Expression Profile of Apoptosis-Related Genes and Apoptosis Rate in Neutrophils from Severely Injured Patients

To identify apoptosis-relevant genes that are modified in their expression in neutrophils after major trauma, we screened the regulation of 94 genes in neutrophils isolated from multiple trauma patients. The cDNA from five healthy volunteers was pooled and used as control. Further, pooled cDNA from six patients, three with sepsis development during the first 10 d after trauma and three with uneventful recovery, was used to analyze changes in the expression of apoptosis-relevant genes at d 1, d 5, and d 10 after major trauma. We found all investigated genes to be differentially expressed in controls and patients already from d 1 until d 10 after major trauma (Table 3). Although the expression of antiapoptotic genes such as *Mcl-1*, *A1*, *Dad1* was found to be strongly upregulated, a simultaneously increase in the expression of *Bax*, *Bad*, *Bid* and *caspase-3*, which are known to favor apoptosis, could be observed in trauma neutrophils. Additionally, there was an upregulation of mRNAs encoding for the death receptors tumor necrosis factor receptor superfamily member TNFRSF1A, and to a lower extent for TNFRSF1B and Fas. However, the expression of death receptor associated adapter molecules, for example, Fas-associated via death domain *FADD*, TNF receptor-associated factor *TRAF2* and *TRAF4*, was markedly reduced compared with their expression found in control neutrophils.

Table 2. Demographics, injury severity, and outcome among subsets of patients.

	All patients	No sepsis	Sepsis
number, *n*	24	12	12
Age, years	41.7 ± 3.8	35.8 ± 3.6	47.6 ± 6.4
ISS	46.7 ± 3.1	43.9 ± 3.8	49.5 ± 4.9
ICU, d	21.1 ± 3.8	12.0 ± 1.8	30.3 ± 6.5[b]
Sepsis,% (*n*)	50.0 (12)	0 (0)	100 (12)
Death,% (*n*)	12.5 (3)	0 (0)	24.0 (3)
SOFA d 1	9.0 ± 0.6	7.6 ± 0.8	10.3 ± 0.7[a]
SOFA d 5	6.9 ± 0.9	3.9 ± 1.1	9.5 ± 1.2[b]
SOFA d 10	4.5 ± 0.9	2.1 ± 0.7	6.5 ± 1.4[a]
MODS d 1	6.7 ± 0.6	5.1 ± 0.8	8.3 ± 0.7[a]
MODS d 5	5.5 ± 0.8	2.7 ± 0.9	8.3 ± 0.8[c]
MODS d 10	3.8 ± 0.8	1.3 ± 0.8	6.3 ± 0.9[b]

Data indicated as mean ± standard error of the mean (SEM). ISS indicates injury severity score; ICU, intensive care unit length of stay; SOFA, Sequential Organ Failure Assessment score; MODS, Multiple Organ Dysfunction Score; [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ between sepsis and no sepsis group.

Table 3. Differential expression of apoptosis-related genes in neutrophils after major trauma.

Well	Gene	Fold change, d 1	Fold change, d 5	Fold change, d 10
A01	*SOD1*	1,672493	1,688146	1,466493
A02	*RIPK1*	–2,39164	–3,962909	–2,77739
A03	*BNIP3*	–3,575141	–4,78779	–3,654895
A04	*UNC5B*	n.d.	–9,223153	n.d.
A05	*E2F2*	4,293041	1,042928	1,996911
A06	*BBC3*	1,37745	3,334892	4,523909
A07	*AKT1*	–3,335724	–1,928875	1,10089
A08	*DAD1*	6,329098	8,947223	6,318885
A09	*DIABLO*	1,780151	–1,014353	–1,206
A10	*PMAIP1*	n.d.	n.d.	n.d.
A11	*HIF1A*	23,785328	58,256212	81,666359
A12	*TNFRSF1A*	9,862819	13,25661	10,087313
B01	*STAT5B*	10,070057	1,162427	–1,421713
B02	*TP53INP1*	1,043912	–1,015127	–1,513858
B03	*HSPA1A*	1,396679	2,220739	3,227395
B04	*TNF*	–6,224681	–7,248387	–1,239648
B05	*BCL2L14*	n.d.	n.d.	n.d.
B06	*LTBR*	2,044857	–4,359492	–2,763947
B07	*BCL6*	4,763427	5,137762	5,715462
B08	*DFFA*	–7,200015	–18,630055	–14,919528
B09	*BAX*	2,832351	1,669412	2,290512
B10	*CD40*	n.d.	–12,787479	–2,564238
B11	*BAK1*	n.d.	n.d.	n.d.
B12	*TRAF2*	–17,851829	–8,492916	–2,2984
C01	*FOXO3*	8,011098	4,789384	3,650692
C02	*PAWR*	–29,815694	–21,330709	–7,159853
C03	*BAG4*	–1,077733	–1,177609	–1,118163
C04	*FGFR3*	n.d.	n.d.	n.d.
C05	*SIRT1*	1,638073	1,452804	–1,540854
C06	*XIAP*	–2,140577	–2,523734	–1,435576
C07	*FAS*	3,923116	1,981977	–1,019054
C08	*AVEN*	n.d.	n.d.	n.d.
C09	*API5*	1,001387	–1,058382	–11,060387
C10	*IGF1*	–1080,88701	–32,522359	–123,231733
C11	*BNIP3L*	n.d.	n.d.	n.d.
C12	*HTRA2*	–1,204137	2,963783	2,925473
D01	*PTEN*	n.d.	n.d.	n.d.
D02	*BCL2L2*	n.d.	n.d.	n.d.
D03	*TNFAIP3*	4,263387	3,366945	5,083316
D04	*APAF1*	1,842928	2,182588	2,243529
D05	HSGenomic	n.d.	n.d.	n.d.
D06	*DDIT3*	2,030732	1,879098	6,899874
D07	*BOK*	–68,974921	–14,483717	–56,682442
D08	*CASP2*	–5,16225	–2,213087	1,217709
D09	*GPX1*	–1,775223	1,40011	3,486719
D10	*GSK3B*	5,359134	–190,303726	–34,314109
D11	*BIRC5*	n.d.	n.d.	n.d.
D12	*BID*	2,316586	4,079918	2,954205
E01	*FADD*	–5,4189	–1,70605	1,479357
E02	*MCL1*	4,03341	2,929471	1,517788
E03	*PRDX2*	–47,11126	–119,532573	–45,630609
E04	*TGFB1*	–1,825131	–1,3051	1,52042

Continued

In previous studies, we have already shown that neutrophils after trauma display a prolonged life span when compared with control cells (10,25). However, as depicted in Figure 1A, there was no significant difference in apoptosis rate at d 5 and d 10 after trauma between neutrophils from patients without or with posttraumatic sepsis development, respectively. In addition, fold change in apoptosis when compared with d 1 was negatively correlated with patient's MOD scores ($\rho = –0.435$, $^*P = 0.04$ [see Figure 1 legend]) and to a lower degree with SOFA ($\rho = –0.417$, $P = 0.059$) at d 10 but not at d 5 after trauma, suggesting a link between the apoptotic status of neutrophils and patient's clinical course (Figure 1B).

As previously reported, inhibition of neutrophil apoptosis after major trauma is mediated by serum factors (10,22). Indeed, treatment of control neutrophils with serum taken at d 1, d 5 and d 10 after admission from the 12 patients with and 12 patients without sepsis development led to a significant downregulation of neutrophil cell death compared with the apoptosis rate of neutrophils incubated with serum from healthy volunteers (Figure 1C). However, no differences in apoptosis could be observed between the sera from the two patient groups.

Regulation of the Neutrophil Apoptosis-Related Factors Mcl-1, A1, Dad1, and Bax during Sepsis Development after Major Trauma

To further elucidate the molecular mechanisms underlying reduced neutrophil apoptosis in patients with and without sepsis development after major trauma, we analyzed the expression of apoptotic factors, especially of those who were already shown to be important regulators of neutrophil survival (20).

The expression levels of the antiapoptotic genes *Mcl-1*, *A1*, *Dad1*, and the proapoptotic gene *Bax* are depicted in Figure 2. These factors were found to be clearly upregulated on mRNA level in neutrophils early after major trauma (Table 3). Analysis of gene expression

Table 3. ***Continued.***

E05	*BFAR*	1,358486	3,001409	3,053936
E06	*CASP9*	1,19914	−1,27709	−1,820999
E07	*BNIP2*	1,582275	−1,249768	1,000742
E08	*CASP3*	3,923116	7,663163	3,420412
E09	*BIRC2*	8,645803	9,273025	7,237894
E10	*TRADD*	−5,09118	−4,538325	−1,180363
E11	*CLU*	n.d.	n.d.	n.d.
E12	*DAPK2*	−1,513617	−1,374551	2,166506
F01	*BNIP1*	n.d.	n.d.	n.d.
F02	*TNFRSF1B*	1,649467	1,448279	1,785181
F03	*HRK*	−15,221092	−16,04954	−7,333643
F04	*TP53*	−6,267977	−3,578512	−1,944002
F05	*HIPK2*	n.d.	n.d.	n.d.
F06	*STAT5A*	1,166349	1,740908	2,572855
F07	*DAP*	4,35297	8,866348	4,893511
F08	*CASP7*	−7,200015	−4,333582	−2,271004
F09	*NAIP*	1,934553	1,783906	1,022053
F10	*TNFRSF10A*	n.d.	n.d.	n.d.
F11	*FASLG*	n.d.	n.d.	n.d.
F12	*LTA*	−7,663482	−2,449114	−2,856661
G01	*TNFRSF11B*	n.d.	n.d.	n.d.
G02	*Hs18s*	−17,606058	−18,415645	−2,365475
G03	*DPF2*	1,961558	11,152846	7,256481
G04	*CARD10*	n.d.	n.d.	n.d.
G05	*BAG3*	−69,45468	−71,459801	−16,247322
G06	*CASP8*	−1,108032	1,036011	3,724044
G07	*BCL2L11*	21,141414	242,768933	352,180202
G08	*BAG1*	−13,529157	−5,131071	−7,894949
G09	*BIRC3*	1,022429	n.d.	n.d.
G10	*PERP*	−6,717852	−10,243664	−71,374162
G11	*E2F1*	n.d.	−1,66389	−1,512285
G12	*BAD*	1,285206	1,121353	3,203103
H01	*DAPK1*	−3,133989	−1,055086	n.d.
H02	*BIK*	3,164549	1,443769	2,875813
H03	*RAD21*	4,293041	−1,598213	−1,316977
H04	*BCL2L10*	−84,918089	−12,292956	−22,321004
H05	*VEGFA*	−1,308578	1,942669	5,462533
H06	*BCL2*	−79,782476	−5,322706	−14,748844
H07	*TRAF4*	−22,284984	−11,440356	n.d.
H08	*BCL2L1*	−2,883858	2,476049	2,418596
H09	*GADD45G*	−1,19582	−1,385455	1,568592
H10	*BCL10*	4,322902	5,964247	3,440146
H11	*NFKB1*	1,150292	−1,040127	−1,585052
H12	*BCL2A1*	17,901394	3,652641	6,525417

n.d., Not detected.

using real-time PCR revealed high interindividual variation in mRNA expression levels. The expression of all genes was commonly found to be upregulated at d 1 after trauma when compared with their expression in healthy controls. The mRNA levels of *Mcl-1*, *A1*, *Dad1* and *Bax* further declined to control levels after 10 d. No differences in mRNA expression between septic and nonseptic patients could be determined (Figure 2).

In general, the mRNA levels do not necessarily correlate to the amount of the expressed protein and many proteins have been demonstrated to be posttranslationally regulated. For instance, Mcl-1 turnover has been already shown to be modified by granulocyte-macrophage colony-stimulating factor (GM-CSF) (32). We further examined the protein expression of the apoptotic factors mentioned above using immunoblot analysis. The protein levels of Mcl-1 peaked at d 1 after trauma and were significantly reduced in the sepsis group at d 10 ($P < 0.01$ versus nonsepsis; Figure 3). Additionally, we found A1 protein levels to be significantly diminished in neutrophils isolated at d 5 and d 10 from patients with sepsis development ($P < 0.01$ and $P < 0.05$, respectively) when compared with neutrophils from the nonsepsis group at these times. These findings argue for a general decline of antiapoptotic Bcl-2 members in neutrophils during sepsis development. Although not being a member of the Bcl-2 family, Dad1 has been shown to interact with antiapoptotic Mcl-1 and loss of Dad1 function is associated with apoptotic cell death (33). In contrast to Mcl-1 and A1, Dad1 levels were not increased in patients when compared with control neutrophils. Whereas Dad1 protein levels in patients with uneventful recovery remained unchanged over time after major trauma, the protein was found to be significantly increased at d 10 after trauma in the sepsis group ($P < 0.01$ versus nonsepsis). Moreover, we found that neutrophils of all trauma patients expressed reduced levels of proapoptotic Bax over the whole observation period of 10 d without any intergroup differences. Altogether, these data suggest that neutrophils in sepsis display an obvious imbalance between pro- and antiapoptotic Bcl-2 members owing to reduced levels of Mcl-1 and A1.

Reduced Mcl-1 Levels Are Associated with Impaired Intrinsic Apoptosis Resistance in Neutrophils during Sepsis

Neutrophil spontaneous apoptosis has been shown to be highly dependent on Mcl-1 protein levels. Besides Mcl-1, A1 has been identified to antagonize Bax activation and mitochondrial membrane depolarization (34). It has been already reported that neutrophils from

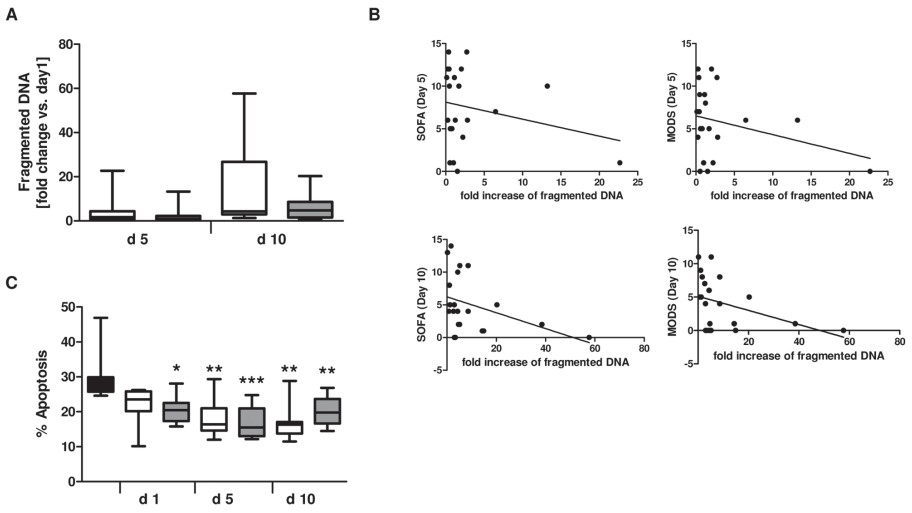

Figure 1. Inhibition of neutrophil spontaneous apoptosis by serum factors. (A) Fold change of DNA fragmentation in neutrophils isolated from patients with (n = 10) or without (n = 10) sepsis development at d 5 and d 10 after major trauma relative to d 1. (B) Correlation of fold change of fragmented DNA with Sequential Organ Failure Assessment (SOFA) score and Multiple Organ Dysfunction Score (MODS) at d 5 and d 10 after major trauma. Spearman rho (ρ) correlation coefficient and P values are indicated. (C) Neutrophils isolated from one healthy control (1×10^6/mL) were incubated with 1% serum from healthy volunteers (control, n = 9) and with patient sera collected at d 1, d 5 and d 10 after major trauma. After 18 h culture, apoptotic neutrophils were quantified by propidium iodide staining and flow cytometry. The white bars indicate apoptosis in cells incubated with sera from patients with uneventful outcome (n = 12); the gray bars indicate apoptosis after treatment with sera from patients with sepsis development (n = 12). *P < 0.05; **P < 0.01; ***P < 0.001 versus control. A, C: □, Nonsepis; ■, sepsis; ■, control. B: SOFA d 5: ρ = –0.23, P = 0.316; SOFA d 10: ρ = –0.417, P = 0.059; MODS d 5: ρ = –0.24, P = 0.29; MODS d 10: ρ = –0.435, P = 0.04.

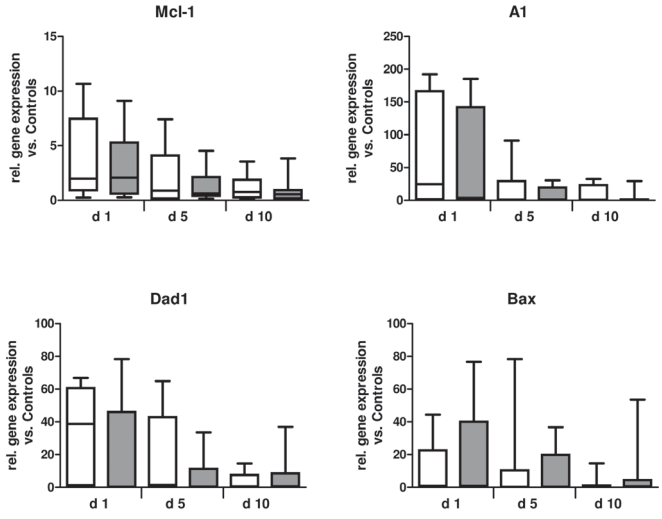

Figure 2. mRNA expression of Bcl-2 family members and *Dad1*. The expression of *Mcl-1*, *A1*, *DAD1* and *Bax* mRNA was quantified by real-time PCR in neutrophils isolated from healthy volunteers (n = 10), as well as from patients with (n = 9–10) or without (n = 10–12) sepsis development after major trauma. Gene expression was normalized to that of the *18S RNA* gene. □, Nonsepis; ■, sepsis.

trauma patients expressing high levels of intracellular Mcl-1 are resistant to mitochondrial membrane depolarization and thus intrinsic apoptosis in response to the proapoptotic stimulus staurosporine (10). In this study, we found neutrophils from patients with sepsis development to express reduced levels of antiapoptotic Mcl-1 and A1. We, therefore, assumed that neutrophils from septic patients might display an impaired intrinsic apoptosis resistance because of reduced Mcl-1 protein levels found in these cells. Indeed, Mcl-1 knockdown in patient neutrophils significantly enhanced sensitivity of the cells to staurosporine, resulting in increased mitochondrial membrane depolarization and apoptosis (Figure 4A). To prove our hypothesis, neutrophils from healthy donors were incubated with patient serum collected at d 1, d 5 and d 10 after severe trauma. We have previously shown that incubation of control neutrophils with patient serum promotes resistance to staurosporine-induced apoptosis by increasing the stability of Mcl-1 protein. This effect was mediated by proinflammatory cytokines, such as GM-CSF (10). Hence, reduced Mcl-1 levels in sepsis, probably because of altered cytokine levels, might be associated with a loss of mitochondrial membrane potential during intrinsic apoptosis induction. As speculated, mitochondrial disruption was significantly increased after treatment with staurosporine in cells incubated with serum from septic patients at d 10 after trauma when compared with cells incubated with serum from patients with uneventful outcome (Figure 4B).

Inhibition of Caspase-8 Activity and Bid Truncation in Neutrophils during Sepsis

However, the reduced neutrophil intrinsic apoptosis resistance found in sepsis does not explain the decreased apoptosis rate of neutrophils from septic patients. We therefore investigated if other mechanisms might be of relevance for the modulation of neutrophil apopto-

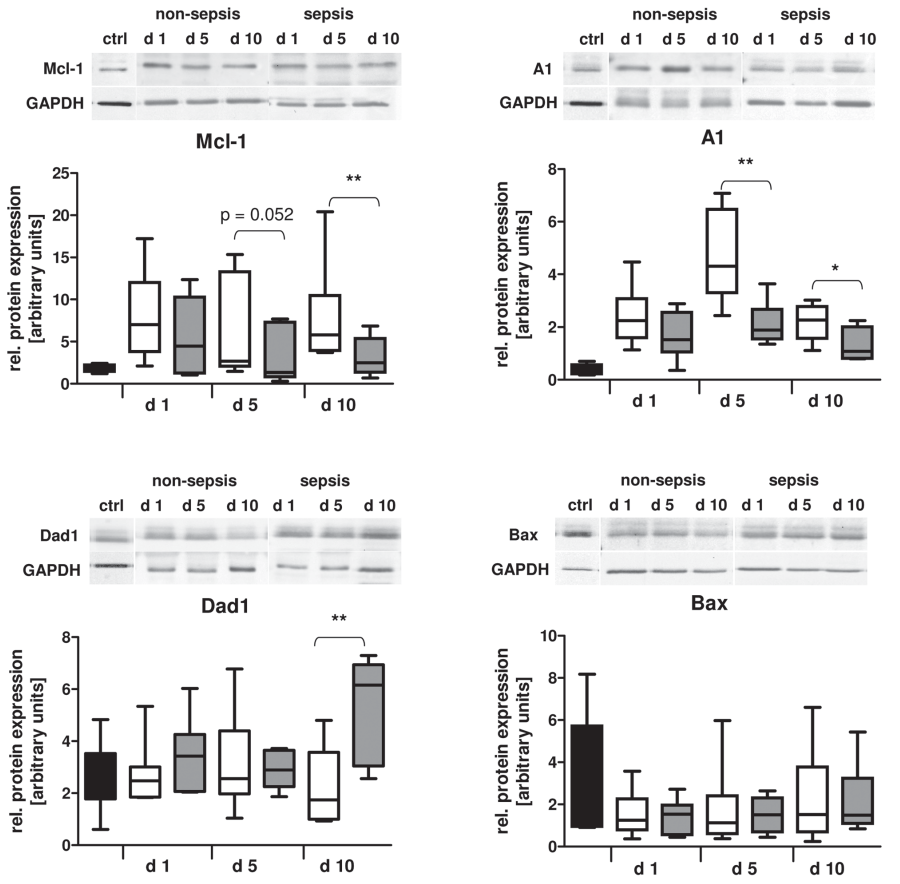

Figure 3. Protein expression of Bcl-2 family members and Dad1. The expression of Mcl-1, A1, Dad1 and Bax protein was determined by Western blot in neutrophils isolated from healthy volunteers (n = 5–13), as well as from patients with (n = 8–9) or without (n = 8–11) sepsis development after major trauma. Blots were analyzed by densitometry and normalized to GAPDH. In each case, representative blots of one healthy control, one patient with and one patient without sepsis development are depicted. *$P < 0.05$; **$P < 0.01$. □, Nonsepis; ■, sepsis; ■, control.

sis. Activation of Fas receptor has been already reported in neutrophils undergoing spontaneous apoptosis. When Fas molecules cluster, they interact with the adapter proteins FADD, which in turn, recruit procaspase-8 molecules to induce their autocleavage and thus production of active caspase-8 (35). Activated caspase-8 then cleaves the proapoptotic Bcl-2 family member Bid (22 kDa) to yield truncated Bid (tBid, 15 kDa). tBid was demonstrated to promote apoptosis by linking the extrinsic to intrinsic pathway in neutrophils and different cell lines (36,37).

As depicted in Figure 5A, neutrophils isolated at d 10 from septic patients displayed significantly reduced caspase-8 activity when compared with neutrophils isolated from patients with uneventful outcome at the same time ($P < 0.05$). In addition, an increased Bid/tBid ratio has been found in neutrophils isolated at d 5 ($P < 0.01$) and d 10 ($P < 0.01$), respectively, from septic patients, indicating a reduced Bid cleavage by caspase-8 due to an impaired extrinsic apoptotic pathway in these cells (Figure 5B).

DISCUSSION

Delayed neutrophil apoptosis combined with cellular hyperactivity is widely accepted to be associated with inflammatory disorders including tissue damage and progressive organ dysfunction. In the current study, we demonstrate that apoptosis-related factors are differentially regulated in neutrophils during sepsis development after major trauma. In addition, we also found an inverse correlation between neutrophil apoptosis rate and the patients' organ dysfunction scores suggesting an influence on sepsis-associated organ damage by delayed neutrophil apoptosis. The reduced neutrophil apoptosis rate in sepsis, which was not related to the expression of the neutrophil antiapoptotic factors Mcl-1 and A1, suggests that prolonged neutrophil life span does not solely depends on their expression levels. We demonstrate here that neutrophil apoptosis in sepsis is rather additionally regulated by factors modulating the extrinsic pathway, as shown by diminished caspase-8 activity and reduced Bid cleavage.

Our results showed many pro- and antiapoptotic genes to be upregulated after major trauma, supporting the regulation of gene activity by inflammatory mediators (38,39). Interestingly, Weber and colleagues have recently described a massive upregulation of proapoptotic factors in circulating blood cells from patients suffering from severe sepsis (40). They further concluded that the proapoptotic pattern of gene expression must be generated by lymphocytes, displaying increased apoptosis in sepsis, rather than neutrophils with prolonged life span (40). However, this work demonstrates that the gene expression of most proapoptotic genes such as *Bax*, *Bad*, and *Bid* is also upregulated in neutrophils after major trauma showing prolonged life span.

Previous studies have suggested that neutrophil spontaneous apoptosis highly depends on the integrity of the mitochondrial membrane and is regulated by Bcl-2 family members (20,22,29). Several lines of evidence indicate that under inflammatory conditions, such as for example, after major trauma, the neutrophil life span becomes modified owing to the action of proinflammatory cytokines

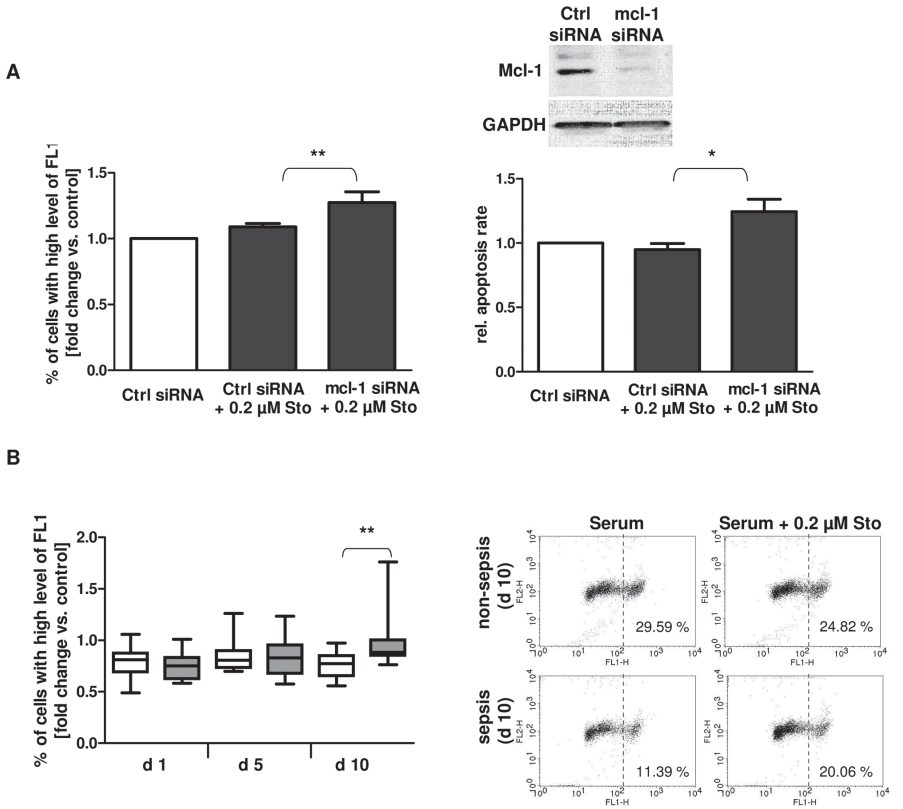

Figure 4. Reduced Mcl-1 protein levels are associated with impaired intrinsic apoptosis resistance during sepsis. (A) Freshly isolated neutrophils from patients (n = 8) at d 1 after trauma were nucleofected with control siRNA (Ctrl) or with mcl-1 siRNA. After 24 h culture, cells were treated with 0.2 μmol/L staurosporine (Sto) or left untreated (Ctrl). Mitochondrial membrane depolarization was quantified by JC-1 staining after 4 h. The percentage of cells with high levels of green (FL1) fluorescence was determined. Fold change versus sample without Sto treatment is depicted (left). After 18 h of culture, apoptotic neutrophils were quantified by propidium iodide staining and flow cytometry (right). Mcl-1 expression was evaluated by Western blot analysis. (B) Freshly isolated neutrophils from one healthy volunteer were preincubated with 1% serum from patients with (n = 12) or without (n = 12) sepsis development after major trauma for 1 h. Then, cells were further incubated with 0.2 μmol/L Sto for 4 h or left untreated. Mitochondrial membrane depolarization was quantified by JC-1 staining (left). Representative dot plots for cells treated with serum isolated at d 10 from one septic or nonseptic patient, respectively, are shown (right). *P < 0.05; **P < 0.01. B: □, Nonsepis; ■, sepsis.

present in the circulation. In this context, it has been already shown by our group and others that GM-CSF– and also interleukin (IL)-18–mediated suppression of neutrophil apoptosis is associated with an increase in antiapoptotic Mcl-1 protein (10,41). So far, it remains speculative whether additional serum factors beside GM-CSF and IL-18 are involved in the regulation of neutrophil apoptotic factors after severe trauma.

The antiapoptotic Bcl-2 members Mcl-1 and A1 are known to impair the intrinsic pathway by maintaining the mitochondrial transmembrane potential (10,42). Both proteins were found to be markedly upregulated in all patients after trauma while Bax protein levels were slightly downregulated when compared with healthy controls. Whereas Mcl-1 protein expression significantly declined at d 10 after trauma in the sepsis group, there was a strong decrease in A1 protein levels from d 5, suggesting a downregulation of antiapoptotic factors in sepsis.

A1 has already been reported to interact with human Bax (43). However, in contrast to the work of Werner and coworkers (42), reporting that A1 blocks the mitochondrial apoptotic pathway, here the reduction in A1 protein alone in the sepsis group at d 5 after trauma did not seem to abrogate intrinsic neutrophil apoptosis resistance as shown by staurosporine treatment and mitochondrial staining. However, it is likely that a simultaneously downregulation of other antiapoptotic Bcl-2 proteins is required to sensitize neutrophils to intrinsic apoptosis inducers.

In a previous study, we have demonstrated that intrinsic apoptosis resistance in neutrophils is accompanied by an increase in Mcl-1 protein levels. Further, incubation of control neutrophils with sera collected from severely injured patients was sufficient to reproduce this effect and to maintain mitochondrial membrane integrity after apoptosis induction with staurosporine (10). Consistent with these previous observations, the reduced Mcl-1 as well as A1 levels found in septic patients at d 10 after trauma were associated with an increased permeabilization of the mitochondrial membrane. In addition, our knockdown experiments using neutrophils expressing elevated levels of Mcl-1 (d 1 after trauma) further confirmed the regulation of the intrinsic apoptotic pathway by this factor. Interestingly, we found here that despite diminished levels of antiapoptotic Bcl-2 members, for example, Mcl-1, the apoptosis of neutrophils from patients with sepsis development after major trauma was still delayed. Based on these findings, we further suggested that additional upstream regulatory mechanisms are involved in the inhibition of neutrophil cell death in sepsis. In spontaneous neutrophil apoptosis Bid is cleaved to tBid which has been reported to activate Bax, thus promoting its translocation from the cytosol to the mitochondria (29,35). In the current study,

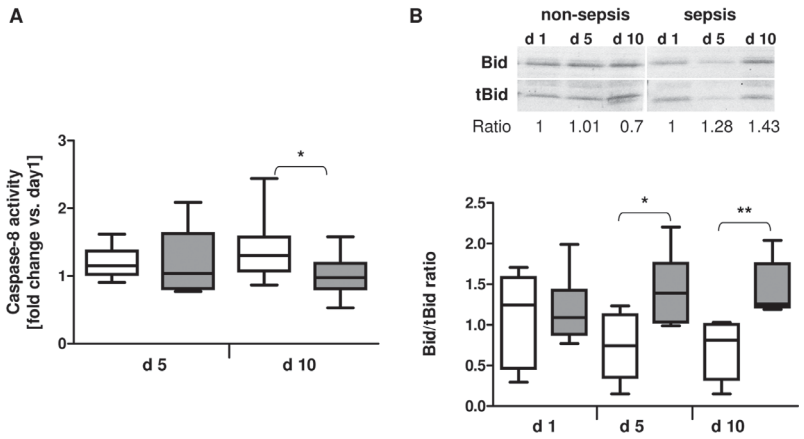

Figure 5. Reduced caspase-8 activity and diminished Bid truncation in patients with sepsis development. (A) Fold change of caspase-8 activity in neutrophils isolated at d 5 and d 10 from patients with (n = 10) or without sepsis (n = 12) development after major trauma versus d 1. (B) Bid and truncated Bid (tBid) protein expression in neutrophils isolated at d 1, d 5 and d 10 from patients with sepsis development (n = 6) or uneventful recovery (n = 6) was analyzed by Western blot. The ratio of Bid/tBid protein expression is depicted for each patient group. *P < 0.05; **P < 0.01. □, Nonsepis; ■, sepsis.

the production of tBid was inhibited in neutrophils isolated from patients with sepsis development at d 5 and d 10 after trauma. This reduced Bid truncation was further associated with significantly reduced caspase-8 activity at d 10. These findings are in line with recently published results demonstrating that prolonged neutrophil life span in septic patients was associated with elevated serum levels of soluble Fas (sFas). sFas may functionally antagonize the Fas/FasL pathway and thus extrinsic caspase-8–mediated apoptosis. Indeed, patient's SOFA and MOD scores showed a positive correlation with serum sFas levels (25) as well as an inverse correlation with neutrophil apoptosis at d 10. Hence, sFas in the circulation of trauma patients could represent another factor responsible for the dysregulation of apoptosis in severely injured patients.

In addition, in this article, we also show for the first time a strong upregulation of Dad1 protein in septic patients at d 10, but its mode of action is not yet understood. Dad1 has largely been implicated in N-linked glycosylation. The inhibition of N-linked protein glycosylation in cells that undergo apoptosis after loss of Dad1 function suggests that loss of N-linked glycoproteins is associated with onset of apoptotic cell death (44). In addition, Dad1 has been also shown to bind to antiapoptotic Mcl-1 protein and his expression in animal cells was reportedly down-regulated prior to the induction of programmed cell death (45). However, it remains unclear whether Dad1 might diminish neutrophil apoptosis by the modulation of Mcl-1 activity or by maintaining accurate N-glycosylation of proteins thus preventing endoplasmic reticulum stress. In fact, diminished neutrophil apoptosis in sepsis seems to be related to both, increased Dad1 levels and impaired extrinsic pathway, respectively.

Overall, most of the effects have been observed at d 10 after trauma. At the earlier time point (d 5), five of twelve patients in our cohort already displayed clinical signs of sepsis. In contrast, at d 10 all patients included in the sepsis group had clinical manifested septic disease.

One important limitation of this study is that we only investigated apoptotic signalling pathways in circulating neutrophils. Because these cells are known to infiltrate tissues and also to retain to the circulation after contact with the inflamed tissue, their function and apoptotic status might change. Being aware that posttraumatic tissue destruction is rather mediated by infiltrated than circulating neutrophils, it was not yet possible to investigate apoptosis of tissue neutrophils in patients, which however is a common limitation in patient studies. Further, we did not differentiate between different subpopulations of neutrophils, for example, newly released cells from the bone marrow, and therefore further discrepancies regarding apoptotic signalling in these cells cannot be ruled out.

CONCLUSION

In summary, our study demonstrates that sepsis development after major trauma is associated with changes in the expression of neutrophil antiapoptotic factors. In the early phase after trauma, neutrophil apoptosis seems to be mainly regulated by antiapoptotic Bcl-2 members which inhibit the intrinsic mitochondria-dependent pathway. Importantly, our data indicate that neutrophil apoptosis do not always correlate with Mcl-1 protein levels. Neutrophils from patients with clinically diagnosed sepsis at d 10 after trauma displayed reduced neutrophil apoptosis despite diminished levels of antiapoptotic Mcl-1 and A1 protein. In these patients, a predominant inhibition of the extrinsic apoptotic pathway could be found. The correlation between reduced neutrophil apoptosis and the severity of illness further supports the importance of neutrophil activity in the pathophysiology of sepsis. On the basis of these findings, drugs designed to target extrinsic rather than intrinsic apoptotic signalling in neutrophils during sepsis may help to modulate neutrophil life span and to prevent host tissue damage under these conditions.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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