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Entwicklung und Anwendung eines *in vitro* Co-Kultur-Modells der Haut zur Identifizierung potentieller Therapeutika bei Schwefellost-Vergiftungen

Development and Application of a *in vitro* Co-Culture Model of the Skin for the Identificaton of Therapeutics against Sulfur Mustard Intoxications

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Zusammenfassung

Schwefellost (S-Lost) ist ein blasenbildender chemischer Kampfstoff der vor 100 Jahren zum ersten Mal militärisch eingesetzt wurde. Als es 2015 nachweislich zur Ausbringung von S-Lost in Syrien kam, rückten die unzulänglichen Therapiemöglichkeiten einer S-Lost-Vergiftungen wieder in den Fokus der Öffentlichkeit. Bei einer Vergiftung mit S-Lost sind besonders Augen, Lunge und Haut betroffen. Abhängig von der aufgenommenen Giftmenge kommt es zu Nekrose, Apoptose und Entzündung in den exponierten Organen. Bis *dato* ist noch keine kausale Therapie möglich und die Behandlung erfolgt lediglich symptomorientiert und zum Teil sogar empirisch.
Ziel der wissenschaftlichen Arbeit war die Etablierung und Erprobung eines *in vitro* Co-Kultur-Modells aus Keratinozyten (HaCaT) und Immunzellen (THP-1), das die Physiologie der Haut besser darstellt als eine HaCaT-Monokultur. Mit diesem Modell ist es möglich potentielle Therapeutika gegen S-Lost-Intoxikation zu identifizieren und die aktuellen Therapievorschläge zu evaluieren, wobei gleichzeitig die Notwendigkeit für Tierversuche verringert wird.
Bei der Etablierung des Co-Kultur-Modells wurden zwei Versuchsansätze gewählt. Zum einen wurden vergiftete HaCaT-Zellen mit ungeschädigten THP-1-Zellen vergesellschaftet, um so das Einwandern von Makrophagen in den Entzündungsherd zu simulieren. Es konnte gezeigt werden, dass unbehandelte THP-1-Zellen die Toxizität von S-Lost nicht erhöhen, sondern im Gegenteil bei den stärker vergifteten HaCaT-Zellen die Toxizität zum Teil signifikant verringern. Zum anderen wurde ein Versuchsansatz gewählt, in dem die HaCaT-Zellen zuerst mit den THP-1-Zellen vergesellschaftet wurden, um dann simultan mit S-Lost vergiftet zu werden. Hier wurde die Vergiftung von in der Haut vorhanden Immunzellen simuliert. Die Ergebnisse zeigten, dass bereits ein Verhältnis von 2 % THP-1-Zellen im Vergleich zur ausgesäten Zahl an HaCaT-Zellen ausreichend ist um die Nekrose, Apoptose und Entzündung nach einer S-Lost-Vergiftung hochsignifikant zu steigern. Das Co-Kultur-Modell lieferte daher Ergebnisse, die näher an den *in vivo* Bedingungen liegen als eine HaCaT-Monokultur.
Das Co-Kulturmodell mit simultaner Vergiftung der HaCaT- und THP-1-Zellen wurde im weiteren Verlauf der Arbeit genutzt um die Effektivität von anti-inflammatorischen Arzneistoffen (Dexamethason, Ibuprofen und Diclofenac) zu testen und Therapieempfehlungen für eine Vergiftung abzuleiten. Dabei wurde zu Vergleichszwecken jeweils eine HaCaT-Monokultur unter gleichen Bedingung parallel behandelt. In den Ergebnissen bewirkte Dexamethason in geringen Konzentrationen eine signifikante Verringerung der Apoptose und der Interleukinausschüttung, die allerdings in der Monokultur stärker ausgeprägt war als in der Co-Kultur. Dies lässt vermuten, dass der protektive Effekt des Dexamethasons nicht in erster Linie durch eine Beeinflussung von Immunzellen zu Stande kommt. Ibuprofen führte in den eingesetzten Konzentrationen zu einer Verstärkung der Zytotoxizität des S-Lostes, sowohl in der Mono- als auch in der Co-Kultur. Daher ist eine Therapie von S-Lost-Vergiftungen mit Ibuprofen nicht empfehlenswert. Diclofenac war in der Lage, die Toxizität von S-Lost zu verringern, wobei diese Effekte in der Co-Kultur besonders deutlich werden, was auf eine Beeinflussung der Immunzellen durch Diclofenac hindeutet. Die Herausarbeitung der protektiven Effekte des Diclofenacs *in vitro* waren nur durch die neu etablierte Co-Kultur aus HaCaT- und THP-1-Zellen möglich, während sie bei einem regulären Screening mit einer HaCaT-Monokultur unterschätzt worden wären.
Insgesamt wurde also ein valides Co-Kultur-Modell der Haut zur Testung potentieller Therapeutika gegen S-Lost-Vergiftung etabliert, wobei sich das Modell bereits in der Erprobung erster Substanzen bewährt hat.

Abstract

Sulfur mustard (SM) is a vesicant agent that had its first military use 100 years ago, at Ypres. Recently, the use of SM in Syria 2015 was verified and moved the difficulties which are linked with the treatment of SM intoxications back into the spotlight. A SM intoxication especially affects eyes, lung and skin and leads dose dependently to necrosis, apoptosis and inflammation. No causal antidote to counteract SM toxicity exits, so far. Therefore, treatment is mainly symptomatic and in some cases even empiric.

Aim of this work was the establishment and testing of a co-culture model of the skin, composed of keratinocytes (HaCaT) and immunocompetent cells (THP-1). This *in vitro* model reflects the physiology of the skin more closely than a monoculture of keratinocytes and can be used to identify potential candidate substances for the treatment of SM intoxications and evaluation of the current therapy recommendations, respectively. Moreover, it diminishes the need for animal trials.

For the validation of the co-cultur-model we used two different strategies. Firstly, we inoculated unexposed THP-1 cells with SM-exposed HaCaT cells. This approach simulates the migration of macrophages from the blood into the inflamed tissue. Our results showed, that healthy THP-1 cells do not aggravate the course of a SM intoxication. On the contrary, the THP-1 cells significantly reduced SM toxicity in the more severely poisoned cells, if the number of THP-1 cells was sufficient. Secondly, adherent HaCaT-cells were inoculated with THP-1 cells before the SM exposure and thus being poisoned simultaneously. The data showed, that a ratio of 2 % THP-1 cells relating to the number of seeded HaCaT cells is sufficient to aggravate necrosis, apoptosis and inflammation in a highly significant manner. In conclusion, the co-culture-model containing HaCaT and THP-1 cells mimics the physiology of the skin *in vitro* more closely than a HaCaT monoculture.

The co-culture model which exposes the HaCaT and THP-1 cells simultaneously to SM has been used in further experiments to ascertain the efficacy of anti-inflammatory drugs against SM intoxication and draw recommendations for SM treatment thereof. For comparability reasons a HaCaT monoculture was treated under the same conditions as the co-culture. In the results, dexamethasone significantly decreased apoptosis and interleukin production when applied in low concentrations. This effect was in the monoculture more clearly than in the co-culture, which led to the conclusion, that the protective effects of dexamethason did not arise from a modulation of immunocompetent cells in first line. Ibuprofen, in the used concentrations, led to a strong increase in SM cytotoxicity in the mono- as well as the co-culture. For that reason, ibuprofen cannot be recommended for the treatment of SM intoxications. Diclofenac on the other hand was able to attenuate SM toxicity. The protective effect was stronger in the co-culture compared to the monocultur, which indicates that diclofenac modulates the response of the immunocompetent cells to a SM exposure. The full protective effects of diclofenac could only be carved out by the use of the new established co-culture model while they would have been underestimated in a HaCaT monoculture screening.

In summary, the establishment of a valid co-culture–model of the skin was successful. The model was also used to evaluate anti-inflammatory compounds which generated treatment recommendations for the therapy of SM intoxications.

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema „Entwicklung und Anwendung eines *in vitro* Co-Kultur Modells der Haut zur Identifizierung potentieller Therapeutika bei Schwefellost-Vergiftungen“ selbstständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 14.12.2018

(Georg Menacher)

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Abkürzungsverzeichnis

ADP	Adenosindiphosphat
AK	Adenylatkinase
ARDS	Acute respiratory distress syndrome
ATP	Adenosintriphosphat
Bis-G	[2-(Guanin-7-yl)ethyl]sulfid
CEES	2-Chloroethylethylsulfid (S-Lost-Analogon)
COPD	Chronisch obstruktive Lungenerkrankung (Chronic obstructive pulmonary disease)
COX	Cyclooxygenase
CDDE	Cell Death Detection ELISA
DNA	Desoxyribonukleinsäure
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	endotheliale Stickstoffmonoxid-Synthase
GM-CSF	Granulozyten-Monozyten-Kolonie-stimulierender Faktor (granulocyte-macrophage colony stimulating factor)
iNOS	induzierbare Stickstoffmonoxid-Synthase
IL	Interleukin(e)
MMP-9	Matrix-Metalloproteinase-9
NAC	N-Acetylcystein
NAD	Nicotinamidadenindinucleotid
NAD^+	Nicotinamidadenindinucleotid (oxidierte Form)
NFκB	Nuklear Faktor kappa B
NSAID	Nichtsteroidales Antirheumatika (non-steroidal anti-inflammatory drug)
OVCW	Organisation für das Verbot Chemischer Waffen (Organisation for the Prohibition of Chemical Weapons)
PARP	Poly-(ADP-Ribose)-Polymerase
PG-E2	Prostaglandin-E2
RNS	reaktive Stickstoffspezies
ROS	reaktive Sauerstoffspezies
RSDL	Reactive Skin Decontamination Lotion
S-Lost	Schwefellost
SM	Sulfur mustard (Schwefellost)
TNFα	Tumornekrosefaktor alpha
TRPA1	Transient receptor potential ankyrin 1

Publikationsliste

Publikationen der kumulativen Dissertation

Frank Balszuweit, **Georg Menacher**, Brunhilde Bloemeke, Annette Schmidt, Franz Worek, Horst Thiermann, Dirk Steinritz, (2014). Development of a co-culture of keratinocytes and immune cells for *in vitro* investigation of cutaneous sulfur mustard toxicity, *Chemico-Biological Interactions*, 223, 117–124.

Georg Menacher, Dirk Steinritz, Annette Schmidt, Tanja Popp, Franz Worek, Thomas Gudermann, Horst Thiermann, Frank Balszuweit, (2018). Effects of anti-inflammatory compounds on sulfur mustard injured cells: recommendations and caveats suggested by *in vitro* cell culture models, *Toxicology Letters*, 293, 91-97.

Weitere Publikationen

Frank Balszuweit, **Georg Menacher**, Harald John, Annette Schmidt, Kai Kehe, Dirk Steinritz, Franz Worek, Horst Thiermann, (2014). Entwicklung und Anwendung eines Zell-Co-Kultur-Systems zur Untersuchung der Schwefellost-Toxizität und potentieller Therapeutika, *Wehrmedizinische Monatsschrift*, 58, 306-310.

Frank Balszuweit, **Georg Menacher**, Annette Schmidt, Kai Kehe, Tanja Popp, Franz Worek, Horst Thiermann, Dirk Steinritz, (2016). Protective effects of the thiol compounds GSH and NAC against sulfur mustard toxicity in a human keratinocyte cell line, *Toxicology Letters*, 244, 35-43.

Simon Lang, Tanja Popp, Christian Silvester Kriegs, Annette Schmidt, Frank Balszuweit, **Georg Menacher**, Kai Kehe, Horst Thiermann, Thomas Gudermann, Dirk Steinritz, (2018). Anti-apoptotic and moderate anti-inflammatory effects of berberine in sulfur mustard exposed keratinocytes, *Toxicology Letters*, 293, 2-8.

Posterpräsentationen

Georg Menacher, Frank Balszuweit, Annette Schmidt, Horst Thiermann, Dirk Steinritz. Aufbau und Anwendung eines *in-vitro* Testsystems zur Identifizierung möglicher Therapeutika nach Schwefellost-Verletzung. 44. Jahreskongress der Deutschen Gesellschaft für Wehrmedizin und Wehrpharmazie e. V. (DGWMP), Rostock Warnemuende, 2013.

Frank Balszuweit, **Georg Menacher**, Annette Schmidt, Brunhilde Bloemeke, Horst Thiermann, Franz Worek, Dirk Steinritz. Evaluation of current and future therapeutics for sulfur mustard injury in immuno-competent co-culture models of human skin. 80. Jahrestagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie e. V. (DGPT), Hannover, 2014.

Frank Balszuweit, **Georg Menacher**, Annette Schmidt, Franz Worek, Horst Thiermann, Dirk Steinritz. Respiratory and cutaneous cell co-cultur models to develop novel treatments against sulfur mustard injury. Military Health System Research Symposium (MHSRS), Fort Lauderdale, Florida, USA, 2015.

Georg Menacher, Dirk Steinritz, Annette Schmidt, Franz Worek, Horst Thiermann, Frank Balszuweit. Wirkung von Necrosulfonamid gegen Schwefellost-Vergiftung. 46. Jahreskongress der Deutschen Gesellschaft für Wehrmedizin und Wehrpharmazie e. V. (DGWMP), Oldenburg, 2015.

Georg Menacher, Frank Balszuweit, Annette Schmidt, Franz Worek, Horst Thiermann, Dirk Steinritz. Effect of Necrosulfonamide on Sulfur Mustard Toxicity *in vitro*. 16th Medical Chemical Defense Conference (MCDC), München, 2017.

1 Schwefellost

1.1 Geschichte

Schwefellost (S-Lost, Bis(2-chloroethyl)sulfid, CAS-Nr.: 505-60-2) ist ein blasenbildender chemischer Kampfstoff [58], der erstmals 1822 von Despretz synthetisiert wurde [29]. Die blasenbildende Wirkung auf die Haut wurde 1860 von Guthrie und Niemann beschrieben [47, 73]. Meyer entwickelte 1866 eine neue Synthesevorschrift und erhielt so S-Lost in größerer Reinheit [71].
Während des Ersten Weltkrieges kam es im Verlauf der Marneschlacht zum Erstarren der Fronten und die Kriegsparteien gruben sich ein. Die herkömmlichen Waffen waren wenig geeignet, einen Gegner in befestigten Stellungen zu bekämpfen. Es wurde nach neuen Kampfmitteln gesucht, die in der Lage waren, den Gegner aus seinen Stellungen zu werfen. Dabei schienen chemische Kampfmittel am geeignetsten [48]. Die Wissenschaftler Lommel und Steinkopf entwickelten eine Methode zur großtechnischen Herstellung von S-Lost. Von ihren Nachnamen ist auch das Akronym Lost abgeleitet worden [109]. Der erste militärische Einsatz von S-Lost erfolgte durch deutsche Truppen in der Nacht vom 12. zum 13. Juli 1917 in der Flandernschlacht bei Ypern [48]. Der Kampfstoff wurde dann im weiteren Kriegsverlauf sowohl von den Mittelmächten als auch der Entente im großen Maßstab eingesetzt (siehe Abbildung 1 auf Seite 2) und war für mehr als 80 % der durch chemische Kampfstoffe verwundeten Soldaten verantwortlich [8].
Während des Zweiten Weltkrieges wurden zwar große Mengen an chemischen Kampfstoffen produziert, kamen aber glücklicherweise nie zum Einsatz [9]. Lediglich im Dezember 1943 wurden im italienischen Bari 600 Menschen mit S-Lost exponiert, als bei der Bombardierung des Hafens durch die deutsche Luftwaffe ein alliiertes Schiff explodierte, das mit ca. 100 Tonnen des Kampfstoffes beladen war [3].
Im Rahmen der Abrüstung wurden hundertausende Tonnen chemischer Kampfstoffe nach dem Zweiten Weltkrieg im Meer verklappt. Vor allem die Küsten von Europa, Japan, Russland und den USA waren betroffen [45]. Beispielsweise wurden in der Ostsee ca. 40.000 t chemischer Waffen im Bornholmer Becken versenkt. Die Eingrenzung der Verklappungsareale ist allerdings nicht exakt möglich, da die Munition teilweise in Holzkisten versenkt wurde, die von der Strömung auf ein weites Gebiet verteilt wurden. Deshalb kommt es trotz des Fischfangverbots im Bornholmer Becken immer wieder zur Exposition von Fischern mit S-Lost, wenn sie mit ihren Netzen in unbeabsichtigter Weise den Kampfstoff bergen [40, 93]. Die durch das Versenken der chemischen Kampfstoffe verursachten Folgen und Probleme sind bis heute noch nicht vollständig abschätzbar [45].
Auch nach dem Zweiten Weltkrieg wurde S-Lost wiederholt eingesetzt, beispielsweise zwischen 1983 und 1988 im Irak-Iran-Krieg [9]. Die aktuelle Präsenz von S-Lost wird dadurch deutlich, dass die 2004 von Libyen deklarierten S-Lost-Lager noch immer nicht komplett vernichtet sind [77]. Syrien hat 2014 seine S-Lost-Bestände der Vernichtung übergeben [75]. Trotzdem kam es 2015 in Marea (nördlich von Aleppo) zur Ausbringung des Kampfstoffes [78, 85].
Die 1997 gegründete Organisation für das Verbot chemischer Waffen (OVCW) überwacht das Chemiewaffenübereinkommen, das den Einsatz chemischer Waffen verbietet [76].

Abbildung 1: Abschuss von französischen Gasgranaten durch US-amerikanische Artillerie während des Ersten Weltkrieges [109].

1.2 Physikochemische Eigenschaften

Reines S-Lost ist eine klare, geruchlose, ölige Flüssigkeit [68]. Das großtechnisch produzierte S-Lost kann dagegen aufgrund von Verunreinigungen gelb bis braun sein und hat einen typischen Geruch nach Senf bzw. Knoblauch, woher auch der Name Senfgas rührt [27, 68, 46, 9]. Eine Zusammenstellung über die physikochemischen Eigenschaften zeigt Tabelle 1 (siehe Seite 3).

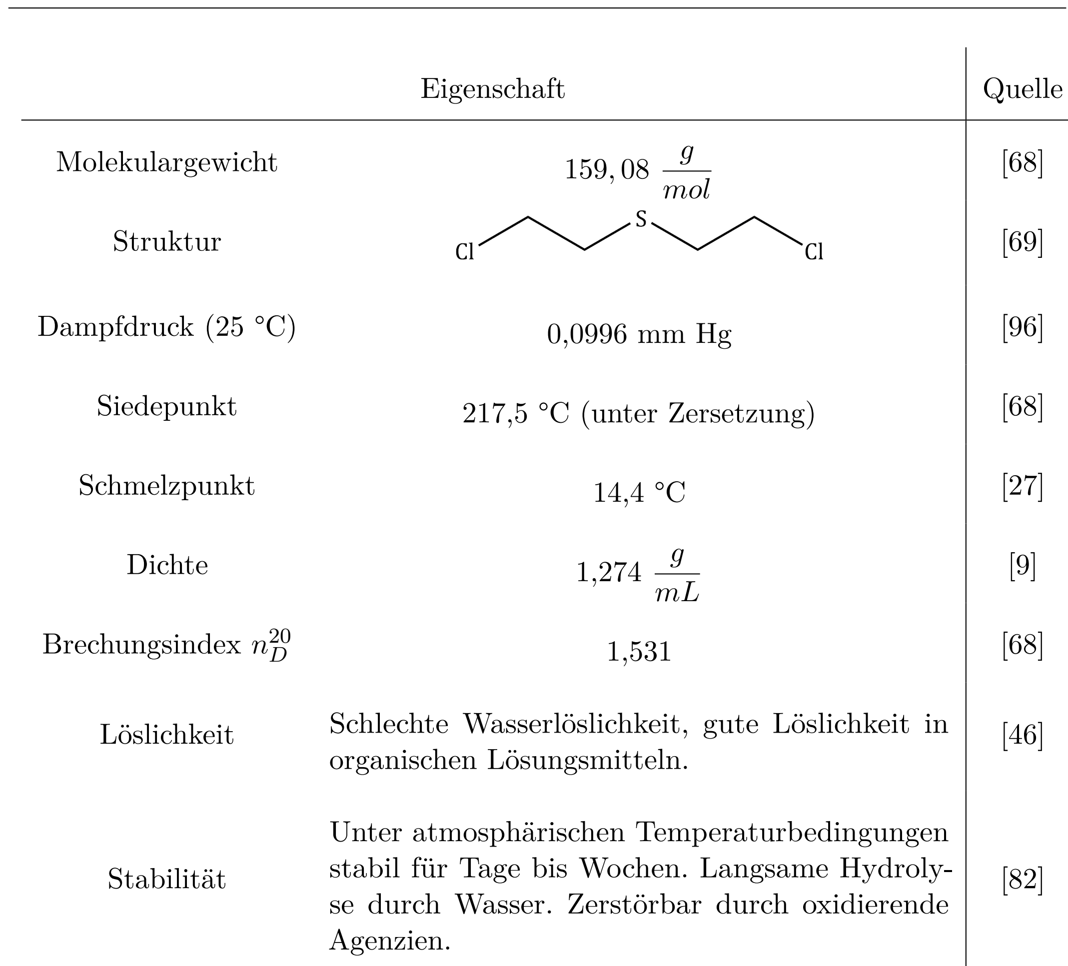
1.3 Klinische Symptomatik von S-Lost-Vergiftungen

Im warmer, trockener Umgebung kommt es zum zügigen Verdampfen von S-Lost, dessen Dämpfe gewöhnliche Kleidung penetrieren. Nach der S-Lost-Exposition tritt ein symptomfreies Intervall auf, das 1 bis 24 Stunden dauern kann. Dabei ist die Dauer des symptomfreien Intervalls umgekehrt proportional zur aufgenommenen Giftdosis. Bei einer Vergiftung sind besonders die Augen, der Respirationstrakt und die Haut betroffen. Zur Störung der Blutbildung und zur Immunsuppression kommt es nur, wenn sehr hohe Giftmengen absorbiert wurden [31, 58].

1.3.1 Augen

Die Augen reagieren besonders empfindlich auf S-Lost. Erste Symptome können bereits eine Stunde nach der Exposition auftreten [9]. Als akute Symptome einer

Tabelle 1: Übersicht über die physikochemischen Eigenschaften von S-Lost.



	Eigenschaft	Quelle
Molekulargewicht	$159,08\ \frac{g}{mol}$	[68]
Struktur	Cl–CH2–CH2–S–CH2–CH2–Cl (Strukturformel)	[69]
Dampfdruck (25 °C)	0,0996 mm Hg	[96]
Siedepunkt	217,5 °C (unter Zersetzung)	[68]
Schmelzpunkt	14,4 °C	[27]
Dichte	$1{,}274\ \frac{g}{mL}$	[9]
Brechungsindex n_D^{20}	1,531	[68]
Löslichkeit	Schlechte Wasserlöslichkeit, gute Löslichkeit in organischen Lösungsmitteln.	[46]
Stabilität	Unter atmosphärischen Temperaturbedingungen stabil für Tage bis Wochen. Langsame Hydrolyse durch Wasser. Zerstörbar durch oxidierende Agenzien.	[82]

Vergiftung manifestieren sich Photophobie, Fremdkörpergefühl, Tränenfluss, lokales Ödem, Blepharospasmus, Konjunktivitis und extreme Schmerzen [35, 79]. Der Blepharospasmus verbunden mit dem Lidödem führt dabei oft zur vorübergehenden Erblindung des Patienten (siehe Abbildung 2 auf Seite 4) [55]. Kommt es zur Exposition mit S-Lost-Konzentrationen größer 400 $mg * min * m^{-3}$ tritt eine Schädigung der Hornhaut in ihrer ganzen Dicke auf, evtl. verbunden mit einer Ulzeration und einem Verschluss von Blutgefäßen der Bindehaut [58]. Schwer geschädigte Augen sind zusätzlich anfällig für Infektionen, etwa mit *Pseudomonas aeruginosa* [79].
Noch Jahrzehnte nach der Augenexposition kann eine verzögerte Keratopathie zum Sehverlust führen [118].

1.3.2 Respirationstrakt

S-Lost schädigt den Respirationstrakt dosisabhängig beginnend mit der Nasenschleimhaut bis hin zu den terminalen Bronchiolen bei sehr hoher Expositionsdosis [9]. Die akute Letalität bei S-Lost-Vergiftungen ist relativ gering (zwischen 1 und 5 %) [35], wird aber hauptsächlich auf die akute Schädigung der Lunge zurückgeführt [112]. Wenn nicht letal, so kann S-Lost doch zu chronisch zerstörten Atemwegen oder zur Manifestierung von chronischen Atemwegserkrankungen führen [39].
Die ersten Symptome einer S-Lost-Vergiftung der Atemwege treten üblicherweise 4 bis 16 Stunden nach der Vergiftung auf und zeigen sich durch Schmerz oder Un-

Abbildung 2: Britische Soldaten, die während des Ersten Weltkrieges S-Lost verursachte Augenschäden erlitten [109].

behagen in der Nase und den Sinusiden, begleitet von einer Rhinorrhoe, Niesen und Halsschmerzen. Werden höherer S-Lost-Mengen inhaliert, tritt zusätzlich eine Schädigung des Kehlkopfes auf, was eine heisere Stimme bis hin zur Aphonie nach sich zieht. Außerdem werden bereits die oberen Atemwege in Mitleidenschaft gezogen (Tracheobronchitis) und ein trockener Reizhusten ist die Folge. Bei der Inhalation sehr hoher S-Lost Mengen werden selbst die terminalen Bronchiolen geschädigt und es kommt zu Husten mit Auswurf, Dyspnoe und evtl. Hämorrhagie in den Alveolen [9]. Schwere Schädigung der unteren Atemwege führt zum ARDS (Acute Respiratory Distress Syndrome), das mit einer hohen Mortalität einhergeht [39]. Wenn das Epithel zerstört wird können sich, ähnlich wie bei der Diphterie, in der Trachea und den Bronchien Pseudomembranen ausbilden, die sich im weiteren Verlauf ablösen, die Atemwege verlegen und zum Tod führen können [35]. In schweren Fällen kann eine bakterielle Infektion zur Gangrän der Lunge führen [39].
Eine pulmonale Spätfolge, die in vielen Patienten auftritt ist *Bronchiolitis obliterans* [38] und im späteren Verlauf die sogenannte „*mustard lung*“ (Senfgaslunge), die symptomatisch die COPD imitiert, sich pathophysiologisch allerdings davon unterscheidet. So ist in der Senfgaslunge beispielsweise die Aktivität von Proteasen nicht erhöht [36].

1.3.3 Haut

Die akuten Symptome einer kutanen S-Lost-Vergiftung sind dosis- und zeitabhängig und reichen von Pruritus, Erythem, Blasenbildung, über Hypo- und Hyperpigmentierung [37]. Auch Inflammation ist in den betroffenen Hautarealen zu beobachten [57]. Schnell proliferierende Zellen werden durch S-Lost besonders stark geschädigt. Des-

halb zeigt die Basalschicht der Haut unter dem Mikroskop die stärkste Schädigung. Diese Schädigung bedingt später die Ablösung der Dermis von der Epidermis und die subepidermale Blasenbildung (siehe Abbildung 3) [83]. Die kleinen Blasen können

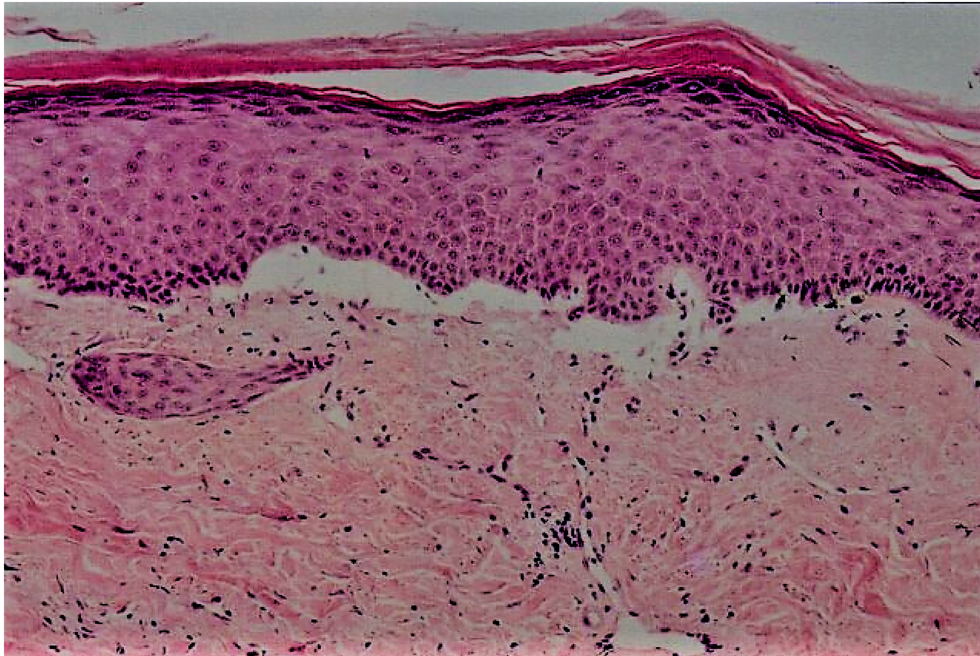


Abbildung 3: Histologischer Schnitt der Haut, 11 Tage nach einer S-Lost Expositon. Gut zu erkennen ist die Trennung von Epidermis und Dermis [113].

im späteren Verlauf zusammenlaufen, nach Erreichen einer gewissen Größe aufbrechen und tiefe Ulzerationen hinterlassen, die mit Wundheilungsstörungen assoziiert sind [118].
Bei Nachsorgeuntersuchungen zwei Jahre nach S-Lost-Exposition beklagten iranische Soldaten vor allem Juckreiz, Brennen und Desquamation der Haut [10]. Außerdem kommt es zur Hyperpigmentierung und im geringeren Umfang Hypopigmentierung der Haut im Bereich des exponierten Areals, was zu charakteristischen Hautbildern führt [90].

1.4 Therapie von Vergiftungen

Bis heute existiert weder ein Antidot noch eine kausale Therapie gegen S-Lost-Vergiftungen. Deshalb ist die Therapie von Verletzungen in erster Linie symptomorientiert und oft sogar empirisch [102]. Im Falle einer Vergiftung von einer oder mehreren Personen steht die Rettung aus dem kontaminierten Bereich und eine schnelle Dekontamination der Betroffenen im Vordergrund, um die aufgenommene S-Lost-Menge möglichst gering zu halten und sekundäre Kontaminationen zu vermeiden. Dabei müssen die Helfer eine geeignete Schutzausrüstung tragen. Zur Dekontamination wird die Kleidung entfernt und eventuell verbleibende Reste auf der Haut mit Seifenwasser oder sofern verfügbar, spezifischen Dekontaminationsmitteln (z. B. RSDL), entfernt [72, 30].

1.4.1 Therapie von Augenverletzungen

Erste Maßnahme ist das ausgiebige Spülen der Augen. Durch eine Fluoreszeinfärbung kann unterschieden werden, ob nur die Bindehaut oder zusätzlich die Hornhaut von der Verletzung betroffen sind. Wenn die Hornhaut in Mitleidenschaft gezogen wurde, ist eine engmaschige Überwachung des Heilungsverlaufs durch einen Ophthalmologen angezeigt. Die Therapie der Augenverletzung umfasst die tägliche Spülung und das regelmäßige Befeuchten der Augen, sowie die topische Gabe von Antibiotika und Corticosteroiden. Eventuell ist die Gabe von Mydriatika und bei zu hohem Augeninnendruck die Gabe von Anti-Glaukom-Tropfen indiziert [44, 13, 100]. Die Therapie der chronischen Beschwerden der Augenverletzung (trockenes Auge, remineszente Inflammation) ist rein symptombezogen [44]. Gegen eine eventuell auftretende Keratitis gibt es kaum Therapiemöglichkeiten [95, 18]. Der Einsatz von befeuchtenden Augentropfen, Corticosteroiden und Immunsuppresiva, sowie das Einsetzen medizinischer Kontaktlinsen können je nach Schwere der Keratitis Linderung bringen [13].

1.4.2 Therapie von Lungenverletzungen

Eine Exposition der Lunge mit S-Lost äußert sich ähnlich wie eine Rauchgasvergiftung [14]. Die Therapie der akuten Verletzung erfolgt symptomatisch. Zum Einsatz kommen Beatmung mit sauerstoffangereicherter Luft, sowie Hydrierung und Gabe von N-Acetylcystein (NAC) zur Viskositätserniedrigung von anfallendem Mucus. Beim Auftreten von Bronchospasmen ist die Gabe von Bronchiodilatoren indiziert. Bei schwer vergifteten Patienten kann eine Intubierung oder eine Tracheotomie notwendig werden [19, 44, 112]. Regelmäßige Bronchoskopien sind notwendig, um auftretende Stenosen der Atemwege und die Bildung von Pseudomembranen frühzeitig zu erkennen und zu beheben, da sie zu lebensbedrohlichen Komplikationen führen können [32, 112]. Die Therapie der *Bronchiolitis obliterans* erfolgt durch pulmonal applizierte Corticosteroide und β2-Agonisten, sowie die Gabe von Azithromycin und NAC [38, 2].

1.4.3 Therapie von Hautverletzungen

Die Schädigung der Haut durch S-Lost ist vergleichbar mit der toxisch epidermalen Nekrolyse und kann ähnlich wie Brandwunden versorgt werden [14]. S-Lost verursachte Hautblasen erfordern ein Laser-Débridement das das komplette, geschädigte Gewebe entfernen muss um Wundheilungsstörungen zu vermeiden. Durch Entfernung größerer Blasen kann außerdem eine Hauttransplantation notwendig werden [42, 43]. Die Inflammation kann gegebenenfalls durch eine topische Kombinationstherapie aus NSAID und Corticosteroid abgemildert werden [26].
Nach einer S-Lost-Verletzung kann chronischer Juckreiz auftreten, der mit Antihistaminika behandelt werden kann. Chronisch trockene Haut kann durch regelmäßiges Eincremen der betroffenen Stellen verbessert werden [95]. Außerdem muss eine adäquate Schmerztherapie durchgeführt werden [102].

1.4.4 Therapieempfehlungen

Oft kommt es bei S-Lost-Geschädigten zu einem behandlungsbedürftigen Pruritus, der teilweise nicht allein durch Antihistaminika behandelbar ist. Studien deuten darauf hin, dass der histaminunabhängige Pruritus durch eine TRPA1-Aktivierung verursacht sein könnte [104, 102], die scheinbar durch die Gabe von NAC behandelbar ist [103]. Weiterhin bildet S-Lost in der Haut ein Depot, von dem es in der Lage ist, nach und nach wieder in den Körper zu diffundieren [49], weshalb die Gabe von NAC

zu empfehlen ist, da es Schäden durch S-Lost abmildert und so zur Rekonvaleszenz beitragen kann [102, 12]. Allerdings ist der Wirkmechanismus von NAC noch nicht abschließend geklärt, da der bisher vermutete Scavenger-Effekt vor kurzem durch *in vitro* Versuche in Frage gestellt wurde [97].

1.5 Toxikodynamik

S-Lost ist ein bifunktionelles alkylierendes Agens, das nach Bildung des Sulfoniumions in der Lage ist, schnell mit Nucleophilen abzureagieren. Nucleophile Gruppen, die physiologisch im Körper vorkommen, sind Sulfhydryl-, Phosphat- und Carboxylgruppen, sowie zyklisch gebundener Stickstoff. S-Lost alkyliert und schädigt so Membranen, Proteine und DNA [8, 94, 34].
Trotz jahrzehntelanger Forschung ist der molekulare Mechanismus der S-Lost-Toxizität immer noch nicht vollständig verstanden. Allerdings war es möglich, mehrere der involvierten Schädigungsmechanismen zu identifizieren [34]. DNA-Alkylierung, Nicotinamidadenindinucleotid-(NAD)-Depletion, Modifikation von sulfhydrylhaltigen Proteinen und die Bildung von reaktiven Sauerstoffspezies (ROS) gelten als die plausibelsten Mechanismen [8].

1.5.1 DNA-Alkylierung

Jede Seitenkette des S-Lostes ist in der Lage, eine intramolekulare Zyklisierung einzugehen, wobei Chlorid als Abgangsgruppe fungiert und das reaktive Sulfoniumion entsteht. Der positiv geladene Ethylsulfoniumring reagiert bereitwillig mit Nucleophilen [58, 94]. S-Lost bildet mit DNA mono- und bifunktionelle Addukte [23]. Abbildung 4 zeigt beispielgebend die Reaktion von S-Lost mit dem N7-Atom von Guanin. Das genannte N7-Guanin-Addukt ist das bevorzugte Reaktionsprodukt und

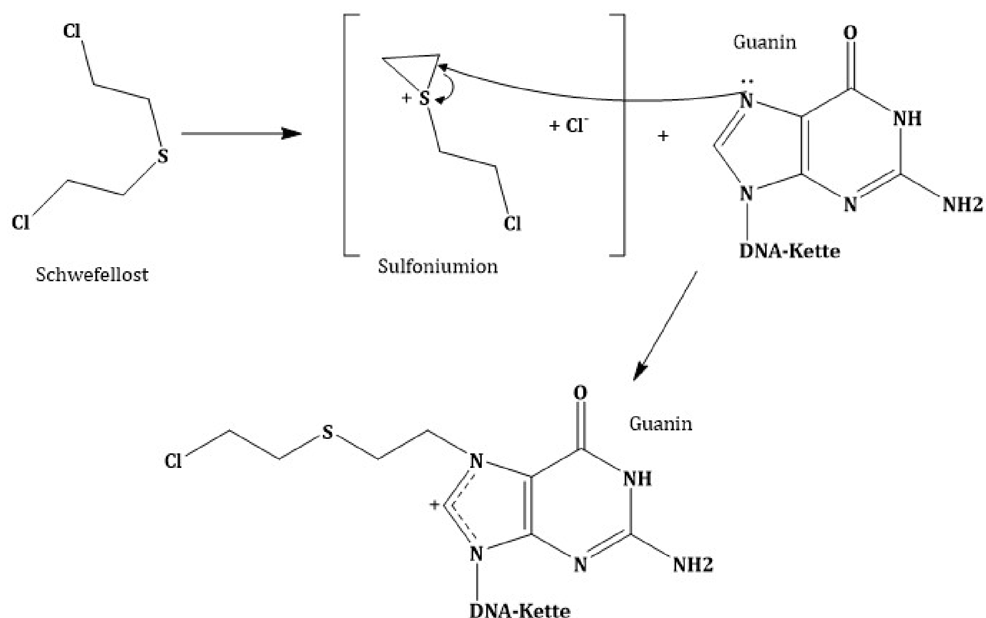


Abbildung 4: Die Reaktion von S-Lost mit Guanin am N7-Atom. Zuerst bildet sich das reaktive Sulfoniumion als Zwischenprodukt, das schnell mit Nucleophilen weiterreagiert. Im Beispiel fungiert das N7-Atom von Guanin als Nucleophil [94].

macht etwa 80 % aller S-Lost-DNA-Alkylierungen aus [117]. Weiterhin kommt es noch zur Alkylierung des Adenins an der N3-Position [64]. Die Alkylierung des Guanins an der O6-Position findet mit ca. 0,1 % nur im sehr geringen Ausmaß statt. Da aber die DNA-Reparaturmechanismen nicht in der Lage sind, diese modifizierte Base zu entfernen, ist diese DNA-Läsion als besonders kritisch anzusehen [66, 65]. Als bifunktionales S-Lost-DNA-Addukt tritt bis[2-(Guanin-7-yl)ethyl]sulfid (Bis-G) auf, das in bis zu 42 % aller Addukte massenspektrometrisch nachgewießen wurde [117, 62, 61]. Die Reaktion der Zelle auf eine S-Lost-Vergiftung ist vom Ausmaß der DNA-Alkylierung abhängig. Je nach Schwere der Vergiftung kommt es zum Zellzyklusarest, zur terminalen Differenzierung, zur Apoptose oder zur Nekrose [55, 99].

1.5.2 PARP-Signalkaskade

Die Familie der Poly-(ADP-Ribose)-Polymerase (PARP) katalysiert nicht nur die translationale Veränderung von Proteinen sondern ist an einer Vielzahl von biologischen Strukturen und Prozessen beteiligt. Unter anderem an der DNA-Reparatur und der Aufrechterhaltung der genomischen Stabilität [24]. Ein Zusammenhang zwischen der supepidermalen Blasenbildung und einer PARP-Aktivierung wurde erstmals 1985 von Papirmeister *et al.* aufgestellt. Er postulierte, dass die durch Endonucleasen ausgelösten Einzelstrangbrüche die PARP übermäßig aktivieren und die Zelle so an NAD^+ verarmt. Diese Verarmung löst dann Vorgänge aus, die zum Zelltod führen [81].
S-Lost kann auf dreierlei Weise einen DNA-Strangbruch auslösen: Erstens durch direkte Reaktion des S-Lostes mit DNA. Zweitens als Zwischenprodukt in der DNA-Reparatur und Drittens durch einen sekundären Anstieg der ROS-Level. Die Strangbrüche führen zur sofortigen Aktivierung der PARP-1 [28]. Auch eine durch S-Lost verursachte NAD-Depletion war nachweisbar und konnte zumindest teilweise auf eine PARP-Aktivierung zurückgeführt werden [52, 98]. Wie diese NAD-Depletion im Detail zur Schadwirkung des S-Lostes beiträgt, ist nicht bekannt. Eine Beteiligung der PARP an der Reparatur von S-Lost bedingten DNA-Läsionen scheint aber sicher [69, 17, 16]. Der Einsatz von PARP-Inhibitoren zur Therapie von S-Lost-Verletzungen sollte allerdings nur nach sorgfältiger Abwägung des Nutzen-Risiko-Verhältnisses erfolgen [69]. *In vitro* Versuche haben gezeigt, dass der Einsatz von PARP-Inhibitoren das allgemeine Zellüberleben nicht erhöht, da die Verringerung der Nekrose durch einen Anstieg in der Apoptose ausgeglichen wird [56, 89, 70]. Außerdem haben Klonogenitäts-Assays ergeben, dass PARP-Inhibitoren die Zellen gegen S-Lost sensibilisieren [69].

1.5.3 Oxidativer Stress

Ein Übermaß an reaktiven Spezies kann das Gleichgewicht zwischen Oxidantien und Antioxidantien in der Zelle stören und so oxidativen Stress verursachen. Reaktive Sauerstoffspezies (ROS) sind in der Lage Makromoleküle zu schädigen und führen zur Oxidation von DNA, Lipiden und Proteinen [74, 60]. Es gibt mehrere Quellen für S-Lost verursachte ROS. Zum einen führt S-Lost direkt zur Bildung von Radikalen mit Kohlenstoffzentrum, welche dann als Vorläufer für Superoxidanion-Radikale dienen [22, 21]. Das S-Lost-Analogon CEES (2-Chloroethylethylsulfid), das eine geeignete Modellsubstanz zur Erforschung von Radikalmechanismen des S-Lostes darstellt [21, 41], führte zur Schädigung der Mitochondrien, was im späteren Verlauf zur erhöhten ROS-Produktion führt [74, 41]. Außerdem führen Neutrophile Granulozyten und Makrophagen, die ins geschädigte Gewebe einwandern, zur Ausschüttung von ROS und reaktiven Stickstoffspezies (RNS) [60]. S-Lost vergiftete Keratinozyten (Ha-

CaT) zeigten *in vitro* die Aktivierung von endothelialen Stickstoffmonoxid-Synthasen (eNOS) und die vermehrte Expression von induzierbaren Stickstoffmonoxid-Synthasen (iNOS), die zur Bildung von RNS und ROS führen könnten [63, 101, 33].

1.5.4 Inflammation

Typisches Zeichen einer dermalen S-Lost-Vergiftung ist die Entzündung, die aufgrund der Konzentrierung oder Ausschüttung von vasoaktiven und chemokinen Mediatoren im betroffenen Gewebe zustande kommt [80, 57, 111]. Eine SM-Intoxikation führt zur Ausschüttung einer Vielzahl pro-inflammatorischer Zytokine. Die Ausschüttung wird größtenteils über den Nuklearen Transkriptionsfaktor kappa B (NFκB) ausgelöst [84]. Beispielsweise kommt es nach S-Lost Exposition zur Ausschüttung von Interleukin-(IL)-1β, -6, -8, Tumornekrosefaktor-alpha (TNFα), Granulozyten-Monozyten-Kolonie-stimulierenden Faktor (GM-CSF), Matrix-Metalloproteinase-9 (MMP-9) [7, 91, 92, 33, 63] und anderen pro-inflammatorischen Mediatoren wie etwa Histamin und Prostaglandin-E2 (PG-E2) [87]. Weiterhin führt eine SM-Exposition zur Induktion der Cyclooxygenase-(COX)-2 [114] und allgemein zur Aktivierung des NFκB-Signalweges [115].

2 Zielsetzungen der Arbeit

Auch wenn derzeit die Gefahr für einen terroristischen Angriff mit chemischen Waffen bei uns gering ist, geben wiederholte Giftgaseinsätze in Syrien doch Grund zur Beunruhigung. Sie verdeutlichen die Notwendigkeit für adäquate Therapiemöglichkeiten von Vergiftungen mit chemischen Kampfstoffen [107, 85]. Die Behandlung einer S-Lost-Vergiftung erfolgt symptomatisch. Eine kausale Therapie ist bis heute nicht möglich [112, 102]. Das Bestreben bzw. das Intersse der pharmazeutischen Industrie Antidote gegen chemische Kampfstoffe zu entwickeln ist gering, da die Kosten für Entwicklung und Zulassung hoch sind und der zu erwartende Ertrag gering ist. Weshalb die Forschung von staatlicher Seite erfolgen muss. Das Screening nach Antidoten bzw. die Evaluierung der aktuellen Therapievorschläge erfolgt vornehmlich in Zellkulturmodellen, um Tierversuche so weit als möglich zu vermeiden. Bei der Planung von *in vitro* Versuchen ist zu beachten, dass Zellen in Co-Kultur mit anderen Zellen anders reagieren können als die entsprechenden Zellen in Monokultur [105]. So stellt eine Co-Kultur aus Keratinozyten (HaCaT) und Fibroblasten (L929) ein besseres Modell für bestimmte Fragestellungen bei S-Lost-Toxizität dar als eine vergleichbare Monokultur [54]. Die Arbeitsgruppe von Frau Brunhilde Blömeke war in der Lage, die Vorhersagbarkeit von Sensibilisierungen durch potentielle Allergene im *in vitro* Modell zu verbessern, indem sie eine Co-Kultur aus Keratinozyten (HaCaT) und immunkompetenten Zellen (THP-1) anlegte [51].

2.1 Erster Teil der Arbeit: Validierung der Co-Kultur

Ziel der Arbeit war es zu verifizieren, ob das propagierte Co-Kultur-Modell aus Keratinozyten (HaCaT) und immunkompetenten Zellen (THP-1) bei S-Lost-Vergiftungen eine von der Monokultur unterscheidbare Reaktion zeigt und so die Physiologie der menschlichen Haut *in vitro* besser darstellt. Bei der Erfassung von S-Lost verursachten Schäden wurde eine bereits etablierte Testbatterie genutzt, die in der Lage ist, Nekrose, Apoptose und Inflammation im selben Zellansatz zu erfassen [50]. S-Lost löst in den Zellen Apoptose aus [53], während der es zur caspase-abhängigen Aktivierung von Endonucleasen kommt, die die DNA in spezifische Fragmente, die Nucleosomen, schneiden. Bei der Nekrose kommt es dagegen zum unspezifischen Abbau der DNA, die dann aufgrund der durchlässigen Zellmembran nach außen abgegeben wird [67, 86, 50]. Die Quantifizierung der apoptosespezifischen DNA-Histonkomplexe im Zelllysat erfolgte mit Hilfe des Cell Death Detection ELISA^{plus}(CDDE) [50]. Adenylatkinase (AK) ist ein ubiquitäres Enzym, dass an der Nukleotoid-Homöostase der Zelle beteiligt ist und jeweils nur in bestimmten Kompartimenten vorkommt [106]. AK katalysiert die Umsetzung von ADP zu ATP [15], was mit Hilfe der Luciferase im ToxiLight Biolumineszenz Assay nachgewiesen wird. Im Zelllysat ist AK direkt proportional zu der Zahl lebender Zellen; im Überstand ist sie direkt proportional zur Zahl nekrotischer Zellen [50]. Als Surrogat für die Inflammation diente die IL-6- und die IL-8-Ausschüttung, die für das Monitoring der Entzündung geeignet sind [6, 59, 11]. Zur Bestimmung der IL-6- und IL-8-Konzentrationen in den Überständen wurden ELISA genutzt [12, 11].

2.2 Zweiter Teil der Arbeit: Anwendung der etablierten Co-Kultur

Im weiteren Verlauf der Arbeit wurde das etablierte Co-Kultur-Modell der Haut seiner ersten Anwendung unterzogen, indem einige Substanzen gegen S-Lost-Vergiftungen getestet wurden. Dazu wurde das Modell mit simultaner Vergiftung heranzogen. D. h., dass die Keratinozyten (HaCaT) und immunkompetenten Zellen (THP-1) zuerst vergesellschaftet wurden um dann simultan vergiftet zu werden. Bei der Wahl der zu testenden Substanzen wurden folgende Überlegungen angestellt:

- Die Gabe eines PARP-Inhibitors war nicht in der Lage das allgemeine Zellüberleben in S-Lost exponierten HaCaT-Zellen zu erhöhen, sondern verursachte lediglich einen Shift von Nekrose zu Apoptose [56], weshalb sich im Projekt gegen die Untersuchung von PARP-Inhibitoren entschieden wurde.

- Die genutzte Testbatterie ist darauf optimiert Nekrose, Apoptose und Entzündungsmediatoren zu erfassen. S-Lost verursachte DNA-Schäden bzw. S-Lost verursachter Oxidativer Stress ist durch die genutzten Analysenmethoden maximal indirekt erfassbar und Aussagen zum Ausmaß der DNA-Alkylierung, bzw. dem oxidativen Status der Zellen sind nicht möglich. Deshalb wurde auf die Untersuchung von DNA-Protektoren und Antioxidantien vorerst verzichtet.

- Eine S-Lost Expositon der Haut löst Entzündungsreaktionen aus [55], weshalb die Gabe von Corticosteroiden [57] und nicht-steroidalen Antirheumatika (NSAIDs) [116, 4, 26] empfohlen wird. Da durch die Erfassung der IL-6- und IL-8-Ausschüttung der Verlauf einer S-Lost verursachten Entzündungsreaktion beurteilt werden kann [6, 59], wurde das evaluierte Testsystem genutzt um die Effektivität von anti-inflammatorischen Arzneistoffen zu evaluieren.

3 Zusammenfassung der Ergebnisse und Publikationen

3.1 Etablierung der HaCaT-THP-1-Co-Kultur

Bei der Etablierung der HaCaT-THP-1-Co-Kultur wurden zwei Versuchsansätze verfolgt. Zum einen wurden HaCaT-Zellen (erstmals 1988 beschrieben von Boukamp *et al.* [20]) im Vorfeld mit THP-1-Zellen (erstmals 1980 beschrieben von Tsuchiya *et al.* [108]) vergesellschaftet und miteinander vergiftet, um so die Physiologie der Haut *in vitro* besser darzustellen, da in der Haut wie in allen Geweben Macrophagen präsent sind und außerdem sind Langerhanszellen über die ganze Haut verstreut [1]. Zum anderen wurde eine HaCaT-Monokultur für eine Stunde mit S-Lost vergiftet und erst nach dem vollständigen Entfernen des Giftes erfolgte die Zugabe der THP-1-Zellen. Hier sollte dargestellt werden, welchen Einfluss gesunde Immunzellen auf den Verlauf der Vergiftung haben, da es im Körper bei Verletzungen oder der Anwesenheit von Pathogenen zum Einwandern von Makrophagen aus der Blutbahn in das Gewebe kommt [88].
Bei der Durchführung wurden 50.000 HaCaT-Zellen pro Well der 96-Well-Platte ausgesät und je nach Versuchsansatz zu unterschiedlichen Zeitpunkten mit einer unterschiedlichen Zahl an THP-1-Zellen beimpft. Die Zahl an THP-1-Zellen wurde jeweils im Verhältnis zu der Zahl ausgesäter HaCaT-Zellen gewählt und betrug 2; 3; 4,5; 6,75 und 10,125 %.

3.1.1 Vergiftung der HaCaT-Monokultur und Zugabe von unvergifteten THP-1-Zellen

Die Beimpfung einer S-Lost-vergifteten HaCaT-Monokultur mit unbehandelten THP-1-Zellen führte zu keiner nennenswerten Erhöhung der S-Lost-Toxizität. Bei der höchsten eingesetzten S-Lost-Konzentration von 300 μM kam es sogar zu einer Abnahme der Nekrose und Inflammation, die mit der THP-1-Konzentration zunahm. Die Abnahme der Nekrose war ab THP-1-Konzentrationen von 4,5 % signifikant. Die Abnahme der Inflammation, d. h. Produktion von IL-6 und IL-8, erreichte ab THP-1 Konzentrationen von 6,75 % Signifikanz. Dies lässt folgende Schlüsse zu:

- Die THP-1-Zellen führen im genutzten Modell *per se* nicht zu einer Schädigung der HaCaT-Zellen, was für die Eignung der THP-1-Zellen für das Zellkulturmodell spricht.
- Ungeschädigte Immunzellen sind in der Lage, den Verlauf einer S-Lost-Vergiftung abzumildern.
- Da der positive Effekt nur bei hohen S-Lost-Konzentrationen und erhöhten THP-1-Konzentrationen signifikant ist kann man vermuten, dass der protektive Effekt der THP-1-Zellen durch deren phagozytotischen Eigenschaften [108] verursacht wird, indem Zelltrümmer und apoptotische Vesikel entfernt werden.

3.1.2 Simultane Vergiftung der HaCaT- und THP-1-Zellen

Bereits die niedrigste Konzentration von 2 % THP-1-Zellen führte zu einer starken Zunahme der Nekrose, Apoptose und Inflammation im Vergleich zur HaCaT-Monokultur. Dieser Effekt war für alle eingesetzten Giftkonzentrationen sichtbar, während die THP-1-Zellen in den unvergifteten Kontrollen nur geringe Veränderungen auslösten. Eine Erhöhung des Verhältnisses von THP-1-Zellen zu HaCaT-Zellen über 2 % führte zu keiner Erhöhung der Toxizität des S-Lostes im Vergleich zu 2 % THP-1-Zellen. Dies führt zu folgenden Schlussfolgerungen:

- Vergiftete Immunzellen verstärken, wie erwartet, die Toxizität des S-Lostes und das Co-Kultur-Modell stellt eine bessere Annäherung an die Physiologie der Haut dar als eine HaCaT-Monokultur.
- Ein Verhältnis von THP-1- zu HaCaT-Zellen von 2 % ist für weitere Versuche ausreichend.
- Die THP-1-Zellen verstärken die S-Lost-Toxizität indem sie mit den HaCaT-Zellen interagieren und so deren Reaktion auf das Gift modulieren. Denn wenn der Effekt allein durch die THP-1-Zellen ausgelöst werden würde, müsste der Effekt mit der THP-1-Konzentration steigen.
- Ein paralleler Ansatz einer HaCaT-Monokultur zur HaCaT-Co-Kultur ist sinnvoll, um die Effekte der Immunzellen besser einschätzen zu können.

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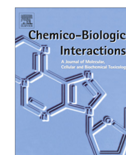

Development of a co-culture of keratinocytes and immune cells for in vitro investigation of cutaneous sulfur mustard toxicity

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A B S T R A C T

Sulfur mustard (SM) is a chemical warfare agent causing skin blistering, ulceration and delayed wound healing. Inflammation and extrinsic apoptosis are known to have an important role in SM-induced cytotoxicity. As immune cells are involved in those processes, they may significantly modulate SM toxicity, but the extent of those effects is unknown. We adapted a co-culture model of immortalized keratinocytes (HaCaT) and immune cells (THP-1) and exposed this model to SM. Changes in necrosis, apoptosis and inflammation, depending on SM challenge, absence or presence and number of THP-1 cells were investigated. THP-1 were co-cultured for 24 h prior to SM exposure in order to model SM effects on immune cells continuously present in the skin. Our results indicate that the presence of THP-1 strongly increased necrosis, apoptosis and inflammation. This effect was already significant when the ratio of THP-1 and HaCaT cells was similar to the ratio of Langerhans immune cells and keratinocytes in vivo. Any further increases in the number of THP-1 had only slight additional effects on SM-induced cytotoxicity. In order to assess the effects of immune cells migrating into skin areas damaged by SM, we added non-exposed THP-1 to SM-exposed HaCaT. Those THP-1 had only slight effects on SM-induced cytotoxicity. Notably, in HaCaT exposed to 300 µM SM, necrosis and inflammation were slightly reduced by adding intact THP-1. This effect was dependent on the number of immune cells, steadily increasing with the number of unexposed THP-1 added. In summary, we have demonstrated that (a) the presented co-culture is a robust model to assess SM toxicity and can be used to test the efficacy of potential antidotes in vitro; (b) immune cells, damaged by SM strongly amplified cytotoxicity, (c) in contrast, unexposed THP-1 (simulating migration of immune cells into affected areas after exposure in vivo) had no pronounced adverse, but exhibited some protective effects. Thus, protecting immune cells from SM toxicity may help to reduce overall injury.

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1. Introduction

Sulfur mustard (SM) is a chemical warfare that was used in several armed conflicts in the 20th century, e.g. World War I and most recently in the Iran–Iraq war in the 1980s. Despite intense efforts on chemical disarmament, large stockpiles still exist. Moreover, the agent is comparatively easy to synthesize. Thus, SM remains reason for strong concern. Even though the mortality among victims of SM exposure is comparatively low (2%, according to historic data), victims suffer from ulcerating, painful injuries, delays in wound healing and chronic illness that may affect eyes, respiratory system and skin [1,2]. Despite decades of medical research, no causative antidote exists. The pathophysiology of SM poisoning is highly complex, involving DNA and protein alkylation, dysfunctional enzymes, altered gene expression, excess formation of radical compounds and energy crisis, leading, dose-dependently to cell cycle arrest, apoptosis or necrosis [3]. All of these stages can be accompanied by inflammation [3]. Interactions on the intercellular level further contribute to this complexity and are likely to have a profound effect on the course of illness. Rosenthal et al. [4] found evidence that extrinsic apoptosis is a predominant pathway of apoptosis and overall cytotoxicity of cutaneous SM injury. Production of the pro-apoptotic cytokine Fas ligand may result in apoptosis of neighboring cells even when the latter had not or only

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reversibly been affected by primary SM-induced damage. A number of authors have demonstrated that interaction between immune cells and the major constituents of the particular tissue (i.e. keratinocytes in skin) is of crucial importance for the clinical course of injury and subsequent chronic illness. Arroyo et al. [5] had demonstrated that 100 μM hemi-mustard stimulates human monocytes to produce TNF-α, another pro-apoptotic cytokine. Gao et al. [6] demonstrated that SM concentration as low as 10 μM SM were sufficient to impair chemotaxis and phagocytosis in monocytes. Macrolide antibiotics were able to partially reverse that effect. Zarin et al. [7] investigated clinical samples from bronchiolitis obliterans (BO) patients that had either been exposed to SM in the 1980s or had BO of different etiology. They found increased levels of transforming growth factor beta-1 and -3 in samples from patients that had been exposed to SM. This was attributed to a more effective efferocytosis (phagocytotic removal of remnants from apoptotic cells) and considered a potential contributor to less severe courses of BO in SM-injured patients (compared to BO of different etiology). In summary, immune cells have an important role in the response to SM injury and may contribute to both aggravating (production of Fas ligand and/or TNF-α, inducing extrinsic apoptosis) and mitigating processes (phagocytosis and, in particular, efferocytosis). Thus, the role of immune cells in SM-injured tissues needs to be studied and/or modeled in cell cultures in order to establish more realistic models and to facilitate the search for effective antidotes. After own previous investigations of SM toxicity and potential therapeutics in HaCaT cells [8,9], our aim was to integrate an immunocompetent cell line into a HaCaT model. HaCaT cells [10,11] are a widely used model for keratinocytes. THP-1 cells are a monocytic cell line, initially isolated from a 1 year old leukemia patient. They were first described by Tsuchiya et al. in 1980 [12] who had listed the following monocytic properties: presence of alpha-naphthyl butyrate esterase activity, lysozyme production, phagocytotic activity and the ability to restore T-lymphocyte response to concanavalin A.

THP-1 monocultures had been used by Gao et al. to investigate SM-induced effects [6]. Tietze and Bloemeke had used THP-1 in sensitization assays [13] and concluded that THP-1 cells were suitable model to investigate immune reactions in skin. THP-1 cells were subsequently integrated into a co-culture model with HaCaT cells, for the first time demonstrating crosstalk between those two cell lines [14]. The aim of our study was to adapt and establish the co-culture model for investigations on SM toxicity. Thus, we had to determine, whether HaCaT monocultures and the HaCaT-THP-1 co-cultures responded differently to SM exposure. We intended to establish a concentration of THP-1 that was sufficient to produce effects significantly different from monocultures and at the same time as close as possible to the physiological ratio of immune cells to keratinocytes in vivo. The immune cells, continuously present in human skin are Langerhans cells and belong to the group of dendritic cells. Thus, their properties are somewhat different from monocytes. However, dendritic Langerhans cells originate from the differentiation of monocytes in vivo [15]. Hennen et al. had shown that a basal and induced cross-talk between THP-1 and HaCaT cells was observed and that this particular co-culture model was considered as a model for dendritic cells [14]. The ratio at which Langerhans cells occur in skin has been described as remarkably constant, close to 2% [16]. Based on that ratio we adjusted the number of THP-1 cells in our model (see discussion, Section 4.1 for details).

A final objective of our study was to model the effects of healthy (unexposed) immune cells, migrating into SM-injured skin. We thus introduced THP-1 into HaCaT populations that had previously been exposed to SM and compared the resulting effects, both to HaCaT monocultures and HaCaT-THP-1 co-cultures in which both cell lines were affected by SM.

2. Materials and methods

A detailed rationale for our methods, including cell lines chosen, exposure protocols and SM dosages is provided in the discussion, Section 4.1.

2.1. Cell culture

2.1.1. Initial co-culture model – resident immune cells

HaCaT cells purchased from cell lines service (cls, Eppelheim, Germany) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Karlsruhe, Germany), supplemented with 10% Fetal Calf Serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2 (subsequently referred to as "standard conditions"). No antibiotics were used. In each experiment 50,000 HaCaT cells per well were seeded on two 96-well-plates which were then incubated for approx. 24 h, allowing cell adherence and proliferation. DMEM was removed from all wells. THP-1 cells had been grown in flasks, suspended in RPMI medium, supplemented by 20% FCS and 0.1% [V/V] mercaptoethanol, subsequently referred to as THP-medium. By gentle wiping, THP-1 cells were fully suspended and the concentration of this cell suspension was determined using a CASY cell counter (Innovartis, Germany). 100 μl of the relevant THP-medium were applied to each well. The number of THP-1 cells per well was 1000, 1500, 2275, 3375 and 5062, respectively in order to co-cultivate HaCaT cells with 2, 3, 4.5, 6.75 and 10.125% THP-1. Wells intended for HaCaT monoculture just received 100 μl THP-medium. HaCaT and THP-1 cells were kept in co-culture for 24 h. As some of the THP-1 cells remain in suspension and adhesion of the other THP-1 cells (that have settled) is fairly weak, cell culture medium was not removed prior to SM exposure. 100 μl of THP-medium, containing the double SM concentrations, were added, exposing cells to 30 μM, 100 μM and 300 μM SM. Control groups received another 100 μl pure THP-medium. Exposed wells and controls were then incubated for 24 h. SM concentrations were chosen in accordance with previous studies and the overall consensus in literature that had established 100 μM SM as an acutely toxic (vesicating dose) [17].

2.1.2. Alternative co-culture model – unexposed immune cells

HaCaT cells were grown and seeded as described above. However, after 24 h of incubation in supplemented DMEM, cell culture medium was removed. HaCaT cells were exposed for 1 h to 30, 100 and 300 μM SM, dissolved in Minimum Essential Medium (MEM). Controls were kept in MEM for 1 h. Afterwards, MEM (with or without SM) was removed. 200 μl cell suspension, containing 1000, 1500, 2275, 3375 or 5062 THP-1 cells per well was added. Wells designated as HaCaT monocultures received 200 μl THP-medium.

2.2. Sampling

After 24 h of incubation, the 96-well-plates were centrifuged at 300*g* and 4 °C for 5 min. Supernatants were collected. Adherent cells were washed twice with phosphate-buffered-saline (PBS). For lysis, 200 μl 0.1% Triton X-100, dissolved in PBS was added. Cells were lysed for 30 min, cooled on ice and gently agitated on a plate shaker at 100 rpm. Lysates were collected.

2.3. Analysis of cell integrity (necrosis), apoptosis and inflammatory response

The detailed methodology of analysis was described previously [8,9]. In brief, the ToxiLight BioAssay (Lonza, Basel, Switzerland) was used to quantify adenylate kinase (AK) both in the supernatant

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($AK_{supernatant}$, representing loss of cell integrity) and in the lysate (AK_{lysate}, representing intact adherent cells). The ratio of $AK_{supernatant}$ and total AK (i.e. sum of $AK_{supernatant}$ + AK_{lysate}, representing the total number of cells) indicated the percentage of necrotic cells. Nucleosomes formed during apoptosis were quantified in the lysate of intact adherent cells using the Cell Death Detection Elisa Plus, CDDE (Roche, Basel, Switzerland). To account for expected variations in cell density induced by SM exposure, we calculated the ratio of nucleosome amount and AK_{lysate}, determined from the identical sample, to obtain the Apoptotic Index value, representing the apoptotic activity in intact cells at the end of the experiment. IL-6 and IL–8 levels were measured in the supernatants using ELISA kits from eBioscience, San Diego, USA according to the manufacturer's protocols. Absolute interleukin levels in the supernatant were measured and correlated to the amount of cells. In detail, the relative interleukin production is the ratio of interleukin levels found in the supernatant and the amount of total AK ($AK_{supernatant}$ + AK_{lysate}), which represents the combined amount of intact and disintegrated cells at the end of the experiment.

2.4. Statistical analysis

Data from three independent experiments with $n = 8$ for exposure groups and $n = 4$ for controls were normalized, using the mean of the sham-treated 300 µM SM group as the 100% reference value. Normalized data were pooled, resulting in $n = 24$ per exposure group and $n = 12$ for control groups. Significance was tested by One-Way ANOVA, followed by Bonferroni's Multiple Comparison test. p-Values below 0.05 were considered significant.

3. Results

All values in Section 3 are given as percentage values of the reference group (HaCaT monoculture, 300 µM SM exposure, e.g. 100% does not indicate a complete necrosis but a necrosis similar to the observation in HaCaT monocultures, exposed to 300 µM SM. Thus, values above 100% are possible, in particular in co-cultures and even for necrosis, when the particular parameter (necrosis, apoptosis etc.) exceeds the corresponding level in the reference group.

3.1. Necrosis in co-culture with SM-exposed THP-1 cells (Fig. 1A)

Necrosis in HaCaT monoculture increased with the dose of SM exposure. In co-cultures with 2% THP-1, necrosis increased significantly. In controls, however, this increase was slight (+5.6%). The increase of necrosis was more pronounced in co-cultures that were exposed to SM (+8.3% after 30 µM SM, +12.9% after 100 µM SM and +30.2% after 300 µM SM.) Any further increase of the number of THP-1 cells did only slightly change the extent of necrosis.

3.2. Necrosis in co-culture with unexposed THP-1 cells (Fig. 1B)

Exposure to SM led to an increased necrosis. 8.8% necrosis was observed in controls of HaCaT monoculture, increasing to 16.6% after 30 µM SM, 44.3% after 100 µM SM and 100.0% (i.e. the reference value, not necessarily a complete necrosis) after 300 µM SM.

At an SM concentration of 30 µM, unexposed THP-1 cells led to a slight increase in necrosis, which became only significant at high THP-1 concentrations (6.75% and 10.125%). At an SM concentration of 100 µM, the presence of unexposed THP-1 cells did not lead to any significant changes in necrosis; even though a tendency towards slight increases of necrosis was observed. At the highest SM concentration of 300 µM, a moderate decrease in necrosis occurred, which was significant at THP-1 concentrations of 4.5% and higher.

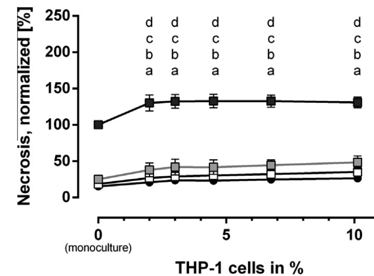

Fig. 1A. SM-exposed THP-1 cells amplify necrosis in co-culture with HaCaT cells. HaCaT cells were co-cultured with THP-1 cells for 24 h and subsequently exposed to 30, 100 or 300 µM SM. Cells culture models were incubated for 24 h. Necrosis was assessed by determination of adenylate kinase. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

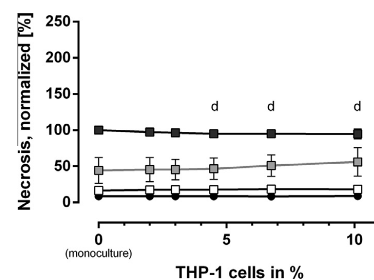

Fig. 1B. Unexposed THP-1 cells do not amplify necrosis in co-culture with SM-exposed HaCaT cells. HaCaT cells were grown for 24 h and subsequently exposed to 30, 100 or 300 µM SM for 1 h. Medium was removed and THP-1 cells suspended in fresh medium were added. Cells culture models were incubated for 24 h. Necrosis was assessed by determination of adenylate kinase. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

3.3. Apoptosis in co-cultures with SM-exposed THP-1 cells (Fig. 2A)

Apoptosis in monoculture controls was weak (3.1%), exposure of monocultures to 30 and 100 µM SM induced 10.7% and 15.5% apoptosis, respectively. Co-cultivation with THP-1 cells led to a significant increase of apoptosis (under control conditions or induced by 30 µM SM) only when using an excessive amount of THP-1 (10.125%). In wells exposed to 100 µM SM, no significant change between HaCaT monoculture and any co-culture was observed.

However, 300 µM SM induced strong apoptosis in monocultures (100%, reference value), i.e. a 6-fold increase compared to the 100 µM SM group and a 33-fold increase in comparison to controls. Co-cultivation with 2% THP-1 cells led to a pronounced and highly significant increase to 194% of the monoculture and reference group. Co-cultivation with higher amounts of THP-1 cells resulted in apoptosis that was still significantly stronger, compared to monocultures, but not significantly changed from apoptosis observed in the 2% THP-1 co-culture. In fact, when the amount of THP-1 was increased, apoptosis was even slightly, yet non-significantly decreased.

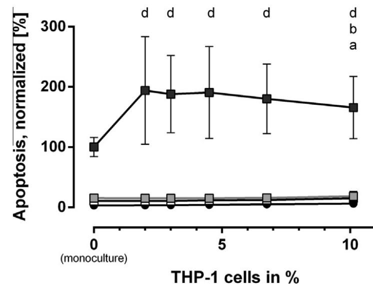

Fig. 2A. THP-1 cells exposed to 300 μM SM amplify apoptosis in co-culture with HaCaT cells. HaCaT cells were co-cultured with THP-1 cells for 24 h and subsequently exposed to 30, 100 or 300 μM SM. Cells culture models were incubated for 24 h. Apoptosis was assessed by determination of nucleosome formation. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

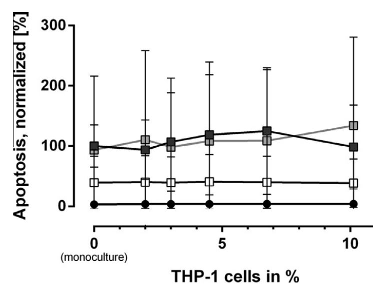

Fig. 2B. Unexposed THP-1 cells do not amplify apoptosis in co-culture with SM-exposed HaCaT cells. HaCaT cells were grown for 24 h and subsequently exposed to 30, 100 or 300 μM SM for 1 h. Medium was removed and THP-1 cells suspended in fresh medium were added. Cells culture models were incubated for 24 h. Apoptosis was assessed by determination of nucleosome formation. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. No significant changes were observed.

3.4. Apoptosis in co-culture with unexposed THP-1 cells (Fig. 2B)

Apoptosis in the controls was weak (3.11%). Exposure to SM led to an increase of apoptosis in monocultures (39.27% after 30 μM SM, 93.57% after 100 μM, and 100.0% after 300 μM SM, respectively), whereas the presence of unexposed THP-1 cells in concentrations up to 10.125% showed no significant influence on apoptosis.

3.5. IL-6 production in co-culture with SM-exposed THP-1 cells (Fig. 3A)

IL-6 production in monoculture controls was weak at 1.1% of the reference value. Exposure of monocultures to 30 and 100 μM SM increased IL-6 production to 2.8% and 9.3% of the reference value, respectively. Co-cultures with THP-1 produced more IL-6 than monocultures (6.7% in controls, 11.7% after 30 μM SM, 51.9% after 100 μM SM). With increasing SM concentration, the difference between co-cultures and monocultures was more pronounced. 2% THP-1 cells were sufficient to induce significant changes in IL-6 production and even though IL-6 productions tended to increase with the amount of THP-1 cells, no significant differences among the co-cultures, exposed to the same SM concentration was observed.

300 μM SM induced a strong IL-6 production, even in monocultures. At 100% of the reference value (per definition), this was an approx. 90-fold increase from monoculture controls and an 11-fold increase from monocultures, exposed to 100 μM SM. However, co-cultivation with just 2% THP-1 cells, resulted in an even more pronounced increase of IL-6 production of up to 209.6%. Any further increase of the amount of THP-1 cells slightly, but steadily, albeit non-significantly decreased IL-6 production from its peak level observed in 2% THP-1 co-cultures.

3.6. IL-6 production in co-culture with unexposed THP-1 cells (Fig. 3B)

In controls, there was only a weak IL-6 production (6.16%). Exposure of monocultures resulted in increases of IL-6 production to 37.85% after 30 μM SM, 171.3% after 100 μM SM and (by definition) 100.0% after 300 μM. Notably, in this protocol IL-6 production was most intense after 100 μM SM. The relative decrease, observed from 100 to 300 μM SM does not reflect an "anti-inflammatory" effect, but severe cell damage after 300 μM SM, reducing their

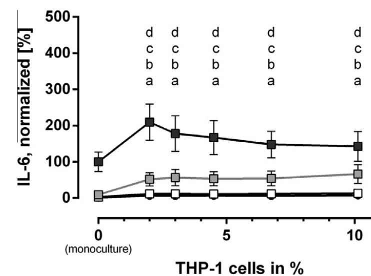

Fig. 3A. THP-1 cells exposed to SM amplify IL-6 production in co-culture with HaCaT cells. HaCaT cells were co-cultured with THP-1 cells for 24 h and subsequently exposed to 30, 100 or 300 μM SM. Cells culture models were incubated for 24 h. IL-6 production was determined by ELISA. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

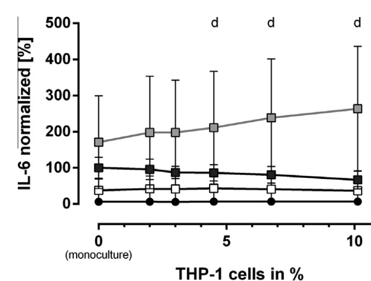

Fig. 3B. Unexposed THP-1 only slightly amplify IL-6 production in co-culture with HaCaT cells exposed to 100 μM SM and significantly reduce IL-6 production after 300 μM SM exposure. HaCaT cells were grown for 24 h and subsequently exposed to 30, 100 or 300 μM SM for 1 h. Medium was removed and THP-1 cells suspended in fresh medium were added. Cells culture models were incubated for 24 h. IL-6 production was determined by ELISA. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

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ability to produce IL-6. The presence of unexposed THP-1 cells did not lead to changes in the IL-6 production in controls and after 30 μM SM exposure. At an SM concentration of 100 μM, there was a tendency towards increased IL-6 production, which, however, did not reach significance. Following exposure to 300 μM SM, there was a decrease in IL-6 production, which was more pronounced at higher concentrations of unexposed THP-1 cells. This effect reached significance at a THP-1 concentration of 6.75% and is highly significant at 10.125%.

3.7. IL-8 production in co-culture with SM-exposed THP-1 cells (Fig. 4A)

Unlike IL-6, IL-8 is produced in considerable amounts (30.1%) even in monocultures under control conditions. Exposure of monocultures to 30 μM left IL-8 production almost unchanged at 30.3%. Exposure of monocultures to 100 μM increased IL-8 to 47.8%. 300 μM SM on monocultures induced 100% IL-8 production (by definition) which is just a 3-fold increase from controls.

Co-cultivation with 2% THP-1 induced a pronounced and significant increase of IL-8 production to 124.7% in controls, 105.7% after 30 μM SM, 129.9% after 100 μM SM and 171.0% after 300 μM. Any further increase of THP-1 cells did not result in significant differences among co-cultures. Under control conditions, 30 or 100 μM SM, there was a tendency of IL-8 production increasing with THP-1. However, under 300 μM SM, co-cultures with 2% THP-1 produced a peak amount of IL-8 per cell and IL-8 production in all the other co-cultures is slightly, albeit non-significantly lower.

3.8. IL-8 production in co-culture with unexposed THP-1 cells (Fig. 4B)

The baseline secretion of IL-8 was already high in controls (60.74%) but still increased after SM exposure, to 86.10% after 30 μM SM; 180.48% after 100 μM and 100% after 300 μM. As seen before (i.e. IL-6), the IL-8 production after 100 μM SM is higher compared to wells exposed to 300 μM SM. The presence of unexposed THP-1 cells did not lead to significant changes in the IL-8 production in controls and wells exposed to 30 μM SM. Following exposures to 100 μM SM, there was a tendency toward increased IL-8 production, which did, however, not reach significance. In cells exposed to 300 μM SM, there was a decrease in IL-8 production, which again was growing with increasing concentrations of unexposed THP-1 cells. This effect reached significance at a THP-1 concentration of 6.75% and was highly significant at 10.125%.

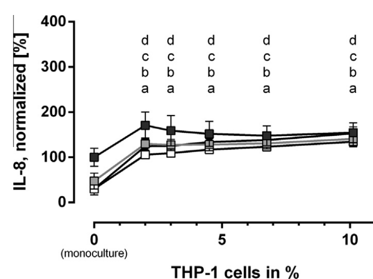

Fig. 4A. THP-1 cells exposed to SM amplify IL-8 production in co-culture with HaCaT cells. HaCaT cells were co-cultured with THP-1 cells for 24 h and subsequently exposed to 30, 100 or 300 μM SM. Cells culture models were incubated for 24 h. IL-8 production was determined by ELISA. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

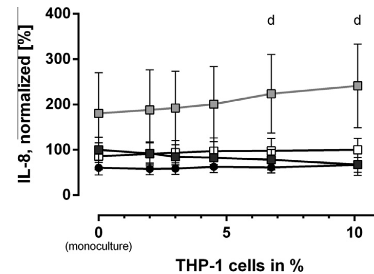

Fig. 4B. Unexposed THP-1 only slightly amplify IL-8 production in co-culture with HaCaT cells exposed to 100 μM SM and significantly reduce IL-8 production after 300 μM SM exposure. HaCaT cells were grown for 24 h and subsequently exposed to 30, 100 or 300 μM SM for 1 h. Medium was removed and THP-1 cells suspended in fresh medium were added. Cells culture models were incubated for 24 h. IL-8 production was determined by ELISA. Symbols represent the mean of each group ($n = 24$ for SM-exposed cultures or $n = 12$ for controls). Error bars indicate standard deviations. Letters indicate significant changes between monocultures and co-cultures exposed to identical SM concentrations. Quantitative data are summarized in the table with bold numbers indicating significant changes. Significance was assessed by One-Way ANOVA and Bonferroni's Multiple Comparison with $p < 0.05$ considered significant.

4. Discussion

4.1. Rationale for the use of cell lines and SM doses

Our aim in this study was to establish a co-culture model, capable to deliver reproducible results. THP-1 cells, derived from a leukemia patient, exhibit somewhat different properties in comparison to monocytes from healthy donors. Nevertheless, THP-1 cells have been well established as an in vitro model for monocytes in cell culture experiments with some advantages compared to healthy donor monocytes. The latter are inevitably associated with inter-individual variations. Moreover, a clinically healthy donor may not be aware of subclinical infections and/or inflammatory processes which may nevertheless influence the properties and mode of action of their monocytes. Thus, the use of THP-1 cells instead of healthy donor monocytes, was favoured, aiming for reproducibility, potentially at the expense of a slight difference to the in vivo situation. The properties of THP-1 described by Tsuchiya et al. [12], closely resembling some key properties of healthy monocytes and the well-established use of THP-1 support this approach.

Prior to our experiments, we had two hypotheses, predicting very different effects of the presence of THP-1. Initially, we expected THP-1 to contribute to extrinsic apoptosis and inflammation, either by directly producing pro-apoptotic or pro-inflammatory cytokines or by stimulating their production in HaCaT cells. In both cases, amplification of apoptosis and inflammation was expected. A contrary hypothesis predicted that THP-1 may have the ability to eliminate cell debris by phagocytosis. The elimination of apoptotic bodies is specifically known as efferocytosis. In clinical situations, Zarin et al. had described that efferocytosis was a beneficial mechanism, contributing to a relatively mild course of illness in patients suffering from the chronic effects of SM exposure. If similar effects were to occur in our model, the addition of THP-1 cells should result in a decrease of cytotoxic parameters. In this study we used two variations of the co-culture model. Immune cells are continuously present in the human skin. In case of local SM exposure those resident immune cells will also be affected by SM toxicity, thus, in the initial protocol they had to be present at the

time of exposure. The ratio at which Langerhans cells occur in skin has been described as remarkably constant, close to 2%. We chose 2% as the minimum ratio for THP-1 in co-cultures. Initially assuming that immortalized THP-1 might exert a weaker activity, compared to primary immune cells, we decided to test higher ratios of THP-1/HaCaT, increasing it repeatedly by the factor 1.5–3%, 4.5%, 6.75% and 10.125%. Another rationale for increasing the number of THP-1 was the assumption that additional immune cells might be attracted by the inflammatory effects of SM injury, even at a time when SM might still be present. In accordance with previous studies, 50,000 HaCaT cells per well were seeded, thus the corresponding number of THP-1 varied from 1000 (2%) to 5062 per well (10.125%). For comparison, a mono-culture of HaCaT cells was kept on the same plate, under identical conditions (including cell culture medium and SM challenge), but in the absence of THP-1 cells. In a variation of the primary protocol, we exposed HaCaT cells to SM, removed the poisoned cell culture medium and added unexposed THP-1 cells in ratios and numbers, similar to the previous protocol. The intention was to investigate the activity of immune cells that had remained unexposed and unaffected by primary SM damage.

4.2. Effect of SM poisoning on resident immune cells

Even under control conditions, 2% THP-1 cells induced slight, yet significant increases of necrosis, apoptosis and IL-6. Exposure of monocultures to 30, 100 and 300 μM SM also resulted in a significant increase of those parameters of cytotoxicity. However, when cell cultures were exposed to SM, the difference between monocultures and co-cultures became much more evident and pronounced and increased further with higher SM concentrations. The combined effects, i.e. increase of cytotoxicity in SM-exposed co-cultures was much larger than the sum of isolated effects, i.e. SM exposure in monocultures plus THP-1 cell in controls. In summary, there was a synergistic, over-additive amplification of SM toxicity when THP-1 cells were also present at the time of exposure. We thus have to assume that resident immune cells, affected by local exposure to SM, amplify tissue damage and certainly cannot contribute to any mitigating mechanisms.

Considering the origin of THP-1 cells and their precursor-like stage of development, their mode of action may be somewhat biased towards a pro-inflammatory macrophage phenotype by producing IL-1. This bias is acceptable for practical reasons.

In future studies testing the effect of anti-inflammatory or other potentially protective drugs, a slight pro-inflammatory bias is equivalent to a conservative approach. If the approach is in fact biased, it is biased on the side of caution. If a candidate drug is capable to counteract inflammation in this rather pro-inflammatory setting, it is likely that this effect will be reproducible in animal studies and – more importantly in a real clinical situation. This conservative approach is even more important, considering that the efficacy of antidotes against SM cannot be assessed in clinical efficacy studies for obvious ethical reasons. Efficacy has to be assessed from animal and cell-culture experiments and a rather conservative approach is justified, even necessary.

Moreover, anti-inflammatory effects of unexposed THP-1 cells suggested that a pro-inflammatory bias should not be excessive, see Section 4.6 for details.

4.3. Effect of SM exposure in co-cultures with artificially high concentrations of THP-1 cells

While cytotoxic effects under identical SM concentrations dramatically increased from monocultures to co-cultures with 2% THP-1, further increase of THP-1 did not produce significant additional effects. Thus, in order to investigate the effect of SM-exposed immune cells in co-cultures, there is no need to increase THP-1 concentrations beyond 2%, a level that corresponds to the physiological concentration of Langerhans cells in healthy human skin. Significant effects of artificially high concentrations of immune cells (in comparison to physiological concentrations) were not observed and it thus it seems unlikely that additional knowledge could be gained from a subsequent use of those artificial conditions.

4.4. Mechanism behind amplified cytotoxicity in HaCaT – THP-1 co-cultures

However, above-mentioned experiments may have provided useful insight into the overall mechanism of amplified SM toxicity in co-cultures. Initially, these two hypotheses seemed acceptable: THP-1 cells may have been interacting actively with HaCaT cells to amplify inflammation, apoptosis and overall cytotoxicity, by either producing pro-inflammatory and pro-apoptotic cytokines or by stimulating their production in HaCaT cells. This could be considered the "active interaction hypothesis". In contrast, one might also assume that THP-1 cells were simply destroyed by SM, releasing highly aggressive digestive enzymes into the supernatant which in turn amplified the damage inflicted on HaCaT cells. This alternative assumption might be considered a "passive mechanism hypothesis". If, however, that passive mechanism had any significant effect on the overall cytotoxicity, we would expect a much stronger correlation between the number of THP-1 cells and the cytotoxic effects, inflicted by identical SM challenges. It would be reasonable to expect that the SM-induced destruction of a 5-fold number of THP-1 (10.125%) cells would release much larger amounts of digestive enzymes and induce a significantly stronger increase in cytotoxicity compared to co-cultures with 2% THP-1. As mentioned above, this was not the case and thus, an active interaction between THP-1 and HaCaT cells is the plausible mechanism to explain amplified cytotoxicity in SM-exposed co-cultures.

Moreover, the evaluation of experiments with varied numbers of THP-1 also indicates, whether HaCaT or THP-1 cells were mainly responsible for the production of interleukins. If those cytokines were, to a significant degree, produced by THP-1 themselves, we could expect a strong, possibly linear correlation between the number of THP-1 and the interleukin production. Again, this was not the case; interleukin production was strongly amplified by 2% of SM-exposed THP-1, whereas cell cultures with large amounts of THP-1 did not differ significantly from the 2% group. When adding healthy THP-1 to severely (300 μM) exposed HaCaT cells, interleukin production even decreased. Thus, we have to assume that IL-6 and IL-8 are mainly produced by HaCaT cells, even in co-culture with THP-1. THP-1 cells initiate a crosstalk with HaCaT cells, stimulating the latter to increase interleukin production. This is in agreement with previous studies by Hennen et al. [14] and Klein et al. [18].

4.5. Pro-apoptotic effects of SM-exposed THP-1 cells

Following exposure of a HaCaT-THP-1 co-culture to 300 μM SM, a distinct amplification of apoptosis was observed in the presence of SM-exposed THP-1 cells. This amplified apoptosis also affected HaCaT cells and thus, it is by definition an extrinsic apoptosis, induced or amplified by another cell (and cell type) than the target cell.

According to Sarkar et al. [19], donor-derived monocytes exerted extrinsic apoptosis in vascular smooth muscle cells. The mechanism was described to be independent of classic pathways of extrinsic apoptosis, e.g. Fas ligand, interleukin-1β or interleu-

kin-18. In fact, a transfer of caspase-1 through microvesicles was observed.

Our experimental setting is different from those used by Sarkar et al.: THP-1 cells were used instead of donor monocytes, HaCaT cells instead of vascular smooth muscle cells and sulfur mustard, rather than endotoxin as the original trigger of cytotoxicity. Thus, we currently cannot rule out Fas Ligand, IL-1β or IL-18 as contributors to the extrinsic apoptosis after SM poisoning. In particular, a number of authors have shown Fas ligand to have an important role after SM poisoning, including Rosenthal et al. [4], Pirzad et al. [20], and Keyser et al. [21]. Clarification of the exact mechanism of extrinsic apoptosis, exerted by immune cells after SM exposure remains a rewarding subject for further investigation.

4.6. Effect of unexposed immune cells on SM-exposed HaCaT cells

In this study, we modeled migration of unexposed, largely intact immune cells into SM-exposed tissue by exposing HaCaT cells to SM, removing poisoned medium and subsequent addition of specified amounts of THP-1 cells. While the number of resident immune cells is fairly constant, migration of immune cells will depend on multiple factors, including extent of inflammation, overall strength of the immune system, migratory ability of immune cells etc., leading to different, and often highly increased populations of immune cells within the damaged tissue. Thus, the varied concentrations of THP-1 used in these experiments were not just useful to validate the model, but might actually reflect clinical situations, following SM injury, inflammation and a strong response of the immune system. The most evident result is that – unlike the previous experiment, when THP-1 cells were exposed to SM – there is no synergistic increase of cytotoxic effects. Addition of THP-1 to controls and HaCaT cells exposed to 30 μM SM did not affect cytotoxicity at all. Necrosis, apoptosis and interleukin production remain unchanged. In HaCaT cells, exposed to 100 μM SM, necrosis, apoptosis and inflammation increased slightly, but not significantly with the number of THP-1 cells. This could be seen as a (relatively weak) indicator that crosstalk between moderately poisoned keratinocytes and healthy immune cells still results in a slight amplification of active cytotoxic processes after SM exposure (in particular, apoptosis and inflammation). The clinical relevance of this trend may be limited, and clinical consequences should be very different from the near-catastrophic amplification of cytotoxicity we observed when THP-1 and HaCaT cells were simultaneously exposed to SM. When healthy THP-1 were added to severely poisoned HaCaT cells (exposed to 300 μM), this even resulted in slight, yet beneficial effects. Necrosis was reduced significantly, when 4.5% or more THP-1 were added. The tendency towards reduced apoptosis did not reach significance. However, IL-6 and IL-8 productions slightly but steadily declined with increasing numbers of THP-1, significance was reached when 6.75% or more THP-1 were added. The largest amount of THP-1 cells decreased IL-6 and IL-8 production by approx. one third. While the effects of lower THP-1 concentrations and the effects on necrosis in general might be considered weak and their clinical relevance questioned, they constitute a most pronounced contrast to the aggravating effects observed from THP-1 that were exposed to SM: while SM-exposed THP-1 strongly aggravate the damage induced by 300 μM SM, healthy THP-1 actually contribute to limit the cytotoxicity. The mechanism responsible for the beneficial effects of healthy THP-1 is not fully understood, but 300 μM SM is certainly a strong SM challenge, inducing both active (apoptosis, inflammation) and passive (excess radical formation, energy crisis, necrosis) processes. THP-1 cells are known to have phagocytotic abilities and the removal of debris (from necrotic cell death) or apoptotic bodies (from apoptotic HaCaT cells) should contribute to reduce inflammation and secondary cellular damage.

Finally, the anti-inflammatory effect of unexposed THP-1 cells in severely (300 μM) SM-exposed HaCaT cells alleviates some of our initial concern about a potential bias of THP-1 that may lead to more pro-inflammatory reaction than healthy monocytes. This potential bias may still exist, but it is apparently not very strong: even the potentially pro-inflammatory THP-1 were in fact able to reduce inflammation in HaCaT cells, immediately after a strong pro-inflammatory challenge, further supporting the notion that a bias may be limited and THP-1 are in fact a close approximation of the in vivo conditions.

4.7. Usability of the co-culture model in subsequent studies on candidate substances

Considering the constant presence of resident immune cells in human skin and the pronounced aggravating effects when immune cells were affected by SM, we have to consider the co-culture model the more valid approach to assess SM cytotoxicity. In subsequent investigations to test candidate substances for their efficacy against SM toxicity, the co-culture model should definitely be used and should produce the more relevant results. This is particularly true for anti-inflammatory compounds, the effects of which in SM-exposed co-cultures will be our subsequent objective of investigation. Moreover, substances that influence extrinsic apoptosis, e.g. antagonists of Fas ligand, caspase inhibitors etc. should also be investigated in this co-culture model. Monoculture experiments, especially, when carried out simultaneously, may still be useful to understand whether any protective effects (or an opposite thereof) result from drug interaction with keratinocytes and/or immune cells.

We also demonstrated that 2% THP-1, closely corresponding to physiological amounts of resident immune cells in human skin significantly aggravated cytotoxicity when exposed to SM and excess concentrations of (resident) immune cells did not act significantly different from the 2% concentration. Thus, the co-culture model can be considered robust, i.e. the overall response being largely independent from the precise number of THP-1 cells. However, in an effort to model in vivo conditions as closely and realistic as possible, the 2% concentration of THP-1 should be preferred to conduct future experiments.

4.8. Potential clinical relevance

The previous findings may have profound clinical consequences as the state of the systemically distributed immune cells, capable of migration to damaged tissues may well determine the clinical course of local effects: while poisoned immune cells are most likely to aggravate cellular and tissue damage, healthy immune cells may contribute to recovery. Efforts to reduce the systemic toxic burden of SM exposure such as swift decontamination, but also systemic administration of scavengers (e.g., n-acetylcysteine) may initially have a limited effects to local tissues already exposed, but at the same time protect the overall immune system. Immune cells affected by systemic poisoning will be likely to aggravate the course of illness while healthy immune cells might indeed facilitate the recovery from SM injury. Thus, therapeutic interventions to protect immune cells against systemic SM toxicity may reduce the severity and duration of SM-induced injuries, both locally and systemically. Moreover, therapeutic compounds, administered after SM exposure should preferably be able to modulate the action of immune cells in order to limit excessive inflammation and extrinsic apoptosis. The efficacy of anti-inflammatory drugs to achieve this, along with their safety in a SM-exposed environment should also be investigated in this co-culture model.

Conflict of Interest

The authors declare that they have no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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References

[1] K. Kehe, H. Thiermann, F. Balszuweit, F. Eyer, D. Steinritz, T. Zilker, Acute effects of sulfur mustard injury – Munich experiences, Toxicology 263 (2009) 3–8.
[2] M. Rowell, K. Kehe, F. Balszuweit, H. Thiermann, The chronic effects of sulfur mustard exposure, Toxicology 263 (2009) 9–11.
[3] K. Kehe, F. Balszuweit, D. Steinritz, H. Thiermann, Molecular toxicology of sulfur mustard-induced cutaneous inflammation and blistering, Toxicology 263 (2009) 12–19.
[4] D.S. Rosenthal, A. Velena, F.P. Chou, R. Schlegel, R. Ray, B. Benton, D. Anderson, W.J. Smith, C.M. Simbulan-Rosenthal, Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard, J. Biol. Chem. 278 (2003) 8531–8540.
[5] C.M. Arroyo, R.L. Von Tersch, C.A. Broomfield, Activation of alpha-human tumour necrosis factor TNF-alpha) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulphide (H-MG), Hum. Exp. Toxicol. 14 (1995) 547–553.
[6] X. Gao, R. Ray, Y. Xiao, K. Ishida, P. Ray, Macrolide antibiotics improve chemotactic and phagocytic capacity as well as reduce inflammation in sulfur mustard-exposed monocytes, Pulm. Pharmacol. Ther. 23 (2010) 97–106.
[7] A.A. Zarin, M. Behmanesh, M. Tavallaei, M. Shohrati, M. Ghanei, Overexpression of transforming growth factor (TGF)-beta1 and TGF-beta3 genes in lung of toxic-inhaled patients, Exp. Lung Res. 36 (2010) 284–291.
[8] A. Heinrich, F. Balszuweit, H. Thiermann, K. Kehe, Rapid simultaneous determination of apoptosis, necrosis, and viability in sulfur mustard exposed HaCaT cell cultures, Toxicol. Lett. 191 (2009) 260–267.
[9] F. Balszuweit, H. John, A. Schmidt, K. Kehe, H. Thiermann, D. Steinritz, Silibinin as a potential therapeutic for sulfur mustard injuries, Chem. Biol. Interact. 206 (2013) 496–504.
[10] P. Boukamp, R.T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, N.E. Fusenig, Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line, J. Cell Biol. 106 (1988) 761–771.
[11] D. Breitkreutz, H.J. Stark, P. Plein, M. Baur, N.E. Fusenig, Differential modulation of epidermal keratinization in immortalized (HaCaT) and tumorigenic human skin keratinocytes (HaCaT-ras) by retinoic acid and extracellular Ca2+, Differentiation 54 (1993) 201–217.
[12] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, K. Tada, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1), Int. J. Cancer 26 (1980) 171–176.
[13] C. Tietze, B. Blomeke, Sensitization assays: monocyte-derived dendritic cells versus a monocytic cell line (THP-1), J. Toxicol. Environ. Health A 71 (2008) 965–968.
[14] J. Hennen, P. Aeby, C. Goebel, T. Schettgen, A. Oberli, M. Kalmes, B. Blomeke, Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization, Toxicol. Sci. 123 (2011) 501–510.
[15] K. Nagao, T. Kobayashi, K. Moro, M. Ohyama, T. Adachi, D.Y. Kitashima, S. Ueha, K. Horiuchi, H. Tanizaki, K. Kabashima, A. Kubo, Y.H. Cho, B.E. Clausen, K. Matsushima, M. Suematsu, G.C. Furtado, S.A. Lira, J.M. Farber, M.C. Udey, M. Amagai, Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin, Nat. Immunol. 13 (2012) 744–752.
[16] J. Bauer, F.A. Bahmer, J. Worl, W. Neuhuber, G. Schuler, M. Fartasch, A strikingly constant ratio exists between Langerhans cells and other epidermal cells in human skin. A stereologic study using the optical disector method and the confocal laser scanning microscope, J. Invest. Dermatol. 116 (2001) 313–318.
[17] W.J. Smith, K.M. Sanders, S.E. Ruddle, Cytometric analysis of DNA changes induced by sulfur mustard, Proceedings of the U.S. Army Medical Defense Bioscience Review, US Department of Commerce, Baltimore, MD, 1993, pp. 189–198.
[18] S.G. Klein, J. Hennen, T. Serchi, B. Blomeke, A.C. Gutleb, Potential of coculture in vitro models to study inflammatory and sensitizing effects of particles on the lung, Toxicol. In Vitro 25 (2011) 1516–1534.
[19] A. Sarkar, S. Mitra, S. Mehta, R. Raices, M.D. Wewers, Monocyte derived microvesicles deliver a cell death message via encapsulated caspase-1, PLoS ONE 4 (2009) e7140.
[20] G. Pirzad, M. Jafari, S. Tavana, H. Sadrayee, S. Ghavami, A. Shajiei, M. Ghanei, The role of Fas-FasL signaling pathway in induction of apoptosis in patients with sulfur mustard-induced chronic bronchiolitis, J. Toxicol. 2010 (2010) 373612.
[21] B.M. Keyser, D.K. Andres, E. Nealley, W.W. Holmes, B. Benton, D. Paradiso, A. Appell, C. Carpin, D.R. Anderson, W.J. Smith, R. Ray, Postexposure application of Fas receptor small-interfering RNA to suppress sulfur mustard-induced apoptosis in human airway epithelial cells: implication for a therapeutic approach, J. Pharmacol. Exp. Ther. 344 (2013) 308–316.

3.2 Testen der Effektivität von anti-inflammatorischen Arzneistoffen gegen S-Lost-Toxizität

In dem zuvor entwickelten Co-Kultur-Modell (simultane Vergiftung der HaCaT- und THP-1-Zellen) wurde nun die Effektivität von anti-inflammatorischen Arzneistoffen getestet. Außerdem wurde der gleiche Versuch mit einer HaCaT-Monokultur durchgeführt, um die Effekte der Immunzellen auf die Keratinozyten besser erkennen zu können. Die Behandlung mit Arzneistoff erfolgte eine Stunde nach der Vergiftung der Zellen mit S-Lost. Getestet wurden Dexamethason, Ibuprofen und Diclofenac in Konzentrationen, die angelehnt sind an Therapieempfehlungen und daraus resultierenden Plasmaspiegeln im Blut.
Dexamethason zeigte dabei leicht positive Effekte in Bezug auf die S-Lost-Toxizität. So war es in der Lage, die Nekrose dosisabhängig zu verringern, wobei nur die höchste eingesetzte Konzentration (9 μM Dexamethason) eine signifikante Verringerung bewirkte. Die Verringerung der Apoptose war bereits bei 3 μM Dexamethason signifikant, konnte aber durch höhere Dexamethason-Konzentrationen nicht weiter gesenkt werden. Die Senkung der Interleukinausschüttung war bereits bei 3 μM Dexamethason nachweisbar, ist allerdings bei 9 μM Dexamethason bereits wieder angestiegen. Es ist daher zu folgern, dass die verhinderte Nekrose durch 9 μM Dexamethason zu einer Rettung von schwerstgeschädigten Zellen führt, die dann im Nachhinein zu einer gesteigerten Inflammation führen, was kontraproduktiv ist. Interessant ist, dass die Effekte des Dexamethasons in der Monokultur HaCaT stärker ausgeprägt waren als in der Co-Kultur, was darauf hindeutet, dass Dexamethason die Immunzellen nicht im besonderen Maße beeinflusst. In niedrigen Konzentrationen (3 - 6 μM) war Dexamethason aber in der Lage, die Toxizität von S-Lost zu verringern.
Ibuprofen führte in den eingesetzten Konzentrationen (300, 600 und 900 μM) zu einer Verstärkung der Zytotoxizität des S-Lostes. Sowohl die Apoptose als auch die Nekrose wurden in der Mono- und Co-Kultur bei S-Lost-Konzentrationen von 200 und 300 μM signifikant erhöht. Die Abnahmen in den Interleukinausschüttungen, sofern sie vorhanden waren, sind höchstwahrscheinlich eine Folge des forcierten Zellsterbens, welche dann nicht mehr in der Lage sind IL zu produzieren. Diese Ergebnisse traten sowohl in der Mono- als auch der Co-Kultur auf, wobei die Effekte in der Nekrose der Monokultur deutlicher sind, was aber vermutlich auf einen Plateaueffekt in der Co-Kultur zurückzuführen ist. Vermutlich war in der Co-Kultur die Nekrose bereits so stark ausgeprägt, dass eine Steigerung durch Ibuprofen kaum mehr möglich war. Auf einen Einsatz von Ibuprofen in der Therapie von S-Lost-Vergiftungen sollte bis zur weiteren Klärung des Sachverhaltes in *in vivo* Tierversuchen verzichtet werden.
Diclofenac zeigte in der HaCaT Monokultur, mit Ausnahme bei der Reduzierung der IL-6-Ausschüttung, nur geringe positive Effekte bei einer S-Lost-Vergiftung. Im Gegensatz dazu ist die Senkung der Toxizität in der Co-Kultur durch Diclofenac viel ausgeprägter. So führt Diclofenac zu einer dosisabhängigen Reduktion der Nekrose und Apoptose, die bei 60 und 90 μM Diclofenac signifikant ist. Die IL-6 Ausschüttung wird bereits bei 30 μM Diclofenac signifikant gesenkt und steigt bei 90 μM Diclofenac tendenziell wieder an. Der Anstieg der IL-Ausschüttung kann auch hier wieder Folge der Rettung von zu stark geschädigten Zellen sein. Dass die positiven Effekte von Diclofenac vor allem in der Co-Kultur deutlich werden spricht dafür, dass die Antwort der Immunzellen auf die Vergiftung im positiven Sinne moduliert wird. Diclofenac hat *in vitro* die Toxizität von S-Lost stark verringert und war Ibuprofen im Zellkulturmodell deutlich überlegen. Die positiven Effekte des Diclofenacs konnten *in vitro* nur aufgrund des neu etablierten Co-Kultur-Modells herausgearbeitet werden.

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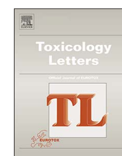

Effects of anti-inflammatory compounds on sulfur mustard injured cells: Recommendations and caveats suggested by *in vitro* cell culture models

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A B S T R A C T

Sulfur mustard (SM) is a vesicant agent who had its first military use 100 years ago, in Ypres. Since then it has been used in several conflicts like the Iran-Iraq war in the 1980s. The use of SM in Syria 2015 indicated the still existing threat. Despite decades of research no causal antidote against SM intoxication is available, so far. A SM intoxication is accompanied by necrosis, apoptosis and inflammation. To counteract the SM-induced inflammation, glucocorticoids and non-steroidal anti-inflammatory compounds (NSAIDs) are recommended. Aim of this study was to evaluate the efficacy of the anti-inflammatory compounds dexamethasone, ibuprofen and diclofenac *in vitro*. For that purpose, two different cell culture models were used. Firstly, a monoculture of keratinocytes (HaCaT) and secondly, an established co-culture of keratinocytes (HaCaT) and immunocompetent cells (THP-1) to identify the role of immune cells in the process and to mimic the dermal physiology more closely. Both models were challenged with different SM concentrations (100, 200 and 300 μM) and treated with different anti-inflammatory compounds one hour after the SM exposure. Analytical analysis of necrosis (ToxiLight), apoptosis (CDDE) and inflammation (IL-6 and −8 ELISAs) followed 24 h thereafter. Dexamethasone provided small but consistent protective effects in the monoculture. For the reduction of apoptosis, 3 μM dexamethasone was sufficient. The most effective reduction regarding interleukin (IL) production was found with 6 μM dexamethasone. Protective effects were less pronounced in co-culture, which implies, that the protective effects of dexamethasone are rather generic and not due to a modulation of the immune cells. Against our expectations, ibuprofen strongly amplified apoptosis and necrosis in SM exposed cells in the monoculture as well as the co-culture. Therefore, use of ibuprofen for treatment of SM intoxication should at least be considered most critically, if not even regarded as harmful. Diclofenac significantly reduced necrosis, apoptosis and inflammation in the co-culture in a dose-dependent manner. The greatest benefit regarding cell survival and reduction of the inflammation-marker IL-6 after a SM treatment was observed after diclofenac treatment. The protective effects of diclofenac were less pronounced in the monoculture which suggests, that diclofenac can modify the response of immune cells to SM. In conclusion, the results of our experiments, showing a benefit for diclofenac after SM exposure are in line with *in vivo* data of other researchers. Though, our *in vitro* results suggest the preferred use of diclofenac over ibuprofen. The benefit of dexamethasone is still equivocal, but low concentrations seem to have some positive effects.

1. Introduction

Sulfur mustard (SM) is a chemical warfare agent that was used in several armed conflicts in the 20th century, *e.g.* World War I and more recently in the Iran-Iraq war in the 1980s. Despite intense efforts on chemical disarmament, large stockpiles still exist. Moreover, the agent is comparatively easy to synthesize. Thus, SM remains reason for strong concern (Kehe et al., 2009b; Rowell et al., 2009), especially since the OPCW confirmed the use of SM in Syria 2015 (OPCWEC, 2015; Patocka, 2016). Even though the acute mortality among victims of SM exposure is comparatively low (2%, according to historic data), victims suffer from ulcerating, painful injuries, delays in wound healing and chronic illness that may affect eyes, respiratory system and skin (Kehe et al., 2009b; Rowell et al., 2009). Despite decades of medical research, no

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causative antidote exists. The pathophysiology of SM poisoning is highly complex, involving DNA and protein alkylation, dysfunctional enzymes, altered gene expression, excess formation of radical compounds and energy crisis, leading dose-dependently to cell cycle arrest, apoptosis or necrosis. All of these stages can be accompanied by inflammation (Kehe et al., 2009a).

For symptomatic therapy, use of glucocorticoids has been suggested, although being discussed controversially (Kehe et al., 2009b). Nonsteroidal anti-inflammatory compounds (NSAIDs) are regarded as basic therapeutics (Amir et al., 2000; Dachir et al., 2004; Wormser et al., 2004b; Amitai et al., 2005, 2006; Kadar et al., 2009; Young et al., 2012). The rationale for their use is that SM injury is accompanied by inflammation and *anti*-inflammatory compounds should be capable of at least reducing inflammation and pain or secondary tissue damage arising thereof. Some successful investigations on the effect of anti-inflammatory compounds after SM exposure have been conducted in animal studies: Dexamethasone and diclofenac were tested in a mouse-model and both compounds reduced SM-induced ear swelling. Moreover, some reduction of tissue damage was shown (Dachir et al., 2004). In another study, Diclofenac significantly reduced edema and subepidermal blisters in SM-exposed mouse ear skin (Amitai et al., 2006). However, studies investigating the effective concentrations and dose-effect relationships at cellular levels are not available. Thus, it was the aim of the present study to assess anti-inflammatory compounds in SM-exposed cell culture models, to establish dose-effect relationships and to quantify the effects on inflammation, necrosis and apoptosis. Along with the well-established HaCaT mono cell culture model, a co-culture model of HaCaT and THP-1, serving as immune cells, was used.

In case of SM exposure, interactions on the intercellular level further contribute to the complex pathophysiology and are likely to have a profound effect on the course of illness. There is evidence, that extrinsic apoptosis is a predominant pathway of apoptosis and overall cytotoxicity of cutaneous SM injury, *i.e.* production of the pro-apoptotic cytokine Fas ligand may result in apoptosis of neighbouring cells even when the latter had not or only reversibly been affected by primary SM-induced damage (Rosenthal et al., 2003). Furthermore, an interaction between immune cells and keratinocytes is of crucial importance for the clinical course of injury and subsequent chronic illness: 100 µM hemimustard stimulated human monocytes to produce TNF-α, another *pro*-apoptotic cytokine (Arroyo et al., 1995). Monocytes that have already been used to study SM toxicity are the cell line THP-1 (Gao et al., 2010). They can be used in sensitization assays and are a suitable model to investigate immune reactions in the skin (Tietze and Blomeke, 2008). Moreover a crosstalk between HaCaT and THP-1 cells has been observed (Hennen et al., 2011). Previous studies have also shown that a co-culture model of HaCaT and 2% THP-1 responded significantly different, with amplified cytotoxic effects to SM exposure (Balszuweit et al., 2014). Therefore, this co-culture model and HaCaT monocultures were chosen to investigate the effect of dexamethasone, diclofenac and ibuprofen. While the co-culture model is a closer approximation of the physiological situation in SM-exposed skin, comparison with monoculture data allowed the elucidation of the role of immune cells in the response to SM and anti-inflammatory treatment.

2. Materials and methods

2.1. Cell culture model

HaCaT cells (Boukamp et al., 1988; Breitkreutz et al., 1993) were purchased from cell lines service (cls, Eppelheim, Germany) and cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Karlsruhe, Germany, catalogue number: 41966-029), containing 4,5 g/l D-glucose, L-glutamine and pyruvate, supplemented with 5% Fetal Calf Serum (FCS), at 37 °C in a humidified atmosphere containing 5% CO_2. No antibiotics were used. In each experiment 50,000 HaCaT cells per well were seeded on two 96-well-plates which were then incubated for approx. 24 h, allowing cell adherence and proliferation. In previous studies, we found a co-culture with a ratio of 2% THP-1 to HaCaT exhibited a significantly stronger response to SM, *i.e.* amplified necrosis, apoptosis and inflammation, compared to HaCaT monocultures. Increasing the number of THP-1 cells any further resulted in no additional effects compared to the results observed with 2% THP-1 (Balszuweit et al., 2014). Thus, and because 2% coincides with the concentration of immuno-competent Langerhans cells in human skin (Bauer et al., 2001), this concentration was chosen for our co-culture model of keratinocytes and immune cells.

THP-1 cells had been grown in flasks, suspended in RPMI medium (catalogue number: 52400-025), containing L-glutamine and 25 mM HEPES, supplemented with 10% FCS and 1% [V/V] mercaptoethanol (subsequently referred to as THP-medium). By gentle wiping, THP-1 cells were fully suspended and the concentration of this cell suspension was determined using a CASY cell counter (Innovartis, Germany). The concentration was adjusted to 12,500 cells per millilitre. 80 µl of THP-1 cell suspension (containing 1000 THP-1 cells) was applied to the HaCaT cell layers. The newly formed co-cultures were incubated for 24 h. For monoculture experiments, cells were treated according to a similar protocol that used pure THP-1 medium instead of actual THP-1 cell suspensions.

2.2. SM exposure

As THP-1 cells are at least partly suspended, cell culture medium was not removed in the subsequent steps. For exposures, 80 µl of SM-containing THP-medium was added exposing cells to 100 µM, 200 µM and 300 µM SM. Control groups received another 80 µl pure THP-medium. Exposed wells and controls were then incubated for 1 h.

Sulfur mustard doses were initially chosen in accordance with our previous studies on SM toxicity in HaCaT cells (Balszuweit et al., 2013) at 30, 100 and 300 µM SM. However, data from initial experiments suggested that SM toxicity was attenuated by the presence of 10% FCS in the new co-culture model, resulting in weak toxic effects in the 30 µM SM group. Therefore, we changed our SM doses to 100, 200 and 300 µM SM, which is also in agreement with literature establishing 100–300 µM SM as the acutely toxic dose (Smith et al., 1993).

2.3. Treatment with anti-inflammatory compounds

Dosages for dexamethasone, ibuprofen and diclofenac were calculated from doses approved for clinical use and their pharmacokinetics, given in the "Summary of Product Characteristics", published by pharmaceutical companies. See the Supplement "Rationale for Treatment dosages chosen" for details.

To avoid any loss of THP-1 cells, SM-treated cell culture medium was not removed after 1 h. The following dilutions of anti-inflammatory compounds were prepared in cell culture medium: 9, 18 and 27 µM dexamethasone, 90, 180 and 270 µM diclofenac or 900, 1800 and 2700 µM ibuprofen. Sham-treated groups received pure THP-1 medium, the other wells were treated with 80 µl of one of the above-mentioned solutions. As 160 µl of THP-1 medium were already present in the wells, anti-inflammatory compounds were diluted to the following concentrations: 3, 6 and 9 µM dexamethasone, 30, 60 and 90 µM diclofenac and 300, 600 and 900 µM ibuprofen.

2.4. Sampling

After 24 h of incubation, the 96-well-plates were centrifuged at 300*g* and 4 °C for 5 min, to clear the supernatant from those THP-1 cells that may still be in suspension. Supernatants were collected. Adherent cells were washed twice with phosphate-buffered-saline (PBS). For lysis, 200 µl 0.1% Triton X-100, dissolved in PBS was added. Cells were lysed for 30 min, cooled on ice and gently agitated on a plate shaker at 100 rpm. Lysates were collected.

2.5. Analysis of cell integrity (necrosis), apoptosis and inflammatory response

The detailed methodology of analysis has been described previously (Heinrich et al., 2009; Balszuweit et al., 2013). In brief, the ToxiLight BioAssay (Lonza, Basel, Switzerland) was used to quantify adenylate kinase (AK) both in the supernatant ($AK_{supernatant}$, representing loss of cell integrity) and in the lysate (AK_{lysate}, representing intact adherent cells). The ratio of $AK_{supernatant}$ and total AK (*i.e.* sum of $AK_{supernatant}$ + AK_{lysate}, representing the total number of cells) indicated the percentage of necrotic cells.

Nucleosomes formed during apoptosis were quantified in the lysate of intact adherent cells using the Cell Death Detection Elisa Plus, CDDE (Roche, Basel, Switzerland). To account for expected variations in cell density induced by SM exposure, we calculated the ratio of nucleosome amount and AK_{lysate}, determined from the identical sample, to obtain the *Apoptotic Index*, representing the apoptotic activity in intact cells at the end of the experiment.

Inflammation is a key mechanism in SM injury (Kehe and Szinicz, 2005) and is mediated – amongst other factors – through the upregulation of IL-6 (Arroyo et al., 2001) and IL-8 (Lardot et al., 1999). Thus, the quantification of IL-6 and IL-8 as a surrogate parameter for SM-induced inflammation is a feasible approach to investigate SM-induced inflammation. IL-6 and IL-8 levels were measured in the supernatants using Sandwich-ELISA kits from eBioscience, San Diego, USA (catalogue numbers: 88-7066-88 and 88-8086-88) according to the manufacturer's protocols. Absolute interleukin levels in the supernatant were measured and correlated to the number of cells. In detail, the relative interleukin production is the ratio of interleukin levels found in the supernatant and the amount of total AK ($AK_{supernatant}$ plus AK_{lysate}), which represents the combined amount of intact and disintegrated cells at the end of the experiment.

2.6. Statistical analysis

Data from three independent experiments were normalized and pooled, resulting in n = 18 per experimental group, except for control groups (n = 9). Significance was tested with GraphPad Prism by one-Way ANOVA, followed by Bonferroni's Multiple Comparison test. p-values below 0.05 were considered significant.

3. Results

3.1. Controls

No unexpected observations were made in control wells, *i.e.* wells not exposed to SM. Apoptosis and IL-6 production was negligible, necrosis was acceptable at approx. 5% in HaCaT monocultures and 10% in co-cultures. There was some baseline secretion of IL-8 which was comparable to previous findings in HaCaT (Balszuweit et al., 2013, 2014, 2016). None of the anti-inflammatory compounds had any dramatic effect in controls. Some changes were statistically significant and have been marked in the figures in due form, but these effects were small in comparison with the effects of SM and treatment effects in SM-exposed cells and thus their biological impact is negligible. Notably, the (statistically significant) effect of ibuprofen in controls was to decrease necrosis. In conclusion, the tested concentrations of anti-inflammatory compounds were regarded as well tolerated by the cells.

3.2. Mono- vs. co-culture

When considering only the sham-treated groups of the experiments (Figs. 1–3) it is obvious, that the presence of THP-1 cells in the co-culture increased necrosis, apoptosis and IL-production, compared to the HaCaT monoculture exposed to the same SM-concentrations. This finding is in agreement with our previous findings (Balszuweit et al., 2014) and proves the proper execution of the cell culture protocols.

3.3. Dexamethasone effects

Protective effects of dexamethasone in monocultures were small but consistent (Fig. 1A). Necrosis was reduced in a dose-dependent way. This effect reached significance after treatment with 9 μM dexamethasone, *i.e.* the highest concentration tested in this study. Apoptosis was also reduced, but with a different dose-effect relationship: 3 μM dexamethasone was sufficient for a significant reduction of apoptosis, higher doses did not result in an additional significant benefit. Interleukin production was also reduced by dexamethasone, 3 μM was in general sufficient for a significant reduction and in most cases 6 μM provided the most effective reduction. 9 μM dexamethasone did not provide an additional reduction of interleukin production, to the contrary, in some cases interleukin production slightly increased, at least in comparison to levels observed under 6 μM dexamethasone.

In summary, dexamethasone influenced both apoptosis and overall cell death. Whereas 3 μM dexamethasone significantly reduces apoptosis, 9 μM dexamethasone is required for an overall reduction of cell death. It is, however, questionable whether those high-dose effects are desirable: 9 μM dexamethasone apparently may allow the survival of some severely damaged cells, which continue to produce *pro*-inflammatory cytokines (as seen in the relative increase of IL-6 and IL-8 production).

In comparison, dexamethasone effects in co-culture (Fig. 1B) were weaker, even though some protective effects were still observed, in particular after the relatively light, yet acutely toxic exposure to 100 μM SM. There is a pronounced contrast with diclofenac, the latter being much more effective in co-cultures while having little effect on monocultures. Dexamethasone, at least in our experimental setting, had no specific effect on immune cells, but a rather generic cytoprotective effect, which was most evident in HaCaT monocultures.

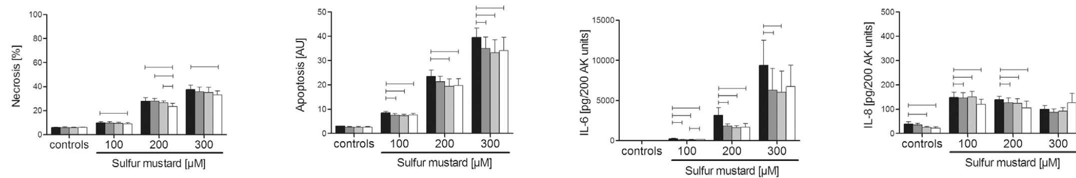
3.4. Ibuprofen effects

Ibuprofen, very much in contrast to the other substances tested, did not reduce, but enhanced apoptosis and overall cell death in cells exposed to SM (Fig. 2). This effect was not observed in controls – to the contrary, necrosis in controls slightly decreased under ibuprofen treatment. Thus, the aggravating effects of ibuprofen in SM-exposed cells cannot be attributed to any potential toxicity of ibuprofen in this cell culture model. Instead, we observed a synergistic effect of SM exposure and ibuprofen treatment that did result in amplified SM toxicity.

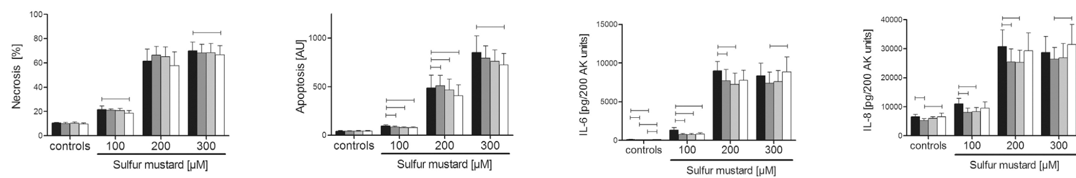
The effect was most pronounced in cells exposed to 200 μM SM, when cytotoxicity continuously increased with the ibuprofen concentration. After 100 μM SM exposure, only high ibuprofen concentrations significantly increased cytotoxicity, while lower doses had no influence on the investigated parameters. In monocultures (Fig. 2A), exposed to 200–300 μM SM, a near-linear increase of necrosis and apoptosis with increasing ibuprofen doses was observed. In co-cultures (Fig. 2B), this correlation was almost linear in 200 μM SM-exposed groups. However, in co-cultures exposed to 300 μM SM, 300 μM ibuprofen provoked a maximum cytotoxicity which did not significantly increase with higher doses of ibuprofen. Likewise, in those severely exposed co-cultures, when THP-1 cells further amplified cytotoxic effects, 300 μM ibuprofen was sufficient to induce a maximum of apoptotic activity that could not be further stimulated by larger concentrations of ibuprofen.

Interestingly, in cells treated with 900 μM ibuprofen, necrosis and apoptosis induced by 200 μM SM were even stronger compared to the effects of 300 μM SM exposure. This may suggest, that ibuprofen amplifies active cytotoxic processes, *e.g.* extrinsic apoptosis and this amplification may be more pronounced under 200 μM SM: In contrast after 300 μM SM, primary SM-induced cellular damage is severe and cytotoxicity is increasingly dominated by a passive breakdown of cell

A) Monoculture



B) Co-culture



sham-treated Dexamethasone 3 µM Dexamethasone 6 µM Dexamethasone 9 µM

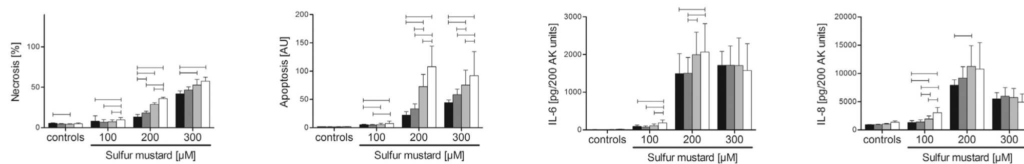
Fig. 1. Effects of dexamethasone on a HaCaT monoculture (A) and a HaCaT THP-1 co-culture (B). The graphs depict the percentage of necrotic cells, the index of apoptotic cells as well as the interleukin-(IL)-6 and IL-8 production. The vertical error bars represent the standard deviation of each experimental group with n = 18 and n = 9 for control groups, respectively. Horizontal bars show significant changes between two particular experimental groups ($p < 0,05$). Axis scaling for apoptosis and IL-8 differs between mono- and co-culture groups to visualize significant differences in the respective experiments.

metabolism and integrity. Under these circumstances, the aggravating effects of ibuprofen (as well as the protective effects of dexamethasone and diclofenac) are limited.

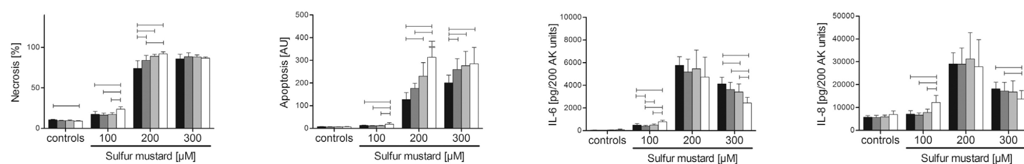
3.5. Diclofenac effects

Even though diclofenac and ibuprofen belong to the family of NSAIDs, effects of diclofenac in SM-exposed cells are radically different from those of ibuprofen. In co-culture experiments (Fig. 3B), diclofenac

A) Monoculture



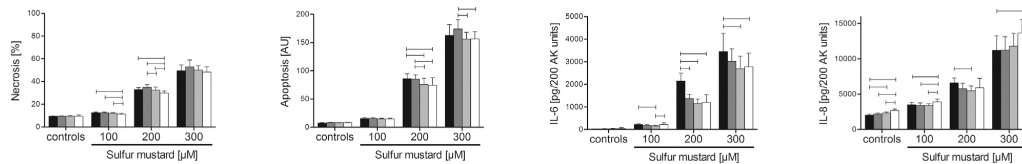
B) Co-culture



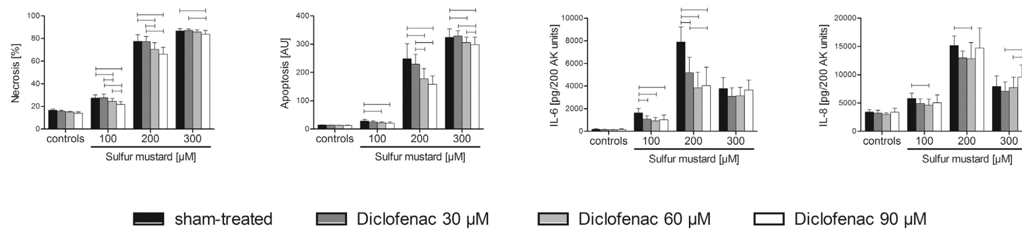
sham-treated Ibuprofen 300 µM Ibuprofen 600 µM Ibuprofen 900 µM

Fig. 2. Effects of ibuprofen on a HaCaT monoculture (A) and a HaCaT THP-1 co-culture (B). The graphs depict the percentage of necrotic cells, the index of apoptotic cells as well as the interleukin-(IL)-6 and IL-8 production. The vertical error bars represent the standard deviation of each experimental group with n = 18 and n = 9 for control groups, respectively. Horizontal bars show significant changes between two particular experimental groups ($p < 0,05$). Axis scaling for apoptosis, IL-6 and IL-8 differs between mono- and co-culture groups to visualize significant differences in the respective experiments.

A) Monoculture



B) Co-culture



sham-treated Diclofenac 30 µM Diclofenac 60 µM Diclofenac 90 µM

Fig. 3. Effects of diclofenac on a HaCaT monoculture (A) and a HaCaT THP-1 co-culture (B). The graphs depict the percentage of necrotic cells, the index of apoptotic cells as well as the interleukin-(IL)-6 and IL-8 production. The vertical error bars represent the standard deviation of each experimental group with n = 18 and n = 9 for control groups, respectively. Horizontal bars show significant changes between two particular experimental groups ($p < 0,05$). Axis scaling for apoptosis, IL-6 and IL-8 differs between mono- and co-culture groups to visualize significant differences in the respective experiments.

reduced necrosis, apoptosis and inflammation in a significant, pronounced and dose-dependent way. This was particularly evident in cells exposed to moderate SM doses (200 µM). Protective effects in lightly exposed cells (100 µM SM) were less pronounced, but nevertheless consistent. In severely exposed cells (300 µM SM), only the largest dose of diclofenac (90 µM) could reduce necrosis and apoptosis slightly but significantly. However, in this group a minor increase of IL-8 was evident while IL-6 was decreased in mono-cultures and remained unchanged in co-cultures. The increase of IL-8 might point to a propagation of an inflammatory response. However, if this very minor increase has a biological relevance is questionable and should not be considered too detrimental.

The comparatively weak effects of diclofenac in HaCaT monocultures (Fig. 3A) do not diminish the potential clinical relevance of our findings; in fact, the co-culture model is a much closer approximation of the *in vivo* situation. They do however suggest, that any generic cytoprotective effect of diclofenac is weak and the pronounced protection, observed in co-cultures is attributed to an interaction of diclofenac and immune cells.

4. Discussion

In general, the anti-inflammatory compounds tested had profound effects on SM cytotoxicity, *i.e.* treatment did not just influence interleukin production, but also apoptosis and overall cell death. Protective effects of dexamethasone were rather weak but consistent, particularly in monocultures. Ibuprofen aggravated SM-induced damage in monocultures and co-cultures. Diclofenac, which had only exhibited weak effects in monocultures turned out to be remarkably protective in co-cultures.

Comparing the three anti-inflammatory compounds tested here, ibuprofen – at least in our *in vitro* experiments – revealed some alarming results as it enhanced SM cytotoxicity significantly. Dexamethasone had some protective effects, but diclofenac significantly improved overall cell survival and reduced the inflammation-marker IL-6 at the same time. It is worth noting, that the use of diclofenac is now consistently supported by both *in vitro* and animal studies. In summary, we have gained substantial insight into clear diverse effects of anti-inflammatory compounds in SM-exposed cells, along with dose-effect relationships which are likely to have an important clinical relevance in case of SM exposure.

4.1. Potential reasons for different effects of diclofenac and ibuprofen

The very different effects of diclofenac and ibuprofen in SM-exposed cells may be explained by the pro-apoptotic effects of ibuprofen that have been repeatedly described (Bonelli et al., 2011; Duncan et al., 2012; Todo et al., 2013). In contrast, the effects of diclofenac on apoptosis depend on the cells involved and the presence of other pro-apoptotic stimuli. A number of authors have shown pro-apoptotic effects of diclofenac (Braun et al., 2012; Martin and Stockfleth, 2012; Valle et al., 2013). However, others found no pro-apoptotic effect of diclofenac in granulocytes at therapeutically relevant pharmacological concentrations (Garcia-Martinez et al., 2003). Nor were any pro-apoptotic effects of diclofenac reported in fibrosarcoma cells (Hoferova et al., 2004). It was suggested, that metabolic activation of diclofenac was required for its pro-apoptotic effects in hepatocytes (Gomez-Lechon et al., 2003). Contrarily, a protective effect of diclofenac against L-leucine-methyl-ester mediated apoptosis was reported (Sawada et al., 2000). And whilst the pro-apoptotic effects of NSAIDs, including diclofenac in tumour cells, are acknowledged, diclofenac was found to inhibit caspase-3 and thus, apoptosis in primary guinea-pig gastric mucous cells (Ashton and Hanson, 2002). Cyclooxygenase-(COX)-2 is involved in the acute phase of SM-induced inflammation, while COX-1 seemed to have a protective effect in the skin (Wormser et al., 2004a). Maybe the different effect on the course of an SM-treatment is due to the different selectivity for COX-isozymes. Diclofenac has a higher selectivity for COX-2 than ibuprofen (Capone et al., 2007).

In this study, apoptosis in controls was not affected by ibuprofen or diclofenac. However, the apoptotic response to SM was synergistically

amplified in the presence of ibuprofen, whereas diclofenac exerted an anti-apoptotic effect. It is worth noting, that the anti-apoptotic effects of diclofenac are much more pronounced in HaCaT THP-1 co-cultures, compared to HaCaT monocultures. This may indicate, that diclofenac modulates extrinsic apoptosis, stimulated by immune cells. A selective (or at least preferred) reduction of extrinsic apoptosis should be beneficial from a clinical point of view: Intact cells and those with reversible damage would be protected from extrinsically induced apoptotic cell death, while intrinsic apoptosis could still eliminate cells that were irreversibly damaged after severe SM exposure. This may also reduce the potential danger of an increased risk for long-term adverse health effects after cell rescue. However, a definite answer to this question cannot be obtained from our *in vitro* experiments. The pro-apoptotic effect of ibuprofen on the other hand is already distinctive in monocultures. Hence, it must be assumed, that ibuprofen aggravates apoptosis in a more unselective way.

4.2. Comparison of monocultures and co-cultures

Dexamethasone exhibited better protective effects in HaCaT monocultures, suggesting a generic cytoprotective effect, rather than a specific interaction with immune cells (THP-1). Moreover, it is possible that dexamethasone allowed the temporary survival of some severely damaged THP-1 which in turn may have stimulated inflammation and extrinsic apoptosis, reversing some of the protective dexamethasone effects on HaCaT which had been observed in monocultures. Ibuprofen aggravated apoptosis of SM-treated cells in mono- as well as co-culture. The mitigating effects of diclofenac were much more pronounced in cocultures. Thus, the substance is likely to modulate the interaction of immune cells with keratinocytes by attenuating active intercellular pathways of SM-induced toxicity.

4.3. Effects on interleukin production

Interleukin production was modulated in a more complex pattern than initially expected from *anti*-inflammatory compounds. In general, the compounds dexamethasone and diclofenac reduced interleukin production, in particular after exposure to 100 and 200 µM SM. They were less effective after exposure to 300 µM SM, often failing to significantly reduce interleukin production. Notably, the low to medium doses of dexamethasone (3–6 µM) and diclofenac (30–60 µM) provided some reduction, but this effect was reversed under high-dose treatment. In the latter case, the temporary rescue of some severely damaged cells may have enabled their continued interleukin production, cancelling the anti-inflammatory effects observed at low-to-medium dose treatment.

Ibuprofen treatment amplified inflammation after 100 µM SM exposure which was in line with its aggravating effects on SM toxicity. Surprisingly however, interleukin production decreased in cells after 300 µM SM exposure and high-dose ibuprofen treatment whereas necrosis and apoptosis were amplified to maximum levels. This cannot be considered a protective effect, but rather a “pharmacological debridement”: the accelerated destruction of SM-exposed cells under the influence of ibuprofen limited subsequent interleukin production. This is also in agreement with previous animal studies (Amitai et al., 2005) which observed reduced edema, but no reduction of severity score or epidermal necrosis after ibuprofen treatment in SM-exposed mouse ear models.

Any clinical application of “pharmacological debridement”, using ibuprofen after SM exposure would thus be risky with limited benefit: in lightly to moderately SM-exposed cells that may have a chance of recovery, ibuprofen also accelerated cell death and amplified inflammation.

4.4. Dosages suggested by in vitro data

Regarding the protective compounds dexamethasone or diclofenac it should be noted, that the medium doses tested, *i.e.* 6 µM dexamethasone or 60 µM diclofenac produced the most beneficial effects. At lower concentrations, protective effects against necrosis and apoptosis were comparatively weak and sometimes failed to reach significance. In contrast, any further dose increase, *i.e.* to 9 µM dexamethasone or 90 µM diclofenac rarely produced additional benefits. In most cases, the medium dose had already provided the best protection against apoptosis. Necrosis could sometimes be further reduced with high dose treatment, but rescue of severely damaged cells often resulted in a relative increase of interleukin production, *i.e.* interleukin production was higher, compared to cells under low- to medium-dose treatment. Frequently, the medium dose of dexamethasone and diclofenac also provided the best reduction of interleukin production, *i.e.* the best anti-inflammatory effect. So, it is to note, that 60 µM diclofenac and 6 µM dexamethasone provided the best *in vitro* effects against SM exposure. If and how the concentrations are transferable for the treatment of humans should be further investigated by *in vivo* experiments with animals.

4.5. Comparison of in vitro and in vivo findings

With regard to the protective effects of dexamethasone and diclofenac, our *in vitro* findings are largely in agreement with results of other researchers, who tested a topical mixture containing 0,58% diclofenac and 0,05% dexamethasone on a mouse-ear-model (Dachir et al., 2004). It had also been noticed, that diclofenac was by trend more beneficial than ibuprofen in matters of subepidermal blister formation when used as commercial creams (Voltaren and Ibulefe) in a mouse-ear-model (Amitai et al., 2006). Even though an aggravating effect of ibuprofen was not discussed in this *in vivo* study, *in vitro* and *in vivo* findings are in agreement to support the use of diclofenac. Moreover, a minor increase of edema after CEES exposure and topical treatment with 1.5 µmol ibuprofen was noted in a mouse ear model, whereas 1.5 µmol diclofenac provided a reduction in the same experimental setting (Young et al., 2012). The effects in this study (which used the analogue CEES instead of SM) were relatively weak. However, they are also in line with our findings, discouraging the use of ibuprofen.

4.6. Clinical relevance

Considering the relatively weak effects of dexamethasone in the coculture model, it is in fact questionable, whether the slight reduction of necrosis, apoptosis and inflammation may have any clinical relevance. In contrast, diclofenac reduced apoptosis by more than 30% and IL-6 production by more than 50%. Overall cell survival after 24 h also improved in a statistically significant manner, but the pronounced reductions of apoptosis and inflammation are probably of greater importance. Cells treated with diclofenac and surviving 24 h after SM exposure are considerably less likely to succumb to apoptosis and a 50% reduction of inflammation should mitigate clinical symptoms and reduce the risk of secondary tissue damage. *In vivo* studies discussed above also noted the benefit of diclofenac, underscoring the probable clinical relevance of these findings.

Regarding ibuprofen, from a precautionary point of view the more than 100% increases in necrotic and apoptotic cell death (after 200 µM SM exposure) should be considered clinically relevant and therefore ibuprofen should not be used after SM exposure.

5. Outlook

Further studies will compare the effects of selective and unselective COX-inhibitors on SM-exposed cells to investigate whether the COX-selectivity determines the influence of a compound on the course of a

SM intoxication.

Moreover, the benefits of a simultaneous treatment with several anti-inflammatory compounds, for example diclofenac and dexamethasone will be regarded in continuing experiments.

Furthermore, it should be elucidated if cells, which survive due to the anti-inflammatory treatment, reveal increased proliferation and migration. This should be addressed in *in vivo* animal studies with a focus on wound healing after SM-exposition and treatment with anti-inflammatory compounds.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.09.003.

References

Amir, A., Turetz, J., Chapman, S., Fishbeine, E., Meshulam, J., Sahar, R., Liani, H., Gilat, E., Frishman, G., Kadar, T., 2000. Beneficial effects of topical anti-inflammatory drugs against sulfur mustard-induced ocular lesions in rabbits. J. Appl. Toxicol. 20 (Suppl.1), S109–14.

Amitai, G., Adani, R., Fishbein, E., Meshulam, H., Laish, I., Dachir, S., 2005. Bifunctional compounds eliciting anti-inflammatory and anti-cholinesterase activity as potential treatment of nerve and blister chemical agents poisoning. Chem. Biol. Interact. 157-158, 361–362.

Amitai, G., Adani, R., Fishbein, E., Meshulam, H., Laish, I., Dachir, S., 2006. Bifunctional compounds eliciting anti-inflammatory and anti-cholinesterase activity as potential treatment of nerve and blister chemical agents poisoning. J. Appl. Toxicol. 26 (1), 81–87.

Arroyo, C.M., Tersch, R.L., von Broomfield, C.A., 1995. Activation of alpha-human tumour necrosis factor (TNF-alpha) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulphide (H-MG). Hum. Exp. Toxicol. 14 (7), 547–553.

Arroyo, C.M., Broomfield, C.A., Hackley, B.E., 2001. The role of interleukin-6 (IL-6) in human sulfur mustard (HD) toxicology. Int. J. Toxicol. 20 (5), 281–296.

Ashton, M., Hanson, P.J., 2002. Disparate effects of non-steroidal anti-inflammatory drugs on apoptosis in guinea-pig gastric mucous cells: inhibition of basal apoptosis by diclofenac. Br. J. Pharmacol. 135 (2), 407–416.

Balszuweit, F., John, H., Schmidt, A., Kehe, K., Thiermann, H., Steinritz, D., 2013. Silibinin as a potential therapeutic for sulfur mustard injuries. Chem. Biol. Interact. 206 (3), 496–504.

Balszuweit, F., Menacher, G., Bloemeke, B., Schmidt, A., Worek, F., Thiermann, H., Steinritz, D., 2014. Development of a co-culture of keratinocytes and immune cells for in vitro investigation of cutaneous sulfur mustard toxicity. Chem. Biol. Interact. 223, 117–124.

Balszuweit, F., Menacher, G., Schmidt, A., Kehe, K., Popp, T., Worek, F., Thiermann, H., Steinritz, D., 2016. Protective effects of the thiol compounds GSH and NAC against sulfur mustard toxicity in a human keratinocyte cell line. Toxicol. Lett. 244, 35–43.

Bauer, J., Bahmer, F.A., Worl, J., Neuhuber, W., Schuler, G., Fartasch, M., 2001. A strikingly constant ratio exists between Langerhans cells and other epidermal cells in human skin. A stereologic study using the optical disector method and the confocal laser scanning microscope. J. Invest. Dermatol. 116 (2), 313–318.

Bonelli, P., Tuccillo, F.M., Calemma, R., Pezzetti, F., Borrelli, A., Martinelli, R., Rosa, A., de Esposito, D., Palaia, R., Castello, G., 2011. Changes in the gene expression profile of gastric cancer cells in response to ibuprofen: a gene pathway analysis. Pharmacogenomics J. 11 (6), 412–428.

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., Fusenig, N.E., 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. 106 (3), 761–771.

Braun, F.K., Al-Yacoub, N., Plotz, M., Mobs, M., Sterry, W., Eberle, J., 2012. Nonsteroidal anti-inflammatory drugs induce apoptosis in cutaneous T-cell lymphoma cells and enhance their sensitivity for TNF-related apoptosis-inducing ligand. J. Invest. Dermatol. 132 (2), 429–439.

Breitkreutz, D., Stark, H.J., Plein, P., Baur, M., Fusenig, N.E., 1993. Differential modulation of epidermal keratinization in immortalized (HaCaT) and tumorigenic human skin keratinocytes (HaCaT-ras) by retinoic acid and extracellular Ca2+. Differentiation 54 (3), 201–217.

Capone, M.L., Tacconelli, S., Di Francesco, L., Sacchetti, A., Sciulli, M.G., Patrignani, P., 2007. Pharmacodynamic of cyclooxygenase inhibitors in humans. Prostaglandins Other Lipid Med. 82 (1-4), 85–94.

Dachir, S., Fishbeine, E., Meshulam, Y., Sahar, R., Chapman, S., Amir, A., Kadar, T., 2004. Amelioration of sulfur mustard skin injury following a topical treatment with a mixture of a steroid and a NSAID. J. Appl. Toxicol. 24 (2), 107–113.

Duncan, K., Uwimpuhwe, H., Czibere, A., Sarkar, D., Libermann, T.A., Fisher, P.B., Zerbini, L.F., 2012. NSAIDs induce apoptosis in nonproliferating ovarian cancer cells and inhibit tumor growth in vivo. IUBMB Life 64 (7), 636–643.

Gao, X., Ray, R., Xiao, Y., Ishida, K., Ray, P., 2010. Macrolide antibiotics improve chemotactic and phagocytic capacity as well as reduce inflammation in sulfur mustard-exposed monocytes. Pulm. Pharmacol. Ther. 23 (2), 97–106.

Garcia-Martinez, J.M., Fresno Vara, J.A., Lastres, P., Bernabeu, C., Betes, P.O., Martin-Perez, J., 2003. Effect of metamizol on promyelocytic and terminally differentiated granulocytic cells. Comparative analysis with acetylsalicylic acid and diclofenac. Biochem. Pharmacol. 65 (2), 209–217.

Gomez-Lechon, M.-J., Ponsoda, X., O'Connor, E., Donato, T., Jover, R., Castell, J.V., 2003. Diclofenac induces apoptosis in hepatocytes. Toxicol. In Vitro 17 (5-6), 675–680.

Heinrich, A., Balszuweit, F., Thiermann, H., Kehe, K., 2009. Rapid simultaneous determination of apoptosis, necrosis, and viability in sulfur mustard exposed HaCaT cell cultures. Toxicol. Lett. 191 (2-3), 260–267.

Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M., Blömeke, B., 2016. Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization. Toxicol. Sci. 123 (2), 501–510.

Hoferova, Z., Soucek, K., Hofmanova, J., Hofer, M., Chramostova, K., Fedorocko, P., Kozubik, A., 2004. In vitro proliferation of fibrosarcoma cells depends on intact functions of lipoxygenases and cytochrome P-450-monooxygenase. Cancer Invest. 22 (2), 234–247.

Kadar, T., Dachir, S., Cohen, L., Sahar, R., Fishbine, E., Cohen, M., Turetz, J., Gutman, H., Buch, H., Brandeis, R., Horwitz, V., Solomon, A., Amir, A., 2009. Ocular injuries following sulfur mustard exposure?pathological mechanism and potential therapy. Toxicology 263 (1), 59–69.

Kehe, K., Szinicz, L., 2005. Medical aspects of sulphur mustard poisoning. Toxicology 214 (3), 198–209.

Kehe, K., Balszuweit, F., Steinritz, D., Thiermann, H., 2009a. Molecular toxicology of sulfur mustard-induced cutaneous inflammation and blistering. Toxicology 263 (1), 12–19.

Kehe, K., Thiermann, H., Balszuweit, F., Eyer, F., Steinritz, D., Zilker, T., 2009b. Acute effects of sulfur mustard injury-Munich experiences. Toxicology 263 (1), 3–8.

Lardot, C., Dubois, V., Lison, D., 1999. Sulfur mustard upregulates the expression of interleukin-8 in cultured human keratinocytes. Toxicol. Lett. 110 (1-2), 29–33.

Martin, G.M., Stockfleth, E., 2012. Diclofenac sodium 3% gel for the management of actinic keratosis: 10+ years of cumulative evidence of efficacy and safety. J. Drugs Dermatol. 11 (5), 600–608.

Organization for the Prohibition of Chemical Weapons Executive Council, 2015. Further Reports of the OPCW Fact-Finding Mission in Syria: EC-M-50/DEC. pp. 1(https://www.opcw.org/fileadmin/OPCW/EC/M-48/ecm48dec01_e_.pdf Accessed 12 February 2016).

Patocka, J., 2016. Syria conflict and chemical weapons: what is the reality? Mil. Med. Sci. Lett. 85 (1), 39–43.

Rosenthal, D.S., Velena, A., Chou, F.-P., Schlegel, R., Ray, R., Benton, B., Anderson, D., Smith, W.J., Simbulan-Rosenthal, C.M., 2003. Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard. J. Biol. Chem. 278 (10), 8531–8540.

Rowell, M., Kehe, K., Balszuweit, F., Thiermann, H., 2009. The chronic effects of sulfur mustard exposure. Toxicology 263 (1), 9–11.

Sawada, T., Hashimoto, S., Tohma, S., Nishioka, Y., Nagai, T., Sato, T., Ito, K., Inoue, T., Iwata, M., Yamamoto, K., 2000. Inhibition of L-leucine methyl ester mediated killing of THP-1, a human monocytic cell line, by a new anti-inflammatory drug, T614. Immunopharmacology 49 (3), 285–294.

Smith, W.J., Sanders, K.M., Ruddle, S.E., Gross, C.L., 1993. Cytometric analysis of DNA changes induced by sulfur mustard. J. Toxicol. 12 (4), 337–347.

Tietze, C., Blomeke, B., 2008. Sensitization assays: monocyte-derived dendritic cells versus a monocytic cell line (THP-1). J. Toxicol. Environ. Health A 71 (13-14), 965–968.

Todo, M., Horinaka, M., Tomosugi, M., Tanaka, R., Ikawa, H., Sowa, Y., Ishikawa, H., Fujiwara, H., Otsuji, E., Sakai, T., 2013. Ibuprofen enhances TRAIL-induced apoptosis through DR5 upregulation. Oncol. Rep. 30 (5), 2379–2384.

Valle, B.L., D'Souza, T., Becker, K.G., Wood, W.H.3, Zhang, Y., Wersto, R.P., Morin, P.J., 2013. Non-steroidal anti-inflammatory drugs decrease E2F1 expression and inhibit cell growth in ovarian cancer cells. PLoS One 8 (4), e61836.

Wormser, U., Langenbach, R., Peddada, S., Sintov, A., Brodsky, B., Nyska, A., 2004a. Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice. Toxicol. Appl. Pharmacol. 200 (1), 40–47.

Wormser, U., Sintov, A., Brodsky, B., Casillas, R.P., Nyska, A., 2004b. Protective effect of topical iodine containing anti-inflammatory drugs against sulfur mustard-induced skin lesions. Arch. Toxicol. 78 (3), 156–166.

Young, S.C., Fabio, K.M., Huang, M.-T., Saxena, J., Harman, M.P., Guillon, C.D., Vetrano, A.M., Heck, D.E., Flowers, R.A.2, Heindel, N.D., Laskin, J.D., 2012. Investigation of anticholinergic and non-steroidal anti-inflammatory prodrugs which reduce chemically induced skin inflammation. J. Appl. Toxicol. 32 (2), 135–141.

4 Diskussion und Ausblick

Der erste militärische Einsatz von Schwefellost (S-Lost) erfolgte 1917 [48] und jährte sich damit zum hundertsten Mal. S-Lost wurde im ersten Weltkrieg, im Iran-Irak-Krieg und zahlreichen weiteren Konflikten des 20. Jahrhunderts eingesesetzt [8, 48]. Aber auch heutzutage ist S-Lost immer noch präsent, was durch die nachweisliche Ausbringung von S-Lost in Syrien 2015 verdeutlicht wird [78, 85].
Bis heute gibt es keine kausale Therapie gegen S-Lost-Vergiftungen [102, 112]. Um Antidote gegen chemische Kampfstoffe zu finden, bzw. um aktuelle Therapievorschläge gegen Vergiftungen zu evaluieren werden überwiegend Zellkulturmodelle genutzt, um die Anzahl an Tierversuchen so gering wie möglich zu halten [105].

In der eigenen Forschungsarbeit war es möglich ein valides Co-Kultur-Modell der Haut, bestehend aus Keratinozyten (HaCaT) und immunkompetenten Zellen (THP-1), zu etablieren. Bei den Untersuchungen wurden zwei verschiedene Modelle getestet, die unterschiedliche Erkenntnisse brachten.
So zeigte die Zugabe von unbehandelten THP-1-Zellen zu vergifteten HaCaT-Zellen, dass diese *per se* nicht zellschädigend sind, sondern im Gegenteil sogar helfen, die S-Lost-verursachten Schäden zu verringern. In weiteren Versuchen wird es so möglich sein, das Einwandern von Makrophagen in den Entzündungsherd *in vitro* darzustellen. Auch das zweite Modell, bei dem die HaCaT- und THP-1-Zellen erst vergesellschaftet wurden, um dann simultan vergiftet zu werden, lieferte aussagekräftige Ergebnisse. So konnte gezeigt werden, dass ein THP-1-Verhältnis von 2 % im Vergleich zur ausgesäten HaCaT-Zellzahl ausreichend ist, um die S-Lost-induzierte Nekrose, Apoptose und Inflammation hochsiginifikant zu steigern. Mit diesem Modell ist es nun möglich, die Effekte der in der Haut vorhanden Makrophagen *in vitro* zu simulieren.
Die etablierten Modelle stellen die Physiologie der Haut *in vitro* besser dar, als eine vergleichbare Monokultur. Damit wird es leichter sein, vielversprechende Substanzen zur Therapie von S-Lost-Vergiftungen zu identifizieren, bzw. die aktuellen Therapievorschläge zu evaluieren, um so mit einer geringen Anzahl gezielter Tierversuche aussagekräftigere Ergebnisse produzieren zu können.

Die Anwendung des Modells mit simultaner Vergiftung wurde zur Testung der Effektivität von anti-inflammatorischen Substanzen genutzt, wobei zu Vergleichszwecken eine HaCaT-Monokultur unter den gleichen Bedingungen parallel angesetzt wurde. Dadurch war es möglich, den Einfluss der anti-inflammatorischen Substanzen auf die vergifteten Immunzellen abzuschätzen. Die Versuche führten zu Erkenntnissen, die Einfluss auf die Therapie von S-Lost-Vergiftungen haben werden.
Die *in vitro* Ergebnisse zeigten einen positiven Effekt von Dexamethason auf den Verlauf einer S-Lost-Vergiftung, wenn die Anwendung in niedrigen Konzentrationen erfolgt. Dabei waren die protektiven Effekte des Dexamethasons in der HaCaT-Monokultur mit denen in der HaCaT-THP-1-Co-Kultur vergleichbar, was den Schluss zuließ, dass die zellprotektiven Eigenschaften des Dexamethasons nicht auf eine Modulation der Immunzellantwort zurückzuführen sind.
Ibuprofen führte sowohl in der Mono-, als auch in der Co-Kultur zu einer Verstärkung der Nekrose und Apoptose und kann daher für die Therapie einer S-Lost-Intoxikation nicht empfohlen werden.

Die stärksten protektiven Effekte gegen eine S-Lost-Exposition zeigte Diclofenac. Es war in der Lage in der Co-Kultur Nekrose, Apoptose und Interleukinausschüttung signifikant zu senken. Die positiven Effekte des Diclofenacs waren allerdings, mit Ausnahme der Interleukinausschüttung, in der Monokultur weniger stark ausgeprägt. Dies ist aber kein Nachteil, da die Co-Kultur aus HaCaT- und THP-1-Zellen die Situation *in vivo* besser darstellt, als die HaCaT-Monokultur. Die Herausarbeitung der positiven Effekte des Diclofenacs war nur durch die neu etablierte Co-Kultur aus HaCaT- und THP-1-Zellen möglich, da die positiven Effekte in der Monokultur unterschätzt werden.
Die gefundenen Resultate stimmen mit den Ergebnissen in Tierversuchen überein und bestätigen so die Effektivität des Co-Kultur-Modells. So hatte die topische Anwendung einer Mischung aus Dexamethason und Diclofenac im S-Lost-exponierten Mausohrmodell einen positiven Effekt [26]. Außerdem ergaben die Arbeiten von Amitai *et al.*, dass Diclofenac im Vergleich zu Ibuprofen tendenziell besser geeignet ist, die subepidermale Blasenbildung nach S-Lost-Exposition zu verringern (jeweils topische Anwendung als kommerziell erhältliche Creme im Mausohrmodell) [5]. Weiterhin zeigte eine Studie mit dem S-Lost-Analogon CEES im Mausohrmodell eine Zunahme der Ödembildung nach topischer Auftragung von 1,5 μmol Ibuprofen, während 1,5 μmol Diclofenac im gleichen Versuchsaufbau die Ödembildung reduzierte [116]. Auch wenn in den letztgenannten Studien für Ibuprofen keine toxizitätssteigernde Wirkung diskutiert wird, wird die Empfehlung, bei einer S-Lost-Intoxikation die Gabe von Diclofenac gegenüber Ibuprofen zu bevorzugen, untermauert.
Wie und in welchen Konzentrationen Diclofenac einzusetzen ist müssen allerdings weitere *in vivo* Studien mit Tieren noch zeigen.
Die Unterschiede zwischen der Ibuprofen- und Diclofenacbehandlung bei S-Lost vergifteten Zellen könnte darauf zurückzuführen sein, dass Diclofenac selektiver die Cyclooxygenase-(COX)-2 hemmt als Ibuprofen, das die beiden COX-Isoenzyme relativ unselektiv hemmt [25]. Studien haben gezeigt, dass die COX-1 positive Auswirkungen auf die Rekonvaleszenz haben könnte, während die Aktivierung der COX-2 die Toxizität von S-Lost im Allgemeinen erhöht [114, 110]. Daher sollten weitere Studien den Einfluss von COX-selektiven Substanzen auf den Verlauf einer S-Lost-Intoxikation beleuchten.
Ferner ist es notwendig, die Effektivität von Kombinationstherapien, beispielsweise Dexamethason und Diclofenac, zu evaluieren.

5 Literatur

1. Abbas, A. K., Lichtman, A. H. & Pober, J. S. *Cellular and molecular immunology* 2. Ausgabe. XIII, 457 S (Saunders, Philadelphia Pa. u.a., 1994).
2. Abtahi, H., Peiman, S., Foroumandi, M. & Safavi, E. Long Term Follow-Up of Sulfur Mustard Related Bronchiolitis Obliterans Treatment. *Acta medica Iranica* **54,** 605–609 (9 2016).
3. Alexander, S. F. Medical report on the Bari Harbor mustard casualties. *Military surgeon* **101,** 1–17 (1 1947).
4. Amir, A., Turetz, J., Chapman, S., Fishbeine, E., Meshulam, J., Sahar, R., Liani, H., Gilat, E., Frishman, G. & Kadar, T. Beneficial effects of topical anti-inflammatory drugs against sulfur mustard-induced ocular lesions in rabbits. *Journal of applied toxicology* **20 Suppl 1,** S109–14 (2000).
5. Amitai, G., Adani, R., Fishbein, E., Meshulam, H., Laish, I. & Dachir, S. Bifunctional compounds eliciting anti-inflammatory and anti-cholinesterase activity as potential treatment of nerve and blister chemical agents poisoning. *Journal of applied toxicology* **26,** 81–87 (1 2006).
6. Arroyo, C. M., Broomfield, C. A. & Hackley JR, B. E. The role of interleukin-6 (IL-6) in human sulfur mustard (HD) toxicology. *International journal of toxicology* **20,** 281–296 (5 2001).
7. Arroyo, C. M., Schafer, R. J., Kurt, E. M., Broomfield, C. A. & Carmichael, A. J. Response of normal human keratinocytes to sulfur mustard: cytokine release. *Journal of applied toxicology* **20 Suppl 1,** S63–72 (2000).
8. *Basic and Clinical Toxicology of Mustard Compounds* (Hrsg. Balali-Mood, M. & Abdollahi, M.) 1. Ausgabe (Springer International Publishing, Cham, 2015).
9. Balali-Mood, M. & Hefazi, M. The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. *Fundamental & clinical pharmacology* **19,** 297–315 (3 2005).
10. Balali-Mood, M. & Hefazi, M. Comparison of early and late toxic effects of sulfur mustard in Iranian veterans. *Basic & clinical pharmacology & toxicology* **99,** 273–282 (4 2006).
11. Balszuweit, F., John, H., Schmidt, A., Kehe, K., Thiermann, H. & Steinritz, D. Silibinin as a potential therapeutic for sulfur mustard injuries. *Chemico-biological interactions* **206,** 496–504 (3 2013).
12. Balszuweit, F., Menacher, G., Schmidt, A., Kehe, K., Popp, T., Worek, F., Thiermann, H. & Steinritz, D. Protective effects of the thiol compounds GSH and NAC against sulfur mustard toxicity in a human keratinocyte cell line. *Toxicology letters* **244,** 35–43 (2016).
13. Baradaran-Rafii, A., Eslani, M. & Tseng, S. C. G. Sulfur mustard-induced ocular surface disorders. *The ocular surface* **9,** 163–178 (3 2011).
14. Barillo, D. J., Cancio, L. C., Hutton, B. G., Mittelsteadt, P. J., Gueller, G. E. & Holcomb, J. B. Combat burn life support: a military burn-education program. *The Journal of burn care & rehabilitation* **26,** 162–165 (2 2005).
15. *Stryer Biochemie* (Hrsg. Berg, J. M., Tymoczko, J. L. & Stryer, L.) (Springer, Berlin, Heidelberg, 2013).
16. Bhat, K. R., Benton, B. J., Rosenthal, D. S., Smulson, M. E. & Ray, R. Role of poly(ADP-ribose) polymerase (PARP) in DNA repair in sulfur mustard-exposed normal human epidermal keratinocytes (NHEK). *Journal of applied toxicology* **20 Suppl 1,** S13–7 (2000).

17. Bhat, K. R., Benton, B. J. & Ray, R. Poly (ADP-ribose) polymerase (PARP) is essential for sulfur mustard-induced DNA damage repair, but has no role in DNA ligase activation. *Journal of applied toxicology* **26,** 452–457 (5 2006).
18. Blodi, F. C. Mustard gas keratopathy. *International ophthalmology clinics* **11,** 15 (3 1971).
19. Borak, J. & Sidell, F. R. Agents of chemical warfare: sulfur mustard. *Annals of emergency medicine* **21,** 303–308 (3 1992).
20. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. & Fusenig, N. E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *The Journal of cell biology* **106,** 761–771 (3 1988).
21. Brimfield, A. A., Mancebo, A. M., Mason, R. P., Jiang, J. J., Siraki, A. G. & Novak, M. J. Free radical production from the interaction of 2-chloroethyl vesicants (mustard gas) with pyridine nucleotide-driven flavoprotein electron transport systems. *Toxicology and applied pharmacology* **234,** 128–134 (1 2009).
22. Brimfield, A. A., Soni, S. D., Trimmer, K. A., Zottola, M. A., Sweeney, R. E. & Graham, J. S. Metabolic activation of sulfur mustard leads to oxygen free radical formation. *Free radical biology & medicine* **52,** 811–817 (4 2012).
23. Brookes, P. & Lawley, P. D. The reaction of mono- and di-functional alkylating agents with nucleic acids. *The Biochemical journal* **80,** 496–503 (3 1961).
24. Bürkle, A. Poly(ADP-ribose). The most elaborate metabolite of NAD+. *The FEBS journal* **272,** 4576–4589 (18 2005).
25. Capone, M. L., Tacconelli, S., Di Francesco, L., Sacchetti, A., Sciulli, M. G. & Patrignani, P. Pharmacodynamic of cyclooxygenase inhibitors in humans. *Prostaglandins & other lipid mediators* **82,** 85–94 (1-4 2007).
26. Dachir, S., Fishbeine, E., Meshulam, Y., Sahar, R., Chapman, S., Amir, A. & Kadar, T. Amelioration of sulfur mustard skin injury following a topical treatment with a mixture of a steroid and a NSAID. *Journal of applied toxicology* **24,** 107–113 (2 2004).
27. Dacre, J. C. & Goldman, M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. *Pharmacological reviews* **48,** 289–326 (2 1996).
28. Debiak, M., Kehe, K. & Burkle, A. Role of poly(ADP-ribose) polymerase in sulfur mustard toxicity. *Toxicology* **263,** 20–25 (1 2009).
29. Despretz, M. Des composes triples du chlore. *Ann. Chem. Phys* **21,** 438 (1822).
30. Devereaux, C. A., Amundson, C. D. E., Parrish, C. J. S. & Lazarus, C. A. A. Vesicants and nerve agents in chemical warfare. *Postgraduate Medicine* **112,** 90–96 (2002).
31. Evison, D., Hinsley, D. & Rice, P. Chemical weapons. *British Medical Journal* **324,** 332–335 (7333 2002).
32. Freitag, L., Firusian, N., Stamatis, G. & Greschuchna, D. The role of bronchoscopy in pulmonary complications due to mustard gas inhalation. *Chest* **100,** 1436–1441 (5 1991).
33. Gao, X., Ray, R., Xiao, Y., Barker, P. E. & Ray, P. Inhibition of sulfur mustard-induced cytotoxicity and inflammation by the macrolide antibiotic roxithromycin in human respiratory epithelial cells. *BMC cell biology* **8,** 17 (2007).
34. Ghabili, K., Agutter, P. S., Ghanei, M., Ansarin, K., Panahi, Y. & Shoja, M. M. Sulfur mustard toxicity: history, chemistry, pharmacokinetics, and pharmacodynamics. *Critical reviews in toxicology* **41,** 384–403 (5 2011).
35. Ghabili, K., Agutter, P. S., Ghanei, M., Ansarin, K. & Shoja, M. M. Mustard gas toxicity: the acute and chronic pathological effects. *Journal of applied toxicology* **30,** 627–643 (7 2010).
36. Ghanei, M. & Harandi, A. A. Molecular and cellular mechanism of lung injuries due to exposure to sulfur mustard: a review. *Inhalation toxicology* **23,** 363–371 (7 2011).

37. Ghanei, M., Poursaleh, Z., Harandi, A. A., Emadi, S. E. & Emadi, S. N. Acute and chronic effects of sulfur mustard on the skin: a comprehensive review. *Cutaneous and ocular toxicology* **29,** 269–277 (4 2010).

38. Ghanei, M., Shohrati, M., Harandi, A. A., Eshraghi, M., Aslani, J., Alaeddini, F. & Manzoori, H. Inhaled corticosteroids and long-acting beta 2-agonists in treatment of patients with chronic bronchiolitis following exposure to sulfur mustard. *Inhalation toxicology* **19,** 889–894 (10 2007).

39. Ghasemi, H., Owlia, P., Jalali-Nadoushan, M. R., Pourfarzam, S., Azimi, G., Yarmohammadi, M.-E., Shams, J., Fallahi, F., Moaiedmohseni, S., Moin, A., Yaraee, R., Vaez-Mahdavi, M. R., Faghihzadeh, S., Mohammad Hassan, Z., Soroush, M.-R., Naghizadeh, M.-M., Ardestani, S. K. & Ghazanfari, T. A clinicopathological approach to sulfur mustard-induced organ complications: a major review. *Cutaneous and ocular toxicology* **32,** 304–324 (4 2013).

40. Glasby, G. P. Disposal of chemical weapons in the Baltic Sea. *The Science of the total environment* **206,** 267–273 (2-3 1997).

41. Gould, N. S., White, C. W. & Day, B. J. A role for mitochondrial oxidative stress in sulfur mustard analog 2-chloroethyl ethyl sulfide-induced lung cell injury and antioxidant protection. *The Journal of pharmacology and experimental therapeutics* **328,** 732–739 (3 2009).

42. Graham, J. S., Chilcott, R. P., Rice, P., Milner, S. M., Hurst, C. G. & Maliner, B. I. *Wound healing of cutaneous sulfur mustard injuries: strategies for the development of improved therapies* DTIC Document, 2005.

43. Graham, J. S., Schomacker, K. T., Glatter, R. D., Briscoe, C. M., Braue JR, E. H. & Squibb, K. S. Efficacy of laser debridement with autologous split-thickness skin grafting in promoting improved healing of deep cutaneous sulfur mustard burns. *Burns* **28,** 719–730 (8 2002).

44. Graham, J. S. & Schoneboom, B. A. Historical perspective on effects and treatment of sulfur mustard injuries. *Chemico-biological interactions* **206,** 512–522 (3 2013).

45. Greenberg, M. I., Sexton, K. J. & Vearrier, D. Sea-dumped chemical weapons: environmental risk, occupational hazard. *Clinical toxicology* **54,** 79–91 (2 2016).

46. *Handbook of toxicology of chemical warfare agents* (Hrsg. Gupta, R. C.) 1. Ausgabe (Elsevier/Academic Press, Amsterdam und Boston, 2009).

47. Guthrie, F. Ueber einige Derivate der Kohlenwasserstoffe CnHn. *Annalen der Chemie und Pharmacie* **113,** 266–288 (3 1860).

48. *Der chemische Krieg* (Hrsg. Hanslian, R.) 3. Aufl. 2 Bde. (ES Mittler & Sohn, Berlin, 1937).

49. Hattersley, I. J., Jenner, J., Dalton, C., Chilcott, R. P. & Graham, J. S. The skin reservoir of sulphur mustard. *Toxicology in vitro* **22,** 1539–1546 (6 2008).

50. Heinrich, A., Balszuweit, F., Thiermann, H. & Kehe, K. Rapid simultaneous determination of apoptosis, necrosis, and viability in sulfur mustard exposed HaCaT cell cultures. *Toxicology letters* **191,** 260–267 (2-3 2009).

51. Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M. & Blomeke, B. Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization. *Toxicological sciences* **123,** 501–510 (2 2011).

52. Hinshaw, D. B., Lodhi, I. J., Hurley, L. L., Atkins, K. B. & Dabrowska, M. I. Activation of poly ADP-Ribose polymerase in endothelial cells and keratinocytes: role in an in vitro model of sulfur mustard-mediated vesication. *Toxicology and applied pharmacology* **156,** 17–29 (1 1999).

53. Kan, R. K., Pleva, C. M., Hamilton, T. A., Anderson, D. R. & Petrali, J. P. Sulfur Mustard-Induced Apoptosis in Hairless Guinea Pig Skin. *Toxicologic Pathology* **31,** 185–190 (2 2003).

54. Kehe, K., Abend, M., Ridi, R., Peter, R. U. & van Beuningen, D. Tissue engineering with HaCaT cells and a fibroblast cell line. *Archives of dermatological research* **291,** 600–605 (11 1999).

55. Kehe, K., Balszuweit, F., Steinritz, D. & Thiermann, H. Molecular toxicology of sulfur mustard-induced cutaneous inflammation and blistering. *Toxicology* **263,** 12–19 (1 2009).

56. Kehe, K., Raithel, K., Kreppel, H., Jochum, M., Worek, F. & Thiermann, H. Inhibition of poly(ADP-ribose) polymerase (PARP) influences the mode of sulfur mustard (SM)-induced cell death in HaCaT cells. *Archives of toxicology* **82,** 461–470 (7 2008).

57. Kehe, K., Thiermann, H., Balszuweit, F., Eyer, F., Steinritz, D. & Zilker, T. Acute effects of sulfur mustard injury–Munich experiences. *Toxicology* **263,** 3–8 (1 2009).

58. Kehe, K. & Szinicz, L. Medical aspects of sulphur mustard poisoning. *Toxicology* **214,** 198–209 (3 2005).

59. Lardot, C., Dubois, V. & Lison, D. Sulfur mustard upregulates the expression of interleukin-8 in cultured human keratinocytes. *Toxicology letters* **110,** 29–33 (1-2 1999).

60. Laskin, J. D., Black, A. T., Jan, Y.-H., Sinko, P. J., Heindel, N. D., Sunil, V., Heck, D. E. & Laskin, D. L. Oxidants and antioxidants in sulfur mustard-induced injury. *Annals of the New York Academy of Sciences* **1203,** 92–100 (2010).

61. Lawley, P. D. & Brookes, P. Interstrand cross-linking of DNA by difunctional alkylating agents. *Journal of molecular biology* **25,** 143–160 (1 1967).

62. Lawley, P. D., Lethbridge, J. H., Edwards, P. A. & Shooter, K. V. Inactivation of bacteriophage T7 by mono- and difunctional sulphur mustards in relation to cross-linking and depurination of bacteriophage DNA. *Journal of molecular biology* **39,** 181–198 (1 1969).

63. Lomash, V., Jadhav, S. E., Vijayaraghavan, R. & Pant, S. C. Time course pathogenesis of sulphur mustard-induced skin lesions in mouse model. *International wound journal* **10,** 441–454 (4 2013).

64. Ludlum, D. B., Austin-Ritchie, P., Hagopian, M., Niu, T. Q. & Yu, D. Detection of sulfur mustard-induced DNA modifications. *Chemico-biological interactions* **91,** 39–49 (1 1994).

65. Ludlum, D. B., Kent, S. & Mehta, J. R. Formation of O6-ethylthioethylguanine in DNA by reaction with the sulfur mustard, chloroethyl sulfide, and its apparent lack of repair by O6-alkylguanine-DNA alkyltransferase. *Carcinogenesis* **7,** 1203–1206 (7 1986).

66. Ludlum, D. B., Tong, W. P., Mehta, J. R., Kirk, M. C. & Papirmeister, B. Formation of O6-ethylthioethyldeoxyguanosine from the reaction of chloroethyl ethyl sulfide with deoxyguanosine. *Cancer research* **44,** 5698–5701 (12 1984).

67. Majno, G. & Joris, I. Apoptosis, oncosis, and necrosis. An overview of cell death. *The American journal of pathology* **146,** 3–15 (1 1995).

68. Malhotra, R. C., Ganesan, K., Sugendran, K. & Swamy, R. V. Chemistry and toxicology of sulphur mustard-a review. *Defence Science Journal* **49,** 97 (2 1999).

69. Mangerich, A., Debiak, M., Birtel, M., Ponath, V., Balszuweit, F., Lex, K., Martello, R., Burckhardt-Boer, W., Strobelt, R., Siegert, M., Thiermann, H., Steinritz, D., Schmidt, A. & Burkle, A. Sulfur and nitrogen mustards induce characteristic poly(ADP-ribosyl)ation responses in HaCaT keratinocytes with distinctive cellular consequences. *Toxicology letters* **244,** 56–71 (2016).

70. Meier, H. L. & Millard, C. B. Alterations in human lymphocyte DNA caused by sulfur mustard can be mitigated by selective inhibitors of poly(ADP-ribose) polymerase. *Biochimica et biophysica acta* **1404,** 367–376 (3 1998).

71. Meyer, V. Ueber Thiodiglykolverbindungen. *Berichte der deutschen chemischen Gesellschaft* **19,** 3259–3266 (2 1886).

72. Neitzel, C. & Ladehof, K. *Taktische Medizin. Notfallmedizin und Einsatzmedizin* (Springer-Verlag, Berlin, Heidelberg, 2012).
73. Niemann, A. Ueber die Einwirkung des braunen Chlorschwefels auf Elaylgas. *Annalen der Chemie und Pharmacie* **113,** 288–292 (3 1860).
74. Nourani, M. R., Mahmoodzadeh Hosseini, H. & Imani Fooladi, A. A. Comparative transcriptional and translational analysis of heme oxygenase expression in response to sulfur mustard. *Journal of receptor and signal transduction research* **35,** 479–484 (5 2015).
75. Organisation for the Prohibition of Chemical Weapons. *Consignment of Sulfur Mustard Delivered to Latakia and Removed from Syria* <`https://www.opcw.org/news/article/consignment-of-sulfur-mustard-delivered-to-latakia-and-removed-from-syria/`> (2017).
76. Organisation for the Prohibition of Chemical Weapons. *About OPCW* <`https://www.opcw.org/about-opcw/`> (2017).
77. Organisation for the Prohibition of Chemical Weapons. *Libya: Facts and Figures* <`https://www.opcw.org/opcw-archive/the-opcw-and-libya/libya-facts-and-figures`> (2017).
78. Organisation for the Prohibition of Chemical Weapons - Technical Secretariat. *Report of the OPCW Fact-Finding Mission in Syria. Regarding Alleged Incidents in Marea, Syrian Arab Republic, August 2015* <`https://www.opcw.org/fileadmin/OPCW/Fact_Finding_Mission/s-1320-2015_e_.pdf`> (2017).
79. Panahi, Y., Rajaee, S. M. & Sahebkar, A. Ocular Effects of Sulfur Mustard and Therapeutic Approaches: A Review. *Journal of cellular biochemistry* (2017).
80. Pant, S. C. & Lomash, V. Sulphur Mustard Induced Toxicity, Mechanism of Action and Current Medical Management. *Defence Life Science Journal* **1** (1 2016).
81. Papirmeister, B., Gross, C. L., Meier, H. L., Petrali, J. P. & Johnson, J. B. Molecular basis for mustard-induced vesication. *Fundamental and applied toxicology* **5,** S134–49 (6 1985).
82. Papirmeister, B. *Medical defense against mustard gas. Toxic mechanisms and pharmacological implications* (CRC Press, Boca Raton, 1991).
83. Papirmeister, B., Gross, C. L., Petrali, J. P. & Meier, H. L. Pathology Produced by Sulfur Mustard in Human Skin Grafts on Athymic Nude Mice. II. Ultrastructural Changes. *Journal of Toxicology: Cutaneous and Ocular Toxicology* **3,** 393–408 (4 1984).
84. Paromov, V., Suntres, Z., Smith, M. & Stone, W. L. Sulfur mustard toxicity following dermal exposure: role of oxidative stress, and antioxidant therapy. *Journal of burns and wounds* **7.** eprint: `18091984` (2007).
85. Patocka, J. Syria Conflict and Chemical Weapons: What ist the Reality? *Military Medical Science Letters* **85,** 1–5 (2016).
86. Raffray, M. & Gerald M., C. Apoptosis and necrosis in toxicology. A continuum or distinct modes of cell death? *Pharmacology & Therapeutics* **75,** 153–177 (3 1997).
87. Rikimaru, T., Nakamura, M., Yano, T., Beck, G., Habicht, G. S., Rennie, L. L., Widra, M., Hirshman, C. A., Boulay, M. G. & Spannhake, E. W. Mediators, initiating the inflammatory response, released in organ culture by full-thickness human skin explants exposed to the irritant, sulfur mustard. *The Journal of investigative dermatology* **96,** 888–897 (6 1991).
88. Rink, L., Kruse, A. & Haase, H. *Immunologie für Einsteiger* (Spektrum Akademischer Verlag, Heidelberg, 2012).
89. Rosenthal, D. S., Simbulan-Rosenthal, C. M., Liu, W. F., Velena, A., Anderson, D., Benton, B., Wang, Z. Q., Smith, W., Ray, R. & Smulson, M. E. PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. *The Journal of investigative dermatology* **117,** 1566–1573 (6 2001).

90. Rowell, M., Kehe, K., Balszuweit, F. & Thiermann, H. The chronic effects of sulfur mustard exposure. *Toxicology* **263,** 9–11 (1 2009).

91. Sabourin, C. L., Petrali, J. P. & Casillas, R. P. Alterations in inflammatory cytokine gene expression in sulfur mustard-exposed mouse skin. *Journal of biochemical and molecular toxicology* **14,** 291–302 (6 2000).

92. Sabourin, C. L. K., Danne, M. M., Buxton, K. L., Casillas, R. P. & Schlager, J. J. Cytokine, chemokine, and matrix metalloproteinase response after sulfur mustard injury to weanling pig skin. *Journal of biochemical and molecular toxicology* **16,** 263–272 (6 2002).

93. Sanderson, H., Fauser, P., Thomsen, M. & Sorensen, P. B. Human health risk screening due to consumption of fish contaminated with chemical warfare agents in the Baltic Sea. *Journal of hazardous materials* **162,** 416–422 (1 2009).

94. Shakarjian, M. P., Heck, D. E., Gray, J. P., Sinko, P. J., Gordon, M. K., Casillas, R. P., Heindel, N. D., Gerecke, D. R., Laskin, D. L. & Laskin, J. D. Mechanisms mediating the vesicant actions of sulfur mustard after cutaneous exposure. *Toxicological sciences* **114,** 5–19 (1 2010).

95. Shohrati, M., Peyman, M., Peyman, A., Davoudi, M. & Ghanei, M. Cutaneous and ocular late complications of sulfur mustard in Iranian veterans. *Cutaneous and ocular toxicology* **26,** 73–81 (2 2007).

96. *Chemical warfare agents. Toxicology and treatment* (Hrsg. Sidell, F. R., Maynard, R. L. & Marrs, T. C.) 2. Ausgabe (Wiley, Chichester, England und Hoboken, NJ, 2007).

97. Siegert, M., Kranawetvogl, A., Thiermann, H. & John, H. N-Acetylcysteine as a chemical scavenger for sulfur mustard: New insights by mass spectrometry. *Drug Testing and Analysis.* <http://dx.doi.org/10.1002/dta.2299> (2017).

98. Smith, W. J., Gross, C. L., Chan, P. & Meier, H. L. The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. *Cell biology and toxicology* **6,** 285–291 (3 1990).

99. Smith, W. J., Sanders, K. M., Ruddle, S. E. & Gross, C. L. Cytometric analysis of DNA changes induced by sulfur mustard. *Journal of Toxicology: Cutaneous and Ocular Toxicology* **12,** 337–347 (4 1993).

100. Solberg, Y., Alcalay, M. & Belkin, M. Ocular injury by mustard gas. *Survey of ophthalmology* **41,** 461–466 (6 1997).

101. Steinritz, D., Elischer, A., Balszuweit, F., Gonder, S., Heinrich, A., Bloch, W., Thiermann, H. & Kehe, K. Sulphur mustard induces time- and concentration-dependent regulation of NO-synthesizing enzymes. *Toxicology letters* **188,** 263–269 (3 2009).

102. Steinritz, D., Striepling, E., Rudolf, K.-D., Schroder-Kraft, C., Puschel, K., Hullard-Pulstinger, A., Koller, M., Thiermann, H., Gandor, F., Gawlik, M. & John, H. Medical documentation, bioanalytical evidence of an accidental human exposure to sulfur mustard and general therapy recommendations. *Toxicology letters* **244,** 112–120 (2016).

103. Stenger, B., Popp, T., John, H., Siegert, M., Tsoutsoulopoulos, A., Schmidt, A., Muckter, H., Gudermann, T., Thiermann, H. & Steinritz, D. N-Acetyl-L-cysteine inhibits sulfur mustard-induced and TRPA1-dependent calcium influx. *Archives of toxicology* (2016).

104. Stenger, B., Zehfuss, F., Muckter, H., Schmidt, A., Balszuweit, F., Schafer, E., Buch, T., Gudermann, T., Thiermann, H. & Steinritz, D. Activation of the chemosensing transient receptor potential channel A1 (TRPA1) by alkylating agents. *Archives of toxicology* **89,** 1631–1643 (9 2015).

105. Szinicz, L., Worek, F., Thiermann, H., Kehe, K., Eckert, S. & Eyer, P. Development of antidotes: problems and strategies. *Toxicology* **233,** 23–30 (1-3 2007).

106. Tanabe, T., Yamada, M., Noma, T., Kajii, T. & Nakazawa, A. Tissue-specific and developmentally regulated expression of the genes encoding adenylate kinase isozymes. *The Journal of Biochemistry* **113,** 200–207 (2 1993).

107. Thiermann, H., Worek, F. & Kehe, K. Limitations and challenges in treatment of acute chemical warfare agent poisoning. *Chemico-biological interactions* **206,** 435–443 (3 2013).

108. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. & Tada, K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International journal of cancer* **26,** 171–176 (2 1980).

109. Tuorinsky, S. D. *Medical aspects of chemical warfare* (Office of the Surgeon General, U.S. Army und Borden Institute, Walter Reed Army Medical Center, Falls Church, Va. und Washington, DC, 2008).

110. Vane, J. R., Bakhle, Y. S. & Botting, R. M. Cyclooxygenases 1 and 2. *Annual review of pharmacology and toxicology* **38,** 97–120 (1998).

111. Vogt, R. F., Dannenberg, A. M., Schofield, B. H., Hynes, N. A. & Papirmeister, B. Pathogenesis of skin lesions caused by sulfur mustard. *Fundamental and Applied Toxicology* **4,** 71–83 (2 1984).

112. Weinberger, B., Malaviya, R., Sunil, V. R., Venosa, A., Heck, D. E., Laskin, J. D. & Laskin, D. L. Mustard vesicant-induced lung injury: Advances in therapy. *Toxicology and applied pharmacology* **305,** 1–11 (2016).

113. Willems, J. L. Clinical management of mustard gas casualties. *Ann Med Mil Belg* **3,** 1–61 (1989).

114. Wormser, U., Langenbach, R., Peddada, S., Sintov, A., Brodsky, B. & Nyska, A. Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice. *Toxicology and applied pharmacology* **200,** 40–47 (1 2004).

115. Yazdani, S., Karimfar, M. H., Imani Fooladi, A. A., Mirbagheri, L., Ebrahimi, M., Ghanei, M. & Nourani, M. R. Nuclear factor kappaB1/RelA mediates the inflammation and/or survival of human airway exposed to sulfur mustard. *Journal of receptor and signal transduction research* **31,** 367–373 (5 2011).

116. Young, S. C., Fabio, K. M., Huang, M.-T., Saxena, J., Harman, M. P., Guillon, C. D., Vetrano, A. M., Heck, D. E., Flowers, R. A. 2., Heindel, N. D. & Laskin, J. D. Investigation of anticholinergic and non-steroidal anti-inflammatory prodrugs which reduce chemically induced skin inflammation. *Journal of applied toxicology* **32,** 135–141 (2 2012).

117. Yue, L., Zhang, Y., Chen, J., Zhao, Z., Liu, Q., Wu, R., Guo, L., He, J., Zhao, J., Xie, J. & Peng, S. Distribution of DNA adducts and corresponding tissue damage of Sprague-Dawley rats with percutaneous exposure to sulfur mustard. *Chemical research in toxicology* **28,** 532–540 (3 2015).

118. Zilker, T. Medical management of incidents with chemical warfare agents. *Toxicology* **214,** 221–231 (3 2005).

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