DISSERTATION

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"Bacterial ghosts as carrier of active substances:
Effects on cell viability and induction of innate immunity"

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Mag. Verena Juliana Koller

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Table of Contents

Outline and Objectives

Bacterial Ghosts (BGs) represent bacterial envelopes which are produced by the controlled expression of the phage derived lysis gene *E*. Because the cytoplasmatic content is expelled through the generated lysis hole, the intracellular space can be filled with any biologically active substances. Like their viable counterpart, BGs provide fully intact surface structures including macromolecular assemblies such as flagella, pili or LPS and are therefore able to stimulate the innate immune system of the host *per se*. Thus, the BG technology provides an innovative system for vaccine, drug or active substance delivery for various cells and tissues of animal, human or plant origin.

Detailed information on the BGs platform system is given in **Chapter 1. "Introduction to the bacterial ghost technology platform"** which consists of the following reviews:
Chapter 1.1. "Bacterial ghosts as Vaccine and Drug Delivery Platforms"
(published in "*Patho-Biotechnology*", 2008).
Chapter 1.2. "The Bacterial Ghost Platform System: Production and Applications"
(accepted for publication in "*Bioengineered Bugs*", 2010).
Chapter 1.3. "Bacterial Ghosts (BGs) – Advanced Antigen and Drug Delivery System"
(accepted for publication in "*Vaccine*", 2010).

The main findings, summarized in Chapter 2. "Application of Bacterial Ghosts, are categorized into three topics which address fundamental issues for the prospective application of the BG system and contain subchapters which are written in the style of discrete manuscripts, containing the following sections: Title, Abstract, Introduction, Materials and Methods, Results, Discussion, Figures and References.

Chapter 2.1. Bacterial ghosts as carrier vehicles for cytostatic drugs

Bacterial ghosts (BGs) are able to target various types of cancer cells and efficient endocytosis of BGs was reported after incubation with colon carcinoma, leukaemia and melanoma cells. Previous studies showed that the colon cell line CaCo2 effectively internalized BGs which were loaded with the cytostatic drug doxorubicin (DOX). Furthermore it was demonstrated that the BG's content was released to the cytoplasm of the target cells and accumulation of the drug was found in the nucleus. Incubation with DOX-loaded BGs significantly reduced the viability as well as the proliferative capacity of the cells and the effects were 2-3 orders of magnitude higher than in comparison to results obtained with free DOX. Similar results were obtained with different leukaemia cell lines.

In this part of the thesis, the chemotherapeutic compounds resveratrol (RV) and its analogues digalloylresveratrol (DIG) and 3,3',4,4',5,5'-hexahydroxystilbene (M8) should be entrapped into BGs. Furthermore, the impact on the viability of the colon cell line HT29 should be determined after long term incubation with the loaded BGs. Additionally, the endogenous drug release of RV-BGs should be confirmed by HPLC measurements.

Chapter 2.2. Bacterial ghosts as targeting vehicles for ocular surfaces

The conjunctival epithelium on the ocular surface serves as a protective barrier against detrimental effects from the environment and the delivery of drugs against ocular diseases is challenging.

As BGs represent a novel carrier and adjuvant system for the delivery of mucosal vaccines, the targeting properties of various BGs, produced from different species, should be investigated in human conjunctiva derived cells. Uptake efficiencies should be determined by flow cytometry. Furthermore, the influence of BGs on the expression of MHC class I and class II molecules as well as of ICAM-1 should be examined.

In order to demonstrate that the LPS content, presented on the BG's surface, does not represent a risk for using them as vaccine candidates, the potential cytotoxic and genotoxic effects of BGs should be investigated in the conjunctival cell line CCL 20.2 by use of the neutralred assay (viability) as well with the single cell gel electrophoresis technique (DNA-damage). A previous study demonstrated that bacteria influence (potentiate or reduce) the damaging effects of drugs. Therefore, it should be investigated whether the toxic impact of the preservative benzalkonium chloride towards the CCL 20.2 cells could be modulated in presence of BGs.

Chapter 2.3. Bacterial ghosts as triggers of the innate immune system

The innate immune system protects the host from invading microorganisms and triggers a series of defense responses. As already mentioned at the beginning, BGs retain all of the surface structural, morphological and antigenic components which are required for the activation of the innate immune system.

Because little is known about the BG's capacity concerning the expression of innate immune modulators of the skin, human keratinocytes should be investigated after application of BGs for their proinflammatory cytokine expression, especially of interleukin (IL)-6 and IL-8. Furthermore, it should be determined whether BGs are still able to induce antimicrobial peptides such as psoriasin and human β defensin-2.

Another defense mechanism of the innate immune system is given by the release of radicals, e.g.. reactive nitrogen species like nitric oxide (NO). This molecule represents a mediator for various cellular functions and is produced through the inducible nitric oxide

synthase (iNOS). It is known that LPS induces iNOS dependent NO formation in phagocytes. Therefore it should be investigated whether BGs can induce NO-accumulation in RAW 264.7 macrophages and whether this could be modified when BGs would be loaded with the iNOS inhibitor resveratrol.

Appendix

Supplementary data and figures that were not included in the respective chapters are subject of the appendix (**A.1.-A.5.**). Furthermore, the loading capacity of BGs with the coffee compounds kahweol and cafestol was investigated by HPLC measurements and the results are shortly discussed in section **A.6.** In the last part of the appendix, the cytotoxic impact of various eye drops towards the conventional conjunctiva cell line CCL 20.2 and human conjunctiva derived primary cells is shown (**A.7.**). This study was included because almost all of the investigated solutions contained BAC, which was already investigated in **Chapter 2.2.2**. in presence of BGs. Furthermore, preliminary experiments were conducted with two selected eye drops and their cytotoxic impact was determined after coincubation with BGs (**A.4.**).

Summary

Bacterial ghosts as carrier vehicles for cytostatic drugs

Many studies demonstrated the chemopreventive and cytostatic effects of the hydroxystilbene resveratrol (RV). Furthermore, the novel analogues digalloylresveratrol (DIG) and 3,3',4,4',5,5'-hexahydroxystilbene (M8) have been shown to exert even more anti-tumor activity than RV and provide therefore new drug candidates for treatment of colorectal cancer.

The advantage that the bioadhesive BGs contain natural immune stimulating compounds on their surface and provide a huge reservoir for substances in the inner lumen, make them optimal drug delivery vehicles which can be used for tumor treatment and therapy. Thus, the cytotoxic impact of BGs which were loaded with the chemotherapeutic compounds mentioned above, on the colon cell line HT29 was investigated. Findings from comparative investigations showed that the drug, delivered by BGs, significantly reduced the cell viability according to the cytotoxic pattern of the compounds alone (DIG > M8 > RV). In order to prove that the cytotoxic effects were caused by intracellular drug delivery, experiments were conducted with the less toxic RV-BGs, and RV and its metabolites were determined by HPLC. The results showed that the entrapped photosensitive RV was highly protected by the BG's interior and that the loaded substance was effectively released within the targeted HT29 cells. Moreover, sustained intracellular levels of RV and its metabolites were found even after coincubation of RV-BGs for up to 24 hrs and higher concentrations of the compound were detected after RV-BG-treatment when compared to treatment with an equivalent dose of the substance alone.

Bacterial ghosts as targeting vehicles for ocular surfaces

In general, conjunctiva cells represent not only a mechanical barrier against pathogens but also protects the eye against allergens and toxic substances. Nevertheless, disorders of the conjunctiva, are affecting people worldwide and there is an extensive need for delivery systems which are capable to effectively transfer medical drugs to the eye's compartment.

In order to investigate whether BGs can be used as carrier systems for future therapeutic applications, the responsiveness of different human conjunctiva-derived cells after coincubation with BGs, generated from different bacterial species, was examined. High uptake BG levels were found with FACS-analyses in primary cells, with efficiencies modulated by the type of the BG species used. Furthermore, increased expression of the molecule ICAM-1 was demonstrated on the surface of the conjunctiva cells that might have an additional stimulatory effect on leukocyte populations or as a costimulatory signal required for T-cell activation and cytokine production.

Benzalkonium chloride (BAC), which is commonly used as a preserving agent in

ophthalmic solutions, is known for its cytotoxic action in conjunctival cells. The performed studies revealed that no toxic effects (neither cytotoxic nor genotoxic) were mediated by BGs in the conjunctiva cell line CCL 20.2. Therefore, the impact of BAC in presence of BGs was investigated. It was found that BGs were highly protective against the BAC induced cytotoxicity in CCl 20.2. cells. Furthermore, results from single cell gel electrophoresis (SCGE) assays show that BAC also induces DNA-damage in conjunctiva cells, probably due to the generation of H_2O_2. As peroxidase activities were detected in BGs, additional experiments were conducted with selected BGs and beneficial effects of BGs were also seen in genotoxicity assays. In addition it was demonstrated that the bactericidal nature of the preservative BAC was not lost in presence of the BGs.

Bacterial ghosts as triggers of the innate immune system

Infectious agents are immediately recognized, inactivated and killed by the innate immune system which is built up by certain cells and humoral factors. The defense mechanisms include phagocytosis of bacteria by neutrophils or macrophages and the release of antimicrobial peptides, proinflammatory cytokines, hydrolytic enzymes or of reactive oxygen/nitrogen species by phagocytes.

The induction of antimicrobial peptides and proinflammatory cytokines, produced by epidermal keratinocytes (KCs), provide a protective barrier to invading microorganisms and are a critical part of the innate immune system of the skin. Since the antimicrobial peptides S100A7c (psoriasin) and human β defensin-2 (hBD-2) are most efficient agents against skin pathogens, their mRNA expression after incubation of KCs with *E. coli* BGs was investigated. In quantitative real-time PCR analyses it was demonstrated that BGs are able to induce the two peptides in a particle dependent manner. It was further shown, that the endocytic activity of BGs (flagellin-deficient and wild-type) by KCs as well as the release of proinflammatory cytokines like interleukin (IL)-6 and IL-8 were dependent on the flagellin content on the surface of the BGs.

The production of nitric oxide (NO) by the inducible NO-synthase (iNOS) plays an important role in infectious diseases. As bacterial lipopolysaccaride (LPS) is known to be a potent inducer of iNOS, the NO formation in a murine macrophage cell line after coincubation with BGs was investigated. It was demonstrated that *E. coli* BGs are rapidly phagocytized by RAW 264.7 cells and induced NO-formation in a particle dependent manner. Furthermore, the BG-induced NO-accumulation was determined with BGs which were loaded with the iNOS inhibitor resveratrol (RV). Indeed, modulated NO-formation was found. Even though, the general uptake mechanism of RV is still unclear it was found that endogenous RV-delivery by BGs led to modified NO release. Because of this, the existence of an internal RV-receptor is postulated which is termed ERVR (endogenous resveratrol receptor).

Gliederung und Zielsetzung

Bakterielle Ghosts (BGs) repräsentieren intakte bakterielle Hüllen, die durch kontrollierte Expression des Lysegens *E* gebildet werden. Während des Lyseprozesses wird der gesamte zytoplasmatische Inhalt durch ein generiertes Loch freigesetzt. Dieser intrazelluläre Bereich kann nun mit jeglichen biologisch aktiven Substanzen gefüllt werden. Oberflächenstrukturen, so auch makromolekulare Verbindungen wie z.B. Flagella, Pili oder LPS, bleiben bei BGs genauso intakt wie beim nativen Bakterium, wodurch sie *per se* die unspezifische Immunabwehr stimulieren können. Aus diesem Grund bietet die BG Technologie ein innovatives System für den Transport von Vakzinen, therapeutischen Wirkstoffen und andere aktive Substanzen für verschiedenste Zellen und Gewebe tierischen, menschlichen oder pflanzlichen Ursprungs.

Detaillierte Informationen über das BG-System sind in **Kapitel 1. "Introduction to the bacterial ghost technology platform "**, bestehend aus folgenden Reviews, zu finden:
Chapter 1.1. "Bacterial ghosts as Vaccine and Drug Delivery Platforms";
dieser Review wurde in "*Patho-Biotechnology*" (2008) publiziert.
Chapter 1.2. "The Bacterial Ghost Platform System: Production and Applications";
dieses Manuskript ist bereits als Publikation in "*Bioengineered Bugs*" (2010) angenommen.
Chapter 1.3. "Bacterial Ghosts (BGs) – Advanced Antigen and Drug Delivery System";
dieses Manuskript ist bereits als Publikation in "*Vaccine*" (2010) angenommen.

Die relevanten Ergebnisse sind in Kapitel 2. „Application of bacterial ghosts" zusammengefasst. Dieses ist in drei Themengebiete unterteilt und beinhaltet fundamentalen Anhaltspunkte für zukünftige Anwendungen des BG-Systems. Die jeweiligen Unterkapitel sind als eigenständige Arbeiten im Stil eines Manuskripts verfasst und enthalten alle folgenden Abschnitte: Titel, Kurzbeschreibung, Einleitung, Material und Methoden, Diskussion, Figuren und Referenzen

Kapitel 2.1. Bakterielle Ghosts als Trägervehikel von Zytostatika

Bakterielle Ghosts (BGs) können zielgerichtet an verschiedene Typen von Krebszellen anhaften und es wurde gezeigt, dass BGs effektiv von Kolonkarzinoma-, Leukämie- und Melanomazellen endozytiert werden. Vorangehende Studien belegen, dass BGs, welche mit dem zytostatischen Wirkstoff Doxorubicin (DOX) beladen wurden, erfolgreich von der Kolonzellline CaCo2 internalisiert wurden. Weiters konnte gezeigt werden, dass die beladene Substanz im Zytoplasma der Zielzelle freigesetzt wurde und akkumulierte Konzentrationen von DOX wurden im Zellkern detektiert. Die Inkubation mit DOX-beladenen BGs führte zu einer

signifikanten Reduktion der Zellviabilität sowie Proliferationskapazität. Die Effekte, welche mit DOX-BGs erzielt wurden, waren um -2-3 Ordnungen höher als im Vergleich zu jenen die mit freiem DOX in äquivalenter Dosis erhalten wurden. Ähnliche Resultate wurden auch mit verschiedenen Leukämiezelllinien erhalten.

In diesem Teil der Arbeit sollten BGs mit den chemotherapeutischen Substanzen Resveratrol (RV) und dessen Analoga Digalloylresveratrol (DIG) und 3,3',4,4',5,5'-Hexahydroxystilben (M8) beladen werden. Weiters sollte der Einfluss auf die Viabilität der Kolonzelllinie HT29 nach Inkubation mit diesen beladenen BGs untersucht werden. Zusätzlich sollte die endogene Freisetzung von RV mittels HPLC bestimmt werden.

Kapitel 2.2. Bakterielle Ghosts als „targeting vehicles" von Augenzellen

Das konjunktivale Epithelium der Augenoberfläche dient als Schutzbarriere gegen schädliche Einflüsse und stellt eine Herausforderung für die Verabreichung von Wirkstoffen gegen Augenerkrankungen dar.

Aufgrund dessen, dass BGs ein neues Träger- und Adjuvans-System für das „delivery" mukosaler Vakzine repräsentieren, sollten die „targeting" Eigenschaften unterschiedlicher BGs, die aus verschiedenen Spezies generiert wurden, in humanen konjunktivalen Zellen untersucht werden. Die Aufnahmeraten sollten dabei mittels Durchflusszytometrie bestimmt werden. Weiters sollte der Einfluss der BGs auf die Expression verschiedenster Moleküle (MHC-I und MHC-II sowie ICAM-1) untersucht werden.

Um zu beweisen, dass Lipopolysaccharid (LPS), welches auf der Oberfläche von BGs vorhanden ist, keinen Risikofaktor für die Verwendung von BGs als Vakzinkandidat darstellt, sollten die potentiellen zytotoxischen und genotoxischen Effekte von BGs in der konjunktivalen Zelllinie CCL 20.2 mittels Neutralrot Assay (Viabilität) sowie mittels der Einzelzellgelelektrophorese Technik (DNA-Schädigung) untersucht werden.

Eine frühere Studie demonstrierte, dass Bakterien einen Einfluss (verstärkend oder reduzierend) auf die schädigende Wirkung von Substanzen haben können. Daher sollte untersucht werden, ob der toxische Einfluss des Konservierungsmittels Benzalkoniumchlorid auf die konjunktivale Zelllinie CCL 20.2 in Anwesenheit von BGs moduliert werden kann.

Kapitel 2.3. Bakterielle Ghosts als Auslöser des angeborenen Immunsystems

Das angeborene Immunsystem schützt den Organismus vor eindringenden Mikroorganismen und löst eine Reihe von Verteidigungsmechanismen aus. Wie bereits anfangs erwähnt, besitzen BGs alle strukturellen sowie morphologischen und antigenischen Komponenten, die für die Aktivierung des angeborenen Immunsystems benötigt werden.

Da nur wenig über die Kapazität der BGs betreffend der Expression von angeborenen Immunmodulatoren der Haut bekannt ist, sollte, nach Applikation von BGs, die Expression von

proinflammatorischen Zytokinen (v.a. Interleukin (IL)-6 und IL-8) in humane Keratinozyten untersucht werden. Weiters sollte festgestellt werden ob BGs antimikrobielle Peptide wie z.B. Psoriasin und humanes β Defensin-2 induzieren können

Ein weiterer Verteidigungsmechanismus des angeborenen Immunsystems ist durch die Freisetzung von Radikalen z.B. reaktiver Stickstoffspezies wie Stickoxid (NO) gegeben. Dieses Molekül dient als Mediator verschiedenster zellulärer Funktionen und wird durch die induzierbare NO-Synthase (iNOS) produziert. Es ist bekannt, dass LPS iNOS abhängige NO-Bildung in Phagozyten auslöst. Daher sollte untersucht werden ob BGs die Akkumulierung von NO in RAW 264.7 Makrophagen induzieren können und ob die Bildung von NO durch die Beladung von BGs mit dem iNOS Inhibitor Resveratrol moduliert werden kann.

Appendix

Zusätzliche Daten und Figuren, die nicht in den jeweiligen Kapiteln inkludiert wurden, sind im Appendix zu finden (**A.1.-A.5.**). Weiters wurden BGs mit Komponenten von Kaffee (Kahweol und Cafestol) beladen und anschließend mittels HPLC analysiert. Die Ergebnisse werden in Sektion **A.6.** kurz diskutiert. Im letzten Teil des Appendix wird die zytotoxische Wirkung verschiedenster Augentropfen auf die konventionelle Augenzellline CCL 20.2 sowie auf konjunktivalen Primärzellen untersucht (**A.7.**). Diese Studie wurde inkludiert, da fast alle untersuchten Lösungen BAC beinhalteten, welche schon in Kapitel **2.2.2**. in Kombination von BGs analysiert wurde. Weiters wurden vorläufige Untersuchungen durchgeführt, in denen die zytotoxische Wirkung zweier Augentropfen in Anwesenheit von BGs ermittelt wurde (**A.4.**).

Zusammenfassung

Kapitel 2.1. Bakterielle Ghosts als Trägervehikel von Zytostatika

In vielen Studien wird die chemopräventive und zytostatische Wirkung des Hydroxystilbens Resveratol (RV) demonstriert. Die neuartigen, RV-Analoga Digalloylresveratrol (DIG) und 3,3',4,4',5,5'-Hexahydroxystilben (M8) zeigen höhere antitumor-Aktivität als RV und sind daher neue Wirkstoffkandidaten für die Behandlung von Kolorektalkrebs.

Der Vorteil, dass die bioadhäsiven bakteriellen Ghosts (BGs) ihre natürlichen immunstimulierenden Komponenten auf ihrer Zelloberfläche behalten, prädestinieren sie als optimalen „drug delivery" Vehikel, die für die Behandlung und Therapien von Tumoren eingesetzt werden können. Daher wurden BGs, die mit diesen chemotherapeutischen Komponenten beladen wurden, auf ihre zytotoxische Wirkung in der Kolonzelllinie HT29 untersucht. Befunde aus vergleichenden Untersuchungen zeigten, dass durch das „BG-delivery" der Substanzen die Lebensfähigkeit der Zellen signifikant reduziert werden konnte – gemäß des zytotoxischen Schemas der freien Substanzen (DIG> M8 > RV). Um zu bestätigen, dass diese Effekte auf intrazelluläres „drug-delivery" zurückzuführen sind, wurden Experimente mit RV-BGs durchgeführt und die Konzentration von RV sowie dessen Metaboliten mittels HPLC bestimmt. Die Ergebnisse dieser Studien zeigen, dass das beladene photosensitive RV, sehr gut durch die BG Hüllen geschützt wurde und dass die beladene Substanz effektiv in den HT29 Zielzellen freigesetzt wurde. Weiters wurden intrazelluläre RV-Konzentrationen und Metaboliten gefunden, die selbst nach Coinkubation von RV-BGs bis zu 24 Stunden detektierbar waren. Zudem wurden nach Behandlung mit RV-BGs höhere Konzentrationen von RV in HT29 gemessen, als im Vergleich zur Behandlung mit freiem RV in äquivalenter Dosis.

Kapitel 2.2. Bakterielle Ghosts als „targeting vehicles" von Augenzellen

Bindehautszellen repräsentieren nicht nur eine mechanische Barriere für Pathogene, sondern schützen das Auge auch gegen Allergene und toxischen Substanzen. Dennoch sind weltweit viele Menschen von Erkrankungen der Bindehaut betroffen und es bedarf eines „delivery" Systems, welches effektiv den Transport von Wirkstoffen in den betreffenden Bereich des Auges gewährleistet.

Um die Anwendung von BGs als Trägersysteme für zukünftige therapeutische Applikationen zu prüfen, wurden grundlegende Reaktionen von konjunktivalen Zellen nach Coinkubation mit BGs analysiert. Die dafür verwendeten Zellen wurden durch Kultivierung primärer humaner Bindehäute erhalten und die zu untersuchenden BGs wurden von unterschiedlichen bakteriellen Spezies produziert. In Abhängigkeit des BG-Typus, konnten bei

konjunktivalen Primärzellen hohe Aufnahmeraten von BGs mittels FACS Analysen nachgewiesen werden. Weiters konnte gezeigt werden, dass BGs zu einer erhöhten Expression von ICAM-1 auf der Oberfläche von Bindehautszellen führen. Es ist anzunehmen, dass dies einen zusätzlichen stimulatorischen Effekt auf die Leukozytenpopulation haben könnte oder als kostimulatorisches Signal dient, das für Aktivierung von T-Zellen und Zytokinproduktion benötigt wird.

Die zytotoxische Wirkung von Benzalkoniumchlorid (BAC), welches häufig als Konservierungsmittel in ophthalmischen Lösungen verwendet wird, ist weitgehend bekannt. Da nachgewiesen werden konnte, dass durch BGs keine toxischen Effekte (weder zytotoxisch noch genotoxisch) ausgelöst werden, wurden BAC-bedingte toxische Effekte in der konjunktivalen CCL 20.2 Zelllinie in Anwesenheit von BGs untersucht. Es stellte sich heraus, dass BGs äußerst protektiv gegenüber BAC induzierter Zytotoxizität in CCL 20.2. Zellen wirkten. Weiters konnte gezeigt werden, dass BAC, vermutlich durch die Produktion von H_2O_2, zu DNA-Schäden in Bindehautszellen führt. Da nachgewiesen werden konnte, dass selbst lyophilisierte BGs Aktivitäten des Enzyms Peroxidase aufweisen, wurden weitere Experimente mit selektierten BGs durchgeführt und BAC-protektive Effekte in Gentoxizitätsmessungen gefunden. Weiters wurden Tests durchgeführt, in denen bestätigt wurde, dass die bakteriozide Wirkung des Konservierungsmittels in Anwesenheit von BGs erhalten bleibt.

Kapitel 2.3. Bakterielle Ghosts als Auslöser des angeborenen Immunsystems

Das angeborene Immunsystem besteht aus bestimmten Zellen und humoralen Faktoren, welche infektiöse Agentien sofort erkennen, inaktivieren und töten. Dieser Verteidigungsmechanismus beinhaltet die Phagozytose von Bakterien durch Neutrophile oder Makrophagen sowie die Freisetzung von antimikrobiellen Peptiden, proinflammatorischen Zytokinen, hydrolytischen Enzymen oder reaktiver Sauerstoff/Stickstoff Spezies durch Phagozyten.

Antimikrobielle Peptide und proinflammatorische Zytokine, welche von epidermalen Keratinozyten (KCs) produziert werden, stellen eine Schutzbarriere für eindringende Mikroorganismen dar und bilden einen wichtigen Teil in der unspezifischen Immunantwort der Haut. Aufgrund der Tatsache, dass die antimikrobiellen Peptide S100A7c (Psoriasin) und humanes Beta defensin-2 (hBD-2) zur Eliminierung von Hautpathogenen führen, wurde die mRNA Expression dieser Defensine nach Inkubation von KCs mit *E. coli* BGs untersucht. Ergebnisse von quantitativen real-time PCR Tests zeigten, dass BGs diese beiden Peptide in einer Partikelzahl-abhängigen Wirkung induzieren. Zudem konnte gezeigt werden, dass die Aufnahme der BGs in KCs wie auch die Freisetzung von proinflammatorischen Zytokinen, wie Interleukin (IL)-6 oder IL-8, auch vom Flagellingehalt auf der Oberfläche der BGs abhängig ist.

Die Freisetzung von Stickoxid (NO), welches durch die induzierbare NO-Synthase (iNOS) produziert wird, spielt eine wichtige Rolle bei infektiösen Krankheiten. Da bekannt ist, dass bakterielles Lipopolysaccharid (LPS) ein starker „Inducer“ der iNOS ist, wurde die Bildung von NO in einer murinen Makrophagenzelllinie nach Inkubation mit BGs untersucht. Es zeigte sich, dass *E. coli* BGs schnell von RAW 264.7 Zellen phagozytiert werden und in Abhängigkeit der Partikelzahl die NO-Akkumulierung induzieren.

Von Resveratrol (RV) weiß man, dass es inhibierend auf die iNOS wirkt. Daher wurden weitere Experimente durchgeführt, in denen die BG-induzierte Akkumulierung von NO im Vergleich zu RV-beladene BGs untersucht wurde. Tatsächlich konnte die NO-Entwicklung auf diese Weise moduliert werden. Obwohl der generelle Aufnahmemechanismus von RV noch unklar ist, konnte gezeigt werden, dass endogenes „RV-delivery“ mittels BGs zu einer Modifizierung der NO-Freisetzung führt. Durch diese Untersuchungen konnte die Existenz eines zellinternen RV-Rezeptors postuliert werden, der als ERVR (Endogener Resveratrol Rezeptor) bezeichnet wird.

List of Abbreviations

ADDS	advanced drug delivery system
ADH	alcohol dehydrogenase
AG	antigen
APC	antigen-presenting cell
BAC	benzalkonium chloride
BG	bacterial ghost
BPL	β-propiolactone
C	cafestol
CFU	colony forming units
CLSM	confocal laser scanning microscopy
CPS	cytoplasmic space
DC	dendritic cell
DIG	digalloylresveratrol
DMBA	7,12-dimethylbenz[a]anthracene
DOX	doxorubicin
EALT	eye associated lymphoid tissue
ETEC	enterotoxigenic E. coli
FITC	fluorescein isothiocyanate
FL1	fluorescence signal 1
FSC	forward scatter
GFP	green fluorescent protein
GST	glutathione S-transferase
hBD-2	human β defensin-2
HCC	human conjunctival cell
HCDEC	human conjunctiva-derived epithelial cell
HPLC	high performance liquid chromatography
ICAM-1	inter-cellular adhesion molecule 1
IL	ionic liquid
IL-6	interleukin-6
IL-8	interleukin-7
IM	inner membrane
iNOS	inducible nitric oxide synthase
IPTG	isopropyl β-D-thiogalactopyranoside

K	kahweol
KC	keratinocyte
LPS	lipopolysaccharide
M8	3,3’,4,4’,5,5’-hexahydroxystilbene
mc	minicircle
MDR	multi-drug resistance
MFI	mean fluorescence intensity
MOI	multiplicity of infection
MPL	monophosphoryl lipid A
NAT	N-acetyltransferase
NLR	NOD-like receptor
NO	nitric oxide
OD	optical density
OM	outer membrane
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PGRP	peptidoglycan-like recognition protein
P-HCl	pilocarpine hydrochloride
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine
PPS	periplasmatic space
PSI	pounds per square inch
pSIP	self-immobilizing plasmid
qRT-PCR	quantitative real-time PCR
RV	resveratrol
SCGE	single cell gel electrophoresis
SD	standard deviation
SNUC	staphylococcus aureus nuclease
TAA	tumor-associated antigen
TFF	tangential flow filtration
TLR	toll-like receptor

Chapter 1.
Introduction to the Bacterial Ghost Technology Platform

Chapter 1.1.
Bacterial ghosts as vaccine and drug delivery platforms

Ulrike Beate Mayr, Verena Juliana Koller, Petra Lubitz, Werner Lubitz *

University of Vienna, Department of Medicinal Chemistry, Althanstr. 14, UZA2 2B522, 1090 Vienna, Austria, and BIRD-C GmbH&CoKEG, Hauptstr. 88, 3420 Kritzendorf, Austria

* corresponding author
University of Vienna
Department of Medicinal Chemistry
Althanstr. 14, UZA2 2B522
1090 Vienna, Austria

Tel.: +43 1 4277 55115
Fax: +43 1 4277 55120
werner.lubitz@univie.ac.at
werner.lubitz@bird-c.at

The Bacterial Ghost (BG) Vaccine Platform Technology represents a particulate carrier system for protein subunit or DNA-encoded antigens endowed with intrinsic adjuvant properties. By all its biological background BG vaccines alert the immune system with signals for a bacterial infection and induce innate and adaptive immune responses against the antigens. Presentation of subunit vaccines within the BG complex is of advantage for the recognition of the target antigens by the immune system. Delivered as particle, to facilitate the uptake by professional antigen presenting cells (APC), BG satisfy the requirement of naturally furnished adjuvant particles for submit vaccine candidates. Such BG particles have a surface make-up which is not denatured and their surface adhesins are fully functional for the interaction with cellular receptors of APCs to induce the release of natural danger signals and cytokines characteristics for infections with real pathogens. The specificity for targeting tissues or cells, its easy method of production and its versatility in entrapping and packaging various compounds in different compartments of ghosts can be used for the creation of Advanced Drug Delivery Systems (ADDS). The original targeting functions of the bacteria ghosts are derived from enable them to bind to and/or are taken up by specific cells or tissues of animal, human or plant origin. The bacterial ghost system represents a platform technology for creating new qualities in non-living carriers which can be used for the specific targeting of drugs, DNA or other active compounds such as tumour cytostatics to overcome toxic or non desired obstacles. The new system is an alternative to liposomes and may have an advantage to its higher specificity for targeting different tissues, its easy way of production and its versatility in entrapping and packaging various compounds in different compartments of the carriers.

Introduction

In analogy to empty erythrocyte ghosts which are devoid of cytoplasmic content and to bacteriophage ghosts which are free of nucleic acids the empty envelope of Gram-negative bacteria produced by PhiX174 gene E-mediated lysis has been named Bacterial Ghosts (BG). BG will be used in the following for a single bacterial ghost as well as for multiple bacterial ghosts. BG is already a common abbreviation for any type of Gram-negative cell envelope produced by expression of controlled expression of cloned PhiX174 gene E. [1] BG have been produced from different bacteria including *E. coli* C, different *E.coli* K12 laboratory strains, enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC), *Salmonella enterica* serovar *typhimurium*, *S. enteritidis*, *Shigella flexneri*, *Vibrio cholerae* O1 and O139, *Helicobacter pylori, Neisseria meningititis, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Pasteurella multocida, Mannheimia haemolytica, and Francisella tularensis* LVS and *Pectobacterium cypripedii*. Except of E.coli C and K12 derivatives and Pectobacterium cypripedii all other bacterial strains used are pathogenic strain causing severe diseases in animals or man. This imbalance of the number of BG produced from pathogenic versus non-pathogenic Gram-negative bacteria reflects one of the major applications of BG as vaccines. Investigations with E.coli C and K12 strains were mostly done for genetic engineering work to establish the BG system, and with the plant-specific P. cypripedii for plant pesticide targeting. [2]

The other major application of BG is in Advanced Drug Delivery Systems (ADDS) is in part an extension of our basic investigations how to produce and to modify BG and to use the empty cell envelops for biomedical applications. BG production is characterized by the introduction of a small hole in the envelope of the bacteria and release of the cytoplasmic content driven by the osmotic pressure difference between the cytoplasma and the outside growth medium of the bacteria. This process of E-mediated lysis of bacteria is driven by gene E expression and co-translational integration of the E-polypeptide into the inner membrane followed by conformational changes of E and represents an endogenous genetic intracellular method to inactivate bacteria. [3] Gram-positive bacteria are simply killed by break down of the membrane potential without loss of cytoplasmic material. [4]

BG have multiple applications in biomedicine as vaccines, as adjuvants, as DNA transfection vehicle for DNA vaccines or somatic gene transfer, as drug carrier for tumour therapy, miniature bioreactors, artificial bacterial life forms, and last but not least as construction modules for molecular machines in micro- and nanotechnology. All these different applications are based on our knowledge how to produce and modify BG for specific purposes. In the following examples the focus are applications of the BG platform technology in vaccines and ADDS where the products are based on common components and production processes.

More information on the BG vaccination trials can be found in several recent reviews where most of the original literature is cited [1, 5-8]as well as for the application as ADDS with schematic illustrations. [9, 10].

Characterization of BG

Studies of the E-lysis process of Gram-negative bacteria has been emerged from basic science addressing both the lysis mechanism of bacteriophage PhiX174 after infection of *Escherichia coli*, and, the specific mode of action of the cloned lysis gene E of the phage [11-15] State to the art BG production requires the transformation of the host bacterium with a plasmid which carries the gene E under an inducible promoter. Since the gene E product is highly lethal the recipient either carries the corresponding repressor system on its chromosome or on a plasmid or to use E-lysis plasmids where the repressor system is also encoded on the very same plasmid which is able to raise a critical repressor concentration in the bacterium to silence gene E expression. Thus, the proper establishment of the genetic repression/expression system in a given Gram-negative bacterium determine the success of BG production.

A series of plasmids have been developed which carry the gene E under an inducible expression control. The most elaborate systems are derived from the phage Lambda left or right promoter/operator system with expression control by the thermosensitive cI857 repressor or derivatives providing the growth of bacteria up to 28, 36 and 39°C, and E-mediated lysis at any temperature 2°C above the maximal repression temperature up to 42-44°C for enterobacteriacae and most other bacteria [16]. The loss of nucleic acids due to E-mediated lysis greatly minimizes the risk of horizontal gene transfer of pathogenic islands or antibiotic resistance genes which have been used for the plasmid system(s) used. As DNA can unspecifically adsorb to the inner membrane of BG “superclean” BG can be produced by expression and activation of the cloned Staphylococcus aureus nuclease (SNUC) which degrades DNA and RNA [2]. The remaining DNA level of such BG preparations is below the real-time-PCR detection level and sets a new quality criterion for inactivated vaccines, and diminishes the risk of horizontal gene transfer in some of our BG preparations. A short characterization of protein E and SNUC is given in **Table 1**.

Minimal amounts of protein E are required to lyse Gram-negative bacteria [17] therefore the challenge in BG production is to assure the complete repression of gene E before induction of gene E expression. Induction of gene E, however, is not enough to achieve proper E-mediated lysis. There are additional requirements such as active growth and functional control elements of cell division and of autolytic activity of the host bacteria. Membrane adhesion sites, FtsZ protein in the septosome [18], cis-trans proline isomerases for conformational change of protein E [19], chaperones, the strength of the membrane potential [20], the activity of the autolytic system [21], the ph and osmotic strength of the medium [22], and other factors also

influence the E-lysis process. A reasonable BG production rate of a growing culture is approximately 99.9 - 99.99% and depends largely on exponential growth of the bacterial culture. Only growing bacteria can be lysed, bacteria entering stationary phase are phenotypically resistant to lysis [23]. Although it seems to be trivial to E-lyse bacteria a good and efficient E-lysis needs experience and stringent process control.

Investigations of the molecular mechanism of E-mediated lysis showed that protein E fuses the inner and outer membranes of Gram-negative bacteria, thereby forming a transmembrane lysis tunnel in the bacterial envelope through which the cytoplasmic content is released. High resolution transmission electron micrographs clearly show the intact structure of the bacterial envelope, and of the continuity of the inner and outer membranes forming the border of the E-specific transmembrane [13].

On average, the diameter of the E-specific transmembrane tunnel varied between 40 and 80 nm [13]. The variation in size and irregular tunnel structures indicated that the E-specific transmembrane tunnel structure is dynamically formed by the strong force ejecting the cytoplasmic content through the E-lysis hole which is most probably formed by E-oligomers. [24] Under normal bacterial growth conditions the osmotic pressure difference between the total solutes of the cytoplasma and the outside growth medium is more than 1 bar.

Due to the integration of protein E in the inner membrane the paracrystaline peptidoglycan net located in the periplasmic space between the inner and outer membrane of the cell envelope structure of Gram-negative bacteria exhibits a higher turn-over rate at potential sites of lysis tunnel formation. As a consequence, the borders of the E-lysis tunnel are determined by the local mash size of the peptidoglycan which is the shape determining rigid structure of the bacteria. Except for the hole BG resemble their natural mother bacteria and in the outer surface of BG pili, flagella and lipopolysaccharide are well retained. The inner side of the BG envelope complex corresponds to the inside of the cytoplasmic membrane and its associated products which are not released by E-mediated lysis. As there is a hole in the envelope the membrane potential of BG is no longer existing whereas membrane integrated enzymes like the ATPases are still functional. [20, 25] The space between both membranes is the periplasmic space (PPS) which by its nature is a gel like environment rich in membrane derived oligosaccharides, specific enzymes, proteins and peptidoglycan. It should be mentioned that because of the fusion of the inner and outer membranes at the E- specific transmembrane lysistunnel the PPS is sealed. [13, 20] Again, enzymes specific for the PPS like alkaline phosphatase and beta-lactamase are still active. The outer membrane and all its appendices are functional preserved which is of major importance for their targeting and adhesion properties. Thus, BG are highly sophisticated designed like their mother bacteria for adhesion and eventually uptake by cells or tissues and better equipped than any engineered liposome, which can be artificially produced. As the nature of BG as well as their architecture is important for

their functions when possible in the following sections both applications as vaccines and ADDS are discussed together.

BG as vaccines

There is no doubt that functional vaccines are the most effective medical interventions to safe lives and reduce costs in healthcare. Some of the traditional vaccines still in use do not meet the following requirements for novel vaccines: (i) to be safe and immunogenic in young children, the adult and the elderly, (ii) include multiple serotypes/species, (iii) be inexpensive (less than a Euro per dose), easy to produce, stable without refrigeration, and amenable for needle-free administration, and, last but not least, (iv) should confirm robust immunity with three or less doses. As far as tested and calculated, BG vaccines meet most the criteria listed and provide improvement and substitution for existing vaccines and follow well the paradigm shift from single subunit vaccines to multiple antigen vaccines. BG is also excellent alternatives to vaccines which use chemicals, heat or irradiation to inactivate the pathogen. All these methods denature essential structural components of the bacteria whereas the E-lysis process for BG production is a genetic/biochemical method leading to a superior preservation of their antigenic properties.

In the simplest form BG vaccines consist of a freeze dried powder of BG particles without any additions of stabilizers or adjuvant. The latter is very important as some of the adverse reactions against vaccine preparations originate from the adjuvants or stabilizers used. The BG system, however, is a potent vaccine delivery system with intrinsic adjuvant properties. Due to the particulate nature of BG and the fact that they contain many well known immune stimulating compounds such as LPS, lipid A and peptidoglycan BG enhance immune responses without any further additions. The results of different *in vivo* and *in vitro* studies confirm the potential of bacterial ghosts as carrier and adjuvant in modern vaccine development addressing key immune cells, such as dendritic cells, macrophages or monocytes via toll-like receptors or opsonized antibody facilitated uptake [26]**.** Oral, intra-nasal, intra-ocular, intra-vaginal, rectal and acrogenic are the preferred routes of needle-free BG vaccine delivery. The oral immunization of rabbits with V. cholerae BG induced protective immunity determined with the RITARD test and conferred cross protection between classical O1 and the recently emerging O139 strain [27]. 100% protection levels against heterologous lethal challenge with EHEC was achieved in mice after two oral immunizations with EHEC BG [28]. The same result could be obtained with a single rectal immunization of mice with EHEC BG (Mayr and Lubitz, to be published elsewhere). Here, it should be emphasized that rectal immunization could have a high value for vaccine delivery in children as accurate dosing is given by the supensorium and parents normally feel comfortable and are familiar with fever reducing supensorium. Intra-nasal immunizations with BG in mice gave good immune protection against Shigella infections.

Aerosol immunization of pigs with *Actinobacillus pleuropneumoniae* BG induced sterile immunity against lethal bacterial challenge [29]. BG have an ideal aerosol diameter of 1-3 um and can be prepared as dry or wet aerosols. [30] The pathogen *A. pleuropneumoniae* and the experimental animal model (pigs) used for the former mentioned study are a good model for human lung immunizations or for the aerosol delivery of drugs deep in the alveolar space.

BG with extended properties

The BG system offers a construction kit with a modular concept where different molecular parcels/bricks are assembled within a living cell by genetic engineering before BG formation is induced. [**Fig. 1**] For specific needs and purposes genetic engineering of the host bacteria which are candidates for BG production can be used to modify their cell envelope to carry foreign proteins and/or to use such recombinant proteins to bind other proteins, DNA or active substances. Some of the applications of such modified BG are in vaccines whereas other find their application in BG as carrier of active substances or in more technical applications. It should be mentioned here that one of our future aims is to develop for therapeutic use BG combinations for drug targeting and vaccines in a single BG. In **Table 1** and in the following vaccine and ADDS properties of engineered BG are discussed together.

The following modules are in our construction kid for BG vaccine and BG carrier design:

i) Plain BG:

Vaccines: The BG envelope is either derived from a pathogen or from a non-pathogenic organism. [**Fig. 1**] In the first case the envelope itself is sufficient to induce a relevant immune response in the host. In the latter case the BG can serve to stimulate the innate immune system

ADDS: The BG from pathogens are also excellent targeting vehicles for primary immune cells, endothelial cells of blood vessels or intestine, or to recognize tumour tissue by specific ligand receptor interaction.

ii) BG as carrier of additional proteins:

Vaccines: The BG envelope can be derived from a non-pathogenic bacterium and serves as carrier and adjuvants in regard to the target antigens, or can be derived from a pathogenic organism carrying out the same functions and represent combination vaccines. The additional protein represents always multi-epitope antigens which can be presented in several ways**,** e.g. anchoring of the foreign protein to the inside of cytoplasmic membrane by fusing it to a N-, C- or N- and C-terminal membrane anchor. [**Fig. 1B**] Fusion of the target antigens with the maltose binding protein or fusing it to an export sequence are methods to export foreign protein or polypeptide constructs to the periplasmic space. For the insertion of target antigens on the surface of the outer membrane OmpA-fusion can be used. Foreign or homologous pili can be inserted in the envelope which can either act as subunit vaccine or to broaden the specific

receptor recognition repertoire of the BG. S-layer protein matrices formed by SbsA or SbsB can be modified to carry foreign inserts [31]. As both proteins form sheet like self-assembly structures they are not expelled with the cytoplasma and remain in the inner cytoplasmic lumen after E-mediated lysis. When SbsA or SbsB fusions are exported as maltose-binding protein fusion to the periplasmic space they still retain their self assembly capacity and fill this space with sheets carrying target antigens.

ADDS: If the foreign protein is an enzyme or a functional active polypeptide the same methods for internalization of the target protein as described above for antigens can be used. In addition, if streptavidin is used as foreign protein other biotinylated ligands can be added to the BG protein/polypeptide carrier. [**Fig. 1B**][32] In one of the application examples biotinylated dextran has been filled into the cytoplasmic lumen of BG exposing membrane-anchored streptavidin.[**Fig. 1C**] Such polymers themselves can be substituted with drugs and represent a slow release system. In a specific formulation with in vivo biotinylated E or E-Streptavidin fusions which are still lysis active the ligand receptor moieties of the fusion constructs are exposed on the BG surface surrounding the E-specific lysis tunnel. [**Fig. 1D**] Any streptavidin or biotinylated new compound, respectively, can be directed to bind to this crown like ligand ring.[**Fig. 1E-1C**] For example, small vesicles have been bound to the BG carrier piggy-backing inside out vesicles of other bacteria. It is also feasible to construct net-like streptavidin-biotin chimneys of variable length on top of the E-lysis tunnel structure to form nano tubular structures [**Fig. 1F**] which can modify the release properties of the BG carrier filled with water soluble substances. [**Fig. 1G**]

BG as carrier of nucleic acids:

Vaccines: BG are derived from non-pathogenic or pathogenic bacteria and serve as carrier of DNA and provide for the DNA vaccine targeting functions for antigen-presenting cells and a highly efficient intracellular DNA release from the endosome-lysosome compartment. The high transformation and target gene expression rates of dendritic cells (75%) and for macrophages (60%) with DNA-BG carriers can compete with commercial transfection systems. The method for filling BG with DNA is rather simple and in its standard version freeze dried BG are resuspended in a DNA solution, and after washing off the excess of DNA which is not bound to the inside of the cytoplasmic membrane, the DNA-BG can either be used immediately for gene transfer experiments or can be stored after freeze drying for later applications. The number of plasmids per BG depends on the concentration of the DNA solution used and as greater the DNA concentration the greater the DNA loaded per BG. Loading of BG with DNA is very efficient as more than 3,000 copies of a medium sized plasmid can be bound per BG [33]. A more sophisticated version of loading BG with DNA uses the specific interaction of an inner

membrane anchored DNA binding protein with the corresponding operator region on target plasmid. [**Fig. 1H**] This allows a one step DNA loading and BG formation process [28].

ADDS: For the application of DNA in somatic gene transfer it is desirable to use DNA constructs devoid of antibiotic resistance cassettes and origin of replication of the production plasmid and to design minimal minicircles including an eukaryotic promoter the gene of interest with poly A tail and transcription stop sequences. The improved version of our self-immobilisation plasmids encodes the par A resolvase which is a specific DNA recombination enzyme recognizing homologous sequences on the plasmid. After expression of the par A resolvase and the inner membrane anchored DNA binding protein only minicircle DNA is bound to the inner membrane. By E-mediated lysis BG are produced with in vivo loaded minicircle DNA [34]. In addition to the functional aspects of DNA encoding genes or siRNA for regulation of gene expression, DNA can also be considered as pure structural element for construction purposes to architecture for instance the inner lumen of BG and to serve as docking molecule for other DNA or RNA tailed molecules.

BG as carrier vehicles for DNA and/or cytostatic drugs in tumour therapy:

DNA and/or drugs can be used as active substances for tumour treatment, and investigations have shown that human melanoma and colon carcinoma cells can be targeted with BG for delivery of DNA or drugs, respectively. In a recent study, eight different human melanoma cell lines which have many functions in common with antigen presenting cells [35] [36] have been investigated for their capacity to bind and phagocytise BG [38]. Bowes cells exhibited roughly 80% expression level of BG delivered marker gene indicating that the BG system is suitable as vehicle for the transfer of DNA encoded functional RNA such as siRNA, enzymes for pro-drug conversion or inducer of other activities with therapeutic effects for the host.

Recent investigations of binding polyphenolic compounds to BG confirm the finding that molecules with organic ring structures bind unspecifically to the membrane compartments of BG and add a group of new compounds to the list of active therapeutic substances for intracellular delivery. Treatment of the human colon cancer cell line CaCo2 with doxorubicin (DOX) loaded BG showed an effect on proliferation inhibition by two log difference compared to the free drug [39]. In extended investigations it was determined that DOX BG reduced the growth inhibitory concentrations of the drug up to more than 300 times compared to the EC50 of the free drug and enhanced the intracellular DOX concentrations up to 42 times compared to the free drug (Mader and Lubitz, to be published elsewhere). These results are promising as DOX for instance has accumulating side effects on heart functions and the tumour therapy often has to be terminated once a critical maximal dose is reached. It is not envisaged to apply DOX

BG intravenously but rather local in combination with surgery and pre- and after surgery by short term rinsing or flushing of the colon to deliver the therapeutic cargo. We hope that with this procedure the time window for DOX therapy can be considerably extended and can also contribute to an increased quality of life for the patients as side reactions to the drug are also less severe. The concept to specifically target tumour tissue in combination with surgery could probably also be applied to other tumours such as head and neck cancer and brain tumours as certain BG have a surface make-up that allow them to bind to and being taken up by the tumour cells. The important benefit of BG drug delivery is the increased bioavailability due to the bioadhesive nature of BG envelope surface targeting tumour tissue and the unique intracellular release mechanism of the drug.

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Tables and Figures

Table 1. Short characterization of PhiX174 protein E and Staphylococcus aureus *nuclease A (SNUC)*

Protein E	Very hydrophobic 91 aa membrane-active structural polypeptide with no enzymatic activity
Mode of action	Fusion of inner and outer membrane of Gram-negative bacteria for release of progeny phages in PhiX174 infected cells or transmembrane tunnel formation for production of BG
SNUC	149 aa cytoplasmic protein from *Staphylococcus aureus* strain Foggi
Mode of action	Phosphodiesterase degrades ssDNA, dsDNA and RNA; addition of Ca-/Mg-ions stimulate DNAse activity for degradation of chromosomal and plasmid DNA in course of BG production when at least 99.9% of bacteria are converted to BG

Table 2. Properties of BG and engineered BG in vaccines, ADDS and technical applications

Type of BG	Vaccine	ADDS	Micro-/Nano-/Pico-/ Femtotech/Other
i) Plain BG	BG envelope vaccine; Stimulants of innate immune system	BG carrier of external loaded substances which have a natural affinity to lipid membranes; water-soluble substances or polymers in cytoplasmic lumen	Micrometer carrier with 250 ft volume in cytoplasmic lumen; E-specific lysis tunnel with diameter of 40-100 nm
ii) BG as carrier of additional protein(s)	BG as carrier of subunit vaccine; combination vaccine; BG piggy-back carrier of membrane vesicles from other bacteria	BG carrier of enzymes; BG carrier with Streptavidin as anchoring device for biotinylated products and vice versa	Micro enzyme reactor; BG with membrane vesicles attached at E-lysis tunnel influencing release properties; BG carrier of nano vesicle
iii) BG plus nucleic acids	BG DNA vaccine	BG DNA delivery vehicle for somatic gene transfer and/or for siRNA	BG microparticle with inside DNA architecture

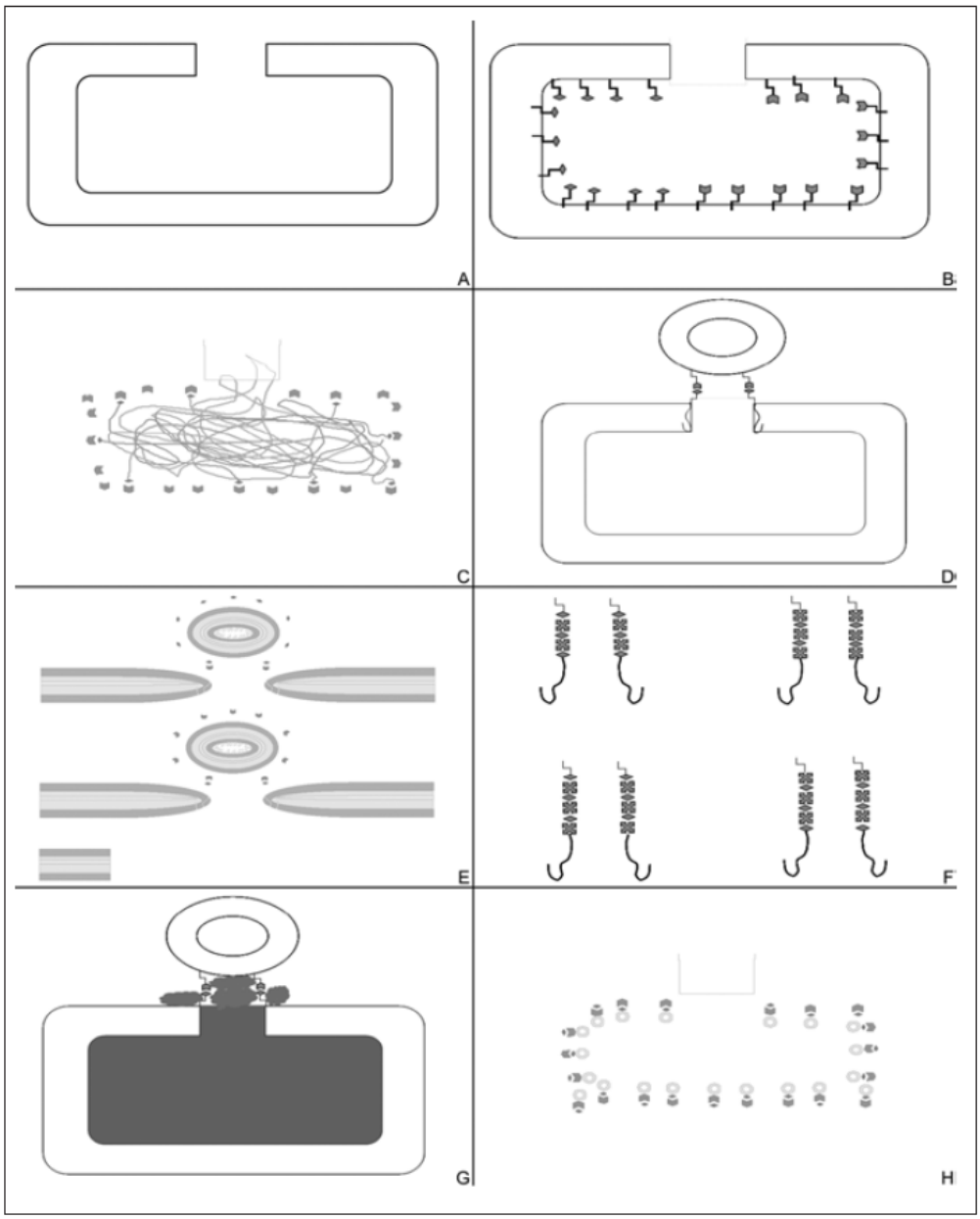

Figure 1. Schematic line drawings of different entities from the BG construction kid. A) Empty BG, inner line corresponds to the inner membrane and outer line to the outer membrane, the space between both lines corresponds to the periplasmic space. B) BG with inner membrane anchored streptavidin or in vivo biotinylation C—terminal amino acid sequence C) BG as carrier of membrane immobilized polymers: , inner membrane anchored streptavidin; , biotinylated polymere, blue and green line represent different polymers, e.g., dextran and polyhydroxybutyric acid. D) BG closed with vesicles attached by specific streptavidin—biotin interaction at E—specific transmembrane lysis tunnel: , protein E with in vivo C-terminal biotinylation site; , inner membrane anchored streptavidin on the outside of inside-out membrane vesicles from Gram-negative bacteria. E) Detail view of in side out vesicles closing the E—specific lysis tunnel. Membrane anchored streptavidin or biotinylated membrane anchor are either expressed on the in side out vesicle or at the E—lysis protein. F) Streptavidin—biotin net-like chimneys of variable length which connect the in side out vesicle with the E—lysis tunnel. G) BG as carrier of water-soluble active substances, water space with solubilized substances leaking out of BG, . H) BG as carrier of inner membrane anchored DNA binding protein, e.g., LacI binding operator site of plasmid or minicircle DNA, .

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Chapter 1.2.
The Bacterial Ghost Platform System: Production and Applications

Timo Langemann [1), 2)], Verena Juliana Koller [1), 2)], Abbas Muhammad [2)], Pavol Kudela [1), 3)], Ulrike Beate Mayr [1)] and Werner Lubitz [1), 2) *]

[1)] BIRD-C GmbH & CoKEG, Hauptstraße 88, 3420 Kritzendorf, Austria

[2)] Department of Medicinal Chemistry, University of Vienna, Althanstraße 14, 2B522, 1090 Vienna, Austria

[3)] Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia

[*] Corresponding author: Tel. +43 (1) 4277 55115 - werner.lubitz@univie.ac.at

The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology. BGs are cell envelopes derived from (recombinant) Gram-negative bacteria that are devoid of all cytoplasmic content but have a preserved cellular morphology including all cell surface structures. Using BGs as delivery vehicles for subunit or DNA-vaccines the particle structure and surface properties of BGs are targeting the carrier itself to primary antigen-presenting cells. Furthermore, BGs exhibit intrinsic adjuvant properties and trigger an enhanced humoral and cellular immune response to the target antigen. Multiple antigens of the native BG envelope and recombinant protein or DNA antigens can be combined in a single type of BG. Antigens can be presented on the inner or outer membrane of the BG as well as in the periplasm that is sealed during BG formation. Drugs or supplements can also be loaded to the internal lumen or periplasmic space of the carrier. BGs are produced by batch fermentation with subsequent product recovery and purification via tangential flow filtration. For safety reasons all residual bacterial DNA is inactivated during the BG production process by the use of staphylococcal nuclease A and/or the treatment with β-propiolactone. After purification BGs can be stored long-term at ambient room temperature as lyophilized product. The production cycle from the inoculation of the pre-culture to the purified BG concentrate ready for lyophilization does not take longer than a day and thus meets modern criteria of rapid vaccine production rather than keeping large stocks of vaccines. The broad spectrum of possible applications in combination with the comparably low production costs make the BG platform technology a safe and sophisticated product for the targeted delivery of vaccines and active agents as well as carrier of immobilized enzymes for applications in white biotechnology.

Introduction

Bacterial ghosts (BGs) are envelopes from Gram-negative bacteria which have been produced by controlled expression of the cloned lysis gene E. After the essential role of gene *E* in the lysis of *Escherichia coli* after infection with bacteriophage ΦX174 was discovered in 1966 [1] and after the eve of genetic engineering 16 years later it could subsequently be shown that after cloning its sole expression is sufficient to cause lysis of *E. coli* [2-3]. E was the first lethal gene for bacteria which could be silenced enough on plasmids to be established in non-host range bacteria of the phage and converts Gram-negative bacteria into BGs whereas Gram-positive bacteria are killed without lysis.

Gene *E* codes for a 91-aa polypeptide [4-5]. In contrast to lytic proteins from other phages protein E has no inherent enzymatic function [6-7] but represents a membrane protein [8-9]. Analysis of the primary structure of protein E revealed a hydrophobic region at its N-terminal end suggesting it is membrane-associated [8] and integrates into the cytoplasmic membrane of *E. coli* [10]. The observation that stationary phase host cells do not respond to E-lysis induction but lyse upon provision of fresh medium as well as the inhibitory effect of non-physiological pH-values on the E-lysis process indicated that E-mediated lysis is dependent on the physiological state of the host cells [11-12] triggers the host cell's autolytic system [9, 13-15].

E has the ability to oligomerize and analysis of the hydropathicity of protein E indicated an E-specific lysis tunnel spanning the inner (IM) and outer membrane (OM) which most probably is located at membrane adhesion sites within the host cell [16]. E-mediated lysis releases all cytoplasmic content to the environment while periplasmic components remained associated with the empty cell envelope [9]. The collapse of the bacterial membrane potential precedes the onset of E-lysis [17]. The E-specific lysis tunnel could be visualized by high-magnification scanning and transmission electron microscopy of E-lysed *E. coli* (**Fig. 1, a**). Moreover, the images illustrated that E- lysis of *E. coli* was accompanied by a fusion of the inner and outer membrane (**Fig. 1, b**) sealing the periplasmic space (PPS) [18]. Several electron-microscopic investigations of E-lysed *E.coli* cells revealed that in more than 90% of the cases the E-specific lysis tunnel is located either at the centre or the poles of the bacteria both of which are potential division zones [19]. Recalling that protein E-mediated lysis is dependent on the physiological state of the host bacterium [11-12] and analysis of E-lysis in *E. coli* mutants with defects in cell division indicated that mechanisms involved in cell division are mandatory for lysis [19-20].

The observed lysis tunnel diameter varies between 40 to 200 nm and does not show any regular structure. The driving force for the rapid discharge of the cell content is the osmotic pressure difference between the cytoplasm and the surrounding medium. However, the native

structure of the peptidoglycan within the envelope complex remains intact and rigid [21]. The observed stimulation of peptidoglycan turnover by about 10% [21] could be in accordance with genetic evidence that protein E inhibits MryA translocase A [22]. Based on these findings and extended experiments using a E-streptavidin fusion protein (E-FXa-StrpA) Schön et al. [23] described the process of E-mediated tunnel formation with a three-phase model: 1. integration of protein E into the IM with the C-terminus facing the cytoplasm - 2. conformational change of protein E translocating the C-terminal domain to the PPS accompanied by oligomerization and targeting of the division initiation complex via lateral diffusion - 3. fusion of IM and OM at membrane adhesion sites induced by exposition of the C-terminus of protein E to the cell surface. This model implies that the lysis tunnel is note solely edged by protein E oligomers but its formation requires protein E-triggered fusion of the inner and outer membrane [23]. A schematic drawing of this model is shown in **Figure 2 (b).**

Upon discovery of the remarkable features of protein E-mediated lysis in *E.coli* the principle of E-lysis could be shown with other Gram-negative bacteria [24-25] but not for Gram-positive bacteria [26]. So far, BGs of numerous Gram-negative strains (different *E. coli* strains, *Salmonella typhimurium, Salmonella enteritidis, Klebsiella pneumoniae, Bordetella bronchiseptica, Heliobacter pylori, Vibrio cholerae, Actinobacillus pleuropneumoniae, Haemophilus influenzae, Mannheimia haemolytica, Pasteurella multocida, Pseudomonas aeruginosa, Pseudomonas putida, Ralstonia eutropha, Pectobacterium cypripedii* and others) have been generated successfully suggesting the BG platform might be extended to any Gram-negative bacterium [25]. The idea of utilizing BGs derived from different Gram-negative bacteria as candidate vaccines emerged due to the demand for both potent and safe new vaccines [27-31]. The BG system offers many advantages over traditional vaccination techniques such as targeting and the intrinsic adjuvant properties of the BG particles. In addition, recombinant DNA technology facilitates the development of multivalent protein or DNA vaccines. Another great feature of BGs is the fact that no denaturing effects occur during E-lysis and hence all antigenic determinants are preserved throughout BG generation. The use of BGs as candidate vaccines and advanced drug carriers can be found in several recent reviews [24, 27, 29, 32-36].

BG - Production Process

Initial cloning and expression studies with gene E used the inducible lac promoter/operator system with an over-expression of the lacI repressor gene (lac PO-lacI^{q1}) [2-3]. Later the temperature sensitive λ-system ($\lambda p_L/\lambda p_R$-cI857) has proven to be more suitable for quick and efficient lysis without the need of any addition of chemical inducers [11]. Since the λ-repressor cI857 shows incipient expression of downstream gene *E* at temperatures above 30°C the temperature sensitivity of the system was optimized to meet more favourable fermentation temperatures of 35°C or higher. Mutations in the O_R2 operator region of the λp_R promoter resulted in tight repression of downstream genes up to 36°C and 39°C, respectively [37-38]. These temperature-inducible E expression cassettes are widely used in current BG production processes since they are robust enough to allow fermentation of the bacterial culture at 35°C and induction of protein E-mediated lysis at 42 or 44°C.

In standard fermentations of various bacteria the quality criterion for a successful E-lysis process is a BG formation of at least 99.9% of the bacterial culture within a time window of 2 h. Depending on the host organism E-lysis efficiencies of more than 99.99% and higher can be achieved in this time frame. In **Figure 3** the time-point of E-lysis induction is defined as time-point zero (0 min) with the preceding growth phase denoted in negative minutes.

BG production has been established in fermentation volumes up to 20 l using Labfors-3 and Techfors-S fermenters (Infors HT, Bottmingen, CH). Starting with a pre-culture that is growing exponentially the production fermenter is inoculated with the inoculum at a volume ratio of 1:10. The standard fermentation process can be divided into three major stages: growth phase (90 min), E-lysis phase (120 min) and downstream processing.

The overall timeline for the production process is designed in a way that from the automatic inoculation of the pre-culture to the final concentration of the product the whole process takes 18 h and can be performed in one working cycle. The key events of the BG production process (**Fig. 4**) are discussed in more detail below.

Growth phase:

The growth phase in an example fermentation with *E. coli* harbouring plasmids for temperature-inducible E-lysis is conducted at 35°C, pH 7.20 and aeration parameters sufficient for exponential growth. To maintain a level of dissolved oxygen (dO_2) of approximately 20% saturation both stirring and aeration rate are adjusted gradually over the course of the growth phase. After 90 min E-lysis of E. coli is induced at cell densities of approximately 1-2 (109) cells/ml.

When recombinant proteins are expressed to be incorporated into the envelope complex before E-mediated lysis expression of the corresponding genes is induced chemically 30 min after inoculation (e.g. lac-, arabinose induction system). In case that the synthesis of the foreign

proteins slows down the growth rate slightly lower cell densities are reached before normal induction of E-lysis and to compensate for BG yield the growth phase may have to be prolonged to 120 min.

E-Lysis phase:

E-lysis of the culture is induced by temperature up-shift from 35 to 42°C (**Fig. 3, (a)**). in our system it takes roughly 10 min to reach the new temperature in the fermenter. At the same time stirring and aeration control is locked to prevent foaming during BG formation. The dO_2-level subsequently drops below 5% and so remains for about 30 min. In the fermentation log visual evidence for E-lysis onset is a sudden signature up-shift of dO_2 (**Fig. 3, (b)**). The E-lysis phase continues for a total of 120 min with its end being characterized by the dO_2 reaching a stationary value of > 95% saturation (**Fig. 3, (c)**).

Down-stream processing:

The BG product is harvested from the fermenter via tangential flow filtration (TFF) in a 0.2 µm hollow fibre module at a temperature of at 15°C. In a first step the fermentation broth (20 l) is concentrated to 2.0 l (**Fig. 5 (a)**) and transferred to a stirred reservoir. Then the concentrate is inactivated with β-propiolactone (BPL). For more detail on BPL see chapter 3. In a second step the BPL-treated broth is washed with sterile, de-ionized water (dH_2O) by diafiltration in a smaller 0.2 µm hollow fibre module. A total of 5.0 l dH_2O displaces the remaining medium and all residual ctoplasmic content. During the non-steady-state diafiltration the product suspension is further concentrated to 400 ml (**Fig. 5 (b)**). The overall concentration factor is 50 while virtually all medium (> 99%) is withdrawn. The final BG concentrate is divided into aliquots and lyophilized. As freeze-dried product BGs are stable at room temperature for many years.

The TFF procedure as described above is an alternative to harvesting and washing BG via centrifugation. Contrary to the filtration process this procedure is more laborious, time-consuming and might lead to BG aggregation because of difficulties with a proper re-suspension of the BG pellet. Furthermore, the implementation of TFF for harvesting and washing of BGs keeps all processes in a closed system and reduces the risk of cross-contamination during the handling procedure.

Process and quality control:

During fermentation all relevant process parameters (T, pH, dO_2, aeration, stirring) are monitored and controlled. Starting from the time-point of inoculation (**Fig. 3: A**, -90 min) samples are taken every 30 min over the course of the fermentation (**Fig. 3: B - H**) and analyzed for optical density (OD600) and cfu. All samples are also examined by light-microscopy and flow

cytometry. Optionally, the biomass is also investigated for DNA content by real-time PCR and the level of protein E expression.

In standard fermentations with *E. coli* the onset of E-lysis is linked to a sudden drop in OD600 of the culture broth and this simple determination is one important indicator of successful E-lysis induction. BG formation can also be observed as the appearance of translucent bacterial bodies in light microscopy. Both methods are good indicators for the quality of E-mediated lysis of *E. coli* but contain no quantitative information. The actual E-lysis efficiency is determined by cfu counting a day after sample collection. Flow cytometry, however, has been established as a reliable real-time tool for the assessment of E-lysis onset and the progress of BG formation [39]. For flow cytometry diluted samples of the culture broth are stained with two fluorescent dyes and run through a CyFlow analyzer (Partec, Münster, Germany). The first dye (RH414) stains phospholipid membranes and with its fluorescence signal defines a gate for the exclusion of all non-cellular background. The discrimination of living cells, dead but non-lysed cells and BGs is obtained by a combination of the forward scatter signal (FSC) and the fluorescence signal (FL1) of the second dye ($DiBAC_4(3)$) which is able to penetrate and stain only cells that have lost their membrane potential. DiBAC-negative cells with a high scatter signal represent living cells, DiBAC-positive cells with a similar scatter signal represent the dead cell fraction. DiBAC-positive cells with a diminished scatter signal are identified as BGs. The general procedure for online monitoring E-lysis of *E. coli* by flow cytometry has been developed by Haidinger et al. [39-40] and was adapted recently. The flow cytometry result for a given sample is available in less than 10 min after sampling. Representative dot-plots of an *E .coli* culture during the E-lysis process is shown in **Figure 6** for the time-points induction (a), course (b) and end of the E-lysis phase (c).

After lyophilization the dry powdery BG product is investigated with respect to sterility and re-suspensibility. For sterility investigations 10 mg of BGs are re-suspended in rich medium and aliquoted for plating as well as for enrichment cultures. All sterility tests are performed in triplets to ensure that the final product does not contain any viable cells. The re-suspensibility is evaluated via flow cytometry with a lyophilized sample that has been re-suspended in dH_2O. Since lyophilized BGs generally are well re-suspensible the sample should give a similar picture and corresponding particle counts as the original sample before lyophilization.

BG – Inactivation

As a new quality criterion set in the last two years the harvested BG product should be free of any living cells before lyophilization. Although the efficiency of BG formation reaches three to five orders of magnitude during the time window of E- lysis (**Fig. 3, 4**) remaining live cells must be inactivated subsequently. The presence of protein E in the envelope complex of bacteria does not necessarily kill all bacteria by E-lysis. However, protein E in the membrane

renders all bacteria more sensitive to killing by lyophilization and in the past no living cell counts could be detected in the lyophilized BG samples. In applications where nucleic acid-free BGs are produced inactivation can be accomplished by the expression of an additional kill gene in the host cells in combination with E-lysis [41]. For this the staphylococcal nuclease A (SNUC) is used which reduces the DNA content below the detection limit of real-time PCR. SNUC activity is also responsible for cleaning up residual DNA in BGs and can lead to complete inactivation of the culture as it degrades the host DNA into fragments no longer than 100 base pairs [41]. Activation of the positive effect of SNUC expression on minimizing both cell viability and residual DNA-content in the BG product is dependent on the addition of Mg^{2+} and Ca^{2+} as well as a shift in pH to 8.0 [41]. **Figure 7** shows a Shigella flexneri 2a culture harbouring plasmid pGLNic for co-expression of temperature-inducible protein E and IPTG-inducible SNUC.

In combination with or as an alternative to SNUC addition of the alkylating agent β-propiolactone (BPL) after harvesting is effective to fully inactivate all viable cells. BPL is known to react with nucleic acids, mainly guanine. BPL is widely used for the inactivation of viruses and further to sterilize vaccines, human tissue implants and plasma [42]. The presence of BPL causes alterations (transition mutations, cross-linking, nicks) in nucleic acids. Besides its reaction with nucleic acids in the presence of water BPL fully hydrolyses at room-temperature into non-toxic β-hydroxypropionix acid [42]. In BG production the amount of BPL for complete inactivation of the BG product suspension depends on three parameters: the amount of DNA present in the suspension, the application time and the application temperature. Due to the expulsion of the cytoplasmic content most DNA in the BG suspension is present in the liquid phase which makes it reasonable to apply BPL in the BG concentrate after harvesting and before diafiltration. At this point approximately 97% of the original fermentation liquid - and therefore 97% of the free DNA - have been withdrawn from the product. Two equal dozes of BPL given at 30 min intervals are sufficient for total inactivation of all surviving cells at 42°C within 60 min. The final BG product is washed with another 5.0 l dH_2O by diafiltration before distribution for lyophilization.

BGs - Applications

BGs solo:

Immunization against pathogenic Gram-negative bacteria can be achieved with the corresponding BGs and has been studied in various animal models [24, 36]. Some of the investigations should be mentioned briefly as they are examples for superior mucosal applications and cross-protection conferred to other subtypes of the pathogens used.

In model investigations for human lung pathogens and for the development of veterinary vaccine candidates vaccination of swine with *A. pleuropneumoniae* (App) BGs not only resulted in protection against aerogenic infection with the potentially lethal pathogen but also prevented colonization of the lungs and tonsils indicating that immunization with BGs is superior to treatment with bacterins [43]. More importantly, no clinical side-effects have been reported. The mucosal application of App BGs as oral immunization [44] or as aerosols [43, 45] induced sterile immunity and cross-protection against other serotypes in pigs [45] whereas intramuscular immunization [46] fully prevented the vaccinated pigs against the disease after lethal challenge but did not confer to the superior sterile immunity as the challenge bacteria could be re-isolated from the tonsils from the vaccinated pigs.

BG produced from *P. multocida* and *M. haemolytica* (formerly: *P. haemolytica*) were used in rabbit and mice models resulting in the production of antibodies effective not only against the strain used for immunization but also against other Pasteurella strains [47]. M. haemolytica BG immunization of cattle offered protective immunity comparable to commercially available vaccines [48].

For *V. cholera* pre-clinical studies have been completed. The ilea loop challenge model revealed full protection of rabbits and more interestingly partial cross-protection between the classical O1 strain and the new upcoming O139 strain [49]. In most models mucosal application has proven to be a favourable route for administration of BG candidate vaccines inducing both humoral and cellular immune response [29, 36].

BGs as adjuvants:

The BG morphology is not subject to denaturation during the lysis process and thus all major immune stimulating constituents are preserved. Those constituents are referred to as pathogen-associated molecular patterns (PAMPs) comprising lipopolysaccharides (LPS), monophosphoryl lipid A (MPL), peptidoglycan or flagellants. As PAMPs are recognized by toll-like receptors (TLR) they trigger the innate immune response. Consequently, independent of the bacterial strain from which the BGs are derived all BGs induce innate immune reactions (Abtin, Koller, Lubitz, personal communication) as first response and also carry intrinsic adjuvant properties which makes them extremely versatile to strengthen the general and specific immune status of the application [29].

BGs as carriers of foreign protein antigens:

Using recombinant DNA technology foreign antigens (AGs) can be incorporated into or associated with the envelope complex of the bacteria before lysis and thus are constituents of the latter BGs (**Fig. 2 (a)**). AGs may be presented on the cell surface via fusion with outer membrane proteins (e.g. ompA) [50] or on the IM as membrane anchor fusions with N-, C- or N/C-terminal targeting [30]. It has been shown that fusion with these membrane anchors does not affect proper folding and assembly or diminish the functionality of enzymes leading to the assumption that AGs are also presented in their proper conformation. In addition to directly fusing the target AG to the membrane anchor a system for subsequent loading of BGs with AGs was developed. In this approach the BGs are equipped with membrane-anchored streptavidin. After lyophilization such streptavidin BGs can be loaded with a desired biotinylated compound [51].

Another method of incorporating foreign proteins into BGs is the directed export to the PPS via MalE fusion proteins or with PPS signal sequences. As the PPS is sealed during lysis and the vast majority of all periplasmic components is retained within the envelope complex [9, 18] the target AG can be expressed as a soluble protein and exported to the PPS. The membrane-derived oligosaccharides [52] of the PPS provide a protective environment against inactivation during lyophilization [34].

Also, for presenting protein AGs in BGs fusion of the target antigen with bacterial surface layer (S-layer) proteins can be used. Genes *sbsA* and *sbsB* of *Bacillus stearothermophilus* code for the corresponding S-layer proteins SbsA and SbsB that form sheet-like self-assembling superstructures within the cytoplasmic space (CPS) when expressed heterologous in Gram-negative bacteria [53-54]. Since S-layers are made up of several 100,000 subunits they are not expelled with the cytoplasm during lysis. Both S-layer genes accept insertion of foreign sequences coding for large foreign proteins [54-55]. Linking MalE to SbsA the protein subunits can also be exported to the PPS prior to S-layer formation [56]. It was further suggested that multiple S-layer subunits each carrying a different AG could be expressed together within the same superstructure [36]. All different options of AG presentation in BG envelopes are summarized in **Figure 2 (a)** as a schematic drawing.

BGs as carrier of biologically active substances:

The BG system provides a new promising platform for the delivery of drugs and other biologically active substances. Since BGs are devoid of any cytoplasmic content attention has been paid on the carrier capacity of the inner cytoplasmic lumen which provides an intracellular space of approximately 250 femtoliter per BG where drugs of interest can be filled as liquid or independent of the inner volume absorbed to the lipid compartment or specifically attached to receptors presented in the BGs.

BGs produced from the colonic commensal to pathogenic *M. haemolytica* were used for the *in vitro* delivery of the moderate hydrophilic cytostatic drug doxorubicin (DOX) to human colorectal adenocarcinoma (Caco-2) cells. Endogenous drug release was confirmed and enhanced cytotoxic and antiproliferative activities in the Caco-2 cells were observed with DOX-loaded BGs as compared to the substance per se with a difference of 2-3 orders of magnitude [57]. The water soluble substance calcein was used in another delivery model, whereby the former lysis holes were plugged with bacterial membrane vesicles [58]. BGs from *P. cypripedii* were used as pesticide delivery systems with the lipophilic fungizide tebuconazole. The investigations demonstrated that this formulation conferred a higher resistance to rainfalls due to adherence of the BGs to the plant with protective and curative effects towards agricultural plant pathogens [59].

It is assumed that organic ring structures bind unspecifically to the membrane compartments of BGs. Recent investigations of loading BGs with polyphenolic compounds, like resveratrol, are in agreement with this presumption. Currently, the modulating ability of such substances bound to BGs on the induction of components of the innate immune system, i.e. i-NOS, was examined (Koller and Lubitz, personal communication). Furthermore, advanced drug cytotoxicity due to stabilization and protection of the UV-labile resveratrol derivates, namely Digalloylresveratrol and M8, by adsorption to the BG-interior could be demonstrated recently (Koller and Lubitz, personal communication).

In a model investigation, in which a substituted matrix on the inner membrane of BGs for an enhanced binding of drugs is created, has been shown by Huter et al. [51]. Membrane anchored streptavidin bound the coupling partner to the inside of the cytoplasmic membrane and allowed biotinylated substances to be targeted. Studies with biotinylated alkaline phosphatase or biotinylated fluorescence-labelled dextrans as an additional matrix displayed successful binding within the inner lumen of BGs [51].

BGs as carriers of DNA vaccines:

Several viral and bacterial delivery systems with high transfection efficiencies bear a risk of reversion to their original pathogenic forms and "safer" non-viral systems such as attenuated bacteria, polycation/DNA complexes, nucleoporation have reduced transfection efficiencies [60-67]. The BG system represents a new highly efficient gene delivery platform as an alternative to current viral and bacterial methods in vaccine development. One of the biggest demands for the new DNA-carrier system is the safety of BGs. Recent investigations proved that BGs have no cytotoxic or genotoxic impact on various histological types of human cells after mutual co-incubation independently of the used BG species (manuscript in preparation).

Recently, DNA vaccines got approval for use in veterinary practice [68]. DNA vaccines still require intensive research and improvements to be considered safe for use in human medi-

cine. One reason for this slow pace in development and licensing approval of DNA vaccines is the requirement of high plasmid dosages and low immunogenicity, most commonly attributed to the absence of efficient delivery system [69-70]. Many experiments have been carried out in order to deliver DNA vaccines using BGs as carriers, and a simple procedure for loading BGs with plasmid DNA has been standardized. Lyophilized BGs are re-suspended in DNA solutions followed by a couple of washing steps to remove unbound plasmid DNA from inside the BGs. It is further observed that the amount of DNA loaded inside the BGs is directly related to the concentration of DNA solution used. This loading procedure proved to be very efficient and upto 6,000 midsize plasmid copies per BG can be loaded [71].

The main advantage of BGs is their non-living character, while still retaining all of the surface morphological, structural and antigenic components of their living counterparts, and their outstanding loading capacity [72]. The inner space of BGs empty envelope can be loaded with a combination of peptides, drugs or foreign DNA which gives us an opportunity to design new types of polyvalent vaccines [57, 71, 73-74]. We have shown that BGs loaded with plasmid DNA encoding green fluorescent protein (GFP) are efficiently internalized and phagocytized by both professional antigen presenting cells (APCs) and tumor cells. BGs were able to deliver the heterologous genes to both non-dividing cells (monocyte-derived dendritic cells) and dividing cells (macrophages and melanoma) with study results showing that up to 82% of cells expressing the plasmid encoded reporter gene delivered by BGs and importantly with no cytotoxic impact on target cells [32, 71, 74-75]. Furthermore, intradermal and intramuscular immunization of Balb/c mice with BGs loaded with pCMV encoding beta-galactosidase stimulate more efficient both humoral and cellular AG-specific immune responses than naked DNA. Moreover, beta-galactosidase-specific immune response was detected after intravenous immunization of mice with autologous dendritic cells (DCs) transfected ex vivo with pCMVbeta-loaded BGs [32]. An increase of IFN-gamma producing AG-specific CD8+ T cells was observed in animals vaccinated with DNA loaded BGs in response to restimulation by APCs pulsed with peptide containing the immunodominant MHC class I epitope. Furthermore, BGs enhanced expression of MHC class I molecules and costimulatory molecules on DCs [32]. Cross-presentation of AGs delivered to DCs by BGs could activate both CD4+ and CD8+ T cells and stimulates immune system to enhance immune response against AGs expressed by target cells. Bacterial LPS enhances maturation of DCs, affects endosomal acidification of DCs and also improves cross-presentation of AGs [76-77]. Inner and outer membrane structures of BGs including LPS remain intact in BGs and the surface LPS effectively stimulates the AG-cross-presentation by DCs [19, 72].

In general the production and loading of BGs with plasmid DNA are two separate tasks. With the introduction of our new self immobilizing plasmids (pSIP) this multistep procedure was simplified into one step in-vivo, cost effective procedure. During this process the plasmid

DNA carrying an operator sequence is bound to a specific DNA binding protein present on the IM of the bacteria [78].

For DNA vaccination and plasmid DNA used in gene therapy the bacterial backbone sequences and antibiotic resistant genes are considered to be a biological safety risk. To overcome this hurdle new more sophisticated versions of pSIP BG-DNA-vaccines are based on minicircle (mc) DNA devoid of such biologically risky remains. This improved version of pSIP is based on the ParA resolvase system to produce mcDNA which is bound to the IM receptor while the sisterpair miniplasmid produced during this process is expelled to the culture media during the gene-E mediated lysis [79]. A modified system for minicircle production, based on endonuclease activity of *I*-SceI gene encoded from parent plasmid, digesting the miniplasmid has been reported [80]. In the new modified pSIP generation currently under construction endonuclease activity is also encoded to digest non-recombinant mother plasmids and the ParA produced miniplasmids.

The main benefit of DNA vaccines is based on the induction of both cellular and humoral immune responses caused by processing of AG through both endogenous and exogenous pathways followed by AG epitopes presentation in the context of both MHC class I class and II molecules [81-83]. Well designed and applied gene therapy should provide successful delivery of desired AG DNA to the APCs followed by its expression, naturally processing and presentation of AG-derived epitopes. T-cells raised against delivered, naturally processed and presented AGs by APCs might be more effective in recognition of the same epitopes presented by cells expressing identical AGs. The expression of a delivered gene should induce strong immune responses or change the behavior of targeted cells.

BGs with their intact envelope structures including peptidoglycan and LPS are not only "waking up" professional phagocytic APCs but are also providing stimulatory impulses to tumor cells. It is known that e.g. melanoma cells have the capacity to behave as non-professional APCs and can phagocyte both apoptotic and live cells [84-87] and as recently shown respond to the challenge by BGs [73]. Despite the high DNA loading capacity of BGs relatively low concentrations of DNA are sufficient for effective gene delivery and its expression by melanoma cells. High transfection efficiencies obtained after incubation of BGs with melanoma cells and with monocyte-derived DCs using the identical type of BGs encourage us to design BGs carrying selected immunogenic and immunodominant AGs usable at the same time for gene transfer to both professional APCs and tumor cells what might lead to induction and/or to increase of AG-specific immune response.

White biotechnology - BGs as micro-bioreactors for enzymatic reactions:

As already mentioned above another possible application for the BG platform is the use of BGs as carrier of enzymes. The lack of cytoplasm and of a membrane potential due to E-

mediated lysis of the bacteria is not accompanied by a total loss of enzymatic activities. The enzymatic activities of membrane-bound β-galactosidase and chloramphenicol acetyl transferase have been described in BGs [88-89]. It has also been shown in BGs that membrane associated enzymes like ATPases are still functionally active. Moreover, even though the cytoplasmic content is expelled during lysis the inside of the cytoplasmic membrane as well as its associated products are retained. As the IM and OM are fused at the border of the E-specific lysis tunnel enzymes from the PPS like alkaline phosphatase and β-lactamase are largely retained and active [9, 19]. As compared to their viable counterparts ATPase and β-lactamase sustained decent activities in suspended BGs after one week storage at 4°C. Furthermore, enzyme activities were also detectable in long-term, ambient temperature stored lyophilized BG-batches. The obtained activities were similar to recently produced freeze-dried samples, e.g. concerning ATPase activity no significant differences were observed within five years of storage (Koller, Lubitz - personal communication). These data show that enzymes of BGs *per se* stay functionally preserved for long time of storage indicating the potential of BGs as reservoirs for biological functions to be used as dietary enzyme substitution or for other use.

Pursuing the idea of Pfründer et al. of producing enantiopure fine chemicals such as e.g. asymmetric synthesis of a 3,5-dicarboxyhydroxylate in biphasic ionic liquid/water systems [90-91] BGs shall act as micro-reactors. Anchoring potent enzymes like specialized alcohol dehydrogenases (ADHs) to the IM the internal lumen of the BG becomes the reaction volume. Re-suspension of the BGs in an aqueous solution with a suitable reduction equivalent allows for proper function of the desired enzyme. Since both product and educts in those kinds of reactions are often poorly water-soluble the use of a non-polar solvent - in this case a suitable ionic liquid (IL) - becomes mandatory. IL are organic salts which are liquid at ambient temperatures; due to their low vapour pressure they are considered as safe ("green solvents") and also feature good in-situ extraction properties for product recovery [92]. Hence, BGs loaded with the reduction equivalent solution are dispersed in the ionic liquid which provides the substrate and receives the product. It was shown that the enzyme activity of β-galactosidase could be vastly increased in an IL environment [93]. Preliminary studies of our lab have demonstrated that β-galactosidase was active when BGs were re-suspended in the IL [Bmim]PF_6.

Qualitative determinations showed successful hydrolysis reactions of the substrates which were delivered in the ionic liquid. These findings give a first estimation for feasibility and attainable enzymatic activity in such approaches. It is suggested that a limited series of reactions could be performed within one BG when a multi-step enzyme system is introduced offering the BG system as a versatile vehicle in white biotechnology.

Acknowledgements:

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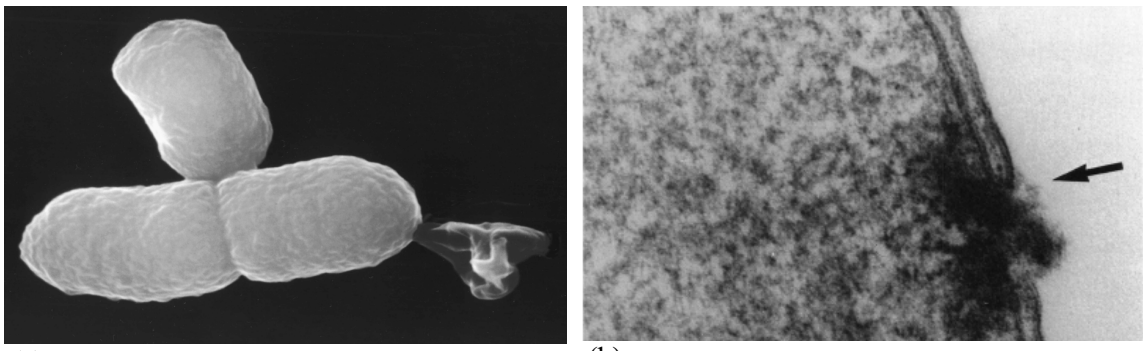
Figures



(a) (b)

Figure 1 (a): Lysis tunnel formation and expulsion of the cytoplasmic contents - reproduced from Ebensen et al. [32].**(b):** Lysis tunnel formation, accompanied by the fusion of IM and OM (arrow) - reproduced from Witte et al. [18]

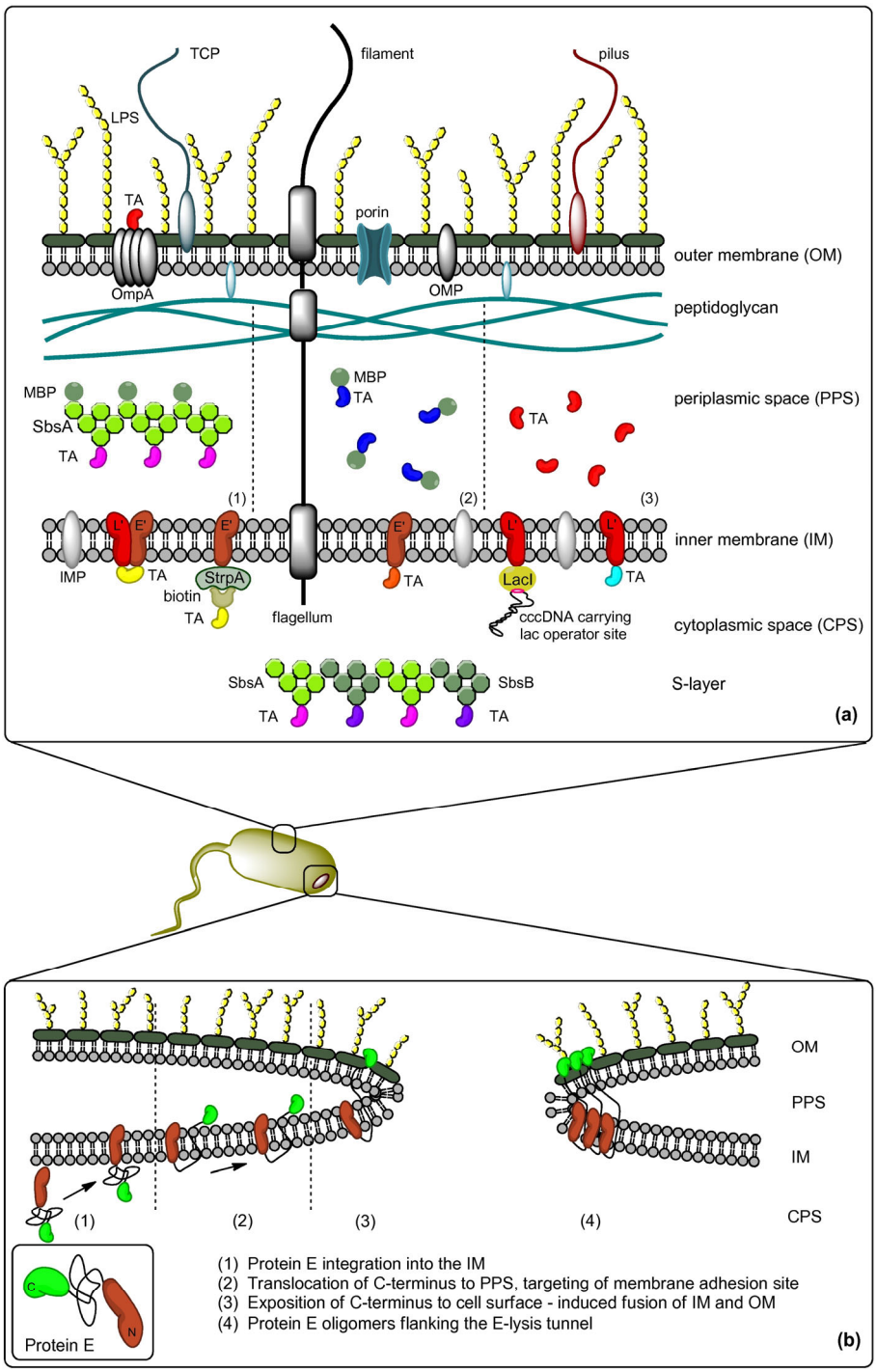


Figure 2 - **(a)**: different methods for AG presentation in the BG envelope complex - BG themselves carry native AG (LPS, OMP, IMP, TCP, flagella, pili) - TA may be presented on the cell surface via fusion with OmpA - the PPS can be loaded with TA via MBP-SbsA-fusion proteins (1), by fusion of the TA with MBP (2) or as sole TA using the gene III signal sequence (3) - Protein TA may be incorporated into the IM via E', L' or E'/L'-anchoring, biotinylated AG can be attached to E'-FXa-StrpA membrane anchors, DNA carrying the lac operator site can be attached to L'-anchored lacI repressor molecules - TA fused with SbsA-/SbsB proteins form S-layers in the PPS. **(b)**: Model of lysis tunnel formation according to Schön et al. [23].

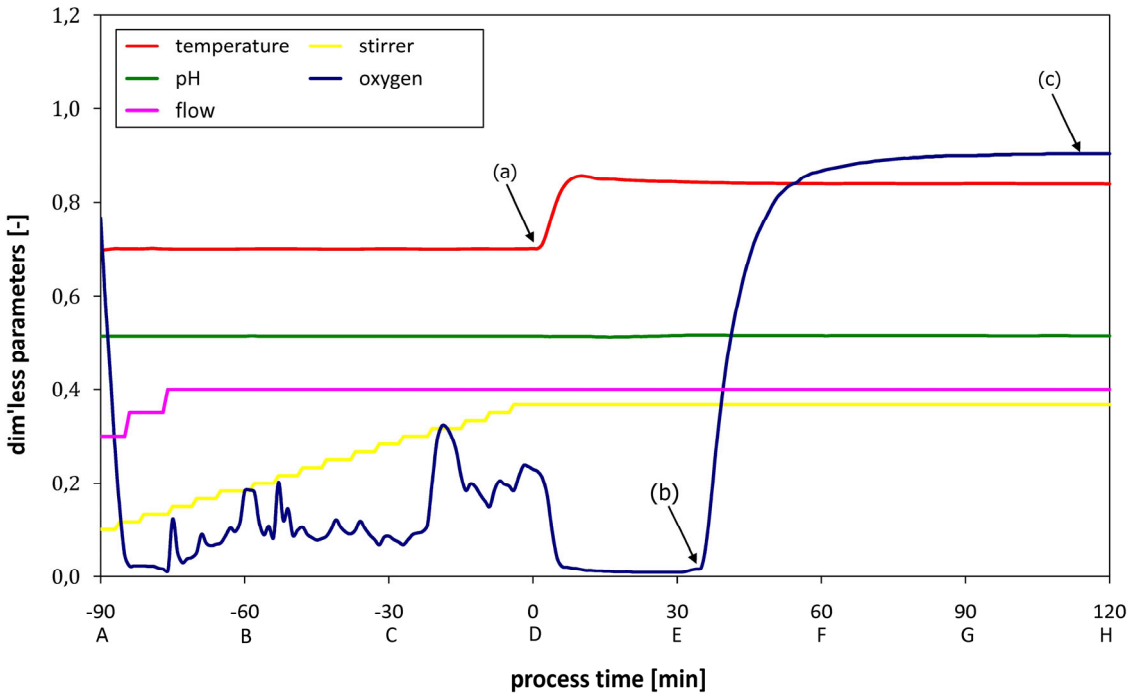


Figure 3: Fermentation protocol (growth/lysis phase) monitoring all relevant process parameters; **(a)** lysis induction, **(b)** lysis onset as indicated by dO_2 up-shift, (c) stationary dO2 plateau indicating end of lysis phase.

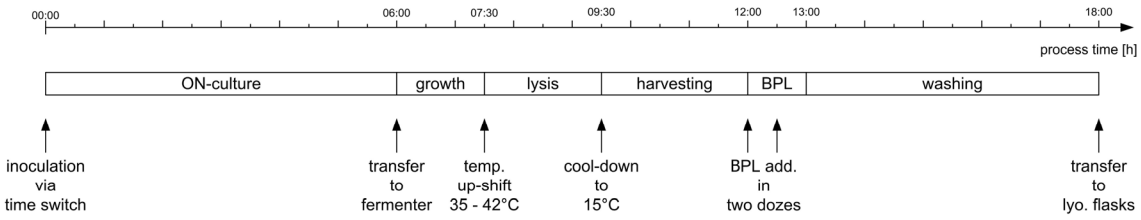


Figure 4: Process timeline for the production of BG including the pre-culture (ON) and down-stream processing.

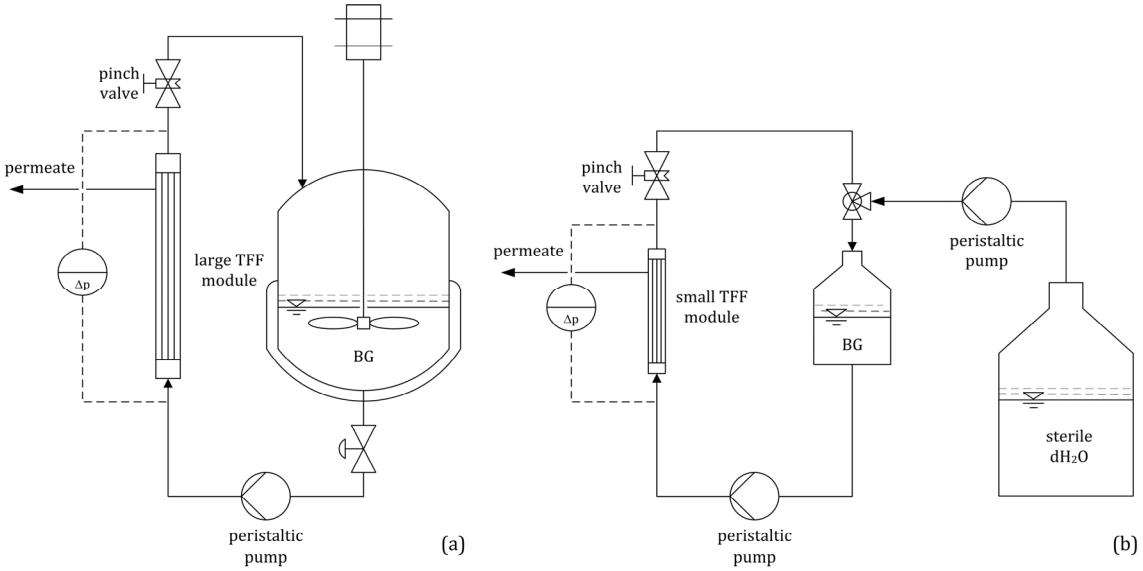


Figure 5 (a): Harvesting of the BG product via TFF; concentration from 20 to 2 l in the fermenter (b) Washing of the BG product with 5.0 l dH_2O via diafiltration; concentration from 2.0 l to 400 ml in a stirred reservoir.

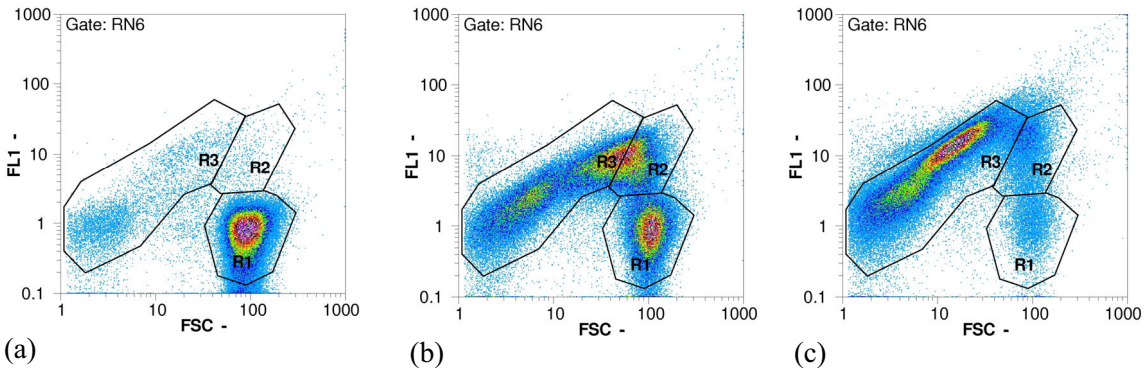


Figure 6: Flow cytometry pictures following the progress of lysis in an *E.coli* NM522 culture (pGLysivb); R1: living cells, R2: dead but intact cells, R3: lysed cells (BG); RN6: exclusion of non-cellular background with RH414 (not shown); FSC - forward scatter, FL1 - fluorescence intensity by DiBAC4(3); **(a)** sample D (0 minutes, lysis induction), **(b)** sample E (30 minutes), **(c)** sample H (120 minutes, end of lysis phase).

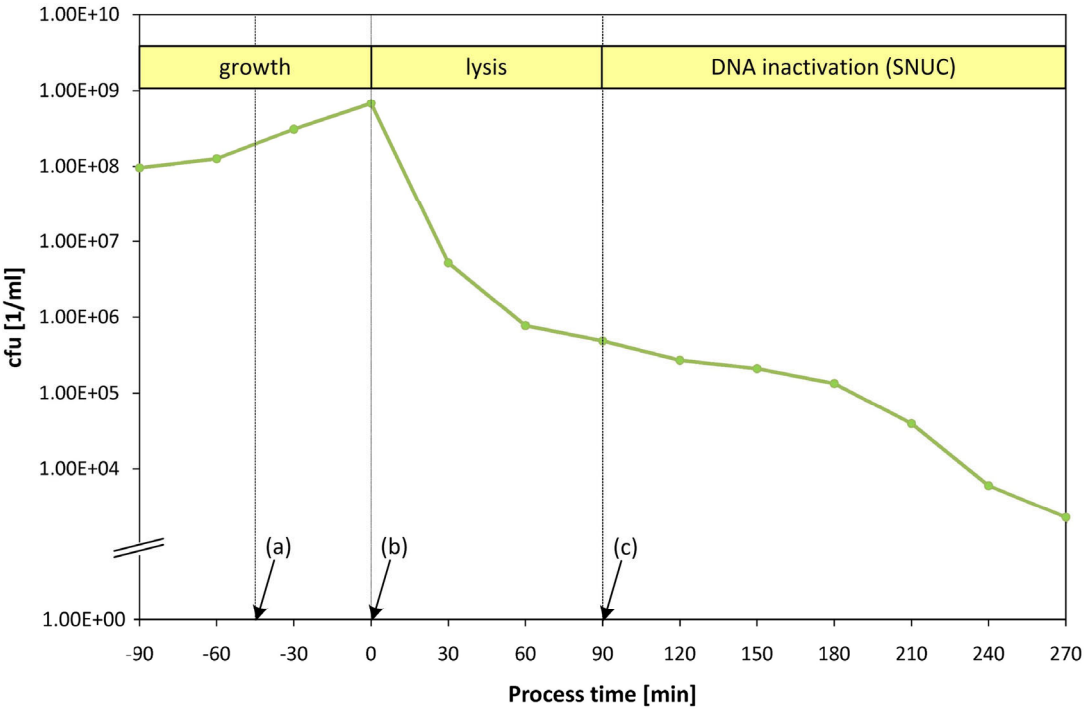


Figure 7: Standard fermentation for a S. flexneri 2a culture harbouring plasmid pGLNic: (a) IPTG-addition at -45 min to induce biosynthesis of SNUC, (b) temperature up-shift to 42°C at 0 min to induce lysis, (c) pH up-shift to 8.0 and addition of Mg2+ and Ca2+ at +90 min to activate the enzymatic function of SNUC .

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Chapter 1.3.
Bacterial Ghosts (BGs) – Advanced Antigen and Drug Delivery System

Pavol Kudela[1,3*] , Verena Juliana Koller[1] and Werner Lubitz[1,2]

[1] BIRD-C GmbH&CoKEG, Kritzendorf, Austria;

[2] University of Vienna, MFPL, Center for Molecular Biology, Vienna, Austria;

[3] Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia.

[*] Corresponding author: Phone: +43-1-427-774-361 - pavol.kudela@bird-c.at

Bacterial ghosts (BGs) are empty bacterial envelopes of Gram-negative bacteria produced by controlled expression of cloned gene E, forming a lysis-tunnel structure within the envelope of the living bacteria. BGs are devoid of cytoplasmic content and possess all bacterial bio-adhesive surface properties in their original state while not posing any infectious threat. BGs are ideally suited as an advanced drug delivery system (ADDS) for toxic substances in tumor therapy. The inner space of BGs can be loaded with either single components or combinations of peptides, drugs or DNA which provides an opportunity to design new types of (polyvalent) drug delivery vehicles. Uptake of BGs loaded with Doxorubicin (Dox) by CaCo2 cells led to effective DOX release from lysoendosomal compartments and accumulation in the nucleus. Viability and proliferative capacity of the cells were significantly decreased (2 to 3 orders of magnitude) after internalization of Dox loaded BGs as compared to cells incubated with free Dox. The same effect was observed with leukemia cells. Melanoma cells also revealed a high capability to internalize BGs. These results indicate that BGs are able to target a range of types of cancer. BGs have also been investigated as DNA delivery vectors. Studies showed DNA loaded BGs are efficiently phagocytosed and internalized by both professional APCs and tumor cells with up to 82% of cells expressing the plasmid encoded reporter gene. Our studies with BGs as an ADDS system contribute (i) to optimize drug delivery for the treatment of cancer; (ii) define specific conditions for selection and preparation of BG formulations; (iii) and provide a background for the clinical application of BGs in cancer therapy.

1. Introduction

Identification of optimal antigen (Ag) forms that are therapeutically effective for the vaccination of patients with cancer opens the door for using new strategies in vaccine development. The competent DNA vaccine should be able to efficiently deliver specific DNA (Ag) to the target cells e.g. DCs in order to elicit strong cellular and humoral immune responses, and a long term immune memory, and should consist of a non-specific adjuvant capable to increase the vaccine immunogenicity. The expression of a delivered gene should either induce strong immune responses or change the behavior of the targeted cells. Several delivery systems have been described, including viral vectors with high transfection efficiencies and "safer" non-viral systems, such as attenuated bacteria, polycation/DNA complexes, nucleoporation, but with reduced transfection efficiencies [1-6]. Although viral vectors provide efficient gene transfer, safety concerns are still present due to the immunogenicity of most viruses which limits the duration of gene expression and the ability to re-administer the genes. Similarly, live bacterial vaccines have been successfully used as carriers for vaccine Ag but their use carries a risk of reversion to their original pathogenic forms [7, 8]. For many different types of tumors the usefulness of highly cytotoxic chemical agents as chemotherapeutics to inhibit fast tumor cell replication is diminished by a number of serious toxic side effects caused by these drugs including increased risk of cardiovascular diseases [9-11]. Many of these toxic effects which are mostly dose-related plus non-specific killing limit repeated drug application in the treatment of cancer patients. Incorporation of highly tumoricidal agents into a non-toxic delivery system capable of transferring the drug to the target cells without modification of the drugs function might lead to improved therapy and significant reduction of toxic side effects. Development of new delivery systems with no cytotoxicity and high efficiency of drug and/or gene medicine delivery as alternatives to current methods is very much needed.

2. Bacterial Ghost System

The bacterial ghost (BG) system developed during the past few years represents a new platform in vaccine development [12]. BGs, empty bacterial shells of Gram-negative bacteria, have been developed as a novel potent carrier and adjuvant system for the delivery of DNA vaccines [13-17]. BGs are produced by protein E-mediated lysis of Gram-negative bacteria. Inducible expression of the E gene causes the fusion of the inner and outer membranes of the bacterial cells to form intermembrane tunnel through which all the cytoplasmic contents of the bacteria is expelled, whilst the inner and outer membrane structures are preserved and remain intact [18] (Fig.1). In contrast to attenuated bacteria there is absolutely no risk of reversal to pathogenic form. Safety profile studies concerning BGs and additional investigations showed

that BGs have no cytotoxic or genotoxic impact on various histological types of human cells (monocyte-derived dendritic cells, macrophages, conjunctival cells, keratinocytes, colon carcinoma cells, melanoma cells, hepatocellular carcinoma cells, promyeloblasts-acute promyelocytic leukemia cells, monocytes-acute monocytic leukemia cells) after mutual co-incubation, independent of the used BG species (Kudela, Koller and Lubitz, manuscript in preparation), and that endotoxicity does not limit the use of BGs as a candidate vaccine [19]. The main advantage of BGs is their non-living character, while still retaining all of the surface morphological, structural and antigenic components of their living counterparts [12]. BGs have been produced from various non-pathogenic, pathogenic and probiotic strains including *E. coli* K12, enterotoxigenic *E. coli*, enterohemorrhagic *E.coli*, *E. coli* Nissle 1917, *Mannheimia haemolytica*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella enterica*, *Helicobacter pylori*, and *Actinobacillus pleurepneumoniae* demonstrating the broad potential of the BG system in vaccine development and tumor therapy (Table 1).

Because of the unique structure of the BG's envelope with intact and preserved pathogen-associated molecular patterns (PAMPs), BGs can be used in biomedicine alone as an adjuvant or as a delivery vehicle for drug or gene medicine. The inner space of BG's empty envelope can be loaded with a combination of peptides, drugs or foreign DNA which gives us an opportunity to design new types of polyvalent vaccines and to use BGs in advanced drug delivery [13] (Fig.2). Uptake of BGs loaded with the anti-neoplastic drug (Doxorubicin) led to its efficient release from lyso-endosomal compartments and accumulation in the nucleus [20] (Fig.3). BGs have excellent DNA-loading capacity varying from 4000 to 6000 plasmids per BG depending on the concentrations of DNA solution used [21-24]. We have shown that BGs loaded with plasmid DNA are efficiently internalized and phagocytized by both professional antigen-presenting cells (APCs) and tumor cells. Cross-presentation of Ag delivered to dendritic cells (DCs) by BGs can activate both CD4+ and CD8+ T cells and stimulate the immune system to enhance the immune response against Ag expressed by target cells. The presence of bacterial lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria enhances maturation of DCs, affects endosomal acidification of DCs and also improves cross-presentation of Ag [25]. Inner and outer membrane structures of BGs including LPS and other PAMPs remain intact after protein E-mediated lysis of Gram-negative bacteria, therefore as well as BGs possessing a high loading capacity; they also carry on their surface highly effective molecules for the stimulation of cross-presentation by DCs [12, 18]. BGs with their intact envelope structures are not only immunostimulatory to professional phagocytes but are also capable of providing stimulatory signals to tumor cells. It is known that melanoma cells have the capacity to behave as non-professional APCs and can phagocyte both apoptotic and live cells [26-28], and we have recently shown that melanoma cells actively respond to exposure to BGs by increasing their rate of phagocytosis. Thus the BG system is an effective DNA carrier with BGs

shown to be very attractive targets for uptake by melanoma cells [21]. Using BGs for gene delivery to the immunocompetent cells, in particular DCs as well as tumor cells, could initiate or restore the immune response against the delivered tumor-associated antigens (TAA) as well as induce and increase expression of target gene by APCs and tumor cells.

2.1 BGs-advanced drug delivery system

A delivery system that transports chemotherapeutic drugs directly to the cytosol and nuclear area of target cells at levels sufficient to inhibit tumor cell proliferation would allow the use of decreased drug dosages, and thus lessen the negative impacts on people already challenged with serious diseases. Recent investigations confirm the recognition of BGs by various types of tumor cells and the capacity of BGs to efficiently target and be internalized by melanoma, leukemia and colorectal carcinoma cells. This phenomenon might be related to the presence of the LPS on the intact and preserved envelope of BGs leading to the activation of tumor cells. Some tumor cells like melanoma cells respond to LPS through the Toll-like receptor-4 *(*TLR-4), a receptor that is constitutively expressed by this type of tumor cell. Activation of melanoma cells by LPS results in enhanced production of IL-8, cell adhesion and might also play an important role in immune escape [29]. Although it has been reported that TLR-4 is not involved in cellular LPS uptake by monocyte or endothelial cells, the connection between TLR-4-mediated melanoma cell activation by LPS and their phagocytic activity remains to be defined [30]. Several studies reported increased tumor progression due to the activation of TLRs expressed on tumor cells with specific ligands, such as LPS [31, 32]. In contrast, other reports showed a protective role of TLR-4 inhibiting lung carcinogenesis [33]. Moreover, *in vitro* activation of tumor cells through TLR-4 promotes an anti-tumoral effect and inhibits tumor growth *in vivo* [34]. Furthermore, systemic administration of attenuated bacteria stimulates a potent immune response in the tumor area and polarizes T cell immune response toward Th1 dominant immunity through TLRs [35]. In general, recent reports indicate both positive and negative influences of tumor cell activation through TLRs but until now without any final clear consensus regarding their role in tumor progression or regression. Therefore, the exact role and the mechanisms involved during both positive and negative activation of TLR signaling in tumor cells has to be determined.

PAMPs present on the surface of BGs help to increase targeting of tumor cells with BGs loaded with chemotherapeutic substances. Our results revealed that melanoma cells have a high capability to bind and internalize BGs without significant effect on their viability and proliferation [21]. Efficient endocytosis of BGs was also observed after incubation with colon carcinoma and leukemia cells (Fig.3 and Fig.4). Doxorubicin (Dox) loaded BGs made by simple resuspension and incubation of lyophilized BGs in Dox solution, were used as a model system

for BGs and drug delivery. It is assumed that Dox binds non-covalently to the inner membrane structure through the interaction of Dox-amine sugar parts and charged membrane proteins or with the antraquinone part of Dox with BG's inner membrane [20]. Attached Dox is slowly released in water from BGs within a period of 8 days, with a detection of approximately 40% release within the first day and then constant release within the remaining days (~10% per day). Incubation of Dox loaded BGs with colon carcinoma cells led to efficient internalization of BGs, their degradation, the release of BG's content to the cytoplasm of target cells and accumulation in the nuclear area. Incubation of tumor cells with Dox loaded BGs led to significantly higher inhibition of target cell proliferation (at least two orders of magnitude) in comparison to results obtained after incubation with pure Dox at the same concentration levels (Fig.5). To achieve the same level of inhibition, the Dox dose could be reduced to 100th when associated with BGs (16 h of co-incubation). The difference in the inhibition of target cell proliferation was even more dramatic when the free Dox and Dox loaded BGs were removed from the cell culture system already after 10 min of incubation in order to simulate a clinical condition which might occur in the medical application of Dox loaded BGs, because it is more likely that the targeting vehicles pass through the target cells or tissues within several minutes than that they remain at the targets for several hours [20].

Similar results were detected in both solid tumors (colon carcinoma) and dispersed tumors (monocytic and myeloid leukemia). Delivery of Dox bound to the inner membrane of BGs enhanced its intracellular concentration within tumor cells (CaCo2) up to 42 times higher than incubation of tumor cells with an equivalent concentration of Dox solution (Winter, Mader and Lubitz, manuscript in preparation). These results also indicate the loading and delivery capacities of BGs may be sufficient to effictively transport chemotherapeutics to cells that highly express P-glycoprotein and MDR1 and overcome Dox resistance [36, 37]. Detailed analysis of Dox loaded BG's cytoxicity showed that drug (Dox)-filled BGs reduced cell proliferation with inhibitory concentrations of the drug (EC50 obtained by MTT-assay) up to 300 times more effectively than when free drug was added to the cell culture system (Winter, Mader and Lubitz, manuscript in preparation). This phenomenon might be caused by degradation of Dox loaded BGs within the endo-lysosome of target cells allowing Dox to bypass the MDR efflux pumps and resulting in enhanced accumulation of Dox in the cytoplasm and then in the nuclear area of target cells. Activation of tumor cells by LPS present on the surface of BGs could also have increased the number of tumor cells in S-phase enhancing DNA intercalation by Dox delivered by BGs to cause a higher cytotoxic effect. However, the exact mechanism of the cytotoxic effect of chemotherapeutic drugs delivered to the tumor cells by BGs and use of this strategy in prospective cancer immunotherapy requires to be further investigated. Together, our results showed that BGs are able to deliver chemotherapeutic drugs without modification of its pharmacological properties. Drug incorporation to the BG's

envelope significantly reduced the concentration necessary to efficiently inhibit tumor cell proliferation [20]. These facts support the potential of the BG system in tumor therapy to provide a desirable reduction in the toxic side effects of current chemotherapeutic agents and hence lead to the improvement and extension of the therapeutic window for patients undergoing chemotherapy.

2.2 BGs-efficient non-toxic DNA delivery system

Gene therapy applications in human medicine focused on delivery of DNA to the target cells may play an important role in the development of effective therapies and vaccines for major human diseases. Precisely designed and applied gene therapy requires a well defined Ag and simple but non-toxic and effective delivery system that successfully delivers the desired DNA (Ag) to the target cells-prefessional APCs e.g. DCs in order to prime effective Ag-specific CD4+ and CD8+ T cells. These induced T cells may be more effective at recognizing the same epitopes presented by cells expressing identical Ag. The expressed genes should induce strong humoral and cellular immune responses or change the behavior of targeted cells. The development of an optimal DNA Ag delivery system that has a suitable safety profile, low production costs and the capacity to positively affect the immune system, opens the door for the use of new strategies in vaccine development and tumor therapy. The efficient delivery of DNA (Ag) to the target cells e.g. DCs needs to elicit a robust immune response. The main benefit of a DNA vaccine is based on its ability to induce both cellular and humoral immune responses due to processing of Ag through both endogenous and exogenous pathways, followed by Ag epitope presentation in the context of both MHC class I and class II molecules [38, 39].

Prospective carrier-adjuvants for gene therapy should be able to deliver functional DNA to the target cells, and combine the capacities to both stimulate cells of immune system and help to increase specific immune response against delivered specific Ag. The selected delivery system should not represent any potential hazard for horizontal gene transfer and must demonstrate a suitable safety profile. Furthermore, in the case of DCs the selected carrier also has to promote cell maturation and the development of both humoral and cellular immune responses [40].

A number of delivery systems with high transfection efficiencies have been successfully used as carriers for DNA-based vaccine Ag but their use bears a risk of reversion to their original pathogenic forms [7, 8]. The development of new delivery systems with no cytotoxicity and high efficiency of gene delivery as an alternative to current viral and bacterial methods is very much needed. The greatest requirement of any new delivery system for vaccine development against human diseases is the safety of the prospective DNA carrier. The BG system offers a new platform that would meet the required safety needs. The safe profile of

BGs was confirmed using a Limulus-assay, where purified LPS (E. coli O26:B6) expressed endotoxic activity values 100-times higher than the BGs [19]. Furthermore, recent investigations of our group proved that BGs have no cytotoxic or genotoxic impact on various histological types of human cells (monocytes, macrophages, monocyte-derived dendritic cells, endothelial cells, keratinocytes) after mutual co-incubation independent of the used BG species (Kudela, Koller and Lubitz, manuscript in preparation). Therefore, endotoxicity does not limit the use of BGs as a candidate vaccine [19].

The major benefit of BGs in vaccine development consists of their non-living character, their ability to retain all of the surface morphological, structural and antigenic components (PAMPs) of their living counterparts as well as their outstanding loading capacity [12]. The inner space of BG's empty shell can be loaded with either single Ag or with a combination of various Ags which opens the possibility to devise and create new types of vaccines [13]. Intensive study of the BG system by our group in the past years concerning also its application as a DNA delivery system revealed that a simple procedure based on mixing of dried lyophilized BGs with a solution of plasmid DNA encoding required Ag followed by several washing steps is sufficient for generation of DNA loaded BGs which are immediately ready to use for gene delivery to different histological types of cells. Our studies confirmed the excellent loading capacity of BGs with final load depending on the concentration of DNA solution used [22-24]. We have shown that BGs loaded with plasmid DNA encoding heterologous gene (green fluorescent protein) are efficiently internalized and phagocytized by both professional mouse and human APCs and tumor cells. BGs were able to deliver the model genes to both non-dividing cells (monocyte-derived DCs) and dividing cells (macrophages and melanoma cells) with study results showing that up to 85% of cells expressed the plasmid encoded reporter gene delivered by BGs (Fig.6 and Fig.7). Principally, incubation of BGs with all tested types of human and mouse cells including DCs, macrophages, epithelial cells and tumor cells caused no toxic impact on target cells [21-24]. Furthermore, intradermal and intramuscular immunizations of Balb/c mice with BGs loaded with pCMV encoding β-gal (pCMVβ) stimulate more efficiently both humoral and cellular β-gal-specific immune responses than immunization with naked DNA. Moreover, increased β-gal-specific immune response was also detected after intravenous immunization of mice with autologous DCs transfected *ex vivo* with pCMVβ-loaded BGs [24]. Production of IFN-γ by Ag specific CD8+ T cells isolated from vaccinated animals was observed after coincubation and recognition of peptide containing the immunodominant MHC class I epitope presented by autologous APCs (Fig.7). Furthermore, stimulation with BGs enhanced expression of MHC class I and class II molecules and costimulatory molecules-CD40, CD54, CD80 and CD86 by DCs [24].

Cross-presentation of Ag successfully delivered to DCs by BGs could activate both CD4+ and CD8+ T cells and thus stimulates the immune system to enhance Ag-specific immune

response leading to elimination of cells expressing target Ag. Bacterial LPS enhances maturation of DCs, affects endosomal acidification of DCs and also improves cross-presentation of Ag [41, 42]. Inner and outer membrane structures of BGs including LPS remain intact after protein E-mediated lysis of Gram-negative bacteria. Thus BGs also expressing LPS on their surface are likely to stimulate the cross-presentation of Ag by DCs [12, 18].

BGs with their intact envelope structures including PAMPs, e.g. peptidoglycan and LPS not only stimulate professional phagocytes but are also capable of providing stimulatory signals to the tumor cells. It is known that melanoma cells have the capacity to behave as non-professional APCs and can phagocyte both apoptotic and live cells [26-28, 43]. Our recent studies showed that melanoma cells actively respond to the challenge by BGs by phagocytosis, thus BGs were shown to represent a very attractive target for melanoma cells [21]. Moreover, a study with melanoma cells revealed that even relatively low concentrations of DNA are sufficient for effective gene delivery and its expression by target cells. BGs loaded with approximately 50 plasmids per BG, when incubated with melanoma cells at the ratio BG:cell-100:1, were sufficient for effective DNA delivery to the target cells and led to transfection efficiency of up to 80%. These observations suggest that BGs could be loaded by multiple plasmids (e.g. plasmids encoding various TAA expressed by certain types of tumor) and used to deliver heterologous genes to different types of cells in particular tumor types (i.e., proliferating and non-proliferating). High transfection efficiencies obtained after incubation of BGs with melanoma cells and with monocyte-derived DCs using the identical type of bacterial strain for preparation of BGs encourage us to design BGs carrying selected immunogenic and immunodominant Ag usable at the same time for gene transfer to both professional APCs and tumor cells. Applications of this type of BGs directly into the tumor microenvironment might lead to induction and/or to an increase of Ag-specific immune response and cytokine milieu alteration resulting in the attraction of the immunocompetent cells participating in the immune response against the tumor cells.

In addition to the simple procedure used for loading of BGs with DNA mentioned above, a technique recently developed by our group showed that loading of BGs with DNA could be simplified even further using self-immobilizing plasmid which is retained by the carrier envelope due to a specific interaction between cytoplasmic membrane anchored proteins with minicircle DNA during and after protein E-mediated lysis. This technique allows the removal of plasmid sequences not required for vaccination, e.g., the origin of replication and the antibiotic resistance marker, resulting in production of BGs containing minicircle DNA with an increased safety profile [44, 45]. Additionally, the cytoplasmic space of BGs can be filled with various substances, e.g., water-soluble proteins, plasmids, drugs, further extending their utility as a potential therapeutic vehicle [13].

3. Conclusions

BGs combine features of the "ideal vaccine candidate and tumor therapy vehicle" (**Table 2**). The production of BGs can be easily and quickly undertaken either in disposable fermenters, small lab steel fermenters or in large scale fermenters from various types of Gram-negative bacteria including pathogens as well as probiotic strains. The production process requires standard technology which allows the use of this system in a broad number of differently developed countries. The BG's high loading capacity, preservation of loaded compounds and stability at room temperature also permit their easy storage and handling in less developed countries. Furthermore, BG's safe profile carries no risk of reversal to the native pathogenic form and no risk of horizontal gene transfer, and together with their ability to act as natural adjuvants due to the presence of all the surface morphological, structural and antigenic components of their living counterparts, especially designate this technology to be intensively studied for further vaccine development and tumor therapy. Altogether, the BG system constitutes a prospective safe and affordable multipurpose vehicle for the therapy of major human diseases including cancer.

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Tables and Figures

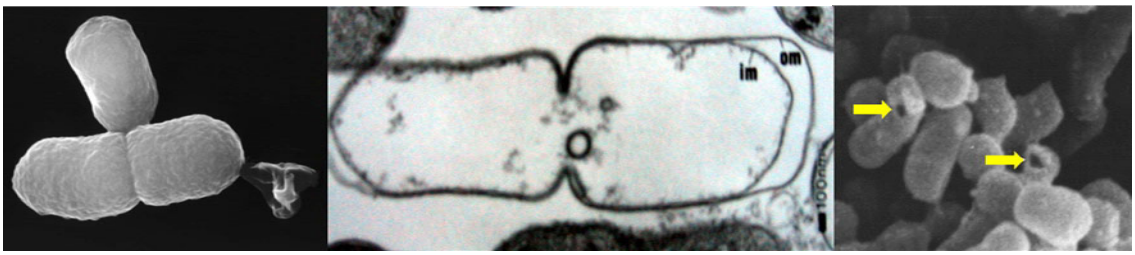

Fig. 1. Bacterial Ghost System. Inducible expression of E causes the fusion of inner and outer membranes of the bacterial cells and forms an intermembrane tunnel. The empty BG envelope is devoid of cytoplasmic content, whereas the inner and outer membrane structures including LPS and peptidoglycan are preserved and remain intact-reproduced from Ebensen et al. 2004. Arrow indicates E-specific lysis hole.

Table 1.
Use of BGs for experimental tumor vaccine and therapy.

BG envelope	Target Antigen/DNA/ Active Compound	Sector	Category of Vaccine	Proof of Principle	Outcome/ Effectiveness	Reference
Helicobacter pylori	Plain BGs	Oncology	Preventive/ Therapeutic	Mouse	Prophylactic oral vaccination of mice with BGs from *H. pylori* showed a significant reduction of the bacterial load in the BGs group. 15 of 20 mice were protected without the use of a mucosal adjuvant. Coadministration of BGs with cholera toxin as mucosal adjuvant resulted in a complete protection of all animals against *H. pylori* challenge, with three animals showing a sterile immunity.	[16]
Escherichia coli	OmpA-HbcAg-149 Protein based	Oncology	Therapeutic	Mouse	Subcutaneous immunizations with BGs containing Ag anchored to either inner or an outer membrane independent of the localization of the Ag on the cell surface or inside of BGs elicit significant Ag-specific humoral immune response.	[17]
Mannheimia haemolytica	Doxorubicin	Oncology	Tumor Treatment	Tissue Culture	Dox loaded BGs have a two orders of magnitude higher cytotoxic capacity to inhibit proliferation of colon carcinoma cells than free DOX at the same concentrations.	[20]
Escherichia coli	Doxorubicin	Oncology	Tumor Treatment	Tissue Culture	DOX loaded BGs enhance intracellular drug concentrations within tumor cells up to 42 times compared to the free Dox.	Winter, Mader, Lubitz; personal communication.

Table 2.
Bacterial Ghost System for vaccine development and tumor therapy.

- **Simple, high dose and low cost production in short time**
- **Stability at room temperature - loaded "merchandise" inside BGs is very well preserved**
- **Safe profile - no risk of reversal to pathogenic form, not a hazard for horizontal gene transfer**
- **Non-living character, but still retaining all of the surface morphological, structural and antigenic components of their living counterparts for tissue and cell targeting**
- **Natural adjuvant - BGs provide direct immunostimulatory effect on various cells of immune system**
- **Mucosal immunization with BGs induce strong humoral and cellular immune responses**

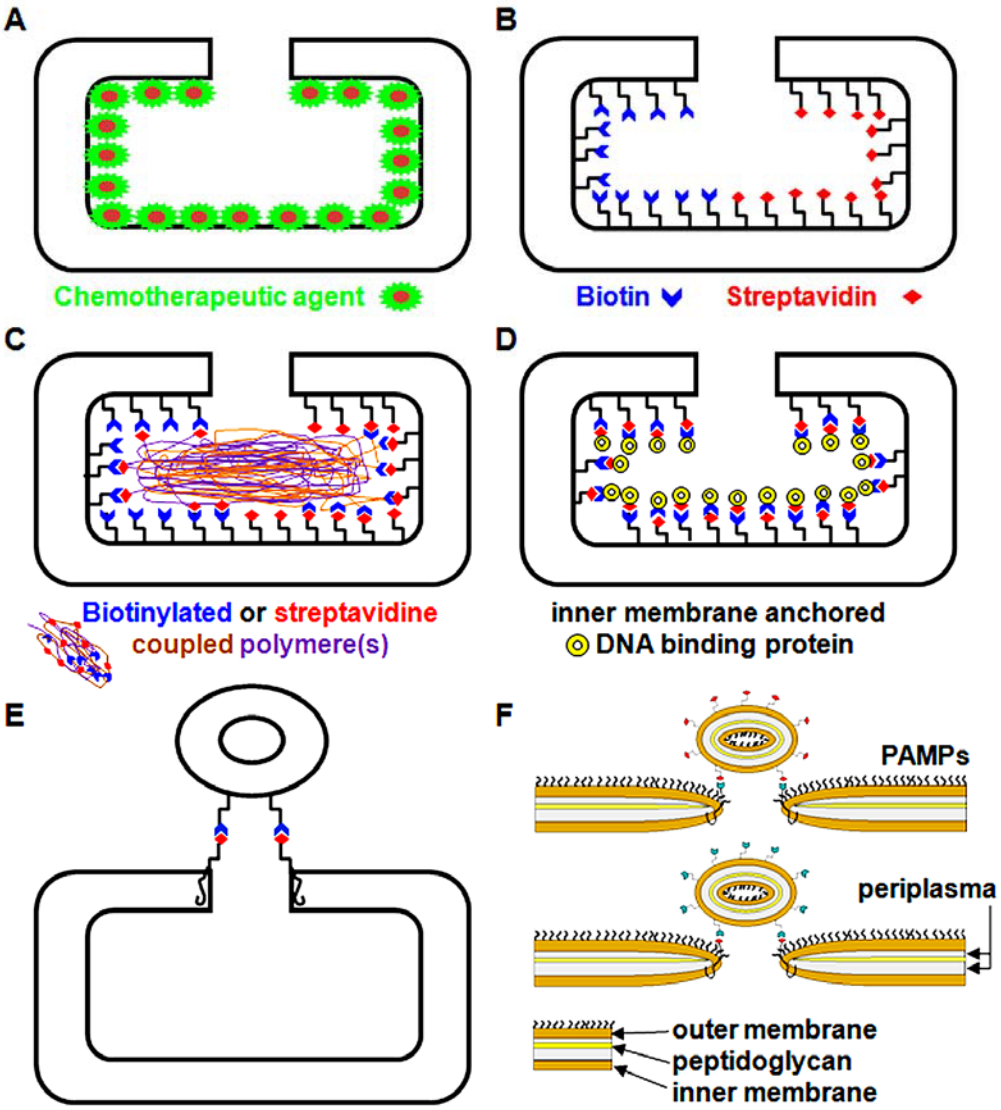

Fig. 2. Modified forms of Bacterial Ghost System. Schematic diagrams showing BG containing intact outer and inner membranes with sealed periplasmic space and inner space loaded with chemotherapeutic agent (A); inner membrane anchored streptavidin or *in vivo* biotinylation C-terminal amino acid sequence (B); membrane anchored biotinylated or streptavidin coupled polymer(s) (C); DNA binding protein anchored to the inner membrane (D). Sealing of BG expressing streptavidin around lysis hole with biotinylated inside-out membrane vesicles from Gram-negative bacteria (E). Detailed view of the inside out vesicles closing E-specific lysis tunnel, membrane anchored streptavidin or biotinylated membrane anchor are expressed either on the inside out vesicles or around lysis hole (F)-reproduced from Mayr et al. 2008.

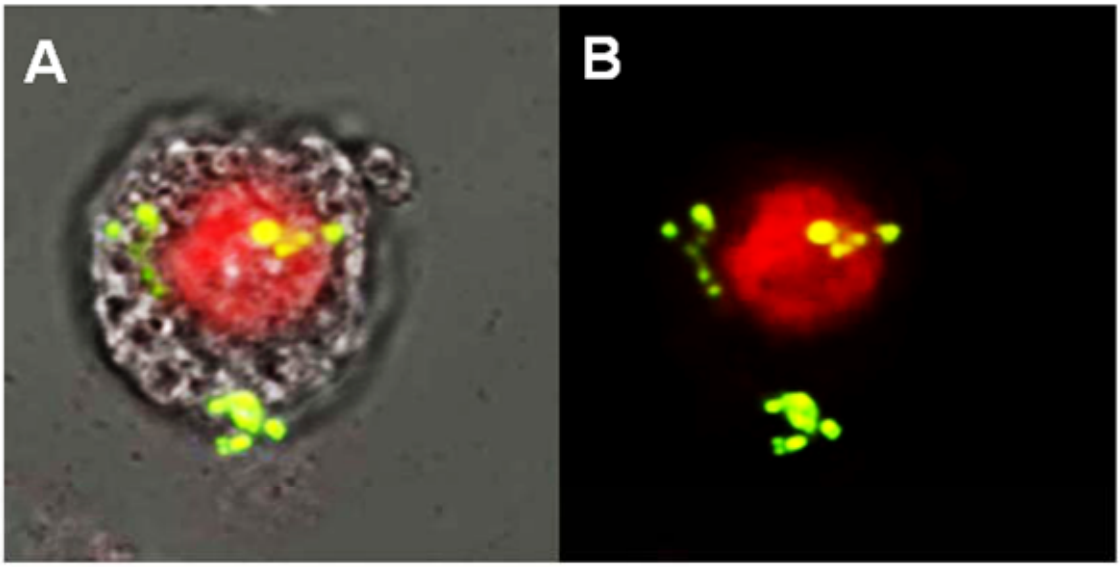

Fig. 3. Release of Dox within colon carcinoma cells after incubation with Dox loaded BGs. The overlay of the transmission light microphotograph (A) and the confocal laser scanning microphotographs (B) show the location of the Dox in the nuclear area or associated with the BGs (yellow as a result of a direct overlay of red and green fluorescence)-reproduced from Paukner et al. 2004.

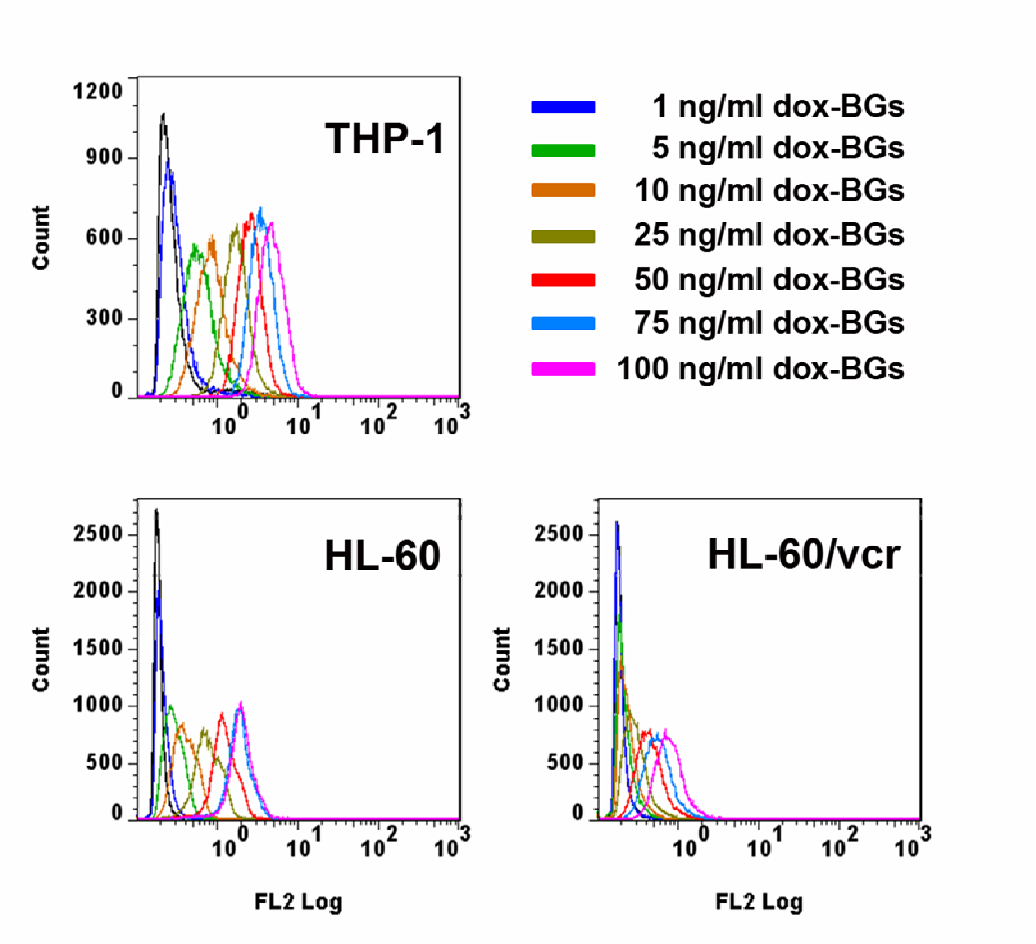

Fig. 4. Release of Dox within leukemia cells after incubation with Dox loaded BGs. Increased fluorescence of leukemia cells caused by incorporation of Dox in the nuclear area after incubation with Dox loaded BGs corresponds to increased concentration of Dox present inside the BGs.

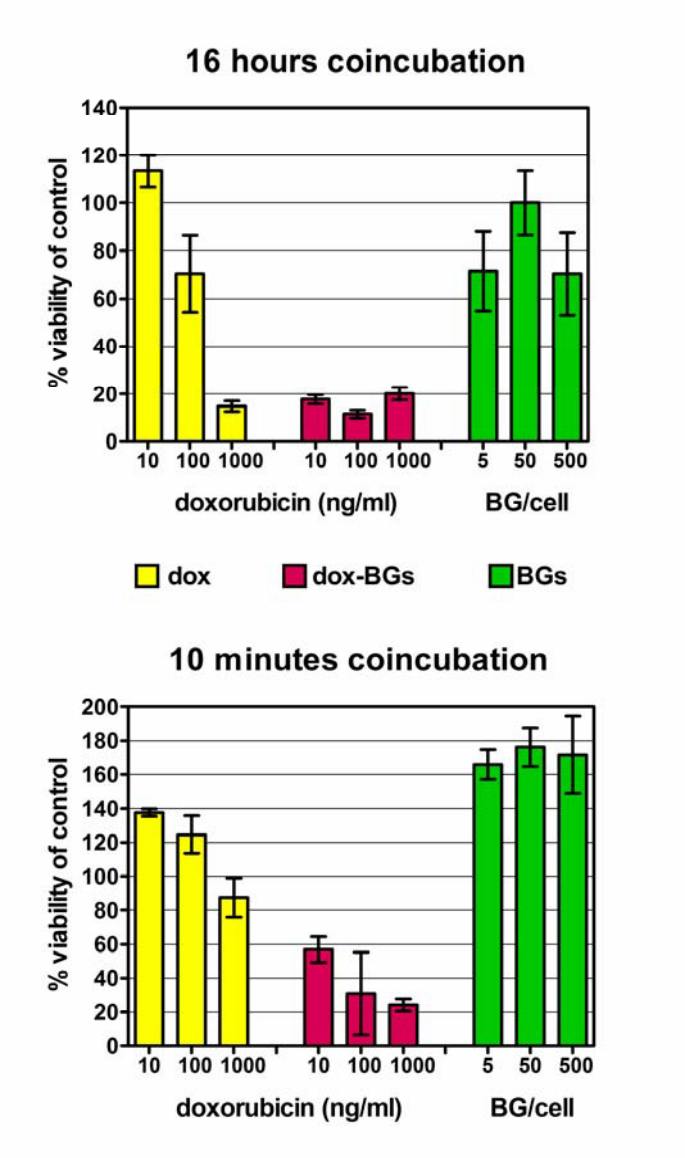

Fig. 5. Inhibition of tumor cell proliferation after incubation with Dox loaded BGs, free Dox and BGs alone. Colon carcinoma cells were incubated with 10, 100 or 1000 ng/ml Dox either free or associated with ghosts (corresponding to ghost/cell ratios of 5, 50, 500) for 16 h or for 10 min followed by removal of free Dox, BGs alone or Dox loaded BGs and incubation for 16h. Subsequently, the BrdU proliferation assay was performed to determine the viability of tumor cells-reproduced from Paukner et al. 2004.

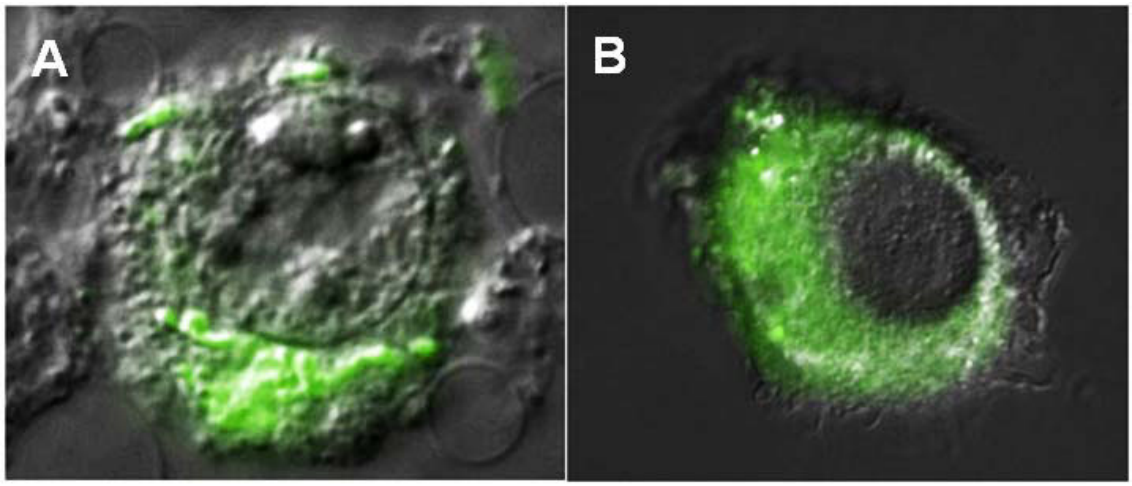

Fig. 6. Bacterial Ghost System as DNA delivery system. Release of fluorescently labeled DNA after incubation of mouse macrophages with BGs loaded with nonhomologous FITC labeled linear double strand DNA (A). Efficient gene delivery and expression of model Ag-GFP 48h after incubation of mouse macrophages with BGs loaded with plasmid DNA pEGF-N1 at the ratio of 500 BGs per cell for 2h (B)-reproduced from Paukner et al. 2005.

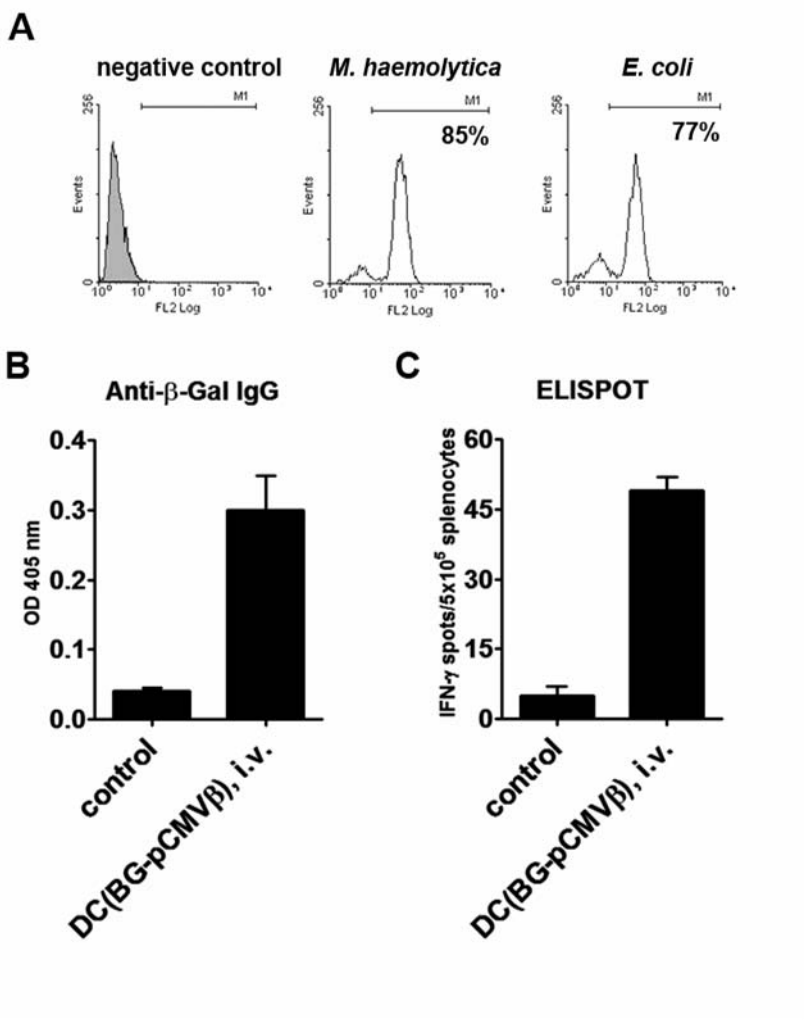

Fig. 7. DNA loaded BGs stimulate Ag-specific immune response. Gene transfer by BGs loaded with pEGFP into human monocyte-derived DCs (A). Immune responses stimulated by DC transfected *ex vivo* with DNA-loaded BGs- Ag-specific serum IgG responses 11 days after vaccination (B), increased number of IFN-gamma producing splenocytes after restimulation with immunodominant MHC class I Ag derived peptide (C)-reproduced from Kudela et al. 2005 and Ebensen et al. 2004.

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Chapter 2.

Applications of Bacterial Ghosts

Chapter 2.1.
Bacterial Ghosts as Carrier Vehicles for Cytostatic Drugs

Chapter 2.1.1.
Cytotoxic impact of resveratrol and resveratrol analogues loaded bacterial ghosts (BGs) on the human derived colon cell line HT-29

Verena Juliana Koller [1,2], Michaela Böhmdorfer[3], Walter Jäger[3], Thomas Szekeres[4] and Werner Lubitz[1,2,*]

[1] University of Vienna, Department of Medicinal Chemistry, Vienna, Austria

[2] BIRD-C GmbH & CoKEG, Hauptstraße 88, 3420 Kritzendorf, Austria

[3] University of Vienna, Department of Clinical Pharmacy and Diagnostics, Vienna, Austria

[4] Medical University of Vienna, Clinical Department for Medicinal and Chemical Laboratory Diagnostics, Vienna, Austria

[*] Corresponding author: Phone: +43-1-2243-28491 - werner.lubitz@univie.ac.at, werner.lubitz@bird-c.at

Colorectal cancer is still the third most common cancer worldwide and new treatment strategies are needed. The aim of the present investigation was to develop a drug delivery model for the polyphenolic compound resveratrol (RV) as well its analogues 3,3',4,4',5,5'-hexahydroxystilbene (M8) and digalloylresveratrol (DIG) which exert anti-tumor activity in a variety of cell lines. For this purpose the bacterial ghost (BG) technology was used. BGs are non-living envelopes that are produced by the plasmid encoded gene E mediated lysis from Gram-negative bacteria. *E coli* BGs were filled with the chemotherapeutic substances and their impact on the colon cell line HT29 was investigated. In a comparative study with the free compounds, it was shown that DIG was quite more potent than M8 or RV. This cytotoxic pattern was also observed when the compounds were entrapped into BGs. Our findings show that the application of loaded BGs significantly reduced the cell viability (up to 80 % with DIG). On the basis of the RV-status of HT29 cells, we were able to prove that the obtained cytotoxic effects were caused by intracellular drug delivery by BGs. Furthermore, more pronounced effects and sustained RV-levels were found after coincubation with RV-BGs than compared to the substance alone. These results indicate that BGs are an effective delivery system for the hydroxystilbene RV and its analogues DIG and M8 and can be used to target diseased human colon cells.

Introduction

The polyphenolic compound resveratrol (RV) is one of the most studied plant derived chemical. The hydroxystilbene shows beside antioxidative properties many chemopreventive activities in various cell lines e.g. leukaemia, breast and colon [1-3]. The exact mechanism of action of its cytostatic and cytotoxic effects is yet unknown. However, it was shown that RV mediates apoptosis through activation of the cell death receptor Fas or via depolarizing mitochondrial membranes followed by activation of caspase 9 [1, 2]. RV was also demonstrated to induce apoptosis via NF-κb inhibition and downregulation of Bcl-2 and promotes *p53* dependent apoptotic responses [2]. Moreover, RV was shown to inhibit the enzyme ribonucleotide reductase (RR) which is found to be highly upregulated in rapidly proliferating tumor cells and catalyzes the rate limiting step of the de novo DNA-synthesis [4].
Even though impressive *in vitro* effects are described, RV gives only limited anti-tumor activity *in vivo* [1, 2, 5]. Therefore, new substances have been developed to enhance the RV-related effects. Among these, higher hydroxylated RV-analogues like the 3,3',4,4',5,5'-hexahydroxystilbene (M8), have been synthesized [6]. It has been demonstrated that M8 induced apoptosis in HL-60 human promyelocytic leukemia cells [6, 7] and impaired melanoma progression was found in a metastatic mouse model [8]. Digalloylresveratrol (DIG) which represents a synthesized resveratrol ester composed of two gallic acid molecules and one RV molecule, showed dose-dependent apoptotic effects in the colon cell line HT29 [9]. Moreover, additive growth inhibition by combinatorial application with the standard chemotherapeuticum 5-fluorouracil and DIG was observed. Increased pro-apoptotic effects of DIG were also found for HL-60 cells [10].

Likewise RV, both chemicals (M8 and DIG) were shown to cause substantial imbalance of deoxyribonucleoside triphosphates (dNTPs), the products of RR, and to inhibit transition from the S- phase of the cell cycle to the G2/M phase [7, 9]. However, these studies demonstrated that both RV-analogues induced more apoptotic effects at lower concentrations than compared to the naturally occurring hydroxystilbene RV.

Because RV as well as M8 and DIG are very UV-labile and are known to be rapidly metabolized, stabilization and enhancement of the bioavailability is desirable. Even though microencapsulation techniques have been developed for RV to solve some of the problems of oxidation and of controlled drug release [11-15], the implementation of the bacterial ghosts (BGs) system would provide new aspects for therapeutic applications.

Over the last decades, the BG system has become an extensive vaccine platform technology for a wide area of applications. BGs are produced from Gram-negative bacteria by controlled expression of the cloned bacteriophage PhiX174 gene E, resulting in empty cell envelopes which are devoid of any cytoplasmic content and contain only minimal amounts of

residual DNA. E-mediated lysis has been achieved for a variety of Gram negative bacteria [16, 17]. Retaining immune stimulating compounds like LPS and peptidoglycan as well as antigenic epitopes, like their living counterparts, BGs are endowed with intrinsic adjuvant properties for boosting innate and adaptive immune responses [18]. This system can be extended by modifying the bacterial envelope prior to lysis or by entrapping various compounds in different compartments of the hosts so that they can be used as Advanced Drug Delivery Systems (ADDS) [17]. Besides the ability to present multiple epitopes due to expression of diverse foreign proteins by BGs, e.g. zona pellucida antigens [19], another advantage is given by the inner cytoplasmic lumen which can be filled with protein, DNA or drugs [17]. Loading and cell-directed delivery of water-soluble substances and emulsions by BGs was demonstrated in cell culture and plant models [20, 21]. The use of BGs as carrier vehicles for cytostatic drugs, like doxorubicin (DOX) was shown with Caco2 cancer cells [22]. It was demonstrated that lower concentrations of this compound, delivered endogenously by BGs, was at least two orders of magnitudes more effective than the free substance.

As recent investigations confirmed that organic ring structures, especially polyphenolic compounds, bind unspecifically to the membrane compartments of BGs [16], we aimed to load *E. coli* BGs with the chemotherapeutic drugs RV, M8 and DIG. The intention of the present study was to determine the cytotoxic impact of the entrapped compounds and to compare their efficiency in the human epithelial colon cell line HT29. To investigate further, whether the obtained effects were caused by endogenous drug delivery by BGs, additional experiments were conducted. For this purpose HT29 cells were treated with RV-loaded BGs and after different coincubation times, intra- and extracellular RV-concentrations were measured by HPLC. Also, the stabilizing and protection properties of RV by the BGs interior were examined. Additionally, long term incubation experiments (6 and 24 hrs) were conducted in which the amount of BG-delivered RV was compared to the free applied compound.

Materials and methods

Cell culture

HT29 cells were cultured under standard conditions (37°C moist atmosphere of 5% CO_2) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10 % heat inactivated fetal calf serum (FCS, Sigma), 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen), 10 mM HEPES buffer (Lonza, Verviers, Belgium), 2 mM L-glutamine (Invitrogen), 0.1 mM MEM Non-Essential Amino Acids (NEAA, Lonza) and 5 µg/ml plasmocin (Lonza). The medium was changed every 2-3 days and cells were grown until they reached confluency. Subculturing was done by detaching the cells with TrypLE Express (Gibco, Invitrogen). Cell numbers were determined by use of a Bürker-chamber. Test media contained only antibiotics.

Loading of E. coli NM522 BGs with RV,DIG and M8

RV was purchased by Sigma and DIG as well as M8 were kindly provided by Dr. Walter Jäger. BGs were produced as described elsewhere [19, 21]. Lyophilized *E. coli* NM522 (pGELys /AB; 071218; $5.4x10^9$ particles/mg) BGs were loaded with different concentrations of RV (1-150 mM), DIG (1-100 mM) and with M8 (1-50 mM) by simple resuspension in the loading solutions. The samples were incubated at 28°C under soft shaking (600-800 rpm) for 30 min. Thereafter, the samples were centrifuged (8 min, 13000 rpm) and washed 5 times with PBS. The BG pellets were stored at -20°C until use. Preparations of M8 and DIG stock solution as well as further loading concentrations were prepared by subsequent dilutions in dimethylsulfoxid (DMSO). RV stock solution and all other dilutions were prepared in methanol. BGs, which were resuspended in DMSO or in methanol and have been treated the same way as the BGs used for loading, served as BG-controls.

Determination of viability by use of the neutralred assay

The neutral red assay has been commonly used for the estimation of vital cells in culture. It is based on the uptake and lysosomal accumulation of the dye in living cells [23]. Prior to treatment, $1*10^5$ cells were seeded in 96-well plates and were allowed to attach overnight. After one washing step with PBS, the cells were treated with 200 µl serum-free (sf) medium containing BGs (either loaded in the solvent control or in different RV, M8 or DIG concentrations) in a BG to cell ratio of 1000:1 ($5x10^8$ particles/ml) or with 50 µM of the substances (RV and RV-analogues) alone. After 24 hrs (37°C, 5% CO_2), the cells were washed twice with PBS and were incubated with 100 µl neutralred dye (80 µg/ml final concentration; Roth) for another two hrs. Subsequently, the cells were washed again and the dye was extracted from the viable cells by use of 100 µl destaining solution (1 ml acetic acid, 73 ml 96 % ethanol and 26 ml deionized water). Absorbance was read with a microplate reader (Opsys MR,

DYNEX Technologies) at wavelength 570 nm (ref. 690 nm). Values, obtained from cells which were treated with serum free medium served as 100 % viability-level. The tests were performed in triplicate and mean values from three independent plates were determined. Data are represented as means + SD.

Determination and quantification of RV extracted from E. coli NM522 BGs and HT29 cells

RV was extracted from *E. coli* NM522 BGs by two times addition of 500 µl ethanol and shaking for 30 min (1000 rpm). After centrifugation (13000 rpm; 8 min), the obtained supernatants were collected and measured by HPLC.

In order to assess the concentration of RV which will be delivered by endogenous uptake of RV-loaded BGs, $2x10^6$ HT29 cells were seeded into 6-well plates and were allowed to attach overnight. The colon cells were incubated either with 2 ml 15 µM serum-free RV-solution or with 2 ml serum-free medium containing RV-BGs in a BG to cell ratio of 500 ($5x10^8$ particles ml^{-1}). Based on the theoretical delivery rate of 40 % (obtained from microscopic studies) of the applied BGs, concentrations of $_t$12 µM for BGs loaded in 100 mM RV were calculated. Cells which were treated with RV-BGs ($_t$12 µM) were harvested after different incubations periods (20 min coincubation of BGs followed by harvesting after 4 hrs, 2, 4, 6 and 24 hrs coincubation). At each endpoint, also the according media were collected to determine the RV-content which was extruded by the cells to the medium through transport proteins during these time periods. For comparison studies, the external as well as internal RV-concentrations were determined after application of 15 µM of pure RV for 6 and 24 hrs.

Immediately after collection of the medium, a centrifugation step (13000 rpm; 10 min) was performed to remove RV-BGs and other cell debris. The obtained supernatants were stored at -20°C until use. Cells were harvested by trypsinization and enzyme activity was stopped by addition of 10% FCS-containing medium. After two washing steps with PBS (15 min, 1100 rpm) the pellets were stored also at -20°C. Cell numbers and viability were determined by trypan blue staining in a Bürker chamber in triplicate.

Methanolic RV-extraction was performed by the freeze-thawing method in liquid nitrogen (three times) and the determination of RV and its metabolites by HPLC was performed using a Dionex "UltiMate 3000" system (Dionex Corp., Sunnyvale, CA). The column oven was set at a temperature of 15°C, and the UV-detector was set at a wavelength at 307 nm. Chromatographic separation of resveratrol and its metabolites was performed on a Hypersil BDS-C_{18} column (5 µm, 250 x 4.6 mm I.D., Thermo Fisher Scientific, Inc, Waltham, MA), preceded by a Hypersil BDS-C_{18} precolumn (5 µm, 10 x 4.6mm I.D.), at a flow rate of 1 ml/min. The mobile phase consisted of a continuous linear gradient, mixed from 5 mM ammonium acetate/acetic acid buffer, pH 7.4 (mobile phase A), and methanol (mobile phase B), to elute RV and its metabolites according to their lipophilicity. The mobile phase was filtered

through a 0.45 µM filter (HVLP04700, Milipore, Vienna, Austria). The gradient ranged from 10% methanol (0 min) to 20% B at 10 min and linearly increased to 35% B at 22 min, followed by another increase to 60% B at 25 min, where it remained constant until 30 min. Subsequently, the percentage of methanol was decreased within 2 min to 10% in order to equilibrate the column for 8 min before application of the next sample. Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed from the peak area of RV and its metabolites to the external standard resveratrol using standard solutions of RV to give a concentration range of 0.1 - 100 µg/ml.

Statistical analysis

All results were analysed by use of GraphPad Prism (version 5, GraphPad Software, Inc; San Diego; CA, USA). Statistical significance was determined by Student's t-test. P-values < 0.05 were considered statistically significant.

Results

Impact of pure and BG-entrapped RV, DIG and M8 on the viability of HT29 cells

It has been already demonstrated that RV loaded BGs have an impact on the viability of murine macrophages (Koller and Lubitz, to be published elsewhere). In this study, analyses were conducted to investigate whether BGs, loaded with RV or its analogues DIG and M8, diminish the viability of the human colon cells HT29. Also the impact of the compounds (RV, M8 and DIG, **Fig. 1**) alone was examined at a concentration of 50 µM, which significantly reduced cell viability (**Fig. 2**). Our findings show that at this concentration RV and M8 significantly decreased the cell viability to a similar extend (20% on average). However, higher effects were obtained after treatment with DIG which caused on average 80 % cell death after 24 hrs..

In order to determine the cytotoxic impact of BG-delivered RV, DIG and M8 (lyophilized *E. coli* NM522 BGs were loaded with the compounds up to the highest possible concentration: RV 150 mM, M8 50mM, DIG 100mM), experiments were conducted with HT29. The cells were treated with the loaded BGs in a MOI (multiplicity of infection) of 1000 for 24 hrs and cell viability was assessed with the neutralred assay. Results are depicted in **Fig. 3A-C**. It can be seen that the viability of the cell line was not impaired after treatment with the ghosts *per se*. However, treatment of HT29 cells with BGs loaded with the three compounds resulted in a dose dependent loss of viability after 24 hrs. The cytotoxic pattern confirm with the results obtained from determination of the effects of the pure substance where the highest impact was found with DIG. Whereas no differences were seen after treatment with 50µM RV and M8 alone (**Fig. 2**), significant differences were observed after delivery by BGs. Comparative investigations of BGs loaded in 50 mM of the adequate solution resulted in the following ranking: RV (91.1±5.6 % viability) <M8 (80.8+11.5 % viability) <DIG (45.6+9.0 % viability).

Quantification of RV extracted from loaded BGs and HT29 cells

Different experiments were conducted in order to investigate the stabilization properties of the BGs envelope for UV-labile chemicals like RV and to examine the extent of RV-delivery into HT29 cells by BGs.

E. coli NM522 BGs revealed after loading (by resuspension in 100 mM RV) and extraction 136.9 ± 15.8 µg RV per $1*10^{10}$ BGs respectively. Under the conditions used in the present study, it has been determined that 1-0.5 % of the total amount of RV was bound in 10^{10} BGs. A representative HPLC-chromatogram of the BG entrapped RV is shown in **Fig. 4A**. It can be seen that no other substances than the pure compound could be determined, indicating that no metabolizing action occurred within the BG and that the substance was protected from UV-damage during handling and storage since no cis-RV was detectable.

As uptake was demonstrated for *E. coli* BGs in the human colon cell line HT29 (Winter and Lubitz to be published), the cells were examined for the delivery of BG-bound RV. The colon cells were incubated with RV-loaded *E. coli* NM522 BGs for different time periods and were further treated as described in materials and methods. Subsequently, the extracted RV-solutions were analysed by HPLC. A representative HPLC-diagram for serum-free media treated control cells is given in **Fig 4B**. Because of the fact that RV was rapidly metabolized by the investigated cells after incubation, the sum of RV and six metabolites (M1-M6) was calculated. No differences could be seen in the representative diagrams which were obtained after incubation with RV (**Fig. 4C**) or RV-BGs (**Fig. 4D**). RV and all metabolites are marked according to their retention time.

From microscopic investigations it has been calculated that after 24 hrs coincubation roughly 40 % of the BGs are taken up by HT29 cells which would give a theoretical concentration of $_t$12 µM for BGs loaded in 100 mM RV.

Short time incubation of HT29 cells with BGs for 20 min, followed by further incubation in sf-medium for 4 hrs until harvesting, showed already detectable concentrations of RV and its metabolites in the cells (1.7±0.7 ng/1x10^6 cells). Increasing intracellular amounts of RV and its metabolites were found after 2 hrs (20.5±1.8 ng/1x10^6 cells) and 4 hrs (29.3+3.7 ng/1x10^6 cells) incubation (**Fig. 5A**).

Since it can be excluded that RV is released from BGs without any uptake by the cells (data not shown), the supernatants of the HT29 cells were investigated. As RV-metabolites can be pumped out from the cells, a time dependent increase in concentration was measured in the medium (**Fig 5B**). After 4 hrs incubation with RV-BGs, 4 times more RV metabolites were detectable in the supernatant medium (4.1±0.8 µg/ml) than found after treatment with BGs for 20 min, followed by further incubation in serum-free medium for four hours until harvesting (1.1±0.1 µg/ml).

Comparative investigations of 6 and 24 hrs treatment either with RV-loaded *E. coli* NM522 BGs ($_t$12 µM) or with 15 µM RV solutions were conducted and results are represented in **Fig. 5C-D**. High significant differences of the intracellular RV and metabolite levels were obtained after treatment with $_t$12 µM BGs when compared with the substance alone after 6 hrs (P=0.0138) and 24 hrs (P=0.0412). On average, 30.5±6.0 ng /1x10^6 cells RV and RV-metabolites were detectable in the BG treated cells after 6 hrs. This represents more than 4-fold of the obtained values by 15 µM RV (7.2+0.8 ng / 1x10^6 cells).

Results obtained from measurements of the supernatant medium of the cells after treatment with 15 µM RV or RV-BGs for 6 and 24 hrs are depicted in **Fig. 3D**. Significant higher concentrations were found with BGs compared to the substance per se after 6 hrs incubation (P=0.0001) and after 24 hrs (P=0.0327)..

Our findings show that RV delivery by BGs caused sustained levels of intra- and extracellular concentrations of the chemical and its metabolites. Furthermore, we could demonstrate a drug release profile in the HT29 cell line depending on the coincubation time of BGs. Moreover, better effects were found with RV when delivered by BGs than with the free applied substance. High differences were also found in regard to the cytotoxic impact of the compound. While neutralred assays as well as trypanblue staining of the cells showed that 15 µM of free RV did not cause reduction of cell viability (data not shown), RV-loaded BGs decreased cell viability about 15 %.

Discussion

More than 655,000 deaths are recorded worldwide per year which are related to colorectal cancer [24]. Therefore, new targeted agents are designed and used in various therapeutic treatments. Several studies showed that RV suppresses the proliferation of various colon cancer cells *in vitro* and that tumor formation was significantly reduced in mice [1]. Also the proapoptotic potential of the RV analogues M8 and DIG has been demonstrated for a variety of rapid proliferating cell lines, including colon and leukaemia cells [9, 10, 25]

To date, there is a high demand for developing a drug delivery system that accounts for substance protection and sustained drug-release. Therefore, encapsulation techniques are now under progress to overcome this problem and researches with diverse biodegradable carrier systems have been conducted. RV has been demonstrated to incorporate into liposomes [13] and the compound was loaded into calcium-pectinate beads having 1-2 mm in diameter [12] or into mPEG-PCL based nanoparticles smaller than 100 nm in size [14].

In the present study, the potential of BGs as a drug delivery system for RV and its analogues M8 and DIG was investigated in the colon cell line HT29. The experiments showed that the cell viability was not affected after long term incubation with *E. coli* BGs *per se* but was reduced in a dose dependent manner when the compounds were entrapped.

As HPLC is a reliable method and was already used for the determination of RV-concentrations in biological systems [26], different experiments were conducted with RV-loaded BGs to investigate if the obtained cytotoxic effects were caused by intracellular delivery.

However, first we examined the stabilization capacity of the BGs interior towards the photosensitive chemical. Comparisons between the standard trans-resveratrol and the extract of the BG-entrapped chemical clearly demonstrate, that no UV-induced alteration had occurred during the encapsulation process as no cis-resveratrol was detectable. This is in analogy to a study in which RV was incorporated into lypophylized *S. cerevisiae* cells which were plasmolyzed before the freeze drying process [15]. As by this method, the cytoplasmic material is mostly removed from the cell, similar to BGs, the encapsulation yield (EY) can be compared with our results. Recalculating for one mg loaded vehicles, the yeast EY was on average 0.0450 $\pm$ 0.005 % compared to an entrapping efficiency of 0.3007 $\pm$ 0.0344 % by BGs. Since the common beaker's yeast sizes in its spherical form 5-10 µm in diameter in contrast to *E. coli* BGs which show on average 1 µm in length, a higher entrapping rate could be assumed. However, a direct opposite can be demonstrated as more than 6 times less RV could be incorporated into *S. cerevisiae*. This can be mainly explained by the efficacy of the lysis process. Compared to cell treatment with a plasmolyser, the ghost production technique represents a controlled lysis system. As nearly all of the cytoplasmic content is expelled in more

than 99.99% of the population through the generated lysis hole (up to 200 nm), the full capacity of the intracellular ghost lumen can be used for the loading of chemicals.

Because of the fact, that some enzymes of the BGs were found to be still active, even after lyophilization, we investigated if any chemical changes take place after BG-encapsulation. Since only high purity peaks were obtained under the chromatographic conditions, we can certify the metabolic inactivity of BGs towards resveratrol.

RV is known to be rapidly metabolized and glucuronidated in the small intestine of rat [27] as well as in the human GI tract and liver [5, 28]. Because sulfotransferase activity was reported to be higher in gut than in liver [29], the GI tract can be considered as the major side for RV metabolism.

To investigated the uptake of RV-loaded BGs and their total-RV metabolite content, the metabolic active HT29 colon cell line was used which expresses multigenic families of enzymes such as sulfotransferases and UDP-glucuronosyltransferases [30, 31]. Furthermore, these cells possess transport proteins like multidrug resistance protein 3 (Mrp3) and ABCG2 (also named BCRP), which have been shown in cell culture studies to mediate the extrusion of metabolites from the cells [30, 32]. Therefore, we examined the intracellular and the extracellular concentrations of RV and their metabolites after applications of RV-loaded BGs in comparison to an equivalent dose of free RV after long term treatment. Due to the high metabolic rates of the used HT29 cells mostly only metabolites were detectable in the cytosolic fractions. However, a higher and sustained release profile could be found, after internal RV delivery by BGs than compared with the substance alone. More than 5 times higher intracellular RV concentrations, mainly metabolites, were detected after 6 hrs application of RV-loaded ghosts than with RV *per se*. After 24 hrs coincubation also differences concerning the cell viability were detectable. In contrast to the free RV solution, drug delivery by BGs caused cytotoxic effects in HT29 cells. Such findings were also obtained in a former study in which increased cytotoxic and antiproliferative activity of the cytostatic drug doxorubicin was found after endogenous release by BGs than compared to the chemical per se [22]. In another investigation with glia cells it was shown that formulation of nanoparticle-bound RV resulted in significant higher cytotoxicity than the free drug in an equivalent dose [14].

Even though RV is known to be metabolized within 8-14 min [12], beneficial effects of dietary RV have been demonstrated in mice [33, 34]. Therefore, it is speculated that its metabolites may still retain some of their pharmacological activities or that due to deconjugation of these metabolites, the pharmacologically active parent compound could be released again. The latter assumption is in agreement by a human study in which new RV plasma peaks were observable again after 6 hrs after intestinal hydrolysis [35]. Furthermore it was shown that the compound interferes with serum albumins *in vitro* [36] and *in vivo* that up to 50 % of RV-metabolites, which were transported into plasma, were noncovalently bound to proteins [37]

However, a previous *in vitro* study demonstrated that RV induces apoptosis only after long term treatment for up to 24 hrs [38] and as we also did not observe any acute toxic events after shorter exposure times, we presume that the cytotoxic effects are mainly related to the originated metabolites.

The results of the present study confirm that BGs represent an effective delivery system for the compounds RV, DIG and M8 by BGs. As RV has been shown to have potential therapeutic efficacy on lower gastrointestinal diseases like colitis and colorectal cancer it could be assumed that the BG bound chemical or its analogues M8 and DIG might enhance the effectiveness of therapy.

Figures

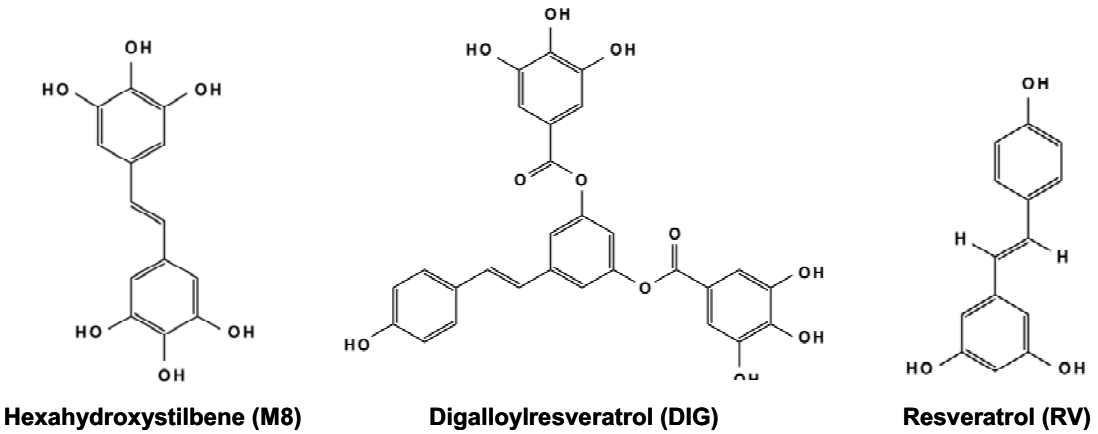

Fig 1. Chemical structures of the 3,3',4,4',5,5'-hexahydroxystilbene (M8, left), digalloylresveratrol (DIG, middle) and resveratrol (RV, right).

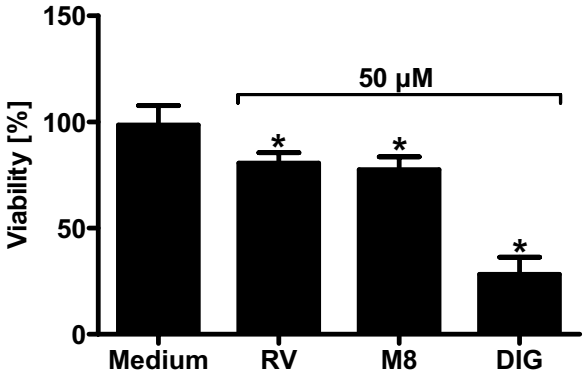

Fig. 2. Comparative cytotoxic effects of RV and RV-analogues M8 and Dig are shown. HT29 cells were treated for 24 hrs. All compounds significantly reduced the cell viability at a concentration of 50 µM. Values are means + SD obtained after 4-6 measurements which were performed in triplicate.

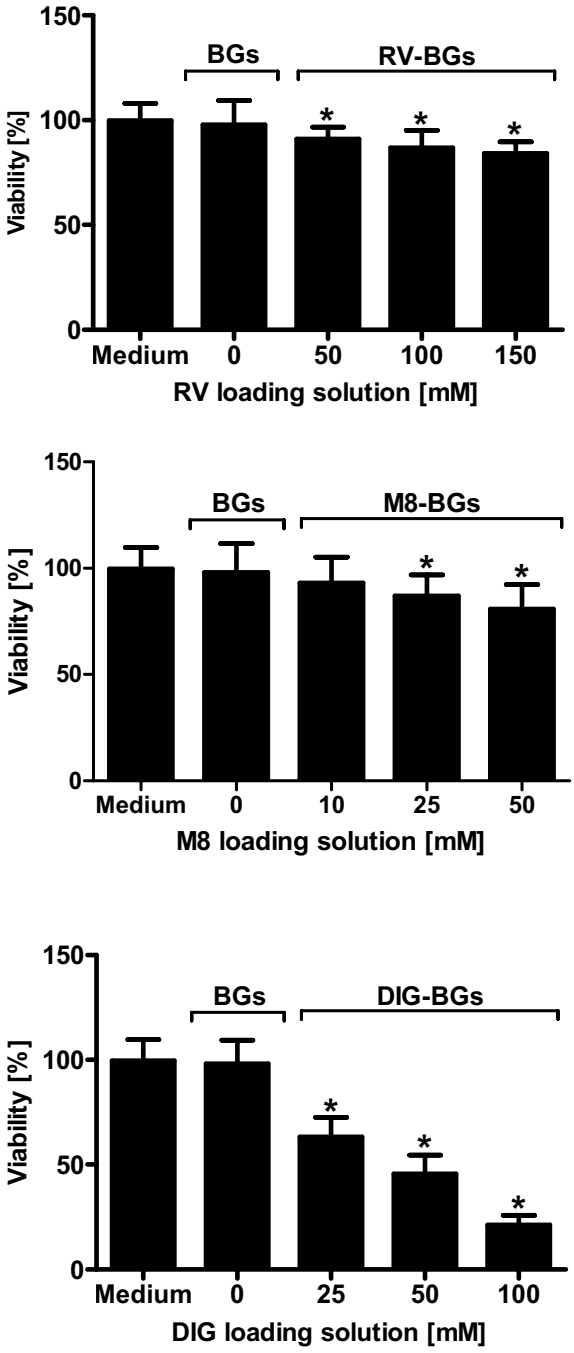

Fig. 3. Impact on the viability of the colon cell line HT29 after treatment with loaded BGs for 24 hrs. Metabolic activity was assessed by neutral red assays. Bars represent mean values + SD of 4-6 determinations measured in triplicate. Asterisks indicate values which were considered significant different from the medium treated controls.

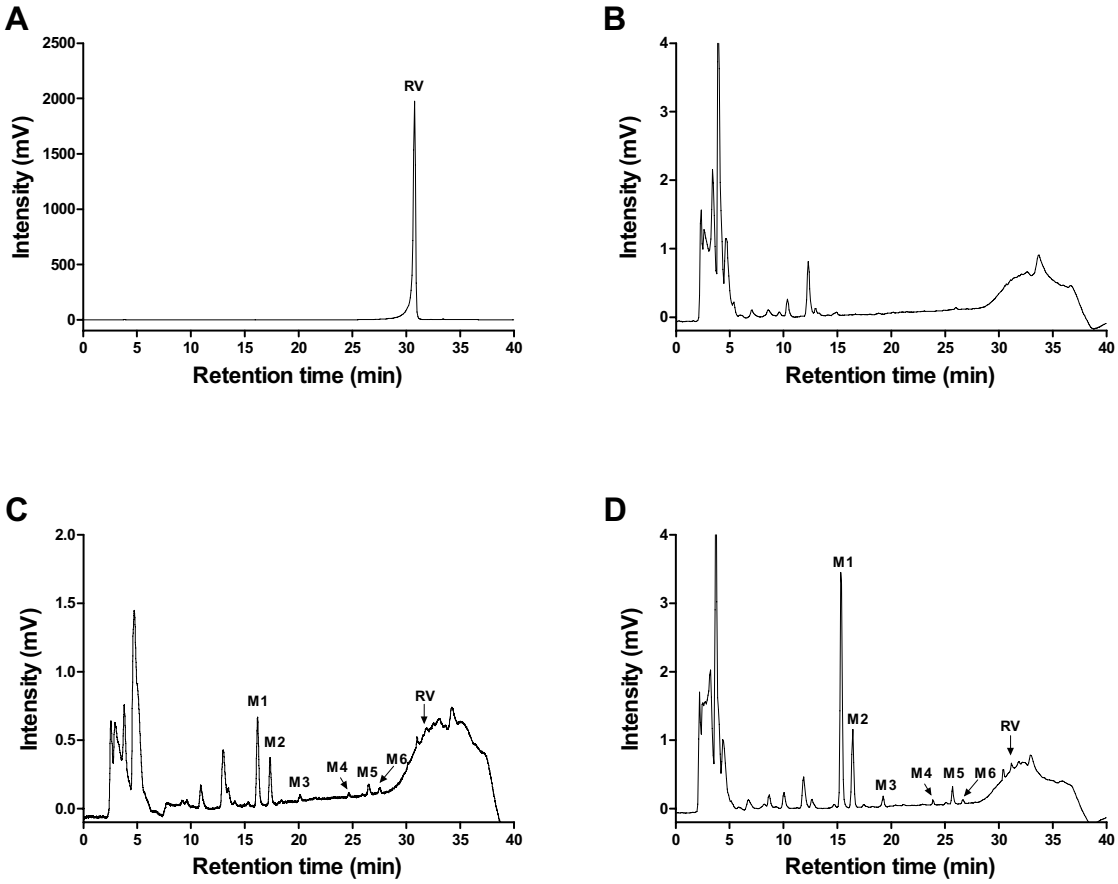

Fig. 4. Representative HPLC chromatograms of RV measurements are depicted. An example for the quantification of RV extracted from BGs is shown **(A)**. It can be seen that no metabolization of the drug occurred within the BG's lumen and that only trans-RV was detected. A diagram of serum-free medium treated HT29 cells is shown in **(B)**. Since RV is rapidly metabolized within the colon cells, also its metabolites were considered for quantification. Representative diagrams after treatment of colon cells with RV alone **(C)** or delivered by BGs **(D)** are depicted.

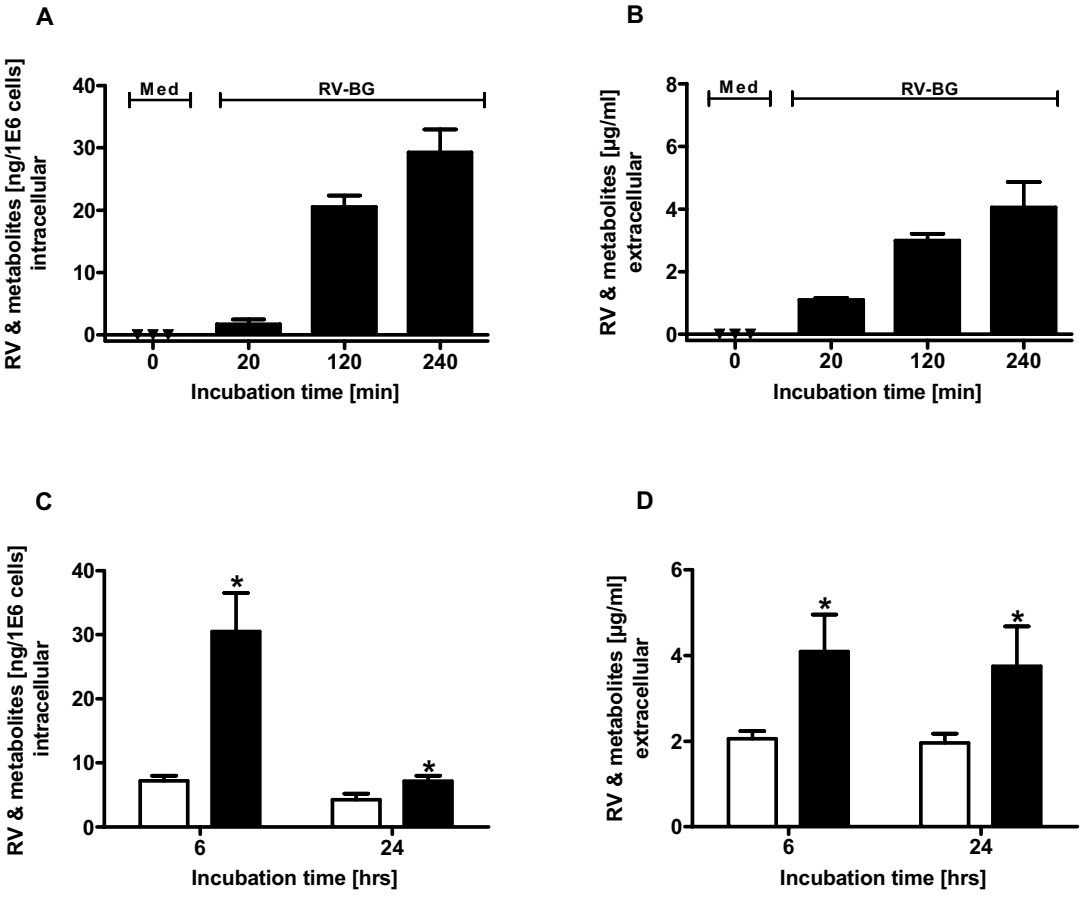

Fig. 5. Time dependent RV delivery by BGs into the colon cell line HT29. RV and its according metabolites were detectable after short term treatment with RV-loaded *E. coli* NM522 BGs for 20 min, followed by further incubation in serum-free medium for 4 hrs until harvesting. Further increase of the concentrations was found after 2 and 4 hours coincubation with BGs in a MOI of 500 ($5x10^8$ BGs/ml). Intracellular RV and RV-metabolite levels are depiced in (**A**) and concentrations which were extruded by the cells into the medium are shown in (**B**). Comparative quantification HPLC analyses were performed to investigate the internal content **(C)** and the extracellular amount **(D)** of RV and its metabolites after 6 and 24 hrs incubation with 15 µM free RV (white bars) or with RV-loaded BGs (black bars). Cells were coincubated with RV-BGs in a MOI of 500 ($5x10^8$ BGs/ml, ~12 µM). Asterisks indicate values which were statistically significant different from those obtained with 15 µM RV *per se*.

Bars represent means + SD measured either in triplicate (internal concentration) or duplicate (external concentration).

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Chapter 2.2.
Bacterial Ghosts as Targeting Vehicles for Ocular Surfaces

Chapter 2.2.1.
Bacterial Ghosts (BGs) as potential antigen and drug delivery system for ocular diseases: Effective endocytosis of BGs by human conjunctival epithelial cells

Pavol Kudela [1,4,6,*], Verena Juliana Koller [1,4], Ulrike Beate Mayr [4], Johannes Nepp [1], Stefan Wagner [2], Karin Hoffmann-Sommergruber[3], Talin Barisani-Asenbauer [1,3], and Werner Lubitz [4,5]

[1] Medical University of Vienna, Department of Ophthalmology and Optometrics,

[2] Center of Physiology and Pathophysiology, Department of Pathophysiology,

[3] Center of Physiology and Pathophysiology, Institute of Specific Prophylaxis & Tropical Medicine, Vienna, Austria;

[4] BIRD-C GmbH&CoKEG, Kritzendorf, Austria;

[5] University of Vienna, Department of Medicinal Chemistry, Vienna, Austria;

[6] Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia.

[*] Corresponding author: Phone: +43-1-2243-28491 - pavol.kudela@bird-c.at

The purpose of the presented investigation was to examine the efficiency of bacterial ghosts (BGs) - empty bacterial cell envelopes of Gram-negative bacteria retaining all the surface morphological, structural and antigenic components in the same quality as in their living counterparts - to target human conjunctival epithelial cells, as well as to test the endocytic capacity of conjunctival cells after co-incubation with BGs generated from different bacterial species, and to foreclose potential cytotoxic effects caused by different types of BGs. The capacity of conjunctival cells to endocyte BGs was investigated using the Chang conjunctival epithelial cell line and primary human conjunctiva-derived epithelial cells (HCDECs) as *in vitro* model. The efficiency of endocytic activity of conjunctival epithelial cells was measured by uptake of fluorescence-labeled BGs and determined by flow cytometry. Potential cytoxicity of BGs was monitored by the WST-1 Proliferation Assay. A high capacity of HCDECs to functionally internalize BGs was detected with the level of endocytosis depending on the type of BG species. Further analysis showed that BGs have no cytotoxic effect on HCDECs after mutual co-incubation, independently of the used bacterial species. Moreover, BGs alone did not enhance expression of both MHC class I and class II molecules, but increased expression of ICAM-1. The high endocytosis rates of BGs by HCDECs with no BG-mediated cytotoxic impact designate this bacterial carrier system to be a promising candidate for an ocular surface drug delivery system. BGs could be useful for future therapeutic ocular surface applications and eye-specific disease vaccine development including DNA transfer.

Introduction

The ocular surface epithelium plays a fundamental role in the protection of the eye from environmental factors and pathogens. First acting as a physical barrier it defends the eye from injury and invasion of pathogens and allergens, and second as part of the eye-associated lymphoid tissue (EALT) it contributes to the homeostasis of the ocular surface [1-2]. This front line role of the ocular surface epithelial cells is supported not just with the capacity to form physical obstacle against microbes or drug agents but also with their capability to produce pro-inflammatory cytokines, chemokines and antimicrobial peptides, and thus to enhance elimination of pathogens, and to protect the eye from uncontrolled inflammatory responses [3-6]. Conjunctival epithelium is about three layers thick and covers vascularized connective tissue built from bone-marrow derived cells which form conjunctiva-associated lymphoid tissue [7-8]. In addition to the presence of CD4+ and CD8+ T cells in conjunctival epithelium; macrophages and Langerhans dendritic cells are also present as numerous populations in the conjunctiva [9-10]. Professional antigen-presenting cells (APCs) capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to the lymphoid organs and secrete cytokines to initiate immune responses [11]. Extracellular antigens present in the conjunctiva are processed by APCs and presented in the context of MHC class II molecules to the lymphocytes in the local lymphoid follicles [9, 12]. Furthermore, intraepithelial T lymphocytes are capable to recognize glycolipids derived from bacterial pathogens which are associated with nonpolymorphic class I MHC-like molecules, and by production of cytokines to activate professional phagocytes [13-14].

Due to the conjunctival epithelium barrier function delivery of drugs/antigens is challenging. Tight intercellular junctions and the constant state of flux of the tear fluid not only hinder the penetration of harmful but also of therapeutic agents. Therefore platforms that overcome this shield enabling effective administration of drugs or antigens to the ocular tissues and EALT would help to progress the development of prospective generic drugs/vaccines/gene therapy against various ocular surface diseases.

Bacterial ghosts (BGs) represent empty bacterial cell envelopes of Gram-negative bacteria which have been developed in the last years as a novel carrier and adjuvant system for the delivery of mucosal vaccines [15-17]. BGs are produced by protein E-mediated lysis of Gram-negative bacteria. Protein E fuses the inner and outer membranes forming a specific small hole in the bacterial envelope through which all the cytoplasmic content is expelled. The empty shell of BGs is devoid of nucleic acids, ribosomes and other constituents, whereas inner and outer membrane structures including the outer membrane proteins, adhesins, LPS, and peptidoglycan are well preserved and remain intact [18].

The main advantage of BGs is their non-living character, while still retaining all of the surface morphological, structural and antigenic components in the same quality as in their living counterparts [19]. The different spaces of BGs can be loaded with a combination of protein antigens, drugs or foreign DNA which gives an opportunity to design new types of polyvalent vaccines or to use BGs as advanced drug delivery system [16]. LPS-lipopolysaccharide (i.e., lipid A, endotoxin) - content of the Gram-negative bacteria cell envelopes presented on the BG's shell does not represent a risk for using of BGs as a vaccine candidate. Previous immunological studies have shown that BGs induce dose-dependent antibody responses against bacterial cell components and LPS without inducing fever. Safe profile of BG was confirmed using a Limulus-assay, where purified LPS (*E. coli* O26:B6) expressed endotoxic activity values 100-times higher than the BGs. Moreover, there is no risk of reversal to pathogenic form in contrast to attenuated bacteria used as bacterial delivery system [20].

Our previously published studies have shown that BGs loaded with plasmid DNA encoding green fluorescent protein are efficiently internalized and phagocyted by both professional antigen-presenting cells and tumor cells. BGs were able to deliver the heterologous genes to both non-dividing cells (monocyte-derived DCs) and dividing cells (macrophages and melanoma) with study results showing that up to 85% of cells expressed the plasmid encoded reporter gene delivered by BGs [21-24].

The purpose of the present study was to examine the capacity of BGs generated from different bacterial species to target primary human conjunctiva-derived cells (HCDECs) and their potential effect on cell viability, as well as to test the endocytic capacity of primary HCDECs after co-incubation with BGs.

Material and methods

Cells and reagents

A human conjunctival cell line (Wong–Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, ATCC *CCL-20.2*) kindly provided by Prof. Bernd R. Binder (Medical University Vienna, Centre of Biomolecular Medicine and Pharmacology; Department of Vascular Biology and Thrombosis Research, Vienna, Austria) was maintained in Dulbecco's modified Eagle's medium (Lonza, Verviers, Belgium) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 10 mM HEPES (Lonza), and 0.1 mM MEM Non-Essential Amino Acids Solution (Lonza) in a 5% CO_2 humidified incubator at +37°C. Human conjunctiva specimen biopsies were obtained from patient donors undergoing eye surgery. Informed consent was obtained in accordance with the Declaration of Helsinki from all subjects prior to surgery. The study was approved by Ethics committee of the Medical University of Vienna (EK-Nr. 598/2008). Primary human conjunctiva-derived epithelial cells (HCDECs) were obtained from biopsy specimen with small modifications of previously described methods [25-27]. Briefly, conjunctival tissues were cut into small pieces and incubated for 1h at +37°C with 1.2 IU/mL dispase II (Invitrogen). Cells were detached, collected and cultured in complete culture medium- Dulbecco's modified Eagle's medium and Ham's F12 (1:1 mixture)(PAA Laboratories GmbH, Vienna, Austria) supplemented with 10% FCS, penicillin/streptomycin, HEPES, MEM Non-Essential Amino Acids Solution, 1 µg/mL bovine pancreas insulin (Sigma), 2 ng/mL epidermal growth factor-EGF (R&D Systems, Inc., Minneapolis, MN), 5 µg/mL hydrocortisone (Sigma), and 0.1 µg/mL cholera toxin (Sigma). The cultures were fed with fresh medium and supplements every 2 to 3 days.

Production of BGs

BGs from *Mannheimia haemolytica* A23, *Escherichia coli* Nissle 1917, *Escherichia coli* NK9373, and *Escherichia coli* NM522 were produced by the controlled expression of the phage-derived lysis protein E, as described previously [18, 28-30]. For safety reasons, to avoid presence of potential non-lysed, non-culturable but viable cells, the BG's preparations were inactivated with gentamycin (50 mg/mL; Invitrogen) and streptomycin (100 mg/mL; Invitrogen). Subsequently the BGs were washed three times with phosphate-buffered saline (PBS, pH~7.4; Lonza), resuspended in distilled water and lyophilized. Lyophilized BGs were stored at +4°C.

FITC-labeled BGs uptake

The efficiency of endocytic activity of conjunctiva cells was measured as described previously [22-23]. Briefly, 5 mg of BGs were resuspended in 500 µL 0.1 M Na_2CO_3 (pH~9.0; Sigma). Stock solution volume of 25 µL FITC (1 mg FITC; Sigma, in 1 mL DMSO; Sigma) was added to the BGs suspension and shaken for 2 h at room temperature. Afterward the BGs were carefully washed with PBS and stored at +4°C protected from light. HCDECs cultured in 24 well plates ($2x10^5$ cells/well; Sarstedt, Nümbrecht, Germany) were incubated with FITC-BGs (1000 per cell) for 10 and 40 min at +37°C. After incubation the cells were washed three times with PBS to remove the excess of BGs. Finally the cells were detached using TrypLE™ Express (Invitrogen), washed 3 times with PBS, fixed in cold 1.5% PFA (Sigma) in PBS and analyzed on BD FACSCanto™ Flow Cytometer (BD Biosciences, Pharmingen, San Jose, USA). Dead cells were excluded according to their forward and side scatter properties. Obtained data were analyzed using FlowJo Software version 7.5 (Tree Star, Inc., Ashland, OR).

Cell proliferation and viability assay

Metabolic activity and viability of HCDECs after incubation with BGs were assessed using premixed WST-1 cell proliferation assay following the manufacturer's instructions (Clontech Laboratories, Inc., Mountain View, CA). HCDECs cultured in 96 well flat-bottom plates ($2x10^5$/well, Sarstedt) were incubated with BGs (1000 per cell) for 40 min at +37°C. After co-culture with BGs the HCDECs were washed to remove the excess of BGs followed by incubation in complete culture medium. After 24 h premixed WST-1 cell proliferation reagent was added to each well and cells were incubated for additional 4h at +37°C in a 5% CO_2 humidified incubator as recommended by the manufacturer. After 1 min of thorough plate shaking, the absorbance was measured at 450 nm (reference wavelength 690 nm) using a DYNEX Opsys MR plate reader (Dynex Technologies, Chantilly, VA). Cytotoxic agent Triton X-100 (0.005%; Sigma) was used as a control.

Flow cytometry analysis of human conjunctiva derived cells

The capacity of BGs to elicit expression of MHC molecules and ICAM-1 on the surface of responding cells was assessed by incubation of HCDECs with BGs (1000 per cell) for 10 and 40 min at +37°C in a 5% CO_2 humidified incubator. After incubation the cells were washed three times with PBS to remove the excess of BGs and fresh complete culture medium was added for additional incubation. Cells were collected 24h, 36h and 72h after stimulation, resuspended in FACs buffer (0.1% BSA (Sigma), 0.01% NaN_3 (Sigma) in PBS) and stained with panel of mAb – FITC conjugated mouse anti-human HLA-ABC (BD Biosciences), PE conjugated mouse anti-human HLA-DR (BD Biosciences), PE-Cy™5 conjugated mouse anti-human CD54 (ICAM-1) (BD Biosciences), and isotype controls (BD Biosciences).

Unstimulated cells incubated without BGs stained with isotype controls and with a panel of described mAbs served as controls. After incubation for 30 min at +4°C the cells were washed with FACs buffer, fixed in cold 1.5% PFA in PBS and analyzed on BD FACSCanto™ Flow Cytometer (BD Biosciences). Dead cells were excluded according to their forward and side scatter properties. Obtained data were analyzed using FlowJo software (Tree Star).

Statistical analysis

Obtained results were analyzed by GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). The statistical significance of the difference between two groups was evaluated by Student's t-test and between more than two groups by the one-way ANOVA. Differences were considered to be significant with $p < 0.05$.

Results

Effective endocytosis of BGs by human conjunctival cells

The endoytosis of BGs by primary HCDECs and conjunctival epithelial cell line CCL-20.2 was determined by flow cytometry after extensive removal of non-internalized BGs by washing with PBS. The detection of efficiently internalized BGs within human conjunctival cells was also confirmed by confocal laser scanning microscopy (data not shown). The obtained results showed that the uptake rates were dependent on the bacterial species from which BGs were prepared and length of co-incubation. The highest internalization of BGs was observed after incubation of human conjunctival cells (HCC) with BGs derived from *M. haemolytica* (**Fig.1**). Up to 80% of conjunctival cells CCL-20.2 effectively internalized *M. haemolytica* BGs at the ratio BG:HCC - 1000:1 after 40 min of co-incubation. Detectable endocytosis of BGs was observed already 10 min after adding of BGs to the culture system. BGs derived from *E.coli* NK9373 revealed approximately the half efficacy to stimulate endocytic capacity of human conjunctival cells compare to *M. haemolytica* derived BGs (~43%). Significant internalization of BGs was also detected after incubation of human conjunctiva cells with BGs derived from *E.coli* Nissle 1917 (~24%). The number of cells with increased fluorescence as a result of endocytosis of FITC-labeled BGs rose with extended time of co-incubation. *E. coli* NM522 derived BGs showed modest positive impact on the capacity of human conjunctival cells to endocyte BGs after 40 min of co-incubation when part of conjunctival cells (~13%) efficiently bound and internalized FITC-labeled empty bacterial shells (**Fig.1A**). Similar results confirming the hierarchy of BG types capable to stimulate endocytic capacity of 6 different human primary conjunctival cells were observed after co-incubation of BGs with HCDECs obtained from various human conjunctival specimen biopsies. The results presented in **Fig.1B** are the average of 6 different primary cells (HCDEC 1-6) tested and thus it is obvious that the standard deviation is higher than the one given for the Wong–Kilbourne derivative of Chang conjunctiva cells (**Fig. 1A**). In the following, all investigations are performed with the 6 different primary conjunctival cells to exclude unspecific effects due to the prolonged culture of the CCL-20.2 cell line resulting in alterations of cell surface properties.

BGs have no cytotoxic impact on primary HCDECs

Analysis of BGs uptake by primary HCDECs revealed various levels of their endocytic capacity depending on the type of bacterial species used for generation of BGs. Cytotoxicity test using premixed WST-1 cell proliferation reagent was performed to investigate if the different types of BGs would affect the viability and metabolic activity of primary HCDECs. Enzymatic cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to water soluble formazan dye by viable cells was analyzed

24 h after incubation of primary HCDECs obtained from different donors (n=6) with BGs (1000 per cell) for 40 min. Released formazan dye was quantitated spectrophotometrically by the measurement of the absorbance at 450/690 nm. The results showed no cytotoxic effects of all the analyzed types of BGs on the viability of primary HCDECs. We detected no significant difference in metabolic activity of analyzed cells incubated with BGs in comparison with the cells incubated without BGs. Significant decrease of viability was detected only after incubation of cells in the presence of cytotoxic agent (Triton X-100) (**Fig. 2**).

BGs stimulate expression of ICAM-1 on HCDECs, but not HLA-ABC and HLA-DR

Human conjunctival cells constitutively express HLA class I antigen-presenting molecules and ICAM-1, but not HLA-DR (HLA class II) without IFN-gamma stimulation.[25] Primary HCDECs were incubated with different types of BGs for 10 and 40 min and after removal of BGs from culture incubated for additional 24h-72h. Increased expression of ICAM-1 molecule on the surface of primary HCDECs was detected already 24h after stimulation with BGs for both 10 and 40 min co-incubation, but no significant changes in HLA class I and class II molecules expression by primary HCDECs were observed after stimulation with BGs (**Fig.3**). The same results for all markers tested were observed 48h and 72h after stimulation of primary HCDECs with BGs (data not shown). Further, no fold change of HLA-ABC and HLA-DR mean fluorescence intensity (MFI) values were noticed 24 h after co-incubation of primary HCDECs with the four different types of BGs for both 10 and 40 min (**Fig. 4A,B**). Stimulation of primary HCDECs generated from different donors (n=6) with the different types of BGs resulted in 6.1, 5.5, 6.4, and 6.4-fold change increase of ICAM-1 (CD54) MFI values after 10 min co-incubation with *E. coli* NK9373, *M. haemolytica*, *E.coli* Nissle 1917, and *E.* coli NM522, respectively. Extended co-incubation of HCDECs with BGs for 40 min did not cause significant changes in expression of ICAM-1 (CD54) compared to results observed after 10 min of co-culture with study results showing 5.9, 5.1, 6.2, and 5,6-fold change increase of MFI values after co-incubation with *E. coli* NK9373, *M. haemolytica*, *E.coli* Nissle 1917, and *E. coli* NM522, respectively (**Fig. 4C**). These results indicate that the stimulation of ICAM-1 expression is independent from the number of BGs being internalized and the signal for the stimulation does not discriminate up to a factor of 4 (*M. haemolytica* and *E. coli* NM522) of the total uptake.

Discussion

In the present study, we investigated the capacity of BGs derived from different bacterial species to target primary HCDECs by evaluating their capacity to endocyte BGs and the impact of BGs on their viability and metabolic activity. Our results demonstrate a high capacity of primary conjunctiva cells to bind and endocyte BGs. In this panel of BGs derived from pathogenic and non-pathogenic bacterial species, the most effective envelopes for uptake by primary HCDECs were determined and identified as BGs from *M. haemolytica*, *E. coli* NK9373, *E. coli* Nissle 1917, and *E. coli* NM522. Subsequent studies revealed that BGs without restrictions to the bacterial species used for their preparation have no cytotoxic effects on conjunctival epithelial cells, but stimulate increased expression of the co-stimulatory molecule ICAM-1 on the surface of primary HCDECs.

Conjunctival epithelial cells in addition to physical protection of the eye are also capable to respond towards bacterial pathogens by production of antimicrobial peptides and pro-inflammatory cytokines [5, 8]. Sensing of pathogens with pattern recognition receptors -TLRs- expressed by conjunctival cells plays a key role in the ocular immune defense mechanism [31]. Probably minor molecular differences in structures of LPS and flagellin presented on the surface of various bacterial species would affect signaling through TLR receptors expressed by conjunctival cells and thus modulate production of proinflammatory cytokines and expression of surface molecules involved in immune response. Our findings about the uptake of different BGs by primary HCDECs presented in this study showed that the selection of bacterial species used for the generation of BGs have a significant role for future use of BGs as carriers for the ocular disease vaccine development and/or drug treatment. From the panel of tested BGs derived from various bacterial species we have observed strong abilities of BGs to be internalized by primary HCDECs (*M. haemolytica*), next we identified BGs with mediate capacity to stimulate endocytosis of primary HCDECs (*E. coli* NK9373), as well as BGs with moderate impact on endocytic capacity of primary HCDECs (*E. coli* Nissle 1917 and *E. coli* NM522). Furthermore, we observed that BGs derived from other bacterial species including *ETEC*, *S. typhimurium*, *S. flexneri*, and *P. multocida* were either ignored or minor recognized by primary HCDECs (data not shown). Entire specific mechanism or identification of BG's surface molecules affecting the endocytic capacity of conjunctival cells and secretion of proinflammatory cytokines by primary HCDECs after co-incubation with BGs should be further investigated in details. Although not having the highest uptake rates, BGs derived from *E. coli* Nissle 1917 seem to have an advantage over the other tested BGs envelopes. *E. coli* Nissle 1917 is a probiotic and therapeutic strain which has a good record for its use in preterm-infants and toddlers, and is registered as therapeutic agent in several European countries [32-33]. With these

findings the way seems to be open to use BGs as carrier of antigens or drugs for preventive and therapeutic use against infectious diseases of the eye.

Besides, for prospective future application of BGs against various ocular surface diseases complete cytokine profile of HCDECs after incubation with BGs needs to be examined. We showed previously that DCs efficiently endocyte BGs derived from various bacterial species and produce high levels of IL-12 and TNF-alpha [34], cytokines very effective in the induction of Th1 type T cell immune response. These cytokines together with IL-6 and IL-8 secreted by conjunctival cells after stimulation with LPS as described Chung et al [35] have the capacity to induce IFN-gamma production by NK and T cells [36]. Moreover, capacity of BGs to stimulate secretion of IL-6 and IL-8 by epithelial cells was observed after incubation of BGs with primary human keratinocytes (Abtin and Lubitz, personal communication). IFN-gamma is critical factor affecting the degree of cellular pathogen infection; furthermore it has the capacity to stimulate expression of antigen-presenting molecules on both professional and non-professional APCs like conjunctival epithelial cells, fibroblasts etc. [25, 37-41].

ICAM-1 is typically associated with adhesion and transmigration of leukocytes, but it also plays an important role in providing of the second or costimulatory signal required for T cell activation and cytokine production after MHC-Ag-complex – TcR ligation [42]. Activation of T cells in ICAM-1/LFA-1 dependent manner stimulates CD8+ T cells with the phenotype of fully differentiated effector IFN-gamma producing cytotoxic T cells capable to lyse antigen specific target cells [43]. Furthermore, increased ICAM-1 expression by tumor cells correlate with increased cytolytic capacity of tumor infiltrating lymphocytes [44]. Besides the impact of ICAM-1 on T cells it was reported recently that cells from stromal microenviroment (fibroblast) are capable to induce maturation of DCs in ICAM-1/LFA-1 dependent manner [45]. These observations open the question if BGs related increased expression of ICAM-1 by human conjunctival cells might have an additional stimulatory effect on leukocyte populations presented in EALT and positive impact on induced antigen-specific immune response.

We proved previously that BGs are able to deliver DNA and drugs to the target cells [21-24, 29, 46]. Excellent BGs delivery capacity, ability to stimulate cytokine secretion by target cells, and capability to increase expression of co-stimulatory molecules on the surface of target cells emphasize BGs as a promising candidate for ocular surface disease treatment. We are aware that using Gram-negative bacteria LPS content of the cell envelopes presented on the BG's shell does not represent a risk for using of BGs as a potential drug delivery candidate [16, 20]. Moreover, we showed that endocytosis of empty bacterial shells did not stimulate increased expression of antigen presenting molecules on the surface of primary HCDECs, but we observed enhanced expression of ICAM-1, which is playing an important role in transmigration of leukocytes and is also capable to provide the activation signal for DCs, T cells and NK cells [43, 45, 47-48]. Future investigations will characterize cytokine production/alteration of primary

HCDECs challenged by BGs like modification of innate immunity markers. Optimization and improvement of the selected prospective model type of BGs would help to progress the development of microbial-mediated ocular disease vaccine and drug carriers, and their application in future clinical trials. However, this concept has to be investigated in details in future studies including *in vivo* models and investigations related to the impact of BGs loaded with DNA and/or drugs on all cell populations forming the target tissue and EALT. In our opinion, these results indicate that BGs have the potential to be used as a carrier for delivery of drugs/antigens/RNA/DNA to human conjunctival cells.

Conflict of interest

JN, SW, KHS disclose no conflict of interest; PK, VJK, UBM, WL are employees of BIRD-C which has licensed the rights to the Bacterial Ghosts Technology; BIRD-C is partner of Laura Bassi Centre for Ocular Inflammation & Infection (OCUVAC); TBA is the project leader of OCUVAC.

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Figures

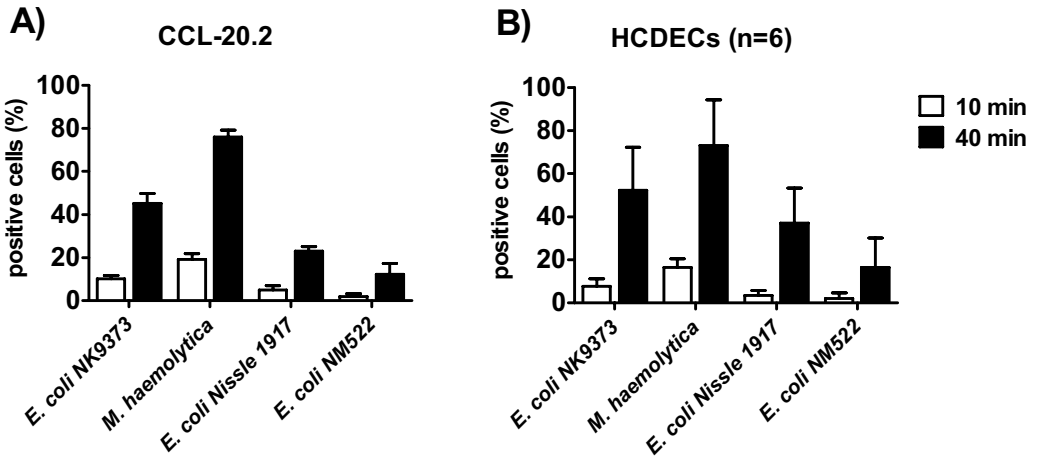

Fig. 1. Endocytosis of FITC-labeled BGs by human conjunctival cells. Human conjunctival cell line CCL-20.2 (A) and primary human conjunctiva-derived epithelial cells (HCDECs; n=6) (B) were incubated with FITC-labeled BGs derived from different bacterial species for 10 and 40 minutes at +37°C. The endocytosis of BGs by conjunctival cells depends on the bacterial species from which the BGs were prepared. Values were calculated as the percentage of cells with increased fluorescence incubated with unlabeled BGs subtracted from the percentage of positive cells incubated with FITC-labeled BGs. Endocytic capacity of conjunctival cells was measured by BD FACSCanto flow cytometer (BD Biosciences). Data represents the mean of three independent experiments ± SD.

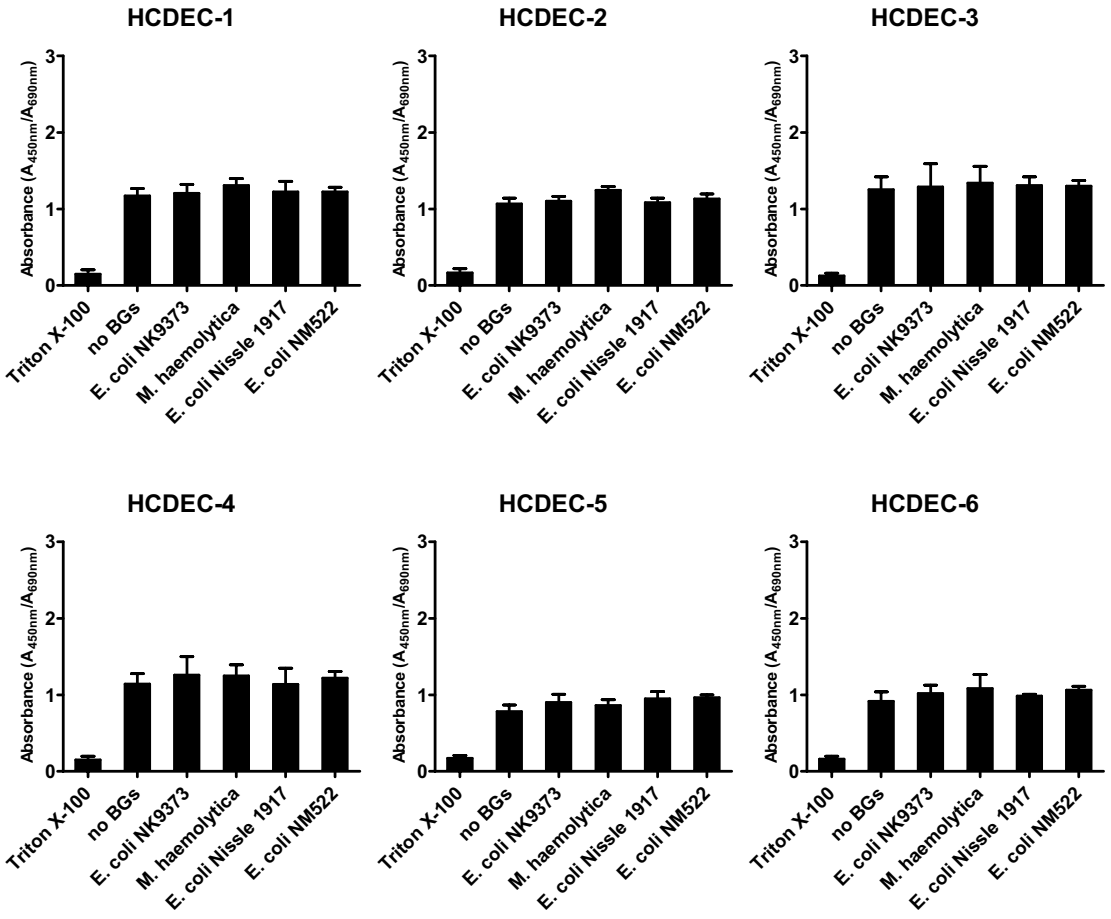

Fig. 2. Impact of BGs derived from different bacterial species on the viability and metabolic activity of the primary human conjunctiva-derived epithelial cells (HCDECs). HCDECs were incubated for 40 min with BGs (1000 per cell) generated from different bacterial species. The colorimetric WST-1 cleavage assay was performed to investigate possible toxic effects of BGs on primary HCDECs. Each bar represents the mean of three independent experiments performed in triplicates ± SD.

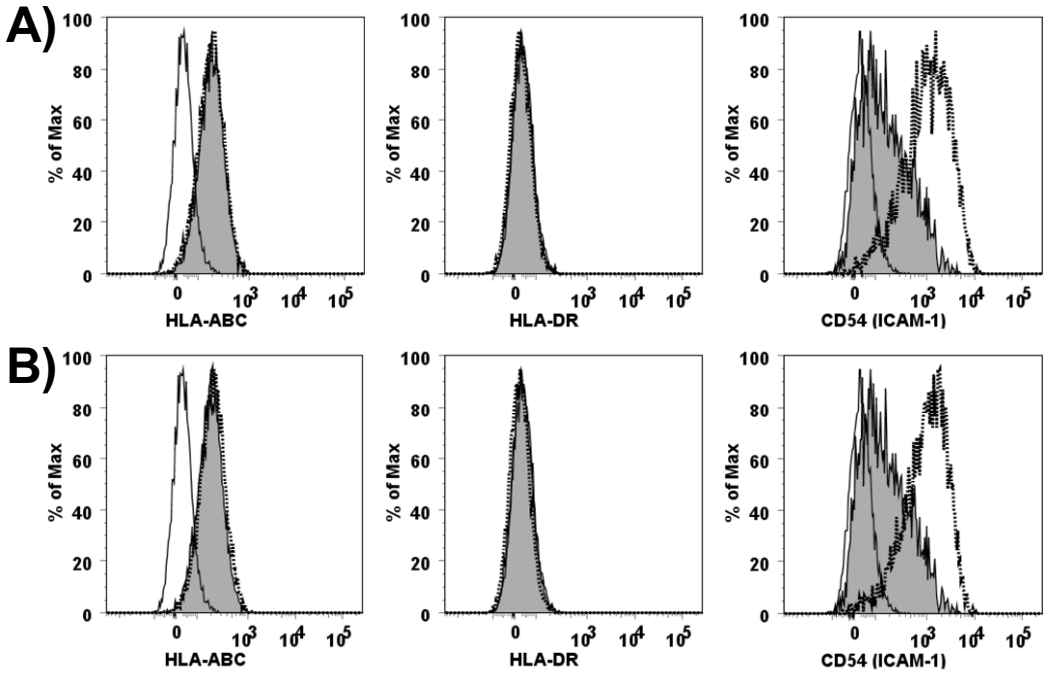

Fig. 3. Representative flow cytometry analysis of HLA-ABC, HLA-DR and CD54 (ICAM-1) expression by the primary human conjunctiva-derived epithelial cells after incubation with BGs. Cells HCDEC-3 were incubated for 10 (A) and 40 (B) min with BGs (empty histograms-dotted line) prepared from *E. coli* Nissle 1917 (1000 per cell) and stained with a panel of monoclonal antibodies. Unstimulated cells incubated without BGs stained with a panel of monoclonal antibodies (full histograms) or isotype control (empty histograms-full line) as described in Material and Methods served as controls.

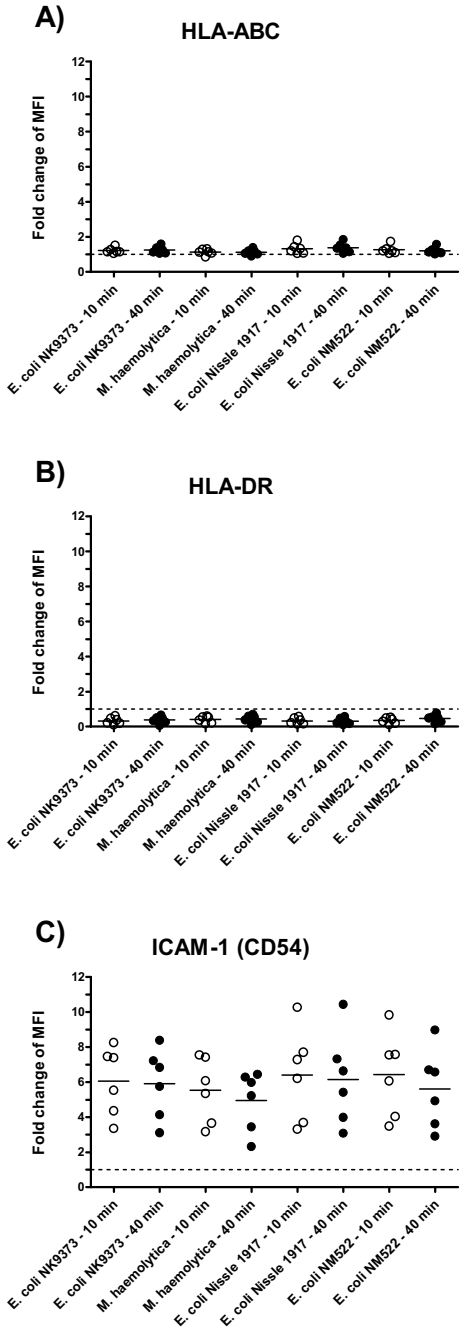

Fig. 4. Incubation of BGs with the primary human conjunctiva-derived epithelial cells (HCDECs) elicits increased expression of ICAM-1 (CD54), but has no impact on the expression of HLA-ABC and HLA-DR by primary HCDECs. Fold change of the MFI of primary HCDECs (n=6) stimulated for 10 and 40 min at +37°C with BGs derived from different bacterial species and stained with mAbs 24h after stimulation as described in Material and Methods. Unstimulated cells incubated without BGs stained with a panel of Abs served as controls. Fold change of BGs-induced HLA-ABC, HLA-DR and ICAM-1 (CD54) MFI values were calculated and shown as the mean of 3 independent experiments.

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Chapter 2.2.2.
Detoxification of the preservative agent benzalkonium chloride (BAC) by bacterial ghosts (BGs)

Verena Juliana Koller[1,2], Maria Eisenbauer[3], Ulrike Beate Mayr[2], Talin Barisani-Asenbauer[4], Werner Lubitz[1,2] and Siegfried Knasmüller[3,*]

[1] University of Vienna, Department of Medicinal Chemistry, Vienna, Austria

[2] BIRD-C GmbH & Co. KEG, Kritzendorf, Austria

[3] Medical University of Vienna, Institute of Cancer Research; Department of Medicine I, Vienna, Austria

[4] Department of Ophthalmology and Optometry, Medical University of Vienna, Vienna, Austria

[*] Corresponding author: Phone: +43-1-4277-65142 - siegfried.knasmueller@meduniwien.ac.at

Bacterial ghosts (BGs) are non-denaturated bacterial envelopes obtained from Gram-negative bacteria produced by E-mediated lysis. In the present study the safety aspects in regard to cytotoxicity and genotoxicity of BGs were examined in the human derived conjunctival cell line CCl 20.2. Further, the toxicity of benzalkonium chloride (BAC), a preservative of numerous medical preparations, was investigated in combination experiments with BGs.

Results obtained from earlier investigations in single cell gel electrophoresis (SCGE) assays showed that bacteria can cause DNA-damage in human derived cells. However, the findings from the present study demonstrate that BGs *per se* no not trigger any toxic effects in CCL 20.2 cells.

In addition, we found that BGs are protective against the acute toxic and genotoxic effects caused by BAC. Presence of BGs increased the viability of the cells after short and long term BAC treatment by 60 %. Furthermore, also the induction of DNA migration by the quaternary ammonium compound was significantly reduced. The protective properties could be partly explained by inactivation of BAC-generated H_2O_2 due to peroxidase activities of BGs. Results of agar diffusion assays in which bacteria were tested with BAC in presence or absence of BGs, showed that the bactericidal nature of the preservative was not affected.

These findings show that BGs are “safe” vehicles that could be used as drug carriers for treatment of conjunctival diseases coevally providing a protection against preservative induced toxicity.

Introduction

BAC is widely used as disinfectant, stabilizer and preservative of a variety of prescriptions as well as over-the-counter-drugs and can be found in cosmetics, infant care products as well as nasal sprays, otic drops and ophthalmic solutions [1]. It is the most commonly used preservative in ophthalmological medications and is added at concentrations ranging between 40 and 250 mg/l to various preparations to prevent bacterial contaminations and disintegration of the active drug [2].

The cytotoxic effects of BAC is well documented with ocular tissues like the cornea, conjunctiva and retina, especially associated with long-term use [3]. Induction of necrosis and apoptosis has been demonstrated in conjunctival cells [4] and it was shown that cell death was caused by direct disruption of the cell membranes [5] or by interaction with membrane G-proteins (Guanine nucleotide triphosphate binding proteins) thereby effecting signal transduction causing apoptotic stimulation [6, 7].

Bacterial ghosts (BGs) are non-degraded bacterial cell envelopes devoid of any cytoplasmatic content produced from Gram-negative bacteria by controlled expression of the plasmid encoded gene E of bacteriophage PhiX174 [8, 9]. These non-living bacterial envelopes maintain the full cellular morphology with preserved peptidoglycan, lipopolysaccharide (LPS) and outer membrane proteins. Furthermore, the inner lumen provides a huge filling volume for drugs, coevally providing a protected environment from outside influences. The uptake and phagocytosis of BGs has been investigated in various cell lines eg. dentritic cells, different human melanoma cells, macrophages and colon cells and the capacity of BGs as drug targeting and delivering vehicles has been demonstrated [10, 11]. BGs can be used for immunization against their own structure, as antigen delivery systems [12] or they can be filled with DNA [13] or drugs [14]. Despite the fact, that BGs were proven to be non-cytotoxic to melanoma cells and macrophages in vitro [15, 16], nothing is known about the safety aspects of BGs concerning epithelial conjunctiva cells.

Aim of the present study was to examine whether BGs themselves cause acute or genotoxic effects in the Wong-Kilbourne derivative of Chang conjunctiva cell line (CCl 20.2). These cells have been widely used for BAC-induced toxicological and functional *in vitro* studies [4, 17, 18] and possess characteristics that are typical for normal tissue and primary cultures [19]. Furthermore, experiments were conducted to find out if the use of ghosts may reverse the toxic effects of BAC and if this has an impact on its bactericidal properties.

The acute toxicity of the drug and of different BGs, prepared from different bacterial species, was determined by use of the neutralred assay and DNA-damage was assessed with the SCGE (single cell gel-electrophoresis) technique. The alkaline SCGE assay is one of the most commonly used methods for identifying substances with genotoxic activity at the single cell

level and is highly sensitive towards ROS [20-22]. This test system is based on the measurement of DNA-migration in an electric field and the size of the comets serves as a measure of the extent of DNA-damage. The SCGE assay has been used in earlier investigations to demonstrate the DNA-damage caused by bacteria [23]. Recent findings showed that BAC causes positive results in SCGE assays with nasal [24] and bronchial cells [25] as well as with primary hepatocytes [26].

Since it has been shown that BAC produces at concentrations in a the range of 50-100 mg/l intracellular reactive oxygen species (ROS), in particular H_2O_2, and leads to cell death of conjunctival cells [17, 18] we examined BAC in solution and determined the possible participation of the bacterial peroxidase in the detoxification. Finally we conducted additional measurements to elucidate if the bactericidal activities of the drug, which we examined by agar disc diffusion assays, are affected by the BGs.

Materials and methods

Cell culture

The American Type Culture Collection (ATCC Manassas, VA) certified cell line (CCL) 20.2 (Wong-Kilbourne derivate of Chang conjunctival cells, clone 1-5c-4) was kindly provided by Prof. Bernd Binder (Medical University Vienna, Center of Biomolecular Medicine and Pharmacology; Department of Vascular Biology and Thrombosis Research, Vienna, Austria). The cells were cultured under standard conditions (37°C moist atmosphere of 5% CO_2) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat inactivated fetal calf serum (FCS, Sigma), 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen), 10 mM HEPES buffer (Lonza, Verviers, Belgium), 2 mM L-glutamine (Invitrogen), 0.1 mM MEM Non-Essential Amino Acids (NEAA, Lonza) and 5 µg/ml plasmocin (Lonza). The medium was changed every 2-3 days and when cultures reached confluency, the cells were washed with phosphate buffered saline (DPBS, Lonza), detached with TrypLE Express (Gibco), centrifuged and subcultured.

Production of bacterial ghosts

The following BGs were produced as described earlier [14, 27] and were used in the present study: *Escherichia coli* NM522 (pGELys), *Escherichia coli* Nissle 1917 (pGLysivb), Enterotoxigenic *Escherichia* coli (ETEC) H10407 (pGLysivb), *Shigella flexneri* CVD1203 (pAW12), *Shigella flexneri* CVD1203-CFAI-CS3 (pGLysivb / pJGX15A / pACCS3), *Shigella flexneri* ATCC700930 (pGLNIc / SNUC), *Salmonella typhimurium* ATCC14028 (pGLysivb) and *Pasteurella multocida* (pSR2).

Cytotoxicity assays

The viability of the cells was determined in neutralred assays. This method is based on the accumulation of the dye in the lysosomes of living cells [28]. To investigate the impact of BGs towards the conjunctival cell line CCl 20.2, $5x10^4$ cells were seeded in 96-well plates and were allowed to attach. Then the cells were treated with 200 µl of different types of BGs (resuspended in serum free medium) in a MOI (multiplicity of infection) of 1000 for 24 hrs. TritonX-100 (0.005 %) served as a positive control and cells treated with serum free medium represent the negative control.

In combination experiments with BAC and BGs, the cells were treated with 200 µl of different BAC-concentrations (ranging from 0.01-0.0001 %) and with BGs (MOI of 1000). For short time incubation experiments (5 min), the cells were divided into two groups: the first were treated only once and the second were re-exposed to BAC 60 min after the initial treatment to mimic a repetitive character of drug treatment. The cell viability was determined after a 24 hrs

recovery period in complete medium. The recovery phase was included to simulate the clinical conditions in which the conjunctival tissue may recover after topical eye drop administration. Long term treatment lasted 24 hrs.

After each treatment, two washing steps with PBS were performed. Subsequently, the cells were incubated with 100 µl of NR (80 µg/ml final concentration; Roth) for two hrs (37°C; 5 % CO_2). After the dye had been discarded, the wells were washed two times with PBS and dye was released by the addition of 100 µl of the destaining solution (1 ml acetic acid, 73 ml 96 % ethanol and 26 ml deionized water). After shaking, the absorbance was measured at 570 nm (reference wavelength 690 nm) with a microplate reader (Opsys MR, DYNEX Technologies).

The tests were performed in triplicate and mean values from 3 independent plates were determined. Data are represented as means + SD. Values obtained with medium treated cells were considered as control and were set as 100 % viability.

SCGE assays

The single cell gel electrophoresis (SCGE) assays were performed according to the guidelines described by Tice at al [22]. In order to test the impact of different BGs on the DNA-stability, $1x10^5$ CCl 20.2 cells were cultured in 6-well plates for 45-48 hrs (37°C; 5% CO_2). Thereafter, the culture medium was replaced by one ml serum free-medium containing BGs (1000 particles / cell) and the cell were incubated for 40 min in the dark (37°C; 5% CO_2).

Analyses, in which the effects of different concentrations of H_2O_2, BAC and BAC in combination with *ETEC* BGs (MOI of 1000) were investigated, were performed with 48-well plates. $2x10^5$ cells were allowed to attach and after removal of the culture medium, the cells were treated with 400 µl of the compounds with or without BGs. Incubation was either performed for 10 min (H_2O_2) or for 5 min and 20 min with the preservative BAC (also in combination with *ETEC* BGs).Thereafter, the medium was discarded and the cells were washed twice with PBS and detached with TripleEx. After two washing steps (1600 rpm, 8 min, 21°C), the pellets were resuspended and viability was determined by trypan blue exclusion [29]. Only cultures in which the viability of the cells was ≥ 80% were used for further analyses. Thereafter, the cells were pelleted (1600 rpm, 8 min, 21°C) and were resuspended in low melting agarose (LMA, 0.5%). Then the cells were spread onto precoated agarose slides (1.5 % normal melting agarose) and lysed in the dark for 1 hour at 4°C. After 20 min of unwinding in electrophoresis buffer, the electrophoresis was carried out for 20 min (300 mA, 25 V) and neutralization was performed twice for 8 min. Air dried slides were stained with ethidium bromide (20 µg/ml) and the percentage of DNA of tail was measured by the use of a computer aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK).For each experimental point, three cultures were made in parallel. From each culture one slide was prepared and from each slide 50 randomly distributed cells were evaluated.

Qualitative determination of H_2O_2-generation by BAC

In order to determine if H_2O_2 is released by BAC, the qualitative method described by Liszkay et al. was used [30]. The assay is based on the development of a blue reaction product when tetramethyl benzidine (TMB) is oxidized by peroxidase in the presence of hydrogen peroxide. For this purpose, three BAC solutions (4, 20 and 40 mg/ml), containing 0.37 % citric acid, 3.5 mM TMB and 1 µg horseradish peroxidase (HRP, Type VI-A from Sigma) were incubated at 37°C. After 3 days, the generation of H_2O_2 was monitored photometrically and absorbance was read at 370 nm. For each point, three experiments were conducted.

Peroxidase activity measurements of BGs

To determine the peroxidase activity of selected BG strains, colorimetric enzyme measurements were conducted as described by Rafii et al. [31]. The enzyme assay was performed in 1 ml PBS containing 1mM TMB, 0.37 % citric acid, 0.3 % H_2O_2 and 10 µl sample. The mixture was incubated for 10 min, centrifuged and the produced oxidized TMB was measured at OD 370 nm.

HRP was used as an internal standard and units were defined as arbitrary units, whereby one mg solid corresponds to 1000 AZBTS units (see information from sigma). Peroxidase activity of BGs was determined from obtained peroxidase activity/used particles*factor. The factor was calculated as µg lyophilized particles per µg protein of the respective BGs. Protein contents were measured with the Bradford protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany)

Agar disc diffusion assays

To monitor the bactericidal effect of BAC, disc diffusion assays were performed with minor modifications [32, 33]. Briefly, overnight cultures of the *E. coli* strain NM522 were spread onto agar plates. After drying, sterile drug-free paper discs (diameter 7.0 mm; No. 2668*;* Schleicher and Schüll, Dassel, Germany) were placed to the surface. Thereafter, 30 µl of different solutions (BAC alone or in combination with $5x10^9$ *ETEC*-BGs / ml) were spotted onto the discs. Then the plates were incubated at 37°C for 48 hrs. The diameter of the growth inhibition zones was determined in triplicate and the obtained area in mm^2 was considered for evaluation.

Statistics

All results were analysed by use of GraphPad Prism (version 5, GraphPad Software, Inc; San Diego; CA, USA). Data are expressed as means + SD. Statistical analysis between more than two groups was performed by the one-way ANOVA and the Dunnett's multiple comparison test as post-test. Difference between two groups was evaluated by the Student’s t-test. P-values $< 0,05$ were considered statistically significant.

Results

Genotoxic and cytotoxic impact of BGs on CCL 20.2 cells

The cell viability was assessed in neutralred assays and no differences were found between the negative controls which were exposed to serum free medium compared to BGs derived different bacterial species (MOI of 1000) after treatment for 24 hrs (**Table 1.**).

Exposure of the cells to the same BGs as used in the cytotoxicity assays, also did not affect the DNA-stability of the cells after 40 min (**Table 1**).

Determination of viability with BGs and BAC

Considering that ophthalmological medications contain up to 250 mg/l BAC and that eye drops are quickly further diluted after application in its environment, e.g. through the tear fluid, different BAC concentrations ranging from 10-100 mg/l were chosen to investigate the impact on the conjunctiva cells after 5 min exposure followed by a 24 hr recovery period (**Fig. 1A**). As it was demonstrated that BAC can be found in the ocular tissues after a single topical application even after a few days [5, 34], experiments were conducted in which the long term application (24 hrs) was studied. Results obtained after 24 hr treatment with ten-fold lower BAC concentration 1-10 mg/l are depicted in **Fig. 1B**. Significant cytotoxic effects were obtained with 50 mg/l BAC after short-term incubation and with 5 mg/l BAC after long-term treatment.

To investigate whether BGs have an impact on the BAC-induced cytotoxicity in CCL 20.2 cells, simultaneous treatment experiments with the preservative and different BGs were conducted. To simulate the situation of multidose application of BAC, further experiments were performed in which the cells were treated twice with the preservative (5 min; 1 hr break) followed by a 24 hr recovery time. It can be seen that double treatment with the chemical alone led to significant decrease in the viability of the cells. As shown in **Fig. 2A**, the percentage of viability dropped from 53 % for the single dose application to 4 % cell viability for 50 mg/l BAC.

However, compared to BAC-treated cells, increased numbers of the cells stayed alive by the presence of BGs. High beneficial effects could be found with BGs after double applications of 50 mg/l BAC. Differences were seen between the *E. coli* BGs and *Shigella* and *Salmonella* BGs. Enhanced viability from 17-fold was found for one of the *Shigella* samples and 5 to 12-fold for E. coli BGs (**Fig. 2A**).

Due to the strong effects seen after double treatment with 50 mg/l BAC, the impact of 10-fold diluted BAC samples for long term exposure (24 hrs) with and without BGs was investigated (**Fig. 2B**). Exposure of CCL 20.2 cells to 5 mg/l BAC for 24 hrs resulted in about 80 % decrease of the viability. Protective effects were found when BGs were present in the

preservative solution. Cytotoxicity was significantly lowered in combination with all different BGs (MOI of 1000) to a similar extent. On average 85 % of the cells survived.

Determination of H_2O_2-generation by BAC and measurement of BG peroxidase acitivity

Experiments were conducted to qualitatively investigate the generation of H_2O_2 by BAC. The development of a blue colour in TMB/HRP supplemented BAC-solution indicated the formation of H_2O_2. Results are depicted in **Fig. 3**.

One explanation of the beneficial effects of BGs towards BAC-induced cytotoxicity (**Fig. 2**) could be the detoxification of ROS due to hydrolysis of H_2O_2 by BG-associated peroxidase. As depicted in **Fig. 4,** all BGs oxidized TMB. The highest enzyme activities were obtained with *ETEC* and *S. flexneri* BGs.

Comparative agar diffusion assays

The mode of cytotoxic action of BAC as preservative and disinfectant is the permeabilization and solubilization of bacterial membranes leading to release of the cell contents [35]. Since Gram-negative bacteria are known to be quite more resistant towards BAC due to their outer membrane than Gram-positives [36], high concentrations of BAC (5, 15 and 40 mg/ml) have been tested with the *E. coli* NM522 strain. For the coincubation of the different BAC solutions with BGs, *ETEC* ghosts have been chosen, as they exhibited good effects on cell viability (**Fig. 2**) and for their peroxidase activity associated with the BGs themselves (**Fig. 4**). A dose dependent inhibition of the bacterial growth could be observed with the used BAC concentrations. The presence of *ETEC* BGs in the three different BAC concentrations had no effect on the inhibition zone, indicating that the peroxidase activity of *ETEC* BGs did not have an impact the bactericidal action of BAC (**Fig. 5**).

Genotoxicity assays with H_2O_2, BAC and combined BAC-ETEC BGs solutions

As H_2O_2 was used as positive control in SCGE assays with BGs (**Table 1**), a dose-response curve was established to investigate the sensitivity of this cell line. A clear dose dependent induction of DNA-damage was observed when cells were exposed to different H_2O_2 concentrations for 10 min. As shown in **Fig. 6A**, significant differences between the percentage of tail intensity were already found at concentrations $\geq$ 50 µM.

Furthermore, the genotoxic impact of BAC towards the conjunctiva cells CCL 20.2 was investigated after short term treatments. Significant DNA-damage was detected already after 5 min incubation with 50 mg/l BAC. Extended time of coincubation of CCl 20.2 and BAC at the same concentration led to a 6-fold increase of percentage of DNA in tail compared to the respective control (**Fig. 6B**)

To elucidate whether BGs could also have an impact on BAC-induced DNA-damage, experiments were conducted with *ETEC* BGs, which already demonstrated protective effects

towards BAC-induced cytotoxicity (**Fig. 2**) and for which high peroxidase activities were obtained (**Fig. 4**). As depicted in **Fig. 6C**, our findings show that coincubation of BAC (50 mg/l) with *ETEC* BGs significantly reduced DNA-migration. On average, the presence of BGs reduced the preservative caused DNA-damage 55% after 5 min and 35 % after 20 min coincubation. A simple explanation for the detoxification seen in the SCGE tests as well as in the cytotoxicity assays could be the possible loading of the BGs with the compound thereby reducing the concentration of BAC in the medium and / or the participation of the active peroxidase system could contribute to the elimination of BAC-generated H_2O_2.

Discussion

This is the first report concerning the "safety" of BGs produced from different bacterial species, and their protective effects towards BAC induced cytotoxicity and genotoxicity in conjunctival cells CCl 20.2.

It was shown that BAC concentrations from 1-100 mg/l led to delayed induction of cell death [4]. Cell proliferation and viability were altered in a dose-dependent time course which worked rapidly for 100 mg/l BAC (within 24 hrs) and gradually for concentration less equal than 50 mg/l. In agreement with the results obtained from their investigations we obtained similar viability values by use of the short time application and recovery model (**Fig. 1**). Further, we conducted double treatment experiments with BAC to mimic a repetitive character as it is used in medical application as eye drop preservative. A strong inhibitory effect of BAC-induced cytotoxicity could be demonstrated when the cells were simultaneously treated with BGs (**Fig. 2A**). More than 10-fold increase in cell viability could be observed when CCL 20.2 cells were concomitantly exposed to the preservative when BGs (in particular *E. coli* or *Shigella* BGs) were present.

Animal studies demonstrated that the turnover of BAC in the eye is very slow and that the compound could be detected in the ocular tissues still after 48 hours up to 9 days after a single topical application [5, 34]. Therefore, experiments were conducted in which the long term application (24 hrs) of BAC was investigated. Findings of treatment experiments with BAC and BGs resulted in high beneficial effects as CCL 20.2 cell viability was 40-60 % enhanced in the presence of BGs (**Fig. 2B**).

The antiseptic effects of BAC at concentrations lower than 500 mg/l have been demonstrated already in 1930s [37, 38]. Resistance to quaternary ammonium chlorides has been reported to be mainly related to the adoption or hyperexpression of multi-drug efflux pumps in gram negative bacteria [39, 40]. Since a membrane potential is needed for functionally efflux systems, these mechanism can be excluded as an explanation for the diminished damage of BAC in combination with BGs.

In former studies it was shown that membrane associated enzymes of BGs like ATPases [27] or enzymes from the periplasmatic space such as alkaline phosphatase and β-lactamase [41] are vastly retained and active. Therefore, lyophilized BGs were investigated for their peroxidase activity and the enzyme was found to be still functionally active (**Fig. 4**). As generation of H_2O_2 by BAC was demonstrated (**FIG. 3**), we assumed that one mechanism of detoxification of BAC with BGs could be due to their peroxidase activity.

BAC treatment has been demonstrated to induce internal ROS production in Chang cells and endogenous H_2O_2-generation was already found at concentrations higher than 10 mg/l BAC [17, 18]. Treatment of human corneal epithelial cells with H_2O_2 concentrations $\geq$ 30 ppm caused

in vitro significant morphological and cytotoxic changes within 5 hrs [42]. Furthermore, a significant decrease in cell viability was shown after exposure to 300 µM H_2O_2 for 24 hrs [43]. To our knowledge, no genotoxic investigations were performed with H_2O_2 in this cell line. In the present study the impact of concentrations 4 to 6-times lower than mentioned above was examined after a short incubation period of 10 min. A dose-dependent induction of DNA-migration could be observed and significant DNA-damage was monitored by use of SCGE already at concentrations ≥ 50 µM (**Fig. 6A**)

Furthermore, results depicted in **Fig. 4B** indicate that BAC induces DNA-lesions at a significant level already with 50 mg/l after short term incubations (i.e. 5 min and 20 min). These findings can be confirmed since high DNA-fragmentation was also observed with an electrophoretic assay system after treatment of CCl 20.2 cells with 100 mg/l BAC for 10 min [4]. Significant DNA-migration caused by the preservative was also found in primary rat hepatocytes when cells were exposed to 1 mg/l for one hr [26]. Another study with the human-derived bronchial epithelial cell line BEAS-2B showed clear cut induction of DNA-damage after 2 hrs treatment with 20-50 mg/l BAC [25].

Since several *in vitro* studies indicated the generation of ROS, in particular H_2O_2 and superoxide anion, after BAC application in conjunctival cells [4, 17, 44], we assume that BAC-induced DNA-migration could be also elicited by ROS, which encounters also a mayor key role in inflammation processes. However, no formation of oxidized DNA-bases was found in SCGE assays with the enzyme FPG in hepatocytes it and was stated that the observed genotoxic effects of BAC may not be related to oxidative stress [26]. Nevertheless, significant decrease of BAC-induced DNA-damage was observed in presence of *ETEC* BGs (**Fig. 6C**). One detoxification mechanism could be the participation of the enzymatic cleavage of BAC-generated H_2O_2 due to the bacterial associated peroxidase or the fact that BGs are loaded with the compound coevally depriving the toxic compound from the medium. Furthermore, as the uptake of BGs used in the present study by CCl 20.2 cells has been determined in a different investigation (Kudela and Lubitz, in press), it can be also assumed that the beneficial effect on cell viability originates also from the intracellular subset and not only from the presence of extracellular BGs.

Due to advantage that BGs also possess intrinsic adjuvant properties, we plan to develop a BG-vaccine against the blindness causing Chlamydia trachomatis pathogen in the near future. As part of the SAFE program (S = Surgery, A = antibiotics, F = Facial cleanliness, Hygiene Promotion, E = Environmental Health Improvements), azithromycin represents the commonly used standard antibiotic which is recommended by the World Health Organization (WHO) to fight these infections [45].

To date a huge spectrum of delivery systems has been developed for ocular drugs [46] and liposomes, polymorphonuclear leukocytes as well as nanoparticles have already been demonstrated as carrier systems for azithromycin [47-49]. However, a recent *in vivo* study

showed that nanoparticles accumulate and induce additional inflammatory reactions due to radical generation causing DNA-damage [50].

As our findings indicate that neither the DNA-stability nor the viability of the conjunctival cells was impaired after treatment with various BGs, the examined strains represent optimal candidates as drug carriers for the treatment of intracellular infectious eye diseases.

As we could further demonstrate that BGs perpetuate the antibactericidal action of the preserving agent (**Fig. 5**), it is conceivable that BGs can be loaded with azithromycin and formulated as eyedrops preserved with BAC. Such applications could have an additional beneficial impact as the ocular surface would be sheltered from preservative related side effects.

Tables and Figures

Table 1

Impact of BGs on the viability and on the DNA-stability of the conjunctival cell line CCL 20.2.[1]

Bacterial ghosts	Viability ± SD [%] [2]	DNA in tail ± SD [%] [2]
Pos. control	4 ± 1	17,690 ± 3,359
Neg. control	105 ± 2	5,952 ± 1,364
***E.coli* NM522**	109 ± 15	4,002 ± 0,831
***E.coli* Nissle**	98 ± 15	6,898 ± 1,459
***ETEC* H10407**	97 ± 3	4,196 ± 0,716
S. flexneri	100 ± 5	7,534 ± 2,500
***S. flexneri* CS3**	111 ± 3	5,416 ± 1,563
***S. flexneri* SNUC**	110 ± 6	7,480 ± 0,703
S. typhimurium	104 ± 2	5,895 ± 2,701
P. multocida	104 ± 1	7,217 ± 1,198

[1] The cells were incubated with different BGs (MOI of 1000) for 24 hrs. Thereafter, the viability was measured with neutralred, and DNA-stability was determined in SCGE assays.

[2] Values represent means + SD of three measurements in parallel.

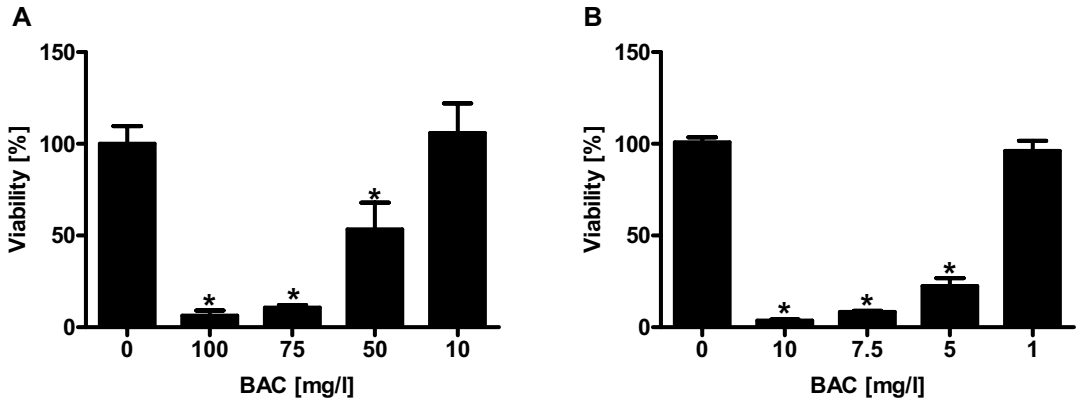

Fig. 1. Impact of BAC on the viability of the conjunctival cell line CCL 20.2. Dose-dependent reduction of viability caused by the preservative BAC in the conjunctival cell line CCL 20.2 is shown after 5 min exposure followed by a 24 hour recovery period **(A)** and after 24 hours incubation **(B).** The viability of conjunctiva cells was determined in neutralred assays. Bars represent values of means + SD obtained from three independent experiments.

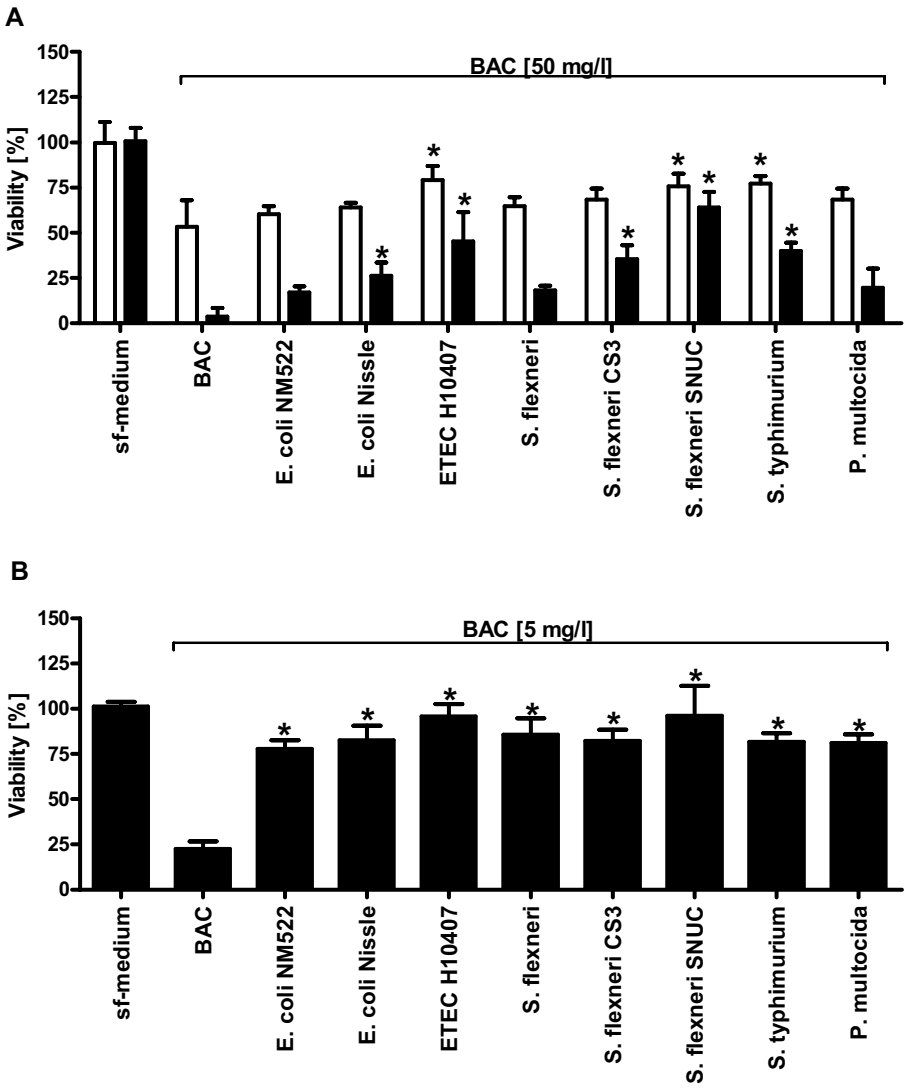

Fig. 2. Impact of BAC alone or in combination with different BGs on the viability of CCL 20.2 cells. Figure **(A)** shows the acute toxic effects toward the conjunctival cell line after short term treatment with either 50 mg/l BAC in combination with different BGs for 5 min (single application white bars; double treatment black bars) followed by a 24 hr recovery period. Figure (**B**) shows the effects on the viability of the conjunctival cell line after long term treatment with 5 mg/l BAC in combination with different BGs for 24 hrs. Cells were treated with BGs in a MOI of 1000 and the viability was determined by the use of the neutralred assay. Bars represent mean data + SD obtained from three independent measurements. Asterisks indicate values which differ significantly from BAC treated cells ($p \leq 0.05$).

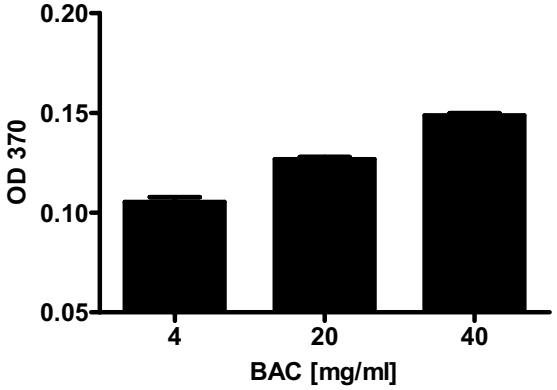

Fig. 3. Dose-dependent generation of H_2O_2 by BAC. The formation of the radicals was monitored by the production of the blue product with the TMB/HRP method. Bars represent mean values + SD of three measurements.

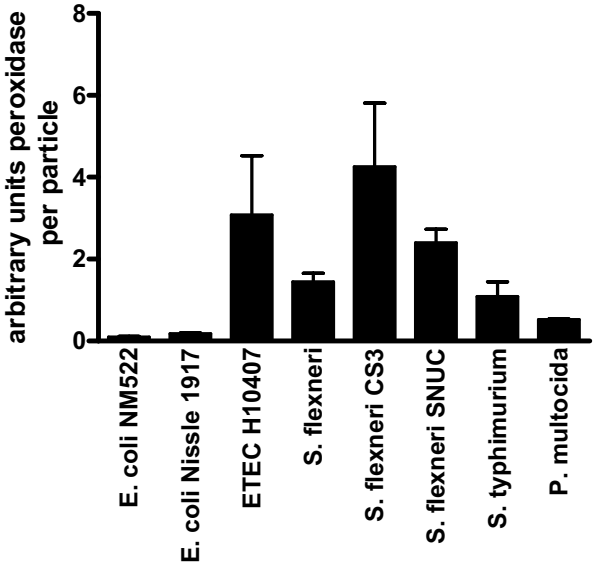

Fig. 4. Determination of peroxidase activity of different BGs. Hydrolysis of H_2O_2 was monitored spectrophotometrically. Enzyme activities are depicted as arbitrary units. The measurements were performed in triplicate and data are depicted as mean values + SD.

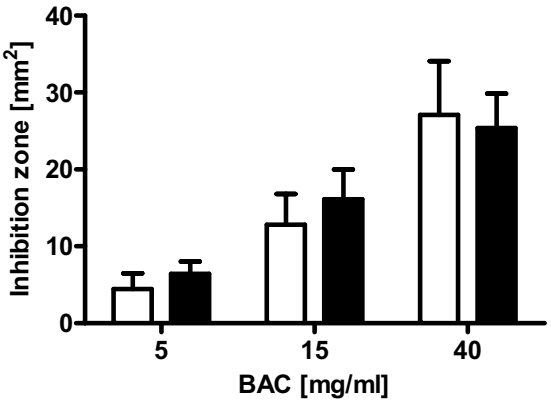

Fig. 5. Comparison of growth inhibition of *E. coli* NM522 caused by different BAC concentrations with or without *ETEC* BGs. Values were obtained by agar disc diffusion assays. Bars indicate the effect caused by the substance alone (white) and in combination with *ETEC* BGs (black).

Data are shown as mean values + SD which were obtained with triplicate measurements.

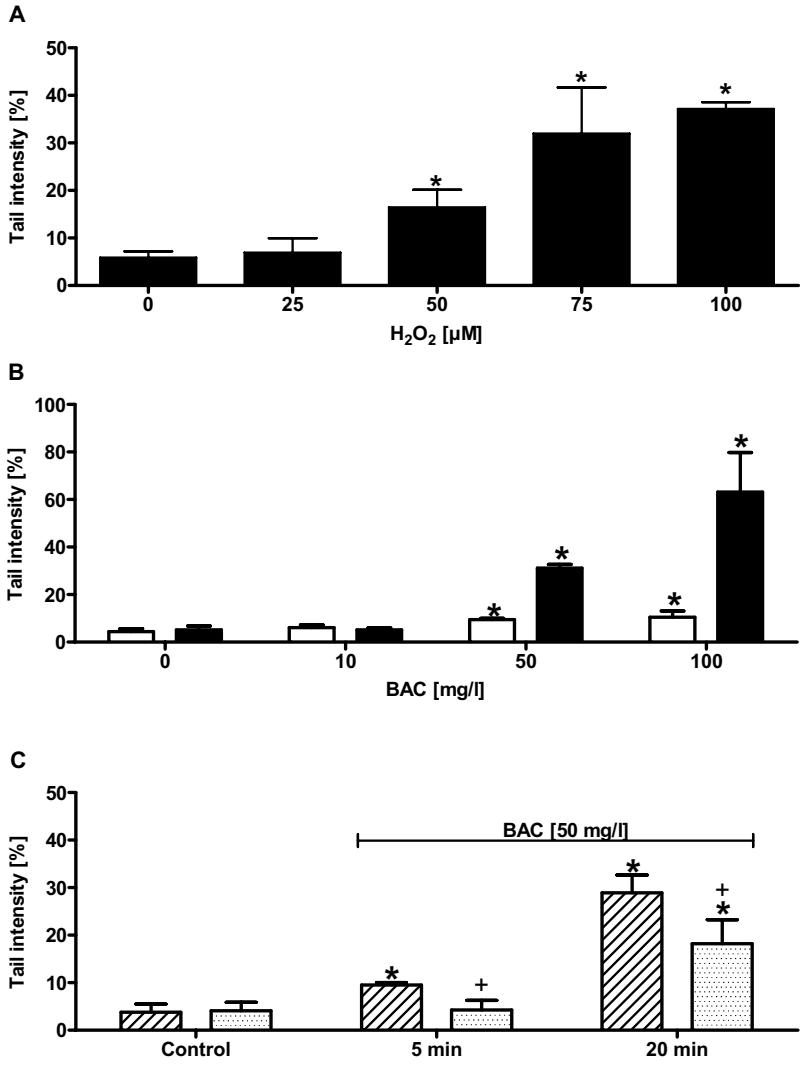

Fig. 6. Dose dependent induction of DNA-damage by H_2O_2 **(A)** and BAC **(B)** in CCl 20.2 cells. The cells were either treated with different doses of H_2O_2 for 10 min, or with BAC for either 5 min (white bars) or 20 min (black bars). In a further combination experiment, the impact of BAC (shaded bars) and *ETEC* H10407 BGs (dotted bars) on the DNA-migration was investigated **(C).**

Bars indicate mean values + SD obtained with three cultures. Asterisks indicate values which differ significantly from the controls and crosses mark statistical significant differences with and without BGs ($p \leq 0.05$).

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Chapter 2.3.
Bacterial Ghosts as Triggers of the Innate Immune System

Chapter 2.3.1.
Bacterial ghosts promote innate immune responses in human keratinocytes

Arby Abtin[1], Pavol Kudela[3, 4], Ulrike Beate Mayr[3], Verena Koller[2, 3], Michael Mildner[1], Erwin Tschachler[1, 5] and Werner Lubitz[2, 3]

[1] Department of Dermatology, Medical University of Vienna, A-1090 Vienna, Austria

[2] Department of Medicinal Chemistry, University of Vienna, Althanstraße 14, UZAII, 2B522, 1090 Vienna, Austria

[3] BIRD-C GmbH & Co. KEG, Hauptstrasse 88, A-3420 Kritzendorf, Austria

[4] Cancer Research Institute, Slovak Academy of Sciences, SK-83391 Bratislava, Slovakia

[5] Centre de Recherches et d'Investigations Epidermiques et Sensorielles (CE. R. I. E. S.), Neuilly sur Seine, France

[*]Corresponding author: Phone: +43-1-2243-28491 - werner.lubitz@univie.ac.at, werner.lubitz@bird-c.at

Bacterial ghosts (BGs) as non-living bacterial envelopes devoid of cytoplasmic content have been used to study the ability of bacterial cell envelop components for the induction of antimicrobial peptides and pro-inflammatory cytokines in human primary keratinocytes (KCs). Quantitative real-time PCR (qRT-PCR) analysis revealed that incubation of KCs with BGs generated from *Escherichia coli* NM522 induced the mRNA expression of two antimicrobial peptides, psoriasin (S100A7c) and human β defensin-2 (hBD-2), in a BGs particle concentration-dependent manner. Using immunoblot analysis we showed that BGs generated from a flagellin-deficient (Δ*fliC*) *E. coli* strain NK9375 were as effective as its isogenic wild-type (wt) *E. coli* strain NK9373 to induce psoriasin protein expression when normalized to BG particles being taken up by KCs. However, results obtained from endocytic activity of BGs by KCs reflect that this uptake is greatly dependent on the presence of flagellin on the surface of BGs. Moreover, BGs derived from wt *E. coli* NK9373 strongly induced the release of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8, compared to Δ*fliC E. coli* NK9375 BGs. Taken together, the data demonstrate that BGs have the capacity to induce the expression of innate immune modulators and that these responses are partially dependent on the presence of flagellin.

Introduction

The innate immune system serves to protect the host from invading microorganisms and upon activation triggers a series of host defense responses (3). A central mechanism of these responses is the production of pro-inflammatory cytokines and antimicrobial defense molecules. Different components of microbial pathogens, referred to pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) of the innate immune system. Among these receptors are the Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and peptidoglycan-like recognition proteins (PGRPs), all contributing to early host defense against pathogens (5,6,12). For example, the bacterial PAMP flagellin, a monomer subunit of the flagellum binds to TLR5 and activates the transcription factor NF-kappaB (10), ultimately leading to the expression of pro-inflammatory cytokines and antimicrobial peptides including psoriasin (S100A7c) and human β-defensin 2 (hBD-2) in human primary keratinocytes (KCs) (1,2,20).

Bacterial ghosts (BGs) are cell envelopes devoid of cytoplasmic content which are produced by the controlled expression of plasmid-encoded lysis gene *E* of bacteriophage ΦX174 in Gram-negative bacteria. Gene *E* codes for a membrane protein, which is able to fuse inner and outer membranes and thus forming an E-specific lysis tunnel through which the cytoplasmic content is expelled (27,28). These non-living bacterial envelopes maintain the full cellular morphology of the native bacteria. All the cell surface structures, including the outer membrane proteins, adhesins, lipopolysaccharide (LPS) and peptidoglycan are preserved in BGs (18).

BGs can be used for immunization either against its own envelop structure or as an antigen delivery system for foreign target antigens (13,18). Immunization with *Vibrio cholerae* ghosts protected against diarrhea and death following challenge with fully virulent *V. cholerae* in a rabbit animal model (7) or incorporation of a core antigen of hepatitis B virus on the surface of *E. coli* BGs resulted in a significant immune response against this core antigen in mice (14). BGs can also be used as delivery vehicles for active substances such as doxorubicin (21) or as a carrier of DNA (15) and enzymes (11). Nevertheless, little is known about the capacity of BGs to induce the expression of innate immune modulators by epithelial cells especially human KCs. In the present study the ability of *E. coli* BGs to induce antimicrobial peptides and pro-inflammatory cytokines expression in human primary KCs has been investigated. The results demonstrate that the presence of flagellin on the surface of BGs enhance the expression of the antimicrobial psoriasin and hBD-2, and the release of interleukin (IL)-6 and IL-8 by human KCs.

Materials & Methods

Cell culture

Human primary KCs prepared from neonatal foreskin were obtained from Clonetics (San Diego, CA, USA) and cultured in serum-free keratinocyte growth medium (KGM, Clonetics) as described previously (23). For stimulation, third passage KCs were cultured in 12-well tissue culture plates (Corning Incorporated, Corning, NY, USA) and used at a confluence of 60-70 %. Stimulation was performed in keratinocyte basal medium (KBM, Clonetics).

RNA isolation and qRT-PCR

After stimulation, cells were washed with phosphate-buffered saline (PBS) and RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For cDNA synthesis RNA was reverse-transcribed with MuLV-reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and oligo dT primers (Roche Diagnostics, Basel, Switzerland). cDNA sequences of the genes under investigation were obtained from the GenBank. Primers were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA, USA). The following forward (F) and reverse (R) intron-spanning primers were used for β-2-microglobulin (B2M): F, 5′-GATGAGTATGCCTGCCGTGTG-3′; R, 5′-CAATCCAAATGCGGCATCT-3′; psoriasin: F, 5′-GGAGAACTTCCCCAACTTCCTT-3′; R, 5′-GGAGAAGACATTTTATTGTTCCT-3′; hBD-2: F, 5′-ATCAGCCATGAGGGTCTTGT-3′; R, 5′-GAGACCACAGGTGCCATTTT-3′; qRT-PCR was performed by the LightCycler technology using the Fast Start SYBR Green I kit for amplification and detection (Roche Diagnostics). In all assays, cDNA was amplified using a standardized program (10 min denaturing step and 55 cycles of 5 sec at 95°C; 15 sec at 65°C, and 15 sec at 72°C; melting point analysis in 0.1°C steps; final cooling step). Each LightCycler capillary was loaded with 1.5 µl DNA master mix; 1.8 µl $MgCl_2$ (25 mM); 10.2 µl H_2O; and 0.5 µl of each primer (10 µM). Determination of the relative quantification of target gene expression and amplification efficiencies were performed using a mathematical model by Pfaffl (22). The expression of the target gene was normalized to the expression of the housekeeping gene β-2-microglobulin. All real-time PCRs were performed in triplicate. The specificity of PCR reactions was confirmed by sequencing of the PCR products.

BGs production and KCs stimulation

E. coli NM522 was obtained from Stratagene, Amsterdam, The Netherlands. *E. coli* NK9373 (wt) and *E. coli* NK9375 (Δ*fliC*), a flagellin-deficient strain having an in frame deletion within the *fliC* gene (4) were kindly provided by Dr. David Bates (Baylor College of

Medicine, Houston, Texas). *E. coli* strains harbouring the lysis plasmid pGLysivb (unpublished) were grown in animal free Lennox Broth (LBv; 10 g/l sojapeptone, 5 g/l yeast extract, 5 g/l NaCl) containing gentamycin (20 µg/ml) at 35°C. Two litre of medium were inoculated with 4 ml glycerine stock, descending from one single transformant colony and used as a preculture for fermentation after over night incubation. Fermentations were performed in 20 l medium using a Techfors S fermenter (Infors Ag, Bottmingen, Switzerland). Following parameters were documented: temperature, flow, stirrer, pH, pO_2, foaming and pumps for acid, base and antifoam. Growth and lysis of the bacteria were followed by measuring the optical density (OD_{600}), by determination of the colony forming units using a spiral plater (WASP system, Don Whitley Scientific Limited, West Yorkshire, UK) and by microscopy of periodically taken samples. Bacteria were grown in LBv medium set to pH 7.2 with aeration and agitation (flow and stirring controlled by a programmed sequence) to mid-logarithmic stage. Expression of the lysis protein E was induced by temperature upshift to 42°C. After the completed lysis process (reaching a plateau in the pO_2 level) the remaining intact bacteria were killed by addition of beta-propiolacton (BPL). A total of 0.075 % BPL was added in two equal doses with a time gap of 30 min. For incubation with BPL the stirring rate was set to 600 rpm. For harvesting by a seperator (CTC1, GEA Westfalia Separator GmbH, Oelde, Germany) the temperature was set to 16°C and a flow rate of about 200 ml/min was used. The system was rinsed with 5 l sterile, distilled water before resuspension of the BG pellet and BGs were subsequently washed by 5 resuspension/centrifugation cycles with distilled water in a total volume of 7.5 l using a Hermle ZK 401 centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany) at 8,000 min^{-1}, 4°C, 15 min. The final pellet was resuspended in 200 ml distilled water, aliquoted into lyophilisation bottles and stored at -80°C. Samples were lyophilized for about 60 h using a Lyolab B (LSL Secfroid, Aclens, Switzerland) lyophilisator.

Lyophilized BGs from *E. coli* NM522, *E. coli* NK9373 (wt) and *E. coli* NK9375 (Δ*fliC*) were resuspended in KBM medium before applying on KCs. For *in vitro* assays recombinant IL-1α (R&D Systems, Minneapolis, MN, USA) were used.

Cytokine measurement

Culture supernatants of stimulated KCs were depleted by centrifugation of detached cells or cell fragments and stored at -20°C until analysis. Concentrations of IL-6 and IL-8 were determined by enzyme-linked immunosorbant assay (ELISA; R&D Systems) according to the manufacturer's instructions.

Immunoblot analysis

For analysis of protein expression, KCs were lyzed in SDS-PAGE loading buffer (50 mM Tris, pH 7.4, 2 % SDS). After sonication insoluble cell debris were removed by centrifugation

and protein concentration was measured by the BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA). Western blot analysis was performed as described previously (19). Equal loading of protein lysates was confirmed by Ponceau S staining of the membrane. The following first step antibody was used: mouse monoclonal IgG_1 anti-psoriasin clone 47C1068 (dilution 1:500; Abcam, Cambridge, UK). The membranes were developed using the Chemiglow reagent (Alpha Innotech, San Leandro, CA, USA) according to the manufacturer's instructions.

Fluorescein isothiocyanate (FITC)-labeled BG uptake

The efficiency of the endocytic activity of the human primary KCs was measured as described previously (15,16). Briefly, human primary KCs cultured in 24 well plates ($2x10^5$ cells/well) were incubated with FITC-BG (1000 per cell) for 2 hours at +37°C. After the incubation cells were washed three times with PBS to remove the excess BGs. Finally the cells were detached using TrypLE™ Express (Invitrogen), washed twice with PBS, fixed in cold 1.5% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS and analyzed on BD FACSCanto™ Flow Cytometer (BD Biosciences, Pharmingen, San Jose, USA).

Results

Expression of antimicrobial peptides by BGs in human primary KCs

It has been reported that *E. coli* culture supernatants induce the expression of the antimicrobial peptides psoriasin and hBD-2 in epidermal KCs (1,8). To investigate the capacity of BGs to elicit innate immune responses by epithelial cells, human primary KCs were stimulated for 24 hours with different particle concentrations of BGs generated from *E. coli* NM522. The relative mRNA up-regulation of the antimicrobial psoriasin and hBD-2 were determined by quantitative real-time PCR (qRT-PCR). Stimulation of KCs by IL-1α (10 ng/ml) served as positive control for the up-regulation of the analyzed antimicrobial peptides (1). As can be depicted from **Fig. 1** the mRNA expression of both peptides was up-regulated in KCs after incubation with BGs. The stimulated psoriasin and hBD-2 mRNA production was dependent on the BG particles and for both peptides the strongest up-regulation was observed at $2x10^8$ BG particles/ml. For psoriasin, BG concentrations below $2x10^8$ particles/ml had minimal to no effect (**Fig. 1A**), whereas for hBD-2 a dose-dependent induction of mRNA was detected (**Fig. 1B**). These data indicate that KCs respond to BGs by the production of antimicrobial peptides in a particle-dependent manner.

We have previously reported that the induction of psoriasin and hBD-2 in KCs is dependent on flagellin expression by *E. coli* (1). To investigate whether flagellin of BGs has a similar effect, we generated BGs from the wild-type (wt) NK9373 and the isogenic flagellin-deficient (Δ*fliC*) NK9375 *E. coli* strains. KCs were stimulated for 48 hours and afterwards analyzed by immunoblot for psoriasin protein production. In contrast to the mRNA data a faint band of psoriasin was detected on the protein level at $2x10^7$ BG particles/ml. Strong induction of psoriasin was observed at $2x10^8$ particles/ml which was even stronger at $2x10^9$ particles/ml by wt (NK9373) *E. coli* BGs. For the isogenic Δ*fliC* (NK9375) strain a faint band of psoriasin was detected starting at $2x10^8$ BG particles/ml with a prominent expression level at $2x10^9$ BG particles/ml (**Fig. 2**).

Expression of cytokines by BGs in human primary KCs

The secretion of the pro-inflammatory cytokines IL-6 and IL-8 by KCs after incubation for 48 hours with BGs were investigated by ELISA. The release of IL-6 and IL-8 by KCs after the incubation with wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs was dependent on the BG source and particle numbers used (**Fig. 3A, B**). Obtained results showed that the effect of wt (NK9373) *E. coli* BGs on IL-6 and IL-8 release was detectable beginning at concentrations $2x10^7$ particles/ml. While concentrations below $2x10^7$ particles/ml were almost ineffective, $2x10^9$ particles/ml strongly enhanced the secretion of IL-6 from 2 pg/ml (untreated) to 600 pg/ml and IL-8 from 60 pg/ml (untreated) to 1350 pg/ml, respectively (**Fig. 3A, B**). However,

Δ*fliC* (NK9375) *E. coli* BGs increased the secretion of both cytokines only when using $2x10^9$ particles/ml to 134 pg/ml and 750 pg/ml of IL-6 and IL-8, respectively, whereas BGs concentrations below that had no effect on the release of both cytokines (**Fig. 3A, B**). The decreased response of KCs towards Δ*fliC* (NK9375) *E. coli* BGs in psoriasin production and IL-6 or IL-8 secretion reflects that flagellin is a major inducing component of these proteins in KCs. The question remains whether other components of the Δ*fliC* (NK9375) *E. coli* BGs have signaling activities for the production of antimicrobial peptides or pro-inflammatory cytokines. For this purpose uptake studies for wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs by KCs were performed.

Endocytosis of wt (NK9373) and ΔfliC (NK9375) E. coli BGs by human primary KCs

As mentioned above, the up-regulation of antimicrobial peptides and enhanced release of pro-inflammatory cytokines by KCs is dependent on the presence of flagellin on the surface of BGs. To further investigate the role of flagellin in the uptake by KCs, endocytosis of wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs were compared and analyzed. Missing flagellin on the surface of BGs caused significant decrease (~6-fold less) of KCs capacity to bind and endocyte BGs (**Fig. 4**). Obtained results clearly indicate the important role of bacterial flagellin in the endocytosis of BGs by KCs as well as in the production of antimicrobial peptides and secretion of pro-inflammatory cytokines.

Discussion

BGs are nonliving cell envelope preparations from Gram-negative bacteria, devoid of cytoplasmic contents, while their cellular morphology and native surface antigenic structures remain preserved (18,24). In this investigation the effect of BGs on the regulation of innate immune modulators using human primary KCs was determined. Applying BGs of the non-pathogenic *E. coli* strain MN522 on human KCs up-regulated the expression of the antimicrobial psoriasin and hBD-2. This was in agreement with earlier reports where cell culture supernatants or disrupted cells of *E. coli* strains enhanced the expression of these two antimicrobial peptides (1,8). Therefore, this investigation indicates that the envelop structure of BGs is recognized by KCs and promotes innate immune responses similar to bacterial compounds used in the former studies.

As reported earlier, the responsiveness towards *E. coli* by KCs is mediated through TLR5 and its ligand flagellin (1). Since we did not have a direct measure of the amount of flagellin present on BGs of *E. coli* NM522, BGs of two isogenic *E. coli* strains NK9373 (wt) and NK9375 (Δ*fliC*) were propagated under the same growth conditions and used in this study. The wt (NK9373) strain exhibited a BG concentration-dependent induction of psoriasin production; whereas the Δ*fliC* (NK9375) strain showed the expression of psoriasin at BG concentrations of one order of magnitude higher than the wt strain (**Fig. 2**). Accordingly, the data obtained from the release of the pro-inflammatory cytokines IL-6 and IL-8 underlined the significance of the presence of flagellin in the BGs preparation when compared to the ΔFliC mutant strain (**Fig. 3**). From the previous studies with supernatants of *E. coli* as source of shad flagellin and/or purified flagellin, it is evident that flagellin is the major inducer of psoriasin in human KCs (1,8). However, since ΔFliC BGs are capable of psoriasin induction there seems to be additional FliC-independent pathways.

Investigation of BGs uptake by KCs derived from wt (NK9373) and Δ*fliC* (NK9375) *E. coli* strains showed that the flagella bearing strain is taken up roughly one order of magnitute better than the ΔFliC strain (**Fig. 4**). This observation suggests that the presence of flagellin on the BG surface contributes to the binding and uptake of BGs into KCs. When the results of psoriasin production are normalized to the number of BG particles being taken up by KCs, the same concentration effects could be seen for the induction of the latter antimicrobial peptide. The release of IL-6 and IL-8 by KCs, however, were more sensitive to the presence of flagellin as no direct correlation with the BGs particle number could be detected (**Fig. 3**).

It is still possible that the acidic degradation of the flagella present in NK9373 BGs in the lysosomal compartment of KCs, monomeric flagellin is able to bind to TLR5 (10) and stimulate the expression of the cytokines IL-6 and IL-8 and the production of psoriasin. As flagellin

binding to TLR5 cannot occur with the ΔFliC BGs and consequently the signalling induced by this binding, other ways of internal signalling leading to NF-kappaB induced expression of psoriasin have to be induced by BGs in KCs. Possible intracellular receptors which might sense the presence of BGs or BGs constituents irrespective of flagellin expression are the NLRs such as NOD1 or NOD2. Recent investigations have reported the functional expression of NOD1 (9) and NOD2 (26) by peptidoglycan fragments in KCs. In particular, NOD1 mediates the sensing of peptidoglycan fragments containing the amino-acid *meso*-diaminopimelic acid and NOD2 mediates the sensing of muramyl dipeptide (3), which both of these fragments are degradation products of the still intact peptidoglycan of *E. coli* BGs (29). For the NOD1/NOD2 dependent induction of hBD-2 (23) the above mentioned peptidoglycan fragments can only originate from the degradation of BGs in the endo-lysosome compartments of KCs. In this context it is worthwhile to note hat the BGs used in this study were all derived from non-pathogenic *E. coli* strains, and thus a discrimination between pathogenic and non-pathogenic strains for their ability to induce hBD-2 via activation of NOD1 through peptidoglycan fragments transported into the effecter cells by a bacterial type IV protein secretion system (25) was not given. The connection between activation of NOD2 by muramyl dipeptde (MDP) and increased hBD-2 production in primary KCs has been reported (23), and in our study the use of BGs to induce hBD-2 and psoriasin has been demonstrated. Therefore, it seems possible to use BGs in therapeutic approaches to enhance the innate immune defence system of the skin. Potential therapeutic effects of BGs can be combined by packaging drugs or other biological active substances into BGs which could also be delivered intracellular for the stimulation of additional beneficial health effects (17,21).

Conflict of interest

The authors state no conflict of interest.

Figures

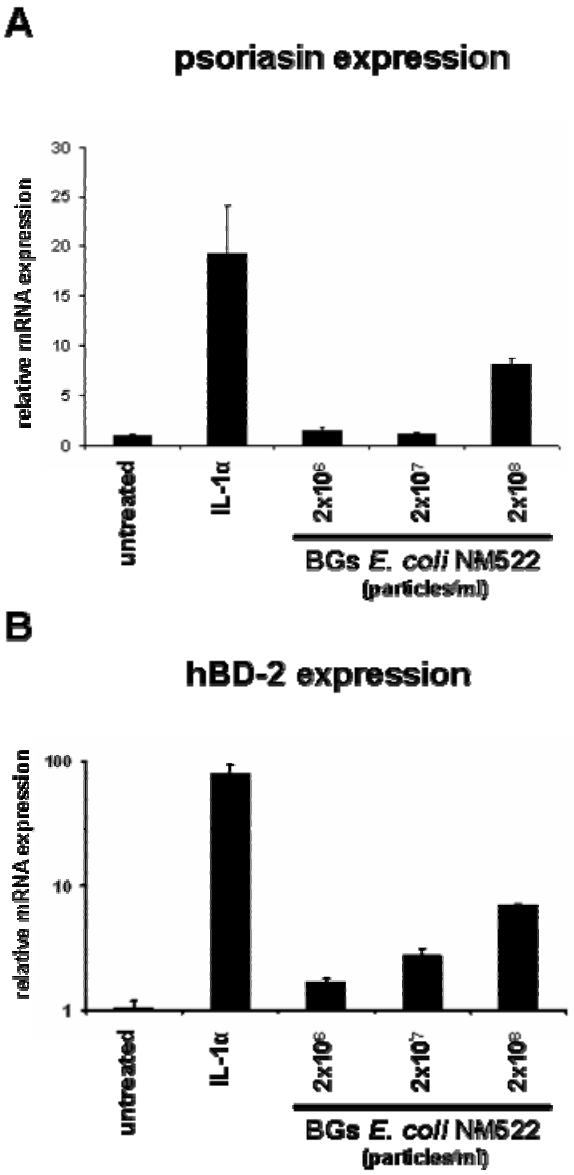

Fig 1. BGs induce the expression of antimicrobial psoriasin and hBD-2 in human primary KCs. KCs were incubated for 24 h with IL-1α (10 ng/ml) or BGs (*E. coli* NM522) varying from $2x10^6$ to $2x10^8$ particles/ml, thereafter total RNA was isolated and reverse-transcribed to cDNA. The relative expression of psoriasin **(A)** and hBD-2 **(B)** was determined by qRT-PCR. The mean values are displayed in relation to untreated cells. Relative gene expression levels were normalized to the expression of the housekeeping gene β-2-microglobulin. Data represent the mean ± SD of triplicate samples.

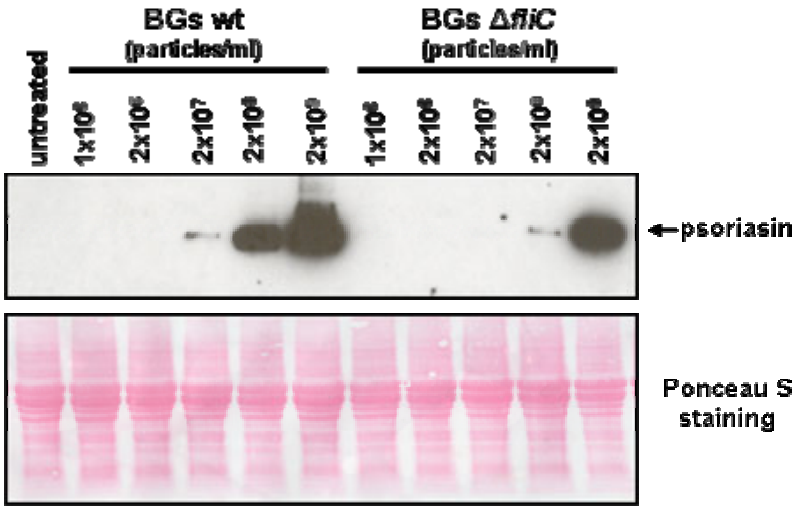

Fig. 2. Induction of psoriasin in KCs by wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs. KCs were incubated for 48 h with wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs from $1x10^6$ to $2x10^9$ particles/ml. After stimulation cell lysates were subjected to immunoblot analysis for psoriasin (upper panel) as described in the Materials and Methods section. Ponceau S staining of the membrane served as loading control of protein lysates (lower panel).

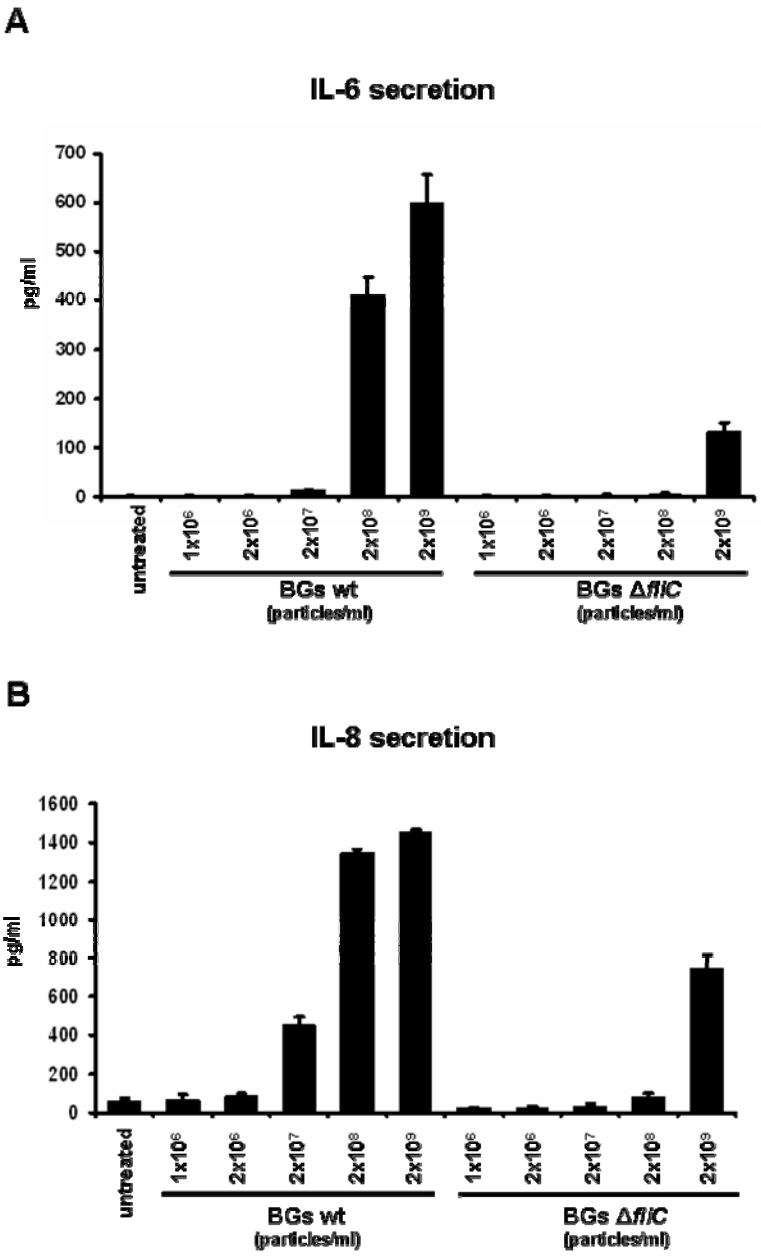

Fig. 3. BGs induce the secretion of pro-inflammatory cytokines. KCs were incubated for 48 h with wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs from $1x10^6$ to $2x10^9$ particles/ml. After the incubation period cell culture medium was collected and the concentrations of IL-6 (A) and IL-8 (B) were determined by ELISA. Data represent the mean ± SD of triplicate samples.

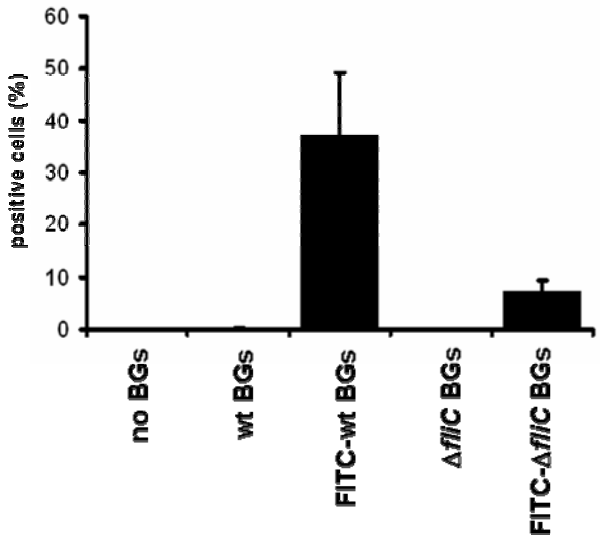

Fig. 4. Comparative flow cytometric analysis of human primary KCs endocytic activity for BGs. KCs were incubated with FITC-labeled BGs (1000 per cell) - wt (NK9373) and Δ*fliC* (NK9375) *E. coli* BGs for 2 h at +37 °C. Cells incubated without BGs or with non-labeled BGs served as the controls. Values were calculated as the percentage of cells with increased fluorescence incubated without or with non-labeled BGs subtracted from the percentage of positive cells incubated with FITC-labeled BGs. Endocytic capacity of KCs was measured by BD FACSCanto™ Flow Cytometer (BD Biosciences). Each bar represents the mean of four independent experiments ± SD.

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Chapter 2.3.2.
Modulation of Bacterial Ghosts (BG)-induced nitric oxide (NO) production in RAW 264.7 macrophages by BG-delivered resveratrol

Verena Juliana Koller[1,2], Hortenzia Beres[3], Oliver Donath[3], Gottfried Reznicek[3], Verena Dirsch[3] and Werner Lubitz[1,2,*]

[1] University of Vienna, Department of Medicinal Chemistry, Vienna, Austria

[2] BIRD-C GmbH & Co. KEG, Kritzendorf, Austria

[3] Department of Pharmacognosy, University of Vienna, Vienna, Austria

[*] Corresponding author: Phone: +43-1-2243-28491 - werner.lubitz@univie.ac.at, werner.lubitz@bird-c.at

In the present study we show that coincubation of RAW 264.7 macrophages with bacterial ghosts (BGs) leads to a dose-dependent induction of the inducible nitric oxide synthase (iNOS).

To determine whether BG-mediated delivery of the known iNOS inhibitor, resveratrol (RV) is an effective way to inhibit NO production in RAW264.7 macrophages we loaded BGs with RV and measured BG-induced nitrite accumulation. Indeed, NO formation was modulated in the presence of BGs loaded with RV and a significant decrease of BG-induced NO production was observed. RV-delivered by BGs was effective in a concentration as low as 0.48±0.25 µM. Externally added RV was still ineffective at a concentration 6-times higher (3 µM). BGs exhibited no cytotoxicity in RAW 264.7 cells even when coincubated for 24 hrs. However, cell viability decreased by about 10 % when macrophages were incubated with RV-loaded BGs dependent on the RV cargo. Since internal cell delivery of RV by BGs seems to increase the RV effect, we postulate an intracellular RV receptor for which we propose the abbreviation ERVR (endogenous resveratrol receptor).

Introduction

The innate immune system consists of special types of cells and humoral factors that rapidly react on infectious agents by inactivation or killing mechanisms in a non-specific manner. Among others, this defense system leads to phagocytosis of bacteria, viruses or protozoa by macrophages, monocytes, neutrophils and dentritic cells resulting in the release of antimicrobial peptides, hydrolytic enzymes and/or reactive oxygen species (ROS) or reactive nitrogen species (RNS). An example of the latter one is given by the production of nitric oxide (NO) through the inducible nitric oxide synthase (iNOS). However, generation of iNOS-dependent NO is not only a feature of phagocytes but also of epithelial cells or keratinocytes [1-3].

NO has been shown to be a versatile molecule, exhibiting an ambiguous endogenously role, with a rapid half life and displays a crucial mediator molecule for various cellular functions [4]. This highly reactive molecule is implicated in the pathophysiology of many diseases and can cause cytotoxic and mutagenic effects when it is produced in excessive amounts, like under oxidative burst conditions [4]. However, due to various effector and immunoregulatory functions, e.g. antimicrobial, antitumorigenic and apoptotic activity or its modulating character of cytokines and T-cell differentiation, NO plays an important role in the immune system. Furthermore, this radical contributes to an antimicrobial activity against certain intracellular bacterial pathogens like *Salmonella enterica*, *Trypanosoma musculi*, *Mycobacterium tuberculosis*, *Legionella pneumophila* or *Leishmania major* [5, 6].

There are three major nitric oxide synthase (NOS) isoforms known, the contitutively expressed neuronal NOS (also known as nNOS or NOS1) and endothelial NOS (eNOS or NOS3) as well as the inducible isoform (iNOS or NOS2). The latter is accountable for the NO production in macrophages and keratinocytes. All three types of NOS oxidize one molecule of L-arginine at a guanidine nitrogen to an intermediate which is oxidized to yield one molecule of NO and L-citrulline. [1-4].

Bacterial lipopolysaccharide (LPS) is among the most important stimuli for murine iNOS induction after binding to toll-like receptor 4 (TLR4) [7].

Bacterial ghosts (BGs) are, in analogy to empty erythrocytes or bacteriophage ghosts, cell envelopes of Gram-negative bacteria which are devoid of cytoplamic content and free of nucleic acids. They are produced by controlled expression of plasmid-encoded lysis gene E of PhiX174 that leads to fusion of inner and outer membranes. Thus, an E-specific lysis tunnel is formed and the cytoplasmic content is expelled due to the osmotic pressure difference between the cytoplasm and the exterior of the bacteria [8, 9]. These non-living, empty bacterial envelopes maintain the full cellular morphology of the native bacteria. The feature, that all the cell surface structures, including the outer membrane proteins, adhesins, LPS and peptidoglycans are well

preserved in BGs emphasizes them as attractive natural adjuvants for vaccination [10]. The fact that BGs share the antigenic determinants from their living counterpart and that additional foreign proteins can be expressed on or within the cell envelope as well as DNA or drugs can be loaded inside the cytoplasmic lumen of the ghosts, highlights them as a novel vaccination system for animals and humans [10-12].

Resveratrol (RV) is a polyphenolic stilbene compound, naturally occurring either in trans- or cis-isomeric forms in various plant species, especially present in skins of grapes, peanuts and berries showing a broad spectrum of immunomodulating activities. Many studies allocate anticancer, antioxidant and cardioprotective properties as well as an extended life-span to this molecule [13-16]. Moreover, RV possesses great therapeutic potential in treatment of a variety of infectious diseases of animals and humans. Its activity has been demonstrated against dermatophytes [17] and viruses like *Herpes simplex* [18]. The growth inhibitory effect of RV has been substantiated for a broad range of bacterial species including intracellular *Chlamydia pneumoniae* that accounts for acute respiratory tract infections [17, 19]. Furthermore, decreased infectivity of protozoa species which cause diseases in fish [20, 21] was achieved and more recently the elimination of the intracellular skin pathogen *L. major*, responsible for cutaneous leishmaniasis, by RV was demonstrated [22, 23].

As shown in earlier studies, BGs have quite an impact on the parameters of the innate immune system like the secretion of cytokines and expression of antimicrobial peptides (Abtin and Lubitz, to be published). Taken into consideration that iNOS-dependent production of NO, a key-player molecule in innate immunity, is stimulated by LPS we aimed, first, to examine the extent of BG-stimulated NO release in the model macrophage cell line RAW 264.7 and, second, to study the impact of RV-loaded BGs on this NO release since RV is reported to inhibit LPS-induced NO production in macrophages [16, 24].

Materials & Methods

Chemicals

If not otherwise stated, all chemicals were obtained from Sigma Aldrich.

Cell culture

RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, USA), were cultured in Dulbecco's Modified Essential Medium with 4.5g/L glucose (Lonza, BioWitthaker®, Verviers, Belgium; DMEM without L-glutamine and phenol red) supplemented with 2 mM L-glutamine (Lonza) and with 10 % heat-inactivated fetal calf serum (FCS, Gibco; Invitrogen, Carlsbad, CA). Additionally, 100 U penicillin/streptomycin (Lonza) was added to the cultivation medium. For the experiments serum-free (sf-) media were used. The cells were grown under standard conditions (37°C, 5% CO_2) and the medium was changed every 2-3 days. The cells were subcultured when they reached their confluent state.

Bacterial ghost production

BGs from *E. coli* NM522 (pGLysivb; 240106-5/6), were produced by the controlled expression of the phage-derived lysis protein E as described elsewhere [9, 25]. Inactivation of the non-lysed bacteria was done by addition of antibiotics. Lyophilized BGs were stored at room temperature (1 mg lyophilized weight contained $1.27x10^{10}$ particles). BGs were resuspended in sf-medium prior to treatment experiments.

Loading BGs with resveratrol

12-24 mg lyophilized BGs were suspended in different RV (Sigma Chemical Co., St. Louis, MO) concentrations (1-35 mg RV/ml methanol) and incubated under vigorous shaking (800 rpm) for 30 min at 28°C. The loaded BGs were collected by centrifugation at 11300 g for 15 min and the pellets were washed three times with water. 1 mg BG aliquots were stored at -20°C until use.

Quantification of RV extracted from BGs

The extraction of RV out of the BGs was performed using 500 µl ethanol 96% (Brenntag CEE GmbH, Vienna) per 1 mg of BGs. After 5 min of ultrasonification the ethanolic extract was diluted 1:1 with H_2O and immediately centrifuged at 11300 g at 4°C for 15 min.

The HPLC analysis was conducted with a PE 200 Series HPLC System, consisting of a pump, an autosampler, a diode-array detector, a column oven and a Turbochrom Navigator software for the control of the equipment and for data evaluation. A Lichrospher 100 RP-18e (5µm) Column (250x4mm) has been used for separation.

The quantification was done by the peak area method applying RV as external standard. A gradient method was performed using diluted acetic acid (pH 2.8) as solvent A and methanol (HiPerSolv for HPLC, gradient grade) as solvent B at a flow rate of 1 ml/min at 25°C. The gradient profile started from 50% B to 60% B within 10 min and a final purge with 95% B. 10 µl of all samples and dilutions were injected and the chromatograms were monitored at 305 nm. The relevant RV peak was identified by comparing the retention time (5.3 min) and the UV of the samples with those of the external RV standard.

Nitrite assay

RAW 264.7 cells were seeded in 96 well plates and cultured for two days. Approximately $3x10^5$ macrophages per well were then stimulated either with 200 µl of a defined concentration of empty BGs (positive control) or with resveratrol-loaded ghosts (4.6 fg RV/BG), or with empty bacterial ghosts plus defined RV concentrations given externally, for 20 min. Thereafter, the bacterial ghost suspensions were removed by washing cells with PBS twice. Then, cells were incubated for another 20 hrs in the dark at 37°C 5% CO_2. In order to investigate the stimulatory impact of pure LPS (Fluka, Sigma Chemical Co., St. Louis, MO; E. coli serotype 055:B5), long-term incubation experiments with various LPS concentrations were conducted for 20 hrs.

Based on the entrapped amount of RV in BGs (**Fig.1A**) and the number BGs which were associated with macrophages (adhered to and taken up by the cells) after 20 min treatment (**Fig. 1B**), the BG-delivered RV particles have been calculated. As stimulation of NO induction in RAW 264.7 cells was performed with BGs having the highest RV-yield (4.6 fg RV/BG) in a multiplicity of infection (MOI) rate of 100 and 1000, calculated RV-delivery values by BGs correspond to 0.48 ± 0.25 µM and 8.86 ± 2.21 µM RV ($1.21x10^7$ RV molecules per single BG).

As an indicator of NO production, nitrite concentration was measured in the supernatant of the macrophages by use of the Griess reaction [26]. Briefly, 100 µl of each supernatant was mixed with 90 µl 1% sulphanilamide (Fluka) in 5 % H_3PO_4 and 90 µl of N-(1-Naphthyl) ethylene diamine dihydrochloride in water. The absorbance was determined at 550 nm with an ELISA reader (Tecan Sunrise).

Application and detection of FITC-labeled BGs

As an indication for endocytic RV delivery, uptake of FITC-labeled BGs was determined in the analyzed cell line. For this purpose, lyophilized BGs (5-15 mg were resuspended in 1.5 ml 0.1M Na_2CO_3; pH ~ 9.0. Thereafter, 25 µl of FITC stock solution (2 mg FITC in 1 ml DMSO) was added to the BG suspension and shaken for 2 hrs in the dark at 16°C. After five washing steps with PBS (5 min; 11300 g) and check for positive labeling, the BG pellets were resuspended again in sodium carbonate buffer and stored at -20°C until use.

Culturing and treatment of RAW 264.7 cells was the same as for the nitrite assay with minor modifications. After 20 min incubation with defined amounts of empty BGs the macrophages were washed twice with PBS and the washing solutions were collected in empty neighbouring wells. Fluorescence was then recorded with a GENios Pro plate reader (Tecan) at excitation and emission wavelengths of 485/535 nm (gain 40). Total fluorescence values were taken as 100% of the applied ghost amount and uptake was calculated from the resulting fluorescence per macrophage.

Internalisation of the bacterial ghosts (BG) was veryfied by use of confocal laser scanning microscopy (CLSM). For this purpose cells were seeded in 8-well chamber slides (Falcon CultureSlide) and were allowed to attach. The medium was replaced by 200 µl sf-medium containing the appropriate FITC-labeled BG giving a BG to cell ratio of 1000 and the slides were further incubated for either 20 min or 40 min. Thereafter, cells were washed twice with PBS and 1% paraformaldehyde (PFA) was added for fixation (20 min, room temperature). In addition, experiments were conducted in which the attached BGs were removed from the cells by trypsinization. After two washing steps with PBS the cells were fixed onto glass slides by cytospin (800 rpm, 15 min) and were fixed with PFA as described before. Thereafter, the cells were washed again for two times with PBS and were permeabilized with 0.5 % Triton X-100 for 20 min, followed by other two washing procedures. Then, 100 µl of freshly prepared PromoFluor 590 (Texas-Red)-conjugated Phalloidin (5 µl methanolic 100 U/ml dye-stock; PBS; 1 % BSA) was added to visualize cell boundaries. After 45 min of incubation, slides were washed again twice with PBS and imaged by CLSM.

Cytotoxicity assays

The neutral red assay was used to test the impact of empty or RV-loaded BGs as well as RV per se on the viability of RAW 264.7 cells. Neutral red (3-amino-m-dimethylamino-2methyl-phenazine hydrochloride) selectively accumulates in lysosomes of living cells [27] and provides therefore a quantitative assessment of viability.

Individual wells of 96-well plates were inoculated with 1.25 x 10^5 cells and were allowed to attach overnight. Macrophages were treated with 200 µl medium containing RV-loaded and unloaded BGs (all resuspended in sf-medium) in different BG to cell ratios (10, 100, 1000) or with RV alone for either 20 min followed by a 20 hour recovery period or 24 hrs. The same conditions were performed with two RV concentrations (15 µM and 30 µM). Triton X-100 (0.01 %) served as positive control and cells treated with sf-medium represent the negative control. After treatment, cells were washed twice with PBS and then incubated either with culture medium for 20 hrs or directly with 100 µl of neutral red (80 µg/ml final concentration) for another two hours (37 °C; 5 % CO_2). Thereafter, the dye was discarded and the wells were washed two times with PBS. Extraction of the dye was accomplished by addition of 100 µl of

the acidic destaining solution (1 ml acetic acid, 73 ml 96 % ethanol and 26 ml deionized water). The plates were shaken for 10 min and the developed color was measured by a plate reader (Dynex OpsysMR) at 570 nm (reference wavelength 690 nm).

Statistics

All results were analysed by use of GraphPad Prism (version 5, GraphPad Software, Inc; San Diego; CA, USA). Data are expressed as means + SD. Statistical analysis was performed by the use of Student's t-test. P-values < 0.05 were considered statistically significant.

Results

Loading of BGs with RV

The loading of lyophilized *E. coli* BGs with RV was performed by resuspension of BGs in RV solutions of different concentrations (1-35 mg/ml RV). To determine the amount of RV in BGs, ethanolic extracts were performed and analyzed via HPLC. The results are depicted in **Fig. 1A.** A clear correlation between the loading concentration of RV and recovered RV was observed (correlation coefficient $r^2 = 0.9980$). The highest loading efficiency was obtained after suspension of lyophilized BGs in a 35 mg/ml RV solution corresponding to 46 µg RV/$1x10^{10}$ BGs (4.6 fg RV/BG).

Adherence and uptake of FITC-labeled BGs by murine macrophages

Fluorometric quantification experiments were conducted in order to investigate the amount of BGs which are either attached and/or taken up by murine macrophages after a short time of coincubation. **Fig. 1B** depicts the result obtained by fluorometric measurements of RAW 264.7 cells coincubated with *E. coli* BGs for 20 min and two washing steps. The amount of associated BGs per cell (attached and intracellular) clearly depends on the ratio of BGs per cell (correlation coefficient $r^2 = 0.998$). Furthermore, no differences where observed when the experiments were performed in 96 or 24 well plates (data not shown). An average of 25 % of the applied BGs were associated with the macrophage population after this short incubation time.

To clarify whether BGs are taken up by murine macrophages within this period, confocal laser scanning microscopy (CLSM) studies were conducted in which the internalization was visually examined. For this purpose, z-stacks were performed after incubation of FITC-labeled *E. coli* BGs with RAW 264.7 cells after 20 and 40 min. Macrophages were stained with Texas-Red Phalloidin, which selectively binds to the F-actin skeleton of cells. Images displaying representative single z-stacks of various optical sections are depicted in **Fig. 2**. It can be seen that FITC-labeled BGs which are already internalized by RAW 264.7 cells stained in red, appear yellow (roughly 65 %). Green colored BGs (less than 35 %) represent those which are not engulfed but attached to cells (**Fig. 2A**). A representative picture of RAW 264.7 cells which were trypsinized to remove attached BGs is shown in **Fig. 2B**. At a BG to cell ratio of 1000, single BGs as well as clusters of internalized BGs were detectable in every macrophage after 40 min.

Induction of NO production

Murine iNOS is induced by LPS. As depicted in **Fig. 3A**, treatment of RAW 264.7 cells with 1-1000 ng/ml LPS for 20 h led to a dose-dependent generation of NO with a significant output starting at 1 ng/ml ($P < 0.0001$).

Due to the fact that BGs fully maintain their LPS molecules bound in the envelope [28], we were curious to which extent BGs are able to stimulate NO production. It turned out that a BG to cell ratio of 10 was sufficient to significantly induce NO generation ($P=0.0005$) to a level similar to 1 ng/ml of free LPS.

Clear particle-dependent stimulation of NO generation was achieved when cells were treated with BGs in a multiplicity of infection (MOI) rate of 100-1000. Higher concentrations of BGs (2000 and 5000 particles per cell) did not show any further impact on NO synthesis (**Fig 3B**).

Modulating capacity of NO stimulation by RV-loaded BGs

As already shown in **Fig. 3B** BGs are able to stimulate NO production in a dose-dependent manner when applied to macrophages in a BG to cell ratio between 10 and 1000.

Many studies showed that RV is capable to reduce LPS-induced NO-production [16, 24]. We, therefore, examined the potential of RV to modulate BG-induced NO production. For this purpose empty *E. coli* BGs were applied to RAW 264.7 cells in a ratio of 500. Coevally, three different concentrations of RV (0.3-30 µM) were added to the cell culture medium. After 20 min coincubation and two washing steps, released NO was measured as accumulated nitrite after 20 hrs. **Fig. 4A** shows that simultaneous treatment of RAW 264.7 cells with *E. coli* BGs and 0.3 µM, 3 µM RV and 30 µM RV, respectively, influenced BG-induced NO generation. A statistically significant decrease of NO production was obtained by 30 µM RV ($P= 0.0078$).

Next, we examined whether RV bound in BGs affects BG-induced NO production. We, therefore, compared NO release in response to empty BGs versus RV-loaded BGs and BGs plus externally added RV. **Fig. 4B** shows that compared to empty BGs NO production was significantly reduced ($P = 0.0048$) in RV-loaded ghosts in a BG to cell ratio of 100 which refers to 0.48 ± 0.25 µM RV. In contrast, a 6-fold higher concentration of externally added RV (3 µM) had no effect. Thus, externally applied RV together with empty *E. coli* BGs did not decrease NO release as effective as RV-loaded ghosts (BG to cell ratio 100).

On the other hand, treatment of RAW 264.7 macrophages in a BG to cell ratio of 1000 with the same RV concentration reduced NO production significantly ($P = 0.0158$) (**Fig. 4C**). Similar effects were found when RV-loaded BGs (BG to cell ration of 1000: 8.86 ± 2.21 µM RV) were applied to RAW 264.7 cells ($P = 0.0002$).

Determination of cell viability

In order to investigate the impact of empty and RV-loaded BGs (4.6 fg RV/BG) on cell viability of RAW 264.7 macrophages two different treatment conditions were examined with three different BG to cell ratios (MOI 10, 100, 1000). The first test was performed analogue to the regimen used to stimulate NO production, i.e. 20 min coincubation of cells with BGs, followed by two cycles of washing and nitrite measurement after 20 hrs. The second analysis determined the effect of BG long-term coincubation (24 hrs) with RAW 264.7 macrophages. As depicted in **Fig. 5A**, *E. coli* BGs *per se* had no cytotoxic impact on RAW 264.7 cells independent of the time of coincubation. No effect was seen after short-term coincubation of RAW 264.7 cells with RV-loaded BGs and with free RV (data not shown). However, enhanced metabolic activity was found after treatment of RAW 264.7 cells with RV-loaded BGs in a MOI of 10 ($P=0.0008$) after 24 h (**Fig. 5B**).

Furthermore, the impact of 15 μM and 30 μM of externally added RV on cell viability was investigated. **Fig. 5B** shows that the lower RV (15 μM) concentration had no impact on cell viability. Viability, however was decreased (81.23 $\pm$ 10.6 %) after treatment with 30 μM RV. An almost similar decrease in cell viability was observed with RV-loaded BGs ($P=0.0462$) at the MOI of 1000 which on average reduced cell viability by 10 % due to the BG delivered RV.

Discussion

In the present study we have demonstrated that BGs are inducing NO formation in macrophages and that this response can be modulated by RV-loaded BGs. Thus, our findings open a new way for therapeutic approaches using BGs to fight macrophage-associated microbial diseases.

It is known in general, that pathogens are phagocytosed by cells of the innate immune system like macrophages and dentritic cells of primary antigen presenting cells (APCs). It has been demonstrated that BGs are preferentially recognized and taken up by various macrophage cell lines and dentritic cells [29-31]. In accordance with earlier findings, uptake was confirmed in the RAW 264.7 macrophage cell line after short-term coincubation of BGs. Interestingly, microscopic observations with CLSM showed that macrophages are capable to engulf single as well as cluster of BGs.

It is known that toll like receptor (TLR) signalling in macrophages is required for anti-pathogen responses, including the biosynthesis of NO radicals [32]. This immediate response is extremely beneficial to cure pathogen-caused diseases, e.g. Leishmaniasis or Tuberculosis which has been demonstrated in several studies [2, 4, 5]. Because LPS is renowned for the induction of iNOS through interaction with TLR-4 [1], we conducted comparative experiments between long-term incubation (20 hrs) with free LPS and short-term incubation with various amounts of BG (20 min followed by determination of nitrite accumulation after 20 hours) in RAW 264.7 cells. It turned out that treatment of macrophages with *E. coli BGs* in a MOI of 10 showed the same effect than 1 ng/ml LPS.

Investigations from Panaro et al. with human macrophages demonstrated a direct correlation between LPS-induced NO generation and the killing of intracellular Leishmania parasites. The efficacy was significantly reduced in both parameters when L -NMMA, a competitive inhibitor of iNOS, was present [33]. As they link NO-production and anti-leishmanial effects we analysed the relation between the observed particle-dependent BG-cell association with the extent of NO-release and found a significant positive correlation (Spearman $r = 1$; P value = 0.0167).

RV, which possesses also antileishmanial activities [22, 23], was shown to influence parameters of innate immunity and prior studies demonstrated its suppressing character towards iNOS induction [16, 24]. In order to investigate whether such a reduction of NO generation can also be achieved by the uptake of RV-loaded BGs into macrophages, the effect of RV, either coincubated together with empty BGs or delivered intracellularly *via* BGs, on BG-induced NO production by RAW macrophages was determined. A highly significant decrease in radical release was obtained by application of RV bound in BGs when compared with empty BGs, demonstrating that RV-loaded BGs were successfully taken up by RAW 264.7 cells and that the

compound retained its activity. Moreover, the externally added pure compound at a concentration of 3 µM, which is roughly 6 times higher than the concentration delivered by BGs in a MOI of 100, had no impact on NO generation in comparison to the BG loaded application which reduced radical release by about 15 %.

As BGs possess all their pathogen-associated molecular patterns (PAMPs) like their natural counterpart, they are very effectively recognized by the corresponding host innate immune receptors. TLRs recognize bacterial LPS with TLR4 and flagellin with TLR5 [7]. Uptake of BGs in the murine RAW 264.7 macrophage cell line is mainly related to TLR4 as they hardly express TLR5 [34]. On the other hand, in a recent study it was shown that *E. coli* BGs enter primary keratinocytes by the flagellin-dependent TLR5 pathway as well as other uptake mechanisms (Abtin and Lubitz, to be published). Moreover, it was demonstrated that BGs lead to induction of the antimicrobial peptides psoriasin (S100A7c) and human β defensin-2 (hBD-2) in a particle-dependent manner. Furthermore, *E. coli* BGs strongly induced the release of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8.

To our knowledge, the cellular uptake mechanism of RV is still unclear. Till now, no specific receptor has been found for the drug. Due to its structure, RV is able to interact with cell surface receptors like estrogen receptors or integrins [16, 35]. Our findings with the intracellular delivery of RV by RV-BGs in macrophages which leads to modification of NO release, leads to the presumption of the existence of an internal RV receptor. Therefore, we propose the term ERVR (endogenous resveratrol receptor) for such a hypothetical receptor.

It is known that bacterial LPS is responsible for manifold pathophysiological effects on a wide variety of mammalian cells. In the worst-case, endotoxic shock and multiple organ failure followed by death could be the consequence. This endotoxicity is mediated through the activation of the host immune and inflammatory cells, especially mononuclear phagocytes, which produce numerous bioactive mediators, including tumor necrosis factor alpha (TNF-alpha), interleukin 1, IL-6 and nitric oxide [36].

As the endotoxin content of Gram-negative bacteria is also present in BGs, safety and toxicity studies with BGs have been performed. It has been shown that BGs derived from pathogenic E. coli O26:B6 and S. typhimurium C5 investigated for their endotoxic activity by the use of standard Limulus amoebocyte lysate (LAL) assay and 2-keto-3-deoxyoctonate (KDO) assay, exhibited only 2-5% of the endotoxic activity compared to free LPS [28, 29]. Cell culture experiments with RAW 264.7 cells revealed 100-fold more BGs were required for the secretion of tumor necrosis factor alpha (TNFα) and prostaglandin E2 (PGE2) synthesis than compared to free LPS [28]

Cytotoxic effects were also investigated after BG application to RAW cells in the present study. No cell killing effects of the *E. coli* BGs *per se* were observed even when incubation was performed up to 24 hrs and cell viability was only reduced when RV was entrapped into BGs

(4.6 fg RV/BG). Assumed that all applied BGs (MOI of 1000) will be taken up by the macrophage population, the level of 30 µM RV would be reached. However, we know from confocal microscopic investigations that roughly 30-50 % of the applied BGs are taken up by the cells which would account for an intracellular concentration of 9-15 µM RV.

Considering that NO-release as well as RV concomitantly contribute to antimicrobial effects and the fact that BGs enhance cellular cytokine secretion and antimicrobial peptides, highlights the BG system after tailoring for its specific purpose as a novel clue for therapy of intracellular pathogens.

Figures

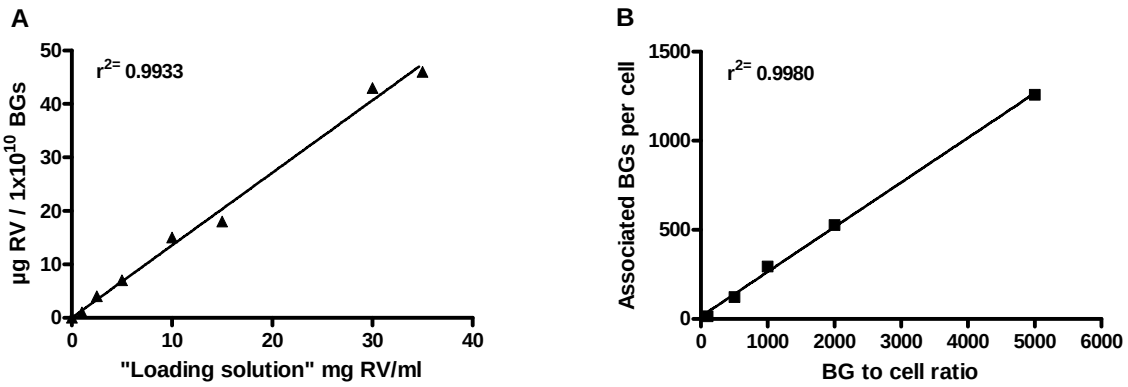

Fig. 1. A, The correlation between the RV concentration used for BG loading ("loading solution") and the recovered amount from loaded *E. coli* BGs (after ethanol extraction) is depicted by linear regression. Values were obtained by HPLC measurements. **B**, Linear regression between applied and cell-associated (attached and intracellular) FITC-labeled BGs after 20 min of BG coincubation with RAW 264.7 cells. Values represent means of four independent measurements and were calculated as described in materials and methods.

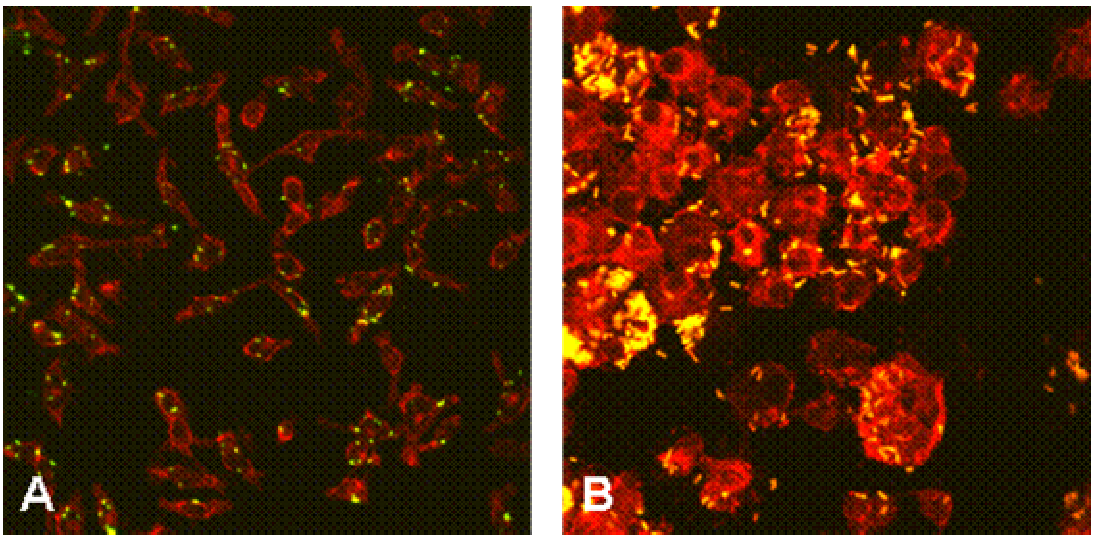

Fig. 2. Adherence and uptake of BGs by RAW 264.7 macrophages. Confocal laser scanning microscopy was performed after incubation of macrophages with FITC-labeled *E. coli* BGs in a BG to cell ratio of 1000. RAW 264.7 cells were stained with Texas-Red Phalloidin. FITC-marked BGs located inside the cell appear yellow, and not engulfed appear green. Images display a representative single z-stack of various optical sections. Pictures were taken with a 20-fold objective after 20 min (**A**) or with a 63x oil objective (**B**) after 40 min incubation. No image processing steps have been performed.

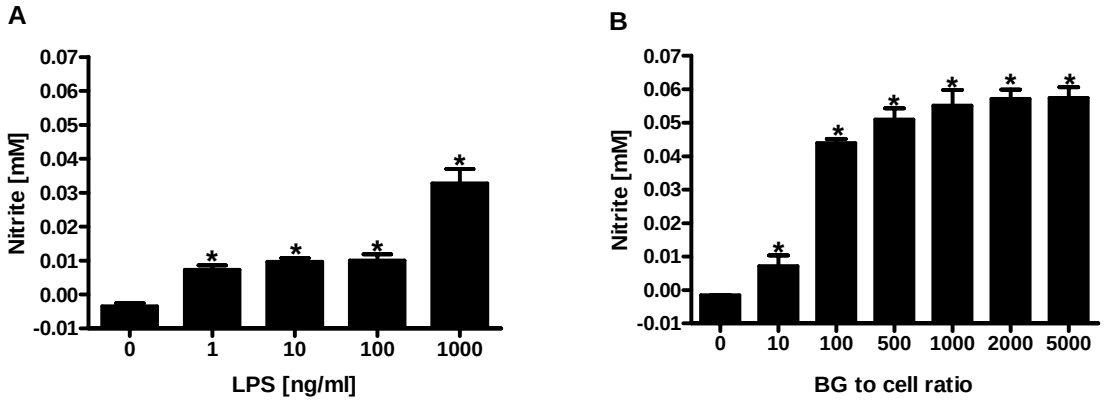

Fig. 3. Induction of NO production. **A**, RAW 264.7 cells were treated with different LPS concentrations (1-1000 ng/ml) for 20 hours. **B**, Impact of increasing amounts of BGs on NO generation by RAW 264.7 cells after 20 min of coincubation followed by 20 hrs cell cultivation in the absence of BGs. Accumulated nitrite concentrations were determined by the Griess assay as described in materials and methods. Bars indicate mean values + SD. Each experimental point represents values form four independent experiments measured in triplicate.

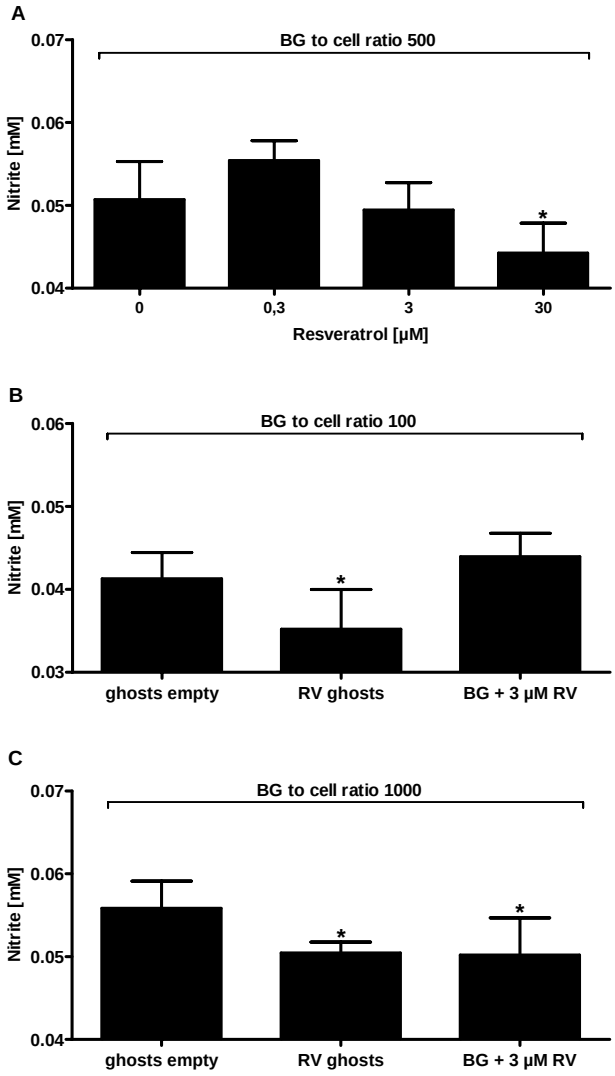

Fig. 4. Impact of RV and RV-loaded BGs on the NO production by RAW 264.7 cells induced by different amounts of BGs. **A**, BG-induced (BG to cell ratio of 500) NO production by RAW macrophages in the absence and presence of different RV concentrations (1-30 µM). **B**,**C** NO generation in response to BGs (BG to cell ratio of 100 (**B**) and 1000 (**C**)) and RV treatment using either RV-loaded BGs (RV ghosts) or empty BGs and externally added RV (3 µM). Based on the calculation from cell associated BGs, a BG to cell ratio of 100 and 1000 would correspond to a RV concentration of 0.48±0.25 µM and 8.86±2.21 µM, respectively. Coincubation was performed in all experiments for 20 min. After 20 hrs accumulated nitrite (**A-C**) was determined by the Griess assay as described in materials and methods. Bars indicate mean values + SD. Each experimental point represents values from four independent experiments measured in triplicate. Asterisks indicate values which are significant different from their respective control ($p < 0.05$).

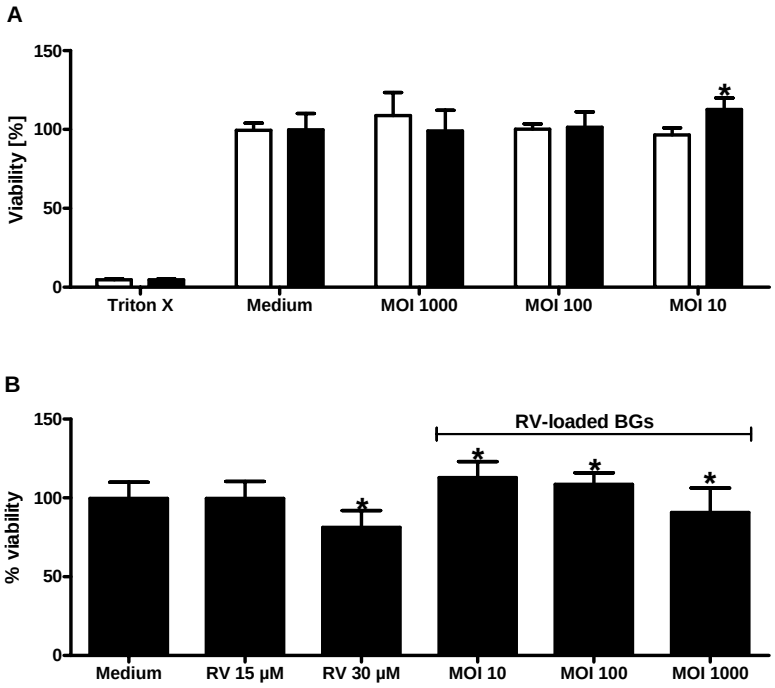

Fig. 5. Influence of empty and RV-loaded BGs on cell viability. **A**, Impact of empty *E. coli* BGs on the viability of RAW 264.7 macrophages. Cells were incubated with three different BG to cell ratios (MOI 10, 100, 1000) for either 20 min followed by a 20 hours recovery period (white bars) or for 24 hours (black bars). **B**, The cytotoxic effect of RV in comparison to the implication of RV-loaded BGs (46 µg RV/1x10^{10} BGs) is depicted. Cell viability was assessed by use of the neutral red assay. Bars indicate mean values + SD. Each experimental point represents values form four independent experiments measured in triplicate. Asterisks indicate values which are significant different from their respective control ($p < 0,05$).

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Appendix

A.1. Supplementary data for Chapter 1.

Impact of freeze drying and lyophilization media on the size of BGs

In order to optimize the BG-production process, different lyophilization solution were analyzed. *E. coli* BGs were lyophilized either in water, saline or in different 1 or 5% sugar solutions (glucose, saccharose, trehalose and lactose). After freeze-drying, the lyophilized BGs were weighted and compared to water dried samples. It turned out that BGs dried in 1% sugar solutions as well as saline were two time heavier than those lyophilized in H_2O and the 5% sugar lyophilization-solution resulted in 8 times higher values (data not shown).

To determine size and clustering behaviour of the samples, BGs were resuspended in 0.2% Tween-20 solution and were analyzed with a Shimadzu laser diffraction particle size analyzer SALD-1100 (Shimadzu Corp., Japan). The samples were measured in triplicate and results are shown in **Fig. 1**.

More or less, the same distribution was found for all investigated BGs and only BGs which were freeze dried in 5% glucose showed a shifted size pattern. As we know from microscopic studies, lyophilized BGs tend to form clusters which can hardly be separated by resuspension. Such a diverse size distribution, single cells and clusters, was also obtained by the laser diffraction analyses. In contrast to this, viable *E. coli* bacteria, which were investigated in their late phase, were completely homogenous in their size (1.77 µM; not shown). As it can be seen in **Fig 1**. only 5 % of the lyophilized BGs were found within the range of 1.3-2.6 µM and maximum peaks were obtained in the range of 11-22 µM (representing more than 30% of the BGs).

APTase activities of lyophilized BGs after long term storage

The activities of the membrane bound ATPase of three batches of lyophilized *E. coli* NM522 (pGLysivb) BGs, with different production dates (2001, 2005, 2006), were investigated Enzyme activity was estimated by the release of inorganic phosphate. The used method is based on the quantification of the green complex formed with acidic malachite green and ammonium-molybdate and free orthophosphate and was performed as described elsewhere [1]. ATPase activity was defined as the amount of enzyme which releases 1nmol of inorganic phosphate per mg protein. The results are depicted in **Fig. 2** and no significant differences were observed within five years of storage at ambient temperature.

Size determination of bacterial membrane vesicles

In order to prevent the leakage of water-soluble substances out of the BGs, BGs can be sealed by fusion of the BG's membrane with bacterial membrane vesicles at the edges of the lysis pore [2].

It has been reported, that the membrane vesicles fractions, produced by the French press method are homogenous in quality and in size (50-200 nm) [2]. As the generated lysis hole of BGs is not uniform in size and reaches up to 200 nm, the vesicle sizes have to be determined to optimize the sealing process.

Therefore, bacterial membrane vesicles were prepared by the use the French press method by which *E. coli* NM522 bacteria were disrupted under high pressure with different psi (pounds per square inch).Due to this treatment method, right-side out (outer membrane out) and inside out (cytoplasmatic membrane out) vesicles are formed which can be separated according to their density by sucrose centrifugation ([2]; **Fig. 3**.)

The obtained samples were investigated for their size with the Zetasizer Nano (Malvern Instruments Ltd., UK). The z-average (d-nm)-values were used for evaluation and results are depicted in **Fig. 4.** It can be seen that the vesicle size in the crude fraction **(Fig 4A.)** was slightly higher (mean value of all samples + SD 258.5+37.67) compared to the inner membrane fraction (155.8+17.31 nm) as shown in **Fig. 4B**. However, results from statistical analyses demonstrated that all the obtained vesicles were homogenous in size and were 7-11 fold smaller than the natural *E. coli* bacterium (1767.3+42.7 nm).

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Figures

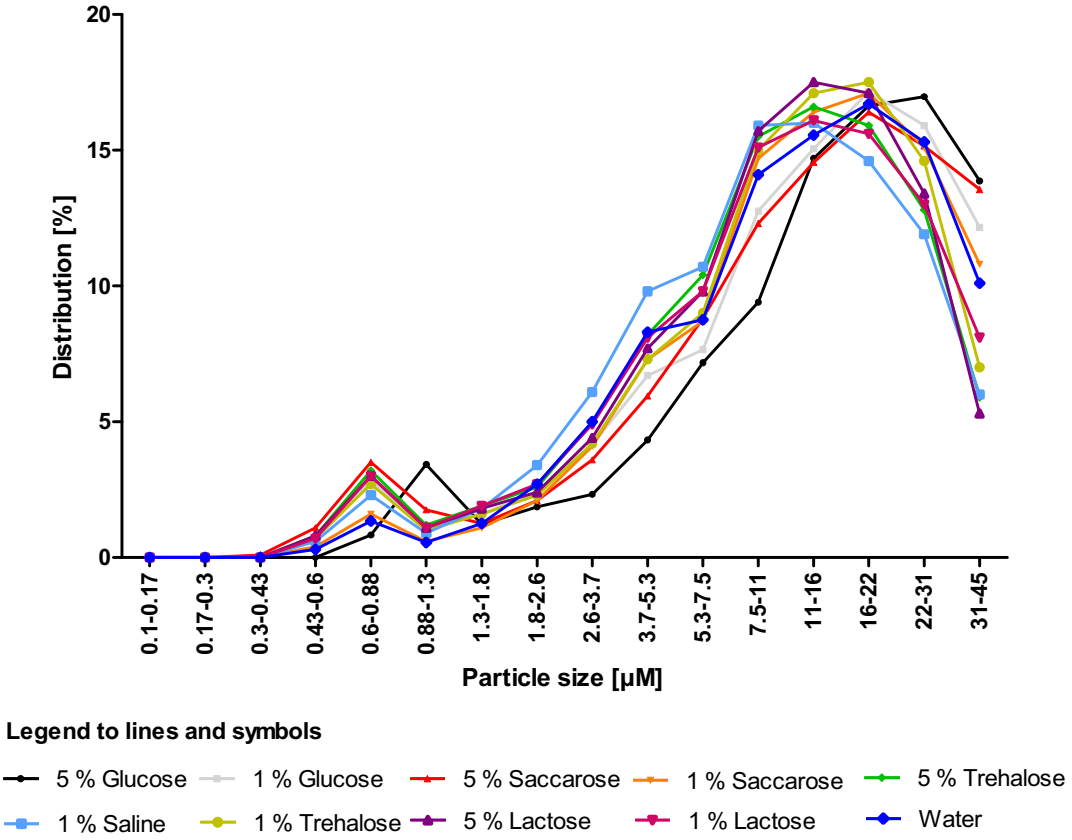

Fig. 1. Size distribution of *E. coli* NM522 (pGLysivb) BGs after lyophilization in different media. The figure shows the particle size distribution measured with the Shimadzu laser diffraction particle size analyzer SALD-1100 after resuspension in 0.2% Tween-20 solution.

Fig. 2. Determination of ATPase activity of lyophilized *E. coli* NM522 (pGLysivb) BGs. Enzyme activities were determined by the release of inorganic phosphate. Bars represent mean values + SD obtained from four independent measurements.

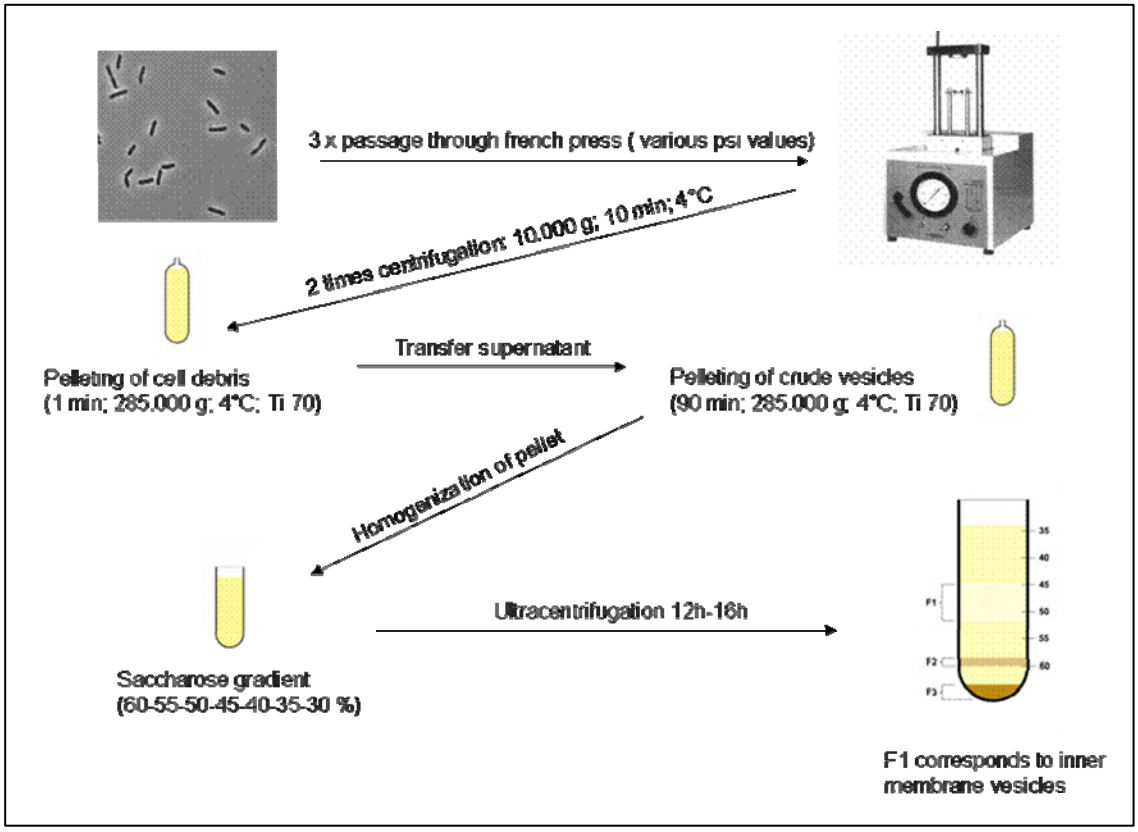

Fig 3. Schematic representation of the experimental design of the preparation of bacterial membrane vesicles.

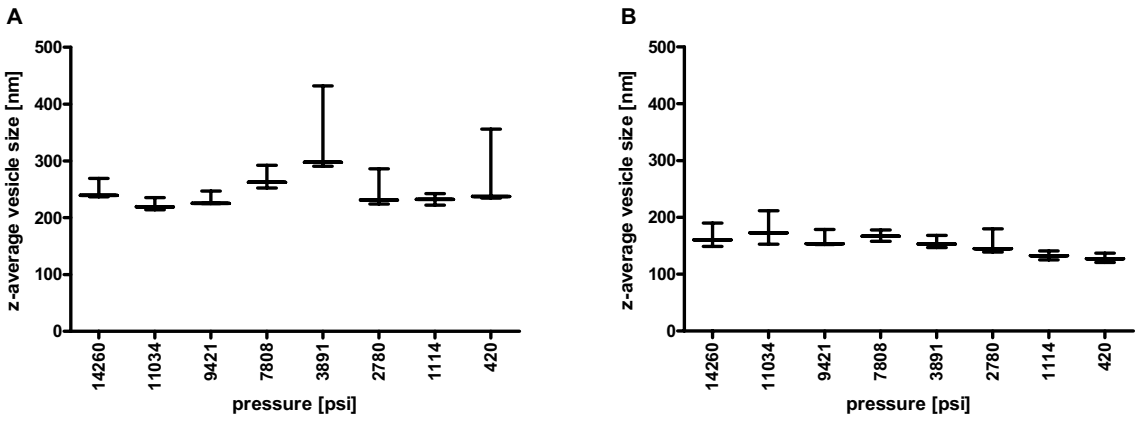

Fig. 4. Determination of bacterial membrane vesicle sizes obtained after disruption of *E. coli* cells by the French press under different pressure conditions. The box and whisker diagrams depict values obtained from three measurements of crude membrane vesicles (**A**) and inner membrane vesicles (**B**) with a Zetasizer.

A.2. Supplementary data for Chapter 2.1.1.

Impact of DIG-loaded BGs on the viability of human cell lines

The cytotoxic effects of empty *Escherichia coli* NM522 (pGELys) BGs and *Escherichia coli* Nissle 1917 (pGLysivb) BGs in comparison to DIG loaded BGs (loading solution 100 mM) was also tested in the following human cell lines: CCL 20.2 (conjunctiva), SK-MEL-28 and A375(melanoma) and HT29 (colon). Cytotoxicity assays were performed as described in Materials and Methods.Treatment with DIG-BGs led to significant decrease in cell viability in all investigated cells after 24 hrs (**Fig. 1).** The highest sensitivity towards the delivered chemical was found for the conjunctival cells CCl 20.2 where only 6-11% stayed alive. Whereas, no differences were found on the impact on cell viability between the delivered DIG by the two *E. coli* BGs in the colon cell line HT29, high variations were observed with the melanoma cell lines and conjunctival cells. High differences were found in CCL 20.2 cells after treatment with DIG-loaded *E. coli* Nissle BGs and *E. coli* NM522 BGs (P=0.0005), whereas the latter one caused more reduction in cell viability. The opposite was observed with the melanoma cell lines. Treatment with DIG-loaded *E. coli* Nissle BG resulted in a significant decrease of cell viability in A375 cells (P=0.0009) and SKMel-28 cells (P=0.0023) than compared to the results obtained with *E. coli* NM522 BG-bound DIG. The biggest variation between the two applied BGs were found for SKMel-28, where on average 50 % more cells were killed by *E. coli* Nissle BG delivered DIG. In each experiment, the impact of the DMSO loaded ghosts, which served as solvent control from loading experiments and were found to do not differ from empty ghosts, was investigated. As depicted in **Fig. 1**, the viability of all cell lines was not impaired after treatment with the BGs *per se* except for the melanoma cell line SKMel-28. Both strains caused cytotoxic effects in these cells to a more or less the same extent (88.22 $\pm$ 9.32 for Nissle and 88.21 $\pm$ 7.39 for NM522).

However, due to the high discrepancies of the two BG strains in SKMel-28 cells concerning their cytotoxic impact when loaded with DIG, it is assumed that this may be cause by different adherence/uptake rates of *E. coli* ghosts. In order to confirm this assumption, qualitative fluorescence adherence assays were conducted. For this purpose, the intensity of the two FITC-labelled BGs was compared after incubation of HT29 and SKMel-28 cells in a MOI of 1000 for 24 hrs. The fluorescence was determined with the Tecan Infinite 200 device (excitation 480 nm; emission 520 nm).

In agree with the obtained results from cytotoxicity studies (**Fig. 1**), no differences were observed between the *E. coli* BGs with the colon cells (**Fig. 2**). However, high differences were found for the melanoma cell line. Adherence/uptake rates were statistically

different (p= 0.0024) between *E. coli* NM522 and *E. coli* Nissle, whereby the latter was giving the highest fluorescence units. (**Fig. 2**).

Figures

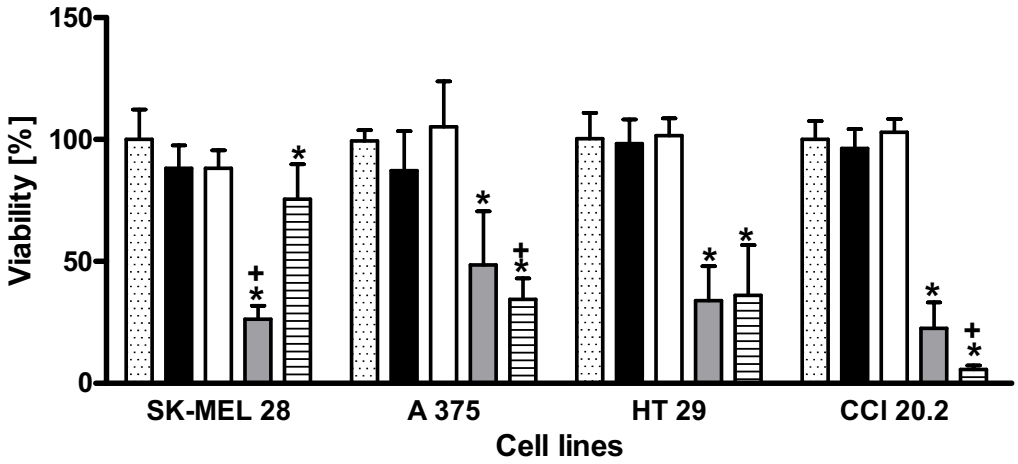

Fig. 1. Cytotoxic impact of DIG-loaded E. coli BGs on various human cell lines. The cells were treated with empty *E. coli* Nissle 1917 BGs (**black bars**), empty *E. coli* NM522 BGs (**white bars**) or with BGs which were loaded in 100 mM DIG (**grey bars**: Nissle and **striped bars** NM522). Serum free-medium treated cells (**dotted bars**) served as controls.
The viability was determined in neutralred assays after treatment for 24 hrs (MOI 1000). Bars represent values of means + SD (N=8-30). Asterisks indicate values which differ significantly from controls ($p \leq 0.05$). Crosses mark differences between the two *E. coli* BGs.

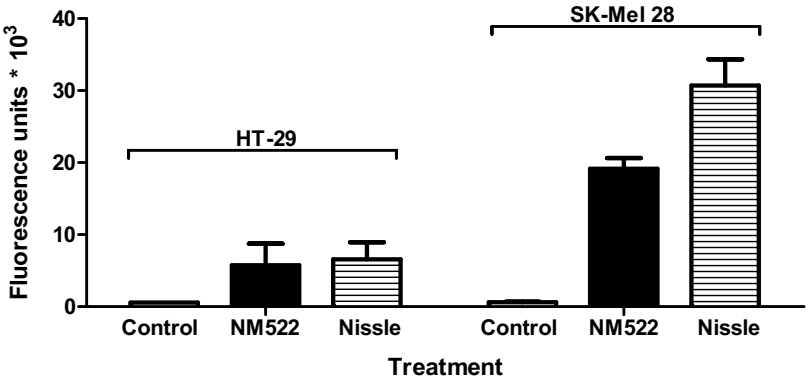

Fig 2. Comparative qualitative adherence assays with FITC labelled *E. coli* BGs indicate the difference of NM522 **(black bars)** and Nissle ghosts **(striped bars)** for their tendency towards for the colon cell line HT29 and melanoma cell line SK-Mel 28. The fluorescence background levels of the cells are depicted as white bars. Values represent means + SD obtained from three independent measurements.

A.3. Supplementary data for Chapter 2.2.1.

Adherence and uptake of BGs by CCL 20.2 cells

Internalization of the BG was verified by use of confocal laser scanning microscopy (CLSM). For this purpose cells were seeded in 8-well chamber slides (Falcon CultureSlide) and were allowed to attach. The medium was replaced by 200 µl sf-medium containing FITC-labeled BG (BG to cell ratio 1000) and the slides were further incubated for 1 hr. Subsequently, the cells were washed twice with PBS and 1% paraformaldehyde was added for fixation (20 min, room temperature). Another two washing steps with PBS were performed before the cells were permeabilized with 0,5 % Triton X-100 for 20 min.; followed by other two washing procedures. Then, 100 µl of freshly prepared PromoFluor 590-conjugated Phalloidin (5 µl methanolic 100 U / ml dye-stock; PBS; 1 % BSA) was added to visualize cell boundaries. After 45 min incubation the slides were washed again twice with PBS and imaged by CLSM. Images displaying representative single z-stacks of various optical sections after coincubation with different BGs (*S. flexneri*, *E. coli* NK9373 and *M. haemolytica*) are depicted in **Fig. 1**. It can be seen that FITC-labeled BGs which are already internalized by CCL 20.2 cells stained in red, appear yellow. Green colored BGs represent those which are not engulfed but attached to cells.

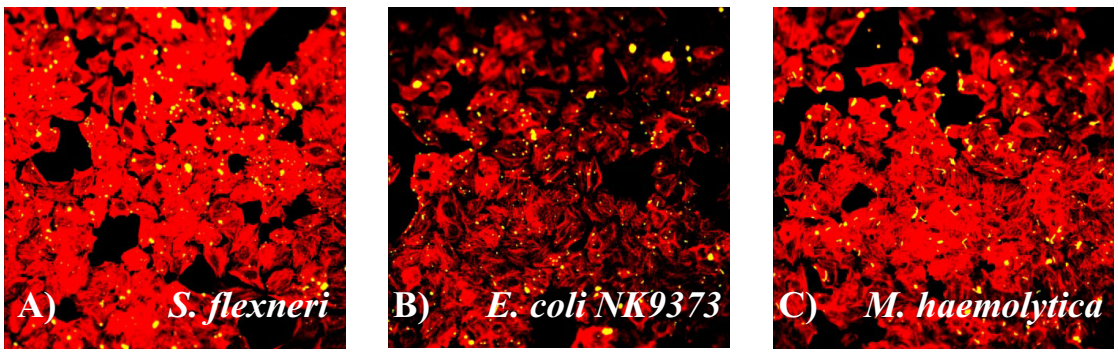

Fig. 1. Adherence and uptake of BGs by the CCL 20.2. cells. Confocal laser scanning microscopy was performed after incubation of conjunctiva cells with FITC-labeled BGs (*S. flexneri* (**A**), *E. coli* NK9373 (**B**) and *M. haemolytica* (**C**))in a BG to cell ratio of 1000. CCL 20.2 cells were stained with Texas-Red Phalloidin. FITC-marked BGs located inside the cell appear yellow, and not engulfed appear green. Images display a representative single z-stack of various optical sections.

A.4. Supplementary data for Chapter 2.2.2.

Detoxification of the anti-glaucoma eye drops Pilocarpine hydrochloride and Cosopt

Since protective effects were observed with various BGs towards BAC induced cytotoxicity in CCL 20.2 cells, preliminary experiments were conducted with the BAC containing eye drops Pilocarpine hydrochloride (0.2 mg BAC/ml) and Cosopt (0.075 mg BAC/ml) in combination with BGs (detailed information about eye drops is provided in the Appendix section A.7. "Comparative *in vitro* studies of the toxicological impact of eye drops used for glaucoma treatment"). Cytotoxicity assays were performed as described in Materials and Methods (Chapter 2.2.2.). Again, the cytotoxic impact of the investigated drugs was found to be significantly reduced in presence of BGs.

Fig. 1. depicts the cytotoxic effects caused by single and double applications of P-HCl (dilution 1:10) alone and in combination with ETEC-BGs for 20 min followed by a 24 hr recovery period. Cell viability dropped from 40 % for a single application to 4 % cell viability after double treatment with P-HCl. Beneficial effects were found when ETEC-BGs were present in the P-HCl solutions. Cell numbers significantly increased after the first ($p=0.0024$) and second ($p=0.0015$) application. Compared to P-HCl treatment alone, 2-4 fold enhanced cell viability was seen with BGs.

In experiments, conducted with the eye drop Cosopt, also other BGs were investigated for their detoxification potential. As shown in **Fig. 2**., this ophthalmological solution (diluted 1:20) dramatically decreased the percentage of viability of CCL 20.2 cells after treatment for 24 hrs (36.67+3.06 % were found to be alive). However compared to Cosopt treated cells, protective effects were found when BGs were present and viability was enhanced up to 57 %.

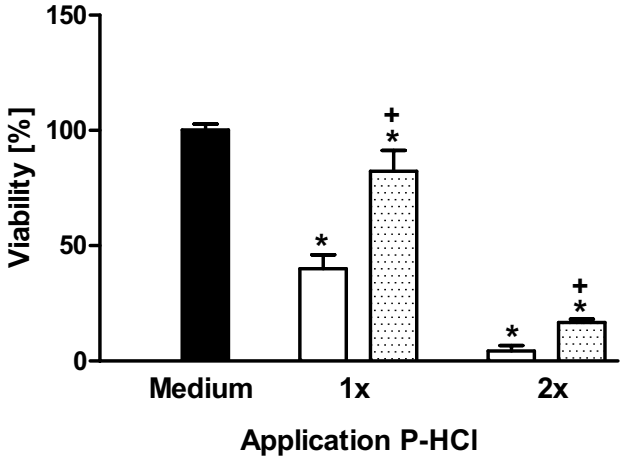

Fig. 1. Impact of Pilocarpine hydrochloride (P-HCl, 2 %) alone or in combination with ETEC-BGs on the viability of CCL 20.2. cells. The figure shows the acute toxic effects towards the conjunctival cell line after single/double application of the drug P-HCl (dilution 1:10, white bars) in combination with BGs (dotted bars) for 20 min followed by a 24 hr recovery period. Cells were treated with BGs in a MOI of 1000 and the viability was determined by the use of the neutralred assay. Bars represent mean data + SD obtained from three independent measurements. Asterisks indicate values which differ significantly medium treated cells (black bar) and crosses mark differences of P-HCl induced cytotoxicity in presence of BGs ($p < 0.05$).

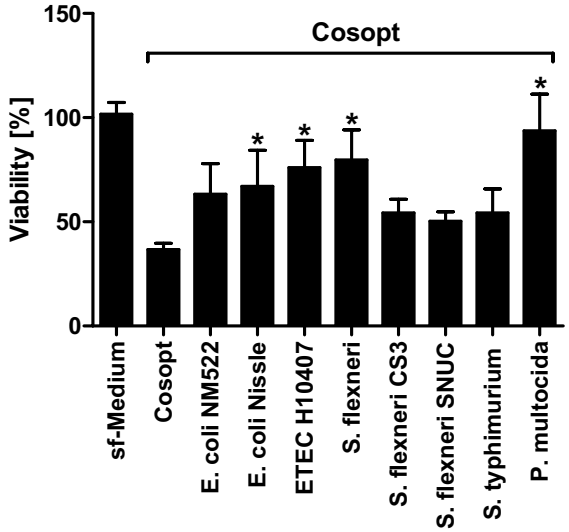

Fig. 2. Impact of Cosopt alone or in combination with different BGs on the viability of CCL 20.2 cells. The figure shows the effects on the viability of the conjunctival cell line after long term treatment with Cosopt (dilution 1:20) in combination with different BGs for 24 hrs. Cells were treated with BGs in a MOI of 1000 and the viability was determined by the use of the neutralred assay. Bars represent mean data + SD obtained from three independent measurements. Asterisks indicate values which differ significantly from Cosopt treated cells ($p < 0.05$, 1way ANOVA).

A.5. Supplementary data for Chapter 2.3.2.

Increased fluorescence of FITC-labeled BGs after coincubation with RAW 264.7 cells

Incubation of the macrophage cell line RAW 264.7 with FITC-labeled *E. coli* NM522 BGs resulted in a time dependent increase of FITC fluorescence.

The experiments were conducted as described in Materials and Methods but were extended for several timepoints. Even though the macrophages were coincubated with the same ratio of BGs, giving the same fluorescence, the fluorescence intensity of the FITC labeled BGs in the media-significantly increased after 12 hrs and 24 hrs ($p<0.0001$). As depicted in **Fig. 1**., the fluorescence intensity was 2-3 fold higher than at the beginning.

In order to determine whether these effects are caused by the pH of the cell's interior or by cell's associated enzymes, FITC-labeled BGs were resuspended in different solutions with different pH values or enzyme (**Table 1**). Fluorescence and pH values were measured at the beginning as well as after 24 hrs incubation.

The results are depicted in **Fig. 2**. It can be seen that citric acid with the lowest pH immediately quenched FITC fluorescence and lasted over 24 hrs. Sodium buffer, which had the highest pH value (~ 10), led to PBS-comparable values and had no further impact on the fluorescence after 24 hrs. The highest fluorescence units were found with the serine proteases proteinase K and TrypleEx. Both digestive enzymes resulted in an increase of fluorescence after the incubation time. However, incubation of FITC-labeled BGs in cell-free lysate-fractions of RAW cells, which were obtained by repeated thawing and freezing in liquid nitrogen, did not result in any fluorescence increase.

As the pH-values did not change significantly after 24 hrs incubation with FITC-labeled BGs (**Table 1**) the increase of the intensity of the fluorochrome appears to be more related to digestive enzymes of the macrophages.

Tables and Figures

Table 1. pH values of different solutions after incubation with FITC-labeled BGs

Treatment	pH (0 hrs)	pH (24 hrs)
PBS	8	7.5
Na-Buffer	10	10
Citric acid	<4.5	4.5
TrypleEx	7	7
Proteinase K	7.5	7.5
RAW-lysate	8.5	8.5

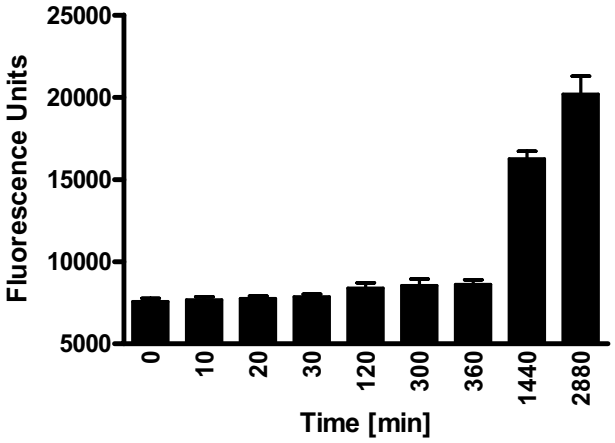

Fig. 1. Time dependent increase of the fluorescence of FITC-labeled BGs after coincubation with macrophages. RAW 264.7 cells were incubated for different timepoints with *E. coli* NM522 BGs. A significant increase in FU can be seen after 24 hrs. Fluorescence values were determined with a Tecan microplate reader. Values represent means + SD.

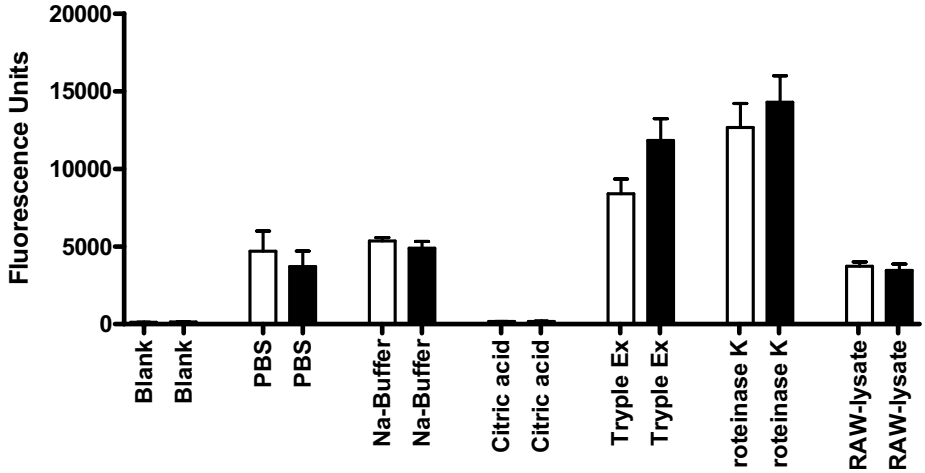

Fig. 2. Fluorescence behaviour of FITC-labeled BGs in different solvents. White Bars indicate fluorescence values measured at the beginning and black bars represent measurements after 24 hrs. Results are shown as mean values + SD.

A.6. Loading capacity of BGs with the coffee compounds kahweol and cafestol

Introduction

Coffee is, after water, one of the most widely consumed beverages in the world. Its consumption has been associated with numerous health benefits, including the prevention of cancer (i.e. in the liver and colon) or other chronic diseases such diabetes, Alzheimer and Parkinson [1, 2].

The diterpenes kahweol (K) and cafestol (C) are two major lipid components of green coffee beans and their chemoprotective effects were demonstrated in several studies. For example, in animal experiments they were found to be antitumorigenic against the carcinogene 7,12-dimethylbenz[a] anthracene (DMBA) in hamster's buccal pouch [3]. Also antimutagenic effects were seen with rat liver preparations against aflatoxin B1 and benzo[a]pyrene [4] and in rat an human cell cultures [5]. Another *in vivo* study demonstrated the chemoprotection of K/C against the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP), the so called cooked food mutagen [6]. It was found, that these coffee components modify the activities of the hepatic enzymes *N*-acetyltransferase (NAT) and glutathione *S*-transferase (GST) and provide therefore protective effects [2].

As in the present thesis, BGs have been already demonstrated for their preservation properties against oxidative damage and for their controlled drug release (Chapter 2.1.1.), we performed additional experiments in which the loading efficiency of BG with the coffee compounds C and K were investigated. Our findings show that all compounds were entrapped in a dose dependent manner. Furthermore we found, that the solvent highly contributes to the loading efficiency.

Materials and methods

Chemicals

Cafestol (purity > 95%) and kahweol /cafestol-palmitate (1:1 blend, i.e. 47% cafestol, 47% kahweol, 5% isomeric derivatives) were a kind gift from Prof. Wolfgang Huber (Institute of Cancer Research, Vienna).

Loading of BGs

25-50 mg lyophilized *E. coli* Nissle 1917 BGs (pGLysivb /BPl; 090421; $1{,}3x10^9$ particles/mg) were suspended in 1.2 ml of different C (5.1-114.7 mM) and K/C (0.7-14.1 mM) solutions. In order to determine the impact of the solvent on the loading efficiency, the compounds were dissolved with two ethanol and DMSO. The samples were incubated under shaking (650 rpm) for 30 min at 28°C. The loaded BGs were collected by centrifugation at 11300 g for 15 min and the pellets were washed five times with phosphate buffered saline. 2-3 mg BG aliquots were stored at -20°C until use.

Extraction and Quantification of cafestol and kahweol

C and K/C concentrations were analysed by reversed-phase high-pressure liquid chromatography (HPLC) as described by Gross et al. [7]

K/C-Palmitate loaded BGs were saponified by the addition of 2 ml methanolic 0.3 M potassium hydroxide solution. After incubation under agitation for 60 min (RT, IKA-VIBRAX agitator, speed 600), 800 µl 2-propanol-ethyl acetate (1:1, v:v) and 800 µl 0.5 M aqueous sodium dihydrogen phosphate were added. After extensive mixing, the solutions were transferred into C18/PRS solid –phase extraction cartridge tandems which were previously conditioned (C18 cartridges were rinsed with 3 ml 2-propanol-ethyl acetate and PRS cartridges were rinsed with 3 ml 1 M HCl, 20 ml water and 3 ml 2-propanol-ethyl acetate). The preparations were slowly eluted by air pressure (applied with a plastic syringe) and were concentrated by use of an exciccator to a final volume of 400 µl. After vortex-mixing the samples were filtered through a Nalgene filter tip (0.2 µM , zero dead volume) direct into HPLC microvials. C was extracted from BGs by two times addition of 500 µl ethanol (100 %) and vortexing for 10 min (1000 rpm). After centrifugation (13000 rpm; 8 min), the supernatants were collected and measured by HPLC.

Chromatographic analysis was performed using a Hewlett-Packard HP 1100 HPLC-FLD model.The mobile phase consisted of water (solvent A) and methanol (solvent B). Separations were carried out using linear gradient elution conditions (0 min: 30% A, 70% B; 20 min: 5% solvent A, 95% solvent B) and the flow rate was 1 ml/min. C was detected at 230 nm and K at 290 nm.

Results and discussion

In order to determine whether BGs can be used as carriers for the coffee diterpenes C and K (**Fig. 1**), loading experiments were performed with BGs, generated from the probiotic *E. coli* Nissle 1917 strain.

The loading of lyophilized BGs was performed by resuspension of BGs in different concentrations of C (5.1-114.7 mM) or K/C-palmitate (0.7-14.1 mM) by the use of different solvents (ethanol and DMSO). To determine the amount of the compounds in BGs, ethanolic extracts were performed and analyzed via HPLC. Clear correlations between the loading concentration of the compounds and recovered amount of the substances were observed. The results and the correlation coefficients (r^2), obtained from linear regression analyses, are depicted in **Fig. 2**. The highest loading efficiency was obtained after suspension of lyophilized BGs in a K/C (DMSO) solutions (**Fig. 3**).

Furthermore, the impact of the solvent on the loading efficiency is exemplified in **Fig.4**. Indeed, in comparison to ethanol, 2-4 fold more of C was bound in BGs when DMSO was used as the solvent.

Our findings show that high molecular weight compounds like the K/C-palmitates (MW 1106.86) can be successfully entrapped into BGs and that the polarity of the used solvent influences the loading capacity.

C and K were shown to cause modifications of the xenobiotic metabolism, such as the induction of GST and inhibition of NAT, thereby leading to chemopreventive effects. Therefore, studies should be conducted in which the impact of loaded BGs on these metabolic enzymes should be investigated. Furthermore, since coffee consumption has been associated with reduced incidence of colon cancer [1] and K/C was found to decrease the formation of colonic DNA-adducts [6], the effects of C and K loaded BGs should be further examined in genotoxicity assays.

The data from the preliminary study open a new application pathway for BGs as functional food ingredients. For example, C or K/C loaded BGs could be added to instant coffee, which contain only very low concentrations of C and K [7].

Figures

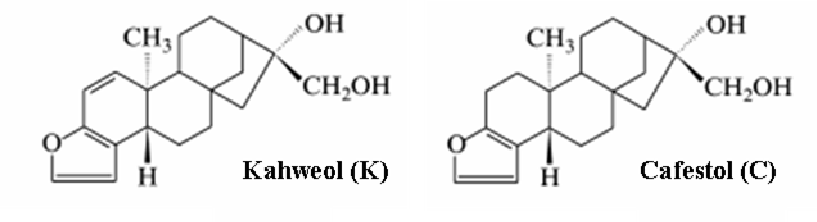

Fig 1. Chemical structures of the diterpenes kahweol (K) and cafestol (C).

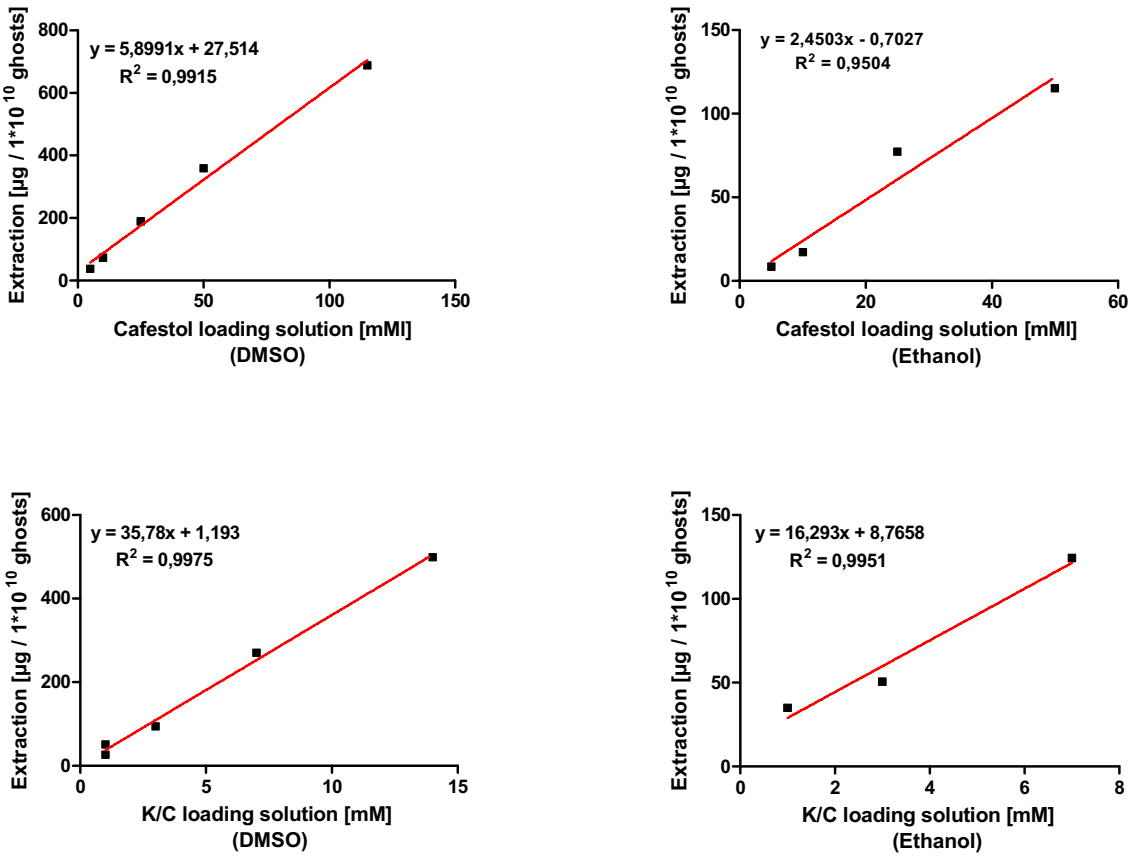

Fig. 2. Standard curves and linear regressions of the of the loading experiments. The plots depict mean values obtained from extraction experiments (µg chemical per $1x10^{10}$ ghosts) versus the loading solution (mg/ml). Squares represent mean values of at least two measurements.

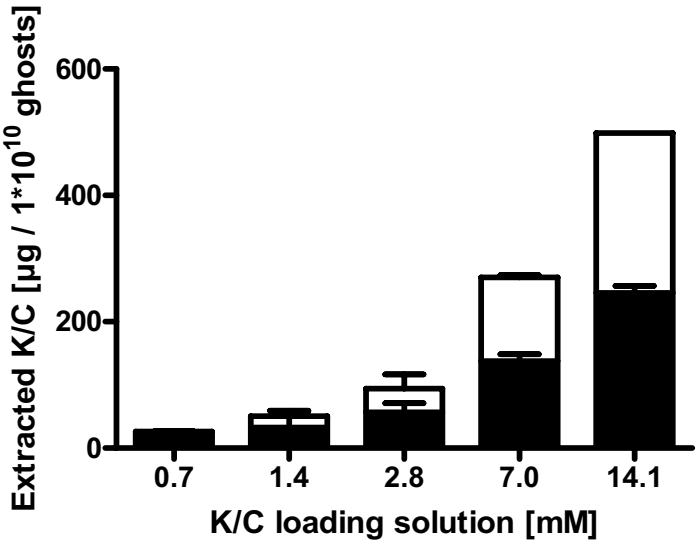

Fig. 3. Dose-dependent loading of *E. coli* Nissle 1917 BGs with the coffee compounds K/C-palmitate (mixture ratio 1:1) in DMSO. Bars represent means + SD obtained from two loading samples. Black bars show the cafestol and white bars the kahweol content of the measured samples.

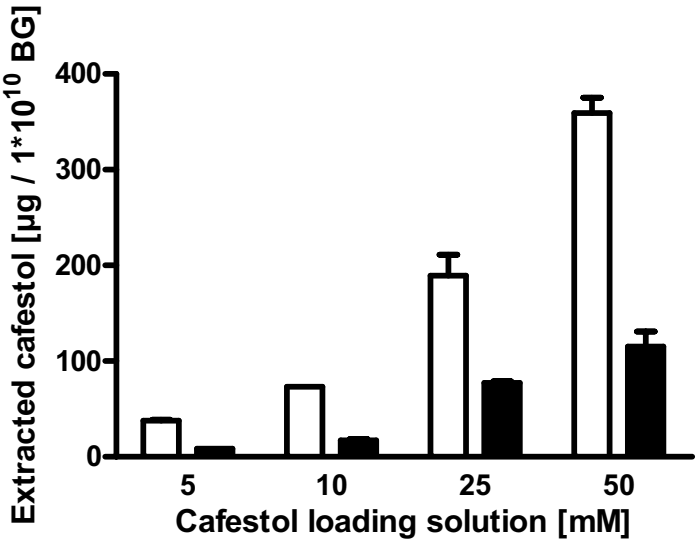

Fig. 4. Solvent dependent loading efficiency of lyophilized *E. coli* Nissle 1917 BGs with cafestol. White bars indicate the extracted values from loading procedures where DMSO was used as solvent; black bars show the results obtained from loading with ethanol as solvent.

References

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4. Cavin, C., et al., *The coffee-specific diterpenes cafestol and kahweol protect against aflatoxin B1-induced genotoxicity through a dual mechanism.* Carcinogenesis, 1998. **19**(8): p. 1369-75.
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6. Huber, W.W., et al., *Chemoprotection against the formation of colon DNA adducts from the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the rat.* Mutat Res, 1997. **376**(1-2): p. 115-22.
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A.7. Comparative *in vitro* studies of the toxicological impact of eye drops used for glaucoma treatment

Introduction

According to the WHO, glaucoma is characterized as a group of diseases which damage the eye's optic nerve and is the third leading cause of blindness in the world at the moment. Latest statistics from the organization show that more than 12 %, equitable to approximately 4.5 million people, of all the global blindness is caused by primary glaucoma. Higher numbers are predicted by Quigley & Broman for the prospective future [1]. They estimate that by 2020 about 80 million people will suffer from these diseases. While the primary prevention strategy of glaucoma remains unclear, early stage diagnosis gives rise to effective methods in surgical and medical treatment. The latter one is predominantly used as first-line therapy and it has been shown that intraocular pressure (IOP)-lowering agents, like prostaglandin analogues, play a major role [2].

As glaucoma is a neuropathy of the optic nerve, topically applied anti-glaucoma drugs have to cross the cornea and the conjunctiva to reach their sites of action. It has been demonstrated that the permeability of the pharmaceutical could be vastly enhanced by addition of an preservative [3].

However, since glaucomatous patients receive topical therapy for many decades, a lot of adverse events and side effects have been reported [4]. Besides pain and discomfort of the patient during instillation, development of cataracts, the leading cause of visual impairment, have been associated with antiglaucoma medication [5, 6]. Furthermore, the epithelium can respond to medication related stress by inflammation reactions which lead to severe damage of the cornea and conjunctiva itself [4]. The loss of vascularization and the exhibition of a spectrum of metaplasia ranging from loss of goblet cells, which is closely related with the dry-eye syndrome, and stratification as well as keratinisation have been demonstrated [4, 7, 8].

It is still a debate if the obtained side effects may be attributable to the active compound *per se* or if they are relatable to the preservatives in the drug solution. The quaternary ammonium compound benzalkonium chloride (BAC) is the most commonly used preservative in ophthalmic medications. Its cytotoxicity is well studied and it is known that the substance is responsible for proapototic and necrotic effects in vitro and in vivo [4, 9, 10]. Moreover, topical long term application of BAC has been shown to decrease aqueous tear basal secretion as well as MUC5AC-related mucin production and leads to deprivation of mucin producing goblet cells [4, 8, 11].

Since overexpression of oxidative stress markers was observed in the aqueous humor and plasma of glautomatous patients and increased levels of 8-oxo-deoxyguanosine were found in the trabecular meshwork [12-14], it has been suggested that oxidative stress could be a key player in glaucoma pathophysiology. It was demonstrated that ocular surface irritations were predominantly higher with preserved than with preservative-free IOP-lowering agents and indicated that reactive oxygen species (ROS) were partly involved [11, 15-18]. Several clinical and experimental studies showed that long-term use of BAC-preserved ophthalmic medications induced overexpression of inflammation-related receptors/molecules such as HLA-DR and intracellular adhesion molecule - ICAM-1 (CD54) expression [11, 19]. A positive correlation was found between class HLA-DR expression and the apoptotic Fas antigen (CD95) [20]. Furthermore, it was shown that BAC caused overexpression of the apoptotic marker Apo2.7 [9] and induced the activation of cell death receptor P2X7 due to ROS overproduction [16]. BAC-related oxidative stress correlated with ocular cytotoxicity [21] and was linked to apoptosis in conjunctival cells [22]. Furthermore, the transcriptional activation of the stress-related genes c-*fos* and c-*jun* after BAC-containing medications was shown [23]..

The conjunctiva represents a mechanical barrier against pathogens and protects the eye against allergens and toxic substances. Because these epithelial cells of the eye's font are the first tissues to be exposed by medical treatment,. a lot of studies were conducted with the commercial available human derived conjunctival epithelial cell line CCL 20.2 [9, 15, 16, 24].

The purpose of the present *in vitro* study was to determine the potential cytotoxic effect of BAC on CCL 20.2 cells and to investigate the cytotoxic from common topical glaucoma medications containing BAC. Moreover, toxicological studies were performed in parallel with human conjunctiva-derived epithelial cells obtained from biopsy material for comparative analysis with the conventional conjunctival cell line.

Materials and methods

Cell culture

The Wong-Kilbourne derivate of Chang conjunctival cells (clone 1-5c-4, ATCC CCL 20.2) were kindly provided by Prof. Bernd Binder (Medical University Vienna, Centre of Biomolecular Medicine and Pharmacology, Vienna, Austria). The cells were cultured under standard conditions (37 °C moist atmosphere of 5% CO_2) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat inactivated fetal calf serum (FCS, Sigma), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 10 mM HEPES buffer (Lonza, Verviers, Belgium), 0.1 mM MEM Non-Essential Amino Acids (NEAA, Lonza) and 5 µg/ml plasmocin (Lonza).

When cultures reached confluency, the cells were washed with phosphate buffered saline (DPBS, Lonza), detached with TrypLE^{TM} Express (Invitrogen), centrifuged and subcultured in the microtiter plates.

Human conjuctival biopsy specimens were obtained from patients who suffered either on chalazion or ectropion and were undergoing eye surgery. Informed and signed consent was obtained in each case in accordance with the Declaration of Helsinki from all subjects prior to surgery. Upon excision, the specimens (approximately 2x2 mm) were placed into sterile ACL-4 (provider) culture medium [25]. The explants were cut into small pieces and were placed into collagen-precoated (|Collagen solution type I from calf skin; dilution 1/20; Sigma-Aldrich) cell culture flasks. The tissue parts were allowed to attach and were covered with ACL-4 medium. The samples were incubated at 37 °C in a humidified atmosphere of 5% CO2. Culture medium was changed every 2-3 days. The development of the cultures was assessed daily by phase contrast microscopy. Confluent cultures were subcultured by trypsinization specify (Invitrogen). The cultured primary cells were named ME-1 (derived from chalazion surgery) and ME-2 (derived from extropion surgery).

Immunocytochemistry

Conjunctival epithelial marker expression was studies by use of antibodies against cytokeratin-7 (CK-7; mouse-anti-cytokeratin-7 FITC-conjugate; clone LP5K; isotype IgG2b) and vimentin (mouse-anti-vimentin PE conjugate; clone VI-RE/1; isotype IgG1); both obtained from Abcam (UK). Samples were stained according to the manufacturer's instructions. Briefly, cells grown on 96-well imaging culture plates (PAA Laboratories GmbH, Pasching, Austria) were fixed in cold methanol and washed twice with PBS. After 10 min of permeabilization with PBS containing 0,25% Triton X-100 the cells were washed three times with PBS and incubated with 1% BSA(provider) in PBS for 30 min. Next, the cells were incubated with antibodies in a final dilution of 1:50 at room temperature in the dark for 1 hr. After extensive washing with

PBS, the fluorescence was analyzed using a Zeiss LSM310 microscope. Obtained digital images were converted to black-and-white images (Adobe Photoshop CS3, Adobe Systems, Mountain View, CA).

Chemical treatment of conjunctival cells

Commercial available eye drop solutions, commonly used in glaucoma therapy, were investigated in the CCL 20.2 cell line as well as in cultured primary cells for their possible cytotoxic and genotoxic actions. The medications contained four different active components (Dorzolamide - carbonic anhydrase inhibitor, Timolol - beta-adrenergic receptor blocker, Brimonidintartrat - alpha2-adrenergic receptor agonist, and Pilocarpine hydrochloride - representative cholinergic agent) alone or in combination with or without different concentrations of the preservative-BAC (**Table 1**). All dilutions and further treatment steps were performed in serum-free media.

Cytotoxicity Assay

The neutral red uptake assay for the estimation of cell viability/metabolic activity was performed as described previously [26]. $1x10^5$ cells per well plated 24 hrs before adding of drug in 96-well plates were incubated for different timepoints in 200 µl solution of the antiglauoma drugs (diluted 1:10) (**Table 1**). To mimic a repetitive character of drug treatment, cells were also re-exposed to the drugs, 60 min after the initial treatment. The cell viability was determined after a 24 hrs recovery period in complete medium. The recovery phase was included to simulate the clinical conditions in which the conjunctival tissue may recover after topical eye drop administration.

After each treatment, two washing steps with PBS were performed. Subsequently, the cells were incubated with 100 µl of NR (80 µg/ml final concentration; Roth) for two hrs (37°C; 5 % CO_2). After the dye had been discarded, the wells were washed two times with PBS and dye was released by the addition of 100 µl of the destaining solution (1 ml acetic acid, 73 ml 96 % ethanol and 26 ml deionized water). After shaking, the absorbance was measured at 570 nm (reference wavelength 690 nm) with a microplate reader (Opsys MR, DYNEX Technologies).

The tests were performed in triplicate and mean values from 3 independent plates were determined. Data are represented as means + SD. Values obtained with medium treated cells were considered as control and were set as 100 % viability.

Statistics

All results were analysed by GraphPad Prism (version 5, GraphPad Software, Inc; San Diego; CA, USA). Data are expressed as means + SD. Statistical analysis was performed by one way ANOVA with Dunnett's multiple comparison test as post-test or by Student's t-test when only two groups were compared. P-values $\leq$ 0,05 were considered statistically significant.

Results

Cultivation of conjunctiva derived cells

Conjunctiva cells obtained from biopsy specimens (ME-1 and ME-2) demonstrated a mixed-shape population of polygonal and more elongated cells with very slow growth. Confluence was reached after 20 days of cultivation and cells were successfully subcultured at an split ratio of 1 to 2.

Cells in passages 10 and 14 (ME-1) and in passage 7 and 9 (ME-2) were used for characterization. The epithelial-cell like character was observed and maintained in every passage of ME-2 whereas ME-1 showed more spindle growth compare to CCL 20.2 cells (**Fig. 1**). At the same time, the expression of the epithelial specific marker Cytokeratin-7 (CK7) was evaluated. Positive reaction against CK-7 antibodies was obtained with the CCl 20.2 cell line (**Fig.1B**) and with ME-2 cells (**Fig. 1F**). ME-1 cells gave negative results (**Fig. 1D**)

Vimentin filaments were described to be detectable in conjunctival cells in culture [27]. Positive reactions were found with ME-1 (**Fig. 1G**) and ME-2 cells (**Fig. 1H**). However, a great difference in the arrangement of the filaments could be observed, showing that ME-1 cells had more fibroblastic appearance.

Cytotoxic impact of eye drops on human conjunctiva derived cells

Due to the lack of an standardized protocol for testing of eye drops in cell cultures, concentrations ranging from the undiluted form to 1:1000 with incubation times varying between 15 min to 24 hours were reported [10, 18, 24]. Based on preliminary studies (data not shown), only short-term exposure times (5 to 30 min) were examined in all cytotoxicity assays and eye drop solutions were tested in 1/10 dilutions.

Comparative investigations of BAC induced cytotoxicity were performed using CCL 20.2 and ME-1 cells. Incubation of conjunctiva cells for 20 min with increased BAC concentrations ($\geq$ 0.01 mg/ml) followed by a 24 hr recovery period resulted in a dose-dependent decrease of cellular viability **(Fig. 2)**. Significant toxic effects ($P<0.05$), were already found after addition of 0.01 mg/ml BAC to the culture system were observed for both CCL 20.2 and ME-1 cells.

Obtained results revealed that cells obtained from tissue biopsy (ME-1) were more sensitive to the treatment with BAC in comparison with CCL 20.2 cells, when 0.025 mg/ml BAC had on ME-1 cells the same toxic effect as 0.05 mg/ml BAC on CCL 20.2 cells.

No acute toxic effects were detected when eye drops were applied for short time period (5-30 min) to the conjunctiva cells at dilutions 1:10 (data not shown). However, the same exposure times (5 or 30 min) resulted in a dose dependent decrease of viability when cells were treated with various eye drop solutions and were cultured further for another 24 hrs. An

example is given in **Fig. 3A** where CCL 20.2 cells were exposed to Pilocarpine hydrochloride (P-HCl, 2%).

Furthermore it was demonstrated that the cytotoxic impact of Pilocarpin was not only time dependent but also dose-dependent. When P-HCl was applied twice (one hour break between the exposure times), followed by a 24 hours recovery period, a dramatically decrease in cell viability was observed. Again, ME-1 cells were more sensitive to the drug than CCL 20.2 cells (as already demonstrated in (**Fig. 2**)). Whereas 62 % of the CCl 20.2 cells stayed alive after a double dose treatment, only 32 % of the primary ME-1 cells were viable (**Fig. 3B**).

Cytotoxic studies of the model conjunctival cell line (CCL 20.2) and the two human conjunctiva derived primary cell cultures (ME-1 and ME-2) incubated with diluted (1:10) ophthalmic medications (Cosopt sine, Cosopt, Pilocarpin Puroptal 1%, Pilocarpin Puroptal 2 %, Timoftal and Alphagan) for 20 min followed by a 24 hour recovery period showed almost in all cases decreased cellular viability of all tested conjunctiva cell types (**Fig. 4).** However, remarkable distinctions were observed between the analyzed cells. While the BAC containing medications (Cosopt and Pilocarpin) dramatically induced decrease of viability in all tested cells, incubation of cells with Timoftal had toxic impact only on cells obtained from conjunctival biopsies (ME-1 and ME-2).

Even though Alphagan contained also the preservative, no harmful impact was observable. As well the preservative-free Cosopt solution did not cause any significant effect in the viability of the cells. However, the impact of BAC could be seen in the in all cell lines, when BAC-containing Cosopt was applied to the cells. In order to the BAC-content of the investigated medications (P-HCl > Timoftal > Cosopt > Alphagan) a significant negative correlation was found for ME-2 under these conditions ($P=0.003$; Pearson $r=-0.9970$).The preservative concentrations of the respective drugs are given in **Table 1.**

Discussion

The conjunctival cell line CCL 20.2 has been shown to have many features in common with primary conjunctival tissues [28] and therefore was used together with primary cells (ME-1 and ME-2) obtained from conjunctival biopsy as model conjunctiva cells for comparative investigations concerning the cytoxic impact of commercial available medications against glaucoma on the conjuctival cells

Many *in vitro* and *in vivo* studies have extensively described a wide spectrum of cytotoxic and inflammatory reactions after administration of antiglaucoma drugs [4, 29]. However these effects were mainly related to the agents used as drug preservative, mostly to the BAC [11, 15, 17-19]. The immortalized human conjunctiva derived cells IOBA-NHC were shown to possess many phenotypic characteristics of the normal conjunctival epithelium and, in contrast to CCL 20.2 cells which are contamined with HeLa cells [30], do not have any other cell type contamination [31]. Recently, also differences concerning the BAC induced cytotoxicity were found between the IOBA and CCL 20.2 cell line [24]. Such discrepancies were also seen in our study where BAC (0.025 mg/ml) caused more cytotoxic effects in ME-1 cells, derived from human conjunctival biopsy, than in CCL 20.2 cells (**Fig 2**).

Pilocarpine, the-antiglaucoma drug with the highest content of BAC of all investigated drugs, was chosen for time-dependent cytotoxicity studies. Although acute toxic effects were negligible after short incubation periods (5 and 20 min) with the drug diluted 1:10, it turned out that after 24 hours of cell recovery the cellular viability dramatically decreased (**Fig.3 and 4**). Surprisingly, even though single dosage of the pilocarpine solution did not induce any lethal effects after 5 min exposure, results from double treatment; having one hour break between the applications, led to extreme cytotoxic consequences after 24 hours cell recovery Viability of CCL 20.2 cells after incubation with Pilocarpin (dilution 1:10) was up to 60% whereas only 30 % of the conjunctiva derived ME-1 population was detected. Obtained results indicate that high toxicity of Pilocarpine is directly connected to the high content of preservative in the drug (0.02 mg/ml BAC in undiluted solution) and may explain the different behaviour of the investigated conjunctival cells.

Our results from comparative cytotoxicity studies using the CCL 20.2 cell line and the two conjunctiva derived primary cells ME-1 and ME-2 (**Fig.4**) are pointing on the negative correlation between BAC containing eye drop preparations and cell viability showed previously [15, 17, 18]. Furthermore, it has been demonstrated that the cytotoxic impact of BAC-containing Pilocarpin was dramatically enhanced when double doses were applied, representing simulations for daily applications of the drug. However, further studies are required to investigate the cytotoxic impact of the pure compounds as well as interactions of combinations with other active drugs or preservatives.

Tables and figures

Table 1. Characteristics of eye drops for glaucoma therapy used in the present study.

Trade name	Active component(s)	Concentration [mg / ml]	BAC [mg/ml]	Company
Cosopt sine	Dorzolamid / Timolol	20 /5	not included	MSD
Cosopt	Dorzolamid / Timolol	20 /5	0.075	MSD
Pilocarpin Puroptal (1%)	Pilocarpine hydrochloride	10	0.200	Agepha
Pilocarpin Puroptal (2%)	Pilocarpine hydrochloride	20	0.200	Agepha
Timoftal 0.25 %	Timolol	2,5	0.100	Agepha
Alphagan 0.2%	Brimonidintartrat	2	0.050	Allergan

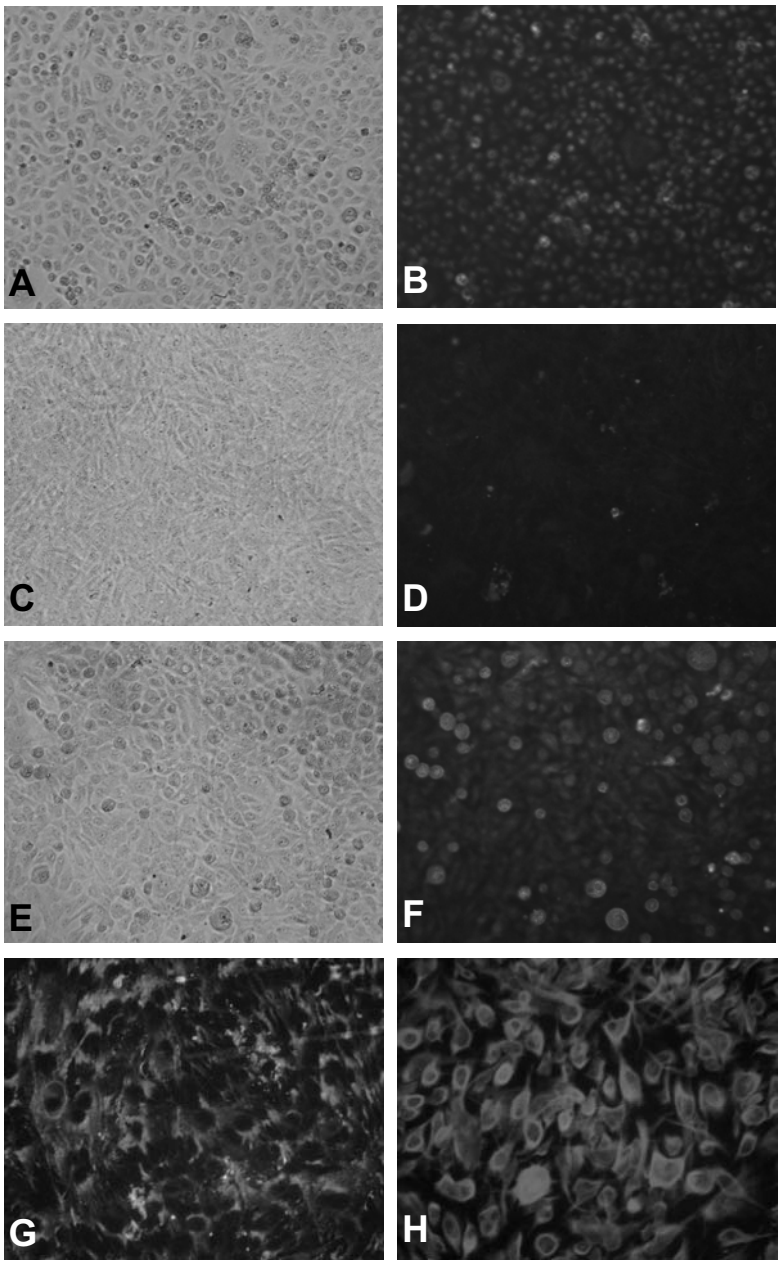

Fig 1. Phase contrast micrographs of confluent cultures from the CCl 20.2 cell line (**A**), from the primary cells ME-1 (**C**) and ME-2 (**E**). CCl 20.2 cells (**B**) as well as ME-2 cells (**F**) showed positive reaction against FITC-labeled anti-CK-7 immunofluorescence staining, whereas ME-1 cells reacted negative (**D**). Magnification x 20. Reactions with the vimentin PE-conjugated antibody are shown for ME-1 cells (**G**) and ME-2 cells (**H**). Note the diffuse arrangement of the filaments in ME-1 cells compared to ME-2 cultures.

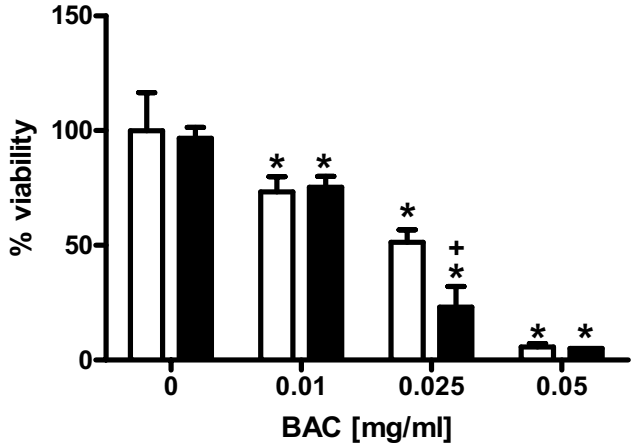

Fig. 2. Results obtained from cytotoxicity assays by the use of the neutralred assay are depicted. CCl 20.2 cells (white bars) and from human conjunctival biopsy derived ME-1 cells (black bars) were exposed to various BAC concentration for 20 min and viability was determined after 24 hours. Bars represent mean values + SD obtained from four measurements. P-values ≤0,05 were considered. As indicated with asterisks, all BAC concentrations led to statistically significant difference compared to their respective control. Statistically significant difference marked by a cross, was also found between CCl 20.2 and ME-1 after treatment with 0.025 mg/ml BAC.

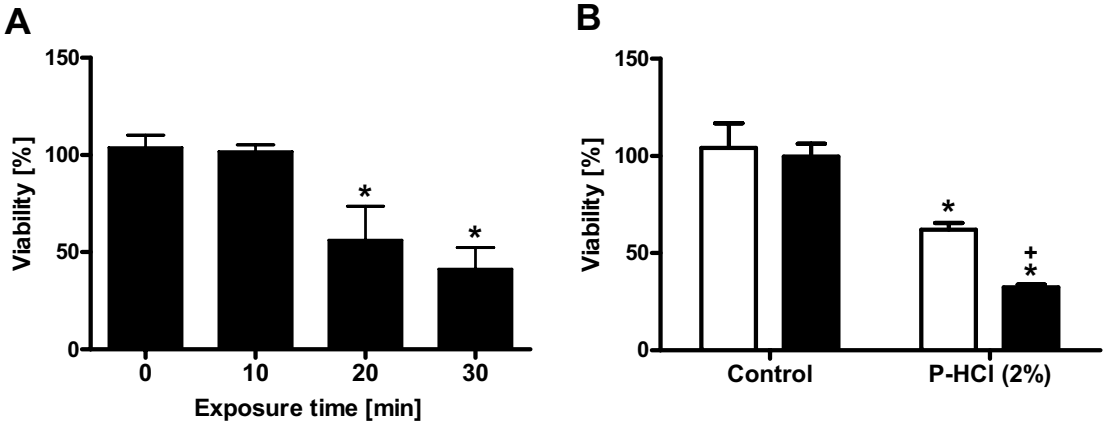

Fig. 3. Evaluation of cytotoxicity obtained after various treatment conditions with the eye drop solution Pilocarpine hydrochloride (P-HCl), 2 %) by use of the neutralred assay. In each experiment a 1/10 dilution of the substance was used. The time dependent effects obtained after different exposure times to CCl 20.2 followed by a 24 hours recovery period are depicted (**A**). The toxicity of a double dose exposure towards CCl 20.2 (white bars) and ME-1 cells (black bars) is shown (**B**). Cells were treated with P-HCl twice for 5 min (one hour break between applications). After 24 hours the viability of the cells was examined. Bars represent mean values + SD obtained after three determinations. Asterisks indicate statistically significant differences compared with control ($p \leq 0.05$)

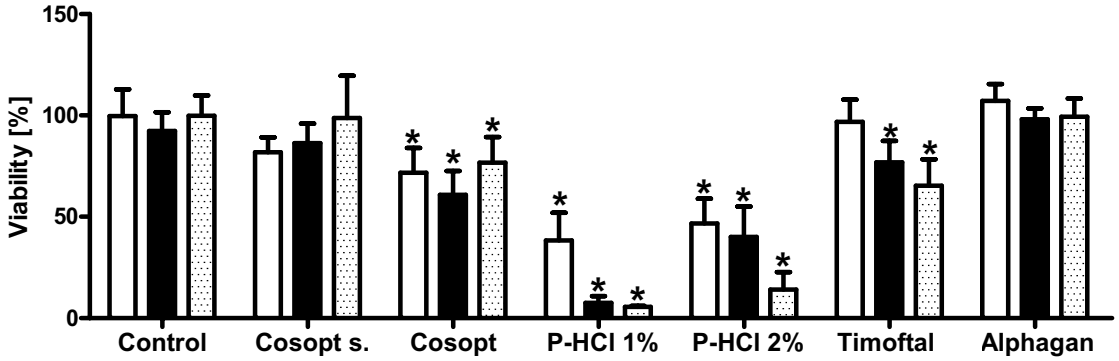

Fig. 4. Determination of various eye drop solutions for their possible cytotoxic effects. The human derived cell line CCl 20.2 (white bars) and primary cells obtained and cultured from conjunctival biopsy tissues ME-1 (black bars) and ME-2 (dotted bars) were examined. Cytotoxicity was evaluated by use of the neutralred assay after 20 min treatment with various eye drops followed by a 24 hour recovery period. Each tested solution was diluted to 1/10. Bars represent means + SD obtained from three different measurements. Asterisks indicate values which are statistically significant different (P-values $\leq$0.05) from their respective control.

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Curriculum Vitae

Personal data

Name:	Verena Juliana Koller
Date / place of birth:	26th April 1981, Güssing
Nationality:	Austrian
Address:	Burggasse 76/29, 1070 Vienna, Austria

Education

Since 2007	Doktorate (Genetics and Microbiology), Faculty of Life Sciences, University of Vienna, "Bacterial ghosts as carrier of active substances: Effects on cell viability and induction of innate immunity"
1999-2006	Diploma study (Genetics and Microbiology), Faculty of Life Sciences, University of Vienna 2004 –2006 diploma thesis: "Investigations concerning the protective effects of lactic acid bacteria towards reactive oxygen species" Graduation: *Magistra rerum naturalium* (Mag.rer.nat)
1995-1999	High school (Bundesoberstufenrealgymnasium), Güssing

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Koller, V. J., Marian, B., Stidl, R., Nersesyan, A., Winter, H., Simic, T., Sontag, G., and Knasmuller, S. (2008) Impact of lactic acid bacteria on oxidative DNA damage in human derived colon cells. *Food Chem Toxicol 46*, 1221-9.

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