ADDENDUM

Optimizing Salmonella enterica serovar Typhimurium for bacteria-mediated tumor therapy

Sebastian Felgner, Dino Kocijancic, Michael Frahm, Roy Curtiss III, Marc Erhardt, and Siegfried Weiss

Department of Molecular Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany; Department of Infectious Diseases and Pathology, University of Florida, Gainesville, USA; Junior Research Group Infection Biology of Salmonella, Helmholtz Centre for Infection Research, Braunschweig, Germany; Institute of Immunology, Medical School Hannover, Hannover, Germany

ARTICLE HISTORY
Received 11 December 2015
Revised 27 January 2016
Accepted 12 February 2016

ABSTRACT
Bacteria-mediated tumor therapy using Salmonella enterica serovar Typhimurium is a therapeutic option with great potential. Numerous studies explored the potential of Salmonella Typhimurium for therapeutic applications, however reconciling safety with vectorial efficacy remains a major issue. Recently we have described a conditionally attenuated Salmonella vector that is based on genetic lipopolysaccharide modification. This vector combines strong attenuation with appropriate anti-tumor properties by targeting various cancerous tissues in vivo. Therefore, it was promoted as an anti-tumor agent. In this addendum, we summarize these findings and demonstrate additional optimization steps that may further improve the therapeutic efficacy of our vector strain.

KEYWORDS
bacteria-mediated tumor therapy; LPS; Lipid A; Salmonella Typhimurium; UK-1

Introduction
Cancer represents a serious health burden for modern societies. Every second individual is expected to receive a diagnosis of cancer within a life time. Although conventional therapies including surgery, radiotherapy and chemotherapy remain common standards for cancer treatment, the 5 y survival rate for many types of cancer remains low despite improvements of such therapies in recent years. Most often, this is due to a lack of tumor specificity and general applicability of the treatment. Not every malignant tissue can be targeted, and even so, it is rarely to a satisfactory level that will prevent regrowth or secondary neoplasias. Therefore, intensive research was performed to broaden the knowledge on cancer and to develop immunotherapies that can raise or strengthen a tumor specific immune response.

Bacteria-mediated tumor therapy (BMTT) represents such an immunotherapy. Interestingly, the intentional use of bacteria as an anti-tumor agent dates back to the 19th century. At the beginning of the 20th century, it was revolutionized by the American physician William Coley. By applying his bacterial mixture to cancer patients, known as Coley’s toxin, he came to notice that an adequate balance between control of infection and therapeutic benefit is essentially required for a successful BMTT. He was only able to achieve this level of balance via heat-inactivation. Nowadays, however, we are able to tailor bacterial strains by means of genetic engineering. Following the conclusions of William Coley, we designed a Salmonella vector strain that should fit the needs for a successful cancer therapy.

In our recent work published in mBio, we investigated the role of the surface molecule lipopolysaccharide (LPS) in regard to the balance between beneficial and harmful immunostimulatory effects of Salmonella enterica serovar Typhimurium (S. Typhimurium). Minor modifications of LPS alone (e.g. Δrfp, Δrfal) did not decrease lethality of Salmonella, however, the core deletion mutants ΔrfpG (ΔwaaG) and ΔrfpD (ΔwaaD) significantly increased the safety level (Fig. 1). Importantly, although these bacteria were able to colonize solid tumors after systemic application, their intrinsic anti-tumor effect was highly reduced i.e. such strains had been over-attenuated.
Thus, the vector was optimized by controlling LPS synthesis using the inducible promoter P_{BAD}.\textsuperscript{12-14} This conditional arabinose dependent attenuation resulted in bacteria with improved anti-tumor effects while keeping a safe phenotype \textit{in vivo}. An ideal balance between therapeutic potency and attenuation was found for this strain although the tumors tested could not be cleared completely. Hence, further optimizations were required.

In this addendum, we provide additional insights into the optimization procedures we have undertaken. We are not simply attempting to discover other suitable mutations but also argue on the genetic background of the \textit{Salmonella} strain we had deployed thus far.

\textbf{Use of S. Typhimurium strain UK-1 improves intrinsic anti-tumor effects}

Many of the laboratory strains considered for vaccination purposes, including the tumor therapeutic agent VNP20009 used in clinical trials, are based on the
ATCC14028 wild type (Wt) strain. In accordance, we constructed our first therapeutic strains on the same background. Despite promising advances, only limited therapeutic potency was observed. Thus, we switched to S. Typhimurium strain UK-1 since this background has very effectively been used in recombinant attenuated Salmonella vaccines (RASV) by the Curtiss lab. The UK-1 strain was shown to be very virulent as its LD50 was lowest among the Salmonella strains tested. For instance, it bears a unique T3SS effector NleC like protein that might add to its virulence. In addition, UK-1 has an improved ability to colonize lymphoid organs indicating strong interactions with the immune system. Consequently, it elicited an improved protective anti-Salmonella immune response. As our therapy relies on the immunogenicity/adjuvant activity of the Salmonella vector strain, the UK-1 background appeared to be a most promising candidate for tumor therapy.

To validate the efficacy of S. Typhimurium ATCC14028 and UK-1 based mutant strains in cancer therapy, we transferred the LPS mutation ΔrfaG to the UK-1 background and compared both strains in vitro and in vivo. As expected, in vitro growth as well as sensitivity towards macrophages and complement was similar (data not shown). For in vivo analyses, CT26 tumor bearing mice were infected with the rfaG mutants and the safety profile was evaluated by analyzing organ colonization, TNF-α induction and body weight loss (Fig. 2). Both rfaG mutants displayed a similar tumor specificity i.e., the bacterial burden at systemic sites and the tumor colonization level was similar (Fig. 2A). Similarly, the body weight was monitored after infection as indicator for the general health status of the mice (Fig. 2B). Again, the profile was similar for both strains. The induction of TNF-α soon after bacterial application is a critical factor for successful cancer therapy. TNF-α induction was similar for both strains (Fig. 2C). Thus, we first reasonably expected that this would also hold for their therapeutic capacity. However, the UK-1 based ΔrfaG mutant caused a significantly enhanced reduction of the tumor size in comparison to its ATCC14028 counterpart (Fig. 2D). Although the CT26 tumor was still able to regrow, the therapeutic effect of the ΔrfaG mutant was significantly boosted when on the UK-1 background.

As intended, the UK-1 background carries a higher intrinsic therapeutic potential than ATCC14028. In agreement, recently genomic profiling of both Wt strains was carried out. It revealed that UK-1 encodes additional virulence genes compared to ATCC14028. This also correlated with a lower LD50 value. Although the UK-1 strain can cause lethal infections in mice, an effectively attenuated strain of UK-1 might provide enhanced immunogenicity relative to those of attenuated derivates of less-virulent Salmonella strains like ATCC14028. As we did not see any differences according to safety issues upon systemic administration, UK-1 indeed might be the preferable bacterial genetic background for cancer therapy.

**Lipid a as suitable target to optimize Salmonella for cancer therapy**

Altogether, the UK-1 derived boost was not sufficient to obtain sustainable anti-tumor effects that were able to completely eradicate the CT26 tumors. Thus, additional optimization of the therapeutic Salmonella mutants was attempted by introducing further genetic modifications. We focused on molecules that play a role in host-pathogen interaction rather than being involved in metabolic or virulence associated pathways. One potential molecule is the hydrophobic anchor of the LPS molecule, the Lipid A. Lipid A is known to directly interact with the Toll-like receptor 4 (TLR-4)-MD2 complex. Salmonella is able to modulate the structure of Lipid A by various genes such as pagP, pagL and lpxR in order to reduce or avoid immune recognition (Fig. 3). For example, a hexa-acylated Lipid A structure stimulates TLR-4 with high affinity while tetra-acylated Lipid A acts as an antagonist. For cancer therapy, we believe that a maximally stimulating bacterium would be therapeutically beneficial. Under in vitro conditions, UK-1 Wt Salmonella express a heterogeneous mixture of hexa- and hepta-acylated Lipid A (Fig. 3A). To avoid in vivo adaptation by expressing a tetra-acylated Lipid A, the 3 genes pagP, pagL and lpxR (denoted as Δ) were deleted. Homogenous expression of hexa-acylated Lipid A was achieved that should exert maximal immune-stimulation (Fig. 3B).

In order to test the effect of this modification, the rfaG mutation was introduced into the mutant strain 3Δ (originally denoted as χ9485) expressing the hexa-acylated Lipid A. CT26 tumor bearing mice were
Figure 2. In vivo comparison of UK-1 ΔrfαG and 14028 ΔrfαG in CT26 bearing BALB/c mice. Mice were infected i.v. with $5 \times 10^6$ CFU. (A) Bacterial burden of blood, spleen and tumor was determined by plating serial dilutions of tissue homogenates at 12 and 36 hpi. (B) Body weight measurement as an indicator of general health after infection of CT26-bearing mice with the different rfαG mutants. (C) TNF-α levels in sera were measured 1.5 hpi. (D) CT26 tumor development after infection with Salmonella variants. PBS served as a negative control. The median and the range are displayed. Results are representative of 2 independent experiments with 5 replicates per group. *, $P < 0.05$.

Figure 3. Lipid structure of Wt Salmonella and 3Δ (ΔlpxR ΔpagL ΔpagP) Lipid mutant. (A) Schematic representation of Lipid A modifications. Left: Wt Salmonella are able to modify the Lipid A molecule according to the environmental situation they encounter. PagL and LpxR are responsible for the removal of the 3-hydroxymyristoyl and 3'-acyloxyacyl chains, respectively, from Lipid A. PagP adds R-3-hydroxymyristoyl to 2-position of Lipid A. Right: Deletion of lpxR, pagL and pagP abrogate this modification resulting in a homogeneously hexa-acylated Lipid A structure (3Δ). (B) Visualization of LPS from Salmonella Wt and 3Δ strains by silver staining of a 16.5% Tris-Tricine SDS-PAGE. The repetitive bands represent the O-Atgs with attached Lipid A. The different electrophoretic mobility of the Wt LPS shows the expected 2 bands for hexa- and hepta-acylated Lipid A (double arrow) per repetitive group. 3Δ only exhibits a single band pattern (arrow) due to the homogeneously hexa-acylated Lipid A structure.
infected with 3Δ ΔrfαG bacteria and tumor therapeutic efficacy was compared to bacteria harboring only the rfaG deletion. We observed a higher stimulatory capacity as reflected in an increased TNF-α response, higher splenic colonization and increased body weight loss during the early stages of infection for the 3Δ ΔrfαG mutant (data not shown). At later stages of infection, however, the rfaG deletion appeared to be dominant for the phenotype observed, since the 3Δ ΔrfαG strain behaved similar to the single mutant strain (Fig. 4A). Importantly, CT26 tumors were completely cleared upon infection with the optimized strain 3Δ ΔrfαG (Fig. 4B). Furthermore, the tumors were not able to regrow at least for 30 d and a rechallenge with freshly prepared CT26 cells at a different site (e.g., abdomen) did not result in new tumor development indicating the sustained anti-tumor effect. Thus, as expected, the additional increased stimulatory capacity of the Lipid A modification was sufficient to induce a sustainable anti-tumor response in the CT26 tumor model.

Taken together, we demonstrate that a tailored Lipid A structure significantly improved the intrinsic bacterial cancer-therapeutic effect. We thus recommend such modifications as effective improvements for Salmonella-based vector systems.

**Conclusion and perspectives**

Adapting Salmonella for therapeutic approaches is a major challenge as an ideal balance between therapeutic benefit and pathogenicity has to be found. Recent examples have shown that commonly used approaches, such as deletion of particular genes or passaging bacteria in vitro and in vivo, might lead to over-attenuation and consequently to reduced therapeutic efficacy.11,28 However, we believe that an optimized balance can be achieved by careful selection of genetic manipulations to achieve appropriate attenuation and optimization of therapeutic benefit within the very same bacterium. In this addendum, we were able to show that modifications of the LPS molecule at 2 specific sites represent a suitable strategy to achieve a proper balance. Nevertheless, the strain remains to be tested with alternative cancer cell lines which exhibit greater resistance to bacterial therapy.11 Although we have shown that the intrinsic anti-tumor effect can be boosted per se, we believe that an optimal therapeutic strain for cancer therapy should, in addition, be utilized as a targeted delivery system to shuttle therapeutic compounds directly into the cancerous tissue. The unique intrinsic ability of bacteria to selectively colonize tumors is a gift which should be exploited further in the future. It may be the key to overcome the limitations of conventional therapies and might provide means for sustainable treatment of cancer.

**Abbreviations**

BMTT bacteria-mediated tumor therapy
CT26 colorectal cancer ATCC CRL-2638
hpi hours post infection
i.v. intravenously
LPS  Lipopolysaccharide
TLR-4  Toll-like receptor 4
TNF-α tumor necrosis factor α
Wt  wild type

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Funding
S.F. was supported within the Ph.D. program Zoonosis by a Lichtenberg Fellowship from the Niedersächsisches Ministerium für Wissenschaft und Kultur (MWK). D.K. was funded in part by the Hannover Biomedical Research School, Center for Infection Biology program (ZIB) and SymbioPharm GmbH.

References
[1] Howlader N, Noone A, Krapcho M, Garshell J, Miller D, Altekruse S, Kosary C, Yu M, Ruhl J, Tatalovich Z, et al. SEER cancer statistics review, 1975–2012. Natl Cancer Institute Bethesda, MD, 2014.
[2] Ringborg U, Platz A. Chemotherapy resistance mechanisms. Acta Oncol (Madr) 1996; 35:76–80; PMID:9142973; http://dx.doi.org/10.3109/02841869609083937
[3] Loeb KR. Significance of multiple mutations in cancer. Carcinogenesis 2000; 21:379–85; PMID:10688858; http://dx.doi.org/10.1039/carcin/21/3.379
[4] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144:646–74; PMID:21376230; http://dx.doi.org/10.1016/j.cell.2011.02.013
[5] Cavollo F, De Giovannoni C, Nanni P, Forni G, Lollini P-L. 2011: The immune hallmarks of cancer. Cell 2011; 60:319–26; PMID:21267721; http://dx.doi.org/10.1007/s00262-010-0968-0
[6] Cheyne WW. Recent essays by various authors on bacterial toxins (the mixed toxins of the Erysipelas and Bacillus prodigiosus). Proc R Soc Med 1910; 3:1-48; PMID:19974799;
[7] Minton NP, Brown JM, Lambin P, Anné J. Clostridia in cancer therapy. In: Clostridia. Wiley-VCH Verlag GmbH; 2005. page 251-70. http://dx.doi.org/10.1002/3527600108.ch8
[8] Hoption Cann SA, van Netten JP, van Netten C. Dr William Coley and tumour regression: a place in history or in the future. Postgrad Med J 2003; 79:672–80; PMID:14707241;
[9] Coley WB. The treatment of inoperable sarcoma with the ‘mixed toxins of Erysipelis and Bacillus prodigiosus. Immediate and final results in one hundred and forty cases. J Am Med Assoc 1898; 16:456–65; http://dx.doi.org/10.1001/jama.1898.92450090022001g
[10] Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med 1910; 3:1-48; PMID:19974799;
[11] Frahm M, Felgner S, Kocijancic D, Rohde M, Hensel M, Curtiss R, Erhardt M, Weiss S. Efficiency of conditionally attenuated Salmonella enterica serovar Typhimurium in bacterium-mediated tumor therapy. MBio 2015; 6 PMID:25873375; http://dx.doi.org/10.1128/mBio.00254-15
[12] Kong Q, Liu Q, Roland KL, Curtiss R. Regulated delayed expression of rfaH in an attenuated Salmonella enterica serovar Typhimurium vaccine enhances immunogenicity of outer membrane proteins and a heterologous antigen. Infect Immun 2009; 77:5572-82; PMID:19805538; http://dx.doi.org/10.1128/IAI.00831-09
[13] Curtiss R, Xin W, Li Y, Kong W, Wanda S-Y, Gunn B, Wang S. New technologies in using recombinant attenuated Salmonella vaccine vectors. Crit Rev Immunol 2010; 30:255–70; PMID:20370633; http://dx.doi.org/10.1615/CritRevImmunol.v30.i3.30
[14] Loessner H, Leschner S, Endmann A, Westphal K, Wolf K, Kochruebe K, Miloud T, Altenbuchner J, Weiss S. Drug-inducible remote control of gene expression by probiotic Escherichia coli Nissle 1917 in intestine, tumor and gall bladder of mice. Microbes Infect 2009; 11:1097-105; PMID:19665575; http://dx.doi.org/10.1016/j.micinf.2009.08.002
[15] Hoiseth SK, Stocker BAD. Aromatic-dependent Salmonella Typhimurium are non-virulent and effective as live vaccines. Nature 1981; 291:238-9; PMID:7015147; http://dx.doi.org/10.1038/291238a0
[16] Clairmont C, Lee KC, Pike J, Ittensohn M, Lowe KB, Pawelek J, Bermudes D, Brecher SM, Margitich D, Turner J, et al. Biodistribution and genetic stability of the novel antitumor agent VNP20009, a genetically modified strain of Salmonella Typhimurium. J Infect Dis 2000; 181:1996-2002; PMID:10837181; http://dx.doi.org/10.1086/315497
[17] Kong W, Brovold M, Koeneman B a., Clark-Curtiss J, Curtiss R. Turning self-destructing Salmonella into a universal DNA vaccine delivery platform. Proc Natl Acad Sci 2012; PMID:23129620; http://dx.doi.org/10.1073/pnas.1217554109
[18] Wang S, Kong Q, Curtiss R. New technologies in developing recombinant attenuated Salmonella vaccine vectors. Microb Pathog 2013; 58:17-28; PMID:23142647; http://dx.doi.org/10.1016/j.micpath.2012.10.006
[19] Zhang X, Kelly SM, Bollen WS, Curtiss R. Characterization and immunogenicity of Salmonella Typhimurium SL1344 and UK-1 delta crp and delta cdt deletion mutants. Infect Immun 1997; 65:5381-7; PMID:9393846;
[20] Luo Y, Kong Q, Yang J, Mitra A, Golden G, Wanda S-Y, Roland KL, Jensen RV, Ernst PB, Curtiss R. Comparative genome analysis of the high pathogenicity Salmonella Typhimurium strain UK-1. PLoS One 2012; 7:e40645; PMID:22792393; http://dx.doi.org/10.1371/journal.pone.0040645
[21] Leschner S, Westphal K, Dietrich N, Viegas N, Jablonska J, Lyszkiwicz M, Lienklaus S, Falk W, Gekara N, Loessner H, et al. Tumor invasion of Salmonella enterica
serovar Typhimurium is accompanied by strong hemorrhage promoted by TNF-α. PLoS One 2009; 4:e6692; PMID:19699266; http://dx.doi.org/10.1371/journal.pone.0006692

[22] Raetz CRH, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem 2002; 71:635; PMID:12045108; http://dx.doi.org/10.1146/annurev.biochem.71.110601.135414

[23] Maeshima N, Fernandez RC. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 2013; 3:3; PMID:23408095; http://dx.doi.org/10.3389/fcimb.2013.00003

[24] Freudenberg M a, Tchaptchet S, Keck S, Fejer G, Huber M, Schütze N, Beutler B, Galanos C. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. Immunobiology 2008; 213:193-203; PMID:18406367; http://dx.doi.org/10.1016/j.imbio.2007.11.008

[25] Needham BD, Carroll SM, Giles DK, Georgiou G, Whiteley M, Trent MS. Modulating the innate immune response by combinatorial engineering of endotoxin. Proc Natl Acad Sci U S A 2013; PMID:23297218; http://dx.doi.org/10.1073/pnas.1218080110

[26] Saitoh S, Akashi S, Yamada T, Tanimura N, Kobayashi M, Konno K, Matsumoto F, Fukase K, Kusumoto S, Nagai Y, et al. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol 2004; 16:961-9; PMID:15184344; http://dx.doi.org/10.1093/intimm/dxh097

[27] Kong Q, Six DA, Roland KL, Liu Q, Gu L, Reynolds CM, Wang X, Raetz CRH, Curtiss R. Salmonella synthesizing 1-monophosphorylated lipopolysaccharide exhibits low endotoxic activity while retaining its immunogenicity. J Immunol 2011; 187:412-23; PMID:21632711; http://dx.doi.org/10.4049/jimmunol.1100339

[28] Broadway KM, Denson EAP, Jensen RV, Scharf BE. Rescuing chemotaxis of the anticancer agent Salmonella enterica serovar Typhimurium VNP20009. J Biotechnol 2015; 211:117-20; PMID:26200833; http://dx.doi.org/10.1016/j.jbiotec.2015.07.010