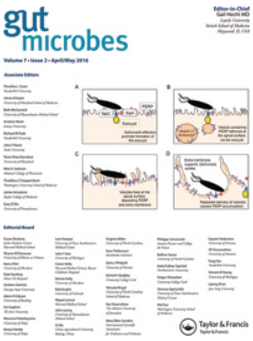

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ADDENDUM

Optimizing *Salmonella enterica* serovar Typhimurium for bacteria-mediated tumor therapy

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ABSTARCT
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.

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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.[1] 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.[2] 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.[3-5]

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.[6,7] 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.[8-10] 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*,[11] 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. Δ*rfaP*, Δ*rfaL*) did not decrease lethality of *Salmonella*, however, the core deletion mutants Δ*rfaG* (Δ*waaG*) and Δ*rfaD* (Δ*waaD*) 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.

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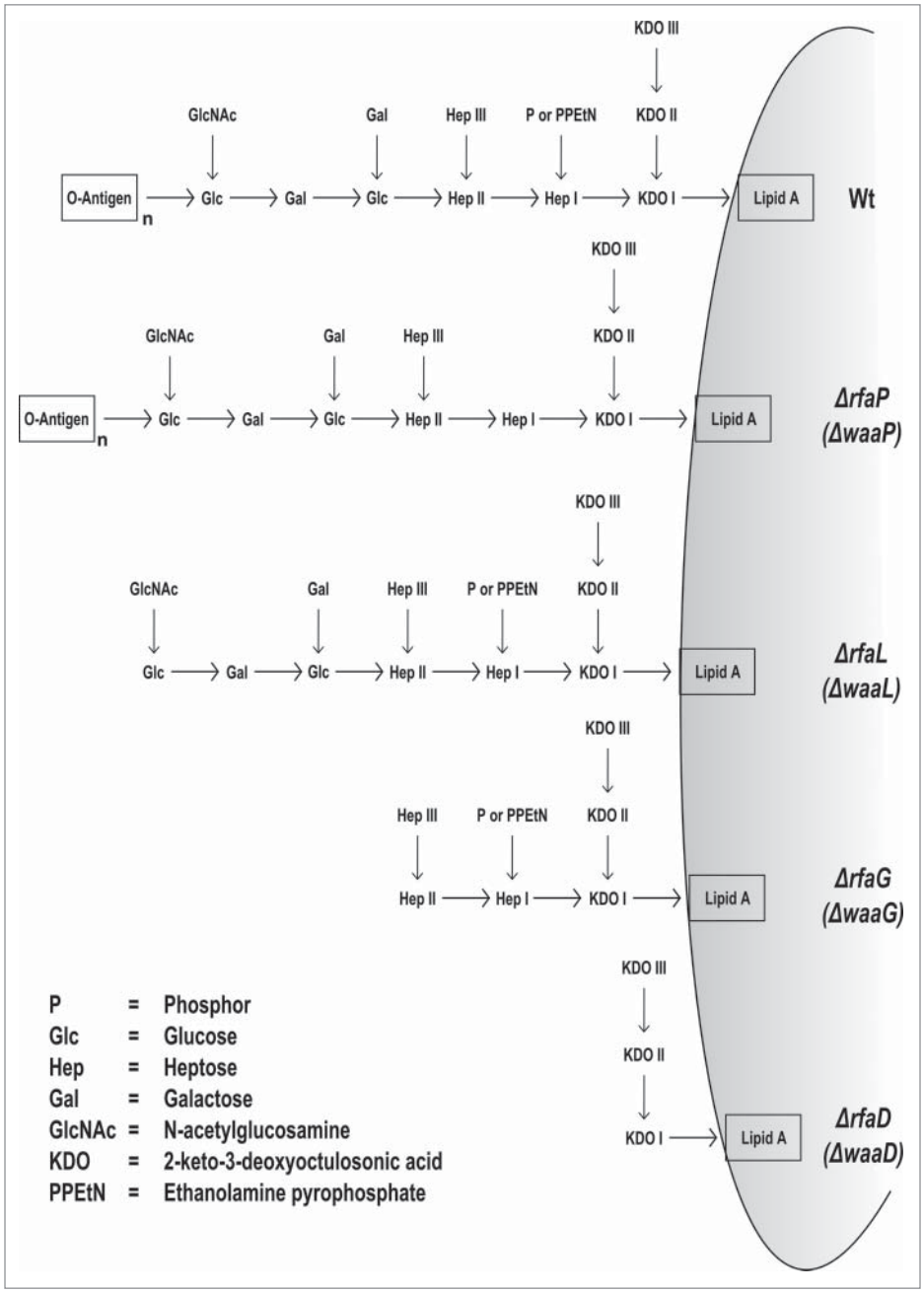

Figure 1. LPS structure of *Salmonella* and corresponding LPS mutants.

Thus, the vector was optimized by controlling LPS synthesis using the inducible promoter P_{BAD}.[12-14] This conditional arabinose dependent attenuation resulted in bacteria with improved anti-tumor effects while keeping a safe phenotype *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 *Salmonella* strain we had deployed thus far.

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.[15,16] In accordance, we constructed our first therapeutic strains on the same background. Despite promising advances, only limited therapeutic potency was observed.[11] 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.[17-19] The UK-1 strain was shown to be very virulent as its LD_{50} was lowest among the *Salmonella* strains tested.[19,20] For instance, it bears a unique T3SS effector NleC like protein that might add to its virulence.[20] 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.[19] As our therapy relies on the immunogenicity/adjuvanticity 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 Δ*rfaG* 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.[21] 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 Δ*rfaG* 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 Δ*rfaG* 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.[20] It revealed that UK-1 encodes additional virulence genes compared to ATCC14028. This also correlated with a lower LD_{50} 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.[19] 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.[22] Lipid A is known to directly interact with the Toll-like receptor 4 (TLR-4-)-MD2 complex.[23,24] *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.[25,26] 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 3Δ) were deleted. Homogenous expression of hexa-acylated Lipid A was achieved that should exert maximal immune-stimulation (Fig. 3B).[25]

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.[27] CT26 tumor bearing mice were

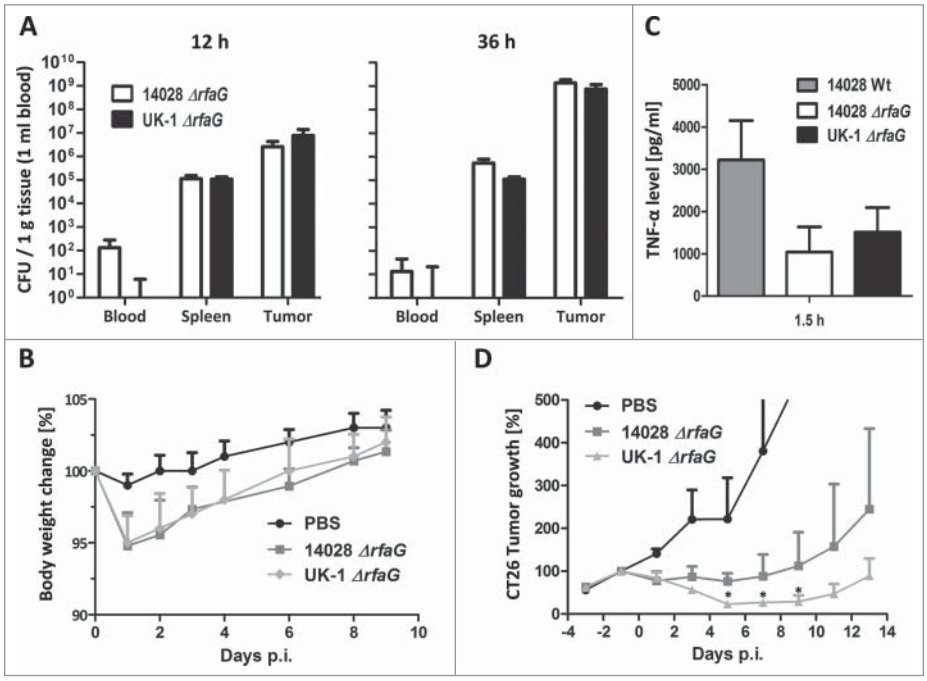

Figure 2. ***In vivo*** **comparison of UK-1 Δ*rfaG* and 14028 Δ*rfaG* in CT26 bearing BALB/c mice.** Mice were infected i.v. with 5×10^6 CFU. (A) Bacterial burden of blood, spleen and tumor was determined by plating serial dilutions of tissue homogenates at 12 and 36 hpi. (B) Body weight measurement as an indicator of general health after infection of CT26-bearing mice with the different *rfaG* 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. *, $P < 0{,}05$.

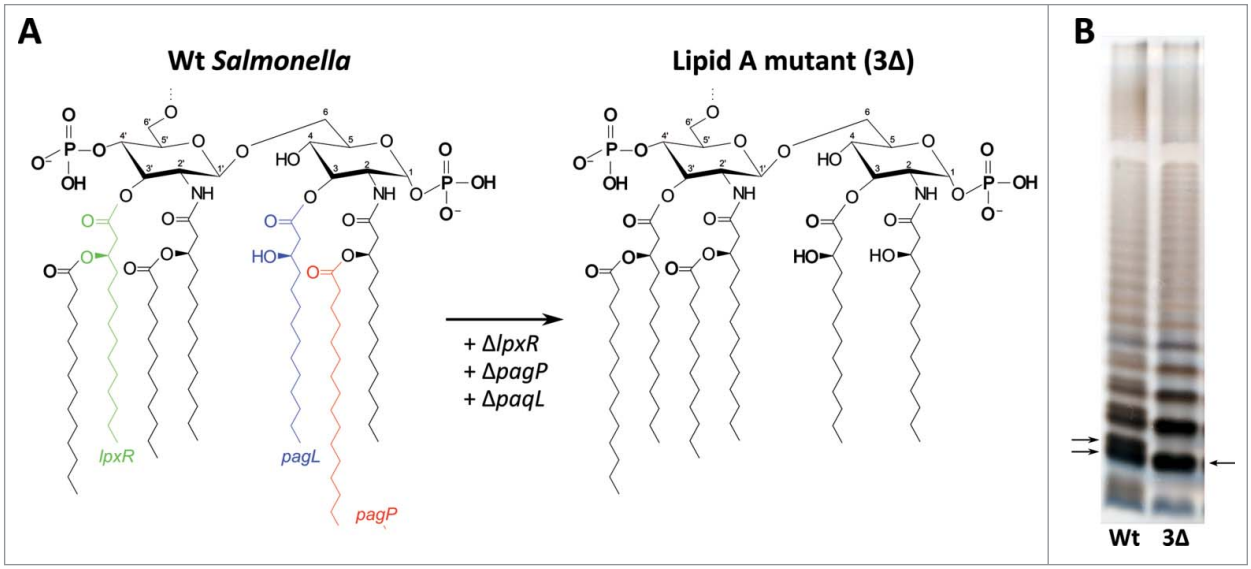

Figure 3. Lipid structure of Wt *Salmonella* and 3Δ (Δ*lpxR* Δ*pagL* Δ*pagP*) Lipid mutant. (A) Schematic representation of Lipid A modifications. Left: Wt *Salmonella* are able to modify the Lipid A molecule according to the environmental situation they encounter. PagL and LpxR are responsible for the removal of the 3-hydroxymyristoyl and 3′-acyloxyacyl chains, respectively, from Lipid A. PagP adds R-3-hydroxymyristoyl to 2-postion of Lipid A. Right: Deletion of *lpxR, pagL* and *pagP* abrogate this modification resulting in a homogenously hexa-acylated Lipid A structure (3Δ). (B) Vizualization 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 Δ*rfaG* 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 Δ*rfaG* mutant (data not shown). At later stages of infection, however, the *rfaG* deletion appeared to be dominant for the phenotype observed, since the 3Δ Δ*rfaG* strain behaved similar to the single mutant strain (Fig. 4A). Importantly, CT26 tumors were completely cleared upon infection with the optimized strain 3Δ Δ*rfaG* (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.

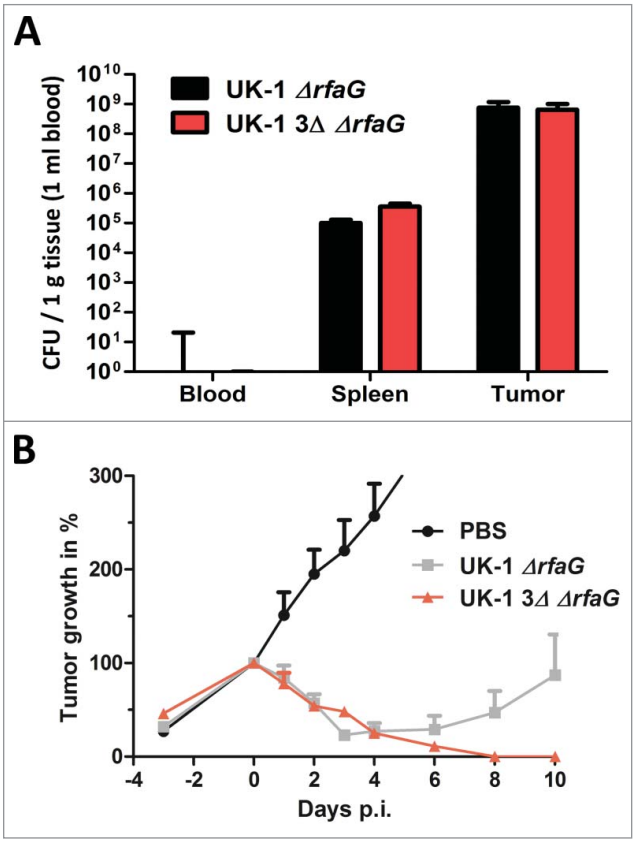

Figure 4. Colonization profile and tumor development upon infection with Δ*rfaG* and 3Δ Δ*rfaG* (Δ*lpxR* Δ*pagL* Δ*pagP* Δ*rfaG*) on UK-1 background. CT26 tumor bearing mice were infected with 5×10^6 bacteria. (A) Bacterial burden of blood, spleen and tumor was determined by plating serial dilutions of tissue homogenates at 36 hpi. (B) Tumor volume was measured by caliper. PBS served as negative control. Tumors were not able to regrow at least for 30 d and a rechallenge with freshly prepared CT26 cells at a different site did not result in new tumor development. The mean with STD is displayed. Results are representative of 2 independent experiments with 5 replicates per group.

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.

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