The immunogenic potential of bacterial flagella for *Salmonella*-mediated tumor therapy

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NOVELTY AND IMPACT: Balancing safety and therapeutic efficacy remains a major challenge for the rational design of bacteria for immunotherapy applications. We demonstrated that manipulating the spatiotemporal regulation of flagella synthesis can confer this balance in *Salmonella*. Unexpectedly, abrogating flagella synthesis at an early stage resulted in outer membrane vesicle formation, which enhanced the immunogenic properties. These engineered *Salmonella* strains might have the potential to serve as vector platform for various therapies ranging from immunization to cancer therapy.

KEYWORDS: *Salmonella* Typhimurium, host-pathogen interaction, flagella, luminex, bacteria-mediated tumor therapy

ARTICLE CATEGORY: Cancer therapy and prevention

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ABBREVIATIONS: PAMP = pathogen-associated molecular pattern, LPS = lipopolysaccharide, TLR = toll-like receptor, PPR = pattern-recognition receptor, UK-1 = Universal Killer 1, Wt = wild-type, MOI = multiplicity of infection, LDH = lactate dehydrogenase, IL = Interleukin, TNF-α = tumor necrosis factor α, hpi = hours post infection, dpi = days post infection, OMV = outer membrane vesicle, CT26 = murine colon carcinoma, RenCa = renal adenocarcinoma, ELISA = enzyme-linked immunosorbent assay, BMDM = bone-morrow derived macrophages

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ABSTRACT:

Genetically engineered *Salmonella* Typhimurium are potent vectors for prophylactic and therapeutic measures against pathogens as well as cancer. This is based on the potent adjuvanticity that supports strong immune responses. The physiology of *Salmonella* is well understood. It simplifies engineering of both enhanced immune-stimulatory properties as well as safety features, thus, resulting in an appropriate balance between attenuation and efficacy for clinical applications. A major virulence factor of *Salmonella* is the flagellum. It is also a strong pathogen-associated molecular pattern recognized by extra- and intracellular receptors of immune cells of the host. At the same time, it represents a serious metabolic burden. Accordingly, the bacteria evolved tight regulatory mechanisms that control flagella synthesis *in vivo.* Here, we systematically investigated the immunogenicity and adjuvant properties of various flagella mutants of *Salmonella in vitro* and in a mouse cancer model *in vivo*. We found that mutants lacking the flagellum-specific ATPase FliHIJ or the inner membrane ring FliF displayed the greatest stimulatory capacity and strongest anti-tumor effects, while remaining safe *in vivo*. Scanning electron microscopy revealed the presence of outer membrane vesicles in the *ΔfliF* and *ΔfliHIJ* mutants. Finally, the combination of the *ΔfliF* and *ΔfliHIJ* mutations with our previously described attenuated and immunogenic background strain SF102 displayed strong efficacy against the highly resistant cancer cell line RenCa. We thus conclude that manipulating flagella biosynthesis has great potential for the construction of highly efficacious and versatile *Salmonella* vector strains.

INTRODUCTION

Despite the exponential growth of biomedical knowledge over the last decades, we are still facing health conditions that are not controllable. Thus, present biomedical research is called upon to provide solutions to this dilemma. In face of the demographic changes within the world population these problems become even more acute with cancer being one of the most pressing problems. Consequently, scientists need to develop novel and/or more effective strategies for vaccines and immunotherapies. Search for proper protective antigens is required for the success of such immune interventions but at the same time efficacious and safe adjuvants are required for such strategies. Employment of pathogens as platforms for many of such approaches is therefore a straightforward possibility. These microorganisms are able to act as potent carriers because they elicit strong immune reactions i.e. they exhibit strong adjuvant properties. Viruses are often employed in this context [1]. However, bacteria have also successfully been tested. For instance, *Salmonella* spp. are known to elicit strong cellular and humoral immune activities which underscore their potential as an effective live carrier [2–4].

Salmonella spp. is a pathogen. It may elicit live threatening disease in the host. Hence, their pathogenic properties have to be attenuated to ensure safe application. In this case, attenuation and immune-stimulation needs to be well in balance to guarantee safety and efficacy [5,6]. This represents the basic problem of live bacterial carriers.

Immune-recognition, immune-stimulation as well as immune-evasion of *Salmonella* are closely connected to the availability of pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) or flagella [7]. Responses to LPS, as an agonist of the Toll-like receptor 4 (TLR-4) and causative agent of sepsis are intensively investigated [8]. LPS has been established as one of driving forces for adjuvanticity [5]. These experiments included generation of conditionally attenuated *Salmonella* carrier strains. A regulatory connection between LPS modifications and

flagella synthesis via the RflP/ClpXP pathway has been suggested [9]. In addition, the impairment of both these two important PAMPs - LPS and flagellum - directly led to a loss of therapeutic potency. Apparently, such molecules are essential assets of the adjuvanticity of the *Salmonella* vector.

However, the contribution of the flagellum to the establishment of a successful infection and its involvement in an efficacious immune stimulation is less well explored. On the one hand, flagellar motility contributes to pathogenesis by promoting bacteria-host interactions, adherence and invasion of host cells [10,11]. On the other hand, as soon as *Salmonella* reaches its anatomical destination in the body, flagella synthesis is a serious burden for the microorganism. Down-regulation to avoid immune recognition by Pattern-Recognition-Receptors (PPR), like extracellular TLR-5 or intracellular caspase-1 is essential for bacterial survival *in vivo* [12,13]. This evasion mechanism already indicates that bacteria constitutively expressing flagella might elicit strong immune activation.

The flagellum is a sophisticated macromolecular apparatus composed of several thousand copies of approximately 25 different proteins. It can be classified into three main parts: i) a basal body embedded in the cytoplasmic membrane that traverses the periplasm and cell wall up to the outer membrane (the engine), ii) a long external filament (the propeller) and iii) a flexible, curved structure known as the "hook", which connects the basal body with the rigid filament [14].

S. Typhimurium usually encodes two antigenically distinct filament proteins (the flagellins FliC or FljB), whose mutually exclusive expression is regulated by phase switching [15]. Of note, strains constitutively expressing the phase-2 flagellin protein FljB were found to be more potent in targeting tumors in a murine tumor model compared to strains expressing phase-1 FliC [16]. In line with these observations, Eom and colleagues observed an enhanced adjuvanticity of *Salmonella* that co-expressed both flagellin proteins FliC and FljB [17]. These results demonstrate that a modulation of synthesis and assembly of flagella might allow to engineer appropriately modified bacterial

vector strains for therapeutic applications. Recent results confirm the importance of FlaB flagella for successful *Salmonella* based cancer therapy [18]. Furthermore, motility, chemotaxis and the presence of flagella as antigen have been shown to be important for tumor therapy as well [18–21]. Thus, manipulations of the various flagellum sub-structures may positively influence the performance of the therapeutic strains.

In the present study, we aimed to systematically unravel the connection between the presence of flagella components and immune stimulatory potency. We hypothesized that manipulating the spatiotemporal onset of flagella synthesis or of various steps in flagellar assembly might represent a valid strategy to increase the adjuvant power of *Salmonella* vector strains without increasing their pathogenicity. Thus, we investigated three groups of flagella mutants (Figure 1): i) flagellin phase locked mutants (FliC-ON, FljB-ON, FliC-ON & FljB-ON), ii) non-filamentous mutants (Δ*flgK*, Δ*fliF*, Δ*fliHIJ*) and flagella overproduction mutants (Δ*rflP*, Δ*rflP* Δ*flgM* and Δ*rflP* Δ*flgM* Δ*rflM*).

In summary, our results highlight the importance of a controlled spatiotemporal regulation of flagella synthesis during host-pathogen interactions. *Salmonella* mutants lacking the inner membrane ring and basal body component FliF or the flagellum-specific ATPase complex FliHIJ displayed the highest therapeutic efficacy in murine tumor models despite the fact that they are unable to assemble flagella. We thus believe that the flagellum of *Salmonella* represents an ideal target for immunomodulatory modifications and it might be possible to generate optimized safe vector strains with improved adjuvant properties for prophylaxis and therapy.

MATERIALS AND METHODS

Ethics statement: All animal experiments were performed according to guidelines of the German Law for Animal Protection and with permission of the local ethics committee and the local authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under permission number 33.9-42502-04-12/0713, 33.9-42502-04-13/1122 and 33.9-42502-04-13/1191.

Strains and preparation of inoculum: Bacterial strains are shown in Table S1. Strain construction was done by P22 phage transduction or λ-red recombination [22]. *Salmonella* strains were grown overnight and sub-cultured to mid-log phase in LB media at 37 °C. The bacteria were washed twice and adjusted to the desired OD_{600} in pyrogen free PBS. Plating served as control.

Cell lines and primary cells: Immortalized RAW264.7 macrophages (RRID:CVCL_0493) were used for invasion assays and obtained from Raschke et al. [23] (Salk Institute, San Diego, USA). CT26 tumor cells (RRID:CVCL_7524, obtained from: Brattain et al. [24], Comprehensive Cancer Center, University of Alabama, USA) and RenCa tumor cells (RRID:CVCL_2174, obtained from: Wells et al. [25], Tumor Biology Center, Freiburg, Germany) were used for the subcutaneous murine tumor model. All cell cultures used in this study were tested and confirmed as mycoplasma-free. Bone-marrow derived macrophages (BMDMs) were isolated from the femur of BALB/c mice and differentiated using 20% (v/v) L929 (RRID:CVCL_0462, obtained from: Monner et al. [26], National Research Center for Biotechnology, Braunschweig, Germany) conditioned medium in RPMI containing 10% FCS. All cells were maintained at 37 °C, 5% CO_2 and 90% rel. humidity.

Motility assay: The motility of the *Salmonella* strains was assessed on semi-solid agar plates containing 0.3% (wt/vol) agar by inoculating 2 µl of a bacterial overnight culture into the agar and incubated at 37 °C. The swarm diameter was measured after 4 h incubation.

Flagella immunostaining: *Salmonella* strains grown to mid-log growth phase were fixed on L-lysine coated microscopy slides using formaldehyde (c_f= 2%, v/v) and glutaraldehyde (c_f = 0.2%,

v/v). Flagellum staining was accomplished using polyclonal rabbit anti-FliC (Difco) as primary and anti-rabbit Alexa Fluor-488 as secondary antibody and the bacteria were stained with DAPI (Sigma-Aldrich). Images were taken using an Axio Observer microscope equipped with an Axiocam HR camera (Zeiss) at 100x magnification and analyzed with ImageJ.

Invasion assays: RAW 264.7 and BMDM mycoplasma-free cells were used for the phagocytic uptake and intracellular replication. The assay was performed as described before [27] using MOIs of 1 and 10. CFUs were determined by plating of serial dilutions and compared to the corresponding parental strains.

Murine tumor model: Six-week old BALB/c mice (Janvier) were intradermally inoculated with $5*10^5$ syngeneic CT26 or $2*10^6$ RenCa tumor cells in the right flank. Tumor development was monitored using caliper measurements. Upon reaching a tumor volume of approx. 150 mm^3, the mice were injected intravenously into the tail vein with $5*10^6$ *Salmonella*.

Therapeutic efficacy: Tumor development was monitored using caliper measurements for as long as tumors persisted or until confronted with a humane endpoint in terms of exceedingly large tumor size (~ 1 cm^3) or morbidity. Body weight as general health indicator was monitored using a scale. A loss of body weight below 80% of the original body weight was incentive to euthanize a subject.

TNF-α ELISA measurement: Supernatant samples of cultivated macrophages were taken 6 h post infection. The TNF-α ELISA Max^{TM} Standard Kit (Biolegend) was used to determine the TNF-α level according to the manufacturer's manual. Three different biological replicates were analyzed and a PBS treated group served as negative control.

Cytokine, chemokine and growth factor detection in supernatants and sera: Cytokine, chemokine and growth factor concentrations in supernatants of 264.7 RAW macrophages cells (6 hpi) or sera (1.5 hpi, 6 hpi and 24 hpi) were quantified by the Luminex-based multiplex technique according to the manufacturer's instructions (Bio-Rad, USA). Standard curves and concentrations were

calculated with Bio-Plex Manager 6.0, the detection sensitivity of all proteins was between 1 pg/ml and 40 µg/ml.

RNA isolation and sequencing: RNA isolation was performed as described previously [16]. Library preparation of planktonic cultures grown to mid-log phase was done using the ScriptSeqTM v2 RNA-Seq Library Prep Kit (Illumina) and the vendor's protocol. Sequence reads were mapped to the genome sequence of the reference strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium UK-1 (Genbank, CP002614.1) using bowtie2 [28]. Differential gene expression was calculated using robust generalized linear models and the quasi-likelihood F-test of the R package edgeR [29]. Genes were considered differentially expressed if the fold change expression was significantly greater than 2 (edgeR function glmTreat) with a false-discovery rate (FDR) cutoff of 0.05. Multidimensional scaling plots (MDS) were visualized using ggplot2 [30].

Genome sequencing and SNP calling: Genomic DNA was extracted from planktonic overnight cultures using the DNeasy Blood & Tissue kit (Qiagen) and sequenced using Illumina HiSeq. Single nucleotide polymorphism (SNP) analysis of DNA sequencing data was carried out using samtools mpileup and python [31].

***Statistics*:** Significance between two groups was determined using the nonparametric Mann-Whitney test, while one-way analysis of variance (ANOVA) with Bonferroni posttest was used to compare two or more groups. Significance levels of $p < 0.05$, $p < 0.01$, or $p < 0.001$ were denoted with asterisks: *, **, and ***, respectively.

***Data availability*:** All raw and processed sequencing data have been submitted to GEO (GSE116623; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116623). All raw and processed genome sequencing data have been submitted to SRA (SRP153811, https://www.ncbi.nlm.nih.gov/sra/SRP153811).

RESULTS

Construction and phenotypic characterization of flagella mutants: Flagella represent a major immune-stimulatory structure. In accordance, introducing flagella of other bacterial species or modulating assembly or expression of flagella has improved the performance of such strains in tumor therapeutic approaches [18,32,33]. The strains still showed exquisite safety features *in vivo.* We therefore intended to systematically test mutants of flagella assembly and expression for their immune stimulatory capacity as well as their therapeutic potential against tumors *in vivo*. This way, we hoped to reveal additional genetic targets that would allow further improvements of therapeutic *Salmonella* strains.

We constructed the following flagella variants on the genetic background *S.* Typhimurium strain UK-1. Deleting the recombinase Hin, which is responsible for flagellin phase variation [15], as well as removing FljA, the repressor of *fliC* translation [34], allowed us to influence the composition of the large antigenic flagellar filament: *Δhin*-5717 (FliC-ON), *Δhin*-5718 (FljB-ON), *ΔfljA Δhin*-5718 (FliC-ON & FljB-ON). The absence of the filament junction protein FlgK (*ΔflgK*), the flagellum-specific ATPase complex FliHIJ (*ΔfliHIJ*), or the inner membrane ring FliF (*ΔfliF*) would interfere with flagellar assembly [35–37]. Various deletions of regulatory proteins would interfere with the spatiotemporal regulation of flagella expression: *ΔrflM* removes the negative, autoregulatory feedback of the flagellar master regulator FlhDC [38], *ΔrflP* prevents post-translational degradation of FlhDC under conditions of cell envelope stress [38,39], and *ΔflgM* allows for premature expression of genes from flagellar Class 3 promoters [40]. The expected phenotypes are shown schematically in Figure 1 and listed in Table S1.

First, we investigated whether the general physiology was affected by such mutations. Thus, planktonic growth was monitored in LB for 24 h (Supplementary Figure S1A). As expected, no significant growth differences were observed for the structural flagella mutants (Supplementary

Figure S1A, left), while the regulatory mutants exhibited a minor growth defect (Supplementary Figure S1A, right). To ensure that the reduced growth rate was caused by increased flagellar biosynthesis and not due to secondary effects, the master regulator *flhDC* was deleted in these strains to abrogate flagella gene expression all together. Deletion of *flhDC* restored the growth of *ΔrflP ΔflgM ΔrflM* to wild-type (Wt) levels (Supplementary Figure S1B). This observation indicates that overexpression of flagellar components, e.g. the many thousand flagellin subunits, probably cause a metabolic burden that results in the observed decreased bacterial growth. The flagellation phenotype of the various flagella mutants was as expected as determined by SDS-PAGE and Western Blot analyses (Figure 2A).

The level of flagellin expression and/or the number of assembled flagella may relate to virulence and immunogenicity. Hence, the flagellar filaments were stained by fluorescent antibodies and the average number of flagella per bacterium was determined for each strain (Figure 2B). On average, the Wt strain and the phase-locked mutants displayed 3.5 flagella per cell under *in vitro* conditions. The overexpressing mutants *ΔrflP* and *ΔrflP ΔflgM ΔrflM* displayed significantly more flagella (4.4 ± 1.1 and 4.9 ± 1.3 flagella per cell, respectively). This confirmed the increased levels of flagellin detected by Western Blot in these mutants. The assembly mutant *ΔflgK* did not exhibit functional flagella, as expected, despite expressing and secreting flagellin. The other mutants, *ΔfliHIJ* and *ΔfliF,* defective in flagella assembly did not display any flagellin in their supernatants (Fig. 2A).

Finally, the motility of the various flagella mutants was tested (Figure 2C). As expected, the mutant strains *ΔflgK*, *ΔfliHIJ* and *ΔfliF* were non-motile. The phase-locked mutants FliC-ON and FljB-ON displayed Wt motility and the hyper-flagellated mutants exhibited increased motility. Only the FliC/FljB co-expressing mutant showed decreased motility although it displayed the same number of flagella as the Wt and FliC-ON and FljB-ON strains.

Interaction of flagella mutants with RAW264.7 macrophages: Since flagella are one of the major PAMPs, we tested the sensitivity and immune stimulatory capacity of the various flagella mutants on macrophages. Macrophages were chosen because they are part of the innate immune system and the first line of defense when the bacteria are systemically administrated. In addition, it had been shown that phagocytosis efficacy and bacterial entry are dependent on flagellar motility [11]. RAW264.7 macrophages were infected with the flagella mutants at MOI 10 (Figure 3) and MOI 1 (Supplementary Figure S2). Except for the non-flagellated mutants *ΔfliHIJ*, *ΔfliF* and *ΔflgK*, the bacterial uptake of the mutants was similar compared to Wt (Figure 3A). This confirms previous studies that functional flagella may be important for bacterial uptake [11,41].

Salmonella are intracellular bacteria and are able to protect themselves from the aggressive environment of the vacuole. Hence, we next determined bacterial survival within macrophages. The hyper-flagellated mutant *ΔrflP ΔflgM ΔrflM* was the only strain that was not able to replicate intracellularly. In this case, the initial bacterial cell count was even reduced after uptake, suggesting that the bacteria died during the duration of the assay (Figure 3B). However, since gentamycin was present in the culture medium, cell death of macrophages would also result in death of the liberated bacteria. To distinguished bacteria-mediated killing of macrophages from killing of bacteria by macrophages, a lactate dehydrogenase (LDH) release assay was performed to monitor lysis of the mammalian cells. LDH levels in supernatants of macrophages infected with the hyper-flagellated mutant were significantly increased in comparison to Wt infections. This suggested that the observed reduced levels of the *ΔrflP ΔflgM ΔrflM* mutant was due to macrophage cell lysis during the survival assay, presumably by pyroptosis [42]. The effect was less pronounced at MOI 1 indicating that the effect was dose-dependent (Supplementary Figure S2B).

In response to bacterial infection, macrophages secrete chemokines and cytokines to recruit and activate immune cells. To evaluate the influence of the flagella on these immune reactions, the

supernatants of macrophages infected with flagella variants were analyzed by Luminex to screen for 23 different cytokines and chemokines (Figure 3C). Interestingly, the non-flagellated mutants *ΔfliF* and *ΔflgK* induced significantly elevated levels of pro-inflammatory cytokines and chemokines like IL-6, G-CSF, TNF-α and RANTES. Similarly, *ΔfliHIJ* elicited increased levels of RANTES and TNF-α. On the other hand, the flagellin phase did not affect the immunogenicity. *In vitro,* no significant change was observed with either phase-locked *Salmonella* strains FliC-ON or FljB-ON, while the *Salmonella* mutant co-expressing FljB and FliC induced increased TNF-α. These results suggest that *Salmonella* variants that can no longer assemble flagella elicit stronger immune reactions. Similar results were obtained for *ΔfliF* and *ΔfliHIJ* at MOI 1 when we tested for TNF-α release (Supplementary Figure S2C).

Immunogenicity of flagella mutants in BALB/c mice: Cytokines and chemokines induced in macrophages *in vitro* provide only limited information on the *in vivo* performance of the bacterial strains. We therefore infected BALB/c mice intravenously with the various flagella variants and monitored the immune reaction in sera 1.5, 6 and 24 hpi (Figure 4). With the exception of the *ΔfliHIJ* and FliC-ON strains, all mutants induced increased levels of IL-17 and Eotaxin at 1.5 hpi (Figure 4A). Interestingly, the *ΔfliF* mutant maintained elevated levels of IL-6, KC and RANTES in comparison to Wt over the entire course of the experiment. Furthermore, the levels of TNF-α, IL-17 and Eotaxin were already significantly increased in the early stages of infection by this mutant (Figure 4A and 4B). This indicates a high immunogenic character of this variant. Although the *ΔfliHIJ* mutant showed no elevated cytokine and chemokine levels at 1.5 hpi, significantly increased levels of IL-6, IL-12 and IL-17 were detected 6 hpi (Figure 4B). The results also indicated that the FljB-locked mutant was more immunogenic than its FliC-locked counterpart. In summary, the Luminex analysis of immune reactions clearly demonstrated that the flagellar phenotype does

influence the immunogenicity of the individual strains. Surprisingly, the variants that lost the ability to functionally assemble flagella represent the most immunogenic strains.

The *ΔfliHIJ* mutant represents the most immunogenic candidate in therapeutic setups. As most of the above-mentioned phenotypes still represented a serious health burden for the mice, the most promising flagella mutations were inserted into our previously established attenuated and immunogenic background strain SF102 (*ΔlpxR ΔpagP ΔpagL ΔaroA*) [16]. As control, we inserted a deletion of the flagellar master regulatory operon *flhDC* (*ΔflhDC)*, which completely abolishes flagella synthesis[43]. First, *in vitro* characterization of these strains revealed that the flagella mutations in the SF102 strain background displayed similar phenotypes as the single mutations described above (Supplementary Figure S3 and Supplementary Figure S4). The hyper-flagellated mutants exhibited increased number of flagella, motility and enhanced macrophage invasion. The assembly mutants were non-motile and their interaction with RAW264.7 macrophages was comparable to SF102. Similar to our findings above, infection of macrophages with the SF102 + *ΔflgK*, SF102 + *ΔfliF* and SF102 + *ΔrflP ΔflgM ΔrflM* mutants resulted in the most pronounced cytokine response (Supplementary Figure S4C).

To extend these *in vitro* findings to the *in vivo* situation, BALB/c mice were infected with $5 \cdot 10^6$ bacteria of the respective strain and the cytokine response was monitored in the serum (Figure 5). In general, the number of differentially secreted cytokines was lower than with the flagella mutants in the Wt background. This was expected as the response was normalized to SF102, which itself is already highly immunogenic[16]. Again, the non-flagellated *Salmonella* variants harboring a *ΔflhDC*, *ΔfliF* or *ΔfliHIJ* deletion exhibited the strongest pattern of cytokine induction. While the *ΔflhDC* strain initially induced a strong response at 1.5 hpi, most of the signals were lost at 6 hpi. However, the *ΔfliHIJ* mutant exhibited high cytokine levels over the entire observation period. Especially, IL-

6 and KC were still significantly present at 6 hpi and may drive an enhanced therapeutic response. In summary, the non-flagellated mutants and in particular the *ΔfliHIJ* deletion may represent promising candidates for improving therapeutic *Salmonella* vectors as judged by the induced cytokine patterns.

Comparison of genetic profiles of SF102 and its *ΔfliF* and *ΔfliHIJ* derivatives: To understand the improved immunogenic potency of the SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* bacteria, we performed transcriptional profiling (Supplementary Figure S5). Principal component analysis revealed that the transcriptome of the SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* strains clustered with the parental strain SF102 and the differences in gene expression were minor (Supplementary Figure S5A). In fact, only 35 genes were significantly regulated (9 upregulated and 26 downregulated) for SF102 + *ΔfliF* and 29 downregulated genes for SF102 + *ΔfliHIJ* in comparison to SF102 (Supplementary Figure S5B and Table S2). Most of the differential expression affected flagella Class 3 genes like the filament proteins or the chemotaxis apparatus. They are expected not to be expressed in the *ΔfliF* and *ΔfliHIJ* mutants due to the incomplete assembly of flagella basal bodies. Interestingly, many genes influencing tRNA biosynthesis were significantly upregulated in SF102 + *ΔfliF*. However, as we detected those genes only in the Δ*fliF* mutant strain and not in *ΔfliHIJ*, it is unclear whether upregulation of these RNAs is directly related to the increase in immunogenic potency. In addition, the direct comparison of both derivative strains only revealed differential presence of transcripts for the genes that were deleted in the alternative strain (i.e. *fliF*, *fliH*, *fliI* and *fliJ*).

OMVs as possible explanation for increased efficacy: The enhanced cytokine induction by the *ΔfliF* and *ΔfliHIJ* bacteria remains unexplained. Therefore, we searched for alternative structures

that may be responsible for this particular phenotype. We employed scanning electron microscopy to search for alterations at the cell surface or bacterial shape (Figure 6). Interestingly, this analysis revealed the presence of outer membrane vesicles (OMV) for SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* mutants while all the other mutants did not exhibit such structures. Importantly, the combination of all features (e.g. *ΔlpxR ΔpagP ΔpagL - ΔaroA - ΔfliF* or *ΔfliHIJ)* was required to promote OMV formation (Supplementary Figure S6). *Salmonella* derivatives that lack at least one of these properties did not display any OMVs under our conditions. As OMVs include high amounts of LPS and are known to contain flagellar proteins [44], thus, the increased immunogenic potential may very well be attributed to these structures.

Another possibility for the observed OMV production was due to an independent, unintentional mutation that may have accumulated during the strain constructions. Therefore, we sequenced the genome of the Δ*fliF* mutant and its parental strains as it exhibited the most pronounced OMV production (Table S3). No apparent SNPs or additional deletions were detected in the SF102 + *ΔfliF* strain that may have contributed to the OMV phenotype. Therefore, the genomic or transcriptional origin of the enhanced OMV formation remains unanswered.

***ΔfliHIJ* and *ΔfliF* mutants display advanced anti-tumor properties:** Next, we evaluated whether the strongly induced cytokine pattern and production of OMVs correlated with the therapeutic efficacy of the flagella mutant strains in a murine tumor model. Of note, tumor colonization itself appears not to be affected by the modifications of the bacterial flagella as shown previously [33] and confirmed by plating (data not shown). CT26 tumor-bearing mice were infected intravenously with $5*10^6$ bacteria and the body weight changes as indicator for the health burden of therapy as well as the therapeutic efficacy was assessed (Figure 7). Upon bacterial application, all infected mice survived the therapy and weight loss was around 10% at most. This can be considered as minor

(Figure 7A). The regulatory mutants SF102 + *ΔflgK* and SF102 + *ΔrflP ΔflgM ΔrflM* appeared to be highly attenuated as the body weight recovered very fast upon an initial drop. In contrast, the structural flagella mutants induced a persistent weight loss that only slowly recovered after 6 days. However, these structural mutants SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* displayed the most pronounced anti-tumor effect. The bacteria cleared all the CT26 tumors within 8 or 4 days, respectively (Figure 7B and Supplementary Figure S7 for individual tumor development). In addition, the parental strain SF102 and its *ΔflhDC* derivative were able to clear 80% of the analyzed tumors (5/6). In contrast, the hyper-flagellated strain SF102 + *ΔrflP ΔflgM ΔrflM* and the assembly mutant SF102 + *ΔflgK* appeared to be over-attenuated. The tumor-clearing capacity was even lower than that of the parental SF102 strain. In summary, the *ΔfliHIJ* mutant strain exhibited the strongest potential to clear CT26 tumors. However, the *ΔfliF* strain exhibited the best balance of safety and efficacy. The mice recovered fastest from effects of the infection when exposed to these bacteria.

Combination of SF102+*ΔfliF* or SF102+*ΔfliHIJ* strongly affect RenCa tumors: Although the cause of the strong production of OMVs in the SF102 + *ΔfliF* or SF102 + *ΔfliHIJ* strains remains unknown, these structures likely contribute to the improved immunogenicity. We therefore wondered how strongly the therapeutic potency of these strains was improved. Thus, we tested these mutants against the highly resistant cancer cell line RenCa (Figure 7C). As shown before, the LPS mutants only retarded the growth RenCa shortly [5]. In contrast, employing SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* for therapy dramatically improved the anti-tumor response and significantly prolonged the survival of the RenCa bearing mice. Thus, the unique combination of mutations and specifically the introduction of the gene deletions *ΔfliF* or *ΔfliHIJ* affecting flagella assembly and regulation resulted in anti-cancer strains of exceptional therapeutic potency.

DISCUSSION

Salmonella exerts unique direct interactions with the host cell via specialized secretion systems, effector proteins or PAMPs. Thus, *Salmonella* displays great potential as a highly versatile targeted delivery system for vaccination and cancer immune therapies [45–48]. Flagella represent one of the major PAMPs of these bacteria. Hence, in the present study we aimed to investigate the effects of the structure, spatio-temporal regulation and synthesis of the bacterial flagellum during host-pathogen interactions and in therapy.

To cover a broad range of flagellar phenotypes, we investigated the behavior of eleven different *Salmonella* variants mutated in various regulatory and structural components of the flagellum. For instance, deleting the *hin* recombinase responsible for flagellin phase variation in *Salmonella* allowed to engineer flagellin phase-locked mutants that expressed either FliC or FljB [49]. In order to generate a mutant that co-expressed both filament proteins, we additionally deleted the negative regulator of *fliC* translation, FljA, in a FljB-ON background [50]. Under these conditions, every individual flagellum presumably consists of a mixture of both proteins. The variant exhibited reduced motility although the number of flagella per cell was normal. It might be possible that the structural differences between both flagellin types decrease the efficiency of flagella function, e.g. by impairing filament bundle formation.

In comparison to the Wt, the FliC-ON phase-locked mutant did not exhibit any superior immunogenic behavior *in vitro* and *in vivo*. In contrast, immune activation by the FljB-locked strain was increased during the early stages of infection. This correlated with previous observations that *Salmonella* expressing FljB exhibit higher adjuvant potential [17,51]. This might also explain why the strain SF102, published previously, represents a highly immunogenic therapeutic vector. It predominately expresses FljB [16]. The beneficial effect of FljB-only expression vanishes at the later stages of infection.

Salmonella in the mammalian host is known to down-regulate flagella expression to avoid recognition by TLR-5 or caspase-1 [13,52]. To counteract this escape mechanism, we investigated the role of regulators of flagella synthesis: *rflP*, *flgM* and *rflM* [38,53,54]. As expected, strains bearing deletions of either gene resulted in a significantly increased flagellation status and enhanced flagellin production. Interestingly, this overproduction affected bacterial growth *in vitro*. Furthermore, when exposing the overproducing triple mutant Δ*rflP* Δ*flgM* Δ*rflM* to macrophages, we detected increased levels of LDH in the supernatants. This suggested that the hyper-flagellation might induce intracellular caspase-1 resulting in pyroptosis and macrophage cell death [55]. Bacterial death would consequently be the result from exposure to gentamycin in the medium. *In vivo*, such flagella-overproducing strains would not be able to hide from immune recognition to establish their intracellular niche. This is consistent with *in vivo* efficacy of this strain. It appeared over-attenuated. The mice recovered already 4 dpi and the tumor clearing efficacy was only slightly above 50%. Thus, hyper-flagellation of the *Salmonella* vector could represent a promising strategy to increase vaccine adjuvanticity but may not be a potent strategy for tumor therapeutic applications.

As hyper-flagellation did not appear to be beneficial due to the metabolic burden and/or the extensive activation of pyroptosis, we next investigated the *ΔflgK* mutant, which is able to secrete monomeric flagellin, but is unable to assemble flagellar filaments [56]. *In vitro*, the *ΔflgK* mutant induced a strong IL-6 and TNF-α response. This may indicate a strong stimulation of the TLR-5 receptor due to the enhanced levels of monomeric flagellin [57]. However, this strong phenotype disappeared *in vivo*. Possibly, soluble flagellin was diluted or digested in the blood or the tissue and did not reach concentrations sufficient to stimulate TLR-5 or intracellular receptors like NOD-like receptors or NLRP3. This also correlated with the low efficacy in the anti-tumor response. Therefore, a *ΔflgK* deletion may not represent a good choice when attempting to optimize a vector strain for therapy.

Finally, we tested strains without extracellular flagella. We deleted the gene coding for FliF, which forms the MS-ring of the flagellar basal body in the inner membrane [37], or FliHIJ, which is an ATPase complex involved in the export of flagella building blocks [58]. Both strains neither expressed nor secreted flagellin and thus were non-motile. Interestingly, both strains were highly immunogenic *in vitro* and *in vivo*. In an otherwise Wt background, the *ΔfliF* mutation induced secretion of high levels of pro-inflammatory cytokines like TNF-α or IL-6. Once the mutation was transferred onto our immunogenic background strain UK-1, the *ΔfliHIJ* mutation displayed the most pronounced cytokine response. As FliC is an important PAMP that confers immunogenicity, this finding was surprising since these mutants are not able to express flagellin and therefore lack the flagellar filament. Importantly, in our therapeutic model the SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* strains were able to clear all CT26 tumors within 8 or 4 days, respectively. All mice survived the therapy. Therefore, these strains exhibit an optimal balance of safety and efficacy.

As putative reason for their superiority, we made the serendipitous discovery that a substantial amount of OMVs was produced by these mutants. Although the mechanism of OMV production still remains elusive, we believe that they significantly contribute to the superior therapeutic efficacy of these strains. The increased levels of pro-inflammatory cytokines may derive from the LPS that predominately forms the vesicles or the putative cargo of the OMVs that might include bacterial RNA and DNA [59]. In addition, recent proteome studies have shown that OMVs can carry flagellar proteins [44].

The MS-ring made of FliF and the ATPase complex FliHIJ are important components of the flagella export apparatus that secretes flagellar building blocks from the intracellular to the extracellular space. The deletion of either the Class 2 genes *fliF* or *fliHIJ* results in defective flagellar basal bodies and therefore accumulate flagellar building blocks in the cytoplasm. Thus, the enhanced OMV production might be a strategy of the cell to remove excessive flagellar proteins. This

hypothesis is supported by the fact that no negative regulator controlling flagella synthesis at the level of Class 2 gene expression was found to be significantly regulated.

Besides their immune-stimulatory capacity, the presence of OMVs could be further exploited as delivery system in future studies [60,61]. Therefore, using deletions of the Class 2 genes *fliF* and *fliHIJ* in combination with other immunomodulatory mutations like the SF102 background could turn *Salmonella* into a very effective delivery vector platform. Various immunogenic cargos or therapeutic agents may be transported directly into tumor cells or immune cells of the host.

Taken together, the correct spatiotemporal regulation of flagella synthesis during host-pathogen interactions exerts direct impact on the therapeutic efficacy of *Salmonella* vector strains. While mutants over-expressing flagella were too sensitive to the immune system of the host, the mutations Δ*fliF* and Δ*fliHIJ* appeared to be highly efficacious in our murine tumor models. Increased immunogenic properties were conferred by these mutations. Especially the unique production of high numbers of OMVs induced by mutations of *fliF* and *fliHIJ* might allow to enhance intrinsic therapeutic features of the bacteria and render *Salmonella* into an efficacious delivery platform. Such *Salmonella*-based vectors might represent potent strains for cancer therapy, which is evidenced by the substantial growth retardation of the very resilient RenCa tumors, which have been highly resistant to this type of therapy thus far.

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AUTHOR CONTRIBUTIONS

S.F., I.S., V.P., D.K., C.F. and M.R. performed and analyzed the experiments. M.P. and S.H. were responsible for the sequencing analyses. S.H., S.W. and M.E. provided ideas and critical comments. S.F., S.W. and M.E. wrote the manuscript. S.W. and M.E. directed the project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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FIGURES

Fig. 1: Schematic representation of the flagellar phenotypes of *Salmonella* employed in this study. Three groups of flagella mutants were constructed to investigate the role of the flagella during host-pathogen interaction. **i)** Phase locked mutants (FliC-ON, FljB-ON, FliC-ON & FljB-ON), **ii)** No filament mutants (Δ*flgK*, Δ*fliF*, Δ*fliHIJ*) and **iii)** Hyper-flagellation mutants (Δ*rflP*, Δ*rflP* Δ*flgM*, Δ*rflP* Δ*flgM* Δ*rflM*).

Fig. 2: Phenotypic characterization of flagella mutants of *Salmonella*. A) SDS-PAGE and Western Blot analysis to detect FliC and FljB in the supernatant of LB overnight cultures of *Salmonella* flagella mutants. **B)** Quantification of the average flagella number based on a Gaussian non-linear regression analysis. Flagella (green) were visualized using immunofluorescence microscopy. Pictures for WT Salmonella and the hyper-flagellated version *ΔrflP ΔflgM ΔrflM* are exemplarily shown. **C)** Assessment of motility relative to *Salmonella* Wt in semisolid (0.3% w/v) agar. Mean ± STD are presented. *, $p < 0.05$; ***, $p < 0.001$.

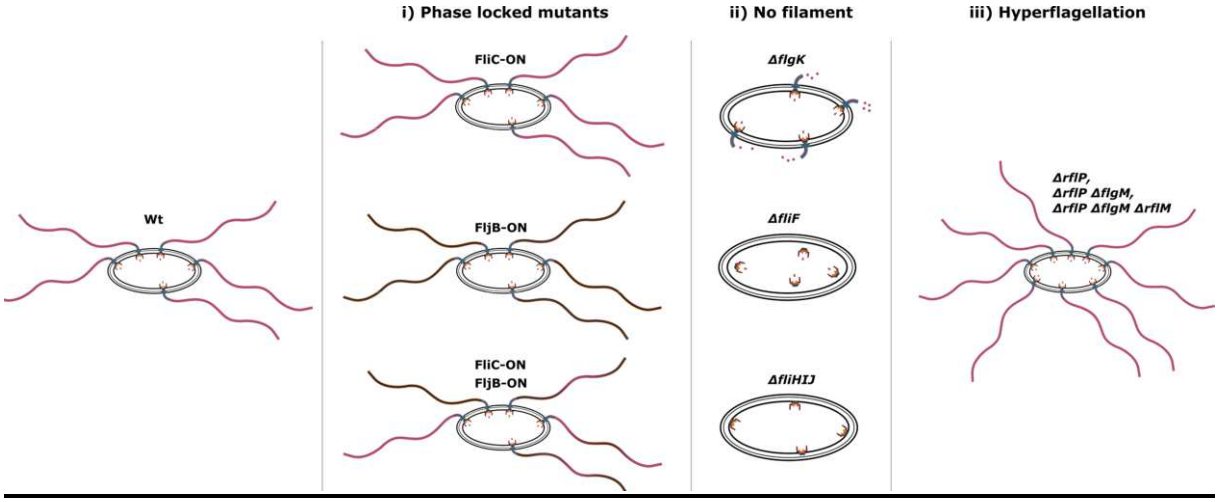
Fig. 3: Exposure of RAW264.7 macrophages to *Salmonella* flagella mutants at MOI 10. RAW264.7 macrophages were infected at MOI 10 with *Salmonella* variants. **A)** Bacterial uptake was determined 2 hpi by serial plating. Mean ± STD are presented. **B)** Intracellular survival and replication was examined 6 hpi. Mean ± STD are presented. **C)** Luminex analysis of supernatant from infected RAW264.7 cells 6 hpi. Mean of representative cytokines relative to Wt are shown with N = 4. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

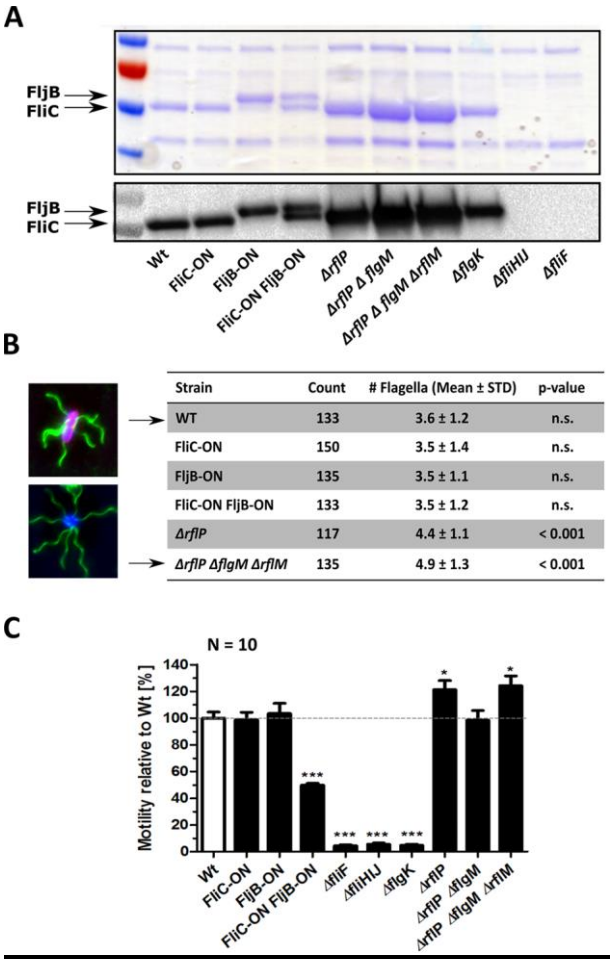
Fig. 4: Luminex analysis of sera from BALB/c mice after infection with *Salmonella* mutants. Mice/c were infected with $5*10^6$ *Salmonella* and sera were obtained at **A)** 1.5 hpi **B)** 6 hpi and **C)** 24 hpi. Cytokines and Chemokines were analyzed using the Bio-Plex Pro Mouse Cytokine 23-Plex kit. PBS treated mice served as control. Mean ± STD are presented. N = 3. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 5: Luminex analysis of sera from BALB/c mice after infection with therapeutic *Salmonella* strains. Promising flagella modifications were inserted into the optimized therapeutic background SF102 (*ΔpagP ΔpagL ΔlpxR ΔaroA*). Mice were infected with $5*10^6$ *Salmonella* and sera were isolated at **A)** 1.5 hpi and **B)** 6 hpi. Cytokines and Chemokines were analyzed using the Bio-Plex Pro Mouse Cytokine 23-Plex kit. PBS infected mice served as control. Mean ± STD are presented. N = 3. *, $p < 0.05$; **, $p < 0.01$.

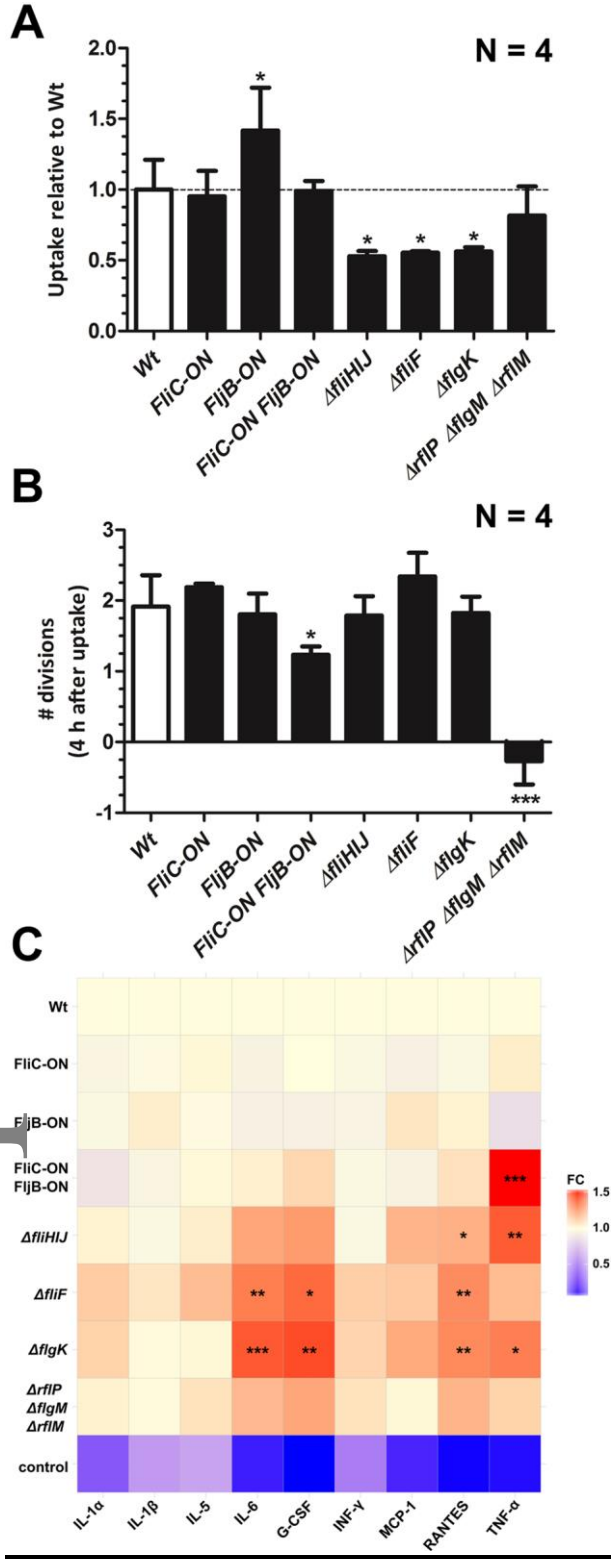
Fig. 6: Scanning electron microscopy reveals OMV production. Therapeutic *Salmonella* strains were analyzed by SEM to find putative explanations for the differential therapeutic efficacy. The strains SF102 + *ΔfliF* and SF102 + *ΔfliHIJ* exhibited OMV production while the other strains did not show this phenotype.

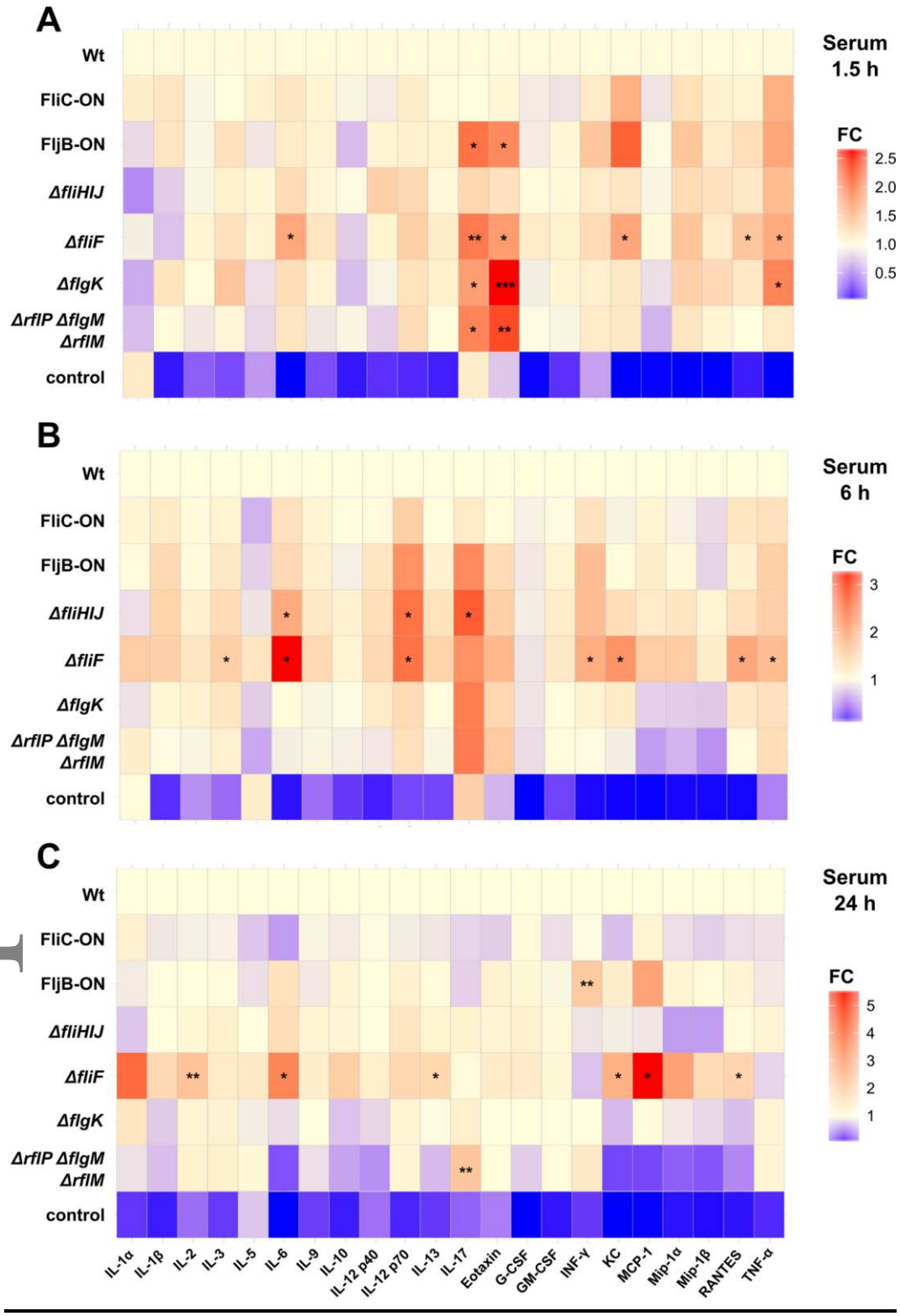
Fig. 7: Therapeutic efficacy of *Salmonella* variants in tumor-bearing BALB/c mice. A) CT26 and tumor-bearing mice were infected intravenously with $5*10^6$ bacteria of the respective *Salmonella* strain. Body weight measurement as indicator of general health status upon infection. **B)** CT26 tumor development after infection with *Salmonella* strains. Number of tumors cleared after 14 dpi are indicated in brackets. Mean values are depicted. N = 6. **C)** RenCa tumor-bearing mice were infected intravenously with $5*10^6$ bacteria of the respective *Salmonella* strain to compare the therapeutic efficacy of the new strains to former strains. Mean values are depicted. N = 5.

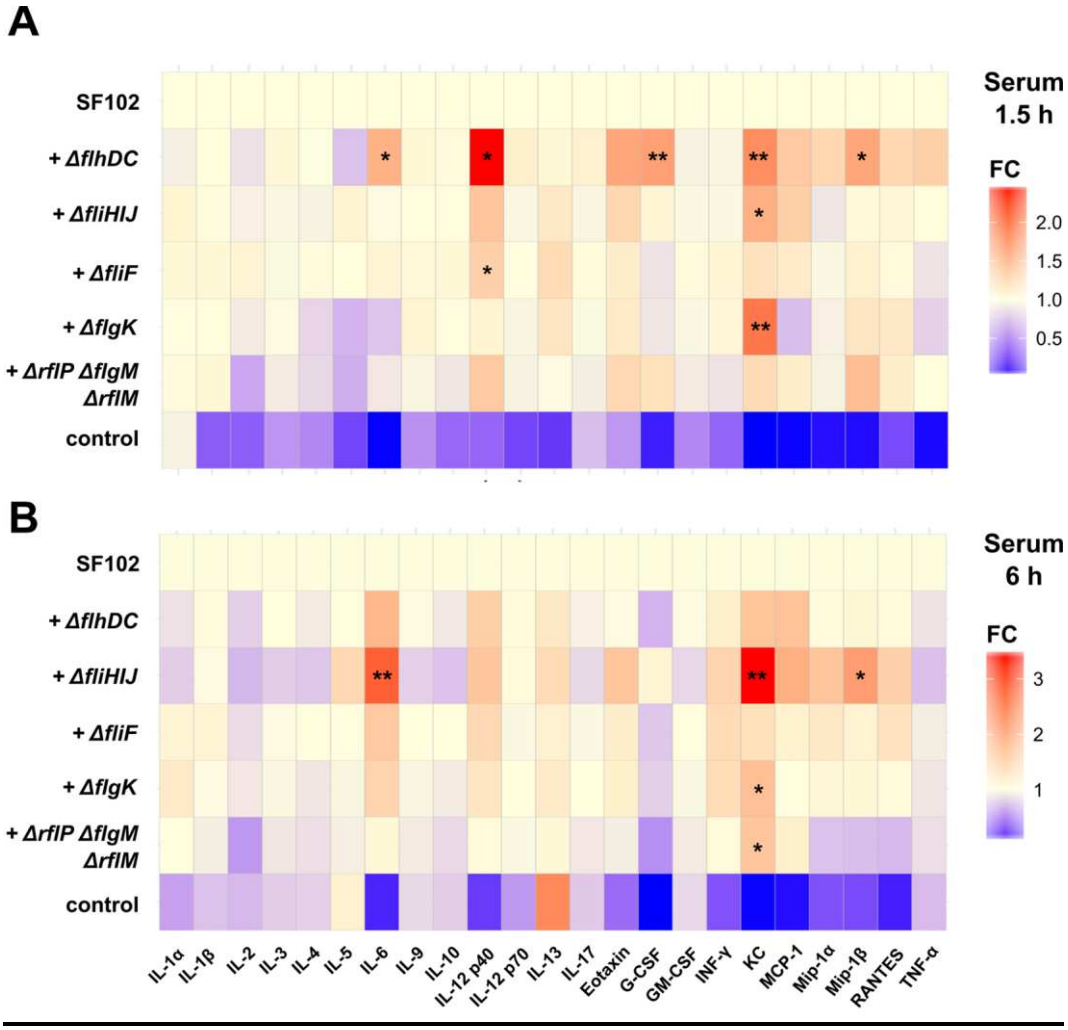


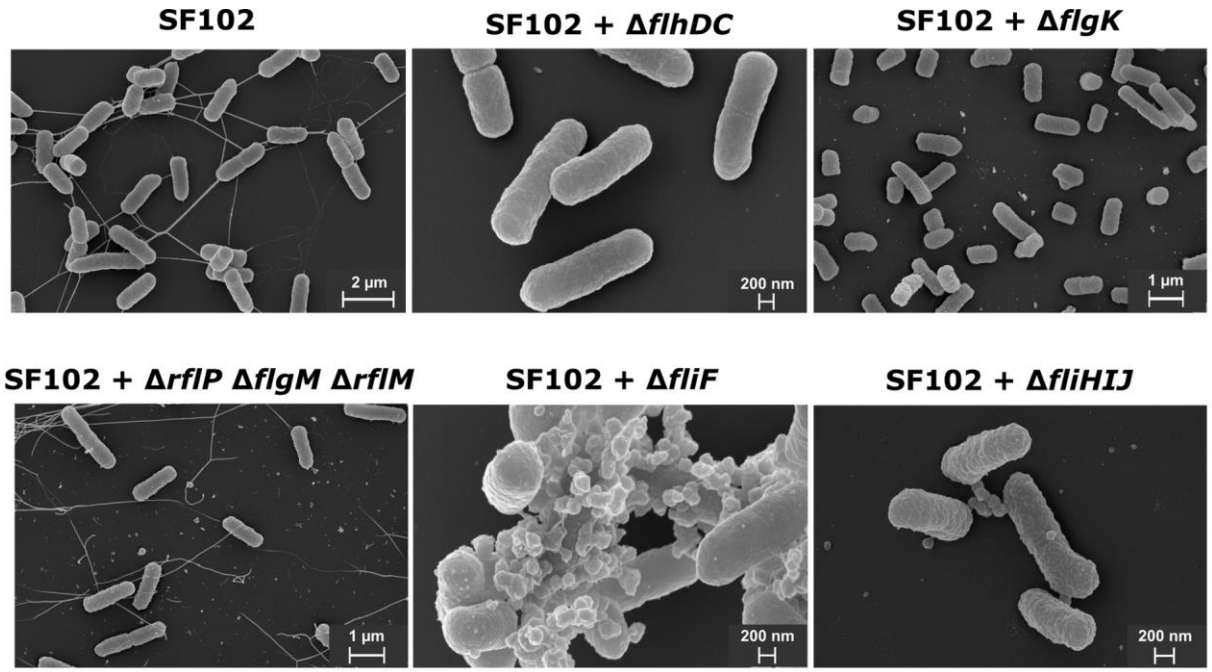


Strain	Count	# Flagella (Mean ± STD)	p-value
WT	133	3.6 ± 1.2	n.s.
FliC-ON	150	3.5 ± 1.4	n.s.
FljB-ON	135	3.5 ± 1.1	n.s.
FliC-ON FljB-ON	133	3.5 ± 1.2	n.s.
ΔrflP	117	4.4 ± 1.1	< 0.001
ΔrflP ΔflgM ΔrflM	135	4.9 ± 1.3	< 0.001









	SF102 + ΔrflP ΔflgM ΔrflM	SF102 + ΔflgK	SF102	SF102 + ΔflhDC	SF102 + ΔfliF	SF102 + ΔfliHIJ
OMVs	-	-	-	-	yes	yes
Therapeutic Effect