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Salmonella Typhimurium
The ’magic bullet’ against cancer?

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To Judith and my family
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List of Abbreviations

BMTT - Bacteria-mediated tumor therapy
CFU - Colony forming unit
CT26 - Colorectal carcinoma, ATCC CRL-2638
CTLA-4 - Cytotoxic T-lymphocyte associated Protein 4
dpi - Days post infection
F1.A11 - Fibrosarcoma cell line
hpi - Hours post infection
IFN-β - Interferon β
iv - Intravenously
IVIS - In vivo imaging system
LPS - Lipopolysaccharide
MAMP - Microbe-associated molecular pattern
MOI - Multiplicity of infection
PAMP - Pathogen-associated molecular pattern
PBS - Phosphate buffered saline
PDL-1 - Programmed cell death ligand 1
PRR - Pathogen recognition receptor
RASV - Recombinant attenuated Salmonella vector
RenCa - Renal adenocarcinoma
RT - Room temperature
S. Typhimurium - Salmonella enterica serovar Typhimurium
SPI - Salmonella pathogenicity Island
T3SS - Type-3 secretion system
TLR-4 - Toll-like receptor 4
TNF-α - Tumor necrosis factor α
UK-1 - Universal Killer
Wt - Wild-type
Summary

*Salmonella* Typhimurium - The 'magic bullet' against cancer?
Sebastian Felgner

Increasing numbers of cancer cases worldwide generate an urgent need for novel and sustainable therapies. Recently, the old concept of using bacteria like *Salmonella* Typhimurium as therapeutic agent was reconsidered. *S.* Typhimurium exhibits a high tumor specificity combined with intrinsic anti-tumor potency. To guarantee successful application, a balance between therapeutic benefit and safety is needed. Hence, the project aimed at tailoring an optimized strain.

Single gene deletions interfering with the LPS synthesis led to safe strains without therapeutic potential. To accommodate efficacy, such genes were expressed under the inducible P<sub>BAD</sub> promoter allowing complementation in culture which is lost *in vivo*. This ‘delayed attenuation system’ reinstalled the therapeutic benefit against CT26 tumors, i.e. growth retardation, while retaining safety. However, efficacy against more resilient tumors like RenCa was less prominent.

To further enhance the therapeutic potency, the LPS constructs were transferred to the more virulent and immunogenic *Salmonella* strain UK-1. The resulting strains showed increased efficacy but no sustainable effect. To further boost the immunogenicity, I genetically modified the Lipid A into a homogenously hexa-acylated structure. This optimization resulted in 100% CT26 rejection and a substantially improved therapy of RenCa tumors.

To increase the safety of the strains, the metabolic attenuation ∆aroA was introduced. Interestingly, extensive pleiotropic effects were observed for fatty acid and amino acid composition and turn over. Also flagellum synthesis was influenced. Most importantly, deletion of *aroA* improved the immunogenicity and virulence of the bacteria as well as their tumor therapeutic potential.

Therefore, by combining aspects of LPS modification with the metabolic mutation of *aroA* and the immunogenic *Salmonella* background UK-1, I was able to establish a new strain that would represent a strong basis for further strain design ultimately aimed for routine clinical application.
Zusammenfassung

*Salmonella* Typhimurium - Die Wunderwaffe gegen Krebs?

Sebastian Felgner


Einzelne Gen-Knockouts innerhalb der LPS Synthese führten zu sicheren Stämmen, jedoch ohne therapeutisches Potential. Deswegen wurden diese Gene mittels des induzierbaren Promoters P\textsubscript{BAD} exprimiert, um sie in der planktonischen Kultur zu komplementieren. Dieser Effekt sollte sich *in vivo* wieder verlieren. Dieses 'Delayed Attenuation System' stellte den therapeutischen Effekt gegenüber CT26 Tumoren (z.B. Wachstumsverzögerungen) wieder her, war aber gegen widerstandsfähigere Tumore wie RenCa wirksam, made.

Um die Effizienz weiter zu verstärken, wurden die Gen-Konstrukte in den wesentlich virulenteren und immunogeneren Salmonellen Stamm UK-1 transferiert. Diese Stämme waren effizienter, zeigten aber noch keine komplette Tumorabstoßung. Infolgedessen wurde zusätzlich das Lipid A Molekül zu einer einheitlichen hexa-acylierten Struktur verändert, um die Immunogenität weiter zu erhöhen. Diese Optimierung führte schließlich zu einer 100%igen Abstoßung von CT26 Tumoren und verbesserte die Therapie gegenüber RenCa erheblich.

Um letztendlich die Sicherheit zu steigern, wurde das metabolische Gen \textit{aroA} ausgeschaltet. Dieser Schritt führte zu globalen pleiotropen Effekten, die den Fettsäuren- und Aminosäurenmetabolismus sowie deren metabolischen Umsatz und die Geißelsynthese betraf. Diese Effekte hatten zudem einen positiven Einfluss auf die Immunogenität und Virulenz der Salmonellen, was ihr therapeutisches Potential zusätzlich verstärkte.

Zusammenfassend kann gesagt werden, dass durch Kombination der metabolischen Veränderung \textit{ΔaroA} mit der LPS Modifikation und dem Wechsel zu UK-1 ein Salmonellen Stamm kreiert wurde, der eine solide Grundlage für zukünftige Anwendungen im klinischen Bereich darstellen sollte.
1 Introduction

Cancer - a word that still spreads fear and anxiety in our modern society. The disease is often associated with extreme pain and suffering not only for the patients but also for their surroundings. According to recent statistics, every second individual will be diagnosed with cancer within its life time [1]. Therefore, nowadays, cancer will affect all of us sooner or later.

Due to the increased life expectancy in our population, cancer has become the second most frequent cause of death in industrialized countries outnumbered only by cardiovascular diseases (Fig. 1.1). The number of new diagnoses is predicted to duplicate within the next twenty years. These frightening numbers and the huge financial burden caused by cancer together with the suffering are the main arguments why cancer is one of the most supported fields of research. However despite of all this intensive research no general cure for cancer is yet available. The reason for this is mainly the complex biology and heterogeneity of this disease.

Fig. 1.1: Leading causes of death in Germany 2014. Cancer was the second most frequent cause of death in Germany in 2014. Total number of deaths was 868,356 (adapted from Statistisches Bundesamt).
1.1 Cancer biology and treatment

Cancer cannot be defined as a single disease. Rather it has to be understood as a group of
diseases. A classical definition describes cancer as neoplasia that is caused by uncontrolled
cell growth. However, the development and establishment of benign (non-invasive) or
malignant (invasive) tumors can be a long progress of clonal selection. It requires the
accumulation of various genetic changes and abnormal gene expression [2,3]. For such
reasons, cancer is also known as a genetic disease. As every cell of the human body can
potentially turn into a cancer cell, every tumor is unique. This explains why a general
therapy is difficult to achieve.

Although every second individual may receive a cancer diagnosis, only every fourth person
will succumb to the disease. This statistic implies that the fight against cancer is not
in vain even though the prognosis for certain cancers like the ones affecting the brain
or pancreas remains rather poor [1]. The factor that appears to matter the most is the
time of diagnosis. The earlier a developing cancer is found the higher is the chance of
curing it. At the moment, routine check-ups like colonoscopy or CT analysis provide the
possibility to detect even small cancers. Thus, many tumors can be removed surgically
before they become malignant or spread. Furthermore, the increasing knowledge on cancer
and tumor development have brought forward a definition of distinct characteristics, or
cancer 'hallmarks', that most of the tumors acquire during oncogenesis. Targeting such
hallmarks in the future may represent a strategy for novel type of therapies [4,5].

Hallmarks of cancer

The hallmarks of cancer summarize ten biological features that are required or impor-
tant for tumor development and tumor progression (see Fig. 1.2) [6,7]. Interestingly, the
number of these characteristics has increased over the last years as more insight into
oncogenesis has been gained. At the beginning of this century, cancer was defined by six
hallmarks that were already known for a long time and rather obvious. These include: i)
sustaining proliferative signaling, ii) evading growth suppressors, iii) resisting cell death and iv) enabling replicative immortality, all of which are connected to the chronic proliferative state of cancer. Most of these abilities are caused by mutations in tumor suppressor genes and oncogenes like p53 or RB influencing the cell cycle [8 - 10] or redirecting growth promoting cytokines like TGF-β [11].

The uncontrolled growth of a cancer cells further requires a constant supply of nutrients and oxygen. Therefore, the fifth hallmark describes the ability of a tumor to initiate the formation of new blood vessels. Angiogenesis, as it is termed, may be induced in response to stimulants VEGF-A (vascular endothelial growth factor A) or TSP-1 (thrombospondin-1) [12,13]. During the last stage of tumor development, typical morphological modifications are known as the 'epithelial-mesenchymal transition’. Moreover, loss of cell to cell

**Fig. 1.2:** Hallmarks of cancer and corresponding treatment options. A tumor can acquire ten distinct capabilities during the course of tumorigenesis that defines it as malignancy. Various therapies specifically interfere with these hallmarks to abrogate tumor development [4].
adhesion molecules promote the activation of invasion and metastasis and constitute the sixth hallmark [11,14].

Recent studies have resulted in expansion of these features introducing two new hallmarks together with additional two enabling characteristics [4,7]. The first additional hallmark outlines the ability to dysregulate cellular energetics. Although glycolysis only provides a small amount of ATP, most cancer cells shift their metabolism to glycolysis (Warburg-Effect), hence describing a unique feature of cancer cells [15,16]. This effect is also driven by mutations in oncogenes like ras or myc [17]. Again, the continuous acquisition of mutations and increasing genome instability is a crucial factor for tumor progression, and has thus been suggested as an important enabling characteristic of cancer development.

Due to the phenotype and genotype of a cancer cell, a tumor represents an anomaly within the human body that should usually be detected and destroyed by the innate and adaptive immune system, in particular by T and B lymphocytes, NK cells or macrophages [18]. However, a potential cancer cell has to overcome immune surveillance as a crucial step of tumorigenesis. Therefore, this ability is also suggested as a hallmark of cancer development. On the other hand, it is known that a tumor can manipulate the 'Cancer-Immunity' by inducing tumor-promoting inflammation [19]. Therefore, the involvement of the immune system in tumorigenesis remains ambiguous. In summary, the understanding of cancer remains incomplete. However, the hallmarks described offer possibilities to develop new therapeutic approaches that may be able to specifically target these checkpoints and abrogate tumor development.

**Cancer therapies**

The mortality rate of late stage cancer has remained almost unchanged during the last century. No general therapeutics are available. Although novel treatment techniques and strategies are continuously introduced into the clinics, the backbone of cancer therapy relies on the classical approaches: surgery, chemotherapy and radiotherapy.
These techniques aim to either remove or destroy cancerous tissue. Although, partial success can be achieved with such conventional treatment options, they also bear many disadvantages. In particular, not every tumor can be removed with a scalpel. Furthermore, the physical and chemical methods cannot distinguish between healthy and malignant tissue [20,21]. In addition, recent studies have shown that radiotherapy can induce tumor-promoting microenvironments that might support tumor re-establishment [22]. Encouraged by these drawbacks of conventional therapies, novel therapies are tested that target the hallmarks of cancer (see Fig. 1.2).

Such novel strategies include immune therapy like the use of monoclonal antibodies or adaptive T-cell transfers as well as gene therapy. Since many hallmarks of cancer originate from malfunctioning genes, gene therapy aims to reinstall the function of such genes. The delivery of functional genes by viral delivery vectors like Adenoviruses represents one potent strategy [23]. Another promising strategy exploits the newly discovered CRISPR/Cas9 technology to repair the affected genes [24,25]. However, these techniques are not specific to cancer and therefore bear a potential risk.

In contrast, immunotherapies are considered to be highly specific and can be separated into passive and active immunotherapy. The passive variant relies on the transfer of effector molecules or cells into a cancer patient. In this context, monoclonal antibodies (mAb) specifically targeting particular molecules of cancer cells like PI3K (phosphatidylinositol-3-kinases), VEGF or MEK (mitogen-activated protein-, extracellular signal-regulated kinases) are explored and already used in clinical trials [26]. The mechanism relies on the activation of the immune system by inducing for instance antibody-dependent cell-mediated cytotoxicity (ADCC) or interfering with signaling pathways. However in many cases, the efficacy of mAb is comparably low as they often do not reach the tumor at high enough concentrations or tumors may develop resistance mechanisms [26].

The adoptive T-cell transfer represents another potent strategy of a passive immunotherapy. Hereby, T cells are extracted from patients, activated by tumor-antigens and rein-
jected into the patients. In this context, it is also possible to genetically engineer T-cell receptors (e.g. CARs - chimeric antigen receptors) that are either tumor-specific or have a broader spectrum of antigen recognition [27,28].

In addition to the passive methods, active immunotherapy aims to reinstall or enhance an already present immune response. Active immunotherapies include for example cancer vaccines or the use of cytokines like IL-2 or GM-CSF [29]. Presently, there is great excitement about antibodies against immune checkpoint inhibitors like PD-1 or CTLA-4. These molecules are known to limit an immune response by inhibiting the activity of T-cells. Typically, this process controls an immune response to avoid over-shooting. However, it also limits the efficacy of an immune response against cancer cells. Blocking these receptors has been shown to enhance the immune response against certain types of cancers, thereby demonstrating the general feasibility of the strategy [30]. Most of these approaches are still under investigation and the road to routine might still be very long. Nevertheless, they may become routine treatments in the future. It is interesting to note that the approach of immune therapy is much older than present conventional therapies. The earliest form of immune therapy relied on the application of bacteria [31]. However, the inability to control bacteria at that time in addition to the general lack of knowledge on cancer development and immune surveillance is amongst the reasons why such immunotherapy was not followed up for almost a century. The following review summarizes the history of bacteria mediated tumor therapy and aims to illustrate the great potential of this unique form of active immune therapy.
References


1.2 Bacteria in Cancer Therapy: Renaissance of an Old Concept

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¹Department of Molecular Immunology, Helmholtz Centre for Infection Research, ²Institute of Immunology, Hannover Medical School

The rising incidence of cancer cases worldwide generates an urgent need of novel treatment options. Applying bacteria may represent a valuable therapeutic variant that is intensively investigated nowadays. Interestingly, the idea to apply bacteria wittingly or unwittingly dates back to ancient times and was revived in the 19th century mainly by the pioneer William Coley. This review summarizes and compares the results of the past 150 years in bacteria mediated tumor therapy from preclinical to clinical studies. Lessons we have learned from the past provide a solid foundation on which to base future efforts. In this regard, several perspectives are discussed by which bacteria in addition to their intrinsic anti-tumor effect can be used as vector systems that shuttle therapeutic compounds into the tumor. Strategic solutions like these provide a sound and more apt exploitation of bacteria that may overcome limitations of conventional therapies.

Contribution:
Sebastian Felgner wrote the manuscript.

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Felgner: Salmonella mediated tumor therapy
1.3 *Salmonella enterica* serovar Typhimurium

As shown in the previous section, the bacterium *Salmonella enterica* serovar Typhimurium (S. Typhimurium, Fig. 1.3) is intensively studied in context of host-pathogen interaction, as potential vaccine carrier and for bacteria mediated tumor therapy (BMTT). As the research focus of this study relies on the application and optimization of *S*. Typhimurium for BMTT, various strategies guiding such optimization procedures will be discussed in this section.

![Fig. 1.3: *Salmonella Typhimurium*. Left: Electron microscopy of negatively stained Wt *Salmonella* UK-1 (white arrows). Right: Scanning electron micrograph of CT26 tumor tissue infected with Wt *Salmonella* UK-1 (copyright Manfred Rohde, HZI).](image)

*S*. Typhimurium is a Gram-negative, facultative anaerobic, rod-shaped bacterium able to cause a variety of disease manifestations ranging from local infections like gastroenteritis to severe systemic infections [1]. Under natural conditions, this zoonotic pathogen is transmitted by ingestion of contaminated food or water. Withstanding the acidity of the stomach and reaching the small intestine, *Salmonella* triggers the uptake into the epithelial cell layer, especially into M-cells, by means of the SPI-I encoded type-3 secretion system (T3SS) [2]. In case of gastroenteritis, the infection remains limited to the lamina propria while during typhoid fever the bacteria escape the GI tract and disseminate via
the blood circulation to vital organs potentially even causing chronic infection [3].

During the course of infection, *Salmonella* encounters the defense mechanisms of the host in form of the innate and the adaptive immune system. Consequently, the host-pathogen relationship is a complex interplay of immune evasion strategies of the pathogen and immune recognition and clearance by host defense mechanisms [4]. Depending on the kind of interplay, immune responses may vary. Immune cells recognize bacteria by their pathogen associated molecular patterns (PAMPs). Therefore, these structures represent targets for optimization of bacterial effectors in BMTT. In case of *Salmonella*, the lipopolysaccharide (LPS) molecule and the flagellum are abundantly expressed PAMPs on the bacterial surface. Both molecules are known to be immune-stimulatory and hence modification of their synthesis and levels of expression can attenuate or optimize the bacteria. Thus, they represent suitable targets to tailor *Salmonella* vector strains for cancer therapy. These alterations mainly affect host-directed defense mechanisms, whereas the fitness of the bacteria should remain unaltered. For such reasons, metabolic mutations were also considered as additional modifications that may aid to install intrinsic attenuation via growth limitations in the mammalian host.

In summary, the efficacy of BMTT relies on an appropriate stimulation of the immune system by the bacteria. Modulating host-pathogen interactions for such required immune stimulation as well as for pathogen attenuation, represents a straight forward strategy to adapt *Salmonella* for BMTT. In the following, a rational strain design is described in that individual mutations and their effect on host-pathogen interactions are highlighted.

**LPS molecule**

The LPS molecule is a dominant constituent of the outer membrane of gram-negative bacteria like *Salmonella* and is known to be essential for the integrity of the bacteria in the host [5]. It consists of a variable O-Antigen, a conserved core structure and the Lipid A (Fig. 1.4). As LPS is highly immune-stimulatory, *Salmonella* has developed strategies
to modify its LPS structures to reduce immunogenicity. Adapting the LPS structure may thus represent a potent strategy to beneficially influence host-pathogen interactions and optimize *Salmonella* for BMTT.

The O-antigen and the core structure are known to confer resistance to effector molecules of the innate immune system like the complement system or phagocytes. For instance, a recent study has shown that the length of the O-Antigen influences the expression of the T3SS and the sensitivity towards complement lysis [7]. In essence, the shorter the O-Antigen becomes, the greater is the sensitivity to complement lysis of the bacteria and the higher the expression of the SPI-I encoded T3SS. Interestingly, once the LPS structure was further truncated by interfering with either the O-Antigen (i.e. ∆rfaL mutant) or the core synthesis (e.g. ∆rfaG or ∆rfaD mutants), *Salmonella* became increasingly more sensitive towards innate immune mechanisms and less invasive for phagocytic cells *in vitro* [8]. Therefore, altering the expression of LPS on the surface of *Salmonella* may represent a potent strategy to design a safe vector for *in vivo* application. The table 1.2 summarizes potential gene targets for the modification of LPS.

However, as commented in the review article, attenuating bacteria by modifying their virulence factors may become easily counterproductive. It could lead to over-attenuation...
1.3 *Salmonella enterica* serovar Typhimurium

Tab. 1.2: Genes of interest for LPS modification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wzzₜ</td>
<td>Length control of O-Antigen</td>
<td>[7]</td>
</tr>
<tr>
<td>rfaL</td>
<td>Connection of O-Antigen with outer core by gene encoded ligase RfaL</td>
<td>[9]</td>
</tr>
<tr>
<td>rfaH</td>
<td>Gene encoded RfaH is an transcriptional anti-terminator for synthesis of surface-associated molecules, thereby controlling, for instance, LPS length</td>
<td>[10]</td>
</tr>
<tr>
<td>rfaG</td>
<td>Connection of outer core sugar glucose with inner core sugar heptose II by gene encoded transferase RfaG</td>
<td>[9]</td>
</tr>
<tr>
<td>rfaD</td>
<td>Gene encoded epimerase RfaD catalyzes the stereochemical inversion of heptose I that is essential required for connecting the inner core to the KDO2-Lipid A molecule</td>
<td>[11]</td>
</tr>
</tbody>
</table>

and low efficacy. For such reasons, the immunogenicity of the LPS molecule was simultaneously increased by optimizing the Lipid A part of the LPS molecule.

The Lipid A molecule is the hydrophobic anchor of the LPS in the outer membrane (see Fig. 1.5). Furthermore, it is an important PAMP that can be recognized either extracellularly by the TLR4-MD2 receptor complex or intracellularly via a non-canonical

![Fig. 1.5: Lipid A molecule.](image)

The Lipid A molecule is the hydrophobic anchor of the LPS molecule in the outer membrane of gram-negative bacteria. The depicted genes represent possible targets for structural modifications.

_Felgner: Salmonella mediated tumor therapy_
inflammasome in a caspase-11 dependent manner [12,13]. Again, *Salmonella* is able to modulate the Lipid A structure by expression of various genes in order to avoid recognition by the immune system (Tab. 2). For instance, the tetra-acylated structure behaves antagonistic while hexa-acylated Lipid A is maximally stimulatory (14). For that reason, the construction of a *Salmonella* mutant harboring a hexa-acylated Lipid A structure should improve the efficacy of the therapy. Taken together, the complex LPS molecule offers possibilities to both attenuate and improve the immunogenicity of *Salmonella* at the very same time. Thus, it is situated in the focus of the *Salmonella* vector strain design in the present project.

Tab. 1.3: Genes of interest for Lipid A modification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>msbB</em></td>
<td>Adds myristic acid to primary linked acyl chain at 3’-position</td>
<td>[15]</td>
</tr>
<tr>
<td><em>lpxR</em></td>
<td>Removes acyloxyacyl residue from 3’-position</td>
<td>[14]</td>
</tr>
<tr>
<td><em>pagP</em></td>
<td>Adds palmitate to primary linked acyl chain at 2-position</td>
<td>[14]</td>
</tr>
<tr>
<td><em>pagL</em></td>
<td>Removes acyl chain from 3-position</td>
<td>[14]</td>
</tr>
<tr>
<td><em>arnT</em></td>
<td>Transfers 4-amino-4-deoxy-L-arabinose to 4’-phosphate group</td>
<td>[14]</td>
</tr>
<tr>
<td><em>eptA</em></td>
<td>Adds phosphoethanolamine to 1-phosphate group</td>
<td>[14]</td>
</tr>
</tbody>
</table>

The flagellar apparatus

The flagellum is a major virulence factor of many pathogenic bacteria, including *Salmonella*. While the nature of LPS as a causative agent of sepsis and agonist of the TLR-4 is well understood, the contribution of the flagellum to *Salmonella* pathogenesis remains ambiguous. On one hand, flagellar motility promotes bacteria-host interactions, adherence and invasion of host cells [16]. On the other hand, once the bacteria have reached their phagosomal destination, flagella synthesis is down-regulated to avoid immune recognition by extracellular TLR-5 or cytosolic by Naip5/6 [1,17]. This protective mechanism already indicates that constitutively expressed flagella have the potential to elicit strong immune reactions.

Felgner: *Salmonella* mediated tumor therapy
The flagellum apparatus consists of a sophisticated macromolecular machine made up by approximately 25 different proteins and can be divided into three main parts: i) a basal body that is embedded in the cytoplasmic membrane and traverses the periplasm and cell wall up to the outer membrane (the engine), ii) a flexible, curved structure known as the 'hook', which connects the basal body with the rigid filament and iii) a long external filament (the propeller, Fig. 1.6) [18].

Fig. 1.6: Flagellar hierarchy. The synthesis of the flagellum can be divided into three flagellar promoter classes that are constitutively expressed and controlled upon encountering various environmental stimuli (copyright Marc Erhardt, HZI).

The synthesis and correct spatiotemporal regulation of flagellar expression is a highly complex process (see Fig. 1.6). In short, various environmental signals drive the expression of the master operon complex FlhD$_4$C$_2$ which controls various gene expression patterns (Class I). In combination with the sigma factor $\sigma^{70}$, FlhD$_4$C$_2$ initiates the expression of class II promoter genes which are responsible for the establishment of the hook basal body (HBB). The synthesis of the HBB is controlled via the anti-sigma factor FlgM until the hook reaches its final length of approximately 55 $\mu$m. Upon reaching this checkpoint, FlgM is secreted, thereby dissociating from sigma factor $\sigma^{28}$ which may now trigger the expression of the class III promoter genes. These genes result in the final synthesis of the filament as well as chemosensory and motor proteins [18, 19].

Felgner: Salmonella mediated tumor therapy
Since flagella are immune-stimulatory *Salmonella* has evolved sophisticated regulatory networks that ensure tight control of flagella synthesis during the course of infection. However, the role of the various regulatory factors and correct spatiotemporal synthesis of flagellar components during host-pathogen interactions remains elusive. In this context, one should note that *Salmonella* bears two antigenically distinct filament proteins, FliC (phase-1) and FljB (phase-2), that are alternatively expressed. The phase-2 protein FljB is co-expressed with the transcriptional repressor FljA which interacts with the *fliC* mRNA transcript, and prevents FliC synthesis. The switch from phase-1 to phase-2 flagellin and vice versa is mediated by the Hin recombinase that can invert the *fljBA* promoter. When transcription via the *fljBA* promoter takes place, *Salmonella* produces FljB and inhibits FliC translation via FljA. On the other hand, once the promoter is inverted *fljAB* transcription is blocked and FliC is produced. Although this flagellar phase variation is controlled by *hin*, the signal inducing this switch is not yet known [20]. Interestingly, a recent study using *Salmonella* as vaccine carrier, has shown that bacteria co-expressing both flagella proteins FliC and FljB exhibit enhanced adjuvanticity [21]. This demonstrates the potential of modifying flagella assembly and synthesis for applications in translational medicine. Thus, it was hypothesized that manipulating components of the regulatory network or the assembly pathway of the flagella will allow to exploit the immunogenic potential of these PAMPs. It should lead to a generation of *Salmonella* vector strains with increased adjuvanticity/immunogenicity (Tab. 1.7).(Tab. 1.4).

**Tab. 1.4: Potential targets to alter flagella appearance.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Gene variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase lock</td>
<td>FliC-ON, FljB-ON, FliC and FljB</td>
</tr>
<tr>
<td>Non-motile</td>
<td>ΔmotA</td>
</tr>
<tr>
<td>No filament</td>
<td>ΔfliHIJ, ΔflgE, ΔflgK, ΔfliF</td>
</tr>
<tr>
<td>Overexpression</td>
<td>ΔydiV, PflhDΔ, ΔlrhA, ΔecnR</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>ΔcheY, ΔcheZ</td>
</tr>
</tbody>
</table>

Felgner: *Salmonella* mediated tumor therapy
In summary, manipulation of flagella synthesis for directed strain design could represent a promising strategy to improve potency of a bacterial vector. For that reason, it was decided to test the modifications shown in Tab. 1.4 for their potential in BMTT.

**Metabolic mutations**

The application of metabolic mutations represents another feasible way to attenuate *Salmonella* for *in vivo* applications. The general aim of this strategy is to limit the growth of *Salmonella* in the body of the treated patient by interfering with essential biosynthesis pathways of compounds like amino acid or cell wall components that are not freely available in the host. For instance, gene deletions of *aroA*, *aroC* and *aroD* are commonly applied in vaccine strains to turn *Salmonella* auxotrophic for aromatic amino acids [22]. Therefore these mutant strains exhibit a significantly reduced *in vivo* fitness and virulence. However, with regard to cancer therapy, this auxotrophic behavior could be exploited to drive tumor colonization and to enhance tumor specificity as the necrotic area of the tumor may represent a source of nutrients from which the bacteria may be able to reinstall their natural physiology. The potency of these genetic alterations for BMTT can already be seen with the prominent tumor targeting candidates VNP20009 and A1-R. VNP20009 carries a deletion in the *purI* loci and therefore is not able to synthesize purines while A1-R was shown to be deficient for leucine and arginine production [23,24]. Nevertheless, both strains have been shown to be highly tumor specific even at the maximal tolerable dose [25].

These findings suggest that the use of metabolic mutations in *Salmonella* could represent a powerful tool to obtain a safe strain with high tumor specificity. This will be considered for strain design especially as a single attenuating mutation will not suffice to hold for a rigid safety profile. The potential of reversion to wild type would be unacceptably high.
References


1.3 *Salmonella enterica* serovar Typhimurium


*Felgner: Salmonella* mediated tumor therapy
1.4 Aims of the project

Within the last decades, cancer therapies including the newly introduced immune therapies have significantly improved. Nevertheless, no general cure is available at the moment. In accordance, novel approaches like bacteria-mediated cancer therapy are extensively explored. In particular the bacterium *Salmonella* Typhimurium has been demonstrated to exhibit a high tumor specificity along with strong intrinsic anti-tumor properties which can result in tumor retardation and prolonged survival of the host. To render the pathogenic *Salmonella* suitable for clinical application, attenuation is required. However, modifications explored thus far have frequently resulted in over-attenuation. Therefore, it is accepted that the major challenge in tailoring a perfect anti-tumor bacterium is to find the adequate balance between safety and therapeutic power.

In most studies, attenuation is achieved by interfering with the synthesis and homeostasis of crucial virulence factors of *Salmonella*. Alternatively, the general metabolism is influenced to reduce their *in vivo* fitness. Unfortunately, this straightforward approach has often led to a reduction of the immune stimulatory properties, which are required to obtain a proper anti-tumor response. The present work was carried out under the hypothesis that clever exploitation of particular host-pathogen interactions may overcome this obstacle. Therefore, the major aim of the current project is to find suitable molecular targets of *Salmonella* that allow for attenuation and improvement of the bacteria at the very same time.

The LPS molecule represents a suitable molecular target. While the outer structures confer resistance to the innate immune system, the inner parts can act immune stimulatory. Therefore, the feasibility of modulating this molecule for BMTT is tested in a model employing murine transplantable tumors in BALB/c mice bearing syngeneic tumors like CT26 (colon carcinoma), RenCa (renal adenocarcinoma) or F1.A11 (fibrosarcoma). It was speculated that an optimized LPS molecule should have beneficial effects for attenuated strains and increase anti-tumor properties due to upregulated/ altered cytokine induction.
In addition to LPS, the role of the flagella as additional PAMP of *Salmonella* should be investigated in the same context. Finally, in order to increase the tumor specificity of the strains, the well-known mutation of *aroA* should be implemented in the strain design as well.

In detail, the construction and investigation of such *Salmonella* vector strains involve the following specific objectives:

1. **Investigating the feasibility of modified LPS structures in *S. Typhimurium* for BMTT.**
   
   The LPS structure should be modified by targeted gene deletions that either affect the length of the LPS (e.g. *rfaD, rfaG*) or optimize the Lipid A (e.g. *lpxR, pagP*). The safety and efficacy of these *Salmonella* mutants should be evaluated *in vitro* and *in vivo*.

2. **Optimizing the *Salmonella* LPS mutants for BMTT by using a delayed attenuation system.**
   
   To increase the therapeutic efficacy of *Salmonella*, LPS synthesis should be placed under the control of an inducible arabinose promoter. The concept relies on the hypothesis that an early wild-type like phenotype would further enhance the outcome of the therapy.

3. **Transfer gene constructs to the more virulent background strain *S. Typhimurium UK-1*.**
   
   Recent studies have shown that the UK-1 strain of *Salmonella* bears an increased intrinsic potential to induce an immune response. Therefore, the feasibility of this strain for BMTT was evaluated after transferring the LPS mutations into this background.
4. Increasing the tumor specificity and safety by applying the metabolic mutation $\Delta aroA$.

Ever since $aroA$ has been described, it is considered as safe. However, a dual role of $aroA$ in vivo was observed. Therefore, the phenotype of *Salmonella* mutants deficient for $aroA$ should be elucidated and its role for BMTT evaluated.

Altogether, this study aims to construct an optimized *Salmonella* vector strain that bears an attenuated character combined with an improved immune stimulatory capacity. Such optimized strains may represent the ’magic bullet’ for effective cancer treatment by using them as a targeted delivery vector system in the future.
2 Efficiency of Conditionally Attenuated *Salmonella enterica* Serovar Typhimurium in Bacterium-Mediated Tumor Therapy

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Increasing numbers of cancer cases generate a great urge for new treatment options. Applying bacteria like *Salmonella enterica* serovar Typhimurium for cancer therapy represents an intensively explored option. These bacteria have been shown not only to colonize solid tumors but also to exhibit an intrinsic antitumor effect. In addition, they could serve as tumor-targeting vectors for therapeutic molecules. However, the pathogenic *S*. Typhimurium strains used for tumor therapy need to be attenuated for safe application. Here, lipopolysaccharide (LPS) deletion mutants (ΔrfαL, ΔrfαG, ΔrfαH, ΔrfαD, ΔrfαP, and ΔmsbB mutants) of *Salmonella* were investigated for efficiency in tumor therapy. Of such variants, the ΔrfαD and ΔrfαG deep rough mutants exhibited the best tumor specificity and lowest pathogenicity. However, the intrinsic antitumor effect was found to be weak. To overcome this limitation, conditional attenuation was tested by complementing the mutants with an inducible arabinose promoter. The chromosomal integration of the respective LPS biosynthesis genes into the araBAD locus exhibited the best balance of attenuation and therapeutic benefit. Thus, the present study establishes a basis for the development of an applicably cancer therapeutic bacterium.

Contribution:
*Michael Frahm and Sebastian Felgner contributed equally to this article. Sebastian Felgner wrote the manuscript.*

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

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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 \textit{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.

Contribution:
*Sebastian Felgner performed the experiments. Sebastian Felgner wrote the manuscript.*

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*Felgner: Salmonella mediated tumor therapy*
AroA deficient *Salmonella* Typhimurium – more than a metabolically attenuated mutant

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Recombinant attenuated *Salmonella* Typhimurium strains are believed to act as powerful live vaccine carriers that are able to elicit protection against various pathogens. Auxotrophic mutations, such as a deletion of *aroA*, are commonly introduced into such bacteria for attenuation without incapacitating immune-stimulation. In this study, the surprising finding is described that deletion of *aroA* dramatically increased the virulence of attenuated *Salmonella* in mouse models. Mutant bacteria lacking *aroA* elicited increased levels of the pro-inflammatory cytokine TNF-\(\alpha\) after systemic application. A detailed genetic and phenotypic characterization in combination with transcriptomic and metabolic profiling demonstrated that \(\Delta aroA\) mutants display pleiotropic alterations in cellular physiology, lipid and amino acid metabolism, as well as increased sensitivity to penicillin, complement and phagocytic uptake. In concert with other immune modulating mutations, deletion of *aroA* affected flagellin phase variation and gene expression of the virulence associated genes *arnT* and *ansB*. Finally, \(\Delta aroA\) strains displayed significantly improved tumor therapeutic activity. These results highlight the importance of a functional Shikimate pathway to control homeostatic bacterial physiology. They further highlight the great potential of \(\Delta aroA\) attenuated *Salmonella* for the development of vaccines and cancer therapies with important implications for host-pathogen interactions and translational medicine.

Contribution:
*Sebastian Felgner performed the experiments. Sebastian Felgner wrote the manuscript.*

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5 Conclusion and Outlook

The present study demonstrates the therapeutic potential of *Salmonella* Typhimurium. It should be considered a step forward towards sustainable optimizations in BMTT. Several essential aspects that are important for such an application of *S.* Typhimurium have been successfully addressed. I confirmed that single gene deletions interfering with crucial virulence factors of *Salmonella* including LPS, such as ∆rfaD or ∆rfaG, can easily lead to over-attenuation. However, it is crucial to retain the therapeutic and immune stimulatory effect of bacteria. Thus, it remains challenging to find ways to install an appropriate balance of therapeutic potency and tolerability. Different strategies concerning this aspect were investigated in this thesis. A delayed attenuation strategy allowed an engineered switch from a Wt-like phenotype in culture to an attenuated bacterial phenotype in the host to accommodate different phases of the therapy. Indeed, this ‘two-phase’ system proved to be more efficient than a range of single gene attenuation.

A second attempt was directed towards optimization of bacterial structures like the Lipid A molecule that is known to interact with the host immune system. In addition, the transfer of the therapeutic features from *S.* Typhimurium 14028 as genetic background strain to the intrinsically more immune stimulatory and virulent strain UK-1 was carried out. Together, these modifications led to increased therapeutic efficacy without compromising the safety of the resulting strain. In result, the *Salmonella* strain represents now a potent vector system able to eradicate CT26 tumors with an efficacy of 100%.

While bacterial therapy was very successful against CT26 tumors using these optimized *Salmonella* strains (i.e. tumor clearance), at best only growth retardation was achieved with more resilient tumors like RenCa or F1.A11. The reason for this discrepancy remains unclear, although for instance F1.A11 is known to modify the cytokine pattern upon infection which could potentially influence the therapeutic susceptibility. Additional genetic modifications of the bacteria addressing the flagellar apparatus did to some extent im-
proven the therapeutic outcome against such tumors. However, a rational strain concept is still indefinable. This was clearly exemplified by the implementation of the aroA deletion into the therapeutic strains. Although intended as a metabolically attenuating mutant, virulence and adjuvanticity was strongly enhanced. Thus, despite attempts of a rational strain design, which has indeed led to improved therapeutic bacteria, a degree of chance or trial and error cannot yet be avoided.

Outlook

Despite successful improvement of the abovementioned strains it becomes clear that a therapy which relies on the intrinsic anti-tumor properties of Salmonella would only be successful in particular cases. Therefore, in addition to the intrinsic therapeutic properties, the vector potential of these bacteria should be exploited. A promising strategy may utilize Salmonella as a specific delivery system to shuttle therapeutically active compounds into the tumor to maximize the local efficacy. This might be helpful as most often chemotherapeutic drugs or monoclonal antibodies do not reach all areas of the tumor or might be deviated by alternative target cells. This presently limits such therapeutic strategies. Applying bacteria that selectively colonize tumors and thereby transport therapeutic compounds to places where their activity is required, could tremendously improve such therapies. The major challenge in this case is to ensure secretion of therapeutic compounds into the tumor as such would provide a continuous supply of the drug over an extended time period. This may appear to be more efficient than a release of the compound by bacterial lysis despite of the recent interest in such a system [1]. Therefore, a flagella based secretion system was investigated and demonstrated to successfully secrete several bacterial toxins and other proteins (data not shown). Moreover, for some of the secreted toxins, biological activity was demonstrated. In order to control the targeted secretion of the compound, either inducible promoters or the recently reported quorum sensing system could be applied [1]. However, improvement of the present therapy by

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such bacteria is still pending. The ultimate goal of these attempts is to apply the bacterial therapy to human cancer patients. As a first step, the model system should be expanded from transplantable syngeneic to spontaneous or autochthonous tumor systems as they resemble more closely the current situation in a human cancer patient with regard to heterogeneity and tumorigenesis. In addition, humanized mice could serve as model system to evaluate the efficacy of our vector strains in more close-to-clinic models. Further optimization and adaptation of our strains in such humanized backgrounds may open the door to Phase I and Phase II clinical trials. Altogether, I believe that exploitation of the unique intrinsic properties of *Salmonella* combined with their potential as a targeted delivery vector system could help pave their way into clinics in the near future.

References

6 Appendix

6.1 List of Publication

Research paper & Reviews


Felgner: Salmonella mediated tumor therapy
Popular scientific article


Submitted article

6.2 Acknowledgement

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