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Review

Salmonella and cancer: from pathogens to therapeutics*

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Bacterial cancer therapy is a concept more than 100 years old — yet, all things considered, it is still in early development. While the use of many passive therapeutics is hindered by the complexity of tumor biology, bacteria offer unique features that can overcome these limitations. Microbial metabolism, motility and sensitivity can lead to site-specific treatment, highly focused on the tumor and safe to other tissues. Activation of tumor-specific immunity is another important mechanism of such therapies. Several bacterial strains have been evaluated as cancer therapeutics so far, *Salmonella* Typhimurium being one of the most promising. *S.* Typhimurium and its derivatives have been used both as direct tumoricidal agents and as cancer vaccine vectors. VNP20009, an attenuated mutant of *S.* Typhimurium, shows significant native toxicity against murine tumors and was studied in a first-in-man phase I clinical trial for toxicity and anticancer activity. While proved to be safe in cancer patients, insufficient tumor colonization of VNP20009 was identified as a major limitation for further clinical development. Antibody-fragment-based targeting of cancer cells is one of the few approaches proposed to overcome this drawback.

Key words: bacterial cancer therapy, immunotherapy, cancer vaccine, tumor targeting, *Salmonella*, VNP20009

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INTRODUCTION

It was at the end of the 19th century that bacteria were for the first time intentionally applied for cancer treatment. William Coley, a bone sarcoma surgeon at the Memorial Hospital in New York, having conducted a thorough literature search, found 47 well-documented cases of beneficial influence of serious bacterial infections in tumor patients (McCarthy, 2006). On the basis of the then-available data he concocted a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* bacteria, which became known as Coley's toxin. Over the next forty years Coley applied his toxin to almost 1000 patients who suffered from various types of inoperable cancers. Striving to improve the effectiveness of the therapy, he used various doses of his toxin, different regimens and durations of treatment, as well as different routes of application and therefore it is difficult to fully comprehend his enormous work. In many cases several-month treatment resulted in complete tumor regression. The five-year survival period for patients suffering from inoperable carcinomas ranged from 34 to 73%, and for those suffering from inoperable sarcomas was in the range between 13 and 79%, varying with the tumor subtype (Green & Hoption Cann, 2007). However, the results of Coley's treatment have been non-reproducible, uncertain, and unpredictable and therefore his therapy often met with strong criticism from the medical community. At the beginning of the 20th century it was gradually displaced by newly developing radiotherapy, which resulted in fast tumor destruction and pain relief, although not necessarily in complete tumor eradication, especially at the stage of advanced, metastatic disease.

With the progress of immunology it became clear that the mechanism of action of Coley's toxin involves activation of the immune system and a multilevel modulation of immune response. This understanding restored interest in possible therapeutic applicability of Coley's approach. Richardson and coworkers (1999) compared the effectiveness of Coley's toxin with contemporary cancer therapies based on published results concerning patients treated with Coley's toxin and matched controls from National Cancer Institute's Surveillance Epidemiology End Result database (Richardson *et al.*, 1999). They found higher rates of ten-year survival of Coley's patients compared to patients subjected to modern treatment in kidney cancer (33 *vs.* 23%), ovarian cancer (55 *vs.* 29%), and sarcoma (50 *vs.* 38%), which gives food for thought. The attempts to re-evaluate Coley's concept are undertaken anew. In 2012 the new phase 1 clinical trial investigating the safety and the dosage of biochemically well-defined and good manufacturing practice (GMP)–compliant Coley's toxin, presently known as MBV (mixed bacterial vaccine) was started in the Ludwig Institute for Cancer Research. It involves patients suffering from cancers expressing NY-ESO-1 antigen, including metastatic melanoma, head and neck carcinoma, sarcoma and prostate cancer (Karbach *et al.*, 2012). The results of the trial have not been published yet.

In line with Coley's concept of using bacteria in cancer therapies, other bacterial species were evaluated

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Abbreviations: BCG, Bacillus Calmette-Guerin; CEA, CarcinoEmbryonic Antigen; CTL, Cytotoxic T Lymphocyte; DC, Dendritic Cell; GFP, Green Fluorescent Protein; HBcAg, core antigen of hepatitis B virus; IFNγ, InterFeroN gamma; IL-12, InterLeukin 12; IL-18, InterLeukin 18; LLO, Listeriolysin; mAFP, mouse Alpha-FetoProtein, MHC, Major Histocompatibility Complex, mPSCA, mouse Prostate Stem Cell Antigen, MTD, Maximum Tolerated Dose; PAMP, Pathogen-Associated Molecular Pattern, PBS, Phosphate Buffered Saline; scFv, single chain variable fragment antibody; SCV, Salmonella-Containing Vacuole; shRNA, short hairpin RNA; Smac, Second mitochondria-derived activator of caspases; SPI, Salmonella Pathogenicity Island; STAT3, Signal Transducer and Activator of Transcription 3; T3SS, Type III Secretion System; TAA, Tumor-Associated Antigen, TNF, Tumor Necrosis Factor, TRAIL, TNF-Related Apoptosis-Inducing Ligand, Treg, regulatory T cells, TRP2, Tyrosinase-Related Protein 2, TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling, VEGFR, Vascular Endothelial Growth Factor Receptor.

for a possible anti-tumor effect. Early experiments on "hemorrhagic allergy" in 1910s and 1920s showed that animals injected with bacterial filtrates developed hemorrhagic necrosis upon re-challenge with bacteria (so called Sanarelli-Shwartzman phenomenon). In particular, this condition was caused by *Bacillus typhosus*, which used to be a synonym for *Salmonella* Typhi at that time (Shwartzman, 1928). Interestingly, this shock syndrome induced a therapeutic effect in tumor-bearing animals (Shwartzman, 1935). However, the studies were discontinued due to very high treatment-related mortality; nowadays the adverse effects can be explained by strong stimulation of proinflammatory cytokines including tumor necrosis factor (TNF) by endotoxin and other bacterial products. Bacillus Calmette-Guerin (BCG), attenuated bovine tuberculosis bacteria (*Mycobacterium bovis*), has been used for decades as a vaccine protecting against tuberculosis. However, it also appeared to be one of the most successful cancer immunotherapeutics. BCG, in the form of repeated intravesical instillations, has already been in use for over 30 years as a standard method to prevent cancer recurrence after endoscopic surgery of intermediate- and high-risk non-muscle invasive bladder cancer (Kawai *et al.*, 2013). It is also effective against inoperable bladder carcinoma *in situ* resulting in a 70–75% complete response rate (Alexandroff *et al.*, 1999). Unfortunately, in other tumor types BCG application has not proved to be more effective than conventional therapies (Alexandroff *et al.*, 1999).

BACTERIAL CANCER THERAPIES CAN ADDRESS THE KEY ISSUES IN CANCER TREATMENT

Starting from 1946, chemotherapy gradually became the principal therapeutic strategy in cancer and bacterial therapies were largely forgotten. The success of small-molecule drugs in cancer treatment was remarkable, but turned out to be incomplete. Chemotherapy of many tumors suffers from limited efficacy towards cancer cells and damaging action towards normal cells. Both phenomena have a significant impact on therapeutic outcomes, being responsible for incomplete tumor killing and adverse effects in other tissues. The two factors responsible for those clinical drawbacks are: low specificity towards cancer tissue and insufficient penetration of the tumor by chemotherapeutics. Cancer cells form a complex and heterogeneous system with areas of high metabolism, rich in nutrients and oxygen, as well as distal regions of poor perfusion, quiescence and necrosis (Saunders *et al.*, 2012).

The use of bacteria as anticancer agents might have multiple advantages over other therapeutic approaches. Bacterial therapies can benefit from microbial metabolism, motility and sensitivity to address a number of issues related to currently used treatment modalities. One of the most important issues, relevant to virtually all chemotherapeutic and biological treatments, is limited accessibility of the tumor tissue to passively-distributed therapeutics. Both in the case of small molecule drugs, as well as larger molecules — cytokines, antibodies or even viruses — the therapeutic agent diffuses from the bloodstream into the periphery, with no transport system that could cross biological barriers, act against haemodynamic gradients or facilitate preferential accumulation in the tumor tissue. In fact systemic delivery of passive therapeutics produces relatively large drug concentrations in the bloodstream and relatively low drug concentrations in the tumor, resulting in limited efficacy and increased extratumoral toxicity. Bacteria offer unique mechanisms that can facilitate site-specific treatment, highly focused on the tumor and safe to other tissues. This can be possible due to several features of bacteria-based therapeutics:

Environmental sensing

Tumor tissue has a complex and heterogenic metabolism that makes it particularly resistant to systemic treatment. The natural ability of bacteria to receive signals via chemoreceptors can be used to effectively target this unique microenvironment. Oxygen concentration is one of the most important signals for anaerobic bacteria and is of particular interest in anticancer therapy since hypoxia is a common feature of tumors. Moreover, auxotrophic bacterial strains that rely on the uptake of certain metabolites can recognize the tumor microenvironment as a source of nutrients. This phenomenon can facilitate specific accumulation of bacteria in the tumor. One of the most effective examples is the use of *Clostridium* spores that can only germinate in oxygen-free tumor regions (Dang *et al.*, 2001).

Motility

The bacterial microorganism is not only capable of detecting chemoattractants, but can also actively follow chemical gradients. This contrasts with passive therapeutics that simply diffuse into tissues from the circulation. Bacteria are able to penetrate deep into the tumor tissue and perform specific actions, e.g. express proteins or transfer genes, to tumor cells localized remotely from the vasculature. This feature can also allow bacteria to cross physiological barriers and accumulate in cellular regions that are either distant and inaccessible for passive therapeutics or quiescent and unresponsive to chemotherapy. For example, motile strains of *Salmonella* were shown to effectively penetrate tumor tissue *in vitro* (Toley & Forbes, 2012). However, the role of bacterial motility in *in vivo* tumor localization is unclear (Stritzker *et al.*, 2010).

Active delivery

Unlike chemical or biological molecules, microorganisms are metabolically active and are able to perform specific metabolic tasks at the tumor site. These include the production of cytotoxic agents (e.g. bacterial toxin), expression of immunomodulatory molecules (e.g. cytokine) or enzymatic conversion of a prodrug into an active therapeutic. Strains derived from intracellular pathogens can infect tumor cells and deliver specific proteins or genes to the tumor tissue (St Jean *et al.*, 2008). Nevertheless, the intratumoral action is not always necessary as bacteria can also express tumor-related antigens to stimulate systemic anticancer immune responses. A *Listeria monocytogenes*-based cancer treatment that has recently entered clinical development is an example of this approach, where the bacterium delivers tumor antigens directly to the antigen presenting cells (Singh & Wallecha, 2011).

Controlled propagation

Preferential growth in tumor tissue exhibited by many bacterial species is not exclusive to bacteria; experimental oncolytic viral therapies are based on a similar principle. However, once administered to the patient viral vectors are beyond external control. In contrast, bacterial therapeutics are susceptible to antibiotic treatment and there-

Table 1. Overview of candidate live attenuated bacteria strains for cancer treatment.

Parental species/strain	General features	
Mycobacterium bovis (BCG)	Obligate aerobe, non-motile, gram-positive, facultatively intracellular, pathogenic to animals	Approved for bladder cancer management as intravesical BCG therapy, proven to be more effective than intravesical chemotherapy (reviewed by Kawai *et al*., 2013).
Salmonella Typhimurium ssp.	Facultative anaerobe, motile, gram-negative, facultatively intracellular, broad-host pathogen	Transplantable and genetic animal tumor models in immunodeficient and immunocompetent mice. Completed and ongoing phase 1 clinical trials in melanoma (*S.* Typhimurium VP20009) (Toso *et al*., 2002) and liver cancer patients (*S.* Typhimurium x4550 expressing IL-2), respectively.
Salmonella Typhi ssp.	Facultative anaerobe, motile, gram-negative, facultatively intracellular, human-adapted pathogen	Growth inhibition of subcutaneous LM3 mammary adenocarcinoma in BALB/c mice after *S.* Typhi CVD 915 multiple treatment (injection into the tumor, peritumoral tissue and draining lymph node areas) (Vendrell *et al.*, 2011). Ongoing phase 1 clinical trial with licensed oral *S.* Typhi Ty21a strain carrying VEGFR2 coding sequence under the control of eukaryotic promoter for anti-angiogenic therapy and immunotherapy of pancreatic cancer (Niethammer *et al.*, 2012).
Clostridium sp. spores	Anaerobic, gram-positive, sporulating, vegetative forms are motile; pathogenicity: *C. sporogenes* — rarely, *C. novyi* — yes, *C. beijerinckii* — no	Strains modified to deliver pro-drug converting enzymes, cytokines, antibodies against tumor antigens. Spores administered systematically germinate and multiply only in hypoxic/necrotic areas of tumors causing significant oncolysis (reviewed by Umer *et al*., 2012) Ongoing phase 1 clinical trial of *C. novyi*-NT spores in patients with solid tumor malignancies[1].
Escherichia coli Nissle 1917	Facultative anaerobe, motile, gram-negative, non-pathogenic probiotic strain for treatment of gastrointestinal disorders	Intravenous administration of bacteria expressing azurin (apoptosis inducing protein) decreased the number of lung metastases in orthotopic 4T1 breast cancer in BALB/c mice and restrained growth of subcutaneous B16 melanoma in C57Bl/6 mice (Zhang *et al*., 2012b).
Escherichia coli K-12/LLO	Strain expressing *hly* gene encoding modified listeriolysin (LLO) lacking its N-terminal signal sequence	*E. coli* MC4100(DE3) modified with plasmid-encoded *hly* gene for truncated listeriolysin (LLO) in order to facilitate antigen export from phagosome to macrophage cytoplasm for antigen presentation in association with MHC class I. Strain co-expressing LLO and truncated WT1 (Wilms tumor gene 1) induced antitumor effect against WT1--expressing tumors in mice (RMA thymoma and MBL2 leukemia) through induction of CTLs and inhibition of Treg. Paraformaldehyde-fixed bacteria were administered in preventive and therapeutic setting (Dai *et al.*, 2009).
Listeria monocytogenes	Facultative anaerobe, motile, gram-positive, facultatively intracellular, pathogenic to animals and human, actively escape into cytosol of infected cell	Bacteria-mediated transfer of plasmid DNA into mammalian cells (bactofection) (reviewed by Tangney *et al*., 2010). Regression of established transplantable tumors and breaking immune tolerance in genetic tumor models in mice administered with *L. monocytogenes dal-, dat-,* and *actA*-deleted strain secreting HPV-16 E7 antigen associated with cervix, head and neck cancer; Her-2/neu breast cancer antigen or prostate specific antigen (PSA) (reviewed by Paterson *et al*., 2010). *L. monocytogenes* HPV-16 E7 (ADXS11-001) = ongoing phase 2 clinical trial for cervical cancer and phase 1 trial for HPV-16+ oropharyngeal carcinoma[2].
Shigella flexneri	Facultative anaerobe, non--motile, gram-negative, facultatively intracellular, human pathogen	Intravenous injection of *S. flexneri* M90T *aroA* mutant strain to mice bearing 4T1 subcutaneous tumors or to spontaneous breast cancer bearing transgenic MMTV-HER2 mice resulted in apoptosis and depletion of tumor associated macrophages (TAMs), which correlated with pronounced 4T1 tumor growth inhibition (Galmbacher *et al.*, 2010).

[1]Please refer to the NCT01118819 clinical trial listed at ClinicalTrials.gov; [2]Please refer to the ClinicalTrials.gov database and clinical trials no. NCT01266460 and NCT01598792, respectively

fore fully manageable in the clinical setting – therapy can be stopped at the onset of adverse effects or when the bacteria are no longer needed. In fact the use of live biotherapeutics that contain antibiotic resistance genes in clinical trials is not recommended by the regulatory agencies[1].

[1]Please refer to: *Guidance for Industry: Early Clinical Trials with Live Biotherapeutic Products*, US Food and Drug Administration, 2012; *Environmental Risk Assessments for Medicinal Products containing, or consisting of, Genetically Modified Organisms*, European Medicines Agency, 2007

Immunostimulation

Bacterial vectors augment the anti-tumor immune response not only because of their cargo but also due to their own potent immunostimulatory activity. A growing tumor creates an immunosuppressive environment and establishes immune escape mechanisms that limit the maturation of dendritic cells (DCs) as well as the priming and migration of specific T cells into the tumor. Bacteria provide a strong danger signal to the immune system. The specific conserved bacterial structures such as components of the cell wall or unmethylated CpG sites in bacterial DNA constitute so-called pathogen-associated

molecular patterns (PAMPs) that are recognized by Toll-like receptors expressed by innate immune cells. PAMPs activate innate- and initiate adaptive immune responses. The induction of TNF, IFNγ and IL-12 results in the recruitment and activation of DCs which upon migration to the lymph nodes may efficiently present tumor antigens to T cells (Chorobik & Marcinkiewicz, 2011). It has been shown that indeed microorganisms colonizing tumors and promoting an inflammatory reaction in the tumor microenvironment potentiate the anti-tumor host response (Avogadri *et al.*, 2005).

The unique features of bacterial therapeutics create the opportunity for novel anticancer strategies, that combine tumor-related molecular gradients, natural bacterial features and genetic engineering. Bacteria meet all the requirements for an ideal tumor-targeting agent and might become a novel tool in the anticancer toolbox (Forbes, 2010). A summary of the bacterial strains studied in cancer treatment is shown in Table 1.

SALMONELLA HAS A NUMBER OF FEATURES FAVORABLE FOR CANCER THERAPY

Salmonella belongs to the *Enterobactericae* family, a group of Gram-negative, facultatively anaerobic and facultatively intracellular pathogenic bacteria. Currently, based on genome sequence similarity, the genus *Salmonella* is categorized into two species *S. bongori* and *S. enterica* which in turn is divided into six subspecies including *S. enterica* subsp. *enterica* (Tindall *et al.*, 2005). According to the White-Kauffman-Le Minor scheme, the subspecies are classified into more than 2500 serovars by serotyping: O-antigens (polysaccharide domain of the cell surface LPS); H1, H2 antigens (flagellin proteins) and the Vi antigen (Guibourdenche *et al.*, 2010). New classification methods including genome-based techniques are currently under development.

In humans, *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Typhi are causative agents of gastroenteritis and typhoid fever, respectively. There are more than 27 million cases of typhoid fever worldwide each year with a mortality rate of 0.8% resulting from intestinal perforation and peritonitis or severe toxic encephalopathy connected with myocarditis and hemodynamic shock (Parry *et al.*, 2002). Infections with *S. enterica* serovar Typhimurium result in an estimated 94 million cases of gastroenteritis worldwide and 0.16% mortality (Feasey *et al.*, 2012). *S.* Typhi is an exclusively human pathogen, while hosts of *S.* Typhimurium include rodents, poultry and cattle.

Salmonella Typhimurium and *Salmonella* Typhi are closely related serotypes of *S. enterica* species which differ in host adaptation and the outcome of infection. Depending on the serotype and host, *Salmonella* colonizes solely the intestinal epithelium leading to gastroenteritis or spreads beyond the gut mainly to liver and spleen causing typhoid fever. *S.* Typhimurium infection in humans is restricted to the digestive tract, with the exception of infants, elderly or immunocompromised individuals in whom it can spread, while in mice *S.* Typhimurium causes enteric fever. On the contrary, *S.* Typhi causes typhoid fever in humans but is not pathogenic to animals. In general, serotypes that lack host specificity, such as *S.* Typhimurium, are more frequently associated with disease in young rather than in adult animals, suggesting their non-optimal adaptation to mature immune system (Baumler *et al.*, 1998).

About 90% of the genes in *S.* Typhi and *S.* Typhimurium serovars are identical (McClelland *et al.*, 2001), but among about 4000 genes of *S.* Typhi, more than 200 are functionally disrupted or inactive, while most of their homologs are still fully functional in *S.* Typhimurium. Genes that differ include virulence factors that determine the pathogenic potential, which can in part explain the restricted host range of *S.* Typhi (McClelland *et al.*, 2001). The majority of virulence factors are encoded by genes grouped in a few clusters in the genome, termed *Salmonella* Pathogenicity Islands (SPIs). *S.* Typhimurium and *S.* Typhi genomes share 11 SPIs, one is specific for *S.* Typhimurium (SPI14) and four are specific for *S.* Typhi (SPI7, 15, 17 and 18) (Kolyva, Waxin, and Popoff, 1992).

Salmonella sp. has an ability to multiply inside phagocytic and nonphagocytic cells including macrophages, dendritic cells (DCs), neutrophils, M cells and epithelial cells (Malik-Kale *et al.*, 2011). The ability of *Salmonella* to invade and survive within a host cell is dependent on two Type III Secretion Systems (T3SS), the multiprotein complexes with a needle-like structure present on the bacteria cell wall. Proteins involved in the assembly of the two major T3SSs of *Salmonella* are encoded by SPI1 and SPI2. T3SS1, encoded by SPI1, is required for efficient invasion of nonphagocytic cells. In contrast, the expression of SPI2-encoded T3SS2 is induced following the internalization of *Salmonella* into host cells and is required for post-invasion processes (Velge *et al.*, 2012). During the invasion, some SPI1 encoded proteins such as InvG, InvJ, PrgH, PrgI, PrgK and SpaO are responsible for the assembly of the needle complex, whereas others, including SipB, SipC and SipD, translocate the effector proteins through this needle. Regulation of gene expression in response to the surrounding microenvironment depends on several two-component systems, such as PhoQ/PhoP whose expression is induced by Mg^{2+} starvation and low pH (Lucas *et al.*, 2000). Another system, OmpR-EnvZ, responds to changes in osmolarity and regulates invasion (Bajaj *et al.*, 1995) as well as intracellular survival. Upon internalization, *Salmonella* modifies the phagosome into a *Salmonella*-Containing Vacuole (SCV), which is characterized by the presence of some lysosomal membrane proteins, low pH, and transient interactions with the endocytic pathways. At early stages of maturation, SCV recruits and quickly looses early endocytic markers, such as the early endosomal antigen 1 (EEA-1) or transferrin receptor (TfR) (Steele-Mortimer *et al.*, 1999). At later points of maturation it acquires several late endosomal markers, including LAMP1 (Lysosomal-Associated Membrane Protein 1) (Steele-Mortimer, 2008). Bacterial replication is accompanied by the formation of dynamic membrane tubules termed *Salmonella*-Induced Filaments, which extend from SCV throughout the cell (Schroeder *et al.*, 2011). The maturation of SCV is controlled by SPI2-encoded effectors which allow bacteria to avoid phagosome-lysosome fusion and degradation and protect them against reactive oxygen and nitrogen species (Chakravortty *et al.*, 2002; Janssen *et al.*, 2003).

After the ingestion of *S. enterica,* the bacteria use different routes to cross the intestinal barrier. The main route leads through the receptor-mediated endocytosis by microfold cells (M-cells) in Peyer's patches (Jepson & Clark, 2001), independently of SPI1 and SPI2 (Martinez-Argudo & Jepson, 2008). Then the bacteria are taken up by the underlying macrophages. Enterocytes of the intestinal epithelium, except for the M cells, engulf *Salmonella* by macropinocytosis in a SPI1-dependent manner. The

bacteria can be also engulfed by DCs (Swart & Hensel, 2012). In epithelial cells *S*. Typhimurium can reside and replicate in SCV, as well as in the cytosol (Malik-Kale *et al*., 2012); however, these two intracellular populations of bacteria are transcriptionally distinct: the intravacuolar bacteria are SPI2-induced, while the cytosolic bacteria are SPI1-induced and flagellated (Knodler *et al*., 2010). *Salmonella* induces caspase-1 and -2 mediated apoptosis of infected macrophages and epithelial cells (Jesenberger *et al*., 2000; Kim *et al*., 1998; Monack *et al*., 1996). Apoptosis depends mainly upon the SPI1 effector protein, SipB, delivered to the host cell by SPI1-encoded T3SS (Jesenberger *et al*., 2000). Bacteria, engulfed by macrophages and DCs spread from Peyer's patches to mesenteric lymph nodes, spleen and liver. The ability of *Salmonella* to elicit systemic disease is serovar-dependent and correlates with its capability to survive and replicate inside the host cell and to avoid the host adaptive immune response (reviewed by Swart & Hensel, 2012).

THERAPEUTIC *SALMONELLA* STRAINS ARE DERIVED FROM *S*. TYPHIMURIUM OR *S*. TYPHI

S. Typhimurium infection in mice remains the dominant animal model of typhoid fever because it leads to comparable systemic disease with dissemination of bacteria to the lymphatic system and peripheral organs. Hence the prevalence of *S*. Typhimurium-based tumor therapeutic vectors studied in murine models of cancer. The choice of *S*. Typhimurium over *S*. Typhi for preclinical research on cancer therapy could be arguable but is legitimated by the availability of suitable animal models. Some improvement was made by the development of a transgenic mouse model of *S*. Typhi infection (Song *et al*., 2010), but it has not been applied to *Salmonella*-based tumor therapy studies yet. Nevertheless, a superior induction of immune response to heterologous antigen delivered by an orally administered vaccine strain of *S*. Typhimurium over *S*. Typhi was shown in a clinical trial (Angelakopoulos & Hohmann, 2000). Both serotypes are amenable to genetic modifications of virulence and in terms of safety are equally eligible as live attenuated therapeutic strains. There has already been some success in the clinical use of attenuated *Salmonella* strains. *S*. Typhimurium VNP20009 was shown to be safe when administered to cancer patients (Toso *et al*., 2002) and *S*. Typhi Ty21a, a live attenuated oral vaccine against typhoid fever, has already been applied for more than 30 years to adults and children above 6 years of age who are at risk of *S*. Typhi exposure. A phase I clinical trial evaluating the safety, tolerability, and effects of the *S*. Typhi Ty21a strain used as a DNA delivery vehicle in cancer patients has been announced (Niethammer *et al*., 2012) but its results are not yet available.

Attenuation of virulence is crucial for the development of new *Salmonella*-based vector strains in order to elicit an appropriate profile of the immune response. Up to date about 50 genes of *Salmonella* spp. have been proven to be feasible for inactivation in order to obtain an attenuated derivative with modified virulence or metabolic functions. Gene inactivation is achieved by laboratory selection of the desired phenotype, for example by passaging bacteria through selective conditions and screening for survivors, or is the result of site-directed- or chemical mutagenesis. Inactivation of genes coding for proteins involved in metabolic pathways generates auxotrophic strains, i.e. dependent on external sources of nutrients, for example aromatic amino acids (*aro* mutants) or purines (*pur* mutants). Direct attenuation of virulence involves inactivation of genes encoding proteins interacting with the infected organism or factors regulating their expression. The latter include: (*i*) *phoP* and/or *phoQ*, which regulate expression of many genes, e.g. those contributing to the resistance against antimicrobial peptides and genes located in pathogenicity islands, (*ii*) *cya* and *crp* genes coding for global regulatory factors involved in expression of many proteins of cellular catabolism, (*iii*) *htrF* gene, which enables survival under stress conditions (Garmory *et al*., 2002; Raupach & Kaufmann, 2001; Raupach *et al*., 2003). The attenuating mutations introduced into *Salmonella* experimental strains are summarized in Table 2.

An interesting example comes from a sophisticated work of Robert Hoffman's group: A1-R is an auxotrophic *Salmonella* strain developed for increased tumor targeting and limited toxicity. First, the researchers obtained an A1 auxotrophic strain dependent on an external source of leucine and arginine by nitrosoguanidine (NTG) mutagenesis of *S*. Typhimurium 14028-GFP. To further enhance tumor targeting of *S*. Typhimurium A1, bacteria were injected *i.v.* into nude mice bearing HT-29 human colon adenocarcinoma. GFP-expressing bacteria isolated from the excised infected tumors, were termed A1-R and had an increased ability to adhere to cancer cells; the number of A1-R bacteria attached to HT-29 cells *in vitro* was approximately six times higher than that of the parental A1 strain (Zhao *et al*., 2006).

S. Typhimurium A1-R was tested in numerous human tumor xenografts in nude mice, such as MARY-X breast tumor (Zhao *et al*., 2006), orthotopic prostate PC-3 cancer (Zhao *et al*., 2007), orthotopic human pancreatic cancer (Nagakura *et al*., 2009), orthotopic U87 spinal cord glioma (Kimura *et al*., 2010), orthotopic MDA-MB-435 breast cancer (Zhang *et al*., 2012a), lung fibrosarcoma (Hayashi *et al*., 2009b) and lung osteosarcoma (Hayashi *et al*., 2009a) resulting in a significant tumor growth inhibition in all tested models, with complete tumor regression in a few of them.

Applicability of A1-R in metastatic disease has also been tested. Hayashi *et al*. (2009b) developed a lymph node metastasis model of human pancreatic cancer (XPA-1) by injecting XPA-1 cells into the inguinal lymph node of nude mice which resulted in tumor growth in the axillary lymph node. Five of six mice had their lymph node metastases eradicated within 7–21 days after intravenous treatment with A1-R in contrast to growing metastases in the untreated control group (Hayashi *et al*., 2009b).

A1-R has also been tested in immunocompetent mice bearing murine Lewis lung carcinoma (Tome *et al*., 2013). The authors proposed to apply a priming dose of 1×10^6 cfu followed, 4 hours later, by a therapeutic dose of 1×10^7 cfu of A1-R. The priming dose resulted in an elevated serum level of TNF and tumor vessel destruction, which could facilitate the invasion of tumor by bacteria.

As it can be expected, the mode of attenuation affects the quality of the anti-*Salmonella* immune response. While cytokines of the Th1 type (IFNγ, IL-12, IL-18) are crucial for protection against *Salmonella* in mice and humans, different mutant *Salmonella* strains require alternative cytokines for the control of infection. Studies with knockout mice showed that IFNγ and TNF were essential for the early control of infection with both wild type and *aroA* mutant strains. In contrast, TNF was not required for the clearance of the *aroA* mutant (Raupach & Kaufmann, 2001).

Table 2. Attenuated strains of *S.* Typhimurium developed as bacterial vaccine vectors.

Parental strain	Modified strain	
ATCC 14028	A1-R	leucine and arginine auxotrophs (Zhao *et al.*, 2005)
SL1344	BRD509	*aroA aroD* aromatic compound dependent (Strugnell *et al.*, 1992)
NCTC12023 (ATCC 14028)	MvP728	*purD htrA* adenine dependent; defective intra-macrophage survival (Xiong *et al.*, 2010)
Not stated	Re88	*aroA dam* (Xiang *et al.*, 2001) DNA adenine methylase mutants are defective in protein secretion, cell invasion and M cell cytotoxicity
SL3261	SB824	*aroA sptP* (encodes virulence protein SptP) (Panthel *et al.*, 2005)
14028s	SHJ2037	*relA spoT* (genes that encode ribosome-bound and cytosolic ppGpp synthetase, respectively) accumulates in infarcted myocardium due to defective ppGpp synthesis (ΔppGpp) (Song *et al.*, 2010)
SL1346	SL3261	*aroA* (Meng *et al.*, 2010)
2337–65 (WRAY)	SL7207	*aroA* dependent on *p*-aminobenzoate and 2,3-dihydroxybenzoate for the synthesis of aromatic amino acids; these two compounds are not available in a mammalian host (Hoiseth & Stocker, 1985)
ATCC 14028	VNP20009 (YS1646)	*msbB purI* purine dependent; reduced ability to induce septic shock due to altered lipid A structure (Low *et al.*, 2004)
SL7207	YB1	*aroA* modified to express *asd* essential gene under hypoxia-induced promoter (Yu *et al.*, 2012)
UK-1	X4550	*cya crp asd* (Saltzman *et al.*, 1996) adenylate cyclase and cyclic AMP receptor protein mutants are avirulent in mice

Attenuated *Salmonella* strains preferentially colonize solid tumors and inhibit their growth in animal models (Bermudes *et al.*, 2002; Pawelek *et al.*, 1997; Zhao *et al.*, 2005). In some therapeutic approaches attenuated strains were used without any further modifications to exert tumor-directed cytotoxic effects and induce proper anti-tumor immune response. Apart from the A1-R strain described above also the application of the *S.* Typhimurium X9241 strain brought successful results. Bacteria injected intratumorally to CT26 tumors or CT26 tumors expressing human tumor antigen, Her-2/neu, significantly inhibited tumor growth. Surprisingly, they did not potentiate tumor-antigen-specific cellular immunity. However, they induced an important functional shift in the phenotype of tumor-infiltrating $CD11b^{+}Gr\text{-}1^{+}$ subpopulation of leukocytes (myeloid derived suppressor cells, MDSC) towards immunogenic TNF-secreting neutrophils. Moreover, the therapy reduced the percentage of regulatory T cells which may promote tumor development (Hong *et al.*, 2013).

Thanks to the intracellular lifestyle and immunomodulatory properties attenuated *Salmonella* strains are also used as vectors for the delivery of therapeutic molecules. The cargo is a genetic material which codes for proteins including tumor antigens (described in the next chapter), cytokines, apoptosis-inducing factors, prodrug-converting enzymes, or short hairpin RNAs (shRNAs) able to silence expression of a protein of choice. Numerous approaches were developed to enhance and navigate the immunomodulatory properties of *Salmonella* through genetic modifications that ensure the delivery of proapoptotic molecules or cytokines straight to the tumor, which limits their potential undesired systemic side effects. The examples are listed in Table 3.

The group of John C. Reed demonstrated a significant increase in tumor growth inhibition by a *Salmonella* strain expressing IL-18 in immunocompetent mice (Loeffler *et al.*, 2008). The modified and parental strains showed comparable toxicity limited to the spleen and liver. IL-18 stimulates T cell and NK cell to proliferation, cytotoxicity and cytokine production. Administration of IL-18-secreting *Salmonella* did not improve tumor infiltration by $CD8^{+}$ T lymphocytes but led to increased infiltration by granulocytes, $CD4^{+}$ T cells and $DX5^{+}$ NK cells, when compared to *Salmonella* control strain (Loeffler *et al.*, 2008).

In order to enhance the proapoptotic activity of *S.* Typhimurium towards infected cancer cells several research groups took an advantage of apoptosis-inducing factors and equipped bacteria with appropriate expression vectors. TRAIL (TNF-related apoptosis-inducing ligand) has a capacity to selectively induce apoptosis in a wide variety of cancer cells but hardly in normal cells which makes it a promising cancer therapeutic (Hylander *et al.*, 2005; Shanker *et al.*, 2008; Walczak *et al.*, 1999; Yagita *et al.*, 2004). Similarly to TNF, TRAIL exerts its proapoptotic effect through the death receptor-dependent pathway which activates the caspase cascade (LeBlanc & Ashkenazi, 2003).

Ganai *et al.* (2009) constructed an expression vector placing *TRAIL* under the promoter of *recA* gene involved in the prokaryotic SOS response to DNA damage (Anderson & Kowalczykowski, 1998). It created a radiation-inducible system, where expression of TRAIL was turned on by genotoxic damage evoked by radiation. Such a system enables temporal and spatial control of the gene expression, additionally increasing the selectivity of the therapy towards cancer cells subjected to radiotherapy. Intravenous administration of *S.* Typhimurium VNP20009 equipped with a P*recA*-TRAIL construct (VNP/pRE-TR) into Balb/c mice bearing subcutaneous 4T1 mammary carcinoma followed by 2 Gy whole body

Table 3. Therapeutic approaches based on *S*. Typhimurium modified to deliver apoptosis inducing or immune modulatory factors.

Vector	Cargo	Mouse strain		Therapeutic scheme	
BRD509	cDNA for TNF fused to 160 amino acid residue-N--terminal fragment of SipB under the control of bacterial *lac* promoter	C57Bl/6	*s.c.* TC-1 cervical tumor, B16F10 melanoma	Two *s.c.* injections of 10^8 CFU/mouse 7 and 14 days after *s.c.* inoculation of 10^5 B16F10	Complete inhibition of tumor cell growth in 90% of animals, but these mice were not protected against second B16F10 challenge. Reduced growth of all listed tumors, although with diverse efficacy (Yoon *et al.*, 2011)
		BALB/c	*s.c.* 4T-1 breast cancer, CT26 colon cancer, and RENCA kidney carcinoma		
SL7207	TRAIL and apoptin genes under the control of eukaryotic promoters	BALB/c nude	*s.c.* SGC-7901 human gastric cancer (5×10^5 cells)	Intratumoral injection 7 days after tumor implantation, repeated every 7 days (2×10^6 CFU)	Cancer cell apoptosis in tumor tissue and tumor regression (Cao *et al.*, 2010)
VNP20009 and MVP728	Stat3 shRNA and survivin expressing plasmid	C57Bl/6	*s.c.* B16F10 melanoma (10^5 cells)	YS1646 with Stat3 shRNA *i.v.* injected at 10^7 CFU when tumor volumes were 50 mm^3 or above (7–8 mm diameter), followed by oral gavage with 10^7 CFU of MVP728 with survivin expression plasmid	Enhancement of CD4+ and CD8+ T lymphocytes infiltration into tumors, increased tumor cell apoptosis, suppression of *s.c.* B16F10 melanoma growth (Manuel *et al.*, 2011)
VNP20009	cDNA for IL-18 fused to N-terminal leader sequence for directing secretion, under the control of bacterial *ompC* promoter	BALB/c	*s.c.* CT26 colon carcinoma (10^5 cells)	*i.v.* injection of 5×10^6 CFU when tumors were visible (14 days after inoculation)	Reduced tumor growth, leukocyte infiltration into tumors, increased intratumoral level of IL-1β, TNF, IFNγ
		BALB/c	*s.c.* D2F2 breast carcinoma (2.5×10^5 cells)	*i.v.* injection of 5×10^6 CFU on day 9, 14 and 19 after tumor inoculation	Significant tumor growth inhibition
		C57Bl/6	*i.v.* Lewis lung carcinoma (5×10^4 cells)	*i.v.* injection 6, 13, and 20 days after tumor cells injection	Reduced tumor burden in lungs (Loeffler *et al.*, 2008)
VNP20009	IDO shRNA (immunosuppressive molecule indoleamine 2,3 dioxygenase 1)	C57Bl/6	*s.c.* B16F10 melanoma (2.5×10^5 cells)	2.5×10^6 CFU injected intravenously twice, 4 days apart, into mice with tumor diameters equal or greater than 7 mm	Synergistic effect of IDO silencing and *S.* Typhimurium on tumor growth suppression. Increased tumor influx of PMNs and intratumoral cell death. Tumor growth suppression occurred also in the absence of functional adaptive immunity (Blache *et al.*, 2012)
VNP20009	TRAIL under the control of bacterial anaerobic-inducible *nirB* promoter	C57Bl/6	*s.c.* B16F10 melanoma (5×10^5 cells) and RM-1 prostate cancer (2×10^5 cells)	*i.p.* injection of 10^5 CFU per mouse on day 7 for B16F10 or day 9 for RM-1 after tumor inoculation	Increased levels of TRAIL in B16F10 tumor but not in liver or spleen and tumor growth inhibition. VNP-TRAIL was not more effective in tumor growth suppression of TRAIL-resistant RM-1 tumors than VNP control strain (Chen *et al.*, 2012)
VNP20009	CCL21 under the control of bacterial *ompC* promoter	BALB/c	*s.c.* CT26 colon carcinoma (2.5×10^5 cells)	Intravenous injection 9, 14 and 19 days after tumor inoculation	Significantly inhibited growth of subcutaneous tumors and pulmonary tumor foci (Loeffler *et al.*, 2009)
			i.v. D2F2 breast carcinoma (5×10^4 cells) for pulmonary tumor model	*i.v.* injection of 5×10^6 CFU at day 6, 13, and 20 after tumor cells injection	

γ-irradiation 2 days later resulted in a significant decrease in tumor growth. The combined treatment increased the tumor size doubling time by 50% compared to VNP/pRA-TR treatment alone and by 100% in comparison to PBS control. The combined therapy has a synergistic effect since irradiation alone barely influenced tumor growth (Ganai *et al.*, 2009).

A similar approach was exploited by Chen *et al.* (Chen *et al.*, 2012) who placed TRAIL cDNA under the control of *nirB* promoter induced by hypoxic conditions (Chatfield *et al.*, 1992). The *S.* Typhimurium VNP20009 strain carrying TRAIL expression vector was intraperitoneally administered to B16F10 melanoma-bearing C57BL/6 mice. Significant inhibition of melanoma tumor growth

and extended survival time were observed. The TUNEL assay showed that the therapeutic effect of modified bacteria was associated with a significant increase in apoptosis of melanoma cells compared to mice receiving the control strain. Importantly, the immunohistochemical study revealed that indeed TRAIL was preferentially expressed in the hypoxic, necrotic area of the tumor, and not in the oxygenated liver or spleen, which may explain limited toxicity of TRAIL in normal tissues.

It has been shown that Smac (second mitochondria-derived activator of caspases) is involved in TRAIL-induced apoptosis and may affect the efficiency of TRAIL-based therapies (Deng *et al.*, 2002; Zhang *et al.*, 2001). Also *in vivo* studies showed that complete regression of established glioma in a mouse model was achieved only when TRAIL therapy was associated with Smac administration (Fulda *et al.*, 2002; Pei *et al.*, 2004). Fu *et al.* (2008) took an advantage of this observation and engineered a modified *S.* Typhimurium SL3261 strain carrying a dual-gene vector coding for both Smac and TRAIL. The cDNAs were placed under the promoter of human telomerase reverse transcriptase, highly active in majority of cancers and generally inactive in normal differentiated cells (Kim *et al.*, 1998). LL/2 Lewis lung carcinoma, B16F10 melanoma and 4T1 mammary carcinoma cells infected with the modified bacteria showed high expression levels of both proteins accompanied by a high rate of apoptosis. Moreover, oral administration of the modified strain significantly suppressed tumor growth in all tested mice models (LL/2, B16F10, 4T1), without any observable side-effects. The volumes of the tumors were lowered approximately by 65–75% compared to groups treated with a control strain and by 90% compared to PBS-treated animals.

Cao *et al.* (2010) applied combination of TRAIL with another apoptosis-inducing factor — apoptin (VP3), a protein of the chicken anemia virus proved to promote tumor cell-specific apoptosis in a p53-independent manner (Zhuang *et al.*, 1995). The study showed that intratumoral administration of *S.* Typhimurium SL7207 carrying apoptin and TRAIL coding sequences under the control of eukaryotic cytomegalovirus immediate early promoter to human gastric tumor xenografts in nude mice resulted in increased effectiveness of bacteria-mediated tumor growth suppression, with complete tumor regression in some animals. TUNEL assays in tissue sections showed a higher apoptotic rate in mice treated with *S.* Typhimurium SL7207 carrying apoptin and TRAIL genes compared to a control strain (Cao *et al.*, 2010).

SALMONELLA CAN BE ALSO USED AS A TUMOR VACCINE VECTOR

The vast majority of tumors express proteins or other antigens that are absent (or present only in very low quantities) in healthy adult tissues. These tumor-associated antigens (TAAs) are potentially immunogenic and tumor development is usually accompanied by specific, although often ineffective, anti-TAA immune response. TAA vaccines used for cancer therapy often fail, probably due to inadequate antigen presentation and insufficient activation of innate immunity. The application of *Salmonella* as a vector for TAAs should result in overcoming both impediments. The first attempts to deliver TAA via *Salmonella* were undertaken in late 1990s. From that time numerous studies utilizing natural (mPSCA, mAFP, survivin, endoglin) or artificial (β-galactosidase) tumor antigens have proved that placing a TAA-coding transgene under strong cytomegalovirus promoter in a plasmid carried by *Salmonella* allows for TAA expression in the cytoplasm of infected cells or dendritic cells which engulfed the infected, apoptotic cells (Paglia *et al.*, 1998; Yrlid & Wick, 2000); TAA expression elicits efficient cell-mediated or both cell-mediated and humoral immune responses (Ahmad *et al.*, 2011; Chou *et al.*, 2006; Fest *et al.*, 2009; Huebener *et al.*, 2008; Jarosz *et al.*, 2013; Paglia *et al.*, 1998). However, this is not always the case. The group of Dai-Ming Fan transformed *Salmonella* with a plasmid, which expressed a fusion protein consisting of a mimotope of the gastric tumor antigen, MG2 with either PADRE T helper epitope (Guo *et al.*, 2003) or HBcAg, a strong Hepatitis B virus T-cell-dependent and T-cell-independent antigen (Meng *et al.*, 2005). Although oral administration of either *Salmonella* strain resulted in a partial inhibition of the growth of the subsequently injected Ehrlich ascites carcinoma, only the humoral but not the cell-mediated response against MG2 could be detected (Guo *et al.*, 2003; Meng *et al.*, 2005).

It is believed that intracellular location of *Salmonella* within phagosomes may limit the ability to generate a MHC class I-restricted immune response towards transgene-encoded proteins. However, *Salmonella* expresses a multi-protein complex, T3SS, to deliver some bacterial effector proteins into the host cell in order to modulate its functions and create a microenvironment favorable for bacterial survival and proliferation (Galan and Collmer, 1999). A special N-terminal signal sequence directs bacterial proteins for export through T3SS to the host cell cytoplasm. This very mechanism was employed by several research groups to deliver *Salmonella* plasmid-encoded tumor antigens to the host cell cytoplasm and thus make them available for the MHC class I presentation. Instead of sequences encoding TAAs alone, the constructs coding for chimeric proteins consisting of a tumor antigen preceded by an N-terminal fragment of a bacterial protein subjected to T3SS export (containing secretion and translocation signals) were placed in the *Salmonella* plasmid. These included peptides derived from the SopE, SseF and YopE proteins (Jellbauer *et al.*, 2012; Manuel *et al.*, 2011; Nishikawa *et al.*, 2006; Zhu *et al.*, 2010). The chimeric transgenes were usually placed under bacterial promoters activated after invasion of the host cell.

This approach was applied by Nishikawa *et al.* in an attempt to generate a therapeutic vaccine against cancers overexpressing the NY-ESO-1 antigen (Nishikawa *et al.*, 2006). This germ cell antigen seems to be a proper target for immunotherapy, since it is expressed by a broad variety of tumors but not by normal somatic cells. Following oral administration of the *Salmonella*-based vaccine that delivered NY-ESO-1 and assured its cytoplasmic localization, $CD8^+$ T cell-dependent regression of established NY-ESO-1-expressing tumors was observed. What is more, the vaccine was able to initiate a process known as epitope spreading and was therefore effective even towards tumors lacking the NY-ESO-1 antigen. Intratumoral injection of this *Salmonella* strain delivering the antigen and engaging pre-existing NY-ESO-1-specific $CD8^+$ T cells resulted in tumor regression and activation of subsets of T cells recognizing at least two different tumor antigens that were not delivered by *Salmonella* (Nishikawa *et al.*, 2006).

A more complex transgene was introduced into a *Salmonella* plasmid by Zhu *et al.*, who worked on an efficient anti-melanoma vaccine (Zhu *et al.*, 2010). It encoded a fusion protein consisting of the secretion and translocation signals of SopE and a fragment of the

melanoma-specific TRP2 (tyrosinase-related protein 2) antigen containing three immunodominant epitopes. The presence of Hsp70, often referred to as immunochaperone, facilitates the proper presentation of antigenic peptides to cytotoxic T cells and may therefore enhance the anti-melanoma immune response. This vaccine induced a specific CTL response against B16F10 melanoma and showed strong protective as well as therapeutic effects (Zhu *et al.*, 2010).

Another modification aiming in augmenting the presentation of *Salmonella*-delivered neuroblastoma antigens was proposed by the group of Lode (Fest *et al.*, 2009; Huebener *et al.*, 2008). The chimeric protein encoded by *Salmonella* plasmid contained a stably ubiquitinated tumor antigen, either tyrosine hydroxylase- (Huebener *et al.*, 2008) or survivin (Fest *et al.*, 2009) fragments. The presence of ubiquitin guaranteed proteasomal degradation of proteins encoded by the transgenes and increased MHC I-mediated peptides presentation. This approach led to significant $CD8^+$ T cell-dependent inhibition of neuroblastoma growth (Fest *et al.*, 2009; Huebener *et al.*, 2008) and decreased the rate of metastasis (Huebener *et al.*, 2008) or tumor growth upon rechallenge (Fest *et al.*, 2009).

Manuel *et al.* also chose survivin as an excellent target for tumor therapy (Manuel *et al.*, 2011). This anti-apoptotic protein is expressed at a low level in normal adult tissues but is abundant in essentially all solid tumors (Altieri, 2003), as well as in endothelial cells during angiogenesis (Tran *et al.*, 1999). However, unlike their predecessors, the researchers utilized bacterial promoter to express a codon-optimized transgene in *Salmonella* and used the SseF secretion signal to transport survivin to the cytoplasm of the infected cells. The combined therapy consisting of sequential intravenous injection of two *Salmonella* strains: the first encoding shRNA targeting a tolerogenic transcription factor, and the second coding for SseF-survivin, turned out to be effective even in the case of large, well-established melanoma tumors. The silencing of STAT3 expression, crucial for this very promising result, led to increased proliferation of intratumoral $CD4^+$ and $CD8^+$ T cells and elevated levels of granzyme B (Manuel *et al.*, 2011).

Not only survivin but also other proteins overproduced in endothelium during angiogenesis, such as VEGFR2, one of the VEGF receptors, or endoglin, a component of the TGFβ (Transforming Growth Factor beta) receptor complex, have been chosen as targets for *Salmonella*-based vaccines (Jarosz *et al.*, 2013; Jellbauer *et al.*, 2012). The induction of the CTL response against these antigens should reduce angiogenesis and destroy tumor vasculature leading to inhibition of tumor development. Indeed, *Salmonella* carrying a plasmid encoding a fusion protein comprising the YopE secretion sequence and the fragment of VEGFR2 containing the $CD8^+$ T-cell epitope efficiently limited the growth of B16F10 melanoma in both prophylactic and therapeutic settings (Jellbauer *et al.*, 2012). The authors hypothesized that the vaccine may also affect the immunosuppresive tumor microenvironment by destroying the VEGFR2-positive subset of Treg lymphocytes.

Similarly, a combined therapy using orally applied *Salmonella* carrying endoglin cDNA with an intratumorally injected plasmid coding for interleukin-12 reduced microvessel density and diminished the number of Tregs within B16F10 tumors resulting in inhibition of the tumor growth and prolonged survival of mice (Jarosz *et al.*, 2013).

Some examples of *in vivo* studies on the efficacy of various *Salmonella* Typhimurium-based anti-cancer vaccines are presented in Table 4.

Recently, a phase 1 clinical trial evaluating the safety of the first *Salmonella* Typhi-based oral vaccine coding for the full length human VEGFR2 and aimed at eliciting an anti-VEGFR2 immune response has started and involves patients suffering from pancreatic cancer. The results of this trial are not yet available (Niethammer *et al.*, 2012).

CLINICAL STUDIES INDICATE TUMOR TARGETING AS THE MAJOR LIMITATION OF *SALMONELLA*-BASED THERAPIES

The mouse is a natural host for *Salmonella enterica* serovar Typhimurium and this species is considered to be the most sensitive to VNP20009 infections. However, the maximum tolerated dose (MTD) of VNP20009 in mice is large and was estimated to be 0.5×10^8 colony forming units per kg of body weight, which makes this attenuated strain at least 50 000 times less virulent than the parental *Salmonella* (Lee *et al.*, 2000). Importantly, similar values of MTD were estimated for other species — dogs, pigs and monkeys (3.0×10^7, 1.9×10^8 and 2.5×10^8 cfu/kg, respectively). Most of the adverse effects observed were transient and related to physiological responses to infection and stress, rather than to the intrinsic toxicity of the bacterial treatment. The studies in pigs found no endotoxic or septic shock reactions. In tumor-bearing mice, VNP20009 accumulates preferentially in tumors over livers at a ratio of 1000:1, but also in dogs with spontaneous neoplasia tumor colonization was detectable in 10 out of 24 *Salmonella*-treated cases (42%) (Thamm *et al.*, 2005). Those promising features of preferential localization and apparent lack of toxicity allowed VNP20009 to enter clinical development in 1999.

In the first-in-man study performed by Vion Pharmaceuticals Inc., a total of 25 patients — 24 with metastatic melanoma and one with metastatic renal cancer — were treated with 30-minute intravenous bolus infusions of VNP20009 dose ranging from 10^6 to 10^9 cfu/m^2. On the basis of dose-limiting toxicity symptoms, the MTD of VNP20009 in humans was estimated to be 3.0×10^8 cfu/m^2. Adverse reactions at higher doses included fever, hypotension, anemia and thrombocytopenia and were probably due to cytokine release. Despite VNP20009 attenuation, significant amounts of TNF were detected in the patients' peripheral blood. Most adverse effects were mild and rapidly reversible, but none of the 25 patients experienced objective cancer regression. Bacteria were rapidly cleared from the peripheral blood and tumor colonization by *Salmonella* could be detected only in three patients (Toso *et al.*, 2002). This result was clearly in contrast with data from the murine tumor models.

In a subsequent clinical study, additional 4 patients with metastatic melanoma received a 4-hour intravenous infusion of VNP20009 at the MTD of 3.0×10^8 cfu/m^2. Adverse effects of the treatment included fever, chills and nausea, but were minor and transient. VNP20009 was detectable in samples of patients' blood up to 2 hours post treatment. No *Salmonella* could be cultured from tumor biopsies taken within 2 weeks of therapy (Heimann & Rosenberg, 2003).

The results of VNP20009 phase I clinical trials did not confirm *Salmonella* accumulation and tumor regression similar to previous preclinical data. However, the important finding is that VNP20009 can be safely ad-

Table 4. Applications of *S.* Typhimurium as a vaccine vector

Plasmid containing:	Promoter	*Salmonella* strain	Tumor	Mouse strain	Route*	P/T**	References
Beta-galactosidase (β-gal)	CMV	SL7207	β-gal$^+$-F1A11 fibrosarcoma	Balb/c	O	P	(Paglia *et al.*, 1998)
MG7-Ag-mimotope-PA-DRE (T_h-epitope)	CMV	SL3261	Ehrlich ascites carcinoma	Balb/c	O	P	(Guo *et al.*, 2003)
MG7-Ag-mimotope-HBcAg	CMV	X4550	Ehrlich ascites carcinoma	Balb/c	O	P	(Meng *et al.*, 2005)
mAFP	CMV	Not stated	CT26-mAFP colon carcinoma Hepa1-6 hepatoma	Balb/C C57J/L	O	P	(Chou *et al.*, 2006)
SopE-NY-ESO-1***	Psop	Δ*phoP* Δ*phoQ*	CMS5a sarcoma	Balb/c	O IT	T	(Nishikawa *et al.*, 2006)
Tyrosine hydroxylase epitopes-Ub	CMV	SL7207	NXS2 neuroblastoma	A/J	O	P + T	(Huebener *et al.*, 2008)
Survivin epitopes-Ub	CMV	SL7207	NXS2 neuroblastoma	A/J	O	P + T	(Fest *et al.*, 2009)
SopE-Hsp70-Trp2	Psop	SL3261	B16F10 melanoma	C57BL/6J	O	P + T	(Zhu *et al.*, 2010)
SseF-CO-Survivin**** + STAT3shRNA	PsseA	MVP728 YS1646	B16F10 melanoma	C57BL/6	O	T	(Manuel *et al.*, 2011)
mPSCA	CMV	SL7207	TRAMPC1 prostate carcinoma	C57BL/6	O	P	(Ahmad *et al.*, 2011)
YopE-VEGFR2	Plac	SB824	B16F10 melanoma	C57Bl/6	OG	P	(Jellbauer *et al.*, 2012)
Endoglin	CMV	SL7207	B16F10 melanoma Renca renal carcinoma	C57Bl/6 Balb/c	O	P + T	(Jarosz *et al.*, 2013)

*O, oral; OG, orogastric; IT, intratumoral; **P, prophylactic-, T, therapeutic approach; ***underlined, *Salmonella* protein fragments serving as secretion signals; ****CO, codon optimized.

ministered to humans in large doses and that the toxicity of this bacterial strain is limited. Moreover, the first-in-man study revealed that low tumor targeting in humans is a crucial therapeutic drawback of VNP20009. Since this feature can be significantly improved using genetic engineering, we made an attempt to enhance the accumulation of *Salmonella* in human tumors by constructing a VNP20009 strain expressing single chain antibody fragments specific to the carcinoembryonic antigen (CEA; Fig. 1). This VNP derivative was able to efficiently target CEA-expressing tumors in mice and could possibly overcome the targeting limitations of VNP20009 in humans, since CEA is present on more than 50% of human carcinomas (Bereta *et al.*, 2007).

In summary, bacterial cancer therapy has recently moved beyond an interesting concept and has by now been supported by solid preclinical and clinical data. The unique features of bacterial therapeutics create the opportunity for novel anticancer strategies as bacteria meet all requirements for an ideal tumor-targeting agent. While the mechanism of action is often unclear, many examples have already proved that cancer treatment with bacteria can be site-specific, highly focused on the tumor and safe to other tissues. Among bacterial strains evaluated as cancer therapeutics so far, *Salmonella* Typhimurium is one of the most promising with first-in-man studies that support feasibility of clinical application. However, excellent tumor colonization observed in murine models was not confirmed in humans, suggesting that tumor targeting is the major obstacle for further development. As

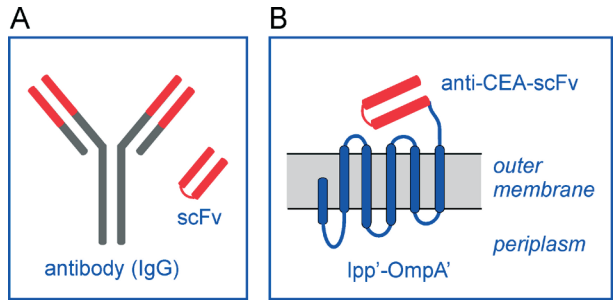

Figure 1. Tumor targeting of *Salmonella via* the anti-CEA antibody fragment.
(**A**) a single chain antibody fragment (scFv) specific to carcinoembryonic antigen (CEA) was derived from a CEA-specific immunoglobulin G molecule; (**B**) A DNA sequence encoding scFv was fused with the gene coding for OmpA, an outer membrane protein of *E. coli*, and expressed in VNP20009, an attenuated strain of *Salmonella* Typhimurium.

many researchers focus on the effector molecules and other antitumor features of *S*. Typhimurium, insufficient localization in human tumors remains to be an unsolved issue. Targeting based on an antibody-fragment specific to TAAs is one of the few approaches proposed to overcome this drawback of therapeutic *Salmonella* strains.

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