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Chapter 3

THE EMERGING ROLE OF *SALMONELLA ENTERICA* AS A THERAPEUTIC AGENT AGAINST CANCER

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ABSTRACT

Cancer has become a disease that annually causes millions of deaths around the world. Although an early diagnosis can achieve tumor eradication with appropriate treatments, failure of conventional chemotherapy and radiotherapy still represents a major issue for complete elimination of tumor cells, and therefore, research on new strategies for cancer therapy is encouraged. One of these alternatives is the use of live-attenuated bacteria with antitumor properties. Among the different types of bacteria analyzed as possible antitumor agents, *Salmonella enteric*a has been the most studied. Although *Salmonella enterica* tumor selectivity remains unclear, pre-clinical and clinical trials have shown its localization in solid, semi-solid and metastatic tumors. Once in the tumor microenvironment, these attenuated bacteria have the ability to induce or activate the innate or specific antitumor immune response, and the potential to be used as a Trojan horse to kill tumor cells by carrying cytotoxic molecules, pro-apoptotic proteins, immunomodulatory molecules, or transferring DNA into eukaryotic cells in a process called bactofection. In this chapter, we review the role of *Salmonella enterica* as a possible therapeutic alternative in the fight against cancer.

Keywords: *salmonella*, cancer, immunotherapy

1. INTRODUCTION

Cancer constitutes a public health problem worldwide, and even though patient's survival has substantially improved with usual treatments, chemotherapeutic agents' toxicity, drugs' resistance and the low accessibility to metastasis, favor failure of these treatments, and make necessary the search for new and more effective antitumor therapeutic alternatives for completely eradicating transformed cells. It is in this context that the use of bacteria with antitumor activity has reappeared as a promising alternative in the fight against cancer [1, 2].

2. BACTERIAL VECTORS AS CANCER THERAPY

It is been more than a century since William Coley's works documented the use of bacteria and their derivatives as cancer treatments [3, 4]. Sarcoma, carcinoma, lymphoma, melanoma and myeloma were treated with Coley's toxins composed of *Streptococcus pyogenes* and *Serratia marcescens* [5]. This was considered the beginning of immunotherapy for cancer [6], which was followed by work done with the *Mycobacterium bovis* attenuated strain (Bacillus Calmette-Guerin, BCG) which was applied as intravesical immunotherapy against superficial transitional cells carcinoma of the bladder [7].

Although the use of live-attenuated bacterial vectors was forgotten for some decades, this concept has recently recovered interest. Some aspects that support the use of bacteria as an alternative for treating cancer are; a) low cost preparation, b) its activity as immunostimulators, c) availability of safe strains that are well tolerated in clinical assays, d) in the case of reversion of the attenuation, antibiotic treatment is a possibility, and e) its great affinity for the tumor microenvironment, which reduce secondary effects [8]. Some bacterial genera such as *Listeria, Mycobacterium, Clostridium, Lactococcus, Bifidobacterium* and *Salmonella* have been evaluated for their antitumor capacity [1].

Listeria

Listeria monocytogenes are the most studied bacteria from the *Listeria* genera; it is an intracellular bacteria that has the ability to enter the cytosol of the infected cell by virulence factors such as listeriolysin O and phospholipase C [9]. Due to this property, it can be used as a tumor antigens carrier for the presentation of antigens to MHC class I for $CD8^+$ cytotoxic T lymphocytes activation, as has been shown in cervical, pancreatic, or head and neck cancer models [10], among others. An alternative mechanism suggests that tumor cells colonized by *Listeria monocytogenes* are killed through generation of high levels of reactive

oxygen species and oxidative stress [11]. Coupling tumor-associated antigens with some of the bacterial virulence factors increase the antitumor effect. Recently, phase I and II clinical assays using *Listeria* monocytogenes as vaccines against cancer have been described [12, 13].

Mycobacterium

This genus is represented by *Mycobacterium bovis* attenuated strain, the Bacillus Calmette-Guerin, BCG. This bacteria was used for the first time as therapy for bladder cancer in 1974 [14], and to date, it is the most successful adjuvant agent for superficial transitional cells carcinoma of the bladder [15]. BCG binds to the urothelial cells through fibronectin and gets into the cancerous cells by micropinocytosis, where it can induce cell death by apoptosis or necrosis. Bladder cancerous cells infected with BCG activate the immune system by regulating antigen presentation to T $CD4^+$ cells, in addition, they secrete cytokines (IL-1, IL-2, IL-6, IL-8, IL-10, 1L-12, TNF-α and IFN-γ), recruit NK cells, cytotoxic T lymphocytes, neutrophils and macrophages that are specifically directed to the tumor cells [16].

Clostridium

The existence of hypoxic regions (< 1% of oxygen or 0-20 mmHg) inside the tumor [17], have allowed the use of strictly anaerobic bacteria in antitumor therapy. The first time that genus *Clostridium* was associated with cancer was in 1813 when tumor regression was observed in patients that contract gaseous gangrene. Later studies in murine models of breast carcinoma showed that *Clostridium tetani* spores administered intravenously spread exclusively inside the hypoxic regions of the tumor [18]. The combination of these bacteria with conventional therapies such as radiotherapy has been also analyzed, for example, the use of *Clostridium novyi*-NT (a mutant strain lacking toxin-α) together with

radiotherapy showed a significant decrease of the tumor compared to the control treatment [19].

Bifidobacterium

Other strictly anaerobic bacteria that have been studied for their antitumor activity correspond to the *Bifidobacterium* genus. Particularly, *Bifidobacterium bifidum* intravenously administered, selectively colonize and proliferate murine models of colon carcinoma, cholangiocarcinoma and melanoma [20]. In this study the number of bacteria in the tumor was 1000 higher than in other organs, mainly in tumors with a diameter above 1.5 cm. *Bifidobacterium longum*, another bacterium from this genus is selectively localized in the hypoxic regions of the tumors [21], and it was observed grouped in the limit between the necrotic and no-necrotic region or only in the necrotic regions [22]. Hypoxic zones in the tumors are the most angiogenic, therefore, this strain has been used for therapeutic administration of proteins such as endostatin, known for suppressing endothelial cell proliferation and for acting as a competitor to inducers secreted by tumor cells [23]. Endostatin expression by a plasmid transported by *Bifidobacterium adolescentis*, in a liver cancer model, inhibited angiogenesis and decreased tumor growth [24].

Lactococcus

Lactococcus lactis, from the Lactococcus genus, known as a live bacterial vector, a carrier of heterologous molecules with vaccine purpose, is neither invasive nor pathogenic [25]. Recently, a *Lactococcus lactis* strain secreting IL-12, proinflammatory cytokine with immunomodulator potential and antitumor activity, administered together with the *Lactococcus lactis* strain which expresses the E7 antigen of HPV (human papillomavirus type 16), in a cervix cancer murine model, significantly reduced tumor size [26].

Salmonella

Salmonella genus is composed of Gram-negative, facultative anaerobic bacteria. *Salmonella enterica* serovar Typhi (*S*. Typhi), that infects human, and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), that infects mice and humans, have caught scientists' attention for their antitumor activity [3]. Some of the characteristics that have made this bacteria a key antitumor vector are; their great capacity for colonizing tumor tissue [27, 28] including metastasis [29], they possess a great affinity for antigen-presenting cells [30, 31], characteristics associated to the induction or activation of the innate [32, 33] and adaptive [34, 35] immune responses, and the availability of strains with diverse mutations that guarantee the biosafety for their use in humans as described below.

3. *SALMONELLA ENTERICA* LIVE-ATTENUATED BACTERIAL VECTOR FOR CANCER THERAPY

Salmonella enterica is the most studied vector in the fight against cancer using live bacteria. To date, there are several strains that were attenuated for minimizing its virulence and pathogenicity [1] (Table 1). Among the mutations performed with *Salmonella enterica* strains, auxotrohic mutations in metabolic pathways that affect purine [36] or amino acid biosynthesis [37] or both stand out [38]. These deletions avoid bacterial replication in environments that lack the aforementioned molecules and therefore do not represent a major risk inside the host. In 1995, Eisenstein et al. used SL3235, a *S.* Typhimurium strain mutant on its metabolic pathways, as immunotherapeutic treatment in a murine model of plasmacytoma [39]. This strain, mutated in the *aro*A gene cannot produce aromatic amino acids such as tryptophan, phenylalanine and tyrosine, and therefore makes bacteria unable to replicate inside the host and needs these amino acids or its precursors for survival. Another *aro*A mutant strain, *S.* Typhimurium SL7207, showed immune antitumor protection in murine

models inoculated with fibrosarcoma and lung adenoma after immunization with the bacteria, resulting in a decrease of the tumor growth [40, 41]. *S.* Typhimurium LVR01 is another strain dependent on aromatic aminoacids with a deletion in the *aroC* gene [42] that has been used in antitumor immunity assays in a murine model of B-cell non-Hodgkin lymphoma (A20). The ability of this *Salmonella enterica* strain for infecting A20 cells was very low, however, it was able to replicate inside the tumor. Antitumor LVR01 administration caused a strong antitumor immunity associated to a late growth of the tumor and a prolonged survival in the mice with lymphoma [43]. Another mutation described in *Salmonella enterica* is the deletion of *purI* gene, which participates in adenine production. An example of this group is the *S.* Typhimurium strain VNP20009, which also has a mutation on the *msb*B gene, which makes it deficient on lipoposaccharide formation, with the objective to prevent a severe septic shock [44] and reduce its toxicity. VNP20009 strain has been evaluated for phase I clinical trials for treating metastatic melanoma in humans. In this study, even though the antitumor activity was very modest, no adverse effects were caused by the strain [45]. Recently, mutations in PhoP/PhoQ system described in VNP20009 have allowed a higher efficiency in releasing RNA interference (RNAi) and reduced bacteria concentration in liver and spleen, significantly increasing tumor selectivity on murine models [46]. Mutant *S.* Typhi strains have been also used for its antitumor activity. An example of this group is CVD915, an attenuated strain obtained from the pathogenic *Salmonella enterica* serovar Typhi Ty2, which has mutations in the *guaBA* operon, interfering with guanine biosynthesis [47]. Its antitumor activity was evaluated in a murine model of breast adenocarcinoma. Mice treated with these bacteria showed an important reduction on tumor growth and lower incidence of metastasis, which was accompanied by an increase of IFN-γ-secreting T $CD4^+$ and T $CD8^+$ cells in the lymphatic nodes close to the tumors and a great number of activated TNF-α-secreting neutrophils [48]. The same strain was also evaluated with good results in a murine model of metastatic T-cell lymphoma [49].

Attenuations in *Salmonella enterica* are not only intended for decreasing toxicity, but also for promoting selectivity and directing to tumors. For this purpose, Zhao et al. generated an attenuated *Salmonella enterica* strain dependent on leucine and arginine, A1 [50], which showed low toxicity and excellent selectivity for the tumor when administered to a murine xenograft model of human prostate cancer (PC-3 cells). The same research group re-isolated the A1 strain from *in vivo* tumor tissue with the aim to improve its selectivity and named it A1-R [50]. Currently, *Salmonella enterica* A1-R antitumor and antimetastatic activity has been tested in different kinds of cancer such as osteosarcoma [51], pancreatic cancer [52], and glioma of the spinal cord [53].

Other mutations in *Salmonella enterica* have achieved increasing tumor selectivity and reducing virulence, which is the case of *S.* Typhimurium SA186 strain with a deletion of the *znu*ABC operon, coding for the high affinity zinc receptor, which gives selective advantage to *Salmonella* growing in poor zinc environments. Deletion of the zinc transport system represents an alternative way for bacterial attenuation that maintains its selective tropism for tumors, as described in a murine model of breast adenocarcinoma where this strain reduced tumor growth and improved mice survival [54]. Another described *S.* Typhimurium strain, ΔppGpp, has mutations in *relA* and *spoT* genes coding for PSI and PSII synthases, respectively, associated to guanosine tetraphosphate (ppGpp) production that regulates the expression of virulence related genes encoded in the pathogenicity island 2 (SPI-2) [55]. This strain achieved an excellent tumor suppression by activating the inflammasome and cytokines inhibiting the tumor growth (IL-1β, IL-18 and TNF-α) in a murine model of colon cancer [56, 57]. Similarly, the *S.* Typhimurium LH340 strain with mutations in the PhoP/PhoQ system, associated with survival inside macrophages, resistance to defensins, and acids resistance, has been successfully tested in a model of mouse prostate cancer [58]. It has been also shown that *S.* Typhimurium X^{4550} strain with mutations in genes encoding adenylate cyclase, Cya-1 and Crp-1, needed for viability and adaptability to osmotic changes, has antitumor and antimetastatic activity in a model of murine adenocarcinoma [59].

Table 1. Attenuated *Salmonella enterica* strains for cancer treatment

SPECIE	STRAIN	MUTATION	MALIGNANCY TREATED	REF.
Typhimurium	SL3235	aroA	Plasmacytoma	[39]
Typhimurium	SL7207	aroA	Fibrosarcoma Lung adenoma	[40, 41]
Typhimurium	SL3261	aroA	Colon cancer	[37]
Typhimurium	LVR01	aroC	Non-Hodgkin lymphoma	[42]
Typhimurium	VNP2009	purI, msbB	Melanoma	[45]
Typhimurium	VNP (PhoP/Q)	purI, msbB, phoP/phoQ	Lung cancer	[46]
Typhimurium	A1/A1R	leu, arug	Osteosarcoma Pancreatic cancer Glioma of the spinal cord	[51] [52] [53]
Typhimurium	SA186	znuABC	Adenocarcinoma	[54]
Typhimurium	ΔppGpp	relA, SPOT	Colon cancer	[55]
Typhimurium	LH340	phoP/phoQ	Prostate cancer	[58]
Typhimurium	X^{4550}	cya-1, crp-1	Adenocarcinoma	[59]
Typhimurium	YS7211/YST212	Pur, Ilv, Arg/Pur, Ilv, Ura	Melanoma	[28]
Typhimurium	SL1344	aroA,hisG/ with cheY, or fiGHI,or InvG,or phoP, or sseD,or ssrB, or purA	Colon cancer	[60]
Typhi	CVD 915	guaBA	Adenocarcinoma	[48]

The attempt to generate safe strains with a higher tropism for the tumor environment has led to the development of *Salmonella enterica* strains with multiple attenuations that have been tested for cancer treatment with encouraging results. Some examples are *S*. Typhimurium strains YS7211 (Pur-, Ilv-, Arg-) and YS7211 (Pur-, Ilv-, Ura-), tested in murine models of melanoma [28] or recombinant *S*. Typhimurium strain SL1344 (aroA, hisG) with deletions in chemiotaxis (cheY), motility (fiGHI), invasiveness (invG, phoP,sseD, ssrB) and in metabolism (purA) that has been successfully tested in a murine model of colon cancer [60].

4. TUMOR SELECTIVITY OF *SALMONELLA ENTERICA*

Since Pawelek JM et al. [28] described the ability of *Salmonella enterica* for infecting and replicating inside tumors implanted in mice in a 1000:1 ratio regarding to normal tissue, *Salmonella enterica* has become the most used bacterial vector as a therapeutic agent in cancer murine models. Even though to date, the mechanisms to explain *Salmonella enterica* selectivity for tumor tissues are not completely understood, it is possible that the microenvironment created by the physiopathology of the tumor, characterized by; a) hypoxia [61], b) acidity, due lactic acid production as a result of the anaerobic metabolism [62], and c) necrosis, resulting from tumor cell death for lack of nutrients and out of control growth, may contribute to bacterial proliferation in the tumor microenvironment [1].

In order to understand the mechanism by which *Salmonella enterica* arrives to the tumor tissue, different models have been developed. In a study, in an *in vitro* tumor model, it was found that *Salmonella enterica* responds to the presence of molecules such as amino acids (aspartic acid and serine) and carbohydrates (ribose and galactose) that act as chemotactic agents by activating the bacteria receptors, aspartic acid receptors participate in the chemotaxis to the tumor, serine receptor is involved in the penetration into the tumor, while ribose and galactose receptors are in charge of directing the bacteria to necrotic areas [63, 64]. A third kind of molecule that favors *Salmonella enterica* chemotaxis to the tumor is ethanolamine, a compound present in prokaryotic and eukaryotic membranes that bacteria need to colonize the gastrointestinal tract [65] and that can be found in high concentrations in different kind of neoplasms [66]. Even though the mechanism by which ethanolamine works as a chemotactic agent for *Salmonella enterica* is not clear, it has been observed that this molecule is recognized and metabolized by the bacteria in a process induced by the activation of the bacterial transcription activation factor EutR [65]. In addition, in a study of *Salmonella enterica* with a deletion on the eutC gene (part of the operon that codes for the enzyme ethanolamine-ammonia-lyase (EAL), in charge of metabolizing

ethanolamine [67]), colonization in a murine model of breast cancer decreased [68].

On another hand, once *Salmonella enterica* has sensed the molecules that favor chemotaxis to the tumor, the proposed mechanism for entering the tumor involves proteins that participate in motility, such as the CheA/CheY system [69] since it has been shown that mutations of this protein cause a lower recruitment in the tumor tissue [63, 64]. This data agrees with results observed for the attenuated *S.* Typhimurium VNP20009 strain (used in phase I clinical trials for treating patients with metastasis of melanoma [45]), which has a polymorphism in CheY that causes lower motility. However, when the *CheY* gene obtained from the 14028s wild-type strain is expressed in the VNP20009 mutant strain, this partially recuperates the capacity to colonize the tumor, although it does not reach the colonization level of 14028s strain [70]. These results suggest that VNP20009 requires another component present in the wild type strain besides the CheA/CheY system to totally restore its motility. It was determined that the second component is lipid A, encoded by the *msbB* gene which was eliminated in the VNP20009 for making it safe for its therapeutic use, when mutations in *CheY* and the deletion of the *msbB* were restored, VNP20009 increased its motility resulting in a higher presence of the bacteria in the tumor [71]. However, to reestablish VNP20009 capacity to produce lipid A would result in a septic shock induced by an increase of TNF-α [72].

Even though this data would explain why the VNP20009 strain did not have a significant effect when used in phase I clinical trials in patients with metastasis of melanoma [45], there is controversial data suggesting that *Salmonella enterica* motility is not necessary for colonizing the tumor. For example, in murine models of breast [68, 73] and colon [60] cancer, deletion of the gene that codes for *CheY* in *S.* Typhimurium did not affect tumor colonization. Additionally, another study showed that deletion of the *trg* gene (that codes for the Trg receptor, in charge of sensing carbohydrates and necessary for CheY function) in *Salmonella enterica*, also did not affect tumor colonization [74].

Also, there is controversy on the participation of proteins such as fliA, fliC and flgE that are needed for bacteria's motility and some studies indicate that they are important for *Salmonella enterica* tumor colonization [73], while other studies suggest that the absence of these proteins does not affect bacteria's capacity for getting into the tumor [60]. Likewise, there are reports showing that mutations in the *motAB* gene, the bacterial flagellar motor, cause a decrease in tumor colonization [68].

Other studies showed that *Salmonella enterica* metabolic pathways for aromatic amino acids (aroA) and purines (purA) are also important for reaching the tumor, since mutations in these pathways cause a lower recruitment in the tumor tissue [60, 73]. Despite these controversies, there is evidence that suggests that the nutrient's presence resulting from cell release in the destruction process by necrosis are necessary for the arrival of bacteria to the tumor microenvironment. In addition, in these studies there are differences in the colonization time and antitumor activity depending on the route of administration of the bacteria (not considering direct administration to the tumor), being the most efficient intravenous and intraperitoneal routes [60].

On the other hand, once *Salmonella enterica* reaches the tumor, the ability to remain on the tissue is related to the low activity of macrophages and neutrophils [75], as well as to the suppression of the immune response by cytokines such as TGF-β and to the difficult access for anti-*Salmonella* antibodies and the factors of the complement system, due to irregular growth of blood vessels in the tumor [76].

Finally, it is important to mention that despite the numerous works on the use of *Salmonella enterica* as antitumor treatment in murine models of cancer, there are very few studies that report internalization of the bacteria in the tumor cell. Instead, the formation of a biofilm in the tumor tissue that allows the bacteria to remain there has been observed [77]. Even though the presence of the biofilm could hinder bacteria eradication, there are reports that show that *Salmonella enterica* can be eliminated with antibiotics in murine models of cancer [78]. Routes of administration and tumor selectivity are depicted in Figures 1A and 1B.

5. INTRINSEC ANTITUMOR ACTIVITY OF *SALMONELLA ENTERICA*

Since the first works of Kurashige S. et al. in murine models of sarcoma [79] and T-cell lymphoma [80] treated with mini cells (vesicles without genomic DNA) obtained from *S.* Typhimurium, several research groups have studied the intrinsic antitumor activity of *Salmonella enterica* using murine models of cancer, including lung carcinoma [81], colon carcinoma [60, 82], prostate cancer [83], metastatic T-cell lymphoma [49] and B-cell lymphoma [84], among others. In most cases, *Salmonella enterica* was able to inhibit tumor growth and its metastasis, prolonging mice survival. Similar results were observed in murine models of xenografts of breast [85] and prostate [50, 86] cancer, using auxotrophic *S.* Typhimurium strains such as A1 (deficient in leucine and arginine synthesis) and A1-R (deficient in leucine and arginine synthesis with a higher capacity for eliminating tumor cells) that maintain antitumor activity, but do not cause damage to the host since bacteria have a higher affinity for tumor tissue [85]. Moreover, in other studies it was shown that A1-R inhibits bone metastasis in murine models of breast cancer [87], as well as metastasis caused by osteosarcoma [88], pancreatic cancer [52], and glioma of the spinal cord [53].

Even though *Salmonella enterica's* intrinsic antitumor activity is widely documented, the mechanism by which bacteria induce tumor cell death without affecting healthy tissue is not yet fully understood. Nevertheless, some proposed mechanism involves manipulation of signal transduction pathways in tumor cells including those associated with cell death [89, 90] and activation of the innate and adaptive immune response of the host. Table 2 summarizes some of the characteristics of the intrinsic *Salmonella enterica* oncolytic activity.

Table 2. Intrinsic antitumor activity of *Salmonella enterica*

SPECIE	STRAIN	TUMOR-BEARING MICE	ANTITUMORAL ACTIVITY	REF.
SIGNAL TRANSDUCTION				
Choleraesuis	HWS 51	Endothelial cells	Inhibition of HIF-1α and VEGF	[91]
Typhimurium	SL7838	Breast cancer	Increased nitric oxide production	[32]
Choleraesuis	ATCC 15480	Melanoma	Downregulation AKT/mTOR pathway	[89, 90]
Typhimurium	STM	Lung cancer	Immunogenic cell death by Calreticulin	[54]
Typhimurium	SL1344 and SR11	Colon cancer	Downregulation of P-Glycoprotein	[92]
IMMUNE ACTIVITY				
Choleraesuis	HWS 51	Melanoma	TLR 4 activation	[93]
Typhimurium	SLΔppGpp	Melanoma and colon cancer	TLR 4 and TLR5 activation	[94]
Typhimurium	SLΔppGpp	Colon cancer	Increased levels of TNF-α and IL-1β	[56]
Typhimurium	SL3261AT	Melanoma	$CD8^+$ activation	[95]
Typhimurium	SL3261AT	Melanoma	Antigen Cross-Presentation by DCs	[96]
Typhimurium	BRD509E	Melanoma	Activation of Myeloid suppressor cells	[33]
Typhimurium	14028	Melanoma	Downregulation of Treg Cells	[97]

Abbreviation. DCs: Dendritic cells; HIF-1α: Hypoxia Inducible Factro-1α; IL-1β: Interleukin- 1β; IL-6: Interleukin- 6; TLR 4: Toll Like Receptor 4; TLR 5: Toll Like Receptor 5; TNF-α: Tumor Necrosis Factor-α; Tregs: T regulatory Lymphocyte; VEGF: Vascular endothelial growth factor.

5.1. Induction of Cell Death Pathways by *Salmonella enterica*

Some of the signaling mechanisms associated with cell death in the tumor that are modified by *Salmonella enterica* are:

1) *Decrease of angiogenesis*. The decrease on new blood vessel formation is induced by the inhibition of the HIF-1α transcription factor and the decrease of the vascular endothelial growth factor (VEGF) [91]. Even though the mechanism by which *Salmonella enterica* reduce these two protein concentrations is unclear, the decrease on angiogenesis avoids a fast development of the tumor.
2) *Apoptotic cell death by nitric oxide (NO)* [32]. NO, a product of the degradation of nitrates and nitrites generated by the hypoxic environment of the tumor [98] by nitrate reductase of *Salmonella enterica* (NirB) [99], would induce the intrinsic apoptosis pathway [100].
3) *Autophagy activation by modifying the AKT/mTOR pathway in the tumor tissue.* The presence of *Salmonella enterica* in the tumor causes a decrease in AKT and mTOR phosphorylation and increases beclin-1 and LC3 (Microtubule-associated protein 1A/1B-light chain 3) expression, favoring autophagy [89, 90].
4) *Immunogenic cell death (ICD).* This kind of cell death is caused by calreticulin (CRT) [54], a protein localized in the endoplasmic reticulum, that when secreted participates in the ICD and the presence of *Salmonella enterica* in the tumor tissue increase this protein concentration [101].

Recently, it has been described that *Salmonella enterica* may promote the sensibility of chemotherapy by inducing a decrease on the expression of molecules such as P-glycoprotein coded by the *mdr*1 gene [92], associated with chemotherapy resistance for different types of cancer [102]. Recent reports show that infecting colon cell lines with a *S.* Typhimurium wild-type strain decrease P-glycoprotein expression [92], suggests that this effect is generated by translocation of *Salmonella enterica* effector protein SipA through T3SS [103]. A decrease of P-glycoprotein would result in tumor cells lower drug-resistance, sensitizing them for the effect of the drugs used in chemotherapy.

5.2. *Salmonella enterica* and Its Intrinsic Antitumor Activity by Activation of the Immune Response

Activation of the host immune system by *Salmonella enterica* plays a very important role in antitumor activity. Once *Salmonella enterica* reaches the tumor microenvironment, some components such as lipopolysaccharide (LPS) are recognized by the toll-like receptor 4 (TLR4), as observed in a murine model of melanoma where TLR4 activation by *S. choleraesuis* contributed to a lower tumor growth, which was associated with neutrophils and macrophages recruitment [93]. *Salmonella enterica* flagellin that activates TLR5 also appears to play an important role in antitumor activity. In a murine model of melanoma, the administration of *S.* Typhimurium flagellin fused with the peptide P10 that belongs to the gp43 protein of *Paracoccidioides brasiliensis*, prevented the development of metastasis [104]. Also, the use of an agonist for TLR5 had antitumor effects in a murine lymphoma model, an effect that was accompanied by activation of $CD8^+$ lymphocytes and NK cells [105]. Zheng et al. confirmed TLR4 and TLR5 participation in antitumor activity by using knocked out mice for these two receptors [94] in which colon or melanoma cancer cells were implanted and subsequently treated with *S.* Typhimurium modified to express flagellin B (FlaB) from *Vibrio vulnificus* (*Salmonella* FlabB). However, there is some controversial data on the involvement of TLR5 in the antitumor response, since administration of *S.* Typhimurium flagellin in a murine model of breast cancer had no significant antitumor effect when the flagellin were administered after tumor formation. When flagellin was administered at the same time as tumor cells, the tumor developed faster [106]. Likewise, a study in cell lines of multiple myeloma showed that TLRs activation, including TLR5, favored cell proliferation [107].

Other studies have documented that TLRs are associated with the recruitment of cells of the immune response, caused by an increase in TNF-α levels due to activation of TLR4 by *Salmonella enterica* LPS [56, 108]. The increase in TNF-α would promote a hemorrhage in the blood vessels in the tumor, thus allowing infiltration of cells associated with the

immune response [109], such as neutrophils [32], macrophages [33], T lymphocytes [35], B lymphocytes [34] and NK cells [105] that would be in charge of eliminating the tumor cells. Additionally, it has been observed that the presence of *Salmonella enterica* in the tumor increases the amount of neutrophils, macrophages, T and B lymphocytes in the spleen [54], that later might migrate to the tumor, contributing to its eradication.

On the other hand, the absence of cells of the immune response favors tumor growth and bacteria dissemination; In this context, it has been shown that depletion of B-lymphocytes affects the ability of *Salmonella enterica* to colonize tumor tissue, and other organs such as spleen and liver, and increased the bacteria presence in blood [34]. But, depletion of $CD4^+$ and/or $CD8^+$ lymphocytes do not affect the preference of bacteria for tumor tissue, but decreases neutrophils and macrophages recruitment [35].

Once recruitment of the immune cells into the tumor has taken place, *Salmonella enterica* also induces T lymphocytes activation and maturation [110]. Even though it is not clear how the bacteria generate this specific response, it would include the participation of dendritic cells (DC). This was shown in a study where *Salmonella enterica* intravenous administration favored the cross-presentation of tumor antigens by DC, and induced activation of $CD8^+$ lymphocytes which recognize the tumor [95], generating a protective effect and avoiding tumor relapse [108]. However, this latter finding is controversial, since in another study using a murine model of melanoma, where a specific response against tumor by the $CD8^+$ lymphocytes was also observed, and immunological memory was not generated [111].

Although to date it is not completely known how *Salmonella enterica* favors recognition and maturation of cells in the immune system, one of the proposed mechanisms suggest the overexpression of gap junction proteins such as connexin 43 (Cx43) [96], a protein that participates in the activation of B and T lymphocytes [112] and facilitates antigens presentation to the DC [113], allowing the transfer of pre-processed peptides from tumor cells to the DC for adequate presentation by MHC class I [96], thus generating a specific antitumor response, as reported in a study by Shilling et al. [114]. These authors showed how *in vitro* activation

of purified DC from mice with cytoplasmic fractions of *S.* Typhimurium and with heat shock proteins derived from the tumor cells prevented tumor formation.

Another factor that would contribute to the tumor regression caused by the presence of *Salmonella enterica* would be to reverse the immunological tolerance of the tumor [115, 116] by two possible ways. The first proposed mechanism would involve the decrease of Treg lymphocytes ($CD4^+$ $CD25^+$) in the tumor tissue [117], due to the effect of LPS and Braun lipoprotein (Lpp) of *Salmonella enterica*, since strains mutated in LPS (msbB) and in Lpp (IppA and IppB) do not decrease the amount of Treg lymphocytes in the tumor [97]. The second mechanism would involve a decrease of the indoleamine 2,3-dioxygenase 1 (IDO1) levels (enzyme involved in tryptophan metabolism and associated with immunological tolerance development by T lymphocytes [118, 119]), avoiding the formation of kynurenine, and therefore favoring T lymphocytes proliferation, capable of recognizing and eliminating the tumor [90].

It has been well documented that the adaptive immunity induced against *Salmonella enterica* antigens [120, 121] is one of the mechanisms to eliminate tumor cells. Infected tumor cells present *Salmonella enterica* antigens, and therefore, are eliminated by cytotoxic T lymphocytes that recognize the bacteria antigens. This mechanism has been proposed for elimination of solid tumors and their metastasis [50, 122] and non-solid tumors [43]. In addition to these findings, there is evidence that the administration of *S.* Typhimurium in a murine model of B-cell lymphoma induced a local and systemic antitumor adaptive immune response characterized by the recruitment of $CD8^+$ and $CD4^+$ T lymphocytes and the lymphocytes from the spleen produced proinflammatory cytokines such as IFN-γ and IL-12 to the specific stimulus by tumor cells. The humoral response analysis demonstrated the presence of specific antibodies against tumor cells [43].

6. *SALMONELLA ENTERICA* AS DELIVERY SYSTEM OF HETEROLOGOUS ANTITUMOR MOLECULES

Although natural oncolytic activity of *Salmonella enterica* is widely documented, the absence of a significant antitumor effect in phase I clinical trials [45] has led several research groups to seek to amplify the bacteria antitumor potential by incorporating molecules with cytotoxic activity, immunomodulators, apoptosis inducers, and tumor-associated antigens with the idea that the bacteria is responsible for producing them and releasing them in the tumor tissue to induce their elimination (Figure 1C) [3, 123]. These heterologous molecules have been inserted into *Salmonella enterica* by genetic engineering, expressing them first in the cytoplasm [124] and later in the periplasm, outer membrane or secreted from the bacterial surface [125, 126]. Table 3 summarizes some characteristics of *Salmonella enterica* strains as carriers of heterologous molecules.

6.1. *Salmonella enterica* as Delivery System of Tumor Associated Antigens

Tumor associated antigens (TAA) or tumor-specific antigens (TSA) [127], proteins expressed in tumor cells responsible for promoting cell transformation and tumorigenesis are some of the heterologous molecules that have been coupled with *Salmonella enterica*. The expression of these antigens is intended to induce or enhance the specific immune response against the tumor, taking advantage of the strong tropism of *Salmonella enterica* for the professional antigen-presenting cells [128]. In the first studies on this subject, *Salmonella enterica* was used to express and release TAA /TSA through its Type 1 Secretion Systems (T1SS) and Type 3 (T3SS). An example of this is the immunization of mice with a strain of *S.* Typhimurium modified to release Prostate Specific Antigen (PSA) through the α-hemolysin A (HlyA) system (T1SS). This strategy generated a $CD8^+$

T lymphocyte mediated immune response that inhibited tumor development [129]. Similarly, immunization of mice with pulmonary adenoma with *S.* Typhimurium expressing the C-Raf antigen, a molecule that plays a central role in carcinogenesis, generated antibodies against this antigen, in addition to a T-cell specific antigen response that contributed to inhibition of tumor growth [40]. Also, the release of tumor antigens through *Salmonella enterica* T3SS in a murine model of fibrosarcoma, which overexpressed peptide 217-225 of the p60 protein of *Listeria monocytogenes*, simulating the presence of a tumor antigen, showed that 80% of the previously immunized mice were protected against the fibrosarcoma tumor cells expressing peptide $p60_{217-225.}$ This effect was associated with the generation of specific $CD8^+$ T lymphocytes against this peptide [130, 131]. Similar results were observed following oral immunization with an attenuated *Salmonella enterica* strain that releases the NY-ESO-1 tumor antigen, a protein from germ cells that is overexpressed in melanoma, lung, esophagus, ovary, bladder, and prostate cancer, through the T3SS [132]. In another study, it was reported that orogastric immunization in a murine model of melanoma by translocation of the Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) through *S.* Typhimurium T3SS, induced an antigen-specific response by $CD8^+$ T lymphocytes, and a reduction of up to 60% of the metastasis in immunized mice [133]. In another murine model of cervical cancer, the release of recombinant E7/SipB protein (E7 protein from the human papillomavirus type 16/ SipB protein secreted through T3SS), inhibited tumor growth by 45% and promoted mice survival up to 70% [134]. Other studies have documented that mice immunization by the release of the antigen of murine melanoma through the *Salmonella enterica* Type V Secretion System (AIDA-I), generated a specific response by $CD4^+$ and $CD8^+$ lymphocyte, in addition to an increase in the secretion of cytokines such as TNF-α and IFNγ, which together prevented tumor development [135].

All these studies have shown that generation of a response against TSA/TAA are able to prevent tumor development due to the induction of a specific immune response and reinforce the use of *Salmonella enterica* as a vaccine agent for prophylactic and therapeutic purposes.

6.2. *Salmonella enterica* as Delivery System of Immunomodulatory Molecules and Molecules That Modulate Apoptosis

Although TSA/TAA transport and release by *Salmonella enterica* produces a specific $CD4^+$ and $CD8^+$ T lymphocyte response to the tumor, this strategy is limited to those immunogenic tumors that express tumor-specific antigens [27], and is not feasible for those tumors that do not express these types of antigens.

An alternative to this strategy is the use of *Salmonella enterica* as a carrier for transporting molecules that modulate the host's immune response, facilitating tumor elimination. In this context, *S.* Typhimurium has been used to transport immunomodulatory molecules such as the cytokine LIGHT [136], interleukin-18 [137], chemokine CCL21 [76] in murine models of breast and colon carcinoma. In all cases the regression of the primary tumor and inhibition of metastasis to lung were observed. Antitumor activity was associated to DC, macrophages, neutrophils, NK cells and lymphocytes recruitment. The use of a *Salmonella enterica* strain that expresses human interleukin-2 prevented the formation of pulmonary metastasis in a murine model of osteosarcoma. Tumor regression was associated with the presence of NK cells [138, 139]. Similar results were obtained in a murine model of melanoma, where a fusion protein of IFN-γ bound to the SipB protein of *S.* Typhimurium was used, and the presence of NK cells induced the antitumor activity [140].

In addition to the expression of immunomodulatory molecules, *Salmonella enterica* has also been used to express and/or secrete molecules that induce tumor cell death by apoptosis, such as Fas Ligand [141], TNF-α [142], or TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) [143], in murine models of colon carcinoma, melanoma or gastric cancer, respectively. In all cases, significant regression of the tumor and increase in mice survival were observed. Additionally, *Salmonella enterica* has also been used to transport and release to the tumor microenvironment molecules that inhibit angiogenesis, such as

endostatin, which decreased tumor growth in a murine model of colon cancer [144].

6.3. *Salmonella enterica* as Delivery System of Cytotoxic Molecules

Some cytotoxic proteins have been expressed in attenuated strains of *Salmonella enterica* to be transported to the tumor microenvironment effectively and selectively. Ryan et al. used a murine model of breast cancer to demonstrate that intravenous administration of a *S.* Typhimurium expressing hemolysin E (Hly E), an *Escherichia coli (E.coli)* protein that makes pores in the cell membrane, under a promoter induced in anaerobiosis, allowed colonization of the tumor, induced necrosis and decreased tumor mass [145]. Another *E. coli* protein evaluated for its anti-tumor activity is cytolysin A (ClyA), which also makes pores in the membrane and has recently been expressed in *S.* Typhimurium under an inducible tetracycline system to avoid damage to normal cells. Administration of this recombinant *Salmonella* as antitumor therapy in a murine model of colon carcinoma induced tumor regression, decreased lung metastasis and promoted mice survival [146-148].

Salmonella enterica has also been used to transport enzymes that once located in the tumor microenvironment, activate cytotoxic compounds (pro-drugs) that eliminate tumor cells [149]. Chen G et al. documented that the expression of the gene encoding enzyme purine nucleoside phosphorylase (sPNP) in *S.* Typhimurium strain VNP20009, activated the non-toxic compound 6-methylpurine-2'-desoxyriboside (6MePdR) in a potent antitumor drug, 6-methylpurine (6MeP), slowing tumor growth and increasing the infiltration of $CD8^+$ T lymphocytes in a murine model of melanoma [150]. Massa P.E. et al. documented the antitumor activity of *S.* Typhimurium SL3262 expressing a single domain antibody against CD20 antigen and at the same time the enzyme thymidine kinase, activator of drugs such as Ganciclovir. This recombinant bacterium increased its specificity for the tumor microenvironment in a xenograft model of human

non-Hodgkin lymphoma due to the presence of the anti-CD20 antibody and induced antitumor activity that increased survival in mice deficient in specific immune responses [151]. In recent studies, *Salmonella enterica* was used to release immunotoxins such as TGFα-PE38, a fusion between a ligand of the epidermal growth factor receptor (EGFR) and Pseudomonas endotoxin A (PE38), a toxin capable of inducing tumor death [152, 153]. In these studies, the release of the TGFα-PE38 immunotoxin increased survival of the murine models of breast cancer, cervical cancer [152] and mouse or human colon cancer [153].

Nemunaitis J. et al. expressed the *E. coli* gene coding for cytosine desaminase in *S.* Typhimurium strain VNP20009. This enzyme is responsible for converting 5-fluorocytosine into 5-fluorouracil, a cytotoxic metabolite that is used in the treatment of gastric, breast, prostate, head and neck cancer [154]. This recombinant strain was used in a clinical trial of three patients with refractory cancer, one with head and neck squamous cell carcinoma and two with esophageal adenocarcinoma. In two of the patients the strain colonized the tumor tissue and cytosine desaminase activity was observed by measuring 5-fluorouracil concentration in the tumor tissue [154].

6.4. *Salmonella enterica* as Delivery System of DNA (Bactofection)

Another characteristic of *Salmonella enterica* that can be used as an antitumor treatment is its ability to transfer nucleic acids to eukaryotic cells [155-157], a process known as bactofection [158]. The ability of *Salmonella enterica* to release genetic material as plasmids has been evaluated in different murine models of cancer including melanoma [159], bladder cancer [160, 161] and lung adenocarcinoma [162]. The strategies to take advantage of this ability include the generation of a specific immune response against TSA/TAA in the form of DNA vaccines [163], the transfer of plasmids encoding immunomodulatory molecules [164], or

the transfer of plasmids encoding RNAs interference against some protein associated to tumor development and progression [165, 166].

HPV16 L1 gene coding for the human papillomavirus type 16 capsid protein is one of the sequences encoding TSA/TAA that has been evaluated in a murine model of cervical cancer. Bactofection of this gene showed tumor regression and increased mice survival [167]. In another murine model of breast cancer, bactofection of the gene encoding the MTDH/AEG1-1 protein, an oncogene associated with angiogenesis that is over expressed in 40% of patients with breast cancer, also promoted tumor regression and mice survival [168]. In a rat colon cancer model, *Salmonella enterica* was used to transport and transfer plasmids encoding the 4-1IBBL molecules, a member of the TNF family, and a CEACAM 6, a cell adhesion molecule, preventing tumor progression, decreasing Treg cells, favoring a Th1-type response and increasing $CD45RO^{+}$ memory T cells [169].

On the other hand, bactofection of plasmids containing gene coding for immunomodulatory molecules includes sequences for interleukin-4 or interleukin-18, which induced a systemic increase of IFN-γ and showed its efficiency to delay tumor growth and prolong survival in a murine model of melanoma [164]. In another study, a strategy that combined the bactofection of the VP3 gene (which codes for the apoptin protein from the chicken anemia virus (CAV) that is very efficient for eliminating tumor cells [170]) and the gene encoding interleukin-24 or MDA7 in a murine model of gastric cancer was used. The combination of these two molecules resulted in a decrease in tumor size, as well as the presence of cytokines IL-6, IFN-γ and TNF-α, an increase in caspase-3 activity and decreased levels of VEGF [171].

Finally, one of the most commonly used strategies is the bactofection of plasmids coding for small interfering RNAs (siRNA), in order to carry out the silencing of genes associated with the development and progression of cancer [165, 166]. An example of this strategy was performed in a murine model of squamous cell carcinoma of the tongue by silencing a protein involved in resistance to chemotherapy, gp-170 encoded by the *mdr* (multi-drug resistance) gene [172]. Transcription factor STAT-3

expression, a molecule associated to tumor cells survival, was also silenced in murine models of prostate cancer [58, 173], and hepatocellular carcinoma [174]. Gene silencing of anti-apoptotic proteins such as Bcl-2 in a murine model of melanoma [175] and survivin in a murine model of laryngeal cancer have been also reported [176]. Also, silencing of oncogenes, such as the *CTNNB1* gene, which encodes β catenin [177] has been performed. In all the aforementioned cases, the bactofection of siRNAs in the different murine models of cancer induced tumor regression.

Table 3. *Salmonella enterica* as delivery system of heterologous antitumor molecules

SPECIE	TUMOR-BEARING MICE	HETEROLOGUS MOLECULES	ANTI-TUMORAL ACTIVITY	REF.
TUMOR ASSOCIATED ANTIGENS/TUMOR SPECIFIC ANTIGENS (TAA/TSA)				
Typhimurium	Prostate cancer	PSA	Cytotoxic CD8+ T-cell response	[129]
Typhimurium	Lung cancer	C-Raf	T cell response and Specific C-Raf antibodies	[40]
Typhimurium	Fibrosarcoma	Peptide p60 from Listeria monocytogenes	Memory CD8+ T cell	[130, 131]
Typhimurium	Melanoma	NY-ESO-1	NY-ESO-1-specific CD8+ and CD4+ T cells	[132]
Typhimurium	Metastatic melanoma	Epitope (KDR2) from VEGFR2	KDR2400–408-specific CD8+ T cells	[133]
Typhimurium	Cervical cancer	HPV16 E7	IFN-γ and TNF-α production and citotoxic T cell response	[134]
Typhimurium	Melanoma	Melanoma class I epitopes TRP-1 and TRP-2	Tumor-specific CD4+ and CD8+ T-cell responses	[135]

Table 3. (Continued)

SPECIE	TUMOR-BEARING MICE	HETEROLOGUS MOLECULES	ANTI-TUMORAL ACTIVITY	REF.
IMMUNOMODULATORS				
Typhimurium	Breast cancer and Colon cancer	LIGHT	Inhibition of tumor growth	[136]
Typhimurium	Breast cancer and Colon cancer	IL-18	Accumulation of T-lymphocytes and NK cells in tumors, granulocytes infiltration and cytokines production	[137]
Typhimurium	Breast cancer and Colon cancer	CCL21	CD4+ and CD8+ T-cell responses	[76]
Typhimurium	Osteosarcoma	IL-2	NK cells response	[138, 139]
Typhimurium	Melanoma	IFN-γ	NK cells response	[140]
INDUCTORS OF APOPTOSIS				
Typhimurium	Breast cancer and Colon cancer	Fas ligand	Proapoptotic effect, neutrophils recruitment	[141]
Typhimurium	Melanoma	TNF-α	Proapoptotic effect and NK cells activation	[142]
Typhimurium	Gastric cancer	TRAIL-Apoptin	Increased the expression of caspase-3 and caspase-9	[143]
Typhimurium	Breast cancer and Colon cancer	Fas ligand	Proapoptotic effect, neutrophils recruitment	[141]
Typhimurium	Melanoma	TNF-α	Proapoptotic effect and NK cells activation	[142]

SPECIE	TUMOR-BEARING MICE	HETEROLOGUS MOLECULES	ANTI-TUMORAL ACTIVITY	REF.
Typhimurium	Gastric cancer	TRAIL-Apoptin	Increased the expression of caspase-3 and caspase-9	[143]
CYTOTOXIC MOLECULES				
Typhimurium	Breast cancer	Haemolysin E (HlyE)	Necrosis	[145]
Typhimurium	Colon cancer	Cytolysin A (ClyA)	Inhibition of tumor growth	[146-148]
Typhimurium	Melanoma	Purine nucleoside phosphorylase (sPNP)	Delayed the growth tumor and increased the CD8+ T-cell infiltration	[150]
Typhimurium	Non-Hodgkin lymphoma	Thymidine kinase and tumor-associated antigen (CD20)	Antitumoral response	[151]
OTHER MOLECULES				
Typhimurium	Colon cancer	TGFα-PE38	Inhibition of tumor growth	[152, 153]
Typhimurium	Colon cancer	Endostantin	Inhibition of angiogenesis	[144]
GENE TRANSFER				
Typhimurium	Cervical cancer	HPV16L1	Systemic and mucosal immunity against HPV16	[167]
Typhimurium	Breast Cancer	MTDH/AEG1-1	CD8+ cytotoxic-T-cell	[168]
Typhimurium	Colon cancer	CEACAM6/4-IBBL	Increased CD45RO+ memory T cells, decreased FOXP3+ cells and promoting Th1 polarization	[169]
Typhimurium	Melanoma	IL-4 and Il-18	Increased levels of IFN-γ	[164]
Typhimurium	Gastric cancer	MDA7-VP3	Increased levels of IL-6, IFN-γ, TNF-α and Caspase 3, Downregulation VEGF	[171]

Table 3. (Continued)

SPECIE	TUMOR-BEARING MICE	HETEROLOGUS MOLECULES	ANTI-TUMORAL ACTIVITY	REF.
Typhimurium	Prostate cancer	STAT3 siRNA	Apoptosis and decreased levels of Bcl-2, cyclin D1, c-Myc and VEGF	[58, 173]
Typhimurium	Melanoma	Bcl-2 siRNA	delayed tumor growth	[175]
Typhimurium	Colon cancer	CTNNB1 siRNA	Decreased expression of c-Myc and cyclin D	[177]
Typhi	Tongue squamous cell carcinoma	MDR1 siRNA	Suppression of P-gp	[172]
Typhi	Laryngeal cancer	Survivin siRNA	Inhibition of tumor growth and induction of apoptosis	[176]

Abbreviation: AEG-1: Astrocyte elevated gene-1; *Bcl-2*: B-cell lymphoma 2 gene; CCL21: Chemokine (C-C motif) ligand 21; CEACAM6: Carcinoembryonic antigen-related cell adhesion molecule 6; Cly A: Cytolysin A; C-Raf1: Serine-threonine kinases of the Raf family; *CTNNB1*: Catenin beta-1 gene; FasL: Fas ligand; Hly E: Hemolysin E; HPV16E7: Human papilloma virus protein E7; HPV16L1: Capsid protein L1HPV16; IFN-γ: Interferon gamma; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-18: Interleukin-18; LIGHT: A member of TNF cytokine family; MDA7: Interleukin 24; MTDH: Metadherin; *MDR1*: Multidrug resistance protein 1 gene; NY-ESO-1: Testis antigen; PE38: Pseudomonas exotoxin A; PSA: Prostate-specific antigen; sPNP: Purine nucleoside phosphorylase; *STAT3:* Signal transducer and activator of transcription 3 gene; TGFα: Transforming growth factor alpha; TK: Thymidine kinase; TNF-α: Tumor necrosis factor α; TRAIL: TNF-related apoptosis-inducing ligand; TRP1 and TRP2: Tyrosinase-related proteins 1 and 2; VEGFR-2: Vascular endothelial growth factor receptor-2; VP3: Apoptin; 4-IBBL: Member of the tumor necrosis factor receptor (TNFR) superfamily.

7. CO-ADMINISTRATION OF *SALMONELLA ENTERICA* AND ANTITUMOR CONVENTIONAL TREATMENTS

Co-treatment of *Salmonella enterica* and conventional antitumor therapies such as chemotherapy, radiotherapy and immunotherapy have also been analyzed in order to improve tumor eradication. Hiroshima Y et al. showed in a murine model of xenotransplantation of human pancreatic cancer resistant to chemotherapy, that *S.* Typhimurium attenuated strain, A1-R, had higher antitumor activity than the chemotherapeutic agents 5-fluorouracil (5-FU), Cisplatin (CDDP), and gemcitabine (GEM), and a synergistic antitumor effect was observed by combining *Salmonella enterica* with 5-FU, suggesting that bacterial treatment induces chemosensitivity in chemotherapy-resistant cells [178]. These results are consistent with studies conducted by Chang WW et al. in murine models of melanoma and breast cancer, where *Salmonella enterica* sensitized the tumor cells to the action of the chemotherapeutic agent cisplatin and showed an additional therapeutic effect in delaying tumor growth and prolonging mice survival [179]. Chemosensitivity induced by *Salmonella enterica* was associated with the overexpression of Conexin 43, a protein that favors adherent unions between tumor cells allowing a better communication and a homogenous distribution of the chemotherapeutic agent [179].

Several studies document chemosensitivity induced by *Salmonella enterica* by the bactofection of sequences coding for proteins, peptides or siRNAs to eliminate tumor cells. In this context, our research group reported that bactofection of a plasmid coding for a peptide of the BH3 region of the pro-apoptotic protein Bax in an *in vitro* model of prostate cancer cells, favored sensitization of prostate cancer cells to treatment with cisplatin [2]. In another study in a murine model of melanoma, it was shown that bactofection of a plasmid encoding a siRNA against ABCB5, a member of the ATP-binding cassette subfamily B member 5, associated with the development of chemotherapy resistance, improved response to cyclophosphamide, favoring tumor elimination [180]. Similar results were obtained in a murine model of prostate cancer xenotransplantation, where

bactofection of a plasmid containing the sequence for a siRNA against HIF-1α and subsequent treatment with cisplatin favored tumor elimination [181].

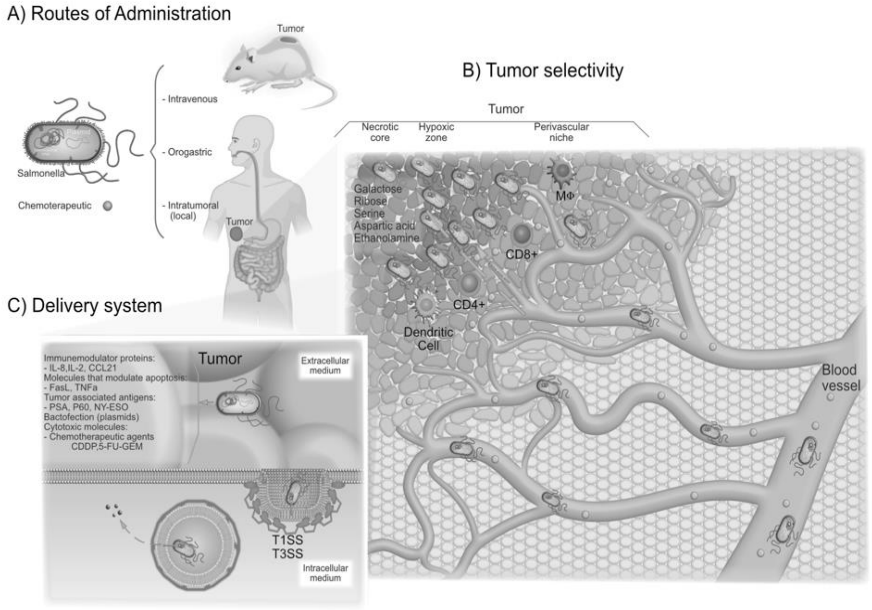

Figure 1. *Salmonella enterica* live-attenuated bacterial vector for cancer therapy. A) Routes of administration. *Salmonella enterica* has been administered by different routes including intravenous, orogastric or locally at the tumor site. B) Tumor selectivity. *Salmonella enterica* has the capacity to migrate, infect and replicate into the tumor tissues, favored by the presence of different components of the tumor microenvironment and low activity of immune cells. Importantly, chemotherapy agents only arrive to perivascular niche, while *Salmonella* arrives until necrotic core of tumor tissue. C) Delivery system. *Salmonella enterica* has been used as delivery system of heterologous molecules to the tumor microenvironment, that includes cytotoxic molecules, immunomodulatory, chemotherapeutic agents, inducers of apoptosis, and tumor associated antigens. Most of those molecules has been expressed in their surface or released through type 1 or type 3 secretion systems.

On the another hand, the combined treatment of trastuzumab, anti-HER2 monoclonal antibody, and *S.* Typhimurium strain A1-R in a murine model of breast cancer xenotransplantation, showed a greater decrease in tumor size, compared to controls only receiving Trastuzumab or A1-R strain [182]. Similar results were observed in the treatment of A1-R strain

together with the chemotherapeutic agent cisplatin in a murine model of human melanoma xenotransplantation [183].

Finally, the combination of *Salmonella enterica* releasing cytotoxic molecules with radiotherapy has also been analyzed. An example of this is the administration of *Salmonella enterica* that releases cytolysin A (ClyA) together with radiotherapy in a murine model of colon cancer [184]. Results showed that the group receiving the combined treatment induced a greater regression in tumor size compared to groups receiving only one of the treatments [185].

CONCLUSION

The use of bacteria in antitumor therapy has been documented for more than a century, and although this alternative was neglected for several decades, the development of live-attenuated bacterial vectors (increasingly safe) for use in humans has promoted the resurgence of their study as therapeutic alternatives against cancer [1]. The research studies described in this chapter document the potential use of attenuated strains of *Salmonella enterica* serovar Typhi and Typhimurium as an effective therapeutic alternative against cancer. These live-attenuated vectors present a high selectivity for tumor tissue and metastasis [3, 123]. Once in the tumor microenvironment, *Salmonella enterica* exerts its intrinsic cytolytic activity and induces innate and adaptive antitumor responses, events that are enhanced by the ability of this bacterium to transport heterologous molecules as tumor antigens, immunomodulatory, cytotoxic, antiangiogenic and pro-apoptotic molecules to the tumor micro-environment. Finally, pre-clinical and clinical studies presented in this chapter prove that *Salmonella enterica* attenuated strains represent a promising therapeutic alternative that should be considered in the fight against cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest of any nature.

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