Cancer Letters xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Mini-review

Targeted cancer immunotherapy with genetically engineered oncolytic *Salmonella typhimurium*

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ARTICLE INFO

Keywords:
Bacteria-mediated cancer therapy
Engineered *Salmonella typhimurium*
Cancer immunotherapy
Tumor immune microenvironment

ABSTRACT

Conventional chemotherapies have some limitations, including the lack of selectivity, high toxicity to normal tissues, multidrug resistance, and tumor relapse. Recently, great progress was made in immunotherapies for anticancer research, with bacteria-mediated cancer therapy one of the most promising approaches among them. Attenuated *Salmonella* have very specific targeting to various solid cancers, making them ideal vectors for the delivery and expression of immunostimulators. They have native bacterial immunogenicity and induce strong anticancer immunity in vivo. In this review, the recent advances in *Salmonella*-mediated cancer immunotherapies and the related mechanisms of *Salmonella*-based cancer therapies are summarized.

1. Current cancer immunotherapy

Cancer is one of the leading causes of death worldwide. Recently, with better knowledge of the complex interactions between cancer cells and the immune system, novel immunotherapy approaches have emerged [1]. Immunotherapy is regarded as the “fourth major therapy” in cancer treatments after surgery, radiotherapy, and chemotherapy. It induces anticancer response by reprogramming or enhancing immune monitoring with reduced immunosuppression [2] and may be classified into “passive” (monoclonal antibodies, adoptive T-cell transfers, and genetically engineered T-cells) or “active” (cancer vaccines or immunostimulators) immunotherapy [3]. Genetically engineered chimeric antigen receptor T cells show considerable therapeutic effect on acute lymphoblastic leukemia, and studies regarding the treatment of solid tumors are also underway [4,5]. However, clinical trials are still in the early stages, and many malignancies become resistant to transferred T cells by evading immune recognition and/or suppressing effector responses, which eventually lead to recurrence [6–9]. Immunological checkpoint therapy, which uses CTLA-4, PD-1, PD-L1, and other monoclonal antibodies, has been approved by the FDA for the treatment of patients with clinical cancer. The mechanism of such drugs mainly involves activating T cells to kill tumor cells by blocking the inhibition signal of T cell activation. Significant clinical effects have been achieved especially in the treatment of patients with cancer, such as melanoma and non-small-cell lung cancer [10,11]. However, immunological checkpoint therapy has no significant effect in tumors with poor T cell infiltration and low tumor antigen mutations, such as pancreatic and ovarian cancers and “cold tumors” [12,13]. In addition, its blockers are non-specific. That is, they can enhance the immune system to kill cancer cells but may also cause damage to normal cells, causing side effects of autoimmune diseases. In this context, we discuss bacteria-mediated cancer therapy (BMCT), which has been described over a century ago and has re-inspired the interest of researchers [14]. Here we made a comparison between *Salmonella*-based immunotherapy and other immunotherapies as presented in Table 1.

2. Bacteria-based cancer immunotherapy

Many studies have shown that bacteria are carcinogenic and pathogenic [15–18]. For example, systemic infection with *Salmonella* can cause septic shock and high mortality in mammals. However, bacteria have also shown great potential antitumor effects when bacterial virulence is attenuated by mutation. The first use of bacteria as anticancer therapeutic agents dates back to the mid-19th century when William Coley used heat-inactivated *Streptococcus pyogene* and *Serratia marcescens* to treat solid tumors [19]. However, the rise of chemotherapy and radiotherapy, along with the criticism of Coley's toxin raised by the medical community due to unpredictable and non-reproducible results, then bacterial cancer therapy was put on hold. With the development of molecular biology and immunology, understanding

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https://doi.org/10.1016/j.canlet.2019.10.033
Received 4 September 2019; Received in revised form 29 September 2019; Accepted 21 October 2019

Table 1
Comparison of conventional immunotherapies and bacteria-based immunotherapies.

Properties	Conventional immunotherapies	Bacteria-based immunotherapies
Tumor specificity	Relatively low	Very specific
Tissue penetration	Limited	Deep penetration to tumor tissues
Immune stimulation	Medium or mild	Robust
Toxicity	Toxic to normal tissues	Less toxic to normal tissues
Cancer type	Limited	Broad-spectrum anticancer activities
Programmability	Poor operability	Easy to operate and remote control
Pharmacokinetics	Rapid clearance with a short half-life, unstable	Stable, maintained high concentration
Costs	Expensive	Inexpensive

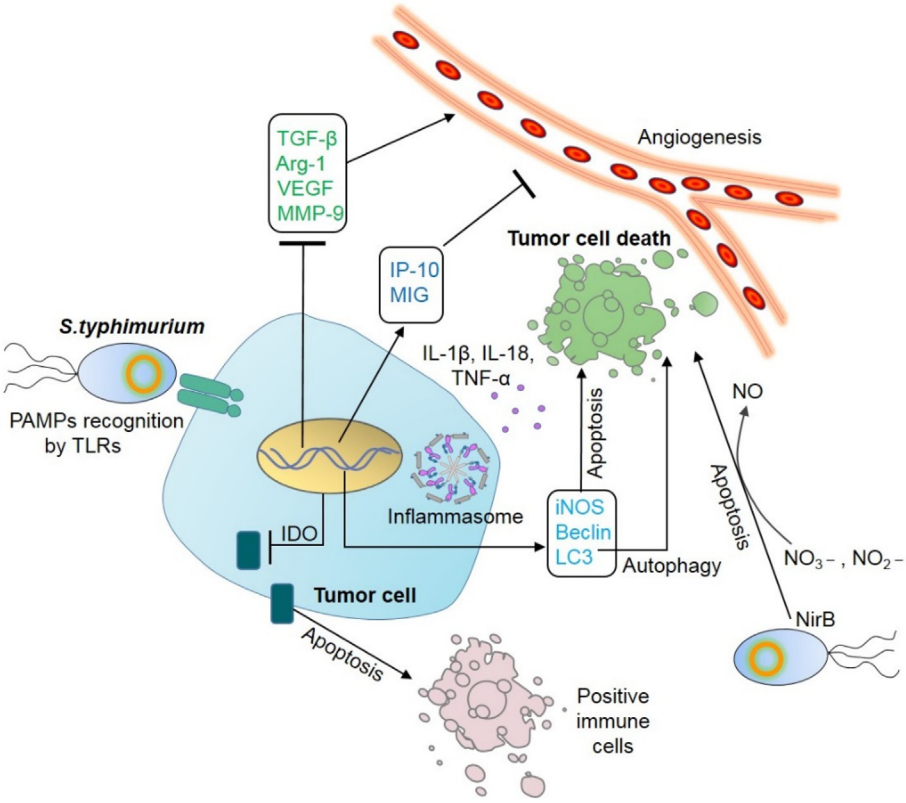

Fig. 1. Oncolytic mechanisms of *Salmonella*. Once *Salmonella* colonize the tumor tissues, it promotes tumor cell death via several mechanism: 1) inhibition of tumor angiogenesis by upregulating the inhibition of angiogenic factors and downregulating the pro-angiogenic factors or 2) direct killing of tumor cell by apoptosis and autophagy via upregulation of related factors. Moreover, it inhibits immunosuppressive molecules, which increases the level of immune cells in the tumor microenvironment and kills tumor cells. PAMPs: pathogen-associated molecular patterns, TLRs: toll-like receptors. TGF-β: transforming growth factor-β, Arg-1: arginase-1, IL-4: interleukin 4, VEGF: vascular endothelial growth factor, MMP-9: matrix metalloproteinase 9, IP-10: interferon-γ-inducible protein-10 (CXCL10), MIG: IFN-inducible chemokines CXCL9, IDO: indoleamine 2, 3-dioxygenase, iNOS: inducible nitric oxide synthase, NirB: nitrite reductase, NO_3^-: nitrates, NO_2^-: nitrites, NO: nitric oxide. Positive immune cells mainly include effector T cells, such as cytotoxic T cells, helper T cells, and NK cells.

on BMCT has increased and BMCT has been identified as promising strategy for treating solid tumors and metastasis [20]. Several bacterial strains, including *Salmonella typhimurium* [21,22], *Escherichia coli* [23–25], *Bifidobacterium* [26], *Clostridium* [27], *Listeria* [28], and *Streptococcus,* have been shown to induce antitumor activity [29]. BMCT is the new generation of cancer therapeutics because of the advantages that anaerobic bacteria and facultative anaerobic bacteria possess. First, such bacteria can colonize and proliferate in hypoxic and/or necrotic regions caused by irregular blood vessels in tumor tissues [30], and this niche is not conducive to conventional anticancer treatment. Second, bacteria have innate conditions that inhibit tumor growth (discussed in Section 3.1). Third, therapeutic agents delivered by bacteria can target tumor tissues to reduce toxicity to normal tissues under a controlled expression system (discussed in Section 4.2). Finally, bacterial combination therapy enhances antitumor potency (discussed in Section 5).

Salmonella own some advantages over other bacteria including high tumor-specificity, deep tissue penetration, native bacterial cytotoxicity, convenient gene modification, and good safety profiles, is widely studied in this field. Many researchers have generated safer and better anticancer activity by reducing bacteria-related toxicity to inhibit the expression of virulent genes. For example, VNP20009 with *purI* and *msbB* gene deletions, two genes necessary for adenine and lipid A synthesis, respectively, have been safely administered to patients with metastatic melanoma and renal cell carcinoma in a phase I clinical study. However, no antitumor effect is observed, which may due be to the over-attenuation of bacteria [31,32]. A1-R, a leucine/arginine auxotrophic strain, show significant anticancer activity in many types of patient-derived orthotopic xenograft models [33–36] and decoy quiescent cells to enter the cell cycle to make them sensitive to chemotherapy [37]. Another strain termed ΔppGpp, which is defective in guanosine 5′diphosphate-3′-diphosphate synthesis [38], substantially increases the median lethal dose (LD50) by 100,000- to 1,000,000-fold. It is virtually avirulent in BALB/c mice [39] and shows highly specific tumor targeting and immune stimulation in the tumor microenvironment [40–42]. Previous studies have shown various mechanisms for bacteria-related cancer therapy. In general, such mechanisms can be summarized into three major aspects. 1) The chaotic vasculature of tumors leads to the formation of hypoxic and necrotic areas, which are ideal environments for obligate anaerobes. 2) Small molecule nutrients (amino acids, carbohydrates, nucleic acids, and ethanolamine), bacterial motility and chemokines [43–45], and the nutrients released from necrotic tumor cells support bacterial growth and proliferation. However, Stritzker et al. demonstrated that chemotaxis and motility do not play important roles in colonization and migration within tumor tissues, but the destruction of aromatic amino acid biosynthesis pathways

and the depletion of macrophages significantly alter bacterial accumulation in murine cancer models [46]. 3) *Salmonella* is forced to flood into tumors following host inflammation to seek protection [47–49], where the tumor microenvironment is highly immunosuppressive, preventing bacteria from being cleared by the host immune system. In short, the features of a tumor microenvironment make tumor tissues an ideal harbor for anaerobic bacteria to survive.

3. Native bacterial immunostimulation

3.1. Activation of inflammasome pathway

Bacterial therapy is an innovative strategy for cancer treatment, and the host immune response induced during bacterial infection is highly complex because of the immunogenic factors derived from bacteria. In Fig. 1, the bacteria-localized tumor shows inhibited tumor growth via various mechanisms.

Salmonella infection is initially sensed by toll-like receptors (TLRs), which can identify various pathogen-associated molecular patterns (PAMPs) of Gram-negative bacteria, such as TLR-activating PAMPs that include lipopolysaccharide (TLR4), flagellin (TLR5), and unmethylated CpG DNA (TLR9), and then stimulate the host immune system and exert antitumor activities [40,49]. Upon *Salmonella* infection, a cascade of cellular signaling events are triggered, eliciting a strong cytokine and chemokine storm followed by the immune cell population's influx into tumor tissues [50] (Fig. 2). The tumor cells damaged by *Salmonella* infection release ATP, which activates the NLRP3 inflammasome and further increases the amount of inflammatory cytokines, such as IL-1β, IL-18, and TNF-α, resulting in tumor regression. Two molecular mechanisms are involved in the activation of inflammasomes. One is directly activated by *Salmonella* (LPS), while the other is activated by signals (ATP) released by damaged tumor cells and/or by the phagocytosis of damaged tumor cells. Extracellular ATP acts on the P2X7 receptor, leading to the activation of NLRP3 inflammasome in macrophages [41]. In a recent study conducted by Kocijancic et al., systemic therapy using LPS purified from *Salmonella* induced tumor-specific $CD8^+$ T cells and high levels of TNF-α and showed strong tumor suppression comparable with viable *Salmonella* treatment [51]. In another study, peritumoral administration of purified *S. typhimurium* flagellin at the time of antigenic tumor implantation promoted tumor growth with decreased IFN-γ:IL-4 ratio and increased $CD4^+CD25^+$ T cell frequency, while late flagellin administration significantly inhibited tumor growth with increased IFN-γ:IL-4 ratio and decreased frequency of $CD4^+CD25^+$ T regulatory cells. Furthermore, tumor growth was completely suppressed when early flagellin therapy was combined with the administration of CpG-containing oligodeoxynucleotides [52]. Metastases in murine melanoma model were inhibited after the flagellin derived from *S. typhimurium* was treated and fused with peptide P10 of the gp43 protein of *Paracoccidioides brasiliensis* [53]. However, the antitumor activity of *Salmonella* was significantly inhibited in $TLR4^{-/-}$ and $MyD88^{-/-}$ mice, and the infiltration of neutrophils and monocytes/macrophages in tumors were decreased in $TLR4^{-/-}$ mice compared with that in wild-type mice. Several studies have shown that the antitumor activity of *Salmonella* is mainly induced by TLR4 activation, while the effect of TLR5 activation is an auxiliary role [42,54,55]. In another study, the colonization of *S. choleraesuis* in tumor tissues upregulated IFN-γ and IFN-inducible chemokines MIG and IP-10 that inhibited angiogenesis. In addition, the apoptosis of cancer cells increased compared with that in TLR4-deficient mice [55].

The studies listed indicate that the intrinsic antitumor activity of *Salmonella* is largely dependent on the innate immunity of the host induced by bacteria. However, adaptive immune responses also play an important role in *Salmonella*-mediated cancer therapy. The accumulation of *Salmonella* in tumor tissues increases the penetration of host immune cells (i.e., neutrophils [56], natural killer (NK) cells [57], macrophages, dendritic cells (DCs), B cells [58], $CD8^+$ T cells [59]) and reduces the number of regulatory T cells (Tregs). Therefore, anticancer activity is exerted by increasing the expression of immunostimulatory factors (e.g., IL-1β, TNF-α, and IFN-γ) [39,40,60] and inhibiting the expression of immunosuppressive factors [e.g., arginase-1 (Arg-1), IL-4, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF)] [54]. TNF-α upregulation accompanied with *S. typhimurium* colonization increased the permeability of intratumoral blood vessels and caused blood vessels in the tumor to hemorrhage, thus enhancing the infiltration of immune cells to strengthen tumoricidal effects [48,51]. Tumor vascularity also influences tumor sensitivity to *S. typhimurium* A1-R therapy. Abundant blood vessels in the tumor increases the damage caused by *S. typhimurium* A1-R to tumors

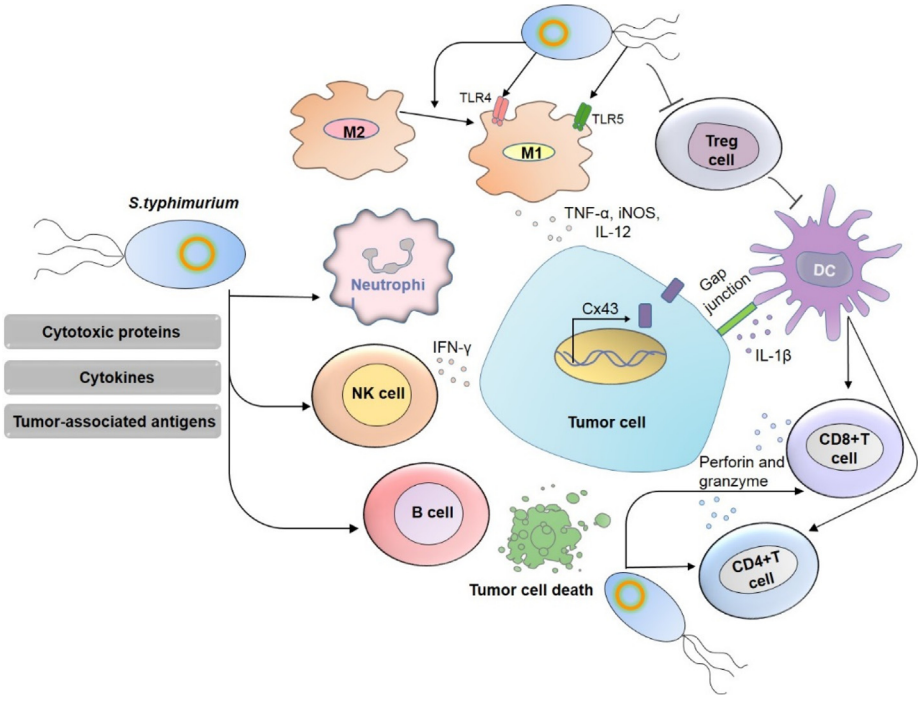

Fig. 2. Activation of immune responses in TME by *Salmonella*. Once *Salmonella* reach the tumor tissues, a massive number of immune cells are recruited to the tumor microenvironment and secrete proinflammatory cytokines to promote tumor cell killing. Immunomodulators delivered by *Salmonella* also further enhances cancer immunotherapy. M1: M1-like macrophage, M2: M2-like macrophage, NK cell: natural killer cell. DC: dendritic cell, TLR4: toll-like receptor 4, TLR5: toll-like receptor 5, TNF-α: tumor necrosis factor-α, IFN-γ:Interferon-γ, Cx43: connexin 43, IL-1β: interleukin 1β.

[61]. When anticancer activity is induced by bacterial infection, a specific immune memory is evoked [51]. However, a study of a murine melanoma model showed a specific response against the tumor mediated by $CD8^+$ T cells but did not induce an immunologic memory, which needs to be further explored [62]. *Salmonella*-colonized tumors also induce the expression of connexin 43 (Cx43) [63,64], which enhances the transfer and cross-presentation of processed tumor antigens by promoting the formation of gap junctions between tumor cells and DCs, thus resulting in enhanced anticancer activity (Fig. 2) [64].

3.2. Phenotype shift of immune cells

Injected bacteria interact with the host immune system to induce anticancer activity. In this process, the immune cell phenotype transformation is involved. Lee et al. reported that *S. choleraesuis* infection polarizes T-cell response to a Th1-dominant state via TLR4 signaling to suppress cancer growth [55]. The administration of attenuated *Salmonella* can also induce changes in myeloid cells in tumor tissues. These changes are consistent with the maturation of macrophage effector factors and shift the tumor-infiltrating M2 macrophages to express immunosuppressive molecules, such as Arg-1 and IL-10 [65,66]. These molecules promote tumor growth and malignancy to M1 macrophages to express immunostimulatory factors, including nitric oxide synthase (iNOS), IL-1β, and TNF-α that promote tumor regression (Fig. 2) [42,54,67,68]. Indoleamine 2,3-dioxygenase (IDO) exerts its immunosuppressive effect by developing immune tolerance in T lymphocytes and activating Tregs, leading to the growth arrest and apoptosis of positive immune cells (mainly effector T cells such as cytotoxic T, helper T, and NK cells) (Fig. 1) [69,70]. However, *Salmonella* infection can lead to tumor regression by reducing the expression of IDO [71,72], thus converting a highly immunosuppressive tumor microenvironment into an immunogenic one.

3.3. Induction of tumor cell death

In addition to the activation of host immune responses, bacteria induce tumor regression by directly killing tumor cells (Fig. 1). *S. typhimurium* is reported to enhance anticancer activity with the induction of apoptosis and autophagy [30,73,74]. However, different bacterial strains cause tumor regression through various mechanisms in tumor microenvironments. In short, bacteria may induce cancer cell death through competition for extracellular nutrients [75], stimulation of immune responses [56], induction of apoptotic or autophagy signal transduction pathways after intracellular accumulation [30,73], and release of bacterial toxins. *Salmonella*-colonized tumor suppresses HIF-1α by downregulating the AKT/mTOR pathway then increases the expression of autophagy-related proteins (Beclin, LC3) to induce tumor cell autophagy and caspase-3-dependent apoptosis [73]. Moreover, the enzyme nitrate reductase (NirB) from lysed *Salmonella* metabolizes nitrates and nitrites [76], which are further converted to nitric oxide (NO) in the tumor microenvironment to induce tumor cell apoptosis [57,77]. *Salmonella* induces macrophages to secrete IL-12, which triggers NK cells for the secretion of IFN-γ and further activation of macrophages. This positive feedback loop induces iNOS in macrophages and the production of massive NO, which exerts microbicidal and tumoricidal capacity [78].

3.4. Reversal of immune tolerance within the tumor microenvironment

One reason cancers are difficult to cure is because the tumor microenvironment is highly immunosuppressive. However, bacteria can relieve this immunosuppression by upregulating immunostimulatory factors and downregulating immunosuppressive factors (Fig. 1). Tregs play a crucial role in maintaining self-tolerance and the resolution of immune responses. Their relatively high population in the tumor microenvironment dampens antitumor responses and serves as a barrier for effective cancer immunotherapy [79]. After *Salmonella* colonization in tumors, tumor regression is triggered by reverting the immune tolerance via decreasing the number of Tregs in tumor tissues [80]. Tumors also need a dedicated blood supply to provide oxygen and other essential nutrients they require for rapid growth. Furthermore, angiogenesis is the most critical part of tumor transformation from benign to malignant. In addition to killing cancer cells, bacteria can delay tumor growth by inhibiting angiogenesis or destroying blood vessels in tumor tissues. *Salmonella* infection inhibits the expression of VEGF, which stimulates the formation of blood vessels. *Salmonella* infection can also induce the upregulation of Cx43 in melanoma models [64] and inhibit angiogenesis through the downregulation of HIF-1α and VEGF [81]. Recent reports showed that the downregulation of VEGF is mediated by the inhibition of HIF-1α via the downregulation of the AKT/mTOR pathway [73]. In addition, the destruction of intratumoral vessels by *Salmonella* is closely related to the distribution of tumor blood vessels. Vascular-rich tumors respond to bacterial therapy earlier than tumors with sparse vessels [61].

3.5. Destruction of tumor stromal cells

Tumor stroma plays an active role in tumor invasion, metastasis, and poor prognosis. Disrupting tumor vasculature required for tumor growth is also effective in bacterial therapy. P-glycoprotein (P-gp) is localized on the cell membrane and pumps chemotherapeutic drugs out of the cells, and its high expression in tumors is associated with drug resistance. *S. choleraesuis* reduces P-gp expression by inhibiting the AKT/mTOR/p70s6K signaling pathway, which makes the tumor sensitive to chemotherapies [82]. Matrix metalloProteinase 9 (MMP-9) in tumors, which may be induced by the tumor microenvironment, contributes to tumor growth, metastasis, and poor prognosis. *Salmonella* inhibit the expression of MMP-9 through the downregulation of AKT/mTOR to prevent epithelium-to-mesenchymal transition [83].

4. Enhanced cancer immunotherapy

4.1. Immunomudulators delivered by Salmonella

In addition to native bacterial anticancer activity, attenuated *Salmonella* strains can also be used as vectors to carry immunomodulators for enhancing cancer immunotherapy. For example, a recent study by Zheng et al. demonstrated that flagellin B (FlaB) of *Vibrio vulnificus* delivered by *S. typhimurium* ΔppGpp strain displays remarkable tumor regression [42]. The main mechanism involved is the activation of TLR4 and TLR5 pathways by LPS and FlaB, respectively, which result in the massive infiltration of macrophages and neutrophils in the tumor microenvironment and shift the tumor-promoting M2-like macrophages to tumor-suppressing M1-like macrophages. This outcome indicates that the anticancer effect of the armed strain is initially mediated through the activation of TLR4, whereas the effect of TLR5 is exerted after priming [42]. *S. typhimurium* expressing CytolysinA (ClyA), a spore-forming bacterial toxin, can penetrate the neutrophil barrier and permeate the proliferating area of tumors, thus retarding tumor growth and promoting the survival of mice with colon cancer [84]. TGF alpha (TGF-α) is recognized by EGFR, which is highly expressed in various kinds of cancers and plays an important role in cancer development. Lim et al. observed that tumor growth is significantly retarded in TGFα-PE38 fusion protein delivered by *Salmonella* compared with that in *Salmonella* alone [85]. The fusion protein TGFα-PE38 is reported to be toxic to EGFR-expressing tumor cells and enhances cytotoxic T-lymphocyte to kill tumor cells [86,87].

In another study by Li et al., the recombinant *Salmonella* harboring either murine (mIL-12 and mGM-CSF) or human (hIL-12 and hGM-CSF) cytokine genes exerted significant antitumor effect by increasing cytotoxic T cells in peripheral blood and cytokine production. However, hGM-CSF and hIL-12 concentrations reach their peaks at 2–4 weeks and

return to normal levels at 10–12 weeks, whereas the mGM-CSF and mIL-12 concentrations remain at high levels at 10–12 weeks. This may explain why the survival duration between murine and human cytokine gene-treated groups is different [88]. IL-18-producing *Salmonella* also inhibits tumor growth without overt toxicity to normal tissues by inducing host inflammatory responses, including increased numbers of T and NK cells, production of certain related cytokines, and massive infiltration of neutrophils [89]. *Salmonella*-based therapy expressing CCL21, which controls the migration of lymphocytes, DCs, and NK and T cells, significantly inhibits the growth of primary tumors and pulmonary metastases in murine carcinomas compared with control groups [90].

Murine melanoma antigen Melan-A and a DNA vaccine element encoding two murine melanoma class I epitopes (TRP-1: TWHRYHLL and TRP-2: SVYDFFVWL) delivered by attenuated *Salmonella* activate both tumor-specific $CD4^+$ and $CD8^+$ T-cell responses, promote T-cell proliferation, and increase cytokine production (TNF-α, IL-12, and IFN-γ) to promote antitumor immunity [91]. In addition, an attenuated *S. typhimurium* strain carries the VEGF receptor 2 (VEGFR2; also known as FLK-1), breaks peripheral immune tolerance, and elicits cytotoxic T cell-mediated immunity against this self-antigen expressing endothelial cells, which lead to the suppression of angiogenesis in the tumor vasculature and the effective inhibition of pulmonary metastasis [92,93]. The recombinant attenuated *S. typhimurium* secreting NY-ESO-1 localized to NY-ESO-1–negative tumors also activates NY-ESO-1-specific $CD8^+$ T-cell response, leading to tumor regression [94]. The widely studied immunodulators delivered by *Salmonella* are listed in Table 2.

4.2. Expression systems for reduced toxicity

Although bacteria itself can effectively inhibit tumor growth, engineered recombinant bacteria carrying therapeutic cargos are more effective. However, bacteria are initially colonized in the liver and spleen via tail vein injection [95,96], so constitutive antitumor therapeutic agents delivered by engineered attenuated *Salmonella* will inevitably result in hepatic or splenic injury. Therefore, remote control of gene expression is necessary for the application of tumor-targeting bacteria in vivo to reduce toxicity to normal organs. Nuyts et al. reported the deliberate induction of gene expression in the host in response to ionizing radiation [97]. However, radiation can cause damage to normal tissues, so some researchers used inducible promoters (e.g., pBAD, pTet, and Pm) that can activate genes through the administration of exogenous transcriptional inducers (e.g., L-arabinose, tetracyclines, and acetyl salicylic acid) in vivo [84,98–100]. Unfortunately, these expression systems have limitations, including the need to administer inducers regularly to maintain continuous expression of oncolytic agents, remote gene control through the diffusion of the injection site containing off-target effects and inhomogeneity, and frequent injections that are stressful to hosts. To improve the convenience and safety of controlling target gene expression, Kim et al. used a cell mass-dependent inducible quorum-sensing system [101]. Given that tumor tissue has a hypoxic environment, flexible hypoxia-inducible promoter systems, such as HIP-1 and NirB, are developed to ensure specific gene expression in hypoxic tumor tissues and reduce toxicity to normal tissues [102,103]. These systems are a promising approach for engineering tumor-targeting bacteria to generate bioluminescence and expression system for the evaluation of therapeutic efficacy through the visualization of bacterial colonization and replication in specific organs (tumor, liver, and spleen). Highly attenuated *S. typhimurium* bioengineered to carry target genes, such as ClyA and reporter genes, can likewise be used for visualized cancer therapy [104].

5. Modification of TME for combinational therapy

Although *Salmonella* exert strong anticancer activity, its therapeutic effect can be further improved when combined with other therapies. In the study conducted by Chen et al., triptolide significantly improved tumor colonization of VNP20009 and expanded the necrotic region in the melanoma by decreasing the number of infiltrated neutrophils, which clear bacteria in infected hosts. The combination therapy also inhibited tumor angiogenesis by reducing VEGF expression and produced a more hypoxic tumor microenvironment that was conducive to bacterial colonization and proliferation [105]. Meanwhile, the infiltration of $CD8^+$ T cells were significantly increased within tumor site

Table 2
Immunomodulators delivered by *Salmonella*.

Immunomodulators	Immune responses	References
Cytotoxic proteins		
FasL	Accumulation of inflammatory cells (particularly neutrophils).	[115]
CD40L	Indispensable for activating antigen presenting cells (APCs).	[116]
FlaB	Accumulation of abundant immune cells via TLR4 signaling and secreting proinflammatory cytokines.	[42]
ClyA	Breaks the neutrophil barrier within tumors.	[84]
TGFα-PE38	Enhanced cytotoxic T-lymphocyte.	[87]
Cytokines		
TNF-α	Activation of NK cells.	[117]
IFNγ	Activation of NK cells.	[118]
IL-2	Infiltration of lymphocytes, activation of NK cells.	[119,120]
IL-4 and IL-8	Increased level of IFN-γ.	[121]
IL-12 and GM-CSF	Increased number of cytotoxic T cells and induced pro-inflammatory cytokines.	[88]
IL-18	Increased number of T cells and NK cells, as well as several cytokine production, massive neutrophil infiltration.	[89]
LIGHT	Infiltration of T lymphocytes and NK cells.	[122]
CCL21	Increased levels of INF-γ, CXCL9, and CXCL10. $CD4^+$ and $CD8^+$ T-cell response are indispensable.	[90]
Tumor-associated antigens		
Melan-A, TRP-1 and TRP-2	Activation of tumor-specific $CD4^+$ and $CD8^+$ T-cell responses, increased T-cell proliferation, and induced cytokine production.	[91]
NY-ESO-1	Activated NY-ESO-1-specific $CD8^+$ T cell response.	[94]
VEGFR-2	Induction of VEGFR2-specific $CD8^+$ T cells.	[92,123]
Antigen PSA	Increased level of IFN-γ.	[124,125]
C-Raf	Induction of humoral and Raf-specific $CD8^+$ T-cell response.	[126]
HPV16 E7	Induction of cytotoxic T lymphocyte (CTL) and cytokine responses.	[127]

FasL: Fas ligand, CD40L: CD40 ligand, FlaB: *Vibrio vulnificus* flagellin B, ClyA: cytolyin A, TGFα-PE38: tumor growth factor α-*Pseudomonas* exotoxin 38, TNF-α: tumor necrosis factor-α, IFN-γ: Interferon-γ, IL-2: interleukin 2, GM-CSF: granulocyte/macrophage colony-stimulating factor, LIGHT: TNF superfamily member 14, CCL21: C–C motif chemokine ligand 21, Melan-A, TRP-1 and TRP-2: melanoma antigen, murine melanoma class I epitopes (TRP-1: TWHRYHLL and TRP-2: SVYDFFVWL), NY-ESO-1: also known as CTG1B, a germ cell protein often expressed by tumor cells, VEGFR-2: vascular endothelial growth factor receptor 2, PSA: prostate specific antigen, C-Raf: serine-threonine kinases of the Raf family, HPV16 E7: human papillomavirus and oncogenic proteins E7.

in combination with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone). The infiltrated $CD8^+$ T cells further enhanced NK cell cytotoxic activity compared with *Salmonella* alone or CHOP treatment alone, thus enhancing tumoricidal activity [106]. Angiogenesis is associated with tumor growth and metastasis. *Salmonella* combined with cyclophosphamide therapy significantly reduced tumor microvascularization and improved tumor regression in murine melanoma models [107]. In another study, a combination of adoptive T cell therapy with live or heat-killed (HK) *S. typhimurium* improved tumor regression compared with OT-1 T cell therapy alone. Combination therapy significantly enhanced the proliferation of adoptively transferred OT-1 T cells, increased the number of neutrophils, and reduced monocytes in the tumor stroma, which has been associated with enhanced T cell therapeutic efficacy [108]. However, one mouse died 16 days post-treatment with HK *Salmonella* combined with adoptive T cell therapy, indicating potentially increased toxicity due to the elevated levels of IL-6 and other pro-inflammatory cytokines, which can be overcome by applying neutralizing antibodies [109]. Chemotherapy resistance is a major factor impairing the efficacy of cancer therapy. *Salmonella* can enhance the sensitivity of cisplatin-resistant cancer cells by upregulating Cx43 mediated by p38 signaling cascade-enhanced gap intercellular communication [110]. Additionally, the combination of *S. choleraesuis* and cisplatin further increased infiltrating neutrophils, $CD8^+$ T cells, and apoptotic cells within tumors compared with *S. choleraesuis* or cisplatin treatment only [111]. P-gp expression was also significantly reduced in tumor cells after *Salmonella* administration in a dose-dependent manner. The downregulation of P-gp mediated by *Salmonella* showed a significant increase in intracellular accumulation of Rho-123, suggesting the suppressed transportation activity of P-gp and resulting in the enhanced sensitivity of tumor cells to 5-FU therapy [82].

6. Conclusions and perspectives

To date, *Bacillus Calmette-Guerin* is one of the most successful bacterial agents applied for intravesical administration in patients with bladder cancer [112]. Some of recent clinical trials of using engineered *Salmonella* have been carried out (Table 3). Although considerable progress has been made in bacterial cancer therapy, many questions have yet to be addressed before bacteria can be applied in the clinic.

1. Genetic instability. The recombinant plasmid carried by bacteria can be lost and mutated after generations in host, which may lead to a variety of potential risks, such as therapy failure and exaggerated infection.
2. Intrinsic bacterial toxicity. The use of bacteria as anticancer agents for patients with clinical cancer and their toxicity and side effects require verification using different clinical trials and long-term follow-up. Although the toxicity of bacteria can be attenuated with gene engineering, any residual toxicity may be a disaster for advanced patients with poor immunity. Moreover, the use of bacteria as anticancer agent to improve the therapeutic effect while reducing the dose remains to be solved.
3. Biosafety. Bacteria are living organisms, and they may mutate in patients and proliferate in the human body. The occurrence of septicemia will be another disaster for patients. As such, the clinical development of bacterial therapy faces substantial hurdles due mainly to the potential adverse effects of infection. Furthermore, unlike other small molecules or clinical agents, live bacteria cannot be sterilized by filtration or heating. The kind of liquid for bacterial dilution, the conditions to be used, and the effect on bacterial activity are problems in clinical translation as well. Therefore, there is still a long way to go for the application of bacterial therapy.
4. Bacterial administration. Systemic infection of bacteria is highly inconvenient, has a high risk of toxicity, and exerts a psychological burden to patients. Therefore, oral administration of bacteria without affecting the efficacy must be developed.
5. Killing of bacteria in vivo. After the tumor has subsided, suitable antibiotics can be used to kill bacteria in the host. However, the amount of antibiotics, resistance of the host, and destruction of balance in the host's microbiota are new problems that need to be solved. Furthermore, antibiotic abuse is a serious problem faced by the whole world. The excessive use of antibiotics accelerates the spread of drug-resistant bacteria. Whether antibiotics can be used to kill bacteria in vivo as expected remains unclear.
6. Tumor recurrence. Although the effect of treating cancer with bacterial therapy is significant, recurrence is still possible. The inhibition of tumor in the treatment of colon cancer mouse tumor model with ΔppGpp *Salmonella* alone is transient, and the tumor subsequently relapses [40]. This outcome may be attributed to the altered tumor microenvironment during ΔppGpp *Salmonella* treatment, leading to bacterial death in tumor masses or activation of immune evasion/tolerance mechanisms to disrupt the immune response mediated by bacteria.
7. Anti-bacterial immunity. Although pre-exposure to the treated bacterial strain improves safety, it reduces immune pathology response and limits the efficacy elicited by bacteria [113]. Therefore, in-depth knowledge of the patients' immunological background is essential before a precise treatment is specified to enhance the immune stimulatory capacity of the bacteria used through targeted genetic manipulation or bacteria-encapsulated delivery system and thus achieve safer and more effective treatment [114].

BMCT has many advantages over conventional cancer therapy, but it still cannot replace traditional cancer therapies. BMCT combined with traditional cancer therapies can kill tumor cells more effectively. The rational application of BMCT in clinics is full of challenges, and considerable work still needs to be evaluated. Nonetheless, the use of live attenuated *S. typhimurium* in cancer immunotherapy is also full of hope.

Declaration of competing interest

The authors have no conflicts of interest.

Acknowledgements

This work was supported by the Fundamental Research Funds for the Central Universities, China.

Table 3
Clinical trials using *Salmonella*-based immunotherapy.

Strain	Cancer type	Species and clinical phase	References
S. typhimurium VNP20009	Metastatic melanoma and renal cell carcinoma	Human, phase I	[31,32]
S. typhimurium VNP20009 TAPET-CD	squamous cell carcinoma and adenocarcinoma	Human, Phase I	[128]
S. typhimurium SalpIL2	Appendicular osteosarcoma	canine, phase I	[129]
S. typhimurium SalpIL2	Liver metastases of solid tumors	Human, phase I	[130] unpublished and completed

S. typhimurium TAPET-CD (VNP20009 expressing cytosine deaminase, an enzyme that catalyzes the chemical reaction); *S. typhimurium* SalpIL2: *S. typhimurium* χ4550 expressing IL-2.

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