# Salmonella-based cancer therapy and the stabilizing methods.

Tianyang Wang s3327523

Supervisor: Wouter Hinrichs

Abstract: Bacteria plays an important role in maintaining homeostasis. The human microbiota can induce and prevent oncogenesis at the same time. Various genetically engineered salmonella (VNP20009, A1-R and ΔppGpp) have been proven effective against cancer. Salmonella-mediated bacterial therapy has great potential due to their diversified mechanism of actions including the direct killing of cancer cells via inducing apoptosis and autophagy pathway; specifically targeting and accumulating in tumor; stimulating the immune system in the body and around the tumor; being used as a vector to deliver various anti-cancer agents; inhibiting angiogenesis; reducing drug resistance by reducing the abundance of p-glycoprotein; combining with and improving the efficacy of chemotherapy and radiotherapy. Two potential stabilization methods freeze-drying and foam drying of salmonella are described.

Key words: Salmonella, bacterial therapy, cancer, mechanism of actions, stability.

Introduction:

Cancer causes numerous deaths annually, various approaches are developed for the treatment of this disease, such as chemotherapy, radiotherapy. These measures have been proven to be effective and capable of improving life expectancy. However, severe side effects usually occur alongside the treatments which influence the quality of life of the patients to a great extent (Armstrong *et al.*, 2016). Chemotherapy is often characterized by low tumor specificity and high toxicity (Schirrmacher, 2019), common side effects include nausea, vomiting and neurotoxicity (Nurgali, Jagoe and Abalo, 2018). Radiotherapy is characterized by dermatitis (Singh *et al.*, 2016) and neurotoxicity (Seward, 2019). Besides side effects, traditional treatments have limitations such as unable to penetrate deep tumor tissue, tumor resistance can be easily developed (Duong *et al.*, 2019), unable to treat metastatic cancer effectively (Forbes *et al.*, 2018).

Novel treatments are being developed to treat cancer, meanwhile avoiding and countering the traditional side effects, one of which is bacterial therapy. Bacterial therapy is defined as the use of natural microorganisms with low toxicity and certain anti-cancer efficacy to treat cancer (Forbes *et al.*, 2018). Bacterial therapy exhibits high specificity to tumour (Forbes *et al.*, 2018), this indicates that there will be less overall toxicity to healthy host cells. The mechanism of action of bacterial therapy can be categorized into 4 different ways. 1) According to Ganai *et al.* (2011), bacteria can be modified so that they can access tumors that are normally inaccessible to small molecule medicine and standard biologics. For example, *Clostridium* can enter the hypoxic tumor microenvironment (TME) due to its anaerobicity (Duong *et al.*, 2019) while normal

medicines can hardly penetrate. 2) Microbial therapy can release cytotoxic compounds within tumor beds which results in the death of cancerous cells (Forbes *et al.*, 2018). 3) Another function is that bacterial therapy can ignite the immune system which is normally suppressed by cancer cells so that the host immune system can participate more in the treatment of cancer (Kocijancic *et al.*, 2017). 4) Bacterial therapy is used to reduce the side effects of other cancer therapy.

*Salmonella typhimurium* is an intracellular facultative bacterium that plays 2 sided roles. On one hand, salmonella is the cause of many food-borne diseases (Herrero-Fresno and Olsen, 2018), on the other hand, the tumor specificity and many other intrinsic anti-cancer effects make it a good candidate for cancer treatment (Jazeela *et al.*, 2020). These anti-cancer properties include but not limited to: inducing autophagy and apoptosis (Uchugonova *et al.*, 2015; Galluzzi *et al.*, 2018); reducing nutrients supply to the tumor via inhibiting angiogenesis (Guo *et al.*, 2020); stimulating the immune system (Kupz *et al.*, 2014); reduce resistance against chemotherapy by downregulating p-glycoprotein (p-gp) (Yang *et al.*, 2018); being used as a vector to deliver therapeutic agents (Zhou *et al.*, 2018); combining with chemotherapy or radiotherapy (Platt *et al.*, 2000; Zhao *et al.*, 2016). Numerous mutated strains of salmonella are capable of carrying out these functions, e.g. VNP20009, A1-R and ΔppGpp.

Preserving bacteria can be done by removing its mobility. Mobility on a molecular level is essential for metabolism to take place, meaning if mobility is removed then there will be less metabolism and the bacteria can be stored for a longer period. Multiple methods can be used such as freeze-drying and foam drying. This review will focus on the relationship between bacteria and cancer, the use of salmonella in cancer treatment and potential mechanisms. In addition, methods to stabilize salmonella to improve the shelf life.

# 1. The relationship between bacteria and cancer, foe or friend?

Bacteria play an important role in the human body. There are 30 trillion human cells whereas there are 38 trillion bacteria cells indicates the essential role of bacteria in maintaining the homeostasis of the human body (Sender, Fuchs and Milo, 2016). It is not surprising that any disbalance or change in the human microbiome (dysbiosis) could lead to severe effects. Microbes are the cause of 15% to 20% of cancer cases (Forbes *et al.*, 2018). Preclinical studies demonstrate that modulation of inflammation, DNA damage, and metabolite production are potential mechanisms of oncogenesis or tumor suppression, which may be altered with changing the microbial composition (Bhatt, Redinbo and Bultman, 2017).

### 1.1Bacterial infection and oncogenesis

Bacteria can manipulate the host cell during the infection process and such manipulation can damage the integrity of the host cell and increase the risk of oncogenesis (Elsland and Neefjes, 2018). Although some of the specific pathogenic pathways are not clear, numerous bacteria have been correlated with different types of cancer and these mechanisms have been studied extensively. CagL is a protein produced by H.pylori which is essential for the adhesion of H.pylori to the gastric epithelial cells, meanwhile, this protein can also promote the production of gastrin which leads to hypergastrinemia and increase the chance of developing gastric adenocarcinoma (Elsland and Neefjes, 2018). *Fusobacterium nucleatum* produced FadA protein can bind with and inhibits E-cadherin whose function is tumor suppression, result in an increasing risk of colorectal cancer (Rajagopala et al., 2017). F. nucleatum can also secrete Fap2 protein which inhibits the natural killer cell mediated immunosurveillance of cancer cells (Gur *et al.*, 2015). Another example is *Salmonella Typhi* and gallbladder cancer. Typhoid is a toxin produced by *Salmonella Typhi*, this toxin induces DNA damage and changes the cell cycles upon infection to induce cancer (Di Domenico *et al.*, 2017). At the same time, *Salmonella Typhi* can form biofilms which is extremely hard to eradicate by antibiotics, the prolonged infection results in chronic inflammation and increases the chance of developing gallbladder cancer. These findings suggest that not only various microbes can initiate and participate in tumor formation, but also each microbe has more than one pathway that can lead to oncogenesis.

### 1.2 The two-sided roles of gut microbiota in cancer

The gut microbiota is associated with cancer development The gut microbiota is composed of around 1000 different species (Nishida *et al.*, 2018). The microbiota in a healthy individual can play various roles, such as protection against pathogens, regulate immunity and metabolism (Nishida *et al.*, 2018). However, the balance between different species of bacteria is relatively fragile and the abundance of bacteria can be changed based on factors such as nutrition, medications and diseases. These factors can lead to dysbiosis which is characterized by an unstable microbiome. Dysbiosis could potentially lead to the growth of opportunistic pathogens which release factors that may lead to DNA damage directly such as reactive oxygen species, these pathogens can also promote an inflammatory environment (Irrazábal *et al.*, 2014) which a risk factor for cancer Such pathogens include but are not limited to *H.pylori, B. Fragilis, E.Coli* and *Fusobacterium nucleatum.* The integrity of the epithelium, which protects the gut from foreign pathogens, can be compromised by dysbiosis, this will inevitably result in more immune cell recruitment and inflammation (Roy and Trinchieri, 2017).

However, the gut microbiota also plays an important role in defense against cancer. Butyrate, which is a metabolite of commensal bacteria, has an anti-inflammatory effect via inducing the production of anti-inflammatory molecules in dendritic cells and macrophages, this will drive the differentiation of the regulatory T cells (T-reg) (Singh *et al.*, 2014). Butyrate can also inhibit the cancer cell histone deaceylase to decrease the risk of oncogenesis. T-reg can limit the proliferation of CD4+ T cells which will limit the

inflammatory responses (Song and Chan, 2019). Other functions of butyrate include increasing the differential of IL-10 producing T cells, active PPARγ which antagonize the NF-κβ signaling pathway, both of which are anti-inflammatory (Knudsen *et al.*, 2018). Another example is *Lactobacillus rhamnosus GG*. This commensal bacterium induces apoptosis in tumor cells (Orlando, Linsalata and Russo, 2016), reduces the production of IL-8 which is pro-inflammatory (Llewellyn and Foey, 2017) and reduces the chance of metastasis (Escamilla, Lane and Maitin, 2012). Moreover, *Salmonella enterica* has monophosphoryl lipid A as LPS, this component can ignite T-cell mediated cancer response. All of these examples demonstrate that it is essential that the microbiome is intact not only because commensal bacteria can prevent tumor formation but also dysbiosis can further induce oncogenesis.

# 2. Genetic modification on Salmonella and corresponding differences.

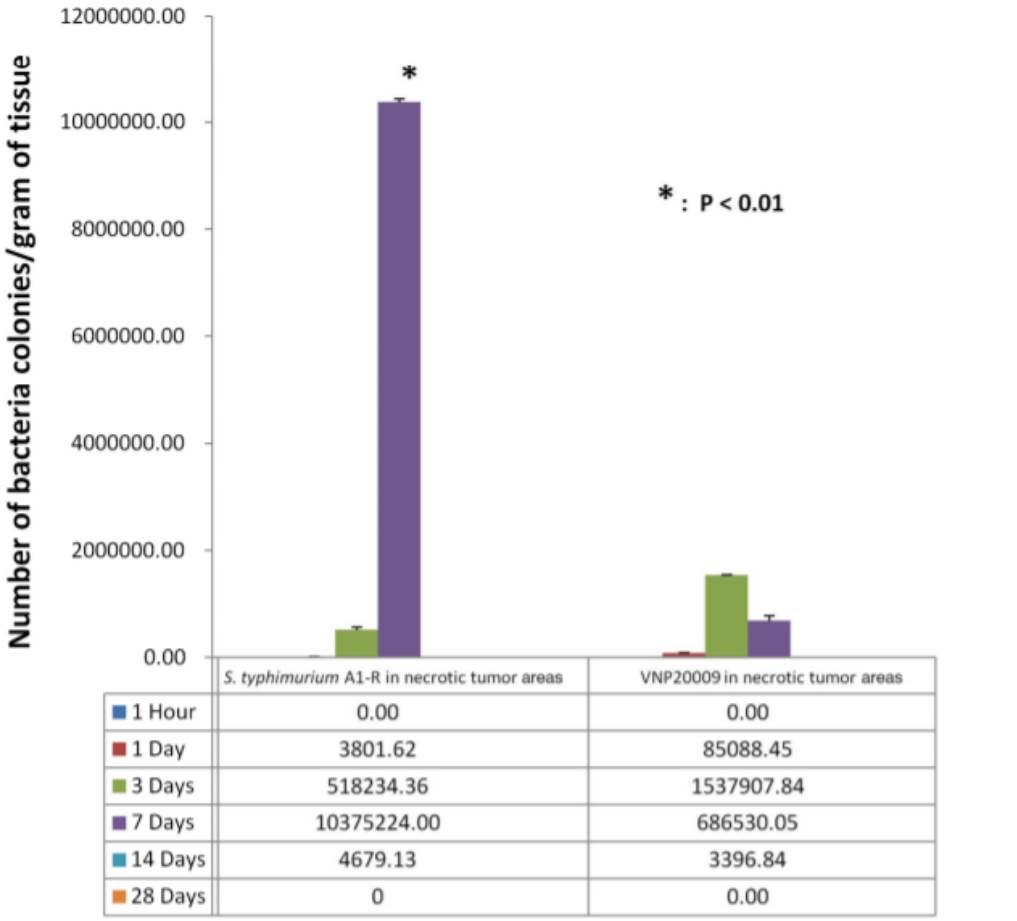
## 2.1 VNP20009

To improve the safety profile and the efficacy of salmonella cancer treatment, multiple genetic modifications were done on salmonella. VNP20009 is a genetically engineered strain of *Salmonella Typhimurium* developed by Yale University and the only salmonella strain that has been used in phase I clinical trials. VNP20009 is less virulent than normal *Salmonella Typhimurium* due to the deletion of *msbB* and *purI* gene (Pangilinan and Lee, 2019). Lipid A is a critical component of lipopolysaccharide (LPS) (Low *et al.*, 1999). Deletion of *msbb* inhibits the addition of a terminal myristyl group to lipid A and lowers the risk of LPS-related septic shock (Liang *et al.*, 2019). Deletion of *purI* reduces the synthesis of adenine and ensure the salmonella only grows in an adenine-rich environment with high cell turnover, death, and cellular debris (malignant area)(Pawelek, Low and Bermudes, 1997). This attenuation significantly improves the safety of salmonella treatment as the $LD_{50}$ compared with the wild type strain has increased around 10000 times (Liang *et al.*, 2019). Despite the attenuation of Salmonella Thyphurium, VNP20009 still shows strong tumor targeting ability, specific accumulation in tumor cells (ratio compared with healthy cell colonization is approximately 1000:1)(Wang, Kazmierczak and Eisenstark, 2016). However, although VNP20009 possesses strong antitumor activity in mouse models (Zhang *et al.*, 2017), Toso *et al.* (2002) demonstrated that VNP20009 does not induce any tumor regression in melanoma patients who received VNP20009 intravenously. This finding is further proved by Heimann and Rosenberg, (2003) where 4 patients with melanoma received VNP20009 intravenously and 3 of them had development of cancer after 1-2 months. Potential reasons could be bacterial production of penta-acylated lipid A, which is an antagonist for TLR4 and caspase-11(Liang *et al.*, 2019); decreased level of *msbB* due to attenuation result in a decreased level of TNF-a production, and TNF-a is important for the influx of blood into tumors. Therefore, tumor

colonization is slowed down and the effect of salmonella is limited (Leschner *et al.*, 2009).

### 2.2 A1-R

The leucine-arginine auxotrophic *Salmonella typhimurium* strain A1-R has been proven effective against cancer. AI-R is made through a process named nitroguanidine mutagenesis where the bacteria are first randomly mutated after nitroguanidine treatment, then placed into mice model to test toxicity and virulence, the final bacteria is isolated from the model and becomes A1-R (Zhao *et al.*, 2005). The use of A1-R in various patient-derived orthotopic xenograft (PDOX) model shows A1-R has great potential against various tumors, e.g. gastrointestinal stroma tumor (Miyake *et al.*, 2018); osteosarcoma (Igarashi *et al.*, 2017); pleomorphic liposarcoma (Kiyuna *et al.*, 2018); melanoma (Yamamoto *et al.*, 2016) and follicular dendritic cell sarcoma(Kiyuna *et al.*, 2016). Like VNP20009, A1-R also shows great tumor specificity and accumulates in tumor cell over a heathy cell in a ratio around 1000:1 (Mi *et al.*, 2019). Zhang *et al.* (2017) compared the use of VNP20009 and A1-R in mouse models in various perspectives. The results suggest that A1-R accumulates better in tumor (Fig.1); have a stronger inhibitory effect on the tumor in terms of tumor size and weight (Fig.2); better clearance in health tissue/organ. The reason for these significant advantages could be A1-R is the only auxotrophic for leu-arg so there is no over-attenuation like VNP20009 (Miyake *et al.*, 2018). The only flaw of AR-1 is that AR-1 leads to a weight loss of the mice after 4 days of administration of $5*10^5$CFU/100ul PBS; however, the body weight comes back to normal after day 11. These findings suggest that if the weight loss of A1-R can be tackled, A1-R seems like a fully upgraded version of VNP20009.




| | *S. typhimurium* A1-R in necrotic tumor areas | VNP20009 in necrotic tumor areas |
|---|---|---|
| 1 Hour | 0.00 | 0.00 |
| 1 Day | 3801.62 | 85088.45 |
| 3 Days | 518234.36 | 1537907.84 |
| 7 Days | 10375224.00 | 686530.05 |
| 14 Days | 4679.13 | 3396.84 |
| 28 Days | 0 | 0.00 |

Fig.1 (Zhang *et al.*, 2017)Differences in growth patterns between A1-R and VNP20009. VNP20009 shows a strong proliferation pattern in the necrotic area before day 3. This is reversed from day 3 onwards. By the time of day 7, the amount of A1-R is around 15 times higher than VNP20009. The clearance of salmonella starts after day 7, by the end of day 28, both A1-R and VNP20009 are cleared from the model.

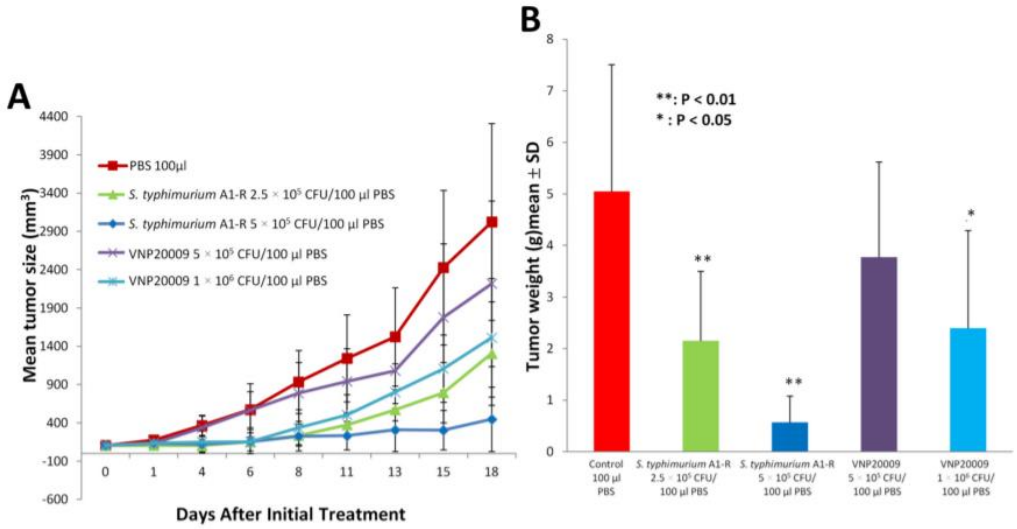



Fig.2 (Zhang *et al.*, 2017)Tumor size (A) and tumor weight (B) after A1-R and VNP20009

treatment. A) After administering $5*10^5$ CFU/100ul of PBS of A1-R, the mean tumor size did not show any significant increase at day 18 compared with day 0. Other doses of A1-R and VNP20009 were not able to achieve the same/better results. B) $5*10^5$ CFU/100ul of PBS of A1-R results in the lowest tumor weight which is the best results compare with other doses of A1-R and VNP20009.

### 2.3.ΔppGpp

Another mutated strain of *Salmonella Typhimurium* that shows great potential is ΔppGpp. ΔppGpp is a genetically engineered salmonella with deleted *relA/spot* gene, thus unable to synthesis ppGpp, a signaling molecule that is required to induce multiple virulence genes (Na *et al.*, 2006), such modification improves the $LD_{50}$ to be $10^5$-$10^6$ times higher than the wild type strain and improves the safety profile to a great extent (Nguyen and Min, 2017). Jeong *et al.* (2008) pointed out that ppGpp is essential for salmonella to invade into the host cell, without ppGpp, salmonella will function as an extracellular bacterium. However, the tumor-targeting effects and anti-tumor effects are not influenced by such attenuation. Multiple reports suggested that ΔppGpp is capable of suppressing tumor growth by various pathways, e.g. activating the inflammasome pathway(Yun *et al.*, 2012); stimulating macrophages and dendritic cells to secrete IL-1B and TNF-a (Kim *et al.*, 2015).

The mentioned 3 mutated strains of salmonella have been studied intensively, but other mutated salmonella strains also show great potential, these include but are not limited to SF200 (Δrfa, LPS modification)(Frahm *et al.*, 2015), SF200 (ΔaroA, amino acids modification) (Felgner *et al.*, 2016), SB824 (sptp+, introduce protein) (Roider *et al.*, 2011).

## 3. Potential mechanisms of Salmonella

Salmonella treatment shows promising outcomes. Salmonella is a facultative bacterium which means they can live in both aerobic and anaerobic environments. This characteristic indicates salmonella can be used to treat both hypoxic and non-hypoxic tumors (Mi *et al.*, 2019). Bacterial therapy is well-suited for hypoxic tumor treatment as hypoxic tumors are normally no accessible by chemotherapy due to its complicated vasculature (Chen *et al.*, 2012). The overview of the mechanism of salmonella can be seen in Fig.3

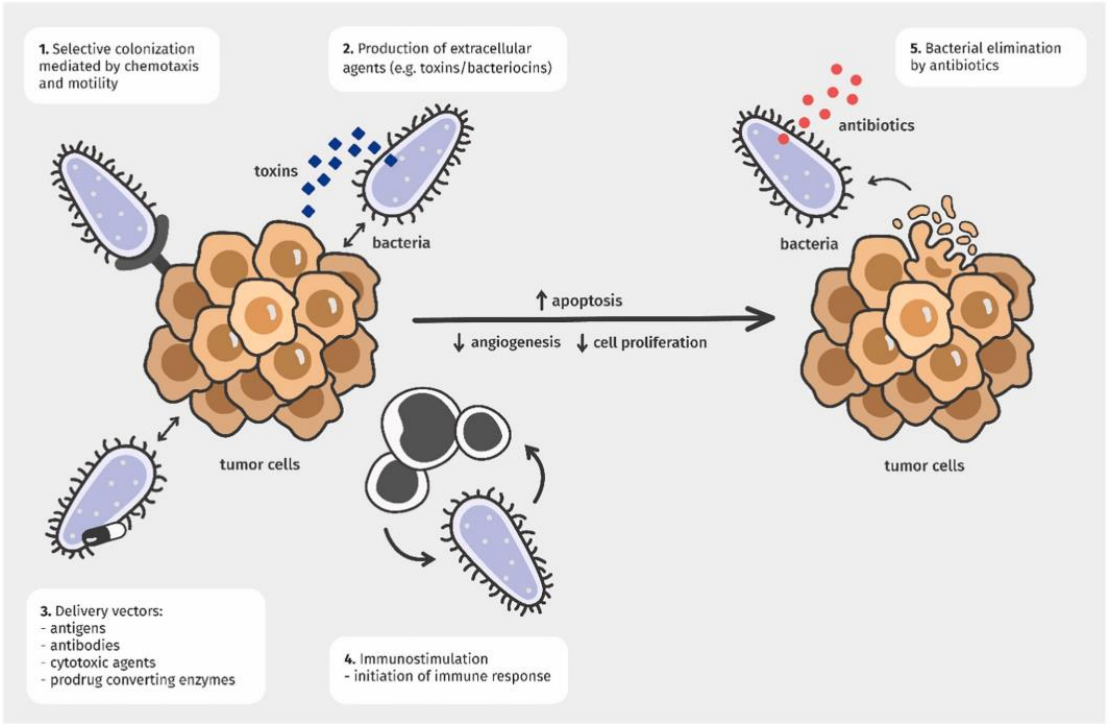



Fig.3 (Dróżdż *et al.*, 2020). Overview of *Salmonella Typhimurium*'s mechanism of actions. 1. Salmonella target and penetrate tumor cells based on the chemotaxis around the tumor and the motility of salmonella. 2. Salmonella produce certain toxins which will directly/indirectly lead to cell death. 3. Salmonella being genetically engineered to deliver therapeutic agents: antibodies, cytotoxic agents, prodrug converting enzymes or deliver tumor antigens to the immune system. 4. Salmonella can be used to ignite the immune system against cancer. By facilitating these mechanisms, salmonella can inhibit angiogenesis, induce apoptosis. The cancer cell growth will be inhibited. After salmonella carried out their functions, they will be cleared by antibiotics, autolysis and the immune system.

### 3.1 Direct killing cancer cells

*Salmonella typhimurium* can lead to direct cancer cell death. A study carried out by Uchugonova (2015) showed that during the infection of *Salmonella Typhimurium* to prostate cancer cells, these bacteria first attached to the cancer cells to a great extent, then invaded inside of the cells and started proliferating, this is followed by massive cancer cell death immediately after 2 hours. They also noticed that the cell membranes of cancer cells were disrupted and apoptotic bodies were found after *Salmonella Typhimurium* was introduced to the tumor model. Moreover, apoptosis and necrosis seem to be a more efficient cell death mechanisms than cell membrane bursting. (Uchugonova *et al.*, 2015).

One of the potential apoptosis mechanisms is the NLRP3 inflammasome pathway. The inflammasome pathway is characterized by IL-1B and IL-18 related apoptosis (Grebe, Hoss and Latz, 2018). Two factors are required to activate the inflammasome. The first

one is the expression of pro-IL-1B, pro-IL18 and inflammasome based on the LPS from *Salmonella Typhimurium* (Franchi, Muñoz-Planillo and Núñez, 2012), and the second is that ATP released from damaged cancer cells caused by salmonella (Phan *et al.*, 2015). These ATP leads to the P2X7 related pore formation on the membrane which allows extracellular ATP to be imported into the cell and activate inflammasome(Phan *et al.*, 2015). Another potential way to activate inflammasome is the concentrated flagellin from bacteria-related IPAF inflammasome activation (Franchi *et al.*, 2006). Once the inflammasome is activated, the inflammasome will activate caspase-1, whose function is to convert proinflammatory cytokines (pro-IL-1B and pro-IL-18) to their active form, IL-1B and IL-18(He, Hara and Núñez, 2016). Both of which result in cell death.

Autophagy is another pathway for the direct killing of cancer cells followed by Salmonella infection. Autophagy is a pro-survival response that degrades and/or recycles potentially harmful/useless intracellular substances such as damaged organelles, misfolded proteins and intracellular bacteria to prolong cell survival (Linder and Kögel, 2019). However, over-activation of autophagy potentially leads to cell death. After salmonella infection, the infected tumor cells activate the autophagy pathway extensively to clear the bacterial infection, but the over-activation eventually results in over digestion of essential molecules for cell survival which ends up with cell death (Galluzzi *et al.*, 2018). Salmonella possesses a protein complex known as T3SS1, whose function is to make pores on the cancer cell membrane, can help the salmonella inject its bacterial effectors and it is essential for bacterial invasion (Finlay and van der Heijden, 2012). The effector protein can initiate the actin rearrangement in the membrane and facilitate Salmonella endocytosis (Engelenburg and Palmer, 2010). The invaded Salmonella resident in the phagosomes of the host cell and change these phagosomes into vacuoles where they can replicate (Stévenin *et al.*, 2019). The T3SS1 now pierce the vacuoles so that the Salmonella can be released into the cytoplasm from the vacuoles and proliferate at an even higher rate. These pathogens will now alarm the ubiquitination system and these bacteria will be conjugated with ubiquitin, the ubiquitinated bacteria will bind to the adaptor protein and eventually be placed onto the anchoring protein LC3 so that they can bind to the autophagosome which will later induce autophagy (Fig.4)(Wu *et al.*, 2020). The second way of inducing autophagy is mediated by damaged vacuoles. B-galatoside is a component on the inside of the vacuoles and they are not accessible by galectin-8 if the vacuoles are intact. Due to the damage, galectin-8 can realize the damage that happened to the vacuole, which will induce the recruitment of adaptor proteins so that the damaged vacuole will be placed in the autophagosome to be ready for autophagy (Fig.4) (Thurston *et al.*, 2012). The third way of inducing autophagy is via cell membrane damage. As mentioned, T3SS1 damages the cell membrane, which results in leakage of amino acids from the cell to the extracellular space (Wu *et al.*, 2020). Now the cell is amino acid deprived and this deprived state can downregulate the mTOR/AKT pathway (whose normal function is downregulating autophagy) and start the autophagy process (Lee *et al.*, 2014).

Autophagy is interconnected with apoptosis. Although the molecular mechanism is not

clear, Lee *et al.* (2014) found that autophagy can enhance apoptosis, and blocking apoptosis results in more autophagy-directed cell death. Therefore, the administration of Salmonella can lead to direct cell death in 2 interconnected pathways, apoptosis and autophagy. This further improves the efficacy of Salmonella.

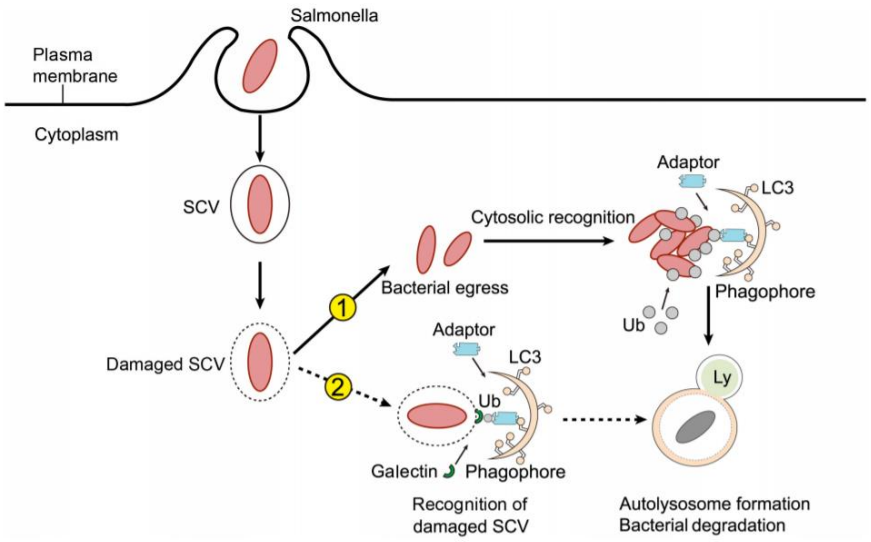



Fig.4 (Wu *et al.*, 2020) 2 potential pathways mediated by salmonella that could lead to autophagy. Salmonella first injects effector proteins to induce host cell endocytosis of salmonella. Then salmonella uses T3SS protein to damage the phagosome/salmonella containing vacuole (SCV) and escape into the cytosol of the host cell. Pathway 1: salmonella gets ubiquitinated and binds with adaptor protein. Then the salmonella-adaptor protein complex binds with docking protein LC3 which are present on the autophagosome. Autophagosome fuses with lysosome and starts autophagy. Pathway 2: Due to the damage on the SCV by T3SS1, galectin can sense B-galatoside which is normally present on the inside of the vacuole. This leads to ubiquitination and adaptor protein recruitment; the adaptor protein complex will bind with LC3 in the autophagosome and start the autophagy pathway.

### 3.2 Targeting tumor and drug delivery as vectors.

Salmonella delivers drugs to the tumor as a vector based on their ability to target cancer cells. Clairmont *et al.* (2000) showed that salmonella preferentially accumulates in tumor cells, the ratio between tumor and healthy liver cell colonization is approximately 1000:1. Therefore, the healthy cells are free from the risk of being infected and cancer cells will be colonized to a great extent. This improves the efficacy of salmonella and limits the toxicity. Tumor colonization can be optimized if salmonella is co-administered with lipid A (Zhang, Swofford and Forbes, 2014). This specificity makes sure that the therapeutic bacteria or the genetically engineered bacteria with medicine will almost selectively grow, proliferate, release medicine or carry out other functions only at the tumor site. The

tumor-targeting property also makes salmonella a perfect agent to treat metastatic cancer. Traditional chemotherapy is not effective against metastatic cancer as a low dose cannot reach therapeutic concentration and a high dose usually comes with toxicity.

### 3.2.1 Tumor targeting and penetration

Chemotaxis might be an important factor for tumor targeting. Ming-Ju Chen, Kreuter, (2006) created a bacterial accumulation model and showed that chemotaxis is one of the key factors that makes sure the model fits into the observed bacterial accumulation pattern. This indicates chemotaxis is one of the reasons why Salmonella is drawn to the tumor. In detail, salmonella is drawn to leucine and arginine which are rich around dying cancer cells (Zhao *et al.*, 2006). This finding is supported by Hoffman (2009), it has been found that auxotrophic salmonella for leucine and arginine infect normal tissue to a very limited extent. Furthermore, aspartate receptor from Salmonella initiate chemotaxis towards tumor cylindroids, serine receptor initiate the penetration and ribose/galactose receptor attracts salmonella towards necrotic tissue ((Kasinskas and Forbes, 2007);(Forbes *et al.*, 2003)). These findings indicate that chemotaxis is critical for successful tumor colonization. However, VNP20009, a mutant strain of salmonella, that has no chemotaxis sensing property, shows no significant difference in colonization compared with VNP20009 with restored chemotaxis sensing ability (Coutermarsh-Ott *et al.*, 2017). This indicates that chemotaxis is not the driving force of salmonella colonization. This principle is further proved by Crull, Bumann and Weiss (2011), they used different strains of salmonella with/without the chemotactic ability to figure out the role of chemotaxis. The results show that regardless of chemotactic ability, similar colonization patterns are seen with different strains. Therefore, salmonella might be targeting the tumor with different mechanisms such as immunosuppressive tumor microenvironment, passive leakage due to immune activation and anaerobic environment.

Motility plays an essential role for salmonella in targeting and penetrating tumors. The tumor microenvironment is characterized by uncontrolled cell growth and angiogenesis. Both of which increase the difficulty for chemotherapeutics to reach the target sites as the distance that the medicine has to diffuse to reach the target site is increased. The use of bacteria overcomes this problem as bacteria can migrate to the target site from the vasculature actively. Toley and Forbes, (2012) compared the level of penetration between E.Coli and Salmonella and found that with higher motility (Salmonella), bacteria can penetrate further into the tumor. Whereas E. Coli can only proliferate nearby the blood vessels. This indicates that when treating solid tumors, bacteria with high motility should be prioritized as solid tumors usually lack of blood vessels. They also found that although both Salmonella and E. coli colonize tumors, the colonization pathway could be entirely different. Salmonella, with higher motility, first penetrates the tumor and then proliferates at a preferential area. E. coli, on the other hand, proliferate first near the blood vessels, and spread towards the tumor once the bacterial density is high enough (Fig.5). This indicates that when it comes to choosing bacteria for tumor penetration, motility should be prioritized over-proliferation rate. Raman *et al.* (2019) pointed out that the high

motility and high invasiveness of Salmonella are based on the protein complex flhDC. The major function of flhDC is to 1) promote flagella expression which plays on an essential role in motility 2) lead to the production of T3SS1 which is important for invasion. Both factors are required for successful treatment as both factors contribute to the accumulation rate. As shown in Fig.6, bacteria with a high accumulation rate tend to resident and colonize the nearby cells whereas bacteria with a low accumulation rate prefer to go back to the bloodstream which limits the efficacy. Should be mentioned that if a bacterium with low motility is chosen for cancer treatment due to its special functions, the immune system should be suppressed in such a way that the bacteria have enough time to proliferate and infiltrate the tumor without being cleared.

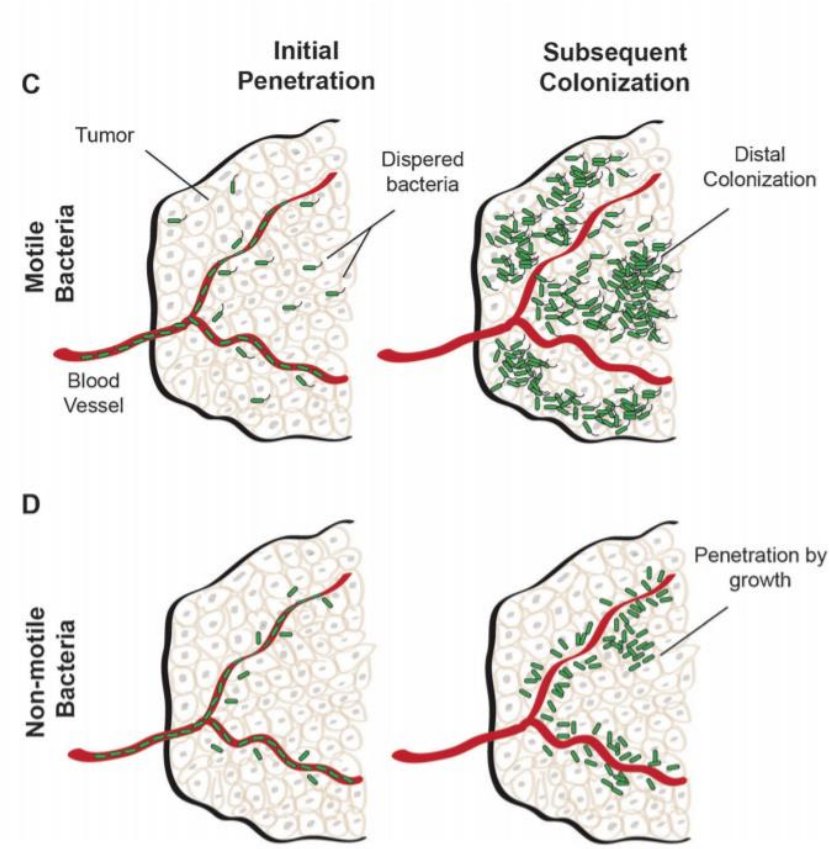



Fig.5 (Toley and Forbes, 2012). Impact of Motility on bacterial penetration. For bacteria with high motility, they leave the blood vessel and find an area that is beneficial for their growth. Then these bacteria start to proliferate and eventually colonize this area. Bacteria with low motility first colonize and proliferate in areas around the blood vessel regardless of whether these areas are suitable for proliferation. After reaching a certain population density, these bacteria start migrating towards a certain area.

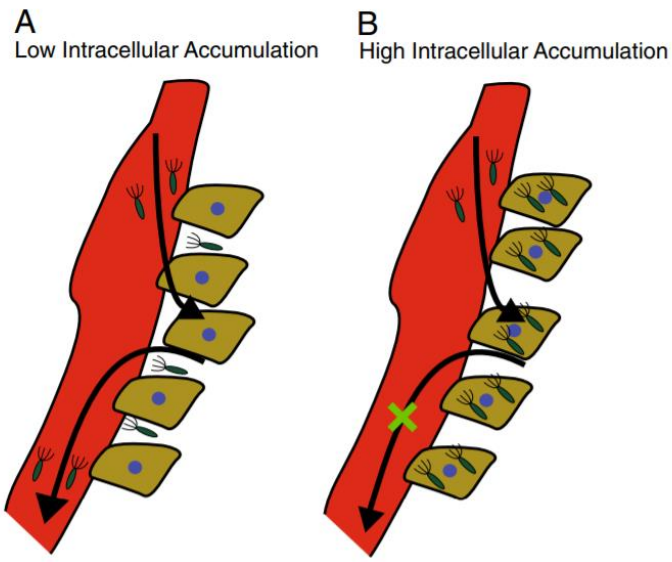



Fig.6 (Raman *et al.*, 2019) Accumulation rate and colonization. Bacteria with a high intracellular accumulation rate leave the blood vessel easily once they found the area that is preferable for growth. It is harder for bacteria with a low intracellular accumulation rate to leave the blood vessel as most of these bacteria are rejected back into the blood.

### 3.2.2 Salmonella as vector

The tumor-targeting effect and the ability to accommodate foreign DNA makes Salmonella a perfect vector to deliver therapeutic agent against cancer (Forbes *et al.*, 2018). Numerous types of therapeutic agents can be integrated into Salmonella, such as cytotoxic compounds, prodrug activating enzymes and immune-stimulating agents (Zhou *et al.*, 2018).

Salmonella Typhimurium can be genetically engineered to produce molecules that lead to apoptosis. Camacho *et al.* (2016) used salmonella as a vector to express and deliver Cp53, a peptide that induces cell death if p53 is present in the cytosol. P53 is a tumor suppressor protein that can easily undergo mutation which results in oncogenesis (Kanapathipillai, 2018). The tumor targeting ability of salmonella makes sure that the healthy cells will not be affected even if they possess p53. The treatment is separated into 2 stages, production of Cp53 and autolysis of bacteria. This is because to induce enough apoptosis, the bacteria must be given enough time to express the peptide to reach therapeutic concentration. Once a certain concentration is reached, an inducer will be given to the patient to induce the autolysis of bacteria so that the medicine can be released from the bacteria to the cancer cell. The autolysis of bacteria also improves the safety of using bacteria because autolysis kills the bacteria and lowers the chance of developing septic shock. Furthermore, tumor necrosis factor alpha (TNF-a) is another factor that has been engineered into salmonella to induce apoptosis. TNF-a is a cytokine that can induce cell death via caspase-8 activation and consequential reactive oxygen species production (Kim *et al.*, 2010). Yoon *et al.* (2011) engineered salmonella to produce TNF-a in a murine melanoma model and tumor regression was around 80-100%. They also found that natural killer (NK) cell seems like an essential factor for TNF-a carrying

Salmonella to work due to NK cell knockout model shows reduced efficacy. Moreover, the gene encoding for Tumor Necrosis factor-related Apoptosis-Inducing Ligand (Trail) has been engineered into salmonella with nirB as a Trail expression promoter (Chen *et al.*, 2012). Trail induces apoptosis via binding to death receptor 4 and 5 (DR4 and DR5) and consequential caspase-8, caspase-3, caspase-6, caspase-7 activation (Yuan *et al.*, 2018). The results from Chen and co-workers shows that when comparing the efficacy between Trail protein carrying Salmonella and Trail gene carrying Salmonella, the latter shows higher efficacy. The reason is most likely that the amount of Trail protein that can be loaded into Salmonella is limited. The efficacy of Trail gene carrying Salmonella was further tested by Cao *et al.* (2010) and the results are promising.

Salmonella can also be used to carry prodrugs converting enzymes. Carboxypeptidase G2 (CPG2) shows prodrug activating ability to many nitrogen mustard L glutamates (Friedlos *et al.*, 2008). Nitrogen Mustard drugs work via binding N7 nitrogen (nitrogen on the seventh position) in guanine and cross-link DNA strands to prevent duplication (Singh *et al.*, 2018). Friedlos *et al.*, (2008) found that Salmonella alone leads to tumor regression and carboxypeptidase G2 gene carrying Salmonella co-administered with nitrogen mustard glutamates shows further antitumor effect. Interestingly, salmonella itself can produce prodrugs converting enzymes as well. E.g. 6-methylpurine 2 deoxyriboside (6MePdR) is a prodrug of antitumor agent 6 methylpurine (6MeP) (Zhang, Kale and Chen, 2015). The activation of 6Mep is dependent on an enzyme named Escherichia coli purine nucleoside phosphorylase (ePNP), although ePNP is an E.coli enzyme, E. coli and Salmonella have 96% homology meaning this enzyme is also present in Salmonella (Chen *et al.*, 2013). The experiments of Chen et al showed that combining 6MePdR with Salmonella has a great antitumor effect.

Immunomodulators or corresponding genes are incorporated into Salmonella to treat cancer. Although tumors possess aberrant antigen expression, this is often ignored by the immune system due to the immune-suppressive environment surrounding the tumor (Gautam *et al.*, 2020). Therefore, presenting the tumor antigen directly to the antigen presenting cells (APC) is critical for cancer treatment. Salmonella can infect macrophages which function as APC. Stegantseva *et al.* (2020) used Salmonella as a vector for oral DNA vaccine loaded with 3 different neuroblastoma antigens (tyrosine hydroxylase, Survivin, PHOX2B). The results show that different antigen has different immunogenicity, and even though the cytotoxicity was high, the tumor regression was not ideal. Potential reasons could be: antigen on the surface of salmonella has better immunogenicity than cytoplasmic antigen(Barat *et al.*, 2012), or immunosuppressive environment might have a stronger impact than this oral DNA vaccine. To present the antigen on the surface of Salmonella, an autotransporter can be used. Mei *et al.* (2017) engineered Salmonella to carry melanoma antigen genes Melan-1 and 2 melanoma epitopes (TRP1 and TRP-2) which activate CD8+ and CD4+ T cells, respectively. The use of autotransporter AIDA shows great CD4+, CD8+ T cell activation and TNF-a production as antigens on the surface of salmonella can be easily presented. Angiogenesis is important for tumor growth as blood provides nutrients to the tumor (Viallard and Larrivée, 2017). Vascular

endothelial growth factor receptor 2 (VEGFR2) is essential for angiogenesis to take place (Behdani *et al.*, 2012). Jellbauer *et al.* (2012) engineered one epitope from VEGFR2 into Salmonella and used T3SS system to direct the antigen into the cytosol of APCs. The results from their study show that activation of CD8+ T-cell destroy cells possess VEFGR2 and result in approximately 62% reduction in angiogenesis and 50% reduction in tumor growth. Although destroying all cells with VEFGR2 might lead to side effects, there was no collateral damage reported in their study. A possible reason could be that normal endothelial cells express less VEFGR2 compared to cells in tumor vasculature (Smith *et al.*, 2010) so that normal cells are less likely to be effected.

### 3.3 Stimulate immune system

Salmonella can boost the immune response against the tumor. Kupz *et al.*, (2014) showed that salmonella's flagellin can activate NK cells to produce INF-gamma, a critical cytokine that is required to activate the toll-like receptor (TLR) signaling pathways. TLR4 signaling pathway is essential for the infiltration of macrophages and neutrophils to the tumor site. TLR4 also works via stimulating myeloid differentiation primary response 88 (MyD88) and results in the release of pro-inflammatory cytokines released by nuclear factor kB pathway (Irfan, Delgado and Frias-Lopez, 2020). TLR5 signaling pathway can further enhance the effect of immune cell recruitment (Zheng *et al.*, 2017). TLR5 signaling pathway also promotes the M2 to M1 macrophages conversion. Although M1 macrophage induced inflammation is beneficial for tumor growth in the beginning, inflammation in the latter stage of tumor is toxic due to the release of ROS, NO and cytotoxic agents. M2 macrophages are characterized by their anti-inflammatory, angiogenesis-stimulating properties, both of which are beneficial for tumor growth (Najafi *et al.*, 2019). Therefore, M2 to M1 conversion induced by salmonella is beneficial. Mi *et al.*, (2019) suggest that salmonella enhance the innate immune system via inducing the maturation of intratumoral myeloid cells to make them less suppressive so the anti-cancer effect is improved. Kim *et al.* (2015) pointed out that the tumor suppression effect of salmonella is based on the production of TNF-a and IL-1B. They found that macrophages and dendritic cells are the major cell types that produce TNF-a and IL-1B.

The T-cell response is strengthened by the use of salmonella. LPS and flagella from salmonella can stimulate T-cell to kill cancer cells (W. Yoon *et al.*, 2014). The dead cancer cell releases its cellular components and the debris is picked up by antigen presenting cells such as macrophages and dendritic cells. The antigen presenting cells present the tumor antigen to activate more CD4+ and CD8+ T-cells which forms a positive feedback loop (Avogadri *et al.*, 2005). Connexin 43 (Cx43) is believed to be the key factor that supports antigen presentation to T-cells. Upon salmonella infection, Cx43 is upregulated and more gap junctions are formed on the cell membrane. These gap junctions allow the tumor antigen peptides to be transferred to nearby dendritic cells and further presented to the CD8+ T-cells (Shilling *et al.*, 2007). The activated cytotoxic T-cell can then mediate its anti-cancer effect. TNF-a induced by salmonella can create openings in the blood vessels which further improves T-cell infiltration. (Leschner *et al.*, 2009). It has been

reported that salmonella infection can downregulate the amount of immunosuppressive molecule IDO, this results in the diminished impact of T-reg (W. K. Wang *et al.*, 2015). The downregulation of IDO is directed by the inhibition of the mTOR and AKT signaling pathway (Kuan and Lee, 2016). M. Christopher (2016) suggests that another critical factor for T-reg downregulation is the LPS from salmonella. The mechanism for this downregulation is based on LPS leads to the reduction of CD44, and CD44 is required for the activation of $CD25^{+}CD4^{+}$Treg; therefore, immunosuppressive Treg is reduced and the anti-cancer effect is improved.

### 3.4 Salmonella has intrinsic ability to inhibit angiogenesis.

Not only salmonella can be engineered to inhibit angiogenesis, but this bacterium itself also possesses an anti-angiogenesis effect. Salmonella carries out this function by downregulating pro-angiogenesis factors and upregulating inhibition of pro-angiogenesis factors (Guo *et al.*, 2020). Cheng *et al.* (2014) suggest that activation of mTOR/AKT signaling pathway upregulates Hypoxic-induced factor-1α (HIF-1a). HIF-1a is a transcription factor whose activation leads to VEGF production (Aldo and Elisabetta, 2019). Wang *et al.*, (2015) demonstrated that downregulation of Cx43 results in increased expression of HIF-1 and boosted transcriptional activity in cancer cells which are beneficial for cancer development. This suggests that Cx43 has anti-HIF-1a effect. Chang *et al.* (2013) proved that not only live salmonella therapy can induce the expression of Cx43 in both aerobic and anaerobic conditions but dead salmonella with the intact cellular component can do the same which further improves the efficacy of salmonella. In general, salmonella inhibits angiogenesis via downregulating mTOR/AKT pathway, result in downregulation of HIF-1a and this led to reduced VEGF and angiogenesis which is beneficial for cancer regression. Salmonella inhibiting angiogenesis is further proven to be effective by Diego *et al.* (2018) who administered salmonella to osteosarcoma in vivo and showed that the angiogenesis was limited (Fig.7). But one drawback is that although the blood vessel growth is limited after Day 28, the tumor area did not show any significant differences from the control group and the tumor area is not inhibited which indicates inhibiting angiogenesis alone may not have a significant impact on tumor regression in osteosarcoma.

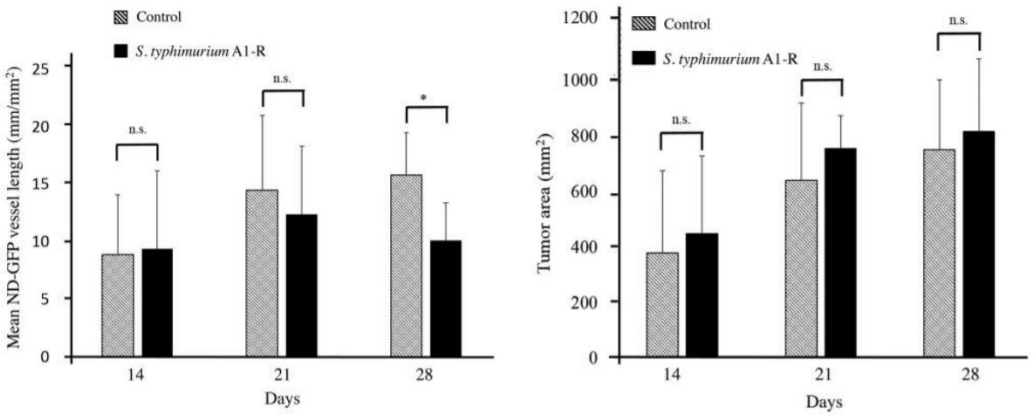



*Fig.7 impact of Salmonella Typhimurium A1-R on angiogenesis (Diego et al., 2018). Left: The inhibitory effect of A1-R on angiogenesis can be seen after day 14, the vessel length is shorter compared with the control group and this difference is more significant after day 28. Right: The effect of A1-R on tumor area: there is no difference compared with the control group and the tumor size increased around 400 $mm^2$ between day 14 and day 28.*

Moreover, the intrinsic ability of salmonella against angiogenesis also indicates salmonella has great potential for the prevention of metastatic cancer as primary tumor requires blood vessels to transport cancer cells to secondary organs. Besides the intrinsic ability to target angiogenesis, salmonella also destroys blood vessels actively which further reduces the chance of metastasis and increases medicine efficacy as more salmonella can infiltrate the tissue following the leak (Tome *et al.*, 2013). A research carried out by Leschner *et al.* (2009) suggested that TNF-a is the key factor for blood vessel destruction. However, Liu *et al* (2010) pointed out that salmonella leads to haemorrhage is not entirely dependent on TNF-a. Nevertheless, the destruction of blood vessels certainly decreases the chance of developing metastatic cancer.

### 3.5 Salmonella's intrinsic ability to suppress p-glycoprotein

Salmonella is capable of suppressing p-glycoprotein (p-gp). P-gp plays an important role in developing multidrug resistance (MDR), which is one of the many reasons why chemotherapy fails to work. P-gp function as an efflux pump that removes medicine from tumor cells. Tumor cells usually have an increased abundance of p-gp on the cell membrane which increases their resistance and prolongs their survival. Salmonella can reduce the number of p-gp via two different pathways, 1) reduce p-gp expression and 2) degrade p-gp on the cell membrane (Fig.8). A recent research carried out by Yang *et al.*, (2018) demonstrates that salmonella has the intrinsic ability to downregulate mTOR/AKT signaling pathway and result in a decrease in the expression of p-gp. Mercado-Lubo *et al.* (2016) suggested that salmonella can lead to the degradation of p-gp on the cell membrane. Their researches pointed out that the effector protein of salmonella, SipA, binds to the SipA transmembrane receptor and result in activation of caspase 3. Caspase 3 can cleave p-gp and cause the p-gp to lose its functionality,

therefore prevent multidrug resistance. To further improve the therapeutic efficacy of SipA, a carrier named AuNPs was conjugated with SipA and the efficacy is around 500 times higher than SipA alone. The intrinsic ability of salmonella against multidrug resistance means salmonella can be administered with medicines that are p-gp substrates under normal circumstances and still achieve strong therapeutic effects. This finding not only suggests that salmonella can improve the efficacy of chemotherapy, but also the dose of chemotherapy can be lowered to reduce toxicity because the medicine accumulates better in tumor cells.

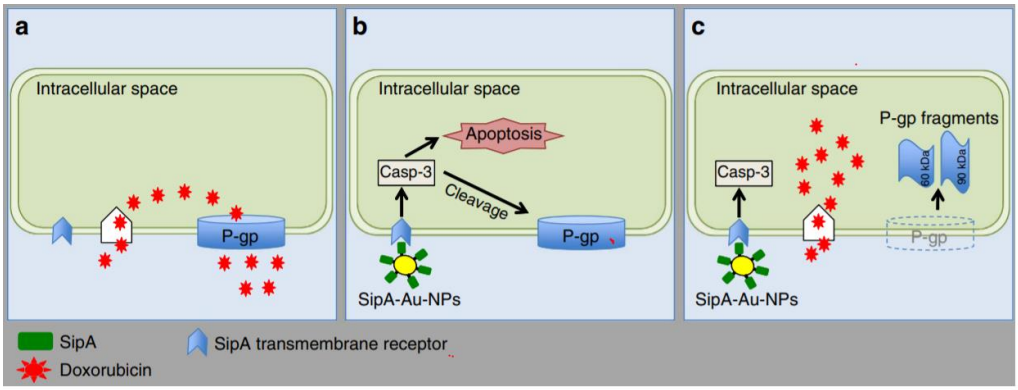



Fig.8 (Mercado-Lubo et al., 2016) Function of p-gp and the removal of p-gp. A) After a therapeutic compound (e.g. doxorubicin) enters the intracellular space of a cancer cell, p-gp facilitates the efflux of doxorubicin. B) SipA is an effector protein of salmonella which is artificially conjugated with AuNPs, this conjugate binds with SipA transmembrane receptor and activate the Caspase-3 signaling cascade and result in apoptosis and p-gp cleavage. The cleaved p-gp becomes 2 fragments with 60kDa and 90kDa. P-gp loses its functionality and doxorubicin can accumulate in cancer and kill cancer cells.

### 3.6 Effector proteins improve the survival of salmonella

To carry out their functions, salmonella has to survive and proliferate first. Effector proteins are essential for the invasion, proliferation and survival of salmonella so that salmonella can have a better therapeutic value. SopE/SopE2/SopB leads to actin assembly, membrane ruffling and bacterial internalization which are essential for infection (Patel and Galán, 2006). SseF promotes salmonella replicating via enhancing the recruitment of dynein (Abrahams, Müller and Hensel, 2006). SipA promotes the replication of salmonella once they reach the cytosol of the host cell (Brawn, Hayward and Koronakis, 2007). SipC prevents the fusion of SCV and lysosome to avoid salmonella being lysed (Myeni, Wang and Zhou, 2013). SifA impairs the function of lysosome so that even if fusion happens, salmonella has a higher chance of survival (Mcgourty *et al.*, 2012). In order to further improve the survival of salmonella, the immune system is inactivated. SteD can inhibit antigen presentation and T-cell activation (Johnson, Mylona and Frankel, 2018); SspH2 improves the colonization of salmonella by downregulating proinflammatory cytokines IL-1β, INF-γ, IL-12, and iNOS (Shappo *et al.*, 2020); AvrA/GogA/GogB/Gtgb are 4 effector proteins that can inhibit the NF-kB signaling pathway to stop inflammation (Liao *et al.*,

2008; Sun *et al.*, 2016; Jennings *et al.*, 2018). These effector protein mediated pathways improve the colonization of salmonella.

### 3.7 Combination therapy of Salmonella with chemotherapy and radiotherapy

In recent years, the center of researches has shifted from either salmonella or traditional therapy alone to combined therapy as not only the efficacy is improved, but also the toxicity is decreased.

Angiogenesis inhibitors alone do not show sufficient anti-tumor effects as tumor stem cells can proliferate with limited presence of oxygen and nutrients. Zhao *et al.* (2016) used *Salmonella typhimurium* VNP20009 carrying sex determining region Y-box 2 (Sox2) shRNA together with angiogenesis inhibitor peptide HM-3 to inhibit the growth of non-small cell lung carcinoma *in vivo*. Sox2 is required for cancer cell migration, proliferation, metastasis and invasion (Novak *et al.*, 2020). Their results (Fig.9) show that combination therapy of VNP20009 and HM-3 generates better anti-tumor effect than each therapy alone (Fig.9). Apoptosis induced by salmonella, Sox2 inhibition by Sox2 shRNA and inhibition of angiogenesis by HM-3 together reduce the tumor size. Besides HM-3, triptolide is another therapeutic agent that can be co-administered with salmonella. Triptolide exert its anti-cancer effect via inducing cell death (apoptosis and autophagy), inhibiting angiogenesis and creating an immune suppressive environment (Noel *et al.*, 2019). Although an immune suppressive environment allows tumor growth, this also prevents salmonella from being cleared by the immune system. Chen *et al.* (2017) administered triptolide together with VNP20009 to treat melanoma *in vivo.* The findings suggest that triptolide and salmonella synergistically inhibit angiogenesis by inhibiting VEGF. However, the dose of triptolide has a controversial impact. A low dose of triptolide promotes CD8+ T-cell infiltration which is beneficial but a high dose of triptolide is immune suppressive which promotes salmonella proliferation. Therefore, whether a low dose or high dose of triptolide should be given should depend on the goal of the treatment. Salmonella with cyclophosphamide can further reduce VEGF and micro vessel density in a tumor significantly. (Jia *et al.*, 2007). A1-R can also inhibit angiogenesis and be combined with gemcitabine and bevacizumab (Fig.10). A research carried out by Hiroshima *et al.* (2014) showed that using A1-R as a following up therapy after anti-VEGF therapy has great potential as the tumor weight decreases.

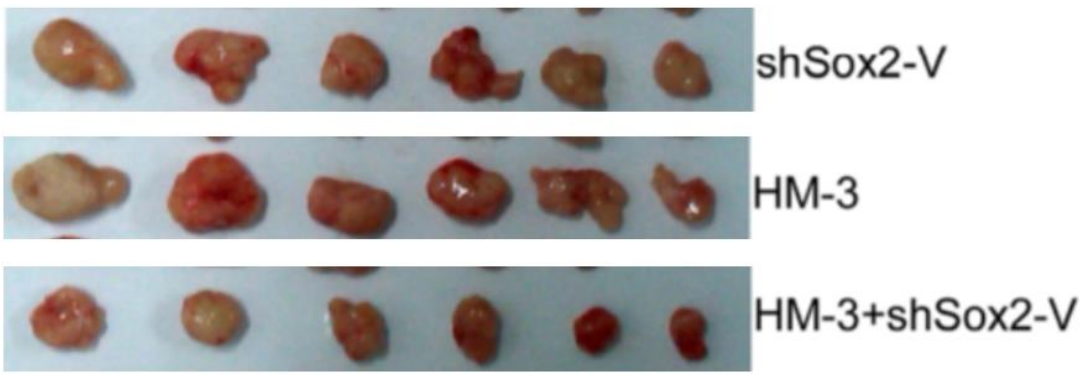



Fig.9 (Novak et al., 2020) Effect of combining chemotherapy with salmonella-based

shSox2 therapy. HM-3+shSox2-V combination therapy inhibits tumor growth better than either alone.

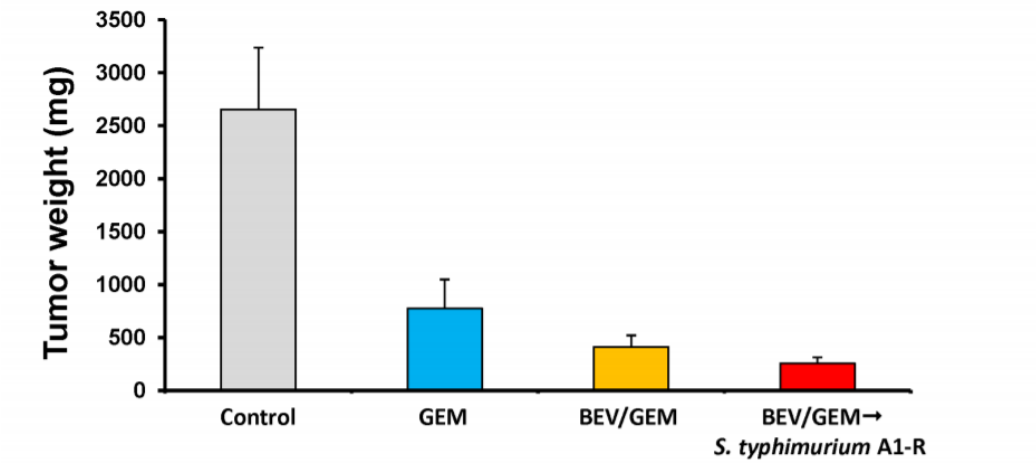



Fig.10 (Hiroshima et al., 2014)Tumor weight after different types of treatment. Gemcitabine alone and gemcitabine+bevacizumab combined therapy reduces the tumor weight. The therapeutic efficacy can be further improved by combining gemcitabine and bevacizumab with A1-R. The tumor weight is around 10 times smaller than the control group.

Similar to chemotherapy, radiotherapy can also be combined with salmonella and shows a better anti-tumor effect than either of the treatments alone. Combination therapy shows better efficacy than the expected efficacy based on numerical additivity (Platt *et al.*, 2000). Although the mechanism of action of this combination is unknown, numerous researches reported that the combined therapy shows a significant tumor suppressive effect. Platt *et al.*, (2000) used salmonella with multiple-dose X-rays to treat melanoma *in vivo.* They found that 20Gy X-ray results in >90% salmonella survival and significant tumor growth retardation. These findings indicate that salmonella is not prone to X-ray radiation at 20Gy. However, different findings reported by Liu *et al.* (2016) suggest that ΔppGpp was significantly influenced by 7Gy X-ray radiation as the cell counts decrease dramatically compared to the control group which might lead to decreased efficacy. The potential reason for the controversial findings is different strains of salmonella were used in these two research and different strains of salmonella might have different sensitivity against X-ray radiation. Despite the differences in X-ray sensitivity found in these 2 pieces of research, they all showed significant tumor suppression when combining radiation therapy. A potential mechanism for the high efficacy of combination therapy could be CD8+ T cell dependent (Avogadri *et al.*, 2008). Radiotherapy increases the expression of MHC class I through induction of NF-kB/IFN-b/MHC I signaling pathway (Wan *et al.*, 2012), the increase in MHC I enhances the tumor antigen presenting to the CD8+ T-cell

by salmonella and result in strengthened combination therapy. Yoon *et al.* (2014) demonstrated that even melanoma, a cancer type that is normally resistant to radiotherapy, showed regression when treated with combined therapy. They proposed that the retardation of tumor growth is due to the increase in production of reactive oxygen species (ROS) and consequential apoptosis.

## 4. Stabilization of salmonella

Freeze-drying is considered a golden standard for bacteria stabilization. To store bacteria for a longer period, the mobility which is essential for metabolism must be minimized so that there is no degradation reaction (Zhang *et al.*, 2020). Water is necessary for many degradation reactions which is the reason why it has to be removed by freeze-drying. Freeze-drying consists of 3 steps, 1) freezing the protected cell culture, 2) drying to remove water in a form of ice (sublimation), 3) drying to remove unfrozen water (desorption) (Fonseca, 2015).

Multiple protective agents are commonly used to improve the survival rate during and after freeze-drying, e.g. sucrose, glucose, trehalose, dextran, lactose, glycerol and skimmed milk (Jałowiecki *et al.*, 2020). These agents can encapsulate and protect the bacteria from freezing, dehydration and oxidative stress, e.g protect bacteria from the ice inside and outside the cell (Fowler and Toner, 2006), replace hydrogen bond between bacteria and water (Milani *et al.*, 2020), shield the bacteria from oxygen and light.

Freezing the bacteria culture is the first step of freeze-drying. The temperature is lowered below its glass transition temperature where ice and glass are formed. The cooling process cannot be performed either too fast or too slow, slow cooling results in water moving to extracellular space and thus osmotic imbalance, whereas fast cooling leads to the formation of intracellular crystals (ice) which is lethal to some bacteria (Seki, Kleinhans and Mazur, 2009). Slow freezing results in crystals with large crystals, vice versa. The size of the crystal determines the speed of the freeze-drying process, larger crystal speeds up the freeze-drying process as a larger crystal creates a network that is beneficial for sublimation. However, slow freezing could result in phase separation between the sugar and the bacteria which means the protective effect of the sugar is gone, also, slow freezing increases the amount of time of bacteria in a concentrated solute which could be lethal to bacteria. Therefore, the freezing process can neither be too fast or too slow, the ideal speed is around 1 $^{\circ}$C/min (Dixit, Kulkarni and Selvam R, 2011).

The drying process consists of primary drying and secondary drying. The sublimation process happens ahead of the desorption process as the sublimation of ice is faster than the glass transition from ice to vapor. Primary drying is carried out at low pressure so that ice can undergo sublimation without including any physical transformation about liquid phase. The temperature should be kept under the glass transition temperature so that there is no sugar crystal formation. Crystal formation means the hydrogen bonding

between the sugar and the bacteria is gone; therefore, the bacteria is no longer stabilized. The crystal itself is sharp thus it might damage the bacteria. The sublimation process should be carried out under 0.006 atm (pressure) and 0.01 °C (temperature). Followed by sublimation, desorption is carried to remove the water from the glass. The temperature can be increased compared to sublimation because the glass transition temperature increases with the decrease in water. However, the overall temperature should still be regulated under the glass transition temperature for the same reason as sublimation.

Despite the use of cryoprotectants, salmonella is still damaged during the process. Rosen *et al.* (2016) used freeze-drying to stabilize salmonella and 32% trehalose to protect salmonella during freeze-drying. However, the results suggest that SOS operon (umuD) expression was significantly elevated. SOS expression is promoted when the cell is under extreme stress, meaning that either during the freeze-drying process or the resuscitation process severe damages were done to the salmonella and these damages could be lethal. SOS gene activation promotes DNA repairing mechanisms, but such processes are usually accompanied by high risk of mutation (Baharoglu and Mazel, 2014). The mutation of genes in salmonella may lead to the loss of virulence and this could be catastrophic to the treatment of cancer as the virulence of salmonella is one of the key factors that salmonella's efficacy is based on.

Foam drying is another potential stabilizing method that has been proven effective. Foam drying whips liquid materials into stable foam and removes the water content at near room temperature (Hardy and Jideani, 2017). Mild temperature can better prevent loss of salmonella during foam drying compared with freeze-drying. Mhatre V. Ho, Ji-Ann Lee (2012) used foam mat drying to stabilize salmonella, the pressure in the freeze dryer was decreased gradually from atmospheric to 100mTorr while the temperature is maintained at 20 °C; the low pressure was maintained for 40 hours and an additional 20 hours at 20 °C for further drying; trehalose-methionine was used for protection. Their results show that: 1) the pressure has to be lowered gradually because a rapid decrease in pressure causes water to freeze which prevent foam forming and increase the loss of viability of salmonella, 2) The storage stability is improved with the usage of plasticizer DMSO compared with glycerol, 3) foam drying salmonella at pH 8 gives better stability than pH6. However, the opposite result has been found by Zeng *et al.* (2009) where they suggest that pH 6-7 provides better stability because, at pH 8, the fluidity of the cell membrane is decreased. But both experiments used NaCl because salt can improve the recovery of salmonella. Zeng *et al* (2009) also pointed out that sucrose is a better stabilizing agent compared with trehalose. Importantly, the immunogenic property of salmonella was not diminished due to foam drying or resuscitation (Mhatre V. Ho, Ji-Ann Lee, 2012). A potential reason for the success is the use of methionine as an additional protective agent so that the bacteria is more stabilized.

## 5. Challenges

The use of salmonella treating multiple types of cancer in animal models has been proven effective, but only to a certain extent. Platt *et al.* (2000) showed that combination therapy of radiotherapy and *Salmonella Typhimurium* leads to tumor retardation but the tumor eventually reoccurred. There are also reports suggesting that combination therapy between the two is effective, but there is no complete regression of tumor cells (Avogadri *et al.*, 2008; Liu *et al.*, 2016). Treating cancer patients who are having chemotherapy with salmonella may not achieve the ideal effect. The reason is that some chemotherapy will suppress the immune system to an extent that the immune system can no longer be stimulated by the LPS from salmonella (Dróżdż *et al.*, 2020). The differences between animals and humans are substantial indicating that whether salmonella-based anti-cancer therapy can function effectively in humans is hard to conclude. For example, the use of mutated salmonella strain VNP20009 in mouse model shows great tumor regression and the very same strain failed to lead to any tumor regression in human clinical trials. Besides the possible limited efficacy of salmonella, the use of bacteria itself comes with certain risks. As mentioned, salmonella can boost the concentration of TNF-a which is beneficial for salmonella migrating towards the tumor, but this mechanism is based on making the blood vessels leakier. The consequence of such an event increases the chance of hemorrhage which could be lethal for cancer patients. Another potential risk is brought up by Yoon *et al.* (2011), where they found that when using salmonella as a vector to carry TNF-a, the efficacy of salmonella is not affected by immune responses and antibiotics. This finding could mean that the immune system and antibiotics fail to respond to salmonella which might increase the chance of having a septic shock in the patient, the outcome could even be worsened if salmonella were given to an immune-compromised patient. Of course, the risk of having septic shock can be lowered by using an inducer to induce the autolysis of bacteria. But it has been reported that when using inducible promoter $P_{BAD}$ for the induction of both tumorlytic gene and lysis gene, the expression of the tumorlytic gene is sub-maximal, which might diminish the antitumoral effect (Jeong *et al.*, 2014). Another way to reduce the risk of having a septic shock is to attenuate salmonella, but the grade of attenuation should be precisely manipulated as over-attenuation in VNP20009 (deleted msbB gene) could diminish the virulence of salmonella and its anti-cancer efficacy. The 1000:1 ratio of accumulation of salmonella in tumor compared to normal tissue indicates that there will still be many healthy cells colonized by salmonella and the risk is increased. After stabilization by freeze-drying, the repair mechanism associated mutation could also increase the virulence of salmonella and causing damage; mutation on the LPS could also compromise the immunogenic ability of salmonella (Abadi, 2016). Besides these potential risks, there is no comprehensive understanding of the mechanism of action of salmonella. The experiments mentioned in this review were using different animal models, different mutated strains of salmonella and different route of administration, treating different types of cancer. To understand better the abilities and limitations of salmonella, more systematic researches need to be done. Moreover, if the tumor is successfully treated by salmonella, antibiotics will be required to remove salmonella from the systematic circulation. But the use of antibiotics could lead to certain risks, 1) dysbiosis, as mentioned

at the beginning of this article, the human microbiota has a protective role against the development of cancer; such use of antibiotics could increase the chance of developing cancer again. 2) abusing antibiotics could create superbugs.

**Conclusion:**

Bacteria play an important role in the human body as a healthy microbiota can maintain homeostasis and a dysregulated microbiota can initiate the progression of many diseases. *Salmonella typhimurium* is a therapeutic agent with great potential. This bacterium can treat multiple types of cancers and improves the life expectancy of the host. The combination use of salmonella with chemo/radiotherapy significantly improves the efficacy and this could be a promising treatment method in the future. However, the mechanism of actions and safety aspects should be further investigated as the current understanding of *Salmonella Typhimurium* is derived from animal models with completely different setups of the experiments. Therefore, whether cancer in humans can be treated as effectively as in animals is inconclusive. Freeze-drying and foam drying are two powerful stabilizing techniques to improve the shelf life of salmonella. Bacterial therapy has great potential waiting to be exploited due to its diversified functions.

References:

Abadi, A. T. B. (2016) 'Comment on "bacteria in Cancer Therapy: Renaissance of an Old Concept"', *International Journal of Microbiology*, 2016. doi: 10.1155/2016/6304150.

Abrahams, G. L., Müller, P. and Hensel, M. (2006) 'Functional dissection of SseF, a type III effector protein involved in positioning the salmonella-containing vacuole', *Traffic*, 7(8), pp. 950–965. doi: 10.1111/j.1600-0854.2006.00454.x.

Aldo, P. and Elisabetta, C. (2019) 'Role of HIF-1 in Cancer Progression : Novel Insights . A Review Role of HIF-1 in Cancer Progression : Novel Insights . A Review', (February). doi: 10.2174/1566524018666181109121849.

Armstrong, T. S. *et al.* (2016) 'The symptom burden of primary brain tumors: Evidence for a core set of tumor-and treatment-related symptoms', *Neuro-Oncology*, 18(2), pp. 252–260. doi: 10.1093/neuonc/nov166.

Avogadri, F. *et al.* (2005) 'Cancer immunotherapy based on killing of Salmonella-infected tumor cells', *Cancer Research*, 65(9), pp. 3920–3927. doi: 10.1158/0008-5472.CAN-04-3002.

Avogadri, F. *et al.* (2008) 'Intra-tumoral Salmonella typhimurium induces a systemic anti-tumor immune response that is directed by low-dose radiation to treat distal disease', *European Journal of Immunology*, 38(7), pp. 1937–1947. doi: 10.1002/eji.200738035.

Baharoglu, Z. and Mazel, D. (2014) 'SOS, the formidable strategy of bacteria against aggressions', *FEMS Microbiology Reviews*, 38(6), pp. 1126–1145. doi: 10.1111/1574-

6976.12077.

Barat, S. *et al.* (2012) 'Immunity to Intracellular Salmonella Depends on Surface-associated Antigens', *PLoS Pathogens*, 8(10). doi: 10.1371/journal.ppat.1002966.

Behdani, M. *et al.* (2012) 'Generation and characterization of a functional Nanobody against the vascular endothelial growth factor receptor-2; angiogenesis cell receptor', *Molecular Immunology*, 50(1–2), pp. 35–41. doi: 10.1016/j.molimm.2011.11.013.

Bhatt, A. P., Redinbo, M. R. and Bultman, S. J. (2017) 'The role of the microbiome in cancer development and therapy', *CA: A Cancer Journal for Clinicians*, 67(4), pp. 326–344. doi: 10.3322/caac.21398.

Brawn, L. C., Hayward, R. D. and Koronakis, V. (2007) 'Salmonella SPI1 Effector SipA Persists after Entry and Cooperates with a SPI2 Effector to Regulate Phagosome Maturation and Intracellular Replication', *Cell Host and Microbe*, 1(1), pp. 63–75. doi: 10.1016/j.chom.2007.02.001.

Camacho, E. M. *et al.* (2016) 'Engineering Salmonella as intracellular factory for effective killing of tumour cells', *Scientific Reports*, 6(March), pp. 1–12. doi: 10.1038/srep30591.

Cao, H. D. *et al.* (2010) 'Attenuated Salmonella typhimurium carrying TRAIL and VP3 genes inhibits the growth of gastric cancer cells in vitro and in vivo', *Tumori*, 96(2), pp. 296–303. doi: 10.1177/030089161009600218.

Chang, W. *et al.* (2013) 'connexin 43 upregulation', 1935(Atcc 15480), pp. 1926–1935. doi: 10.1002/ijc.28155.

Chen, G. *et al.* (2013) 'Tumor-targeting Salmonella typhimurium, a natural tool for activation of prodrug 6MePdR and their combination therapy in murine melanoma model', *Applied Microbiology and Biotechnology*, 97(10), pp. 4393–4401. doi: 10.1007/s00253-012-4321-8.

Chen, J. *et al.* (2012) 'Salmonella-mediated tumor-targeting TRAIL gene therapy significantly suppresses melanoma growth in mouse model', *Cancer Science*, 103(2), pp. 325–333. doi: 10.1111/j.1349-7006.2011.02147.x.

Chen, J. *et al.* (2017) 'Modulation of Salmonella tumor-colonization and intratumoral anti-angiogenesis by triptolide and its mechanism', *Theranostics*, 7(8), pp. 2250–2260. doi: 10.7150/thno.18816.

Cheng, S. *et al.* (2014) 'trained immunity', 345(6204). doi: 10.1126/science.1250684.

Clairmont, C. *et al.* (2000) 'Biodistribution and genetic stability of the novel antitumor agent VNP20009, a genetically modified strain of Salmonella typhimurium', *Journal of Infectious Diseases*, 181(6), pp. 1996–2002. doi: 10.1086/315497.

Coutermarsh-Ott, S. L. *et al.* (2017) 'Effect of Salmonella enterica serovar Typhimurium VNP20009 and VNP20009 with restored chemotaxis on 4T1 mouse mammary carcinoma progression', *Oncotarget*, 8(20), pp. 33601–33613. doi: 10.18632/oncotarget.16830.

Crull, K., Bumann, D. and Weiss, S. (2011) 'Influence of infection route and virulence factors on colonization of solid tumors by Salmonella enterica serovar Typhimurium',

*FEMS Immunology and Medical Microbiology*, 62(1), pp. 75–83. doi: 10.1111/j.1574-695X.2011.00790.x.

Diego, S. *et al.* (2018) 'Tumor-targeting Salmonella typhimurium A1-R Inhibits Osteosarcoma Angiogenesis in the In Vivo Gelfoam ® Assay Visualized by Color-coded Imaging', 164, pp. 159–164. doi: 10.21873/anticanres.12203.

Dixit, M., Kulkarni, K. P. and Selvam R, P. (2011) 'Review on: freeze drying as a crystallization technique', *Elixir Pharmacy*, 41(January 2011), p. 5717. Available at: https://www.researchgate.net/publication/283447469.

Di Domenico, E. G. *et al.* (2017) 'Biofilm producing Salmonella typhi: Chronic colonization and development of gallbladder cancer', *International Journal of Molecular Sciences*, 18(9). doi: 10.3390/ijms18091887.

Dróżdż, M. *et al.* (2020) 'Obligate and facultative anaerobic bacteria in targeted cancer therapy: Current strategies and clinical applications', *Life Sciences*, 261(August), p. 118296. doi: 10.1016/j.lfs.2020.118296.

Duong, M. T. Q. *et al.* (2019) 'Bacteria-cancer interactions: bacteria-based cancer therapy', *Experimental and Molecular Medicine*, 51(12). doi: 10.1038/s12276-019-0297-0.

Elsland, D. and Neefjes, J. (2018) 'Bacterial infections and cancer', *EMBO reports*, 19(11), pp. 1–11. doi: 10.15252/embr.201846632.

Escamilla, J., Lane, M. A. and Maitin, V. (2012) 'Cell-free supernatants from probiotic lactobacillus casei and lactobacillus rhamnosus GG decrease colon cancer cell invasion in vitro', *Nutrition and Cancer*, 64(6), pp. 871–878. doi: 10.1080/01635581.2012.700758.

Felgner, S. *et al.* (2016) 'Aroa-deficient Salmonella enterica serovar Typhimurium is more than a metabolically attenuated mutant', *mBio*, 7(5), pp. 1–12. doi: 10.1128/mBio.01220-16.

Finlay, B. B. and van der Heijden, J. (2012) 'Type III effector-mediated processes in Salmonella infection', *Future Microbiology*, 7, pp. 685–704.

Forbes, N. S. *et al.* (2003) 'Sparse initial entrapment of systemically injected Salmonella typhimurium leads to heterogeneous accumulation within tumors', *Cancer Research*, 63(17), pp. 5188–5193.

Forbes, N. S. *et al.* (no date) 'White paper on microbial anti-cancer therapy and prevention'. doi: 10.1186/s40425-018-0381-3.

Fowler, A. and Toner, M. (2006) 'Cryo-injury and biopreservation.', *Annals of the New York Academy of Sciences*, 1066, pp. 119–135. doi: 10.1196/annals.1363.010.

Frahm, M. *et al.* (2015) 'Efficiency of conditionally attenuated Salmonella enterica serovar typhimurium in bacterium-mediated tumor therapy', *mBio*, 6(2), pp. 1–11. doi: 10.1128/mBio.00254-15.

Franchi, L. *et al.* (2006) 'Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in salmonella-infected macrophages', *Nature Immunology*, 7(6), pp. 576–582. doi: 10.1038/ni1346.

Franchi, L., Muñoz-Planillo, R. and Núñez, G. (2012) 'Sensing and reacting to microbes through the inflammasomes', *Nature Immunology*, 13(4), pp. 325–332. doi: 10.1038/ni.2231.

Friedlos, F. *et al.* (2008) 'Attenuated Salmonella targets prodrug activating enzyme carboxypeptidase G2 to mouse melanoma and human breast and colon carcinomas for effective suicide gene therapy', *Clinical Cancer Research*, 14(13), pp. 4259–4266. doi: 10.1158/1078-0432.CCR-07-4800.

Galluzzi, L. *et al.* (2018) 'Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018', *Cell Death and Differentiation*, 25(3), pp. 486–541. doi: 10.1038/s41418-017-0012-4.

Ganai, S. *et al.* (2011) 'In tumors Salmonella migrate away from vasculature toward the transition zone and induce apoptosis', *Cancer Gene Therapy*, 18(7), pp. 457–466. doi: 10.1038/cgt.2011.10.

Gautam, S. K. *et al.* (2020) 'MUCIN-4 (MUC4) is a novel tumor antigen in pancreatic cancer immunotherapy', *Seminars in Immunology*, 47(January), p. 101391. doi: 10.1016/j.smim.2020.101391.

Grebe, A., Hoss, F. and Latz, E. (2018) 'NLRP3 inflammasome and the IL-1 pathway in atherosclerosis', *Circulation Research*, 122(12), pp. 1722–1740. doi: 10.1161/CIRCRESAHA.118.311362.

Guo, Y. *et al.* (2020) 'Targeted cancer immunotherapy with genetically engineered oncolytic Salmonella typhimurium', *Cancer Letters*, 469(September 2019), pp. 102–110. doi: 10.1016/j.canlet.2019.10.033.

Gur, C. *et al.* (2015) 'Binding of the Fap2 protein of fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack', *Immunity*, 42(2), pp. 344–355. doi: 10.1016/j.immuni.2015.01.010.

Hardy, Z. and Jideani, V. A. (2017) 'Foam-mat drying technology: A review', *Critical Reviews in Food Science and Nutrition*, 57(12), pp. 2560–2572. doi: 10.1080/10408398.2015.1020359.

He, Y., Hara, H. and Núñez, G. (2016) 'Mechanism and Regulation of NLRP3 Inflammasome Activation', *Trends in Biochemical Sciences*, 41(12), pp. 1012–1021. doi: 10.1016/j.tibs.2016.09.002.

Herrero-Fresno, A. and Olsen, J. E. (2018) 'Salmonella Typhimurium metabolism affects virulence in the host – A mini-review', *Food Microbiology*, 71, pp. 98–110. doi: 10.1016/j.fm.2017.04.016.

Hiroshima, Y. *et al.* (2014) 'Efficacy of tumor-targeting Salmonella typhimurium A1-R in combination with anti-angiogenesis therapy on a pancreatic cancer patient-derived orthotopic xenograft (PDOX) and cellline mouse models', *Oncotarget*, 5(23), pp. 12346–12357. doi: 10.18632/oncotarget.2641.

Hoffman, R. M. (2009) 'Tumor-targeting amino acid auxotrophic Salmonella typhimurium', *Amino Acids*, 37(3), pp. 509–521. doi: 10.1007/s00726-009-0261-8.

Igarashi, K. *et al.* (2017) 'Intra-arterial administration of tumor-targeting Salmonella typhimurium A1-R regresses a cisplatin-resistant relapsed osteosarcoma in a patient-derived orthotopic xenograft (PDOX) mouse model', *Cell Cycle*, 16(12), pp. 1164–1170. doi: 10.1080/15384101.2017.1317417.

Irfan, M., Delgado, R. Z. R. and Frias-Lopez, J. (2020) 'The Oral Microbiome and Cancer', *Frontiers in Immunology*, 11(October), pp. 1–18. doi: 10.3389/fimmu.2020.591088.

Irrazábal, T. *et al.* (2014) 'The multifaceted role of the intestinal microbiota in colon cancer', *Molecular Cell*, 54(2), pp. 309–320. doi: 10.1016/j.molcel.2014.03.039.

Jałowiecki, Ł. *et al.* (2020) 'Effect of the freeze-drying process on the phenotypic diversity of Pseudomonas putida strains isolated from the interior of healthy roots of Sida hermaphrodita: Phenotype microarrays (PMs)', *Cryobiology*, 96(July), pp. 145–151. doi: 10.1016/j.cryobiol.2020.07.004.

Jazeela, K. *et al.* (2020) 'Nontyphoidal Salmonella: a potential anticancer agent', *Journal of Applied Microbiology*, 128(1), pp. 2–14. doi: 10.1111/jam.14297.

Jellbauer, S. *et al.* (2012) 'CD8 T-cell induction against vascular endothelial growth factor receptor 2 by Salmonella for vaccination purposes against a murine melanoma.', *PloS one*, 7(4), pp. 71–80. doi: 10.1371/journal.pone.0034214.

Jennings, E. *et al.* (2018) 'Structure-function analyses of the bacterial zinc metalloprotease effector protein GtgA uncover key residues required for deactivating NF-κB', *Journal of Biological Chemistry*, 293(39), pp. 15316–15329. doi: 10.1074/jbc.RA118.004255.

Jeong, J. H. *et al.* (2008) 'Salmonella enterica serovar gallinarum requires ppGpp for internalization and survival in animal cells', *Journal of Bacteriology*, 190(19), pp. 6340–6350. doi: 10.1128/JB.00385-08.

Jeong, J. H. *et al.* (2014) 'Anti-tumoral effect of the mitochondrial target domain of noxa delivered by an engineered Salmonella typhimurium', *PLoS ONE*, 9(1), pp. 1–11. doi: 10.1371/journal.pone.0080050.

Jia, L. J. *et al.* (2007) 'Tumor-targeting Salmonella typhimurium improves cyclophosphamide chemotherapy at maximum tolerated dose and low-dose metronomic regimens in a murine melanoma model', *International Journal of Cancer*, 121(3), pp. 666–674. doi: 10.1002/ijc.22688.

Johnson, R., Mylona, E. and Frankel, G. (2018) 'Typhoidal Salmonella: Distinctive virulence factors and pathogenesis', *Cellular Microbiology*, 20(9), pp. 1–14. doi: 10.1111/cmi.12939.

Kanapathipillai, M. (2018) 'Treating p53 mutant aggregation-associated cancer', *Cancers*, 10(6). doi: 10.3390/cancers10060154.

Kasinskas, R. W. and Forbes, N. S. (2007) 'Salmonella typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis', *Cancer Research*, 67(7), pp. 3201–3209. doi: 10.1158/0008-5472.CAN-06-2618.

Kim, J. E. *et al.* (2015) 'Salmonella typhimurium suppresses tumor growth via the pro-inflammatory cytokine interleukin-1β', *Theranostics*, 5(12), pp. 1328–1342. doi:

10.7150/thno.11432.

Kim, J. J. *et al.* (2010) 'TNF-α-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-XL', *Cell Death and Differentiation*, 17(9), pp. 1420–1434. doi: 10.1038/cdd.2010.19.

Kiyuna, T. *et al.* (2016) 'High efficacy of tumor-targeting Salmonella typhimurium A1-R on a doxorubicin- and dactolisib-resistant follicular dendritic-cell sarcoma in a patient-derived orthotopic xenograft PDOX nude mouse model', *Oncotarget*, 7(22), pp. 33046–33054. doi: 10.18632/oncotarget.8848.

Kiyuna, T. *et al.* (2018) 'Tumor-targeting Salmonella typhimurium A1-R arrests a doxorubicin-resistant PDGFRA-amplified patient-derived orthotopic xenograft mouse model of pleomorphic liposarcoma', *Journal of Cellular Biochemistry*, 119(9), pp. 7827–7833. doi: 10.1002/jcb.27183.

Knudsen, K. E. B. *et al.* (2018) 'Impact of diet-modulated butyrate production on intestinal barrier function and inflammation', *Nutrients*, 10(10). doi: 10.3390/nu10101499.

Kocijancic, D. *et al.* (2017) *Therapeutic benefit of Salmonella attributed to LPS and TNF-α is exhaustible and dictated by tumor susceptibility*. Available at: www.impactjournals.com/oncotarget (Accessed: 17 February 2021).

Kuan, Y. D. and Lee, C. H. (2016) 'Salmonella overcomes tumor immune tolerance by inhibition of tumor indoleamine 2, 3-dioxygenase 1 expression', *Oncotarget*, 7(1), pp. 374–385. doi: 10.18632/ONCOTARGET.6258.

Kupz, A. *et al.* (2014) 'In vivo IFN-γ secretion by NK cells in response to Salmonella typhimurium requires NLRC4 inflammasomes', *PLoS ONE*, 9(5), pp. 8–12. doi: 10.1371/journal.pone.0097418.

Lee, C. H. *et al.* (2014) 'Salmonella induce autophagy in melanoma by the downregulation of AKT/mTOR pathway', *Gene Therapy*, 21(3), pp. 309–316. doi: 10.1038/gt.2013.86.

Leschner, S. *et al.* (2009) 'Tumor Invasion of Salmonella enterica Serovar Typhimurium Is Accompanied by Strong Hemorrhage Promoted by TNF- a', 4(8). doi: 10.1371/journal.pone.0006692.

Liang, K. *et al.* (2019) 'Genetically engineered Salmonella Typhimurium: Recent advances in cancer therapy', *Cancer Letters*, 448(2), pp. 168–181. doi: 10.1016/j.canlet.2019.01.037.

Liao, A. P. *et al.* (2008) 'Salmonella type III effector AvrA stabilizes cell tight junctions to inhibit inflammation in intestinal epithelial cells', *PLoS ONE*, 3(6). doi: 10.1371/journal.pone.0002369.

Linder, B. and Kögel, D. (2019) 'Autophagy in cancer cell death', *Biology*, 8(4), pp. 1–13. doi: 10.3390/biology8040082.

Liu, F. *et al.* (2010) 'vascularity Vessel destruction by tumor-targeting Salmonella typhimurium A1-R is enhanced by high tumor vascularity', 4101. doi: 10.4161/cc.9.22.13744.

Liu, X. *et al.* (2016) 'Radiotherapy combined with an engineered Salmonella typhimurium

inhibits tumor growth in a mouse model of colon cancer', *Experimental Animals*, 65(4), pp. 413–418. doi: 10.1538/expanim.16-0033.

Llewellyn, A. and Foey, A. (2017) *Probiotic modulation of innate cell pathogen sensing and signaling events*, *Nutrients*. doi: 10.3390/nu9101156.

Low, K. B. *et al.* (1999) 'Lipid a mutant Salmonella with suppressed virulence and TNFα induction retain tumor-targeting in vivo', *Nature Biotechnology*, 17(1), pp. 37–41. doi: 10.1038/5205.

M. Christopher, A. M. L. S. (2016) '乳鼠心肌提取 HHS Public Access', *Physiology & behavior*, 176(1), pp. 100–106. doi: 10.1038/cgt.2009.58.An.

Mcgourty, K. *et al.* (2012) 'Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate', *Science*, 338(November), pp. 963–967.

Mei, Y. *et al.* (2017) 'CombiningDNAVaccine and AIDA-1 in Attenuated Salmonella Activates Tumor-Specific CD4+ and CD8+ T-cell Responses', *Cancer Immunology Research*, 5(6), pp. 503–514. doi: 10.1158/2326-6066.CIR-16-0240-T.

Mercado-Lubo, R. *et al.* (2016) 'A Salmonella nanoparticle mimic overcomes multidrug resistance in tumours', *Nature Communications*, 7. doi: 10.1038/ncomms12225.

Mhatre V. Ho, Ji-Ann Lee, and K. C. M. (2012) '基因的改变NIH Public Access', *Bone*, 23(1), pp. 1–7. doi: 10.1038/jid.2014.371.

Mi, Z. *et al.* (2019) 'Salmonella-mediated cancer therapy: An innovative therapeutic strategy', *Journal of Cancer*, 10(20), pp. 4765–4776. doi: 10.7150/jca.32650.

Milani, S. *et al.* (2020) 'Hydroxypropyl beta cyclodextrin: a water-replacement agent or a surfactant upon spray freeze-drying of IgG with enhanced stability and aerosolization', *Drug Development and Industrial Pharmacy*, 46(3), pp. 403–411. doi: 10.1080/03639045.2020.1724131.

Ming-Ju Chen, Kreuter, J. Y.-T. K. (1996) 'Nanoparticles and microparticles for drug and vaccine delivery.', *Journal of anatomy*, 189 ( Pt 3(Ii), pp. 503–505. doi: 10.1002/bit.

Miyake, K. *et al.* (2018) 'Tumor-targeting Salmonella typhimurium A1-R suppressed an imatinib-resistant gastrointestinal stromal tumor with c-kit exon 11 and 17 mutations', *Heliyon*, 4(6), pp. 1–22. doi: 10.1016/j.heliyon.2018.e00643.

Modified, L. G. and Melanoma, M. (2003) 'Letter to the Editor Typhimurium in Patients With', 26(2), pp. 179–180.

Myeni, S. K., Wang, L. and Zhou, D. (2013) 'SipB-SipC Complex Is Essential for Translocon Formation', *PLoS ONE*, 8(3), pp. 1–7. doi: 10.1371/journal.pone.0060499.

Na, H. S. *et al.* (2006) 'Immune response induced by Salmonella typhimurium defective in ppGpp synthesis', *Vaccine*, 24(12), pp. 2027–2034. doi: 10.1016/j.vaccine.2005.11.031.

Najafi, M. *et al.* (2019) 'Macrophage polarity in cancer: A review', *Journal of Cellular Biochemistry*, 120(3), pp. 2756–2765. doi: 10.1002/jcb.27646.

Nguyen, V. H. and Min, J. J. (2017) 'Salmonella-Mediated Cancer Therapy: Roles and

Potential', *Nuclear Medicine and Molecular Imaging*, 51(2), pp. 118–126. doi: 10.1007/s13139-016-0415-z.

Nishida, A. *et al.* (2018) 'Gut microbiota in the pathogenesis of inflammatory bowel disease', *Clinical Journal of Gastroenterology*, 11(1), pp. 1–10. doi: 10.1007/s12328-017-0813-5.

Noel, P. *et al.* (2019) 'Triptolide and Its Derivatives as Cancer Therapies', *Trends in Pharmacological Sciences*, 40(5), pp. 327–341. doi: 10.1016/j.tips.2019.03.002.

Novak, D. *et al.* (2020) 'SOX2 in development and cancer biology', *Seminars in Cancer Biology*, 67(August 2019), pp. 74–82. doi: 10.1016/j.semcancer.2019.08.007.

Nurgali, K., Jagoe, R. T. and Abalo, R. (2018) 'Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae?', *Frontiers in Pharmacology*, 9(MAR), pp. 1–3. doi: 10.3389/fphar.2018.00245.

Orlando, A., Linsalata, M. and Russo, F. (2016) 'Antiproliferative effects on colon adenocarcinoma cells induced by co-administration of vitamin K1 and Lactobacillus rhamnosus GG', *International Journal of Oncology*, 48(6), pp. 2629–2638. doi: 10.3892/ijo.2016.3463.

Pangilinan, C. R. and Lee, C. H. (2019) 'Salmonella-based targeted cancer therapy: Updates on a promising and innovative tumor immunotherapeutic strategy', *Biomedicines*, 7(2). doi: 10.3390/biomedicines7020036.

Patel, J. C. and Galán, J. E. (2006) 'Differential activation and function of Rho GTPases during Salmonella-host cell interactions', *Journal of Cell Biology*, 175(3), pp. 453–463. doi: 10.1083/jcb.200605144.

Pawelek, J. M., Low, K. B. and Bermudes, D. (1997) 'Tumor-targeted Salmonella as a novel anticancer vector', *Cancer Research*, 57(20), pp. 4537–4544.

Phan, T. X. *et al.* (2015) 'Activation of inflammasome by attenuated Salmonella typhimurium in bacteria-mediated cancer therapy', *Microbiology and Immunology*, 59(11), pp. 664–675. doi: 10.1111/1348-0421.12333.

Platt, J. *et al.* (2000) 'Antitumour effects of genetically engineered Salmonella in combination with radiation', *European Journal of Cancer*, 36(18), pp. 2397–2402. doi: 10.1016/S0959-8049(00)00336-1.

Rajagopala, S. V. *et al.* (2017) 'The human microbiome and cancer', *Cancer Prevention Research*, 10(4), pp. 226–234. doi: 10.1158/1940-6207.CAPR-16-0249.

Raman, V. *et al.* (2019) 'The motility regulator flhDC drives intracellular accumulation and tumor colonization of Salmonella', *Journal for ImmunoTherapy of Cancer*, 7(1), pp. 1–16. doi: 10.1186/s40425-018-0490-z.

Roider, E. *et al.* (2011) 'Invasion and destruction of a murine fibrosarcoma by Salmonella-induced effector CD8 T cells as a therapeutic intervention against cancer', *Cancer Immunology, Immunotherapy*, 60(3), pp. 371–380. doi: 10.1007/s00262-010-0950-x.

Rosen, R. *et al.* (2016) 'SOS gene induction and possible mutagenic effects of freeze-

drying in Escherichia coli and Salmonella typhimurium', *Applied Microbiology and Biotechnology*, 100(21), pp. 9255–9264. doi: 10.1007/s00253-016-7751-x.

Roy, S. and Trinchieri, G. (2017) 'Microbiota: A key orchestrator of cancer therapy', *Nature Reviews Cancer*, 17(5), pp. 271–285. doi: 10.1038/nrc.2017.13.

Schirrmacher, V. (2019) 'From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review)', *International Journal of Oncology*, 54(2), pp. 407–419. doi: 10.3892/ijo.2018.4661.

Seki, S., Kleinhans, F. W. and Mazur, P. (2009) 'Intracellular ice formation in yeast cells vs. cooling rate: Predictions from modeling vs. experimental observations by differential scanning calorimetry', *Cryobiology*, 58(2), pp. 157–165. doi: 10.1016/j.cryobiol.2008.11.011.

Sender, R., Fuchs, S. and Milo, R. (2016) 'Revised Estimates for the Number of Human and Bacteria Cells in the Body', *PLoS Biology*, 14(8), pp. 1–14. doi: 10.1371/journal.pbio.1002533.

Seward, E. (2019) 'Recent advances in colonoscopy [version 1; peer review: 2 approved]', *F1000Research*, 8. doi: 10.12688/f1000research.18503.1.

Shappo, M. O. E. *et al.* (2020) 'SspH2 as anti-inflammatory candidate effector and its contribution in Salmonella Enteritidis virulence', *Microbial Pathogenesis*, 142(February), p. 104041. doi: 10.1016/j.micpath.2020.104041.

Shilling, D. A. *et al.* (2007) 'Salmonella typhimurium stimulation combined with tumour-derived heat shock proteins induces potent dendritic cell anti-tumour responses in a murine model', *Clinical and Experimental Immunology*, 149(1), pp. 109–116. doi: 10.1111/j.1365-2249.2007.03393.x.

Singh, M. *et al.* (2016) 'Radiodermatitis: A Review of Our Current Understanding', *American Journal of Clinical Dermatology*, 17(3), pp. 277–292. doi: 10.1007/s40257-016-0186-4.

Singh, N. *et al.* (2014) 'Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis', *Immunity*, 40(1), pp. 128–139. doi: 10.1016/j.immuni.2013.12.007.

Singh, R. K. *et al.* (2018) 'Therapeutic journery of nitrogen mustard as alkylating anticancer agents: Historic to future perspectives', *European Journal of Medicinal Chemistry*, 151, pp. 401–433. doi: 10.1016/j.ejmech.2018.04.001.

Smith, N. R. *et al.* (2010) 'Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers', *Clinical Cancer Research*, 16(14), pp. 3548–3561. doi: 10.1158/1078-0432.CCR-09-2797.

Song, M. and Chan, A. T. (2019) 'Environmental Factors, Gut Microbiota, and Colorectal Cancer Prevention', *Clinical Gastroenterology and Hepatology*, 17(2), pp. 275–289. doi: 10.1016/j.cgh.2018.07.012.

Stegantseva, M. V. *et al.* (2020) 'Multi-antigen DNA vaccine delivered by polyethylenimine

and Salmonella enterica in neuroblastoma mouse model', *Cancer Immunology, Immunotherapy*, 69(12), pp. 2613–2622. doi: 10.1007/s00262-020-02652-2.

Stévenin, V. *et al.* (2019) 'Dynamic Growth and Shrinkage of the Salmonella-Containing Vacuole Determines the Intracellular Pathogen Niche', *Cell Reports*, 29(12), pp. 3958-3973.e7. doi: 10.1016/j.celrep.2019.11.049.

Sun, H. *et al.* (2016) 'A Family of Salmonella Type III Secretion Effector Proteins Selectively Targets the NF-κB Signaling Pathway to Preserve Host Homeostasis', *PLoS Pathogens*, 12(3), pp. 1–19. doi: 10.1371/journal.ppat.1005484.

Thurston, T. L. M. *et al.* (2012) 'Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion', *Nature*, 482(7385), pp. 414–418. doi: 10.1038/nature10744.

Toley, B. J. and Forbes, N. S. (2012) 'Motility is critical for effective distribution and accumulation of bacteria in tumor tissue', *Integrative Biology*, 4(2), pp. 165–176. doi: 10.1039/c2ib00091a.

Tome, Y. *et al.* (2013) 'Primer Dosing of S . typhimurium A1-R Potentiates Tumor-Targeting and Efficacy in Immunocompetent Mice', 102, pp. 97–102.

Toso, J. F. *et al.* (2002) 'Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma', *Journal of Clinical Oncology*, 20(1), pp. 142–152. doi: 10.1200/JCO.20.1.142.

Uchugonova, A. *et al.* (2015) 'Imaging the different mechanisms of prostate cancer cell-killing by tumor-targeting Salmonella typhimurium A1-R', *Anticancer Research*, 35(10), pp. 5225–5230.

Viallard, C. and Larrivée, B. (2017) 'Tumor angiogenesis and vascular normalization: alternative therapeutic targets', *Angiogenesis*, 20(4), pp. 409–426. doi: 10.1007/s10456-017-9562-9.

Wan, S. *et al.* (2012) 'Chemotherapeutics and radiation stimulate MHC class i expression through elevated interferon-beta signaling in breast cancer cells', *PLoS ONE*, 7(3). doi: 10.1371/journal.pone.0032542.

Wang, C. Z., Kazmierczak, R. A. and Eisenstark, A. (2016) 'Strains, mechanism, and perspective: Salmonella -based cancer therapy', *International Journal of Microbiology*, 2016. doi: 10.1155/2016/5678702.

Wang, W. *et al.* (2015) 'Connexin 43 Suppresses Tumor Angiogenesis by Down-Regulation of Vascular Endothelial Growth Factor via Hypoxic-Induced Factor-1 α', pp. 439–451. doi: 10.3390/ijms16010439.

Wang, W. K. *et al.* (2015) 'The treatment of mouse colorectal cancer by oral delivery tumor-targeting Salmonella', *American Journal of Cancer Research*, 5(7), pp. 2222–2228.

Wu, S. *et al.* (2020) 'Salmonella Interacts With Autophagy to Offense or Defense', *Frontiers in Microbiology*, 11(April), pp. 1–13. doi: 10.3389/fmicb.2020.00721.

Yamamoto, M. *et al.* (2016) 'Efficacy of tumor-targeting Salmonella A1-R on a melanoma

patient-derived orthotopic xenograft (PDOX) nude-mouse model', *PLoS ONE*, 11(8), pp. 1–12. doi: 10.1371/journal.pone.0160882.

Yang, C. J. *et al.* (2018) 'Salmonella overcomes drug resistance in tumor through P-glycoprotein downregulation', *International Journal of Medical Sciences*, 15(6), pp. 574–579. doi: 10.7150/ijms.23285.

Yoon, W. *et al.* (2014) 'Engineered Salmonella typhimurium expressing E7 fusion protein, derived from human papillomavirus, inhibits tumor growth in cervical tumor-bearing mice', *Biotechnology Letters*, 36(2), pp. 349–356. doi: 10.1007/s10529-013-1370-8.

Yoon, W. S. *et al.* (2011) 'Antitumor therapeutic effects of a genetically engineered Salmonella typhimurium harboring TNF-α in mice', *Applied Microbiology and Biotechnology*, 89(6), pp. 1807–1819. doi: 10.1007/s00253-010-3006-4.

Yoon, W. S. *et al.* (2014) 'Salmonella typhimurium with γ-radiation induced H2AX phosphorylation and apoptosis in melanoma', *Bioscience, Biotechnology and Biochemistry*, 78(6), pp. 1082–1085. doi: 10.1080/09168451.2014.905173.

Yuan, X. *et al.* (2018) 'Yuan2018_Article_DevelopingTRAILTRAILDeathRecep.pdf', pp. 733–748.

Yun, M. *et al.* (2012) 'Effect of Salmonella treatment on an implanted tumor (CT26) in a mouse model', *Journal of Microbiology*, 50(3), pp. 502–510. doi: 10.1007/s12275-012-2090-9.

Zeng, Y. *et al.* (2009) 'Towards development of stable formulations of a live attenuated bacterial vaccine: A preformulation study facilitated by a biophysical approach', *Human Vaccines*, 5(5), pp. 322–331. doi: 10.4161/hv.5.5.7559.

Zhang, H. *et al.* (2020) 'Vacuum lyophilization preservation and rejuvenation performance of anammox bacteria', *Journal of Bioscience and Bioengineering*, 129(5), pp. 519–527. doi: 10.1016/j.jbiosc.2019.10.007.

Zhang, J., Kale, V. and Chen, M. (2015) 'Gene-Directed Enzyme Prodrug Therapy', *AAPS Journal*, 17(1), pp. 102–110. doi: 10.1208/s12248-014-9675-7.

Zhang, M., Swofford, C. A. and Forbes, N. S. (2014) 'Lipid A controls the robustness of intratumoral accumulation of attenuated Salmonella in mice', *International Journal of Cancer*, 135(3), pp. 647–657. doi: 10.1002/ijc.28700.

Zhang, Y. *et al.* (2017) 'Toxicology and efficacy of tumor-targeting Salmonella typhimurium A1-R compared to VNP 20009 in a syngeneic mouse tumor model in immunocompetent mice', *Oncotarget*, 8(33), pp. 54616–54628. doi: 10.18632/oncotarget.17605.

Zhao, C. *et al.* (2016) 'Enhanced therapeutic effect of an antiangiogenesis peptide on lung cancer in vivo combined with salmonella VNP20009 carrying a Sox2 shRNA construct', *Journal of Experimental and Clinical Cancer Research*, 35(1), pp. 1–11. doi: 10.1186/s13046-016-0381-4.

Zhao, M. *et al.* (2005) 'Tumor-targeting bacterial therapy with amino acid auxotrophs of

GFP-expressing Salmonella typhimurium', *Urologic Oncology: Seminars and Original Investigations*, 23(5), p. 380. doi: 10.1016/j.urolonc.2005.06.008.

Zhao, M. *et al.* (2006) 'Targeted therapy with a Salmonella typhimurium leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice', *Cancer Research*, 66(15), pp. 7647–7652. doi: 10.1158/0008-5472.CAN-06-0716.

Zheng, J. H. *et al.* (2017) 'Two-step enhanced cancer immunotherapy with engineered Salmonella typhimurium secreting heterologous flagellin', *Science Translational Medicine*, 9(376), pp. 1–11. doi: 10.1126/scitranslmed.aak9537.

Zhou, S. *et al.* (2018) 'Tumour-targeting bacteria engineered to fight cancer', *Nature Reviews Cancer*, 18(12), pp. 727–743. doi: 10.1038/s41568-018-0070-z.