LITERATURE REVIEW

The Trojan Bacillus: Transgenic Bacteria in Cancer Therapy

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Abstract

A classic conundrum in oncology is the identification of cancer-specific, druggable molecules which can be targeted with minimal systemic toxicity. A novel candidate for administering cancer therapeutics has emerged in bacteria, which may prove to be excellent delivery vehicles for biologics whose systemic delivery causes severe and unacceptable adverse effects. Bacteria are well-suited to this role due to their ability to colonise tumour microenvironments, synthesise drug molecules, and potentiate innate and adaptive immune responses. Genetic clockwork in the form of quorum sensing mechanisms allows these bacteria to lyse on demand, releasing therapeutic payloads into tumours. Recent in vivo evidence outlined here support this hypothesis, yet there is a great deal of research and refinement still to be done.

Keywords: Synthetic biology, Oncology, Targeted therapy

Introduction

In the late nineteenth century, American surgeon Dr. William Coley noted that injection of bacteria directly into sarcoma induced a number of remissions, some permanent[1]. Recently, the use of bacteria as delivery systems for immunotherapy biologics has emerged. The advantages of bacterial delivery systems are numerous. Bacteria may localise to tumour microenvironments autonomously via chemotactic receptors and release therapeutic biologics within tumours, limiting systemic toxicities. Bacteria can sense population growth and lyse *en masse* through engineered lysis circuits. Furthermore, they can be controlled by external signals while being engineered to produce a range of therapeutic molecules. Such advantages are reviewed elsewhere[2].

Several murine assays demonstrate the efficacy of bacterial delivery of anti-phagocytic and immune checkpoint inhibitory biologic drugs, each inducing tumour remission and affecting systemic immunity, thus demonstrating proof of concept for intratumoural delivery of modified bacteria[3,4]. Further evidence and refinement is required before this can be tested in humans, however. This review will examine the role of lysis circuits in localising bacterial colonisation of tumours, the benefits, and limitations of bacterial drug delivery in cancers, focusing on the drugs which can be delivered, and current in vivo evidence of bacterial drug delivery systems in murine cancer models.

Lysis Circuits

Orchestrating Bacteria: Quorum Sensing

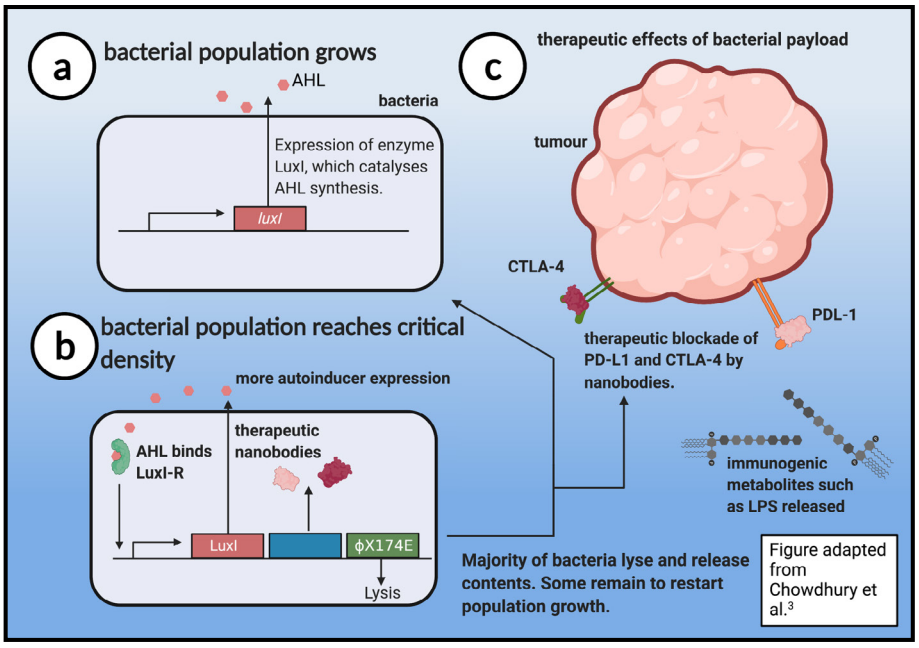
Bacteria functioning alone is both costly and likely ineffective in numerous instances, and consequently bacterial populations coordinate certain activities on a population wide basis, in a system called quorum sensing. This system allows for modulation of gene expression based on the population density of a bacterial population, with each individual simultaneously releasing and sensing a signal molecule, called the autoinducer. Given that these autoinducers are constitutively expressed, their concentrations rise in a stepwise manner with population density[5]. The behaviours controlled by quorum sensing would be ineffectual if undertaken by a single bacterium, and therefore must be coordinated among a larger population of bacteria.

Wild type bacteria use quorum sensing to affect bioluminescence, virulence factor production, biofilm formation and the uptake of DNA[6]. In gram-negative bacteria, the autoinducers are generally acyl-homoserine lactones, which diffuse freely across bacterial cell membranes, activating receptors present either on the plasma membrane or free in the cytosol of neighbouring bacteria. Receptor binding induces gene expression underpinning the aforementioned functions, as well as the expression of the autoinducer resulting in feed forward signalling. Thus, gene expression across individual-bacteria can be coordinated in concert upon the bacterial population reaching a critical population size[7]. It is precisely these characteristics of quorum sensing; that it acts on whole populations of bacteria and affects altered gene expression, that it holds value in bacterial drug delivery. Quorum sensing can induce bacterial drug synthesis, release, and population containment all through expression of transfected genes, whose expression is coordinated among the bacterial population.

How to Control a Bacteria: Synchronised Lysis Circuits

Therefore, we need not rely on natural quorum sensing

Figure 1. **Bacterial Circuitry in Action (Adapted from Chowdhury et al.[3])**



The diagram illustrates the operation of a Synchronised lysis circuit: A) Constitutively expressed autoinducer concentration increases with population density. B) Autoinducer/receptor interaction results in expression of autoinducer, therapeutic payload and lysis protein. C) Therapeutic payload released into tumour microenvironment with concurrent immune activation via release of bacterial metabolites.

mechanisms as we can modify existing ones to generate more efficient bacterial drug delivery systems. This was first demonstrated by Danino et al.[8], with their simple circuit consisting of three genes flanked by identical upstream promoter regions, *luxI*. The first gene codes for LuxI, which catalyses acyl-homoserine lactone (AHL) synthesis. AHL diffuses extracellularly and binds to the constitutively expressed intracellular receptors, LuxI-R. Once bound, these bind the luxI promoter region thus making a positive feedback loop-potentiating more AHL synthesis, and consequently more receptor binding and consequent gene expression (**Figure 1**).

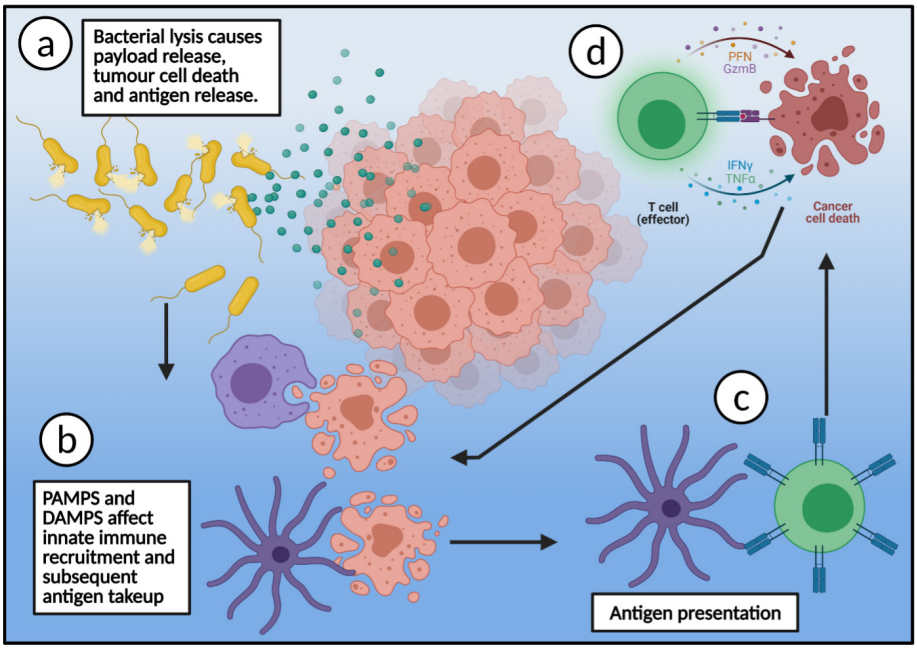
Moreover, this complex also induces the expression of the gene *AiiA*, which forms a negative feedback loop by catalysing acyl-homoserine lactone degradation. Finally, AHL-LuxI-R dimers induce the expression of the reporter gene, green fluorescent protein (GFP). These authors noted periodic oscillations in fluorescence, demonstrating increasing and decreasing GFP expression[8]. Thus, these three genes allowed for population-wide changes in gene expression, showing successful establishment of a genetic circuit constituting artificial quorum sensing. In subsequent assays, GFP is replaced by bacteriophage lysis genes. Benefits of synchronised bacterial lysis in cancer therapy are threefold. Firstly, release of bacterial cytoplasmic and membrane material into the tumour microenvironment potentiates pattern recognition receptor signalling, innate immune activation, and antigen presentation thus driving a cycle of progressive anti-tumour immunity as adaptive immune cells drive tumour cell killing and further antigen release. Secondly, quorum sensing circuits may include genes for anti-tumour biologics, that are released into the tumour as the bacteria lyse. Lastly, mass lysis of the bacterial population limits its size and prevents systemic toxicity from excessive release of bacterial pathogen-associated molecular patterns (PAMPs) (**Figure 2**).

Engineered groups of genes that are based on quorum sensing and which result in bacterial lysis are called synchronised lysis circuits (SLC). Such circuits are similar in composition to the previously mentioned oscillatory circuit, but carry genes for drug production as well as lysis, both of which contain the *luxI* promoter. The phage lysis gene φX174E is used in such circuits, and several different payloads can be expressed by the transgenic bacteria (**Figure 1**). An example is haemolysin E, a pore forming anti-tumour toxin. Din et al., used such a system to demonstrate the necessity of the SLC by incubating HeLa cells with haemolysin E expressing *Escherichia coli* (*E. Coli*) strains either with or without a SLC[9]. The SLC+ stain induced almost total loss of viability in these cells, whereas the bacteria without a SLC only induced a small rise in nonviability, thus demonstrating the efficient delivery of therapeutic payloads allowed for by SLCs[9]. While this result was seen in cell cultures, further in vivo evidence of SLC efficacy is outlined below.

Drug Synthesis

Having examined quorum lysis, its immunostimulatory effects and its benefit in drug release, our attention must now turn to bacterial synthesis of immunotherapeutic or oncolytic agents. Of particular interest is the bacterial expression of nanobodies. These are camelid-single domain immunoglobulins, whose expression by programmable bacteria is advantageous for several reasons. For one, while numerous licensed cancer therapies consist in monoclonal antibodies, these are not readily expressible in bacterial vectors, particularly due to lack of bacterial glycosylation systems. Despite this, IgG antibodies lacking glycosylation have been

Figure 2. **The Cycle of Antigen Release, Presentation and Effector Response**



A) Release of bacterial payload directly induces tumour cell death. B) PAMPs released by the bacteria, and damage-associated molecular patterns (DAMPs) released by tumour cells recruit immune cells like macrophages and dendritic cells that phagocytose tumour antigens and migrate via afferent lymphatics to local lymphoid organs. C) Antigens are presented to naïve T-cells, thus activating and mounting of effector response. D) Effector T-cells induce tumour cell death and antigen release, beginning the cycle again.

expressed in *E. coli*, although not in high yield and crucially, not correctly folded[10]. Thus, while bacteria have several advantages as drug delivery systems, this will likely not extend to delivery of monoclonal antibodies, which need eukaryotic expression systems to function properly. If we want bacteria to synthesise therapeutic compounds within the tumour microenvironment, we must look beyond monoclonal antibodies.

These challenges are not present in the expression of nanobodies, however. Moreover, the large size of antibodies, at 150kDa, restricts their access to tumour antigens with only 20% of administered monoclonal antibodies, eliciting their desired effects. Nanobodies sidestep this by virtue of their relatively small size allowing for greater tumour penetration[11]. This smaller size renders conventional infusion difficult however, as nanobodies are rapidly cleared by glomerular filtration, necessitation high dosing frequency[12]. This, however, may not apply in SLC+ bacterial delivery vectors, where expression of the nanobody occurs autonomously and continuously. In this context, rapid nanobody clearance is advantageous, abrogating excessive nanobody accumulation and resulting systemic toxicities.

Examples of therapeutic nanobodies, expressible in bacteria systems and efficacious in cancer, are those directed against immune checkpoints. Immune checkpoints are inhibitory signals that regulate the action of lymphocytes and are regularly exploited by cancers as a means of evading immunosurveillance. The most notable of these checkpoints are cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death ligand-1 (PDL-1), and PDL-1 receptor PD-1. These are all targeted by the FDA approved monoclonal antibodies, for instance Ipilimumab and Pembrolizumab, and have been proven efficacious in several cancers and are currently being investigated by numerous clinical trials for a wide range of indications. Mutation burden in cancer increases with time, and this results in truncated or otherwise altered proteins expressed at the plasma membrane. These are referred to as neoantigens, and because the immune system was not tolerised to them during thymic selection, it is possible to mount effective anti-tumour immune responses against them. Indeed, neoantigen burden is a predictive marker in checkpoint inhibitor treatment and perhaps will be beneficial in selecting patients in which bacterial delivery of such interventions will be of most benefit[13].

Proof of Concept

Immune Checkpoint Inhibitor Delivery

Drugs targeting CTLA-4 and PDL-1 are efficacious individually but have been proven to be of greater benefit in combination than as monotherapies[14]. Despite the benefit in efficacy, severe toxicities are observed in combination regimens. Moreover, the majority of patients that discontinued combination therapy in one trial had seen objective benefit, but the off-target effects were such that monotherapy was favoured[15]. Owing to these severe off-target effects, more targeted delivery systems are required to maximise efficacy with concurrent reduction in harm. This has been demonstrated in murine models using programmable, non-pathogenic bacteria. Gurbatri et al.[4] infected mice with A20 cells, a murine model of sarcoma that has shown response in past experiments to anti CTLA-4 and PD-L1 agents. In this study, these mice developed tumours in their hind flank and were treated with bacteria expressing a SLC and two nanobodies, each targeting either PDL-1 or CTLA-4.

When this strain was compared against a control with multiple intratumoural injections of bacteria,

significant therapeutic effects were observed with tumours partially or fully regressing[4]. Furthermore, the study showed that there was an increased survival benefit and no visible liver metastasis. These results were highly statistically significant (P<0.0001). In tumours treated with the programmed bacteria, flow cytometry demonstrated increased infiltration by CD8+ T-cells and proliferation of CD4+[4]. Further, the necessity of the SLC was demonstrated by comparing regression in mice treated with SLC+ and checkpoint inhibitor expressing bacteria, SLC- bacteria and lysate from SLC+ bacteria, which again showed significant results (P<0.0001)[4], illustrating that therapeutic payload as well as lysis circuits are required for effective induction of cancer remission.

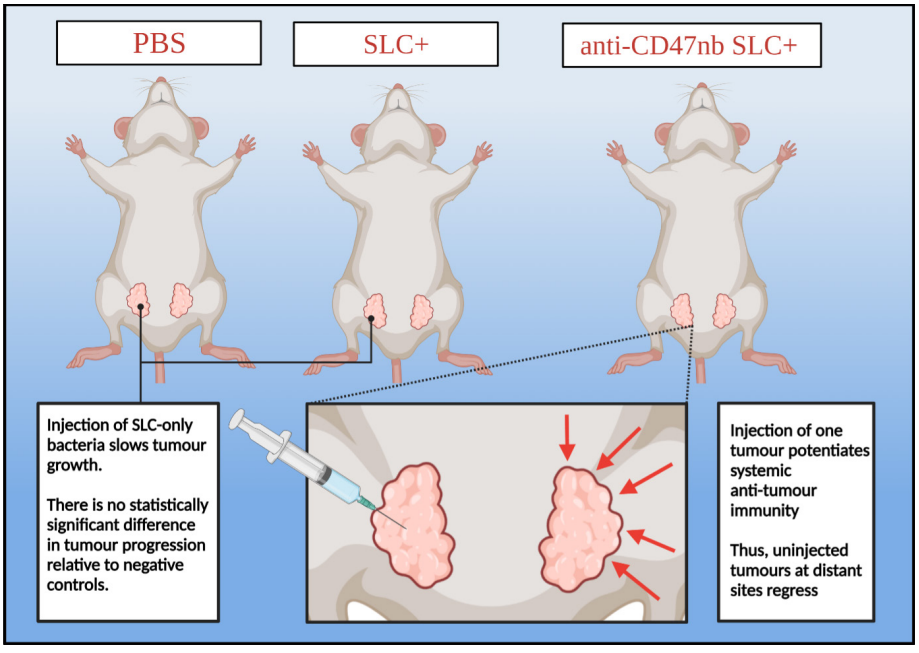
Furthermore, a systemic immune response was seen, with non-treated tumours regressing in mice with one other treated tumour. Finally, two weeks after administration, serum samples taken from treated mice showed no increased titre of TNF, demonstrating the lack of systemic inflammation. The authors attribute this lack of pathological systemic inflammation to the SLC containing the bacteria, and the success to the constitutive expression of nanobodies intratumourally[4]. Additionally, it is likely that the nanobodies used in this assay were being more readily cleared from the blood, therefore they were less likely to cause off target toxicities, further emphasising the potential benefits of this delivery system. In summary, intratumoural delivery of SLC+ bacteria that expressed checkpoint inhibitory nanobodies affected regression of the infected tumour as well as distant tumours, demonstrating these bacteria potentiated both local and systemic anti-tumour immunity. This occurred in the absence of systemic inflammation, bolstering the notion that SLCs maintain the bacterial population with the confines of the tumour microenvironment.

Anti-CD47 Delivery

Another in vivo assay evaluating bacterial delivery of immunotherapeutics was carried out by Chowdhury et al.[3], investigating nanobody mediated blockade of tumour-expressed CD47. CD47 is an antiphagocytic cell surface receptor understood to be expressed in numerous human malignancies. The authors noted however, that while blockade of CD47 does increase tumour cell phagocytosis and antigen cross presentation in murine models, it also caused anaemia and thrombocytopaenia in human trials[3]. As such, the rationale underlying this experiment was to localise CD47 blockade to avoid such systemic toxicities. The authors developed an *E. coli* strain expressing an SLC and a CD47 targeting nanobody. Balb/c mice were infected with A20 lymphoma cells on both hind flanks and subsequently were treated with either phosphate-buffered saline (PBS), SLC *E. coli*, or SLC *E. coli* expressing CD47 nanobody[3]. Initially, the cohort treated with SLC+ bacteria showed slowing of tumour growth, owing likely to the release of bacterial PAMPs and thus innate immune activation. Ultimately, tumour progression in these mice showed no statistically significant difference to the PBS treated tumours[3] **(Figure 3)**.

By contrast, the nanobody treated group (anti-CD47nb SLC+) showed marked clearance of established A20 tumours. Unlike the other groups, these animals rarely developed liver metastases. 80% of these cohort survived more than 90 days and these animals did not develop tumours when rechallenged by injection of A20 cells, as opposed to the naïve mice which developed

Figure 3. **Summary of Chowdhury et al. (2019)[3]**



While SLC+ bacteria slow tumour progression, this is not statistically significantly greater than PBS-treated tumours. In contrast, anti-CD47nb SLC+ bacteria elicit regression in the tumour into which they are injected, as well as distant tumours and tumours established after bacteria injection.

tumours within one week of injection[3]. Further to these results, mouse tumours were injected with either recombinant anti-CD47 nanobody, sonicated SLC nanobody expressing *E. coli* or live SLC+ and nanobody expressing bacteria. Tumour growth was slowed in the first two groups but abolished entirely with whole SLC+ bacteria. These results illustrate the importance of drug delivery and the delivery system by which it uses. The self-renewing, immunogenic nature of the SLC+ bacteria doubtless played a crucial role in potentiating the immune responses to these tumours. Evidence for this was provided when the authors infected mice with A20 cells on either flank and treated only one side.

Remission was induced in both tumours and no therapeutic bacteria were detectable in the untreated tumour, indicating that tumour shrinking was induced by an adaptive immune response[3]. This is further evidence attesting to the necessity of bacterial population fluctuation punctuated by waves of PAMP, drug, and DAMP release. Potentiation of systemic immunity is itself evidence that the bacteria were in fact driving antigen release, uptake, and presentation. The fact that this was only seen in SLC+ bacteria further consolidates the notion that if bacteria are to be efficient delivery vehicles of targeted therapies to the tumour microenvironment, they have to persist for some duration, continuously deliver their gene products, and drive anti-tumour immunity.

Conclusion

Bacterial delivery of biologics is far from fantasy. Synchronised lysis circuits have been developed successfully to limit and manage the synthesis and deposition of these therapeutics to the tumour site. Furthermore, these circuits are self-limiting in nature, allowing for targeted delivery of otherwise systemically toxic agents into the tumour microenvironment. Promising in vivo evidence suggests that this method can induce tumour regression coupled with innate and adaptive immune responses. There have been some studies to date in humans, though admittedly without SLCs[16,17]. The in vivo evidence presented is currently limited to a small number of assays in murine models and requires replication. Further cancer models should be investigated, as should other therapeutic molecules. Only then will SLCs be fit for phase I human trials. Similar to a Trojan horse, we now know bacteria may infiltrate and colonize the tumour microenvironment, and now we know how to arm them. Challenges remain in finding suitable targets to differentiate malignant cells from self, and to generate efficacious nanobodies against them. In time, we may lay siege to tumours utilising these tiny Trojan bacilli. ◄

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Declarations

The author declares no conflicts of interest.

References

1. Coley WB. Contribution To the Knowledge of Sarcoma.' I. a Case of Periosieal Round-Celled Sarcoma of the Metacarpal Bone; Amputation of the Forearm; Gen-Eral Dissemination in Four Weeks; Death Six Weeks Later. Ii. the General Course and Prognosis of Sarcoma, Based. *Ann Surg* [Internet]. 1891;14(3):199–220. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1428624/pdf/annsurg01156-0040.pdf
2. Forbes NS. Engineering the perfect (bacterial) cancer therapy. *Nat Rev Cancer* [Internet]. 2010;10(11):785–94. Available from: http://dx.doi.org/10.1038/nrc2934
3. Chowdhury S, Castro S, Coker C, Hinchliffe TE, Arpaia N, Danino T. Programmable bacteria induce durable tumor regression and systemic antitumor immunity. *Nat Med* [Internet]. 2019;25(7):1057–63. Available from: http://dx.doi.org/10.1038/s41591-019-0498-z
4. Gurbatri CR, Lia I, Vincent R, Coker C, Castro S, Treuting PM, et al. Engineered probiotics for local tumor delivery of checkpoint blockade nanobodies. *Sci Transl Med*. 2020;12(530).
5. Abisado RG, Benomar S, Klaus JR, Dandekar AA, Chandler JR. Bacterial quorum sensing and microbial community interactions. *MBio*. 2018;9(3):1–14.
6. Mukherjee S, Bassler BL. Bacterial quorum sensing in complex and dynamically changing environments. *Nat Rev Microbiol* [Internet]. 2019;17(6):371–82. Available from: http://dx.doi.org/10.1038/s41579-019-0186-5
7. Papenfort K, Bassler BL. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol* [Internet]. 2016;14(9):576–88. Available from: http://dx.doi.org/10.1038/nrmicro.2016.89
8. Danino T, Mondragón-Palomino O, Tsimring L, Hasty J. A synchronized quorum of genetic clocks. *Nature*. 2010;463(7279):326–30.
9. Din MO, Danino T, Prindle A, Skalak M, Selimkhanov J, Allen K, et al. Synchronized cycles of bacterial lysis for in vivo delivery. *Nature* [Internet]. 2016;536(7614):81–5. Available from: http://dx.doi.org/10.1038/nature18930
10. Lee YJ, Jeong KJ. Challenges to production of antibodies in bacteria and yeast. *J Biosci Bioeng* [Internet]. 2015;120(5):483–90. Available from: http://dx.doi.org/10.1016/j.jbiosc.2015.03.009
11. Bannas P, Hambach J, Koch-Nolte F. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. *Front Immunol*. 2017;8(1603):1–13.
12. Hu Y, Liu C, Muyldermans S. Nanobody-based delivery systems for diagnosis and targeted tumor therapy. *Front Immunol*. 2017;8(NOV):1–17.
13. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Exp Mol Med* [Internet]. 2018;50(12):1–11. Available from: http://dx.doi.org/10.1038/s12276-018-0191-1
14. Kwang Chae Y, Arya A, Lams W, Cruz M, Chandra S, Choi J. Current landscape and future of dual anti- CTLA4 and PD-1/PD-L1 blockade immunotherapy in cancer; lessons learned from clinical trials with melanoma and non- small cell lung cancer (NSCLC). *J Immunother Cancer*. 2018;6(39):1–27.
15. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *N Engl J Med*. 2015;372(21):2006–17.
16. Toso JF, Gill VJ, Hwu P, Marincola FM, Restifo NP, Schwartzentruber DJ, et al. Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma. *J Clin Oncol*. 2002;20(1):142–52.
17. Duong MTQ, Qin Y, You SH, Min JJ. Bacteria-cancer interactions: bacteria-based cancer therapy. *Exp Mol Med* [Internet]. 2019;51(152). Available from: http://dx.doi.org/10.1038/s12276-019-0297-0