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Review

REVIEW – Pathogens & Pathogenicity

Oncolytic bacteria: past, present and future

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One sentence summary: This review presents a historical perspective of oncolytic bacteria together with the main oncolytic determinants and ongoing clinical trials on the oncolytic bacteria field.

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ABSTRACT

More than a century ago, independent groups raised the possibility of using bacteria to selectively infect tumours. Such treatment induces an immune reaction that can cause tumour rejection and protect the patient against further recurrences. One of the first holistic approximations to use bacteria in cancer treatment was performed by William Coley, considered the father of immune-therapy, at the end of XIX century. Since then, many groups have used different bacteria to test their antitumour activity in animal models and patients. The basis for this reactivity implies that innate immune responses activated upon bacteria recognition, also react against the tumour. Different publications have addressed several aspects of oncolytic bacteria. In the present review, we will focus on revisiting the historical aspects using bacteria as oncolytic agents and how they led to the current clinical trials. In addition, we address the molecules present in oncolytic bacteria that induce specific toxic effects against the tumors as well as the activation of host immune responses in order to trigger antitumour immunity. Finally, we discuss future perspectives that could be considered in the different fields implicated in the implementation of this kind of therapy in order to improve the current use of bacteria as oncolytic agents.

Keywords: cancer; oncolytic bacteria; cancer therapy; clinical trials in cancer; innate immune response; bacteria recognition

USE OF BACTERIA AGAINST TUMOURS, A HISTORICAL PERSPECTIVE

Oncolytic bacteria can be used to treat cancers by acting directly on tumour destruction and/or by stimulating the patient´s own immune system. As a consequence, this immune stimulation induces a reaction against the tumour cells. Oncolytic bacteria, like other microorganisms used to treat cancer have to be safe enough to specifically kill the tumour while sparing the patient's life.

Several bacterial genus have a preference for accumulating inside tumors, developing an oncolytic action. Over recent decades, different groups have studied the preferential tumour replication of *Salmonella* (Pawelek, Low and Bermudes 1997), *Streptococcus* (Maletzki *et al.* 2008), *Listeria* (Kim *et al.* 2009), *Escherichia* (Yu *et al.* 2004), *Clostridium* (Malmgren and Flanigan 1955), *Bifidobacterium* (Kohwi *et al.* 1978), *Caulobacter* (Bhatnagar *et al.* 2006), *Proteus* (Arakawa *et al.* 1968), *Lactobacillus* (Motevaseli *et al.* 2018), *Klebsiella* (Hetz *et al.* 2002) or *Mycobacterium* (Morales 2017). Specific oncolytic bacteria species and strains that will

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be described later have been characterised, and it is likely that many more yet to be characterised have similar or alternative antitumour activities. In recent years, immunotherapy has revisited the use of microorganisms to treat cancer since conventional approaches such as surgery, chemotherapy and radiotherapy are not powerful enough to cure all patients.

The idea of using microorganisms as a therapy against tumours is ancient. The first known report describing a connection between infections and cancer tissue was documented by the Egyptian physician Imhotep 2600 BC described in the Ebers Papyrus. Around 1320 Saint Pellegrino Laziosi suffered a cancer of the tibia that required amputation of the limb. Before the operation, his leg had been seriously infected, but this was followed by a complete spontaneous remission (Mager 2006; Jessy 2011). In the eighteenth and nineteenth centuries, independent observations described how some cancer patients experienced 'spontaneous remissions' of their malignancies after episodes of erysipelas, tuberculosis, malaria or syphilis or even intentionally opened wounds (Hoption Cann, Van Netten and Van Netten 2006). Many cases of spontaneous cancer cure have already been reviewed elsewhere (Hoption Cann *et al.* 2002).

In 1866, Wilhelm Busch in Germany documented the cure of a neck cancer in a patient after streptococcal infection of a cauterized wound (Kienle 2012). After this report, there were attempts to intentionally stimulate erysipelas in cancer patients, some of them effective, but dangerous for the patients. In 1882, Fehleisen was able to treat a tumour by the inoculation of *streptococci* causing erysipelas (Fehleisen 1882). Other examples of cancer tissue regression were reported in patients suffering from gas gangrene caused by *Clostridium perfingens* (Minton 2003). Unfortunately, the inability to control the bacteria inoculated in the patients makes this treatment unattractive and difficult or impossible to apply under today´s current patient safety regulations (Hoption Cann, Van Netten and Van Netten 2003).

Soon after, and probably unaware of the European reports, the American physician William Coley (1862–1936), became conscious of the potential use of streptococcal infections to treat cancer patients. Although his first experiments were performed using live *Streptoccocus pyogenes*, later successful attempts used a heat-inactivated mix of *Streptococcus pyogenes* with *Serratia marcensens*, creating the so called 'Coley toxin', also more recently named mixed bacterial vaccine (MBV) (Karbach *et al.* 2012). Coley´s toxin was well described by Stephen Hall in 'A commotion in the blood' (Hall 1997), and reviewed by many, including McCarthy (McCarthy 2006). Some formulations of Coley´s toxin were proven to be effective in many patients with operable and inoperable cancers. Coley's daughter, Helen Coley Nauts, documented the results of her father's work (Nauts, Swift and Coley 1946; Nauts and McLaren 1990). She revisited hundreds of microscopically confirmed cases treated with Coley's toxin. Reports of the effectiveness of bacteria treatment in different tumours have been summarised elsewhere (Kienle 2012).

The efficiency of Coley´s toxin seems to depend on several factors (Coley 1910; Pelner and Fowler 1959a,b; Johnston 1962; Nauts 1989), including stage of tumour progression, degree of tumour burden, immune-competence of the patient, effectiveness of the preparation, tumour localisation, injection site, dosage, regularity and increase of doses, timing in relation to surgery, febrile reactions achieved after injection and treatment of patients after radiation and/or chemotherapy. In the case of Coley´s toxin, most of these properties are directly connected to the ability of the treatment to activate patients' immune responses against the tumour.

Coley´s therapy was gradually abandoned during the middle of XX century. As compared to Coley´s approximation, new therapies at that time such as radiation and chemical therapies could be better reproduced and show more consistent results (Hoption Cann, Van Netten and Van Netten 2003). William Coley is recognised as laying the intellectual basis for actual immunological approaches against cancer. Coley´s therapeutic approximation was revisited in the 1970s, 1980s and 1990s and his work has been the inspiration for current immunotherapeutic approaches, including those that propose the use of microorganisms such as viruses or bacteria or stimulatory molecules derived from them. Immunotherapy has proved to be an effective therapeutic approach in a variety of cancers (Chen and Mellman 2017).

The activation of the patient's own immune response to react against tumour cells has been extensively explored. However the use of live, attenuated or inactivated bacteria as antitumour agents (with the exception of the *Mycobacterium bovis* bacillus Calmette-Guerin or BCG) has not been developed enough to be brought as a general strategy against cancer in the clinic. The success (and failure) in using bacteria as oncolytic agents may depend on patient factors that immunology could describe and characterise using current tools. Much work is needed to understand and improve bacterial oncolytic properties. Bacteria-specific oncolytic properties such as virulence factors or toxins are poorly characterised in the oncolytic context and nowhere close to being considered as tools by immunologists or clinicians. However, bacterial pathogen-associated molecular patterns (PAMPs) are recognised as one of the most potent tools able to enhance tumour immune reactivity (Linnebacher *et al.* 2011; Karbach *et al.* 2012).

Bacteria and viruses are highly immunogenic agents that can be used to trigger an immune response towards tumoral tissue. These organisms have particularly conserved structures that are recognised by the innate immune systems activating a proinflammatory response. These detection mechanisms lead to an inflammatory response against the tumour (Blander and Sander 2012). PAMP structures are recognised by the pattern recognition receptors (PRR). There are various PAMPs that are common for almost all bacteria. One of the most important is bacterial DNA, which triggers cGAS/STING signaling and is able to detect it when located in the cytoplasm of cells, and unmethylated GpG motifs common in bacterial DNA, which can activate the intracellular toll-like receptor 9 (TLR9). Lipoproteins are able to activate the toll-like receptor 2 (TLR2), and the N-Formylmethionine (f-Met) of the new synthetised bacteria proteins is detected and activates f-Met receptors 1 and 2. In addition to these universal PAMPs, different bacteria species have their own specific PAMPs that can trigger inflammation. The main bacteria PAMPs and PRRs of oncolytic bacteria are indicated in Table 1.

MYCOBACTERIUM BOVIS BACILLE CALMETTE–GUÉRIN (BCG), THE FIRST BACTERIUM APPROVED AS ONCOLYTIC AGENT

Coley´s therapeutic approach has been reexamined in the light of the success of the BCG bladder instillation therapy. BCG is an attenuated strain of, *Mycobacterium bovis*. At the present, intravesical BCG instillation is one of the best therapies for treating bladder cancer. However, despite its success, BCG is the only life bacterial therapy approved for clinical purposes against bladder cancer for almost 30 years (Morales 2017).

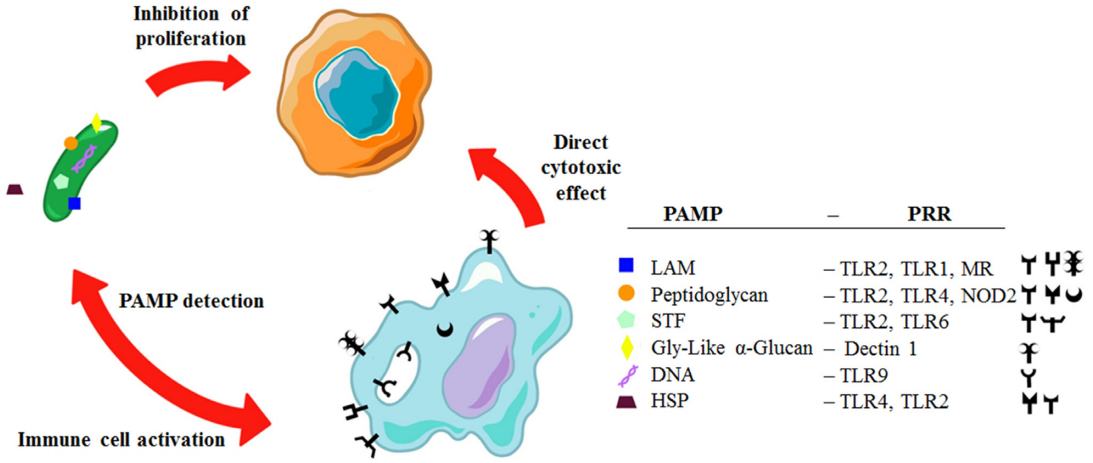

Figure 1. Mycobacterium bovis, a tool already in the clinic. The main known direct effect that was produced by BCG against the tumour cells is cell cycle arrest and cytoestaticity leading to a decrease in proliferation. Activation of immune cells takes place after the recognition of the multiple mycobacterium PAMPs and ending in a direct cytotoxic response against the tumour. In the images are listed the main BCG PAMPs and the PRRs implicated in its recognition.

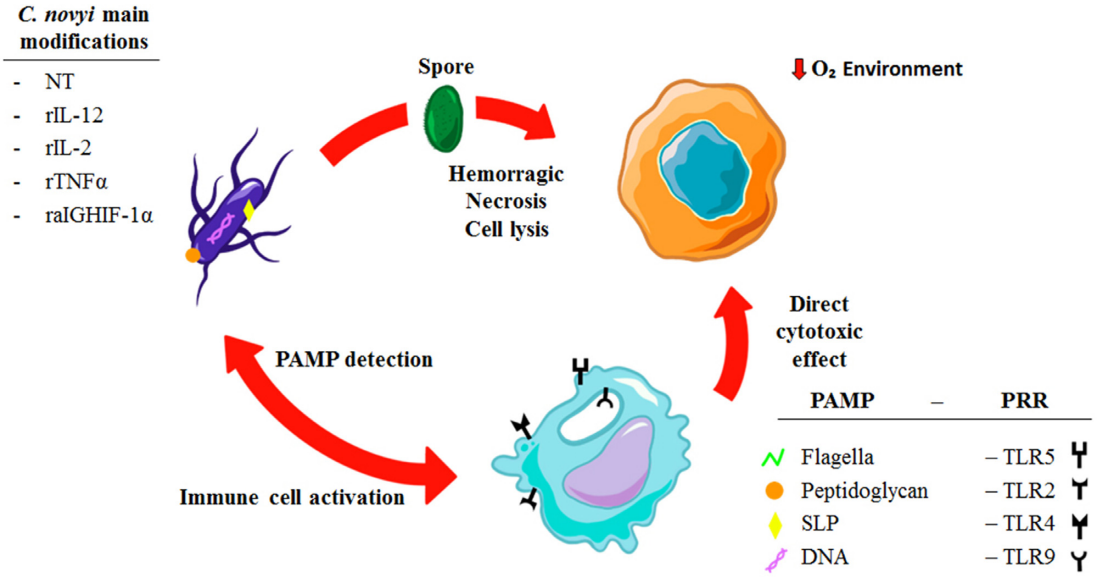

Figure 2. *Clostridium novyi*, reaching the tumour necrotic areas. *C. novyi* has motility, which allows the bacteria to grow in the most inaccessible zones. In addition, the bacteria can grow in the anoxygenic tissue, causing hemorrhagic necrosis and cell lysis. Immune cells are able to recognise the bacteria that are on the surface, becoming activated and reacting against the tumour cells. In the picture are represented the genetic modification done over *C. novyi* for oncolytic purposes and the PRRs that are able to detect its main PAMPs.

The most frequent etiological agent causing tuberculosis is the species *Mycobacterium tuberculosis*. *Mycobacterium bovis* is another species of the *Mycobacterium genus* that has been reported to cause tuberculosis in humans, (Cosivi *et al.* 1998). Both *Mycobacterium tuberculosis and Mycobacterium bovis* can multiply inside the alveolar macrophages, hidden to antibodies, producing significant tissue damage (Guirado, Schlesinger and Kaplan 2013).

Tuberculosis was a devastating disease in the 19th century, prevalent in Europe and America (Otis 1909). After Robert Koch´s discovery of *Mycobacterium tuberculosis* as the tuberculosis causing agent, there was great interest in developing vaccines against this deadly bacterium. Albert Calmette and Camille Guérin at the Institute Pasteur in Lille, were able to develop a low virulent strain of *Mycobacterium bovis* that was attenuated after repeated sub-culture of the bacterium over the years. BCG was proven to be safe in animals and was administered for the first time in humans in 1921 to neonates, proving its safety and efficacy (Luca and Mihaescu 2013). Soon after, BCG vaccine use was extended to the rest of Europe. Unfortunately 10 years later, dozens of infants died in Lübeck, Germany, after receiving a contaminated vaccine. This tragedy created a great distrust of BCG and other vaccines.

Several accidental findings in the first years of XX century reported a coincidental absence of cancer in patients that had died of tuberculosis (Pearl 1920). Studies using BCG by Old *et al.* after World War II demonstrated a strong BCG antitumoral activity in animals (Old, Clarke and Benacerraf 1959). In addition, BCG was also proven to have oncolytic activities against leukemias (Mathe *et al.* 1969) and melanomas (Morton *et al.* 1974), including a melanoma bladder metastasis response after intratumoural injection with BCG (Silverstein, Dekernion and Morton 1974).

In 1970, Zbar *et al.* proposed that for successful systemic BCG oncolytic therapy, direct contact between the tumour and

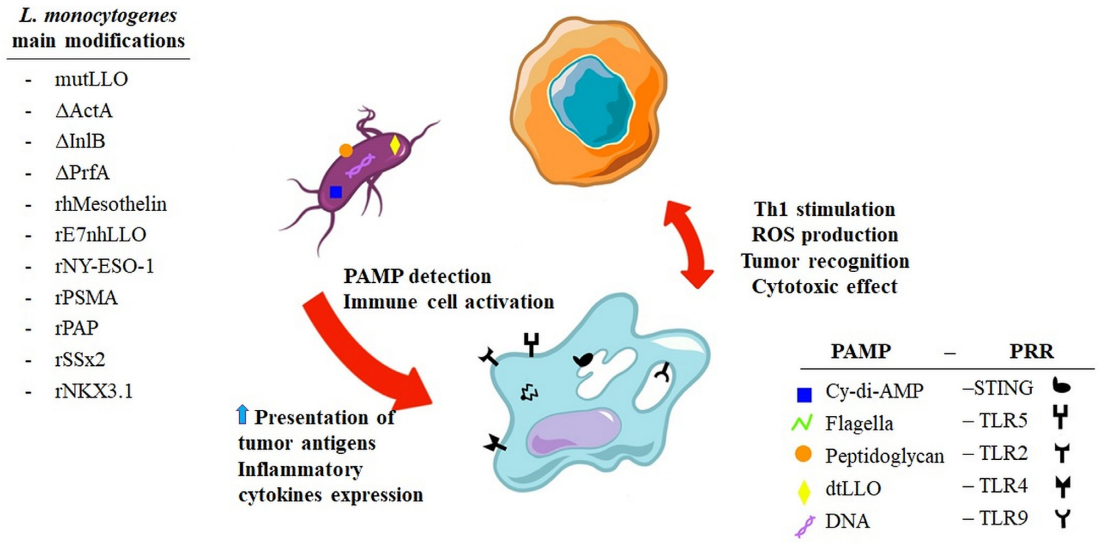

Figure 3. *Listeria monocytogenes*, triggering the immune system from inside. *L. monocytogenes* oncolytic properties rely in its ability to infect mainly immune cells. Genetic modifications of this bacterium allow the production of inflammatory cytokines or the presentation of tumour antigens directly from inside of the colonised cells, making easier the recognition of the tumour by the immune cells. In the picture are represented the main genetic modification used in *L. monocytogenes* as oncolytic treatment and the PRRs that are able to detect its major PAMPs.

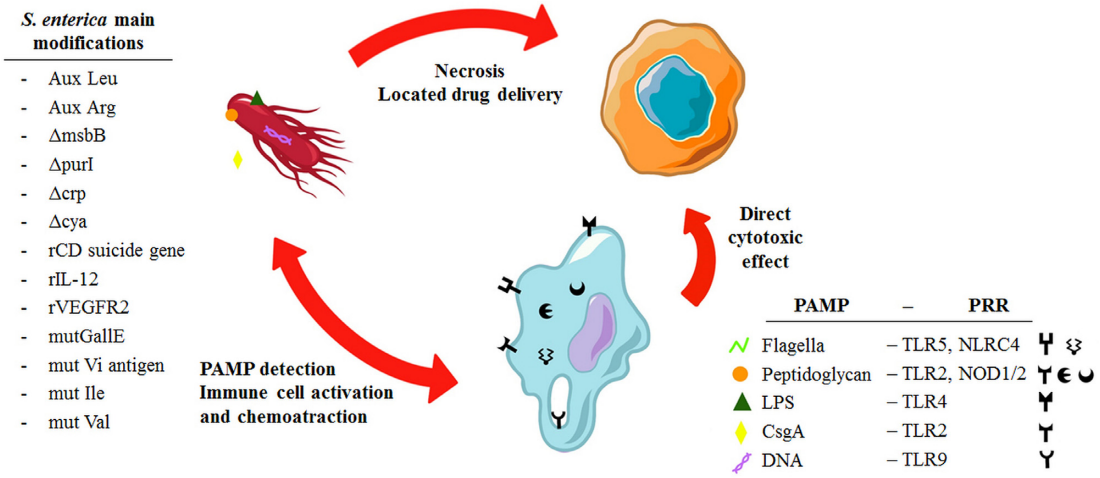

Figure 4. *Salmonella enterica*, a versatile tool for a huge range of tumors. *S. enterica* is able to colonise both aerobic and anaerobic tumours, growing inside them until they produce the cell bursting. The easy manipulation of *S. enterica* genome makes it a suitable tool for intratumoural drug delivery or cytokine expression. The detection of its PAMPs or the recombinant cytokines and chemokines produced by the bacteria are able to trigger an antitumoural response by the immune cells. The major modifications done to *S. enterica* and the main PAMPs recognised by the host cell PRRs are listed in the image.

bacteria was required (Zbar, Bernstein and Rapp 1971). In addition, important factors to be considered in this kind of therapy were an immune-competent patient, a sufficient number of living bacteria and a close proximity of the tumour to the injection area of the bacteria (Zbar, Bernstein and Rapp 1971). Later, further trials verified the efficacy of repeated doses of BCG against non-muscle invasive bladder cancer (Morales, Eidinger and Bruce 1976).

The National Cancer Institute sponsored two clinical trials at the University of Texas in San Antonio (UTSA) and The Memorial Sloan Kettering in New York that confirmed the efficacy of BCG. These trials showed a significant reduction in recurrence and longlasting protection, respectively (Buonaiuto *et al.* 1980; Lamm *et al.* 1980). In 1990, the US Food and Drug Administration approved the use of BCG and nowadays it is one of the best examples of immune-based treatments against cancer.

The precise mechanism by which BCG exerts its bladder cancer therapeutic effect is not fully understood. Upon administration, BCG can induce a local inflammation within the bladder mucosa that is enhanced following repeated instillations. Triggering of the inflammatory response is dependent on BCG attachment and internalisation into the urothelial cells. Fibronectin extracellular matrix protein is recognised by the BCG´s fibronectin activating protein (FAB) present in the tumour epithelium, inducing a caveolae-dependent endocytic mechanism (Coon *et al.* 2011). This BCG internalisation together with the recognition by macrophages triggers an influx of inflammatory cells and production of inflammatory cytokines which stimulates the activation of neutrophils, natural killer cells and reactive lymphocytes (Maruf, Brancato and Agarwal 2016). Among the activation of inflammatory response, some studies have seen a direct effect of BCG in the proliferation of tumour cells.

Table 1. Pathogen-associated molecular patterns and their receptors for the oncolytic bacteria used in clinical trials.

		ORGANISM				
PAMP	RECEPTOR	*M. bovis*	*C. novyi*	*L. monocytogenes*	*S. enterica*	*K. pneumoniae*
Peptidoglycans	TLR2	+	+	+	+	+
LPS	TLR4	NA	NA	NA	+	+
Flagella	TLR5, NLR2	NA	+	+	+	NA
DNA	TLR9	+	+	+	+	+
CsgA	TLR2	NA	NA	NA	+	NA
Lipoarabinomannan	TLR2, TLR1, MR	+	NA	NA	NA	NA
Glycogen-Like Alpha-Glucan	Dectin 1	+	NA	NA	NA	NA
Heat Shock Proteins	TLR2, TLR4	+	NA	NA	NA	NA
Soluble Tuberculosis Factor	TLR2	+	NA	NA	NA	NA
Muramyl Dipeptides	NOD2	+	NA	NA	NA	NA
Surface Layer Proteins	TLR4	NA	+	NA	NA	NA
Capsular polysaccharides	DC-SIGN, MR	NA	NA	NA	NA	+
Outer Membrane protein A	TLR2	NA	NA	NA	NA	+
Cyclic di-AMP	STING	NA	w	+	NA	NA
ActA	Increase antigen presentation	NA	NA	+	NA	NA
Listeriolysin O	TLR4	NA	NA	+	NA	NA

The BCG fibronectin-mediated cell adhesion induces the integrin receptor cross-linkage leading to cell cycle arrest. Mycobacteria treatment of tumour cells is related with an increase of cells in G0/G1 interphase inducing a cytostatic effect (Chen *et al.* 2005).

DETERMINANTS OF *MYCOBACTERIUM BOVIS* BACILLUS CALMETTE-GUÉRIN REACTIVITY AND IMMUNOGENICITY

In addition to the direct cytolytic effect of BCG on the tumoral tissue, this bacterium is able to trigger the activation of the innate immune system that triggers a localised inflammation. This localised proinflammatory state allows the immune system to recognise tumoral antigens enhancing the antitumoral immune response. Cell recognition by the innate immune system seems to occur mainly through the mycobacteria cell wall (Fig. 1). PAMPs located at the cell wall activate TLR2 and TLR4 in macrophages and trigger the proinflammatory responses (Tsuji *et al.* 2000). Moreover, the muramyl dipeptides of the different species of mycobacteria are recognised in the macrophage cytoplasm by NOD2 (Ferwerda *et al.* 2005; Ferwerda *et al.* 2007; Schenk *et al.* 2016). In addition, mycobacterial glycolipid lipoarabinomannan (LAM), located on the cell surface, especially the uncapped ones, such as AraLAM, as well as the phosphatidylinositol mannoside precursors of LAMs have the ability to activate TLR2 and TLR1 (Tapping and Tobias 2003). The mannose-modified LAMs (ManLAM) are able to induce the activation of the Mannose Receptor (MR) that belongs to the C-Type lectin receptor family (Kleinnijenhuis *et al.* 2011). Finally, some mycobacteria produce a glycogen-like α-glucan that can trigger Dectin 1 activation (Dinadayala *et al.* 2004).

Besides the cell surface PAMPs, there are some secreted proteins that are able to trigger the proinflammatory response. The soluble tuberculosis factor (STF) is able to induce the interaction with TLR6 and activate TLR2 (Bulut *et al.* 2001). *Mycobacterium tuberculosis* also secretes the Heat Shock Proteins (HSP) that are a set of proteins that play an important role in the pathogenesis of *M. tuberculosis*. The HSP60 and 65 are recognised by the TLR4 while the HSP70 can activate both TLR4 and TLR2 (Winters and Cederbaum 1992; Ohashi *et al.* 2000). As in almost all bacterial pathogens, TLR9 has been shown to be activated in response to unmethylated CpG motifs in mycobacterial infections (Bafica *et al.* 2005). Some studies have reported the activation of TLR8 to pulmonary tuberculosis and BCG treatments, but the specific PAMPs that could be triggering this activation remain unknown (Davila *et al.* 2008).

MYCOBACTERIUM BOVIS BACILLUS CALMETTE-GUÉRIN IN CLINICAL TRIALS

BCG has been used since 1976, mainly in the treatment of superficial bladder cancer where it has been shown to inhibit its proliferation and recurrence (Lamm 1985; Shinka *et al.* 1989). Nevertheless, for reasons yet to be clarified, around 40% of patients have tumour recurrence after BCG therapy (Malmstrom *et al.* 2009). Current efforts in the treatment of those patients are focused on the combination of BCG with other oncolytic therapies including surgery, chemotherapy, radiotherapy and more recently immunotherapy (Zheng *et al.* 2015). BCG is by far the bacterium under the largest number of clinical trials in cancer malignancies. Near to one hundred recent or ongoing clinical trials have explored or are exploring the improvement of BCG by combining the use of the bacteria with other oncolytic agents against non-disseminated bladder cancer.

In addition to bladder cancer, BCG is being studied as a treatment for other cancer types. Different clinical trials have explored the use of BCG in the treatment of neoplasias such as lung cancer (NCT02333474, NCT00003279, NCT00006352, NCT00037713), melanoma (NCT01838200, NCT01729663, NCT01013623, NCT00671554, NCT00477906, NCT00052156, NCT00052130, NCT00003715), prostate cancer (NCT00514072), colorectal cancer (NCT00427570, NCT00016133, NCT00007826), ovarian cancer (NCT00003386), breast cancer (NCT00003184), neuroblastoma and sarcoma (NCT00003023).

RECENT AND CURRENT USE OF OTHER BACTERIA IN CLINICAL TRIALS

In recent years there has been an increasing interest in the use of bacteria against some tumours that cannot be easily

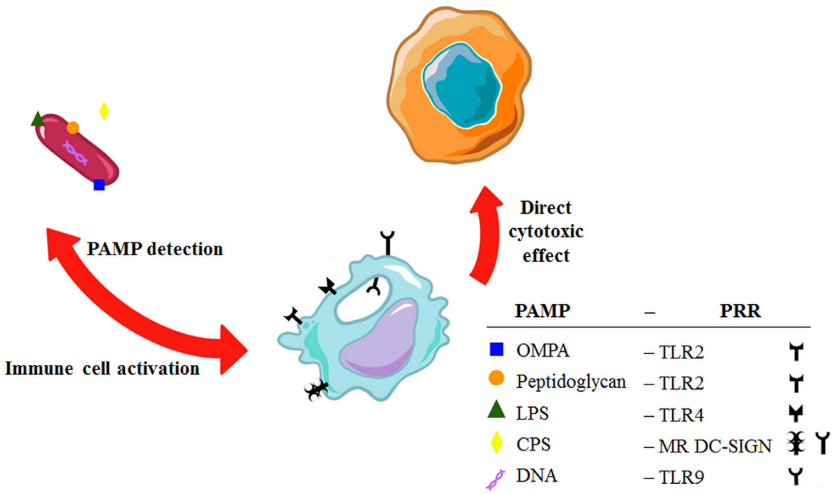

Figure 5. *Klebsiella pneumoniae*, a new oncolytic possibility to be better characterised. The PAMPs of *K. pneumoniae* are able to trigger the immune activation and its direct reactivity against the tumour cells. In the image are shown the main PAMPs of *K. pneumoniae* and the PRRs capable of recognize them.

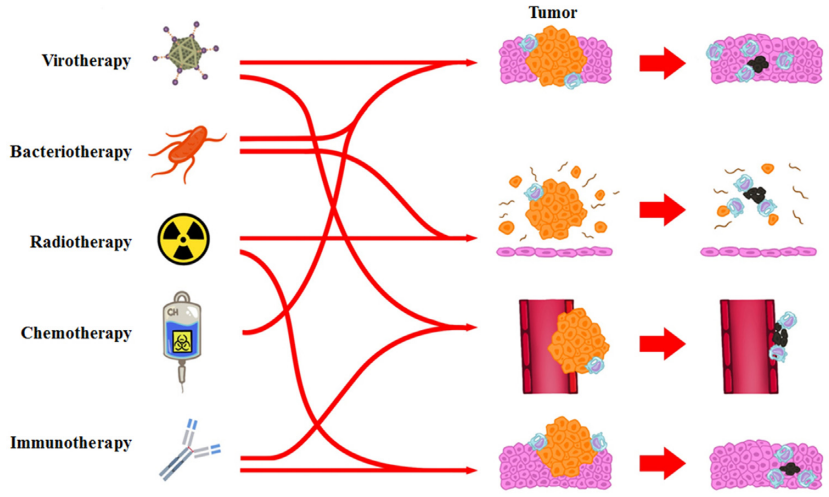

Figure 6. Different tumour, different strategy. The many possible effective treatments against cancer seem to be a work of multiple therapies, being the new techniques compatible with the traditional ones. Due to the huge difference between tumour types, the use of a different combination of therapies for each one, or a group of them, looks like the better approximation will be determined by the cancer type, the tumour type and the best possible set of therapies in each case.

treated using conventional therapies or if treated, have a window of improvement that could be enhanced using live, attenuated or inactivated bacteria. *Lactobacillus, Bifidobacterium* or specific strains of *Escherichia coli* have been used in different clinical trials in patients to improve gastric conditions, where gastric mucosa and gastric microbiota have been altered by aggressive anticancer treatments. Although this approach may have a dual effect, both, improving the gastric function and the immune-stimulation of the patient against the tumour cells, we are not going to refer to such bacteria since they are not yet considered as direct oncolytic agents. The use of such probiotics in patients has been reviewed elsewhere (Motevaseli, Dianatpour and Ghafouri-Fard 2017).

Notwithstanding BCG, several bacteria are currently under study as oncolytic agents. Some of them have had very promising results and may be an alternative or complementary treatment to current therapies in the near future. The possibility of modifying current strains and 'domesticating' those bacteria to treat tumours is an interesting challenge for bacteriologists and immunologists. We will describe here the bacteria used in recently finished or ongoing clinical trials. Some of the current clinical trials are also testing the possibility of combining different microbial agents. In Table 2 is listed the Gram-positive bacteria used in clinical trials and in Table 3 the Gram-negative ones.

CLOSTRIDIUM, AN ANAEROBIC BACTERIUM WITH PREFERENCE FOR NECROTIC TUMORS

Blood access to certain parts of hypoxic tumors could be a handicap in some oncolytic treatments. When blood in cancerous tissue is insufficient, the tumour environment becomes hypoxic and acidified and there is a reduction in the nutrients availability for tumor cells (Allen *et al.* 2016).

Clostridium spp. are Gram-positive, motile, obligate anaerobic bacterium, that can form spores (Maclennan 1962). They usually inhabit soils, water and the intestinal tract of animals, including humans (Maclennan 1962). Some species cause human pathologies such as *Clostridium botulinum*, which is the etiologic agent of botulism, *Clostridium tetani* or *Clostridium difficile that* cause

Table 2. Main Gram-positive oncolytic bacteria together with their specific characteristics and the clinical trials where they have been utilised.

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Mixed vaccines								
Mixed Bacteria Vaccine (MBV):*Streptococcus pyogenes* and *Serratia marcescens*	*Serratia marcescens* and *Streptococcus pyogenes* (W. Colley).	NCT00623831	A Phase 1 Study of Mixed Bacteria Vaccine (MBV) in Patients With Tumors Expressing NY-ESO-1 Antigen	Melanoma. Sarcoma. Gastrointestinal Stromal tumour. Head and Neck cancer. Transitional cell carcinoma. Prostate cancer. Ovarian Carcinoma. Esophaheal cancer. Breast cancer	Phase 1	2007/2013	Completed	Total: 12 Partial respose: 1 No evidence of disease: 2
Mixed vaccine:(MV): DTP, BCG, Measles virus, *Serratia marcescens* and S. *pneumoniae*.	Lipid suspension with 5 vaccines, including DPT (diphtheria, pertussis, and tetanus), BCG (Bacille Calmette-Guerin vaccine), Measles virus, *Serratia marcescens* and *Streptococcus pneumoniae*.	NCT02333474	Safety and Efficacy of Mix Vaccine in Lung Carcinoma Patient	Lung Neoplasms	Phase 1 and Phase 2	2015	Completed	Total: 20 No results posted
Clostridium spp.								
Clostridium novyi NT	*C. novyi* toxin A deficient. Spores	NCT00358397	One-Time Injection of Bacteria to Treat Solid Tumors That Have Not Responded to Standard Therapy	Tumors	Phase I	2006/2008	Terminated (Design Problem)	Total: 2 No results posted
Clostridium novyi NT	*C. novyi* toxin A deficient. Spores	NCT01118819	Safety Study of *Clostridium novyi*-NT Spores to Treat Patients With Solid Tumors That Have Not Responded to Standard Therapies	Solid tumour malignancies	Phase I	2013/2016	Terminated (The study was repeted at NCT01924689)	Total: 5
Clostridium novyi NT	*C. novyi* toxin A deficient. Spores	NCT01924689	Safety Study of Intratumoral Injection of *Clostridium novyi*-NT Spores to Treat Patients With Solid Tumors That Have Not Responded to Standard Therapies	Solid tumour malignancies	Phase I	2013/2018	Completed	Total: 24 Results under submission
Combination of *Clostridium novyi* NT combined with with Pembrolizumab	*C. novyi* toxin A deficient.	NCT03435952	Pembrolizumab With Intratumoral Injection of *Clostridium novyi*-NT	Neoplasm of Breast, digestive organs, eye, CNS, Female and male genital organs, lip, oral and Pharinx, Mesothelial and soft tissue, respiratory organs, endocrine galnds, urinary tract.	Phase I	2018/2021	Recruiting	Estimated total: 18

Table 2. Continued

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Listeria								
Listeria monocytogenes ANZ-100	Attenuated double-deleted *Listeria monocytogenes* ANZ-100 has engineered deletions of two genes encoding virulence determinants ActA and Internalin B (inlB, *Lm* Δ*actA*/Δ*inlB*)	NCT00327652	Study of Safety and Tolerability of Intravenous CRS-100 in Adults With Carcinoma and Liver Metastases	Study of Safety and Tolerability of Intravenous CRS-100 in Adults With Carcinoma and Liver Metastases	Phase I	2006/2008	Completed	Total: 9 No results posted
Listeria monocytogenes CRS-207	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT00585845	Study of Safety and Tolerability of Intravenous CRS-207 in Adults With Selected Advanced Solid Tumors Who Have Failed or Who Are Not Candidates for Standard Treatment	Malignant Epithelial mesothelioma. Adencarcinoma of pancreas. Carcinoma,. Non-small-cell lung cancer. Adenocarcinoma of the ovary.	Phase I	2007/2009	Terminated (No reason listed)	Total: 17 No results posted
Listeria monocitogenes ADXS 11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT01116245	An Assessment of an Attenuated Live Listeria Vaccine in CIN 2+	Cervical Intraepithelial Neoplasia	Phase II	2010/2016	Terminated (Study stopped due to lack of enrollment)	Total: 81 No results posted
Listeria monocytogenes. ADXS11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT01266460	Vaccine Therapy in Treating Patients With Persistent or Recurrent Cervical Cancer	Cervical Adenocarcinoma. Cervical Adenosquamous Carcinoma. Cervical Squamous Cell Carcinoma, Not Otherwise Specified. Recurrent Cervical Carcinoma.	Phase II	2010/2018	Active, not recruiting	Estimated total: 61
Listeria monocytogenes CRS-207 combined with GVAX (whole tumour cells genetically modified to secrete GM-CSF) and Cyclophosphamide.	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT01417000	Safety and Efficacy of Combination Listeria/GVAX Immunotherapy in Pancreatic Cancer	Metastatic Pancreatic Cancer	Phase II	2011/2017	Completed	Total: 93 Serious AE: 39 AE: 93
Listeria monocytogenes. ADXS11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT01598792	Safety Study of Recombinant *Listeria monocytogenes* (Lm)Based Vaccine Virus Vaccine to Treat Oropharyngeal Cancer	HPV-16 oropharyngeal carcinoma.	Phase I	2012/2014	Terminated (Patient 2 suffered DLT post-vaccination) Dose limit toxicity??	Total: 2 No results posted

Table 2. Continued

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Listeria monocytogenes CRS-207	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT01675765	Safety and Efficacy of Listeria in Combination With Chemotherapy as Front-line Treatment for Malignant Pleural Mesothelioma	Malignant Pleural Mesothelioma	Phase I	2012–2018	Active, not recruiting	Actual total: 60
Listeria monocytogenes CRS-207 combined with GVAX (whole tumour cells genetically modified to secrete GM-CSF)	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT02004262	Safety and Efficacy of Combination Listeria/GVAX Pancreas Vaccine in the Pancreatic Cancer Setting	2nd-line, 3rd-line and Greater Metastatic Pancreatic Cancer	Phase II	2013/2018	Completed	Total: 303 slight increase in life expectancy for CRS-207 treated patients
Listeria monocitogenes ADXS 11–001 combined with 5FU, Mitomycin and IMRT radiotherapy.	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT01671488	A Phase I/II Evaluation of ADXS11–001, Mitomycin, 5-fluorouracil (5-FU) and IMRT for Anal Cancer	Anal Cancer	Phase I and Phase II	2013/2018	Terminated (pilot data to be used for upcoming larger trial)	Total: 11 No results posted
Listeria monocytogenes ADU-623	live-attenuated *Listeria monocytogenes* vaccine (ΔactA/ΔinlB) expressing EGFRVIII and NY-ESO-1	NCT01967758	Phase I Study of Safety and Immunogenicity of ADU-623	Astrocytic Tumors. Glioblastoma Multiforme. Anaplastic Astrocytoma. Brain Tumor.	Phase I	2013/2018	Completed	Total: 11 No results posted
Listeria monocitogenes ADXS 11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT02002182	ADXS 11–001 Vaccination Prior to Robotic Surgery, HPV-Positive Oropharyngeal Cancer	Head and Neck Cancer, Squamous Cell Carcinoma of the Head and Neck, HPV Positive Oropharyngeal Squamous Cell Carcinoma.	Phase II	2013/2019	Recruiting	Estimated total: 30
Listeria monocytogenes CRS-207 combined with GVAX (whole tumor cells genetically modified to secrete GM-CSF), Cyclophosphamide and Nivolumab	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT02243371	GVAX Pancreas Vaccine (With CY) and CRS-207 With or Without Nivolumab	Previously Treated Metastatic Adenocarcinoma of the Pancreas	Phase II	2014/2019	Active, not recruiting	Actual total: 96
Listeria monocytogenes JNJ-64 041 809	Live attenuated *Listeria monocytogenes* by the deletion of the coding sequences of two wild-type virulence determinants (ActA and Internalin B.	NCT02625857	Safety & Immunogenicity of JNJ-64 041 809, a Live Attenuated Double-deleted Listeria Immunotherapy, in Participants With Metastatic Castration-resistant Prostate Cancer	Prostatic Neoplasms, Castration-Resistant	Phase 1	2015/2018	Completed	Total: 26 No results posted

Table 2. Continued

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Listeria monocytogenes JNJ-64 041 757	Recombinant live attenuated *Listeria monocytogenes* by the deletion of the coding sequences of two wild-type virulence determinants (ActA and Internalin B and heterologous gene expression by stable integration of epidermal growth factor variant III (EGFRvIII) human mesothelin (hMeso) expression cassette into the tRNAArg locus of the Lm ΔactA/ΔinlB chromosome.	NCT02592967	Safety & Immunogenicity of JNJ-64 041 757, Live-attenuated Double-deleted Listeria Immunotherapy, in Subjects With Non Small Cell Lung Cancer	Carcinoma, Non-Small-Cell Lung	Phase I	2015/2020	erminated (Discontinued due to lack of efficacy)	Total: 18 No results posted
Listeria monocitogenes ADXS 11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT02164461	ADXS11–001 High Dose HPV + Cervical Cancer	Effects of Immunotherapy, Metastatic/Recurrent Cervical Cancer, Cervical Adenocarcinoma, Cervical Adenosquamous Cell Carcinoma, Cervical Squamous Cell Carcinoma, Cervical Small Cell Carcinoma, Stage III Cervical Cancer, Stage IVA Cervical Cancer, Stage IVB Cervical Cancer.	Phase I and Phase II	2015/2018	Completed	Total: 25 No results posted
Listeria monocitogenes ADXS 11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT02399813	Phase 2 Study of ADXS11–001 in Subjects With Carcinoma of the Anorectal Canal	Anal Cancer, Rectal Cancer	Phase II	2015/2022	Active, not recruiting	Total: 55
Listeria monocitogenes ADXS 11–001 combined with Pemetrexed.	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT02531854	A Study of Pemetrexed Maintenance With or Without ADXS11–001 Immunotherapy in Patients With Human Papillomavirus Positive (HPV +), NSCLC Following First-Line Induction Chemotherapy	Carcinoma, Non-Small-Cell Lung	Phase II	2016	Unknown (Not yet recruiting)	Estimated total: 124

Table 2. Continued

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Listeria monocytogenes CRS-207 combined with Pembrolizumab and Epacadostat.	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT02575807	Safety and Efficacy of CRS-207 With Epacadostat in Platinum Resistant Ovarian, Fallopian or Peritoneal Cancer	Platinum-resistant Ovarian Cancer. Platinum-resistant Fallopian Cancer. Platinum-resistant Peritoneal cancer.	Phase I and Phase II	2016/2018	Terminated (Study was stopped due to low enrollment and lack of clinical activity)	Total: 35 REVISAR
Listeria monocitogenes ADXS 11–001	Recombinant live attenuated *Listeria monocytogenes* (Lm) with a deletion in transcription factor PrfA encoding human papillomavirus (HPV) type 16 E7 fused to a non-hemolytic listeriolysin O.	NCT02853604	Study of ADXS11–001 in Subjects With High Risk Locally Advanced Cervical Cancer	High Risk Cervical Cancer, Advanced Cervical Cancer	Phase III	2016/2021	Active, not recruiting	Estimated total: 450
Listeria monocytogenes pLADD.	Attenuated double-deleted *Listeria monocytogenes* in actA and internalin B genes strains that have been engineered to express tumor-associated antigens.	NCT03189030	Study of Personalized Immunotherapy in Adults With Metastatic Colorectal Cancer	Colorectal neoplasm	Phase I	2017/2020	Active, not recruiting	Total: 28
Listeria monocytogenes CRS-207 combined with Pembrolizumab	Recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT03122548	Safety and Efficacy of CRS-207 With Pembrolizumab in Gastric, Gastroesophageal Junction or Esophageal Cancers	Gastric Adenocarcinoma, Gastroesophageal Junction Adenocarcinoma and Esophageal Adenocarcinoma	Phase II	2017/2019	Terminated (Low enrollment and lack of clinical activity in other CRS-207 studies)	Total: 5 No response in any patient
Listeria monocytogenes CRS-207 combined with Pembrolizumab	Recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT03175172	Evaluation of CRS-207 With Pembrolizumab in Previously Treated MPM	Malignant Pleural Mesothelioma	Phase II	2017/2019	Terminated (Study was stopped due to low enrollment and lack of clinical activity)	Total: 10 No response in any patient Stable disease: 3
Listeria monocytogenes CRS-207 combined with GVAX (whole tumor cells genetically modified to secrete GM-CSF), Cyclophosphamide, Ipilimumab and Nivolumab.	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT03190265	Study of CRS-207, Nivolumab, and Ipilimumab With or Without GVAX Pancreas Vaccine (With Cy) in Patients With Pancreatic Cancer	Pancreatic Cancer	Phase II	2017/2019	Recruiting	Estimated total: 63

Table 2. Continued

Bacteria used in the trial	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Listeria monocytogenes JNJ-6 404 175 in combination with Nivolumab	Recombinant live attenuated *Listeria monocytogenes* by the deletion of the coding sequences of two wild-type virulence determinants (ActA and Internalin B and heterologous gene expression by stable integration of epidermal growth factor variant III (EGFRvIII) human mesothelin (hMeso) expression cassette into the tRNAArg locus of the Lm ΔactA/ΔinlB chromosome.	NCT03371381	An Efficacy and Safety Study of JNJ-64 041 757, a Live Attenuated *Listeria monocytogenes* Immunotherapy, in Combination With Nivolumab Versus Nivolumab Monotherapy in Participants With Advanced Adenocarcinoma of the Lung	Carcinoma, Non-Small-Cell Lung	Phase I and Phase II	2018/2022	Terminated (Discontinued due to lack of clinical benefit observed in the Phase 1b portion of the study)	Total: 12 No results posted
Listeria monocytogenes CRS-207 combined with GVAX (whole tumor cells genetically modified to secrete GM-CSF), Pembrolizumab, Cyclophosphamide and Epacadostat	recombinant *Listeria monocytogenes* with a deletion in actA and internalin B genes and expressing human mesothelin	NCT03006302	Epacadostat, Pembrolizumab, and CRS-207, With or Without CY/GVAX Pancreas in Patients With Metastatic Pancreas Cancer	Metastatic Pancreatic Adenocarcinoma	Phase II	2018/2023	Recruiting	Estimated total: 70

Table 3. Main Gram-negative oncolytic bacteria together with their specific characteristics and the clinical trials where they have been utilised.

Bacteria used in the trial Salmonella	Bacteria characteristics	Trial number	Name of the Trial	Treatment	Clinical Phase	Starting/Ending	Status	Participants
Salmonella typhimurium VXM01	Recombinant VXM01 attenuated *Salmonella typymurium* Ty21a (defficient in multiple genes s, including the genes responsible for the production of Vi) encoding VEGFR2	NCT02718443	VXM01 Phase I Pilot Study in Patients With Operable Recurrence of a Glioblastoma	Glioblastoma	Phase I	2016/2018	Completed	Total: 14 No results posted
Salmonella typhimurium VXM01	Recombinant VXM01 attenuated *Salmonella typymurium* Ty21a (defficient in multiple genes s, including the genes responsible for the production of Vi) encoding VEGFR2	NCT01486329	VXM01 Phase I Dose Escalation Study in Patients With Locally Advanced, Inoperable and Stage IV Pancreatic Cancer	Stage IV pancreatic cancer	Phase I	2011/2015	Completed	Total: 72 No results posted
Salmonella typhimurium VXM01	Recombinant VXM01 attenuated *Salmonella typymurium* Ty21a (defficient in multiple genes s, including the genes responsible for the production of Vi) encoding VEGFR2	NCT02718430	VXM01 Phase I Study in Patients With Metastatic Colorectal Cancer With Liver Metastasis	Colorectal Cancer	Phase I	2016/2018	Completed	Total: 6 No results posted
Salmonella typhimurium VNP20009	*Salmonella typhimurium* VNP20009 attenuated by chromosomal deletion of the purI and msbB genes	NCT00006254	VNP20009 in Treating Patients With Advanced Solid Tumors	Unspecified Adult Solid Tumor,	Phase I	2000/2008	Completed	Estimated total: 15–45 No results posted
Salmonella typhimurium VNP20010	*Salmonella typhimurium* VNP20009 attenuated by chromosomal deletion of the purI and msbB genes	NCT00004216	VNP20009 in Treating Patients With Advanced or Metastatic Solid Tumors That Have Not Responded to Previous Therapy	Unspecified Adult Solid Tumor,	Phase I	1999/2008	Completed	Estimated total: 12–40 No results posted
Salmonella typhimurium VNP20009	*Salmonella typhimurium* VNP20009 attenuated by chromosomal deletion of the purI and msbB genes	NCT00004988	Treatment of Patients With Cancer With Genetically Modified *Salmonella typhimurium* Bacteria	Cancer. Neoplasm. Neoplasm and metastasis.	Phase I	2000/2002	Completed	Total: 45 No results posted
Attenuated *Salmonella typhimurium* (virulent strain x4550)	Recombinant Attenuated *Salmonella typhimurium* (virulent strain x4550) expressing human IL-2	NCT01099631	IL-2 Expressing, Attenuated *Salmonella typhimurium* in Unresectable Hepatic Spread	Liver Neoplasms. Biliary Cancer	Phase I	2010/2014	Completed	Total: 22 No results posted
Klebsiella *Klebsiella pneumoniae* QBKPN SSI	QBKPN SSI	NCT02256852	Exploratory Study of QBKPN SSI in Non-Small Cell Lung Cancer	Non-Small Cell Lung Cancer	Phase II	2014/2016	Completed	Total: 6 No results posted

tetanus and diarrhea, respectively. The main virulence factors of the species of *Clostridium* are the different secreted toxins that produce numerous alterations in the host. However, there are other *Clostridium* species that do not cause human pathologies.

Clostridium spp. are one of the most promising agents in cancer treatment due to their obligate anaerobic growth, spore forming abilities and potential restriction for the tumor. Moreover, systemic administration of spores has a very interesting property, since they will remain dormant till they reach hypoxic environments like the ones created in the necrotic areas inside of solid tumors. This property could be employed in the treatment of some hypoxic tumors. In this context, Malmgren and Flanigan demonstrated that anaerobic bacteria could survive and replicate in necrotic tumor tissue which had low oxygen content (Malmgren and Flanigan 1955).

The oncolytic mechanism triggered by Clostridium is based on the specific ability of these bacteria to replicate in anaerobic and necrotic tumour microenvironments. *Clostridium sp*. can secrete exotoxins like phospholipases, haemolysins or lipases that can damage tumour cells (Adkins *et al*. 2012; Brito *et al*. 2018). Upon infection of the tumour microenvironment, clostridia infection also induces the activation of macrophages and neutrophils preventing the spread of bacteria into surrounding tissues and increases the expression of cytokines, chemokines and the activation of the adaptive immune response (Staedtke *et al*. 2015).

Among the *Clostridium* genus members, *Clostridium novyi* has been selected for oncolytic therapy based on its particular properties. This bacterium has been considered the etiologic agent of a variety of diseases in man and animals (Hatheway 1990). *Clostridium novyi* synthesises five different toxins (α, β, γ, ε, ι). Its status as a strict anaerobic organism gives *C. novyi* a very high specificity against non-vascularised tumors, where anoxic conditions favour the replication of the bacterium. Spores do not have optimal germination conditions till oxygen is absent (Dang *et al*. 2001). *C. novyi* has a potent cytotoxic effect against tumour cells, generating hemorrhagic necrosis and cell lysis *in vivo* (Roberts *et al*. 2014). The *C. novyi´s* genome has been modified in order to inactivate toxins such as the α-Toxin. This artificial mutant, named *C. novyi*-NT has lower toxicity in the recipient of the therapy, preserving its oncolytic features (Dang *et al*. 2001).

Administration of large doses of *C. novyi*-NT spores directly into the blood stream is rapidly eliminated by the organisms. Twenty-four hours after administration, 90% of the spores disappear from blood stream, but some can germinate in tumor tissues. The associated toxicity to this inoculation is almost absent at a systemic level (Diaz *et al*. 2005). However, the number of infective spores that reach the tumor tissues is much lower than that obtained through direct intratumoral injection (Roberts *et al*. 2014). The number of spores inside the tumor can be increased with the administration of vascularisation inhibitors such as combretastatin A-4 phosphate (Theys *et al*. 2001). Due to the presence of flagella, the distribution of *C. novyi* in the less vascularised areas of the tumour occurs homogeneously, a quality that provides this bacterium with an advantage over other non-motile anaerobic organisms (Dang *et al*. 2001).

The presence of necrotic tissue is one important restriction factor of access for some conventional cancer treatments such as chemotherapies (Varna *et al*. 2014). Since advanced solid tumours usually present hypoxic areas, in many cases, they eventually evolve into necrotic tumours. Clostridia therapy could thus be used for the treatment of this type of advanced and hypoxic tumors such as colorectal cancer (Pollheimer *et al*. 2010), melanoma (Ladstein *et al*. 2012) or lung cancer (Zunzov *et al*. 1975) among other solid tumours that present necrotic features. In addition, necrotic areas may prevent the effectiveness of alternative treatments such as the use of oncolytic viruses, since they require living cells to replicate, and are more difficult to reach. In this scenario, clostridia can proliferate and grow using necrotic material as a source of nutrients. At some point, the immune system should detect the bacteria outgrowth and become activated upon clostridia detection.

DETERMINANTS OF *CLOSTRIDIUM NOVYI* REACTIVITY AND IMMUNOGENICITY

Besides the direct oncolytic effect of the bacterium, it is important to mention the immunotherapeutic characteristics of *C. novyi*. Antitumour immune stimulation by *C. novyi* is important from at least two different points of view. In order to prevent bacterial dissemination and undesired side effects, the bacteria have to trigger innate immune response mechanisms that can initiate the host immune defence. In parallel, this recognition of the microorganism, only replicating within the tumour, will trigger an immune response not only against the bacteria, but also against tumour-specific antigens, also present in the area of replication (Fig. 2).

As said previously, initiation of the immune recognition occurs though the so-called pathogen-associated molecular patterns or PAMPs. In addition to the general PAMPs mentioned above, *Clostridium spp*. express abundant peptidoglycans and lipoteichoic acids in the cell wall. These PAMPs, are recognised by TLR2 (Schwandner *et al*. 1999). TLRs family activation triggers the production of several proinflammatory cytokines such as IFN-γ, IL-1β, IL-6, IL-8, TNF-α or IL-12, that mediate the activation of a T-cell specific response and tumour clearance. The flagellin present in *C. novyi*'s flagella is also a recognised PAMP involved in the activation of the TLR5 and the activation of a proinflammatory response (Batah *et al*. 2017). A large number of the known Clostridia have a series of highly immunogenic proteins called Surface Layer Proteins (SLP) in their membrane (Desvaux *et al*. 2006). The *Clostridium difficile* SLP, which have accumulated the largest number of studies, are recognised by TLR4, activating the production of proinflammatory cytokines (Mori and Takahashi 2018).

C. NOVYI-NT IN CLINICAL TRIALS

Clostridium novyi is the bacterium that has been used the most from the clostridia sp. in clinical trials. *Clostridium novyi*-NT is capable of selectively colonising some tumors in addition to having diminished adverse effects caused by the toxin (Dang *et al*. 2001). It was used in experimental models with pancreatic cancer (Maletzki *et al*. 2010), gliomas (Staedtke *et al*. 2015) and sarcomas (Roberts *et al*. 2014) in order to observe its selective colonisation, immune cell infiltration and cytokine release. The use of *Clostridium novyi*-NT in cancer animal models has been reviewed elsewhere (Staedtke *et al*. 2016).

In humans, *Clostridium novyi*-NT has been used in clinical trials alone (NCT00358397, NCT01118819, NCT01924689) or in combination with Pembrolizumab (NCT03435952). Pembrolizumab is a therapeutic antibody that blocks the programmed cell death protein 1 receptor (PD-1) located on lymphocytes. This receptor is responsible for preventing the immune system from attacking the body's own tissues (Francisco, Sage and Sharpe 2010). All clinical trials using clostridia are so far in Phase I. Those already

finished, NCT00358397 and NCT01118819 have proved that the bacterium was safe and demonstrated promising results (Krick *et al.* 2011). Future use of *C. novyi*-NT, could be improved by genetic modification in order to express recombinant proteins to increase immunogenicity or stimulate the immune response against the tumour. Some preclinical approaches have introduced immune stimulatory genes such as IL-12 (Zhang *et al.* 2014), IL-2 (Barbe *et al.* 2005) and TNF-α (Nuyts *et al.* 2001) or have expressed antibodies against hypoxic-related protein HIF-1α (Groot *et al.* 2007).

LISTERIA MONOCYTOGENES: THE IDEAL ANTIGENIC VECTOR BACTERIUM

Listeria monocytogenes is a rod-shaped, facultative anaerobic, Gram-positive bacterium. It reaches host intestinal epithelium by the ingestion of contaminated food or raw milk, and invades the gastrointestinal epithelium. In this tissue, the bacterium enters into the host's monocytes, macrophages, polymorphonuclear leukocytes, surviving and multiplying inside them, and then disseminates into the entire organism (Hamon, Bierne and Cossart 2006). The main health alterations caused by *Listeria monocytogenes* are meningitis, septicemia and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (Chlebicz and Slizewska 2018).

The invasion of host cells by *L. monocytogenes* occurs via a process known as the 'zipper' mechanism in which a bacterial surface ligand interacts with a receptor on the host cell, inducing actin cytoskeletal rearrangements and the phagocytosis of the bacterium. Two different surface proteins, internalin A and B (InlA and InlB), can each act as ligands and interact with the host surface receptors E-cadherin and Met (also known as the hepatocyte growth factor receptors), respectively (Hamon, Bierne and Cossart 2006). *L. monocytogenes* escapes from the phagolysosomes into the cytoplasm of antigen presenting cells by disrupting the phagosome membrane through the action of the virulence factor listeriolysin O (LLO) and some lipases (Birmingham *et al.* 2008). In the cytoplasm, *L. monocytogenes* can replicate and spread to other cells (Michel *et al.* 1990).

The main oncolytic mechanism of action of *Listeria monocytogenes* relies on its ability to colonise macrophages. Listeria infection induces the activation of the mechanism for antigen presentation and the expression of Th1-related cytokines, helping T cells to destroy the tumour (Makino *et al.* 2005). Being an intracellular pathogen makes this bacterium an excellent presenter of antigens though both MHC class I and class II molecules (Ikonomidis *et al.* 1994; Gunn *et al.* 2001). In addition, Listeria can infect myeloid-derived suppressor cells against metastatic breast cancer in young and old mice (Chandra *et al.* 2013). The activation of immune cells and the antigen presentation stimulates lymphocyte recruitment and the activation of the adaptive immune response, causing a specific lytic effect of tumor cells. Furthermore, Listeria can selectively colonise the tumour microenvironment (Kim *et al.* 2009), killing tumor cells as a result of activation of the NADPH-oxidase pathway through the action of reactive oxygen species (ROS).

Among LLO, other *L. monocytogenes* virulence factors are Actin A (ActA) and the aforementioned IntB. Act A allow the bacterium to move across the cell cytoplasm as well as to 'jump' from cell to cell due to its ability to produce a nucleation of actin filaments (Welch *et al.* 1998). IntB is the virulence factor responsible for the infection of non-phagocytic cells. *L. monocytogenes* has been attenuated by deletion of these two genes, resulting in a strain that is more than 1000 less toxic than the corresponding wild type (Brockstedt *et al.* 2004). This attenuated strain of *Listeria* has been generated in order to prevent the propagation at the systemic level, causing disease retaining at the same time keeping its immunogenicity properties and reducing its virulence. Although the prfA was deleted in the background strain, the prfA was later on included in the Listeria vector, although mutations were included in the prfA to improve safety (Gunn *et al.* 2001).

DETERMINANTS OF *LISTERIA MONOCYTOGENES* REACTIVITY AND IMMUNOGENICITY

L. monocytogenes has been proven to be an excellent immune-stimulating agent (Johnson *et al.* 2011; Eypper *et al.* 2013; Lin, Van and Smooker 2015). Direct intratumoural administration triggers specific antitumour immune reactions. Direct injection of the bacteria into the tumour microenvironment infects both tumour cells and tumour resident antigen presenting cells (Fig. 3). Intratumoural *L. monocytogenes* promotes the expression of proinflammatory signals, a strong stimulation of reactive oxygen species (Makino *et al.* 2005) and the inhibition of the immune-suppressive environment inside the tumour (Wallecha, Singh and Malinina 2013) leading to T-cell response and antitumoor programme activation (Pan *et al.* 1995; Jahangir *et al.* 2017).

L. monocytogenes has the ability to induce MHC class I and class II peptide presentation in antigen-presenting cells. In the cytoplasm, peptides secreted by the bacterium can be presented both, through MHC class I antigen presentation, inducing CD8 T-cell responses and also by MHC class II presentation (Portnoy *et al.* 1989) resulting from phagosomal degradation, inducing CD4 T-cell responses (Starks *et al.* 2004).

The immune-stimulatory properties of *Listeria spp.*, makes an ideal vector for the expression of recombinant cytokines and antigens. *Listeria* has been used as a vaccine to induce specific immune responses in the tumour environment through the expression of stimulants such as cytokines (Ikonomidis *et al.* 1994; Shen *et al.* 1995; Mata *et al.* 2001), or tumor antigens (Pan *et al.* 1995; Gunn *et al.* 2001; Prins *et al.* 2006). The new generation of attenuated *Listeria spp.* vectors lacking virulence genes but retaining immunogenicity has shown promising results in several models (Angelakopoulos *et al.* 2002; Brockstedt *et al.* 2005; Li *et al.* 2005).

As a Gram-positive bacterium, *Listeria monocytogenes* is able to induce the activation of TLR2 through the detection of peptidoglycans and lipoteichoic acids (Schwandner *et al.* 1999). The presence of flagella implies the activation of TLR5 through the extracellular detection of flagellin (Gewirtz *et al.* 2001) and the activation of NLRC4 (also known as Ipaf) through the detection of flagellin in the cytoplasm of the host cell (Amer *et al.* 2006).

Listeriolysin O can also be considered a PAMP due to its high immunogenicity. The problem with LLO is that it has the capacity to lyse and induce apoptosis in lymphocytes (Carrero, Calderon and Unanue 2004). For this reason the protein has been truncated or mutated at the cholesterol binding sites in order to disable this toxicity and be used in a therapeutic treatment. These 'detoxified' variants of Listeriolysin O are named dtLLO (Michel *et al.* 1990). The dtLLO has been shown to activate the production of IL1, IL6, TNF-α and IL12 among other inflammatory-related cytokines through the activation of TLR4 (Park *et al.* 2004).

Another of the immunogenic proteins present in *Listeria monocytogenes* infection is ActA. This protein has been shown to

be an excellent adjuvant for the production of vaccines (Wood *et al*. 2010) and can be fused to tumour antigens to enhance the proinflammatory responses against the tumour. ActA immunogenicity is associated with the presence of numerous proline, glutamic acid, serine and threonine (PEST)-rich domains at its N-terminal end. These domains improve antigen immunogenicity, increasing the processing and presentation of the antigens to which they are fused (Moors, Auerbuch and Portnoy 1999).

The cyclic di-AMP, is a well-known PAMP that acts as a second messenger used by *L. monocytogenes* to modulate bacterial functions such as cell growth and motility (Witte *et al*. 2013). It is secreted after infection, representing a potent PAMP that induces the production of type 1 IFN and other proinflammatory cytokines through activation of the cytoplasmic immune-sensor STING (Ishikawa and Barber 2008).

LISTERIA MONOCYTOGENES IN CLINICAL TRIALS

Several strains of *Listeria monocytogenes* have been tested in clinical trials. The first reported clinical trial (NCT00327652) started in 2006 and used the *L. monocytogenes* ANX-100 strain in a phase I clinical trial in patients with carcinoma and liver metastasis refractory to other treatments. This attenuated strain was generated by deleting ActA and InlB genes from the *L. monocytogenes* CERS 382.20 strain (Le *et al*. 2011). Administration of bacteria caused transient fever and mild gastrointestinal symptoms, otherwise treatment was well tolerated (NCT00327652).

This phase I trial was followed by another (NCT00585845) in patients with malignant epithelial mesothelioma, adenocarcinoma of pancreas, non-small-cell lung cancer or adenocarcinoma of the ovary. It was performed using a modified version of ANX-100 named CRS-207 that expresses human Mesothelin, an antigen that is frequently overexpressed in multiple solid tumors (Hassan *et al*. 2000; Frierson *et al*. 2003). As observed previously, only mild symptoms were observed upon bacteria administration, and there was a T-cell specific cell response stimulation in 37% of the patients (Le *et al*. 2011). A further phase I clinical trial (NCT01675765) was performed in malignant pleural mesothelioma patients in combination with standard chemotherapy. Thirty-eight patients enrolled on the study. CRS-207 in combination with chemotherapy exhibited encouraging anti-tumor activity with a 59% response rate and median progression-free survival was 8.5 months showing good activation of specific antitumour immune responses (Jahan *et al*. 2016).

Based on these promising results, CRS-207 was also used in two phase II clinical trials (NCT01417000 and NCT02004262) in combination with other oncolytic agents. In both cases, bacteria were inoculated in combination with the immune suppressor cyclophosphamide and the vaccine expressing GM-CSF GVAX (vaccine prepared with irradiated cancer cells expressing GM-CSF). In the first case, 90 patients with 2nd and 3rd line and greater metastatic pancreatic cancer enrolled on this trial (Le *et al*. 2015). Patients receiving the combined treatment experienced an overall survival of 9.7 versus 4.6 months in the patients without the bacterium. Final results in the second case have not yet been published.

CRS-207 is also under study in a parallel clinical phase I/II trial (NCT02575807) in patients with ovarian, fallopian or peritoneal platinum-resistant tumours. In this case, CRS-207 is administered in combination with the IDO1 inhibitor Epacadostat and the anti PD-1 antibody Pembrolizumab. Using a similar treatment, another trial NCT03006302 in metastatic pancreatic adenocarcinoma has recently been initiated (NCT03006302). Other phase II trials are testing the effect of CRS-207 in combination with Pembrolizumab to treat gastric cancers (NCT03122548) or pleural mesothelioma (NCT03175172). Finally, CRS-207 is in a phase II clinical trial in combination with Epacadostat and Pembrolizumab in platinum-resistant ovarian, fallopian or peritoneal tumours (NCT02575807). No results of these trial shaves have yet been published. An additional phase II trial is using a combination of CRS-207 and the anti-PD-1 antibody Nivolumab alone (NCT02243371) or in combination with Ipilimumab (NCT03190265) to treat metastatic pancreatic cancer.

Listeria monocytogenes ADXS 11–001 is another live attenuated bacterium with mutations in the prfA gene to improve safety, although this gene was added back in a mutated form into the bacteria. This *L. monocytogenes* has been engineered to encode a fusion of E7 protein of the human papillomavirus (HPV) type 16 fused to a non-hemolytic Listeriolysin O protein (Gunn *et al*. 2001). ADXS 11–001 safety has been tested in a phase I clinical trial (Maciag, Radulovic and Rothman 2009) showing mild to moderate side effects. Phase II clinical trials assessing the efficacy and safety in patients with oropharyngeal cancer (NCT01598792) were suspended after one patient developed systemic listeriosis (Sacco *et al*. 2015), which underscores the need to further attenuate *L. monocytogenes* used in immunocompromised patients. Since this episode, ADXS 11–001 has been used with additional caution in several other clinical trials, including NCT01116245 (terminated due to lack of recruitment), NCT01266460 (ongoing), NCT02002182 (ongoing), NCT02164461 (ongoing), NCT02399813 (ongoing) and NCT02853604 (ongoing). Additionally, ADXS 11–001 is also under study to treat HPV-related papilloma in combination with other therapies. One clinical trial is combining ADXS 11–001 with the antiproliferative drugs 5-Fluorouracil and Mitomycin and radiotherapy (NCT01671488). This clinical trial has shown very promising results (Howard *et al*. 2017). Another clinical trial combines ADXS 11–001 with Pemetrexed (NCT02531854). In this case, no results have yet been published.

There is another recombinant oncolytic strain of *Listeria monocytogenes* with a deletion in ActA and InlB genes named ADU-623. ADU-623 expresses human Mesothelin, EGFRVIII and NY-ESO-1 antigens. This strain is being tested in a phase I clinical trial (NCT01967758) in patients with glioma. No results have yet been released. A similar strain named ADU-214 but expressing EGFRVIII and human Mesothelin is in a phase I clinical trial for non-small-cell lung cancer treatment alone (NCT02592967) or in combination with Nivolumab (NCT03371381). ADU-741 is another version of the bacterium expressing four prostate associated antigens (PSMA, PAP, cancer testis antigen SSX2 and the prostate associated transcription factor NKX3.1). ADU-741 is in a phase I clinical trial for prostate cancer metastasis (NCT02625857). A version of the bacterium with the double deletion but without recombinant gene expression is named pLADD. This strain is currently under phase I clinical trials (NCT03189030) for the treatment of metastatic colorectal cancer.

Overall, these attenuated versions of *Listeria monocytogenes*, expressing tumour-specific antigens are probably the closest oncolytic bacterium after BCG that will be used in the clinic to treat human tumors.

SALMONELLA ENTERICA, A FACULTATIVE INTRACELLULAR BACTERIUM EASY TO MANIPULATE GENETICALLY

Gram-negative bacteria have not been extensively considered as oncolytic agents with the exception of salmonella. One

important limiting factor of Gram-positive bacteria is the difficulty of controlling the potent proinflammatory effect of the lipopolysaccharides (LPS) induction of TLR4. The list of Gram-negative bacteria and the clinical trials using these bacteria is indicated in Table 3.

Salmonella spp. are Gram-negative rod-shaped bacterium, of the *Enterobacteriaceae* family. There are two species of *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies that include over 2600 serotypes. Only some *S. enterica* subspecies cause human pathology. Non-typhoidal serotypes usually invade the gastrointestinal tract and cause salmonellosis, but typhoidal serotypes can cause typhoid fever, a bloodstream infection, and also invade organs.

Salmonella is a facultative anaerobic microorganism. In addition, *Salmonella* is a facultative intracellular bacterium. Characteristic of salmonella allow it to colonise both large tumours, in which situations of anaerobiosis occur, and small tumors, resulting from metastasis. *Salmonella* is a flagellated bacterium which ensure movement in order to access nutrients such as ribose or amino acids, which are often present in hypoxic and necrotic tumors (Kasinskas and Forbes 2007; Park *et al.* 2013).

As an intracellular pathogen, the mechanism of action by *Salmonella spp.* implies a direct oncolytic effect produced by its ability to kill tumour cells by growing inside them until the cell bursting (Uchugonova *et al.* 2015). Among this cytolytic effect the infection of tumor cell types that preserve pathogen detection and signaling mechanisms should trigger the inflammatory response. This bacterium is able to increase the infiltration of activated macrophages and neutrophils into the tumor enhancing the antitumor response (Chen *et al.* 2017).

The genome of *Salmonella spp.* is relatively easy to manipulate, which raises the possibility of it being used to create conditional mutants or vectors for gene expression delivery. This strategy has been used for the generation of recombinant bacteria able to release cytotoxic factors in tumour tissue, cytokines that facilitate recognition by the immune system and enzymes capable of enhancing or localising the treatment more efficiently than conventional treatments (al-Ramadi *et al.* 2009; Moreno *et al.* 2013; King, Itterson and Bermudes 2009).

Some *Salmonella enterica* strains have been selected over the years for clinical purposes. Those serotypes with better qualities have been modified to obtain suitable bacteria with low pathogenicity and still with good immunogenic properties. The Typhimurium serotype seems to be the most promising in this sense due to its attenuated pathogenesis and sensitivity to antibiotics (Wall, Srikanth and Mccormick 2011). *Salmonella typhimurium* modifications have allowed also the generation of specific strains for tumor targeting (Clairmont *et al.* 2000).

One example is the VNP20009 strain which has been genetically engineered by the elimination of metabolic genes creating an auxotrophic strain for certain metabolites that are common in the tumor microenvironment (Clairmont *et al.* 2000). The best example from *S. typhimurium* is the A1-R strain, which is auxotrophic for leucine and arginine (Zhao *et al.* 2006; Hiroshima *et al.* 2015b). In addition, the toxicity of this strain can be reduced by modifications in genes responsible for the synthesis of lipopolysaccharides (LPS) present in its outer membrane (Freudenberg *et al.* 2001; Nallar, Xu and Kalvakolanu 2016). Another strategy is creating mutants such as one with an hypoxic-specific promoter controlling the expression of an essential gene (*asd*) that allows the bacteria to colonise tumour but not normal tissues (Yu *et al.* 2015). The LPS of the external membrane of *Salmonella spp.* could be highly toxic for the patients because of their inflammatory properties. Mutants expressing a less toxic LPS structure have been generated with modified fatty acids that weaken inflammation (Kong *et al.* 2011) and thus, are less dangerous for the patient.

DETERMINANTS OF *SALMONELLA SPP.* REACTIVITY AND IMMUNOGENICITY

Salmonella spp. can be both extracellular and intracellular pathogens, can be recognised by several extracellular and intracellular PRRs of the host defences, inducing a powerful proinflammatory response (Haraga, Ohlson and Miller 2008). One example of an intracellular and extracellular PAMP is the flagellin of *Salmonella spp.* (Fig. 4). Flagellin can activate membrane-bound TLR5, as has been described for other flagellate bacteria (Gewirtz *et al.* 2001), and also NLRC4PRR, in the cytoplasm of the infected cell (Mariathasan *et al.* 2004; Amer *et al.* 2006).

One of the main *Salmonella´s* extracellular PAMPs is CsgA. This protein is a subunit that forms part of the amyloid curly fibres of *Salmonella typhimurium* biofilm. CsgA is recognised by TLR2, triggering an immune response (Tukel *et al.* 2005).The LPS produced by Gram-negative bacteria are also classic proinflammatory PAMPs recognised by the membrane-bound PRR TLR4. Additionally, in the intracellular space *Salmonella spp.* peptidoglycans are able to induce NOD1 and NOD2 PRRs which trigger the inflammasome and the production of IL-1β and other proinflammatory cytokines (Geddes *et al.* 2010).

SALMONELLA SPP. IN CLINICAL TRIALS

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium* o *S. typhimurium*) is one of the most studied and modified *Salmonella*, which has also been tested as oncolytic bacterium. A previous revision covered the main modifications introduced in this bacterial strain in order to use it as an oncolytic (Nallar, Xu and Kalvakolanu 2016). *S. typhimurium* is the principal *Salmonella* serotype used in clinical trials, demonstrating interesting antitumour properties (Zheng and Min 2016). All the clinical trials performed with *S. typhimurium* have been at phase I. The first trials were performed using genetically modified bacterial strains called VNP20009 (NCT00006254 and NCT00004988) and VNP20010 (NCT00004216). These strains were genetically modified by deletions in the *msbB* and *purI* genes. The *msbB* gene is required for lipid A synthesis and its deletion reduces LPS-related toxicity (Low *et al.* 1999). Deletion of *purI* gene creates an auxotrophic mutant for adenine that bacteria can obtain from necrotic areas inside tumours, making bacteria replication more tumour-specific (Clairmont *et al.* 2000). Tumours with purine-rich activities would be ideal for colonisation by this *Salmonella* strain. A clinical trial using VNP20010 showed moderate treatment toxicity but also a weak tumor colonisation (Toso *et al.* 2002).

Following these initial attempts, VNP20009 was further modified to express the *E.coli* suicide gene Cytosine deaminase. This new recombinant was named TAPET-CD (Nemunaitis *et al.* 2003). This enzyme is able to convert 5-Fluorocytosine (5-FC), an antifungal agent with limited toxicity into at least three toxic intracellular metabolites: 5-FdUMP, 5- FdUTP and 5-FUTP, which induce apoptosis (Chabner and Longo 2011). Three patients received intratumoral injection with scaling doses of the bacteria and 5-FC. Two of the three showed signs of bacterial colonization and one patient showed a prolonged survival (Lee *et al.* 2001).

Another alternative of oncolytic *S. typhimurium* is the A1-R auxotrophic strain, which is restricted to replicate in tumours. A1-R oncolytic activity has been proved in several cancer models in mice such as prostate (Toneri *et al.* 2015), breast (Fu, Le and Hoffman 1993), lung (Miwa *et al.* 2014), pancreas (Hiroshima *et al.* 2014), bone (Hayashi *et al.* 2009), ovary (Matsumoto *et al.* 2014), gastrointestinal (Miyake *et al.* 2018), melanoma (Yamamoto *et al.* 2016), cervical (Hiroshima *et al.* 2015a) and colorectal cancers (Hoffman 2016). *S. typhimurium* A1-R is a more specific tumour coloniser and less toxic than the VNP20009 strain in a CT26 colorectal tumor model in syngeneic Balb/c mice (Zhang *et al.* 2017) or Lewis lung tumor model in nude mice (Zhang *et al.* 2015). A1-R (and other oncolytic *S. typhimurium* bacteria strains) present as a more convenient agent for treatment since they can be administered systemically (Zhao *et al.* 2005; Hiroshima *et al.* 2015b). Although A1-R in a promising oncolytic agent, no reports of use in humans or reference of ongoing clinical trials have yet been reported.

One of the first recombinant attenuated oncolytic bacteria was the *Salmonella typhimurium* strain X4550 mutant (Δcrp, Δcya) expressing human IL2 (X4550-pIL2) (Saltzman *et al.* 1997). X4550-pIL2 has oncolytic activity in a MC38 syngeneic mouse hepatic cancer model (Saltzman *et al.* 1996). X4550-pIL2 was used in a phase I clinical trial in patients with unresectable liver cancer (NCT01099631). The study was terminated owing to slow accrual and results have not yet been published.

Another attenuated strain, *Salmonella typhi* Ty21a, with the commercial name VXM01 (used as a prophylactic oral life attenuated vaccine to temporarily protect from typhoid fever), has been also tested as an antitumor agent. VXM01 is deficient in multiple genes, including mutations in the GalE gene, Vi antigen biosynthesis, isoleucine and valine biosynthesis (Germanier and Furer 1983; McKenna *et al.* 1995). VXM01 has been manipulated to express VEGFR2 (Chen *et al.* 2015) and has been used in three clinical trials to date. The first one (NCT01486329) was recently evaluated on 45 patients with stage IV pancreatic cancer, showing a requirement of preexisting immunologic memory to achieve an antitumor effect (Schmitz-Winnenthal *et al.* 2015). Although results have not yet been published, VXM01 is currently used against glioblastoma (NCT02718443) and metastatic colorectal cancer with liver metastasis (NCT02718430). These studies, although in phase I, should clarify the potential of this strain in selective tumor colonisation and as an immune response inducer.

In recent attempts to improve VXM01, immunogenicity genetic modifications have been carried out in the bacteria to improve its type III secretion system (Xu *et al.* 2014). This new strategy will allow a better antigen secretion and could be modified in order to express recombinant genes to increase tumour-specific toxicity.

REVISITING 'COLEY TOXIN'

Serratia marcescens/Streptococcus pyogenes

Serratia marcescens and *Streptococcus pyogenes*, were the two organisms used by William B. Coley in his studies. *Serratia marcescens* is a Gram-negative bacterium which belongs to the family *Enterobacteriaceae*, and although it is widespread in the environment, it is a rare cause of human disease. It does not cause primary invasive diseases, but produces an infection when it gains access to a suitably compromised host. It causes 1–2% of the nosocomial infections which are mostly confined to the respiratory tract, the urinary tract, surgical wounds and soft tissues (Khanna, Khanna and Aggarwal 2013).

Streptococcus pyogenes is a Gram positive non-motile coccus. This bacterium has several virulence factors that enable it to attach to host tissues and evade the immune response. Although *S. pyogenes* is considered an extracellular pathogen, this bacterium can also enter into human epithelial cells, including oropharyngeal keratinocytes, at least *in vitro* (LaPenta *et al.* 1994; Schrager, Rheinwald and Wessels 1996; Molinari *et al.* 1997).

The most frequent pathologies produced by *S. pyogenes* are pharyngitis, skin damage and septic shock, when some proteins act as superantigens and trigger a strong immune reaction. Other times, it has been detected as an asymptomatic pharyngeal bacterium.

Determinants of Coley´s toxin reactivity and immunogenicity

Due to the high mortality associated with the administration of living organisms, Coley chose to inactivate the inoculum with heat, thus administering mainly endotoxins (Coley 1910; McCarthy 2006). The sequential and reiterative inoculation of the toxin causes an antitumour immune response accompanied by fever (Nauts, Swift and Coley 1946).

The main active components of the vaccine seem to be prodigiosin produced by *S. marcescens* and the SpeA, SpeB and SpeC proteins produced by *S. pyogenes* (Kramer *et al.* 2018). The LPS component from *S. marcescens* may also play a role in triggering TLR4 (Zhou *et al.* 2012). Prodigiosin is a toxin, produced by several species of *Serratia* sp. and *Vibrio sp.,* which has shown antitumour activity, through the induction of p53-dependent apoptosis (Montaner *et al.* 2000; Montaner and Perez-Tomas 2001; Hong *et al.* 2013). This activity does not seem to be altered in tumors that possess multiresistant pumps (Soto-Cerrato *et al.* 2004; Elahian *et al.* 2013). On the other hand, the SpeA, SpeB and SpeC exotoxins of *S. pyogenes* are pyrogens that are closely related to toxic shock syndrome related proteins (Babbar 2015). They have classic superantigen structures that make them capable of binding to MHC-II and TCR producing non-specific activation of CD4 lymphocytes.

Over the years, up to 16 different Coley toxins have been used. They differ on how the solution was inactivated or purified, as well as different modes of administration that resulted in totally different outcomes (McCarthy 2006; Kramer *et al.* 2018), probably due to the bacteria differences in the activity of these PAMPs and toxins present in the different preparations. In 1962 the FDA eliminated Coley's toxin from the list of drugs approved for clinical purposes (Hoption Cann, Van Netten and Van Netten 2003).

Coley toxin in clinical trials

We have previously described William Coley´s approximation to create a bacteria-based formula to treat cancer patients. One of the main problems in this approach was being able to have consistent bacteria preparations. In fact other laboratories trying to reproduce Coley´s results were not as successful, probably due to a deficient bacteria inoculum preparation. There was an attempt in 2007 to revisit Coley´s strategy in trying to reproduce his bacteria 'toxin' also named mixed bacteria vaccine (MBV). This new attempt was carried out in patients with different tumours expressing the NY-ESO-1 antigen (Karbach *et al.*

2012) in a phase I clinical trial (NCT00623831) that has been completed. Seventeen patients were enrolled and 15 of them completed the study. Patients developing fever could be associated in some cases to tumor regression. MBV could be verified as a potent immune modulator at higher dose levels. MBV could be an interesting treatment in combination with other antigen-specific cancer vaccines or with other alternative or conventional cancer treatments.

KLEBSIELLA PNEUMONIAE, A HUMAN PATHOGEN THAT COULD BE ADAPTED FOR LUNG TUMOUR TREATMENT

Klebsiella pneumoniae is a Gram-negative, capsulated Enterobacteria, traditionally considered as commensal, but also as an opportunistic pathogen (Lau, Huffnagle and Moore 2008). *Klebsiella pneumoniae* colonises the mucosal surfaces in humans, including the nasopharynx and the gastrointestinal tract (Podschun and Ullmann 1998) and, depending on the strain virulence factors and host immunity, can invade and produce infections such as respiratory and urinary tract infections.

DETERMINANTS OF *K. PNEUMONIAE* REACTIVITY AND IMMUNOGENICITY

Like the other Gram-negative bacteria, *Klebsiella* LPS can trigger TLR4 (Schurr *et al.* 2005; Wieland *et al.* 2010). In addition, *Klebsiella pneumoniae* can produce and secrete outer membrane vesicles (OMV) rich in LPS, which are able to trigger the expression of proinflammatory cytokines (Lee *et al.* 2012). Moreover, the capsular polysaccharides (CPS) that form part of the *Klebsiella*'s biofilm are able to activate MR and DC-SIGN, belonging both to the C-type Lectin receptors family (Kabha *et al.* 1995; Zamze *et al.* 2002; Sahly *et al.* 2008) in macrophages and dendritic cells (Fig. 5). Another PAMP able to induce a proinflammatory response is the outer membrane protein A (OMPA) that is conserved in *Enterobacteriaceae* and has been shown to be an activator of TLR2 (Jeannin *et al.* 2005). It has been shown that the activation of TLR9 by the unmethylated CpG motifs of the microbial DNA is crucial in the immune response against *Klebsiella pneumoniae* infection (Bhan *et al.* 2007).

CLINICAL TRIALS USING *K. PNEUMONIAE*

The Canadian company Qu Biologics is working on the use of a specific and attenuated strain of *Klebsiella pneumoniae* named QBKPN SSI. This strain was isolated from a patient. Since *K. pneumoniae* can target lung cells, it has been proposed to treat lung cancers such as non-small lung cancer. This approach is currently under study in a phase II clinical trial (NCT02256852).

MIXED VACCINE TREATMENT

There is an experimental phase I/II clinical trial (NCT02333474) in patients with lung carcinoma using a combination of common human vaccines. The patients enrolling in this trial received the so-called mixed vaccine (MV), which is an intravenous suspension containing five different vaccines, including DPT (diphtheria, pertussis, and tetanus), BCG, measles, *Serratia* and *Streptococcus pneumoniae*. No results of this trial are yet available.

FUTURE OF ONCOLYTIC BACTERIA: RECOMBINANT BACTERIA AND MOLECULAR APPROXIMATIONS TO IMPROVE CANCER IMMUNE-THERAPY

The use of one bacterium or another for a particular treatment using infectious or immune stimulating agents against cancer cells will depend on several factors such as tumour accessibility, tumour state, tumor type, anoxic or necrotic state of the tumour, or factors that may determine the immune-reactivity of the particular tumour. In the case of using bacteria, it also depends on the ability of the bacterium to induce an immune response. Some bacteria are excellent inducers of the innate and adaptive immune system depending on specific PAMPs. Those properties should be accompanied by bacteria that fulfill criteria such as safety, easy to manipulate, immune-stimulatory capacity, effectiveness and restriction to the tumour tissue.

Tumours can be divided into immunogenic and non-immunogenic (Blankenstein *et al.* 2012). Characterisation of the immunoreactivity of tumors infected with different types of oncolytic bacteria or oncolytic viruses will improve the understanding of what would be the best options when microorganisms are assessed in therapy. There are examples of bacteria that are pathogenic in animals but not in humans. In fact BCG, the only bacterium approved in non-disseminated bladder cancer is a bovine adapted bacterium. In addition, most tumours present an immune suppression in the tumour microenvironment to prevent the immune system to recognise and react against certain tumor-specific antigens that they may express (Whiteside *et al.* 2016; O'Donnell, Teng and Smyth 2018; Togashi, Shitara and Nishikawa 2019). Initially, immune suppression may allow bacteria to survive and multiply in these tumor microenvironments, but not in normal tissues that lacks immune suppression (Fig. 6).

Although bacteria were the first microorganisms proven to have oncolytic activity, viruses have also received much more attention as oncolytic agents. Compared to bacteria, viruses have several properties that make them attractive. They have smaller genomes, most of their virulence factors have been characterised and with the use of molecular techniques (genetic modification by reverse genetics), their genomes can be easily manipulated and pathogenic associated characteristics modified in order to make the oncolytic virus safer or modified to increase tumor tropism. In addition, expression of recombinant proteins by the infected cell can be directed to stimulate CD8 T cell responses that are important in order to activate the immune reactivity of the tumor.

However, there is a great disadvantage in the use of oncolytic viruses as compared to bacteria. Since viruses are obligated intracellular pathogens, they require living cells in order to replicate and destroy tumours. This characteristic is a problem, especially in large solid tumours that are difficult to reach, and thus inoperable. In such tumours, hypoxia-related tumour necrosis may mask many cells that are unable to be reached by viruses or the immune cells. In addition tumuor cells have to have receptors for the virus and be competent of replicating them. Moreover, tumour cells should have defects in the intracellular innate immune response against virus in order to restrict the virus to the tumor area. If any of these factors is not optimal, virus-mediated tumor oncolysis specificity and efficacy will be diminished.

Oncolytic virus therapy is limited since a repeated administration triggers specific T cell and antibody responses against

these infectious agents. Systemic readministration of the infectious agent induces a boost of the adaptive immune response that at the end limits the replication of the agent and the expected therapeutic effect.

Oncolytic bacteria have several properties that make them the microorganism of choice as compared to viruses. A central property of bacteria as compared to viruses is the ability to grow extracellularly and reach areas of the tumor that are not accessible to viruses. Although some of the bacteria used in clinical trials, such as *Mycobacterium spp*., *Salmonella spp*. or *Listeria spp*., are facultative intracellular microorganisms, bacteria can survive in the absence of cells and some replicate in hypoxic or anoxic conditions. In addition, some bacteria have motility and can penetrate necrotic areas and use them as a source of nutrients and energy, able to clean the tumor of necrotic mass and make it accessible to other cells or other therapies.

Unlike viruses, bacteria that are not intracellular do not need to use cell receptors to invade cells in the tumor and should be limited to the tumor area by the tumor microenvironment. Oncolytic bacteria should be attenuated enough, so if they escape from the tumor, they will be easily controlled by patients' immune responses. Usually, solid tumours present fibrous barriers between tumour compartments (Sriraman, Aryasomayajula and Torchilin 2014). Tumour isolation with fibrotic tissue is one of the protective methods helping to slow down tumor growth (Ohlund, Elyada and Tuveson 2014). Safety of oncolytic bacteria is one of the main issues for their use in cancer therapies. Safety could be improved by selecting bacteria or strains that would be able to be retained inside tumor structures. Alternative approaches could be made so that bacteria can disseminate in the organisms without compromising patients' lives, ideally replicating in distant metastatic tumours.

Bacterial genomes are much bigger than those of viruses. In general they contain a larger arsenal of weapons in order to antagonize and escape the immune response as compared to viruses. Although they can be more difficult to control, we can use specific antibiotics to control their replication. Bacterial genomes can be easily manipulated and engineered to overcome some of the restrictions of existing cancer therapies. There is a room for improvement in making recombinant bacteria already used in patients like BCG. Bacteria can also be engineered to deliver radioactive molecules such as radionuclide ^{188}Rhenium and ^{32}P or immune adjuvants like alphagalactosylceramide (Quispe-Tintaya *et al*. 2013, PNAS, Chandra *et al*. 2017, Oncotarget; Singh *et al*. 2013). Another recent review article (Zhou *et al*. 2018) has addressed the complexity of specifically targeting the tumor microenvironment and provides examples to illustrate different ways to engineer bacteria for improved safety and efficacy.

Impressive progress in cancer treatments has been achieved in recent years, however the therapies are not optimal and may have side effects. In addition, cancer treatments may only increase the patient's survival but not be totally effective. Many authors have proposed combined therapies in order to achieve effective treatments and increase the rates of complete cure (Juergens *et al*. 2012; Cohn *et al*. 2013; Fuchs *et al*. 2015; Bayat Mokhtari *et al*. 2017). Some current treatments using chemotherapy and radiotherapy are toxic to normal tissue and sometimes cannot completely destroy all the cancer cells (Minchinton and Tannock 2006). Incomplete tumour targeting can be due to inefficient tissue penetration and limited toxicity to all cancer cells (Jain 1998). Using oncolytic bacteria should be considered in such cases, especially in those where the tumour is compartmentalized.

Combination of oncolytic bacteria and viruses has already been tested with interesting results (Cronin *et al*. 2014; Krzykawski 2015). Likewise, combination with other selected microorganisms (i.e.: unicellular parasites, or fungi) could be a possibility to explore in the future. In addition, bacteria can be combined with other treatments such as antibodies (Klier *et al*. 2012), conventional chemotherapeutic drugs: (Dang *et al*. 2001; Dang *et al*. 2004; Lee *et al*. 2005) radiation (Platt *et al*. 2000; Bettegowda *et al*. 2003; Jiang *et al*. 2010) or toxic substances (Gericke and Engelbart 1964; Jia *et al*. 2005; Shilling *et al*. 2007). Additional strategies could be employed to specifically deliver toxic or toxic inducible systems in bacteria that could combine their oncolytic activity (Forbes 2010; Gardlik *et al*. 2011).

As previously mentioned, bacteria themselves will produce or carry PAMPs during their replication or administration. However, some bacteria may induce a weak response upon exposure to PAMPs. Combination with compounds such as pI:pC, Imiquimod or cy-di-GAMP could be an interesting approach to enhance such response. These and other possible molecules delivered by bacteria inside of tumours have been reviewed elsewhere (Song, Vuai and Zhong 2018).

In the light of better characterized mechanisms that may explain the immune response against infections, an increasing interest in the innate immune response memory, also renamed as trained innate immunity (Netea *et al*. 2016), may explain some of William Coley´s observations. Trained immunity creates an epigenetic memory in antigen presenting cells after a first exposure to a PAMP or a danger signal that determines the level of stimulation upon a second exposure to the same or different agonist. This memory may enhance the response, or attenuate it, depending on the sequence of stimuli. Symptoms during treatment such as fever and increase of inflammatory markers are indicators of the antitumor reactivity. This reaction is a sign of an inflammatory process that may induce training in some antigen presenting cells of the patient. In the context of an immune-dormant environment, such as that of many tumours, the use of different infectious agents may change the fate of the trained immunity in antigen-presenting cells. A strong danger signal in the tumor microenvironment can induce a reaction opposed to the tolerant condition of the patient against tumor. Modification of trained macrophages with infection or bacteria-derived PAMPs could be used to improve the antitumor response. Moreover, in addition to PAMPs, danger signals produced both by the pathogen and the infected cells during infection, such as reactive oxygen species can also trigger inflammation and alter training immunity.

As mentioned above, one of the key aspects in the use of microorganisms such as oncolytic bacteria or oncolytic viruses is safety. In the case of viruses, the use of non-human adapted viruses has been an interesting characteristic in order to prevent dissemination of the virus in areas other than the tumour. Newcastle disease virus (NDV) or Vaccinia virus are examples of animal viruses that do not replicate well in humans but to replicate well in tumour cells (Hastie and Grdzelishvili 2012; Zamarin and Palese 2012). Likewise, bacteria that may replicate well inside tumours but do not possess virulence factors in humans could be an interesting area to explore for new safer strains.

In coming years, a revision of the use of oncolytic bacteria in combination with current oncolytic therapies in the clinic is expected. We should see oncolytic bacteria as a window of opportunity to improve current cancer treatments. Improvement in the knowledge and control of inflammatory responses as well as understanding the biology of different cancers will

give clues to the correct treatment in each case. The use of bacteria in some of those scenarios is awaited.

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