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REVIEW ARTICLE

Bacterial immunotherapy of gastrointestinal tumors

Michael Linnebacher · Claudia Maletzki · Ulrike Klier · Ernst Klar

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Abstract
Background Cancer immunotherapy using bacteria dates back over 150 years. The deeper understanding on how the immune system interferes with the tumor microenvironment has led to the re-emergence of bacteria or their related products in immunotherapeutic concepts. In this review, we discuss recent approaches on experimental bacteriolytic therapy, emphasizing the specific interplay between bacteria, immune cells and tumor cells to break the tumor-induced tolerance.
Results Experimental research during the last decades demonstrated beneficial but also adverse influence of bacteria on tumor growth. There is a strong correlation between chronic infections and tumor incidence. However, acute bacterial infections have favourable effects on tumor growth often contributing to complete remission. Tumor regression is usually attributable to both direct tumor cell killing (via apoptosis and/or necrosis, depending on the applied bacteria) and indirect immune stimulation. This includes (I) elimination of immunosuppressive immune cells (i.e. tumor-associated macrophages, myeloid-derived suppressor, and regulatory T cells), (II) suppression of Th2-directed cytokine secretion (TGFα, IL10), (III) providing a pro-inflammatory micro-milieu (tumor infiltrating neutrophils) and (IV) supporting the influx of cytotoxic T cells into tumors. This finally forces the development of an immunological memory and may provide long-term protection against cancer.
Conclusion Immunotherapy using bacteria is still a double-edged sword. Experiences from the last years have substantially contributed to when bacteria and defined components thereof might be integrated into immunotherapeutic concepts. Attempts in transferring this approach into the clinics are on their way.

Keywords Bacterial immunotherapy · Gastrointestinal cancer · Inflammation · Orchestrated immune response

Abbreviations

BCG	Bacillus Calmette-Guérin
BG	Bacterial ghost
CCL	Chemokine (C-C motif) ligand
COBALT	Combination bacteriolytic therapy
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cells
EGFR	Epidermal growth factor receptor
G-CSF	Granulocyte colony stimulating factor
IFN	Interferon
IGF	Insulin growth factor
IL	Interleukin
mAb	monoclonal antibody
MDSC	Myeloid-derived suppressor cells
NK cells	Natural killer cells
PAMP	Pathogen-associated molecular patterns
TAM	Tumor-associated macrophages
TGF	Transforming growth factor
Th	T-Helper
TLR	Toll-like receptors
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

This review is dedicated to Prof. Dr. med. J. Emmrich, an active promoter of the bacterial therapy concept at our university hospital.

Michael Linnebacher and Claudia Maletzki contributed equally.

M. Linnebacher (✉) · C. Maletzki · U. Klier · E. Klar
Department of General, Vascular, Thoracic and Transplantation Surgery, University of Rostock,
Rostock, Germany
e-mail: Michael.linnebacher@med.uni-rostock.de

Introduction

The interaction of mammals and especially of humans with bacteria is among the most interesting investigative fields. Acute bacterial infection is still one of the most feared killers with an estimated death toll of 75,000 per year in Germany. In the last decades it has been clearly established that chronic infections with bacteria may additionally contribute to this negative picture. *Helicobacter pylori*-associated gastric cancer is the most prominent example. Contrary to that, commensal microbia living on human body's surfaces help us in various ways by ensuring immunity against pathogenic microorganisms. Indeed, the number of bacteria colonizing mucosal and skin surfaces exceeds the number of cells forming the human body by far. The symbiotic host–commensal microflora interactions maintain homeostasis by (i) protecting against harmful microbiotic pathogens (viruses, bacteria, fungi), (ii) providing a barrier against invading infectious or immunogenic components present on the mucosa into the circulation and (iii) exerting tolerance against harmless antigens present on the mucosal surface [1, 2]. The growing attention to the role of the intestinal microflora in human health has stimulated efforts to optimize its composition by probiotics. These orally applied live microbial cultures (e.g. *Lactobacilli*, *Bifidobacteria*, *Escherichia coli* Nissle) positively influence the microflora, inhibit pathogenic colonization, affect the mucosal barrier and stimulate the immune system [1].

Not so obvious, bacteria and especially bacteria-derived products are also directly involved in protecting human beings from various diseases since they are widely used for vaccination purposes.

From an evolutionary point of view, it is completely logical that the presence of bacteria stimulates an inflammatory response that finally helps to initiate memory immune responses. The mammalian innate immune system is armed by sensors able to recognize a plethora of biomolecules produced exclusively by microbes. Potentially the most important class of these molecular sensors are the so-called toll-like receptors (TLRs). They recognize pathogen-associated molecular pattern (PAMP) including bacterial DNA, viral RNA, lipoteichoic acid and lipopolysaccharides. Upon stimulation, an intracellular signaling cascade is initiated—involving inflammatory cytokines and activation of the innate immune system—that mediates cross-activation of the adaptive immune system thus helping to establish antigen-specific immune responses which have long-lasting character.

This strong potential of PAMP substances to unspecifically activate the immune system has been transferred to experimental cancer immunotherapy. Today, it is well known that bacteria as well as bacterial components, such as lipoteichoic acid, bacterial DNA and exotoxins/endotoxins mediate antitumor activities not only by the earlier characterized indirect activation of the immune system but additionally by direct tumoricidal effects [3–5]. However, there is growing experimental evidence that PAMPs may have the opposite effect, i.e. stimulating tumor growth under conditions of chronic inflammation [6].

In this review, we highlight recent approaches on using bacteria as anticancer agents and discuss its potential clinical applicability. In particular, we will focus our attention on the controversial role of bacteria and inflammation by having the potential of eliminating even solid tumor masses on the one hand while driving carcinogenesis on the other.

The historical idea of using bacteria for cancer immunotherapy

Immunotherapy is based on the idea that the patient's immune system can be stimulated or enhanced to attack malignant tumors. Long before detailed knowledge on how the immune system acts on tumors and is thus potentially involved in inflammation-mediated tumor shrinkage, some reports suggested that having an infection might cause tumor regression [7]. This observation dates back 150 years when German physicians W. Busch and F. Fehleisen separately noticed tumor regression in cancer patients after accidental erysipelas infection [8, 9]. Hence, Busch was the first who intentionally inoculated a cancer patient with erysipelas. Some years later, Fehleisen identified *Streptococcus pyogenes* as the causative agent of erysipelas [9].

At the same time, the New York surgeon William B. Coley observed that malignant tumors, particularly sarcomas, regressed in patients suffering from concurrent bacterial infection whilst hospitalization [10]. He then started to systematically treat bone and soft tissue sarcoma patients with infectious erysipelas and in fact repetitively reported infection-associated tumor regression. Patients usually experienced infectious disease processes, including high fever, chills and malaise [11]. In his first experiments, Coley focused on treatment with live streptococci. However, due to lethal systemic *Streptococcus* infection, he subsequently used heat-killed streptococcal organisms combined with heat-killed *Serratia marcescens* (formerly known as *Bacillus prodigiosus*). This bacterial vaccine became known as "Coley's toxin". Coley injected more than 1000 cancer patients with bacteria or bacterial products. He hypothesized that an immune reaction against a "toxin" present in the microbial material cross-reacted with and destroyed the tumor cells [12]. Coley reported a high success rate in treating patients with sarcomas and other malignancies, including carcinomas (e.g. breast and renal cancer), lymphomas and melanomas. Thus, the inoculation of this

vaccine became the cornerstone in development of cancer immunotherapy.

During the 1920s it was insisted that the excellent responses reported by Coley were often because the patients had the wrong diagnoses. By 1952, the Coley's toxin was no longer produced, and in 1962 the Food and Drug Administration refused to acknowledge Coley's toxin as a proven drug [7]. In the 1960s and 1970s, commercial preparations of Coley's toxin were tested on small patient cohorts. However, results obtained by Coley could not be reproduced, and with the emergence of modern chemotherapy and radiotherapy, his work gradually fell out of favor.

Recent approaches on bacterial immunotherapy

Experimental studies

Coley's historical idea on inducing a strong inflammatory response that leads to tumor reduction provided the basis for developing different forms of immunotherapy. The ideal anti-cancer therapy should selectively eradicate tumor cells, whilst minimizing side effects to normal tissue. Today, it is known that both direct tumoricidal effects and immune activation force antitumor activities. During the immune response, Toll-like receptors (TLRs) sense a diversity of PAMPs to organize the body's immune defense. This includes (i) stimulation of pro-inflammatory immune cells capable of lysing (infected) target cells in an antigen-independent manner (neutrophils, macrophages) and (ii) inhibition of the tumor-induced immune suppression (tumor-associated macrophages, myeloid-derived suppressor cells and regulatory T cells) that ideally (iii) leads to the development of potent cellular immune responses—often dominated by activated cytotoxic T cells—finally mediating long-term protection against cancer.

Over the past 50 years, several strains of facultative and obligate anaerobic bacteria were applied as oncolytic agents due to their capacity to selectively proliferate in oxygen-starved environments [13–17]. Based on the observation that necrotic regions exist only within tumors and not in normal tissues, the group of Vogelstein demonstrated that lethal toxin-free *Clostridium novyi* NT spores are very efficient in eradicating established tumors [14, 18]. They showed that anaerobic bacteria specifically and preferentially target solid tumors, leading to an inflammatory reaction within the tumor that is followed by tumor regression in about 30% of cases [19, 20]. In a preclinical regimen, anaerobic *C. novyi* NT spores were combined with conventional drugs or radiotherapy. This strategy, referred to as combination bacteriolytic therapy (COBALT), was shown to mediate dramatic and prolonged regression of subcutaneous tumors following a single systemic administration [14]. Similarly, bacteria were found to improve the efficacy of radiotherapy in several mouse models [21]. The authors explained their findings with the fact that efficient tumor cell killing by radiation requires oxygen. Hypoxic cells are more resistant to ionizing radiation than normoxic cells, and hence hypoxic zones in poorly vascularized or necrotic tumors are a major handicap in cancer therapy [22, 23]. By using anaerobic bacteria, these hypoxic regions can be targeted and destroyed, making tumors vulnerable to radiotherapy.

Of particular importance, bacteriolytic therapy was reported to induce immunological memory. *C. noyvi* infection is associated with inflammation (secretion of neutrophil-directed cytokines) at the tumor periphery leading to development of an effective cellular antitumoral immune response (monocytes and lymphocytes) [14, 17, 18]. Lymphocyte transfer from cured animals into naïve tumor-bearing mice revealed that $CD8^+$ cytotoxic T cells are the main cell type involved in this process [20]. However, in a subsequent study performed by our group, using the very same treatment protocol, similar results could not be observed [24]. The bacterial treatment predominantly activated the innate immune system's arm. NK cells, but not (tumor) antigen-specific T cells, have been activated by bacteriolytic therapy.

Beyond that, at least in our hands, applicability of this approach was imperfect due to troubles in standardization. Regarding toxicity, tumor size and spore dose, we observed that (I) small tumors (< 150 mm^3) were completely unaffected; (II) very large tumors (> 450 mm^3) responded with substantial necrosis followed by shrinkage but significant animal mortality and (III) an optimal treatment window exists for tumors of approximately 250–300 mm^3 [24]. The comparably high mortality rate in large-tumor bearing mice was most likely attributable to the so-called "tumor lysis syndrome" [25]. Similar experiences have already been described by Diaz and colleagues in a large study on evaluating pharmacology and toxicity of *C. novyi* NT spores [20]. Nevertheless, a clinical trial has been initiated in 2006, but it was terminated due to design problems. Attempts in re-emergence of clostridia-based therapies in the clinical setting are on their way. In August 2011, the BioMed Valley Discoveries Inc. started a pilot study to investigate the safety of *C. novyi* NT spore administration in patients with treatment-refractory solid tumor malignancies. The primary aim of this study is determination of the optimal time point to initiate antibiotic treatment in these patients. Results of this study will finally help to estimate whether *C. novyi* NT-based immunotherapies are clinically safe and feasible (Table 1).

Besides *C. novyi*, genetically modified strains of *Salmonella typhimurium* have gained interest as potential anticancer agent, either alone or in combination with radiotherapy and chemotherapy [26]. Zhao and coworkers designed a

Table 1 Ongoing clinical trials on bacteriolytic cancer immunotherapy

Treatment regimen	Tumor entity	Specifications	Phase
C. novyi NT spores	Treatment-refractory solid tumor malignancies	None	I
Mixed bacterial vaccine (MBV)	Melanoma, sarcoma, gastrointestinal stromal tumor, head and neck cancer, transitional cell carcinoma, prostate cancer	NY-ESO-1 expression	I
Pseudomonas exotoxin A (immunotoxin MOC31-PE)	Tumor type not specific	Conjugated to anti-Ep-CAM/ epithelial glycorprotein 2	I
Urocidin (EN3348; mycobacterial cell wall–DNA complex)	Recurrent or refractory non-muscle invasive bladder cancer	BCG-pretreatment	III
CpG ODN	Glioma	None	II

Data are taken from www.clinicaltrials.gov

tumor-targeting strain auxotrophic for the amino acids arginine and leucine, termed A1 [3]. In contrast to strictly anaerobic bacteria, *S. typhimurium* growth is not confined to necrosis and can be found throughout the tumor, including viable regions [27]. There, bacteria invade and replicate intracellular in cancer cells. But growth is not sustained in normal tissue, indicating its potential applicability against small tumors or maybe even micrometastases. If finally necessary, tumor-colonizing salmonella can be readily controlled by early post-infection systemic administration of the antibiotic ciprofloxacin. Tumor lytic effects and accompanying specific antitumor immune response development is preserved [28]. Thus, *S. typhimurium*-mediated tumor therapy might be applied safely when combined with early antibiotic treatment. To increase the tumor targeting ability and killing efficacy, the A1 strain was further modified by re-isolation from a tumor growing in a nude mouse and termed A1-R [27]. Of note, this strain was able to eradicate metastatic lesions in orthotopic models of breast, prostate and pancreatic cancer, both after local as well as systemic administration [27–30].

These observations have contributed to the initiation of a clinical phase I trial [31]. Twenty-five cancer patients received bolus infusions of a lipid A-attenuated *S. typhimurium* (VNP20009). This strain was safely administered to the patients. Focal tumor colonization was observed in some patients receiving high bacterial numbers. None of the patients experienced objective tumor regression, including those with colonized tumors [31]. This is a striking contrast to the results obtained with rodent models. A conceivable explanation is that differences with respect to tumor vasculature, bacterial entry into and growth within tumors, as well as clearance of bacteria exist between rodents and patients. Further studies are required to finally judge if this regimen may still be successful in the clinics.

Irrespective of the presence or absence of intratumoral necrotic lesions, our group showed in a series of experiments that viable as well as lysed and avitalized gram-positive facultative anaerobic bacteria have a direct impact on tumor growth in immunocompetent and in immunocompromised hosts. In an initial work, we explored the antitumoral potential of a bacteriolytic therapy based on *S. pyogenes* (4). This strain was chosen as a bacterial model because (i) it exerts direct cytolytic effects on eukaryotic cells by secreting several cytotoxic enzymes and pore-forming toxins, (ii) it effectively induces the secretion of proinflammatory cytokines (TNFα, IFNγ) and chemokines (G-CSF), (iii) it mediates high influx of inflammatory cells (e.g., neutrophils, macrophages and NK cells) into the focus of infection and (iv) it initiates the generation of an adaptive, cell-mediated immune response [32, 33] (Fig. 1).

S. pyogenes binds target cells via fibronectin or collagen. Bound fibronectin acts as a bridging molecule towards host cell integrins, which in turn initialize the uptake process that leads to internalization (Fig. 2) [34]. Direct tumor cell contact is thus necessary for *S. pyogenes* infection. This finally leads to the induction of tumor cell apoptosis. Accordingly, in vivo bacteriolytic therapy in tumor-bearing mice was performed by local injection. A single application of viable bacteria resulted in complete pancreatic carcinoma regression that was accompanied by massive immune activation secondary to infection. As a consequence, immunological memories, as determined by in vitro functional tests and in vivo rechallenge experiments, developed [4]. However, as for potential clinical application, inactivation prior to administration seemed necessary. The streptococcal lysate used in a subsequent study was also shown to mediate substantial growth arrest when injected intratumorally. Again, this antitumoral effect could be attributed to a massive stimulation of immune response mechanisms including a strong systemic elevation of granulocyte numbers and an increase in tumor infiltrating cytotoxic T cells [35]. Taking into consideration that the streptococcal lysate had striking antitumoral activity even when administered alone, it might thus be useful as immunotherapeutic adjuvant in combination with conventional chemotherapy.

Having in mind that the innate immunity is considered to be the sentinel of the first line defense that contributes to the containment and elimination of microbes, we next focused

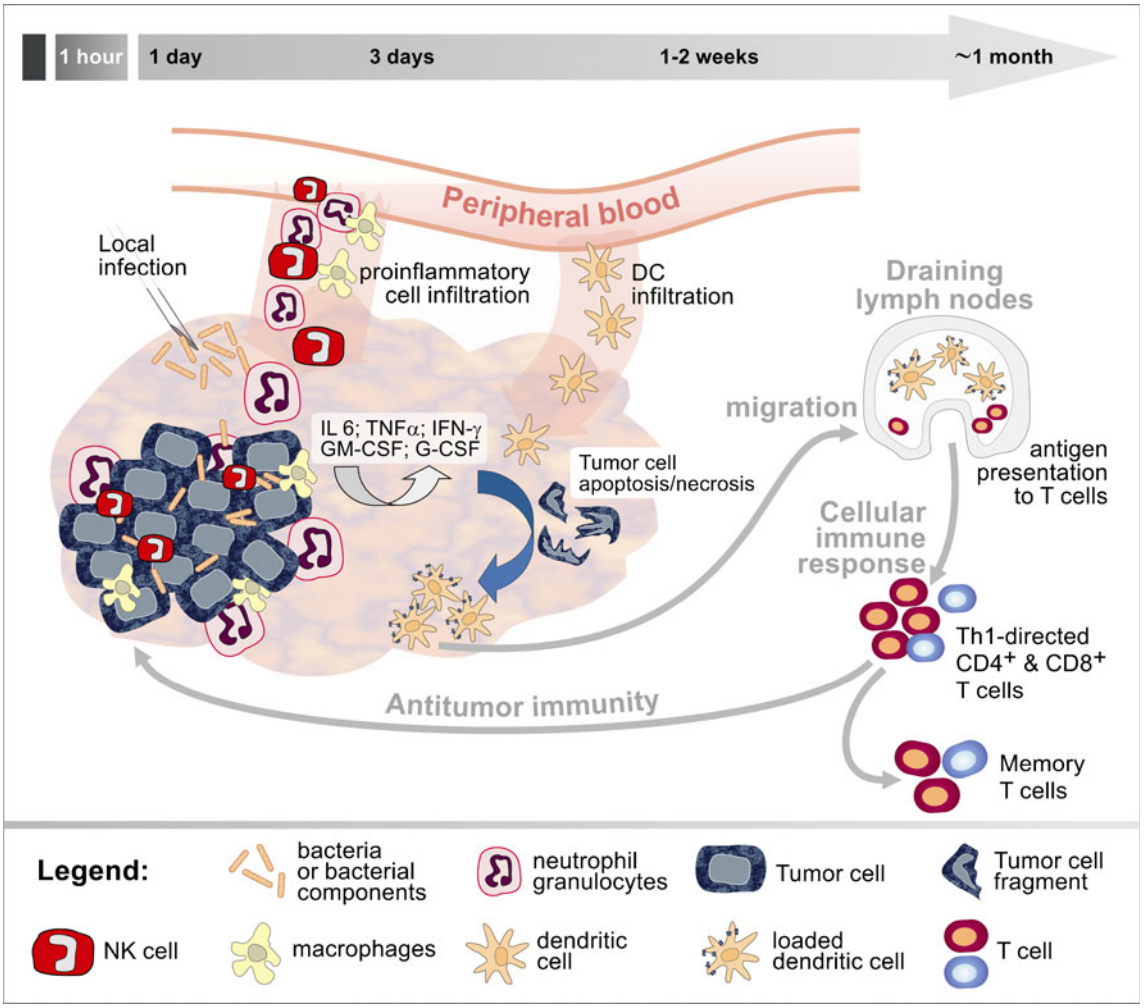

Fig. 1 Scheme: bacterial immunotherapy's mode of action. Presence of intratumoral bacteria or bacterial components is sensitized by innate immune cells (NK cells, macrophages, neutrophils). Tumor destruction takes place to a varying degree followed by secretion of proinflammatory cytokines and chemokines. This attracts immature DCs into the focus of infection. They take up bacterial material together with tumor fragments, mature while migrating to draining lymph nodes, where they present tumor antigens (in addition to bacterial antigens) to T cells. Activated and expanded T cells subsequently infiltrate the proinflammatory tumor microenvironment where they efficiently kill tumor cells. Long-lasting antitumoral immunity with the potential to control micrometastases forms when part of these T cells becomes memory cells

on stimulation of the unspecific immune system, i.e. macrophages, granulocytes, dendritic cells (DC) and NK cells. NK cells in particular can promote tumor regression either directly through the anti-tumor activity of type I interferons or through cell-mediated tumor cell killing. In our study on using avitalized staphylococci, effective tumor growth delay was found to be accompanied by increased numbers of tumor-infiltrating immune cells (NK cells and DC) [36]. The importance of the innate immune system on tumor control could further be corroborated in T cell deficient mice using human colorectal carcinoma (CRC) xenografts. However, despite the observed significant tumor growth control, *S. aureus* did not provoke complete cure, independent from the treatment regimen [36]. When comparing these findings with previous results using live and lysed bacteria, the adjuvant stimulus of avitalized bacteria was probably strong enough to break the tumor-induced tolerance; however, it is not strong enough to induce long-term immunological responses.

To improve this regimen with respect to specific tumor targeting, we performed experiments using conjugates of bacteria and therapeutic monoclonal antibodies (mAb) for experimental immunotherapy (*manuscript in preparation*). This strategy is based on the idea that clinically successful therapeutic mAb may have an additional or synergistic impact on tumor growth. In first line treatment, therapeutic mAbs like cetuximab and panitumumab specifically targeting epidermal growth factor receptors (EGFR) are applied in combination with chemotherapies [37–39]. MAb can have effects on tumor cells by (i) disturbing receptor-mediated

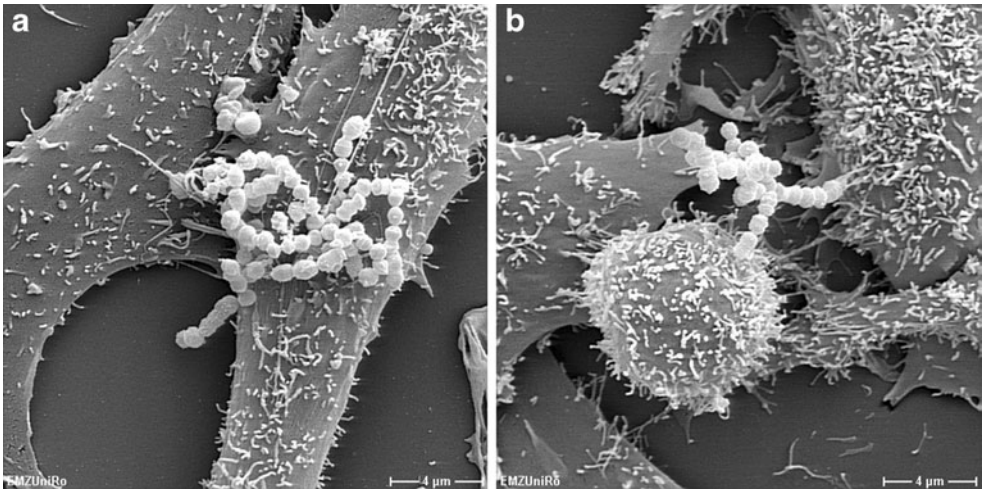

Fig. 2 Electron microscopy showing *S. pyogenes* binding to tumor cells. **a** Murine pancreatic carcinoma cell line (Panc02). **b** Human pancreatic carcinoma cell line (AsPC1). Bacteria adhere to tumor cells via surface molecules. This process, known as bacterial adherence, is the first step of tumor infection thus providing the basis for subsequent tumor cell lysis

signaling, (ii) complement-mediated tumor cell lysis and (iii) cell-mediated tumor cell attack. This may finally lead to a boosted overall immune response. By using immunocompromised mice, we performed clinically relevant systemic injections based on the hypothesis that bacteria coupled to tumor-specific mAb shall permit effective tumor cell targeting in vivo thus concentrating the lytic effects of bacteria on the tumor. Beyond that, potential allergic reactions might be minimized. Although we could not experimentally prove tumor-targeting by bacteria–mAb conjugates, i.e. neither bacteria nor mAbs were detectable in tumors, we observed a substantial delay in tumor growth. This result correlated well with immunological parameters. We found massively increased numbers of circulating monocytes, DCs and NK cells. This provides a basis to refine the concept on bacteria-based combination therapies in the future.

Taken together, these studies contribute to the deeper understanding of how bacteria or their related products act on tumor and immune cells. Hence, bacteriolytic immunotherapy is still a promising alternative strategy for cancer treatment, either alone or in combination with cytostatic drug or antibody therapy. Future investigations will show if these experiments stand clinical trials.

Current clinical approaches using bacteria or bacterial preparations

Up to now, Bacillus Calmette–Guérin (BCG) is still the most successful bacterial agent used for superficial bladder cancer treatment [40]. BCG was introduced by Morales in 1976, based on the original development in 1921 as an attenuated strain of *Mycobacterium bovis* for tuberculosis vaccination. BCG as a live bacterium can exert local (irritative voiding symptoms) as well as systemic (fever, chill, malaise) effects. A functioning immune system is required to prevent excessive reaction, and consequently immunosuppressed patients are not considered for bacterial therapy. Patient's treatment is conducted on weekly intravesical injections for 6 consecutive weeks. In some cases adjuvant cytokines (IFN) may be added.

An inflammatory and immunological reaction, i.e. leukocytosis, granulomas and urinary cytokines (IL2, IL6, IL8, IL10, IFNγ, TNFα), was found to be positively correlated with patients outcome. The mechanism of action is not yet fully understood. There is, however, some degree of tumor-specific killing by BCG, which is exclusively seen when live bacteria are used—heat-killed bacteria have no effect. This is due to the behavior of *M. bovis* as an obligate intracellular pathogen. Hence, the mechanism of infection can here be used for tumor therapy: BCG is taken up in a fibronectin-dependent manner. The infected cell responds with an inflammatory cascade (cytokine and chemokine secretion) followed by cell lysis (in a TNF-related apoptosis inducing ligand-specific manner), the initiation of an unspecific neutrophils-directed reaction and the induction of a significant Th1 response. This lastly provides long-term adaptive immunity.

The cell wall skeleton of *M. bovis* is supposed to be the major immune stimulating component. BCG–cell wall skeleton induces IFNγ secretion and stimulates skin Langerhans cells to convert to DCs [41, 42]. Thus, it may act as an ideal adjuvant for immunotherapy. By taking advantage of the favorable immune effects, *Mycobacterium tuberculosis* strain H37Ra, a heat-killed and dried bacterial preparation, has been widely applied as part of the complete Freund's adjuvant to attract macrophages and neutrophils to the injection site. Complete Freund's adjuvant is typically used for initial injections and incomplete Freund's adjuvant (without H37Ra) for subsequent boosts [43, 44]. Of note, many new candidate immunoadjuvants are tested in clinical trials in order to treat several tumor entities. An overview of actual recruiting trials is given in Table 2.

Table 2 Ongoing clinical studies using bacterial-based immunologic adjuvants for cancer therapy with or without defined antigens

Adjuvant	Tumor entity	Antigen	Phase
TLR3 agonist Poly-I:C (+/− Montanide and GM-CSF)	Colorectal carcinoma	NY-ESO-1	I/II
Peptide (URLC10-177 and TTK-567) vaccine (+TLR9 agonist CpG ODN, +/− Montanide)	Esophageal cancer	None specified	I/II
Mifamurtide (L-MTP-PE, a synthetic bacterial cell wall component) (+/− chemotherapy)	High grade osteosarcoma	None specified	I
TLR9 agonist PF-3512676 (+ radiation)	Low grade B-cell lymphoma	None specified	II
TLR3 agonist Poly-I:C (+/− Montanide and GM-CSF)	Melanoma	NY-ESO-1	I/II
TLR7/8 agonist resiquimod + peptide vaccine (gp100)	Melanoma	gp100 and MAGE-3	II
TLR9 agonist CpG ODN + autologous tumor cell vaccine (+ chemotherapy, if necessary)	Metastatic colorectal carcinoma	None specified	I
TLR7 agonist imiquimod (+ laser therapy)	Metastatic stage III or stage IV melanoma	None specified	I
TLR9 agonist CpG ODN (+/− Montanide and GM-CSF)	Pretreated stage II or stage III breast cancer	Her2/neu/MUC1	I
TLR9 agonist EMD 1201081 + cetuximab	Recurrent or metastatic head and neck squamous cell carcinoma	None specified	II
TLR7/8 agonist resiquimod + peptide vaccine	Stage II, stage III, or stage IV melanoma after surgery	NY-ESO-1	I
CpG ODN + (multiple) peptides (+/− Montanide)	Stage III/IV melanoma patients	Melan-A, Mage-10 and NY-ESO	I
TLR3 agonist Poly I:C + peptide vaccine	Stage IV melanoma	MAGE-A3	II
OK-432 (Picibanil) + mixed vaccine	Esophageal, lung, stomach, breast and ovarian cancer	HER2/neu and/or NY-ESO-1	I
TLR8 agonist VTX-2337 + cetuximab	Locally advanced, recurrent or metastatic squamous cell cancer of the head and neck	None specified	I

Data are collected from clinical trials listed in www.cancer.gov/clinicaltrials

Bacteria as vectors for gene therapy

Besides their capacity to directly act on tumor cells, bacteria are widely applied as delivery systems for tumor-associated antigens in tumor immunotherapy [45]. Bacteria serve as vectors or vehicles for (i) producing the protein of interest, (ii) delivering cytostatic drugs or prodrug converting enzymes and (iii) expressing cytotoxic peptides or therapeutic proteins. Unlike viral gene delivery systems, bacteria have a large capacity for genetic material insertion without affecting the production of infectious offspring. Even multiple different plasmids encoding for distinct antigens and immune enhancing elements can be introduced [46, 47]. *S. typhimurium* as a facultative intracellular pathogen is one of the most attractive vehicles that guide the way for bacterial oncolytic therapy. Salmonellae can be taken orally, cross the lumen of the gut via M cells of Peyer's Patches and are taken up by local macrophages and DCs [48]. An innate immune response can be generated to promote the development of adaptive immune responses against the carried tumor-specific antigens. Similarly, *Listeria*, *Clostridium*, *Shigella* and *E. coli* can be used as excellent vehicles for the production and targeted delivery of therapeutic molecules into cancer cells [49]. A comprehensive overview on how bacteria are currently used as gene delivery vectors, the mechanisms of action and successes at preclinical and clinical levels is reviewed by Baban et al. 2010 [5].

Additionally, bacterial ghosts (BGs) are under experimental investigation for advanced drug delivery systems of toxic substances in tumor therapy. BGs are empty bacterial envelopes of Gram-negative bacteria, devoid of the cytoplasmic content. They possess all bacterial bio-adhesive surface properties in their original state while not posing any infectious threat. The inner space of BGs can be loaded with either single components or peptide combinations, drugs or DNA which provides an opportunity to design new types of (polyvalent) drug delivery vehicles [50].

In summary, oncolytic bacterial therapies have been examined in combination with clinically applicable treatments such as radiotherapy and chemotherapy [45, 46]. Many of the nascent bacterial delivery platforms described have entered human clinical trials.

An inflammatory milieu—protumoral or antitumoral effects?

Bacteria, either used as direct anticancer agent or as a vehicle for cytotoxic drugs, mediate strong pro-inflammatory reactions that have beneficial effects for tumor therapy. On the contrary, there is strong evidence that chronic viral or bacterial inflammation initiates or triggers tumorigenesis [51, 52]. Epidemiological studies showed that a number of chronic infections predispose to various tumor types. In this regard, infection by *H. pylori* is associated with gastric cancer and mucosal lymphoma, while viral infections are related to cervical and liver cancer [53–58]. Vaccination against such viruses has proven efficient in preventing cancer development [59, 60].

The underlying mechanism on how bacteria and viruses may instigate malignant transformation is in part attributable

to the expression of several toll-like receptor (TLR) agonists, a broad variety of structurally conserved molecules derived from microbes (an overview is given in Fig. 3). On the one hand, TLR stimulation mediates tumor cell apoptosis and accelerates innate immune activation that is often followed by cellular Th1-directed immune responses [61–64]. On the other hand, TLR stimulation exerts protumoral effects by favoring (i) tumor initiation, development and invasion, (ii) resistance to chemotherapy and (iii) immune tolerance [6, 65–67]. Indeed, some types of tumor cells exhibit increased proliferation rates upon TLR stimulation [own unpublished data]. These findings fit well with a recent study performed by Cherfils-Vicini and colleagues, showing that TLR7 or TLR8 stimulation of primary lung tumor cells activates NF-kappaB, upregulates Bcl-2 expression, and increases tumor cell survival and chemoresistance [68].

Besides microbial-induced tumorigenesis, non-pathogenic triggers of chronic inflammation, including autoimmune diseases (e.g. inflammatory bowel disease), enhance the risk for malignant transformation (Fig. 4). Accordingly, non-steroidal anti-inflammatory treatments are supposed to decrease tumor incidence [69]. However, a very recent publication describes controversial effect on long-term "chemoprevention" against cancers. The authors showed in a series of large-scale nested case–control studies that long-term use of selective cyclooxygenase 2 inhibitors was associated with a reduced risk of colorectal cancer (CRC), while the risk for breast cancer and haematological malignancies (particularly lymphomas) was increased [70]. These observations underscore the bivalent role of inflammation on cancer, favoring tumor regression in the acute phase while increasing the risk after chronic manifestation.

Two pathways describe the relationship between inflammation and cancer: an intrinsic pathway, driven by genetic alterations that cause inflammation and neoplasia (such as oncogenes), and an extrinsic pathway, driven by inflammatory leukocytes in the context of chronic infectious or persistent inflammatory conditions that augment cancer risk [71]. The latter one is attributable to microbial-induced tumorigenesis. This facilitates a "vicious circle" characterized by (i) local cytokine production (e.g. TNFα, which has controversial roles in cancer, serving as a tumor-promoting or tumor-destructive factor), (ii) chemokine production (CCL7, CCL20, CCL25, CXCL1 and CCL26), (iii) secretion of growth factors (EGF, IGF, TGFα, VEGF), (iv) suppressing adaptive immunity and T cell function and (v) secreting matrix-degrading enzymes (matrix-metalloproteinases, e.g. MMP2 and 9). In solid tumors, immune cells are localized both at the periphery and in the tumor stroma, occasionally invading cancer cell nests. Tumor-associated macrophages (TAM) and T lymphocytes are the most abundant immune population in the tumor-microenvironment, although some eosinophils, mast cells, NK cells and rare DC can be found [72].

Most of the infiltrating leukocyte subsets are effective suppressors of antitumoral Th1-directed immune responses. TAM (also M2 or 'alternatively' activated macrophages, phenotype: IL-12^{low}/IL-10^{high}) inhibit T-cell activation via secretion of different suppressive mediators, such as IL4, IL13, IL10, TGFβ and indoleamine 2.3-dioxygenase (IDO) [73, 74], as well as MHC class II down regulation. The currently accepted concept implies the M2-polarized myeloid cells promote tumor angiogenesis and invasion [75]. In many but not all human tumors, a high frequency of infiltrating TAM is associated with poor prognosis. But macrophages

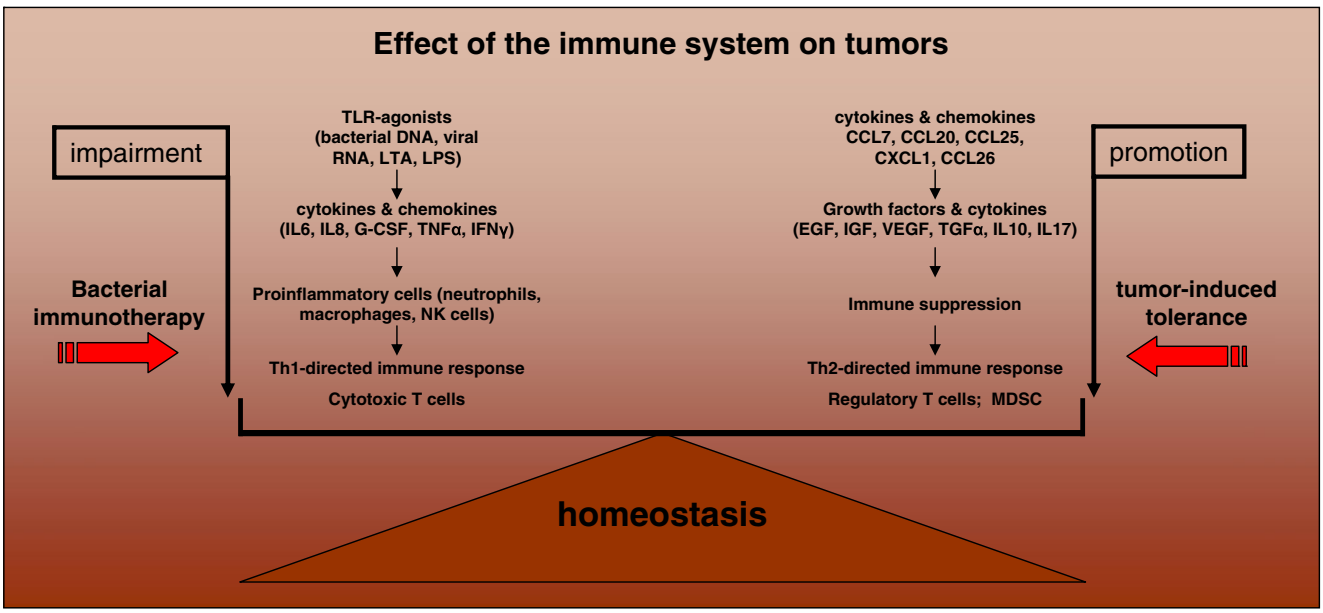

Fig. 3 Scheme: balance of physiological immunity in the tumor context. LTA (lipoteichoic acid), LPS (lipopolysaccharide)

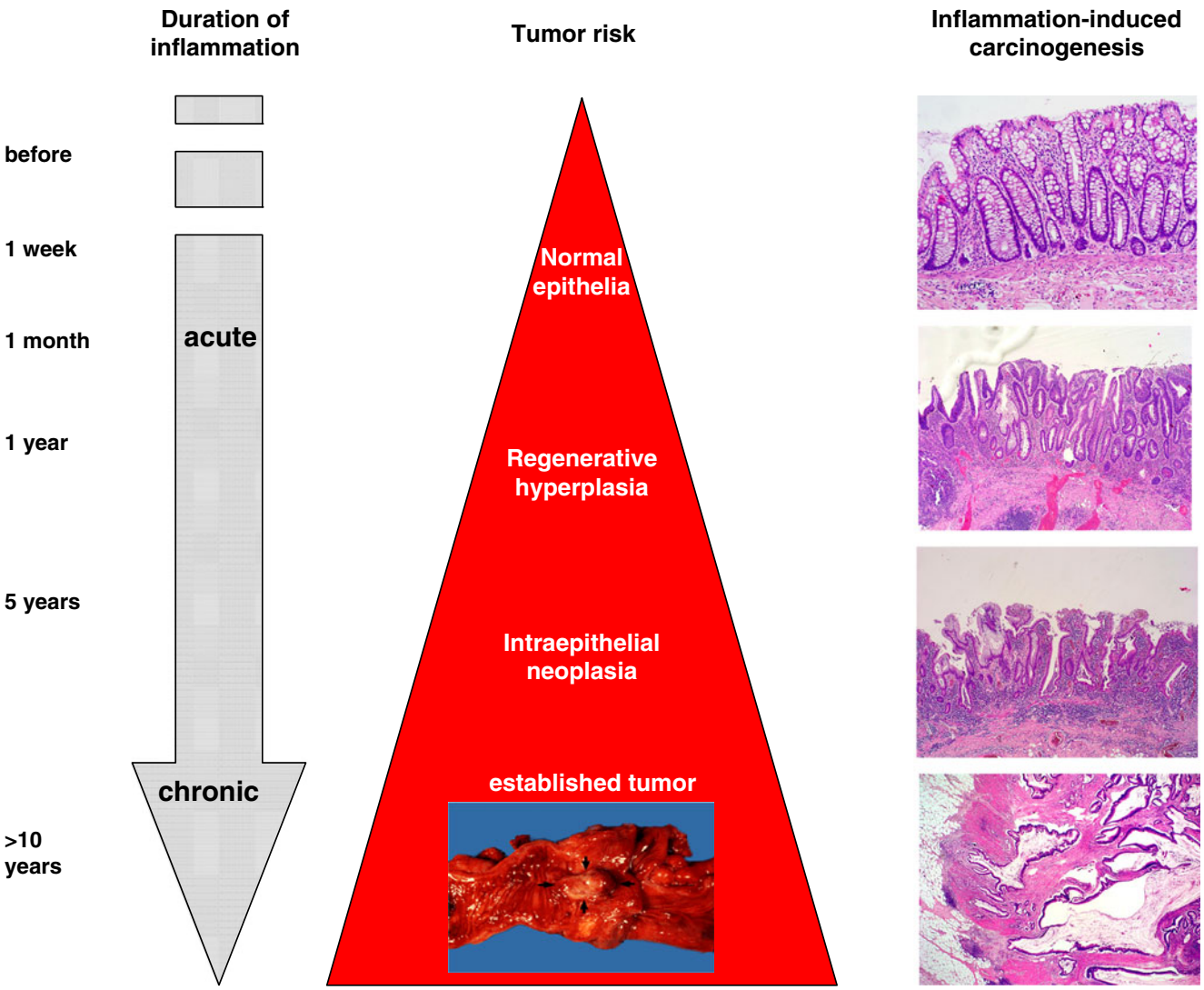

Fig. 4 Scheme: relationship between inflammation and tumor development. Ulcerative colitis associated tumors develop over decades in a multistep process characterized by defined morphological alterations. Regenerative hyperplasia characterizes the body's attempts to restore gut function after an acute inflammatory episode. Intraepithelial neoplasia develops when this regenerative stimulus becomes chronic. Finally genetic alterations triggered by inflammation-induced DNA damage force tumor development

may act as a double-edged sword in cancer, since these cells can be re-educated to exert antitumor activity [76, 77]. It was demonstrated that IL12 injection induced a pro-immunogenic potential of TAM while reducing tumor-supportive activities [78].

In the context of an inflammatory response, myeloid cells are the primary recruited effectors. In tumors, these cells are represented by myeloid-derived suppressor cells (MDSCs). Murine MDSCs are referred to as $CD11b^{+}Gr1^{+}$, which are the predominant population of tumor-associated myeloid cells. Similar to TAMs, increased numbers appear in the blood during tumor growth in mice (Fig. 5), with some being recruited to the tumor site to promote angiogenesis [73, 79]. Of note, myeloid cells infiltrating tumors may induce tumor cell resistance to CTLs by modifying the peptide–MHC complexes on tumor cells via reactive oxygen species [79]. These modifications reduce the capacity of the tumor cells' MHC to bind antigenic peptides and subsequent recognition by CTLs. This provides a novel concept regarding tumor escape associated with inflammation and might have consequences for therapeutic approaches focusing on pharmacological reactive oxygen species inhibition [79].

Finally, regulatory T cells (phenotype: $CD4^{+}CD25^{+}FOXP3^{+}CD127^{low}$) which have been identified long time ago contribute to the general tumor-specific T cell tolerance. Tregs are activated in an antigen-specific manner but are believed to suppress T cells in an antigen-non-specific

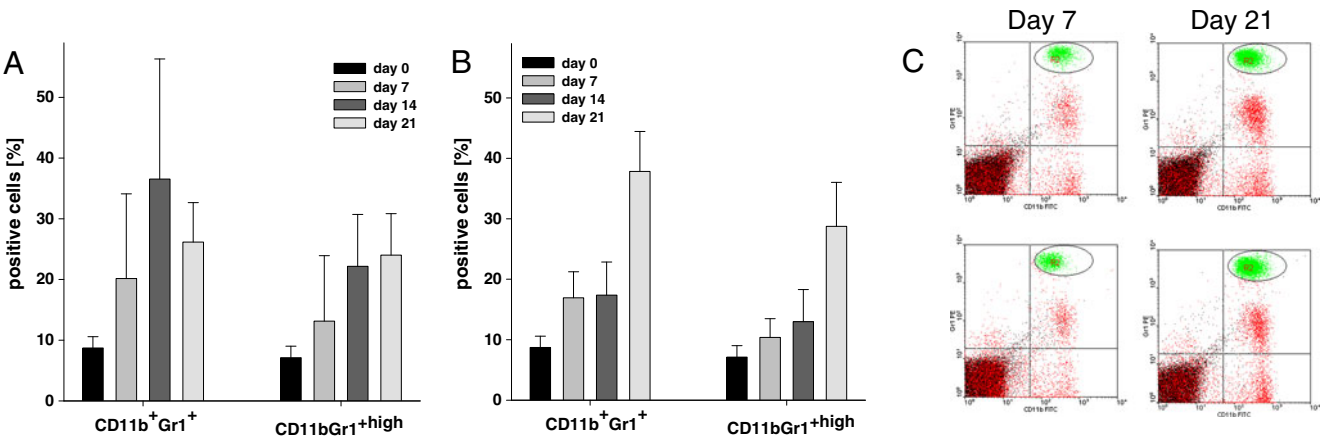

Fig. 5 Myeloid-derived suppressor cells in the peripheral blood of mice harboring murine Panc02 tumors. Tumor cells were either injected (**a**) subcutaneously into the right hind flank or (**b**) orthotopically into the pancreas. Blood samples were taken weekly for a period of 3 weeks, and amounts of $CD11b^{+}Gr1^{+}$ cells were assessed by flow cytometry. **c** Representative dot plots showing positive staining for MDSC marker CD11b and Gr1 at days 7 and 21 post-tumor cell injection either subcutaneously (*upper panel*) or orthotopically (*lower panel*). Mean + SEM. $n = 3–5$ mice per group

manner [71]. While Treg accumulation at high density in tumors is generally related to a poor outcome, in patients suffering from CRC, high Treg infiltration is associated with a favorable clinical prognosis [80]. To explain this irony, the dense microbiological flora present in the gut requires a T-cell-mediated inflammatory anti-microbial response that, among others, involves Th17 cells [81]. This Th17-cell-dependent proinflammatory and tumor-enhancing response can be attenuated by Tregs, thus reconstituting the balance between pro-inflammatory and anti-inflammatory effects in the gut that may contribute to the favorable role in CRC prognosis. The link between a high density of FOXP3-positive Tregs in CRC may lead to a paradigm shift and thus help to decide when immunotherapy based on the integration of bacteria or microbial structures is feasible and safe.

Summary and outlook

Bacterial-induced inflammation is a double-edged sword. In an acute phase, bacteria massively activate the immune system initiating an unspecific, often neutrophil-directed reaction that is followed by a Th1 or cytotoxic T cell directed cellular response. This lastly provides long-term protective immunity. However, when starting to be chronic, inflammation triggers tumorigenesis by suppressing adaptive immunity and T cell function. An immune status characterized by tolerance towards self but altered cells arises that finally drives tumor progression. Experiences from the last years helped to get deeper insight into the mechanisms, and hence attempts in dealing with the fine line between protumoral and antitumoral effects to finally re-establish homeostasis are on their way. However, questions remain for which tumor type bacterial-based therapy will be applicable, since differences exist between tumor entities with a tendency towards better responses in tumors that arise in a sterile microenvironment compared to those coming from a non-sterile, bacterial-experienced background (i.e. the gut).

Bacteria and their components, i.e. mainly defined TLR-ligands (PAMPs) can be safely applied in humans with limited adverse side effects and are thus established in the clinic as immunostimulatory adjuvants. Combination therapies are also being investigated for potential future applications.

Finally, several findings argue in favor of bacteria for immunotherapy compared to viruses. These include (i) safety of application; even when using live bacteria, secure control can be guaranteed due to their antibiotic sensitivity; (ii) several bacterial ligands (PAMPs) are known to directly act on immune and tumor cells; (iii) fewer problems in terms of practicability of usage, production and prize; and (iv) fewer size restrictions when generating transgenics expressing tumor target genes, therapeutic proteins or prodrug converting enzymes. Bacterial ghosts may even be used to delivering anticancer agents or cytotoxic peptides, directly.

These findings provide a ready basis for further priming the concept of bacterial cancer immunotherapy for the clinical setting.

Conflicts of interest None.

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