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Engineered bacteria as therapeutic agents

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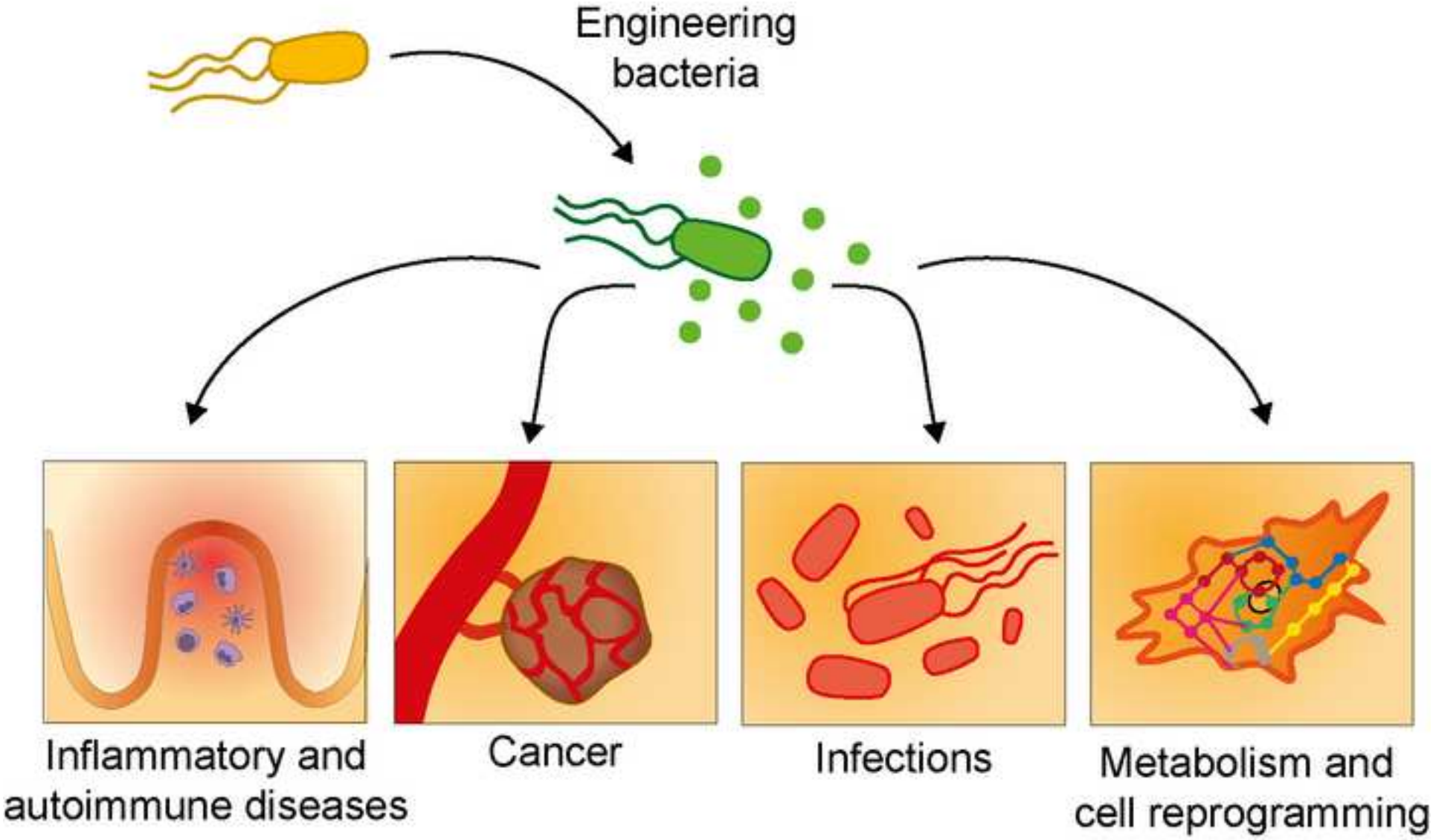
Abstract

Although bacteria are generally regarded as the causative agents of infectious diseases, most bacteria inhabiting the human body are non-pathogenic and some of them can be turned, after proper engineering, into “smart” living therapeutics of defined properties for the treatment of different illnesses. This review focuses on recent developments to engineer bacteria for the treatment of diverse human pathologies, including inflammatory bowel diseases, autoimmune disorders, cancer, metabolic diseases and obesity, as well as to combat bacterial and viral infections. We discuss significant advances provided by synthetic biology to fully reprogram bacteria as human therapeutics, including novel measures for strict biocontainment.

Graphical abstract (for review)



Introduction

Bacteria are key elements for human health. Two evidences support this notion, the existence of a stably population of microbes, termed microbiota, in healthy individuals and the number of health disorders associated to axenic (germ-free) animals [1,2]. In addition, bacteria have been actively administered in patients suffering from different illnesses for over a century. These intentional administrations are in general carried out with natural isolates obtained from the microbiota of healthy individuals and are referred to as probiotics. In most cases they belong to lactic acid bacteria (LAB) and in a lesser extent to *Escherichia coli* strains [3]. The development of efficient DNA technologies for manipulation of microbial genomes and the increasing knowledge of the molecular basis of diseases are allowing the engineering of tailored bacteria for the treatment of human disorders. Bacteria can be altered to produce a continuous and inexpensive supply of heterologous molecules of biomedical interest, such as human hormones, interleukins (ILs) and antibodies (Abs) within specific organs or tissues. We have restricted this review to bacterial engineering for human therapies, but similar concepts can be applied for the development of live bacterial vaccines [4].

Engineered bacteria against inflammatory and autoimmune disorders

Inflammatory bowel diseases (IBDs) have been prototypical targets of probiotic therapies, given the immunomodulatory effects that certain strains are able to exert in the gastrointestinal tract (GIT). Patients suffering from IBDs frequently have alterations in pattern recognition receptors and pro-inflammatory genes, which elicit an abnormal activation of the immune system in the gut and lead to chronic intestinal inflammation and to an increased rate of oxidative stress [5]. Different bacteria, mostly LAB, have been engineered to express a wide variety of compounds *in situ* (i.e. anti-inflammatory cytokines, anti-oxidant enzymes) to prevent the appearance of these symptoms [6]. These strategies are summarized in Figure 1. At least three of these engineered microorganisms have been tested in clinical trials. A *Lactococcus lactis* strain, engineered to secrete the anti-inflammatory cytokine IL-10 [7] showed a clinical benefit

in 8 out of 10 patients tested with Crohn's disease [8], probably by inducing regulatory T cells (Tregs) through activation of dendritic cells (DCs) [9]. Another approach involved the use of engineered *L. lactis* strain secreting single domain antibody fragments (nanobodies) [10] against the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNF-α). This study showed promising results in murine models of IBD [11] and the bacterial strain has been tested in a phase 1 clinical trial by Actogenix (http://www.actogenix.com/). Lastly, *L. lactis* expressing human Trefoil Factor 1 (hTFF1) - a cytopeptide involved in epithelial wound healing - has been formulated as a mouthwash for the treatment of oral mucositis [12], which is a common complication found in patients that are subjected to chemo- and radiotherapies. This therapy has already passed through a phase 1 pharmacokinetic study and a phase 1b clinical trial involving cancer patients receiving chemotherapy, which showed alleviation of ulcerative oral mucositis symptoms in 30% of cases [13].

Besides the treatment of IBDs, engineered LAB secreting specific peptide antigens have been delivered orally to induce mucosal immune tolerance to antigens involved in food-allergies (e.g. ovalbumin) [14] or to gliadin antigens in the celiac disease [15]. Interestingly, this approach has been also applied to treat type 1 diabetes (T1D), an autoimmune disorder that arises from insufficient tolerance to self-antigens of pancreatic β-cells. An engineered *L. lactis* strain secreting the auto-antigen proinsulin and the anti-inflammatory cytokine IL-10, combined with subtherapeutic doses of systemic anti-CD3 Abs, was administered to non-obese diabetic (NOD) mice [16]. The immunosuppressive effect of anti-CD3 Abs (targeting the T-cell receptor) hampers immune system activation, whereas IL-10 avoids inflammation in the gastrointestinal mucosa and induces the development of Tregs, providing altogether an ideal scenario for the induction of tolerance. After 6 weeks of daily oral administration of the engineered strain, approximately 60% of the mice stably reverted diabetes, probably as a consequence of the infiltration of Tregs into pancreatic islets. Responding mice showed normal glucose levels in blood, and remained responsive to other antigens,

suggesting an antigen-specific immunosuppression. A similar combination therapy based on anti-CD3 Abs and the oral delivery of engineered *L. lactis* secreting IL-10 and the diabetes-related auto-antigen glutamic acid decarboxylase (GAD)-65 has been reported [17]. This combination was effective in the treatment of advanced stages of T1D with severe hyperglycemia. Induction of tolerance against heat shock proteins (HSPs) has been also achieved using LAB. HSPs partipate in the control of autoimmunity and in the cellular response to stress and are overexpressed in diseases involving inflammation and autoimmunity mechanisms, such as atherosclerosis and encephalomyelitis [18]. Oral administration of *L. lactis* strains secreting different forms of HSP-65 exerted a protective effect against endothelial damage and against the formation of atherosclerotic lesions in a low-density lipoprotein receptor-deficient (LDL-RD) mouse model [19]. This approach also prevented the development of encephalomyelitis in mice, showing reduced inflammatory cell infiltrate in the spinal cord [20]. These effects were associated with increased production of IL-10 and reduced levels of IL-17 and interferon gamma (IFN-γ).

Engineered bacteria against cancer

Firsts reports of bacterial treatments against solid tumors date from the end of the 19th century. Over the years many genera of (facultative and strict) anaerobic bacteria (e.g. *Salmonella*, *E. coli*, *Clostridium, Bifidobacterium*) were reported to proliferate preferentially within solid tumors [21] due to a combination of mechanisms. These include enhanced bacterial entry and retention in tumors caused by their chaotic and leaky vasculature [22,23] and unimpeded bacterial replication in the anoxic and immune-deficient tumor microenvironment, which lacks macrophage and neutrophil clearance mechanisms [24]. Chemotaxis and active motility of bacteria has also been shown to have a positive influence in the colonization of tumors [25,26]. Intra-vesicle administration of Bacillus Calmétte-Guerin (BCG) has been used in the clinic during the last decades as the standard treatment for high-grade non-muscle invasive bladder cancer [27], representing a good example of a current anti-cancer therapy in which

bacteria is employed to stimulate the immune system in order to promote the killing of cancer cells by a mechanism that is not yet fully understood.

Bacteria can bypass problems associated with poor selectivity and limited tumor penetrability of conventional cancer chemotherapies, and can be finely engineered to sense and respond to the tumor microenvironment [28]. However, the antitumoral effect of bacterial growth within tumors is generally weak, and different strategies have been followed to improve their therapeutic potential against cancer. One of them is the direct destruction of tumor cells through the secretion of bacterial toxins *in situ* (e.g. *Staphylococcus aureus* alpha hemolysin) [29,30] or the expression of pro-drug converting enzymes that locally convert non-toxic prodrugs into drugs, like *E. coli* cytosine deaminase (CD) that transforms non-toxic prodrug 5-Fluorocytosine into toxic 5-Fluorouracil, resulting in a bacterial-directed enzyme prodrug therapy (BDEPT) localized in tumor areas [31]. BDEPT provides an excellent tumor selectivity since the drug is produced *in situ*, however, and similarly to conventional chemotherapy, its efficiency is highly dependent on physico-chemical properties of the prodrug that will define its ability to reach deep areas of the tumor in which bacteria (and therefore the converting enzyme) are located. In another strategy, engineered bacteria compete with the mechanisms that foster tumor formation (i.e. angiogenesis, resistance to apoptosis, evasion from the immune system, etc.) through the *in situ* delivery of polypeptides with pro-apoptotic activity (e.g. TNF-related apoptosis-inducing ligand -TRAIL-, Fas ligand, Noxa), anti-angiogenic factors (e.g. Endostatin, Thrombospondin 1), and cytokines (e.g. IL-2, LIGHT) that induce the immune system against tumor cells [28,32]. These strategies are shown in Figure 2. Interestingly, an additional level of specificity is provided by controlled gene expression with promoters that specifically respond to small chemical inducers (e.g. L-arabinose, anhydrotetracycline, salicylate) [33,34], tumor environment [35], hypoxia [36], or γ-irradiation [37]. Remarkably, the expression of the desired therapeutic protein can be specifically triggered within tumors using bacterial promoters responding to quorum sensing molecules released by the high-

density of bacteria in tumors [38]. A similar strategy has also been employed to amplify the expression of the desired protein induced with small molecules (e.g. L-arabinose) that diffuse poorly within the tumor mass [39]. Bacteria have also been engineered to silence the expression of important genes related to tumor development through RNA interference. For instance, *E. coli* was engineered to transfer to host cells plasmids encoding short-hairpin RNAs (shRNAs) silencing catenin beta-1, whose overexpression is involved in several types of cancer [40]. This therapy has been granted by the FDA as orphan drug for the treatment of familial adenomatous polyposis and Marina Biotech (http://www.marinabio.com/) is currently developing clinical trials to analyze the safety and tolerability of its oral administration.

First reported clinical trials with refractory cancer patients using systemic administration of engineered *Salmonella* strains highlighted the trade-off between safe and effective dose, since the highest tolerated dose was insufficient for effective tumor colonization [41]. Hence, improving bacterial tumor colonization at low bacterial doses is an area of great interest. A promising advance is the constitutive and non-toxic expression on the bacterial surface of synthetic adhesins, which contain nanobodies targeting antigens expressed on the surface of tumor cells [42]. Expression of synthetic adhesins in *E. coli* allowed a significant reduction of two-orders of magnitude in the dose of bacteria needed for efficient colonization of target solid tumors in mice.

Engineered bacteria for the treatment of metabolic disorders

Given that the intestinal microbiota influences the susceptibility of an individual to develop metabolic disorders, it seems reasonable that the introduction of properly engineered bacteria could ameliorate their symptoms. In the case of obesity it has been demonstrated that the composition of the microbiota in lean mice is different from the one found in obese mice [43,44]. Furthermore, experiments involving transplantation of the intestinal microbiota from human identical twins discordant for obesity to germ-free mice revealed that only those mice receiving microbiota from the obese human

developed obesity [45]. Recently, an approach to control obesity has been developed based on an engineered *E. coli* Nissle 1917 (EcN) strain expressing an N-acyltransferase from *Arabidopsis thaliana* that synthesizes N-acylphosphatidylethanolamines (NAPEs) [46]. NAPEs are precursors of the N-acylethanolamide (NAE) family of lipids, which are naturally synthesized in the small intestine in response to feeding and cause a reduction in food intake and obesity. Incorporation of NAPE-expressing EcN in drinking water diminished food intake in mice, reducing body fat and weight gain, with no signs of adverse effects due to bacterial administration. Treated mice also had lower levels in plasma of insulin and leptin. Interestingly, the protective effect of the engineered bacteria persisted for at least 4 weeks after their removal from drinking water, suggesting a non-transient colonization of the GIT.

Engineered bacteria have been also administered to lower the elevated blood glucose levels (hyperglycemia) in T1D. The gut hormone Glucagon-like peptide (GLP)-1 was known to induce insulin production in epithelial cells [47]. An engineered *Lactobacillus gasseri* strain secreting GLP-1 has been able to reprogram intestinal cells into insulin-secreting cells [48]. Reprogrammed rat intestinal epithelia expressed important markers of pancreatic β cells, and the insulin secretion kinetics were similar to those of healthy control rats, indicating that insulin production was glucose-responsive.

Engineered bacteria to combat viral and bacterial infections

Intervention against viral and bacterial infections has been addressed with engineered bacteria, which have been recently reviewed [49]. A wide variety of approaches have been followed, including binding of toxins, interference with quorum sensing molecules and adhesion mechanisms, release of neutralizing antibodies and antimicrobial factors, which are summarized in Figure 3. For instance, an engineered *Streptococcus mutans* strain deficient for lactic acid production - therefore unable to produce dental caries - and secreting a bacteriocin capable of killing virtually all other streptococci strains, was shown to displace cariogenic *S. mutans* in a rat model [50]. More recently, strains of *L.*

lactis [51] and *E. coli* [52] have been engineered to secrete different bacteriocines in response to multiresistant *Enterococcus faecalis* pheromones and *Pseudomonas aeruginosa* quorum sensing molecules, respectively, showing bactericidal activity *in vitro*. Furthermore, an engineered *Lactobacillus casei* strain secreting human lactoferrin was demonstrated as effective to reduce the load of pathogenic *E. coli* in the duodenal fluid of infected mice, thus improving their illness score compared with untreated mice [53]. Production of antiviral molecules by engineered bacteria has been also investigated. *Lactobacillus jensenii*, a microorganism that is part of normal flora in human vagina, was engineered to secrete cyanovirin-N, a cyanobacterial protein with antiviral activity against HIV. Notably, trials in Rhesus macaques treated with the engineered *L. jensenii* strain and later challenged with simian HIV, showed a 63% reduction in HIV infection [54]. Modified *Lactobacillus* strains have been also used to deliver nanobodies interfering with rotavirus infection [55]. Lastly, there is a recognized potential in the incorporation of engineered bacteria into the microbiota of vector insects that mediate the transmission of human pathogens such as *Trypanosoma cruzi*, the causative agent of Chagas disease [56].

Stem cell reprogramming and genome edition using engineered bacteria

Although new technologies for genome edition such as transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 endonuclease, are minimizing the risk of undesired genome modifications [57], reprogrammed stem cells obtained through DNA transfer techniques have a limited clinical applicability due to the risk of insertional mutagenesis during the differentiation process. In addition, it has been reported that successfully reprogrammed induced pluripotent stem cells (iPSCs) show a pronounced silencing of the transgenes encoding for reprogramming factors, suggesting that these factors are required only transiently to mediate cell reprogramming [58]. Transient protein delivery represents a good alternative to mediate differentiation of stem cells, since completely avoids the risk of insertional mutagenesis and provides a measure for the temporal control of cell

exposure to reprogramming factors. The Type III Secretion System (T3SS) found in different Gram-negative pathogenic strains (*Salmonella*, *Shigella*, *Yersinia*, *E. coli*) is a needle-like macromolecular complex on the bacterial cell envelope that directly translocates effector proteins into the cytoplasm of infected host cells [59]. Attenuated strains carrying a functional T3SS have been used to deliver vaccine polypeptides and proteins of therapeutic potential (e.g. antibodies) into mammalian cells [60,61]. Bacteria with T3SS have also been employed to deliver enzymes and transcription factors that can edit the mammalian genome and reprogram cell differentiation. An attenuated strain of *Pseudomonas aeruginosa* harboring a functional T3SS has been shown to deliver Cre-recombinase into the nucleus of mouse embryonic stem cells (or iPS) [62] triggering loxP mediated excision of the nuclear reprogramming cassette carrying c-Myc, Klf4, Oct4 and Sox2 [63]. The T3SS of this bacterial strain has also been used to translocate TALENs into human cells, which are capable of genome edition [64]. The same group reported the differentiation of mouse embryonic fibroblasts into myocites through the T3SS-mediated injection of MyoD, a transcription factor that acts as master regulator of myogenesis [65].

Synthetic Biology approaches improving bacterial therapies

Synthetic biology aims to rationally design bacteria for therapy and other applications through the development of computational tools and techniques for extreme genetic manipulation. By these means, designed biological modules, devices and regulatory circuits of predictable behavior can be integrated into a bacterial chassis genome with strict biocontainment measures [66]. In recent years there have been multiple reports describing modular parts and more complex devices that can be used to program important aspects of the designed bacteria: controlled expression of payload proteins [67,68], programmable adhesion to target surfaces and cells [42], or the incorporation of stable memory into the engineered bacteria [69,70] that can be used to detect small molecules in the gut [71]. Programming of bacterial tropism has also been demonstrated by engineering *E. coli* chemotaxis toward areas in which a pathogen, such as *P.*

aeruginosa, is present [72]. Recent advances in engineering chemoreceptors and chemoeffectors [73] may allow a programmable chemotaxis to other molecules of interest in the future.

Ideal engineered bacteria for therapy should be sensitive to antibiotics and be free of mobile elements such as transposons and plasmids. Stable integration of the recombinant DNA in the chromosome is the simplest way to minimize gene flow, but there are other strategies available, like a mutually dependent host-plasmid platform based on conditional origins of replication, auxotrophies and toxin anti-toxin pairs [74]. In addition, engineered bacteria must have own containment strategies resistant to environmental supplementation, mutagenic drift and horizontal gene transfer. All these safeguards should avoid the spreading of these microorganisms into the environment and the proliferation of deleterious bacteria. Classically, biocontainment has been achieved through either engineered auxotrophies (e.g. strains deficient for thymidylate synthase) or induced lethality, which have been applied in clinical trials [8,13]. Synthetic biology is providing stricter biocontainment of the engineered bacteria. Minimal genomes encoding only the genes needed to sustain life might preclude unexpected evolution of engineered microbes. These minimal genomes could be generated through genome reduction techniques [75] and provide an excellent genetic chassis to implement the designed therapeutic gene devices (Fig. 4). However, the definitive firewall for biocontainment might be the use of artificial genetic languages. Indeed, there are already available engineered *E. coli* strains that either incorporate a non-standard amino-acid in the core of essential proteins [76] or the synthetic thymine analogue 5-chlorouracil instead of the natural thymine nucleotide in the DNA [77]. These strains exhibit strong resistance to evolutionary escape through mutagenesis or horizontal gene transfer, and cannot be supplemented with natural compounds.

Concluding remarks

Engineered bacteria represent an effective method to deliver therapeutic molecules *in*

vivo allowing the development of novel treatments against infections and major human diseases such as inflammatory, autoimmune and metabolic disorders, and cancer. In addition, engineered bacteria could help to reprogram host cells by delivering transcription factors and enzymes for genome modification. Next-generation therapeutic applications of bacteria will be based on the conscious design of microorganisms with defined properties for specific applications, rather than on the finding of probiotic isolates. This fine engineering can complement a genetic bacterial chassis with modules and parts designed *de novo* or based on the engineering of the plethora of devices that bacteria have evolved to interact with human cells, such as immuno-modulatory and effector proteins, chemotactic sensor systems, adhesins and protein delivery machineries. Human origins are those of nomad hunters and fruit pickers that flourished when they learned how to breed and cross wild animals and plants into the variants that still feed ourselves today. Similarly, microbiology started from microbe hunters studying wild bacteria and is now evolving to the rationale design of safe engineered bacteria able to monitor our bodies and fight against human diseases.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

** of outstanding interest

1. Smith K, McCoy KD, Macpherson AJ: **Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota**. *Semin Immunol* 2007, **19**:59-69.

2. Sekirov I, Russell SL, Antunes LC, Finlay BB: **Gut microbiota in health and disease**. *Physiol Rev* 2010, **90**:859-904.

3. Behnsen J, Deriu E, Sassone-Corsi M, Raffatellu M: **Probiotics: properties, examples, and specific applications**. *Cold Spring Harb Perspect Med* 2013, **3**.

4. Unnikrishnan M, Rappuoli R, Serruto D: **Recombinant bacterial vaccines**. *Curr Opin Immunol* 2012, **24**:337-342.

5. Xavier RJ, Podolsky DK: **Unravelling the pathogenesis of inflammatory bowel disease**. *Nature* 2007, **448**:427-434.

6. Martin R, Miquel S, Ulmer J, Kechaou N, Langella P, Bermudez-Humaran LG: **Role of commensal and probiotic bacteria in human health: a focus on inflammatory bowel disease**. *Microb Cell Fact* 2013, **12**:71.

7. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E: **Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10**. *Science* 2000, **289**:1352-1355.

8. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L: **A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease**. *Clin Gastroenterol Hepatol* 2006, **4**:754-759.

9. Huibregtse IL, Zaat SA, Kapsenberg ML, Sartori da Silva MA, Peppelenbosch MP, van Deventer SJ, Braat H: **Genetically Modified *Lactococcus lactis* for Delivery of Human Interleukin-10 to Dendritic Cells**. *Gastroenterol Res Pract* 2012, **2012**:639291.

10. Muyldermans S: **Nanobodies: natural single-domain antibodies**. *Annu Rev Biochem* 2013, **82**:775-797.

11. Vandenbroucke K, de Haard H, Beirnaert E, Dreier T, Lauwereys M, Huyck L, Van Huysse J, Demetter P, Steidler L, Remaut E, et al.: **Orally administered *L. lactis* secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis**. *Mucosal Immunol* 2010, **3**:49-56.

12. Caluwaerts S, Vandenbroucke K, Steidler L, Neirynck S, Vanhoenacker P, Corveleyn S, Watkins B, Sonis S, Coulie B, Rottiers P: **AG013, a mouth rinse formulation of *Lactococcus lactis* secreting human Trefoil Factor 1, provides a safe**

and efficacious therapeutic tool for treating oral mucositis. *Oral Oncol* 2010, **46**:564-570.

13. Limaye SA, Haddad RI, Cilli F, Sonis ST, Colevas AD, Brennan MT, Hu KS, Murphy BA: **Phase 1b, multicenter, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects with locally advanced head and neck cancer receiving induction chemotherapy**. *Cancer* 2013, **119**:4268-4276.

* This paper reports a recent clinical trial of an engineered *L. lactis* strain secreting human Trefoil Factor 1 to combat oral mucositis.

14. Huibregtse IL, Snoeck V, de Creus A, Braat H, De Jong EC, Van Deventer SJ, Rottiers P: **Induction of ovalbumin-specific tolerance by oral administration of Lactococcus lactis secreting ovalbumin**. *Gastroenterology* 2007, **133**:517-528.

15. Huibregtse IL, Marietta EV, Rashtak S, Koning F, Rottiers P, David CS, van Deventer SJ, Murray JA: **Induction of antigen-specific tolerance by oral administration of *Lactococcus lactis* delivered immunodominant DQ8-restricted gliadin peptide in sensitized nonobese diabetic Abo Dq8 transgenic mice**. *J Immunol* 2009, **183**:2390-2396.

16. Takiishi T, Korf H, Van Belle TL, Robert S, Grieco FA, Caluwaerts S, Galleri L, Spagnuolo I, Steidler L, Van Huynegem K, et al.: **Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified *Lactococcus lactis* in mice**. *J Clin Invest* 2012, **122**:1717-1725.

17. Robert S, Gysemans C, Takiishi T, Korf H, Spagnuolo I, Sebastiani G, Van Huynegem K, Steidler L, Caluwaerts S, Demetter P, et al.: **Oral delivery of glutamic acid decarboxylase (GAD)-65 and IL10 by *Lactococcus lactis* reverses diabetes in recent-onset NOD mice**. *Diabetes* 2014, **63**:2876-2887.

18. Colaco CA, Bailey CR, Walker KB, Keeble J: **Heat shock proteins: stimulators of innate and acquired immunity**. *Biomed Res Int* 2013, **2013**:461230.

19. Jing H, Yong L, Haiyan L, Yanjun M, Yun X, Yu Z, Taiming L, Rongyue C, Liang J, Jie W, et al.: **Oral administration of *Lactococcus lactis* delivered heat shock protein 65 attenuates atherosclerosis in low-density lipoprotein receptor-deficient mice**. *Vaccine* 2011, **29**:4102-4109.

20. Rezende RM, Oliveira RP, Medeiros SR, Gomes-Santos AC, Alves AC, Loli FG, Guimaraes MA, Amaral SS, da Cunha AP, Weiner HL, et al.: **Hsp65-producing *Lactococcus lactis* prevents experimental autoimmune encephalomyelitis in mice by inducing CD4+LAP+ regulatory T cells**. *J Autoimmun* 2013, **40**:45-57.

21. Pawelek JM, Low KB, Bermudes D: **Bacteria as tumour-targeting vectors**. *The Lancet Oncology* 2003, **4**:548-556.

22. Forbes NS, Munn LL, Fukumura D, Jain RK: **Sparse initial entrapment of systemically injected *Salmonella typhimurium* leads to heterogeneous accumulation within tumors**. *Cancer Res* 2003, **63**:5188-5193.

23. Maeda H: **The link between infection and cancer: tumor vasculature, free radicals, and drug delivery to tumors via the EPR effect**. *Cancer Sci* 2013, **104**:779-789.

24. Westphal K, Leschner S, Jablonska J, Loessner H, Weiss S: **Containment of tumor-colonizing bacteria by host neutrophils**. *Cancer Res* 2008, **68**:2952-2960.

25. Toley BJ, Forbes NS: **Motility is critical for effective distribution and accumulation of bacteria in tumor tissue**. *Integr Biol (Camb)* 2012, **4**:165-176.

26. Zhang M, Forbes NS: **Trg-deficient *Salmonella* colonize quiescent tumor regions by exclusively penetrating or proliferating**. *J Control Release* 2015, **199**:180-189.

27. Gandhi NM, Morales A, Lamm DL: **Bacillus Calmette-Guerin immunotherapy for genitourinary cancer**. *BJU Int* 2013, **112**:288-297.

28. Forbes NS: **Engineering the perfect (bacterial) cancer therapy**. *Nat Rev Cancer* 2010, **10**:785-794.

29. Jiang SN, Phan TX, Nam TK, Nguyen VH, Kim HS, Bom HS, Choy HE, Hong Y, Min JJ: **Inhibition of tumor growth and metastasis by a combination of *Escherichia coli*-mediated cytolytic therapy and radiotherapy**. *Mol Ther* 2010, **18**:635-642.

30. St Jean AT, Swofford CA, Panteli JT, Brentzel ZJ, Forbes NS: **Bacterial delivery of *Staphylococcus aureus* alpha-hemolysin causes regression and necrosis in murine tumors**. *Mol Ther* 2014, **22**:1266-1274.

31. Lehouritis P, Springer C, Tangney M: **Bacterial-directed enzyme prodrug therapy**. *J Control Release* 2013, **170**:120-131.

32. Jeong J-H, Kim K, Lim D, Jeong K, Hong Y, Nguyen VH, Kim T-H, Ryu S, Lim J-A, Kim JI, et al.: **Anti-Tumoral Effect of the Mitochondrial Target Domain of Noxa Delivered by an Engineered *Salmonella typhimurium***. *PLoS ONE* 2014, **9**:e80050.

33. Loessner H, Leschner S, Endmann A, Westphal K, Wolf K, Kochruebe K, Miloud T, Altenbuchner J, Weiss S: **Drug-inducible remote control of gene expression by probiotic *Escherichia coli* Nissle 1917 in intestine, tumor and gall bladder of mice**. *Microbes Infect* 2009, **11**:1097-1105.

34. Royo JL, Becker PD, Camacho EM, Cebolla A, Link C, Santero E, Guzman CA: **In vivo gene regulation in *Salmonella* spp. by a salicylate-dependent control circuit**. *Nat Methods* 2007, **4**:937-942.

35. Arrach N, Zhao M, Porwollik S, Hoffman RM, McClelland M: ***Salmonella* promoters preferentially activated inside tumors**. *Cancer Res* 2008, **68**:4827-4832.

36. Mengesha A, Dubois L, Lambin P, Landuyt W, Chiu RK, Wouters BG, Theys J: **Development of a flexible and potent hypoxia-inducible promoter for tumor-targeted gene expression in attenuated *Salmonella***. *Cancer Biol Ther* 2006, **5**:1120-1128.

37. Ganai S, Arenas RB, Forbes NS: **Tumour-targeted delivery of TRAIL using *Salmonella typhimurium* enhances breast cancer survival in mice**. *Br J Cancer* 2009, **101**:1683-1691.

38. Swofford CA, Van Dessel N, Forbes NS: **Quorum-sensing *Salmonella* selectively trigger protein expression within tumors**. *Proc Natl Acad Sci U S A* 2015, **112**:3457-3462.

** This work describes the engineering of *lux* QS system from *Vibrio fischeri* to generate a gene circuit in *Salmonella* that triggers the expression of a target protein specifically with the high bacterial densities reached within solid tumors, thus reducing nonspecifc damage of healthy tissues.

39. Dai Y, Toley BJ, Swofford CA, Forbes NS: **Construction of an inducible cell-communication system that amplifies *Salmonella* gene expression in tumor tissue**. *Biotechnol Bioeng* 2013, **110**:1769-1781.

40. Xiang S, Fruehauf J, Li CJ: **Short hairpin RNA-expressing bacteria elicit RNA interference in mammals**. *Nat Biotechnol* 2006, **24**:697-702.

41. Toso JF, Gill VJ, Hwu P, Marincola FM, Restifo NP, Schwartzentruber DJ, Sherry RM, Topalian SL, Yang JC, Stock F, et al.: **Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma**. *J Clin Oncol* 2002, **20**:142-152.

42. Piñero-Lambea C, Bodelón G, Fernández-Periáñez R, Cuesta AM, Álvarez-Vallina L, Fernández LA: **Programming controlled adhesion of *E. coli* to target surfaces, cells, and tumors with synthetic adhesins**. *ACS Synth Biol* 2015, **4**:463-473.

** This work demonstrates that *E. coli* can be programmed to attach to target surfaces and cells expressing a specific antigen on their surface. Engineered bacteria efficiently colonize target tumors at low bacterial doses.

43. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI: **Obesity alters gut microbial ecology**. *Proc Natl Acad Sci U S A* 2005, **102**:11070-11075.

44. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI: **Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome**. *Cell Host Microbe* 2008, **3**:213-223.

45. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, et al.: **Gut microbiota from twins discordant for obesity modulate metabolism in mice**. *Science* 2013, **341**:1241214.

46. Chen Z, Guo L, Zhang Y, R LW, Pendergast JS, Printz RL, Morris LC, Matafonova E, Stien X, Kang L, et al.: **Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity**. *J Clin Invest* 2014, **124**:3391-3406.

** This work generate a therapy against obesity based on an engineered *E. coli* strain that express precursors of a family lipids synthesized in the small intestine in response to feeding.

47. Suzuki A, Nakauchi H, Taniguchi H: **Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells**. *Proc Natl Acad Sci U S A* 2003, **100**:5034-5039.

48. Duan FF, Liu JH, March JC: **Engineered commensal bacteria reprogram intestinal cells into glucose-responsive insulin-secreting cells for the treatment of diabetes**. *Diabetes* 2015, **64**:1794-1803.

* This paper demonstrates that oral administration of an engineered *Lactobacillus gasseri* strain secreting Glucagon-like peptide 1 (GLP-1) mediates *in vivo* reprogramming of intestinal cells into glucose-responsive insulin-secreting cells.

49. Goh YL, He H, March JC: **Engineering commensal bacteria for prophylaxis against infection**. *Curr Opin Biotechnol* 2012, **23**:924-930.

50. Hillman JD, Brooks TA, Michalek SM, Harmon CC, Snoep JL, van Der Weijden CC: **Construction and characterization of an effector strain of *Streptococcus mutans* for replacement therapy of dental caries**. *Infect Immun* 2000, **68**:543-549.

51. Borrero J, Chen Y, Dunny GM, Kaznessis YN: **Modified lactic acid bacteria detect and inhibit multiresistant enterococci**. *ACS Synth Biol* 2015, **4**:299-306.

52. Saeidi N, Wong CK, Lo TM, Nguyen HX, Ling H, Leong SS, Poh CL, Chang MW: **Engineering microbes to sense and eradicate *Pseudomonas aeruginosa,* a human pathogen**. *Mol Syst Biol* 2011, **7**:521.

53. Chen HL, Lai YW, Chen CS, Chu TW, Lin W, Yen CC, Lin MF, Tu MY, Chen CM: **Probiotic *Lactobacillus casei* expressing human lactoferrin elevates antibacterial activity in the gastrointestinal tract**. *Biometals* 2010, **23**:543-554.

54. Lagenaur LA, Sanders-Beer BE, Brichacek B, Pal R, Liu X, Liu Y, Yu R, Venzon D, Lee PP, Hamer DH: **Prevention of vaginal SHIV transmission in macaques by a live recombinant *Lactobacillus***. *Mucosal Immunol* 2011, **4**:648-657.

55. Pant N, Hultberg A, Zhao Y, Svensson L, Pan-Hammarström Q, Johansen K, Pouwels PH, Ruggeri FM, Hermans P, Frenken L, et al.: ***Lactobacilli* expressing variable domain of Llama heavy-chain antibody fragments (Lactobodies) confer protection against rotavirus-induced diarrhea**. *Journal of Infectious Diseases* 2006, **194**:1580-1588.

56. Hurwitz I, Fieck A, Durvasula R: **Antimicrobial peptide delivery strategies: use of recombinant antimicrobial peptides in paratransgenic control systems**. *Curr Drug Targets* 2012, **13**:1173-1180.

57. Smith C, Gore A, Yan W, Abalde-Atristain L, Li Z, He C, Wang Y, Brodsky RA, Zhang K, Cheng L, et al.: **Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs**. *Cell Stem Cell* 2014, **15**:12-13.

58. Papapetrou EP, Tomishima MJ, Chambers SM, Mica Y, Reed E, Menon J, Tabar V, Mo Q, Studer L, Sadelain M: **Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation**. *Proc Natl Acad Sci U S A* 2009, **106**:12759-12764.

59. Galán JE, Lara-Tejero M, Marlovits TC, Wagner S: **Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells**. *Annu Rev Microbiol* 2014, **68**:415-438.

60. Panthel K, Meinel KM, Sevil Domenech VE, Trulzsch K, Russmann H: ***Salmonella* type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors**. *Int J Med Microbiol* 2008, **298**:99-103.

61. Blanco-Toribio A, Muyldermans S, Frankel G, Fernández LA: **Direct injection of functional single-domain antibodies from *E. coli* into human cells**. *PLoS One* 2010, **5**:e15227.

62. Bichsel C, Neeld DK, Hamazaki T, Wu D, Chang L-J, Yang L, Terada N, Jin S: **Bacterial Delivery of Nuclear Proteins into Pluripotent and Differentiated Cells**. *PLoS ONE* 2011, **6**:e16465.

63. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K: **Virus-free induction of pluripotency and subsequent excision of reprogramming factors**. *Nature* 2009, **458**:771-775.

64. Jia J, Jin Y, Bian T, Wu D, Yang L, Terada N, Wu W, Jin S: **Bacterial Delivery of TALEN Proteins for Human Genome Editing**. *PLoS One* 2014, **9**:e91547.

65. Bichsel C, Neeld D, Hamazaki T, Chang LJ, Yang LJ, Terada N, Jin S: **Direct reprogramming of fibroblasts to myocytes via bacterial injection of MyoD protein**. *Cell Reprogram* 2013, **15**:117-125.

* This paper reports the differentiation of mouse embryonic fibroblasts into functional myocites through the transient injection of MyoD protein with an attenuated *P. aeruginosa* carrying a functional T3SS.

66. Cameron DE, Bashor CJ, Collins JJ: **A brief history of synthetic biology**. *Nat Rev Microbiol* 2014, **12**:381-390.

67. Huh JH, Kittleson JT, Arkin AP, Anderson JC: **Modular design of a synthetic payload delivery device**. *ACS Synth Biol* 2013, **2**:418-424.

68. Green AA, Silver PA, Collins JJ, Yin P: **Toehold switches: de-novo-designed regulators of gene expression**. *Cell* 2014, **159**:925-939.

69. Farzadfard F, Lu TK: **Synthetic biology. Genomically encoded analog memory with precise in vivo DNA writing in living cell populations**. *Science* 2014, **346**:1256272.

70. Siuti P, Yazbek J, Lu TK: **Synthetic circuits integrating logic and memory in living cells**. *Nat Biotech* 2013, **31**:448-452.

71. Kotula JW, Kerns SJ, Shaket LA, Siraj L, Collins JJ, Way JC, Silver PA: **Programmable bacteria detect and record an environmental signal in the mammalian gut**. *Proc Natl Acad Sci U S A* 2014, **111**:4838-4843.

*This study employs a stable memory device based on cI/Cro region of phage lambda to engineer an *E. coli* strain that records the presence of a chemical in the gut.

72. Hwang IY, Tan MH, Koh E, Ho CL, Poh CL, Chang MW: **Reprogramming microbes to be pathogen-seeking killers**. *ACS Synth Biol* 2014, **3**:228-237.

*This study reports the development of a genetic circuit in *E. coli* that allow detection of QS molecules, directing the motility toward *P. aeruginosa* and the killing of both planktonic and biofilm-associated *P. aeruginosa*.

73. Bi S, Lai L: **Bacterial chemoreceptors and chemoeffectors**. *Cell Mol Life Sci* 2014.

74. Wright O, Delmans M, Stan GB, Ellis T: **GeneGuard: A modular plasmid system designed for biosafety**. *ACS Synth Biol* 2015, **4**:307-316.

75. Csorgo B, Feher T, Timar E, Blattner FR, Posfai G: **Low-mutation-rate, reduced-genome Escherichia coli: an improved host for faithful maintenance of engineered genetic constructs**. *Microb Cell Fact* 2012, **11**:11.

76. Mandell DJ, Lajoie MJ, Mee MT, Takeuchi R, Kuznetsov G, Norville JE, Gregg CJ, Stoddard BL, Church GM: **Biocontainment of genetically modified organisms by synthetic protein design**. *Nature* 2015, **518**:55-60.

** This paper describes the engineering of an *E. coli* strain that incorporates a non-natural amino acid in the core of essential proteins. The engineered strain provides a strict measure for biocontainment of engineered microorganisms.

77. Marliere P, Patrouix J, Doring V, Herdewijn P, Tricot S, Cruveiller S, Bouzon M, Mutzel R: **Chemical evolution of a bacterium's genome**. *Angew Chem Int Ed Engl* 2011, **50**:7109-7114.

Figure Legends

Figure 1. Engineered bacteria against inflammatory disorders. Scheme depicting major strategies followed against inflammatory disorders in the gastrointestinal tract. Bacteria (green) can release locally antibody fragments (brown Ys) against pro-inflammatory cytokines (red dots), anti-oxidant enzymes (yellow dots), or anti-inflammatory cytokines (blue dots) acting on lymphocytes, and other cells of the mucosal immune system.

Figure 2. Engineered bacteria in cancer therapies. Scheme showing common approaches in bacterial interventions against tumors. Bacteria preferentially accumulate and replicate within solid tumors allowing localized expression of reporter genes, bacterial toxins, pro-drug converting enzymes, pro-apoptotic molecules and cytokines. In addition, bacteria can harbor plasmids that, once transferred into cancer cells, may encode shRNAs for gene silencing.

Figure 3. Strategies to combat infections with engineered bacteria. A pathogenic bacterium is represented in the center releasing toxins (red molecules) and quorum sensing signals (orange molecules) to the medium and expressing surface adhesins (light blue lines). Engineered bacteria (brown) over the microvilli of host cells (yellow) show different strategies against the pathogen: (A) Toxin neutralization using modified surface components. (B) Production of antimicrobial factors (green molecules) upon detection of quorum sensing signals from the pathogen mediating killing of pathogenic bacteria. (C) Interference with quorum sensing mechanisms by the release of alternative quorum sensing signals (grey molecules) triggering repression of virulence genes. (D)

Prevention of colonization. Bacteria can be engineered to secrete antibodies and adhesin subunits that competitively inhibit pathogen adhesion to host cells.

Figure 4. Synthetic bacteria for biomedical applications. Scheme of an engineered bacterium based on a minimal chassis (grey) carrying diverse genetic modules of interest for therapy. A chemotactic module (light blue) could control bacterial migration in response to environmental signals of interest (orange molecules). A sensory module (purple) could detect environmental signals and respond by activating the transcription of a payload (yellow) and/or reporter (orange) modules. An adhesion module (green) could facilitate binding of the engineered bacteria to a specific target cell or tissue. A delivery module (pink) may allow the release of therapeutic molecules. A containment module (red) prevents the environmental spread of the synthetic bacterium.

Figure 1

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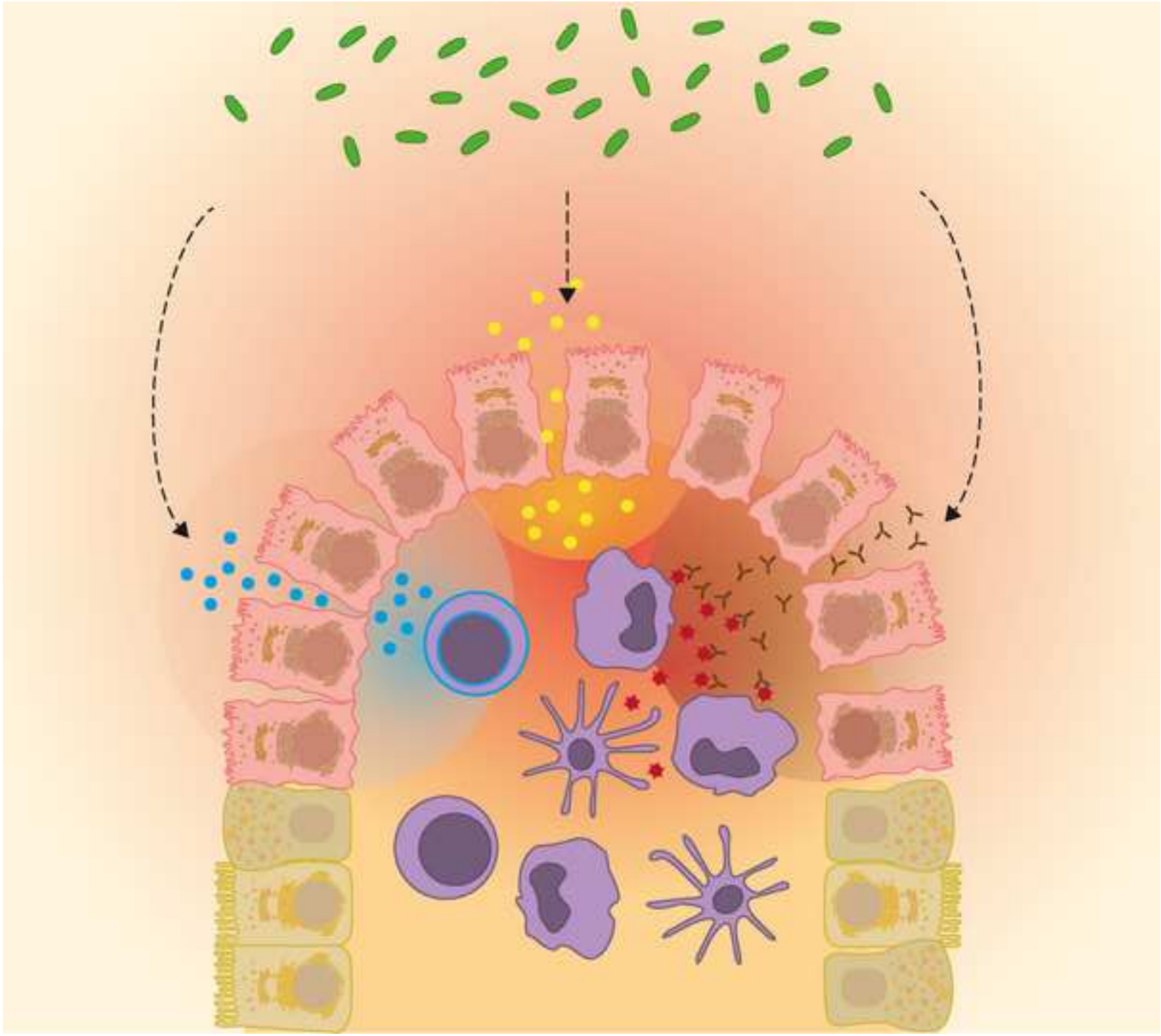

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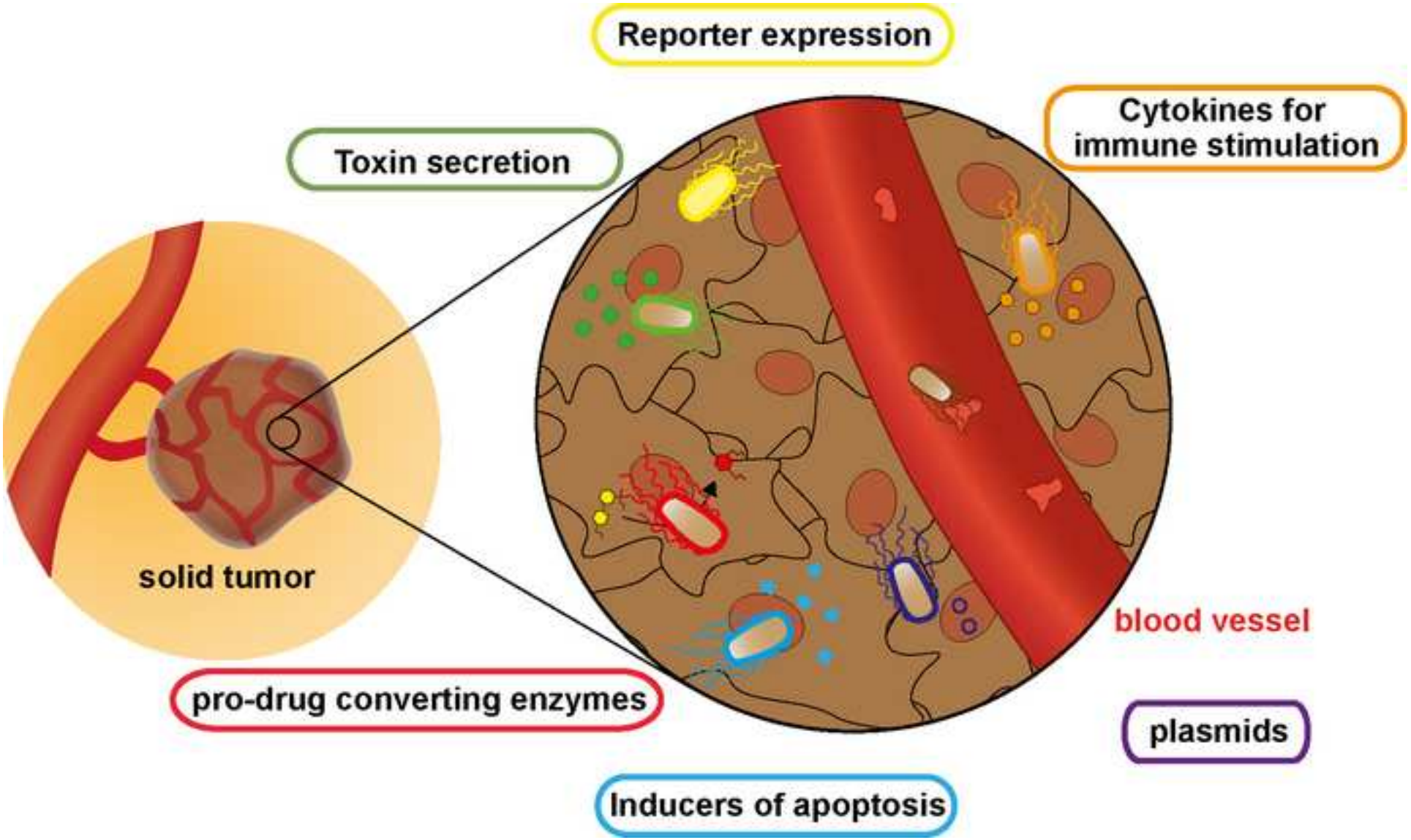

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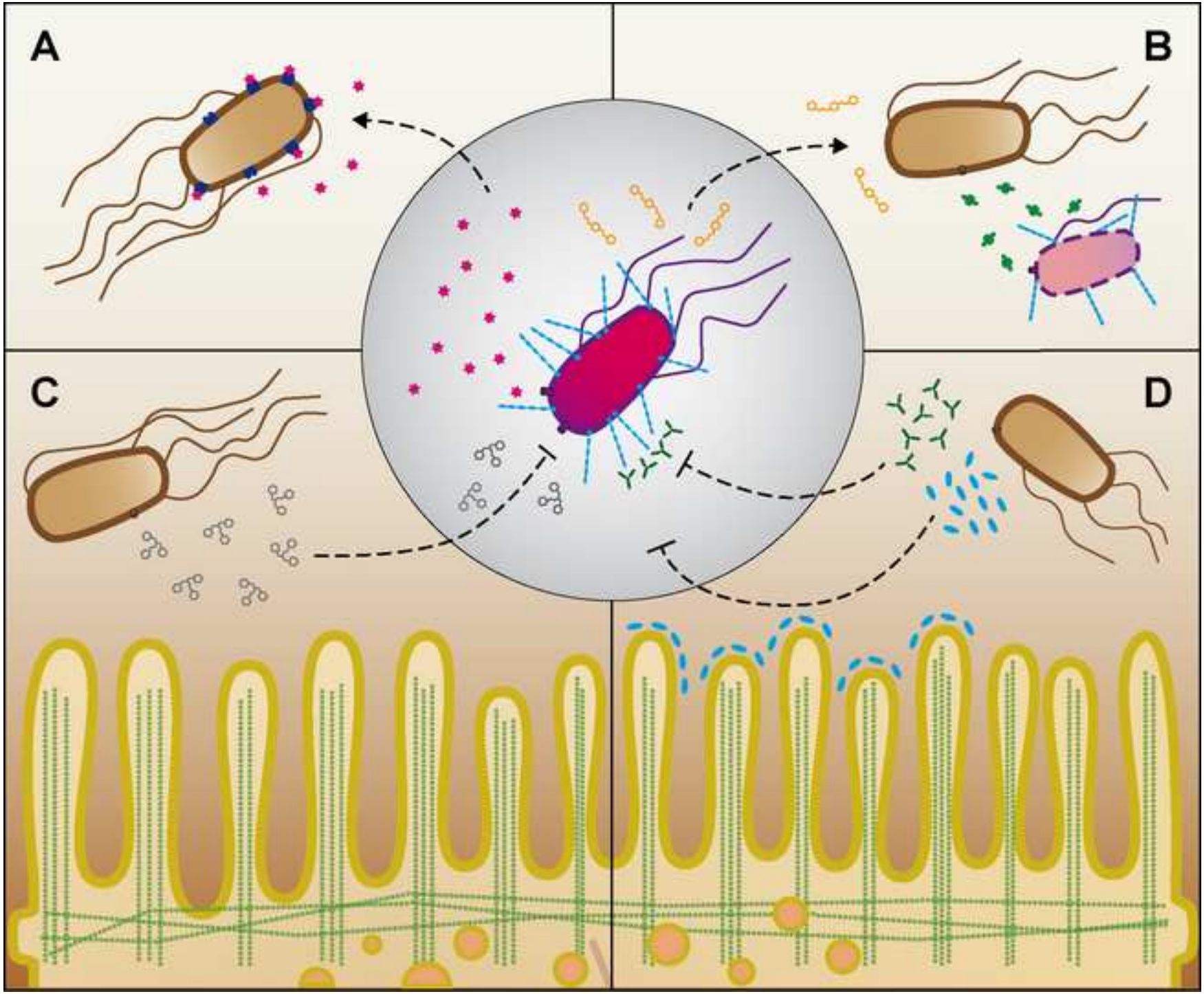

Figure 4

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