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Gene therapy using bacterial vectors

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1. ABSTRACT

Bacteria can be used for gene therapy via two strategies – either by transfection of eukaryotic host cells using bacteria – bactofection or by alternative gene therapy that does not alter the host genome, but uses the prokaryotic expression system that can be controlled or stopped from outside. While bactofection is optimal for gene substitution and DNA vaccination, alternative gene therapy is suitable for *in situ* delivery of proteins and treatment with intracellular bactochondria. A specific form of bacteria-mediated gene therapy is the transkingdom RNA interference. In this review advantages and issues related to bacterial vectors as well as the major applications in biomedical research are summarized. Despite numerous published experiments, especially in the treatment of solid tumors and gut infections, the progress in the clinics lags behind and major improvements in the safety and even more in the efficiency of these approaches are needed.

2. INTRODUCTION

The field of gene therapy has undergone a tremendous development with many ups and downs. The first clinical trial approaching severe combined immune deficiency was covered in detail even by lay media. The enthusiasm alternating with disappointment followed lastly by sober expectations mirrored the development of the whole field of gene therapy. It took roughly 20 years – similarly to any other drugs, but finally, the first gene therapy is in the clinical use in Europe and further gene therapy products are in the pipeline (1). Throughout the whole history of gene therapy, the major issue to solve is the transport of DNA into the body and especially into the target cell (2).

The so called vectorology looks for new vectors for gene transfer and improves the pharmacokinetics of gene therapy (3). We have published a review about the vectors used in gene therapy in the past and described the pros and cons of the available vector types (4). Two most common approaches for gene transfer are viral and non-viral vectors. Bacterial vectors are used very rarely and in most statistics they even can be found in the category “Other” showing that they are simply ignored by many scientists from the community (2). Viruses have been developed by nature to transfer their DNA and so, their efficiency in gene transfer is very high. Most of them are true or opportunistic pathogens and, thus, their use is associated with some risks. Immune system activation leads to antibody production that very often prevents repeated use. And, even more important, previous infections with viruses with similar epitopes greatly decrease the efficiency of viral gene therapy (5).

Non-viral vectors is an artificial term covering the use of free DNA in form of plasmids, PCR products and oligodeoxynucleotides. The numerous non-viral vectors used for gene therapy have been reviewed and summarized in the past (6). The efficiency of DNA transfer via non-viral vectors into cells is limited and must be

enhanced in most applications by *in vivo* electroporation, high pressure or by chemical transfection agents (4). Cell-free DNA activates the innate immune system via toll-like receptor 9 (7, 8). The resulting inflammation can be modulated by several characteristics of the nucleic acid such as the content of CpG islands, methylation, fragmentation etc. Although in most cases immune system activation is to be prevented, DNA vaccination requires an additional inflammatory signal and benefits from the effects of extracellular DNA on the immune system (9). A major issue with non-viral vectors is the targeting of the treatment (10). Systemic administration leads to more or less systemic expression of the transgene which is rarely adequate. The low efficiency of gene transfer, however, remains the main obstacle for wider use of non-viral vectors in the clinics.

3. BACTERIAL VECTORS

Decades ago it has been shown that DNA can be transferred from bacteria into eukaryotic cells (11). Prokaryotic vectors have several advantages over both, viral and non-viral vectors. These include easy inexpensive production and amplification, tropism to specific tissues as well as the ability to limit or stop the treatment if needed by administration of antibiotics (12). Large cargo capacity is virtually not limited, which is a major difference in comparison to commonly used viral vectors. Another important advantage is the knowledge of the bacterial physiology and genomics enabling advanced genetic engineering and control over the production of therapeutic molecules but also proteins important for immune reactions of the host against the vector or the transgene (13).

There are, of course, also disadvantages of bacteria as vehicles for genes. Immune system activation, relatively low transfection efficiency and the mechanism of DNA transfer, which is not completely clear (14). Clinical studies have uncovered further issues to be solved – the need for an improved targeting and reduced toxicity of the used bacterial strains (15, 16). Another issue is the difference between results of animal experiments and human studies. This might be due to low reproducibility of the published data, publication bias, but also due to immunologic differences between rodents and human patients (17). Our last review on bacteria-mediated gene therapy summarizing the advantages and disadvantages of bacterial vectors as well as their applications was published several years ago and requires this update (18).

4. BACTOFECTION

Bacteria can actively transfer genetic material into eukaryotic cells after lysis upon entry of the host cell cytoplasm (19). Especially Listeria and Shigella as intracellular pathogens can be used for such purposes (20). Salmonella transfers DNA passively when taken up by phagocytes (21). Other bacteria can be artificially prepared to transfer genetic material, even Escherichia. This is achieved by introducing the invasin gene from Yersinia and listeriolysin gene from Listeria into the bacterial vector (22). Invasin is important for the interaction with integrins on target host cells, lysteriolysin enables the bacteria to escape the phagolysosome (21). These abilities are of importance for the so called bactofection – the transfection of eukaryotic cells using bacteria (Figure 1).

Inflammation is always associated with oxidative stress, either due to increased free radical production of activated immune cells or due to a decrease in total antioxidant capacity of the surrounding cells. In the colitis model we have tested bactofection mediated by attenuated Salmonella with an antioxidant and anti-inflammatory strategy (23). Both approaches partially improved the course of colitis and both seemed to act via increase of the antioxidant status. However, this effect was gender-specific. We could not reproduce the results in female rats, especially because we were not able to induce the same severity of colitis (24). We have later described sex differences in the model of colitis that seem to be caused by sex hormones, especially by the different estrogen concentrations (25).

Bactofection-mediated gene therapy was tested widely in the treatment of cancer making use of the targeted colonization of hypoxic areas of solid tumors by anaerobic bacteria (26). Another application is the prevention of infections. We have partially prevented kidney colonization and pyelonephritis by uropathogenic Escherichia in a mouse model of urinary tract infection (27). This was achieved by a preventive administration of Salmonella containing a eukaryotic expression plasmid with the gene for toll-like receptor 4 as a major regulator of the innate immune system. Paradoxically, infection with an attenuated pathogen was able to prevent infections with virulent pathogens. Importantly, the potential vaccination mechanism was not involved in this beneficial effect.

Bactofection can be coupled with other current promising genetic technologies. In the past, we have hypothesized that bacteria can be used for the transfer of the Yamanaka factors into target cells to induce pluripotency potentially enabling regeneration of the diseased tissue (28), such the gut that is affected in the course of colitis (29). In an animal experiment we have shown that at least partially this might work. The clinical scores in a mouse model of colitis were reduced by the treatment with bactofection-mediated pluripotency induction (30). Although the effect size was limited, further studies and especially optimization of the dosing could show benefits useful for other disease models beyond colitis.

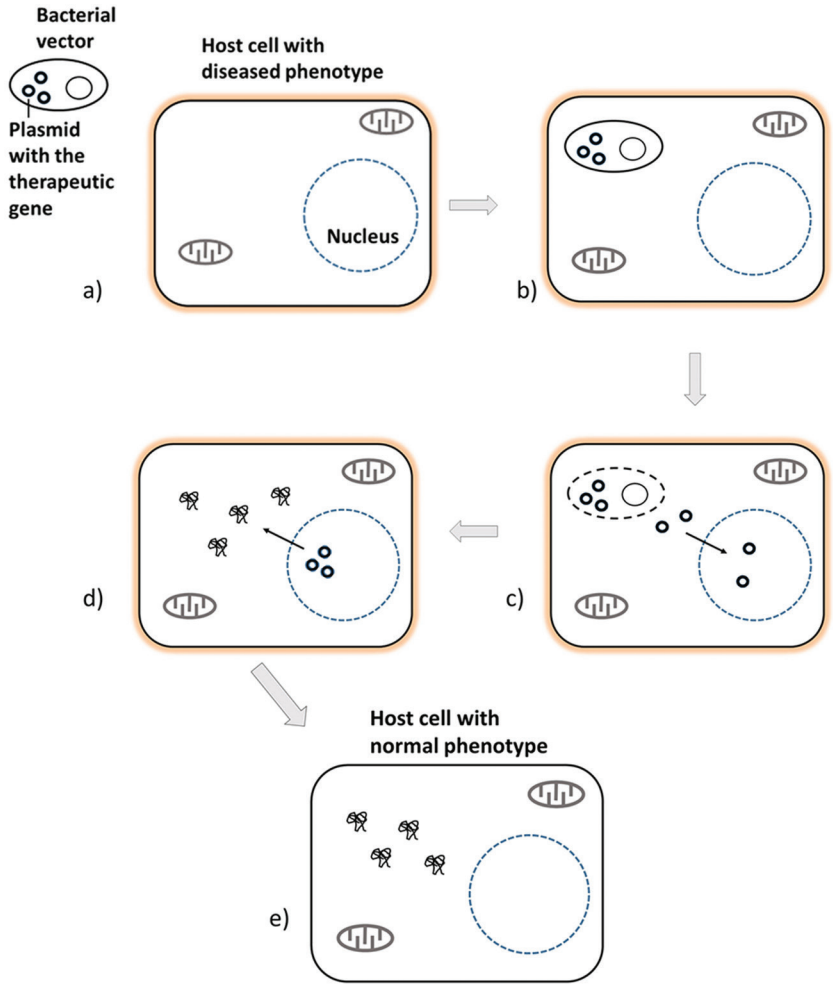

Figure 1. Bactofection – gene therapy. Bacteria are used as vectors for the delivery of plasmids with therapeutic genes into the host cells with diseased phenotype (a). An invasive bacterial vector enters the host cell (b). After bacterial escape from the phagolysosome, plasmids are released into the cytoplasm of the host cell and transported into the nucleus (c). Therapeutic gene encoded on the plasmid is expressed by the host cell in the form of a therapeutic protein (d). The mutated/deleted gene product is replaced by a transgene-encoded protein and the cell regains normal phenotype (e).

5. ALTERNATIVE GENE THERAPY

An alternative approach to bactofection is the use of genetically modified bacteria for direct *in situ* production of therapeutic molecules – bacterial protein delivery (Figure 2). We have suggested the term alternative gene therapy because the host genome is not affected and, so, it represents an alternative to the standard gene transfer to the nucleus of the target cell (31). A major advantage is that off-target genomic effects known to cause complications with the use of viral vectors are irrelevant. The treatment can be easily stopped anytime by administration of antibiotics. The inability to stop the treatment in standard gene therapy approaches is often neglected, but for clinical applications this represents a major issue. Finally, in alternative gene therapy the production of the therapeutic molecules can be fine-tuned using expression system regulated from outside such as the arabinose or lactose operon (32, 33). The remote control of transgene expression in the bacterial vectors has been proved *in vivo*, which is very important because of specific pharmacokinetics of the particular inducers (34). In a methodological study tetracycline was used for the control of transgene expression. The production of the therapeutic protein was shown to be regulated similarly to the marker gene luciferase that can be used for *in vivo* imaging (35). This approach basically combined diagnostics – the imaging of the tumor where the Salmonella vector can survive, and therapy – using bacterial cytotoxin with an antitumor effect. The possibilities of *in vivo* imaging of bacterial gene therapy vectors with simultaneous tumor-targeting therapy have been reviewed in detail (36). The final therapeutic molecule in alternative gene therapy is mostly a protein, but it can also be RNA such as short-hairpin RNA for RNA interference to downregulate the expression of specific genes. The latter approach is termed transkingdom RNA interference.

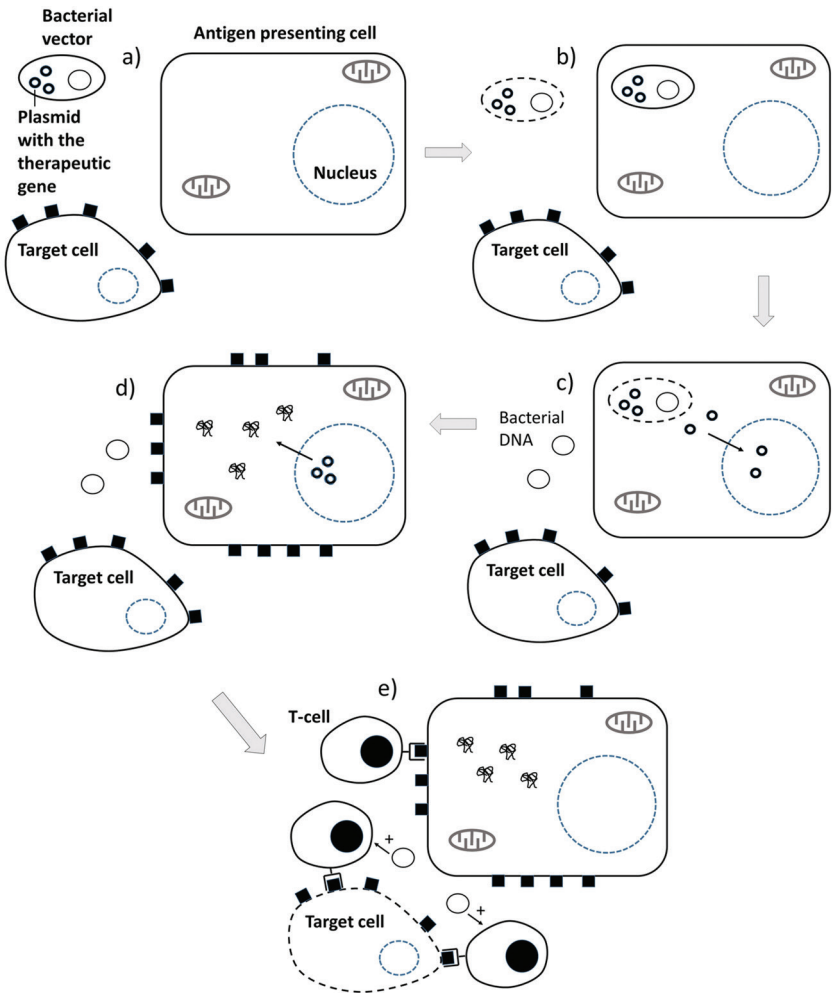

Figure 2. Alternative gene therapy – bacterial protein delivery. Bacteria are used as vectors for the delivery of recombinant proteins into target cells with a diseased phenotype. The therapeutic gene is encoded on a plasmid or bacterial chromosome (a). An invasive bacterial vector enters the host cell. The therapeutic gene is expressed by bacterial expression machinery and the therapeutic protein is secreted into the cytoplasm of the target cell (b). A non-invasive bacterial vector stays outside the target cell. The therapeutic gene is expressed by the bacterial expression machinery and the therapeutic protein is secreted into the extracellular space (c). Bacterial vectors can survive both, extracellularly or in the target cell (as so-called bactochondria), but can also be killed by the immune system and antimicrobial agents (d). The therapeutic protein is produced inside or outside the target cell that subsequently regains normal phenotype (e).

One of the commonly used strategies in alternative gene therapy is the application of an enzyme that converts a safe prodrug to a toxic agent. This has been achieved with Clostridium that converts a prodrug to a toxic molecule using the enzyme nitroreductase (37). Such a genetically modified Clostridium with potential anti-tumor effects has already went to a phase 1 clinical trial (38). The use of bacteria for gene therapy of cancer has its rationale in the additional direct toxic effect of bacteria against the tumor cells (39). Bacteria can also carry genetic information for other bacterial or eukaryotic toxins to further improve the anti-tumor effect. In one study a toxin from Staphylococcus aureus – the alpha hemolysin was cloned into Escherichia and injected into tumor-bearing mice. Although the bacteria were injected systemically, the volume of the tumor decreased due to the toxic effect of the transgene product that diffused to the target tissue very efficiently (40). Although in the field of bacteria-mediated gene therapy the focus is still on the improvement of the vectors, a well-chosen transgene can bring promising results despite the use of simple laboratory strains without major genetic engineering. On the other hand, we and others have repeatedly seen in experiments that control groups receiving mock vectors very often showed a difference to the groups receiving no treatment at all. It suggests that the vectors have an immunomodulatory effect (41-43). This is not a negative finding per se, but it complicates the design of experiments to prevent bias in interpretation (44). It is likely that this non-specific effect is mediated by binding of bacterial antigens including DNA to toll-like receptor 9 (45).

There are several differences between alternative gene therapy and bactofection. Although both approaches use genetically modified bacteria, different mechanisms and targets are needed. While in bactofection DNA is transferred into host cells and the survival of the bacteria is needed only until the plasmid DNA is released into the cytoplasm, in alternative gene therapy bacteria have to survive longer, ideally in the target tissue either in the extracellular space or inside cells depending on the characteristics of the therapeutic gene product. In general, bacteria can be targeted to tumors or other hypoxic areas by simply choosing anaerobic strains as vectors. This is of special interest for the therapy of solid tumors as shown for example in an animal experiment for the nonpathogenic Bifidobacterium after systemic intravenous administration. The bacteria survived and replicated in the hypoxic tumor tissue (46). Similarly, Clostridium was able to colonize a solid tumor with high specificity (47).

Ligands of membrane-bound receptors such as growth factors, hormones, their inhibitors or apoptotic signals should be produced into the extracellular space. It is unclear whether such an approach of extracellular alternative gene therapy can be called gene therapy at all (18). The recombinant probiotics are a product of genetic engineering and although the genome of the target host cells is not changed the genetic material of the bacteria is modified according to the therapeutic purposes (48). If RNA or proteins that interact with intracellular counterparts are to be produced by bacteria, the approach is intracellular alternative gene therapy and the genetically modified bacteria are called bactochondria (18). The term bactochondria is a remembrance of the similarities to mitochondria that are also of prokaryotic origin and survive intracellularly relatively independent of the host cells while helping them to survive under specific conditions.

There are risks related to the use of genetically modified bacteria as recombinant probiotics for the treatment of gut diseases. Horizontal gene transfer could lead to changes of the phenotype of the bacterial vector, but might also change the metagenome of the gut microflora. Some researchers even suggest to ban the use of recombinant probiotics (49). However, horizontal gene transfer can occur with no relation to previous genetic modification and although studies on the consequences are surely needed, this should not hinder the experiments and progress of the therapeutic potential. In contrast, the use of bacteria generally recognized as safe from the group of lactic acid bacteria such as Lactococcus for therapeutic purposes is favorable in terms of safety in comparison to modified pathogens used for other applications (50). Various therapeutic genes were added to Lactococcus for expression *in situ*. Some to prevent diabetes mellitus type 1 (51), some to treat oral mucositis (52), but mostly to reduce colitis-associated inflammation (53).

Bacterial strains used for gene therapy have to be attenuated to decrease the risk of complications. Especially, bacteria must not produce lipopolysaccharide or other strong antigens. Some are engineered not to produce the bacterial cell wall at all and some are programmed to die after escaping from vacuoles in the cytoplasm by various mechanisms of auxotrophy (54). It has been shown that such bacteria designed for bactofection do not survive in the tissue for longer than 24-48 hours, but the expression of the transgene can be detected for much longer, up to several months depending on the tissue type and the turnover of the cells (21). This is, however, dependent on the vector type and the used expression system including the promoter.

6. RNA INTERFERENCE USING BACTERIA

Transkingdom RNA interference is a very specific application of bacteria in gene therapy (Figure 3). In 2006 scientists from Harvard have published the first report on the use of bacteria producing short hairpin RNA that is further processed by the host cell machinery in the cytoplasm to small interfering RNA and can alter the expression of target proteins by translation inhibition (55). This theoretical framework works *in vitro* and *in vivo* (56). It is especially useful in diseases of the intestines as the gut is inhabited by microbes and further addition of genetically modified bacteria does not represent an additional signal for immune system activation. The advantages of bacteria-mediated gene therapy and RNA interference are combined and can be specifically applied in some conditions such as colitis or colorectal cancer. In addition, the production of small interfering RNA can be done by bacterial and host cells and does not have to be done artificially before administration (57). This makes the treatment cheaper and the pharmacokinetics easier, while targeting of treatment depends on the bacterial strain chosen for the therapy and on the treated condition.

Colitis is an ideal target disease for transkingdom RNA interference. The inflammatory process has several regulatory control points that can be blocked by small interfering RNA. In a recent study an Italian group showed that by inhibiting the production of cyclooxygenase-2 the colitis severity can be significantly reduced (41). Interfering RNA was produced by invasive Escherichia applied via enema. The authors have confirmed the effect on a macroscopic and microscopic level, but they have also analyzed the microbiome of the animals. Interestingly, administration of the vectors with the therapeutic RNA gene resulted in a decrease of abundance of potentially pathogenic microbes suggesting an additional mechanism of action.

RNA interference can be used to modulate angiogenesis in the tissue by decreasing the expression of the main angiogenesis regulator – vascular endothelial growth factor. In patients with solid tumors blockage of

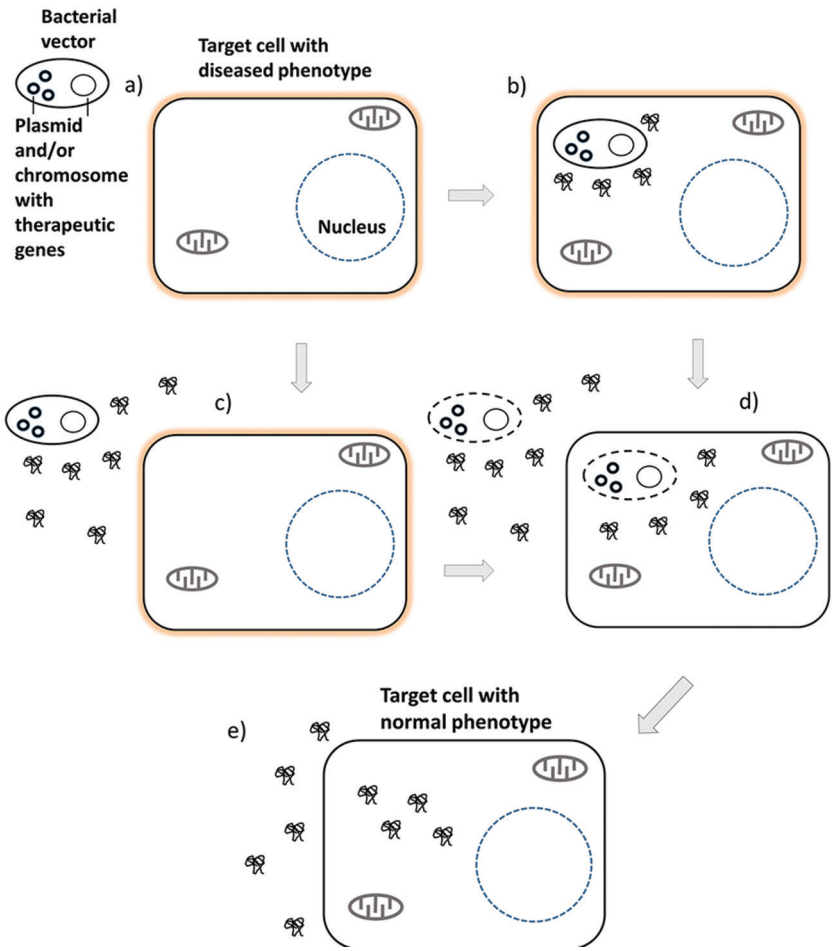

Figure 3. Alternative gene therapy – Transkingdom RNA interference. Bacteria are used as vectors for the delivery of RNA interference-mediating sequences (short hairpin RNAs – shRNAs) into the target cells. The cell has a mutated gene and produces excessive amounts of its mRNA that is in turn translated into a protein (a). An invasive bacterial vector enters the target cell and uses the shRNA cassette in the cytoplasm of the cell (b). The shRNAs produced by the bacteria mediate RNA interference against the target gene that results into its knock-down (c). Bacterial vectors can survive in the host cell if needed, but can be destroyed by the immune system or antimicrobial agents as well. The shRNAs present in the target cells suppress the expression of the diseased gene and the target cell regains normal phenotype (d).

angiogenesis prolongs survival. This is clinically achieved with monoclonal antibodies or decoy receptors, but a gene therapy approach, especially via RNA interference might be more suitable and cost-effective (58, 59). The various bacteria-mediated anti-angiogenesis approaches have been reviewed by our group few years ago (60). Bacteria-mediated gene therapy can, however, also be used for stimulation of angiogenesis in ischemic diseases. We have published the proof-of-principle experiment via alternative gene therapy using Escherichia producing vascular endothelial growth factor (31), but we were less successful with angiogenesis stimulation by hypoxia inducible factor 1 alpha delivered via bactofection (43).

7. MICROBIOME MODIFICATIONS

Another application of bacteria-mediated therapy is the targeted short-term or long-term change in the microbiome. It has been shown that microbiome transplantation is an efficient way for the treatment of Clostridium infections of the gut (61). Recently, such a microbiome modification using a collection of probiotic strains has been shown to reduce hyperammonemia in an animal model of liver disease (62). Changes in the microbiome can be improved by antimicrobial genes or other selective advantages added to the genetic arsenal to improve survival of the transplanted microbes. In addition, antibiotic resistance can be modulated either to add selective advantage over the actual gut microbes, or to enable ending the treatment with antibiotics when needed.

One typical application of microbiome modification using recombinant probiotics is gut inflammation. Crohn disease and ulcerative colitis are both associated with altered gut microbiome composition (63). Although the causality is not clear and the etiology of these unspecific inflammatory diseases

remains unknown. Treatment with probiotics has shown some beneficial effects, but these are lacking behind the standard anti-inflammatory treatment associated with severe side effects (64). Recently, the well-known and widely used probiotic strain E. coli Nissle 1917 was modified to produce nematode cystatin – a known immunomodulator that seems to be partially responsible for the beneficial effect of concurrent parasitic infections on the course of colitis (65). Importantly, the authors have confirmed the effect of the recombinant probiotic on both, a murine and a porcine colitis model. We have conducted a similar experiment with E. coli Nissle 1917 expressing the immunosuppressive cytokine interleukin 10, but the effects were not yet proved on a larger animal model (66). Lothar Steidler was more successful with Lactococcus producing interleukin 10 in the gut of mice and patients with colitis (67, 68).

The probiotic strain E. coli Nissle 1917 is used very often when microbiome of the gut has to be modified. This is because this strain is one of the few strains where the kinetics after oral administration is described and known (69). When genes for the production of N-acylphosphatidylethanolamines were added to this probiotic strain, it caused a reduction of food intake in mice as these molecules are precursors for signaling molecules that report satiety to the brain (70). This very well conducted study pointed towards the possibility that genetically modified functional food could once be a cornerstone for a really functioning diet. This might be of great importance for the obesity epidemics if confirmed in human studies.

The current bioengineering enables researchers to literally program bacteria using the tools of synthetic biology. Synthetic regulatory networks in the bacteria can be designed to function as a memory element that monitors the environment and based on the external signal changes its state (71). This could have enormous implications for the clinical diagnosis in gastroenterology. The interactions and communications between bacteria and eukaryotic host cells, the so-called interkingdom signals are complex and might be involved in the pathogenesis of diseases (72). On the other hand, they could be used for improvements of gene therapy such as precise regulation of transgene expression (73). Escherichia has been also modified to produce deoxyribonuclease I and the antimicrobial peptide microcin S to destroy the extracellular scaffold of a biofilm and to kill surrounding non-resistant bacteria, respectively (74). To make it even more sophisticated, the authors have put these transgenes under the regulation of an expression system than is activated by molecules secreted by Pseudomonas. The same signal was used for the stimulation of the locomotor activity of the bacteria towards the pathogen. Although the system has not yet been proved *in vivo*, the approach is extremely interesting and shows the potential of synthetic biology for gene therapy using bacteria.

8. DNA VACCINATION

Although bacterial vectors are attenuated to decrease the risk of immune system activation, this still represent a major disadvantage in most gene therapy applications. However, this disadvantage can be highly beneficial in DNA vaccination (Figure 4). Vaccines are mostly made of proteins from the pathogens or inactivated viral particles. In DNA vaccination, the genetic information for the production of the particular protein is transferred into host cells that produce the protein on their own, but can be achieved by direct prokaryotic expression as well (75). The expression by host cells improves the immune response. Additional adjuvans is often needed in the vaccine preparations. DNA vaccination can be conducted using plasmid DNA (76). CpG islands in the plasmid DNA activate the innate immune system. Bacteria add to the immune activation with pathogen-associated molecular patterns that include bacterial DNA, plasmid DNA, but also parts of their outer membrane. Bacterial vectors also partially provide targeting of the gene transfer as they are often engulfed by antigen-presenting cells (21). These characteristics make bacterial vectors especially useful for DNA vaccination.

The first demonstration of DNA vaccination using bacteria was published 20 years ago for an attenuated Shigella strain (77). In the pioneer experiment the strain was attenuated by a deletion of aspartate beta-semialdehyde dehydrogenase – an enzyme required for cell wall synthesis. The authors have shown that a marker gene under the eukaryotic cytomegalovirus promoter was expressed by the host cells. Intraocular and intranasal routes were used for *in vivo* administration. Both led to transgene expression in the cornea and the spleen, respectively. This caused an immune reaction and specific antibody production as shown by the same team later (78). Similar effects were found when Salmonella was used for oral vaccination. Both, humoral and cellular antigen-specific immunity were confirmed in several mouse strains (79, 80). Beyond immunological reasons bacteria-mediated DNA vaccination is usable also due to relatively cheap production costs.

The efficiency of DNA vaccination has been proved in experiments showing that such vaccination not only results in immune reactions but also protects mice from infections with the particular pathogen (81). Bacterial strains differ in their efficiency to induce antigen-specific immune reactions. Immunity against measles was induced by DNA vaccination with Shigella much better than with Salmonella in a direct comparison (82). Shigella was also a suitable vector for inducing musical immunity against influenza (83). Salmonella has been recently, on contrary, shown to be a very efficient vector for a DNA vaccine against Yersinia (84).

Both, Salmonella and Listeria were tested as potential carriers of anti-cancer vaccines (85-87). The

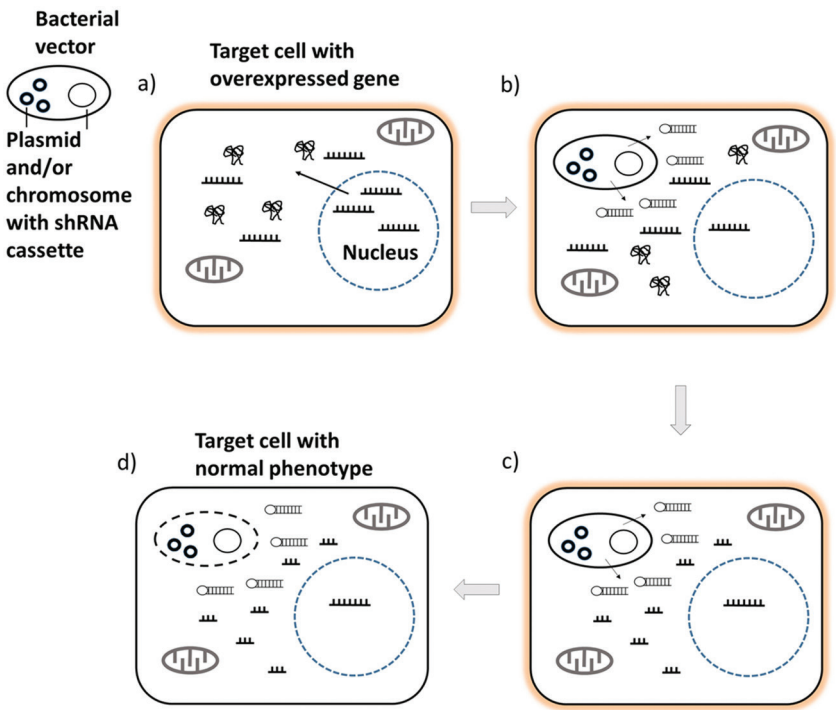

Figure 4. Bactofection – DNA vaccination. Bacteria are used as vectors to deliver genes encoding antigens to antigen presenting cells (APCs) that mediate the immune response against target cells (e.g. tumor cells) (a). Invasive bacterial vector enters the APC. Some of the bacterial vectors are destroyed by the innate immune system before they enter the cell (b). Bacteria are disrupted in the host cell and the plasmids encoding the target cell-expressed antigen are released and transported into the nucleus. Bacterial DNA, which is present outside the cell, acts as an adjuvant to stimulate the immune reaction (c). Target cell-expressed antigen is expressed by the APC and presented on its surface (d). T-cells are primed by the antigen presented on the APC and initiate cellular and humoral immune reaction to the tumor cells (e).

immune reaction induced by the two vectors differed due to differences in their life cycles in the host cell (88). Salmonella induced a Th2 immune response, while Listeria induced a Th1 immune response due to its active escape from the phagolysosome (89). This shows that details of the prokaryotic physiology can affect the efficiency and, thus, the application of the particular vector. Salmonella-mediated DNA vaccination against prostate cancer-specific antigen resulted in breaking of immune tolerance against this antigen and in a slower xenograft growth in mice (90).

DNA vaccination using bacteria does not have to be mediated via bactofection, but can be conducted as alternative gene therapy with direct prokaryotic expression of the antigens. However, this approach was inferior to bactofection-mediated DNA vaccination (91). Of course, such a comparison is always context-dependent. Lactococcus was modified to produce an outer membrane protein of a periodontopathogen Fusobacterium (92). Oral administration of this Lactococcus resulted in vaccination against Fusobacterium and subsequently to a reduced number of gingival abscesses in mice. The need for stimulation of the immune reaction and the need to limit toxicity of the vector to the host are contradictory and represent one of the many difficulties in DNA vaccination (93). DNA vaccination is the most developed application of bacteria-mediated gene transfer. It has been recently reviewed and is associated with great hopes for future clinical use (94).

9. FUTURE OUTLOOK

The recent revolution in gene therapy is caused by the available gene editing strategies, especially using the versatile, very efficient and cheap CRISPR/Cas system, which is derived from bacteria (95, 96). It is possible that in the near future bacterial vectors will be used for the *in vivo* genome editing using this system. Induction of pluripotency as a promising tool for regenerative medicine has also been suggested for *in vivo* application via bacterial vectors (28). Finally, genetically modified probiotics have the potential to improve the prevention and therapy of gastrointestinal diseases (97). The differences between gene therapy and functional food will very likely disappear and the regulatory organs will have to make difficult decisions. Gene therapy using bacterial vectors stands at a crossroad with many possible pathways to go. The question is which if these will lead the field into successful clinical application. Until now none of the bacterial vectors has been approved for clinical use. But taking a lesson from viral vectors, things can change very fast (2). Phase I trials have been conducted or are ongoing with Listeria, Salmonella,

Shigella, Bifidobacterium, Clostridium and others for DNA vaccination, bactofection and alternative gene therapy against cancer (98). One phase I trial is testing the safety of transkingdom RNA interference targeting beta-catenin with E. coli as the vector, another trial is analyzing the effects of Lactococcus producing interleukin 10 against colitis in phase II (99). Numerous trials are testing bacteria-mediated DNA vaccination, but major progress can be expected after the latest improvements of the vectors will be implemented (100). Unfortunately, most of the clinical studies were not published and it can only be speculated that the results were negative. Thus, whether any of these approaches will make it to the routine clinics remains to be seen. It is likely that further improvements will be needed.

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Abbreviations: CRISPR/Cas: clustered regularly interspaced short palindromic repeats/CRISPR-associated genes

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