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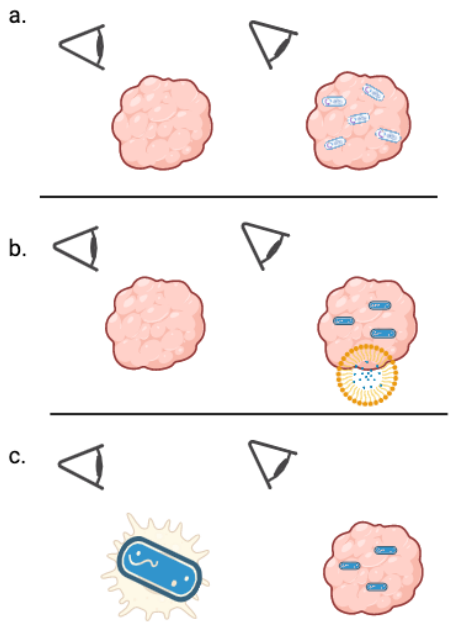
Engineering Bacteria to Expose Tumors

Bacteria-based immunotherapies serve as a potential tool to suppress tumor growth.[1–5] Tumor cells are unique in their ability to avoid immune detection via several mechanisms. These include, but are not limited to, the release of inhibitory molecules, recruitment of myeloid-derived suppressor cells, and exploitation of immune checkpoints.[1] This silences key components of the immune system and as a result, uncontrolled tumor growth can occur. In contrast, bacteria present several immune activating features. Components such as bacterial DNA/RNA, and peptidoglycan/lipopolysaccharide found in cell walls trigger immune signaling pathways.[1] Therefore, researchers are exploring methods to deliver bacteria to the tumor environment, which can "wake-up" the immune system and kill tumor cells (figure 1).

Figure 1: Immune Activation Schematic



a) Delivery of bacteria to tumor environment awakens immune system b) Delivery of liposomal antibiotics to endogenous bacteria awakens immune system c) Expression of extracellular polymer evades immune detection until it is turned off

Delivery of wild-type bacteria to tumors have reduced tumor size in previous studies.[1] However, the use of recombinant DNA technology in bacteria can allow researchers to expand on the existing genome. For example, bacteria can be engineered to express pro-inflammatory immune molecules and immune checkpoint inhibitors.[1,3,4] These molecules can reactivate the immune system in the tumor environment.[1] Despite the potential benefits of these molecules, uncontrolled expression of any inflammatory molecule can incur damage to the host. To work around this, artificial gene circuits can be designed for spatiotemporal control of gene expression. [1,3,4]

Chen *et al* designed a gene circuit to thermally induce a pro-inflammatory cytokine (interferon-gamma) in a non-pathogenic strain of *Escherichia coli*.[4] The main components of the gene circuit include the following: a heat-sensitive repressor, temperature-regulated promoter elements, and the inducible gene coding for interferon-gamma. At baseline physiological temperatures of mice, the repressor binds to the promoter elements and prevents transcription of interferon-gamma. However, when focused ultrasound irradiation is targeted towards the tumor area (target-site), the temperature increases to 42-45 °C. This denatures the repressor, and it can no longer bind to the promoter element, allowing for the transient expression of interferon-gamma. One concern in this model is that non-target sites are activated by the ultrasound as well.[4] For example, they used fluorescence imagery to show where the gene circuit is activated. The goal was to target the liver, but off-target sites such as the spleen and lungs showed low levels of fluorescence as well. This could be dangerous because pro-inflammatory molecules in the off-target sites may incur damage to healthy tissue.[3] It may be worth exploring liposomes as

a method to better target the bacteria to tumor sites. Alternatively, the *E. Coli* can be engineered to express bacterial membrane proteins that specifically bind to tumor cells.

In a related study conducted by Albedi *et al.*, the gene circuit design allowed for continuous expression of immune checkpoint inhibitors upon ultrasound irradiation.[3] They achieved this by inserting an enzyme under a temperature-regulated promoter which inverts two upstream sites. Then, the promoter region within the inverted sites activates the continuous expression of immune checkpoint inhibitors. Although continuous expression may be desirable for some cancer therapies, transient expression may be safer for clinical studies. The latter provides the ability to fine-tune the therapeutic expression and customize it to individual tolerances.

To evaluate the effect of interferon-gamma expression from engineered *E.coli* in tumor environments, Chen *et al.* created murine tumor models.[4] First, cancer cells were inoculated into the mice for tumor growth. Then engineered *E. Coli* were systemically administered into the mice. Lastly, the target sites were irradiated with focused ultrasound to activate interferon-gamma. At the end of the study period, they evaluated tumor volume (mm^3). A total of six groups were included in the study: the first received no bacteria (saline injection), the second received wild-type *E. Coli* without focused ultrasound, the third received wild-type *E. Coli* with focused ultrasound, the fourth received engineered *E. Coli* without focused ultrasound, the fifth received engineered *E. Coli* with focused ultrasound, and the sixth received focused ultrasound only. This is a good way to ensure that the change in tumor growth is not an artifact of the ultrasound irradiation or the *E.* Coli itself. The final average tumor volume was less than 250

mm^3 In the group that received focused ultra-sound irradiation and engineered *E. Coli*, while averages were over 1000 mm^3 in the other groups (p-value < 0.001).[4] The study conducted by Albedi *et al.* also resulted in similar suppression of tumor growth.[3]

Quantifying immune response can also help explain the changes in tumor volume. In the tumor environment, M2 macrophages are tumor-promoting, while M1 macrophages are tumor-suppressing.[1] Chen *et al* used flow cytometry to evaluate cell phenotype of macrophages isolated from the spleen.[4] The study found that the ratio of M1:M2 was highest in the group that received engineered *E. coli* and focused ultrasound, and lowest in the control group (saline injection).[4] The additional datapoints further support that the tumor suppression is not an artifact of the methods and is a key analysis in the study.

When the immune system is activated once, it can also create specialized cells that identify and kill previously identified threats.[6] This is known as an adaptive or secondary immune response.[6] By inoculating cancer cells into two opposing sites of the body (right and left), and only treating one side, Chen *et al.* were able to assess the adaptive immune response for tumor cells.[4] Significant tumor suppression compared to the control was observed in both the primary tumor which received treatment (engineered E.coli and focused ultrasound), and the secondary tumor which did not receive treatment.[4] The average secondary tumor size was less than 250 mm^3, while the tumor volume in the control ranged from ~500 mm^3 to over 1000 mm^3. In addition, metastasis foci found in the lung was greater than 20 in the control, and less than 10 in the treatment group. This model is significant because the therapy can potentially prevent

future recurrence of tumors unlike in surgery and radiation therapy. And it may also provide some promising results in difficult to treat metastatic cancers.

The studies described above used a non-pathogenic strain of *E.coli* for clinical safety.[3,4] However, there is still a maximum tolerated dose of bacterial injection for the host. [1,2] Harimoto *et al.* have designed an encapsulation system to increase this maximum tolerated dose and avoid immune detection until it is delivered to the tumor site.[2] The expression of an extracellular polymer effectively hides bacterial recognition sites from the immune system (figure 1c).[2] This could be useful in clinical studies because unlike in laboratory mice, patient size and tumor size can be variable. Therefore, the ability to increase the maximum tolerated dose may be helpful for fine-tuning the therapy.

Besides engineered bacteria, there are some therapies involving endogenous bacteria as well.[5] *Wang et al.* found that liposomal antibiotics delivered to oncogenic bacteria (*Fusobacterium nucleatum*) can expose epitopes and activate the immune system (figure 1b).[5] Taking advantage of existing bacteria can avoid many of the side effects associated with administration of engineered bacteria. However, the tumor microbiome is not well characterized, and results from past sequencing efforts have been inconclusive.[7] But combining the efforts from engineered bacteria with liposomal delivery of antibiotics could provide additional safety measures. The future of bacteria-based immunotherapy will most likely integrate multiple tools, including the ones described in this paper.

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