Offsetting The Effects of Microbiome-Derived Butyrate on Natural Killer Cells in Pancreatic Ductal Adenocarcinoma Through CRISPR Knockout of MCT1

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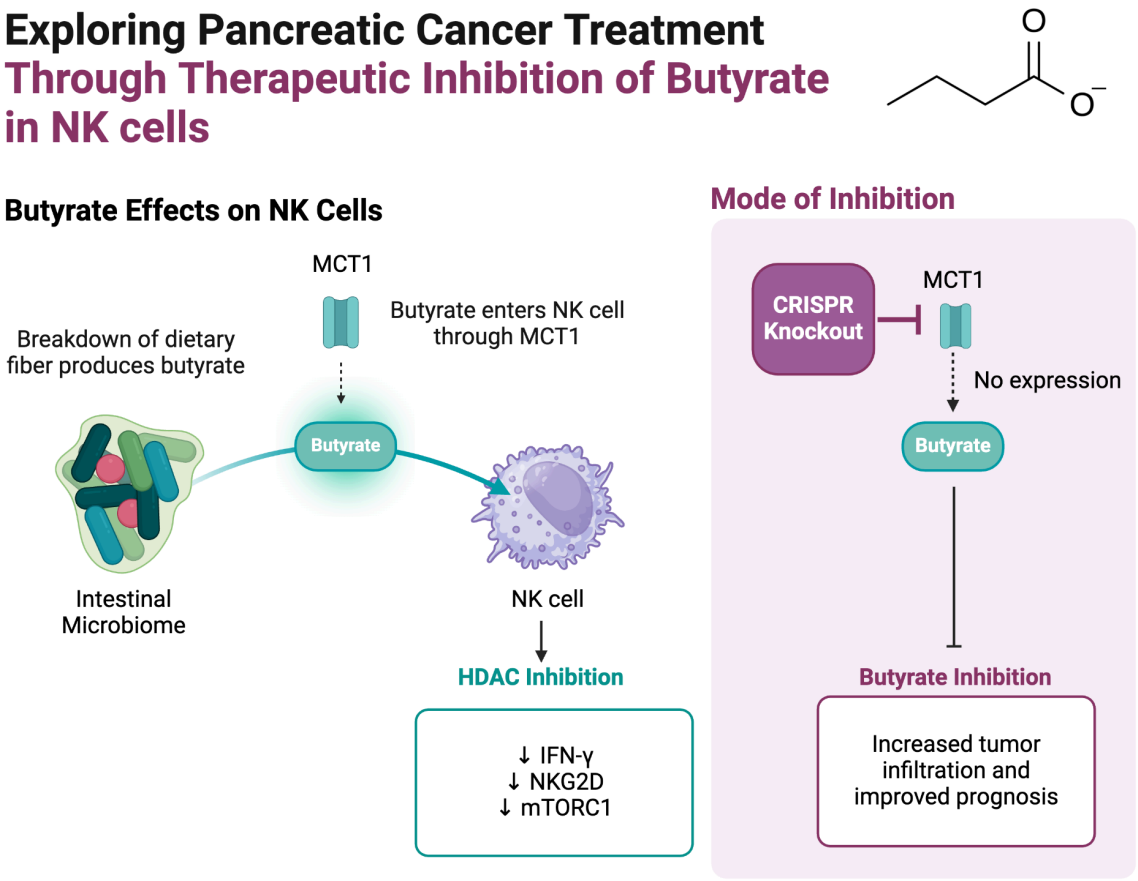
Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) is characterized by an immunosuppressive tumor microenvironment with low tumor infiltration. In the past few years, a method gaining more potential for treating PDAC is immunotherapy. However, many immunotherapies have fallen short because they fail to regard an important component of the body: the interface between the immune system and the intestinal microbiome. In this proposal, the mechanism suggested to worsen the prognosis of PDAC patients happens through the short-chain fatty acid (SCFA) butyrate. This chemical has been shown to reduce cytokine production and cytotoxicity of natural killer (NK) cells, with the cytokine levels of IFN-γ and perforin being decreased in both microbiome and butyrate-modulated NK cells. This proposal takes advantage of monocarboxylate transporter 1 (MCT1), the primary mediator of butyrate transport into NK cells. With a CRISPR knockout of the MCT1 gene, NK cell tumor infiltration can be increased, improving patient prognosis.

Visual Abstract



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1. Introduction

Pancreatic ductal adenocarcinoma is the most prevalent form of pancreatic cancer and makes up 90% of all cases [2]. While pancreatic cancer itself is relatively rare, it is relatively deadly compared to other forms of cancer, being the third leading cause of cancer-related death in the United States [3]. With a less than 12% 5-year survival rate for patients with PDAC [4], its low detection rate creates very stark outcomes for patients.

PDAC forms through various genetic mutations that often mimic pancreatic development [15,17]. For example, the mutation common in almost all cases of PDAC is the activation of the Kras oncogene [12,15], normally silenced after the formation of the pancreas. This promotes the creation of a PanIN lesion [15], the first step to the development of PDAC. After various other mutations [15], which can differ from patient to patient, PDAC is fully formed. As cancer research evolved, PDAC is still a difficult carcinoma to treat [12]. This is primarily due to its

metastatic potential, a trait that makes it difficult to treat by radiation or surgery [12]. For this reason, cancer immunotherapy has been looked upon as a potential method for PDAC treatment [12]. This process engineers the immune system to respond more potently and specifically towards the targeted tumor [12,24]. The potential of this treatment is greater in PDAC as this carcinoma is characterized by an effective escape from immune-surveillance [12]. After developments with T cells, researchers have looked to a different cell for executing these therapies: natural killer (NK) cells [37]. Their ability to quickly identify cancerous cells, along with their safety profile, makes them ideal for use in immunotherapy [37]. Recently, therapies have looked to expand the potency of these therapeutics by using CRISPR systems [13]. The potential of CRISPR systems to effectively enhance anti-tumor proteins while repressing exhaustive genes in T and NK cells makes it a valuable tool for the development of immunotherapeutics [13].

Recently, another area of interest is discovering the influence of the microbiome on the immune system [5]. It has been shown that the gut microbiome can influence several aspects of the body due to the byproducts of bacterial processes [7]. However, its influence on immunotherapy has not been well-thought out into the design of effective therapies [13,37]. In this research article, the analysis bringing together the results of multiple papers can drive a conclusion that butyrate is affecting the response of natural killer (NK) cells to PDAC tumors. Multiple factors play a part in this process from the intestinal microbiome to the metabolism of NK cells. From this, a solution can be suggested to combat the problem and offer a new method of treating PDAC. Using CRISPR systems, it can be shown that the inhibition of butyrate through the removal of its transporter can lead to a response in PDAC patients.

2. Analysis

2.1. The Microbiome's Effect on PDAC

The intestinal microbiome is an environment of different bacteria and other living organisms that have coevolved inside our body [6]. They are primarily located inside the intestinal lumen and have various functions in our body. Many bacteria have a symbiotic relationship with our body and can do things like produce vitamin K for our bodies from the food we eat [6]. However, these bacteria in particular have had to set up defenses against our immune system to prevent our body from killing them. This is done through many regulatory components such as immunosuppressive compounds and special inhibitory molecules called PAMPs [5]. In a healthy body, this prevents inflammation of the gastrointestinal (GI) tract. However, in the case of cancer, this may be a negative as suppressing immune cells can reduce their ability to infiltrate the tumor microenvironment.

Researchers at the University of Florida, Yu et al., hypothesized that this could be the case with natural killer cells in PDAC [6]. When comparing mice that had an intact microbiome

and mice that had been given antibiotics, the researchers found that the mice with an intact intestinal microbiome had a 57.4% reduction in NK cell-mediated tumor infiltration. The study found that these NK cells lacked several qualities of healthy cells, most notably a decrease in cytokines IFN-γ and perforin, inhibiting the quality of the response to a PDAC tumor [6].

2.2. The Production of Butyrate by the Microbiome

Butyrate, or butanoate, is an organic ion with the chemical formula $C_4H_7O_2^-$ [10]. Bacteria produce this short-chain fatty acid (SCFA) in the intestinal microbiome from the fermentation of dietary fiber. Two of the most prominent bacteria in the microbiome, *Faecalibacterium prausnitzii* and *Eubacterium rectale*, which combined make up 27% of all fecal gut microbiota, have a major role in butyrate production [11,20].

Butyrate is produced from dietary fiber, which is a carbohydrate made up of many monomers. After it passes through the digestive tract and into the colon, the aforementioned bacteria will start the fermentation process which breaks down this fiber [18].

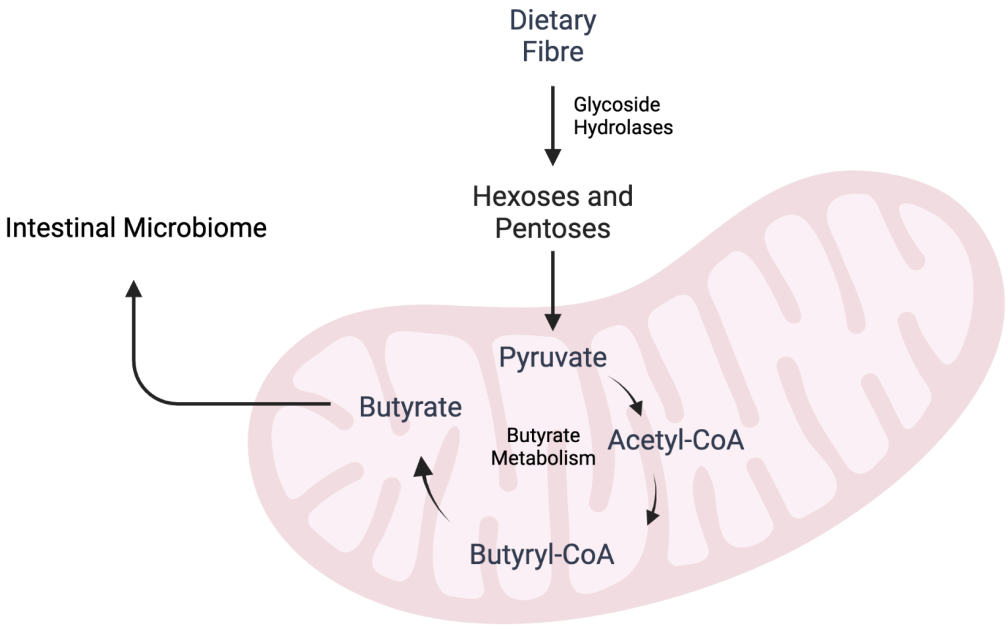

Figure 1 | Dietary fiber is broken down into simpler substrates such as hexoses and pentoses by the bacteria's enzymes called glycoside hydrolases [18]. Once the hexoses and pentoses enter the bacterial cytoplasm, they are degraded into pyruvate [18]. The pyruvate is then converted to acetyl-CoA. Acetyl-CoA is converted to butyryl-CoA until finally, butyryl-CoA is converted to butyrate by a butyrate kinase or butyryl-CoA:acetate-CoA transferase [18]. Finally, butyrate is

released from these bacteria into the GI tract, where it can have several effects on surrounding cells [18]. Created with Biorender.com.

2.3. The Effect of Butyrate on NK Cells

Butyrate is known to have several effects in cells due to its role as a histone deacetylase (HDAC) inhibitor [1]. These molecules are responsible for tightening the wrapping of histone proteins around DNA, reducing gene transcription [36]. In NK cells, this has the potential to reduce the production of cytokines and stimulants of NK cell activity [1].

Recently, Zaiatz-Bittencourt et al. ran a study in which NK cells stimulated by the cytokines IL-12 and IL-15 were exposed to butyrate in culture. What the researchers found is that NK cells had reduced levels of the activating receptors TRAIL, NKp44, NKp30, and NKG2D in response to butyrate stimulation [1]. With a lower level of activating receptors, NK cells cannot exhibit their anti-tumor properties nearly as well. Another role of NK cells is signaling other parts of the immune system when there is distress. This is done through the use of cytokines. In the same study, the researchers found that the crucial cytokines IFNγ, TNF-α, IL-22, and granzyme B were all greatly reduced when NK cells were exposed to butyrate. With lower expression of cytokines, NK cells are unable to communicate distress to other parts of the immune system, creating a more viable environment for tumors [1]. Finally, the researchers tested how NK cell metabolism is affected by butyrate. This process is regulated by the mTORC1, c-Myc, and HIF1-α dependent signaling pathways. When the NK cells were exposed to butyrate, it disrupted the first two signaling pathways. This created a situation where the energy of the NK cells was depleted and they got exhausted more easily.

2.4. Connecting the Literature

These three effects that were studied have drastic consequences on the ability of NK cells to fight PDAC. From what has been looked at here, it can be correlated that the production of butyrate from the microbiome is one cause for the progression of PDAC through lowered NK cell function. Therefore, the solution to this problem is to inhibit the effect of butyrate on NK cells using our knowledge of how it enters the cell.

In the study done by Yu et al. [6], it was shown through qPCR analysis that IFNγ and perforin were greatly reduced in NK cells affected by the microbiome. Similarly, both of these cytokines were reduced in the study by Zaiatz-Bittencourt et al. [1] as well, showing a connection between the two. These cytokines control the expression of multiple other proteins, explaining the other altered expressions in each study. Therefore, it can be concluded that butyrate is the chemical responsible for the weakness of natural killer cells in pancreatic cancer [1,6].

3. Methods

3.1. NK Cell-Specfic Inhibition

To remove the negative effects of butyrate on NK cells, it is necessary that this method is specific to those cells. This is important for two reasons: butyrate-dependent metabolism, as well as its variable effects on different cells. Colonocytes, differentiated cells in the colon, require butyrate as one source of their energy [20]. When butyrate enters these cells, it undergoes rapid β-oxidation and can then enter the TCA cycle inside mitochondria to produce ATP for the cell's energy. If butyrate was inhibited from these cells, the colonocytes would lose their primary energy source, as butyrate metabolism is used for 70% of ATP production in these cells [20]. Butyrate may be inhibited from NK cells as these cells use a glycolytic metabolism, not needing butyrate for ATP production [19].

The second reason NK cell specificity is needed is because certain cells may benefit from exposure to butyrate [1,6]. It has been shown that cytotoxic T lymphocytes are positively stimulated by butyrate molecules with the expression of more effector molecules [1]. To not remove any stimulus from the immune system, NK cells must specifically be targeted for maximum potency against PDAC tumors.

3.2. Targeting MCT1 to Inhibit Butyrate Uptake

Monocarboxylate transporter 1 (MCT1) is a proton-link plasma membrane transporter responsible for the uptake of butyrate in NK cells [23]. However, this transporter is also responsible for the transport of several other molecules, including lactate, pyruvate, acetoacetate, β-hydroxybutyrate, and GHB [23].

In many cases, this means that inhibiting butyrate would not be possible due to potential side effects. However, another transporter in MCT4 can mitigate these issues [23]. This is a similar transporter to MCT1 that is used to transport molecules of similar structure [23]. This transport protein can intake the following molecules: lactate, pyruvate, acetoacetate, and β-hydroxybutyrate [23]. In addition, butyrate is not uptaken by MCT4 [23], making this method a valid course of action. If MCT1 is inhibited, every molecule except for GHB can be transported while still inhibiting butyrate [23]. GHB is a neurotransmitter that is present in the body in low concentrations [26]. It has been shown to have either no effect or even a negative one on natural killer cells [26], making this method of butyrate inhibition effective.

The literature suggests that this method is a viable option for inhibiting butyrate. The colonocytes of patients with inflammatory bowel disorder have been noted to have lower levels of MCT1 present on their surface. It has been shown that this led to lowered butyrate oxidization for energy, pointing to how butyrate was inhibited from the cells when MCT1 was

downregulated [22]. Another area of concern is the potential for intracellular lactate build-up in the MCT1-knockout cells. As MCT1 is also responsible for the transport of lactate, this may pose issues for the cell in managing the release of lactate. When this molecule builds up in NK and CD8+ T cells, it has been shown to greatly reduce their cytotoxicity [25,27]. In one study, it was shown how inhibiting MCT1 didn't lead to lactate buildup, as MCT4 was still available for transport. It was only when both of these transporters were inhibited that lactate started building up and the T cell cytotoxicity was reduced [33].

3.3. CRISPR Knockout of MCT1

Clustered regularly interspaced short palindromic repeats (CRISPR) is a system used by bacteria to provide immunity against bacteriophages and viruses [31]. This system works by using Cas9 guide RNAs and cleaving targeted double strand DNA [31]. In human cells, this system can be used to target certain sequences within genes, effectively altering or removing their expression from the cell [32]. This method can be used in natural killer cells to remove expression of MCT1.

According to current literature, the exon most targeted in the knockout of MCT1 is Exon 2 [33]. The sg-RNA used for this knockout is 5'-TATCCATGACACTTCGCTGG-3'.

Position	Strand	Sequence	PAM	Specificity Score	Efficiency Score
27872	-1	TATCCATGACACTTCGCTGG	TGG	85.50461	69.03967454
27915	1	ATGTTGGCTGTCATGTATGG	TGG	63.9165885	66.46853565
27750	1	GGATACACCCCCCCAGATGG	AGG	73.0730359	63.4911785
27715	-1	ACTGGACCTCCAACTGCTGG	TGG	67.3705772	63.47900094
27797	-1	AAATGCATAAGAGAAGCCGA	TGG	65.6931903	61.64319619
27880	1	CCACCACCAGCGAAGTGTCA	TGG	52.0464814	60.74277947
27875	-1	GGATATCCATGACACTTCGC	TGG	88.5141912	59.80957068
27899	1	ATGGATATCCTCCATAATGT	TGG	69.5953876	59.79242908
27729	1	CCAGCAGTTGGAGGTCCAGT	TGG	70.3309652	59.57132052
27869	-1	CCATGACACTTCGCTGGTGG	TGG	72.4526918	59.02228093

Table 1 | Benchling's CRISPR score prediction software [35] provided 10 potential sequences for the sgRNA of this CRISPR knockout. The first sequence was chosen for having the highest efficiency score and the second-highest specificity score.

This sg-RNA and the Cas9 enzyme can be delivered to the cell via several delivery systems, including AAV, lentiviral, and adenoviral vectors [34]. To test if this hypothesis is true,

there can be several tests done to confirm the conclusions of this idea. The first step is to test if MCT1 knockout will lead to lowered butyrate levels in NK cells.

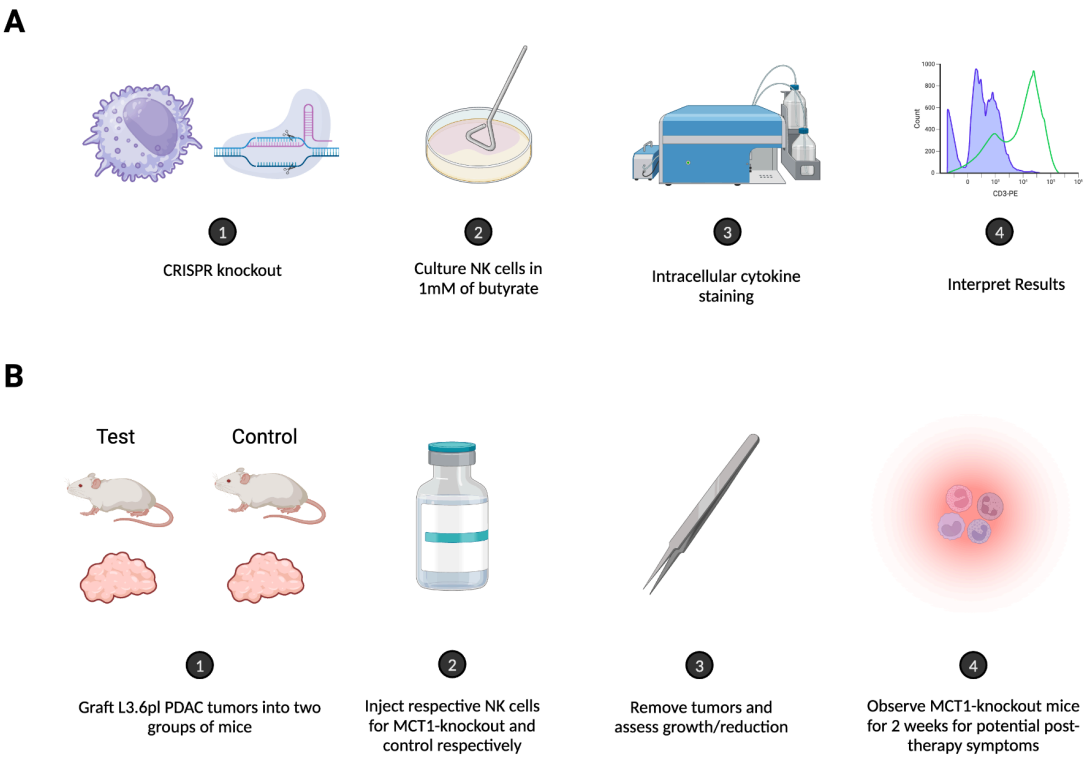

Figure 2 | (A) Perform the CRISPR knockout in NK cells from the NK92MI cell line and culture. Include a culture of wild-type NK cells as a control group for this experiment. Culture NK92 cells in 1mM of butyrate for both groups [1]. Perform intracellular cytokine staining to analyze the cytokine levels of perforin and IFNγ in both groups. Furthermore, the levels of mTORC1 metabolism can be tested through pS6 intracellular flow cytometry staining. If metabolism and cytokine production are higher in the knockout-NK cells, it can be confirmed that the loss of the MCT1 transporter will inhibit butyrate from entering the NK cells. (B) Testing is needed to confirm the effects these more effective NK cells would have against PDAC tumors. This could be tested in the following method. First, graft L3.6pl PDAC tumors into two groups of mice [6], one control group and one testing group. Administer the same volume of NK cells to both groups of mice, one with wild-type NK cells and the other with the MCT1-knockout NK cells. After one week, extract tumors from mice and measure their size. If the tumors of the MCT1-knockout mice are smaller than the wild-type mice, this strategy is more effective and viable against pancreatic cancer. These methods will help design the NK cells and confirm their positive effect against PDAC. Created with Biorender.com

4. Discussion

PDAC is one of the deadliest forms of cancer in the world and is the third-highest cause of cancer-related death despite being relatively low in occurance [3]. It is characterized by a hyper-immunomodulatory tumor microenvironment, posing challenges in the infiltration of the immune system [12, 14]. With the growth of immunotherapy, there have been advancements in overcoming these hurdles in fighting PDAC [12]. However, many immunotherapies do not account for the microbiome in their design, an important aspect of the body [7]. The microbiome is a complicated system that has coevolved with our bodies, introducing the opportunity for several mechanisms of homeostasis [6,7]. For this reason, there must be careful consideration when attempting to manipulate this balance. While the effect it has on the immune system has only recently become a topic of interest, the microbiome's influence on every system in the body is massive [7]. Studying its characteristics is crucial to the development of effective therapies as shown in this idea.

In this paper, it is reasoned that the SCFA butyrate is the cause of a lack of NK cell-mediated tumor infiltration. Through one studies, it was shown that the microbiome plays a role in influencing the conscription of NK cells to PDAC tumors [6]. In a separate study, it was shown that the chemical butyrate, produced by the gut microbiome, plays a role in reducing the effector function of NK cells [1]. With the cytokines IFN-gamma and perforin both being affected in these studies, major contributors to the expression of other immunological proteins, it can be concluded that butyrate is the molecule responsible for the weakening of NK cells in PDAC. The suggested solution in this article is through a CRISPR knockout of the MCT1 gene, the transporter responsible for the transport of butyrate into NK cells [23]. The sgRNA sequences were determined using Benchling [35] and used to calculate the scores needed to check the effectiveness of the system. After confirming its efficacy, the system is ready for use to improve NK cell function against PDAC.

Despite strong evidence behind this treatment, there are still some limitations that have to be addressed. One of these limitations is that we may not fully understand the role of MCT1, leading to potential adverse effects. In the evidence given, it is shown that with our current knowledge of the chemicals MCT1 transports, there should be little issue in performing a CRISPR knockout. However, this will change if we discover other chemicals which MCT1 may be responsible for. Another limitation is that we cannot know for sure the full effects of butyrate on NK cells. Despite both cytokines IFNγ and perforin being affected, it is not known what other chemicals may play a role in the suppression of NK cells. For example, in the same study by Zaiatz-Bittencourt et al. [1], it was shown that another SCFA called propionate has similar effects to butyrate in NK cells. A third limitation is not fully understanding the homeostatic balance of

the immune-microbiome interface. Disrupting the intake of butyrate may possibly lead to inflammation of the intestinal lumen if the NK cells become overstimulated.

In conclusion, this idea has the potential to open a lot of doors in the treatment of PDAC, a cancer that has been feared for a long time. By incorporating the effects of the microbiome into consideration, inhibition of MCT1 can be used to inhibit butyrate, a chemical shown to have negative effects on the cytotoxicity of NK cells. This method can potentially increase the stark chances patients have against PDAC, hopefully increasing the quality of life for these people.

5. Supplementary Details

The sequence for the sg-RNA of the CRISPR-Cas9 system targeting Exon 2 of MCT1 was taken using Benchling's [35] CRISPR design tool. The predicted on-target score is 69.0 and the predicted off-target score is 85.5.

6. Conflict of Interest

The author declares no conflict of interest.

7. Funding

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