Old Herborn University Seminar Monograph 36: The microbiome and cancer.
A summary of the lectures given during the 36th Old Herborn University Seminar
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THE IMPACT OF GUT MICROBIOTA-DERIVED METABOLITES ON THE TUMOUR IMMUNE MICROENVIRONMENT

Introduction

The topic of the presentation of *Prof. Dr. Alexander Visekruna* (Institute for Medical Microbiology and Hospital Hygiene, Philipps-University, Marburg, Germany) was "the impact of gut microbiota-derived metabolites on the tumour immune microenvironment".

Under homeostatic conditions, intestinal microbes produce and modify many small substances collectively termed "microbial metabolites", which serve as a very efficient means of communication between intestinal bacteria and the host. These small substances might profoundly affect human health, but many of them are poorly characterized and some of them are even unknown.

The Human Microbiome Project

At the moment, there is a lot of research performed on the microbiome with many publications as result. Twenty years ago it was different, at that time only a few researchers worked on the microbiome. This changed in 2006, when new powerful technologies were developed, like next-generation sequencing, mass spectrometry, metabolomics, etc. In 2007, the National Institutes of Health (NIH) initiated one of the biggest and most interesting research projects that would run until 2016: The Human Microbiome Project. It had many collaborators worldwide. The aim was to improve understanding of the microbiota involved in human health and disease. Launched in 2007, the first phase (HMP1) focused on identifying and characterizing human microbiota. The second phase, known as the Integrative Human Microbiome Project (iHMP), was launched in 2014 with the aim of generating resources to characterize the microbiome and elucidating the roles of microbes in health and disease states.

Gut microbiota-derived metabolites

In his presentation, special attention was given by Prof. Visekruna to "gut microbiota-derived metabolites". These small molecules are very interesting targets for clinical translation. In the gut there is only one thin layer of epithelial cells between the host and the harsh environment with all kind of bacterial metabolites. Bacteria should not cross the epithelial barrier, but the small molecules produced by the bacteria can diffuse across the epithelial barrier and reach the lamina propria (Figure 1).

These small bacterial metabolites can even enter the circulation and can impact on remote organs. The main group of microbial metabolites is called short-chain fatty acids. these are products of bacterial fermentation of non-digestible carbohydrates. In 2013, three publications showed the importance of microbial metabolites for mucosal immunity (*Furosawa* et al., 2013; *Arpaia* et al., 20013, *Smith* et al., 2013). Furosawa and colleagues found that gut commensal microbes, especially *Clostridia*, influence mucosal immunity by promoting differentiation and expansion of various T-cell types. Butyrate, a microbial fermentation product in the large intestine, induces Treg-cell differentiation in mice. Among short-chain fatty acids, butyrate is the most effective inducer of Treg-cell differentiation, both *in vitro* and *in vivo*. Their findings illuminate how microbial metabolites help maintain immune homeostasis in the gut by promoting regulatory T-cell development (Figure 2; *Furosawa* et al., 2013).

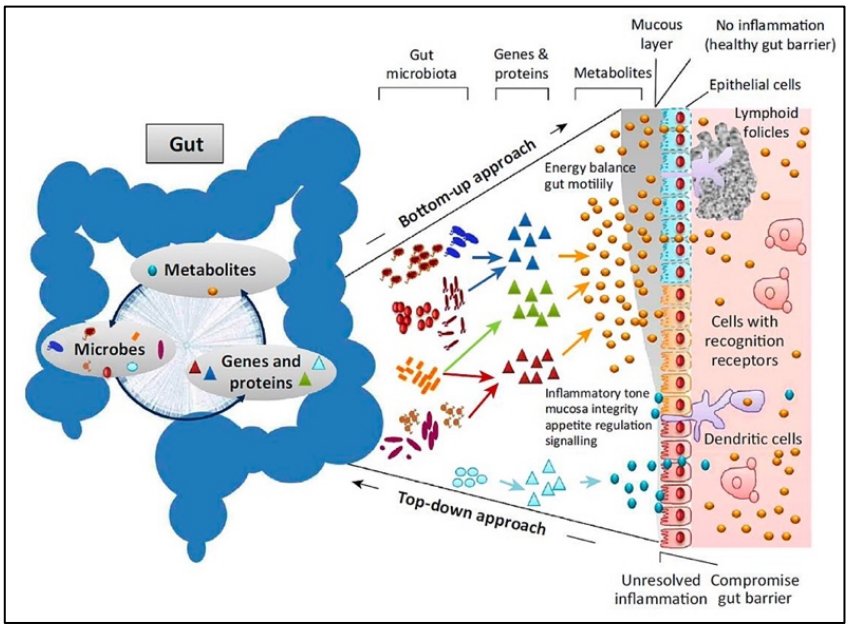

Figure 1: Bacterial metabolites can cross the epithelial gut barrier. (Figure from *Moya* and *Ferrer*, 2016).

Pentanoate is a microbiota-derived metabolite, it is present (0.4 - 0.6 µMol/g) in the coecum and the colon of germ-free mice (but is absent in the small intestine). The amount is 2.8% of all the short-chain fatty acids that can be found in the colon. Although the amount of pentanoate is very low when compared to acetate, propionate or butyrate, Prof. Visekruna's research was directed at

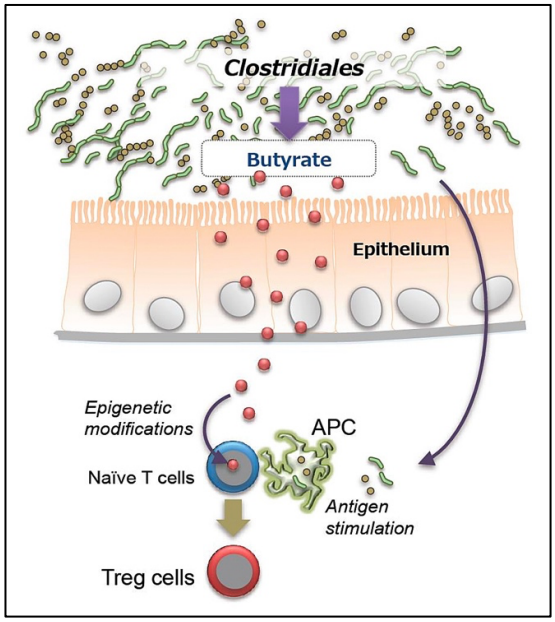

Figure 2: Butyrate induces differentiation of colonic Treg cells in mice. (Figure from *Furusawa* et al., 2013).

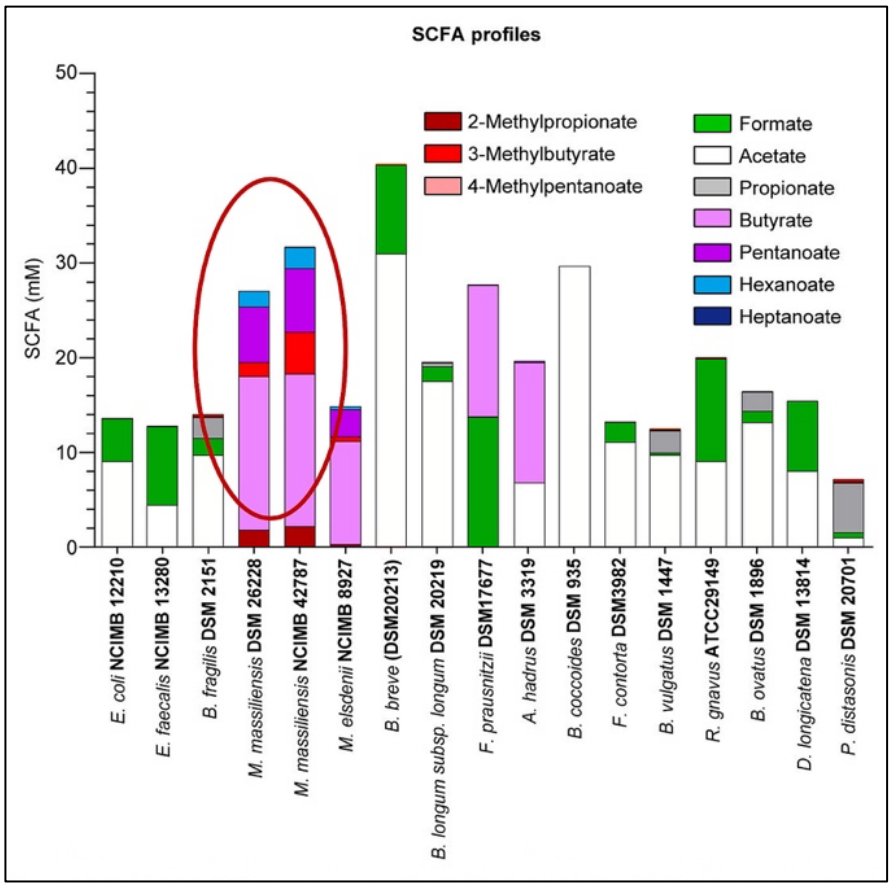

Figure 3: Microbiota-derived short-chain fatty acids from human gut bacteria. (Figure from *Luu* et al., 2021).

pentanoate, a short-chain fatty acid that has not received much attention in the literature. They concentrated on this molecule because they had the idea that this molecule could have therapeutic properties. They started a cooperation with the University of Aberdeen in Scotland which are experts in cultivating intestinal bacteria.

All together they screened 67 intestinal bacteria from humans, and only one, *Megasphaera massiliensis*, turned out to be able to produce pentanoate (Figure 3). This bacterium also produces high amounts of butyrate (*Luu* et al., 2021).

Histone acetylation, catalysed by histone acetyltransferases (HATs), adds an acetyl group to lysine residues, neutralizing the positive charge and loosening DNA-histone interactions to regulate gene expression.

Microbiota-derived short-chain fatty acids

Microbiota-derived short-chain fatty acids are strong class I histone deacetylase (HDAC) inhibitors. Pentanoate strongly increase the acetylation of histone 3 and histone 4, which modifies epigenetic regulation. This effect was confirmed in an *in vitro* assay by testing more than nine histone deacetylases.

Short-chain fatty acids have immuno-modulatory effects, but there is limited knowledge about the role of pentanoate in regulating immune cell functions. Th17 cells are pro-inflammatory immune cells that are implicated in the

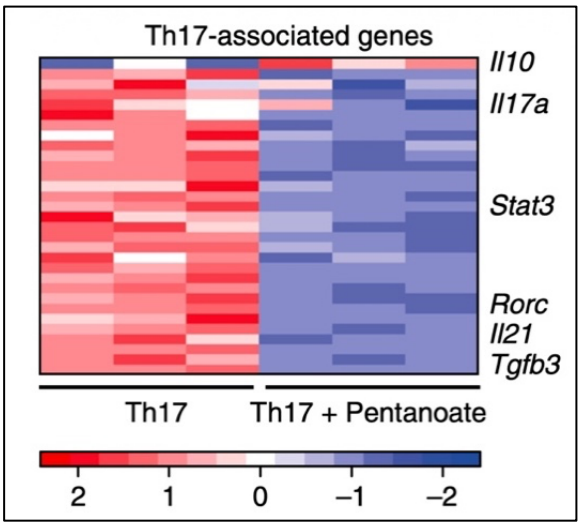

Figure 4: RNA-seq analysis of pathogenic Th17 cells in the presence or absence of pentanoate. (Figure from *Luu* et al., 2019).

pathology of many auto-immune diseases, such as multiple sclerosis and Crohn's disease. The effect of pentanoate from the supernatant of *Megasphaera* cultures on Th17 cells was tested. The global RNA-seq analysis revealed that pentanoate upregulated IL-10 expression and downregulated most of the Th17-associated genes including Rorc, IL-21, Stat3, and predominantly Transforming Growth Factor beta-3 (TGFß-3), which is endogenously produced by pathogenic Th17 cells (Figure 4).

Fax analysis of Th17 cells in the absence or presence of pentanoate showed a cytokine switch, treatment of the Th17 cells with pentanoate caused the loss of the pro-inflammatory cytokine IL-17A (Figure 5).

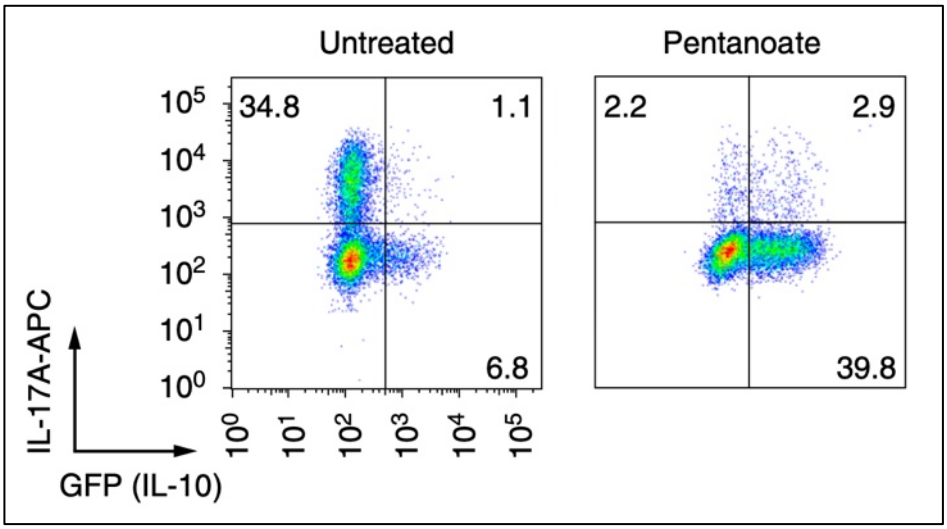

Figure 5: Flow-cytometry results of analysing CD4+ T cells isolated from FIR x tiger mice, polarized under Th17-inducing conditions and treated with pentanoate (5 mM). (Figure from *Luu* et al., 2019)

Not the presence of intestinal bacteria, but the presence of certain strains in the intestinal microbiome is important for the defence against intestinal pathogens. By using mono-contaminated germ-free mice it was shown that the presence of segmented filamentous bacteria (SFB) in the small intestine is sufficient to trigger the development of Th17 cells which are crucial for defence against intestinal pathogens (*Ivanov* et al., 2009).

Effect of pentanoate on the immune system

To investigate whether pentanoate could modulate inflammation of the central nervous system (CNS), experimental autoimmune encephalomyelitis (EAE) was induced in FIR × tiger reporter mice. The treatment of mice with pentanoate ameliorated EAE severity and reduced the number of infiltrating CD4+ and CD8+ T-cells in the CNS. Treatment with pentanoate of SFB-mono-colonized germ-free mice also significantly ameliorated EAE and led to reduced cell numbers and frequencies of Th17 cells in the CNS (*Luu* et al., 2019).

Thus, the microbial product pentanoate has a drastic effect on $CD4_+$ and $CD8_+$ T-cells. CD4+ and CD8+ T-cells are crucial components of the adaptive immune system; CD4+ T-cells (helper T-cells) coordinate the immune response, while CD8+ T-cells (cytotoxic T-cells) directly kill infected or cancerous cells. Both cell types are used in immunotherapy to fight cancer.

Extracts from the contents of the ileum, coecum and colon of SPF and germ-free mice were added to cytotoxic (CD8+) T-cells and incubated for 3 days. The extracts from SPF mice, in contrast to those from germ-free mice, stimulated the CD8+ T-cells to differentiate into cytotoxic T lymphocytes (CTLs), showing the role of bacterial metabolites in this process. CTLs produce interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and granzyme B (GrB), all involved in killing virus infected cells and tumour cells. The extracts of the coecum and the colon of SPF mice significantly increased the amount of measured IFN-γ and TNF-α when compared to extracts obtained from germ-free mice, while the extracts form the ileum did not significantly differ in measured IFN-γ and TNF-α between SPF and germ-free mice. Short-chain fatty acids could only be detected in the extracts from the coecum and colon of SPF mice, not in the ileum of SPF and germfree mice.

Having shown the effect of short-chain fatty acids *in vitro*, a mouse model was used to test the effectivity of the CTLs obtained by stimulation of CD8+ T-cells by pentanoate compared to CTLs from CD8+ T-cells that had not been stimulated by pentanoate. B16-OVA mouse melanoma cells were injected subcutaneously in wild-type mice and after 5 days of tumour growth the animals were i.p. injected with both types of CTL's. The tumour volume was measured every second day during 15 days after tumour transplantation, starting at day 5 after tumour transplantation.

The tumour growth after injection with the untreated CTLs was lower than the growth in animals that had not received CTL's, but the tumour growth in the mice injected with the CTL's that were obtained after stimulation with pentanoate was significantly lower than the growth in both the untreated mice and the mice injected with the untreated CTLs (Figure 6).

This experiment was repeated by transplanting "Panc OVA" cells into the mice. Panc OVA is a murine pancreatic cancer cell line. The modified cell line, Panc-OVA, is used in cancer research to study immune responses to tumours

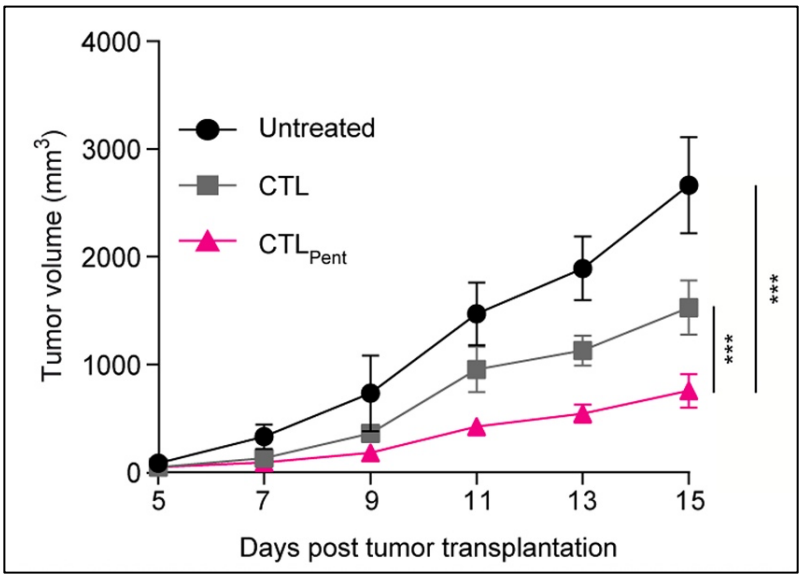

Figure 6: Growth of B16-OVA mouse melanoma in mice injected with untreated CTLs and CTLs obtained after stimulation with pentanoate, compared with tumour growth in untreated mice. (Figure from *Luu* et al., 2021).

because it allows researchers to track specific T-cell responses against the OVA antigen. In this second experiment, the results were even better, showing complete tumour suppression after injection with the CTL obtained from CD8+ T-cells that had been stimulated by pentanoate (Figure 7).

These promising data show that the short-chain fatty acid pentanoate can be a candidate to be used for cancer treatment, possibly in combination therapy.

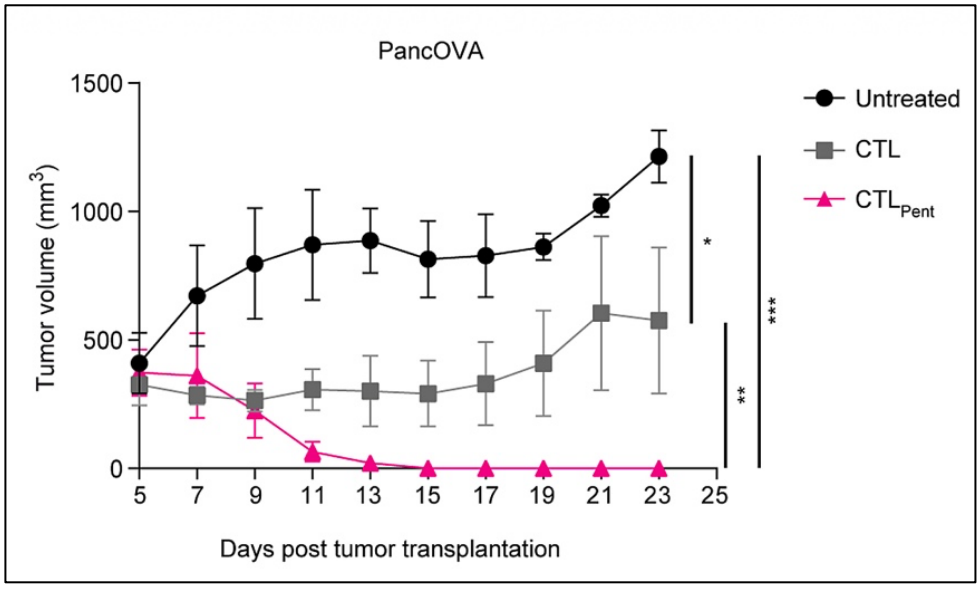

Figure 7: Growth of Panc OVA pancreatic tumour cells in mice injected with untreated CTLs and CTLs obtained after stimulation with pentanoate, compared with tumour growth in untreated mice. (Figure from *Luu* et al., 2021).

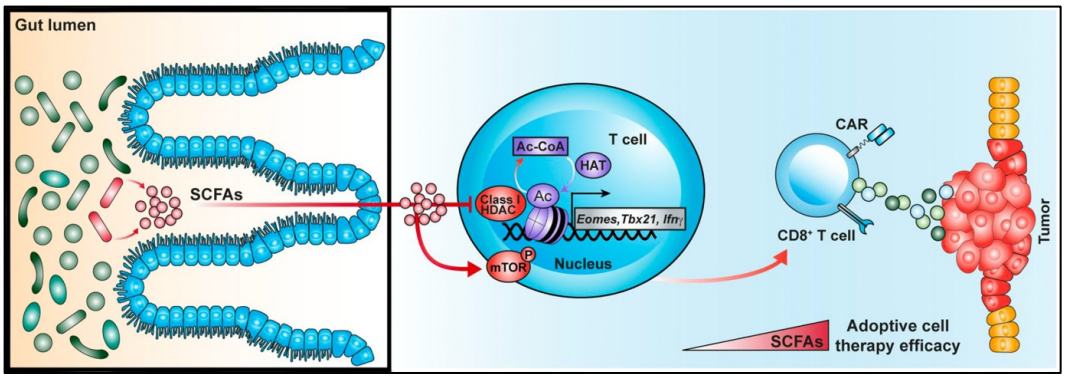

Figure 8: The crosstalk between microbial metabolites and T cells improves cellular immunotherapy. (Figure from *Luu* and *Visekruna*, 2021)

Immunotherapy for cancer

Mocetinostat is a histone deacetylase (HDAC) inhibitor that targets the class I HDACs, and it is being investigated for cancer therapy. TMP-195 is a selective class IIa histone HDAC inhibitor being also investigated for its potential in cancer therapy and other diseases. Both HDAC inhibitors were investigated for their impact on cytotoxic T lymphocytes (CTLs); they can *in vitro* activate these cells. Recent data show that pentanoate is a class I HDAC inhibitor and is more effective in activating CTLs than mocetinostat. Since there are indications that mocetinostat (and TMP-195) may play a role in treating Alzheimer's disease by reducing the production of amyloid-ß and tau protein, there possibly might also be a role for pentanoate in treating this neurodegenerating disease.

ROR1-specific CAR (Chimeric Antigen Receptor) T-cell therapy is a type of immunotherapy that genetically engineers a patient's T-cells to recognize and attack cancer cells that express the ROR1 protein. ROR1 is highly expressed in various cancers like leukaemia, lymphoma, and some solid tumours, but is minimally expressed in normal adult tissues, making it an attractive target.

Pentanoate improves the efficacy of human CAR T-cells by enhancing their anti-tumour activity through metabolic and epigenetic reprogramming. It increases the function of the mTOR pathway and inhibits histone deacetylases (HDACs), leading to increased production of effector molecules like CD25, IFN-γ, and TNF-α. This results in better performance against tumours.

A publication of Luu and Visekruna in 2021 summarizes how the molecular interaction of microbial metabolites (SCFAs) with T-cells improves immunotherapy for cancer (Figure 8; *Luu* and *Visekruna*, 2021).

In the human intestinal tract the low-abundant human bacterium *Megasphaera massiliensis* is able to produce high amounts of the SCFAs butyrate and pentanoate. Several CTL-associated effector molecules are upregulated following pentanoate- or butyrate-mediated inhibition of HDACs, which results in augmented anti-cancer potency of CTLs and CAR T-cells. This is a promising example of the therapeutic potential of microbial metabolites to improve immunotherapy by reprogramming the metabolic and epigenetic status of CTLs.

This paper was reviewed by Prof. Dr. Alexander Visekruna before publishing.

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