

## The Path of Life: On the Role of Microbiota in Mucosal Immunity

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### Abstract

The human immune system develops during fetal life in the sterile environment of the uterus. Directly after birth the baby, and its immune system, are exposed to an outside world, teeming with bacteria and viruses. The gastro-intestinal tract, also still sterile at birth, becomes rapidly colonized with large numbers of a large variety of bacteria. During development, the mucosal immune system becomes tolerant to resident, commensal bacteria. Via interaction with specific bacterial species, such as segmented filamentous bacteria and *Bacteroides fragilis*, as well as via bacterial metabolites such as butyrate, the neonatal immune system develops in a balanced and well-regulated manner. The importance of interaction of gut bacteria with the immune system also becomes evident in the outcome of check-point inhibition immunotherapy for cancer. Through mechanisms that need to be defined, gut bacteria improve the functionality of cytotoxic T-lymphocytes and thus enforce the anti-tumor response.

**Keywords:** Breast milk; Butyrate; Checkpoint inhibition; CTLA-4; Development; IgA; Microbiota; Mucosal immune system; PD-1; Regulatory T lymphocytes

**Abbreviations:** CD21: Complement Receptor Type 2; CTLA-4 : Cytotoxic T-Lymphocytes-Associated Antigen 4; DC: Dendritic Cell; FcRn: Neonatal Fc Receptor For IgG; FMT: Fecal Microbiota Transplantation; GF: Germ-Free; PFS: Progress-Free-Survival; PSA: Polysaccharide A; SCFA: Short Chain Fatty Acids; SFB: Segmented Filamentous Bacteria; TGF- $\beta$ : Transforming Growth Factor- $\beta$ ; Treg: Regulatory T-Lymphocytes.

### Introduction

Hieronymus Bosch (ca 1450-august 1516) was the most important medieval painter from the Netherlands. Of his works, only 24, that can be attributed to his name with certainty, have survived. Bosch painted on wood, and in many of his works also the outer panels were used. The Haywain triptych, showing a world of greed and desire, when closed reveals a poor wayfarer on his Path of Life. The wayfarer, or pedlar, carries a basket of merchandise on his back, and is en route on his path of life. He is holding a stick to

ward of threats, like an evil dog (Figure 1).



**Figure 1:** The Path of Life by Jheronimus Bosch. Outer panels of The Haywain (1510-1516). Museo del Prado, Madrid, Spain. [https://commons.wikimedia.org/wiki/File:Hieronymus\\_Bosch\\_070.jpg](https://commons.wikimedia.org/wiki/File:Hieronymus_Bosch_070.jpg) Assessed May 1, 2019.

The path of life itself also is full of other dangers, illustrated by a small, unsteady bridge that the pedlar is about to cross. The meaning of the painting is to depict the dangers and temptations during life which can be avoided by staying on the straight but narrow path. In the under drawing of this painting, as revealed by infrared reflectography analysis, a cross was placed at the small and unsteady bridge, but this was eliminated by Bosch in the final version. The pedlar is the same figure as in another Bosch' painting, The Wayfarer, which was completed before The Path of Life. Both paintings may have a similar intention which is to make the viewer reflect on their own path of life and to avoid all sinful temptations. In The Path of Life, the pedlar uses his stick as a form of protection against hostile attacks. In the context of the current manuscript, the stick could be regarded as the immune system, which also has as function to protect against hostile attacks by micro-organisms. Not all animals in Bosch's paintings have hostile intentions; the Path of Life also includes birds and sheep. Likewise, not all micro-organisms that are encountered by the immune system during development and throughout life have hostile intentions. Most of the micro-organisms in the gastro-intestinal tract have a positive effect on the development and regulation of the immune system, as will be discussed below.

## Development and maturation of the immune system

Human embryonic and fetal development takes place in the sterile environment of the uterus. As such, the immune system wouldn't be needed during that period. Indeed, most infections in newborns are obtained through maternal delivery. The most important infections in newborns are caused by *Escherichia coli*, group B streptococci, and *Listeria monocytogenes* [1]. The increased susceptibility to infections of newborns is determined by several factors. The barrier between bloodstream and meninges is not yet perfect, so that meningitis is relatively easy to develop [2], and different parts of both the innate and the acquired immune system are still immature, as will be discussed below. Although the number of granulocytes in the circulation of a newborn is high in absolute terms, the bone marrow production is still limited, especially during infection [3]. The binding to endothelial cells and the chemotactic activity of the granulocytes is very low, making it more difficult for them to go to tissues. That could possibly explain the high number of granulocytes in the blood. The bactericidal activity and their reduced response to inflammation together explain the reduced activity of neutrophils and thus the increased risk the child has of develop a bacterial infection [3].

The expression of TLR4 and thereby the cytokine production of monocytes is reduced, but their antigen presentation is also still insufficiently developed, resulting in reduced tissue repair, reduced phagocytosis of potential pathogens and reduced secretion of bioactive molecules [4,5]. Dendritic Cells (DCs) are less common in the blood and tissue. They have a reduced expression of co-

stimulatory (CD80, CD86) and MHC class II molecules and they make fewer cytokines (IL-12p70) [6]. Moreover, the Th1 and CD8<sup>+</sup> T lymphocyte responses are reduced, making the newborn susceptible to infections with e.g. *Mycobacterium tuberculosis* and *Salmonella* spp [7]. Natural Killer cells (NK cells) react less to activation by cytokines (IL-2 and IL-5) are susceptible to inhibition by transforming growth factor- $\beta$  (TGF- $\beta$ ) and have a reduced cytolytic activity, as a result of which protection against virus infections is also reduced [8]. Complement factors (mainly factors from the classical route) are only present in low concentrations in neonates [9]. Moreover, their own antibody production also still has to start. This means that the neonate is dependent on the alternative route and the lectin route of the complement system, which can be activated directly by microbial components such as polysaccharides and endotoxins. In newborns almost all IgG is obtained transplacentally from the mother [10]. Because antibodies of the IgM and IgA class cannot pass the placenta, they are initially almost completely absent in newborns. The moderate antibody formation results in poor opsonization of both encapsulated and un-encapsulated micro-organisms. It is striking that especially the T-lymphocyte-dependent antibody formation against polysaccharide antigens (TI-2 antigens) is deficient for the first one and a half to two years of life [11].

The reason for this is the low expression of the Complement Receptor type 2 (CD21) on the B lymphocytes of newborns and young children [12]. On marginal zone B lymphocytes in the spleen, CD21 seems to be completely absent for the first few years of life [13]. These B lymphocytes seem to be ultimately targeted against polysaccharide antigens. The response against polysaccharide antigens is particularly important for the defense against encapsulated microorganisms (such as pneumococci and *Haemophilus influenzae*). In newborns the majority of T-lymphocytes, like B-lymphocytes, are still naïve. Memory lymphocytes are logically not yet present at first. Due to the presence of TGF- $\beta$  during fetal life, about 3% of all CD4<sup>+</sup> T lymphocytes at the time of birth are Foxp3<sup>+</sup>CD25<sup>+</sup> regulatory T-lymphocytes (Treg) [14]. Furthermore, cytokine production is not balanced: the newborn exhibits a relatively high production of Th2-cytokines, while the production of IFN- $\gamma$  is still low. Because of this combination of T-lymphocytes, an anti-inflammatory profile with tolerogenic activity, reduced recognition of alloantigens of the mother's MHC and reduced responses to external antigens prevails on young children [15,16]. At birth there are many B-lymphocytes in the blood that produce IL-10 and TGF- $\beta$  and thus control the maturation of Th2-lymphocytes [17,18]. Gradually, more conventional B- lymphocytes that can generate a broad repertoire of antibody specificities arise. Neonatal B lymphocytes, however, exhibit less somatic hyper mutation and thus less affinity maturation, even though memory cells arise. There are also hardly any long-living plasma cells in the bone marrow, and IgG antibodies

disappear from circulation relatively quickly after vaccination. For neonate's breast milk is of great importance to support the immune system. Breast milk contains antibodies, especially ones from the IgA class [19].

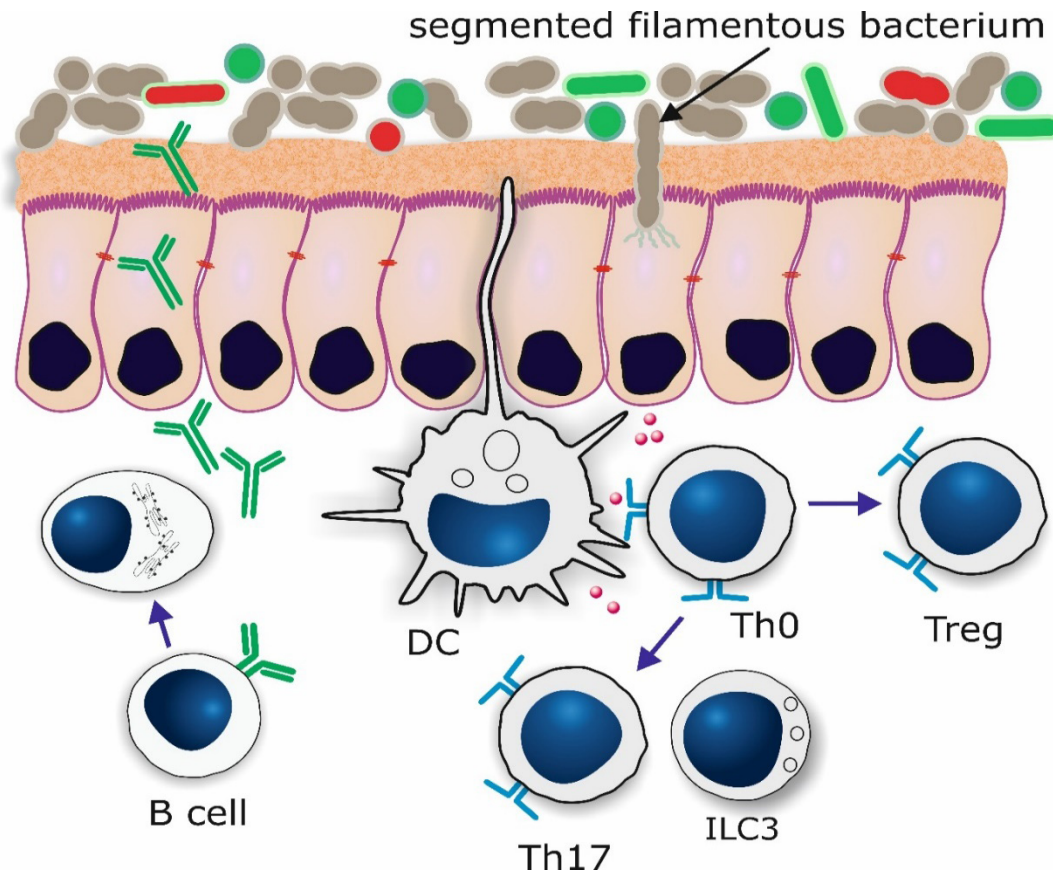
These antibodies inhibit adhesion of pathogenic micro-organisms to mucous membranes, thereby preventing invasion. In addition, breast milk contains a large number of other factors that contribute to the defense of the newborn. The lactoferrin, lysozyme, and  $\beta$ -defensins in breast milk have antibacterial effects. Cytokines and growth factors in breast milk reinforce the barrier function of the intestinal epithelium. In addition, breast milk contains a large number of sugar compounds (at least 80 different kinds), which promote the growth of bifidobacteria and lactobacilli (probiotic bacteria) in the baby's gut [20]. This contributes to the formation of a balanced intestinal microbiota. The non-optimal defense of the newborn is supported for up to several months after birth by the transplacentally obtained IgG and via breast milk obtained IgA antibodies (and other defense-promoting factors). In the blood, the neonatal Fc receptor for IgG (FcRn) is expressed on endothelial cells and circulating monocytes. This receptor ensures the internalization of IgG by binding in the acid endosomal compartment. FcRn recycles IgG molecules into the circulation. In such a way, the half-life of IgG is extended to about 21 days [21]. In the intestine, FcRn is expressed on enterocytes and antigen-presenting cells in the lamina propria. IgG is transported via enterocytes to the lumen where it can bind to antigens. Thus formed immune complexes then pass to the DCs in the lamina propria either directly via ingestion or indirectly via uptake by epithelial cells. After three to six months, the maternal IgG has totally disappeared from the circulation of the baby and breastfeeding is usually stopped by that time. During that period, however, the child's own antibody production capacity has not yet fully matured, so at this point in time a transient hypogammaglobulinaemia can occur. This can be considered to be an age-dependent immune deficiency [22]. Following this period, the immune system further develops into maturity.

### **Interaction between gut microbiota and the developing immune system**

The anatomical sites of the human body that are exposed, either directly or indirectly, to the outside world such as the skin, upper airways and some parts of the genital tract are permanently

colonized by diverse populations of microbiota. The greatest numbers and diversity of bacteria, however, are found in the gastrointestinal tract [23,24]. This creates a challenge for the developing intestinal immune system, as it must regulate the exposure between micro-organisms and host tissues, while also avoiding over-activation of the immune system at these mucosal surfaces. The mucosal immune system regulates this exposure to micro-organisms through compartmentalization and DCs play an important role in this process [25]. DCs reside in the lamina propria, and directly sample bacteria by opening the tight junctions in between epithelial cells, extending their dendrites across the epithelial monolayer to face the gut lumen [26]. The few bacteria that infiltrate this epithelial monolayer are captured by DCs and transported to the mesenteric lymph nodes where B cells are induced to produce IgA. Secretory IgA is transcytosed across the epithelium, where it binds intestinal bacteria and avoids their infiltration of the epithelial monolayer [25]. Secretory IgA is thus important for intestinal barrier function and has additionally shown to alter the composition of gut microbiota. Gut bacteria, in turn, upregulate the production of IgA, as a decrease of intestinal IgA-producing cells has been demonstrated in Germ-Free (GF) mice [27].

As indicated above, the mucosal immune system of the human newborn is exposed immediately after birth to great numbers of diverse commensal bacteria as well as to potential pathogens. In order to discriminate between "The good" (commensals) and "The bad and ugly" (pathogens), toll-like receptors are expressed which can recognize pathogen associated molecular patterns [28]. During the first days and weeks after birth, the mucosal immune system of the newborn becomes tolerant for commensal bacteria [29]. The mucosal immune system further develops and matures in close interaction with gut microbiota [30-34]. This interaction is vast and complex. The many different microbial species residing within the digestive tract produce a number of different metabolites, which can shape the mucosal immune system [35,36]. It should be noted that this interaction is not only of importance in neonatal development, but also during later stages of life [37]. The interaction between the developing mucosal immune system and the gut microbiota will be illustrated below with 3 examples for which the molecular mechanisms have been elucidated. The first example is the immune regulatory role of the so-called Segmented Filamentous Bacteria (SFB) (illustrated in Figure 2).

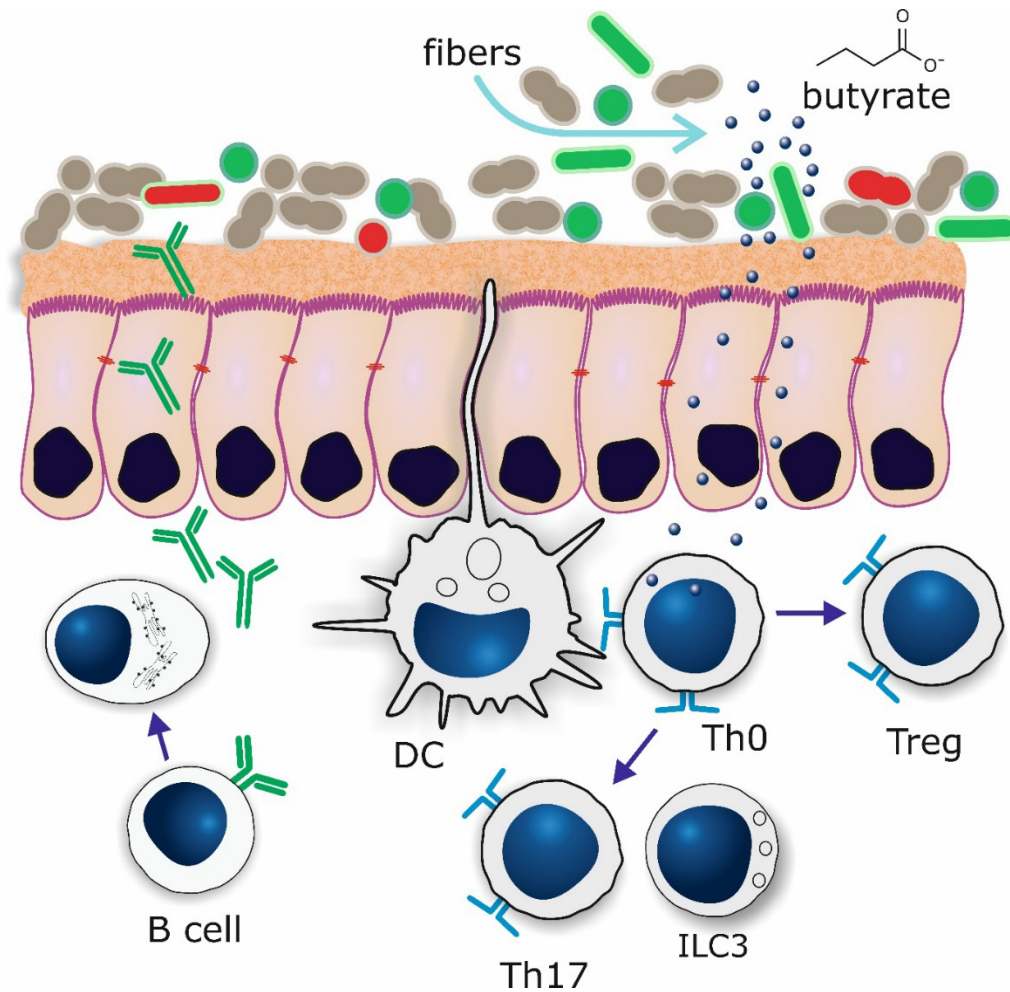


**Figure 2:** Regulation of the mucosal immune system by segmented filamentous bacteria. Modified from reference [30] See text for further explanation.

SFB adhere firmly to epithelial cells of the intestines, owing to their unique structure, which also enables filamentation, and the production of spores and intracellular offspring. The presence of SFB has been established in fecal samples and intestinal fluid of children up to the age of three [38,39]. Experiments in mice have revealed that SFB is a potent inducer of intestinal IgA-producing B-lymphocytes [40]. Moreover, SFB is a selective inducer of IL-17 [26,41]. Colonization of mice with SFB results in the production of serum amyloid A in the terminal ileum, which in turn prompts DCs in the lamina propria to stimulate Th17 cell differentiation [42]. This colonization of mice with SFB was also associated with increased protection against a certain pathogenic bacterium. The production of ATP by commensal bacteria is similarly correlated with Th17 differentiation, and upregulation of Th17

cells is associated with the production of the antimicrobial protein REGIII $\gamma$ . Th17 cells play important roles in several inflammatory and autoimmune diseases, and inhibition of Th17 differentiation is subsequently associated with amelioration of/increased resistance to those diseases [43]. It has been demonstrated that GF mice with a genetic predisposition to spontaneous arthritis do not develop the disease. Colonization with SFB, however, results in a progression to arthritis. The manipulation of this commensal may thus influence the development of Th17 cell-associated diseases [44,45].

The second example deals with the immune regulatory role of bacterial metabolites. Of the many bacterial metabolites with an effect on the immune system of the host, the Short Chain Fatty Acid (SCFA) butyrate stands out (Figure 3) [46].

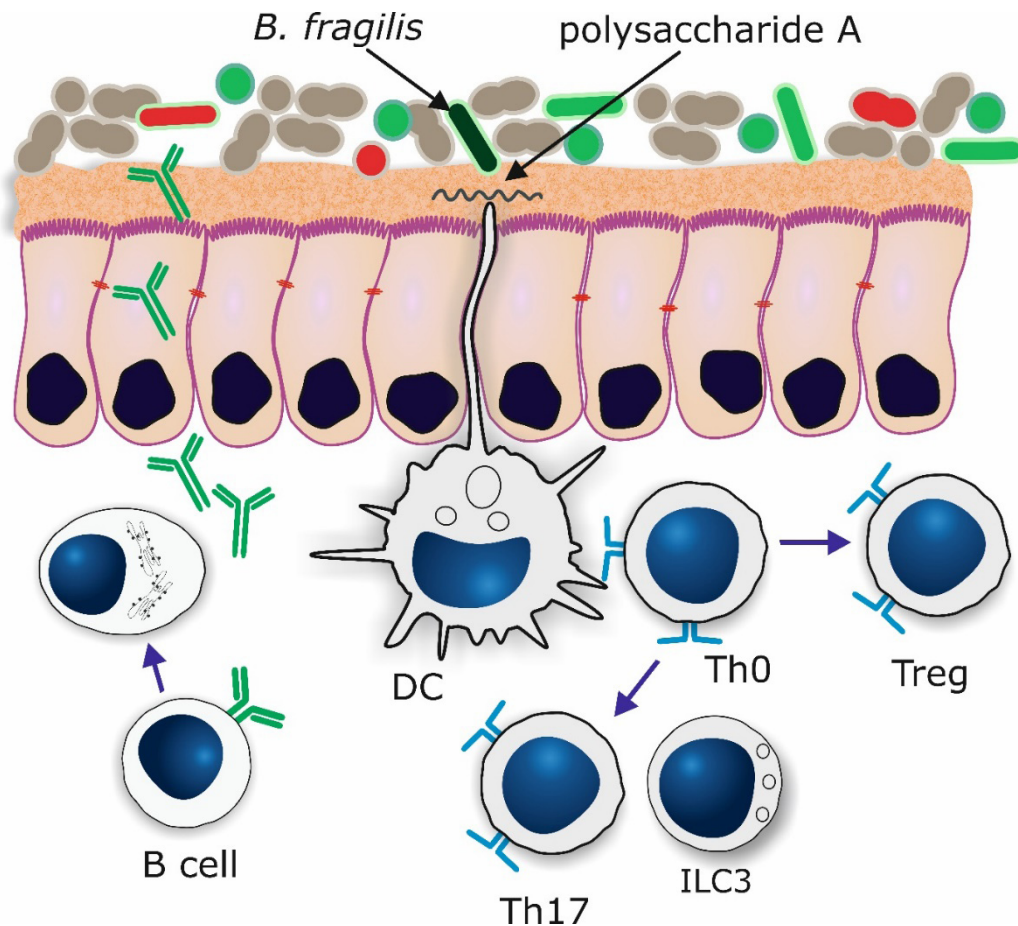


**Figure 3:** Induction of regulatory T cell differentiation by butyrate. Modified from reference [30]. See text for further explanation.

SCFA result from the fermentation of carbohydrates, and acetate, propionate and butyrate are the major SCFA with anti-inflammatory properties [47]. The production of IL-10 by Tregs is pivotal in the suppression of inflammatory responses in mucosal tissues. Commensal microbes, such as *Clostridium* spp, have shown to induce such IL-10 producing Tregs in the colon [48,49]. This differentiation of colonic Tregs in murine models has shown to be induced by butyrate. The SCFA stimulates upregulation of the Foxp3 gene in CD4<sup>+</sup> T-lymphocytes and thus induce the differentiation of Tregs [50,51]. Butyrate has been compared with other SCFAs such as propionate and acetate, both *in vitro* and in specific pathogen-free mice but no metabolite was as potent as butyrate in inducing Foxp3<sup>+</sup> Tregs. In murine models of chronic intestinal inflammation, a diet containing butyrylated high-amylase maize starches was associated with amelioration of colitis development. The SCFA also mediates intestinal inflammation in humans. Down-regulation of the butyrate transporter has been

observed in mucosal tissues of patients with Inflammatory Bowel Disease (IBD), along with a reduction of butyrate-producing bacteria [52,53].

The third and final example is that of a bacterial polysaccharide: polysaccharide A (PSA) (Figure 4). PSA is produced and secreted by *Bacterioides fragilis*, and has shown to protect against animal's models of colitis induced by *Helicobacter hepaticus* [54]. PSA modulates the mucosal immune response by suppressing the secretion of IL-17 by cells of the mucosal immune system of the gut. In addition, it stimulates the production of the anti-inflammatory cytokine IL-10 by CD4<sup>+</sup> T-lymphocytes [55,56]. Above examples illustrate how gut microbiota (in a number of cases via their metabolites) regulate the function of the mucosal immune system. Recent data from studies in patients treated with checkpoint inhibition therapy for various forms of cancer now show that the composition of gut microbiota may also play a decisive role under these circumstances.



**Figure 4:** Induction of mucosal regulatory T cells by polysaccharide A from *Bacteroides fragilis*. Modified from reference [30]. See text for further explanation.

### Modulation of the anti-tumor response by gut microbiota during checkpoint inhibition therapy.

The function of the immune system is not only to protect the host against infections but also to detect and destroy tumor cells. The traditional view (prevailing paradigm) thus was that in patients with cancer, there must be some kind of immunodeficiency. Thanks to the increasing knowledge of tumor cell phenotypes and of the immune system, specifically in the field of T-lymphocyte activation regulation this view has radically changed. Thanks to these insights and new biotechnological possibilities, the last 5 years have seen a real breakthrough in the immunotherapy of cancer [57]. This is reflected in Nobel Prize for Medicine 2018, awarded to Honjo and Allison for their groundbreaking research on PD1 [58,59] and CTLA4, respectively [60,61]. Tumor cells use various ways to escape from attack by the immune system and one of this is to express PD-L1, a ligand for PD1 in order to block activation of tumor infiltrating T-lymphocytes. The basis of the immunotherapy is to disrupt the receptor-ligand engagement of

PD-1 using antibodies, and thus enable an enhanced T-lymphocyte mediated antitumor response [62]. PD-1 is a cell surface receptor, with two distinct, but related ligands PD-L1 and PD-L2. It delivers inhibitory signals to activated T-lymphocytes, and upon interaction with its ligands, provides a regulatory mechanism for preventing overstimulation of an immune response and (thus) autoimmunity.

Interaction between PD-1 and PD-L1 is used in the tumor microenvironment to enable tumor cells to resist anti-tumor activity from the immune system. PD-L1 ligation initiates various pathways leading to suppression of T-lymphocyte activation, such as apoptosis, energy and exhaustion. Immunotherapy directed against the blockade of PD-1, takes away the inhibition, which allows for the formation of an effective antitumor response. Indeed, treatment of melanoma, non-small cell lung carcinoma, or ovary carcinoma patients with pembrolizumab or nivolumab (both anti-PD-1 antibodies) is effective, but not in all patients. CTLA-4 also has a regulatory role for cytotoxic T-lymphocytes. CTLA-4 provides regulatory feedback inhibition by inhibiting co-

stimulation by CD28. CD28 on T-lymphocytes can bind to CD80 on antigen presenting cells and provides a co-stimulatory signal, next to the primary stimulatory signal induced by interaction between the T cell receptor and antigen presented by MHC. CTLA-4 has much higher affinity for CD80 on APCs, and it is thus believed that much of the antitumor function of CTLA-4 blockade takes place in the secondary lymphoid tissue, as opposed to the tumor microenvironment itself [63,64]. CTLA-4 acts via multiple inhibitory pathways, including the inhibition of IL-2 mediated transcription in CD4<sup>+</sup> T-lymphocytes, as well as recruitment of Tregs. CTLA-4 therefore is another appropriate target for the development of checkpoint inhibition therapy and the monoclonal antibody ipilimumab has been used since 2011 for treatment of several types of cancer, such as late-stage melanoma. Combination therapy with antibodies directed against PD-1 and CTLA-4 increases the therapeutic effect. Checkpoint inhibition immunotherapy (unfortunately) is not successful in every patient, because detectable, and functional tumor infiltrating T-lymphocytes are required. Furthermore, there is a price to be paid for taking away the breaks of the immune system: the treatment with checkpoint inhibitory drugs can cause autoimmune reactions [60].

While it is possible that non-responsiveness to checkpoint inhibition therapy is due to quantitative and/or qualitative deficiency of cytotoxic T-lymphocytes, it now has been found that non-responsiveness is correlated with intestinal dysbiosis. Certain gut commensals (discussed below) have a positive modulatory effect on immune checkpoint inhibition therapy [65]. Routy and his colleagues have studied the influence of the microbiome on the anti PD-1 therapy [66]. They identify primary resistance to anti-PD-1 inhibition, which is observed at levels of 60-70% of the patients as partly a function of malignancy-associated intestinal dysbiosis. They use quantitative metagenomics to explore the microbiome composition of the responders and non-responders. The main finding was that *Akkermansia muciniphila* was enriched in patients with Progress-Free-Survival (PFS) longer than three months, along with other bacterial species such as *Ruminococcus* spp [66]. In another study in melanoma patients, also significant differences between the gut microbiome composition and response to checkpoint inhibition were found. Importantly, patients with a high diversity of gut microbiota had significantly longer PFS than those with reduced microbial diversity. Responsiveness was associated with the presence of *Clostridiales* and *Ruminococcae*. Non-responders had an enrichment of *Bacteroidales* and *E. coli* [67]. Comparison of bacterial metabolic pathways shows that in responders, there is a prevalence of anabolic functions, including amino acid biosynthesis which may promote host immunity. Furthermore, significant differences in tumor microenvironment were found, including significant positive correlation between the CD8<sup>+</sup> T-lymphocyte infiltrate and the relative abundance of *Faecalibacterium*, *Ruminococcae* and *Clostridiales* species. In

contrast, patients with higher load of *Bacteroidales* displayed a Treg dominated, cytokine-dampened profile.

Finally, Fecal-Microbiota Transplant (FMT) in GF mice of (human) responders resulted in an enhanced anti-tumor response in the mice [67]. In an independent study, again in melanoma patients on PD-1 checkpoint inhibition therapy, also significant association between the gut microbiome composition and clinical response was found [68]. In this case *Bifidobacterium longum*, *Collinsella arofacins* and *Enterococcus faecium* were correlated with response to therapy [68]. Above studies, as well as comparable studies reviewed in reference [69] underscore the critical role of gut microbiota in the success of checkpoint inhibition therapy [69]. Given the positive outcome of FMT in mouse models, it could be argued that FMT could be used in human patients as additional treatment during checkpoint inhibition immunotherapy. Better understanding of the mechanics that govern the interaction between gut microbiota and host immune system can contribute to further development and improvement of future therapeutic procedures.

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