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Gut microbiota – architects of small intestinal capillaries

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1. ABSTRACT

The commensal gut microbiota is an environmental factor that exerts manifold effects on host physiology. One obvious trait is the impact of this densely colonized ecosystem on small intestinal mucosal vascularization. At present, the microbiota-triggered signaling pathways influencing small intestinal renewal, angiogenesis, and vascular remodeling are largely unexplored. While the interplay of gut microbial communities with pattern recognition receptors, such as Toll-like receptors, in intestinal homeostasis is increasingly understood, it is unresolved how commensal microbiota affect the signaling pathways responsible for the formation of capillary networks in the intestinal mucosa. It is evident that intestinal vascular remodeling and renewal is disturbed in case of dysbiosis of this densely colonized microbial ecosystem, in particular under conditions of intestinal inflammation, but the effects of individual components of the gut microbiota are elusive. This review article provides an overview on the revealed microbiota-host interactions, influencing angiogenesis and vascular remodeling processes in the small intestine.

2. INTRODUCTION

Inherent factors such as genes along with environmental factors interact in tandem in myriad ways to influence, modulate, and modify the biology of all living organisms. This makes one wonder whether genetic and environmental factors can ever truly act independently of each other. Now we know that environmental exposures and experiences can have a direct influence on the expression of genes through epigenetic regulations or on the function of gene products through post-translational modification (1). Likewise, genetic factors influence the consequences of environmental exposures or stresses on the organism.

Dietary substances represent key environmental factors that influence the host and its resident, coevolving microbial communities (2). Microbiota, or the microbes that colonize each of us, populate all body surfaces (e.g. skin, vagina, lung, oral cavity, and gut). The corresponding collection of bacterial genes of this complex bacterial population provide a panoply of genomic material - the microbiome (3). The largest and most complex of these host-associated microbial communities resides within the intestine. The metagenomic potential of this internal microbial community coevolved with the human host and has increasingly been shown to interact with the host genome in development, health, and in diseases, ranging from periodontal disease to rheumatoid arthritis to inflammatory bowel disease to cancer (4).

The intestinal microflora serves three major functions: metabolic, trophic and protective (5). It produces short-chain fatty acids (SCFAs) and vitamins, thereby ensuring host health and metabolic functions. The microflora is also involved in intestinal epithelial cell growth, turn over and differentiation (6). The gut microflora induces maturation of host immunity; stimulate the intestinal epithelium in order to protect the host against invasion by pathogens and transforms carcinogens. With these including many other different functions, the gut microbiome serves as an 'organ', no wonder therefore, this symbiotic association of host–microbe being considered as a 'superorganism' (7, 8) that influences human health and development (9). The development of the intestinal microflora occurs during early infancy (10), and a distortion in any of the microbiota functions could potentially contribute to a wide range of diseases. The trillions of microorganisms that colonize the human body control many aspects of both innate and adaptive immune responses (11, 12), and a healthy microbiota plays a crucial role in maintaining immune homeostasis. Accordingly, dysbiosis of the gut microbiota is associated with many diseases characterized by chronic gut inflammation, including inflammatory bowel diseases (13). As the commensal gut microbiota is an environmental factor that is a driving force in postnatal gut development (14), including the development of intricate capillary networks in small intestinal villus structures (15), we here review the signaling mechanisms triggered by commensal microbiota that impact remodeling and renewal processes, angiogenesis, and vascularization of the small intestine.

3. PATHWAYS INVOLVED IN GUT DEVELOPMENT

The human intestinal tract is considered the organ with the most rapid renewal rates in the body. The underlying molecular pathways are usually involved in gut development and need to be tightly controlled in order to preserve vital organ functions, such as efficient nutrient uptake and transport, digestion, gut barrier function, excretion and detoxification of catabolites, and protection from infections. Small intestinal villus morphogenesis is induced at E15.5. (16) and major changes in morphology occur after birth and at weaning, indicating that this may coincide with the formation and changes of the gut microbiota, impacting normal gut development (17). The serosal mesothelium was shown to respond to Hedgehog signals, undergoing epithelial-to-mesenchymal transition and migrating into the gut tube at E11.5, differentiating into endothelial cells, vascular smooth muscle cells, and pericytes (18). The intestinal microbiota is an environmental factor that profoundly impacts on mucosal morphology and cellular renewal in the gut (19), but little is known on the exact mechanisms how this microbial ecosystem affects renewal of the epithelial lineage from the crypt stem cell niche, differentiation of mesenchymal cells, mucosal angiogenesis and vascular remodeling (20). If the control on intestinal cell renewal is lost, this can result in dysbiosis, malnutrition, intestinal inflammation, and even in the occurrence of intestinal cancers. The most central signaling pathways involved in gut development, intestinal morphogenesis and renewal are the Hedgehog pathway (21), the transforming growth factor-β (TGF-β)/Smad pathway, the WNT pathway (Wingless/Int-1), the Notch pathway as well as several tyrosine kinase pathways (e.g. EGF signaling). The gut microbial ecosystem may impact on several morphogenic pathways in the intestinal mucosa, thus shaping its habitat and host (patho)physiology (22).

4. GUT MICROBIAL PRODUCTS STIMULATING TOLL-LIKE RECEPTORS AND INTESTINAL REMODELING

Microbes are recognized by pattern recognition receptors, e.g. Toll-like receptors (TLRs), ubiquitously expressed by multiple cell types, leading to physiologic or pathologic responses. To study the role of TLRs in the intestinal epithelium and to explore the relationship between intestinal epithelial cells (IECs) and commensal bacteria, researchers first aimed to determine whether the intestinal epithelium expresses TLRs under normal physiological conditions and, if so, what is the regional and spatial localization of TLR expression in the intestine. Considering the diversity of whole intestine, it is difficult to pinpoint expression of TLRs by IECs using whole intestinal lysate as the intestine homes range of different cell types that can express TLRs, e.g. epithelial cells, macrophages, dendritic cells, B cells, T cells, and various stromal cell types. Hence, immunohistochemistry, enzymatic separation of IECs, and laser capture microdissection of the intestinal epithelium were used to show that TLR2 and TLR4 are expressed at low levels by IECs in normal human colon tissues (23–25). TLR3 seems to be abundantly expressed in normal human small intestine and colon, whereas TLR5 is expressed predominantly in the colon (23). Almost all TLRs are expressed, at least at the mRNA level, in the human colon. The expression of TLR1, TLR2, TLR3, TLR4, TLR5, and TLR9 has also been detected in IECs of the human small intestine (25). Moreover, IECs from patients with inflammatory bowel diseases have higher expression of TLRs, especially TLR4, and comparable expression of TLR2, TLR3, TLR5, and TLR9 than IECs from control individuals (23, 26–28). Inflammatory cytokines have been shown to regulate the expression of TLRs by IECs (27, 29–31). Early studies showed that interferon-γ (IFNγ) and tumor necrosis factor (TNF) induce the transcription of TLR4 and its co-receptor MD2 (also known as LY96) (27, 30). Cytokine-mediated induction of TLRs may allow their selective expression during times of danger perceived by the host (32). Studies comparing germ-free (GF) mice with conventionally housed mice indicated that

commensals induce the expression of certain TLRs (TLR2, TLR3, TLR4, and TLR5), as assessed in mucosal scrapings (33). Using immunohistochemistry, TLR9 was shown to be expressed on the apical surface brush border of the colon of mice with conventional flora but not that of GF mice (34). In addition, we have recently shown that mice colonized with conventional microflora from birth showed TLR2-dependent increased small intestinal renewal and apoptosis compared with GF controls with elevated mRNA levels of the proliferation markers Ki67 and Cyclin D1, elevated transcripts of the apoptosis marker Caspase-3, and increased numbers of TUNEL-positive cells per intestinal villus structure (35), suggesting a role for TLR signaling in intestinal epithelial renewal. On the other hand, we have also reported gut microbiota independent TLR5 expression in the small intestine that is dependent on the MyD88 and TRIF adaptors (36).

Over the decades of scientific work, it is now well established that even in the absence of dysbiosis, pathogen-associated molecular patterns (PAMPs) derived from gut microbial communities, such as peptidoglycan (PG) (37) and lipopolysaccharides (LPS) (38) constantly leak into tissues and the portal circulation (39), triggering adaptive TLR signaling in the host. However, intestinal epithelial cell lines are unresponsive to purified, protein-free LPS as measured by NF-kB activation and IL-8 secretion (24, 40). This unresponsiveness was explained by the low expression of TLR4 and its co-receptor MD-2 in intestinal epithelial cell lines (24). Expression of both TLR4 and MD-2 restores the ability of intestinal epithelial cells to respond to LPS, suggesting that the intracellular signaling pathway leading to NF-kB is intact in these cells. Even at remote sites, gut microbial products may contribute to disease pathogenesis by affecting endothelial cell function in conditions such as atherosclerosis and liver diseases (41, 42). TLR signaling is not restricted to innate immune cells (43), but involves potentially other vascular cell types, including endothelial cells (TLR2, 4, and 9) (44–46) and platelets (TLR1, 2, 4, 6, and 9) (47–50). The consequences of TLR activation on epithelial and immune cells have been investigated extensively (51, 52), but little information is available on the effect of microbial products on non-immune cells, microbiota-triggered remodeling processes in the small intestine, and particularly on mucosal endothelial cells.

5. IMPLICATIONS OF THE GUT MICROBIOTA IN REMODELING AND RENEWAL OF THE SMALL INTESTINE

The gut microbiota is a complex microbial ecosystem that forms immediately after birth and is shaped by numerous environmental factors (e.g. mode of birth, mother's milk, nursing personnel, nutrition, antibiotics, stress) (10). So far, more than a thousand bacterial species have been identified most of them belonging to the *Firmicutes* and *Bacteroidetes* phyla. This ecosystem was estimated to consist of 395 bacterial phylotypes with most of the species never cultivated (53). The microbial communities in this intestinal ecosystem provide a multitude of functions that the host did not have to develop. Thus, this forgotten organ exerts profound effects on remodeling and cell renewal in the intestine mucosa, but also on host metabolism (22). It is known for several decades that colonization with a gut microbiota impacts on mucosal morphology and epithelial cell renewal rates across various phyla of the animal kingdom (19, 54, 55). Our recent work has revealed protease-activated receptor-1 (PAR1) mediated coagulation factor signaling pathways that trigger remodeling of intricate capillaries in the small intestinal villus architecture (20) (Figure 1). Now, we began to understand how the intestinal architecture and cell renewal is controlled (56). Nevertheless, the microbial signals that affect morphogenic pathways, the various morphogenic pathways in the intestine that are regulated by the microbiota, and the complex interplay between these pathways remain enigmatic. Also the role of the gut microbiota in the regulation of pathways regulating cell renewal and tissue repair in inflammatory disease states like necrotizing enterocolitis in newborns, inflammatory bowel disease (IBD), and radio or chemotherapy-induced mucositis of cancer patients is largely unexplored.

6. INNATE IMMUNE SIGNALING AFFECTS INTESTINAL VASCULAR REMODELING

As evident from experiments with GF animal models, normal development, especially of the gastrointestinal tract, is influenced by the presence of commensal microbiota (57). GF mice show an altered immune phenotype, with deficits in both innate and adaptive immune components of the gut mucosa (58, 59). Reintroducing microorganisms postnatally partially corrects many of these defects, although even a brief GF neonatal period can induce immunological changes that persist into adulthood (58, 60). Notably, different bacterial species have been shown to distinctly modulate the host immune system, indicating that the presence of specific bacteria within a given developmental window is important for normal patterning of host immunity (60–62).

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is a complex process involving endothelial as well as various mesenchymal cell types, which are in close proximity to the microvasculature (63). Fibroblasts release various pro-angiogenic factors upon cytokine stimulation and express functional TLRs (64). The TLR adaptors MyD88 and TRIF are essential elements that are required for renewal and villus vascularization in postnatal gut development (65). Recently, it was shown that the gut microbiota can selectively activate mucosal endothelial and mesenchymal cells to

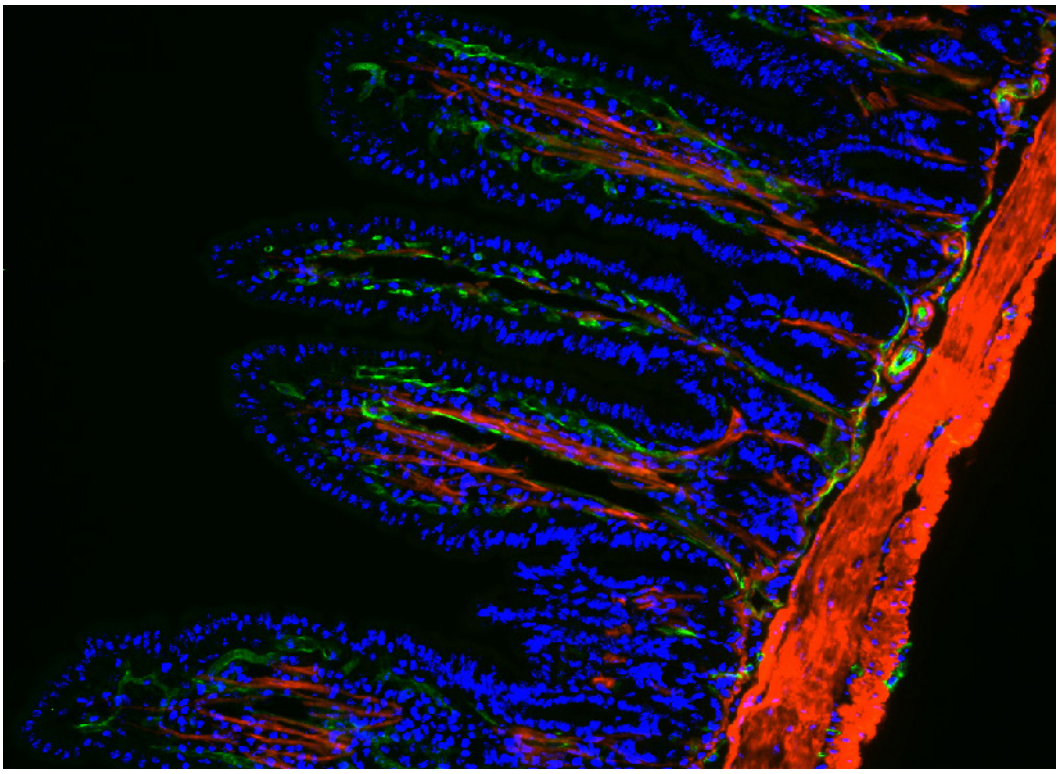

Figure 1. Immunofluorescence image of the mid small intestinal villus architecture of a conventionally raised mouse. Cell nuclei (blue), the vascular marker PECAM-1 (green), smooth muscle actin (red); 10x magnification.

promote specific angiogenic responses in a TLR- and NOD-like receptor-dependent fashion (Figure 2). This innate immunity-mediated response may expand the mucosal microvascular network, foster immune cell recruitment, and contribute to chronic intestinal inflammation (66). Leaking of microbial products in the inflamed intestine allows interaction with mucosal cells bearing their specific receptors (67), including endothelial and other mesenchymal cells (64, 68, 69). In addition to microbial derived products, microbiota help breaking down complex dietary macromolecules in much simpler and absorbable micromolecules that result in stimulation of a wide range of host genes involved in the uptake of theses digestion products benefiting the host (3, 70–76). Simultaneously, by increasing the intestine's absorptive capacity through promotion of angiogenesis, the microbiota provide excellent mutual beneficial associations to the host. Endothelial cells, including human intestinal microvascular endothelial cells (HIMEC), produce their own pro-angiogenic factors, acting in an autocrine fashion, and gut mucosal extracts contain pro-angiogenic factors (77, 78).

Not only development of blood microvasculature but also lymphatic vascularization extends beyond postnatal development and various proteins were shown to regulate this process. The multifunctional protein fasting-induced adipose factor (Fiaf), also known as angiopoietin-like protein 4 (Angptl4), has been shown as an important regulator for functional partitioning of postnatal intestinal lymphatic and blood vessels (79). It was observed that Fiaf-deficient GF mice exhibited a similar phenotype as conventionally raised (CONV-R) Fiaf knockouts and Fiaf mutants die within a few weeks of birth with dilated and blood-filled lymphatics that are aberrantly connected to blood vessels (79). It is interesting to note that Fiaf expression is higher in the villus epithelium of GF mice compared with CONV-R wild-type mice (80, 81). Moreover, transcriptional profiling of mice monoassociated with *S. boulardii* showed upregulation of 'non-immune' signatures. The majority of those signatures were derived from vascular genes (82). This yeast along with enteric microbiota modulates angiogenesis to limit intestinal inflammation and promotes mucosal tissue repair by regulating VEGFR signaling during the acute phase of intestinal inflammation (83).

Taken together, under basal conditions, several immune and non-immune pathways are regulated in the intestinal epithelium. These signals selectively induce specific pro-angiogenic pathways that promote intestinal angiogenesis by activation of mucosal endothelial and mesenchymal cells.

7. MICROBIOTA-INDUCED VASCULAR REMODELING OF THE SMALL INTESTINE

Immediately after birth, the intestine undergoes rapid and dramatic postnatal remodeling. The complexities in the intestine in terms of villus architecture and vascular branching grows extraordinarily during postnatal development. The quantum of complexities measured in the intestine of GF mice as opposed to CONV-R mice was 50% less in terms of vascularization, which was recovered within 10 days of colonization with commensal microbiota (15). Interestingly, in the same study it was also established that monocolonization of GF mice with *Bacteroides thetaiotaomicron* was sufficient to recapitulate normal vascular development (15). Throughout host development, this coordination between the microbiota and the host intestine grows in parallel to fulfill the need of nutrient requirements. This regulation was demonstrated to be coordinated to some extent by Paneth cells (15). The appearance of Paneth cells coincides with initial colonization of the gut and their strategic positioning coordinate development of both the microbiota and the microvasculature. It is of note that commensal microbiota influence the subsequent differentiation of Paneth cells, while at the same time their secreted antimicrobial peptides/proteins effect microbial ecology (84, 85). With sufficient evidence it can be said that colonization increases angiogenesis-related gene expression in the intestine, e.g. angiogenin-3 along with secreted proteins with known pro-angiogenic activity (86, 87). qRT-PCR and microarray data suggested that angiogenin-3 mRNA is largely expressed in crypt epithelium, which increases upon colonization (88). Furthermore, monoassociation with either *Bacteroides thetaiotaomicron* or *Bifidobacterium infantis* or *E.coli K12* is sufficient to restore angiogenin-3 expression in the ileum of GF mice to comparable levels as measured in CONV-R counterparts (88). The influence of gut microbiota on intestinal injury healing innvolving angiogenesis is evident in yet another study of fecal microbiota transplantation (FMT), where gut microbes were shown to not only alleviate and protect against radiation induced intestinal injury but also improved survival rate in a murine irradiation model via upregulating VEGF expression levels in the small intestine of irradiated mice (89). In summary, colonization with gut microbiota or select gut resident microbes evokes transcriptional responses that shape intestinal development and microvasculature expansion.

8. ROLE OF MICROBIAL PROTEASES AND HOST PROTEASES IN PROTEASE-ACTIVATED RECEPTOR (PAR)-MEDIATED INTESTINAL REMODELING PROCESSES

The activity of serine proteases and matrix-metalloproteases (MMP) can impact morphogenic signaling pathways in the intestine and in turn may alter the cell-type specific expression of proteases that act on paracellular junctions or extracellular matrix components of the basal lamina thus affecting intestinal function (90). For instance, it has been demonstrated that TGF-β enhances the migration of intestinal epithelial cells by up-regulating their MMP-1 and MMP-10 expression (91). Members of the PAR family of heptahelical G-protein-coupled receptors are expressed in most tissues and are also active in the intestinal mucosa (92–97). These receptors primarily mediate the cellular actions of coagulation proteases, but they also fulfill important non-hemostatic functions during development and are mediators of tissue remodeling and repair processes. Ample evidence exists for that activation of PAR signaling pathways improves wound healing (98, 99). In this context, the pro-angiogenic function of activated Protein C in cutaneous wound healing has been demonstrated (100). In line with the importance of tyrosine kinase signaling in intestinal remodeling, we have recently revealed that tissue factor (TF)-dependent coagulation factor signaling via PAR1 augments angiopoietin-1 (Ang-1) expression and Tie-2 signaling in the distal small intestine. This microbiota triggered signaling loop enhances vascular remodeling in small intestinal villus structures (20) (Figure 2). On the other hand, the various proteases expressed by gut microbes can also act directly on PARs. For instance, *Staphylococcus aureus* has become an early colonizer of the infant gut (101) and Staphylocoagulase (102) can activate prothrombin, the prototypic serine protease that activates PAR1. Moreover, it has been suggested that proteases from *Porphyromonas gingivalis* can induce β-defensin-2 expression via gingival epithelial PAR2 receptor signaling (103). There is emerging evidence for an interplay between innate immune signaling and PAR signaling since NFκB signaling can be enhanced by the physical interaction of TLR4 with PAR2 (104). The information on bacterial activation of PARs is sparse, but it appears plausible that beyond pattern recognition this could be a relevant mode of action of how gut microbial communities can shape their habitat.

9. DYSBIOSIS AND GENE MUTATIONS AFFECT INTESTINAL REMODELING IN INTESTINAL DISEASE

Microbial dysbiosis is associated with a number of diseases, including inflammatory disorders, but it is currently unclear whether dysbiosis occurs as a consequence of an inflammatory process or if other factors, such as diet or host genetics, induce dysbiosis, which then leads to inflammation. Obesity is one known factor that leads to dysbiosis and is linked to an increased risk for cancer. Obese individuals exhibit increased proportions of *Firmicutes* and decreased proportions of *Bacteroidetes* in the gut (105) as well as an overall reduction in microbial genetic abundance (106). Inflammation driven by gut bacteria is also

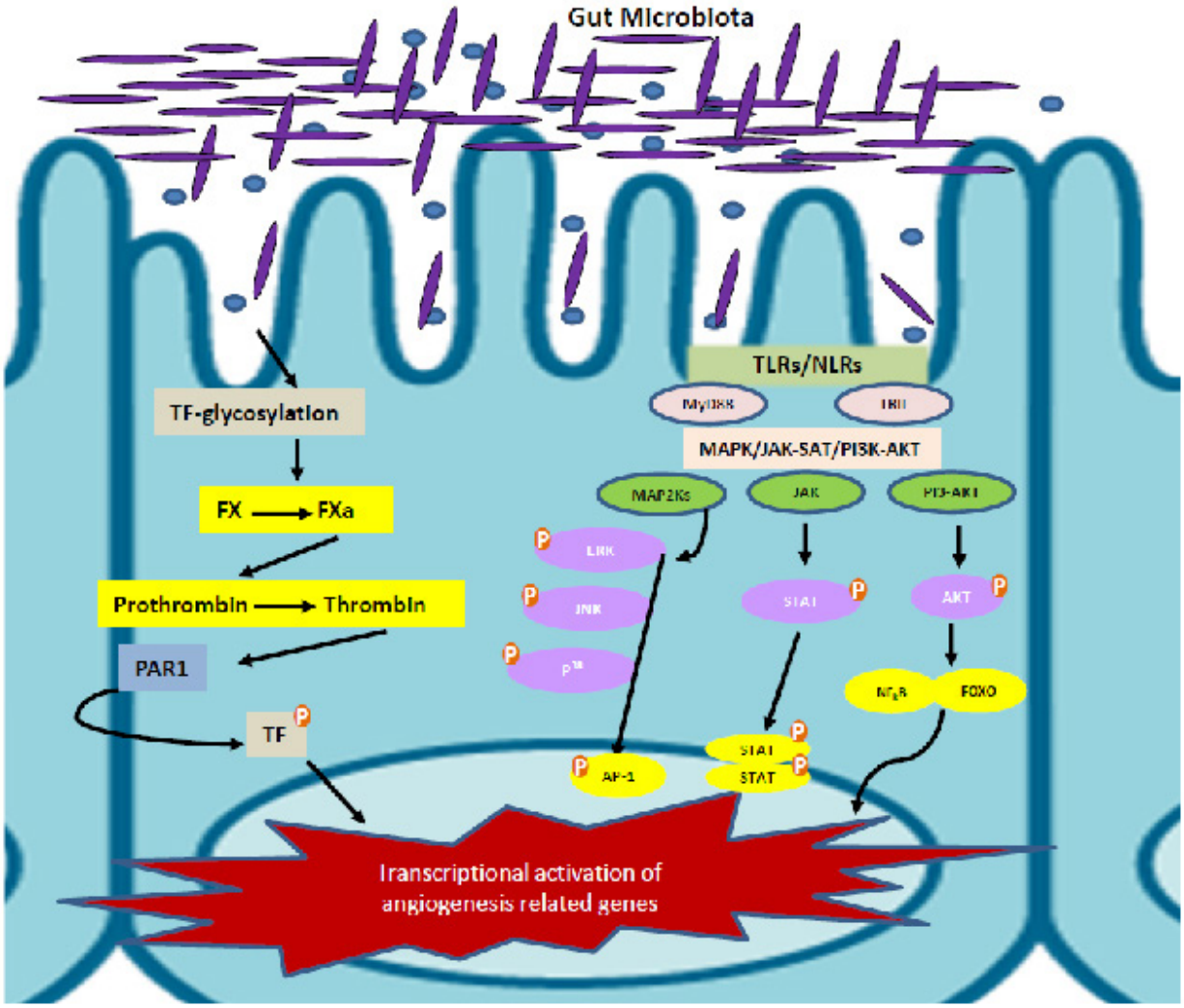

Figure 2. Schematic view on relevant pathways influencing vascularization in the small intestine. (I) Gut microbiota promote N-glycosylation of tissue factor (TF) on enterocytes, triggering coagulation factor signaling via protease-activated receptor-1 (PAR1) resulting in TF phosphorylation and expression of a pro-angiogenic genes. (II) Microbiota-induced pattern recognition receptor signaling augmenting the expression of genes involved in vascular remodeling in the small intestinal mucosa.

thought to have an impact on carcinogenesis. In some cases, inflammation promotes tumorigenesis by generating a dysbiotic environment within the gut that favors the expansion of tumorigenic bacterial strains. Recent work showed that intestinal inflammation in the IL-10-deficient mouse model modifies gut microbial communities and promotes the growth of genotoxic bacteria (107). These findings support the idea that cancer in the colon can be caused by particular microbes that are fostered within an inflammatory environment.

Inflammatory bowel disease (IBD) is an autoimmune condition that is believed to be caused by an excessive immune response against normal constituents of the gut microbiota (108). Important, recent studies provide compelling evidence that this diseases can result from dysbiosis since it can be transmitted by transfer of the microbiota from a T-bet(-/-)×Rag2(-/-) ulcerative colitis (TRUC) mouse to adult WT mice (109). During IBD and celiac disease altered TGF-β signaling is observed (110–112), but the influence of the gut microbiota or single gut bacterial species on these pathways are unexplored. Of note, impaired TGF-β signaling in T-cells results in a reduced number of regulatory T-cells and higher susceptibililty to dextran sodium sulfate-induced colitis (113). Expression of TGF-β was found increased in various mouse colitis models, but TGF-β signaling was impaired due to elevated levels of Smad 7 (114). In fact, TGF-β signaling is defective in IBD (115). In mouse models Smad 7 overexpression increased the severity

of DSS-induced colitis (116). In healthy individuals, the gut is primarily populated by a core microbiota composed mainly of obligate anaerobic bacteria within two phyla, the *Firmicutes* and *Bacteroidetes*. However, when there is a disturbance that shifts the composition of the normal microbial community, there is an increase in facultative anaerobic bacteria, that can lead to various inflammatory processes by including potentially harmful microorganisms (117). From a therapeutic point of view, it draws an attention to correct dysbiosis. So far, the efficacy of bacteria based therapies, such as probiotics or antibiotics, were proven to be inefficient to overcome complex intestinal inflammatory conditions, such as IBD. However, on certain instances like recurrent *Clostridium difficile* infection (CDI), FMT has been successfully used for several years as a treatment regime with proven randomized control trial (118). More recently, FMT was proven to be a method of choice to treat IBD. FMT not only restored the deficient microbiota but also established the crosstalk of the host immune system with indigenous microflora, which is affected during complex disease etiologies such as IBD (119, 120). It appears plausible that complex microbiota derived diseases like IBD would require combinatorial treatment, on the one hand to restore the host microbiota crosstalk and on the other to suppress the exacerbated immune activation.

10. CONCLUSION AND PERSPECTIVE

Growing evidence suggests the implication of the gut microbiota in various facets of health and disease and it now appears to influence the host at nearly every level and in every organ system (121, 122). The impact of gut microbial communities on intestinal renewal and the expansion of intricate capillary networks in the small intestine is one pivotal trait modulating various immunological and metabolic functions. Determining the details of the gut microbiome's involvement in host development, and its function in both health and disease holds promise of translational applications, from optimizing healthy nutrition to offering new tools in our fight against the pandemics of cancer and obesity.

With further advances and the use of available technologies, such as metagenomics and metabolomics, keystone microbes should be characterized and their interaction with the host understood, which will allow the creation of a database of potential pathobionts to target in order to modulate the microbial community. However, to reach this stage, research efforts must pose and answer concrete questions detailing specific aspects of host-microbe relations and the mechanisms underlying them.

We are now entering in an era when the use of antibiotics is increasingly restricted, while probiotics can expect a promising future. Besides, the selection of excellent strains and improved processing techniques, more research is needed to evaluate the functionality and efficacy of select strains and their substrates related to host nutrition. Therapeutically, probiotic-based approaches have been used with some success as have the more drastic and cruder approach of wholesale microbiota replacement strategies based upon fecal transplantation.

11. ACKNOWLEDGEMENT

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