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Intracellular bacteria in cancer—prospects and debates

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Recent evidence suggests that some human cancers may harbor low-biomass microbial ecosystems, spanning bacteria, viruses, and fungi. Bacteria, the most-studied kingdom in this context, are suggested by these studies to localize within cancer cells, immune cells and other tumor microenvironment cell types, where they are postulated to impact multiple cancer-related functions. Herein, we provide an overview of intratumoral bacteria, while focusing on intracellular bacteria, their suggested molecular activities, communication networks, host invasion and evasion strategies, and long-term colonization capacity. We highlight how the integration of sequencing-based and spatial techniques may enable the recognition of bacterial tumor niches. We discuss pitfalls, debates and challenges in decisively proving the existence and function of intratumoral microbes, while reaching a mechanistic elucidation of their impacts on tumor behavior and treatment responses. Together, a causative understanding of possible roles played by intracellular bacteria in cancer may enable their future utilization in diagnosis, patient stratification, and treatment.

INTRODUCTION

The prospect of bacteria potentially impacting cancer gained traction in the late 19th century, with reports such as that of William Russell and colleagues suggesting that microorganisms may reside within tumors[1,2]. After a period of early excitement and attempts to identify a unified bacterial cause of cancer[3], reproducible research on carcinogenic bacteria proved elusive for almost a century. Consequently, the bacterial theory of cancer was abandoned, and the fields of microbiology and oncology independently evolved for several decades with little intersection. In cancer research, genomic aberrations and deregulated signaling pathways have, for long, taken center stage amongst other modulating factors, as key drivers of tumorigenesis[4]. Conversely, vastly improved culturing and identification methods have revealed early reported "cancer microbes" to be likely contaminants[5]. While groundbreaking discoveries on *Helicobacter pylori* (*H. pylori*) in stomach ulcers and consequent gastric neoplasms transformed our view of microbes in cancer[6], it is only in the past 15 years that the prospect of cancer microbiome research has been revisited on a larger scale[7]. Technological developments in next-generation sequencing (NGS) that enabled the characterization of microbiome-rich samples, such as fecal, vaginal and oral microbiomes, have revealed distinct stool microbial signatures associated with different stages of colorectal cancer (CRC)[8–11] and possibly other cancers[7,12].

Beyond changes in the stool microbiome, analyses of tumor sequencing data recently suggested that specific microbes may reside within tumors and their microenvironment[13–16]. Interestingly, some of these cancer-associated low-biomass bacteria were suggested to reside inside tumor and immune cells. Ample evidence attests to the enrichment of intracellular bacteria, such as *Fusobacterium nucleatum* (*F. nucleatum*) in CRC[16–18]. Generalization of such findings to other tumors that had been previously considered sterile is suggested by some studies[14,15] and debated by others[19,20], therefore meriting future confirmatory studies.

Intracellular pathogens have been extensively studied in infectious diseases. For many bacterial pathogens, intracellular localization constitutes an important, at times obligatory, component of their lifestyle. Intracellular localization bestows numerous advantages on the invading microbe, ranging from immune escape to a favorable nutritional environment and a platform for replication and dissemination. In contrast to infectious pathogens, interactions and target cells of cancer-specific intracellular bacteria remain understudied. Addressing the critical question of whether low-biomass microbial inhabitants are consistently present in tumors, and whether they bear functional implications on cancer development, progression, and therapy response is faced by multiple technical and conceptual challenges. Nevertheless, it is already yielding insightful discoveries. For example, certain bacteria detected in tumors, such as genotoxic *Escherichia coli* (*E. coli*) and enterotoxigenic *Bacteroides fragilis* (*B. fragilis*) have been linked to the production of metabolites that promote inflammation and DNA damage, contributing to the initiation and growth of tumors[21–23]. Moreover, some bacteria may influence the response of tumors to chemotherapy and immunotherapy[24,25]. Some of these effects may be explained by bacteria metabolizing and inactivating chemotherapeutic drugs[26]. Others can alter an immune response in the context of immune checkpoint blockade in modulating the response to immunotherapy[27,28].

While more than a century has passed since the first reports suggesting that microorganisms may exist within cancer cells, the notion of intracellular bacteria in cancer is being revisited. As microbiology and oncology come to intersect anew, we aim to describe in this review the challenges facing the field and highlight where lessons may be learned from decades of microbiology research in a non-cancer context. Here, we provide an overview of intratumoral bacteria research, focusing on intracellular bacteria that have been suggested to colonize different tumor types and cancer models (Fig. 1). The number of studies focusing specifically on intracellular bacteria in cancer remains limited, and proper separation between intratumoral and intracellular effects is often lacking. For this reason, in this review, we refer to "intratumoral" microbes as tumor-associated microorganisms whose precise localization is unclear, while denoting them "intracellular" if they are suggested to reside within cells of

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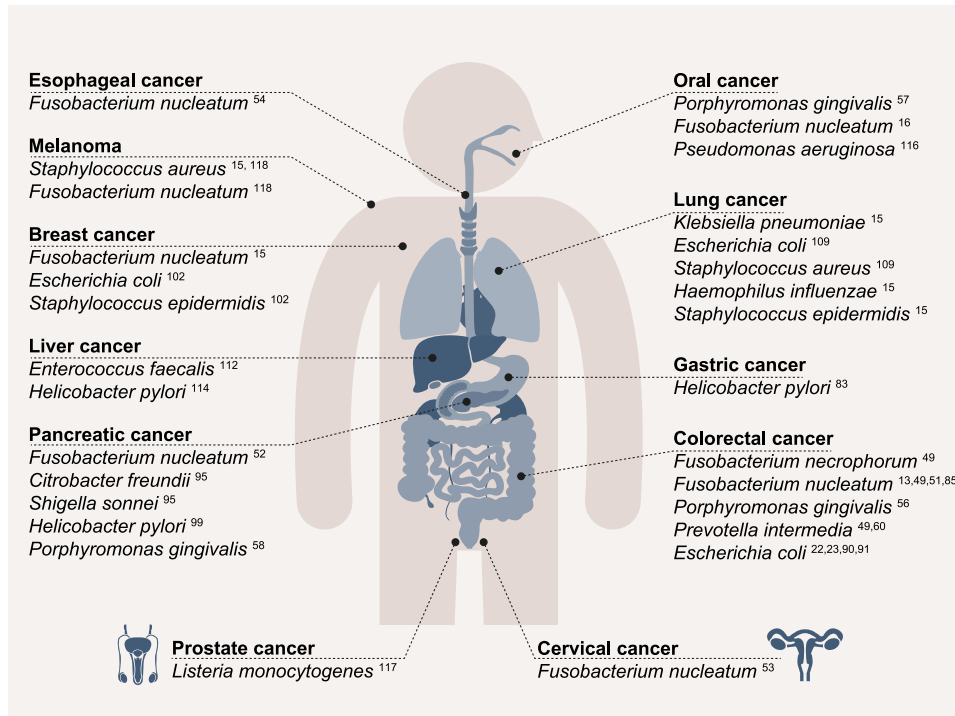
Fig. 1 Tumor-associated bacterial species suggested to occur intracellularly. An overview of bacterial species that are suggested to be enriched in various cancers while exhibiting the capacity to invade host cells.

the tumor microenvironment (TME). The broader relationships of the bacterial gut microbiome[7,14,27,29] and other microbial kingdoms[30,31] with cancer are reviewed elsewhere.

LIFESTYLES OF INTRACELLULAR BACTERIA

Intracellular pathogens have been studied for decades and yielded key insights into the mechanisms driving bacterial invasion, induction of host damage and evasion of host defense. Concepts gained from these studies, summarized below, could prove useful to the study of similar bacterial behaviors and host impacts in the cancer setting.

Strategies of bacterial invasion

Pathogen interaction with host receptors drives their adherence to host cells and triggers actin cytoskeleton rearrangements, leading to cellular invasion. Intracellular bacteria may reside in isolation, or in mixed consortia, depending on the species[32,33]. Bacteria that are classified as intracellular occur inside the host cell for at least parts of their lifecycle. Obligate intracellular bacteria, such as *Rickettsia spp.* and *Chlamydia spp.*, have an exclusively intracellular lifestyle, while facultative intracellular bacteria, such as *Salmonella spp.* or *Listeria monocytogenes* (*L. monocytogenes*), can also proliferate extracellularly under certain conditions. A pathogen's path into the host cell fundamentally varies between active invasion and passive phagocytosis, of which only active invasion will be discussed here. Bacteria can enter the host cell actively via so-called zipper or trigger mechanisms (Box 1)[34]. In some cases, actin polymerization and depolymerization lead to the formation of membrane protrusions, which ultimately result in the uptake of bacteria within a vacuole. Target cells of invasion are diverse and include among others epithelial cells, endothelial cells, keratinocytes, and different types of immune cells such as macrophages[34–39]. Intracellular localization provides bacteria with several advantages, such as protection from circulating immune cells and nutrient availability[40].

Box 1: Overview of the trigger and zipper mechanism

Trigger and Zipper mechanism
Intracellular bacteria invade non-phagocytic host cells via the trigger or zipper mechanism.
Zipper: The bacterium enters the cell in a receptor-mediated, zipper-like manner. Surface invasins and adhesins of the bacterial membrane interact with receptors on the host membrane. This interaction triggers signaling cascades that lead to reorganization of the host cytoskeleton by actin polymerization. This results in the formation of membrane protrusions that enclose and internalize the bacterium.
Trigger: The bacterium inserts effector molecules into the host cell, where they lead to actin rearrangement and then ultimately membrane protrusions, allowing the bacterium to enter the host. The effector molecule application typically occurs through type III (T3SS) and type IV (T4SS) secretion systems.

Host bacterial sensing

To counter pathogenic bacterial engagement and associated cellular and tissue damage, the host has developed sophisticated systems to recognize and combat intracellular bacteria. Recognition by the innate immune system of the host is mostly mediated by pattern-recognition receptors (PRRs) such as surface-bound Toll-like receptors (TLRs) or cytosolic NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I) receptors, among others. These enable constant surveillance of different compartments of the cell for signs of infection. Activation of PRRs by invading bacteria triggers a variety of inflammatory cascades, including nuclear factor kappa B (NF-κB) activation, inflammasome formation, and cytokine release, coupled with induction of an inflammation-induced infected cell death termed pyroptosis, which collectively elicit broad rejection responses to bacterial invasion[41,42]. In addition, upon bacterial sensing, microRNAs (miRNA) play a role in regulating various downstream processes such as cell cycle, autophagy or immune responses[43] and hence can favor or combat infections. The adaptive immune system is also able to sense intracellular bacteria by host presentation of bacteria-derived peptide fragments on major histocompatibility

complexes (MHC), sometimes challenged by bacterial antigenic variation[44]. Interaction of MHC peptide complexes with effector T cells leads to activation and proliferation of these cells, which in turn initiates differentiation and activation of other immune cells, ultimately triggering a multi-channeled antimicrobial immune response[45].

Survival and proliferation strategies

To counter these defense mechanisms in a constant "arms race", intracellular bacteria have evolved mechanisms of adaptation and resistance[41]. After entering the host by either the trigger or zipper mechanism, the bacteria end up in a phagosome. Phagosomes normally fuse with lysosomes to phagolysosomes, which digest their contents. Bacteria have devised intricate mechanisms of blocking this fusion, thereby escaping the phagosome, as well as other means of neutralizing bacteriolytic function, while surviving inside the host cell. For example, *Mycobacterium tuberculosis* (*M. tuberculosis*) uses an interplay of an impenetrable cell envelope, detoxification, and radicals and acquires the Ras-related protein (Rab-5A), which blocks fusion with the lysosome in enabling survival in the phagosome[45]. Listeria, Rickettsia, and Shigella escape from the vacuole and replicate in the cytosol[46] through movement facilitated by hijacking of the host cytoskeleton via actin polymerization. Additionally, intracellular bacteria, such as Rickettsia, Burkholderia, *L. monocytogenes*, and *Shigella flexneri* (*S. flexneri*), can exploit the actin cytoskeleton for spreading between host cells. These pathogens utilize the host cell actin network to form protrusions into adjacent cells, leading to a double-membrane vacuole forming in the new host cell, composed of the membrane of the previous and the new host[47]. Other bacteria may inhibit autophagy, a degradation and recycling process that is effective against invading bacteria[48]. Similar invasion, evasion, and survival mechanisms may be used by intracellular bacteria in the cancer setting, and merit future studies.

COMPOSITION AND LOCALIZATION OF INTRATUMORAL BACTERIA

Some tumors, and in particular CRC, are convincingly shown to harbor intratumoral and even intracellular bacteria. Indeed, most CRC-associated microbes, such as *F. nucleatum*[15–18,49–55], *P. gingivalis*[56–59], and *Prevotella intermedia* (*P. intermedia*)[49,60,61] are suggested to reside intracellularly. Other, traditionally considered "sterile" tumors are also suggested to feature low-biomass bacterial communities, as shown by several genomic-based[14,15,20,62], and imaging-based approaches[15,16]. Of note, some of the genomic approaches utilized by these studies have been recently challenged[19,63], with a definite resolution of these debates, and high-resolution characterization of intratumoral bacteria meriting future studies.

NGS, ranging from characterization of the 16S rDNA gene to global characterization of the bacterial pan-genome using shotgun metagenomic sequencing, is extensively utilized in unraveling fecal cancer-associated microbiome signatures at low host DNA contamination levels[8,9]. In contrast, efforts to profile the low-biomass tumor-resident microbiome prove to be more challenging, given a substantial excess of human reads, imperfections in reference databases, experimental and computational contaminations, and severe batch effects caused by varying collection methods, sample processing, and sequencing pipelines[14,62,64]. Suggested solutions include novel low-biomass sequencing approaches, in which five regions of the 16S rRNA gene are simultaneously amplified and sequenced, enabling higher coverage and resolution[15,65]. These amplicons can then be computationally combined using Short MUltiple Regions Framework (SMURF)[65], a platform built specifically for this method. Of note,

5 region 16S rRNA sequencing is highly sensitive and bacterial DNA can be found in many sources of contamination, making it challenging to distinguish between low-biomass bacterial signals originating from the tissue and those originating from environmental contamination[66,67]. Such distinction requires careful assessment by multiple controls to bioinformatically filter out interfering signals[15]. Additional efforts to improve disentangling of true low-biomass signals from contamination and noise focus on improved harmonization of experimental and computational pipelines[68], the use of multiple technical and biological controls, and validation of sequencing-based results with additional, non-genomic modalities. With these modalities providing some solution to the low-biomass microbial challenges, future development is needed to further disentangle true microbial signals from noise.

Metatranscriptomic analysis can provide further functional insights into microbial consortia and their relationships with their host[69]. Dual RNA sequencing approaches have emerged in 2012[32] to gain insights into simultaneous gene expression of host cells, invading bacteria and their interactions[32]. These modalities have evolved to enable the assessment of co-infections of different bacterial species, and even viruses and bacteria, in decoding inter-species and inter-kingdom interactions[70]. Single-cell RNA sequencing may further minimize bulk contamination by cellular and genetic heterogeneity and reveal the extent to which individual cells are targeted and shaped by invading bacteria. For example, Galeano Niño et al. established a method called invasion–adhesion-directed expression sequencing (INVADEseq), focusing on cell attachment and invasion with spatial resolution. In INVADEseq, primers targeting a conserved region of the bacterial 16S rRNA locus enable the generation of cDNA libraries containing bacterial transcripts from human cells associated with bacteria. While good resolution may be achieved, distinction between invaded and cell-adjacent bacteria still remains a major obstacle[16]. Using this method in oral squamous cell carcinoma (OSCC), Fusobacterium and Treponema colonization were associated with macrophages and aneuploid epithelial cells[16,71]. Another tool, Single-cell Analysis of Host–Microbiome Interactions (SAHMI)[52], relies on recovering and denoising microbial signals from single-cell sequencing data of the host tissue and mapping it to a microbial reference genome. In addition, computational prediction tools such as Host–Microbe Interaction PREDictor (HMI-PRED), used for prediction of protein–protein interactions, may enable further elucidation of host–microbe interactions[72].

Imaging techniques are increasingly used to complement genomic low-biomass microbial signals[15]. These include immuno-histochemistry (IHC), FISH and high-resolution electron microscopy. The recently introduced correlative focused ion beam/scanning electron microscopy (c-FIB/SEM) combines volume electron microscopy and fluorescence microscopy, thereby enabling better understanding of host-microbe interactions at the 3D ultrastructural level[73]. Utilization of fluorochrome-conjugated bacteria-specific antibodies coupled with permeabilization of host samples may enable intracellular microbial imaging[18,74]. However, killing of both bacteria and host cells limits the use of this approach to endpoint assays. Stable labeling of intracellular bacteria, such as *F. nucleatum*, is challenged by difficulties in genetic modification, despite recent progress[75]. Bacterial chemical labeling[50,76], while beneficial for short-term analyses, suffers from attenuation of the signal by serial dilution, promoted by cell division over time. Fluorescently labeled D-alanine may offer a solution to this challenge, as it is metabolically incorporated into the bacterial cell wall and thus may enable the detection of living, metabolically active tumor-associated bacteria upon incubation with fresh tumor samples[77].

Recent work from the Bullman group incorporated several state-of-the-art spatial profiling methods in better characterizing bacterial niches in tumors[16]. RNAscope-fluorescence in situ

hybridization (RNAscope-FISH) enabled the visualization of bacterial RNA within individual host cells, thereby revealing locations of bacteria within tumors. Using the digital spatial profiling platform GeoMX, expression of 77 proteins related to anti-tumor immunity was correlated with tumor characteristics. Through application of 10X Visium spatial transcriptomics, positional information could also be documented in tumor niches. Using these methods, it was suggested that most intratumoral bacteria are located in micro-niches at the tumor margin, featuring an immunosuppressive and poorly vascularized microenvironment[16].

Suggested intracellular bacteria across tumor and tissue types
Studies utilizing the above technologies suggest that tumors may harbor unique microbial signatures. The most extensive evidence demonstrating such tumor-residing bacteria and their tumor-modulatory function involves gastric and colorectal cancer. A definite level of proof regarding presence, extent, and possible functions of intratumoral microbiomes, and the prospect of their utilization in cancer diagnosis[14], remains debated[19] and awaits future validation.

Gastric cancer. *H. pylori* was the first, and currently only bacterium recognized by the World Health Organization as a carcinogen to date. *H. pylori* constitutes a prototypical driver of gastritis and subsequently gastric cancer via diverse mechanisms such as induction of chronic inflammation[78–80] and modulation of wingless-related integration site (Wnt) signaling[81], amongst others. The many direct and indirect contributions of *H. pylori* to gastric cancer development have been reviewed elsewhere[82]. Of interest to intratumoral microbiome research, some of the effects conferred by *H. pylori* have been suggested to be linked to attachment and potential invasion of gastric epithelial cells[74,75], supported by the presence of *H. pylori* in gastric cancer tissue[83].

Colorectal cancer. Colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer deaths in the US[84]. Due to its proximity to a rich luminal microbiome, it is unsurprising that it has been studied extensively for intratumoral and intracellular bacteria. As noted above, among the CRC-associated bacteria with intracellular localization, *F. nucleatum* is the best studied species to date. Several studies have detected an enrichment of *F. nucleatum*, in human adenomas and CRC compared to healthy colon tissue samples[13,51,85]. The invasive behavior of patient-derived *F. nucleatum* subspecies was validated by functional assays in CRC cell lines[49,74,86]. Intracellular *F. nucleatum* is also enriched in inflammatory bowel disease (IBD), a condition predisposing to CRC, especially in patients suffering from an active disease compared to those in remission[87]. *F. nucleatum* strains from patients featured an invasive behavior in 2D cell line assays, which correlated with IBD status[74]. *Fusobacterium necrophorum* (*F. necrophorum*), another member of the Fusobacterium genus with an invasive behavior[88], has also been associated with CRC[49]. Another periodontal-linked bacterium, *P. gingivalis*, was enriched in CRC compared to healthy adjacent tissue[56] and suggested to reside intracellularly[16] while featuring an invasive behavior[89]. *P. gingivalis* dominantly occurs in the oral cavity, where it resides in gingival epithelial cells and is linked to periodontitis and OSCC[57,89]. Comparing paired adenocarcinoma and polyp samples, a higher abundance of *P. intermedia* was detected in adenocarcinoma samples[60], and it was suggested to reside, at least partly, intracellularly[16,49,61]. Pathogenic strains of *E. coli* are also recurrently found in CRC[21–23,90]. Some of those are able to thrive within macrophages[91] and could be cultured from tumor tissue after gentamycin treatment[92], suggesting that they may feature a capacity to intracellularly survive. Of note, a recent report suggests a close interplay between attachment and genotoxicity for some of these strains[92].

Pancreatic cancer. Pancreatic ductal adenocarcinoma (PDAC) constitutes the most common form of pancreatic cancer, accounting for more than 85% of all cases[93]. In PDAC, Campylobacter, Leptotrichia, *F. nucleatum* and *Clostridioides difficile* were suggested as intratumoral species based on single-cell sequencing data[52]. Further studies detected *Citrobacter freundii* and *Shigella sonnei*[94] as part of the pancreatic cancer TME, while other studies suggested that these bacteria occur intracellularly[95,96]. A meta-analysis from 2011 concluded that *H. pylori*, which was shown to be capable of invading host cells[97], enhances the risk of pancreatic cancer[98]. *P. gingivalis* has also been reported to be dominantly enriched in patients with pancreatic adenomas and possibly correlated with PDAC disease progression[58]. PDAC was also suggested to bear fungi[15,99,100], but some of these findings were recently contested[63], meriting future research.

Other cancer types. *F. nucleatum*[15], *Staphylococcus epidermidis* (*S. epidermidis*), *E. coli*[101], and other members of the genus Staphylococcus, Streptococcus, and Lactobacillus[102] were suggested to be present in human **breast cancer** samples. The abundance and potential intracellular localization of microbes in breast cancer awaits definite resolution. *F. nucleatum* was suggested to be enriched in **cervical cancer**[53] and **esophageal cancer**[54] compared to adjacent healthy tissue. Associations and potential functional roles for intracellular *Chlamydia spp.* have been described in **cervical** and **ovarian cancer**[103–105]. This pathogen has been shown to decrease p53 signaling and DNA damage response[106], in facilitating the pathogen's intracellular replication, induction of ROS production and DNA damage[107]. Analysis of a TCGA lung adenocarcinoma dataset, a subtype of **lung cancer**, suggested that *E. coli* and *Staphylococcus aureus* (*S. aureus*) may reside within these tumors[108]. *S. epidermidis*, *Haemophilus influenzae* (*H. influenza*), and *Klebsiella pneumoniae* (*K. pneumoniae*)[15], were suggested to reside in lung cancer samples in separate studies[109]. Using transmission electron microscopy, intracellular bacteria could be visualized in intrahepatic cholangiocarcinoma, a form of **liver cancer**, and surrounding tissue, but no further characterization of the bacteria was performed[110]. A presence of *Enterococcus faecalis* (*E. faecalis*) was demonstrated in hepatocellular carcinoma samples and suggested to have a prominent role in liver carcinogenesis[111], also in the in vivo setting[112]. In hepatocellular carcinoma, the presence of *H. pylori* was detected only in cancer samples, while it was absent in healthy controls[113]. Based on spatial analysis, Galeano Niño et al. revealed that the genera Parvimonas, Peptoniphilus and Fusobacterium were most abundant in an **oral cancer** subtype, OSCC[16]. *Pseudomonas aeruginosa*, which is capable of an intracellular lifestyle[114], was detected in the same cancer subtype[115]. *L. monocytogenes* was detected in the **prostate cancer** TME[116], while *S. aureus* was suggested to be present in **melanoma**[15,117]. It is noteworthy that in these studies, low-biomass approaches utilized different protocols, isolation methods and sample handling techniques, which can collectively lead to variations and inconsistencies between studies. For example, *B. fragilis* was suggested to be more abundant in CRC in one study[118], while other studies reported no significant differences in its abundance[119], which could stem from differences in participant characteristics and varying experimental designs. Given recent debates, these low-biomass findings merit future validation.

Synergies between intracellular bacteria
Many microbiome-modulated diseases are not driven by single pathogens, but rather by synergistic consortia that can evolve into a dysbiotic state that impairs host homeostasis via concerted activities[120]. For example, gingival *P. gingivalis* infection in germ-free mice induces periodontal disease only in the presence of

commensal communities, through joint promotion of polymicrobial biofilms via regulation of cytokine levels[121]. Similar concepts could also be relevant in the cancer context. Initial studies show that *F. nucleatum*-positive CRC tissues featured non-random co-colonization with commensals such as *B. fragilis* or *P. intermedia*, whereas tissues lacking *F. nucleatum* featured different bacterial colonization patterns[49]. These co-colonization patterns may bear functional importance. For example, communities containing both *F. nucleatum* and *P. gingivalis* featured a higher rate of invasion of gingival epithelial cells as compared to those featuring either of these species without the other[122]. Further investigation is needed to determine the extent to which bacterial composition and co-occurrence patterns causally shape their modulatory activities impacting cancer.

Routes of bacterial colonization into tumors

Routes by which intratumoral bacteria may reach and persist in the TME remain elusive to date. It is conceivable that bacteria from the adjacent normal tissue become enriched at tumor sites during tumorigenesis due to the changed microenvironment and easier tissue access upon disruption of epithelial and mucus barriers, for example, the gastrointestinal tract. For many other tumor types and even most gastrointestinal tumors, additional routes are plausible, including transmission of bacteria to distant sites via the bloodstream or the lymphatic system[123,124].

Oral cavity-to-gut translocation of bacteria constitutes an active field of investigation in CRC and other cancer research. Bacteria of the oral cavity are involved in local inflammatory diseases such as periodontal disease and are also enriched in various, seemingly unrelated, tumor tissues. Intriguingly, a 10-year study found periodontal patients to have an increased risk of cancer development, particularly pancreatic cancer[125], but also other cancers such as lung cancer, head and neck cancer, abdominal and esophageal cancer, breast cancer, and CRC[126]. Several periodontal bacteria, such as *F. nucleatum* and *P. gingivalis*, feature an invasion and colonization capacity even in their gingival tissue of origin[127,128]. Elucidation of effector similarities of such oral microbiome bacteria upon migration into tumor sites merits future mechanistic research. *F. nucleatum*, a well-studied invasive cancer-related bacterium, is believed to migrate from its natural habitat within the oral cavity[124] via the bloodstream. From the bloodstream, local enrichment in colorectal adenocarcinoma is suggested to be mediated by binding between bacterial fibroblast activation protein-2 (Fap2) and host epithelial D-galactose-β(1-3)-N-acetyl-D-galactosamine (Gal-GalNAc), which is overrepresented at CRC sites[129]. In addition, *F. nucleatum* can bind to the salivary protein statherin via FomA, which functions in biofilm formation and is known to bind especially to *P. gingivalis*[51,130]. In PDAC, gut bacteria can migrate from the anatomically connected upper gastrointestinal tract into the tumor[131]. Of note, translocation of gut microbes to sites like the liver can precede tumor formation[111]. After entering the TME, factors such as nutrient-rich niches, low pH, necrotic foci, hypoxia, or abundant blood supply may support bacterial colonization[132–134]. Similar considerations may apply to microbial colonization of metastases. In a murine breast cancer model, bacteria detected in circulating tumor cells were also enriched in lung metastasis sites, suggesting that some bacteria, and specifically intracellular bacteria, may migrate to metastatic sites within tumor cells via the systemic circulation[102]. Further evidence suggests that certain strains of *E. coli* can disrupt the gut vascular barrier and support CRC cells at multiple stages of their metastatic dissemination[135].

POTENTIAL IMPACTS OF INTRACELLULAR BACTERIA ON CANCER PHENOTYPES

The above observations, suggestive of the occurrence of tumor-associated bacteria, constitute an intriguing starting point of investigation of potential causation, e.g., whether tumor-residing or even intracellular bacteria may impact tumorigenesis, cancer progression, and treatment responsiveness. This "chicken-and-egg" dilemma constitutes a formidable challenge that requires sophisticated experimental models and techniques to detect causes and mechanisms.

Bacterial invasion models

To mechanistically study bacterial invasion, in vitro models of varying complexity are often combined with antibiotic protection assays that use non-penetrant antibiotics, such as gentamicin, to enrich viable intracellular microbes[86,136–141]. There are different approaches and models to study invasion that vary in their ease of use and the representation of in vivo physiology, a summary of which is depicted in Fig. 2. One of the most commonly used invasion assays is based on the co-incubation of 2D cell lines, such as Caco-2 cells derived from CRC, with microbes that actively invade the host cells[18,25,50,74,86,138,139]. Such 2D studies revealed important interactions between surface components of bacteria and the host, invasion, and the induction of oncogenic and inflammatory pathways upon infection[18,50,86,129]. For example, the use of such platforms uncovered the importance of the lectin Fap2, expressed on *F. nucleatum*, binding to Gal-GalNAc on host cells. This interaction, in turn, drives an accumulation of Fusobacteria in CRC[50,129], upregulation of inflammatory markers upon invasion[13,18,50], and direct modulation of the TME[138]. Moreover, *F. nucleatum* is suggested to enhance CRC progression through its FadA adhesin, which attaches to epithelial cadherin (E-cadherin) and may activate the Wnt/β-catenin signaling pathway[86] leading to enhanced proliferation[86]. Such 2D invasion assays have also proved invaluable in testing means to interfere with bacterial tumor cell invasion. For example, galactosides were discovered to interfere with Fap2-mediated invasion of *F. nucleatum* into CRC cells[50]. However, many proposed cancer-associated bacteria thrive in anaerobic conditions, making it difficult to mimic a suitable 2D environment that would phenocopy their in vivo niche. In addition, 2D in vitro invasion models remain limited to timeframes of a few hours, which may be too short for the effects of viable intracellular bacterial invasion and related metabolic crosstalk to become apparent[142–144], while the immortalized character of most cell lines makes it difficult to elucidate differences between healthy and cancer cells.

To overcome these limitations, new models enable more physiologically relevant conditions for such invasion studies, which are summarized in Fig. 2. Organoids represent a useful model to study pathogen-host interactions because they mimic the polarized epithelial cell layer enclosing a luminal compartment[138,139]. There are various ways to use organoids as co-culture platforms, including microinjection of bacteria into the hypoxic lumen from where they can actively invade the surrounding epithelial layer in the right orientation. Early steps of invasion of bacteria such as *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) were decoded using such platforms. Further usage of invasion platforms, including spheroids[145], and transwell invasion assays[146] is likely to reveal additional functional impacts of intracellular bacteria in cancer.

Organs-On-Chip (OOC) models, like CRC OOCs, enable the study of bacterial tumor invasion at an enhanced level of complexity, by replicating environmental features, including stretch, flow, and even complex gut microbial compositions[76,147,148]. Using Caco-2 colonized OOCs[149], factors such as crypt-like structures, peristalsis, and flow were shown to have a substantial impact on Shigella invasion. Mouse models can further elucidate the impacts of intracellular and intratumoral bacteria on tumor growth in a complete host environment. As one example, *F. nucleatum*-positive colorectal tumors were subcutaneously transplanted into immunodeficient mice and tracked over time. Using this model, viable *F. nucleatum* could be maintained over time, while
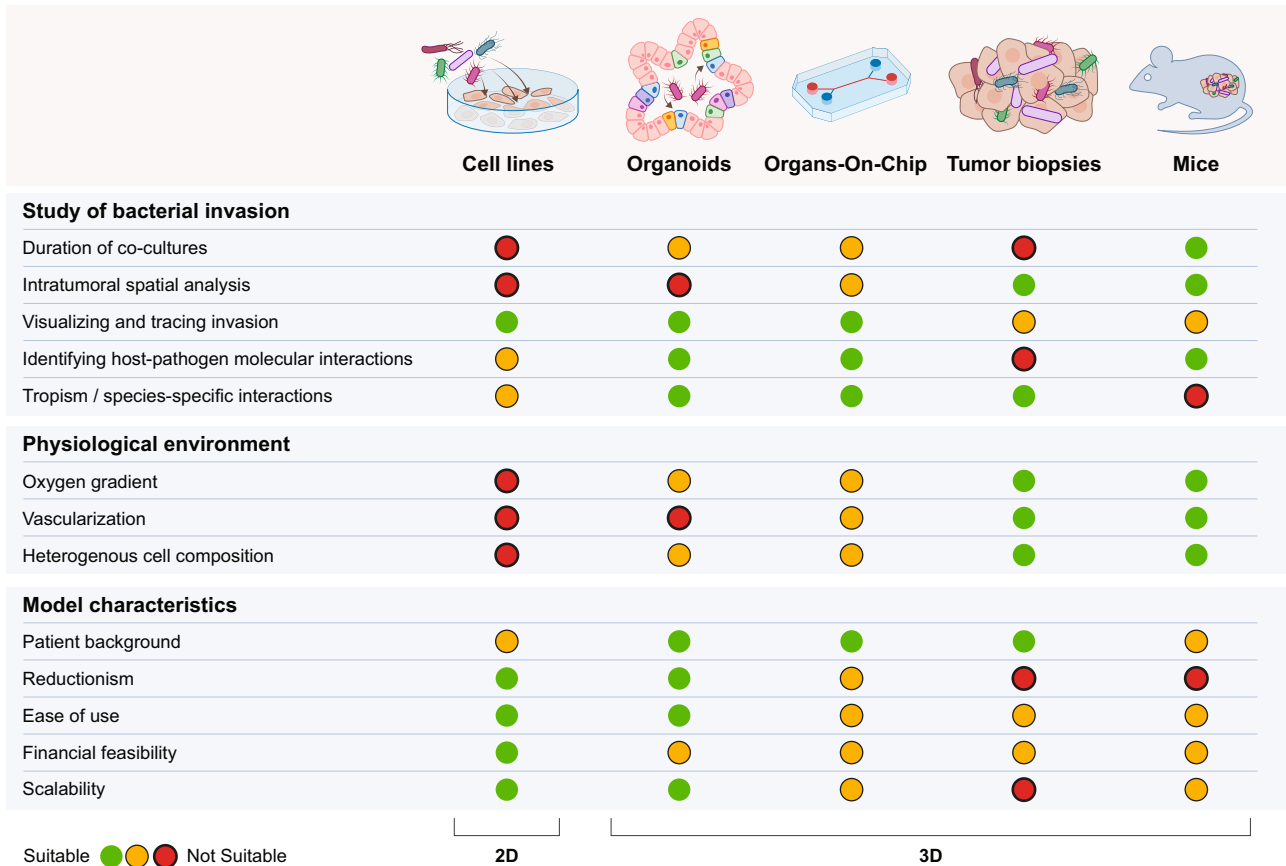
Fig. 2 **Experimental models and approaches.** Key features of cell-based models with varying complexity focusing on the study of bacterial invasion, the physiological environment, and model characteristics. Advantages and disadvantages for multiple parameters are compared and evaluated by a traffic light system.

antibiotic administration reduced tumor growth[49]. In the C57BL/6 Apc[min/+] CRC mouse model, increased tumor formation occurred upon gavage of *F. nucleatum*, while demonstrating a key role of FadA in its tumor-promoting activity[140]. Enhanced tumorigenesis could be likewise demonstrated upon transplantation of the *P. gingivalis* pre-infected pancreatic cancer cell line PANC1, suggested to be mediated by enhancement of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling[128].

Modulation of immune responses

Infection of host cells often generates potent immune responses. For example, endothelial cells invaded by the obligate intracellular bacterium *Orientia tsutsugamushi* elicit an interferon response[69] promoting immune cell recruitment[150]. *S. Typhimurium* that infects macrophages can replicate intracellularly, resist host reactive oxygen species (ROS) responses and actively induce macrophage polarization[150]. Within the cytosol, *F. nucleatum* can be sensed via PRRs such as RIG-I[151] or alpha kinase 1 (ALPK1), leading to NF-κB activation. This leads to various signaling cascades such as expression of the proinflammatory cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor alpha (TNF-α), upregulation of adhesion molecules[152], enhanced proliferation or autophagy[51,151]. Suggested mechanisms of intra- and extracellular F. nucleatum affecting proliferation, autophagy and TME modulation of the host are depicted in Fig. 3. Autophagy is modulated by various intracellular bacteria, affecting their niche and enabling intracellular survival. *F. nucleatum*, for example, may promote initiation of autophagy through downregulation of miR-18a* and miR-4802[43], while autophagosome fusion with the lysosome may be inhibited via intracellular *F. nucleatum*-

mediated upregulation of miR-31[48,153]. An expansion of myeloid-derived immune cells including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs) and myeloid-derived suppressor cells (MDSCs) in a *F. nucleatum*-inoculated CRC mouse model was accompanied by T-cell suppression and increased expression of immunosuppressive molecules such as CTLA4 and arginase-1[17,154]. Recruitment of myeloid cells by intratumoral bacteria may also contribute to inflammation via activation of the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway and secretion of interleukins and chemokines[87]. Modulation of natural killer (NK) cells by bacteria in the TME can further promote an immunosuppressive environment. For example, interaction of Fap2, expressed by *F. nucleatum*, with the human inhibitory receptor T-cell immunoreceptor with Ig and ITIM domains (TIGIT) expressed on immune cells such as NK cells and T cells, abrogated NK cell-mediated killing of human tumor cells[138]. Similarly, *F. nucleatum*-induced Fap2-dependent mechanisms can drive lymphocyte apoptosis[155,156]. Tumor regions featuring a high bacterial load may correlate with reduced vascularization, thereby contributing to the formation of an immunosuppressive environment[16]. Intratumoral bacteria may also modulate the TME adaptive immune response via the presentation of bacteria-derived peptide fragments on host antigen-presenting cell HLA complexes. Indeed, in melanoma, peptides of species such as *F. nucleatum* and *S. aureus* were presented on TME antigen-presenting cells. Some of these peptides appear to be potentially immunogenic, thereby bearing a capacity to activate adaptive immune responses while promoting tumor infiltration of lymphocytes and generating an inflammatory immune response[117].
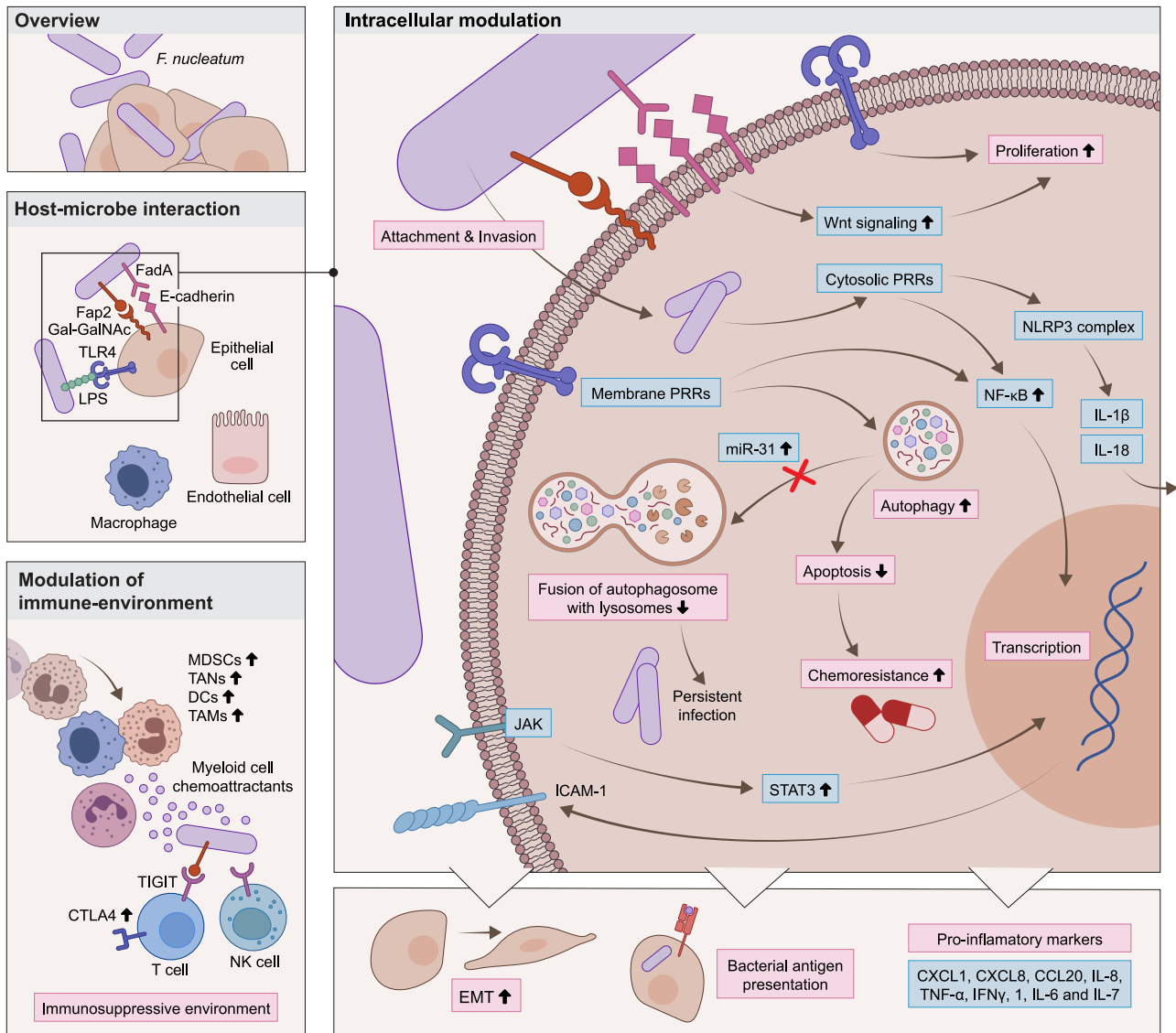
Fig. 3 Modulation of host cells by *F. nucleatum*. The opportunistic pathogen *F. nucleatum* impacts host cell behavior via numerous extra- and intracellular mechanisms. *F. nucleatum* interacts with the host via protein interactions such as Fap2-Gal-GalNAc or FadA-E-cadherin and can invade cell types such as epithelial cells, endothelial cells or macrophages. Intracellular *F. nucleatum* is sensed by membrane-bound and cytosolic PRRs, mediating NF-κB activation and expression of inflammatory molecules such as precursors of IL-1b and IL-18 that are activated by cleavage by inflammasomes and then released from the host cell. Activation of cytosolic PRRs also affects increased expression of ICAM-1. The binding of LPS to TLR4 causes upregulation of autophagy, linked to chemoresistance. *F. nucleatum* also leads to the upregulation of miR-31 which inhibits the fusion of autophagosomes with the lysosome and enables persistent infection. Consequences of *F. nucleatum* invasion include increased JAK-STAT signaling, enhanced secretion of proinflammatory markers, EMT phenotype and bacterial antigen presentation on HLA molecules. The binding of FadA to E-cadherin influences downstream Wnt signaling, leading to enhanced proliferation. Extracellularly, *F. nucleatum* induces secretion of myeloid chemoattractants that recruit TANs, DCs and TAMs, leading to suppressed activity of CD4+ T cells. Direct interaction of *F. nucleatum* with immune cells via Fap2-TIGIT binding, expressed on T and NK cells, mediates decreased cytotoxicity and ultimately inhibited killing activity. CCL20 C-C Motif Chemokine Ligand 20, CTLA4 cytotoxic T-lymphocyte associated protein 4, CXCL C-X-C motif chemokine ligand, DCs dendritic cells, E-cadherin epithelial cadherin, Fap2 fibroblast activation protein-2, *F. nucleatum Fusobacterium nucleatum*, Gal-GalNAc ᴅ-galactose-β(1-3)-N-acetyl-ᴅ-galactosamine, IFN interferon, ICAM-1 intercellular adhesion molecule 1, IL interleukin, JAK Janus kinase, LPS lipopolysaccharide, miRNA-31 microRNA-31, NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells, NK natural killer, NLRP3 NLR family pyrin domain containing 3, PRRs pattern-recognition receptors, STAT signal transducer and activator of transcription, TAMs tumor-associated macrophages, TANs tumor-associated neutrophils, TIGIT T-cell immunoreceptor with Ig and ITIM domains, TLRs Toll-like receptors, TNF tumor necrosis factor, Wnt wingless-related integration site.

Similar findings are emerging in glioblastoma multiforme[157], where multiple bacteria-derived antigens were suggested to be recognized by T cells of the TME. However, several of the suggested microbe-harboring tumors appear to be cold, or relatively immunotolerant. This apparent lack of tumor antibacterial immune reactivity, despite the suggested presence of highly immunogenic bacteria, merits future studies.

Impact on tumor metastasis

Intratumoral and intracellular microbes may also regulate the metastatic cascade. Whole genome sequencing revealed a similarity of 99.9% between *F. nucleatum* isolates from primary CRC and liver metastasis, indicating likely bacterial migratory characteristics between these tumor sites[49]. Microbial regulation of the metastatic process may involve a variety of mechanisms.
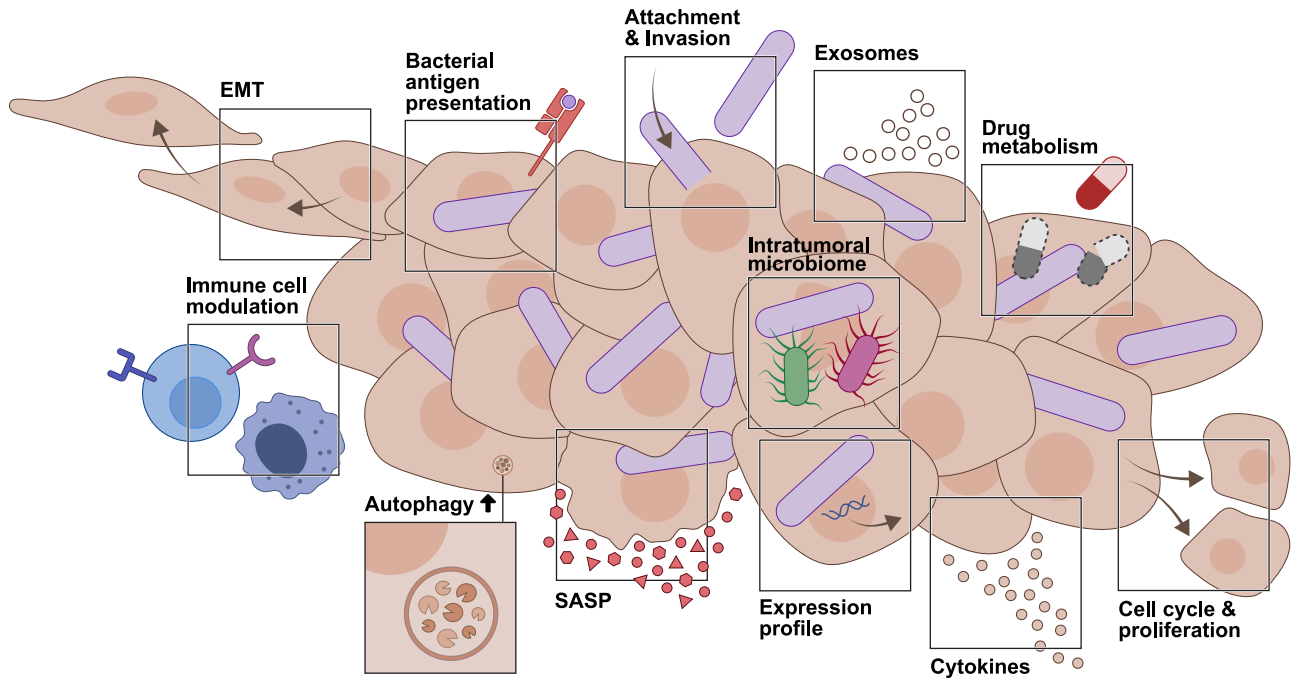
Fig. 4   Cancer-related processes possibly influenced by intracellular bacteria. Bacteria colonizing host tumor cells may alter a multitude of cancer-related features. These include gene expression changes triggered by host bacterial sensing, SASP or alterations in proliferation, and induction of metastasis-linked behavior such as migration, invasion, EMT or release of exosomes. Intracellular bacteria may modulate autophagy, immune cell recognition and antigen presentation, potentially impacting cancer immune recognition. Ultimately, bacteria-driven host modulations can lead to alterations in drug metabolism and consequently therapy response, thereby impacting the outcome of cancer therapies. EMT epithelial–mesenchymal transition, SASP senescence-associated secretory phenotype.

Epithelial–mesenchymal transition (EMT) is a dynamic process of epithelial conversion to a mesenchymal phenotype, characterized by a gradual loss of epithelial features such as epithelial cell adhesion molecule (EpCAM) expression or strong cell-cell contacts[158]. F. nucleatum may induce transcriptional features of EMT such as upregulation of Vimentin, Snail or Slug[16,159,160]. Higher intratumoral F. nucleatum abundance is associated with an increase in ALPK1, which in turn results in upregulation of intercellular adhesion molecule 1 (ICAM-1) on the host cell surface. This leads to increased attachment of CRC cells to endothelial cells which is suggested to promote EMT and ultimately metastasis[152]. Other suggested microbial mechanisms impacting host tumor cell metastatic potential include the secretion of bioactive products, as exemplified by F. nucleatum-derived short-chain fatty acid formate that was recently linked to CRC cell stemness and invasion[161]. In addition, F. nucleatum may induce the expression of pro-migratory and pro-metastatic genes in a CRC cell line, impact the motility of cancer cells, and modulate the virulence factor Fap2-driven host cell binding and suppress T-cell infiltration[16]. Keratin7 (KRT7) is reported to be upregulated in tumors upon F. nucleatum presence and was suggested to enhance lung metastasis in a murine CRC model[162]. In addition, tumor-associated bacteria may modulate cell-cycle signaling pathways[16] and induce senescence in the host. Unlike apoptotic cells, senescent cells, which undergo irreversible cell-cycle arrest, remain viable. The senescence-associated secretory phenotype (SASP) involves the release of factors, which promote inflammation and malignancy (Fig. 4). In CRC, invasive P. gingivalis is enriched and correlated with higher levels of butyrate, possibly inducing SASP[59]. Regarding the secretion profile, F. nucleatum invasion also induced increased exosome secretion of CRC cells carrying metastasis-related miRNAs and proteins, which, in turn, were internalized by uninfected cells and activated Wnt/β-catenin signaling, enhancing migration and proliferation[163].

Effects on drug metabolism and treatment responsiveness

In addition to their ability to disrupt endogenous cellular processes, bacteria can metabolize drugs, thereby potentially affecting tumor responses. Several studies suggest that bacteria-mediated drug metabolism may induce drug activation or promote adverse effects. For example, gut bacteria-derived beta-glucuronidase can metabolize the chemotherapy irinotecan in a manner promoting increased mucosal toxicity[164]. In a larger in vitro screen in which 30 FDA-approved drugs were co-incubated with bacteria, 10 compounds were found to feature a decreased efficacy, while 6 featured an increased efficacy[165]. Geller et al. reported that Gammaproteobacteria may confer gemcitabine resistance to CRC cell lines by metabolizing the drug to its inactive form by the bacterial enzyme cytidine deaminase. Consequently, antibiotic treatment promoted a regained responsiveness to gemcitabine in a CRC mouse model. Importantly in this study, the most common species in human PDAC belonged to the Gammaproteobacteria class, while 14 out of 15 PDAC-derived bacterial cultures mediated gemcitabine resistance upon co-culture with CRC cell lines. Recently, E. coli-mediated inactivation of the nucleoside analog 5-fluorouracil (5-FU) was associated with a poorer therapy response. Interestingly, 5-FU was also suggested to inhibit the growth of F. nucleatum[166], which might further contribute to its therapeutic activity. Neoadjuvant chemoradiotherapy with fluoropyrimidines (which are activated to 5-FU) decreased the prevalence of F. nucleatum from 58 to 26% in locally advanced rectal cancers, while the persistence of F. nucleatum following treatment predicted a relapse[167]. Co-cultivation of 5-FU and Oxaliplatin with F. nucleatum suggested that an altered chemotherapeutic drug response was linked to a decrease in two miRNAs (miR-4802 and miR-18a*) driving enhanced expression of autophagy-related proteins[25]. Understanding the effects of microbial colonization in tumors on treatment effectiveness and adverse effects may enable tailoring treatment to the individual

based on their microbial profiles, in optimizing dosing while minimizing adverse effects.

Targeting intracellular bacteria in cancer treatment

Intratumoral bacteria may represent potential therapeutic targets in cancer treatment. For example, antibiotic treatment of mice transplanted with *F. nucleatum*-positive patient-derived CRC xenografts reduces tumor size and cancer cell proliferation, indicating that bacterial suppression may support tumor growth suppression[49]. However, targeting intratumoral pathogens remains challenging, as in many cases internalized antibiotics do not reach the minimum inhibitory concentration in the TME[168], while high doses are associated with adverse effects on the host and its microbiome. Currently tested intracellularly acting antimicrobials include sulfonamides, quinolones, tetracyclines, and beta-lactams[169]. However, emerging antibiotic resistance requires the development of further therapeutic strategies that overcome low cellular permeability and microbial resistance. Explored modalities include intracellularly acting antimicrobial peptides (AMPs), such as fungal plectasin, which suppresses intracellular methicillin-resistant *S. aureus* (MRSA). Nonetheless, AMP activity may be abrogated upon reaching the host cell cytoplasm. Cell-penetrating peptides (CPP) can translocate to the nucleus, mostly via endocytic pathways, where they may exert antibacterial effects. CPP can also be used as translocation backbones to direct drugs into infected cells. As an example, the CPP Tat was coupled to the antibiotic gentamicin, leading to a reduction of intracellular *E. coli* K1[170]. Singh et al. proposed to conjugate the antibiotics polymyxin B sulfate and sushi peptide to gold nanoparticles, in effectively targeting intracellular *Salmonella typhi*[171]. Utilization of bacteriophages represents another experimental approach in specifically eliminating tumor-associated bacteria. For example, phages targeting *F. nucleatum*, combined with irinotecan were able to reduce tumor growth, while selectively inhibiting CRC-associated bacterium *F. nucleatum* in vivo[172]. Similarly, phages directed against adherent invasive *E. coli* reduced tumor burden in a CRC mouse model[173]. These pioneering efforts may be further optimized by the use of phage combinations suppressive target bacterial strains through different receptors, as was recently shown in the context of IBD[174]. However, phage treatment of intracellular bacteria is challenged by limited access into host cells, a changed environment altering intracellular phage-bacterial engagement, and the necessity for phages to transit between host cells in targeting multiple intracellular bacteria.

Utilization of intracellular bacteria in cancer treatment

Intracellular bacteria may be potentially utilized as medical interventions or treatment delivery vectors. For example, Salmonella used as a nonpathogenic delivery system targeting therapeutic proteins into the cytoplasm induced a reduced tumor growth and metastasis in mouse models[175]. Modification of the *Photorhabdus asymbiotica* type VI secretion system, which plays a role in macrophage invasion and intracellular survival, enabled the delivery of custom payloads into human cells[176–178]. Additionally, intratumoral bacterial peptide human leukocyte antigens (HLA) presentation on host cells may enable the harnessing of antibacterial immunity in cancer immunotherapy. As the bacterial antigens are recognized as non-self, they could serve as targets for immunotherapy stimulating an immune response[117]. In 2004, an attenuated *S. Typhimurium* systemically administered to a mouse model of melanoma featured a sustained ability to invade tumor cells. Prior vaccination of mice that generated Salmonella-specific T cells resulted in a substantial improvement in tumor load. Tumor cell invasion was suggested to play a role in this effect, as the use of non-invasive *S. Typhimurium* resulted in a marked attenuation of the anti-tumor response[179].

LIMITATIONS AND CHALLENGES IN THE STUDY OF INTRACELLULAR TUMOR-RESIDING BACTERIA

Despite recent advances in our collective understanding of the possible presence and activities of microbes in cancer, the field remains in its infancy and faces major challenges and obstacles. While the intracellular presence of Fusobacterium is well documented[15–18,49–55], the presence of microbial communities, consisting of bacteria[14,15,62] and fungi[14,15,99] within different tumors that were commonly considered sterile has been recently debated[19,63,180] and merits definite characterization by future studies. Such low-biomass tumor microbiome characterization is challenged by a variety of technical and biological challenges and potential biases stemming from contaminations, batch effects, erroneous read allocation, and imperfection in analytical pipelines, while lacking resolution to identify intracellular localization of bacteria in conjunction with tumor phenotype measurements. Examples of the challenges facing such research are presented in Box 2. The study of bacterial invasion using animal models and organoids will further contribute to our understanding of the true extent of intratumoral bacterial colonization and its potential functions. In such future experiments, the importance of addressing experimental and computational contaminations through the use of robust and diverse controls and analytical tools cannot be overstated and is highlighted by the long-standing debate as to the existence of a placental microbiome which has only recently been resolved (as likely not being present) by a large consortium effort[181].

Disentangling the effects of extracellular microbes from those of true tumor cell-penetrating intracellular bacteria may enable to quantify their true impact. Importantly, recent advances have highlighted the likely heterogeneous distribution of bacteria between different specimens and even within the same sample[16]. Despite advances in bacterial identification, variations on a species and subspecies level are often not easy to decode, especially in the low-biomass microbial setting, with different strains often grouped solely by their genus, disregarding their diversity and presence of virulence factors in specific strains.

An additional major challenge stems from the crucial need to transform our level of understanding from correlation, association, and prediction to experimental determination of causation and molecular mechanisms. Even for some of the most commonly

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Box 2: Challenges and open questions facing the prospect of intracellular bacteria in cancer

Technical challenges:

1. …robustly characterize the presence, abundance, and composition of intratumoral microbiomes in diverse cancer types.
2. …account for environmental contaminants, batch effects, read misalignment, and reference database imperfections.
3. …resolve the presence of genomically identified taxa that are unlikely to colonize the human body.
4. …elucidate the precise (intracellular) localization of specific members of the intratumoral microbiome.
5. …integrate in vivo and next-generation in vitro models to accurately reflect human bacteria-tumor interactions.
6. …achieve single-cell resolution of bacterial invasion effects.
7. …distinguish between intracellular and extracellular effects.

Biological questions:

1. Do bacterial signals identified within tumor cells represent intact, live organisms?
2. How stable is bacterial localization within tumor cells?
3. Is bacterial tumor colonization random or pre-determined?
4. Which bacteria actively invade cancer and TME immune cells, and which are passively ingested by tumor-associated phagocytes?
5. Can intratumoral bacteria causally impact cancer and its treatment?
6. How do bacterial strains differ in their invasion and tumorigenic capacity?
7. Which microbe–environment and microbe–microbe interactions matter in the context of invasion and cancer biology?

reported intracellular bacteria, like *F. nucleatum* in CRC, conflicting reports suggest that some strains lack stable gut colonization, coupled with a lack of CRC-promoting effects[182,183]. Careful evaluation of bacterial engraftment, invasion and downstream impacts on the invaded host, assessed across multiple experimental platforms, may be necessary to draw robust conclusions on an intratumoral or even intracellular microbial causative roles in cancer. Beyond single-species bacterial studies, elucidation of bacterial-bacterial interactions, as well as trans-kingdom interactions with viruses[184–186], fungi[30,187,188] and eukaryotic microorganisms will constitute fascinating topics of research in the coming decade. Progression from the use of cellular systems and animal models to clinical translation will be complex but presents opportunities to harness the gained knowledge in developing novel diagnostics and therapeutics.

In conclusion, some studies suggest that seemingly sterile tumors may harbor a unique low-biomass microbiome, whose bacterial constituents may partially reside within host tumor and immune cells. Future advances in imaging, experimental models, sequencing and data analysis may validate these findings, while accounting for confounders and possible contaminations. Even such definite proofs will only form the basis for the exploration of many unknowns. The rules of engagement by which bacteria populate tumors in specific intratumoral niches remain to be determined. Causal temporal and spatial relations between bacteria and their invaded TME host cells remain elusive, and their downstream effects on tumorigenesis and treatment response are only beginning to be unraveled. The coming decade will likely see a deepening research effort exploring these exciting topics, while exploiting the gained knowledge towards a putative development of novel cancer interventions.

Reporting summary

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L.S. and M.M. are co-first authors. L.S. and M.M. conceptualized and wrote the manuscript. E.E. and J.P. wrote the manuscript and supervised the review.

COMPETING INTERESTS

E.E. is a scientific founder of Daytwo and BiomX, and a paid consultant to Igen, PurposeBio, Aposense, and Zoe. The remaining authors declare no competing interests.

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