WNT SIGNALING IN IMMUNE CELL REGULATION DURING MICROBIAL INFECTION AND CANCER

EDITED BY: Malini Sen, Dennis A. Carson, Antje Blumenthal, Elena Martin-Orozco and Jere W. McBride

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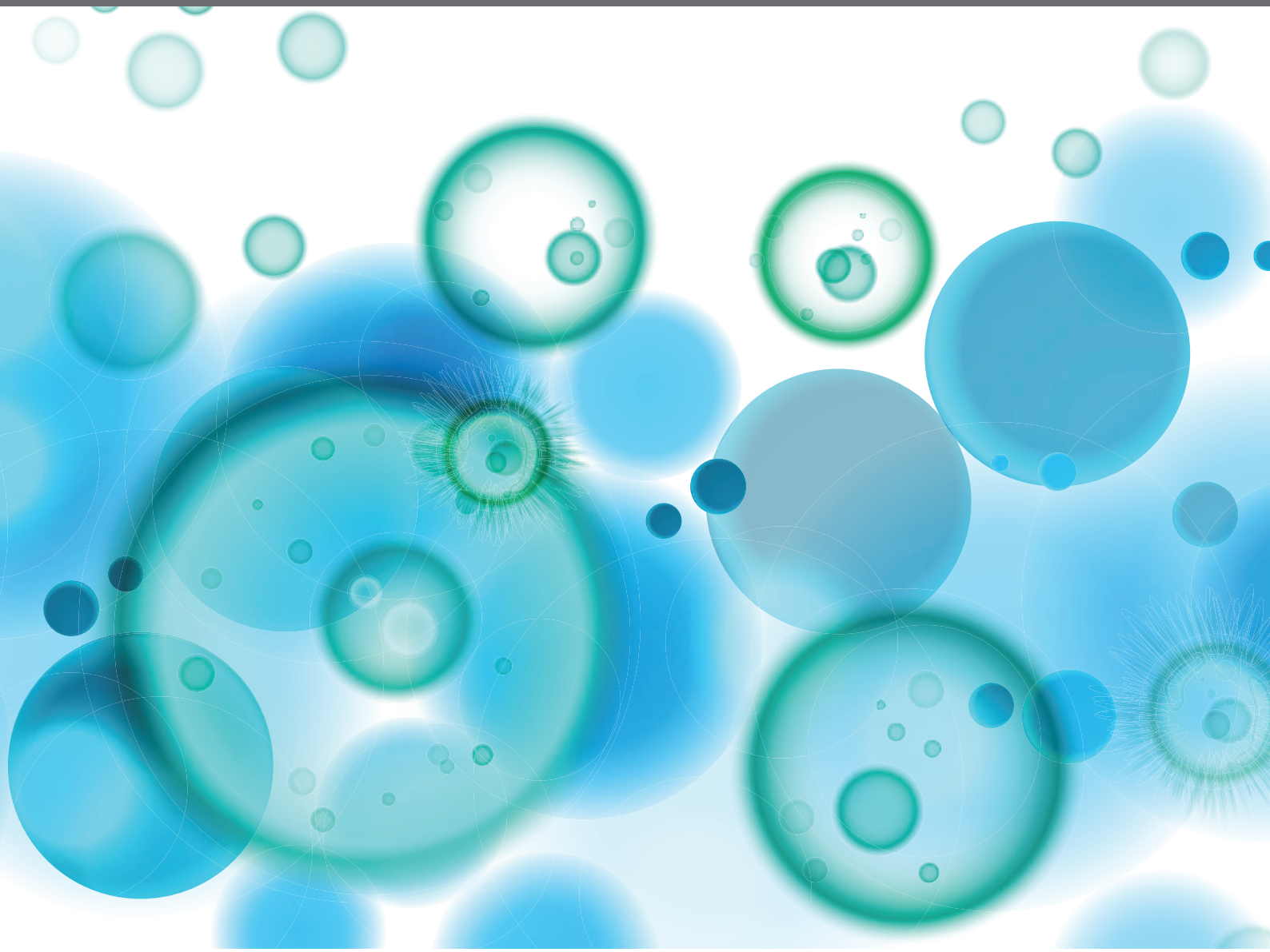

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WNT SIGNALING IN IMMUNE CELL REGULATION DURING MICROBIAL INFECTION AND CANCER

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EDITORIAL
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Editorial: Wnt Signaling in Immune Cell Regulation During Microbial Infection and Cancer

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Editorial on the Research Topic

Wnt Signaling in Immune Cell Regulation During Microbial Infection and Cancer

OVERVIEW

WNT ligands interact with distinct families of cell surface receptors to initiate signaling in response to the various environmental cues that orchestrate cell physiology and tissue homeostasis. For example, WNT signaling directs cytoskeletal modulations, organelle dynamics and specific transcriptional programs that regulate cell growth, differentiation, and migration. While WNT signaling was initially best studied in embryogenesis and carcinogenesis, quite naturally, this important aspect of life was also discovered to orchestrate interactions between the immune system and invading pathogens and tumors, as well as the microbiota that co-exist with the host. Many gaps remain in our understanding of how WNT signaling is involved in immune defense, host cell interactions with tumors and phylogenetically diverse microbes. This Research Topic comprises articles that provide a comprehensive picture of the current understanding of WNT signaling in response to pathogens, commensals, and tumors. With a view toward exploiting these novel insights for therapeutic applications, considerations of targeting specific WNTs and WNT signaling intermediates in the various scenarios are discussed.

WNT SIGNALING SCHEME

WNTs comprise a family of secreted glycol-lipo-protein ligands that bind to cell surface receptors including Frizzled G protein-coupled receptors, as well as ROR and Ryk tyrosine kinases to elicit signal transduction. Although WNT signaling is broadly categorized into 2 principal types, β-catenin-dependent and β-catenin independent, considerable crosstalk exists among the signaling intermediates of WNT signaling pathways in complex modes that are mostly context dependent. The intricate features of the diverse WNT signaling cascades under physiological states and different pathophysiological settings have been covered categorically in the articles by Mukherjee et al., Ljungberg et al., Silva-Garcia et al., Jati et al., Rogan et al., Li et al., Cosin-Roger et al., Patel et al., and Martin-Orozco et al..

WNT SIGNALING IN RELATION TO MICROBIAL PATHOGENESIS

Being an important component of cell and tissue homeostasis, WNT signaling is often exploited by pathogens for their own survival. For example, *Salmonella* Typhimurium depresses WNT signaling in the endothelium by virtue of its T3SS effector system and utilizes the vascular leakiness to enhance its dissemination (Rogan et al.). Some pathogens, for example *S. flexneri* on the other hand, take advantage of the inflammatory component of WNT signaling for propagation of infection (Mukherjee et al.). Along the same lines, Silva-Garcia et al. have outlined the role of various bacterial virulence factors in the regulation of the WNT-β-catenin pathway during disease pathogenesis. Ljungberg et al. provide an overview of bacteria-induced WNT responses in experimental systems and patient samples, and the functional consequences in the context of immune responses. Some examples include impact on immune cell differentiation, antimicrobial defense, inflammation, and the cross-talk between innate and adaptive immunity. Adding another twist to this topic, Jati et al. have described how actin cytoskeleton organization by WNT5A signaling dictates the killing of some pathogens by utilizing the host autophagy machinery. Jati et al. also raise how WNT signaling might facilitate differentiating between pathogenic and non-pathogenic microbes, given the plethora of beneficial commensals that have evolved with our system.

In view of the ongoing tug of war between the host and pathogens for WNT signaling components during the onset and progression of infections, it would be fair to state that appropriate and targeted inhibition or activation of WNT signaling may be beneficial for the host's ability to control pathogens. Such operations, however, would naturally vary case to case.

WNT SIGNALING IN TUMORIGENESIS AND ANTI-TUMOR IMMUNE RESPONSES

Consistent with an important role in directing transcription, cytoskeletal motility and cell proliferation and differentiation, dysregulated WNT signaling is associated with the development of tumors. With the discovery that WNT signaling also shapes immune cell functions, it is increasingly appreciated that the immune response to tumors is shaped by WNT signaling in both tumor and immune cells. As explained by Cosin-Roger et al., evidence of the association of WNT signaling with tumorigenesis was first reported in 1991, when mutations in the Adenomatous Polyposis Coli (APC) gene were linked with the development of colorectal cancer. Anomalous expression and function of the APC gene product, which regulates β-catenin functions as a transcriptional co-activator, is now known to be associated with uncontrolled cell proliferation in cancer. Cosin-Roger et al. and Li et al. project WNT-β-catenin signaling as a high priority target for therapeutic intervention in the treatment of cancers. In their review, Patel et al. describe the role of tumor infiltrating macrophages in colorectal cancer, commenting on the high level of expression of WNT2 and WNT5A in the macrophages and its correlation with cancer progression. They further describe how WNT-β-catenin signaling is involved in the functional polarization of tumor-associated macrophages that facilitates tumor growth. Martin-Orozco et al. discuss the role of WNT-β-catenin signaling in augmenting drug resistance of tumors through expression of drug export pumps. Importantly, reports of the role of WNT signaling in tumor regression also exist. For example, evidence has been presented that WNT5A acts as a tumor suppressor in various cancers. In fact Foxy-5 is a hexapeptide mimic of WNT5A, which is currently being studied in clinical trials as a tumor suppressor (Patel et al.).

As dysregulated WNT signaling is explored as a therapeutic target in cancer, it is important to consider that the applied interventions will define and re-shape the associated immune response. With pro- and anti-tumorigenic functions of WNT signaling in specific tumor settings it will be important to evaluate the therapeutic benefits and potential risks of WNT-targeted interventions in different cancers.

PERSPECTIVE AND FUTURE DIRECTIONS

Activation of WNT signaling is an integral part of host responses to microbial encounter and tumorigenesis. Given the complexity of WNT signaling, special emphasis should be placed on the nature of the WNT response, as well as the understanding of functional impact of individual WNT ligands and their concerted action in response to infection and tumorigenesis. This will be essential to evaluate strategies for WNT-directed therapeutic interventions that might prove particularly valuable considering the significant challenges posed by drug resistance in pathogens as well as tumors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The WNT Framework in Shaping Immune Cell Responses During Bacterial Infections

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A large proportion of the world is inflicted with health concerns arising from infectious diseases. Moreover, there is a widespread emergence of antibiotic resistance among major infectious agents, partially stemming from their continuous dialog with the host, and their enormous capacity to remodel the latter toward a secure niche. Among the several infection-driven events, moderation of WNT signaling pathway has been identified to be strategically tuned during infections to govern host-pathogen interactions. Primarily known for its role in arbitrating early embryonic developmental events; aberrant activation of the WNT pathway has also been associated with immunological consequences during diverse patho-physiological conditions. Here, we review the different mechanisms by which components of WNT signaling pathways are exploited by discrete bacterial agents for their pathogenesis. Furthermore, recent advances on the cross-talk of WNT with other signaling pathways, the varied modes of WNT-mediated alteration of gene expression, and WNT-dependent post-transcriptional and post-translational regulation of the immune landscape during distinct bacterial infections would be highlighted.

Keywords: Wnt, infectious diseases, epigenetics, nuclear Wnt signaling, therapeutics

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INTRODUCTION

The Discovery of WNT Ligands and Receptors

Three decades have passed since Dr. Roel Nusse cloned and characterized the first mammalian Wnt gene in 1982. This continued with the congregation of additional 18 ligands and 10 receptors into the large family of Wnt genes. The series of these discoveries have been elaborated by Dr. Nusse in his essay commemorating 30 years of Wnt genes (1). Wnt derives its name from the amalgamation of two lines of research being pursued in the 1980s. On one hand, in an attempt to identify the genes that induced mammary tumors upon infection with mouse mammary tumor virus (MMTV), Nusse and Varmus discovered a single "integration site" that could lead to disease phenotype, and named the same as *int1*. Later it was shown that overexpression of *int1* is sufficient to induce tumorigenesis in mice. At a similar time, *int1* homolog *Wingless* was identified in a Drosophila mutant screen; wherein the gene was found to be essential for segment polarity and for the formation of wing tissue. By the end of 1980s, enormous screens were carried out to decipher other integration sites that may contribute to tumors (*int2, int3, int4*). However, those tumorigenic integration events were diverse and had least homology with *int1*; necessitating the nomenclature to be revisited. The researchers working then with *int1* and *Wingless* consented to the hybrid name "Wnt" (Wingless-related integration site), and *int1* as the pioneer was called Wnt1; whereas *int2* is known today as FGF3, *int3* as NOTCH4 and *int4* as Wnt3A (1).

The Wnt family has only been expanding with the youngest member being added to the list in 2002 (2). In view of simplification, the Wnt members and their signaling mechanisms have now been broadly categorized into canonical, non-canonical and alternate, as briefly discussed in the following section.

The Wnt Signaling Pathway

The Wnt pathway is evolutionarily conserved in all metazoans, where it contributes to critical fate decisions such as polarity, axis formation, organogenesis, tissue homeostasis, and stem cell renewal. Wnt ligands (19 in number), secretory glycoproteins, upon being translated undergo palmitoylation by the ER-resident *Porcupine* acyl transferases, and are then transported through the Golgi network to be docked onto the plasma membrane. When stimulated, the extracellular domain of Wnt is cleaved to release the bioactive ligand into the extracellular milieu, which binds to corresponding Frizzled (Fzd) receptors (10 in humans) in an autocrine or paracrine manner. Fzd form a class of seven-pass transmembrane proteins, showing topological homology to G-protein coupled receptors (GPCRs). The ligand binding occurs at the N-terminal cysteine-rich extracellular domain, bringing about conformational alterations that subsequently activate the adaptor molecule *Disheveled* (Dsh/Dvl). The phosphoprotein Dvl is activated by kinases such as Casein Kinase1, Casein Kinase2, and Protein Kinase C (PKC) among others. Apart from Wnt and Fzd, co-receptors (lipoprotein receptor-related protein, LRP5/6) are critical for tuning the transduction of signal through Wnt receptor. Physiological and biochemical characterization of Wnt ligands, their receptors, co-receptors and the corresponding signaling mechanisms have identified at least three distinguishable consequences of Wnt interaction with specific Frizzled (Fzd) receptors: the canonical β-CATENIN-dependent signaling pathway; and the non-canonical β-CATENIN-independent Planar Cell Polarity and Wnt/Ca^{2+} pathways. Further, the signaling cascades are kept under tight control by various negative regulators operating both intracellularly and extracellularly (briefly described in **Figure 1**). Owing to the modular architecture, Dvl stands as a single molecule past the ligand-receptor interface to be common in all the three pathways (3–5).

Wnt/β-CATENIN Signaling Pathway

This forms the first pathway to be identified by genetic screens in Drosophila and validated in worms, frogs, fishes, and mice. The central objective of the pathway ensures the stabilization and activation of the transcription co-activator β-CATENIN. When unstimulated, β-CATENIN is trapped in a destruction complex composed of Axin, Adenomatous Polyposis Coli (APC), Casein kinase1α (CK1α), glycogen synthase kinase3 (GSK3). Here, GSK3/CK1α phosphorylates β-CATENIN, thereby priming it for ubiquitination-mediated proteasomal degradation. Upon signaling activation, Axin is translocated to the membrane to bind to LRP5/6, which leads to a series of relatively uncharacterized events activating Dvl and inactivating GSK3. These steps release the repression from β-CATENIN and allow it to translocate to the nucleus along with specific nuclear localization signal (NLS)-containing proteins. β-CATENIN, then effectuates Wnt-specific gene expression by interacting with the TCF/LEF (T cell factor/ lymphoid enhancer factor) family of DNA-binding transcription factors, while eliminating the negative regulator Groucho from the site.

Planar Cell Polarity (PCP) Pathway

The non-canonical pathways activate Fzd and utilize specific domains of Dvl to transduce the signals. LRP5 and LRP6 do not participate in the non-canonical pathways, and are reportedly replaced by NRH1, Ryk, Ror as co-receptors. In the PCP pathway, activated Dvl adopts a route through GTPases Rho and Rac to activate ROCK and JNK, respectively. These events majorly culminate in cytoskeletal re-organization, and mechanisms of the pathway's contribution to transcriptional rewiring still require investigation.

Wnt/Ca^{2+} Pathway

This pathway came to light with the observation that discrete Wnt signals can lead to Ca^{2+} release from the endoplasmic reticulum (ER) in a GPCR-dependent manner. Though the precise GPCR has not been elucidated, it is clear that the activated trimeric G proteins lead to Ca^{2+} release and activation of Ca^{2+}-dependent kinases such as Calcium/ calmodulin Kinase II (CamKII) and PKC, and transcription factor NFAT. Interestingly, CamKII can also intercept β-CATENIN-driven regulation of gene expression, thereby altering the canonical arm of Wnt signaling.

WNT PATHWAY IN INFECTIONS

Conventionally, Wnt genes have been correlated with carcinogenesis, retrovirus-mediated tumorigenesis and developmental processes, however, its contributions in the context of various infections is manifold (5). Sentinel macrophages, epithelial cells and other immune cells adopt a number of defense mechanisms to defy the invading pathogens that include autophagy, apoptosis, inflammation, antigen presentation, ER-stress regulation, inflammasome activation, and priming of cell-mediated immune responses such as T helper or cytotoxic phenotype. This review will give a brief account of the same with respect to bacterial infections in the light of Wnt signaling pathways.

Regulation of Inflammation and Adaptive Immune Responses During Bacterial Infections

Inflammation is a complex process encompassing the differential activation of pathways leading to the expression and secretion of a set of cytokines by the host cells themselves or by host cell-mediated priming of the adaptive immune system. Skewing cytokine response to establish successful infection is adopted by several pathogens such as *Mycobacterium tuberculosis*, *Shigella flexneri*, *Salmonella typhimurium*, *Citrobacter rodentium* etc. In this context, it was recently shown that canonical Wnt signaling is essential to maintain an inflammatory equipoise during *S. flexneri* infection. It was found that

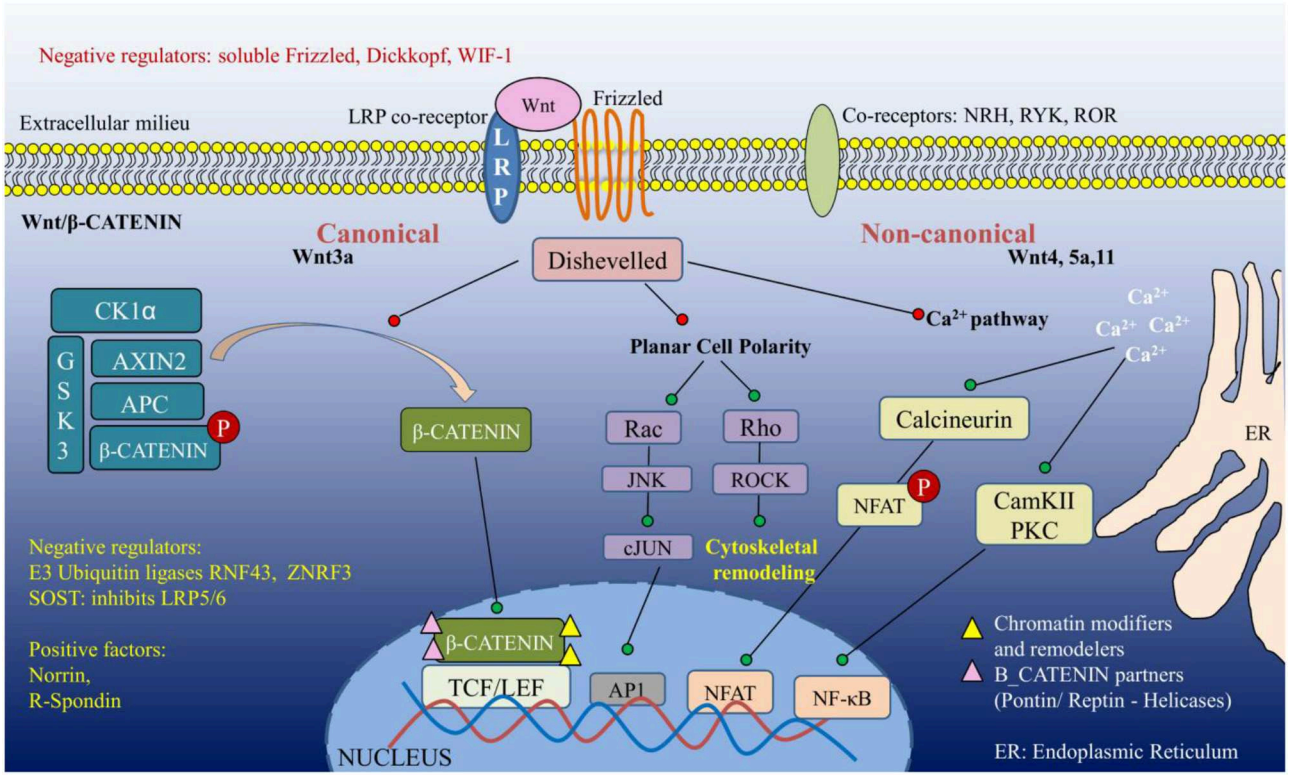

FIGURE 1 | An overview of Wnt signaling pathway.

Shigella-induced inflammation is maintained by NOD2-driven Epidermal Growth Factor Receptor (EGFR)-dependent activation of Wnt/β-CATENIN axis, leading to the production of the anti-inflammatory molecule IDO1 (indoleamine 2, 3 dioxygenase1). The perturbation of Wnt signaling compromised IDO1 expression and pharmacological inhibition of IDO1 in mice exacerbated Shigellosis by inducing intestinal hyper-inflammation (6). Another instance of NOD2-driven inflammation was replicated in an acute arthritis model wherein Singh et al. utilized NOD2 agonist muramyl dipeptide (MDP) to demonstrate Wnt/β-CATENIN activation through LYPD6. It was found that the resultant Wnt signaling orchestrates β-CATENIN-dependent XIAP expression that mediated NLRP3 inflammasome activation, Caspase1 maturation and IL-1β secretion, thereby leading to the development of the inflammatory condition of acute arthritis in mice (7). Therefore, in contrasting conditions the same components of the canonical Wnt pathway generate intermediates that influence net inflammation.

Apart from the direct interception of Wnt pathway, certain pathogens alter Wnt signaling events by modulating the expression of the regulators of the pathway. For instance, in a model of *C. rodentium* infection in resistant C57BL/6J mice vs. susceptible C3H/HeOuJ and FVB mice, the authors found a robust expression of R-Spondin (Rspo2) in the susceptible strains (8). Rspo2 is known to activate Wnt pathway by negatively regulating E3 ubiquitin ligases ZNF43 and ZNRF3 that allow proteasomal degradation of Wnt pathway components. The authors show that Rspo2 is associated with pathological Wnt/β-CATENIN activation-driven loss of intestinal differentiation, resulting in susceptibility to *C. rodentium* infection. Further studies by the same group found Rspo2 to be associated with enhanced MHC-II-driven antigen presentation and generation of Th1/ Th17 in susceptible mice (9). This could be attributed to the pronounced ability of proliferating intestinal cells to sense the pathogen because of an apparent loss of functionally differentiated goblet cells in Rspo2-driven Wnt active intolerant/susceptible mice.

Modulation of Host Cell Death Pathways

The regulated execution of specific cell death pathways enables bacterial pathogens to either localize in host cells or disseminate systemically during the course of infection. Therefore, it can be surmised that the dynamics of these pathways would be carefully tapped during host-pathogen interactions. Wnt signaling has been significantly implicated in apoptotic events during development, and has subsequently been found to contribute to distinct cell death phenomena associated with infections. It has been reported that canonical Wnt3a-β-CATENIN signaling enhances apoptotic host cell death during *Mycobacterium bovis* BCG (BCG) infection via mitochondrial cytochrome c-driven Caspase3 activation. This involved the

upregulation of the pro-apoptotic Bax and downregulation of anti-apoptotic Mcl1 proteins (10). Alongside, another study reported the ability of Wnt3a to inhibit BCG-induced necrosis (another cell death pathway) by restricting the ROS-dependent PARP1-AIF cascade (11). These studies employed the vaccine strain BCG, which might present immunologically diverse regulatory outcomes compared to virulent mycobacteria. However, the parallel results of enhanced apoptosis and restricted necrosis may be explained by the ability of BCG to utilize Wnt pathway-driven apoptosis as a strategy to establish infection at early stages and therefore the dissemination-prone necrotic pathway is limited. In another instance, Liu et al. demonstrated that *Salmonella* infection enhances Wnt2 expression at the transcript and protein level, partially by AvrA-mediated regulation, which is responsible for inhibiting *Salmonella* infection-driven apoptotic/necrotic host cell death, thereby enabling pathogen survival (12). Recent advances have identified multiple forms of regulated cell death mechanisms, apart from apoptosis, such as RIPK1-RIPK3-MLKL-dependent necroptosis and iron-mediated ferroptosis, however, these have not been explored in the context of Wnt signaling and infections.

Wnt-Dependent Regulation of Cellular Homeostatic Processes

Among different cellular events, autophagy forms a homeostatic process initiated to unload host cells of non-functional/unfolded proteins, worn-out/excess organelles and exogenous agents, including intracellular pathogens. The processing of intracellular pathogens, referred to as xenophagy, displays an efficient host response to infections. The implication of Wnt pathway in the inhibition of autophagy in the context of mycobacterial infection has been demonstrated. It was found that infection with pathogenic bacteria, including BCG, *S. flexneri* and *Listeria monocytogenes* stimulate the expression of Wnt ligand Wnt5a, receptor Fzd4 and co-receptor Lrp5 to bring about the inhibition of IFNγ-induced autophagy. The detailed molecular mechanism implied an mTOR-dependent miR31- and miR155-driven downregulation of the phosphatase PP2A, thereby stabilizing the inhibitory phosphorylation on the negative regulator of Wnt pathway i.e., GSK3β. However, non-pathogenic bacteria such as *E. coli* failed to execute the string of events (13). Interestingly, in another study with *Ehrlichia chaffeensis*, Lina et al. demonstrated the ability of the pathogen to utilize Wnt-driven mTOR-PI3K pathway to inhibit autophagy by restricting the fusion of amphisomes (bacteria-containing endosomes decorated with autophagy markers BECLIN1, p62, LC3BII) with lysosomes. The authors suggest that the virulence factor Etf1 allows amphisome formation in order to acquire nutrients, while, another set of virulence determinants TRP120, 32 and 47 act in concert to activate Dvl/Wnt pathway to mediate mTOR-driven TFEB cytosolic retention and consequent inhibition of amphisome-lysosome fusion (14). These studies clearly indicate the ability of pathogens to hijack the Wnt pathway to evade host innate defense of autophagy. In addition to these reports, Jati et al. demonstrated that pathogens such as *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* indeed downregulate Wnt5a in order to suppress autophagy and establish a successful infection (15). Thus, Wnt signaling is regulated disparately by diverse pathogens to execute similar immune responses, indicating toward the specificity of virulence mechanisms employed by infectious agents.

Non-canonical Wnt in Infectious Diseases

The non-canonical components of the Wnt pathway have distinct roles in patho-physiologies, and their association with infectious diseases has started to garner significant attention. For instance, Luo et al. investigated the ability of the obligate intracellular bacterium *E. chaffeensis*, causing human monocytotrophic ehrlichiosis (HME) to utilize the non-canonical Wnt pathway for its pathogenesis. The authors showed that the unusual bacterium upregulates several components of the Wnt pathway, including ligands Wnt6 and 10a; receptors Fzd5 and 9; co-receptor LRP6 and TCF7 early during infection. Interestingly, *E. chaffeensis* tandem repeat proteins (TRPs) were shown to assist bacterial internalization through non-canonical Wnt components, where they interacted with Wnt pathway regulators like ARID2, KDM6B, IRF2BP2, PPP3R1, and VPS29, thus influencing Wnt signaling activation and aiding in bacterial survival by both canonical β-CATENIN and non-canonical Ca^{2+}-CAMKII-NFAT routes (16). Apart from this, several reports have focused on enteric pathogens for their association with the non-canonical Wnt pathway. Wnt11 has been reported to be enhanced during intestinal bowel disease (IBD), and Liu et al. demonstrated its relevance in the context of bacterial inflammation for the first time. They found that infection of host epithelial cells with *S. typhimurium* leads to an elevated expression of Wnt11, dependent on the *Salmonella* effector protein AvrA. Further, the *in vivo* studies showed a relocalization of Wnt11 to the lower crypts during infection and also demonstrated that overexpressing Wnt11 shows inhibitory effects on *Salmonella* internalization and IL-8 expression-mediated intestinal inflammation (17). Another instance is detailed with the kinome array conducted for *Salmonella enteritidis* infection in chicken, wherein the authors find an infection-driven activation of non-canonical components of the Wnt pathway in Fzd1-dependent manner. This was shown to converge to the phosphorylation of Fzd1-CamKII/β-CATENIN/Protein Kinase C that eventually activates NFAT and suppresses canonical Wnt signaling. This aids the expression of anti-inflammatory IL-10 and TGFβ, thereby generating a tolerogenic state and allowing persistent colonization of *Salmonella* in the chicken gut (18). In the realm of mycobacterial infection, the contribution of canonical Wnt/β-CATENIN pathway has been detailed in several studies (13, 19). Interestingly, mycobacterial infection has also been reported to mediate the expression of non-canonical Wnt components (Wnt5a and Wnt6). In one instance, Wnt5a-Fzd5 axis was shown to be activated in myeloid cells of the TB lesion in order to prime cell-mediated pro-inflammatory immune responses such as IFNγ production from T cells (20). Conversely, in another study, Wnt6 was found to be expressed specifically by lipid-laden macrophages in TB granulomas and was associated with

Arginase1 expression, while being negatively correlated with pro-inflammatory TNFα production (21). Although both the studies were conducted in the mouse model of chronic TB infection, it appears that the cell type expressing specific Wnt ligands and their spatio-temporal abundance strongly influences the immune cell polarization and the net immune outcome.

Cross-Talk of Wnt With Other Signaling Pathways During Infections

Infection with pathogens triggers several signaling pathways that act in concert to define the immune outcome. The Wnt cascade itself establishes communication with intracellular signaling intermediates in physiological and infectious scenarios. This is achieved by the presence of common upstream regulators or downstream target molecules. It has been shown that Wnt interacts with Sonic Hedgehog (SHH) signaling through GSK3β, which acts as a negative regulator of both the pathways. In the context of mycobacterial infection in macrophages, miRNAs 31 and 155 were found to repress the expression of the phosphatase PP2A, thereby retaining the inhibitory phosphorylation of GSK3β, and consequently allowing the activation of Wnt and SHH through β-CATENIN and NUMB, respectively (13). Further, in another study, a pathogenic encounter with Mtb, *Salmonella* or *Staphylococcus* was found to induce both NOTCH (Notch1, Jagged1) and Wnt (Wnt5a, Fzd4, Lrp5) signaling pathways. This pathogen-specific TLR2-dependent activation of Wnt and NOTCH relied on a Wnt/β-CATENIN-driven expression of NOTCH pathway ligand Jagged1 in iNOS/NO-dependent manner. Critically, such studies broaden the repertoire of signals responsible for canonical β-CATENIN activation to include non-canonical Wnt ligands, such as WNT5a. Such an orchestration was observed to mediate regulatory T cell expansion during pathogenic bacterial infections (19). The signaling cross-talks are witnessed across phyla and species, ranging from Drosophila and zebrafish to mice and pigs. In this light, a study by Huan et al. showed the synergistic effects of Wnt attenuation (assessed by cytosolic localization of β-CATENIN and downregulation of target gene expression) and NOTCH pathway activation in regulating goblet cell maturation, mucosal integrity and crypt cell proliferation in pigs infected with *Lawsonia intracellularis*, thus providing insights into the pathogenesis of this bacterial agent (22).

Recent reports demonstrate Wnt pathway to respond to *S. flexneri* infection-activated EGFR pathway. It was shown that the pathogen utilizes EGFR pathway to initiate a cascade comprising the engagement of c-Abl and HIPK2 to mediate the activation of the Wnt/ β-CATENIN axis and the subsequent expression of anti-inflammatory IDO1 (6). In the context of another intestinal infection with *C. rodentium*, Wnt signaling has been reported to coordinate with AHR signals to maintain a balance of intestinal stem cell renewal and differentiation. Metidji et al. demonstrated that AHR pathway transcriptionally upregulates Wnt negative factors RNF43 and ZNRF3, thereby limiting Wnt signaling activation. This check on Wnt pathway allows intestinal stem cell repair and differentiation; and prevents from intestinal infections (*C. rodentium*) and formation of colorectal cancers (23).

Hijacking Wnt Components for Gaining Access to Host Cells

The intracellular events post-receptor-driven activation of Wnt pathway are well appreciated. The independent roles for the receptors themselves during infections form a relatively recent development in the functional spectra of this signaling cascade. In 2016, Tao et al. demonstrated the implication of Fzd receptors in the invasion and thereby pathogenesis of the Gram positive bacterium *Clostridium difficile*. They found that the pertinent TcdB toxin of the pathogen is infused into host cells via Fzd2 receptor, alongside Fzd1 and Fzd7. Single knockout of either Fzd1/2/7 or triple knockout in HeLa cells compromised TcdB-mediated host cell rounding and cell death; while complementing any of the three Fzd in the triple knockout conditions restored the host cell sensitivity to the toxin. Having validated the same in colorectal cancer cell line HT-29 and colonic organoids and having confirmed its relevance in detecting TcdB in mouse colon epithelium *in vivo*, it is intriguing to consider Fzd receptors as potential targets for minimizing the detrimental effects of this infection (24). Another interesting study by Zhang et al. (25), demonstrated the effect of Axin1 in *Salmonella* invasion and resultant inflammation. They found that the pathogen attaches to the host cell membrane irrespective of the presence of Axin1; however, the invasion of *Salmonella* is severely compromised in cells overexpressing Axin1. Mechanistically, the authors found that *Salmonella* strategizes the depletion of Axin1 early during infection by post-translational mechanisms through ubiquitination and SUMOylation (25). Of note is that a proportion of overexpressed Axin1 is found localized to the plasma membrane upon *Salmonella* infection, suggesting a possible inhibitory effect being generated at the entry point.

Wnt-Dependent Epigenetic Regulation of Infection-Driven Immune Responses

The paradigm of regulation of gene expression also includes accurate and swift alterations mediated by epigenetic mechanisms. These epigenetic changes result from the covalent modifications of DNA/ histones or the structural reorganization of chromatin. The bearings of such modifications are immense, as recruitment of the transcriptional co-activator, β-CATENIN, alone is not sufficient to orchestrate WNT target gene expression. Therefore, a major focus of the current research invests in exploring the epigenetic interventions. In this front, it is known that upon nuclear translocation, β-CATENIN interacts with multiple factors through its N- and C-terminal domains. The N-terminus interacts with LEF to allow its DNA binding, while also associating with a number of factors such as Legless (Lgl) and Pygopus (Pygo). The C-terminus is more diverse in the repertoire of chromatin binding proteins that include histone methyl transferases such as MLL, PRC2 complex (EZH2), demethylase LSD1, histone acetyl transferase like p300, and chromatin remodelers such as BRG1 and ISW1. A study by Yakulov et al. has compared the chromatin partners of β-CATENIN in the presence or absence of the canonical ligand, WNT3a (26, 27). Although, some of these association

studies have been prominent in cancers and development, the implications of the same in bacterial infections is in its infancy.

In a model of *C. rodentium* infection in mice, Roy et al. proposed the activation of WNT signaling-driven crypt hyperplasia and tumorigenesis to result from EZH2-dependent downregulation of the negative Wnt regulator WIF1. They demonstrate an enhanced expression of EZH2 upon infection with *Citrobacter* (28). However, there is a lack of evidences that compare the infection-moderated chromatin profile or alterations of associating partners of β-CATENIN. Such instances may be borrowed from studies elucidating distinct pathologies. For instance, the type-II protein arginine methyltransferase, PRMT5, activates Wnt signaling and Wnt/ β-CATENIN target gene expression by differentially modulating the promoter occupancy by co-activators and co-repressors in lymphoma cells. Chung et al. specifically demonstrated that PRMT5 inhibition reduces the recruitment of co-activators p300, MLL1, while enhancing the co-repressors HDAC2 and LSD1 in lymphoma cell lines. Interestingly, the occupancy of β-CATENIN over the promoters of these target genes does not alter significantly upon PRMT5 inhibition making it an interesting supposition to assess if PRMT5 associates with β-CATENIN to mediate differential recruitment of activators/repressors in Wnt-specific gene expression even during infections (29).

Future Perspectives

Infectious diseases account for a vast proportion of disability associated life years (DALYs) as well as mortality in the global context. This stems from the ability of major infectious agents to adopt strategies in order to co-evolve with their host, and exhibit immune evasion and subversion mechanisms as a part of their survival tactics. With the early reports of the implications of Wnt pathway in development and tumorigenesis, it could be foreseen that the pathway might be hijacked by infectious entities to disrupt homeostasis and establish infection. In this light, we discussed the many facets that are exploited by pathogens along the Wnt signaling axis, including Wnt ligands, receptors, coreceptors, transcription factors, and certain epigenetic alterations (summarized in **Table 1**). However, the increasing complexities being identified for the Wnt pathway provides an array of avenues that still remain untapped in the realm of bacterial infections.

The canonical and non-canonical Wnt signaling pathways are majorly distinguished by the nuclear translocation of the transcriptional co-activator β-CATENIN, which alters immune gene expression. Notably, several other Wnt components such as APC, Axin2, and Dvl proteins harbor nuclear import and export signals and have been found to shuttle to the nucleus in order to fine tune the outcome of the Wnt pathway (30–33). However, it is unclear as to how these cytosolic/membrane-localized Wnt components are targeted to the nucleus. Since the infection outcomes depend on the transcriptional landscape, filling the gaps in nuclear Wnt signaling may assist the understanding of gene expression in a more cohesive fashion. It would be interesting to assess if bacterial pathogens or their virulence factors associate with these accessory components to invade into the host cell nucleus or differentially drive their shuttling to enhance their pathogenesis.

Chromatin modifications contribute significantly in defining the infection-induced transcriptome. With the premise that β-CATENIN is decorated with several chromatin modifiers and remodelers once inside the nucleus, exploring the epigenetics along Wnt signaling axis during bacterial infections, as a cause or consequence, requires further investigation. Moreover, it is reported that certain epigenetic factors can also act on host cytosolic proteins to alter their functions. In a very recent series of research outcomes, it was identified that the arginine methyl transferase PRMT1 is important for Wnt signaling activation. Mechanistically, the asymmetric arginine dimethylation conferred by PRMT1 on many of the GSK3 target proteins either primed them for GSK3-mediated phosphorylation or packaged them along with GSK3 into multi-vesicular bodies (MVBs), which stands as a pre-requisite for β-CATENIN nuclear translocation and Wnt activation (34). Further development showed that PRMT1-Wnt axis is crucial for governing macropinocytosis- (referring to the uptake of extracellular fluid with particles $>0.2\,\mu m$)-driven endosomal trafficking and lysosomal degradation to increase the bioavailability of free amino acids (35). This attribute of Wnt signaling can be extended to infectious scenarios, where pathogens derive a large portion of nutrients from the host. It might possibly posit a critical persistence mechanism as many of the infections deal with innate sentinels such as macrophages, which sample their microenvironment and may collect nutrients by macropinocytosis upon infection-driven Wnt activation.

Finally, the extensive repertoire of Fzd receptors still require detailed investigation for their possible implications in pathogen uptake and ensuing microautophagy/macropinocytosis events. The endless possibilities of Wnt functions, given the apparent cross-talks and the overlaps of the canonical and non-canonical components, can levy contrasting immune outcomes during host-pathogen interactions as may be observed with the ability of Mtb to induce canonical Wnt/ β-CATENIN signaling to inhibit autophagy, while the non-canonical Wnt being downregulated by *P. aeruginosa* and *S. pneumoniae* infections to execute the same (13, 15). Wnt was primarily recognized to be essential for cell proliferation and differentiation processes, which also included the modulation of host cell death. It may be noted that Wnt-directed host cell death during infections has only been restricted to apoptosis and necrosis. Advances in the understanding have revealed the existence of multiple regulated cell death pathways, such as necroptosis, ferroptosis, and pyroptosis. Though Wnt has been associated with components of these cell-death events, it has not been studied in infections. For instance, necroptosis that occurs via the formation of "necrosome" consisting of RIPK1-RIPK3-MLKL axis has been linked with Wnt pathway. In a study with colorectal cancer, it was shown that RIPK3 knockout leads to the excessive activation of various pathways including Wnt/ β-CATENIN (36). Moreover, recent evidence succinctly demonstrated that GPX4 interacts with canonical Wnt transcription factors TCF3

TABLE 1 | Interactions of bacterial pathogens with Wnt pathway components.

Pathogens	WNT component involved	Effect on infection-driven processes	References
Shigella flexneri	Canonical β-CATENIN pathway	Maintaining inflammatory equipoise through the regulation of IDO1	(6)
Citrobacter rodentium	R-spondin 2-mediated Wnt activation	Involved in loss of intestinal differentiation	(8)
		Leads to increased MHC-II responses and Th1/17-driven hyper-inflammation	(9)
Mycobacterium bovis BCG	Wnt3a	Induction of apoptosis	(10)
Mycobacterium bovis BCG	Wnt3a	Inhibition of necrosis	(11)
Salmonella	Wnt2	Inhibition of necrosis	(12)
BCG, *Shigella flexneri*, *Listeria monocytogenes*	Wnt5a, Fzd4, Lrp5	Inhibition of autophagy	(13)
Ehrlichia chaffeensis	Canonical Wnt/Dvl-mTOR axis	Inhibition of autophagy	(14)
Pseudomonas aeruginosa/ *Streptococcus pneumoniae*	Downregulation of Wnt5a	Inhibition of autophagy	(15)
Ehrlichia chaffeensis	Wnt6, Wnt10a, Fzd5, Fzd9, Lrp6, Tcf7	Assist bacterial internalization and survival	(16)
Salmonella typhimurium	Wnt11	Inhibition of *Salmonella* internalization and IL-8 expression-mediated inflammation	(17)
Salmonella enteritidis	Fzd1	Production of anti-inflammatory cytokines IL-10, TGFβ	(18)
Mycobacterium tuberculosis	Wnt5a-Fzd5	Induction of pro-inflammatory immune response	(20)
Mycobacterium tuberculosis	Wnt6	Expressed in lipid droplets and associated with production of anti-inflammatory Arginase1	(21)
Mycobacterium tuberculosis, *Salmonella*, *Staphylococcus*	Wnt5a, Fzd4, Lrp5	Regulatory T cell expansion	(19)
Lawsonia intracellularis	Downregulated Wnt signaling (cytosolic β-CATENIN)	Inhibition of goblet cell maturation at peak of infection	(22)
Citrobacter rodentium	Upregulation of negative regulators of Wnt pathway - RNF43 ZNRF3	Intestinal stem cell renewal and differentiation, thereby preventing intestinal infections	(23)
Clostridium difficile	Fzd1, 2, 7	Assist in pathogen internalization	(24)
Salmonella	Axin1	Limits invasion into host cells	(25)
Citrobacter rodentium	Epigenetic downregulation of WIF1, leading to Wnt activation	Crypt hyperplasia and tumorigenesis	(28)

and TCF4, occupies the promoters of Wnt target genes and suppresses their expression (37). GPX4 acts as a major regulator of ferroptosis (iron-induced programmed cell death) by buffering peroxide levels, and is downregulated by infectious agents like Mtb (38). These observations offer a vast avenue for probing into the mechanistic insights of Wnt-associated cell death events during infections.

A major proportion of research on Wnt pathway in the realm of infectious diseases limits to intestinal bacterial infections and subsequent inflammation. However, as may be noted now that the potential implications of a versatile and complex pathway like Wnt has so far not been appropriately exploited in distinct infections and associated cellular consequences. With the expanding dynamics of antibiotic resistance among

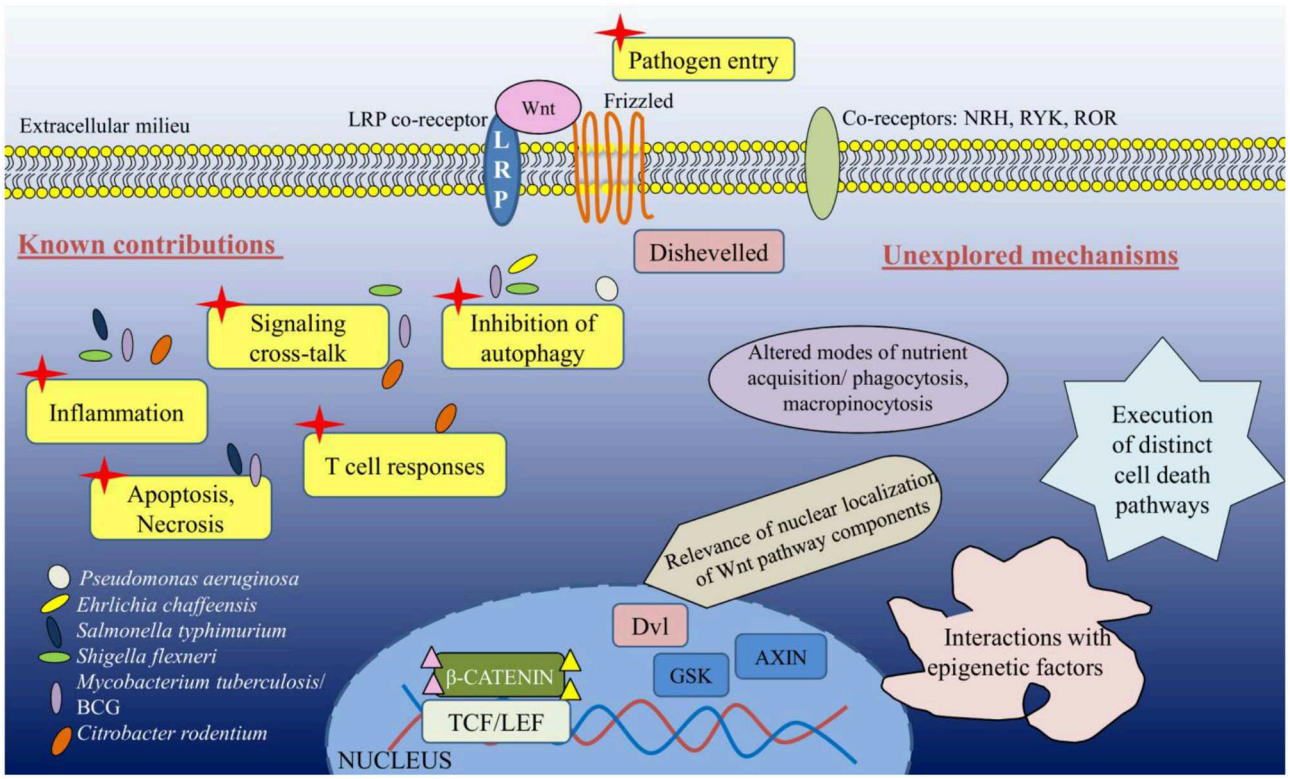

FIGURE 2 | The unexplored facets of Wnt signaling during bacterial infections.

globally relevant pathogens, host-directed therapeutics form an essential alternative (39), and we believe that unraveling the contributions of Wnt pathway would provide critical cues for the same. Several platforms are being synthesized for targeting the Wnt pathway in distinct pathologies, however, it is imperative to understand that the numerous ligand-receptor permutations along with their downstream cross-talks present an unavoidable drawback for Wnt-directed therapeutics (40). Moreover, the ubiquity of kinases like GSK3 would compromise the specificity of such attempts. Overall, though it would be highly challenging to design Wnt-specific therapies, it must be envisioned that a thorough analysis of the pathway with respect to its interactome, including chromatin modifiers/remodelers, cell death factors to name a few, and how they are redefined during infections would offer a novel paradigm for combinatorial adjuncts (**Figure 2**).

AUTHOR CONTRIBUTIONS

KB constructed the framework of the manuscript. TM wrote the initial draft. TM and KB finalized, edited, and proofread the manuscript.

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REVIEW
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Macrophages as an Emerging Source of Wnt Ligands: Relevance in Mucosal Integrity

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The Wnt signaling pathway is a conserved pathway involved in important cellular processes such as the control of embryonic development, cellular polarity, cellular migration, and cell proliferation. In addition to playing a central role during embryogenesis, this pathway is also an essential part of adult homeostasis. Indeed, it controls the proliferation of epithelial cells in different organs such as intestine, lung, and kidney, and guarantees the maintenance of the mucosa in physiological conditions. The origin of this molecular pathway is the binding between Wnt ligands (belonging to a family of 19 different homologous secreted glycoproteins) and their specific membrane receptors, from the Frizzled receptor family. This specific interaction triggers the activation of the signaling cascade, which in turn activates or suppresses the expression of different genes in order to change the behavior of the cell. On the other hand, alterations of this pathway have been described in pathological conditions such as inflammation, fibrosis, and cancer. In recent years, macrophages—among other cell types—have emerged as a potential source of Wnt ligands. Due to their high plasticity, macrophages, which are central to the innate immune response, are capable of adopting different phenotypes depending on their microenvironment. In the past, two different phenotypes were described: a proinflammatory phenotype—M1 macrophages—and an anti-inflammatory phenotype—M2 macrophages—and a selective expression of Wnt ligands has been associated with said phenotypes. However, nowadays it is assumed that macrophages *in vivo* move through a continual spectrum of functional phenotypes. In both physiological and pathological (inflammation, fibrosis and cancer) conditions, the accumulation and polarization of macrophages conditions the future of the tissue, facilitating various scenarios, such as resolution of inflammation, activation of fibrosis, and cancer development due to the modulation of the Wnt signaling pathway, in autocrine and paracrine manner. In this work, we provide an overview of studies that have explored the role of macrophages and how they act as a source of Wnt ligands and as mediators of mucosal integrity.

Keywords: macrophage, mucosal homeostasis, regeneration, fibrosis, cancer, Wnt ligands

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INTRODUCTION

Wnt signaling is a molecular pathway found across species and cell lines and which is absolutely necessary for development and for the growth, homeostasis, and regeneration of most tissues in an organism. This molecular cascade plays a pivotal role determining cell fate by controlling critical processes such as migration, polarity, regulation of the neural pattern, and organogenesis during embryonic development (1). Evidence of its central role in the development and homeostasis of several tissues is the fact that aberrant alterations of this molecular pathway have been involved in multiple human disorders and pathologies such as birth defects, autoimmune diseases, metabolic diseases, and cancer.

In the present review we summarize our current understanding of the main participants in Wnt signaling and the role of this pathway in mucosal homeostasis, regeneration, fibrosis, and cancer. In addition, we will describe the different sources of Wnt ligands in mucosal tissue, with a special focus on the role that macrophage-derived Wnt ligands play in physiological and pathological conditions.

WNT SIGNALING COMPONENTS

The interaction between a Wnt-secreted protein and a Frizzled receptor triggers the activation of several downstream pathways, which, in turn, has a bearing on the future of the cell and, indeed, that of the organism. One reason for the huge complexity of this pathway is the presence of multiple Wnt genes in any animal genome. In fact, in vertebrates, 19 different Wnt proteins have been identified and their expression is known to be temporally regulated during the development of the organism. Each Wnt protein is crucial, since the deletion of a given ligand from the genome triggers different phenotypes (2). Indeed, studies have demonstrated the role of specific Wnt ligands in the development of the organism. For instance, a lack of Wnt4 affects the normal development of the lung (3), whereas the absence of Wnt5a causes several alterations in the gastrointestinal tract (4).

Wnt ligands belong to a huge family of secreted glycoproteins of ∼40 kDa, which are highly hydrophobic and cysteine-rich. Although it has been reported that Wnt ligands can be expressed in different cell types, one feature of these proteins is cell and tissue specificity (5). These ligands have the ability to mediate cell-cell communication not only between two cells that are in contact, but also over short distances. Wnt proteins are made in a cellular compartment called the endoplasmic reticulum. There, an acyl group from palmitoleic acid is added to Wnt by the membrane-spanning enzyme Porcupine. This modification allows Wnt ligands to become hydrophobic and, consequently, to be secreted through exocytic vesicles or exosomes into the extracellular microenvironment. Following their secretion, they can act in either an autocrine or a paracrine manner in cells, where they bind specifically to a heterodimeric receptor complex (6–8). All Wnt ligands have been classified in two groups depending on their downstream effects: canonical Wnt ligands, which induce a β-catenin dependent pathway (Wnt1, 2, 3, 8a, 8b, 10a, and 10b), and non-canonical Wnt ligands, which induce β-catenin-independent pathways (Wnt4, 5a, 5b, 6, 7a, 7b, and 11) (9).

There are molecules that inhibit the Wnt signaling pathway. These can be subdivided into: secreted frizzled-related proteins (SFRP1-5), Dickkopf family proteins (DKK1-4), Wnt inhibitory factor (Wif1), Wise/SOST, and Cereberus. The mechanism of action involved in the inhibition of this pathway varies: while SFRPs, Wif1, and Cereberus are able to sequester Wnt agonists in the extracellular environment, DKK proteins and Wise/SOST compete with Wnt agonists and prevent their binding with the receptor LRP5/6 (10).

The activation of Wnt signaling initiates when a Wnt ligand binds specifically to a cell surface heterodimeric complex composed of two components: a member of the Frizzled receptor (FZD) family and a second receptor, which can be any one of the following: the low density lipoprotein receptor-related protein 5/6 (LRP5/6), receptor-like tyrosine kinase (RYK), the Neurotrophin Receptor Homolog 1 (NRH1), the receptor tyrosine kinase like orphan receptor 2 (ROR2), or Tyrosine-protein kinase-like receptor 7 (PTK7). In vertebrates, the FZD family includes 10 different receptors (FZD1-10), all of which are composed of an extracellular region with an N-terminal sequence and a cysteine-rich domain, a seven-pass transmembrane domain and an intracellular domain with the C-terminal sequence. One of the most striking properties of these receptors is the fact that one FZD receptor can interact with different Wnt ligands to activate either canonical or non-canonical pathways. This characteristic, together with the presence of several Wnt ligands, makes it very difficult to understand this cascade (11). Indeed, the selective binding of each Wnt protein with a given FZD receptor is still poorly understood, and further research need to be performed in order to further our knowledge.

As mentioned above, Wnt signaling is classically subdivided into canonical and non-canonical pathways. In the canonical pathway, the activation of a FZD receptor and its co-receptor LRP5/6 by a canonical Wnt ligand triggers the inhibition of the complex, which destroys β-catenin due to the recruitment of the disheveled protein (Dsh) (12). This inhibition provokes the accumulation of the non-phosphorylated β-catenin in the cytosol and its translocation into the nucleus, where it interacts with Tcf/Lef transcription factors and some co-activators, thereby activating the transcription of the target genes involved in this molecular pathway (13).

On the other hand, the non-canonical pathway is initiated when a non-canonical Wnt ligand binds to a Frizzled receptor as well as with another co-receptor, which could be NRH1, Ryk, PTK7, or ROR2. In this case, the Wnt/Ca+2 or Planar Cell Polarity (PCP) pathways are activated depending on the cell type, meaning that this branch of the Wnt pathway is even more complex and less studied than the canonical pathway. Despite these differences, it is important to point out that both canonical and non-canonical pathways are crucial to essential processes such as apoptosis, proliferation, survival, cell motility, or cell fate (14).

WNT PATHWAY IN MUCOSAL HOMEOSTASIS

The importance of the Wnt signaling pathway in embryogenesis has been demonstrated by murine β-catenin knock-out embryos, which fail to develop the endodermal and mesodermal germ layers and are unviable (15). Wnt signaling is also required for the further differentiation of embryonic stem cells. Specifically, this pathway is temporarily regulated, so that it is activated to differentiate toward the mesoderm lineage or is inactivated to create the neuroectoderm (16).

The Wnt cascade also constitutes a key molecular pathway in the homeostasis of different tissues throughout the life cycle of adult mammals (17). In the next part of this review, we will discuss in more detail the relevance of this molecular cascade in the maintenance of mucosal integrity, specifically in the intestine and lung.

Intestine

The intestinal epithelium is the most rapidly-renewing tissue in the body of adult mammals, regenerating every 4–5 days, while the Wnt signaling pathway is closely implicated in the homeostasis of this tissue. The first hints of the relevance of the Wnt pathway arose during genetic experiments with mice. Korinek et al. reported that the lack of TCF4, one of the central downstream effectors of Wnt signaling, caused an absence of proliferative crypts in neonatal mice (18). In line with this, the maintenance of the intestinal crypts in adult mice is dependent on Wnt signaling, since the conditional ablation of TCF4 triggers the loss of most of the proliferative crypts (19). Furthermore, the role of Wnt antagonists and Wnt agonists has also been demonstrated, with the overexpression of the Wnt antagonist DKK1 provoking a complete loss of proliferation (20). On the other hand, the transgenic expression of the strong Wnt agonist R-spondin 1 exacerbates the hyperproliferation of the intestinal crypts (21).

This rapid self-renewal is guaranteed by the presence of Intestinal Stem Cells (ISCs) at the base of the intestinal crypts. Specifically, two types of ISCs have been identified: crypt-based columnar stem cells (CBCs), which express the cell-surface marker leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and continually proliferate under homeostasis and an alternative reserve ISC population, which are quiescent ISCs and located in position +4 (22). Given that stem cells are located in the lower part of the crypt, Wnt signaling is present to a greater extent in the bottom of the crypt, and its activation gradually ameliorates toward the top of the crypt. It is widely assumed that Lgr5+ CBC stem cells are the driving force of tissue renewal, since they proliferate and generate a subpopulation known as transit-amplifying cells (TA cells), which quickly divide and migrate toward the upper part of the crypt, where they finally differentiate into secretory, absorptive, or enteroendocrine lineages (23–25). Therefore, as a consequence of the high rates of proliferation and differentiation among intestinal crypts, the Wnt signaling pathway coexists with several molecular pathways, such as Notch and Hedgehog, and the homeostasis of this tissue depends on the correct balance of all these pathways (26).

The intestinal stem cell niche provides the optimal environment to sustain the self-renewing and multipotent behavior of the ISCs (27). This intestinal niche is maintained due to the presence of the subepithelial mesenchyme, which is composed of myofibroblasts, fibroblasts, neuronal, and smooth muscle cells. Among all the essential factors secreted by these mesenchymal cells, Wnt ligands constitute one of the most abundant. In this way, it has been recently reported that blockage of the Wnt ligands secreted by subepithelial telocytes impairs epithelial renewal and alter intestinal integrity (28). In the same way, Wnt ligands from subepithelial Gli1+ mesenchymal cells ensure the presence of stem cells, since the specific blockage of said Wnt ligands provokes a loss of stem cells, thus altering the integrity of the intestinal epithelium and resulting in epithelial death (29).

Apart from the importance of mesenchymal cells in maintaining the intestinal stem niche, Paneth cells also secrete essential growth signals, including Wnt3, EGF, and Notch ligands. Specific Paneth are located between the CBCs, since these cells do not migrate upwards like the rest of the differentiated epithelial cells, but rather migrate downwards and constitute a niche for intestinal stem cells at the base of the intestinal crypts. The importance of these specialized intestinal epithelial cells was demonstrated by the loss of Lgr5+ stem cells due to the genetic ablation of Paneth cells *in vivo* (30). Interestingly, among all the Wnt components, these cells exhibit a pronounced and specific expression of the Wnt3 ligand. The importance of this Wnt ligand specifically from Paneth cells seems differ between *in vitro* and *in vivo* conditions. In fact, Wnt3 from Paneth cells acts as an essential niche factor for the growth and development of organoid cultures *in vitro*, while mesenchymal Wnt ligands compensate for the lack of Wnt3 from Paneth cells *in vivo* (31). In addition, Paneth cells are dependent on Wnt, which is evident in the presence of an autocrine loop. In this sense, the Wnt signaling pathway is important for the development, differentiation and maturation process of the epithelial cells that regulated the expression of the alpha-defensins HD5 and HD6 (32).

Lung

The lung is another organ whose development and homeostasis is regulated by the Wnt pathway. In 1990 Gavin et al. identified six new members of the Wnt family that are involved in the development of the lung in the fetus (33). That report spawned several further studies using different knock-out mice of several Wnt ligands which confirmed the relevance of this molecular pathway in lung development by (34–36). Recent reports have highlighted an emerging role of the non-canonical Wnt pathway in lung development. In line with this, experiments with Wnt5a knock-out mice have demonstrated that the absence of this non-canonical ligand results in several defects in the lung, such as abnormal capillary formation and endothelial differentiation and an impaired differentiation of alveolar epithelial cells (37–39).

On the other hand, the Wnt signaling pathway is not only essential in the development of the lung, but also in lung morphogenesis and homeostasis (40). Several reports have

demonstrated—by both *in vitro* and *in vivo* approaches—that the Wnt pathway is part of a tightly-honed interplay between all the different cell types present in the lung, including the epithelium, mesenchyme, and endothelium. Indeed, the lack of Wnt2, Wnt4, or Wnt7b causes lung hypoplasia (3, 34, 41). In addition, receptors such as LRP-5/6 and Wnt antagonists such as sFRP-1 and Dkk-1 have also exhibited an important role in lung morphogenesis. In this sense, the genetic deletion of LRP-5 has been shown to impair alveolar formation and angiogenesis in neonatal mice (42), whereas the accumulation of nuclear β-catenin in SFRP-1 knock-out mice increases mesenchymal proliferation and impairs alveolar formation (43).

As occurs in the intestine, the Wnt signaling pathway of the lungs manages the successful proliferation and differentiation of the stem cell progenitors located in the lung into airway smooth muscle cells (ASMCs). In fact, Cohen et al. demonstrated that Wnt7b and β-catenin control the differentiation and development of ASMCs through the Platelet-derived growth factor receptor (PDGFR) pathway (44). Although most studies demonstrate the relevance of the Wnt pathway in mice, few studies have revealed the importance of this molecular cascade in human lung morphogenesis and homeostasis. The expression of several Wnt ligands (Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8b, Wnt10b, Wnt11, and Wnt16), Wnt receptors (FZD1, FZD2, FZD3, FZD4, FZD6, FZD7, FZD8, LRP5, and LRP6), β-catenin and members of the TCF/LEF family has been detected in human epithelial cells (40).

WNT PATHWAY IN MUCOSAL REGENERATION

One of the molecular pathways involved in the complex process of regeneration is the Wnt signaling pathway. In non-regenerating vertebrates, the Wnt pathway is active only in specific tissues with high turnover, such as the intestine, hematopoietic compartment, and epidermis, where it preserves the self-renewal of stem cells necessary for homeostasis. However, when a tissue is injured, a cascade of molecular events is activated in order to regenerate the damaged tissue as soon as possible (45). In the following section, we will review current understanding of the role of the Wnt pathway in mucosal regeneration specifically in the intestine and lung.

Intestine

Due to its elevated turnover the intestinal epithelium has the ability to regenerate after multiple stresses or harmful agents that disrupt the tissue, such as gamma radiation, cytotoxic drugs, or after surgical resection. The process of intestinal regeneration is mainly characterized by an enhanced proliferation of the intestinal crypts. Indeed, when the intestinal tissue is damaged, the crypts close to the injury increase their proliferation in order to close the wound as soon as possible. The regenerative process has been widely studied and can be subdivided into four steps: an apoptotic phase, characterized by a continuous loss of crypts; a reduction of the crypt size and shortening of the villi, a regenerative phase during which crypts close to the injury proliferate and close the wound; and the normalization phase, in which the size and length of the new crypts are normalized (46).

Several studies have demonstrated that the activation of the Wnt signaling pathway plays a pivotal role in the induction of intestinal regeneration. In response to the damage, this pathway is activated and the expression of *c-Myc*, one of the most well-known Wnt targets, is increased and promotes the regeneration of the epithelial cells through Focal adhesion kinase (Fak) and Akt/mTOR signaling (47). Furthermore, the Wnt5a from the macrophages present in the mesenchyme activates the regeneration of the intestinal crypts through TGF-β signaling (48). Recently, it has been reported that Wnt2b from intestinal epithelial cells is essential for intestinal regeneration because it induces the proliferation of the subpopulation of the quiescent intestinal stem cells located at position +4 Tert+ stem cells. In the study in question, Suh et al. showed that, although the conditional ablation of these Tert+ stem cells did not alter intestinal homeostasis, tissue regeneration was significantly impaired after the injury (49). Hence, it is clear that intestinal regeneration is a complex process regulated by several non-redundant Wnt ligand sources that are crucial for the activation of the Wnt pathway and the complete regeneration of the intestine after insult.

Given the central role of the Wnt pathway in intestinal regeneration, this molecular cascade acquires a huge importance in patients with Inflammatory Bowel Disease (IBD), who suffer continuous episodes of intestinal inflammation and recovery (50). We have previously reported that this pathway is more activated specifically in the damaged mucosa of IBD patients compared with the non-damaged mucosa of the same patients. Moreover, we have shown how the activation of the Wnt signaling pathway with a Wnt agonist accelerates mucosal regeneration after acute TNBS-induced colitis (51, 52). In line with our studies, Tao et al. have recently demonstrated that microRNA-31 promotes the regeneration of intestinal epithelium following injury by regulating the Wnt signaling pathway (53).

Lung

The epithelium of the lung can be injured by insults such as inflammation, infection, allergic reactions (asthma), physical trauma, or inhaled particles coming from cigarettes or pollen. All of these stresses provoke damage to the pulmonary epithelium, characterized by the disruption of the tight junctions between epithelial cells and impaired epithelial barrier function, which in turn leads to increased permeability, debilitated cell-cell adhesion, necrosis, and apoptosis of epithelial cells. After an injury, the regenerative mechanism is initiated in order to restore the pulmonary epithelium (54). In this context, the Wnt signaling pathway is also essential for the regeneration of the lung. It is important to consider that, although the cellular proliferation in this organ is weaker than in others, the presence of an injury still triggers the activation of this molecular cascade. The Wnt signaling pathway regulates the activity and control of lung stem cells, which differentiate toward either mesoderm or endoderm progenitor cells as a result of this molecular cascade (55). In line with this, McCauley et al. have recently published an elegantly performed study consisting of experiments with

airway organoids generated from single-cell lung progenitors, demonstrating that the Wnt signaling pathway plays a central role in the regulation of the proximal and distal epithelial pattern in both murine and human pluripotent stem cells. Their results have underlined the essential role of this pathway in lung regeneration, revealing a significant reduction in tissue repair when the Wnt pathway was absent (56).

The central component of the canonical Wnt pathway, β-catenin, mediates the regeneration of the lung, thus acting as a transcription factor which activates the expression of genes involved in epithelial regeneration and regulating the tight junctions of the epithelial cells in the lung. In this respect, Cai et al. demonstrated that overexpression of β-catenin enhanced the differentiation of mesenchymal stem cells of the lung into alveolar epithelial type II cells, which improved the permeability of the lung epithelium and reduced the acute inflammation and lung fibrosis caused by administration of lipopolysaccharide (57). The activation of the Wnt pathway after injury to the pulmonary epithelium stimulates the proliferation of epithelial cells and the secretion of FGF-10, which subsequently activates β-catenin in different epithelial cells and enhances the proliferation and regeneration of the damaged epithelium (58). As previously mentioned, β-catenin also contributes to pulmonary regeneration, acting as an adherens junction protein. Finigan et al. reported that the central protein of the Wnt signaling pathway mediates pulmonary epithelial permeability, showing that the human epidermal growth factor receptor-2 (HER-2) interacts with β-catenin to trigger the dissolution of the adherens junction, reduce the cell-cell adhesion and alter pulmonary epithelial barrier function (59).

As occurs in the intestine, due to the pivotal role of the Wnt signaling pathway in the homeostasis and regeneration of the lung, this molecular cascade acquires huge protagonism in pathologies of the lung, such as pulmonary arterial hypertension (PAH), asthma and Chronic obstructive pulmonary disease (COPD) (60). GWAS studies have identified several polymorphisms in genes of the Wnt signaling pathway in asthmatic patients. Specifically, a single nucleotide polymorphism (SNP; rs2929973) in the WISP1 gene has been associated with asthma in children (61). In addition, a more recent GWAS edition has identified SNPs close to the genes FZD3 and FZD6 in asthmatic patients (62). In addition to genetic analysis, an enhanced expression of some Wnt components such as WNT5a and FZD5 has been demonstrated in patients with Th2-high asthma (60).

WNT PATHWAY IN FIBROSIS

Fibrosis is characterized by an exacerbated accumulation of components of the extracellular matrix (ECM), mainly collagen fibers. This pathogenic mechanism is associated with a huge number of diseases, and can appear in several tissues, eventually culminating in organ failure. Several molecular pathways, including transforming growth factor-β (TGF-β), Notch, BMP, NF-κB, and Wnt signaling, have been associated with the development of fibrosis (63–66). In recent years, Wnt signaling has emerged as a central pathway that regulates different steps of this complex process. In addition, a profound crosstalk between TGF-β (the master regulator of fibrosis) and Wnt signaling pathways is present in both extracellular and intracellular compartments. In this regard, TGF-β and Wnt ligands can regulate each other, whereas TGF-β in the cytoplasm can induce the translocation of β-catenin into the nucleus through Smad3 (67).

In normal conditions, in response to an insult that causes damage, both epithelial and endothelial cells secrete pro-inflammatory cytokines, which leads to the formation of blood clots and a provisional extracellular matrix. Subsequently, epithelial, endothelial cells and myofibroblasts secrete matrix metalloproteinases, which degrade the basement membrane and several cytokines and chemokines that recruit and activate neutrophils, leukocytes, macrophages, T cells, B cells, and eosinophils. This initial inflammatory stage is followed by a remodeling stage during which activated myofibroblasts promote the closure of the wound. These active myofibroblasts can originate local fibroblasts recruited from bone marrow, or from epithelial or endothelial cells derived from epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndEMT), respectively (68). The persistence or chronification of these processes leads to fibrosis (69). In the next section of this review, we will describe the specific relevance of the Wnt signaling pathway in fibrosis originating in the intestine and lung.

Intestine

Intestinal fibrosis is one of the most frequent complications in Crohn's Disease (CD) patients, and leads to an irreversible stenosis of the colon. It is believed that intestinal fibrosis in these patients is the consequence of chronic inflammation, and the therapeutic goal has been to reduce the inflammatory process. Nevertheless, anti-inflammatory drugs are incapable of resolving this complication, so surgery is currently the only option to remove fibrotic tissue, and does not necessarily prevent recurrence (70). To our knowledge, not a single study has analyzed the relevance of the Wnt signaling pathway in intestinal fibrosis. We have recently reported that, compared with non-IBD patients, Crohn's sufferers whose condition is characterized by stenotic and penetrating behavior exhibit higher levels of profibrotic markers, enhanced gene expression of FZD4 and Wnt target genes such as C-MYC, LGR5, and CYCLIN D1, and higher protein levels of β-catenin. In the same study we have also demonstrated that WNT2b activates EMT in intestinal epithelial cells through FZD4. In this way, we have demonstrated for the first time that there is an association between the Wnt signaling pathway and intestinal fibrosis, which suggests that the Wnt signaling pathway is also important in the development of intestinal fibrosis (71).

Nevertheless, the functional role of the Wnt pathway in the development of intestinal fibrosis *in vivo* is still not clearly determined. Therefore, further studies should be performed in order to analyse the specific role of Wnt signaling in each stage of intestinal fibrosis. In light of the body of evidence from studies performed in fibrosis in other organs, such as lung or kidney, we

hypothesize that pharmacological inhibition of the Wnt pathway will also be beneficial in intestinal fibrosis. However, future studies are needed to confirm this hypothesis. As we will describe in the following section, there is considerable data about the role of Wnt signaling in lung fibrosis, while, as far as we know, no studies have been published about its role in intestinal fibrosis.

Lung

Idiopatic pulmonary fibrosis (IPF) constitutes an important complication characterized by damage to the lung epithelium and activation of fibroblasts with an excessive accumulation of extracellular matrix components, all of which triggers a progressive loss of the functions of the lung. In this pathological scenario, an overactivation of the canonical Wnt/β-catenin pathway has been described in both human patients and experimental mice models. In line with this, the lungs of IPF patients exhibit an increased gene expression of WNT1, WNT7b, WNT10b, FZD2, FZD3, β-catenin, and LEF1 compared with subjects without IPF. These results have been reinforced with immunohistochemical studies which have revealed an accumulation of WNT1, WNT3a, and β-catenin specifically in the bronchial and alveolar epithelium and in myofibroblasts in IPF patients (72, 73). The functional relevance of WNT1-inducible signaling protein-1 (WISP1) was demonstrated by Königshoff et al., who reported an attenuation of lung fibrosis through WISP1 neutralization *in vivo*. In their elegantly performed study, the authors also demonstrated that WISP1 regulates alveolar epithelial cell function and the reprogramming of myofibroblasts (73). WISP1 has also been described as a downstream mediator of some profibrotic factors, such as miR-92A, Transforming growth factor β (TGFβ) and Tumor necrosis factor α (TNFα) in human lung fibroblasts, which points to a possible pharmacological target for IPF treatment (74). Of interest, the implication of the non-canonical Wnt pathway has been reported in pulmonary fibrosis. Indeed, it has been described that WNT5b activates profibrotic signaling in a β-catenin-independent pathway through the FZD8 receptor. Vuga et al. expanded these observations by demonstrating that the non-canonical Wnt ligand Wnt5a regulates the deposition of ECM by fibroblasts in the lung and protects them against apoptosis induced by oxidative stress (75). On the whole, the literature offers firm evidence that both canonical and non-canonical Wnt pathways mediate the development of fibrosis in the lung.

Given the pivotal role of the Wnt signaling pathway in the development of pulmonary fibrosis, huge efforts have been made in order to prevent the over activation of this molecular pathway by targeting this cascade at different molecular levels, with promising results having been reported to date. In this sense, the effectivenss of tankyrase inhibitors as pharmacological agents was demonstrated by Wang et al. when they reported improved survival and amelioration of the lung fibrosis induced by bleomycin after administration of the tankyrase inhibitor XAV939. In said study, the authors proposed that this inhibitor affords benefits by reducing fibroblast proliferation, impairing differentiation of myofibroblasts and enhancing differentiation of bone marrow-derived mesenchymal cells into epithelial cells (76). Cao et al. recently reported that the inhibition of Wnt signaling pathway with a peptidomimetic small-molecule inhibitor called ICG-001 impairs myofibroblast differentiation of resident lung mesenchymal stem cells and attenuates lung fibrosis (77). The therapeutic option of inhibiting the Wnt signaling pathway has recently been strongly reinforced when Chen et al. showed that blocking the Wnt pathway with a glycoprotein called *Thymocyte differentiation antigen-1* improves pulmonary fibrosis through a reduction in the proliferation of fibroblasts in cases of acute interstitial pneumonia (78).

Nevertheless, it is important to take into account that, whereas the inhibition of this pathway produces anti-fibrotic effects, the activation of the Wnt pathway in pulmonary epithelial cells during the early stages of injury is necessary in order to repair and regenerate the tissue. Therefore, further studies should be performed in order to modulate this molecular cascade, specifically at optimal moments.

WNT PATHWAY IN CANCER

Cancer englobes a group of pathologies characterized by an uncontrolled growth of some cell types, which spread throughout the whole organism and invade different tissues. An aberrant Wnt signaling pathway has been closely related to a broad range of cancers, from their initiation to their development, due to the dysregulation of the Cancer Stem Cells (CSCs) (79). Apart from its relevance in CSCs, the Wnt pathway plays an important role in the activation of EMT in tumor cells, thus promoting cancer metastasis and progression (80).

Intestine

Colorectal Cancer (CRC) is a frequent and lethal disease originating from an alteration of the epithelial cells that line the colon or rectum as a result of the most common mutations of the Wnt signaling pathway, which triggers an uncontrolled proliferation and migration of these cells around the organism. The first evidence of a role for Wnt signaling in cancer was published in 1991, when Nishisho et al. reported that the gene known as Adenomatous Polyposis Coli (APC), which encodes a protein implicated in the control of nuclear β-catenin, was mutated in patients with non-inherited forms of CRC (81). Since then, accumulated evidence has confirmed that an over activation of this molecular pathway leads to the formation of adenomas and, subsequently, to the development of colon carcinoma. In fact, 90% of colon carcinoma patients present acquired mutations in APC, and these mutations determine differences in the location of tumors along the large intestine (82, 83). Of interest, the reversible knockdown of APC with shRNA in a murine model of carcinogenesis promotes regression from an adenoma to normal tissue, which highlights the importance of this pathway in the maintenance of the tumor (84). A feature of CRCs that is still a mystery is the abundance of mutations specifically in APC, rather than in other WNT components. Indeed, mutations in AXIN or β-catenin genes are present only in a small fraction of CRC patients (around 5–6%) (85). This special characteristic—described only in CRC, and not in other types of cancer—needs to be addressed in the future.

The survival of these patients has increased considerably in recent years, but this has been achieved due to advances in surgery, adenoma detection, chemotherapy, and recent drugs targeting VEGF and EGFR signaling pathways. Despite the clearly pivotal role of the over activation of the Wnt signaling pathway in CRC patients and the huge amount of different strategies to block this molecular cascade, no therapy has yet obtained significant results in CRC treatment (85). The Wnt signaling pathway has been targeted at three different points: blocking the interaction between the Wnt ligand and its receptor; targeting the destruction complex of the β-catenin; and targeting the transcriptional activity of β-catenin. The first strategy has not obtained significant advances in CRC treatment, since most CRCs activate the Wnt pathway independently of a specific Wnt ligand. Hence, although some drugs, such as Vanticumab or Ipafricept, have progressed in clinical trials in several cancers (e.g., breast, pancreatic, and ovarian), none of them have achieved good results in CRC (86). Regarding the second strategy, some studies have shown that inhibition of Tankyrase enzymes (TNKS) improves and synergizes with approved chemotherapies and drugs which target PI3K-AKT and RAS-MAPK signaling pathways (87, 88). However, these TNKS inhibitors have a high toxicity and also interfere with the stability of proteins essential in the elongation of telomeres and cell division (89). Finally, with respect to the third strategy, the drug PRI-724, which blocks the interaction between β-catenin and the CREB-binding protein, is currently in Phase I trials, and at Phase 2 in a randomized study in which it is being used in combination with FOLFOX/Bevacizumab to treat metastatic CRC (85). As a whole, it is clear that further studies must be performed in order to identify safer molecules to target over activation of the Wnt signaling pathway, the driving force of this type of cancer. We can only hope that the huge research efforts currently being made will provide significant clinical benefits and improve the quality of life of CRC patients.

Lung

Lung cancer is one of the most devastating forms of cancer, and can originate as one of two types depending on clinical, molecular, histological and endocrinological characteristics: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (90). Unlike colon cancer, lung cancer is rarely associated with APC mutations, and the accumulation of Wnt proteins is mainly caused by dysregulation of the transcription of Wnt ligands. In this context, an exacerbated activation of either canonical or non-canonical Wnt pathway has also been detected in lung cancer, specifically in patients with non-small cell lung cancer. In fact, an accumulation of several Wnt participants, including WNT1, WNT2, WNT3, WNT5a, FZD8, Dsh, Porcupine, and TCF4, has been reported in patients with lung cancer, and this accumulation is associated with a poor prognosis (91). In addition, in certain NSCLC subtypes, a shift from the canonical to the non-canonical Wnt pathway has been detected, while both Wnt pathways have been involved in lung cancer metastasis, since the metastatic stage of lung tumors has been associated with EMT linked to β-catenin dependent signaling and the expression of the non-canonical WNT5a, which increases the expression of fibroblast growth factor (FGF) 10 and sonic hedgehog (SHH) (92, 93). Moreover, the expression of matrix metalloproteinases, whose levels increase at the metastatic stage, can be activated by both canonical and non-canonical Wnt signaling pathways (94). It is important to point out that the combination of the enhanced Wnt pathway with a constitutive activation of different molecular pathways (such as the KRAS pathway) caused by the mutations in KRAS triggers an increase in tumor size and a poor prognosis (95). The central role of the Wnt pathway in lung cancer has recently been endorsed by Wagner et al., who have just shown for the first time that activation of the canonical Wnt is involved in resistance to chemotherapy in SCLC (96).

As occurs in the intestine, given the pivotal role of the aberrant activation of the Wnt pathway in all stages of lung cancer development, several compounds have been designed and tested. These Wnt inhibitors can be subdivided into two different categories: small molecule inhibitors and biologic inhibitors. The first group of molecules includes several Wnt antagonists (e.g., ICG-001, XAV939, AV-65, PRI-724, NCTD, etc.), whereas biologic inhibitors include antibodies such as OMP-18R5, OMP-54F28, OTSA101, small interfering RNA, short hairpin RNA, and recombinant proteins such as adenoviral sLRP6E1E2 (97). The strategies used in order to block the Wnt pathway have been basically the same ones used in other cancer types, such as colon carcinoma. Several studies have demonstrated that several small molecule compounds which target β-catenin, such as XAV939, ICG-001, or TNKS inhibitors, reduce the proliferation of lung cancer stem cells (98–100). The beneficial role of some natural compounds (e.g., curcumin or 25-hydroxyprotopanaxadiol) that reduce NSCLC cell proliferation and invasion through the inhibition of the Wnt pathway has also been reported (101, 102). On the other hand, antagonist monoclonal antibodies against the Wnt ligands Wnt1 and Wnt2 have provided beneficial results in various cancers, including NSCLC (103). The positive role of monoclonal antibodies in combatting FZD receptors has also been demonstrated in lung cancer. In this context, the antibody Vantictumab (OMP-18R5), which can undermine FZD1/2/5/7/8 receptors is currently being used in combination with docetaxel in Phase I of a Clinical Trial with patients with NSCLC (104). Despite all the progress achieved in the treatment of patients with lung cancer by specifically targeting the Wnt signaling pathway, this pharmacological strategy is still at a very primary stage, and further studies must be performed in the coming years in order to improve the safety and efficacy of all these drugs. Nevertheless, the results obtained in preclinical studies and clinical trials to date endorse the Wnt signaling pathway as a promising pharmacological target in the treatment of lung cancer.

SOURCES OF WNT LIGANDS

It is logical to consider that, given the diversity of the non-pathological and pathological processes that are regulated by the Wnt signaling pathway, Wnt ligands have become the subject of study for many years. Since the first Wnt ligand was identified more than 30 years ago, several Wnt proteins

have been identified, but the specific role of each ligand is still not well-characterized. Although there is a growing body of literature regarding the role of Wnt pathway components in several cell types, very little is known about the different sources of Wnt ligands and the relevance of those sources in different pathological scenarios. Therefore, we will now describe the sources of Wnt ligands and will focus specifically on macrophages as a source of Wnt ligands, mainly in inflammatory conditions.

Epithelial cells constitute an important source of Wnt ligands in several tissues, including intestine, bone, and kidney. In the intestine, the role of epithelial-secreted Wnt ligands remains controversial; several reports demonstrate that these epithelial Wnt ligands are redundant and non-essential (31, 105, 106), while a recent study from Zou et al. has demonstrated that Wnt proteins in epithelial cells are necessary for the expansion of stem cells after virus-induced villus damage (22). The authors of the aforementioned study proposed that epithelial Wnt ligands are important for the repair of the intestinal epithelium, in contrast to the redundant role of epithelial Wnt proteins in intestinal homeostasis. Epithelial Wnt ligands are essential for the maintenance of the bone, since these proteins activate the Wnt signaling pathway in osteoblasts and osteoclast precursors, thus preventing bone resorption (107). Regarding the role of epithelial Wnt ligands in the kidney, it is known that Wnt9b from epithelial cells is involved in nephrogenesis, whereas epithelial Wnt11 regulates the control of ureteric bud branching (108). However, Wnt ligands from epithelial cells not only regulate physiological functions, but are also involved in pathological conditions. In this sense, specifically in the kidney, tubular epithelial cells are the predominant source of dickkopf 3 (DKK3), which acts as a profibrotic and immunosuppressive molecule that promotes renal fibrosis (109).

Mesenchymal cells have also been identified as an important source of Wnt ligands in most tissues. In the intestine, unlike that which happens with epithelial Wnt ligands, mesenchymal-derived Wnt proteins play a key role in the regulation of the proliferative status of the stem cells (105, 110, 111). Specifically, in the kidney, the Wnt4 ligand of the metanephric mesenchyme is required for tubule formation (112). Mesenchymal cells in the lung are an important source of Wnt ligands, since stromal fibroblasts secrete, Wnt ligands, including Wnt5a, which maintains alveolar stem cells and their capacity for proliferation (113). In addition, other cellular sources of Wnt ligands have been reported, such as endothelial cells and adipocytes. In the liver, Wnt ligands from endothelial cells, specifically Wnt2 and Wnt9b, help to maintain the progenitor niche (114), while frizzled-related protein 5 secreted from adipocytes controls the white adipose tissue under metabolic stress (115).

Immune cells such as macrophages, dendritic cells (DCs), T cells, and platelets can also act as cellular sources of Wnt ligands. DCs secrete Wnt ligands, whose functions include the modulation of the immune responses mediated by B and T cells. Indeed, Kim et al. demonstrated that follicular dendritic cells secrete Wnt5a, which protects isolated germinal center B cells from apoptosis through activation of the Wnt/Ca+2 pathway (116). Reinforcing this observation, Valencia et al. reported that Wnt5a from DCs exerts a regulatory effect on both dendritic cells and T cells and increases secretion of IL-2 and IFN-γ, in an autocrine and paracrine manner (116, 117).

Platelets play important roles in inflammation and act as a source of molecules that impair the activation of Wnt signaling in different cells. Among them, DKK1 derived from platelets is the most well-characterized, having been implicated in several pathological conditions. Platelets are able to inhibit Wnt signaling pathway in alveolar epithelial cells through DKK1, thus contributing to pulmonary inflammation by inducing the adhesion of both macrophages and neutrophils to alveolar epithelial cells (118). Another pathological role for DKK1 secreted from platelets has been reported in *Leishmania major* infection, since pharmacological blockage of DKK1 with the inhibitor WAY-262611 was found to weaken the cytokine production of Th2 cells and leukocyte infiltration, which protected mice from the infection (119).

T cells, one of the key protagonists of the adaptive immune response, can also act as a source of molecules that antagonize Wnt signaling. These T cell-derived Wnt antagonists modulate the immune system, triggering changes in the immune response in several conditions. Treg cells can prevent T cell-mediated colitis through Dickkopf-1 (120), while DKK3 from T cells have been implicated in the regulation of the tolerance of CD8 T cells (121).

MACROPHAGES AS A SOURCE OF WNT LIGANDS

Macrophages are cells involved in the innate immune response, and play a variety of roles in the inflammatory process. They are essential in the release of inflammatory mediators, but also modulate wound healing and fibrosis development. These varying functions are a result of the way these cells change their phenotype depending on the microenvironment. Accumulative evidence demonstrates that macrophages constitute a significant source of Wnt proteins in adult tissues (122–127), and the synthesis of these ligands seems to depend on their phenotype (51). These observations, joined with the fact that these cells usually accumulated in chronic inflammation, point to the relevance of macrophage-derived Wnt ligands in mucosal regeneration and in complications such as fibrosis and cancer.

Macrophages display remarkable plasticity and can adopt both pro- and anti-inflammatory phenotypes, allowing them to switch between homeostatic and tissue repair functions. The differentiation programs involved in macrophage polarization have been categorized as classic M1-type activation and alternative M2-type activation. As a consequence of several stresses, such as inflammatory pathway activation or the presence of pathogenic organisms, monocytes can adopt an M1 pro-inflammatory phenotype, exerting a relevant role in host defense, and promote a T_H1-like immune response. M1 polarization is characterized by upregulation of cell surface activation markers and molecules involved in antigen presentation, such as major histocompatibility complex class II, CD16, CD32, CD80, CD86, and IL-1 receptor, as well as the production of

a range of pro-inflammatory molecules (IL-1, IL-6, IL-12, Il-23, inducible nitric oxide synthase, matrix metalloproteinase 12, and macrophage-inducible C-type lectin). During the repair phase, M2 macrophages predominate, originating from *in situ* proliferation, differentiation from infiltrating monocytes, or phenotype switch from M1 macrophages. M2 macrophages have a wide range of functions, which include the suppression of inflammation and promotion of tissue repair. The M2 phenotype has been classified into three different M2 subpopulations (M2a, M2b, and M2c) depending on the basis of their phenotype and function. Thus, macrophages are alternatively activated *in vitro* in the following manners: a) IL-4/IL-13 are designated as M2a macrophages; b) immune complexes and Toll-like receptor (TLR) and/or IL-1R agonist are designated as M2b; and c) IL-10, transforming growth factor-β (TGF-β) and glucocorticoids are designated as M2c. M2 markers, including CD206, CD163, arginase 1, Dectin 1, IL-10, IL-4Ra, and TGF-β_1, are more highly expressed during late inflammation and granulation (128).

In the following sections, we describe the role of macrophages as a source of Wnt ligands in mucosal regeneration, fibrosis and cancer. As a general observation, the synthesis of these ligands seems to be associated with an M2 macrophage phenotype, while other mediators involved in mucosal proliferation and differentiation, such as Notch ligands, have recently been associated with a subpopulation of pro-inflammatory macrophages (51, 129, 130).

Macrophage-Derived Wnt in Mucosal Regeneration

The specific expression of Wnt ligands by the macrophage phenotype was first analyzed by our group. We demonstrated that the *in vitro* polarization of human macrophages toward a M2 phenotype by means of treatment with IL4 was associated with increased levels of Wnt1 and Wnt3a with respect to non-polarized or M1 (LPS + IFNγ-treated) macrophages (51). In addition, we detected activation of the Wnt signaling pathway in intestinal epithelial cells co-cultured with M2 macrophages. *In vitro* experiments performed with human hypoxic macrophages also showed that epithelial cells impaired autophagy through Wnt1, when they are co-cultured with hypoxic macrophages (131). In contrast to this observation, Wang et al. showed that blocking Wnt secretion, by either treatment with the IWP12 porcupine inhibitor or knockdown of WLS, did not modulate autophagy or ER stress in hepatocellular carcinoma cell lines or colorectal cancer cell line (132). It seems that the specific role played by Wnt ligands in autophagy regulation deserve further investigation but of interest, we found the co-localization of Wnt1 with CD206, a marker of M2 macrophages in the inflamed intestine of patients with Ulcerative Colitis in which an impaired autophagy was detected (51, 131).

The functional relevance of macrophage-derived Wnt in intestinal regeneration has recently been demonstrated in murine models of colitis or intestinal damage. First, we demonstrated STAT6-dependent overexpression of *Wnt2b*, *Wnt7b*, and *Wnt10a* in IL4-treated murine macrophages *in vitro*, in parallel with defects in mucosal regeneration in STAT6 knockout mice. We showed that the administration of a Wnt agonist—as well as transfer of polarized M2a macrophages to STAT6$^{-/-}$ mice—activated the Wnt signaling pathway in the damaged mucosa and accelerated wound healing (52). In support of these observations, the study by Saha et al., who used macrophage-restricted ablation of Porcupine (133), demonstrated that macrophage-derived extracellular vesicle-packaged Wnts are essential for the regenerative response of the intestine to radiation.

The role of Wnt signaling in lung macrophages has been studied in infectious and inflammatory processes. It has been described that Wnt3a inhibits proinflammatory cytokine secretion of murine macrophages (134), and Wnt6 has been identified as a novel factor driving macrophage polarization toward an M2-like phenotype (135). However, other studies have highlighted the role of the Wnt5a ligand as a novel marker of inflammation with intrinsic pro-inflammatory properties (122, 136). For instance, the Wnt5a ligand regulates inflammatory cytokine secretion, polarization, and apoptosis in *Mycobacterium tuberculosis*-infected macrophages (137).

The role of macrophage-derived Wnt proteins in lung regeneration is incipient and requires further study. Hung et al. demonstrated a reduced expression of Wnt4 and Wnt16 in Trefoil factor 2 (TFF2)-deficient macrophages, and reconstitution of hookworm-infected CD11c^{Cre} TFF2^{flox} mice with rWnt4 and rWnt16 restored proliferation in lung epithelia post-injury. Their work revealed a mechanism wherein lung myeloid phagocytes utilize a TFF2/Wnt axis as a mechanism that drives epithelial proliferation following lung injury (138).

Under healthy physiological conditions, the kidney macrophage compartment includes a population of long-living tissue-resident macrophages, as well as macrophages that have differentiated from circulating monocytes produced in the bone marrow. RNA sequencing shows that acute kidney injury causes transcriptional reprogramming of macrophages resident in the kidneys, and they are enriched in the Wingless-type MMTV integration site family (Wnt). Relative expression levels measured by RNAseq have shown that levels of Wnt4 are higher than other Wnts in kidney-resident macrophages (139). In the same line, Wnt7b derived from wound-healing or pro-reparative macrophages has been shown to promote tubular epithelial cell proliferation, angiogenesis, and kidney repair. The report in question showed that kidney injury results in an up-regulation of Wnt ligands (*Wnt4*, *7b*, *10a*, and *10b)* in macrophages and the canonical Wnt response in epithelial cells. Furthermore, macrophage ablation during repair of the injured kidney results in a reduced canonical Wnt response in kidney epithelial cells. Indeed, compromise of Wnt receptors or conditional deletion of Wnt7b in the macrophage lineage has been shown to undermine the repair response and persistent injury (127, 140, 141).

Macrophage-derived Wnts have been shown to affect blood vessel formation by regulating VEGF and angiopoietin signaling in vascular endothelial cells (142). For instance, during eye development, macrophages secrete Wnt7b to induce blood vessel regression (143–145).

There is growing evidence regarding the role of both macrophages and Wnt signaling in infarct healing. In line with this, Palevski et al.'s recent work shows that Wntless-deficient macrophages in myocardial infarction present a unique subset of M2-like macrophages with anti-inflammatory, reparative, and angiogenic properties, and these mice exhibit an increased vascularization near the infarct site compared with wild-type mice. They conclude that loss of macrophage Wnt secretion improves remodeling and function after myocardial infarction in mice (146).

Macrophage-Derived Wnt in Fibrosis

In the context of intestinal fibrosis we have described that CD16+ CD206+ macrophages accumulate in the mucosa of patients with Crohn's Disease, and that these macrophages express high levels of Wnt2b compared with controls and patients with a stenotic pattern (147, 148). In a similar manner, Salvador's work demonstrated the accumulation of CD16+ macrophages, a subgroup of macrophages that express Wnt6, in the mucosa of STAT6 knockout mice treated with TNBS to induce intestinal fibrosis. It seems likely that a pro-fibrotic macrophage phenotype expressing CD16 accumulates in both human and murine intestinal fibrosis, although the specific Wnt ligand expressed by these cells may differ depending on the species analyzed (147).

Although macrophages, particularly the alveolar type, are important for host defenses in the lung, they can also contribute to tissue fibrosis. The role of macrophage-derived Wnt proteins in pulmonary fibrosis has been analyzed in a co-culture system in Hou's study, which showed that M2 macrophages (IL-4–stimulated RAW cells, $CD68^+$ $CCR7^-CD206^+$), but not M1 macrophages (LPS-stimulated, $CD68^+$ $CCR7^+CD206^-$), promote myofibroblast differentiation of lung-resident mesenchymal stem cells through the release of Wnt7a (149).

Traditionally, macrophages have been recognized as key players in kidney fibrosis. Feng et al. have described that Wnt5a promotes kidney fibrosis by stimulating Yap/Taz-mediated macrophage M2 polarization (150). It is important to note that Wnt ligands have also been reported to modulate macrophage polarization toward a pro-fibrotic phenotype. In accordance with this, treatment of macrophages with Wnt3a exacerbated IL-4- or TGFβ1-induced alternative macrophage polarization (M2) (151), and activation of Wnt/β-catenin signaling can promote macrophage proliferation and macrophage accumulation, which may play a role in kidney fibrosis (152).

Macrophage-Derived Wnt and Cancer

Tumor associated macrophages (TAMs) constitute one of the most abundant components of the tumor microenvironment. These macrophages originate in mononuclear cells present in blood vessels, which, after their extravasation, penetrate the tumor tissue and respond to all the signaling substances released from the tumor cells (153). Once there, TAMs polarize preferably toward the M2 phenotype, thus playing an important role in the suppression of the immune system, the promotion of infiltration, increase of tumor size, angiogenesis, and metastasis. Therefore, the abundance of these TAMs is an indicator of bad prognosis in cancer patients (154).

The relevance of TAMs in tumor progression has been widely studied, but the molecular mechanisms involved are not well-elucidated. There is growing evidence to support an important crosstalk between tumor cells and macrophages due to the action of many kinds of soluble factors, including Wnt ligands (155).

Oguma et al. demonstrated that infiltrated macrophages are needed for the development of intestinal tumors and are associated with activation of the Wnt signaling pathway. *In vitro*, they showed that macrophages can activate this cascade through the secretion of TNF-α. Although they did not confirm that macrophage-derived Wnt ligands are also responsible for this effect, they speculated that other soluble factors might also be involved, since they could only demonstrate a partial role of TNF-α in Wnt activation (156). In order to better characterize the expression pattern of of tumor-associated macrophages, Ojalvo et al. analyzed the gene expression signature of TAMs by subdividing them into two subpopulations: non-invasive and invasive macrophages. Among other pathways, they identified for the first time that the Wnt signaling pathway was increased specifically in an invasive subpopulation of TAMs that promotes tumor metastasis and angiogenesis. In addition, they highlighted a specific increase in the expression of Wnt5b and Wnt7b in this subpopulation of invasive TAMs (157). To our knowledge, the aforementioned study provided the first confirmation of an increased expression of Wnt components specifically in invasive TAMs. In line with this, the tumor-associated macrophage-derived Wnt7b ligand has also been involved in cholangiocarcinoma growth *in vivo* (158).

In line with the above studies, Pukrop et al. demonstrated that the expression of Wnt5a is enhanced in TAMs in breast cancer and lymph node metastasis, and non-canonical Wnt activation by this ligand increases the invasiveness of cancer cells (124). Of interest, another Wnt ligand has recently been associated with tumor progression. In fact, Linde et al. have just shown that intraepithelial macrophages secrete higher levels of Wnt1 and activate epithelial-to-mesenchymal transition in cancer cells, thus triggering and fuelling metastasis (159). Therefore, it is important to point out that tumor-associated macrophages promote tumor progression and metastasis through both canonical and non-canonical Wnt pathways.

On the other hand, TAMs can also activate the Wnt pathway in epithelial cells through several soluble factors other than Wnt ligands. These macrophages can activate Wnt signaling in colon cancer cells through the secretion of the proinflammatory cytokine IL-1β, thereby increasing the survival of cancer cells (160). TAMs can also activate epithelial-to-mesenchymal transition (EMT) in several cancers (e.g., breast, lung and pancreas) through the secretion of prostaglandin E2 and IL-10, which increases the translocation of β-catenin into the nucleus (161–163). Chen et al. have recently reinforced these observations by demonstrating that TNF-α derived from TAMs activates EMT and cancer stemness

TABLE 1 | Summary of macrophage-derived Wnt ligands and their relevance in homeostasis and different pathologies.

Macrophage-derived Wnt ligand	Relevance	References
Wnt1	Activates EMT and promotes metastasis in breast cancer	(159)
	M2 (IL4) macrophages act as a source of Wnt1 and promote Wnt signaling in intestinal crypts in IBD	(51)
	Hypoxic macrophages impair autophagy in epithelial cells through Wnt1	(131)
Wnt2b	M2 (IL4) macrophages overexpress Wnt2b in a STAT6-dependent manner and accelerate intestinal wound healing in mice	(52)
	M2 (CD45+CD64+CD206+CD16+) macrophages act as a source of Wnt2b and promote EMT in CD	(148)
Wnt3a	M2 (IL4) macrophages act as source of Wnt3a	(51)
	Inhibits proinflammatory cytokine secretion of murine macrophages	(134)
	Drives parenquimal regeneration of hepatocytes	(168)
Wnt4	Restores proliferative defects in post-injury lung epithelial cells produced by TFF2-deficient macrophages	(138)
Wnt5a	Enhances the invasiveness of cancer cells in breast cancer	(124)
	Regulates inflammatory cytokine secretion, polarization, and apoptosis in mycobacterium tuberculosis-infected macrophages	(137)
	Promotes kidney fibrosis by stimulating Yap/Taz-mediated macrophage M2 polarization	(150)
Wnt5b	Induces tumor progression of breast cancer	(157)
Wnt6	Up-regulated in the colonic mucosa and in $CD16^{+}$ macrophages of STAT6 knockout mice in an IBD model	(147)
	Drives macrophage polarization toward the M2 phenotype	(135)
Wnt7a	M2 (IL4) macrophages act as a source of Wnt7a and promote lung fibrosis	(149)
Wnt7b	Induces tumor progression of breast cancer	(157)
	Promotes cholangiocarcinoma growth	(158)
	M2 (IL4) macrophages overexpress Wnt7b in a STAT6-dependent manner and accelerate intestinal wound healing in mice	(52)
	Wound-healing macrophages promote tubular epithelial cell proliferation, angiogenesis and kidney repair	(127)
	Affects blood vessel formation	(143, 144)
Wnt10b	M2 (IL4) macrophages overexpress Wnt10b in a STAT6-dependent manner and accelerate intestinal wound healing in mice	(52)
Wnt16	Restores the proliferative defect in lung epithelial cells post-injury produced by TFF2-deficient macrophages	(138)

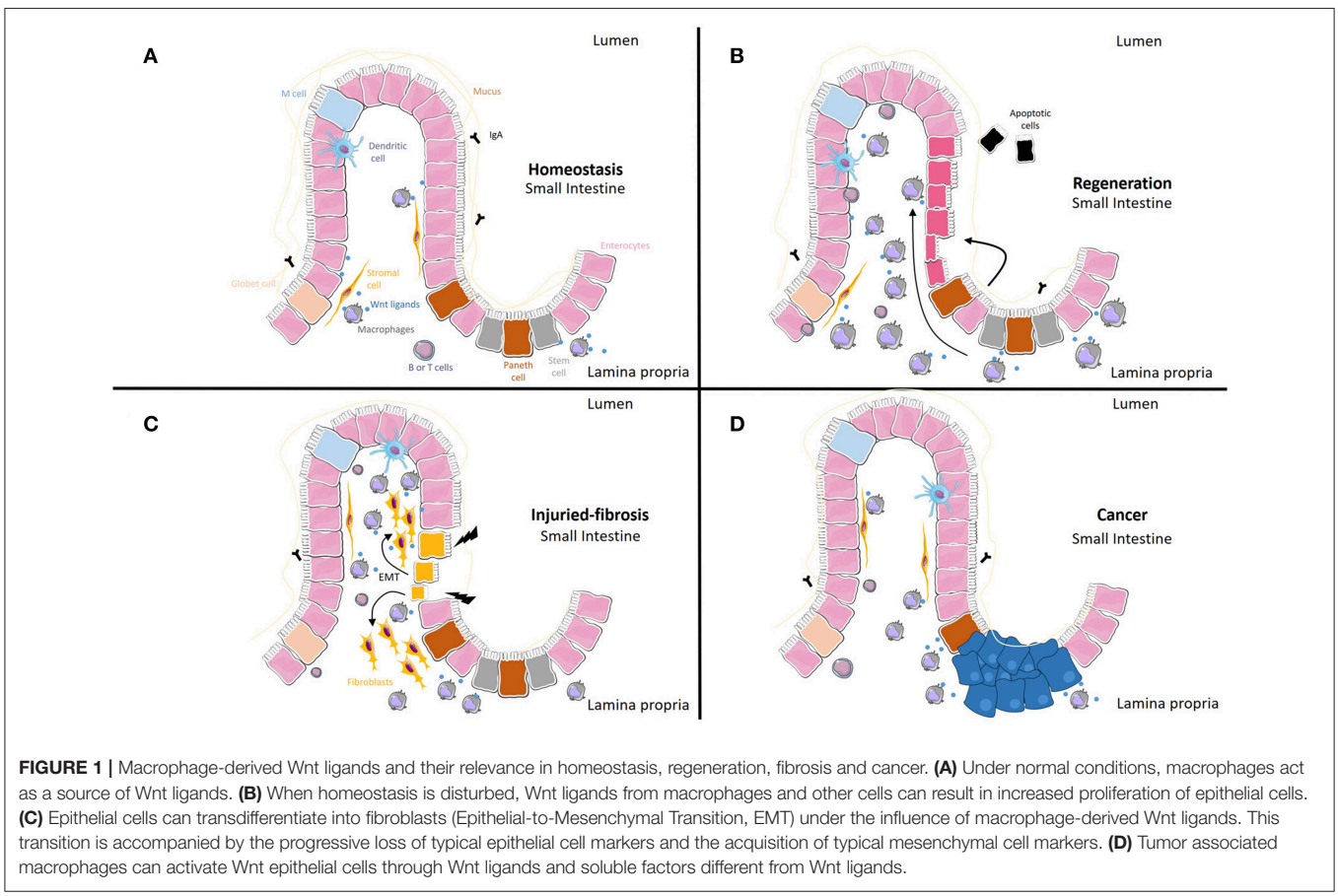

FIGURE 1 | Macrophage-derived Wnt ligands and their relevance in homeostasis, regeneration, fibrosis and cancer. **(A)** Under normal conditions, macrophages act as a source of Wnt ligands. **(B)** When homeostasis is disturbed, Wnt ligands from macrophages and other cells can result in increased proliferation of epithelial cells. **(C)** Epithelial cells can transdifferentiate into fibroblasts (Epithelial-to-Mesenchymal Transition, EMT) under the influence of macrophage-derived Wnt ligands. This transition is accompanied by the progressive loss of typical epithelial cell markers and the acquisition of typical mesenchymal cell markers. **(D)** Tumor associated macrophages can activate Wnt epithelial cells through Wnt ligands and soluble factors different from Wnt ligands.

in hepatocellular carcinoma cells through activation of the Wnt signaling pathway (164). In line with this, it has recently been reported that tumor-associated macrophages enhance the proliferation and migration of osteosarcoma cells due to the activation of Wnt signaling through the secretion of CCL18 (165).

CONCLUSION AND FUTURE PERSPECTIVES

The present review supports a central role of Wnt signaling in mucosal homeostasis and regeneration, but it also highlights how this pathway is involved in fibrosis and cancer, which complicated the pharmacological modulation of this cascade. Nevertheless, the inhibition of Wnt signaling has become a pharmacological strategy, and several compounds have been or are being designed in order to treat several diseases. A wide spectrum of different compounds has been tested in several cancers, including porcupine inhibitors, antibodies against Wnt family proteins, Tankyrase inhibitors, Disheveled inhibitors, TCF/beta-catenin Transcription Complex Inhibitors, Wnt co-activator antagonist, and Gamma Secretase Inhibitors (166). In the context of fibrosis, some inhibitors of CBP/β-catenin interaction, such as ICG-001 and PRI-724, have been tested, and promising results have been obtained *in vitro* and *in vivo* with animal models (167). Nevertheless, these compounds are yet to be approved specifically for fibrosis treatment in clinical trials. In spite of all the evidence accumulated, we still lack conclusive results regarding these compounds, and the potential effects of pharmacological inhibition of the Wnt signaling pathway are yet to be determined.

The Wnt signaling pathway is activated mainly by Wnt ligands, small proteins that are synthetized and released from different cell types. In inflammatory conditions, in which mucosal integrity is altered and macrophages accumulate, the synthesis of Wnt ligands from these cells seems to modulate mucosal regeneration, but the persistence of these ligands in chronic situations can also lead to fibrosis or cancer. In **Table 1** and **Figure 1**, we include all macrophage-derived Wnt ligands and their relevance in homeostasis and a range of different pathologies. A better characterization of the specific ligands released from these cells, as well as the specific receptors involved in each scenario, will help to define more selective pharmacological approaches.

AUTHOR CONTRIBUTIONS

JC-R and MO-M organized and wrote the draft manuscript. MB critically reviewed the draft. All authors contributed to the writing of the manuscript and approved the final version.

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REVIEW
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WNT/β-Catenin Signaling Pathway Regulating T Cell-Inflammation in the Tumor Microenvironment

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Immunotherapy with checkpoint inhibitors has greatly prolonged the overall survival of cancer patients in melanoma and many other cancer types. However, only a subset of patients shows clinical responses from these interventions, which was predicated by the T cell-inflamed tumor microenvironment. T cell-inflamed phenotype is characterized by the infiltration of $CD8^+$ T cells, CD8α/CD103-lineage dendritic cells (DCs), as well as high density of forkhead box P3 $(FoxP3)^+$ regulatory T cells (Tregs) that are associated with the efficacy of immune checkpoint blockade. A number of regulators has been associated with T cell-inflammation in the tumor microenvironment, and WNT/β-catenin signaling is one of the best characterized. The tumor-intrinsic WNT/β-catenin signaling activation is frequently associated with poor spontaneous T cell infiltration across most human cancers. In this article, we review the essential roles of WNT/β-catenin signaling in the T cell-inflamed and non-T cell-inflamed tumor microenvironment, including the development and function of immune cells, activation of immune exclusion of tumor cells, and cancer immunosurveillance. We also discuss the impact of this pathway in driving the non-T cell-inflamed tumor microenvironment in other tumor types. To improve immunotherapy efficacy, we argue that targeting Wnt/β-catenin signaling should be a high priority for combinational cancer therapy to restore T cell infiltration.

Keywords: WNT/β-catenin signaling pathway, T cell-inflammation, tumor microenvironment, $CD8^+$ T cells, immune exclusion, immunotherapy

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INTRODUCTION

Immunotherapy with immune checkpoint inhibitors (ICIs) is now common for melanoma and many other cancer types (1). An important property of the immune system is to distinguish "foreign" cells from normal cells in the body, which allows the body to attack foreign cells while retaining normal cells. Therefore, it uses "checkpoints"; specifically, molecules on certain immune cells need to be activated (or inactivated) to initiate an immune response (2). As a checkpoint protein on T cells, programmed death-1 (PD-1) normally acts as a type of "off switch" to ensure that T cells do not target other cells in the body (3). Upon binding to PD-L1, which is a ligand protein expressed by some normal (and cancer) cells, it signals T cells to abstain from attacking. Antibodies that target either PD-1 or PD-L1 block this binding and boost the immune response against cancer cells (2). The therapeutic targeting of this axis has shown impressive

activity in various tumors including melanoma of the skin, non-small cell lung cancer, head and neck squamous cell carcinoma, renal cell carcinoma, urothelial carcinoma, Hodgkin lymphoma, Merkel cell carcinoma, and microsatellite unstable or mismatch repair-deficient tumors (2). CTLA-4 is another type of checkpoint inhibitor, and monoclonal antibodies that target this marker can also boost the body's immune response against cancer cells (4). For ICIs, and especially CTLA-4 antibodies, one concern is their side effects in some patients as they permit the immune system to attack some normal organs in the body (4).

Despite improvements in clinical outcomes, only the minority of patients responds to ICIs (5). In patients susceptible to immunotherapy, an active immune response is usually observed prior to treatment, which is characterized by infiltrating antigen-specific T cells (6). This phenotype has been described as a T cell-inflamed tumor microenvironment (TME) and can be used to predict responding and non-responding tumors (7). The TME is the environment surrounding the tumor and consists of peripheral blood vessels, immune cells, fibroblasts, signaling molecules, and extracellular matrix (8). Tumors are closely related to the surrounding microenvironment and interact with it continually. Specifically, by releasing extracellular signals, promoting tumor angiogenesis, and inducing peripheral immune tolerance, tumors can affect the microenvironment; in turn, the growth and evolution of cancer cells are also affected by immune cells in the microenvironment (9). In the TME, chemokines support the influx of $CD8^+$ effector T cells, which subsequently are functionally inhibited by PD-L1, IDO, Treg cells, and anergy. The development of TME is promoted in part by type I interferon signaling and the $CD8a^+$ dendritic cell (DC) lineage (10). In normal tissue microenvironments, chemokine expression is poor and T cell infiltration is lacking, and this is also associated with the minimal presence of defined immune inhibitory pathways (11). In non-T cell inflammatory tumors, T cell inflammatory gene expression is significantly lower than that in matched normal tissues, which is associated with the loss of the native immune phenotype (6). Notably, a recent TCGA database study found that most tumor types are inversely associated with a T-cell-inflamed gene expression signature, and that WNT/β-catenin pathway activation is one potential causal pathway (6).

A correlation between WNT/β-catenin signaling and the T-cell-inflamed TME has been established (6). Moreover, identification of molecular factors associated with non-T cell-inflamed TME is important for the development of combinational immunotherapy regimens. Mechanistic studies based on genetically-engineered mice have also shown that activation of tumor cell-intrinsic β-catenin prevents spontaneous T-cell priming and infiltration into the TME, rendering cells resistant to combinational ICI therapy (12, 13). Thus, pharmacologically targeting the WNT/β-catenin pathway could potentially improve the efficacy of immunotherapy.

Here, we review the mechanisms wherein WNT signaling interrupts immune functions in the T cell-inflamed and non-T cell-inflamed TME, including the development and function of immune cells, activation of immune exclusion in tumor cells, and cancer immunosurveillance. We also discuss the therapeutic potential of harnessing currently available WNT modulators to augment cancer immunotherapy.

CANCER IMMUNOTHERAPY AND THE TME

The T cell-inflamed subset of tumors is dominated by T cell markers and chemokines, which might mediate effector T cell recruitment (14–16). The expression of chemokines C-C motif chemokine ligand (CCL) 2, CCL3, CCL4, CCL5, C-X-C motif chemokine ligand (CXCL) 9, and CXCL10 is associated with the infiltration of T cells, and each of these chemokines is sufficient to recruit $CD8^+$ effector T cells *in vitro* (16). Previous studies have confirmed that the T cell-inflamed subset contains variable numbers of $CD8^+$ T cells and CD8α/CD103-lineage DCs, but also possesses the highest density of $FoxP3^+$ regulatory T cells (Tregs) (16). Additionally, many conventional T cells have a dysfunctional anergic phenotype. It has been found that CXCR3-binding chemokines (such as CXCL9 and CXCL10) are critical and essential for the recruitment of activated $CD8^+$ T cells to tumor sites (17). As a major driver of Treg recruitment, CCL22 is partially produced by activated $CD8^+$ T cells (18). Despite the presence of specific adaptive immunity in this subset of patients, the cause of tumor progression is likely secondary to immunosuppressive mechanisms that act to some extent in the TME (19). Furthermore, T cell dysfunction in the TME is antigen-specific and restricted to tumor reactive T cells (19).

In contrast, T cell markers and chemokines that mediate T cell recruitment in the non-T cell-inflamed TME are lacking. Macrophages, vascular endothelial cells, fibroblasts, extracellular matrices, and immature DCs in some cases are still present in these tumors (20–24). Moreover, both the priming and effector phases of the anti-tumor immune response are deficient in non-T cell inflammatory tumors (19). Effector T cell trafficking into the TME is complex and dependent on adhesion molecules and homing receptors on vascular endothelial cells, consistent with the fact that chemokines are produced by tumor cells and stromal cells within the TME (19). In most cases, this process is necessary for the clinical response of immunotherapy.

The T cell-inflamed phenotype is associated with the efficacy of immune checkpoint blockade, whereas non-T cell-inflamed tumors rarely benefit. Recently, a series of studies has linked alterations in WNT signaling to oncogenesis, disease progression, and resistance to treatment in the TME (25, 26). Furthermore, dysregulated WNT signaling supports malignant transformation and disease progression through a variety of mechanisms in the TME (27). The high expression of specific immune cell genes in the TME, known as the T-cell-inflamed phenotype, has been associated with response to multiple immunotherapies including therapeutic vaccines and checkpoint blocking antibodies (11, 15, 16, 28–31). In contrast, the non-T-cell-inflamed TME appears to be closely related to a lack of clinical benefit from immunotherapy, particularly in relation to anti-PD-1 antibodies (30, 31). Despite a variety of molecular mechanisms that could be theoretically detrimental to the T-cell-inflamed microenvironment, several studies have indicated

that oncogenic molecular aberrations are sufficient to drive the immune exclusion phenotype in some cases (6). In a study using a genetically-engineered mouse model, tumor cell-intrinsic WNT/β-catenin signaling in melanoma was found to be the first somatic alteration associated with the non-T-cell-inflamed TME in patients (13). In addition, the transcriptional repression of key chemokine genes leads to a lack of basic leucine zipper ATF-like transcription factor 3 (Batf3)-lineage DC recruitment, and the subsequent failure to prime and recruit $CD8^+$ T cells appears to be involved in this effect (12, 13). This effect is dominant in the TME and results in decreased pre-clinical efficacy for checkpoint blockade, tumor antigen vaccination, and adoptive T-cell transfer immunotherapy approaches (12, 13). In addition, blocking the β-catenin pathway enhances the influx of $CD8^+$ T cells and increases IFNγ-related gene targets in syngeneic murine models of B16F10 melanoma, 4T1 mammary carcinoma, Neuro2A neuroblastoma, and Renca renal adenocarcinoma (32). Therefore, strategies to overcome barriers that restrict T cell migration into tumor sites might ultimately promote immunotherapy efficacy in non-T cell-inflamed tumors. The Wnt/β-catenin pathway could thus represent a high-priority target for combinational cancer immunotherapy.

WNT/β-CATENIN SIGNALING AND THE DEVELOPMENT AND FUNCTION OF IMMUNE CELLS

The WNT signaling pathway is highly conserved between species and has been shown to play an important role in controlling multiple developmental processes including asymmetric cell division, stem cell pluripotency, and cell fate specification (33, 34). In addition to the importance of WNT signaling in stem cells and hematopoiesis, its role in the development of T lymphocytes in the thymus is indispensable (35).

T cell factor (TCF), the effector transcription factor of the WNT signaling pathway, was named for its indispensable role in T cell development and proliferation in the thymus (36). The TCF family consists of four members, specifically TCF-1 (encoded by the *TCF-7* gene), TCF-3, TCF-4, and LEF-1 (37, 38). TCF/LEF transcription factors are the major end-point mediators of Wnt signaling throughout metazoans. Although there are other transcription factors that can bind β-catenin and activate transcription, TCFs are major nuclear effector molecules of this pathway (39).

Wnt/β-Catenin Signaling and $CD8^+$ T Cells

$CD8^+$ T cells are key effectors of the tumor-immune cycle. In the tumor immune system, $CD8^+$ T cells are activated by DCs and co-stimulatory molecules, and then infiltrate into the tumor site to kill target cancer cells (40). By preventing the infiltration of $CD8^+$ T cells during tumor progression, tumor cells evade immune elimination, excluding or inactivating $CD8^+$ T cells (41). Owing to prolonged antigen exposure and the suppressive TME, tumor-infiltrating $CD8^+$ T cells affect the progressive loss of effector functions (42). Wnt/β-catenin signaling is essential for T cell differentiation, effector functions, and migration (43). The activation of TCF-1/β-catenin signaling can result in stem cell-like phenotypes resulting in the formation of memory $CD8^+$ T cells or differentiation into a Tfh cell-like gene expression profile *in vivo* (44). Naive $CD8^+$ T cells differentiate into effector T cells and kill tumor cells in the tumor-immune cycle (45). Moreover, the activation and maintenance of memory T cells is indispensable to maintain anti-tumor immunity. It has been confirmed that Wnt/β-catenin signaling and TCF1 are highly activated and expressed in undifferentiated $CD8^+$ T and memory $CD8^+$ T cells, and that TCF1 is downregulated when naive $CD8^+$ T cells differentiate into effector $CD8^+$ T cells (46).

Collectively, the differentiation of naïve $CD8^+$ T cells into $CD8^+$ T effector cells is inhibited, the development of memory precursor and central memory $CD8^+$ T cells is promoted, and expansion is stimulated by TCF-1 (**Figure 1**). However, the requirement for β-catenin in memory T cell functions, as a coactivator of TCF-1, remains controversial.

Wnt/β-Catenin Signaling and $CD4^+$ T Cells

WNT/β-catenin signaling also regulates the differentiation of $CD4^+$ helper T (T_H) cells. TCF-1 and β-catenin support Th2 polarization through activation of the expression of the Th2 master transcription factor GATA binding protein 3 (GATA3) via a special AT-rich sequence binding protein-1 (SATB1), which is a chromatin organizer that plays pivotal roles in T cell development (47, 48). A subsequent study confirmed that the sustained activation of β-catenin in mouse $CD4^+$ thymocytes results in the up-regulation of RAR related orphan receptor C (RARC) and consequent Th17 polarization, ultimately resulting in the production of pro-inflammatory cytokines that favor tumorigenesis (49). Another recent study suggested that Wnt10b regulates type 2 inflammation and the activation of Th2 cells, since Wnt10b deficiency was found to exacerbate house dust mite-induced asthma in mice (50). In addition, the selective ablation of TCF-1 or LEF-1 via two plausible mechanisms leads to Tfh deficiency in the LCMV acute infection model (51).

Overall, the differentiation of naïve $CD4^+$ T cells into Th1 and Th17 cells is inhibited, whereas differentiation into Th2 and Tfh subsets is promoted, by TCF-1. Further, unlike the effects of TCF-1, all types of T cell differentiation and function are enhanced by the expression of β-catenin (**Figure 1**).

Wnt/β-Catenin Signaling and $FoxP3^+$ T Cells (Tregs)

It is been shown that the infiltration of Treg cells into the TME results in inhibition of the anti-tumor immune response (41). Treg cells are recruited into the TME by chemokines (such as CCL-28) secreted by tumor cells and innate immune cells (52). Wnt/β-catenin signaling limits the immunosuppressive activity of those cells by modulating the TCF-1-dependent inhibition of FoxP3 transcriptional activity (53). Production of the negative immunomodulators Foxp3, TGFβ, and IL-10 can be reduced by blocking the Wnt/β-catenin signaling pathway in Treg cells (54). Further, if wild-type Treg cells are inactivated, the functional integrity of Tregs will be ensured by the direct activation of genes encoding PD-1 and GITR to increase β-catenin expression, whereas β-catenin levels are reduced and

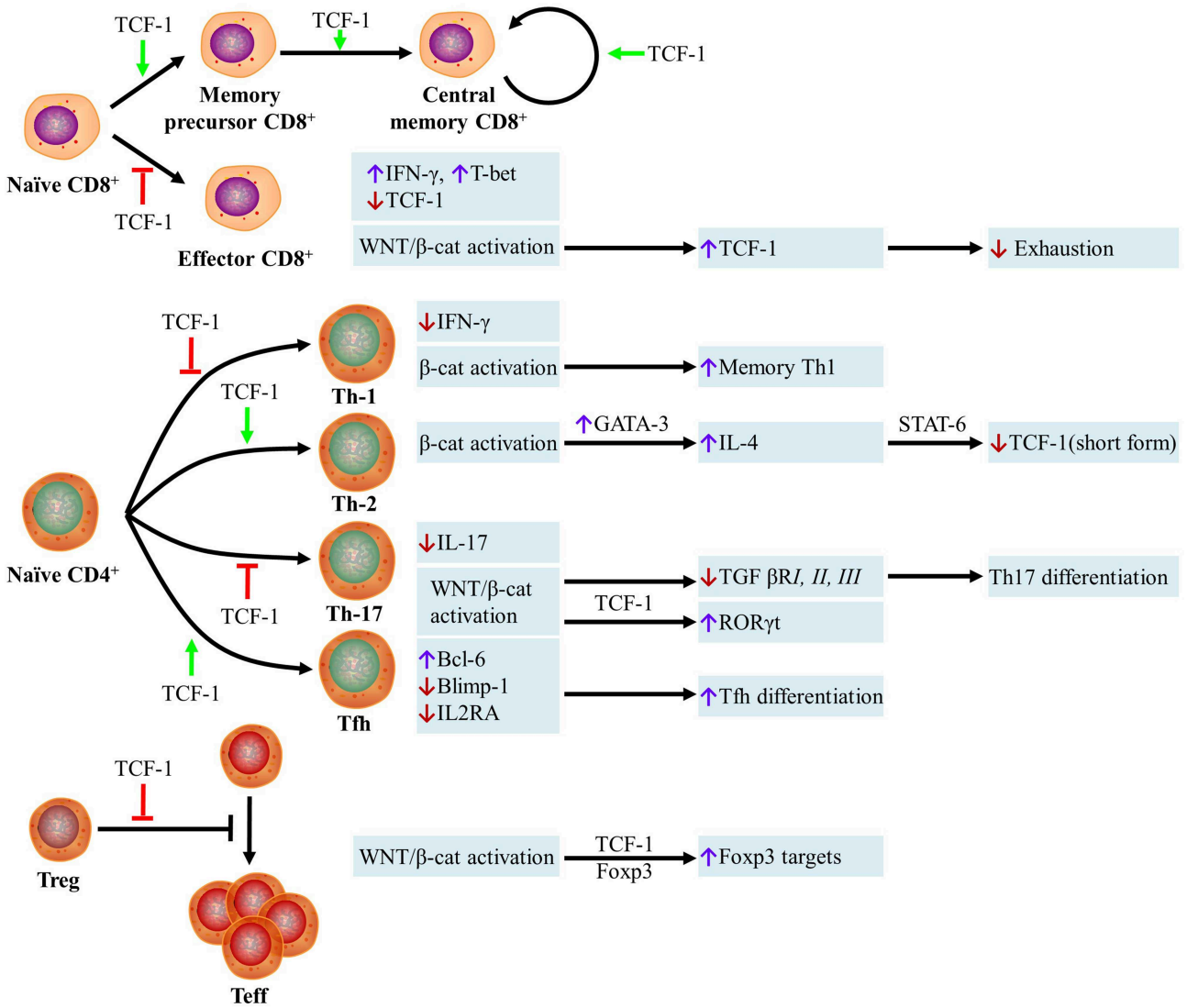

FIGURE 1 | Role of Wnt Proteins in T cells. (i) The differentiation of naïve $CD8^+$ T cells into $CD8^+$ T effector cells is inhibited, the development of memory precursor and central memory $CD8^+$ T cells is promoted, and the expansion is stimulated by TCF-1. Naïve $CD8^+$ T cells are resistant to becoming CD8 effector T cells if they are TCF-1^{high} cells, whereas TCF-1^{low} cells become active IFN-γ^+ T-bet^+ effector T cells. WNT/β-catenin-mediated activation of effector $CD8^+$ T cells can stimulate the transcription of TCF-1, which inhibits type I IFN-mediated cell exhaustion. (ii) The differentiation of naïve $CD4^+$ T cells into Th1 and Th17 cells is inhibited and differentiation into Th2 and Tfh subsets is promoted by TCF-1. Unlike TCF-1, all types of T cell differentiation and function are enhanced by the expression of β-catenin. The transcription of TCF-1 is inhibited by T-bet, which is the Th1 hallmark transcription factor, thus preventing the inhibition of T-bet-mediated transcription of IFN-γ. Beta-catenin activation enhances memory Th1 cell formation. In Th2 cells, the transcription of GATA-binding protein-3 (GATA-3) can be induced by β-catenin. GATA-3 stimulates the expression of IL-4, which can bind IL-4R and suppress transcription of the short form of TCF-1 via STAT6. In Th17 cells, IL-17 transcription can be suppressed by TCF-1 directly. Wnt signaling activation inhibits the transcription of TGF-βRI, II, and III, which has an important effect on Th17 differentiation. Upon β-catenin activation, RORγt transcription can be increased by TCF-1 directly. TCF-1 promotes Tfh differentiation by increasing Bcl-6 expression and inhibiting the transcription of Blimp-1 and IL2RA. (iii) TCF-1 inhibits the Treg cell-mediated suppression of effector T cell (Teff) proliferation. In the absence of WNT, the transcriptional repressor Foxp3 inhibits many genes including IL-2. TCF-1 activity is prioritized over Foxp3 when WNT is present, which results in the expression of various transcriptional targets shared by Foxp3 and TCF-1. TCF-1, T-cell factor 1; IFN-γ, interferon gamma; GATA3, GATA binding protein 3; IL-4, interleukin 4; TGFβRI, transforming growth factor beta receptor I; TGFβRII, transforming growth factor beta receptor II; TGFβRIII, transforming growth factor beta receptor III; Blimp-1, B lymphocyte-induced maturation protein-1; IL2RA, interleukin 2 receptor alpha.

Treg-mediated immune suppression is impaired by liver kinase B1 (LKB1) deletion (55).

In summary, TCF-1 inhibits the Treg cell-mediated suppression of effector T cell proliferation. When WNT is present, TCF-1 activity is prioritized over Foxp3, which results in the expression of various transcriptional targets shared by Foxp3 and TCF-1 (**Figure 1**).

Wnt/β-Catenin Signaling and CD8α/CD103-Lineage DCs

The TME regulates DCs through a variety of mechanisms, inhibiting their ability to induce anti-tumor responses. It has been confirmed that the Wnt/β-catenin signaling pathway plays a critical role in crosstalk between tumor cells and DCs within the TME (56). Upon conditional knockout of the Wnt co-receptors LRP5 and LRP6 on DCs, DC-mediated anti-tumor immunity is enhanced, ultimately resulting in delayed tumor growth (57). The recruitment of T cells and DCs into solid tumors is inhibited by β-catenin signaling in melanoma cells (13). In tumor-bearing mouse studies, the activation of β-catenin in DCs resulted in more tolerogenic phenotypes than those mediated by the DC vaccine-induced cross-priming inhibition of anti-tumor $CD8^+$ T cells by IL-10 (58). Furthermore, it has been demonstrated that melanoma induces DC-mediated tolerance to inhibit the efficacy of immunotherapy via Wnt5a-mediated mechanisms in mice (59). Notably, DC-specific β-catenin ablation was found to improve anti-PD-1 immunotherapy efficacy in a syngeneic tumor model (59). In antitumor immunotherapy, DCs are crucial subsets that can modulate T cell responses, and studies have summarized the potential to therapeutically target the Wnt pathway in DCs.

The role of the Wnt-β-catenin pathway might be related to the generation of tolerogenic DCs. However, caution is needed before concluding that the Wnt pathway plays a major role in DC function.

WNT Signaling and Immune Exclusion in the TME

Understanding the mechanisms of T cell exclusion in the TME is critical to improve cancer immunotherapy. The Wnt/β-catenin pathway has been identified as one of the most important oncogenic signaling pathways associated with immune evasion (58, 60). The pan-cancer association between WNT/β-catenin signaling and immune exclusion was previously confirmed by analyzing activated WNT/β-catenin signaling based on somatic mutations, copy number alterations, gene expression, and reverse-phase protein arrays (6). In addition to WNT/β-catenin signaling, other oncogenic events can also contribute to immune exclusion (19, 61). Accordingly, complex interactions among multiple oncogenic events are coupled to mediate more potent immune exclusion in some tumors, such as colorectal cancer, which exhibits the concurrent activation of WNT/β-catenin, MYC, and RAS (62–64).

Mutations in Wnt/β-catenin lead to a non-T cell inflammatory tumor phenotype and might be a biomarker to predict resistance to immunotherapy with ICIs in hepatocellular carcinoma (HCC) (65). Activation of β-catenin reduces T cell infiltrations, increases progression in immunocompetent hosts, and suppresses ICIs in a mouse model of hepatocarcinogenesis (66). The Wnt/β-catenin pathway is also involved in the regulation of innate immunity, such as DCs (67). One study detected an association between activation of Wnt/β-catenin signaling and the loss of T cell gene expression in human metastatic melanoma (13). Beta-catenin activation occurs in 48% of non-T cell inflammatory melanomas (6), and thus, other oncogenic pathways likely contribute to immune exclusion in the remainder of these tumors. Tumor-cell intrinsic activation of the WNT/β-catenin pathway is associated with a lack of T cells in the microenvironment of metastatic melanoma and other cancer types (68). Beta-catenin induces expression of the transcriptional repressor ATF3 and inhibits the transcription of CCL4 in mouse models (69). Furthermore, the defective production of CCL4 results in the impaired infiltration and activation of Batf3-lineage $CD103^+$ DCs, reduced $CD8^+$ T cell priming and infiltration, and a subsequent lack of responses to immune checkpoint blockade (69). In the absence of active β-catenin signaling, the normal production of CCL4 was found to be restored, resulting in $CD103^+$ DC activation and the infiltration and proficient priming of $CD8^+$ T cells (69). Wnt/β-catenin signaling within intestinal DCs regulates the balance between inflammatory and regulatory responses in the gut (69). In intestinal DCs, β-catenin was found to be associated with anti-inflammatory mediators, such as retinoic acid-metabolizing enzymes, IL-10, and transforming growth factor-β, as well as Treg-induced stimulation (70).

It has also been shown that Wnt/β-catenin signaling is involved in crosstalk between cancer cells and tumor-associated macrophages. One study demonstrated that interleukin-1β, secreted by tumor-associated macrophages (TAMs), might increase the availability of β-catenin via the phosphorylation of GSK3β in colon cancer cells, thereby disrupting the function of the β-catenin destruction complex (71). Colorectal cancer cells stimulate IL-β production in macrophages via Snail, which is a soluble factor and product of Wnt target genes (72).

In summary, the Wnt/beta-catenin pathway mediates immune exclusion via three mechanisms as follows: (i) inhibiting the production of CCL4 in Batf3-lineage $CD103^+$ DCs through induction of the expression of the transcriptional repressor ATF3, which in turn reduces the initiation and infiltration of $CD8^+$ T cells; (ii) increasing the interaction between Snail (a soluble factor and product of a Wnt-regulated gene) and TAMs, which in turn increases β-catenin activity via IL-1β; (iii) enhancing Treg survival (**Figure 2**).

WNT SIGNALING AND IMMUNOSURVEILLANCE IN THE TME

It has been widely accepted that WNT/β-catenin signaling affects cancer immunosurveillance across cancer types (27). By modulating various aspects of tumor-immune cell interactions including the immunogenicity of cancer cells and the ability of immune cells, such as DCs, natural killer (NK) cells, Treg cells, myeloid-derived suppressor cells (MDSCs), and cytotoxic T lymphocytes (CTLs) to elicit effective tumor-targeting immune responses, it has been discovered that WNT signaling positively or negatively affects anticancer immunosurveillance (**Figure 3**).

Tumor-intrinsic WNT/beta-catenin signaling affects the immunogenicity of carcinoma cells. Several components of the WNT signal transduction cascade that are overexpressed in carcinoma cells as tumor-associated antigens can be recognized

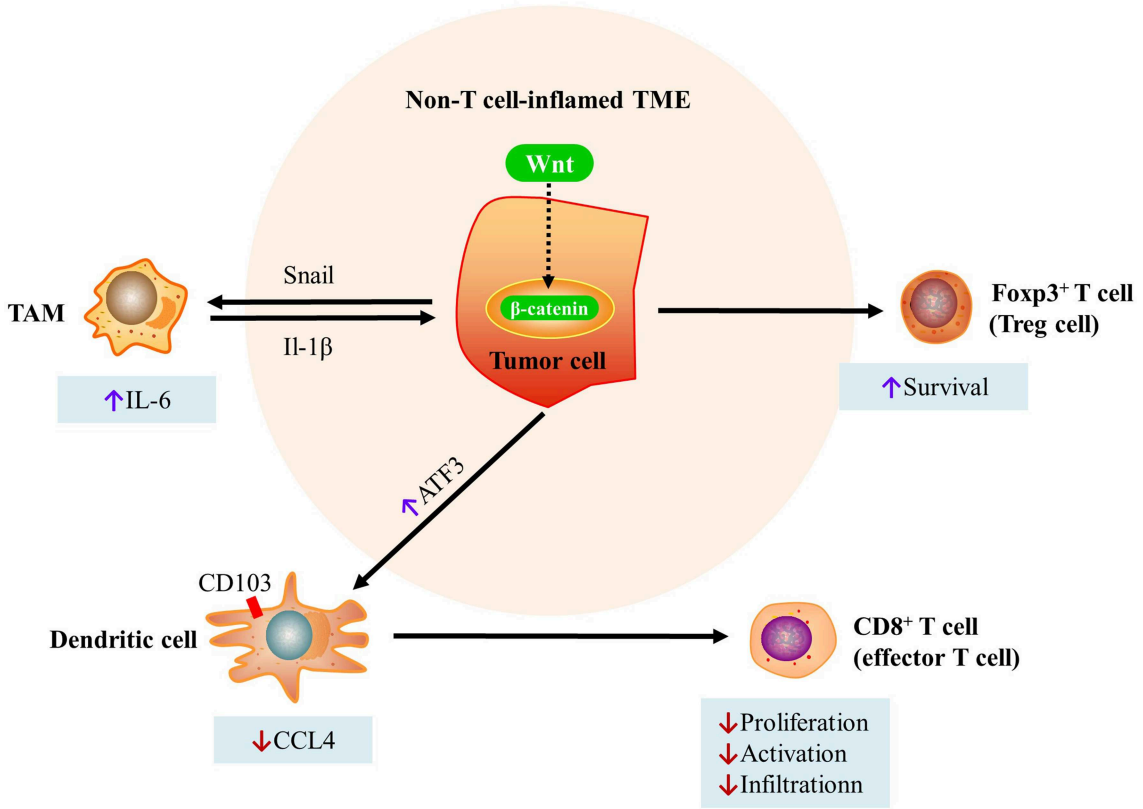

FIGURE 2 | Mechanisms of immune exclusion through the Wnt/beta-catenin pathway. (i) Inhibition of the production of CCL4 in Batf3-lineage CD103$^+$ DCs through induction of the expression of the transcriptional repressor ATF3. This in turn reduces the initiation and infiltration of CD8$^+$ T cells. (ii) Increases in the interaction between Snail (a soluble factor and product of a Wnt-regulated gene) and TAMs, which in turn increases β-catenin activity via IL-1β. (iii) Enhanced Treg survival [modified from Pai et al. (73)]. DC, dendritic cell; TAMs, tumor-associated macrophages; CCL4, C-C motif chemokine ligand 4; ATF3, activating transcription factor 3; TME, tumor microenvironment.

by the immune system (74). Moreover, it has been confirmed that WNT signaling is a major regulator of tumor immune tolerance. Active forms of β-catenin promote immune evasion and resistance to immunotherapy with anti-PD-1, which was found to involve the deficient recruitment of DCs and impaired T cell activity in a novel mouse model of HCC (75). A previous study showed that tumor-induced β-catenin signaling plays a role in DCs, resulting in inhibition of the DC-dependent cross-sensitization of anti-tumor CTLs and infiltrating immune effector cells into a tolerant state (76).

The accumulation of β-catenin in human melanoma cells or DCs leads to the expression of IL-10, which diminishes the capacity of DCs to cross-prime CD8$^+$ CTLs (77). In melanomas, canonical or non-canonical WNT signaling also limits antitumor immunity in DCs, which is associated with metabolic immunosuppression by IDO1 (78). This indicates that the activation of this pathway in DCs is important to maintain clonal amplified antigen-specific CTLs, although most studies indicate that classical WNT signaling inhibits antitumor CTL cross-priming. These studies indicate that the role of WNT signaling in the regulation of anticancer responses during CTL activation is stage-dependent (27).

Excluding CTL infiltration into the TME is the prominent mechanism of WNT-mediated immunoevasion in multiple types of cancer. Therefore, the conditional expression of oncogenic Braf plus Pten deletion and the engineered expression of β-catenin in mouse melanomas results in cells that are unable to express CCL4, leading to DC recruitment defects (13). Due to the absence of CD103$^+$ DC-derived chemokines, such as CXCL9 and CXCL10, tumor infiltration by CTLs is prevented and antitumor immune responses are impaired (13). It is generally accepted that effector cell deficiency in the TME is associated with active classical WNT signaling, which is also the main cause of resistance to cancer immunotherapy (79). Actually, tumor-intrinsic, active β-catenin signaling in human melanoma causes resistance to monoclonal antibodies including anti-PD-L1and anti-CTLA4 via T cell exclusion (13). In the absence of WNT ligands, GSK3β mediates the phosphorylation-dependent proteasomal degradation of PD-L1 by β-TrCP, and tumor infiltration is ultimately increased by interferon-producing CTLs (80).

WNT signaling exerts significant anticancer effects. For example, limiting β-catenin signaling prevents carcinogenesis

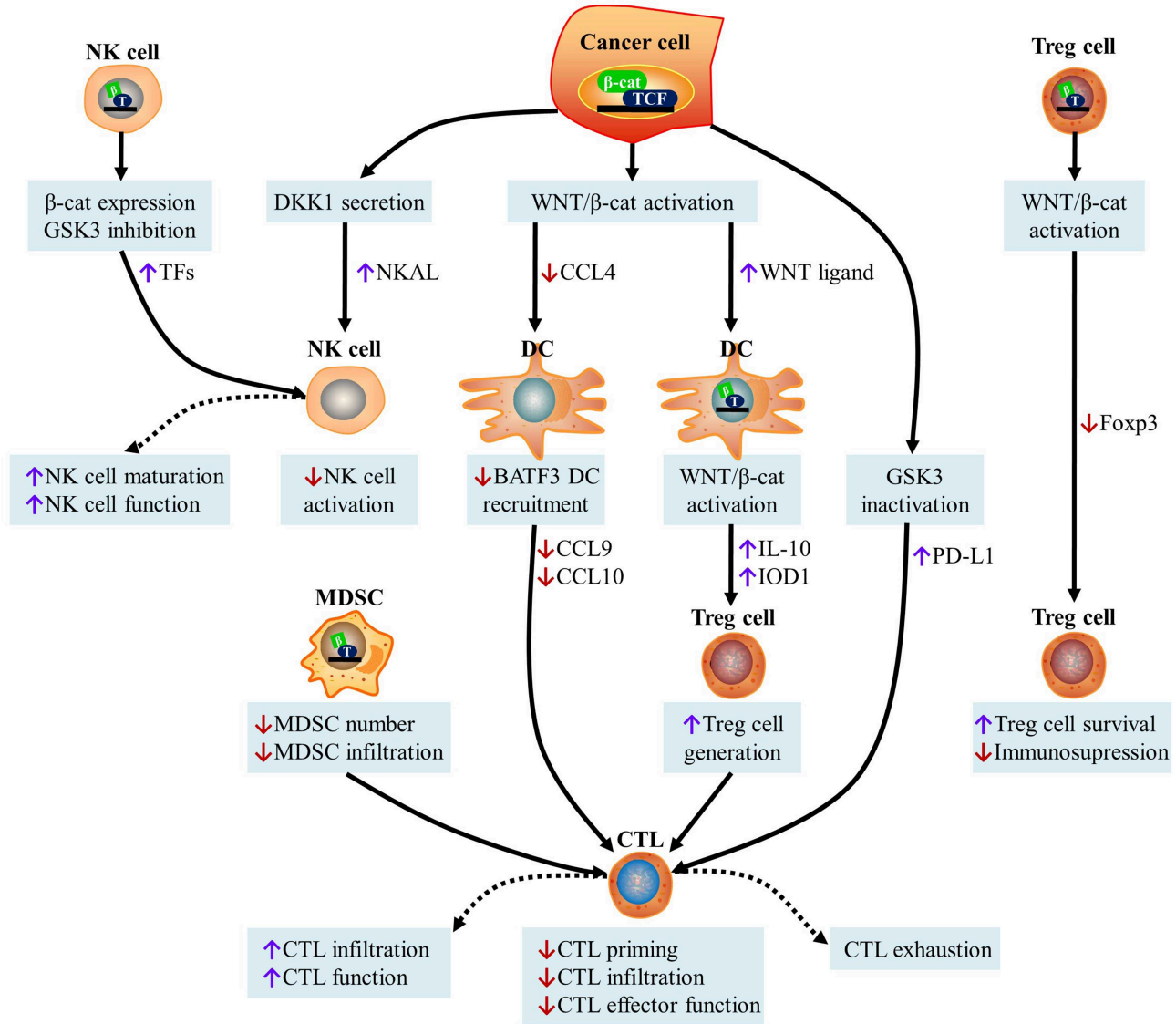

FIGURE 3 | Effect of WNT signaling on cancer immunosurveillance. (i) Activation of the WNT/β-catenin pathway in cancer cells inhibits the secretion of CCL4 and recruits BATF3-dependent DCs to the TME. This causes a decrease in DC-derived CXCL10 levels and limits $CD8^+$ CTL infiltration, leading to defective cross-priming. WNT ligands released by cancer cells can induce the canonical WNT signaling pathway in DCs, leading to the increased secretion of IL-10, restricted production of IL-12, and up-regulation of indoleamine IDO1. This contributes to the production of Treg cells and the subsequent inhibition of CTL activity. The inhibition of GSK3 in cancer cells promotes β-catenin activation and subsequently stabilizes PD-L1, which drives CTL exhaustion upon interactions with PD-1. Canonical WNT/β-catenin in MDSCs inhibits their ability to accumulate and infiltrate into malignant lesions, leading to the increased recruitment of CTLs. (ii) DKK1 secreted by latent metastatic cells prevents NK cell-dependent cancer cell lysis, which is caused by the low expression of NKALs, which engage NKARs. GSK3 inhibition and β-catenin overexpression in NK cells lead to the up-regulation of transcription factors responsible for NK cell maturation, thereby stimulating the function of NK cells as effectors. (iii) Although the expression of β-catenin is beneficial for the survival of Treg cells *in vitro*, the activation of TCF-1, downstream of canonical WNT signaling, blocks Foxp3 transcriptional functions and impairs the immunosuppressive activity of Treg cells. CTL, cytotoxic T cell; DKK1, Dickkopf WNT signaling pathway inhibitor 1; NKALs, NK cell activating ligands; NKARs, NK-cell-activating receptors; MDSCs, myeloid-derived suppressor cells; DC, dendritic cell; CCL4, C-C motif chemokine ligand 4; TCF, T cell factor; BATF3, basic leucine zipper ATF-Like transcription factor 3; GSK3, glycogen synthase kinase 3; PD-L1, programmed cell death ligand 1; NK, natural killer; IL-10, interleukin 10; IDO1, indoleamine 2,3-dioxygenase 1; TFs, transcription factors; Foxp3, forkhead box p3.

and the accumulation of $CD11b^+Gr\text{-}1^+$ MDSCs (81). Beta-catenin inhibits the downstream deletion of *Plcg2* (82), *Cul4b* (83), or *Mucl* (84), as well as the increased availability of DKK1 in the microenvironment (85), leading to MDSC amplification and recruitment into the TME, attenuating specific immune responses in different types of tumors. Quiescence in lung metastatic cancer cells is caused by autocrine DKK1, which leads to the downregulation of certain cell surface markers recognized by NK cells, allowing cancer cells to evade innate immunity (86).

In summary, cancer immunosurveillance is influenced by WNT signaling in a complex and context-dependent manner.

WNT MODULATION FOR CANCER IMMUNOTHERAPY

The WNT/β-catenin pathway should be a high-priority molecular target for new drug development, in an effort to restore T cell infiltration and potentially expand the efficacy of immunotherapy. Several specific inhibitors have been developed and have entered preclinical trials (**Table 1**); however, the activity of monotherapy has not been sufficient to warrant further advances to registered trials (87). Beta-catenin signaling is widely utilized by many cell types, and thus on-target, off-tumor effects limit the potential of therapeutics that target this protein. The immune evasive mechanisms of β-catenin activation, including the inhibition of chemokines and cytokine gene expression by tumor cells (13), provide the opportunity to develop agents with more restricted activities that predominantly augment immunity while maintaining other essential cellular functions.

Over the past few years, a variety of agents that target main components or modulators of WNT signaling have been developed and used as tumor therapies. Different clinical trials listed in the National Library of Medicine database are testing the effectiveness and safety of these drugs, which have not been approved by the regulatory agencies for clinical use. At first, drug development focused on disrupting WNT-driven tumor growth, including monoclonal antibodies to frizzled class receptor (FZD) receptors (e.g., OMP-54F28, OMP-18R5) (88), porcupine O-acyltransferase (PORCN) inhibitors (e.g., WNT974, RXC004, ETC1922159) (89), AXIN1 activators (e.g., Niclosamide, XAV939) (90), and β-catenin inhibitor (e.g., PRI724, PKF115-584) (91). Even though the immunomodulatory effects of these drugs have been neglected for a long time, there is increasing evidence suggesting that WNT inhibitors can also help to reestablish anticancer immunity. For example, the β-catenin inhibitor PKF115-584 effectively stimulates DC suppression, leading to a robust therapeutic response (77). Furthermore, both IWP-L6 and XAV939 deplete Treg cells from the TME, thus achieving a therapeutically-relevant immune response in an animal model of melanoma and lymphoma (57, 92). Various mechanisms associated with the cancer immunomodulatory effects of WNT inhibitors are listed in **Table 1**.

The immunological context of the TME has an important effect on the clinical efficacy of cancer immunotherapy, including its composition, activation status, and localization (93). Actually, the inhibition of local or systemic immunosuppression is the target of immunotherapy that is used to restore CTL depletion and reinstate the immunological control of tumor growth (94). ICIs limit the capability of co-inhibitory receptors including PD-1 and CTLA4, which are used to establish CTL depletion. However, when CTLs are absent or unable to infiltrate malignant cell niches, the efficacy of immunotherapy with ICIs is severely reduced (95). Since this phenotype is common in tumors that present with WNT activation, inhibitors of canonical WNT signaling can be combined with CTLA4-blocking mAbs to suppress the progression of melanoma in mice (78). Notably, molecular profiling of HCC has identified that patients with ICIs and activating alteration in WNT/β-catenin signaling have lower disease control rates, shorter median progression-free survival, and shorter median overall survival (96). One recent study developed potent, selective inhibitors targeting the interaction of β-catenin/Bcl9, which overcome resistance to ICIs by modulating Treg cells (97).

The activation of anticancer immune responses largely determines the efficacy of several conventional therapeutic strategies for the treatment of cancer, including a variety of chemotherapeutics, radiation therapy, and some targeted anticancer agents (98). Different anticancer molecules, such as oxaliplatin and doxorubicin can result in a noticeable immunogenic variant of cell death to establish adaptive anticancer immunity (99). Further, it has been confirmed that WNT signaling limits the efficacy of multiple anticancer therapies in this manner (10). Accordingly, it has been found that efficient local Wnt5a trapping results in significant remodeling of the immunosuppressive TME and promotes immunogenic cell-death-mediated immunotherapy (10).

The activity of chemotherapy and immunotherapy with ICIs can be boosted by WNT inhibitors. Studies have shown that $CD8^+$ T cells and $CD4^+$ T_H17 cells can differentiate into stem-like cells with advantageous anticancer functions upon the intervention with GSK3 inhibitors (46, 100); they also stimulate NK cell-dependent anticancer immunity (86). The restoration of β-catenin expression in MDSCs and the use of an anti-DKK1 mAb also limit tumor growth by establishing a TME that is beneficial for immunocompetency (85). Therefore, based on the existing preclinical evidence, WNT activation can constitute a therapeutic target.

CONCLUSION AND FUTURE PROSPECTS

This review highlights the role of WNT signaling in tumor initiation, malignant tumor progression, and resistance to therapeutics. In the context of combinatorial treatment regimens, a promising candidate WNT modulator, to improve the efficacy of various immunotherapeutic agents, is currently being validated in a variety of preclinical tumor models.

Many preclinical studies have demonstrated that the efficacy of ICIs is affected by WNT/β-catenin signaling, which reduces the infiltration of CTLs into the TME. In contrast, the insensitivity of melanoma patients to immunotherapy with ICIs affects the expression levels of certain components of the WNT signaling cascade (3). In terms of the future

TABLE 1 | WNT inhibitors for cancer immunotherapy currently in clinical development.

Mechanism of action	Agent	Stage of clinical development	Status	Details	Trial phase; Identifier
Anti-FZD7 antibody	OMP18R5	Phase 1	Completed	Metastatic breast cancer with locally recurrent or metastatic; in combination with paclitaxel	NCT01973309
AXIN1 activator	Niclosamide	Phase 1	Recruiting	Colon cancer subjected to primary tumor resection	NCT02687009
		Phase 1	Recruiting	Metastatic prostate carcinoma, recurrent prostate carcinoma, and stage IV prostate cancer	NCT03123978
		Phase 2	Recruiting	Metachronous or synchronous metastases during colorectal cancer progression under standard therapy	NCT02519582
AXIN1 activator	XAV939	Preclinical			
COX2 inhibitor	Celecoxib	Phase 2	Withdrawn	Breast carcinoma	NCT03185871
DVL2 inhibitor; PORCN inhibitor	IWP-L6	Preclinical		Favors tumor infiltration by IFNG-producing $CD4^+$ and $CD8^+$ T cells; depletes intratumoral Treg cells	
FZD10-targeting ARC	OTSA101	Phase 1	Terminated	Doxorubicin and ifosfamide-refractory synovial sarcoma	NCT01469975
FZD8-Fc Decoy receptor	OMP-54F28	Phase 1b	Completed	Locally advanced or metastatic hepatocellular cancer, in combination with sorafenib	NCT02069145
		Phase 1b	Completed	Recurrent platinum-sensitive ovarian cancer, in combination with paclitaxel and carboplatin	NCT02092363
		Phase 1b	Completed	Untreated stage IV metastatic pancreatic cancer, in combination with gemcitabine and nab-paclitaxel	NCT02050178
		Phase 1	Completed	Metastatic and unresectable refractory solid tumors	NCT01608867
PORCN inhibitor	C59	Preclinical		Synergizes with CTLA4-targeting antibodies in mouse melanoma models	
PORCN inhibitor	ETC1922159	Phase 1a/1b	Active, not recruiting	Locally advanced or metastatic solid tumors	NCT02521844
PORCN inhibitor	RXC004	Phase 1	Not yet recruiting	Advanced malignancy not considered appropriate for further conventional treatment	NCT03447470
PORCN inhibitor	WNT974; LGK974	Phase 1/2	Completed	BRAF-mut mCRC and WNT pathway mutations; in combination with LGX818 and cetuximab	NCT02278133
		Phase 2	Withdrawn	Metastatic head and neck squamous cell carcinoma	NCT02649530
		Phase 1	Recruiting	Documented BRAF mut for mCRC and pancreatic cancer; tumors of any histological origin with documented genetic alterations upstream of Wnt signaling	NCT01351103
Unclear	Artesunate	Phase 2	Recruiting	Single primary site colorectal adenocarcinoma or high-grade dysplasia plus unequivocal radiological evidence of invasive cancer	NCT02633098
Unclear	SM08502	Phase 1	Recruiting	Advanced solid tumors who are refractory to or intolerant of established therapy	NCT03355066
WNT decoy	OMP54F28	Phase 1	Completed	Solid tumor with metastasis or unresectable	NCT01608867
WNT inhibitor	CGX1321	Phase 1	Recruiting	Locally advanced or metastatic solid tumors	NCT02675946
		Phase 1	Recruiting	Advanced GI tumors, such as colorectal adenocarcinoma, gastric adenocarcinoma, pancreatic adenocarcinoma, bile duct carcinoma, hepatocellular carcinoma, esophageal carcinoma	NCT03507998
WNT5A inhibitor	WNT5A trap	Preclinical		Modulates the immunological tumor context; favors doxorubicin-driven immunogenic cell death	
β-Catenin inhibitor	PKF115-584	Preclinical		Restores CTL activation *in vivo*	
β-Catenin inhibitor	PRI724	Phase 1b	Completed	Advanced or metastatic pancreatic adenocarcinoma, in combination with gemcitabine in the second line of treatment	NCT01764477
		Phase 1/2	Completed	Advanced myeloid malignancies	NCT01606579
		Phase 2	Withdrawn	Advanced mCRC; in combination with mFOLFOX6 + bevacizumab, in the first line of treatment	NCT02413853
		Phase 1a/1b	Terminated	Phase 1a: any advanced neoplasm; Phase 1b: only patients with mCRC	NCT01302405

mCRC, metastatic colorectal cancer; ARC, antibody-radionuclide conjugate; COX2 (official name PTGS2), prostaglandin-endoperoxide synthase 2; DVL2, disheveled segment polarity protein 2; FZD7, frizzled class receptor 7; PORCN, Porcupine O-Acyltransferase; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CTL, cytotoxic T lymphocyte.

direction, it is important to identify biomarkers associated with the non-T cell inflammatory tumor microenvironment. Although WNT modulators are in development for use in combination immunotherapy regimens for cancer, further preclinical and clinical studies are needed to confirm their utility.

AUTHOR CONTRIBUTIONS

XK and XL conceived and wrote this manuscript. XL, YX, FL, CY, BL, and XK discussed the literature and contributed to the revision of the manuscript. CY contributed to the revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW
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Wnt/β-Catenin Signaling as a Molecular Target by Pathogenic Bacteria

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The Wnt/β-catenin signaling pathway is crucial to regulate cell proliferation and polarity, cell determination, and tissue homeostasis. The activation of Wnt/β-catenin signaling is based on the interaction between Wnt glycoproteins and seven transmembrane receptors—Frizzled (Fzd). This binding promotes recruitment of the scaffolding protein Disheveled (Dvl), which results in the phosphorylation of the co-receptor LRP5/6. The resultant molecular complex Wnt–Fzd–LRP5/6-Dvl forms a structural region for Axin interaction that disrupts Axin-mediated phosphorylation/degradation of the transcriptional co-activator β-catenin, thereby allowing it to stabilize and accumulate in the nucleus where it activates the expression of Wnt-dependent genes. Due to the prominent physiological function, the Wnt/β-catenin signaling must be strictly controlled because its dysregulation, which is caused by different stimuli, may lead to alterations in cell proliferation, apoptosis, and inflammation-associated cancer. The virulence factors from pathogenic bacteria such as *Salmonella enterica* sv Typhimurium, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Citrobacter rodentium*, *Clostridium difficile*, *Bacteroides fragilis*, *Escherichia coli*, *Haemophilus parasuis*, *Lawsonia intracellularis*, *Shigella dysenteriae*, and *Staphylococcus epidermidis* employ a variety of molecular strategies to alter the appropriate functioning of diverse signaling pathways. Among these, Wnt/β-catenin has recently emerged as an important target of several virulence factors produced by bacteria. The mechanisms used by these factors to interfere with the activity of Wnt/β-catenin is diverse and include the repression of Wnt inhibitors' expression by the epigenetic modification of histones, blocking Wnt–Fzd ligand binding, activation or inhibition of β-catenin nuclear translocation, down- or up-regulation of Wnt family members, and inhibition of Axin-1 expression that promotes β-catenin activity. Such a variety of mechanisms illustrate an evolutionary co-adaptation of eukaryotic molecular signaling to a battery of soluble or structural components synthesized by pathogenic bacteria. This review gathers the recent efforts to elucidate the mechanistic details through which bacterial virulence factors modulate Wnt/β-catenin signaling and its physiological consequences concerning the inflammatory response and cancer.

Keywords: bacteria, Wnt, β-catenin, infectious disease, signaling transduction, frizzled

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INTRODUCTION

The Wnt/β-catenin signaling pathway is an evolutionarily conserved mechanism that plays a preeminent role in maintaining cellular homeostasis. It regulates embryo development, cell proliferation and differentiation, apoptosis, and inflammation-associated cancer (1). In basal conditions, the protein transcriptional co-activator β-catenin is constantly phosphorylated by casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) in a process mediated by the Axin complex (also known as *destruction complex*) constituted by the scaffolding protein Axin, the tumor suppressor gene product adenomatous poliposis coli (APC), CK1α, and GSK3β. Such phosphorylation adds a chemical label to β-catenin that is recognized and polybuquitinated by the Skp1-Cdc53-F-Box E3 ubiquitin ligase complex ($SCF^{\beta-TRCP}$) and degraded by the proteasome 26S. The activation of the canonical pathway is dependent on the binding of Wnt glycoprotein ligands to Frizzled (Fzd) receptors and its co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6) on the plasma membrane. Several molecular events take place after the formation of the ternary complex Wnt–Fzd–LRP5/6. One of them is the translocation of the scaffolding protein disheveled (Dvl) to the complex, and the phosphorylation of LRP5/6 followed by the recruitment of the *destruction complex* to the receptor. The consequence of this series of protein–protein interactions is the inhibition of Axin-mediated β-catenin phosphorylation, which leads to β-catenin stabilization, cytoplasmic accumulation, and nuclear translocation. Once in the nucleus, β-catenin displaces the corepressor Groucho and binds to the DNA-bound transcription factors T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to activate Wnt-dependent gene expression (**Figure 1**).

The inflammatory response (IR) is one of the main defense mechanisms of the innate immune system that protects us against physical, chemical, or biological aggressions (2). The activation of different signaling pathways plays a fundamental role to promote, in the first place, or control, in later stages, the IR. Some of the most relevant transcription factors activated by these signaling mechanisms are the nuclear factor κB (NF-κB), the nuclear factor erythroid 2-related factor 2 (Nrf2), and the hypoxia-inducible factor 1α (HIF-1α) (3, 4). Interestingly, pro-inflammatory and anti-inflammatory roles have also been recently assigned to the Wnt/β-catenin signaling in different tissues stimulated with Wnt glycoproteins or infected by pathogenic bacteria. For example, the pro-inflammatory function was documented in pre-adipocytes and microglia cells stimulated with Wnt1 and Wnt3a, respectively (5, 6).

This review highlights new findings that strongly support the regulatory role of Wnt/β-catenin signaling components in the bacterial-induced IR and cancer. Each section contains concise but detailed descriptions on how pathogenic bacteria activate or inhibit the Wnt/β-catenin signaling and what physiological alterations they may cause. We have chosen to present pathogenic bacteria according to their impact on human health and the number of reports cited. **Table 1** shows the most important effects on Wnt/β-catenin of each pathogenic bacteria discussed in text. It is our hope that discussing the activation or inhibition mechanisms of pathogenic bacteria on Wnt/β-catenin signaling will incentivize researchers to explore these molecular processes and propose new therapeutic targets.

Salmonella enterica SV TYPHIMURIUM: A CHRONIC GASTROENTERITIS THAT MAY BECOME CANCER

Salmonella enterica is one of the major public health issues in third- and first-world countries. According to statistical data in the United States, the number of illnesses caused by this enteric bacterium every year is about 1.2 million, with 23,000 hospitalizations and 450 deaths (25). Recent *S. enterica* outbreaks, linked to the consumption of a variety of foods such as tahini, raw chicken, and ground beef and having hedgehogs as pets, have been well-documented (25). Symptoms of acute *S. enterica* infection include fever and gastroenteritis; however, if *S. enterica* colonization becomes chronic, it may lead to other gastrointestinal disorders such as chronic inflammation and cancer. To deliver soluble protein virulence factors in the cytoplasm of host cells, this enteric bacterium is equipped with a type three secretion system (TTSS) (26), which is a nanostructure composed of a hollow needle that serves as a channel to inject the pathogenic virulence factors (called effectors). These *S. enterica* effectors can take on the role of eukaryotic molecules, changing the proper function of host-cell signaling pathways (26).

The best characterized *S. enterica* effector is the anti-virulence factor A (AvrA), a protein related to *Yersinia* acetyltransferase effector YopJ and the *Xanthomonas campestris* pv *vesicatoria* protein AvrBsT (27). AvrA promotes the inhibition of NF-κB activity by enhancing β-catenin deubiquitination in epithelial cells colonized with the non-pathogenic strain *S. enterica* SL1344 PhoPc $AvrA^-$ or genetically complemented wild-type $AvrA^-/AvrA^+$ (28, 29). Direct interaction between the NF-κB p50 subunit and β-catenin caused a reduction of NF-κB-DNA binding and transcriptional activity, which inhibits the IR in *S. enterica* infections (30) (**Figure 2**). Apart from its stabilizing effect on β-catenin, AvrA is also an inducer of several Wnt glycoproteins expression including Wnt2, Wnt3, Wnt6, Wnt9a, and Wnt11 (13). The increase in Wnt2 and Wnt11 causes important reductions in IL-8 expression and *S. enterica* invasion, respectively [as reviewed in (31)]. Wnt3, Wnt6, and Wnt9a, which are important ligands that modulate the activity of stem cell differentiation, were also upregulated in mouse intestinal epithelial cells (IECs) (13). Recently, another member of the Wnt family, Wnt1, was shown to be affected by *S. enterica* (32). Expression of Wnt1 was decreased by ubiquitination and degradation in the proteasome 26S. Because AvrA is a deubiquitinase, this experimental finding may indicate a different mechanism used by AvrA to reduce the Wnt1 protein levels. This Wnt1 expression reduction in tumors of colon mucosa from mice led to an increase in the pro-inflammatory cytokines GM-CSF (granulocyte macrophage–colony stimulating factor), IL-6, and IL-8, which may promote

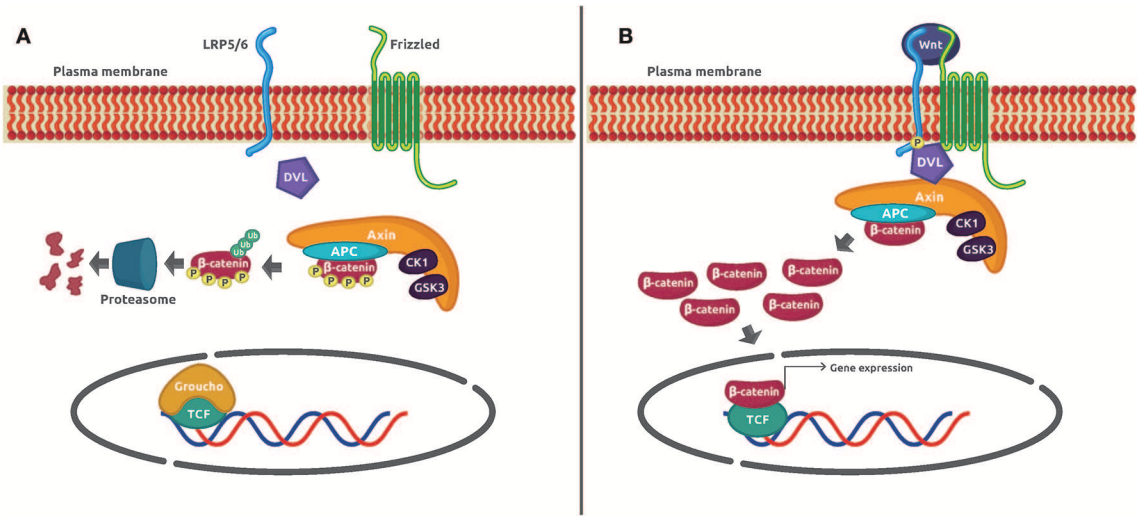

FIGURE 1 | Wnt/β-catenin signaling mechanism. **(A)** In unstimulated cells, β-catenin forms a macromolecular complex with Axin, APC, CK1α, and GSK3 (*destruction complex*). Phosphorylation by CK1α and GSK3 prepares β-catenin to be ubiquitinated and degraded in the proteasome 26S. **(B)** The interaction of Wnt glycoprotein ligands with Frizzled receptors and LRP5/6 co-receptors recruits DVL to the plasma membrane. Then, GSK3 phosphorylates LRP5/6 and β-catenin is no longer phosphorylated and ubiquitinated because the *destruction complex* disassembles. The β-catenin accumulated in the cytoplasm is translocated to the nucleus where it displaces the co-repressor Groucho and interacts with TCF to activate gene expression.

TABLE 1 | Effects of bacterial virulence factors on Wnt/β-catenin signaling pathway.

Bacterium	Bacterial effector	Wnt/β-catenin pathway effect	Pathway component affected	References
Clostridium difficile	Toxin B	↓ Reporter activity	↓FZD 1, 2, and 7 function	(7)
	Toxin A	↓ c-Myc expression ↓ Reporter activity	↓β-catenin stability	(8)
Citrobacter rodentium	Unknown	↑ c-Myc and Mmp7 expression	↓ WIF1 expression ↑ β-catenin stability ↑ R-Spondin 2	(9) (10)
Pseudomonas aeruginosa	LecB	↓ c-Myc and cyclin D-1 expression	↓β-catenin stability	(11)
Bacteroides fragilis	Toxin	↑ c-Myc expression ↑ TCF reporter activity	↑ β-catenin stability	(12)
S. enterica typhimurium	AvrA	↑ TCF reporter activity	↑Wnt2, Wnt3, Wnt6, Wnt9a, and Wnt11 ↑β-catenin stability	(13)
Helicobacter pylori	CagA	↑ c-Myc expression ↑ TCF reporter activity	↑ β-catenin stability ↑ FZD7 expression ↓ WIF1 expression ↓ SFRP expression ↓ DKK expression ↓ Runx3/TCF4 dimer formation	(14–18)
Mycobacterium tuberculosis	Unknown	↑ c-Myc expression ↑ TCF reporter activity	↑ Wnt5a ↑FZD1 ↑β-catenin stability	(19)
Escherichia coli	LPS	↓ TAZ / WWTR1 expression	↓ β-catenin	(20)
Haemophilus parasuis	Virulent strain	↓ β-catenin–E-cadherin interaction	↑ β-catenin nuclear translocation	(21)
Shigella dysenteriae	Virulent strain	↑ IL-8 ↑ TNFα	↑ phospho-β-catenin ↑ phospho-GSK3β	(22)
Lawsonia intracellularis	Virulent strain	↓ MUC2 ↑ Ki67 ↑Caspase-3	↑ β-catenin	(23)
Staphylococcus epidermidis	LP78	↓ TNFα ↓ IL-6	↑ phospho-β-catenin	(24)

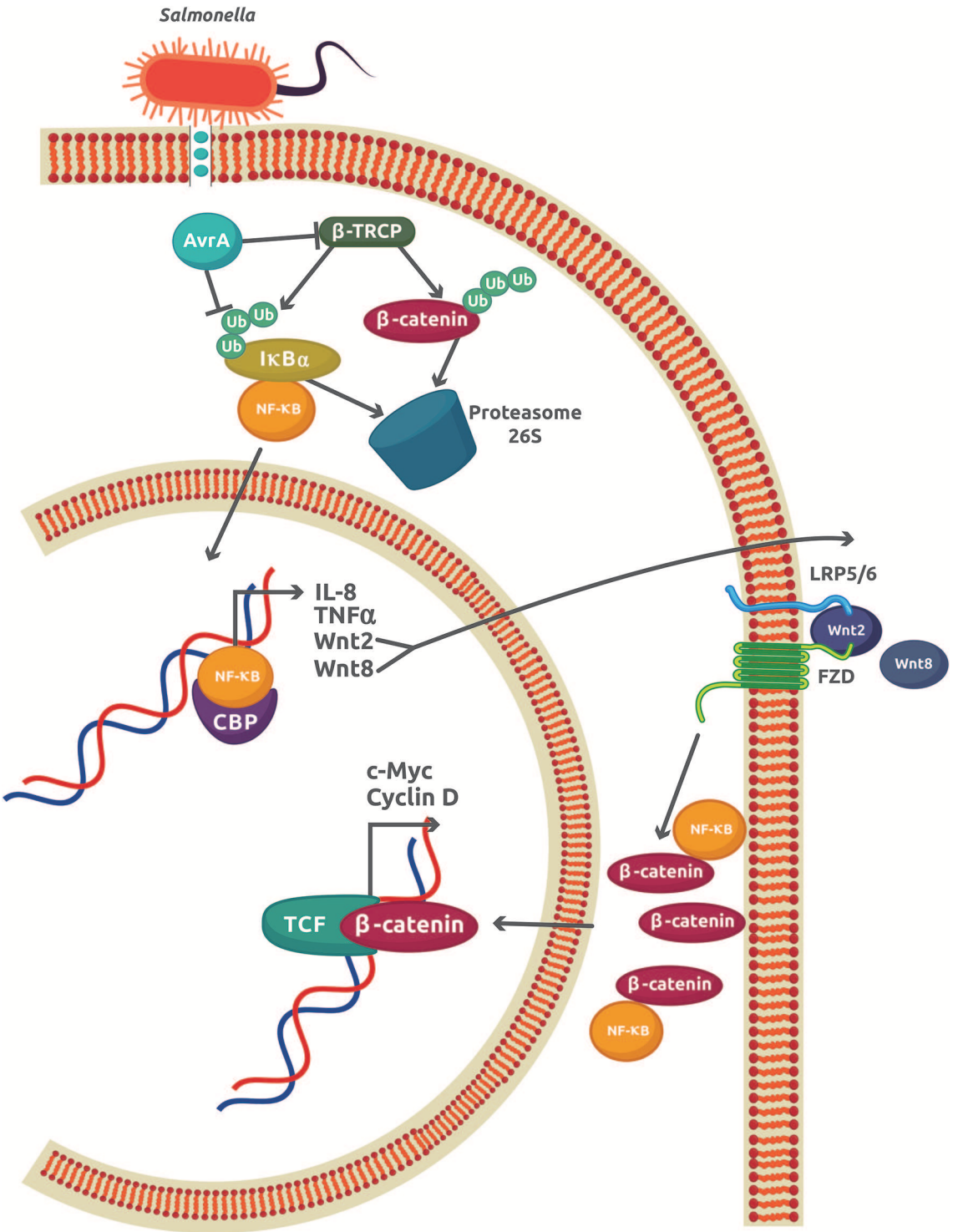

FIGURE 2 | The AvrA virulence factor from *S. enterica* sv Typhimurium inhibits the inflammatory response in epithelial cells. The initial inflammatory response promoted by interaction of *S. enterica* with TLRs leads to an increase in the NF-κB activity and expression of the classical genes, IL-8 and TNFα, and also to Wnt2 and Wnt8. The autocrine effect of Wnt2/8 is to promote the cytoplasmic accumulation of β-catenin. Part of this β-catenin reduces the amount of free NF-κB by forming a complex with the NF-κB p50 subunit. *S. enterica* injects the virulence factor Avra through a type 3 secretion system. AvrA is also able to both inhibit the activity of β-TRCP and directly deubiquitinate IκBα, which increases the stability of the IκBα-NF-κB cytoplasmic complex and reduces the free NF-κB.

a colitis-associated tumorigenesis (32). Thus, AvrA exerts a regulatory role on the host IR by inhibiting the NF-κB-dependent pro-inflammatory gene expression, inducing the activation of stem cells, and increasing the expression of pro-inflammatory cytokines in intestinal tumors as a consequence of Wnt1 expression inhibition. These effects observed in *S. enterica* infections may contribute to chronic disease and cell proliferation, leading to tumor development and cancer.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) regulates many fundamental biological processes including cell proliferation and differentiation, inflammation, and apoptosis (33). In homeostatic conditions, STAT3 maintains under control the inflammation caused by indigenous gut microbiota. In chronic inflammation, the continuous stimulation of IECs by STAT3-activating cytokines (i.e., IL-6 and IL-15) increases the rate of cell proliferation, generates tumorigenic IECs, and causes loss of gut inflammation control (33). The activation of JAK-STAT3 signaling has also been associated with inflammatory bowel disease and colon cancer (34). Interestingly, AvrA activates the expression of the STAT3 target genes *MMP7* (matrix metalloproteinase-7) and *SOCS3* (suppressor of cytokine signaling 3) in a colon cancer mouse model (35). It is tempting to speculate that chronic activation of STAT3 synergizes with β-catenin to promote uncontrolled IEC proliferation and tumorigenesis.

AvrA structural and biophysical data were obtained last year by Labriola et al. (36). Its crystal structure showed many similarities with two YopJ acetyltransferases HopZ (a suppressor of immunity in *Arabidopsis thaliana*) from *Pseudomonas syringae* (37) and Pop2 (an inducer of immunity and inhibitor of proteasome activity in *A. thaliana*) from *Ralstonia solanacearum* (38). The 2.4-Å crystal resolution of AvrA allowed the identification of the secondary arrangement of the regulatory as well-as the catalytic site, and the binding sites of hexakisphosphate and acetyl-CoA (36). More importantly, they characterized the role of an α-helix near the catalytic site and a Leu^{140} residue as a determinant of substrate specificity. Such AvrA structural details have opened new possibilities to develop inhibitory molecules that would help block the harmful effects of this *S. enterica* effector.

Helicobacter pylori: A RISK FOR COLORECTAL AND GASTRIC CANCER

Helicobacter pylori is a spiral-shaped bacterium that causes more than 90% of duodenal ulcers and ∼80% of gastric ulcers and cancer (39), and is present in about 67% of the population worldwide (40). Chronic infection by *H. pylori* is a strong risk factor for gastric cancer development. The virulent strains of *H. pylori* (Type 1 strains) contain the genomic cytotoxin-associated gene A pathogenicity island (*cagA*PAI) that mediates the assembly of a syringe-like structure type IV secretion system (T4SS) (40). Injecting *cagA* through the T4SS initiates a complex series of, as yet, not well-defined interactions with host cell molecules that are thought to be correlated with gastric diseases and cancer. Phosphorylated CagA can interact with many intracellular proteins such as Abl kinase (Abelson murine leukemia viral oncogene homolog 1), Crk (a proto-oncogene adaptor protein), Csk (C-terminal Src kinase), Shp2 (a protein tyrosine phosphatase encoded by the gene *PTPN11*), and others that contain an SH-2 domain (41). These CagA-SH-2 domain interactions influence the cytoskeleton rearrangement and increase cell mobility and size. The interaction of non-phosphorylated CagA with beta-1-integrin, E-cadherin, c-Met (a protein tyrosine kinase), P120-catenin, and ZO-1 (zonula occludens-1), just to cite a few (41), triggers inflammation and alterations in mitogenic signals, and disrupts cell junctions' structure. These phosphorylation-independent CagA activities and other mechanisms not related to CagA are directly associated with the accumulation of β-catenin in nucleus and gastric cancer emergence and development.

The infection by *H. pylori* causes colorectal and gastric cancer because it can activate the Wnt/β-catenin signaling pathway mainly by translocating the virulence factor CagA to the cytoplasm of epithelial cells. Once inside the host cell, CagA interacts and phosphorylates the oncoprotein c-Met receptor that, in turn, activates NF-κB and the expression of numerous pro-inflammatory cytokines and chemokines, enzymes, and angiogenic factors (41). The functional ternary complex CagA-c-Met-CD44 also induces nuclear β-catenin accumulation by activating the PI3K/Akt signaling (14, 42). This pathway inhibits the apoptotic cell death and is associated with colorectal cancer (43). The direct interaction of CagA with the gastric tumor suppressor transcription factor Runx3 (runt related transcription factor 3) labels it for ubiquitination and proteasome degradation (44). Runx3 is normally found interacting with Tcf4 and therefore repressing the β-catenin-dependent gene expression. The interaction of CagA with Runx3 exposes the Tcf4 binding site for β-catenin, which activates the upregulation of β-catenin target genes and induces stomach carcinogenesis (15) (**Figure 3**). Another tumor suppressor that is negatively affected by *H. pylori* is TFF1 (trefoil factor 1) (45). Akt inhibition by TFF1 increases the phosphorylating activity of GSK3β on β-catenin activating PP2A (protein phosphatase 2A). The net effect is the reduction of β-catenin nuclear translocation and Tcf4 transcriptional activity (45). In epithelial cells, *H. pylori* promotes the *TFF1* gene hypermethylation, leading to an increase in β-catenin-dependent gene expression (16). Promoter methylation and mRNA downregulation of Wnt/β-catenin antagonist genes secreted FRP (frizzled-related protein), DKK (DICKKOPF), and WIF1 (Wnt-inhibitory factor 1) have recently been demonstrated during gastric carcinogenesis and affected by *H. pylori* infection (17). As a result of this genetic modification, β-catenin nuclear translocation was increased in gastric epithelial cells. This study confirms that epigenetic modification is an important factor for gastric cancer, in which *H. pylori* plays a significant role. Apart from epigenetic modifications, *H. pylori* infection promotes cancer stem cell characteristics in cancer gastric cells by activating Wnt/β-catenin signaling in a process dependent on CagA (46). It was also found that Nanog and Oct4, two transcription factors associated with epithelial–mesenchymal transition (EMT), increased their expression in the gastric

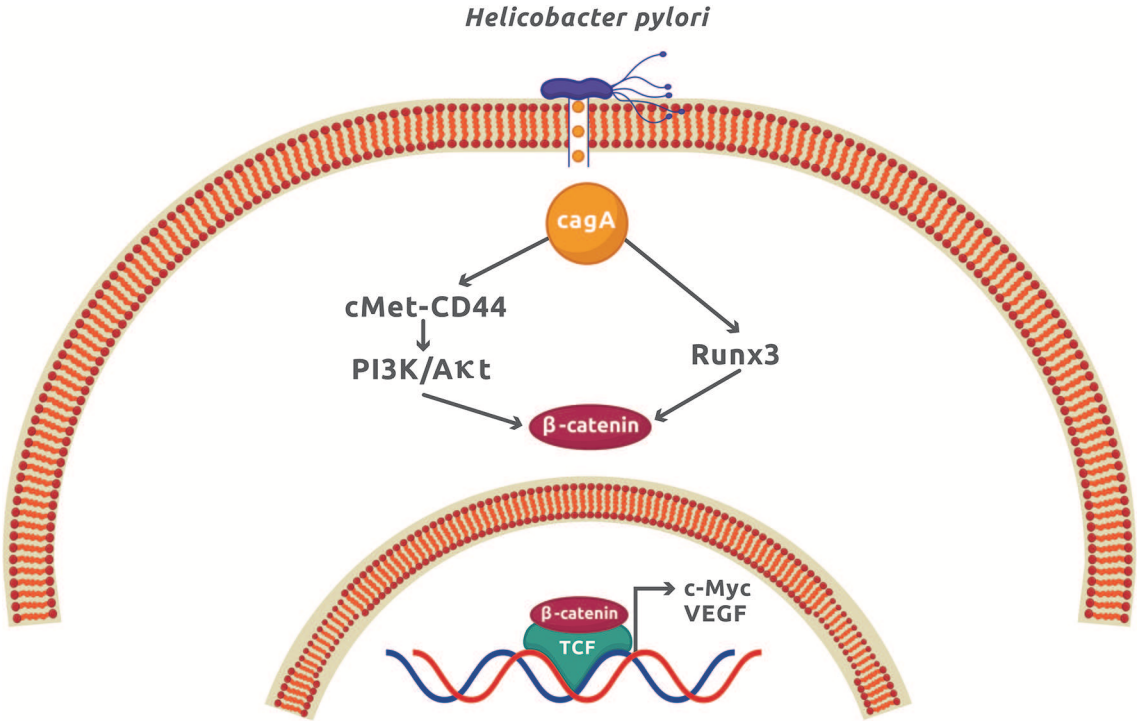

FIGURE 3 | The CagA virulence factor from *Helicobacter pylori* promotes gastric cancer by activation of the Wnt/β-catenin signaling. Once CagA enters the cytoplasm by injection through the type 4 secretion system, it forms a molecular complex with c-Met and CD44, which activates the PI3K/Akt signaling. CagA also interacts with Runx3, which promotes its ubiquitination and proteasome degradation. These two CagA-dependent mechanisms, along with others independent of CagA described in the text, produces a net increment of cytoplasmic β-catenin and expression of β-catenin-dependent genes.

cancer samples from patients infected with *cagA*-positive *H. pylori* (47).

Helicobacter pylori can induce gastric cancer by activating cell proliferation, cell invasion, and angiogenesis (48). The activation of Wnt/β-catenin signaling is mainly responsible for cell proliferation in gastric tumorigenesis (49). Interestingly, a new mechanism to activate Wnt signaling was recently discovered by Geng et al. (18). These authors showed that *H. pylori* upregulates the Wnt receptor Fzd7 and simultaneously represses miR-27b. The inactivation of miR-27b, which has been identified as a tumor suppressor (50, 51), resulted in the overexpression of Fzd7 followed by Wnt signaling activation. Moreover, Wnt/β-catenin activation in *H. pylori* infection has been linked to angiogenesis in the gastric mucosa, which is an important process for tumorigenesis and development of gastric cancer (52). This mechanism involves the upregulation of cyclooxygenase 2 (COX-2) and inhibition of β-catenin phosphorylation. As a result, β-catenin accumulates in the cytoplasm and then translocates to the nucleus. Finally, COX-2/Wnt/β-catenin signaling activation leads to an increase in vascular endothelial growth factor (VEGF) expression, a strong proangiogenic molecule (52).

Mycobacterium tuberculosis: A WNT–FZD INTERACTION THAT DEFINES THE INFECTION OUTCOME

Tuberculosis (TB), the name of the infection caused by *M. tuberculosis* in humans, is one of the most widespread and deadly diseases worldwide with approximately 25% of the whole population infected. The number of TB cases in 2017 around the world was about 10 million, with 1.3 million TB-associated HIV patient deaths (53). Once *M. tuberculosis* invades the lungs, it infects the alveolar macrophages in a process associated with, but not strictly dependent on, several membrane receptors including C-type lectin receptors, mannose receptors, scavenger receptors, CD14, CD43, and LSPA (lung surfactant protein A) [as reviewed in (19)]. The success of *M. tuberculosis* as an intracellular pathogen depends on its ability to manipulate many signaling mechanisms activated by the host cell. For example, after infection, a burst of pro-inflammatory cytokines and chemokines like TNFα (tumor necrosis factor α), IL-1β, and IL-6 is observed followed by the expression of anti-inflammatory cytokines such as TGF-β (transforming growth factor-β) and IL-10 [as reviewed in (54)]. It is assumed that the attenuation of the inflammatory and adaptive response through production of IL-10 is critical

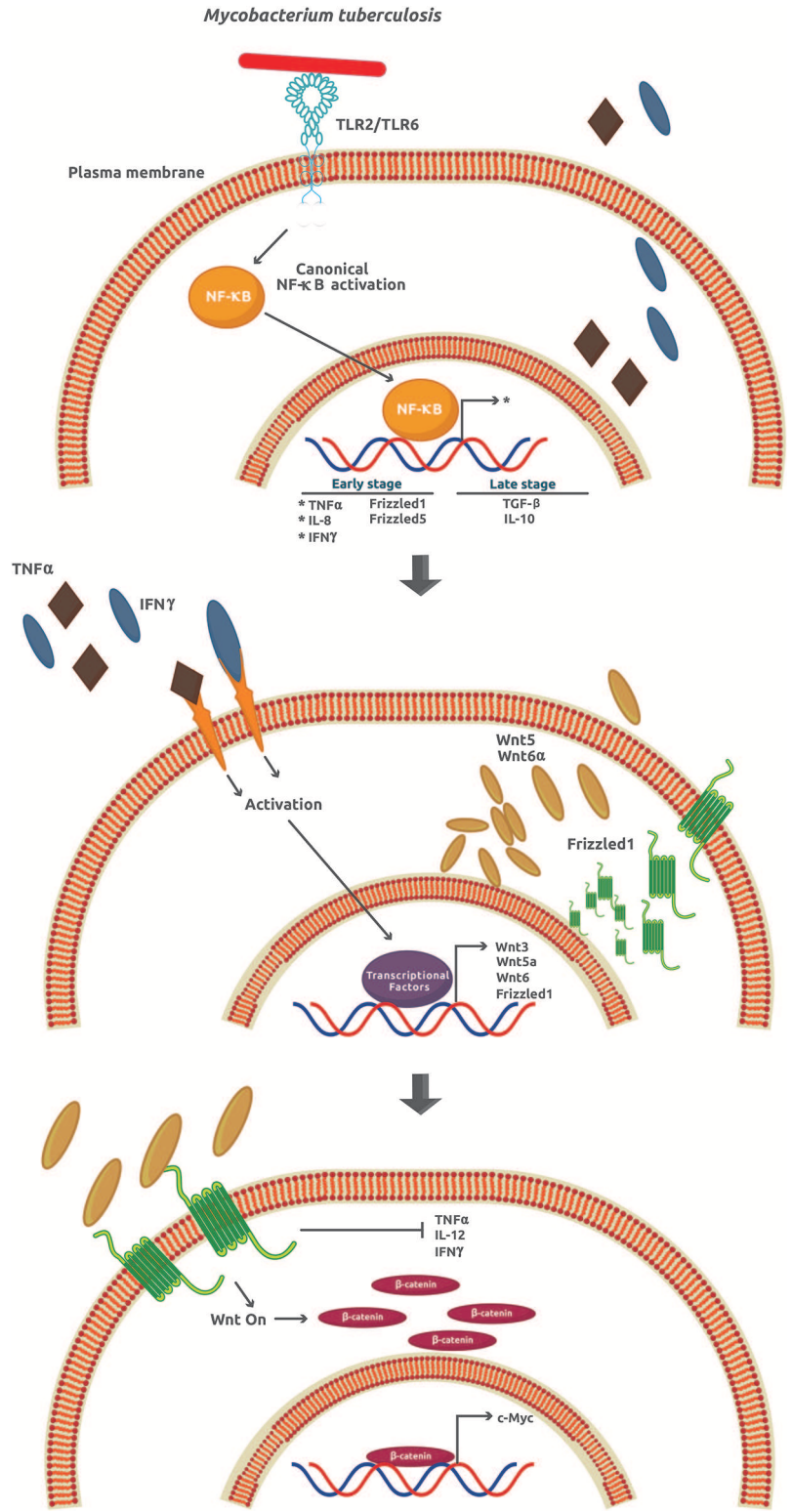

FIGURE 4 | *Mycobacterium tuberculosis* activates several signaling pathways that lead to an inflammatory response inhibition. During the early phase of infection, *M. tuberculosis* induces the expression of TNFα, IL-6, IFNγ, Frizzled1, and Frizzled5. However, at later stages of infection, there is an increase of the anti-inflammatory cytokines TGFβ and IL-10. The secreted TNFα and IFNγ promote the paracrine expression of Wnt3, Wnt5a, Wnt6, and Frizzled1 that in turn activates the Wnt/β-catenin signaling.

for *M. tuberculosis* survival and granuloma formation (**Figure 4**, upper panel).

Wnt glycoproteins play a functional role as pro- or anti-inflammatory factors depending on the cellular type and context, the stimuli, and the kind of cytokines previously secreted (1). In particular, when cells are infected by *M. tuberculosis*, Wnt3a and Wnt5a downregulation and Wnt6 upregulation promote an anti-IR, *via* canonical (Wnt3a) and non-canonical (Wnt5a and Wnt6) signaling in mouse and human models (19). In contrast to Wnt3 that prevents infection by *M. tuberculosis*, Wnt5a and Wnt6 promote it. Except for Wnt5a, Wnt3, and Wnt6, expression is not directly induced by *M. tuberculosis* (**Figure 4**, upper and middle panel). However, the upregulation of Fzd1 and Fzd5 is a process directly related to the effect of *M. tuberculosis* (19). The downregulation of Fzd3–4 and Fzd6–10 has been correlated with reduced β-catenin-dependent gene expression (55). Apart from the effects on inflammation, Wnt3, Wnt5a, and Wnt6 inhibit necrosis, TNFα secretion, IL-12 and IFNγ production, phagolysosome formation, promotion of apoptosis and phagocytosis, cellular adhesion, and cellular proliferation, among others (**Figure 4**, lower panel). Collectively, these effects help *M. tuberculosis* survive and damage the intracellular milieu and surrounding cells (19).

Pseudomonas aeruginosa: TARGETING WNT/β-CATENIN TO PROMOTE BACTERIAL SURVIVAL

P. aeruginosa is an opportunistic bacterial pathogen associated with severe intra-hospital pneumonia (56). One of its most common pathogenic mechanisms leading to the recurrence and chronic infections is to damage the epithelial monolayer structure in the lung alveoli. Also, *P. aeruginosa* avoids elimination by macrophages, which promotes its prevalence and produces chronic inflammation (57).

Experiments with murine macrophages RAW264.7 infected with *P. aeruginosa* 19660 have demonstrated a time-dependent increase in β-catenin degradation (58). In contrast, the overexpression of the β-catenin gene *CTNNB1* in macrophages enhances bacterial intracellular killing by counteracting cell auto-phagocytosis without affecting reactive oxygen species synthesis. However, *P. aeruginosa* can survive inside macrophages by promoting β-catenin degradation (58). These experimental observations indicate that, in macrophages, β-catenin promotes an auto-phagocytosis-breakresistant phenotype.

In an acute lung injury model, *P. aeruginosa* infection of epithelial H1299 and H1975 cell lines promotes NF-κB activation by stimulating its nuclear translocation and β-catenin degradation (11). Interestingly, this degradation was linked to the virulence factor LecB because incubating H1299 cells with recombinant purified LecB was sufficient to induce β-catenin degradation and repress the expression of the β-catenin-dependent genes *c-Myc* and *Cyclin-D1*. Thus, LecB causes a reduction in cellular proliferation by arresting cell cycle in G1 to S phase transition. LecB also promoted β-catenin intracellular reorganization by destabilizing its molecular complex with α1β3 integrin in the plasma membrane and β-catenin degradation in a process dependent on GSK3α/β but independent on PI3K/Akt (11). In agreement with these data, Wnt3a, a prototypical regulator of β-catenin destruction complex, prevented LecB-induced β-catenin degradation (11). These results suggest a role for β-catenin in modulating cellular functions directly related to *P. aeruginosa* clearance.

Citrobacter rodentium: AN UNKNOWN BACTERIAL EFFECTOR PROMOTING HYPERPLASIA

This bacterium is an enteric pathogen of mice that shares mechanistic similarities with the classical enteropathogenic and enterohemorrhagic strains of *Escherichia coli* (EPEC and EHEC). This feature constitutes a useful model to investigate the characteristic attaching/effacing (A/E) injuries of the gut epithelium induced by *E. coli* in humans (59). Apart from the A/E lesions, *C. rodentium* induces hyperplasia and tumorigenesis of the crypt cells (60). A detailed analysis has revealed that mesenchymal stem cells are the primary target of hyperplasia and the activation of Wnt/β-catenin signaling has been correlated to the self-renewal phenotype and proliferation (60). Wnt/β-catenin signaling also promotes an increase in R-spondin-2 expression, an agonist of the leucine-rich repeat G-protein coupled receptor (Lgr), that, along with Fzd and LRP5/6, regulates the expression of Mmp7 and c-Myc genes involved in cell regeneration (9). Although a specific bacterial effector responsible for these mechanisms has not been identified, the hyperplasia caused by *C. rodentium* infection requires an intact T3SS to promote cellular proliferation and Wnt/β-catenin activation (60). Infecting mice with the T3SS defective mutant ΔEscV (an inner membrane T3SS assembly related protein) fails to induce the expression of proliferation markers and attenuates hyperplasia (10, 61).

C. rodentium infection also promotes Wnt/β-catenin-mediated hyperplasia through the repression of the Wnt/β-catenin inhibitor factor 1 (WIF1) expression in two different ways. The first mechanism involves epigenetic modification of the enhancer region of the methyltransferase zeste homolog-2 (*EZH2*) gene that, in turn, promotes the H3 lysine-27-trimethylation (H3K27me3) in the promoter region of the WIF1 gene (10). The second mechanism involves the downregulation of WIF1 gene by the upregulation of miRNA203. Interestingly, these mechanisms are dependent on bacterial effectors because the infection of mouse colonic primary cells with the *C. rodentium* mutants ΔEscV showed a reduced expression of *EZH2* and *H3K27me3* genes and an increased expression of *WIF1* compared with their non-mutant counterparts (10). So far, it is uncertain why *C. rodentium* promotes stem cell hyperplasia. It is likely that elevated levels of β-catenin may block the NF-κB pathway (62), which is one of the main defense mechanisms against bacterial infections.

Clostridium difficile: HIJACKING WNT–FZD INTERACTION

This bacterium is a human commensal and opportunistic pathogen related to pseudo-membranous colitis and antibiotic-associated diarrhea, which can be life-threatening diseases among elderly patients in developed countries. The most virulent strains secrete toxins A and B. These toxins bind to chondroitin sulfate proteoglycan 4 (CSP4) in HeLa cells and inhibit small GTPases by glycosylation resulting in cell rounding and death (63, 64); however, CSP4 was not detected in colonic epithelium where *C. difficile* inhabits, indicating the existence of additional receptors involved. The evidence obtained by genetic screening analysis suggests that the Wnt receptors Fzd1, 2, and 7 from HeLa, Caco-2, and HT-29 cells interact with toxin B (7, 65). When toxin B binds to Fzd receptors, Wnt3a and probably other Wnt ligands cannot activate the Wnt/β-catenin pathway (7, 66). The inhibition of Wnt/β-catenin signaling by toxin B from *C. difficile* represses the TCF-dependent gene expression.

Toxin A, on the other hand, can attenuate Wnt/β-catenin signaling by repressing target genes expression like *c-Myc* even in the presence of the GSK3 inhibitor LiCl, indicating the existence of additional steps in its inhibitory mechanism (8). A precise role for Wnt/β-catenin pathway inhibition during *C. difficile* infections remains unclear. Further research on these pathogenic mechanisms is strongly recommended because this bacterium is difficult to control and may cause fatal diseases (63).

Bacteroides fragilis: TARGETING β-CATENIN FROM THE ADHERENS JUNCTIONS

This bacterium constitutes around 1 to 2% of the gut microbiota and approximately 30% of the anaerobic organisms cultured from human feces. It is considered as the most frequent anaerobic pathogen capable of infecting soft tissues and an important etiological agent of diarrhea (67). The genome of *B. fragilis* virulent strains contains a pathogenic island that encodes a *B. fragilis* toxin (BFT), which is a heat-labile metalloprotease. Rabbits challenged with high concentrations of BFT show tissue damage and hemorrhage, an increase in IL-8 expression, NF-κB activation, and disruption of the epithelial barrier (68, 69).

BFT was the first bacterial effector reported to activate the β-catenin-dependent gene expression (12). The protease-mediated activity of BFT on the extracellular domain of E-cadherin has been suggested as the mechanism to disrupt epithelial cell-to-cell contact. As a result, BFT causes the dissociation of β-catenin from E-cadherin and, once released, β-catenin translocates to the nucleus where it forms a complex with TCF4, leading to *c-Myc* expression and cellular proliferation in the APC mutant cell lines HT29/C1 and SW480 (12). β-Catenin released from E-cadherin interaction do not translocate directly to the nucleus but to the perinuclear endocytic recycling compartment in A431 cells that express full APC protein (70). This observation indicates that the impaired function of the β-catenin *destruction complex* is a condition needed for BFT to induce β-catenin-mediated effects. However, the effects induced by *B. fragilis* infection on the β-catenin *destruction complex* proteins in non-APC mutant cells are still unknown and deserve further investigation. In agreement with this reasoning, *Bordetella pertussis* toxin, which has been reported to induce the dissociation of β-catenin from the complex with VE-cadherin of adherens junctions, also promotes GSK3-specific phosphorylation at Thr41 and Ser45 of the released β-catenin that marks it for degradation (71).

E. coli: OSTEOGENIC DIFFERENTIATION OF HUMAN PERIODONTAL STEM CELLS BY LPS

Periodontitis is an inflammatory disease caused by microbial populations in oral microbiota, which implies biofilm formation and deregulation of the IR from the host (72). Gram-negative anaerobic bacteria are among the most prominent bacterial species found in periodontal disease. Lipopolysaccharide (LPS) is a common cell surface antigen present in Gram-negative bacteria. Recently, Xing et al. (20) found that LPS from *E. coli* stimulated osteogenic differentiation of human periodontal ligament stem cells (HPDLSCs) but not their proliferation, viability, or cell cycle regulation. They showed that this stimulation was driven by the activation of Wnt/β-catenin pathway. Osteogenesis and osteogenesis stimulated by LPS were confirmed by RT-PCR of osteogenesis-related genes ALP (alkaline phosphatase), RUNX2 (Runt-related transcription factor 2), OCN (osteocalcina), and OSX (osterix) (20). TAZ (also known as WWTR1 [domain containing transcription regulator 1]) is a key transcriptional co-activator with a PDZ binding motif that mediates cell proliferation, differentiation, and stem cell renewal through the Wnt/β-catenin. Its mRNA and protein levels were also increased during LPS-stimulated osteogenesis. The depletion of TAZ by an antiviral transfection with a shTAZ construction reduced both LPS-stimulated and independent osteogenesis *in vitro* and also blocked the induction of osteogenesis-related genes, emphasizing its major role in osteogenesis (20). To demonstrate the role of Wnt/β-catenin in the induction of TAZ and osteogenesis, LPS stimulation was performed in the presence of DKK1 (DIKKOPF1), a known inhibitor of the Wnt/β-catenin transduction pathway that blocks the interaction between Fzd and LRP5/6 with Wnt ligands. ALP or alizarin red staining assays showed that DKK1 inhibited osteogenesis *in vitro*, in the presence or absence of LPS. This was accompanied by a decrease in the accumulation of TAZ and β-catenin in the presence of LPS, and a slight but significant increase in phosphorylated TAZ at Ser89. DKK1 also reversed the induction of osteogenesis related-genes by LPS (20).

Haemophilus parasuis: INITIATION OF EMT

Glässer's disease (Gd) is an acute infection in pigs characterized by a combination of meningoencephalitis, polyserositis,

pericarditis, polyarthritis, and, in some cases, primary pneumonia. It is caused by specific serovars of *H. parasuis*, a bacterium that normally resides in the upper respiratory tract. In certain host stress conditions (i.e., weaning, antibiotic treatment, and transportation), *H. parasuis* may turn pathogenic, causing Gd or an acute respiratory disease, depending on the site of infection. Sometimes, an acute inflammatory systemic response causes massive fibrin exudates in the pleuroperitoneal cavity leading to sudden death (73).

The disruption of adherens junctions is a major alteration of epithelial cells that leads to the modification of epithelial permeability. Recently, a role for Wnt/β-catenin in the EMT that contributes to the loss of epithelial permeability caused by *H. parasuis* was reported (21). The virulent *H. parasuis* strain SH0165 was observed to increase β-catenin and reduce phospho-β-catenin at Ser33/37/Thr41 as compared with the non-virulent strain HN0001 in pig kidney (PK-15) cell line and newborn pig tracheal (NPTr) epithelial cell lines. Relative luciferase expression assays showed that the Wnt/β-catenin pathway was activated by increasing doses of the virulent strain and by LiCl, a known GSK3β inhibitor that stimulates the Wnt signaling pathway. This activation correlated well with an increase in β-catenin and a decrease in phospho-β-catenin. β-Catenin was also translocated from the membrane to the cytoplasm and to the nucleus by inoculation with the virulent strain and treatment with LiCl (21). E-cadherin was also degraded upon the interaction of epithelial cells with the virulent strain or by stimulation with LiCl; this was reversed by adding Wnt/β-catenin inhibitors ICG001 and IWR-endo-1, demonstrating the role of the signaling pathway in maintaining the stability of adherens junctions (21). Furthermore, nuclear immunolocalization of β-catenin and reduction of E-cadherin levels in the membrane were confirmed by confocal laser scanning microscopy (CLSM), and these correlated with alterations in epithelial monolayer permeability. The final demonstration of Wnt signaling activity in EMT was conducted by applying siRNA technology against several Wnt target genes such as *MMP7* (membrane palmitoylated protein 7), *PAI-1* (plasminogen activated inhibitor 1), and *COX2* (cyclooxygenase 2), which significantly produced a recovery of the expression levels of the EMT-related genes *E-cadherin*, *collagen IV*, *cytokeratin*, *N-cadherin*, *Snail*, *Vimentin*, and *S100A4* (21).

Lawsonia intracellularis: ALTERATION OF HOMEOSTASIS AND PROMOTION OF CELL PROLIFERATION DURING INFECTION OF INTESTINAL CRYPT CELLS

Proliferative entheropathy (PE) is an infectious disease caused by *L. intracellularis* in which bacteria invade the intestinal crypt cells inducing extensive cell proliferation and loss of the integrity of intestinal mucosa and mucosal thickening. The disease may have two forms—an acute one characterized by hemorrhagic diarrhea and sudden death, and a chronic one with no hemorrhagic diarrhea that causes wasting and loss of control (74).

Wnt/β-catenin and Notch signaling regulate intestinal stem cell (ISC) proliferation and differentiation. These pathways transit amplifying (TA) progenitor cells from which secretory cells and intestinal enterocytes differentiate, regulating in that way the intestinal homeostasis. The evaluation of Wnt/β-catenin and Notch signaling during the disruption of intestinal homeostasis caused by *L. intracellularis* infection was investigated by Huan et al. (22). They detected fluorescent *L. intracellularis* in the crypts of ileum sections and observed an increase in bacterial number from 3 to 14 days post-colonization (dpc) and a drastic decrease by 28 dpc. Fluorescence and mRNA levels of MUC2 (intestinal mucin 2) reached the lowest levels by 14 dpc, consistent with the maximum of *L. intracellularis* fluorescent signal in the ileum. A cell proliferation marker Ki67 and the apoptosis marker caspase-3 also reached their highest fluorescent signals by 14 dpc. These assays demonstrated the correlation between *L. intracellularis* ileum crypt colonization and the increase in cell proliferation and apoptosis, which result in PE tissue damage. β-Catenin also increased in the upper and lower halves of the crypt by 14 dpc, which was in agreement with the repression of the Wnt/β-catenin-dependent genes *ASCL2* (achaete-scute family BHLH transcription factor 2), *LGR5* (leucine-rich repeat containing G-protein receptor 5), *SOX9* (SRY box 9), and *Cyclin D1* (22). The intercellular Notch-1 receptor domain/C-terminal (NICD1) immunodetection in crypts also reached its highest level at 14 dpc, correlating with the transcript repression of Notch-regulated genes *ATOH1* (atonal BHLB transcription factor 1; 14 dpc), *HES1* (hairy and enhancer ff split1; 21 dpc), and *OLFM4* (olfactomedin 4; 28 dpc) (22). Changes in Wnt/β-catenin- and Notch-regulated gene expression were consistent with previous reports on their role in intestinal homeostasis, allowing authors to suggest the participation of these major signaling pathways in PE lesions.

Shigella dysenteriae: IR THROUGH WNT/β-CATENIN AND NF-κB PATHWAYS

Shigella dysenteriae is one of the four species of the genus. It is integrated by 15 serotypes, which are one of the main causative agents of dysentery in developing countries. It destroys the colonic epithelium without causing systemic infection. The typical symptoms produced are diarrhea with blood and mucus accompanied by abdominal cramps and fever (75). Colon infection is associated with an acute IR mediated by IL-8. Recently, Gopal et al. (23) explored the involvement of the Wnt/β-catenin and NF-κB pathways in regulating IL-8 levels and the IR. A rat ileal loop infection model was used to test the IR and the signaling pathways involved. The infection of the ileal loop with *S. dysenteriae* increased both the protein and mRNA expression levels of proinflammatory cytokines IL-8 and TNFα. Immunohistochemical detection reveals that both β-catenin and NF-κB transcription factors increased the level of expression and its localization (from cytoplasmic to nuclear) after infection with *S. dysenteriae* (23). Phospho-IκBα, NF-κB, phospho-β-catenin, and GSK3β phosphorylated

at Y216, and proteins showed increased levels after infection, whereas unphosphorylated β-catenin and phospho-GSK3β at S9 were reduced. Co-immunoprecipitation with GSK3β and β-catenin antibodies demonstrated the association of β-catenin and NF-κB through GSK3β. Overall, these data suggest that *S. dysenteriae* negatively regulates β-catenin pathway by modulating GSK3β activity; also, the formation of a β-catenin/GSK3β complex promotes β-catenin degradation and release of NF-κB from the ternary complex, leading to inflammation (23).

Staphylococcus epidermidis: INHIBITION OF SKIN INFLAMMATION BY β-CATENIN ACTIVATION

Staphylococcus epidermidis protects the skin from pathogen infections by secreting antimicrobial peptides (76), although, in some specific conditions, it may become an opportunistic pathogen. Lactic acid bacteria have also been associated with skin wound healing because they can produce bioactive metabolites that control pathogen bacteria and modulate the immune response (77). The ability of commensal *S. epidermidis* to contribute to wound healing was recently examined by Li et al. (24). These authors isolated lipopeptide 78 (LP78; a peptide of 23 amino acids with an heneicosanoic acid bound to the N-terminal aspartic acid D1) from culture medium and tested its effects on IR in a neonatal human epidermal keratinocytes (NHEKs) cell line and in streptozotocin-induced type 1 diabetic mice. When NHEKs were pre-treated with poly(I:C) and different LP78 concentrations, a significant reduction of TNFα and IL-6 was observed (24). The inhibitory effect of LP78 was potentiated by the addition of lipoteichoic acid (LTA), one of the main proinflammatory structures present in the cell wall of Gram-positive bacteria.

When TNFα and IL-6 protein levels were estimated in the wounds of normal mice or in the wounds of *Tlr-3*$^{-/-}$ deficient mice, LP78 repression of cytokine expression was observed in normal but not in TLR3-deficient mice (24). Immunolocalization by CLSM and quantification of the NF-κB p65 subunit suggested that LP78 does not block NF-κB nuclear translocation induced by poly(I:C). In NEHKs, LP78 induced accumulation of phospho-β-catenin at Y654 and translocation of β-catenin to nucleus in a time-dependent manner. The LP78-mediated repression of poly(I:C)-induced cytokines is lost in NEHK cells with shRNA-reduced levels of β-catenin and in wounds treated with the β-catenin inhibitor FH535, which also caused a decrease in LP78-induced phospho-β-catenin (Y654) accumulation (24). The TLR2 inhibitor OxPAPC (oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine) caused a reduction in β-catenin nuclear translocation and LP78 repression of poly(I:C)-induced cytokines. LP78 effect is also lost in a TLR2-deficient genetic background in the ear intradermic injection model and in primary culture of murine keratinocytes and in the skin wound model. These data suggest that LP78 activates TLR2. To elucidate the TLR2 pathway, inhibitors such as PP2 (specific for non-receptor tyrosine kinase family Src), Bay11 (specific for NF-κB), SB431542 (specific for TGF-β), and FH535 (specific for β-catenin) were tested (24). PP2 and FH535 blocked LP78-induced β-catenin phosphorylation. Therefore, these data suggest that LP78 activates TLR2-Src to induce β-catenin activation. GW9662, an inhibitor of PPARγ (peroxisome proliferator-activated receptor gamma), inhibited poly(I:C)-induced TNFα and IL-6. As it is known that PPARγ interacts with p65 and that this interaction is disrupted by β-catenin, experiments were conducted to elucidate if LP78 inhibits TLR3-mediated IR by β-catenin-mediated impairment of p65–PPARγ interaction (24). NEHK cells treated with poly(I:C) and stimulated with LP78 showed reduced amounts of p65. Under these conditions, β-catenin amounts decreased in the cytoplasm and increased in the nucleus. Altogether, these data suggested that LP78 induces β-catenin nuclear translocation. Once in the nucleus, β-catenin disrupts the interaction between TLR3-activated p65 and PPARγ, thus inhibiting TLR3-mediated inflammation in skin wounds.

CONCLUDING REMARKS

The *golden age* of antibiotics therapy against the most important human pathogenic bacteria is approaching a dead end. Nosocomial infections caused by several bacteria cited in this review are difficult to eliminate because of multiple mechanisms that make them resistant to antibiotics specifically recommended for their treatment (78). This antibiotic resistance has generated the appearance of *superbugs*, which are responsible for epidemics with enhanced morbidity and mortality.

One of the most promising alternatives to overcome the bacterial resistance to antibiotics is to search for the host cell mechanisms that are activated or inhibited by virulence factors secreted during pathogenic bacteria infections and then identify the protein molecules involved. Protein kinases (i.e., p38, JNK) and specific transcription factors (i.e., NF-κB), as well as cytoskeletal targets activated during the pro-IR, have been characterized in a number of bacterial infections. More recently, signaling pathways like Wnt/β-catenin and Notch, whose main function is not related to inflammation, were added to the arsenal of molecular mechanisms touched by pathogenic bacteria. The complexity is even greater because the classical NF-κB pro-inflammatory signaling molecules cross-talk with other signaling pathways such as the Wnt/β-catenin, which are not apparently related to inflammation. This interrelationship frequently gives unexpected phenotypes that need to be taken into account to understand the temporal stages of infection. An integration of signaling at each stage and its proper interpretation may help researchers identify the important clues to fight lethal bacteria. Once signaling molecules are identified, the next step would be to design specific drugs to deactivate the virulence factors. In order to accomplish this ambitious goal, the structural details of virulence factors need to be elucidated. Such biochemical studies combined with bioinformatics, molecular dynamics approaches, and traditional methods (i.e., vaccines) will undoubtedly pave the way to win the battle against infectious diseases.

AUTHOR CONTRIBUTIONS

OS-G and JV-A helped with the writing of the manuscript. VB-A conceived, wrote, edited, and reviewed the manuscript.

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REVIEW
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Bacterial Manipulation of Wnt Signaling: A Host-Pathogen Tug-of-Wnt

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The host-pathogen interface is a crucial battleground during bacterial infection in which host defenses are met with an array of bacterial counter-mechanisms whereby the invader aims to make the host environment more favorable to survival and dissemination. Interestingly, the eukaryotic Wnt signaling pathway has emerged as a key player in the host and pathogen tug-of-war. Although studied for decades as a regulator of embryogenesis, stem cell maintenance, bone formation, and organogenesis, Wnt signaling has recently been shown to control processes related to bacterial infection in the human host. Wnt signaling pathways contribute to cell cycle control, cytoskeleton reorganization during phagocytosis and cell migration, autophagy, apoptosis, and a number of inflammation-related events. Unsurprisingly, bacterial pathogens have evolved strategies to manipulate these Wnt-associated processes in order to enhance infection and survival within the human host. In this review, we examine the different ways human bacterial pathogens with distinct host cell tropisms and lifestyles exploit Wnt signaling for infection and address the potential of harnessing Wnt-related mechanisms to combat infectious disease.

Keywords: Wnt, β-catenin, bacteria, pathogen, innate immunity, immunoevasion

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INTRODUCTION

The innate immune response is the first, and in many successful cases, the primary barrier between bacterial invader and human host. Fine-tuned through co-evolution with microbial insults and largely evolutionarily conserved across the Metazoa, the innate immune system specializes in both pathogen recognition and the formation of a rapid response (1). At the foundation of the innate cellular antimicrobial response are families of germline-encoded pattern recognition receptors (PRRs) expressed by professional phagocytes, including Toll-like, C-type lectin, NOD-like, and RIG-I-like receptors (2, 3). Interaction of receptor and ligand, which include lipoproteins, polysaccharides, nucleic acids, and other conserved microbial molecular patterns, results in an inflammatory response involving cytokine, and chemokine gene transcription, pathogen clearance through various mechanisms such as lysosomes, antimicrobial peptides, or membrane attack complexes, and coordination of the adaptive immune response (2, 4). Unsurprisingly, bacterial pathogens have evolved an armament of immunoevasion mechanisms as carefully selected for as the immune system that defends the host. The very foundation of bacterial virulence is the ability to subvert host defenses in order to establish a replicative niche. This involves mechanisms beneficial

to pathogens that replicate in the extracellular niche, such as serum resistance, tissue adherence, and motility; mechanisms beneficial to the pathogens that replicate in an intracellular niche, such as controlling host cell fate and avoiding lysosomal destruction; and mechanisms employed by pathogens of both niches including competitive nutrient acquisition systems and secretion of effector proteins to modulate the host.

The means by which pathogens subvert the host innate immune responses have helped expand our knowledge regarding how eukaryotic cellular signaling pathways cooperate to modulate innate immunity. Often, these pathways moonlight as branches of the immune system, as they were originally discovered in the context of cell development or cancer. For example, eukaryotic Notch signaling is a well-characterized regulator of cell fate that is highly active in development and tissue homeostasis (5). This pathway has been shown to function within innate immunity through regulation of PRR expression. The obligately intracellular pathogen *Ehrlichia chaffeensis* utilizes a type 1 secretion system (T1SS) effector to activate Notch signaling which indirectly downregulates PU.1, a transcriptional activator of TLR2 and 4 (6). The discovery of xenophagy as a type of autophagy is another example of a moonlighting innate immune pathway. Years of research have demonstrated that a process originally thought only to function as a cellular starvation and stress response also functions within the innate immune system (7). Xenophagy is deployed against Group A *Streptococcus* as a method of bacterial clearance, while other pathogens express effector proteins to escape xenophagy, as is the case with *Listeria monocytogenes* expression of xenophagy evasion protein ActA (8, 9). As evidence linking conserved eukaryotic cell pathways to the immunosubversion of human pathogens continues to grow, as does our model of the innate immune system and the networks that comprise it.

Mounting research within the last two decades has demonstrated that the conserved eukaryotic signaling pathway Wnt is a significant part of the interplay between the human host and both extracellular and intracellular bacterial pathogens. The discovery of the Wnt pathway began with the identification of the murine oncogene *int-1* that was found to be homologous with a *Drosophila* gene that controlled body segmentation during development (10, 11). Later renamed Wnt proteins, these gene products are a family of 19 highly conserved, secreted, lipidated glycoproteins that regulate metazoan development and tissue homeostasis (12). Wnt proteins participate in paracrine and autocrine signaling through binding of 1 of 10 homologs of the seven-pass transmembrane receptor Frizzled (Fzd1-10) and a cognate coreceptor expressed on the surface of the signal-receiving cell. The signal is transduced through the intracellular mediator Disheveled (Dvl) which, depending on its phosphorylation state, activates either canonical or non-canonical pathways (13). Canonical Wnt signaling, also known as β-catenin-dependent signaling, is the most well-studied Wnt pathway (**Figure 1**) (14). In the pathway off state, the β-catenin destruction complex consisting of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1) facilitates the phosphorylation of β-catenin by GSK3β which induces ubiquitination of β-catenin by the β-TrCP-SCF E3 RING-type ubiquitin ligase complex (β-TrCP) and subsequent proteasomal degradation. When Dvl is activated through interaction of a Wnt ligand with a Fzd receptor and the canonical pathway coreceptor lipoprotein receptor-related protein 5/6 (LRP5/6), the destruction complex is recruited to the Frizzled-Dvl complex at the plasma membrane, freeing β-catenin from degradation. Accumulation of the cytoplasmic pool of β-catenin induces its translocation into the nucleus where it binds with T-cell factor (TCF) transcription factor at the Wnt response element (WRE) DNA sequence and activates transcription of target genes involved in processes such as development (*SNAIL*, *ENGRAILED*, *SLUG*) and cell proliferation (*CMYC*, *CCND1*, and *MMP7*).

Non-canonical, β-catenin-independent signaling can be divided into two pathways: the Wnt/Ca^{2+} pathway and the planar cell polarity (Wnt/PCP) pathway. In the Wnt/Ca^{2+} pathway, Wnt ligands signal through Fzd and the coreceptor receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/2) to induce Dvl-dependent phospholipase C (PLC) cleavage of phosphatidylinositol 4,5-biphosphate (PIP_2), producing inositol triphosphate (IP_3) and diacyl glycerol (DAG) (**Figure 2A**) (15). IP_3 acts on Ca^{2+} channels at the endoplasmic reticulum resulting in a wave of cytosolic Ca^{2+} that drives protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) activity. This controls nuclear translocation of nuclear factor of activated T cells (NFAT) for target gene transcription and actin polymerization through the Rho GTPase CDC42. NFAT target genes have been most thoroughly researched in the context of osteoclast formation and T cell regulation. In the transcription-independent Wnt/PCP pathway, cell polarity and migration are regulated through the direct interaction of Dvl and Dvl-associated activator of morphogenesis (DAAM1), G protein activation of the small GTPase Rac, and Dvl activation of phosphoinositide 3-kinase (PI3K) (**Figure 2B**) (16). Non-canonical pathways demonstrate a high amount of crosstalk and together regulate events such as filopodia formation, cell movement, and establishment of cell polarity.

Pathogen manipulation of Wnt signaling as a mechanism of innate immune subversion takes advantage of three main outcomes of the signal cascade: cell fate determination (including maintenance of epithelia and endothelia), anti-inflammatory effects, and phagolysosome formation. In this review, we highlight mechanisms of Wnt pathway manipulation by several extracellular, and obligate intracellular pathogens, with a specific focus on the virulence factors involved and the consequences on innate immune subversion. **Table 1** summarizes the role of Wnt signaling in the pathogenesis of the bacteria reviewed herein. Ultimately, our understanding of host-pathogen interaction at the cellular and molecular levels will highlight potential targets for therapeutic intervention and expand our model of the many roles of Wnt signaling within the eukaryotic cell.

INTRACELLULAR PATHOGENS

Salmonella enterica

The Gram-negative, facultative intracellular bacillus *Salmonella enterica* causes typhoid fever or non-typhoidal salmonellosis in

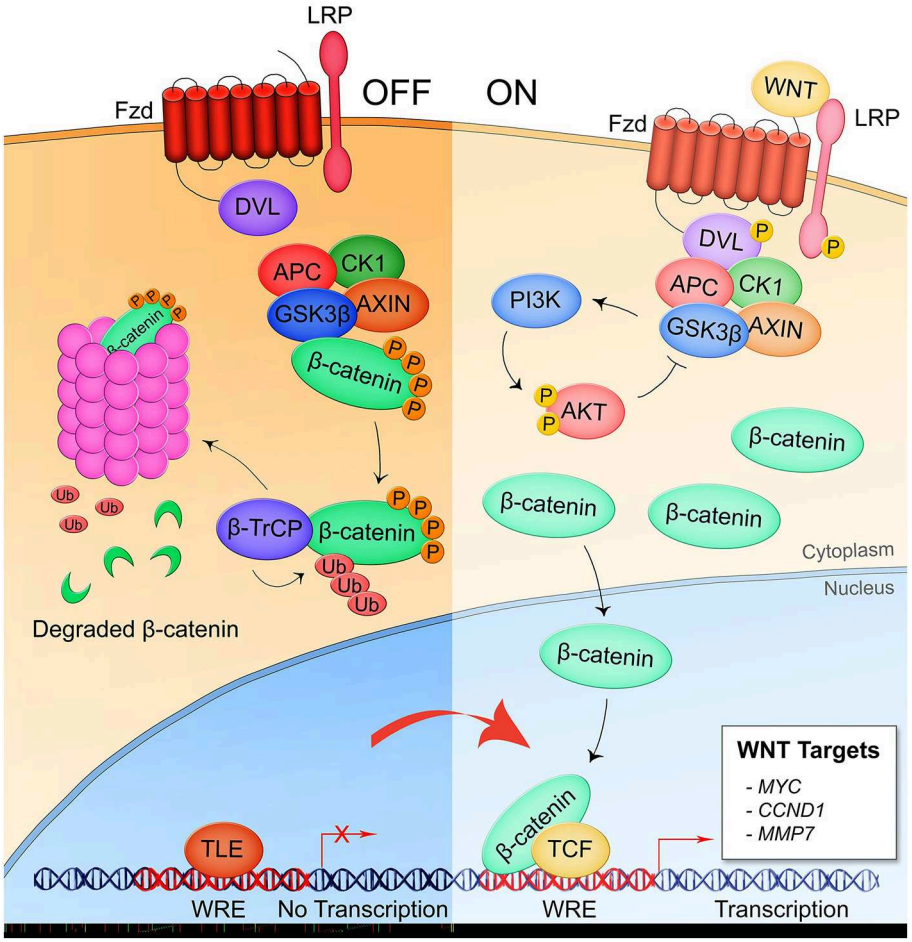

FIGURE 1 | Canonical/β-catenin-dependent Wnt signaling. In the pathway off state, the β-catenin destruction complex consisting of APC, CK1, GSK3β, and AXIN binds β-catenin leading to its phosphorylation by GSK3β and subsequent ubiquitination by the E3 ubiquitin ligase complex β-TrCP which targets β-catenin for proteasomal degradation. Thus, the Wnt response element (WRE) located in the promoter region of Wnt pathway target genes remains bound by transcriptional repressor TLE. When a secreted Wnt ligand originating from the same or a nearby cell binds Fzd and the coreceptor LRP5/6, Dvl is phosphorylated and sequesters the destruction complex, preventing the phosphorylation of β-catenin. Accumulation of β-catenin is the cytoplasm leads to nuclear translocation of the protein where it binds with co-activator TCF at the WRE and drives expression of Wnt target genes.

humans. The bacteria are commonly foodborne pathogens but can also be transmitted fecal-orally (53). *Salmonella* establish infection in the gut where they replicate within the lumen until sufficient numbers induce their entry into M cells which is triggered by T3SS effectors. *S. enterica* are also phagocytosed by various phagocytic cells but can survive phagolysosome acidification and replicate within the intracellular vacuole. Dissemination to other organs is accompanied by a robust immune response and the potential for persistent infection within various cell types. Crossing of the gut barrier and infection of infiltrating immune cells including neutrophils, monocytes, and macrophages are essential to dissemination.

Nearly two decades of research have created the model of *Salmonella enterica* serovar Typhimurium manipulation of canonical Wnt signaling to promote infection (**Figure 3A**). Curiously, *S.* Typhimurium suppresses Wnt signaling in intestinal epithelium and the underlying capillary endothelium but activates the pathway in intestinal stem cells in what appears to be a cell type- or temporally-specific mechanism. In transformed T84 colon carcinoma cells, wild type *S.* Typhimurium represses pathway activation as identified by significant suppression of β-catenin levels that correlates with decreased formation of the TCF-β-catenin complex in the nucleus, dampened expression of the Wnt target gene *CMYC*, and suppression of cellular proliferation (17). This is contrasted with colonization by non-pathogenic *S.* Typhimurium strain PhoP^{C} which possesses an attenuating, constitutively active PhoP-PhoQ two-component system and does not suppress β-catenin-TCF complex formation, implicating a role for this response regulator in suppression of Wnt signaling by pathogenic salmonellae in the colonic epithelium, both in cell culture and in a mouse model (18).

The effects of *S.* Typhimurium suppression of Wnt signaling in the intestines have created two models for

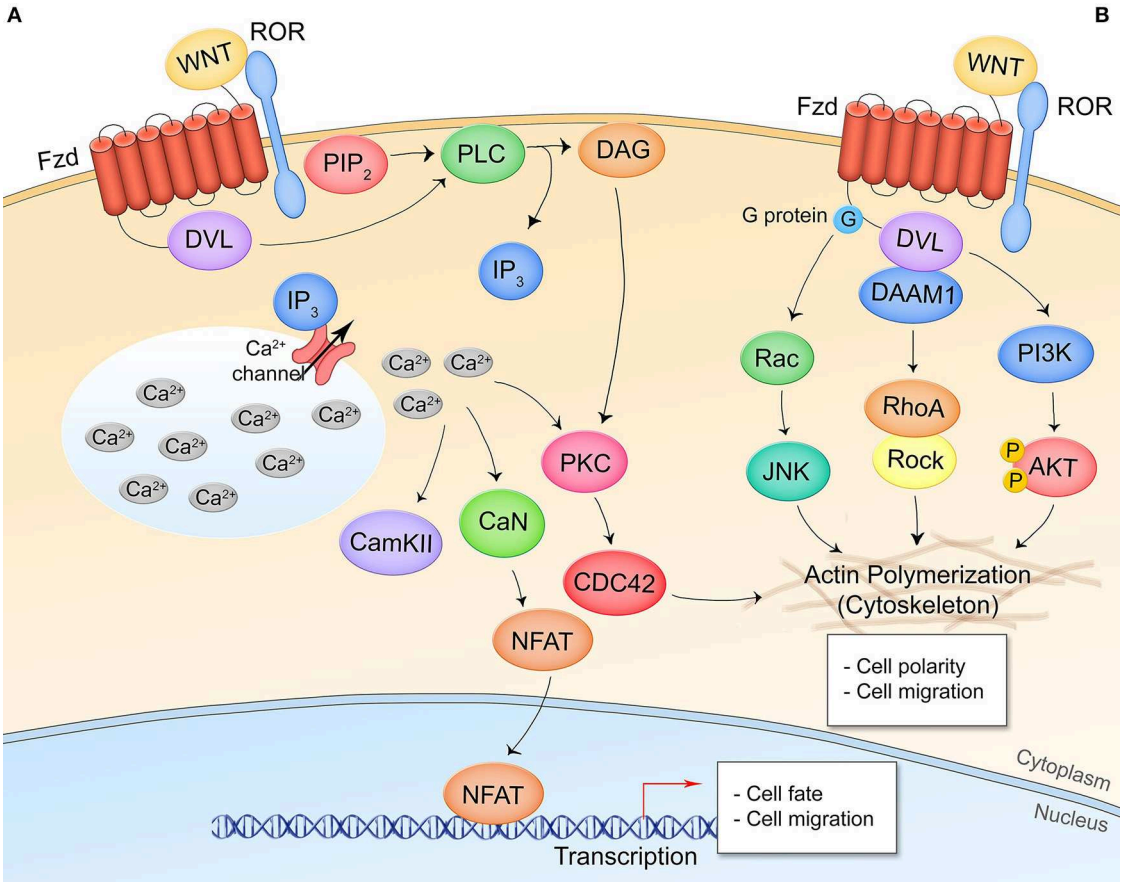

FIGURE 2 | Non-canonical/β-catenin-independent Wnt signaling pathways. **(A)** Wnt/Ca^{2+} signaling. A Wnt ligand interaction with Fzd and the non-canonical coreceptors ROR1/2 activates Dvl which activates PLC, leading to production of IP_3 and opening of cytosolic Ca^{2+} stores. CAMKII, CaN, and PKC are activated by the increased cytosolic Ca^{2+} levels. CaN dephosphorylates NFAT leading to its nuclear translocation and expression of NFAT target genes. PKC crosstalks with Wnt/PCP signaling. **(B)** Wnt/planar cell polarity signaling. Interaction of Wnt and respective receptor/coreceptor leads to cytoskeletal reorganization through G-protein activation of Ras-JNK signaling, Dvl-DAAM1 association and Rho activation, and Dvl activation of PI3K and Akt. This pathway is transcription-independent.

bacterial host manipulation by Wnt signaling modulation. First, *Salmonella* studies have revealed a mechanism behind the immunosuppressive effects of β-catenin signaling. IκBα negatively regulates NF-κB transcriptional activity through cytosolic sequestration of NF-κB. It has been shown that β-catenin associates with this complex, indirectly stabilizing IκBα through an unknown mechanism (19). Activation of the NF-κB pathway causes ubiquitination of IκBα by the ubiquitin E3 ligase β-TrCP, the same ligase that targets β-catenin for degradation to suppress Wnt pathway activity, which results in ubiquitin-dependent degradation of IκBα (54). Activation of this E3 ligase therefore results in silencing of Wnt signaling through loss of β-catenin and activation of NF-κB signaling through loss of IκBα repression, while suppression of β-TrCP has the opposite effect. During *S.* Typhimurium infection of colonic epithelial cells, the NF-κB target genes *IL6*, *IL8*, and *TNFA* are induced, corresponding with enhanced ubiquitination of IκBα as well as β-catenin degradation which demonstrates the reciprocal activation of these two pathways by infection (18, 19). Infection in the presence of lithium chloride, a β-catenin stabilizing agent, or a constitutively active *β*-catenin mutant $CTNNB1^{-/\Delta 45}$ attenuates this effect. Activation of NF- κB signaling through direct inhibition of β-catenin-dependent Wnt signaling induces the proinflammatory response which recruits dendritic cells and macrophages. While TNF-α is important for controlling replication and spread of bacteria, the cells producing it are also host cells for both replicative and persistent salmonellae, thereby supporting survival and dissemination of the bacteria (53, 55, 56).

Recently, another pathogenic mechanism was discovered by which salmonellae inhibit canonical Wnt signaling at the gut-vascular barrier (GVB), a system of tight and adherens junctions in the capillaries underlying the gut epithelium that functions as a size-selective barrier to molecules traversing the gut barrier (20). Similar to the blood-brain barrier, the GVB is regulated by canonical Wnt signaling (20, 57). This protective barrier is impermeable to bacteria, but infection by *S.* Typhimurium results in downregulation of Wnt signaling in the endothelium

TABLE 1 | Summary of the role of Wnt signaling pathways and respective bacterial factors involved in the pathogenesis of representative bacteria.

Pathogen	Pathway	Effect	Tissue/cell type	Bacterial determinants	Mechanism	Outcome	References
Salmonella enterica	Canonical	Inhibition	Intestinal epithelium	PhoP-PhoQ	Unknown	NF-κB activation; inflammation	(17–19)
			Intestinal capillaries	Spi2	Unknown	Gut-vascular barrier disruption	(20)
		Activation	Crypt-localized epithelial cells	AvrA	β-catenin deubiquitination	Cell proliferation; NF-κB inhibition	(21–23)
			Enterocytes	SopB	β-catenin de-phosphorylation	*RANKL*-mediated epithelial-mesenchymal transition	(24)
Chlamydia spp.	Canonical	Activation	Reproductive tract epithelium	Unknown	Adherens junction disruption	Host cell proliferation; *OLFM4* upregulation; chlamydiae development	(25, 26)
			Respiratory epithelium	Cpn1027	Caprin2, GSK3β sequestration	*BCL2*-mediated apoptosis inhibition	(27)
Rickettsia spp.	Canonical	Activation	Endothelium	Unknown	Adherens junction disruption; DKK1 inhibition	*IL6*, *IL8* suppression	(28, 29)
Ehrlichia chaffeensis	Canonical	Activation	Monocytes	TRP120, TRP32, TRP47	Direct interaction with pathway components, target genes	Autophagy inhibition	(30–35)
	Wnt/PCP	Activation				Phagocytosis; lysosome biogenesis suppression; mTOR-mediated autophagy inhibition	
	Wnt/Ca^{2+}	Activation				Phagocytosis	
Mycobacterium tuberculosis	Canonical	Activation	Macrophages	Unknown	Wnt3a-Fzd1 signaling	Pro-inflammatory cytokine suppression	(36–38)
	Wnt/Ca^{2+}	Activation		Unknown	Wnt5a-dependent PIAS1 and SOCS1 expression; Ca^{2+}-regulation of phagocytosis	Inhibition of TLR signaling; inhibition of phagosome-lysosome fusion	(39, 40)
	Non-canonical	Activation		Unknown	Wnt6-G protein-ERK-induced *MYC* expression	Anti-inflammatory M2 macrophage phenotype	(41)
Clostridium difficile	Canonical	Inhibition	Colonic epithelium	TcdA	Inhibition of Rac1-mediated β-catenin nuclear transport; β-catenin degradation	Suppression of cell proliferation	(42)
				TcdB	Fzd binding	Intestinal epithelium weakening; inflammation	(43, 44)
Helicobacter pylori	Canonical	Activation	Gastric epithelium	CagA; T4SS (CagA-independent)	E-cadherin cleavage; methylation of Wnt antagonist genes	Cell proliferation; intestinal transdifferentiation	(45, 46)
	Wnt/Ca^{2+}	Activation			Unknown	Intestinal transdifferentiation	(47)
Pseudomonas aeruginosa	Canonical	Inhibition	Intestinal epithelium	PAI	Adherens junction disruption	Epithelium weakening	(48, 49)
			Lung epithelium	LecB	β-catenin degradation	NF-κB activation; cell cycle arrest; delayed tissue recovery	
Escherichia coli	Wnt/Ca^{2+}	Inhibition	Bladder epithelium	Unknown	Wnt5a suppression	Cell Differentiation	(50–52, 153)
	Wnt/Ca^{2+}	Activation		Unknown	EZH2-mediated Wnt5a expression	Cell proliferation	
	Canonical	Activation	Intestinal epithelium	Unknown	EZH2-mediated WIF1 repression	Crypt hyperplasia	
			Bladder epithelium	HlyA	β-catenin degradation	NF-κB inhibition; immunosuppression	

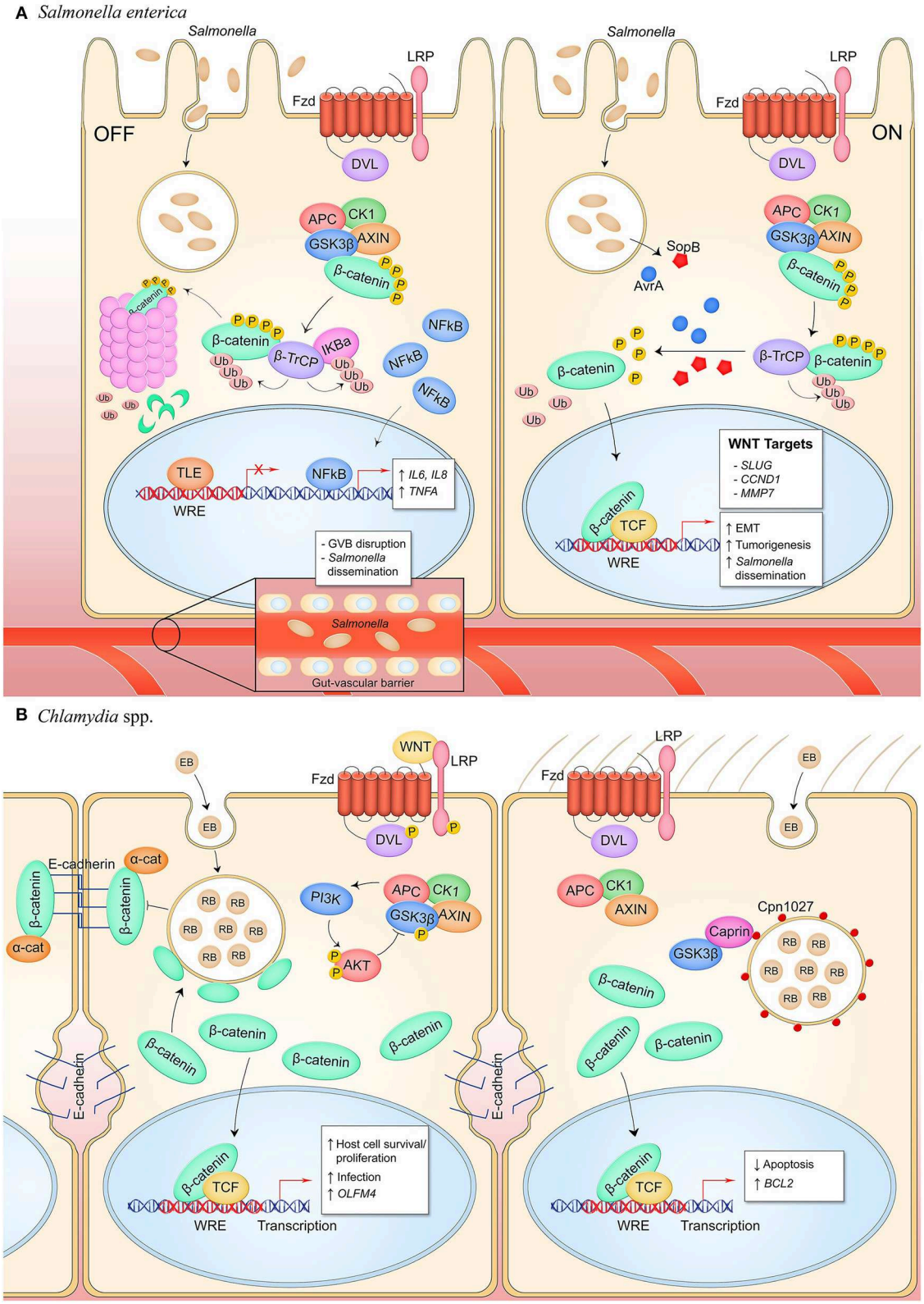

FIGURE 3 | Canonical Wnt signaling manipulation during bacterial infection of epithelial cells. **(A)** *S. enterica* infection of intestinal epithelial cells inhibits Wnt signaling through an unknown mechanism (left). Activity of the E3 ubiquitin ligase β-TrCP, which targets both β-catenin and IκBα for proteasomal degradation, stabilizes NF-κB

(Continued)

FIGURE 3 | levels which causes nuclear translocation of the protein and expression of pro-inflammatory target genes. Wnt signaling is also inhibited in the GVB during infection, promoting bacterial access to vasculature by increasing vascular permeability. *S. enterica* secretes the T3SS effectors AvrA and SopB into intestinal M cells and crypt-localized epithelial cells which causes activation of canonical Wnt signaling (right). Deubiquitinase AvrA and phosphatase SopB induce pathway activation through reversal of β-catenin posttranslational modifications, promoting expression of β-catenin-dependent genes that drive EMT in M cells, induce intestinal stem cell proliferation, and inhibit NF-κB activity in stem cells. **(B)** *C. trachomatis* infection of reproductive tract epithelium induces breakdown of adherens junctions and accumulation of β-catenin in the cytoplasm (left). β-catenin localizes to the chlamydial inclusion and translocates into the nucleus to activate transcription. Pathway activity promotes the bacterial developmental cycle through unknown mechanisms as well as drives expression of *OLFM4* which is known to inhibit NF-κB signaling. *C. pneumoniae* expresses inclusion membrane protein Cpn1027 during infection of respiratory epithelium (right). Cpn1027 recruits Caprin2, a scaffold protein of the β-catenin destruction complex, as well as GSK3β, thereby reducing β-catenin turnover and allowing nuclear translocation for expression of target gene *BCL2* to inhibit host cell apoptosis.

which enhances vascular leakiness and promotes bacterial dissemination (20). In the presence of recombinant canonical pathway ligand Wnt3a, canonical signaling is activated and *S.* Typhimurium-induced leakiness is reduced. *S.* Typhimurium possess two pathogenicity islands (Spi1 and Spi2) that together encode the T3SS. A strain lacking Spi2 is unable to induce vascular permeability, indicating this pathogenic mechanism is Spi2-dependent. This model demonstrates how salmonellae permeabilize endothelial layers utilizing T3SS factors without directly manipulating cell-cell contacts, a mechanism that is employed by other endothelial pathogens such as *Rickettsia*. Whether this implicates activation of Wnt signaling as a potential therapeutic for *S. enterica* infection, and whether specific *S. enterica* pathogenicity island effectors are responsible for Wnt pathway inhibition remains to be investigated.

Studies using a *Salmonella* colitis mouse model have demonstrated that *S.* Typhimurium is able to stimulate Wnt signaling in intestinal stem cells in contrast to the inhibitory mechanism deployed in the epithelium and endothelium. The T3SS effector AvrA, a deubiquitinase, has been shown to activate canonical Wnt signaling in intestinal stem cells through deubiquitination and subsequent stabilization of β-catenin (21, 58). Additionally, AvrA alone, in the absence of infection, can activate pathway activity. In the colon of the *Salmonella* colitis mouse model, stem cells demonstrate β-catenin nuclear localization and expression of Wnt target genes *MMP7* and *CCND1* (22). Hyperactivation mediates pathological effects, and studies have shown that in mice with induced inflammation, infection with $AvrA^+$ *S.* Typhimurium drives tumorigenesis (22). In addition to AvrA, T3SS effector SopB has tumorigenic properties through inducing cellular transformation of follicular-associated epithelial enterocytes into microfold cells (24). This has also been linked to a canonical Wnt-dependent mechanism. The phosphatase dephosphorylates β-catenin and Akt in primary rectal epithelial cells, resulting in β-catenin-dependent signaling and expression of target gene *SLUG* which activates RANKL expression, a critical cytokine for M cell development (24, 59). Indeed, increased M cells is a phenotype of *S. enterica* infection and proliferation of this cell type promotes *S.* Typhimurium invasion of the intestines for enhanced survival and dissemination.

S. Typhimurium has proven to be a model pathogen for understanding the role of canonical Wnt signaling in both gut tissue maintenance and suppression of inflammatory pathway signaling, physiological processes that are applicable to a range of pathogens that occupy a similar niche. Insight to *Salmonella* inhibition of signaling to perturb the GVB brings to light the therapeutic potential of Wnt ligands to maintain endothelial barriers. R-spondin3, a Wnt homolog, has been shown to exhibit an anti-inflammatory effect in an ischemia/reperfusion mouse model and induces tightening of endothelial junctions and loss of vascular leakiness (60). Furthermore, understanding how effectors SopB and AvrA contribute to pathway manipulation during infection highlights the potential of such mechanisms as therapeutics for infection or other disease states mediated by dysregulated Wnt signaling.

Chlamydia spp

Chlamydia trachomatis and *Chlamydia pneumoniae* are obligately intracellular, anaerobic pathogens that typically target the human genital and respiratory tract mucosal epithelial cells, respectively. Chlamydiae undergo a developmental cycle in which they transition between two ultrastructural forms at different phases of infection (61). The elementary body (EB) is the infectious form of the bacteria that triggers entry into the host cell. Once intracellular, the chlamydiae transition into the replicative reticulate body (RB) form within the inclusion, the membrane-bound microcolony. The intracellular chlamydiae interact with the host cell through both T3SS effector proteins as well as inclusion membrane-localized proteins that interface with the host cell cytoplasm. Environmental cues such as drug presence or immunological stress can induce the EB to enter a persistent form in which they evade immune detection by entering a dormant state within the inclusion (62). At the end of the infection cycle, the RB transition back into and EB and leave the cell through lysis or exocytosis to infect a neighboring cell.

C. trachomatis targets epithelial cells of the genital tract, including the endometrial and fallopian epithelium. Wnt/β-catenin signaling is known to maintain epithelial cell homeostasis through regulating tissue renewal and cell proliferation, and through facilitating epithelial barrier integrity by the β-catenin-E-cadherin complex that constitutes adherens junctions (63–65). *C. trachomatis* infection in the fallopian tube has been shown to disrupt adherens junctions and cause redistribution of β-catenin from the plasma membrane to the chlamydial inclusion (25) (**Figure 3B**). It is unclear if disruption of these junctions amplifies β-catenin nuclear localization and target gene expression, but infected epithelium does demonstrate increased Wnt pathway activity evidenced by phosphorylation-dependent inactivation of GSK3β and redistribution of APC which indicates inactivation of the β-catenin destruction complex (25). Additionally, inhibition of Wnt signaling through either

RNA silencing of β-catenin or a small molecule inhibitor reduces infectivity of the chlamydiae and impairs chlamydiae intracellular development (25, 26). Thus, signaling is beneficial to chlamydiae and may be synergistically activated through inhibition of the β-catenin destruction complex and disruption of adherens junctions. The known beneficial phenotypes of Wnt signaling for the chlamydial niche are 2-fold. First, infection causes Wnt signaling-dependent host cell proliferation, a critical survival strategy for an obligately intracellular pathogen that can maintain persistent infection (25). Second, Wnt signaling upregulates the stem cell marker OLFM4 which is a suppressor of NOD1/2 and NF-κB-dependent pro-inflammatory cytokine expression (23). Therefore, Wnt signaling appears to be an active mechanism of pathogenesis by which *Chlamydia* establishes infection and suppresses NF-kB-mediated innate immune mechanisms.

C. pneumoniae inclusion protein Cpn1027 is the only chlamydial protein known to directly interface with the canonical Wnt pathway. During *C. pneumoniae* infection of respiratory epithelium, Cpn1027 directly binds the Caprin2, an adaptor protein within the β-catenin destruction complex (27, 66). GSK3β also localizes to the Cpn1027-Caprin2 complex and demonstrates decreased kinase activity. Consequently, β-catenin translocates to the nucleus and drives expression of the anti-apoptotic *BCL2* gene, linking the infection phenotype of apoptosis inhibition to manipulation of Wnt signaling for enhanced intracellular survival. This mechanism of pathway manipulation is unique to *C. pneumoniae* as Cpn1027 is not expressed by other species of the *Chlamydia* genus (67).

Several questions remain regarding the role of Wnt signaling during *Chlamydia* spp. infection. The consequence of β-catenin localization to the *C. trachomatis* inclusion concurrent with adherens junction disruption is unclear, as signaling is not inhibited in the host cell, and β-catenin is necessary for the chlamydial intracellular life cycle. The chlamydial deubiquitinase ChlaDub 1 has been shown to deubiquitinate NF-κB inhibitor IκBα in order to suppress the NF-κB-dependent expression of proinflammatory cytokines (68). β-catenin and IκBα are both substrates of the E3 ligase $SCF^{\beta-TrCP}$, raising the question of whether β-catenin localizes to the chlamydial inclusion to also serve as a ChlaDub 1 substrate which would lead to pathway activation (69). Identifying interactions between chlamydial secreted effectors or inclusion proteins and components of the Wnt pathway will define novel host-bacterial pathogenic interactions that can be targeted by therapeutics. In addition to understanding pathogenic mechanisms, further research will shed light on chlamydial mechanisms of cellular transformation. *Chlamydia trachomatis* is associated with cancer development through infection-induced degradation of p53 and dysregulated ROS production (70–72). It is well-known that canonical Wnt signaling is a driver of tumorigenesis in multiple human cancers, including cervical cancer (73). However, a model for cervical cancer development through *C. trachomatis*-induced hyperactivation of Wnt signaling has not been investigated.

Rickettsia spp

The *Rickettsia* genus comprises 27 species of obligately intracellular Gram-negative bacteria, over half of which are human pathogens primarily transmitted by various arthropod vectors (74). *R. conorii* and *R. rickettsii* are both members of the spotted fever group of *Rickettsia* and causative agents of the human diseases Mediterranean spotted fever and Rocky Mountain spotted fever, respectively. These pathogens establish infection in the endothelium which induces an inflammatory response consisting of increased vascular permeability, recruitment and activation of natural killer cells and macrophages, and ROS- and cytokine-mediated vascular damage. The bacteria escape from their endocytic vesicle and replicate in the cytosol where they utilize actin-based motility for cell-to-cell spread. Robust production of cytokines IL-6 and IL-8 correlate with infection lethality, and clearance of the bacteria is typically mediated by PRR engagement (75).

Wnt signaling has a complex role in the endothelium, and activation of the pathway induces endothelial cell proliferation and enhanced interaction between endothelial cells and monocytes (76, 77). Additionally, β-catenin is present at endothelial adherens junctions, regulating cell-cell contacts (78). The Wnt signaling pathway controls neovascularization during development but demonstrates decreased activity in adult vasculature (79). However, activation in certain disease states including infection and cancer can induce Wnt-dependent vascular endothelial growth factor A (VEGF-A) expression followed by angiogenesis (76). Activated endothelial cells also release DKK1, a member of the Dickkopf family of secreted Wnt signaling antagonists that exert their effect by outcompeting Wnt ligands for binding of coreceptor LRP5/6 (80). DKK1 is a β-catenin target gene that functions through feedback inhibition of Wnt signaling, thereby decreasing neovascularization induced by VEGF-A (81, 82).

In a HUVEC model of *R. conorii* infection, β-catenin rapidly localizes to the nucleus within 2 hpi, indicating activation of Wnt signaling early in infection (**Figure 4**) (28). It is unclear how the pathway is activated by rickettsiae, as other markers of signaling activity have not been investigated. In a HUVEC model of *R. rickettsii* infection, adherens junctions are disrupted and β-catenin is redistributed from primarily membrane-localized to diffuse localization throughout the cell (29). A correlation was identified between vascular permeability through loss of adherens junction integrity as well as increased expression of NF-κB-dependent inflammatory cytokines, but whether this is related to canonical Wnt signaling reciprocal regulation of the NF-κB signaling pathway is unknown. Increased nuclear entry of β-catenin during rickettsial infections has not been specifically investigated, but consistent with other models of infection-induced adherens junctions remodeling, it is likely that *Rickettsia*-induced cell contact disruption can activate canonical Wnt signaling through increasing the cytoplasmic pool of β-catenin.

In accordance with Wnt signaling activation, DKK1 secreted protein level is significantly reduced in a HUVEC model of *R. conorii* infection relative to uninfected controls from 5 to 180 hpi (28). Infection activates endothelial cells and causes significant increase in secreted protein levels of inflammatory cytokines IL-6 and IL-8. However, RNA-mediated silencing of DKK1 in infected HUVECs significantly reduces production

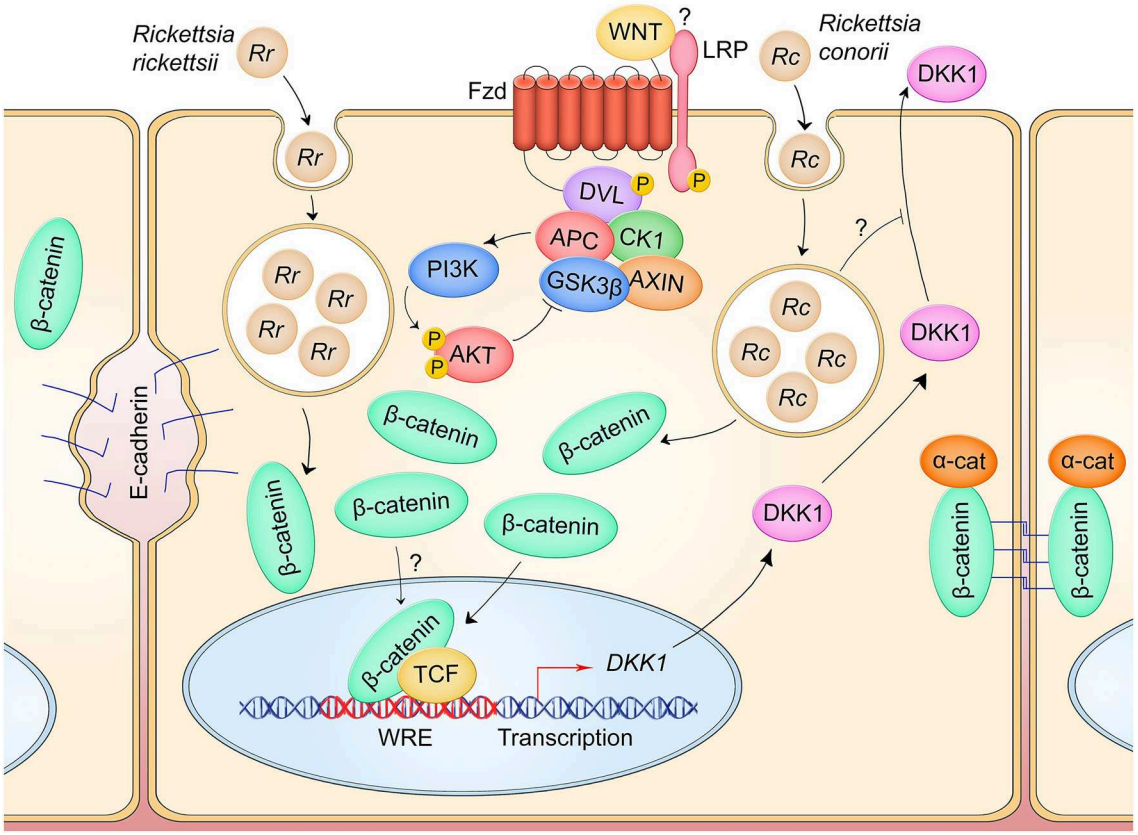

FIGURE 4 | Canonical Wnt signaling manipulation during *Rickettsia* spp. infection of endothelium. *R. rickettsii* (Rr) infection of endothelial cells induces breakdown of adherens junctions and accumulation of cytoplasmic β-catenin which may drive pathway activity (left). *R. conorii* (Rc) infection leads to a suppression of secreted Wnt antagonist DKK1, which may facilitate activation of Wnt signaling to induce an anti-inflammatory environment during infection. IL-6 and IL-8 secretion are suppressed during infection in a DKK1-dependent manner.

of these cytokines, suggesting that DKK1 plays a role in modulating the inflammatory response. It is hypothesized that *R. conorii* suppresses DKK1 to prevent negative regulation of Wnt signaling, thereby keeping proinflammatory cytokine expression relatively low during infection.

These studies highlight a unique role of Wnt signaling feedback inhibitors in pathogenic mechanisms of infection. In seeking to augment Wnt signaling for its anti-inflammatory effect, *Rickettsia* may possess active mechanisms for reducing the activity of Wnt pathway antagonists like DKK1. A critical gap in this model is whether DKK1 is inhibited at the transcriptional, translational, or post-translation level. Evidence suggests *Rickettsia* may be inhibiting DKK secretion, although further studies are needed to confirm this. This research also presents the therapeutic potential of harnessing the Wnt pathway to mitigate disease. Recombinant DKK1 has been shown to reduce pathological Wnt signaling activation in a mouse model of neovascularization-induced blindness (81). DKK1 levels in *R. conorii*-infected patient sera indicate a decline in secreted DKK1 throughout infection, but whether this is a direct result of pathogen manipulation has not been investigated (28). Altogether, these results propose a complex model in which intracellular pathogens act on feedback inhibition mechanisms of Wnt signaling to interfere with pathway activity, and they propose a unique role for DKK1 in influencing the proinflammatory response to *Rickettsia*.

Ehrlichia chaffeensis

Ehrlichia chaffeensis is the causative agent of the tick-transmitted disease human monocytic ehrlichiosis (HME). The pathogen is an obligately intracellular bacterium and infects mononuclear phagocytes including monocytes. *E. chaffeensis* enters the host cell through phagocytosis and replicates within a membrane-bound vacuole to form a microcolony known as a morula (83). The morula resembles an autophagosome, but through pathogenic mechanisms never fuses with the lysosome (31). Similar tochlamydiae, ehrlichiae undergo a biphasic developmental cycle within the host cell (84). The infectious dense-cored cell (DC) ehrlichiae invade the host cell and transition into replicating reticulate cells (RC) that divide through binary fission. The ehrlichiae complete their infection cycle by transitioning back into DC, rupturing the host cell, and spreading hematogenously to the next host cell. Throughout infection, ehrlichiae secrete a variety of T1SS and T4SS effectors,

including the TISS tandem repeat protein (TRP) effectors TRP32, TRP47, and TRP120 that induce pathogenesis through direct interactions with numerous host proteins and host DNA (34, 35, 85, 86). Through unknown mechanisms, the TRPs also localize to the surface of the ehrlichiae and decorate the outer membrane, facilitating interactions with the host cell leading to invasion (87, 88).

E. chaffeensis has emerged as a model organism for pathogenesis mediated by the hijacking of conserved cell signaling pathways including Wnt signaling (87). Both canonical and non-canonical Wnt signaling are active early during infection and are necessary for enhancement of ehrlichial infection, as inhibitors or gene silencing of canonical and non-canonical pathway components significantly reduce *E. chaffeensis* survival within the monocyte (**Figure 5**) (30, 32). Of note, RNA silencing of antagonist DKK3 results in increased infection, while RNA silencing of canonical and non-canonical pathway components such as CK1, CAMKII, NFAT, and β-catenin significantly reduces infection. *E. chaffeensis* enters the host cell via phagocytic pathways regulated by Ca^{2+} signaling and actin filamentation, and the morula labels with autophagosomal markers LC3 and beclin-1 even though autophagy appears to be inhibited and lysosomal marker LAMP2 never localizes to the morula (31, 89, 90). The Wnt pathway has been shown to involved in *E. chaffeensis* phagocytosis because microspheres coated in ehrlichial surface TRPs can stimulate phagocytosis by monocytes but are unable to do so in the presence of a small molecule inhibitor of Wnt signaling (30). Additionally, RNA silencing of Wnt receptors Fzd5 and Fzd9, and Wnt coreceptor LRP6, significantly reduces the number of intracellular bacteria, indicating a potential role for Fzds as *E. chaffeensis* receptors. The hypothesis that *E. chaffeensis*

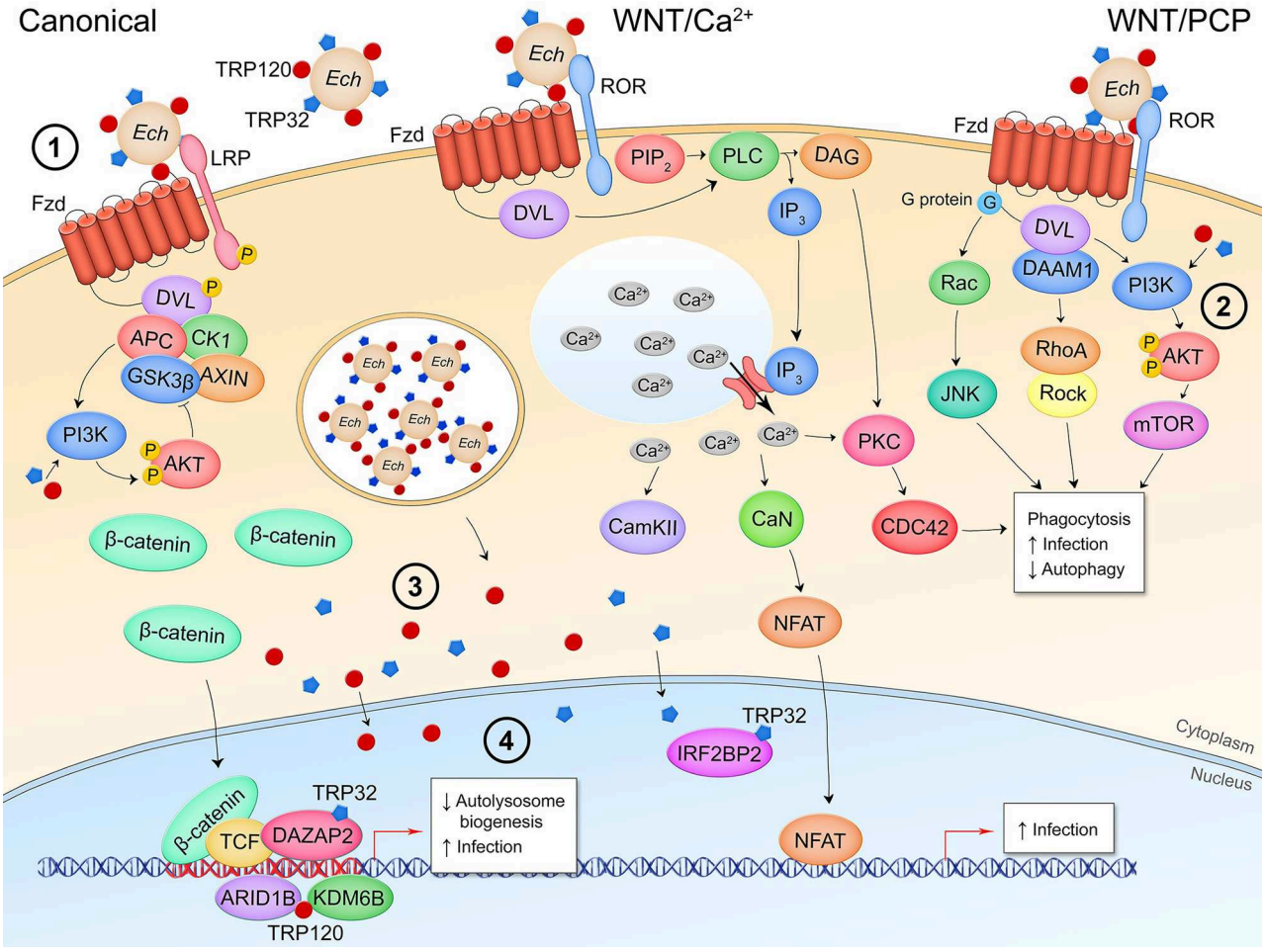

FIGURE 5 | Canonical and non-canonical Wnt signaling manipulation during *E. chaffeensis* infection of monocytes. (1) *E. chaffeensis* dense-cored cells express surface proteins TRP32 and TRP120 that stimulate phagocytosis in a Wnt signaling-dependent manner, potentially through interaction with the Wnt receptor complex and stimulation of non-canonical Wnt pathways to induce cytosolic Ca^{2+} flux and cytoskeletal reorganization. (2) Pathway activity during bacterial entry as well as T1SS secreted effectors TRP32 and TRP120 direct stimulation of PI3K-AKT signaling facilitates mTOR activation which prevents fusion of the bacterial replicative autophagosome with the lysosome. During infection, the secreted TRP effectors localize to the host cytoplasm where they interact with proteins involved in Wnt signaling (3) as well as translocate to the nucleus where they bind host DNA within the promoter regions of Wnt target genes or directly interact with transcriptional regulators of Wnt target genes (4).

uses activation of non-canonical Wnt signaling to drive actin filamentation and bacterial uptake is supported by evidence demonstrating Wnt5-Fzd5-PI3K non-canonical Wnt signaling induces uptake of non-pathogenic *E. coli* without leading to bacterial killing, as well as the role of non-canonical Wnt signaling in cytoskeletal control (91–94). *E. chaffeensis* avoidance of lysosomal fusion was also found to be dependent on ehrlichial activation of Wnt-PI3K signaling, mediated by TRP32 and TRP120, to stimulate mTOR inhibition of autophagy as well as inhibition of TFEB-dependent lysosome synthesis genes (31). Accordingly, inhibition of Wnt signaling following *E. chaffeensis* infection in monocytes promotes colocalization of lysosomal marker LAMP2 with the ehrlichial cytoplasmic vacuole (31). β-catenin activation is known to repress the autophagy protein p62, indicating another potential mechanism by which ehrlichial activation of canonical Wnt signaling drives intracellular survival (95).

In addition to manipulation of Wnt pathway activation early during infection to facilitate phagocytosis and intracellular survival, *E. chaffeensis* maintains pathway activity throughout infection of the monocyte (30). Yeast-two-hybrid data identified multiple interactions between TRP32 or TRP120 and Wnt signaling pathway components and regulators in the host cytoplasm and nucleus, implicating intracellular pathway manipulation through direct interference with Wnt signaling regulators (85, 86). These interacting partners include pathway negative regulators CEP164 (96), KLHL12 (97), ILF3 (98), and LMO2 (99); and positive regulators PPP3R1 (100) and VPS29 (101). RNA-mediated knockdown of these interacting targets results in significant, differential regulation of THP-1 monocyte infection which demonstrates these TRP-host protein interactions are relevant to *E. chaffeensis* establishment of infection (32). TRPs also interact with nuclear proteins involved in Wnt target gene epigenetic modification and expression, including the Wnt response element transcriptional repressor TLE4 (102), co-activator DAZAP2 (103), and histone remodeling proteins ARID1B, KDM6B, and IRF2BP2 (104–106). Furthermore, TRP32, TRP47, and TRP120 DNA-binding motifs are within the promoter region of numerous Wnt target genes, indicating the TRPs may directly influence Wnt gene transcription through nucleomodulin activity (33–35). Although these specific interactions have not yet been investigated, it is likely that the nucleomodulin activity of TRP effectors or the recently discovered ubiquitin ligase activity of TRP120, plays a role in manipulation of these target proteins, facilitating Wnt pathway activity and ultimately enhancing *E. chaffeensis* intracellular survival (107).

E. chaffeensis stands out among human pathogens as a bacterium that targets Wnt signaling at both canonical and non-canonical levels and takes advantage of a wide range of pathway outcomes. Gaps that remain to be filled in the *E. chaffeensis* monocyte infection model include how both signaling branches are activated. The studies reviewed demonstrate that *E. chaffeensis* utilizes an extracellular mechanism of pathway activation, potentially through surface TRPs interaction with the Wnt receptor complex. While many bacteria use secreted effectors to activate Wnt signaling through manipulation of midstream signaling components, ehrlichial TRPs are the only bacterial factors that are known to trigger entry into a cell in a Wnt-dependent mechanism which suggests the surface proteins mimic Wnt ligands and utilize Wnt control of the cytoskeleton for phagocytosis. *E. chaffeensis* demonstrates a potential for intracellular activation of signaling through the secretion of TRP effectors that interact with Wnt pathway-related proteins and DNA in the cytosol and nucleus. These dual modes of extra/intracellular pathway activation may be necessary for temporal regulation of signal activity to facilitate the bacterium's complex intracellular developmental cycle. Understanding the relevance of these vast interactions will shed light ehrlichial pathogenesis while also serving as a model for other intracellular bacteria that reprogram the host cell to establish a replicative niche.

Mycobacterium tuberculosis

The causative agent of one of the most prevalent infectious diseases in the world, *Mycobacterium tuberculosis* establishes infection in the lower respiratory tract and infects alveolar macrophages as well as epithelial cells. The outcome of infection involves a delicate balance between the innate immune response control of intracellular bacterial killing early in infection and containment of infection by the adaptive immune response in lung granulomas to prevent dissemination. However, this can result in persistent infection with the ability to transition into an acute infection even decades after the initial exposure.

Canonical Wnt signaling in infected macrophages in the lung has been shown to be part of the innate immune response to *M. tuberculosis* and is regulated by the Wnt3a-Fzd1 signaling axis, with significant crosstalk from other Wnt ligands as well as TLR signaling. Although canonical signaling is involved in activation of macrophages, it has also been shown to lead to suppression of pro-inflammatory cytokine expression (36–38). It is hypothesized this anti-inflammatory effect contributes to tissue renewal following activation of the inflammatory response, but canonical pathway activation prior to complete clearance of the bacteria can also facilitate persistent infection. Several non-canonical pathways also play a part in *M. tuberculosis* infection, including Wnt/Ca^{2+} signaling which may be responsible for Ca^{2+} signaling that is necessary for recruitment of phagosome coat protein TACO which allows the bacterium to avoid the lysosomal pathway (39). Wnt5a signaling drives expression of PIAS1 and SOCS1 which inhibit TLR signaling, thereby controlling innate immune coordination (40). A non-canonical signaling pathway in which Wnt6 signals through G proteins to activate ERK and drive *MYC* expression has also been identified as a mechanism of induction of an anti-inflammatory M2 macrophage phenotype which may cooperate with canonical Wnt signaling suppression of inflammation to not only permit tissue renewal but also allow pathogen persistence.

Wnt signaling plays a large role in the processes that both permit and control mycobacterium survival. For further detail, the authors would like to direct the reader to a publication from 2017 that thoroughly reviews the mechanisms controlled by various Wnt ligands during *M. tuberculosis* infection (108).

EXTRACELLULAR PATHOGENS

Clostridium difficile

The anaerobic, Gram-positive bacterium *Clostridium difficile* is part of the human intestinal normal flora. It is the causative agent of pseudomembranous colitis (PMC), a severe inflammatory disease of the colon that arises from opportunistic infection by *C. difficile* typically following antibiotic-induced disturbances in the normal flora population proportions (109). The bacteria are transmitted fecal-orally in the form of spores and upon environmental triggers, such as exposure to bile acids, enter a vegetative state in the intestines. *C. difficile* pathogenesis is highly dependent on the expression of a family of exotoxins that includes toxin A (TcdA) and toxin B (TcdB) (110). The toxins are secreted from *C. difficile* through non-canonical secretion mechanisms and are endocytosed by target intestinal epithelial cells (111). These toxins are glucosyltransferases that glucosylate host small GTPases including Rho and Ras. Initially, TcdA, and TcdB together were thought to be responsible for symptoms of PMC. Alone, TcdA, but not TcdB, in small animal models can induce clinical signs of PMC including tissue damage, intestinal endothelial leakage, and inflammation (112). However, $TcdA^{-}/TcdB^{+}$ clinically relevant strains have been isolated, demonstrating TcdB is sufficient for induction of PMC in humans (113).

TcdA and TcdB exert enterotoxigenic effects including weakening of the epithelial barrier through disruption of tight junctions caused by the glucosylation of small GTPases that regulate actin filamentation, and stimulation of epithelial cells to recruit immune cells and program a pro-inflammatory response (110). Additionally, *C. difficile* is known to inhibit cell proliferation within the intestinal epithelium, a process typically regulated by canonical Wnt signaling, through the direct inhibitory effect of TcdA and TcdB on the Wnt pathway (**Figure 6A**) (114). TcdA has been shown to induce β-catenin degradation in the presence of pathway stimulation by Wnt3a, as well as prevent stimulation of pathway activity following pretreatment of intestinal epithelial cells with Wnt3a. Consequently, *CMYC* transcription is suppressed resulting in cell cycle arrest (42). TcdA-mediated degradation of β-catenin is independent of the β-catenin destruction complex, as TcdA stimulation of cells expressing a GSK3β phosphorylation-resistant β-catenin mutant cannot rescue pathway inhibition. It is speculated that TcdA glucosylation of Rac1 also inhibits canonical Wnt signaling as Rac1 expression is necessary for β-catenin nuclear translocation in certain cell types (115).

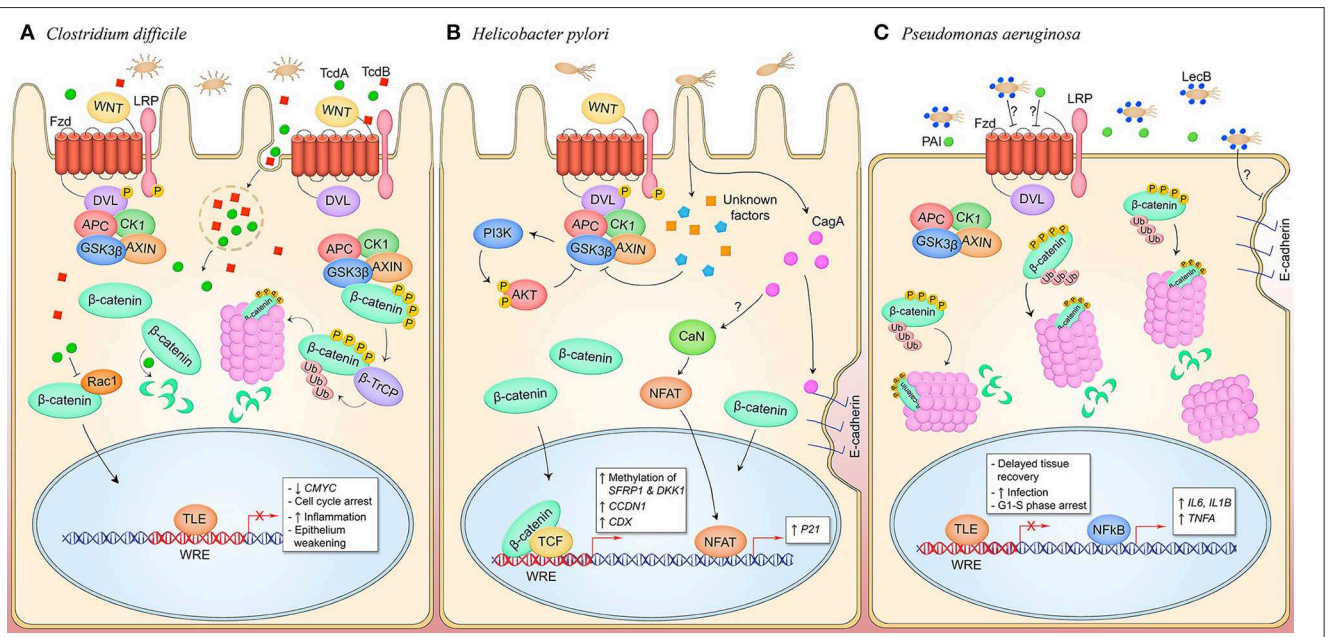

FIGURE 6 | Canonical and non-canonical Wnt signaling manipulation by pathogens occupying an extracellular niche. **(A)** *C. difficile* secretes toxins TcdA and TcdB which are phagocytosed by host cells in the intestinal and colonic epithelia. TcdA glucosyltransferase activity inhibits small GTPases like RacA which may inhibit pathway activity through inhibiting nuclear translocation of β-catenin. TcdB interacts with Wnt pathway components LRP6, Wnt5a, and GSK3β, and Fzd1, 2, and 7. Direct binding of Fzds prevents binding of endogenous Wnt ligands and silences pathway activity. This promotes epithelium permeability and a pro-inflammatory state. **(B)** During *H. pylori* infection of gastric mucosa, CagA activates canonical Wnt signaling through the breakdown of adherens junctions, and non-canonical Wnt signaling through stimulation of CaN and NFAT nuclear translocation by an unknown mechanism. Canonical pathway signaling is also activated through unknown T4SS effectors that induce phosphorylation of LRP and DVL and inhibit GSK3β, promoting cytosolic accumulation of β-catenin and subsequent nuclear translocation to activate genes that drive cell proliferation. Furthermore, unknown mechanisms induce methylation of Wnt pathway inhibitory genes *SFRP1* and *DKK1* to hypothetically facilitate Wnt signaling activity during infection. **(C)** *P. aeruginosa* is decorated with lectin LecB which induces β-catenin degradation through an unknown extracellular mechanism, resulting in decreased cell proliferation. Coincidentally, NF-κB signaling is activated and inflammatory target genes *IL6*, *IL1B*, and *TNFA* are expressed. LecB as well as the quorum-sensing molecule PAI both induce disruption of adherens junction in infected epithelia, contributing to prolonged tissue damage in the host.

Recent studies have identified a unique role for TcdB in inhibition of Wnt signaling. Through sgRNA screening, TcdB has been shown to directly bind Fzd receptors in the intestinal epithelium, including Fzd1, Fzd2, and Fzd7 (43). This results in inhibition of LRP and Dvl phosphorylation, indicating a TcdB mechanism of Wnt inhibition through direct manipulation of the Wnt receptor complex. Wnt ligands interact with Fzd through two distinct binding sites, as well as hydrophobic interactions between a groove on the Fzd-CRD and the palmitoleic acid moiety (PAM) of Wnt (116). TcdB interacts with the Fzd-CRD as well, although the binding site is distinct from the Wnt binding sites as revealed by the co-crystallization of TcdB Fzd-binding domain and Fzd-CRD (44). In the pathway off state under normal physiological conditions, Fzd-CRD binds an endogenous lipid which becomes displaced by the Wnt PAM when the signal-initiating ligand binds. The endogenous lipid acts as coreceptor for TcdB that TcdB directly engages and locks in place, preventing displacement by the Wnt PAM and inhibiting pathway activation. It was also demonstrated that TcdB can bind Fzd when a Wnt ligand has already bound, with the Wnt PAM acting as a TcdB coreceptor, suggesting TcdB may have other inhibitory mechanisms including inhibition of receptor dimerization which has been shown to be stimulated through Wnt binding and necessary for signal transduction to occur (44, 117). This blockade of Wnt signaling is hypothesized to be a major underlying cause of weakening of the intestinal epithelium, tissue damage, and inflammation, hallmarks of PMC and *C. difficile* infection (44).

The unique inhibitory mechanisms of toxins TcdA and TcdB on Wnt signaling in host cells has expanded the model of pathogen manipulation of Wnt signaling through the identification of both intra- and extra-cellular molecular interactions that contribute to *C. difficile* pathogenesis. It remains to be discovered if TcdA glucosyltransferase activity modulates non-canonical Wnt signaling in the intestinal epithelium due to the major role of small GTPases in non-canonical pathways (118). Further investigation of TcdB interference with Fzd receptors will also lend insight into the critical interactions for Wnt pathway signaling initiation under normal physiological conditions. Of note, the canonical Wnt pathway components APC, GSK3β, Wnt5a, and LRP6 were also identified as potential targets of TcdB in an sgRNA screen (43). Whether the pool of TcdB that is endocytosed has a relevant role in Wnt signaling manipulation through interactions with these intracellular components remains to be determined. Of particular interest is the exploitation of TcdB-Fzd interaction for rational drug design. A recombinant TcdB mutant lacking Fzd-binding ability can induce significantly less swelling, epithelial damage, and infiltration of immune cells in a mouse cecum injection model, demonstrating that modulation of this toxin activity in the intestines has strong potential to reduce disease severity (44).

Helicobacter pylori

Helicobacter pylori is a Gram-negative bacilli and most common infectious agent found in both adults and children worldwide. The bacterium is transmitted fecal-orally and oral-orally and replicates within the mucin layer in the stomach adhered to gastric epithelium. Pathogenic *H. pylori* strains possess the *cag* pathogenicity island, a genetic locus that consists of 31 genes including the effector CagA and the T4SS that secretes it. CagA is injected into gastric epithelial cells and directly interferes with cellular signaling pathways such as NF-κB and MAPK signaling, alters cell-cell contacts, and stimulates a proinflammatory response, thereby promoting the *H. pylori* replicative niche (119). CagA induction of the cell cycle and host survival pathways including canonical β-catenin-dependent Wnt signaling induce cellular transformation and replication which is why Cag^+ strains of *H. pylori* increase host predisposition to tumorigenesis and gastric carcinoma, and *H. pylori* is classified as a type I carcinogen (120, 121).

While it is well-documented that *H. pylori* infection activates β-catenin-dependent Wnt signaling in the gastric epithelium, the role of CagA in Wnt activation is controversial. An early observation in the field determined that during infection of human gastric epithelia, β-catenin is stabilized. This was associated with infection by $CagA^+$ strains (**Figure 6B**) (122). The mechanism for activation of β-catenin has been shown to be in part mediated by CagA disruption of adherens junctions. CagA directly interacts with E-cadherin which causes β-catenin dissociation from the adherens junctions complex and subsequent translocation to the nucleus (45). CagA induction of β-catenin target gene CDX and transactivation of p21 links disruption of adherens junctions and Wnt pathway activation with *H. pylori* CagA carcinogenesis as these gene products are required for transformation of gastric epithelium to intestinal epithelium which is a critical event in the development of intestinal metaplasia. An earlier finding connected activation of non-canonical NFAT-dependent Wnt signaling with CagA induction of p21, demonstrating regulation of Wnt signaling by CagA at multiple levels during infection (47). In a mammary epithelium model of infection, adherens junctions proteins were shown to dissociate upon infection in a CagA-independent mechanism resulting in loss of junctional integrity (123). While this group demonstrates β-catenin is stabilized, they do not detect nuclear localization of the transcription factor, but rather propose the hypothesis some other factor is able to cleave E-cadherin and manipulation junctions (123). This could indicate $CagA^-$ strains of *H. pylori* stimulate β-catenin signaling through other mechanisms, or these results could suggest host factor-dependent predisposition to pathway hyperactivation.

Thus, a CagA-independent mechanism of Wnt activation may be active during *H. pylori* infection in addition to manipulation of the signaling pathway through interference with adherens junctions. LRP5/6 and Dvl are both phosphorylated in a Cag-independent, T4SS-dependent manner in a gastric epithelial infection model (124). *H. pylori* has also been shown to inhibit GSK3β activity which suppresses β-catenin degradation and is shown to induce Wnt pathway target gene *CCND1* (125, 126). Intracellularly, β-catenin activation and Wnt target gene expression is linked to activation of PI3K and Akt which inhibit GSK3β during infection, but the role of the Wnt receptor complex protein LRP6 in this mechanism remains to be investigated. Ultimately, there may be cell type- and temporally-specific deregulation of Wnt signaling during infection, as well as diverse effects of different effectors, that promote a tightly controlled differentiation vs. proliferation profile during *H. pylori*

infection that ultimately serves to facilitate bacterial survival and dissemination.

H. pylori is well-known to modify target cell epigenetics which can also promote infection and tumorigenesis (127). A recent report linked *H. pylori* infection with modulation of Wnt signaling through epigenetic modification of Wnt pathway-related genes. Human *H. pylori*-positive gastric carcinoma samples display significantly increased methylation at the host genes *SFRP1* and *DKK1*, inhibitors of Wnt signaling (46). This corresponded with slight to moderate increases in β-catenin nuclear localization. Patient samples that demonstrated *H. pylori* eradication at a one-year follow-up did not have significantly altered epigenetic footprints, indicating *H. pylori* can induce long-lasting changes to host genomics and impact Wnt signaling activity in hosts post-eradication (46).

Understanding the connection between *H. pylori* secreted effectors and changes to host cell signaling pathways provides insight into potential avenues for therapeutics, infection or gastric cancer biomarkers, and pathogen-mediated tumorigenesis. Both CagA-dependent and -independent mechanisms are at play during infection to manipulate Wnt signaling and facilitate an environment within the host that supports bacterial replication. The role of LRP in activation of signaling and CagA cleavage of E-cadherin demonstrates *H. pylori* may utilize secreted proteins to manipulate Wnt signaling at the level of the receptor complex to induce pathway activity, a model of pathway activation applicable to intracellular and extracellular pathogens alike. Interestingly, pathways active during infection including PI3K-AKT and Wnt signaling both converge at the inactivation of GSK3β, indicating redundant mechanisms in place to ensure the different outcomes that are mediated by inhibition of the kinase. Further understanding of how the kinase activity of GSK3β is a crux for infection may lead to the development of therapeutics that act on cellular signaling pathways and have the potential to delay pathogen-induced tumorigenesis.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen and a causative agent of microbial keratitis, burn wound infections, and hospital-acquired pneumonia. The bacteria is found in biofilms that are essential for host attachment and survival (128). *P. aeruginosa* utilizes quorum-sensing (QS) systems to sense bacterial population density and regulate biofilm development, mammalian host defense, host-microbe interactions, virulence and metabolite acquisition (129, 130) *P. aeruginosa* pathogenesis also encompasses disruption of epithelial barrier function, increased inflammatory response, and bacterial virulence factors.

Canonical Wnt signaling plays a major role in epithelial barrier integrity and proliferation during *P. aeruginosa* infection. Host response to *P. aeruginosa* infection leads to activation of the Wnt signaling pathway to regulate the pro-inflammatory response and promote bacterial clearance; therefore, inhibition of Wnt signaling is advantageous for persistent infection and survival. A recent study has shown that macrophages stimulated with Wnt3a conditioned media inhibits production of pro-inflammatory cytokines IL-6, IL-1β, MIP2, and TNF-α during *P. aeruginosa* infection. Wnt3a treatment also promotes apoptosis of macrophages at 12 and 24 hpi and enhances intracellular bacterial killing via upregulation of anti-microbial peptides CRAMP and BD1 (131). During *P. aeruginosa* infection inhibition of Wnt3a upregulates expression of pro-inflammatory cytokines, likely to delay tissue recovery and promote *P. aeruginosa* colonization (**Figure 6C**) (49). In a *P. aeruginosa* keratitis model of infection, melting of the cornea occurs due to bacterial proteases, activation of matrix metalloproteases, and deregulated immune response (132). β-catenin inhibits pro-inflammatory cytokines and decreases bacterial burden in the corneal stroma to reduce the severity of *P. aeruginosa* keratitis (133). Overexpression of β-catenin in B6 corneas results in suppression of *IL1B*, *MIP2*, and *TNFA* in phagocytes, but not in corneal epithelial cells, for host resistance of *P. aeruginosa* infection. Bacterial load is also decreased during overexpression of β-catenin in B6 corneas infected with *P. aeruginosa*. Collectively these studies suggest inhibition of Wnt signaling, specifically by downregulation of Wnt3a and β-catenin, increases bacterial burden and the inflammatory response during *P. aeruginosa* infection. Along with these findings, lithium chloride (LiCl) was shown to promote bacterial clearance. LiCl promotes canonical Wnt pathway activity through the inhibition of GSK3β (134). Various studies have determined that GSK3β is important in regulating inflammatory responses during bacterial infections (135). For example, *Klebsiella pneumoniae*-infected mice provided with LiCl-treated drinking water display increased survival and decreased liver injury due to a decrease in bacterial burden and cytokine production in blood and liver tissues (136). During *P. aeruginosa* infection, LiCl has been shown to inhibit proinflammatory cytokine TNF-α, enhance production of anti-inflammatory cytokine IL-10, and ultimately lead to host resistance against *P. aeruginosa* infection in the cornea (137). In correlation with previous listed studies, a reduction in inflammatory infiltration is observed in LiCl-treated cells, and increased apoptosis of phagocytes occurs. Taken together, *P. aeruginosa*-mediated inhibition of Wnt/β-catenin signaling is important for recruitment of infiltrating phagocytes and increased inflammatory response to promote dissemination and survival of the bacteria.

As previously described, β-catenin is a major contributor to the integrity of adherens junctions and may play an indirect role in the integrity of tight junctions and epithelial barrier during *P. aeruginosa* infection. *P. aeruginosa* contains two quorum-sensing systems which regulate specific signaling molecules that contribute to infection and survival (138). Adherens junctions integrity is decreased by a *P. aeruginosa* acyl-homoserine lactone (PAI) quorum-sensing molecule (48, 139). Treatment of CaCO-2 cells with the PAI results in a decrease in adherens junction-associated proteins E-cadherin and β-catenin expression during early infection. Hyperphosphorylation of occludin, ZO-1, E-cadherin and β-catenin are detected on tyrosine residues during early infection; however, occludin and β-catenin are dephosphorylated on serine and threonine residues. These outcomes are responsible for dissociation of E-cadherin-β-catenin complexes and association of ZO-1-occludin complexes ultimately leading to disassembly of adherens junctions (48). Of note, the expression levels of

E-cadherin and β-catenin increase back to normal levels at later time points of infection (5–24 h), demonstrating a reversible effect of PAI during *P. aeruginosa* infection. Taken together, distinct phosphorylation events on adherens junction proteins, including β-catenin, play an important role in *P. aeruginosa* PAI-mediated tissue damage.

Along with *P. aeruginosa* PAI, soluble carbohydrate-binding lectin LecB is regulated by both *las* and *rhl* QS systems and reprograms the Wnt signaling pathway for infection and survival. LecB is one of two lectins expressed on the outer bacterial membrane of *P. aeruginosa* and is important for adhesion, biofilm formation, pilus biogenesis, and protease activity (140). LecB acts antagonistically to Wnt signaling through proteasomal degradation of β-catenin in a GSK3β-dependent manner, resulting in a G1-S phase cell cycle arrest in lung epithelial cells (49). LecB reduces β-catenin at adherens junctions and causes accumulation of the protein at centrosomes expressing high levels of proteasomes. Furthermore, co-incubation of Wnt3a and LecB repressed nuclear translocation of β-catenin, indicating infection actively suppresses canonical Wnt signaling activation. In addition, Ser536 phosphorylation of p65, a marker for increased transcriptional activity and acetylation of NF-κB, increases during *P. aeruginosa* infection, resulting in activation of NF-κB and subsequent elevation of downstream target genes *TNFA*, *IL6*, and *IL1B* which are linked to delayed tissue recovery (49). Taken together, these results indicate that *P. aeruginosa* manipulates the Wnt/β-catenin signaling pathway via β-catenin degradation and NF-κB activation to delay tissue recovery and promote infection.

P. aeruginosa is an opportunistic pathogen that utilizes various approaches to cross the epithelium at cell-cell junctions, including disruption of the epithelial cell barrier via PAI induction of phosphorylation of E-cadherin-β-catenin complexes (141). *P. aeruginosa* PAI also modulates mechanisms of immune tolerance to provide a niche for bacterial survival (142). Targeting the quorum sensing abilities of *P. aeruginosa* has been explored as an antimicrobial strategy, so understanding the extent of PAI in manipulating the host cell may identify mechanisms by which anti-quorum sensing drugs can also target mechanisms involving Wnt signaling. As previously described, *S. typhimurium* infection results in simultaneous β-catenin degradation and increased NF-κB activity in a murine model (18). *P. aeruginosa* LecB degrades β-catenin to decrease proliferation of lung epithelial cells, ultimately leading to delayed tissue recovery and persistent infection. Further investigation of the role of LecB-mediated β-catenin degradation inducing activation of NF-κB should also be explored. LecB is localized to the outer membrane of *P. aeruginosa* and binds to host cell plasma membrane receptors stimulating changes to epithelial barrier function and increased inflammatory response (143). Therefore, LecB-dependent β-catenin degradation may be stimulated by direct binding to extracellular portions of the Wnt receptor complex or extracellular adherens junction components. Understanding the mechanisms utilized by LecB to manipulate Wnt signaling to decrease proliferation and tissue recovery of lung epithelial cells may serve as a potential therapeutic approach to *P. aeruginosa* infection.

Escherichia coli

Uropathogenic *Escherichia coli* (UPEC) is an extraintestinal pathogenic isolate of *Escherichia coli* and the causative agent of 40% of hospital- and 80% of community-acquired urinary tract infections (144). UPEC infects the urothelium, a highly specialized epithelium lining the lower urinary tract that is composed of basal, intermediate, and superficial cell layers (145). The superficial apical cell layer provides the primary urinary barrier and is composed of large, hexagonal cells known as umbrella cells which are exfoliated and regenerated upon infection (145, 146). Upon infection, UPEC enters and ascends into the urinary tract, enters the urethra, and migrates into the bladder lumen where it engages with bladder umbrella cells by binding to mannose-containing glycoprotein receptors via the adhesin FimH (50). Engulfment into the host cell is followed by the formation of intracellular bacterial communities (IBCs) that demonstrate biofilm-like properties (147, 148). UPEC detach from the intracellular biofilm and establish intracellular colonization leading to eruption of bladder epithelial cells. Dissemination and detachment lead to UPEC mobilization to the bladder lumen which increases the risk of bacteremia and septicemia (148).

UPEC has evolved several molecular-based strategies to subvert the innate immune response for efficient colonization and persistent infection in the urinary tract. One mechanism of host response to UPEC infection is the process of exfoliation of bladder epithelial cells that are overwhelmed with bacteria (146, 147). To renew exfoliated superficial epithelium, genetic programs are activated for proliferation and differentiation of urothelial cells (149). Despite the role of exfoliation in bacterial clearance, this outcome also leads to exposure of immature bladder epithelial cells which are more susceptible to UPEC infection (50). Within these compartments, bacteria are in a quiescent state; however, as the immature urothelial cells differentiate into mature urothelial cells, UPEC replicates for recurrent infection (147). Modulation of Wnt signaling has been linked to differentiation and proliferation of basal/intermediate cells during UPEC infection. More specifically, expression of Wnt5a during UPEC infection leads to proliferation of basal/intermediate cells while suppression of Wnt5a expression results in differentiation (50). A recent study demonstrated suppression of Wnt5a/Ca^{2+} signaling promotes differentiation of basal/intermediate cells prior to exfoliation (50). Infection by $FimH^{+}$ UPEC results in a decrease in Wnt5a expression and subsequent decrease of non-canonical Wnt pathway targets PKCδ and CamKIIδ up to relatively late infection timepoints (50). This suggests that prior to exfoliation, non-canonical Wnt signaling is suppressed to promote differentiation of umbrella cells upon initiation of UPEC infection. However, it has also been shown that UPEC induces infected cells to secrete paracrine factors that cause alterations in the expression of the epigenetic writer EZH2 which enhances Wnt5a expression, promoting proliferation of infected cells at relatively early infection time points (51). Further studies must be done to determine if there is temporal regulation of Wnt5a expression during UPEC infection. Despite these discrepancies, research suggests that modulation of Wnt signaling during UPEC infection promotes exfoliation

and cell proliferation for dissemination and survival, respectively. Consistent with these findings, EZH2 has been shown to be involved in Wnt-mediated proliferation in *Citrobacter rodentium* infection (52). *C. rodentium* is a Gram-negative, murine enteric bacterial pathogen and closely related to enteropathogenic (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (150–152). EPEC, EHEC, and *C. rodentium* are members of the attaching and effacing (A/E) family of bacterial pathogens due to the destructive effect of colonization of the intestinal epithelium and enterocytes (150, 151). The natural host range and genetic make-up of *C. rodentium* makes it a good *in vivo* model for A/E pathogens. EZH2 represses Wnt inhibitory factor WIF1 resulting in activation of Wnt/β-catenin signaling and ultimately *C. rodentium*-induced crypt hyperplasia and tumorigenesis (52). Therefore, modulation of the Wnt signaling pathway by A/E pathogens would appear to be a useful regulatory mechanism by providing a niche for adaptation and survival in the host.

Along with a diverse range of adhesin molecules, UPEC utilizes a variety of toxins to increase invasiveness and facilitate virulence. Of these secreted toxins is a pore-forming, T1SS toxin α-hemolysin (HlyA). HlyA is inserted into epithelial and macrophage membranes and triggers rapid degradation of various host proteins involved in the proinflammatory response and cell-cell and cell-matrix interactions, including β-catenin (153). HlyA mediated degradation of β-catenin in infected BECs occurs simultaneously with loss of IκBα. Additionally, the NF-κB subunit RelA (p65) is also degraded in HlyA-intoxicated bladder epithelial cells. HlyA-mediated degradation of RelA (p65) correlates with reduced *IL6* expression (153). This study suggests a novel mechanism by which UPEC inhibits NF-κB-mediated inflammatory response via β-catenin degradation, and independent of IκBα-mediated proteasomal degradation. Similar results were shown with macrophages, demonstrating a significant role of HlyA in modulating phagocytes and epithelial cell function for UPEC survival. Investigation of the cross-regulation of β-catenin and NF-κB signaling pathways during UPEC infection would demonstrate if UPEC utilizes Wnt signaling as a means of immunosuppression.

Several gaps in knowledge remain in reference to the role of Wnt signaling in subversion of innate immunity during UPEC infection. While exfoliation and proliferation are mechanisms of host response to UPEC infection, this response is beneficial for establishing a niche for persistent infection (153, 154). Identifying the role of Wnt signaling and possible UPEC effectors involved in pathway activation to induce exfoliation and proliferation for survival may allow for better understanding of how UPEC infections can persist in the face of host defenses. Inhibition of Wnt5a-induced non-canonical signaling and EZH2 may potentially inhibit proliferative processes necessary for UPEC infection and may therefore serve as potential therapeutic targets.

CONCLUDING REMARKS—WINNING THE TUG-OF-WNT

Pathogens' respective niches and modes of dissemination shape how they avoid innate immune pressure during infection. Wnt signaling is a host cell pathway targeted by both obligately intracellular and extracellular bacterial pathogens alike, demonstrating the breadth of the pathway's control over eukaryotic cellular processes. A consistent theme among the intracellular pathogens discussed in this review is activation of the pathway in the host cell. Evidence from *Salmonella, Chlamydia*, *Rickettsia,* and *Mycobacteria* demonstrates canonical pathway activation contributes to an anti-inflammatory state which mediates immunosuppression and may prolong infection. The former two pathogens also appear to stimulate canonical Wnt signaling to prolong host cell survival by inducing either proliferation or differentiation of the host cell. *Ehrlichia* is unique in that studies have shown the bacterium activates non-canonical pathways which govern cytoskeletal reorganization and autophagy regulation, permitting phagocytosis of the bacterium without destruction by the lysosome. Inhibition of the canonical Wnt pathway by *Salmonella* and *C. difficile* occurs in intestinal epithelium and the intestinal capillary endothelium for the former and in the colonic epithelium for the latter, and contributes to pathogen manipulation of tissue barriers, facilitating dissemination into the bloodstream. In the case of *Salmonella*, inhibition of Wnt signaling may also lead to an inflammatory response that recruits target cells for Salmonella and promotes dissemination. Studies have shown that *C. difficile* and *P. aeruginosa* utilize multiple effector molecules to interfere with canonical Wnt signaling activity of the surrounding tissue during infection. In both cases, viability of the cell within the infected tissue is reduced which may contribute to nutrient accumulation or dissemination for the bacteria. *E. coli* and *H. pylori*, contrastingly, activate canonical and non-canonical Wnt signaling from their extracellular niches, resulting in increased proliferation or differentiation of cells in the surrounding epithelium. Although evidence is yet to demonstrate a clear phenotype, it is likely that pathway activation also contributes to an anti-inflammatory state in the bacterial replicative niche due to reciprocal regulation of the transcription factors NF-κB and β-catenin.

Studies have implicated a range of bacterial effectors with the capability to manipulate canonical and non-canonical Wnt signaling, including T1,3,4SS effector proteins of *S. enterica, C. pneumoniae, E. chaffeensis, E. coli,* and *H. pylori*; lectins and quorum-sensing molecules of *P. aeruginosa*; two-component regulatory systems of *S. enterica*; and likely many more unknown factors. Shared mechanisms involve disassembly of adherens junctions as is the case for *C. trachomatis*, *R. rickettsii*, *H. pylori*, and *P. aeruginosa* which may be a means for increasing the amount of cytosolic β-catenin available to activate the Wnt response element, or a means of inactivating the protein and facilitating its degradation. Cpn1027 of *C. pneumoniae* and TcdB of *C. difficile* both inactivate pathway components through direct binding. Such may be the case for *E. chaffeensis* TRP32, 47, and 120, as many host-bacterial interactions between negative regulators of Wnt signaling and the TRPs have been identified for which mechanisms have yet to be described. Pathogens also deploy enzymes during infection to manipulate Wnt signaling components at the post-translation modification level, as is the case with *C. difficile* TcdA and *S. enterica*

AvrA and SopB. Research identifying Wnt pathway regulatory mechanisms and foreign molecules that can interfere with the pathway has applications ranging from research tools to potential therapeutics.

As research detailing mechanisms of pathogenesis and bacteria-host interaction accumulates, our knowledge of how various cellular mechanisms participate in innate immune signaling expands. Wnt signaling regulates stem cell renewal, cell proliferation, and cellular morphology, but the ability of pathogens to usurp these processes to establish infection demonstrates that Wnt signaling is also a target for bacterial immunoevasion strategies. By drawing a parallel between pathway dysregulation during infection and dysregulation in "classical" diseases of Wnt signaling, we can identify targetable pathway components for drug intervention of infectious disease. One such strategy involves the use of the existing armaments of small molecule inhibitors and activators that target various components of canonical Wnt signaling thereby overriding the pathogen reprogramming strategy. Another approach is the use of antivirulence therapy to target the pathogen-host molecular interactions that are beneficial to bacterial virulence, thereby weakening the pathogen through impairing its ability to manipulate the Wnt pathway.

Multiple small molecules have been identified that modulate Wnt signaling activity through activation or inhibition of pathway components or regulators (14). Use of these drugs as an approach for infection control is most suitable for bacteria for which stimulation or repression of pathway activity significantly impairs establishment of infection or a damaging inflammatory response. GSK3 promotes Wnt signaling through inactivation of the destruction complex and a number of small molecules target GSK3 activity. These inhibitors have been used in mouse infection models for *Streptococcus* and *Porphyromonas gingivalis* and demonstrate efficacy in reducing bacterial burden or excessive inflammation (155–157). GSK3 represents an attractive target for drug design because of its suppressive effect on NF-κB signaling, and upregulation of its kinase activity by different pathogens makes it a potential point for therapeutic intervention during infection (158). Modulators of extracellular components of Wnt signaling include pathway agonists that mimic Wnt ligands and neutralizing antibodies that target secreted pathway inhibitors (159–161). These types of drugs may be utilized when infection causes suppression of Wnt signaling which leads to tissue damage. *Salmonella* perturbation of the GVB represents a case in point—stimulation of Wnt signaling reverses infection-induced endothelial weakening (20). The effectiveness of these potential Wnt-modulating therapeutics will be impacted by the particular points within the Wnt signal cascade that different pathogens exert manipulation, which prospective mechanism-oriented studies should address. Ultimately, the vast amount of signaling proteins within the Wnt pathway suggests that while this cellular signal cascade can be targeted by drugs, such intervention should be thoroughly researched to anticipate off-target and adverse side effects (162).

Studies of how pathogens interact with the host signalosome allows us to exploit this knowledge to target crucial interactions between bacterial and host factors that enable bacterial virulence (163). Antivirulence therapy has risen as a strategy to combat pathogenic bacteria by disarming them of virulence factors, and an in-depth understanding of the interplay between virulence factors and Wnt signaling enables the development of such therapeutic strategies. For example, *C. difficile* infection has been shown to be controlled by small molecules and monoclonal antibodies that target critical enzymatic or protein-protein interaction-mediating domains within TcdB (164, 165). The identification of a Fzd-binding domain within TcdB uncovers a target against which small molecules or neutralizing antibodies can be directed, thereby reducing the pathological effects of bacterial inhibition of Wnt signaling within the intestines. A similar strategy could be directed toward *E. chaffeensis* infection, as surface TRPs including TRP120 mediate phagocytosis of the bacterium through stimulation of Wnt pathway activity. As further research identifies the molecular determinants of such pathological events, interference with TRP-Wnt receptor complex could reduce the ability of *E. chaffeensis* to establish infection within monocytes. *P. aeruginosa* utilizes surface protein LecB to extracellularly modulate β-catenin activity. Antivirulence drugs in preclinical development that target LecB demonstrate how this protein can be manipulated to reduce infection, and suggest that the efficacy of these drugs is, at least in part, due to the disruption of *P. aeruginosa* modulation of Wnt signaling (166).

The era of Wnt signaling research has shifted from understanding the basic molecular biology of the pathway to identifying how the pathway is manipulated by infectious agents. The ultimate goal is to use this knowledge to repurpose or develop novel therapeutics for infectious disease that are relevant in the age of antibiotic resistance. Pathogens have coevolved with the human host and are master manipulators of signaling pathways that have been in place throughout the evolution of metazoans. Understanding the interactions that dictate how pathogens can usurp the Wnt signal cascade to benefit their survival and dissemination facilitates the development of counter-defense strategies that enable the host innate immune system to control and clear infection.

AUTHOR CONTRIBUTIONS

MR and JM conceived the work. MR gathered information and contributed all sections except for *P. aeruginosa* and *E. coli* which were contributed by LP. JW performed artwork. All authors participated in editing of the final draft.

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Wnt Signaling: Pathogen Incursion and Immune Defense

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Wnt ligands interact with the transmembrane cell surface receptors Frizzled and ROR/RYK to initiate complex signaling cascades that are crucial for cell physiology and the proper functioning of the immune system. Wnt signaling is instrumental in maintaining immune surveillance and during infections by pathogenic microbes helps mount host resistance to infection. Some pathogens, however, utilize Wnt signaling to build a niche for their survival. The goal of this review is to summarize current and developing concepts about the tug of war between Wnt signaling and pathogens for deployment of host resources, focusing mostly on macrophages and cytoskeletal actin dynamics. An additional objective is to outline the interrelation between Wnt signaling and the host microbiota, which is vital for immune defense, discussing in the same perspective, how Wnt signaling could be differentiating pathogen from non-pathogen.

Keywords: Wnt, frizzled, pathogen, microbiota, actin, cytoskeleton, macrophage, immunity

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INTRODUCTION

Macrophages, major sentinels of immune defense utilize the Wnt signaling scheme to sustain immune homeostasis, maintain immune surveillance, and combat infections with several pathogens. Some microorganisms, however, outmaneuver Wnt signaling and exploit it to create a niche for their survival. Wnt associated cytoskeletal modulations and transcriptional programs partake of such host pathogen interactions (1–8). Wnt signaling is also linked with the colonization of distinct groups of microbiota, which coexist with the host and bolster immune defense by inhibiting the growth of pathogenic microbes (9, 10).

Wnt proteins constitute a family of about 19 different secreted cysteine-rich glycoproteins in mammals, which are highly conserved among different species. Nusse et al. first identified the mammalian counterpart of Drosophila Wingless and termed it Wnt1, which nomenclature wise is a combination of Drosophila Wingless and the mouse proto-oncogene Int1 (11–13). The role of Wnt (Wingless) signaling was first documented during Drosophila development (11, 13–15). Subsequently, with the identification of Wnt homologs in mammals the importance of Wnt signaling was recognized in the context of different cellular functions ranging from cell proliferation and migration to cell polarity and tissue homeostasis (16). Wnt signaling initiates when Wnt ligands interact with the Frizzled and ROR or RYK family cell surface receptors. While the Frizzleds (about 12 in number) are transmembrane proteins resembling heterotrimeric G protein coupled receptors, ROR1, ROR2, and RYK resemble tyrosine kinases (12, 17–22). Due to considerable homology among different members of the Wnt and Frizzled families, it is possible for a particular Wnt ligand to interact with multiple Frizzled receptors (23). Thus, the outcome of Wnt-Frizzled signaling depends on the prevailing stoichiometry of the Wnt and Frizzled proteins and their mutual accessibility (24). Wnt-ROR/RYK signaling, mostly described as independent of Wnt-Frizzled signaling has also been shown to support Wnt-Frizzled signaling in some cases (21). A general scheme for Wnt signaling has been described in several review articles (25, 26).

Wnt signaling can be categorized into two main classes—canonical (β-catenin dependent) and non-canonical (β-catenin independent). In canonical Wnt signaling, β-catenin, which accumulates in the cytoplasm, enters into the nucleus acting as a transactivator for LEF/TCF transcription factors to initiate expression of β-catenin responsive genes (27). In non-canonical Wnt signaling, transcriptional activation is mostly associated with transcription factors such as NFκB, NFAT, and AP1 (6, 28, 29), and cell polarity is linked with modulations of the cytoskeleton that involve actin and actin associated proteins (30–32). The ligands Wnt3A and Wnt5A are usually considered as representatives of the canonical and non-canonical modes of Wnt signaling, respectively (33). Since Wnt signaling is guided and controlled by fairly homologous receptors and intracellular signaling intermediates that may be shared by both canonical and non-canonical modes of Wnt signaling, overlap between these two signaling pathways is not uncommon. Quite interestingly, the intracellular adaptor molecule Disheveled and Daple are required for both modes of Wnt signaling (34–36). Disheveled utilizes cholesterol, and the heterotrimeric G proteins coupled to Frizzled receptors interact with Daple for switching between the canonical and non-canonical modes (37). It is important to know if cooperation of the heterotrimeric G proteins is required for Disheveled function during signaling (36, 38). Cell surface co-activator receptors such as Lipoprotein Receptor-like Protein (LRP) 5/6 are usually associated with only the canonical mode of Wnt signaling. Although LRP5/6 has been shown to interact with the non-canonical Wnt signaling ligand Wnt5A (24, 39), whether it is actually required for non-canonical Wnt signaling or acts to strike a balance between the canonical and non-canonical modes remains unsettled. A recent report suggests that Wnt can also signal by binding to TLR4/2 receptors (40). While Wnt-TLR signaling is important in view of the evolving role of Wnts in immunity to infections, the mode of this signaling pathway in relation to the already established intracellular signaling adaptors and intermediates remains to be documented.

Although it is now clear that Wnt signaling is closely associated with infection, sustenance of host microbiota and immune regulation (3–6, 9), interrelations between Wnt signaling and host immunity to different types of infection are complex and the molecular details therein are not yet settled. The goal of this review is to summarize current and developing concepts relating to the role of Wnt signaling in microbial infections with special emphasis on macrophages and cytoskeletal actin. The idea is to address unanswered yet important questions based on existing knowledge and paradigms to help bridge gaps in our understanding of the Wnt signaling scheme in the context of host immune defense programs.

INTERRELATION BETWEEN WNT SIGNALING AND INFECTION WITH PATHOGENIC MICROBES

Wnt signaling in macrophages plays a crucial role in shaping the outcome of infections by different pathogens (3, 4, 41–43). While Wnt signaling facilitates elimination of certain infections by disabling the causative pathogens, it also favors the progression of other infections by allowing the causative pathogen to build its niche within macrophages. It is expected that such interactions of host Wnt signaling with the encountered pathogens would involve actin cytoskeletal dynamics, an integral component of host pathogen interactions (32, 44, 45). We will focus on some representative microbial infections in this regard.

Bacterial Infection

Infection by bacterial pathogens such as *Pseudomonas* sp. and *Streptococcus* sp. is inhibited by Wnt signaling. Wnt induced bacterial clearance in macrophages occurs by phagocytosis and subsequent xenophagy through utilization of the host actin associated autophagy circuit. In this context, the function of Wnt5A, which mediates cytoskeletal actin modulations through activation of the actin associated proteins Rac1 and Disheveled holds considerable significance (3, 4, 36). It has been demonstrated that Wnt5A signaling in macrophages not only facilitates internalization of pathogenic *Pseudomonas* sp. and *Streptococcus* sp., but also enhances their killing. Wnt5A-Rac1/Disheveled dependent cytoskeletal actin rearrangements facilitate the formation of bacteria containing autophagosomes that are destined for lysosomal fusion, thus enabling bacterial clearance (3). Rac1 and Disheveled inhibitors, as well as Cytochalasin D which inhibits actin assembly, suppress Wnt5A mediated killing of *Pseudomonas* and *Streptococcus*. Signaling by Wnt3A and Wnt11 in macrophages and macrophage associated cells of the gut has also been linked with inhibition of infections by pathogenic *Pseudomonas* and *Salmonella* species (43, 46). It is important to know if cytoskeletal modulations as observed in the case of Wnt5A signaling are also associated with reduction of bacterial pathogen load by Wnt3A and Wnt11.

The Human Monocytotropic Ehrlichiosis (HME) causing bacterium *Ehrlichia chaffeensis* is yet another bacterial pathogen that interacts with Wnt signaling in macrophages. Unlike some of the other bacteria, *E. chaffeensis* utilizes both so called canonical and non-canonical Wnt signaling intermediates for niche building and multiplication within macrophages. Infection by *E. chaffeensis* is accompanied by increase in activation of the Wnt signaling intermediates Rac1 and Disheveled (8). Blockade in expression of Wnt5A, its putative receptor Frizzled5 and Rac1 furthermore, leads to decrease in *E. chaffeensis* infection (8), suggesting that Wnt5A signaling is needed for promoting *E. chaffeensis* intracellular niche formation. Interestingly, blockade in Wnt5A signaling also inhibits infection by the non-pathogenic lab strain of *E. coli*, DH5-α, which gets internalized but not killed by activation of Wnt5A signaling (5). Wnt5A induced internalization of *E. coli* DH5-α is dependent upon alterations in actin assembly, which are not conducive to bacterial killing (5). Whether similar alterations in actin assembly are also associated with *E. chaffeensis* infection and niche formation will be important to decipher.

Wnt signaling in macrophages also regulates infection by different *Mycobacterium* species. Mycobacterial infections are associated with altered expression of several Wnts (47–51). While Wnt5A has been reported to promote both pro inflammatory and anti-inflammatory cytokine signatures in mycobacterium

infected macrophages, both Wnt3A and Wnt6 have been reported to skew the intracellular milieu of infected macrophages toward an anti-inflammatory cytokine signature (47, 49, 52, 53). Recently, Gao et al. has demonstrated that Wnt5A restricts niche formation in macrophages by both *Mycobacterium tuberculosis* and *Mycobacterium bovis* through activation of autophagy (54). In light of the documented role of Wnt5A signaling in cytoskeletal modulations and autophagy / xenophagy (3, 4, 30, 31), and the crucial involvement of actin and actin binding proteins during mycobacterial infections (55–57), it is important to examine how Wnt mediated cytoskeletal modulations regulate the sustenance vs. inhibition of mycobacterial infections.

Parasitic and Fungal Infection

Several lines of evidence indicate that Wnt signaling in macrophages regulates parasitic and fungal infections. Wnt5A signaling suppresses infection by the parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis by blocking the sustenance of *L. donovani* containing parasitophorous vacuoles within macrophages. Transmission electron microscopy of *L. donovani* infected macrophages has revealed the predominance of degraded parasitophorous vacuoles in autophagosome like intracellular vesicles upon activation of Wnt5A signaling (4). Wnt5A mediated cytoskeletal alterations correlate with parasitophorous vacuole degradation and inhibition of infection. As in the case of bacterial infection, Wnt5A mediated inhibition of *L. donovani* infection is blocked by inhibitor of Rac1 activation, which is linked with Wnt5A mediated cytoskeletal alterations (4). Inhibition of Wnt5A production *in vivo* by intravenous administration of the inhibitor IWP2 into mice accordingly results in increased susceptibility to *L. donovani* infection (4). *L. donovani* infection in mice reportedly is sustained through a skewed hematopoiesis/myelopoiesis program marked by the predominant prevalence of inflammatory monocytes (58–60). Myelopoiesis and macrophage differentiation under normal conditions are supported by a complex Wnt signaling network that includes Wnt5A (6, 61–64). Thus, sustenance vs. inhibition of *L. donovani* infection is perhaps determined by the diverse traits of Wnt signaling in the context of the infection load. It is important to carry out a detailed evaluation of the role of Wnt5A in the context of other Wnts in this respect, especially in light of the documented antagonism of Wnt5A—actin network toward *L. donovani* infection (4).

The parasite *Trypanosoma cruzi*, which causes Chagas disease, unlike *L. donovani* exploits Wnt signaling for survival as is evident from the diminution of the intensity of *T. cruzi* infection through inhibition of transcriptional activation by β-catenin. *T. cruzi* infection increases expression of several Wnt ligands and Frizzled receptors, including Wnt5A, Wnt3A, Frizzled 4, Frizzled 8, Frizzled 9, and Frizzled 6 (7). Detailed analysis of Wnt-Frizzed signaling pathways in relation to *T. cruzi* infection should unveil the molecular mechanism of intracellular niche building by the parasite and the involvement of cytoskeletal actin therein.

Similar to *L. donovani* infection, and unlike *T. cruzi* infection, fungal infection of macrophages by *Aspergillus fumigatus* is inhibited by Wnt5A signaling. Migration and accumulation of neutrophils caused by Erk1/2 and JNK mediated increase in production of Wnt5A after Dectin1/Lox1 assisted intracellular entry of the fungus have been identified as a cause for the fungal clearance (65). It is important to examine if secreted Wnt5A and actin alterations are directly involved in recruiting neutrophils and activating macrophages for phagocytic clearance of *A. fumigatus*.

Overview of Wnt—Pathogen Interactions

In summary, it appears from the cited examples of different host pathogen interactions that the effect of Wnt signaling on pathogen infection is dependent on the type of the pathogen. **Table 1** summarizes the different interrelations between Wnt signaling and pathogen infections. Although *Pseudomonas* sp. /*Streptococcus* sp., *L. donovani* and *A. fumigatus* are genotypically dissimilar and exercise different modes of action to infect the host (66–68), activation of Wnt5A signaling inhibits initiation of infection by all of these pathogens independent of their genotypic differences perhaps by inducing alterations in actin assembly which are incompatible with pathogen survival. Wnt mediated cytoskeletal alterations may also be crucial for the regulation of infection by *T. cruzi* and *Mycobacterium.*

In light of reports of existing links between cytoskeletal modulation and transcription factor translocation in immune cells (69, 70), it remains to be seen if activation of immune response associated transcription factors such as NFκB, NFAT, or AP1 correlates with cytoskeletal actin alterations during pathogen clearance facilitated by Wnt signaling. Wnt5A signaling, nevertheless, has been described in separate studies to sustain nuclear translocation of NFκB in macrophages both in the steady state and in response to mycobacterial infection, thereby maintaining expression of NFκB responsive immune response genes as a potential means of host resistance (6, 50). The requirement of NFκB mediated gene transcription for immunity to bacterial infections has, furthermore, been independently documented (71–73). Wnt5A signaling can also influence transcriptional regulation of immune response genes such as TNFα, IFNγ, and IL6, which have interrelation with actin dynamics and are known to work toward inhibition of pathogen infection (2, 5, 6, 74–77). It will be interesting to find out how cytokine associated actin dynamics directly links with inhibition of pathogen infection.

Cytoskeletal alterations and transcriptional networks associated with the interaction of pathogens with different degrees and types of Wnt signaling are clearly complex. Careful analyses of further in depth studies with special attention to the different study approaches used by different laboratories are needed in order to establish how the different Wnt molecules work to accommodate or annihilate different pathogens through diverse signaling pathways.

INTERRELATION BETWEEN WNT SIGNALING AND THE HOST MICROBIOTA

Several anatomical locations in the human host serve as home to distinct congregations of bacteria (microbiota) that synchronize

TABLE 1 | Wnt signaling and host-pathogen interaction.

S.no.	Pathogen	Disease	Associated Wnt homolog	Host-pathogen interaction	References
1.	*Pseudomonas* sp., *Streptococcus* sp.	Respiratory diseases (e.g., COPD, sepsis etc.)	Wnt5a, Wnt3A	Wnt5a-RAC1-Disheveled mediated cytoskeletal actin rearrangement facilitates autophagy and containment of infection.	(3)
				Wnt3A mediated increase in antimicrobial peptides causes killing of *Pseudomonas*.	(46)
2.	*Salmonella* sp.	Inflammatory bowel disease (IBD), Typhoid	Wnt11	Wnt11 signaling protects the host from bacterial infection and inhibits apoptosis in intestinal cells.	(43)
3.	*Ehrlichia chaffeensis*	Human monocytotropic ehrlichiosis (HME)	Wnt5a, Wnt10, Wnt6	Wnt ligands and associated signaling pathway (β-catenin mediated, NFAT-C1 mediated and others) promotes survival of the pathogen inside the host.	(8)
4.	*Mycobacterium tuberculosis*	Tuberculosis	Wnt5a, Wnt3A, Wnt6	Mycobacterium infection promotes Wnt5A expression in human PBMC and blockade of Wnt5A signaling results in inhibition of IL-12p40 and IFNγ secretion. Mycobacterium infection downregulates Wnt5A expression in mouse lungs.	(47, 50)
				Enhanced IL36γ secretion during infection induces Wnt5A expression which aids in controlling infection through COX-2 mediated autophagy.	(54)
				Wnt3A promotes an anti-inflammatory effect in murine macrophages during infection in lungs.	(49, 52, 53)
				Mycobacterium infection induces Wnt6 expression and promotes anti-inflammatory phenotype of macrophages through Arginase-1 expression.	(47)
5.	*Leishmania donovani*	Visceral leishmaniasis (Kala azar)	Wnt5a	Wnt5A-Rac1-Rho mediated cytoskeletal alteration promotes enhanced fusion of parasitophorous vacuole with lysosome which helps in restraining infection.	(4)
6.	*Trypanosoma cruzi*	Chagas disease	Wnt3a, Wnt5a	*T. cruzi* early infection increases expression of Wnt5A, 3A, several Frizzled receptors, and Wnt signaling intermediates.	(7)
				Activation of β-catenin promotes inhibition of inflammatory cytokine secretion and replication of parasite.	
7.	*Aspergillus fumigatus*	Fungal keratitis	Wnt5a	Host PRR activates Wnt5A expression through ERK and JNK pathway. Wnt5A attracts neutrophils for clearance of *Aspergillus fumigatus*.	(65)

with host immune programs against infections (78–81). In this context, the microbiota of the gut deserve special mention on account of their prominent prevalence in several niches of the gut especially the Peyer's patches and the *lamina propria* (82). These microbiota exist both in the gut lumen as well as in close proximity with different kinds of macrophages and contribute to immune regulation in the host. For instance, although not clearly understood how, gut microbiota are required for the maintenance of sIgA and a steady state cytokine milieu in the gut lumen, which can potentially serve to fight off pathogens (78, 83, 84). Gut microbiota are also known to secrete antibacterial peptides, which are required for immune defense (10, 85).

Wnt signaling is important for gut organogenesis (86, 87) and sustains intestinal homeostasis through maintenance of specific microflora. Several lines of evidence suggest that colonization of microbiota in specific gut regions correlates with differential expression of Wnts such as Wnt5A and Wnt3A, and Wnt signaling intermediates for example β-catenin (9). Wnt signaling components such as Axin and Disheveled on the other hand, have been shown to act synergistically with Synbindin, a syndecan-2 binding protein, to influence gut microbiota composition (88). How the apparent symbiosis between Wnt signaling and gut microbiota composition relates to immune defense, however remains undocumented. In view of the fact that Wnt signaling, in particular Wnt5A signaling facilitates internalization and destruction of several pathogens through its influence on the cytoskeletal dynamics and autophagy machinery of the host macrophage (3, 4), but coordinates with the resident host microbiota (9), it is quite evident that Wnt signaling is able to differentiate pathogen from non-pathogen. But, how does this happen? Although there is evidence that some microbiota are present within the resident phagocytes (89, 90), it is unclear if the microbial niche is created or promoted by Wnt signaling and how it relates to the cytoskeletal actin network. It also remains to be seen if host resident microbiota influences the phagocytosis and clearance of pathogenic microbes and whether such interrelation involves Wnt signaling.

CONCLUSION AND FUTURE DIRECTIONS

Complex Wnt signaling schemes that are intrinsically associated with macrophage mediated immune functions (e.g., phagocytosis, autophagy/xenophagy) conform to the in-built maneuvering of macrophages as they confront with different kinds of pathogens (1, 91). Several lines of evidence substantiate that Wnt signaling, in particular Wnt5A signaling,

is important for the cytoskeletal modulations and transcriptional programs inherent to macrophages during immune surveillance (3, 4, 6). While many pathogens are disabled through activation of Wnt5A signaling, some pathogens utilize it for their own survival within the host macrophage (3, 4, 7, 8, 65). Moreover, although not known exactly how, colonization of distinct microbiota within the lumen and macrophage associated niches within the gut and other anatomical locations correlate with differential expression of different Wnts and their signaling intermediates (9). The different nuances of Wnt signaling that annihilate some microbes, yet allow the growth and proliferation of others, at the same time sustaining the colonization of diverse microbiota, remain largely uncharacterized. In this regard one may come up with several explanations. In light of the fact that Wnt5A signaling alters actin assembly (3, 4, 30), a vital component of host pathogen interaction, it is possible that Wnt5A induced alterations in actin assembly influence different pathogens differently, depending on the nature of virulence factors, thereby either inhibiting or facilitating their survival. The situation perhaps is guided by the extent of Wnt5A signaling in macrophages, as we observed inhibition of infection by pathogenic microbes through activation of Wnt5A signaling and increased infection through its blockade (3, 4). The decision for pathogen destruction vis. a vis. survival is possibly based on the nature of the tussle between host induced and pathogen induced modulations (conformations) of actin and actin binding proteins in the very early stages of infection wherein host Wnt5A signaling plays a fundamental role. In this connection, knowledge of the status of the macrophage associated resident microbiota, in relation to Wnt signaling and cytoskeletal dynamics is vital.

In future, the influence of Wnt signaling on actin modulations at the initial stages of infection needs to be deciphered at the molecular level in the context of different microbes, ranging from virulent pathogens to the resident microbiota, which may be deemed as non-pathogens. Additionally, the potential links between the cytoskeletal actin dynamics and transcriptional programs need to be carefully assessed. In this connection, a good understanding of the cytokine milieu that correlates with the interaction of Wnt signaling with different microbial infections will be important, especially in view of its connection with the different stages of sepsis associated with pathogenic infections (92). A thorough evaluation of Wnt signaling in the context of microbial pathogenesis and colonization of resident microbiota may lead to the development of new modes of therapeutic interventions for the drug resistant refractory microbial infections.

AUTHOR CONTRIBUTIONS

MS organized the layout of the article and wrote the article. DN contributed to the layout organization and writing. SJ and TS worked on the table and references and assisted in writing.

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Functions of the WNT Signaling Network in Shaping Host Responses to Infection

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It is well-established that aberrant WNT expression and signaling is associated with developmental defects, malignant transformation and carcinogenesis. More recently, WNT ligands have emerged as integral components of host responses to infection but their functions in the context of immune responses are incompletely understood. Roles in the modulation of inflammatory cytokine production, host cell intrinsic innate defense mechanisms, as well as the bridging of innate and adaptive immunity have been described. To what degree WNT responses are defined by the nature of the invading pathogen or are specific for subsets of host cells is currently not well-understood. Here we provide an overview of WNT responses during infection with phylogenetically diverse pathogens and highlight functions of WNT ligands in the host defense against infection. Detailed understanding of how the WNT network orchestrates immune cell functions will not only improve our understanding of the fundamental principles underlying complex immune response, but also help identify therapeutic opportunities or potential risks associated with the pharmacological targeting of the WNT network, as currently pursued for novel therapeutics in cancer and bone disorders.

Keywords: WNT signaling, antigen presenting cells (APCS), infection, inflammation, anti-microbial defense

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THE WNT SIGNALING NETWORK

The WNT signaling network is a central regulator of embryonic development and tissue homeostasis. WNT proteins are phylogenetically highly conserved secreted, cysteine-rich glycolipoproteins (1). Nineteen individual WNT proteins have thus far been described in mammals (2). Best known functions of WNT proteins include regulation of cell cycle, cellular differentiation, cell motility, cellular polarity, and cell death (3). WNT proteins act as directional growth factors that orchestrate patterning, expansion and differentiation of tissues in the organized formation of body plans, and are central regulators of stem and progenitor cell development and maintenance both during embryogenesis and adult homeostasis (4, 5). Dysregulation of WNT signaling is implicated in a multitude of diseases, including cancer, fibrosis, bone density disorders, metabolic and neurodegenerative diseases (6).

WNT proteins are highly hydrophobic due to post-translational modification by palmitoleic and palmitic acid at conserved cysteine residues. This is afforded through action of the acyltransferase Porcupine (PORCN) in the endoplasmic reticulum (**Figure 1**). WNT acylation has been shown to be required for the release, receptor interactions, and functions of WNTs (1). The chaperone Wntless (WLS) facilitates transport of acylated WNT ligands to the plasma membrane and aids in

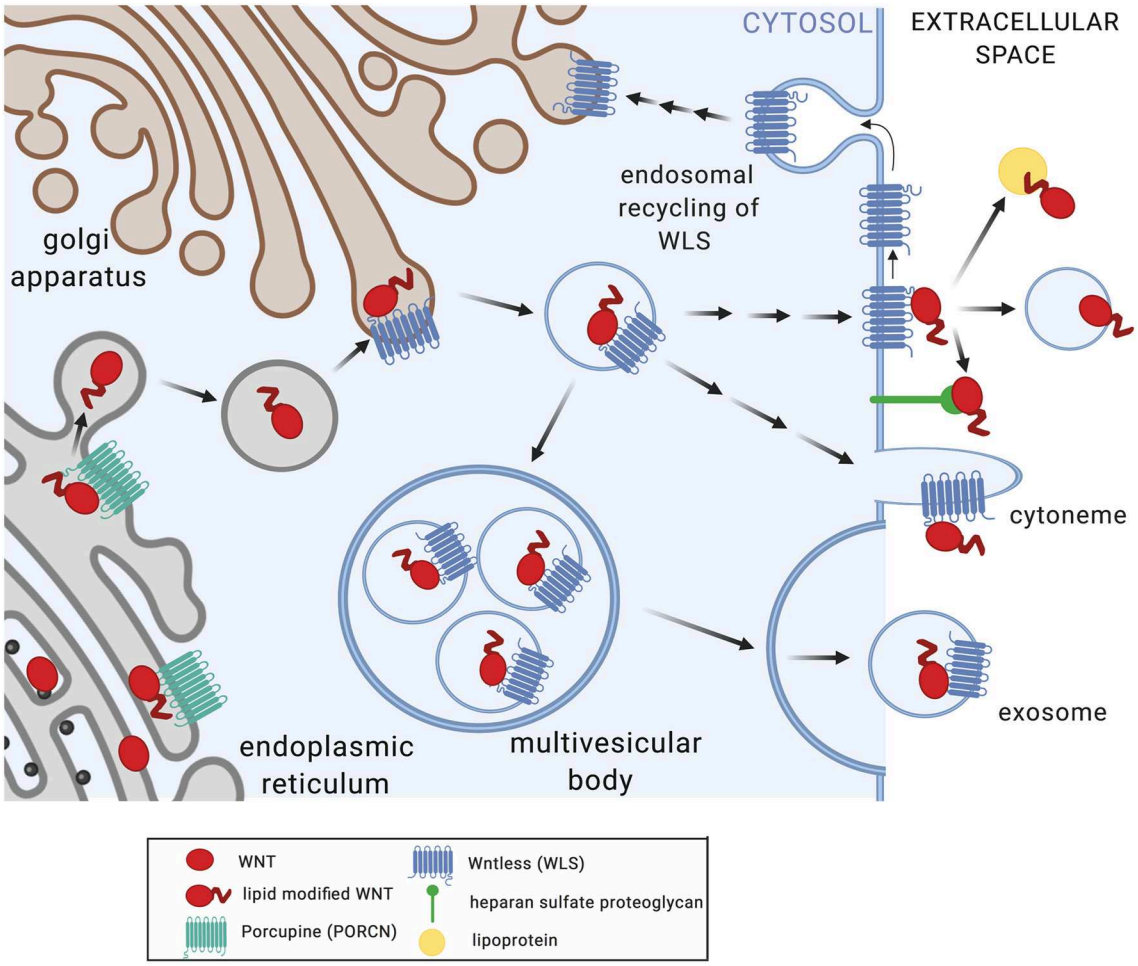

FIGURE 1 | Posttranslational modification and secretion of WNT ligands. Upon translation, WNT proteins undergo acylation in the endoplasmic reticulum by Porcupine (PORCN), a modification required for WNT release (7), receptor interactions (8), and functions (7, 9). Wntless (WLS) facilitates transport of acylated WNT ligands to the plasma membrane and aids in WNT release (10–12). WNT secretion and extracellular transport appears to occur via multiple mechanisms including multi-vesicular bodies and exosomes, cytonemes, lipoproteins, and heparan sulfate proteoglycans (1). WLS protein is recycled via clathrin-mediated endocytosis (13). Figure created with Biorender.com.

WNT release (14). WNT proteins act as morphogens in a concentration-dependent manner through the formation of gradients within tissues. How the hydrophobic WNT ligands act at short distances as well as at longer ranges is incompletely understood. Multiple mechanisms that facilitate WNT transport beyond the boundaries of the producing cell have been described, including chaperones, lipoproteins, exosomes, and cytonemes (1). Macrophages infected by viruses or intracellular bacterial pathogens release exosomes and microvesicles that contain pathogen-derived components alongside host membrane proteins (e.g., MHC-I, MHC-II) and immune mediators (e.g., cytokines) that modulate functions of bystander cells (15–18). Observations of elevated WNT5A protein levels in serum of patients with severe sepsis (19) highlights the possibility that WNT proteins produced in response to infection may act not only locally but also systemically, and thereby shape immune cell differentiation and functions at distant sites.

WNT ligands initiate intracellular signaling by binding to cell surface-expressed WNT receptors and co-receptors, including Frizzled (FZD) 7-transmembrane domain receptors, low-density lipoprotein-related proteins (LRP5, LRP6), as well as receptor tyrosine kinases ROR and RYK (20). Cytoplasmic scaffolding proteins of the disheveled family (DVL) are central to initiating intracellular signaling downstream of FZD receptors (21). The functional outcome of WNT interactions with target cells is decided at the level of receptor engagement. Depending on the receptor context, WNT ligands activate distinct intracellular pathways, which can be grouped into β-catenin-dependent and β-catenin-independent signaling events (**Figure 2**). Individual modalities of β-catenin-dependent and β-catenin-independent WNT signaling have been reviewed in detail elsewhere (3, 5, 20). Briefly, β-catenin-dependent WNT signaling is mediated by cytoplasmic stabilization of β-catenin, which is controlled by the β-catenin destruction complex. The destruction complex

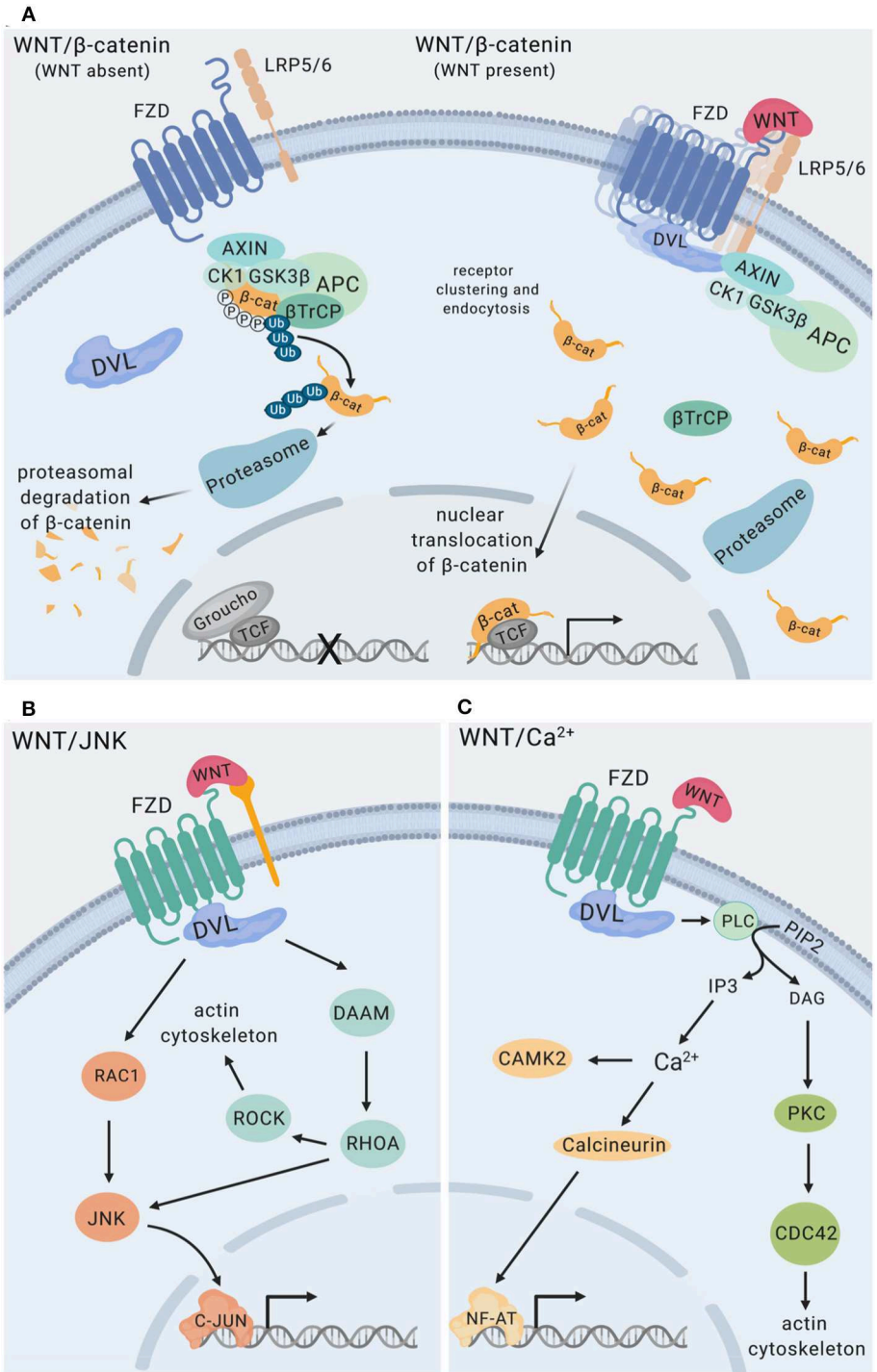

FIGURE 2 | WNT signaling pathways. **(A)** WNT/β-catenin signaling. The destruction complex is comprised of APC, AXIN1, CK1, and GSK3β. Phosphorylation of β-catenin by CK1 and GSK3β within the destruction complex results in β-catenin ubiquitination mediated by β-TrCP resulting in proteasomal degradation of β-catenin (22, 23) The transcriptional repressor Groucho suppresses expression of genes controlled by TCF/LEF transcription factors. Binding of WNT ligands to Frizzled (FZD) receptors and LRP co-receptors promotes recruitment and clustering of DVL, forming signalosomes (21, 24), facilitating recruitment of the destruction complex and stabilization of cytoplasmic β-catenin. Nuclear translocation of β-catenin enables its functions as a transcriptional co-activator for transcription factors of the TCF/LEF family (3). **(B)** WNT/JNK signaling via FZD, alone or in conjunction with co-receptors (e.g., ROR RYK) activates the small GTPases RAC1 and RHOA engaging the actin cytoskeleton, as well as JNK MAP kinase activation (25–27). **(C)** WNT/Ca^{2+} signaling downstream of FZD receptors is mediated by phospholipase C (PLC) activation leading to enhanced levels of cytosolic Ca^{2+}, resulting in calmodulin/calmodulin-dependent kinase II activation and NF-AT-regulated transcriptional responses (28), and engagement of the actin cytoskeleton. RYK has been implicated as a co-receptor for WNT/Ca^{2+} signaling. Figure created with Biorender.com.

is comprised of scaffolding proteins adenomatous polyposis coli (APC), axis inhibition protein (Axin), and the kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). In the absence of WNT ligand binding to FZD and LRP co-receptors, phosphorylation of β-catenin by CK1 and GSK3β within the destruction complex results in β-catenin ubiquitination by beta-transducin repeat-containing E3 ubiquitin protein ligase (βTrCP), fueling continuous degradation of β-catenin by the proteasome (**Figure 2A**). Binding of WNT ligands to FZD/LRP results in recruitment of DVL and the destruction complex, inhibiting GSK3β and CK1 activity and stabilization of cytoplasmic β-catenin. This enables nuclear translocation of β-catenin where it functions as transcriptional co-activator for transcription factors of the TCF/LEF family (**Figure 2A**). WNT/JNK- [described as planar cell polarity (PCP) pathway in *Drosophila*] and WNT/Ca^{2+}-signaling are modes of β-catenin-independent WNT signaling. WNT/JNK signaling results in FZD/DVL-mediated activation of the small GTPases RAC1 and RHOA, directing cytoskeletal rearrangements, cell polarization and motility. Activation of JNK can drive c-Jun- and AP-1-controlled transcription (**Figure 2B**). WNT/Ca^{2+} signaling downstream of FZD receptors and DVL leads to phospholipase C (PLC) activation and enhanced levels of cytosolic Ca^{2+}, which activates calmodulin/calmodulin-dependent kinase II and NFAT-regulated transcriptional responses (**Figure 2C**).

Tight regulation and precise targeting of WNT signaling is essential, as emphasized by the evolutionary investment in multiple layers and modes of WNT pathway modulation. WNT signaling is negatively regulated by secreted Frizzled-related proteins (sFRP) and WNT inhibitory factor 1 (WIF-1), which directly bind WNT proteins interfering with receptor interactions (3). The palmitoleoyl-protein carboxylesterase Notum was shown to facilitate serine de-palmitoleoylation of WNT ligands, thereby negatively regulating WNT functions (29). Members of the Dickkopf (DKK) and Sclerostin/SOST families, as well as the glycoprotein Dorsal Inhibitory Axon Guidance Protein (DRAXIN) interact with LRP5/6 and interfere with WNT binding (30–32). FZD receptor surface availability is regulated through the E3 ubiquitin ligases, Zinc and Ring Finger 3 (ZNRF3) and Ring Finger protein 43 (RFN43), which ubiquitinate FZD receptors destining them for proteasomal degradation (33). ZNFR3 and RFN43 serve as negative feedback regulators for WNT signaling, as they themselves are encoded by WNT target genes (5).

WNT RESPONSES TO INFECTION

Early studies identified *WNT5A* as a highly responsive gene in human macrophages upon microbial encounter (19, 34). *WNT5A* has also been found to be highly expressed by tumor-associated macrophages (35), synoviocytes in rheumatoid arthritis (36), macrophages in atherosclerotic plaques (37), and adipose tissue-resident macrophages in obesity (38). This has directed initial attention toward elucidating immune functions of WNT5A. However, it is increasingly evident that the host response to infection encompasses differential expression of multiple WNT ligands, receptors and regulators (39–43). Thus, detailed understanding of how the concerted actions of WNT ligands and potentially concurrent WNT signaling events define host responses to infection is key to firmly establishing immune functions of the WNT signaling network.

Bacterial Infections

Gram-Negative Bacteria

WNT responses to infection have been studies in the context of experimental infection with a limited number of Gram-negative bacterial pathogens (**Table 1**). WNT pathway activation and functions in the context of *Salmonella* infection have largely been focused on in a model of gastroenteritis in antibiotic-pretreated mice, as well as in epithelial cell lines *in vitro*. *Salmonella (S.) enterica* serovar Typhimurium infection of streptomycin-pretreated mice increased mRNA expression of *Wnt3*, *Wnt6*, *Wnt9a*, and protein expression of Wnt2 and Wnt11 in intestinal tissues (43, 50, 56). *In vitro* studies indicated that colonization of murine intestinal epithelial cells with *S.* Typhimurium induced elevated mRNA expression of *Wnt2* and *Wnt11* (also confirmed at protein level), *Fzd2, Fzd4, Fzd6, Fzd7, Fzd8, Fzd9*, with limited or no effects on the expression of other *Wnt* and *Fzd* genes (50, 56). Induction of Wnt2 and Wnt11 expression was attributed at least in part to *Salmonella* AvrA (50, 56), a bacterial effector that has been implicated in the regulation of β-catenin ubiquitination and stabilization (64–67). With an increasing understanding of the complex WNT response in *Salmonella* infection, future studies should explore WNT network activation in macrophages, innate immune cells that are important in the host control of *Salmonella* infection. Thus far, it has been noted that *Wnt5a* and *Fzd4* expression in *S.* Typhimurium-infected murine peritoneal macrophages was modestly increased, albeit the impact on the expression of other WNT signaling components was not explored in this study (44).

Ehrlichia (E.) chaffeensis infection of human THP-1 macrophage-like cells transiently increased mRNA expression of *WNT6*, *WNT10A, FZD5*, and *FZD9,* while decreasing expression of *WNT5B, WNT7B,* and *FZD7,* as determined by pathway-specific qPCR arrays (42). Expression of WNT regulators such as *DKK3* and *sFRP2* was suppressed or enhanced, respectively, and a significant number of WNT-target genes were differentially expressed (42).

WNT responses upon encounter of pathogenic and non-pathogenic *Escherichia (E.) coli* have been investigated to some extent in mouse models *in vivo*. Mono-colonization of mice with *E. coli* F18 enhanced expression of *Wnt2* in the intestine compared to germ-free mice (50). Bladder infection with uropathogenic *E. coli* (UPEC) induced rapid downregulation of *Wnt5a* expression in the urothelium of infected mice, which was partially attributed to the bacterial virulence and adhesion factor, FimH (58). This observation seems to contrast a small increase of *WNT5A* expression described in a human urothelial cell line infected with UPEC *in vitro* (59). Yet, exposure of mouse thioglycolate-elicited peritoneal macrophages exhibited a marked decrease in *Wnt5a* mRNA expression when exposed to a non-pathogenic *E. coli* strain, while expression of all other WNT ligands remained unaltered at the time point

TABLE 1 | Bacteria-induced WNT responses in experimental systems and patient samples.

	***Mycobacterium* sp.**	***S. aureus***	***S. pneumoniae***	***E. chaffeensis***	***E. coli***	***P. aeruginosa***	***S.* Typhimurium**	**LPS**
WNT1	↑ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	↓ mRNA *mom-2 C. elegans* IEC (45) unaffected mRNA mPM (44) unaffected *L. vannamei* HP, LO, HT (46)	–	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	↓protein HCT116 (47)	↑ protein THP-1 (48) ↑ mRNA mBMDM (40) ↓ mRNA fetal ovine lungs (49) n.d. mRNA murine spleen (39)
WNT2	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	↑ mRNA *cwn-2 C. elegans* IEC (45) unaffected mRNA mPM (44) unaffected *L. vannamei* HP, LO, HT (46)	–	n.d. mRNA THP-1 (42)	↑ mRNA and protein CMT93 (50) unaffected mRNA mPM (44)	–	↑ mRNA and protein IEC-18 (50)	n.d. mRNA murine spleen (39) ↓ mRNA fetal ovine lungs (49)
WNT2B	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44)	↓ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	unaffected mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	unaffected mRNA IEC-18 (50)	↑ mRNA mBMDM (40) n.d. mRNA murine spleen (39)
WNT3	unaffected mRNA *Mtb* murine lung tissue (40)	–	↓ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	unaffected mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	↑ mRNA mIEC (43)	↑ mRNA hMDM (52) unaffected mRNA murine spleen (39)
WNT3A	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44)	–	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	unaffected mRNA IEC-18 (50)	n.d. mRNA mBMDM (40) ↑ mRNA BEAS-2B (53) n.d. mRNA murine spleen (39)
WNT4	↓ mRNA *Mtb* murine lung tissue (40) ↓ mRNA *M. marinum* infected zebrafish (54) unaffected mRNA *M. bovis* BCG mPM (44)	↑ mRNA *D. melanogaster* (55) unaffected mRNA mPM (44) unaffected *L. vannamei* HP, LO, HT (46)	↑ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	unaffected mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	unaffected mRNA IEC-18 (56)	↓ mRNA fetal ovine lungs (49) unaffected mRNA murine spleen (39)
WNT5A	↑ mRNA *Mtb* hMDM (34) ↓ mRNA *Mtb* murine lung tissue (40) ↑ mRNA *Mtb* hPBMC and *M. bovis* (BCG) mPM (44) ↑ mRNA *M. marinum* zebrafish (54) WNT5A expressing macrophages in human tuberculosis granulomas (34)	↑ mRNA mPM (44) ↑ mRNA (*LvWnt5*) *L. vannamei* HP, LO, HT (46)	↓ protein RAW264.7 (57)	n.d. mRNA THP-1 (42)	↓ mRNA mPM (44) ↓ mRNA murine urothelium (58) ↑ mRNA human urothelium (59)	↓ protein RAW264.7 (57)	↑ mRNA mPM (44) unaffected mRNA IEC-18 (56)	↑ mRNA hMDM (34) ↑ mRNA BEAS-2B (53) ↑ mRNA hPBMC and hBMDM during sepsis (19) ↑ mRNA THP-1 (60) ↑ mRNA primary human monocytes (61) ↑ mRNA RAW264.7 (37) unaffected mRNA murine spleen (39) unaffected mBMDM (40)

(Continued)

TABLE 1 | Continued

	Mycobacterium sp.	*S. aureus*	*S. pneumoniae*	*E. chaffeensis*	*E. coli*	*P. aeruginosa*	*S.* Typhimurium	LPS
WNT5B	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) ↑ mRNA (*LvWnt5*) *L. vannamei* HP, LO, HT (46)	↑ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	↓ mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	unaffected mRNA IEC-18 (56)	↑ mRNA mBMDM (40) ↑ mRNA murine spleen (39)
WNT6	↑ mRNA *Mtb* mBMDM (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) ↑ mRNA *L. vannamei* HP, unaffected LO, HT (46)	↓ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	↑ mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	↑ mRNA mIEC (43)	↓ mRNA murine spleen (39) ↑ mRNA mBMDM (40)
WNT7A	↓ *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA (*LvWnt7*) *L. vannamei* HP, LO, HT (46)	↑ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	n.d. mRNA mBMDM (40) n.d. mRNA murine spleen (39)
WNT7B	unaffected mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA (*LvWnt7*) *L. vannamei* HP, LO, HT (46)	↑ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	↓ mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	n.d. mRNA mBMDM (40) ↑ mRNA fetal ovine lungs (49) n.d. mRNA murine spleen (39)
WNT8A	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA (*LvWnt8*) *L. vannamei* HP, LO, HT (46)	–	unaffected THP-1 (42)	unaffected mRNA mPM (44)	–	–	n.d. mRNA mBMDM (40) n.d. mRNA murine spleen (39)
WNT8B	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA (*LvWnt8*) *L. vannamei* HP, LO, HT (46)	–	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	n.d. mRNA murine spleen (39)
WNT9A	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) ↑ mRNA (*LvWnt9*) *L. vannamei* lymphoid organ (46) unaffected mRNA (*LvWnt9*) *L. vannamei* HP, LO, HT (46)	↓ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	unaffected mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	↑ mRNA mIEC (43)	n.d. mRNA murine spleen (39)
WNT9B	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) ↑ mRNA (*LvWnt9*) *L. vannamei* lymphoid organ (46) unaffected mRNA (*LvWnt9*) *L. vannamei* HP, LO, HT (46)	↓ mRNA murine *S. pneumoniae* Δpep27 lung tissue (51)	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	n.d. mRNA murine spleen (39)

(Continued)

TABLE 1 | Continued

	***Mycobacterium* sp.**	***S. aureus***	***S. pneumoniae***	***E. chaffeensis***	***E. coli***	***P. aeruginosa***	***S.* Typhimurium**	**LPS**
WNT10A	↑ mRNA *Mtb* murine lung tissue (40) ↓ mRNA *M. marinum* zebrafish (54)	unaffected mRNA (*LvWnt10*) *L. vannamei* HP, LO, HT (46)	–	↑ mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	↑ mRNA murine spleen (39) unaffected mRNA mBMDM (40)
WNT10B	↓ mRNA *Mtb* murine lung tissue (40) unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA (*LvWnt10*) *L. vannamei* HP, LO, HT (46)	–	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	unaffected mRNA IEC-18 (50)	↑ mRNA murine spleen (39) ↑ mRNA mBMDM (40)
WNT11	unaffected mRNA *M. bovis* BCG mPM (44)	unaffected mRNA mPM (44) unaffected mRNA *L. vannamei* HP, LO, HT (46)	–	unaffected mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	↑ mRNA and protein IEC-18 (56)	unaffected mBMDM (40) ↑ mRNA murine spleen (39)
WNT16	↓ mRNA *Mtb* murine lung tissue (40) ↓ mRNA *M. marinum* zebrafish (54)	unaffected mRNA *L. vannamei* HP, LO, HT (46)	–	n.d. mRNA THP-1 (42)	unaffected mRNA mPM (44)	–	–	unaffected mRNA murine spleen (39)
Effects not specific to individual WNT proteins	↑ *Fzd1 Mtb* murine lung tissue and mBMDM (41) ↑ *Fzd4*, *Lrp5,* β-catenin stabilization through GSK3 phosphorylation in *M. bovis* BCG mPM (44) ↓ *Axin2, Dvl3, Fzd8,9,10* ↑ *Dvl2 M. marinum* zebrafish (54)	↓ *mom-5*, *mig-1* (FZD homologues) *C. elegans* IEC (45) ↑ *Fzd4* m. PM (44) ↑ mRNA *LvWntA L. vannamei* HP, LO (46)	↓ β-catenin activity murine lung tissue (62)	↓ *FZD7* ↑ *FZD5, 9* ↓ *DKK3* ↑ *sFRP2* THP-1 (42)	-	↓ *Ctnnb1* RAW264.7 (63)	β-catenin degradation IEC-18 (64) ↑ *Fzd4, 6, 9, Fzd3, 10* unaffected IEC-18 (50) ↑ *FZD2, 7, 8 FZD5* unaffected HCT116 (56) ↑ *Fzd4* mPM (44)	↑ *Fzd1, 5, Wisp1,* β-*catenin* ↓ *Fzd7, 8 Fzd3, 6, 9,* and *10* unaffected murine spleen (39) ↓ *Ctnnb1* fetal ovine lungs (49) ↑ LRP6 phosphorylation BEAS-2B (53) ↑ *DVL3* hMDM (34)

mRNA or protein expression of WNT ligands and signaling components in response to infection or LPS exposure. ↑, upregulation; ↓, downregulation; –, indicative of no data; n.d., not detectable; protein, protein expression; mRNA, mRNA expression; m, murine; h, human; PM, peritoneal macrophages; BMDM, bone marrow-derived macrophages; IEC, intestinal epithelial cells; MDM, monocyte-derived macrophages; PBMC, peripheral blood mononuclear cells; SMC, spleen mononuclear cells; HP, hepatopancreas; LO, lymphoid organs; HT, hemocytes; Mtb, Mycobacterium tuberculosis.

analyzed (44). Decreased Wnt5a protein and decreased *Ctnnb1* mRNA expression in the mouse macrophage cell line RAW264.7 have also been reported upon infection with *Pseudomonas (P.) aeruginosa* (57) (63).

While several studies reported WNT5A expression to be responsive to macrophage encounter with Gram-negative bacterial pathogens, it remains to be defined whether opposing directions of the regulation of *WNT5A* expression reflect pathogen-specific responses, cell-type-dependent variations, or species-specific differences between humans and mice. Some indication that the latter apect might indeed be of importance comes from studies of macrophages stimulated with lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. Increased *WNT5A* expression has been noted in LPS-stimulated human monocytes, the human monocytic THP-1 cell line, as well as human bronchial epithelial BEAS-2B cells (19, 34, 53, 60, 61). LPS-induced *WNT5A* expression in human macrophages occured downstream of Toll-like receptor signaling and nuclear factor kappa B (NF-κB) activation, and was amplified by inflammatory cytokines such as tumor necrosis factor (TNF) and interferon γ (IFNγ) (19, 34, 60). In contrast, LPS stimulation did not significantly increase the relatively low *Wnt5a* mRNA expression of mouse bone marrow-derived macrophages (40), yet LPS stimulation did enhance *Wnt5a* mRNA expression by mouse RAW264.7 macrophages (37). Nevertheless, the amplitude of the *WNT5A* response might also be governed by the nature of the invading bacterial pathogen as suggested by observations that *Porphyromonas gingivalis* LPS induced *WNT5A* mRNA expression in THP-1 cells to a greater extent compared to *E. coli*-derived LPS (60).

Importantly, however, the LPS-induced WNT response encompasses more than WNT5A. LPS stimulation elevated expression of *WNT3* in human monocyte-derived macrophages (52), and *WNT1* in differentiated human THP-1 cells and murine bone marrow-derived macrophages (40, 48). The latter cells also displayed increased expression of *Wnt2b*, *Wnt5b*, *Wnt6*, and *Wnt10b* upon LPS stimulation, whereas expression of *Wnt5a, Wnt10a,* and *Wnt11* remained unchanged, and expression of the remaining *Wnt* genes was below the detection limit (40). Systemic challenge of mice with a sub-lethal dose of LPS *in vivo* induced rapid elevation of *Wnt5b, Wnt10a, Wnt10b, Wnt11, Fzd1,* and *Fzd5* mRNA expression in spleen tissue, accompanied by elevated expression of the WNT target gene *Wisp1*. In contrast, expression of *Wnt6*, *Fzd7,* and *Fzd8* was decreased, whereas *Wnt3, Wnt4, Wnt5a, Wnt16, Fzd3, Fzd6, Fzd9,* and *Fzd10* were not differentially expressed (39). In an ovine model of fetal intra-amniotic LPS exposure, elevated expression of *Wnt7b*, and reduced mRNA expression of *Wnt1, Wnt2, Wnt4,* and *Ctnnb1* were observed in fetal lung tissue (49).

With patterns beginning to emerge in the WNT response to Gram-negative bacteria (e.g., WNT5A expression by macrophages), more detailed insight is required to delineate the impact of cell type-, species-, and pathogen-specific contributions to the amplitude and composition of infection-associated WNT responses.

Gram-Positive Bacteria

WNT responses upon infection with Gram-positive bacteria are just beginning to be explored (**Table 1**). *Staphylococcus (S.) aureus* infection of *Drosophila melanogaster* led to enhanced expression of *Wnt4* (55). Expression of other WNT ligands was not determined in this study, leaving it to be determined how *S. aureus*, and other pathogens, affect WNT expression in *Drosophila*. *S. aureus* infection of *Caenorhabditis elegans* induced elevated expression of the WNT2 homolog *cwn-2*, and suppressed expression of the WNT1 homolog *mom-2* and the FZD homolog *mom-5* (45). A comprehensive analysis of WNT expression in the Pacific white shrimp *Litopenaeus vannamei* revealed pronounced upregulation of the mRNA expression of multiple WNT ligands, including *LvWnt5, LvWnt6, LvWnt9,* and *LvWntA* in different organs upon *S. aureus* infection (46). Expression of *Wnt5a* and *Fzd4* by murine macrophages marginally increased upon infection with *S. aureus* (44). In contrast, infection of RAW264.7 mouse macrophages with *Streptococcus (S). pneumoniae* has been reported to suppress Wnt5a protein expression (57). Sequencing analyses of lung tissue of mice vaccinated intranasally with *S. pneumoniae* deficient for the autolysis-inducing factor pep27 revealed enhanced expression of *Wnt4*, *Wnt5b, Wnt7a,* and *Wnt7b*, and impaired *Wnt2b, Wnt3, Wnt6, Wnt9a,* and *Wnt9b* mRNA expression (51). Kinase activity profiling in mouse lung tissue of *S. pneumoniae*-infected mice indicated a reduction in β-catenin-stabilizing signals associated with a decrease in β-catenin protein expression (62). Thus, due to the paucity of information it is currently largely unknown if host cell encounter with pathogenic Gram-positive bacteria directly modulates WNT responses and signaling capabilities.

Mycobacteria

Mycobacterial infections induce significant alterations in the expression of WNT signaling components in infected tissues of a variety of host organisms (**Table 1**). Macrophage-associated *WNT5A* expression was initially described in tuberculosis lung granulomas (34), and *WNT5A* and *FZD4* mRNA expression was significantly elevated in peripheral blood mononuclear cells of tuberculosis patients (44). *Mycobacterium (M.) tuberculosis* infection of C57BL/6 mice enhanced lung mRNA expression of *Wnt1, Wnt6, Wnt10a, Fzd1,* and *Fzd5*, while reducing expression of *Wnt2, Wnt2b, Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt8a, Wnt10b*, as well as *Fzd3, Fzd7, Fzd8, Fzd9*, and *Fzd10* (40, 41). *M. marinum* infection of zebrafish enhanced expression of *wnt5a*, yet suppressed expression of multiple other WNT ligands, receptors and WNT pathway regulators (54). Regulation into opposing directions was noted for some WNT network components, depending on the virulence of the infecting *M. marinum* strain (54). Macrophages are major host cells for mycobacteria and have been identified as a significant source of WNT expression during mycobacterial infection. *In vitro* studies showed that infection of monocytes and macrophages of human and mouse origin with mycobacteria across a virulence spectrum (*M. tuberculosis*, *M. avium, M. bovis* Bacillus Calmette-Guérin) greatly enhanced expression of *WNT5A* (34, 44). Importantly, expression and induction of *WNT5A* in human macrophages

was more pronounced compared to mouse cells. In *M. tuberculosis*-infected mice, expression of Wnt6 was localized to macrophages in lung granulomas, and *Wnt6* mRNA expression was significantly elevated in murine bone marrow derived macrophages infected with *M. tuberculosis* or *M. avium* (40). Taken together, the experimental evidence to date suggests that upregulation of WNT5A by mycobacteria-infected macrophages may be evolutionarily conserved between humans, mice and possibly other species. Nevertheless, expression of other WNT ligands by infected macrophages remains to be explored more systematically across species. Moreover, WNT/WNT receptor expression in infected tissues requires cellular context for more detailed understanding of where WNT responses occur upon encounter of pathogenic mycobacteria.

Toward Defining Patterns in the Host WNT Response to Bacterial Infections

A WNT response consistently reported for human, and to some extent murine, macrophages to diverse microbial challenges appears to be regulation of *WNT5A* expression. Yet, as it becomes clear that host WNT responses to bacterial infection reach well beyond differential expression of WNT5A, it will be essential to delineate whether patterns of WNT pathway activity are stereotypical responses of distinct host cell types and tissues to microbial insult, and/or how these responses are defined by the nature of the invading pathogen. With increasing insights into WNT responses to infection arises the need to understand WNT responses in human disease. Studies in patients with severe sepsis and septic shock highlight the complex nature of the host WNT response to microbial insult. Comparisons of blood gene expression patterns in patients with septic shock compared to healthy controls, revealed elevated expression of *WNT5B* and *WNT11*, whereas the expression of *WNT1, WNT2B, WNT3, WNT6, WNT7A, WNT9A, WNT10A, WNT10B*, and *WNT16* was significantly reduced (39). Patients with severe sepsis had elevated WNT5A serum levels, and patients with sepsis-associated acute respiratory distress syndrome displayed elevated WNT5A protein expression in lung tissue (19, 68, 69). An increase of WNT5A protein serum concentrations appeared to correlate with disease progression, whereas a decrease was associated with recovery in critically ill sepsis patients (68). However, *WNT5A* mRNA expression in whole blood was very low and not significantly different between healthy controls and septic shock patients, whereas alterations in the expression of other WNT ligands was more readily detectable (39). Whether dynamic changes in the expression of WNT pathway components accompanying severe acute infections can be exploited for the development of easily assessible biomarkers remains to be determined. Signatures that might enable patient stratification or rapidly identify classes of causative bacteria are worth exploring.

Protozoal and Fungal Infections

WNT responses to infections with protozoa and fungi are less well-explored (**Table 2**). In mice intraperitoneally inoculated with the protozoan parasite *Trypanosoma (T.) cruzi*, protein expression of Wnt3a, Wnt5a, and β-catenin in splenic mononuclear cells increased with disease progression (74). Similar patterns were observed for Wnt3a and Wnt5a mRNA and protein expression in murine bone marrow-derived macrophages (BMDMs) (74). *In vitro* experiments indicated enhanced expression of *Wnt3a* and *Wnt5a*, *Fzd4*, *Fzd6*, *Fzd8,* and *Fzd9* upon *T. cruzi* infection of murine BMDMs. In contrast, *Leishmania donovani* infection of mouse RAW264.7 macrophages resulted in diminished expression of *Wnt5a*, whereas other WNT ligands and sigaling components were not assessed (80). In human corneas infected with the fungus *Aspergillus (A.) fumigatus*, *WNT5A* expression was found to be significantly higher than in uninfected corneal tissues. WNT5A mRNA and protein expression were also enhanced by *A. fumigatus* infection of human THP-1 macrophages (78). Murine peritoneal macrophages infected with *Candida albicans*, *A. fumigatus,* or *A. flavus* or stimulated with the fungal and bacterial cell wall component Curdlan displayed elevated Wnt5a expression (79). More comprehensive profiling of the WNT network will be required to assess the quality of WNT responses by protozoal and fungal infections and determine to what extent WNT expression and signaling are defined by the host cell vs. the nature of the encountered pathogen.

Viral Infections

WNT responses to viral infections have been studied in the context of a limited number of viral infections (**Table 2**). HIV infection elevated *WNT2B* and *WNT10B* expression by human primary astrocytes (71), whereas expression of *WNT1, WNT3, WNT5B, WNT9A, WNT9B,* and *WNT16* remained unaffected, and *WNT2, WNT3A, WNT4, WNT5A, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT10A,* and *WNT11* expression was below the detection limit of the assay (71). HIV infection of mouse neuronal cells of the spinal dorsal horn elevated *Wnt5a* mRNA expression (77). *WNT5A* expression was also upregulated in Epstein Barr virus (EBV)- infected nasopharyngeal carcinoma epithelial cells (75). Influenza A infection of mice resulted in impaired expression of *Wnt2, Wnt3a, Wnt10b, Fzd2, Lrp4,* and *Tcf3* in infected lung tissues (72). Human cytomegalovirus (HCMV) infection of human foreskin fibroblasts was associated with *WNT5A* and *WNT5B* downregulation (76), whereas HCMV infection elevated *WNT2* expression in human mesenchymal stem cells (73). HCMV infection of dermal fibroblasts, placental extravillous trophoblasts, and foreskin fibroblasts was associated with degradation of β-catenin (83). In contrast, β-catenin stabilization was observed in human B cells infected with EBV (86), vaccinia virus-infected HEK293T cells (87), hepatitis B virus-infected Huh7 cells (84), and hepatitis C virus-infected HEK293T cells (85). These reports indicate responsiveness of the WNT signaling network to viral infections. Modulation of β-catenin stabilization might be indicative of viral exploitation of host cell replication and apoptosis. Yet, the WNT responses associated with viral infection noted thus far show no discernible patterns, likely due to the paucity of comprehensive analyses. Systematic comparisons of host cells and different viral classes are required to assess whether there are WNT network signatures that are indicative of a viral infection.

WNT FUNCTIONS IN THE HOST RESPONSE TO INFECTION

The realization that the WNT network is responsive to infections has driven significant interest in delineating its roles in host defense and immune responses. There is increasing evidence that WNT ligands (and other ligands for WNT receptors) contribute to the host control of phylogenetically diverse pathogens in non-vertebrates and vertebrates (57, 74, 80, 88, 89). Some associations between polymorphisms in WNT network genes, and susceptibility and quality of the immune response to infection have been suggested (90–93). Professional antigen-presenting cells (APCs) such as macrophages and dendritic cells have been studied intensively as sources and targets of WNT ligands (19, 40, 44, 94, 95). Roles for WNT ligands in orchestrating phagocytosis, antimicrobial defense and inflammatory cytokine responses have been indicated (**Figure 3**, **Table 3**) (48, 98, 100). WNT ligands have also been implicated in the cellular differentiation and functional polarization of APCs and T cells, bridging of innate and adaptive immune responses (34, 94), and shaping lymphocyte functions (107–112).

Considerations for Experimentation

Experimental approaches to deciphering WNT ligand-driven immune functions include utilization of mouse models with genetic deletion of individual WNT ligands or receptors. Use of cell-specific deletion (95, 113) or heterozygous mice (40, 57) is often indicated due to the deleterious impact of global deletion of individual WNT ligands on embryonic development. SiRNA-mediated knock-down of endogenous WNT components (104, 114), interference with WNT/WNT receptor interactions using neutralizing antibodies and recombinant WNT regulators (e.g., sFRPs, DKK) (34, 115), as well as plasmid-based overexpression of WNT ligands, receptors and regulators (63, 116) are commonly utilized, in particular in *in vitro* cell-based studies. Conditioned media from WNT-overexpressing cells and recombinant WNT proteins have also been proven as valuable tools for deciphering WNT functions. Of note, some biological responses of innate immune cells observed upon exposure to recombinant WNT protein preparations have been attributed to Toll-like receptor activation, rather than known WNT receptors (61, 117). The biological importance of this requires further clarification.

As it becomes increasingly evident that multiple WNT ligands are differentially expressed in response to microbial insults, and that WNT ligands are likely to arise from different cellular sources during infection, strategies that broadly target the WNT response as opposed to individual WNT ligands are increasingly employed. Cell-targeted conditional deletion of WLS and PORCN in mouse models, and the use of small molecule

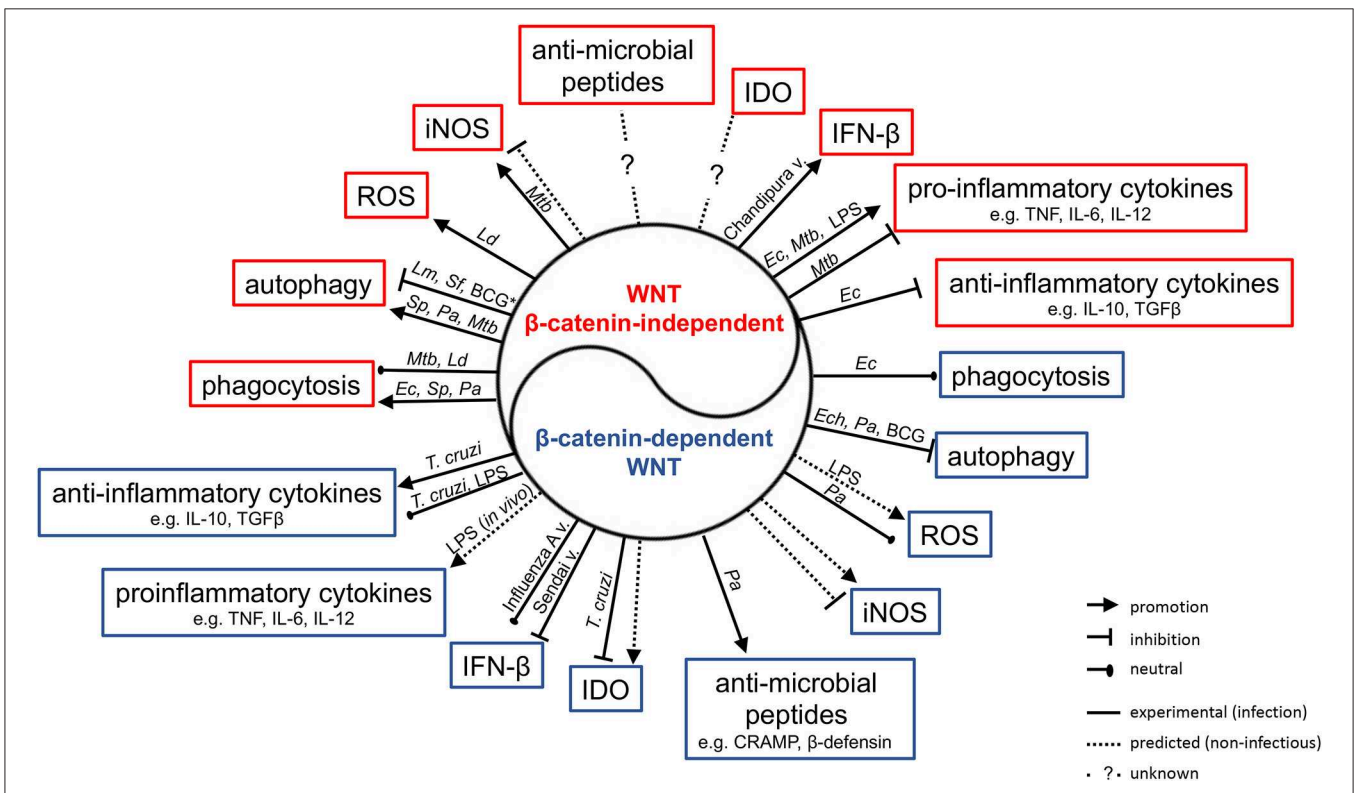

FIGURE 3 | Impact of WNT ligands that activate β-catenin-independent vs. β-catenin-dependent signaling on the functions of macrophages and myeloid DCs in the context of infection with phylogenetically diverse pathogens. *Mtb, Mycobacterium tuberculosis; Ld, Leishmania donovani; Ec, Escherichia coli; Sp, Streptococcus pneumonia; Pa, Pseudomonas aeruginosa; Ech, Ehrlichia chaffeensis; Lm, Listeria monocytogenes; Sf, Shigella flexneri; T. cruzi, Trypanosoma cruzi*; v., virus; *Autophagy in IFNγ-activated macrophages.

TABLE 2 | WNT responses induced by viral, fungal, and protozoal infection in experimental systems.

	Viruses	Fungi	Protozoa
WNT1	↑ mRNA HepC Huh7 (70) unaffected mRNA HIV hPdA (71)	–	–
WNT2	↓ mRNA IVA murine lungs (72) ↑ mRNA HCMV hMSC (73) n.d. mRNA HIV hPdA (71)	–	–
WNT2B	↑ mRNA HIV hPdA (71)	–	–
WNT3	unaffected mRNA HIV hPdA (71)	–	–
WNT3A	↓ mRNA IVA murine lungs (72) n.d mRNA HIV hPdA (71)	–	↑ protein *T. cruzi* mSMC and mBMDM (74) ↑ mRNA *T. cruzi* mBMDM (74)
WNT4	n.d. mRNA HIV hPdA (71)	–	-
WNT5A	↑ mRNA EBV hNC (75) ↓ mRNA HCMV hFF (76) ↑ mRNA HIV mSDH (77) n.d mRNA HIV hPdA (71)	↑ mRNA, protein *A. fumigatus* THP-1 and human corneas (78) ↑ mRNA *C. albicans*, *A. flavus*, *A. fumigatus,* Curdlan mPM (79)	↓ mRNA *L. donovani* RAW264.7 (80) ↑ protein *T. cruzi* mSMC, mBMDM (74) ↑ mRNA *T. cruzi* mBMDM (74)
WNT5B	↓ mRNA HCMV hFF (76) unaffected mRNA HIV hPdA (71)	–	–
WNT6	n.d mRNA HIV hPdA (71)	–	–
WNT7A	n.d mRNA HIV hPdA (71)	–	–
WNT7B	n.d mRNA HIV hPdA (71)	–	–
WNT8A	n.d mRNA HIV hPdA (71)	–	–
WNT8B	n.d mRNA HIV hPdA (71)	–	–
WNT9A	unaffected mRNA HIV hPdA (71)	–	–
WNT9B	unaffected mRNA HIV hPdA (71)	–	–
WNT10A	n.d mRNA HIV hPdA (71)	–	–
WNT10B	↓ mRNA IVA murine lungs (72) ↑ mRNA HIV hPdA (71)	–	–
WNT11	n.d mRNA HIV hPdA (71)	–	–
WNT16	↑ mRNA HPV18 HaCat (81) unaffected mRNA HIV hPdA (71)	–	–
Effects not specific to individual WNT proteins	↓*Fzd2*, *Lrp4, Tcf-3* mRNA IVA murine lungs (72) ↑ WNT/β-catenin signaling Rift Valley fever virus HEK293T (82) ↑ β-catenin degradation HCMV hDF and hPET (83) ↑ β-catenin protein HepB Huh7 (84) β-catenin stabilization HepC HEK293T (85), EBV hB-cells (86), Vaccinia virus HEK293T (87) ↓ protein β-catenin, no effect on DVL2,3 HCMV hFF (76)	–	↑ β-catenin protein *T. cruzi* mSMC and mBMDM (74) ↑ *Fzd4, 6, 8, 9* mRNA *T. cruzi* mBMDM (74)

mRNA and protein expression of WNT signaling molecules in response to infection. ↑, upregulation; ↓, downregulation; –, indicative of no data; n.d., not detectable; protein, protein expression; mRNA, mRNA expression; m, murine; h, human; MSC, mesenchymal stem cells; PdA, primary-derived astrocytes; SMC, spleen mononuclear cells; BMDM, bone marrow-derived macrophages; NC, nasopharyngeal carcinoma; FF, foreskin fibroblasts; SDH, spinal dorsal horn; DF, dermal fibroblasts; PET, placental extravillous trophoblasts.

inhibitors targeting PORCN activity have proven useful for *in vitro* and *in vivo* studies (39, 42, 80, 98, 112, 118). Similarly, genetic and pharmacologic interference with β-catenin functions as a transcriptional co-activator have been employed to delineate functions of β-catenin-mediated WNT signaling (39, 112, 119). It is important to note, however, that β-catenin stabilization is not exclusively indicative of WNT/WNT receptor engagement, and that microbial ligands and growth factors can induce β-catenin stabilization (120–122). Thus, here we chose to focus mainly on studies that link WNT ligands, their receptors and regulators with host defense to infection.

Phagocytosis

WNT-induced engagement of the actin cytoskeleton suggests that WNT ligands may play an active role in phagocytosis. Indeed, the *D. melanogaster* glypican dally is a co-receptor in wingless signaling and has been implicated in promoting phagocytosis of a non-pathogenic virus (white spot syndrome virus) by S2 phagocytes. Functional interactions of dally with frizzled 2 and wnt2 in this process were deduced from gene expression analyses (96). In mouse RAW264.7 macrophage-like cells, it has been reported that exposure to Wnt5a-conditioned medium or recombinant Wnt5a enhanced uptake of non-pathogenic *E. coli* DH5α, as well as latex beads. In contrast, Wnt3a-conditioned medium did not enhance phagocytosis (80, 98). Fzd5, as well as Rac-1, PI3K and IKK signaling were implicated in mediating the Wnt5a-driven phagocytic activity. Treatment with liposome-encapsulated Inhibitor of WNT Production-2 (IWP-2), a small-molecule PORCN inhibitor (123), impaired macrophage uptake of *E. coli* DH5α (98). A follow-up study

TABLE 3 | WNT functions in shaping host cell defense mechanism.

	Phagocytosis	Autophagy	ROS	NOS	Antimicrobial peptides	Inflammatory cytokines
WNT1	–	–	–	↑ iNOS LPS stimulated THP-1 (48)	–	↑ IL-6 ↑ TNF LPS stimulated THP-1 (48)
WNT2	↑ virus uptake *D. melanogaster* S2 (96)	–	–	–	–	–
WNT2B	-	–	–	–	–	↓ Sendai virus-induced *IFNβ1* HEK293T (97)
WNT3A	unaffected *E. coli* DH5α RAW264.7 (98)	↓ *M. bovis* BCG RAW.264.7 (99)	unaffected *P. aeruginosa* RAW264.7 (100) ↑ NADPH oxidase and hydrogen peroxide HUVEC (101)	-	↑ mRNA β-defensin 1 and CRAMP *P. aeruginosa* RAW264.7 (100)	↓ TNF, IL-6, IL-1β *P. aeruginosa* RAW264.7 (100)
WNT5A	↑ *E. coli* DH5α RAW264.7 (98) ↑ latex beads RAW264.7 (80) ↑ *S. pneumoniae and P. aeruginosa* RAW264.7 (57) unaffected *L. donovani* RAW264.7 (80) unaffected Mtb hMDM (102)	↑ *P. aeruginosa* and *S. pneumoniae* RAW264.7 (57) ↑ *Mtb* infected hMDM (102) ↓ *M. bovis* BCG, *S. flexneri*, *L. monocytogenes*, but not *K. pneumoniae*, *S. aureus* or *E. coli* (IFNγ-induced autophagy) RAW264.7 (103)	↑NADPH oxidase-mediated ROS production RAW264.7 (80) ↑ ROS ↑ Wnt5a mPM and RAW264.7 (79)	–	–	↑ mycobacteria-induced IL-12 and IFNγ in PPD and mycobacteria-stimulated PBMC (34) ↑ TNF, IL-6, but not IL-10 RAW264.7 ± *E. coli* (98) ↑ TNF, IL-6, IFNβ *E. coli* RAW264.7 (104) and hPBMC (19) ↓ IFNβ Chandipura virus $Wnt5a^{KD}$ RAW264.7 (104) ↑ IL-8, IL-6, IL-1β in hPBMC (19) ↑ IL-10 hMDM, ↑IL-10 promotor RAW264.7 (105) ↑ IL-6, IL-8, IL1β hPBMC (19)
WNT6	–	–	–	–	–	↓ TNF *Mtb* mBMDM (40)
WNT7A	↓ mMDM (106)	–	–	–	–	↓ IL-10, IL-12 ↑IL-6 mMDM (106)
WNT9B	–	–	–	–	–	↓ *IFNβ1* Sendai virus HEK293T (97)
WNT11	–	–	–	–	–	↓IL-8 mIEC (56)

↑, upregulation; ↓, downregulation; –, indicative of no data; protein, protein expression; mRNA, mRNA expression; m, murine; h, human; HUVEC, human umbilical vein endothelial cells; MDM, monocyte-derived macrophages; PBMC, peripheral blood mononuclear cells; BMDM, bone marrow-derived macrophages; IEC, intestinal epithelial cells; MIM, myocardial infarct macrophages; Mtb, Mycobacterium tuberculosis.

described that exogenously added recombinant Wnt5a also enhanced RAW264.7 cell phagocytosis of *S. pneumoniae* (Gram-positive) and *P. aeruginosa* (Gram-negative) mediated by Rac-1 and Dvl (57). Mice pre-treated with IWP-2 displayed enhanced bacterial burden within peritoneal cells at 2 h post-intraperitoneal infection, and within lung homogenates 5 h after intranasal infection with *P. aeruginosa*. Similarly, more viable *P. aeruginosa* were recovered from peritoneal cell lysates of $Wnt5a^{+/-}$ mice compared to $Wnt5a^{+/+}$ mice (57). These observations further suggest a role for Wnt5a, and potentially other WNT ligands in host cell uptake of *P. aeruginosa*. However, treatment of RAW264.7 cells with recombinant Wnt5a did not alter internalization of *L. donovani* (80), and siRNA-mediated knock-down of endogenous WNT5A did not impair phagocytosis of *M. tuberculosis* by human monocyte-derived macrophages (102). Thus, the effects of WNT5A on phagocytosis of bacterial pathogens requires further investigation, including comparisons of extracellular alongside intracellular pathogens and macrophages of different origins.

E. chaffeensis is an obligate intracellular pathogen that infects mononuclear cells through caveolae-mediated endocytosis and resides in intracellular vesicles that retain characteristics of early endosomes (124). *E. chaffeensis* tandem repeat proteins (TRPs) are secreted effectors of *E. chaffeensis* that have been shown to interact with host cell proteins, including components of the WNT signaling network (125, 126). Phagocytosis of TRP120-coated microspheres by human monocytic THP-1 cells was impaired by small molecules targeting intracellular signaling components that are also part of the WNT signaling network, such as β-catenin/TCF interactions (FH535), CamKII (KN93), and Rac-1 (NSC23766) (42). In contrast, a PORCN inhibitor (IWP-2) did not impair TRP120-microsphere phagocytosis, suggesting that secreted WNT proteins may not have been directly involved in driving this process. In contrast to phagocytic

cells, WNT11 over-expression, but not WNT2 overexpression, by human intestinal epithelial HCT116 cells has been suggested to decrease invasion by *Salmonella enterica* Typhimurium (50, 56). The cellular mechanisms facilitating this protection are unknown and it remains to be established how induction of WNT11 expression by *Salmonella* infection might contribute to pathogenesis *in vivo*.

Autophagy

Several studies have begun to address how WNT ligands might affect the ability of host cells to control pathogenic bacteria. In the case of non-pathogenic *E. coli* DH5α, exogenous addition of Wnt5a enhanced phagocytosis, but did not alter the ability of RAW264.7 macrophages to rapidly kill the engulfed bacteria (98). In contrast, RAW264.7 macrophages exposed to recombinant Wnt5a displayed a more rapid decline in viable intracellular *S. pneumoniae* and *P. aeruginosa* within the first 2–3 h of infection. Wnt5a-induced killing within the first hours of infection was suggested to be mediated by Rac-1 and Dvl. Mechanistically, the authors implicated enhanced autophagy as the mechanism of Wnt5a-induced enhanced control of engulfed *S. pneumoniae* and *P. aeruginosa* (57). While *S. pneumoniae* is targeted by autophagy in non-phagocytic cells (127), the contribution of autophagy in macrophages to controlling this bacterium had not been reported previously. In contrast, the contributions of autophagy to macrophage control of *P. aeruginosa* require further clarification as beneficial effects for the host as well as the bacteria have been suggested (63, 128–131). It is noteworthy, however, that after the sharp initial decline of viable intracellular *S. pneumoniae* and *P. aeruginosa* in Wnt5a-treated RAW264.7 macrophages, from day 1 onwards the intracellular bacterial burden declined more slowly and at a similar rate in both Wnt5a- and control-treated cells (57). Thus, the cellular mechanisms accelerating the initial bacterial killing might be transient, and could be specific to some pathogens as they did not affect macrophage killing of non-pathogenic *E. coli* DH5a (98). With Wnt5a expression reported to be suppressed by *S. pneumoniae* and *P. aeruginosa* infection of macrophages (57), roles of other WNT ligands responsive to infection (e.g., Wnt4, Wnt5b, Wnt7a, Wnt7b) (51) and the net-outcome of WNT signaling in infected cells will need further exploration. Of note, overexpression of β-catenin in RAW264.7 macrophages has been reported to accelerate killing of engulfed *P. aeruginosa*, which was associated with suppression of autophagy (63).

Beneficial or detrimental impact of WNT-autophagy-crosstalk might be defined by a pathogen's ability to exploit intracellular niches for replication and survival. Intracellular bacterial burden in *E. chaffeensis*-infected THP-1 cells was diminished when cells were exposed to IWP-2, as well as the β-catenin/TCF-1 inhibitor FH535, or the CamKII inhibitor KN93. Small interfering RNA-mediated knock-down of WNT pathway components, including *WNT5A, FZD5, FZD9, LRP6, CTNNB1,* and *DVL2* diminished intracellular bacterial burden over 1-2 days of infection, further supporting the notion that intracellular survival of *E. chaffeensis* in this cell line was facilitated by the action of endogenous WNT ligands (42). A subsequent study indicated that DVL signaling suppressed autophagy and phago-lysosomal maturation in *E. chaffeensis*-infected cells (132). WNT pathway activation (e.g., by Wnt5a) upon infection with *M. bovis* BCG has been reported to interfere with IFNγ-induced activation of autophagy in mouse macrophages, a process facilitated by arachidonate lipoxygenase. The same mechanisms have also been implicated for *Shigella flexneri* and *Listeria monocytogenes* infection (103). A recent study suggested that in human monocyte-derived macrophages infected with *M. tuberculosis*, WNT5A contributed to enhancing autophagy resulting in a small decrease in intracellular bacterial burden. In this study, WNT5A-mediated autophagy was suggested as an effector mechanism of IL-36γ (102). However, as *WNT5A* expression in human macrophages is rapidly induced by *M. tuberculosis* infection (34), this mechanism might represent an amplification of the WNT5A response of these cells as indicated for other cytokines such as TNF (19). Exogenous addition of Wnt3a conditioned medium suppressed association of intracellular *M. bovis* BCG with autophagy machinery in RAW264.7 macrophages, which was associated with impaired mRNA expression of autophagy effectors (e.g., *Atg5, Atg7, Atg12, p62*) (99). With evidence for bi-directional regulation between WNT signaling and autophagy (133–135), and the notion that some pathogens might exploit this for their intracellular survival, the functional consequences of this cross-talk for pathogen control is an area for future pursuit.

Reactive Radicals

Additional cell-intrinsic host defense mechanisms that may be regulated by WNT signaling include the formation of reactive radicals. Treatment of RAW264.7 macrophages with recombinant Wnt5a induced NADPH oxidase-mediated ROS production, which has been suggested to contribute to the macrophage control of *L. donovani* (80). Exogenous addition of recombinant Wnt3a or Wnt3a-conditioned medium to human umbilical vein endothelial cells induced elevated expression of endothelial NADPH oxidase and production of hydrogen peroxide (101), and GSK3β has been implicated as a negative regulator of LPS-induced NADPH-oxidase 1 expression and production of reactive oxygen species production by macrophages (136). These observations could implicate β-catenin-stabilizing WNTs as drivers of ROS production. Yet, treatment of RAW264.7 macrophages with Wnt3a-conditioned medium did not affect ROS production upon *P. aeruginosa* infection (100). Thus, contributions of WNT ligands, in particular endogenously expressed WNTs to ROS production as an anti-microbial defense mechanism require further investigation.

Wls-deficiency in BMDMs of $Wls^{fl/fl}$-Lyz2-Cre mice has been reported to significantly increase mRNA expression of inducible nitric oxide synthase (iNOS, encoded by *Nos2*) (137), a phenotype also observed in macrophages isolated from myocardial infarct tissue of *cfms-icre* $Wls^{fl/fl}$ mice (138). This may be reflective of suppression of iNOS expression by autocrine/paracrine WNT signaling. The human iNOS promoter has TCF-4 binding sites and *Nos2* expression and nitric oxide production were shown to be positively regulated by β-catenin and TCF-4 (139). These observations suggest that the balance of β-catenin-dependent and -independent WNT signaling could

be important for fine-tuning iNOS expression and activity. Whether this bears consequences for pathogen control needs to be investigated. Nevertheless, enhanced iNOS expression by *Wls*-deficient macrophages may indicate compensatory mechanisms associated with the inability to release WNT proteins from producing cells and significant elevation of WNT gene expression observed in these cells (137). However, such alterations in WNT expression may be cell specific as F4/80$^+$ liver macrophages of *Wls*$^{fl/fl}$-Lyz2-Cre mice did not show significant differences in *Wnt4* and *Wnt6* expression (118).

With some indication that WNT ligands may determine a cell's ability for production of reactive oxygen and nitrogen species, there is also evidence that ROS and NO produced in response to microbial insult may regulate WNT responses. For example, peritoneal macrophages isolated from *Nos2*$^{-/-}$ mice showed lower induction of *Wnt5a*, *Fzd4,* and *Lrp5* mRNA expression upon *M. bovis* BCG infection compared to wild type control cells. Treatment with an NO-donor restored *Wnt5a*, *Fzd4,* and *Lrp5* expression in *Nos2*-deficient macrophages (44), implicating reactive nitrogen species as potentiators of WNT signaling initiation. Dectin-1/Syk-mediated ROS production by murine RAW264.7 macrophages contributed to β-catenin stabilization (79), although how this might intersect with WNT-driven cellular activation remains to be explored.

Antimicrobial Peptides

Beta-catenin-stabilizing WNT ligands may also play a role in the expression of antimicrobial peptides. A recent study reported that Wnt3a-conditioned medium elevated the *P. aeruginosa*-induced mRNA expression of cathelicidin-related antimicrobial peptide (CRAMP) and β-defensin 1 in RAW264.7 mouse macrophages, which correlated with a small increase in bacterial killing by these cells (100). Stabilization of β-catenin has also been linked to production of the α-defensins cryptdin-1 and cryptdin-6 by murine intestinal crypts (140). In *C. elegans,* it has been shown that expression of the antimicrobial peptide *clec-60* (human homolog RegIIIγ) upon *S. aureus* infection is dependent upon the β-catenin homolog *bar-1* (45). These observations implicate β-catenin in the transcriptional control of a range of antimicrobial peptides. This encourage analyses on the potential roles of infection-responsive endogenous WNTs in the expression of antimicrobial peptides by infected cells.

Tryptophan Metabolism

Indoleamine 2,3dioxygenase (IDO) catalyzes the first rate-limiting step in the catabolism of the tryptophan for the formation of active metabolites (141). IDO activity is essential for host resistance to some infections where IDO activity limits the pathogen's access to the essential amino acid tryptophan (142, 143). The PORCN inhibitor IWP-L6 and the β-catenin inhibitor iCRT14 enhanced IDO expression and activity in *T. cruzi*-infected murine macrophages, which was associated with enhanced control of intracellular parasites (74). This suggests that endogenous WNT expression and associated β-catenin stabilization in *T. cruzi*-infected macrophages suppressed IDO expression in this context. It will be interesting to explore whether induction of WNT/β-catenin signaling by *T. cruzi* is an active strategy of subverting host defense mechanisms. Importantly, β-catenin activity in CD11c$^+$ APCs has been associated with induction of IDO expression and the attainment of a tolerogenic phenotype in DCs (144, 145). Whether these apparent differences are reflective of the cellular context (macrophages vs. CD11c$^+$ dendritic cells) or the immune responses (parasite infection vs. sterile inflammation) are worth further investigations.

Anti-viral State and Type I Interferon Responses

GSK3β activity and β-catenin functions have been implicated in the positive or negative regulation of type I interferon (IFN) responses associated with protection or susceptibility of cells to viral infection (82, 97, 146–152). In some studies, direct contributions of endogenous WNT ligands has been confirmed. For example, siRNA-mediated knock-down of Wnt5a in mouse bone marrow-derived macrophages and RAW264.7 cells impaired Chandipura virus-induced IFNβ production associated with enhanced viral load in infected cell cultures (104). WNT2B and WNT9B were identified as negative regulators of Sendai virus-induced interferon beta (*IFNβ1*) expression, and inhibition of GSK3β-controlled virus-induced type I IFN responses in a β-catenin-dependent manner in a range of human cell lines and primary cells (97). SiRNA-mediated knock-down experiments in human bronchio-epithelial cells (HBECs) identified WNT5A and DKK1 as positive, and FZD5, DVL3, SFRP5, WNT7B, WNT9B as negative regulators of influenza A PR8 replication (114). Knock-down of *WNT2* and *WNT3* (but not *WNT1, CTNNB1, or LEF1*) impaired infection of HeLa cells by Dengue virus (153). Enhanced control of flaviviruses was associated with enhanced type I IFN signaling via interferon regulatory factor (IRF)-3 activation and interferon response gene expression. It was proposed that this was facilitated by cross-regulation and physical interactions between TANK-binding kinase-1 (TBK-1, which phosphorylates IRF-3) and GSK3β (153). However, examples of β-catenin-stabilizing WNTs not affecting virus-induced interferon responses also exist (154).

Inflammation

WNT signaling has been ascribed both pro-inflammatory and immune-regulatory properties. The paradigm developed over the past decade or so suggests that WNT ligands triggering β-catenin-independent signaling exert pro-inflammatory functions, whereas WNT ligands driving β-catenin stabilization have anti-inflammatory or immune-modulatory effects. These emerging concepts of WNT ligands orchestrating inflammation and immune cell functions have been reviewed and commented on extensively over time (19, 120, 155–163). Here we have chosen to specifically focus on examples for pro-inflammatory and regulatory effects of endogenous WNT ligands.

It is increasingly recognized that the WNT response upon infection or microbial challenge comprises complex changes across multiple WNT ligands, receptors and regulators (**Tables 1**, **2**). Moreover, WNT receptors exhibit a degree of promiscuity for WNT ligands (164, 165). Thus, the concerted action of WNT ligands and the consequences for local and systemic inflammation in the context of infection require careful

consideration. Use of small molecule inhibitors of PORCN (e.g., IWP-2) indicated net pro-inflammatory roles of WNT ligands in mouse models of LPS-induced endotoxemia and *E. coli*-induced bacterial peritonitis (39, 98). Moreover, two studies utilizing small molecule inhibitors of β-catenin functions as transcriptional co-activator (ICG001, iCRT3) independently revealed pro-inflammatory functions of β-catenin in LPS-induced endotoxemia and cecal ligation and puncture (CLP)-induced peritonitis (39, 119). This challenged the current paradigm of anti-inflammatory roles of β-catenin stabilization and urges further studies to understand the contributions of β-catenin in different (immune) cells to inflammatory responses *in vivo*. Moreover, which of the individual WNT ligands responsive to infection are responsible for the pro-inflammatory functions *in vivo*, and what role selective downregulation of regulatory WNTs might play in this context remains to be explored in more detail.

Significant focus by some of the earliest studies has been on WNT5A, a WNT family member implicated in driving pro-inflammatory cytokine responses by myeloid cells via β-catenin-independent signaling (19, 34–36, 166). Endogenous WNT5A has been shown to positively contribute to pro-inflammatory cytokine production by monocytes and macrophages in the context of *Mycobacterium* and *E. coli* infection, as well as LPS stimulation (19, 34, 104). Knockdown of WNT5A in primary human bone marrow stromal cell also impaired basal and LPS-induced release of pro-inflammatory cytokines and chemokines (167). Inhibition of endogenous Wnt5a in a mouse model of HIV-induced neuroinflammation reduced gp120-induced pro-inflammatory cytokine responses *in vivo* (77). However, Wnt5a has also been implicated in impairing dendritic cell functions and creating an immune suppressive environment in a mouse melanoma model. Importantly, this was attributed to Wnt5a mediated β-catenin stabilization (168), which contrasts the pro-inflammatory roles of Wnt5a affected by β-catenin-independent signaling upon microbial challenge. This highlights that the receptor/signaling context rather than the WNT ligand might guide the functional outcome of WNT signaling.

Evidence for net anti-inflammatory functions of WNT ligands can be deduced from enhanced pro-inflammatory cytokine release and decreased release of regulatory TGF-β by *T. cruzi*-infected murine macrophages in the presence of PORCN (IWP-L6) and β-catenin/TCF inhibitors (iCRT14) (74). In this study, it was noted that neither PORCN nor β-catenin inhibitors affected *T. cruzi*-induced IL-10 production by infected macrophages *in vitro* (74). Similar results were observed in an *in vivo* LPS-induced endotoxemia model (39). These observations highlight that IL-10 may not be susceptible to WNT regulation in all contexts.

An example of infection-induced expression of a specific endogenous WNT ligand being associated with suppression of pro-inflammatory cytokine responses comes from *M. tuberculosis*-infected mouse macrophages. Bone marrow-derived macrophages from *Wnt6*-deficient mice displayed elevated TNF expression and secretion upon *M. tuberculosis* infection (40). That immune-suppressive roles of individual WNT ligands could be vital for host survival upon bacterial infection has been demonstrated for WntD in *Drosophila*. WntD-deficiency rendered flies more susceptible to *L. monocytogenes* infection and this was attributed to WntD curbing lethal inflammation by negatively regulating expression of the inflammatory mediator edin via suppression of Dorsal, an NF-κB family member (88). Inhibition of intracellular cell signaling cascades that drive pro-inflammatory cytokine expression (e.g., NF-κB) has been implicated as one of the mechanisms by which β-catenin-stabilizing WNT ligands negatively regulate inflammation (169, 170). Evidence on how this contributes to shaping cellular immune responses and inflammation during infection in complex *in vivo* settings will be invaluable to further affirm this regulatory feedback mechanism.

FUNCTIONAL FATE OF MACROPHAGES AND DENDRITIC CELLS WITH IMPLICATIONS FOR T-CELL RESPONSES

WNT ligands have been implicated in defining the functional polarization and differentiation of macrophages and dendritic cells. These innate immune cells are critical in shaping inflammation and antimicrobial defense, and in instructing adaptive immune responses in their role as professional antigen presenting cells (APCs).

Macrophage Polarization

Macrophages exhibit functional plasticity along a multi-dimensional spectrum directed by external and internal stimuli such as microbial products, cytokines, oxygen availability and cellular metabolism (171, 172). Accordingly, phenotypic classification of macrophages based on relative induction or suppression of the transcription of individual genes has limitations. Nevertheless, expression of iNOS is commonly associated with (M1-type) inflammatory macrophages, whereas elevation of arginase 1 (Arg1) expression has been associated with (M2-type) alternatively activated macrophages. Nevertheless, Arg1 activity is also found in M1 macrophages regulating NO production by iNOS (171). *Wls* deletion in resting mouse bone marrow-derived macrophages was accompanied by elevated expression of *Nos2, Tnf, and Il6,* and reduced expression of the M2-associated gene *Mrc1* (macrophage mannose receptor), without affecting *Arg1* expression (137). This suggests that basal *Wls* activity (and by inference the net impact of released WNT ligands) contributed toward M2 polarization of these macrophages. In contrast, several studies indicated that *Arg1* expression is regulated by WNT ligands in macrophages upon pathogen encounter. For example, the PORCN inhibitor IWP-L6, but not the β-catenin inhibitor iCRT14, decreased *Arg1* expression in *T. cruzi*-infected mouse macrophages, yet without impacting production of reactive nitrogen intermediates (74). *Wnt6*-deficient macrophages expressed less *Nos2* and *Arg1* in response to *M. tuberculosis* infection, yet reactive nitrogen production was not impaired relative to wild type controls (40). Exogenous addition of Wnt3a-conditioned medium promoted the expression of *Arg1* in *M. tuberculosis*-infected murine

BMDMs (41). sFrp1-overexpression, which was accompanied by impaired β-catenin signaling, led to reduced expression of *Arg1* and macrophage mannose receptor, CD206 (173). Albeit not evident of endogenous WNT ligands contributing to macrophage polarization, it is worth considering that *in vitro* exposure of macrophages to recombinant WNT ligands (including Wnt1, Wnt3a, Wnt5a, Wnt7a) have returned varying results on their ability to elicit phenotypic changes indicative of alternatively activated macrophages or macrophages tolerized against LPS activation (61, 105, 106, 117).

Dendritic Cell Maturation and Functions

The impact of exogenously added or endogenously released WNT ligands and contributions of β-catenin signaling on the expression of functional surface markers of DCs (e.g., MHC-I and MHC-II, co-stimulatory molecules, PD-L1, PD-L2) and DC endocytic capacity has been analyzed in a number of studies returning varying results (115, 137, 174–182). Such variability is likely governed by the use of cells from different species; differentiation and culture conditions; use of exogenous modulation through recombinant WNTs, conditioned media, WNT regulators vs. perturbation of endogenous WNT ligands and signaling events, for example by using small molecule inhibitors or genetic perturbations. Moreover, the utility of recombinant proteins and the possibility of alternative receptors interacting with WNT ligands requires further validation (61, 95, 117, 183, 184).

Nevertheless, β-catenin activity in myeloid cells has emerged as a rheostat in immune-regulation and tolerance, specifically elucidated in *in vivo* models of autoimmunity, gut mucosal homeostasis and cancer (95, 120, 162, 183–185). Recent studies implicate direct roles for WNT ligands that act via engagement of LRP co-receptors in this regulatory mechanism. Selective deletion of LRP5/6 in $CD11c^+$ APCs (which includes DC and macrophage populations in the intestinal mucosa) rendered mice more susceptible to dextran sodium sulfate (DSS)-induced colitis (95, 144). This was associated with elevated expression of pro-inflammatory cytokines (e.g., TNF, IL-6, IL-1β) and reduced expression of anti-inflammatory/regulatory effectors (e.g., IL-10, IDO), and functional bias toward fostering Th1 and Th17 responses at the detriment of T regulatory cells (Tregs). The microbiome has been implicated as a driver of inflammation in mice with LRP5/6-deficient $CD11c^+$ APCs with expression of a stabilized form of β-catenin specifically in $CD11c^+$ APCs ameliorating disease pathology and pro-inflammatory responses in the DSS colitis model (144). Similar experimental approaches confirmed a regulatory role for β-catenin expression in $CD11c^+$ APCs in mouse models of experimental autoimmune encephalitis (EAE), collagen-induced arthritis, and tumorigenesis (94, 183–185). It is interesting to note that the adjuvant utilized in the EAE model contains mycobacterial antigens and that LRP5/6-deficient DCs exhibited reduced pro-inflammatory and enhanced regulatory cytokine responses upon mycobacterial stimulation *in vitro* (94), suggesting that infection-associated WNT responses might direct APC functions in Treg vs. Th1 and Th17 differentiation. In an OVA-expressing tumor model, Wnt1-overexpression by DCs was associated with reduced T cell receptor stimulation, granzyme B secretion and cytotoxicity by $CD8^+$ T cells (186), whereas conditional knockout of LRP5/LRP6 in $CD11c^+$ cells resulted in an increase in granzyme B production by $CD8^+$ T cells (185). Thus, WNT-mediated activation of APCs also bears consequences for subsequent T cell functionality. Of note, there is some evidence indicating that WNT-mediated β-catenin signaling also orchestrates the differentiation of plasmacytoid DCs (187–190), but consequences for pDC functions remain to be explored.

The aforementioned studies support the view that β-catenin-stabilizing WNT signaling engaging LRP5/6 co-receptors can mediate an immune-regulatory profile of DC functions. In contrast, inducible deletion of Wnt5a and one of its receptors, Ror2, rendered mice more resistant to DSS-induced colitis (113). This was accompanied by diminished pro-inflammatory cytokine responses, including IL-12 expression, and selective impairment in the differentiation of IFNγ-producing $CD4^+$ T cells, without impact on IL-17- and IL-10-producing $CD4^+$ T cells (113). It was implicated that Wnt5a in this context arose from non-hematopoietic cells such as fibroblasts, whereas Ror2 signaling occurred in the hematopoietic compartment including DCs. Nevertheless, cultured Wnt5a-deficient and Ror2-deficient colonic DCs showed impaired pro-inflammatory cytokine profiles upon LPS stimulation including enhanced IL-12 production and increased responsiveness to IFNγ (113). These observations support the notion of pro-inflammatory roles of Wnt5a expressed by myeloid cells. They also align with data indicating that myeloid cell-derived WNT5A, and likely other WNT ligands, bridge innate and adaptive immunity by perpetuating the IL-12-IFNγ axis in T cell and natural killer T (NKT) cell responses (34, 112, 174). Importantly, however, the roles Wnt5a plays in shaping DC functions may be defined by the receptor/signaling output. This is highlighted by findings that melanoma-derived Wnt5a effected a metabolic shift in DCs from glycolysis to oxidative phosphorylation, which was attributed to β-catenin- and PPARγ-mediated cellular activation. This resulted in tolerogenic DCs that promoted IDO activity and regulatory T cell differentiation. Relevance of this mechanism was translated into an *in vivo* melanoma model in mice (145). It will be important to delineate whether factors specific to the pathophysiological context (e.g., immune regulatory molecules, cytokine milieu) explain the apparently opposing outcomes of WNT exposure on DC functions in melanoma vs. inflammatory disorders.

T Cell Functions During Infection

Genetic deletion of β-catenin in $CD11c^+$ cells was associated with only a small increase in the frequency of $CD4^+$ T cells, but no significant changes in the frequency of $CD8^+$ T cells, $TCR\gamma\delta^+$ T cells, NKT cells, Tregs, or T follicular helper cells were observed (183, 191). These findings suggest that β-catenin functions in $CD11c^+$ myeloid cells define the quality of T cell responses

due to the functional capabilities of APCs, rather than by significantly affecting lymphocyte differentiation. Nevertheless, β-catenin and TCF activation play distinct roles in the development, differentiation and function of innate-like and adaptive lymphocytes, and direct contributions of WNT ligands to these processes have been shown (110, 192, 193).

In a mouse model of lymphocytic choriomeningitis virus (LCMV) infection, TCF-1-deficiency had no effect on the expansion and functions (e.g., IFNγ production and cytolysis) of effector $CD8^+$ T cells (194, 195), whereas others reported an increase in effector $CD8^+$ T cells associated with enhanced IFNγ and TNF expression (196). In contrast to the apparently opposing observations for effector T cells, these studies consistently showed reduced numbers of memory $CD8^+$ T cells, reduced IL-2 expression, and impaired expansion of memory cells upon rechallenge (194–196). However, it was suggested that these TCF-1-mediated effects may not be attributable to β-catenin functions, as conditional knockout of β-catenin in mature T cells did not affect memory T cell numbers or functions upon LCMV and *L. monocytogenes* infection (197). Yet, in a transgenic mouse model of constitutively activated β-catenin/TCF-1-signaling, an increased proportion of memory $CD8^+$ T cells and increased IFNγ expression during LCMV, vaccinia virus and *L. monocytogenes* infection were reported (198). These studies indicate that TCF-1 is likely required for $CD8^+$ T cell memory formation and functions after infection. The role β-catenin might play in this and whether WNT ligands have a direct contribution to these signaling events requires further investigation.

In an *in vitro* system, depletion of WNT1, 2B, 3 and 5B from astrocyte-conditioned medium reduced the differentiation of $CD8^+$ T cells toward a $CD4^{dim}CD8^{bright}$ T phenotype in cultures of human peripheral blood mononuclear cells. $CD4^{dim}CD8^{bright}$ T cells in the central nervous system are thought to be effector memory T cells important in the control of HIV (71). While this study implicated direct involvement of WNT ligands in the formation of this $CD8^+$ T cell subset, it remains to be determined whether WNT ligands mediated this differentiation by acting on the $CD8^+$ T cells, or indirectly via APCs (e.g., by shaping the cytokine milieu). To our knowledge, there are thus far only very few links between WNT ligands and $CD4^+$ T cell functions during infection. In a susceptible mouse model of *Leishmania major* infection, an inhibitor of Dkk1, which should increase WNT/β-catenin signaling, exhibited reduced numbers of $CD4^+$ T cells in the draining lymph node, with subsequent reduced IL-4 and IL-10 expression after *ex vivo* stimulation (199). An *in vitro* study utilizing neutralizing antibodies against WNT5A and FZD5 showed impaired antigen-specific IFNγ production by human PBMCs of antigen-experienced donors re-stimulated with *M. tuberculosis* antigen. As human T cells expressed FZD5, it was hypothesized that WNT signaling can facilitate memory T cell activation (34). However, these studies did not demonstrate that these effects were driven directly by WNT signaling in $CD4^+$ T cells, nor did they exclude WNT effects on APC functions. Detailed analyses of the WNT receptor and WNT regulator repertoire of different T cell lineages and subsets should guide targeted interventions with WNT signaling events to delineate the roles infection-associated WNT responses play in shaping T cell effector and memory formation and functions.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The WNT signaling network has been firmly established as an evolutionary conserved integral component of host responses to infection. In-depth understanding of how WNT ligands define immune cell functions is beginning to offer mechanistic insights into the contributions of WNT responses to pathogen control and inflammation. Experiments establishing how infection-associated endogenous WNT responses shape immune cell functionality *in vivo* will be key to deciphering WNT functions in shaping complex immune responses. Thus far, macrophages and DCs, as well as T cells have been a major focus of delineating WNT-mediated immune functions. Knowledge of how WNT ligands shape the functions of other immune cells, including neutrophils, mast cells, natural killer cells, natural killer T cells, innate lymphoid cells, B cells, etc. is required to begin to understand the complexity of immune-related WNT responses.

Considering that the WNT signaling outcome is largely decided by the cellular context at the level of receptor engagement (20), functional redundancy of WNT ligands, or lack thereof, in orchestrating cellular responses of functionally diverse cells in complex tissue environments is an important factor. With a clearer understanding of the WNT receptor and WNT regulator repertoire expressed by different immune- and non-immune cells in responses to infection, it will be important to determine if there are species-specific differences in the consequences of WNT exposure of functionally similar cells. This is especially critical when investing in utilizing animal models for understanding human pathology and calls for systematic analyses of WNT responses in infected tissues across different species. Reporter mice for WNT ligand and receptor expression as well as WNT signaling activity (200, 201) will be invaluable for the temporal and spatial documentation of WNT responses in complex *in vivo* settings, including infections. Comparisons with human specimens, wherever possible, will be critical.

While some consistent patterns of WNT responses begin to arise (e.g., WNT5A regulation in human macrophages), it remains largely unclear whether stereotypical WNT responses to infection exist regardless of the invading pathogen, or whether the nature of the pathogen dictates the WNT response. Comparative studies using phylogenetically diverse pathogens covering spectra of virulence and pathogenesis mechanisms will be essential to distinguish stereotypical and selective responses to microbial infection. In depth understanding of the molecular drivers and regulators of WNT ligand and receptor expression during infection will be invaluable in delineating which microbial factors drive WNT responses. Whereas our understanding of WNT responses and functions during viral and bacterial

infections is taking shape, WNT contributions to parasitic and fungal infections remain to be explored in more breadth and depth. Knowledge of the investment of pathogens into actively manipulating the WNT signaling network (202–204) will inform our understanding of pathogenesis mechanisms and roles of WNT signaling in the host defense against infection. Such insights will be essential when exploring WNT response patterns as biological indicators supporting diagnosis, prognosis and choices for clinical management of infectious diseases (205).

Due to the central role of WNT signaling in maintaining tissue homeostasis, including epithelial barrier functions, consequences of immune-related WNT responses reach beyond leukocyte functions. Indications that WNT/WNT receptor interactions shape chemokine responses (186) and cellular metabolism (145) deserve particular attention in the context of immune responses to infection and beyond. Aberrant WNT expression and/or WNT signaling underlying carcinogenesis, fibrosis, and osteoporosis has generated considerable interest in pharmacologically targeting the WNT signaling network (206–209). Understanding the functional nature and temporal regulation of WNT responses in the host response to infection, and other immune settings, is essential for identifying therapeutic opportunities, but also potential risks of pharmacologically targeting the WNT signaling network.

AUTHOR CONTRIBUTIONS

JL, JK, and AB wrote the manuscript. TT contributed sections to the manuscript. JL and AB conceived and designed figures and tables. All authors read and approved the final version of the manuscript.

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REVIEW
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Wnt Signaling and Its Significance Within the Tumor Microenvironment: Novel Therapeutic Insights

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Wnt signaling is one of the central mechanisms regulating tissue morphogenesis during embryogenesis and repair. The pivot of this signaling cascade is the Wnt ligand, which binds to receptors belonging to the Frizzled family or the ROR1/ROR2 and RYK family. This interaction governs the downstream signaling cascade (canonical/non-canonical), ultimately extending its effect on the cellular cytoskeleton, transcriptional control of proliferation and differentiation, and organelle dynamics. Anomalous Wnt signaling has been associated with several cancers, the most prominent ones being colorectal, breast, lung, oral, cervical, and hematopoietic malignancies. It extends its effect on tumorigenesis by modulating the tumor microenvironment via fine crosstalk between transformed cells and infiltrating immune cells, such as leukocytes. This review is an attempt to highlight the latest developments in the understanding of Wnt signaling in the context of tumors and their microenvironment. A dynamic process known as immunoediting governs the fate of tumor progression based on the correlation of various signaling pathways in the tumor microenvironment and immune cells. Cancer cells also undergo a series of mutations in the tumor suppressor gene, which favors tumorigenesis. Wnt signaling, and its crosstalk with various immune cells, has both negative as well as positive effects on tumor progression. On one hand, it helps in the maintenance and renewal of the leucocytes. On the other hand, it promotes immune tolerance, limiting the antitumor response. Wnt signaling also plays a role in epithelial-mesenchymal transition (EMT), thereby promoting the maintenance of Cancer Stem Cells (CSCs). Furthermore, we have summarized the ongoing strategies used to target aberrant Wnt signaling as a novel therapeutic intervention to combat various cancers and their limitations.

Keywords: immune response, signaling, immunotherapy, β-catenin, anti-tumor response

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INTRODUCTION

Tumorigenesis is a multifaceted process largely occurring due to the accumulation of mutations in the tumor suppressor genes and oncogenes. These mutations lead to uncontrolled proliferation and resistance to cell death. The sustenance and fate of a tumor is dictated by the tumor microenvironment, which fulfills the needed requirements of energy, growth-factors, chemokines, cytokines, and autocrine/paracrine signals, thereby attracting a wide variety of cell types (1). The tumor microenvironment consists of fibroblasts, immune cells, endothelial cells, and the extracellular matrix. The fine balance between these cells and the transformed cells is decided

by the variety of signaling pathways; one such critical pathway is the WNT signaling cascade. Wnt signaling controls a plethora of functions with the help of 19 Wnt proteins, 2co-receptors, 10 Frizzled (Fzd) receptors, and various non-Fzd receptors, for example the Receptor Tyrosine Kinase-like Orphan Receptor and the Ryk Receptor-like Tyrosine Kinase (2). Wnt ligands are secreted lipid-modified glycoproteins and have varied functions that include hematopoietic stem cell maintenance, cell migration, cancer stem cell survival and maintenance, and inflammation and immune tolerance (3–5). These ligand–receptor interactions activates various signaling cascades that are important for cellular homeostasis, oncogenic transformation, tumor progression, and metastasis (3, 6). The Wnt signaling pathway is known to be critical in T cell and dendritic cell development and maturation, which are the epicenter of the adaptive immune response, making it a vital signaling pathway for fighting various pathophysiological disorders (5). Due to its involvement in diverse functions, any aberration or de-regulation of this pathway causes several types of cancers and/or developmental defects. It regulates the anticancer immune response and has shown correlation with poor prognosis and survival of cancer patients. The tumor microenvironment (TME) is a source of both canonical and non-canonical Wnt ligands and can induce aberrant signaling pathways in the cancer cells and immune cells, leading to EMT and altered immune response (7, 8). For several years, it has been established that the Wnt/β-catenin pathway is indispensable for cancer cell survival and maintenance, making it a lucrative target for an anticancer therapy regimen. Various Wnt inhibitors are undergoing clinical trials for therapeutic purposes (alone or in combination with other anti-cancer drugs). This review highlights the role of Wnt/β-catenin signaling cascade in tumor microenvironment and its effects in cancer progression and survival. We conclude by accentuating the potential of Wnt/β-catenin inhibitors in the harnessing of new anticancer therapeutics by targeting cancer microenvironment.

WNT SIGNALING

In 1973, thewingless gene was discovered during a mutagenesis screening for temperature-sensitive mutants in *Drosophila melanogaster* (9). Consequently, many other genetic components involved in embryonic pattern formation were identified (10). The foundation research for Wnt signal transduction was carried out in the 1980s and 1990s, and it was established that the gene products of the Drosophila wingless (wg) and murine proto-oncogene Int1 (now called Wnt1) are orthologous (11). The term "Wnt1" is an amalgamation of *wingless* and *Int1* (12).

WNTs are a large family of secreted, hydrophobic, and Cys-rich glycolipoproteins that direct developmental processes, stem cell proliferation, and tissue homeostasis throughout the metazoans (13, 14). As a result, any abnormality in the Wnt signaling pathway causes pathological conditions such as birth defects, cancers, and other diseases (15). In humans, there are 19 genes encoding WNTs that connect to various receptors and stimulate different intracellular signal transduction pathways (16). Based on different studies, these pathways have been roughly divided into either canonical (β-catenin dependent) or non-canonical (β-catenin independent) signaling pathways (16), as is described in the subsequent section. Depending upon their potential to induce morphological transformation in a murine mammary epithelial cell line (C57MG), the Wnt family has been categorized into different types (17). Wnt1, Wnt3, Wnt3a, and Wnt7a fall under the category of highly transforming members, and Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt11 are grouped under intermediately transforming or non-transforming members (13). In general, Frizzled proteins function as common receptors for both canonical as well as non-canonical pathways (16).

Canonical Wnt Signaling

The canonical Wnt signaling pathway is a well-studied pathway that is activated by the interaction of Wnt with a Frizzled (Fz) receptor and LRP5/LRP6, where LRP stands for lipoprotein receptor-related protein (which is a single-span trans-membrane receptor) (16). Once bound by Wnt, the Fz/LRP co-receptor complex stimulates the canonical signaling pathway. Upon activation, Fz can interact with a cytoplasmic protein called Disheveled (Dsh), which acts upstream of β-catenin GSK3β (15). Research studies have identified Axin as a protein that interacts with the intracellular domain of LRP5/6 through five phosphorylated PPPSP motifs in the cytoplasmic tail of LRP (18, 19). GSK3 phosphorylates PPPSP motifs, whereas Casein kinase 1-γ (CK-1γ) phosphorylates multiple sites within LRP5/6, which in turn promote the recruitment of Axin to LRP5/6. CK-1γ isoforms within the CK-1 family carry putative palmatoylation sites at the carboxy terminal (20).

In unstimulated situations when Wnt is inactive, the transcriptional co-activator β-catenin is rendered inactive due to its phosphorylation by GSK-3. Inactivation of β-catenin is characterized by the formation of a "destruction complex" that comprises of GSK3, adenomatosis polyposis coli (APC), Axin, and casein kinase Iα (CKIα) (16). This destruction complex leads to the ubiquitination of β-catenin by an E3 ubiquitin ligase called β-TrCP and targets it for proteasomal degradation (21). As a result, β-catenin is not translocated to the nucleus and the repressor complex containing T-cell specific factor (TCF)/lymphoid enhancer-binding factor (LEF) and transducing-like enhancer protein(TLE)/Grouche binds and represses the activity of the target gene (14, 22, 23). Following the binding of Wnt to Frizzled-Axin-LRP-5/6 complex, cytosolic GSK-3β (Glycogen synthase kinase-3 beta) is sequestered, and the phosphorylation of β-catenin is blocked. The accumulation of hypo-phosphorylated β-catenin in the cytosol allows its migration to the nucleus, where it regulates target gene expression by interacting with the TCF/LEF family of transcription factors (**Figure 1**). This signaling is implicated in the regulation of cell differentiation and proliferation (3, 24).

Non-Canonical Wnt Signaling

The β-catenin-independent pathway does not involve β-catenin–TCF or β-catenin–LEF components but utilizes alternative means of downstream signaling, which may elicit a transcriptional response. These pathways are categorized

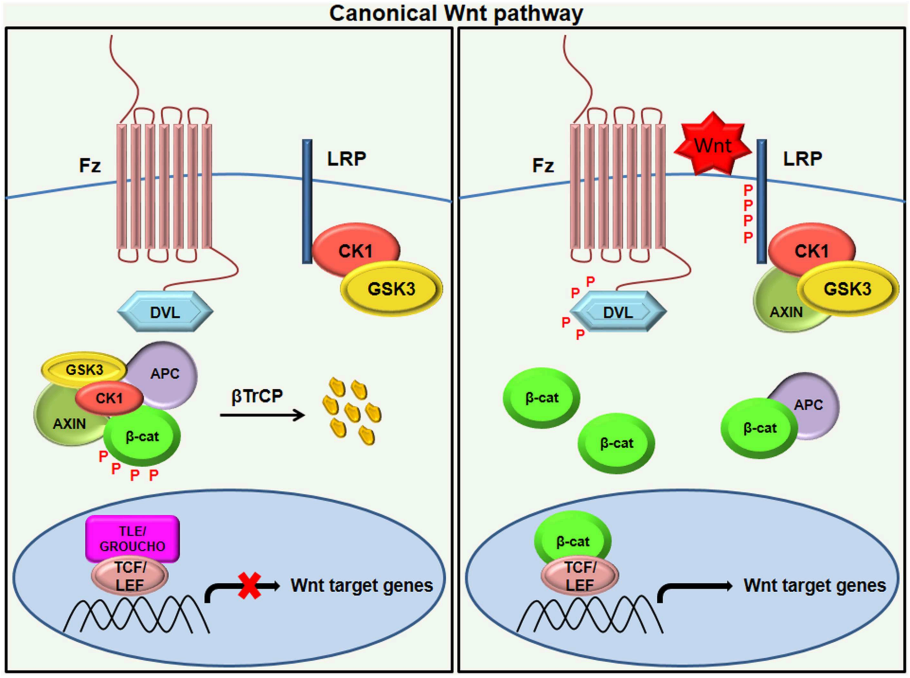

FIGURE 1 | Canonical Wnt signaling. In the absence of a Wnt ligand (left), the phosphorylation of β-catenin by destruction complex (composed of axin, APC, CK1, and GSK3β) leads to its ubiquitination by β-TrCP targeting it for proteasomal degradation. The absence of β-catenin in the nucleus results in the binding of the repressor complex containing TCF/LEF and TLE/Grouche to the target gene and thereby repressing its activity. Once the Wnt ligand binds to the Frizzled receptor and LRP co-receptor (right), LRP receptors are phosphorylated by CK1 and GSK3β, resulting in the recruitment of Dvl proteins to the plasma membrane where they activate and scaffold the β-catenin destruction complex. This results in the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus where it forms a complex with TCF/LEF and transcribes target genes.

depending on the type of Wnt receptor and co-receptor they employ and the downstream receptors they pair with (16). The non-canonical signaling majorly activates PCP, RTK, or Ca^{+2} signaling cascades through FZD and/or ROR1/ROR2/RYK co-receptors (25). The typical example of the β-catenin independent pathway is the PCP signaling pathway. Human non-canonical WNTs generally include Wnt5A, Wnt5B, and Wnt11, which transduce PCP (Planar Cell Polarity) signals through the receptors; FZD3 or FZD6; and co-receptors ROR1, ROR2, or PTK7 (26). In the PCP pathway, the Frizzled receptor activates a cascade involving a small Rho family of GTPases (Rho, Rac, and Cdc42) and Jun-N-terminal kinase (JNK) [**Figure 2**; (27, 28)]. The PCP pathway is involved in regulating cell polarity during morphogenesis. Another example of β-catenin-independent signaling is the Wnt-Ca^{+2} pathway. NFAT (nuclear factor of activated T cells) and TAK1-induced Nemo-like Kinase (NLK) are calcium regulated transcription factors of the non-canonical pathway (29, 30). The binding of Wnt ligand to a Fz receptor results in the activation of phospholipase C, which is located on the plasma membrane of the cell. This, in turn, stimulates the production of certain signaling molecules, such as diacylglycerol (DAG) and 1, 4, 5-triphosphate (IP3). IP3 triggers the intracellular release of Ca^{+2} ions and activation of effector molecules like protein kinase C (PKC), calmodulin-dependent kinase II (CAMKII), and calcineurin. This consequently activates the transcriptional regulator NFAT (**Figure 2**). The Wnt/Ca^{+2} pathway is implicated in cancer, inflammation, and neurodegenerative diseases (31).

TUMOR MICROENVIRONMENT

The cooperative interaction between cells and their microenvironment is imperative for normal tissue homeostasis as well as for tumor growth (32). The tumor microenvironment (TME) has a pivotal role in modulating the metastatic properties of cancer cells and, thus, cancer progression. A range of stromal cells in the adjoining environment are recruited to tumors and facilitate the metastatic distribution to even the most remote organs (33). Solid tumors are not just the arbitrary combination of cells and the extracellular matrix (ECM), they also include adjoining blood vessels, i.e., vasculature and multiple cell types (fibroblasts, endothelial, immune cells, etc.), signaling molecules, and ECM components, which develop complex interactions and start utilizing processes that are similar to those used by developing organs (34). In addition to the abovementioned components of TME, some other components also exist; for example, adipose cells and neuroendocrine cells within the tumor microenvironment also have an important role in tumor aggression and invasiveness. Recent developments in tumor

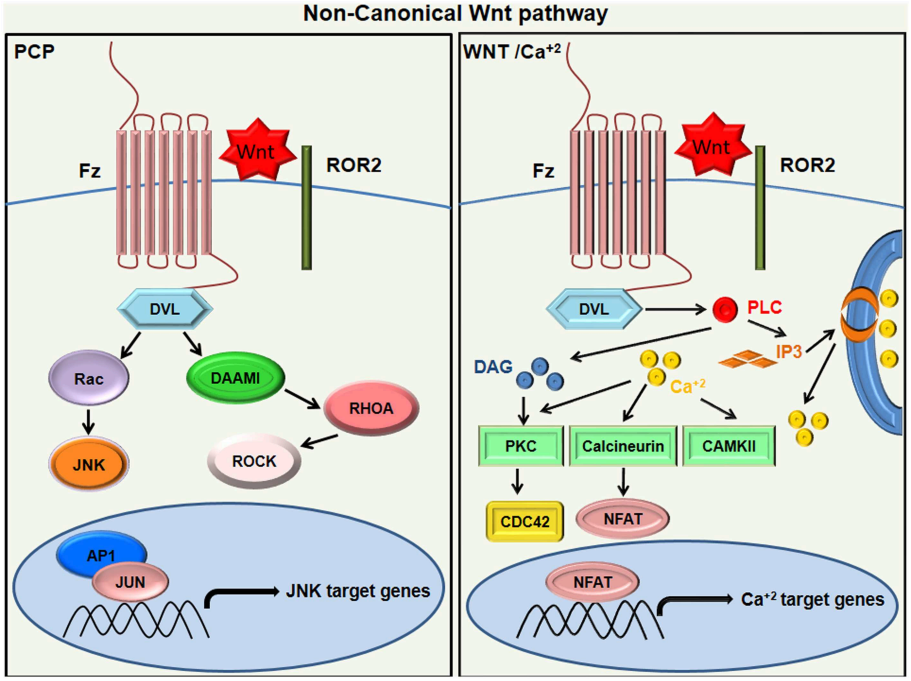

FIGURE 2 | Non canonical Wnt signaling. In Wnt/PCP signaling (left), the binding of Wnt ligands to ROR-Frizzled receptor complex results in the activation of Dvl. The activated Dvl triggers the activation of small GTPase Rho by the de-inhibition of cytoplasmic protein DAAM. Rac1 and Rho together trigger ROCK and JNK and promote polarized cell migration. On the other hand, the WNT/Ca+2 pathway (right) activates PLC to produce DAG and IP3, leading to intracellular calcium fluxes that activate PKC isoforms other than calcineurin and calcium-modulated kinases (CAMKII), which then exhibit an NFAT-dependent transcriptional response.

immunology and immunotherapy have described IL-8 as a potent biomarker in different tumors (35, 36). This may have a profound effect on the tumor microenvironment as IL-8 receptor expression is not only found in cancer cells but also in endothelial cells, TAMs, and neutrophils (37). The function of different cells and their markers, along with the aforementioned components, are compiled in the tabular form (**Table 1**).

Wnt Signaling and the Tumor Microenvironment

The epithelial mesenchymal transition (EMT) is an indispensable process throughout morphogenesis in which epithelial cells lose cell–cell contact, polarity, and other properties of epithelial cells and acquire the properties that are distinctive of mesenchymal cells (like increased motility). Characteristic features of EMT include the loss of E-cadherin at the plasma membrane, the gain of vimentin and fibronectin, and the increased accumulation of nuclear β-catenin. The transition from an epithelial to mesenchymal cell type requires a range of inter- or intra-cellular changes. In addition to performing normal developmental processes, EMT also plays a key role in tumor growth and progression if it goes unchecked (53, 54). The tumor microenvironment (TME) plays a crucial role in assisting cancer metastasis by inducing EMT in the tumor cells. Many different signaling pathways are known to be involved in EMT, such as TGF-β, NF-κB, Notch, Wnt, and receptor tyrosine kinase (55). In this section, we discuss the role of TME in the activation of the Wnt/β-catenin signaling pathway and, thus, epithelial to mesenchymal transition.

Besides Wnt ligands, the growth factors secreted by stromal cells of TME are also responsible for the activation of Wnt signaling in the nucleus (56). For example, stimulation of hepatocyte growth factor (HGF) in colorectal cancer cells (CRCs) promotes phosphorylation of β-catenin in tyrosine residue and its dissociation from Met (HGFR is encoded by proto-oncogene MET) and thus upregulates β-catenin expression via the PI3-K pathway. Moreover, augmented HGF levels enhance the activity of the β-catenin-regulated TCF family of transcription factors. Studies suggest that Met and β-catenin also assist the entry of cells into cell cycle and prevent them from undergoing apoptosis. For example, c-Met overexpression is significantly correlated with cervical cancer progression (57). Therefore, the crosstalk between HGF released from TME and Wnt/β-catenin in CRCs encourages tumor growth and invasion (58).

Another growth factor responsible for the activation of Wnt/β-catenin signaling is the platelet-derived growth factor (PDGF). A study by Yang et al. suggests that PDGF treatment led to the phosphorylation of p68 (a member of the DEAD box family of RNA helicases) at Y593 residue in the cell nucleus (59). Y593 phosphorylated p68 promotes the nuclear translocation of β-catenin by blocking its phosphorylation by GSK-3β and dislodging axin from β-catenin. Subsequently, β-catenin interacts with LEF/TCF in the nucleus and initiates the EMT process (59). Likewise, EGF and TGF-β also induce p68 phosphorylation at

TABLE 1 | Components of the tumor microenvironment: main markers and their key functions.

S. no.	Component of TME	Main markers	Key functions	Reference(s)
1	Vasculature	Vascular endothelial growth factor (VEGF), CD31, CD34, Placental growth factor (PlGF), Platelet derived growth factor-β (PDGF-β), TGFα	Blood vessel formation and nutrient and oxygen supply. Evacuate metabolic waste and CO_2. Help to escape immune surveillance	(38–40)
2	Cancer associated fibroblasts (CAFs)	Epidermal growth factor (EGF), Fibroblast growth factor (FGF), MMP2, CXCL12, CXCL14, Hepatocyte growth factor (HGF), VEGF, PDGF, stromal cell derived factor-1 (SDF-1) and constituents of ECM (OPN)	Integrate collagen and protein to form the Extracellular matrix (ECM), participate in wound healing, and angiogenesis. Regulate inflammation and escape damage to tissues.	(38, 40–42)
3	Inflammatory cells	HMGB1, Foxp3+, TNF-1α, IL-10, IL-12, IL-6, TGF-β, CD163+, KIR, PD-1+, IL-8, IL-4, IL-19, IL-17	Sustained immunosuppression, clearing cellular debris, and treatment of wound healing and infection. Expression of PD-L1 in TME and activation of NK cells and T lymphocytes	(35, 36, 40, 43–45)
4	Extracellular matrix (ECM)	Collagen, fibronectin, proteaglycans, laminin, laminin, vitronectin, tenascin-C, SPARC	Provides mechanical strength. Makes it difficult for drug to penetrate tumor	(46, 47)
5	Tumor associated endothelial cells (TECs)	VEGFR, EGFR, VEGF, PGE_2, TGF-β, IL-6 and IL-10, IL-8	Increased proliferation and migration properties, angiogenesis, and immune suppression	(35–37, 48–50)
6	Adipose cells	Aromatase inhibitors (AIs), methyl-CpG-binding protein 6 (MBD6)	Produce circulatory blood estrogen, vasculogenesis, inflammation, fibrosis, source of adipokines (leptin, adiponectin), remodeling ECM, recruitment of immune cells, IL-6, IL-8, CCL2, and COX2	(40, 51)
7	Neuroendocrine cells	Ki-67, IL-2, KE108, Delta-like canonical notch ligand 3 (DLL3), EGF, Chromogranin A (CgA)	Regulate secretion and motility, inflammation, and angiogenesis	(40, 52)

tyrosine and require p68 for EMT initiation. Therefore, the p-68-β-catenin axis may correspond to a common output for various signaling pathways (54).

The vascular endothelial growth factor (VEGF) is another growth factor that is regarded as the archetype molecule in malignant phenotype (38). VEGF expression and micro-vessel density are regarded as the prognostic factors for poor outcomes in various cancers (60). For instance, the overexpression of VEGFA in aggressive oral squamous cell carcinoma (OSCC) may serve a vital prognostic factor for this kind of cancer (61). In addition to VEGF, a transcription factor known as the ETS-related gene (ERG) belonging to ETS (E26 transformation-specific) family is also implicated in angiogenesis and vascular development. Overexpression of ERG in a mouse model reduces the vascular permeability and increases VEGF-dependent angiogenesis through Wnt/β-catenin signaling. This happens because ERG controls the transcription of Fzd4 receptors and stabilizes β-catenin levels in endothelial cells (62).

Cancer-associated fibroblasts (CAFs) are also known to play a vital role in shaping the immunosuppressive environment within the tumor, specifically in oral squamous cell carcinoma (OSCC), wherein CAF-educated cells suppress the T cell population more efficiently than the control cells (63). CAFs are also considered to be a main source of Wnt2 in colorectal cancer where FZD8 acts as a putative receptor of Wnt2 and is responsible for tumor growth, invasions, and metastasis (64).

Prostaglandin E2 (PGE2) is an effective mitogen that is secreted by TECs of the tumor microenvironment. It activates β-catenin signaling and help in the proliferation of colon cancer cells. Once stimulated, PGE2 can trigger EP2 receptors linked to the heterotrimeric G protein of Gs family. The activated α-subunit of Gs binds to the RGS domain of axin and promotes the dissociation of GSK-3β from its complex with axin. Consequently, free βγ subunits stimulate the activity of PI3K and Akt, thereby resulting in phosphorylation and inactivation of GSK-3β. All these processes lead to translocation of β-catenin to the nucleus and stimulation of growth-promoting genes and, thus, cancer progression (65).

The interaction of inflammatory cells with cancer cells is well-studied. In colorectal cancer, the infiltrating macrophages express high levels of Wnt2 and Wnt5a in progression from normal colorectal adenoma to carcinoma. *In-situ* hybridization studies showed that transcripts of Wnt2 and Wnt5a were majorly present in the lamina propria/stroma region within the macrophages. This suggests that paracrine Wnt activation by macrophages may result in cancer progression (66). Wnt7b is another Wnt ligand that is produced by macrophages residing in the tumor (67). One study proposed a mechanism wherein macrophages produced Wnt7b and initiated canonical the Wnt signaling pathway in vascular endothelial cells (VECs) expressing LRP5 and Frizzled in a paracrine fashion. This ultimately lead to the stabilization of β-catenin and entry of VECs in the cell cycle. In the absence of death/apoptotic signal to VECs, this provided a mechanism for the stimulation of VECs via macrophages and tumor angiogenesis (68). Also, Wnt ligands secreted by tumor cells could stimulate the polarization of TAMs to M2 subtype via the canonical Wnt signaling pathway, resulting in tumor growth and migration (69). Moreover, macrophage-derived soluble factors also induced canonical Wnt signaling pathway and promoted tumor growth and metastasis. For example, tumor cells induced the release of IL-1β from macrophages, thereby inducing the phosphorylation of GSK3β and stabilizing β-catenin. This results in higher expression of Wnt target genes in cancer cells. The

constitutive expression of STAT1 in macrophages is required for activation of IL-1β, which is essential in order for these macrophages to induce Wnt signaling (70).

Components of the extracellular matrix (ECM) also regulate tumor cells, particularly of the colon. Wnt ligands are expressed in both epithelial and mesenchymal cells of the colon. Moreover, aberrant Wnt signaling is also associated with the development of colorectal cancer (71). Mesenchymal forkhead transcription factors, *Foxf1* and *Foxf2*, can promote ECM production in the gut and can limit paracrine Wnt signaling. A study by Ormestad et al. suggested a crosstalk between stromal cells and parenchymal cells, which involves Wnt signaling. According to their findings, deletion of *Foxf1* and *Foxf2* resulted in the enhanced expression of Wnt5a in the mesenchymal cells and nuclear translocation of β-catenin in epithelial cells. This resulted in over-proliferation of intestinal cells, which are also resistant to apoptosis (72).

Altogether, these studies bring about the crucial role of different components of the tumor microenvironment in activation of Wnt/β-catenin signaling and, therefore, tumor invasion and metastasis. The effect of Wnt/β-catenin signaling on tumor immunomodulation is depicted in **Figure 3**.

WNT SIGNALING IN HAEMATOPOIESIS AND IMMUNE CELL MAINTENANCE

WNT signaling works in a context-dependent manner. Under different physiological conditions, it can positively or negatively regulate immunosurveillance mechanisms in the tumor microenvironment. Wnt signaling is an important pathway for immune cell maintenance and renewal. It regulates the progenitor cell homeostasis, thereby controlling hematopoiesis. Various Wnt ligands such as Wnt5a, Wnt10b, and Wnt16 have been reported in regulating hematopoiesis (73–75). Apart from Wnt ligands, downstream molecules of Wnt signaling also control the development and differentiation of various hematopoietic cells. β-catenin is one of the most indispensable molecules for both canonical and non-canonical Wnt signaling and has been shown to positively regulate hematopoiesis, which confers an increased population of hematopoietic stem cells both *in-vitro* and *in-vivo* (76). GSK-3β inhibitors activates Wnt signaling by blocking degradation of β-catenin in murine and human HSCs. *In-vivo* administration of GSK-3β inhibitors has shown potential effects in enhancing the HSCs engraftment during bone marrow transplantation models (77). Besides its role in hematopoiesis, Wnt signaling is also demonstrated to play a role in thymocyte development where it helps in proliferation of immature thymocytes (78). Wnt signaling is involved in consecutive thymic selection events leading to maturation of naive T cells. Wnt inactivation by Dickkopf-related protein 1 (DKK1) in postnatal mice resulted in the loss of progenitor thymic epithelial cells and thymic degeneration (79), highlighting the importance of the presence of Wnt in the microenvironment. Wnt signaling also plays a crucial role in the B-cell development where it controls proliferation and survival of progenitor B-cells. Lef-1-deficient mice showed defects in progenitor B-cell proliferation and survival (80). Recently, it has been shown that the canonical and non-canonical Wnt signaling could contribute to the maintenance and differentiation of B-1 cells, a subpopulation of B cells localized in the peritoneum (81). Thus, Wnt signaling plays a pivotal role in maintenance of hematopoiesis and renewal of immune cells in circulation and thereby positively regulates the anticancer immune response.

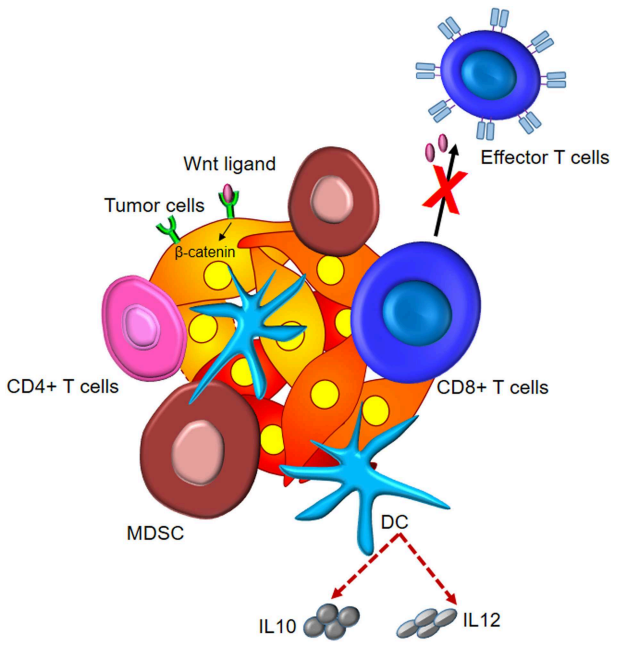

FIGURE 3 | Tumor immunomodulation by Wnt/β-catenin signaling. Wnt signaling induces elevated levels of intracellular β-catenin in malignant cells. This leads to the subsistence of Tregs, differentiation of CD4+ T cells to Th17 subtype, and secretion of IL10 and IL12 by DCs and dampened effector differentiation.

Wnt Signaling Dampens the Antitumor Immune Response in the Tumor Microenvironment

Wnt signaling also play a crucial role in dampening antitumor immune response in tumor microenvironment. There are several reports, which suggest that Wnt signaling encourages tumor progression by promoting tolerance and the immune-escape mechanism. The cumulative anti-tumor immune response mediated by T cells, dendritic cells, B cells, macrophages, neutrophils, and NK cells results in tumor regression caused by elimination of cancer cells (82). Mutations and epigenetic changes developed by tumors due to continuous stimulation by carcinogens or the environmental factors alter the signaling cascade in the tumor microenvironment (1). These changes alter the phenotypic expression of chemokines, cytokines, and some important ligands for the immune cells in the tumor microenvironment resulting in tolerance and immune escape. Recognition and elimination of the cancer cells is largely dependent on antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages, and B cells, which have the ability to present tumor associated antigens (TAAs) (83). Suppressor

cells present in the tumor microenvironment, such as Treg, myeloid-derived suppressor cells (MDSC), DC suppressor cells, etc., induce tolerance and tumor progression (84, 85). Wnt signaling has shown a contradictory role in the regulation of MDSCs; on one hand it inhibits MDSC maturation, but, on the other hand, it promotes VEGF expression, which positively regulates MDSCs (86, 87). Also Wnt 5a induces IL12 expression by the dendritic cells altering the suppressive function of MDSCs (88, 89). Despite MDSCs suppression by Wnt signaling, its presence in the Wnt active tumor microenvironment is highly contradictory. This was partially explained with the presence of Dkk1 secreted by the tumor stroma, which inhibits Beta-catenin in MDSCs (90). To understand this puzzle of interplay between Wnt signaling and MDSCs, further study is needed that can correlate various driving factors working in different context. Wnt signaling shows a negative correlation with TAA presentation. Tumor-induced β-catenin signaling functions in DCs to execute an exhaustive immune-effector phenotype in the infiltrating antitumor CTLs (91). Wnt3a regulates canonical β-catenin signaling in DCs, whereas the non-canonical signaling cascade is regulated by Wnt5a, leading to a tolerogenic DC phenotype (92, 93). Canonical Wnt signaling can potentially regulate DC activation and maturation (94). It has been shown that β-catenin deletion in DCs increased the surface expression of co-stimulatory markers CD80 and CD86 and decreased surface expression of co-inhibitory molecules like PDL1 and PDL2 (95). This phenotype of DC exhibits a positive correlation with antitumor immunity. On the contrary, β-catenin-active tumors do not react to anti-CTLA-4/anti-PD-1 immunotherapy (96). Therefore, a combinatorial therapy of the Wnt inhibitor specifically targeting DCs along with PD1 and CTLA4 immune therapy can work better for patients not responding to cancer immune therapy.

WNT-β-CATENIN PATHWAY: A TARGET FOR THERAPEUTIC INTERVENTIONS IN CANCER

Apart from playing a vital role in various cellular activities like organogenesis and stem cell regeneration, the Wnt/β-catenin pathway is also associated with cancers, such as colorectal, cervical, breast, lung, oral squamous cell carcinoma, and hematopoietic malignancies and their recurrence. In this context, targeting aberrant Wnt/β-catenin signaling as a therapeutic intervention to combat the abovementioned cancers seems a lucrative approach. However, the challenge here lies in identifying effective agents that can target the Wnt pathway without tampering the normal cellular functions like tissue repair and homeostasis, the renewal of stem cells, and survival. The detailed action of Wnt signaling in both canonical and non-canonical pathway has been summarized in the prior sections.

Wnt pathway is observed to be upregulated in cancers. Activation of Wnt leads to the loss of function of APC, which is a negative regulator of cell proliferation. Inhibitors of the Wnt signaling pathway can therefore have therapeutic values in cancer treatment, and multiple such targets have been identified wherein inhibitors act at different steps of Wnt signaling pathway. Broadly, these inhibitors can be classified into two categories: 1. Inhibitors of Wnt-receptor complex, and 2. β-catenin destruction complex inhibitors. The detailed mechanism of action of these inhibitors and their subtypes has been briefed below. Also, a list summarizing such drugs and their clinical trial status has been appended in **Table 2**.

Inhibitors of the Wnt-Receptor Complex

Porcupine Inhibitors

Porcupine, abbreviated as PORCN, is an O-acyltransferase (MBOAT) and plays a crucial role in Wnt ligand secretion by providing the Wnt proteins with a palmitoyl group. It recently became a highly druggable target for inhibiting Wnt signaling pathways (97, 98). Moreover, PORCN is the only enzyme specific to the Wnt cascade that is found to be upregulated in mouse cancer models, and it is often regarded as a poor prognosis marker for head and neck squamous cell cancers (99). *In-vivo* studies have shown that selectively inhibiting PORCN by using LGK974 blocks Wnt signaling and subsequently tumor growth (100). Independent study has shown effectiveness of LGK974 *in-vitro* on head and neck cancer cells with NOTCH1 mutations. This molecule is now undergoing phase 1 and phase 2 clinical trials. Another promising molecule for PORCN inhibition is an oral, selective small molecule inhibitor, ETC-159, of porcupine, which has shown favorable results in preclinical studies and has now entered phase 1 of clinical trials (99).

Antibodies Against Wnt Family Proteins

Several tumors have been marked to be overexpressing Wnt ligands and/or their receptors. This specific interaction is also an attractive target, and many groups are working on antibodies that can act at this point and lead to the inhibition of further downstream signaling. Monoclonal antibodies (MAbs) designed to bind Wnt1 and Wnt2 have shown to lead to tumor suppression in a plethora of malignancies, including, but not limited to, melanoma, colorectal cancers, and non-small cell lung carcinoma (101).

OncoMed Pharmaceuticals/Bayer manufactured a MAb that can target 5 Frizzled receptors and named it as OMP-18R5 or Vantictumab (101). Its safety and efficacy in many cancers are being evaluated alone or combined with other chemotherapeutic regimes. A phase Ib study of a combination of OMP-18R5 with other drugs was carried out in patients with stage IV pancreatic adenocarcinoma and metastatic HER2-negative breast cancer (102, 103). Another recombinant fusion protein that blocks the Wnt signaling is OMP-54F28 (or Ipafricept). A first-in-human phase I study of this decoy receptor for Wnt ligands is presently being carried out in patients with advanced stages of solid tumors (104). It binds to the Wnt ligand through the adomain present in the extracellular part of the human Frizzled 8 receptor (fused to a human IgG1 Fc fragment). This also binds to Wnt ligands and blocks the downstream signaling. In xenograft models of ovarian cancer, Ipafricept has shown a reduction in the frequency of stem cells, suppressed tumor formation, and stimulate differentiation. Also in a combinatorial approach, treatment with OMP-54F28 prior to taxane chemotherapy displays synergy and, therefore, superior antitumor efficacy (105).

TABLE 2 | List of drugs for specific diseases under clinical trials and their targets.

S. no.	Name	Company	Target	Disease	Clinical phase
1.	OMP18R5 (vantictumab)	OncoMed Pharmaceuticals	frizzled	Solid tumors	Phase I (dose escalation study)
2.	OMP-54F28	OncoMed pharmaceuticals/bayer	Wnt	Solid tumors	Phase I
3.	LGK974	Novartis pharmaceuticals	Porcupine	Melanoma, breast cancer, and pancreatic adenocarcinoma	Phase I
4.	CWP232291	JW pharmaceutical	β-catenin	Acute myeloid leukemia	Phase I
5.	PRI-724	Prism/Eisai pharmaceuticals	β-catenin/CBP	Advanced myeloid malignancies	Phase I (dose escalation study)
6.	IWR1	Tocris bioscience	Tankyrases 1, 2 inhibitor	Osteosarcoma	Preclinical
7.	XAV939	Novartis	Tankyrases 1, 2 inhibitor	Neuroblastoma	Preclinical
8.	NSC668036	Tocris bioscience	Disheveled	Fibrotic lung disease	Preclinical
9.	ICG-001	Prism pharma	CREB binding protein/ CBP	Acute myeloid leukemia Chronic myeloid leukemia	Phase I Phase II
10.	DKN-01	Leap therapeutics	DKK, dickkopf-related protein	Multiple Myeloma	Phase I, II

β-Catenin-Destruction Complex Inhibitors

Tankyrase or PARP5 Inhibitors

The Wnt/β-catenin pathway is associated with the PARP [Poly (ADP-ribose) polymerases] family of proteins. Of the PARP family, two isoforms, namely PARP5a (Tankyrase 1) and PARP5b (Tankyrase 2), are known to degrade the axin by the ubiquitin–proteasome dependent pathway (106). Inhibitors of these isoforms, XAV939 and IWR-1, help in maintaining the optimum Axin levels. Another inhibitor- NVP-TNKS656 used in murine xenografts models and colorectal cancer patient-derived sphere culture studies showed a high β-catenin level even in the presence of AKT and PI3K inhibitors signifying that the tankyrase inhibitor could overcome resistance to AKT and PI3K inhibitors. It was also associated with high FOXO3A (Forkhead box O3) activity. The setback of this study lies in the trepidations of gastrointestinal toxicity associated with these inhibitors, and further studies are needed to verify their safety and efficacy. Selective tankyrase inhibitors, MN-64 and CMP8, with the capacity to bind to the nicotinamide subsite of tankyrases are amongst the best inhibitors and have demonstrated nanomolar potencies (107).

Disheveled Inhibitors

Disheveled binds to the frizzled receptor on its C-terminal region via its PDZ domain. This Frizzled–disheveled interaction is a target for some notable inhibitors like NSC668036, FJ9, and 3289–8625, leading to inhibition of the Wnt signal transduction pathway and thereby causing cancer regression (108). Sulindac is a FDA approved disheveled inhibitor which can inhibit the proliferation of lung cancer A549 cells (108, 109).

Inhibitors of Transcription Complex

Wnt signaling involves a plethora of intermediate steps, and there is therefore a constantly evolving pursuit to find agents that can target the downstream steps of this pathway. A high-throughput ELISA-based screening was carried out to shortlist small molecules that could possibly target the interaction between β-catenin and the transcription factor TCF4. Eight inhibitors were identified to be effective in perturbing the β-catenin/TCF complex in a dose-dependent manner. LF3, a 4-thioureido-benzenesulfonamide derivative, possesses the capacity to perturb this interaction in colon cancer (110). However, this interaction is not very specific, causing many off-target effects.

Antagonists of Wnt Co-activator

The prerequisite step for Wnt activation is the interaction of β-catenin with its transcriptional co-activator CBP. Small molecule antagonists that have the capacity to inhibit this interaction can serve as a potential therapeutic agent. CBPPRI-724 is a first-in-class antagonist that acts in line with this approach. This molecule has shown promising results in the preclinical stage with pancreatic cancer cells. It has been proven to promote differentiation of CSCs, inhibition of stroma formation, and decrease the metastatic potential of cancer cells (111).

Wnt5a Mimetics

Wnt5a acts as a tumor suppressor in various cancers, and its downregulation is often associated with lower disease-free survival in primary breast cancers and also in hematopoietic, prostate, and colon cancers. Foxy-5 is a hexapeptide mimic of Wnt5a which is synthesized to possess Wnt5a-like properties that can impair cancer cell migration. After a successful phase 1 study in colon/breast/prostate cancer patients showing no toxicity, phase 1b trials are ongoing and expected to show promising therapeutic value (112). Also, peptides derived by modifying the Wnt5a ligand sequence have shown the capability to mimic the Wnt5a molecule. It can bind to the Fzd-5 receptor in a human breast tumor cell line and impair metastatic ability (113).

Gamma Secretase Inhibitor

Wnt signaling has now been shown to be closely associated with other pathways, like the Notch signaling pathway, and Notch1 is thought to be acting as a connecting link between them.

Treatment of CSCs with GSI agents leads to the induction of apoptosis and inhibition of tumor sphere formation of $CD44^{+}$ CSCs. MK-0752 in combination with ridaforolimus (MK-8669) is currently in phase I trial of patients with solid tumors (114). MK-0752 with docetaxel has entered phase II trials in breast cancer patients (115). Another selective GSI, PF-03084014, showed a reduction in tumor cell migration and mammosphere formation *in vitro*, and a marked decrease in tumor cell self-renewal ability *in vivo* (116). The phase I trials in triple negative breast cancer patients in combination with docetaxel, however, showed gastrointestinal toxicity.

Hedgehog Inhibitors

Another pathway involved in crosstalk with the Wnt pathway is the sonic hedgehog pathway with sFRP-1 being the mediator between them. FDA-approved SMO (Smoothened) inhibitor-Vismodegib binds directly to SMO and inhibits the progression of advanced basal cell cancers (117). It is also being studied in the context of other cancers, like gastric and prostate cancer, and is currently undergoing phase I and II trials. Another FDA-approved SMO antagonist is NVP-LDE225 (Erismodegib or sonidegib), used for advanced cases of basal cell carcinomas (118). Erismodegib is also being studied in phase I and II clinical trials for other cancer types.

Apart from different chemical inhibitors, many natural compounds have shown to target the Wnt pathway directly or indirectly. For example Indole-3-carbinol (I3C), a natural compound present in broccoli can inhibit WWS1-mediated proteasomal degradation of PTEN, which has a direct interaction with the Wnt/beta-catenin pathway, leading to tumor regression

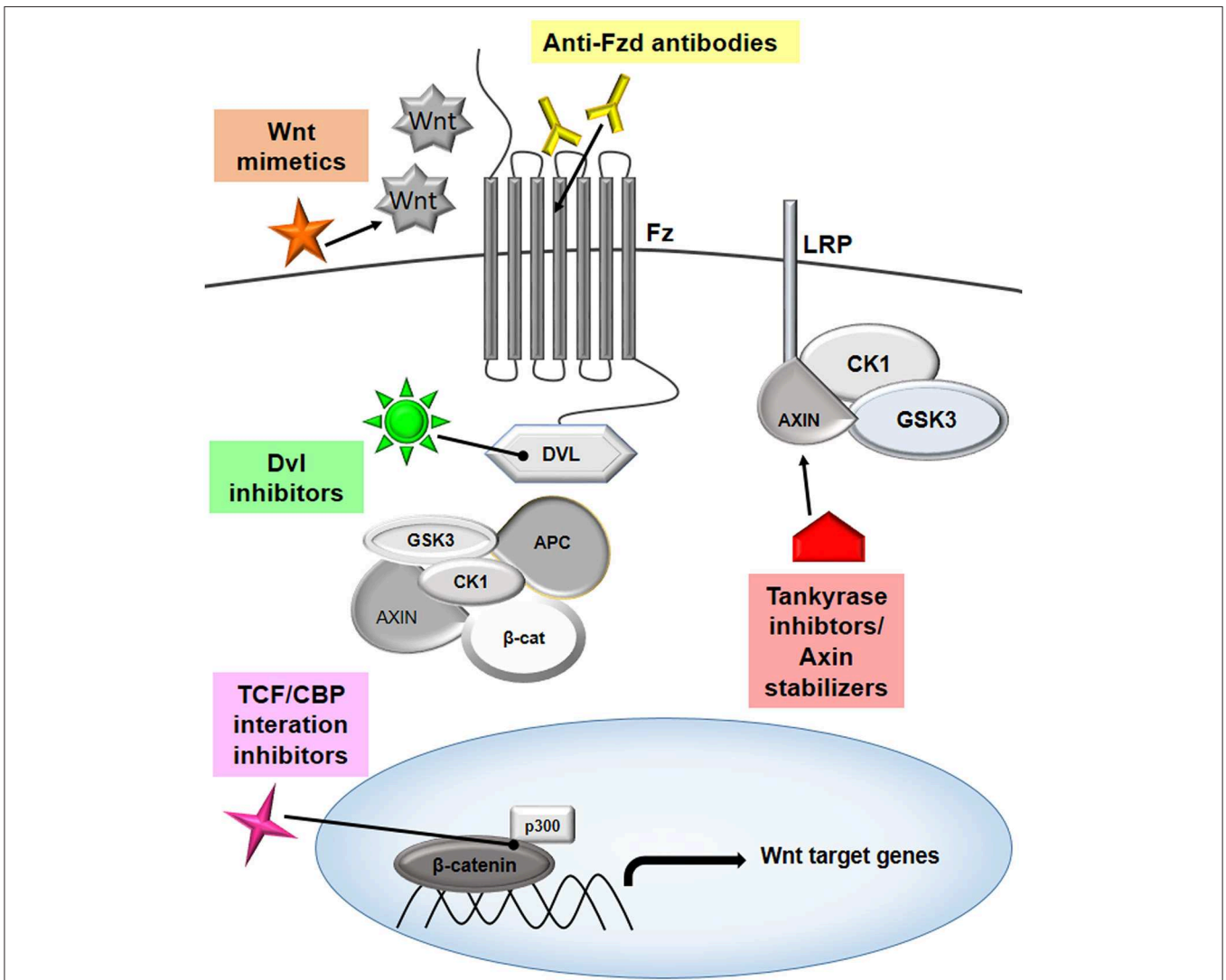

FIGURE 4 | Targeting Wnt signaling to combat cancer. The Wnt pathway has multiple players that can be effectively targeted to modulate the signaling cascade, thereby inhibiting cancer proliferation. The targets that are showing promising results are highlighted in the figure. Several therapeutic molecules, such as anti-Frizzled antibodies, mimetics of Wnt molecules, disheveled inhibitors, tankyrase inhibitors, axin stabilizers, and inhibitors of TCF/CBP interactions are being evaluated in different phases of clinical studies.

both *in vitro* and *in vivo* (119, 120). Also, there are various repressor proteins reported to downregulate β-catenin. One such protein is SMAR1, a tumor suppressor known to be downregulated in higher grades of cancer and that inhibits β-catenin transcription (121, 122). It inhibits β-catenin transcription as well as negatively regulate mir371-373, which is known to target DKK1—an inhibitor of Wnt signaling (121, 123, 124). Aberrant Wnt signaling or CDC20 mediated degradation, downregulate SMAR1 expression thereby promoting tumorigenesis and cancer progression (125). Compounds such as I3C (119) and putative SMAR1 stabilizing compounds can be potential therapeutic targets to inhibit Wnt/β-catenin pathway in different types of cancer cells, thereby regressing tumors.

To conclude, Wnt signaling can be targeted at various steps to develop potential therapy against cancer, and this is summarized in **Figure 4**.

Challenges of Wnt Inhibition-Based Therapies

Inhibiting cancer by targeting the Wnt signaling pathway has been a hotspot for the last four decades. Various molecules involved in this pathway have been studied in depth and proposed as innovative targets for anti-tumor therapy, and some have also found uses in the treatment of neurodegenerative disease, such as Parkinson's disease. Despite showing promising results, no such drugs have been approved for clinical use. This may be attributed to the fact that Wnt/β catenin signaling is crucial for stem cell pool maintenance and also in the regeneration of tissues and organs. Thus, tweaking this pathway can also affect the normal Wnt-dependent activities. Some of the drugs targeting Wnt signaling have shown dose-limiting gastrointestinal toxicity and upregulation of markers for bone formation and growth. Apart from direct roles, Wnt signaling is also known to cross functions with other pathways that are involved in cell signaling. A better understanding of these crosstalks and the development of combination therapy that can target specific molecules without disrupting normal cellular functions or using natural compounds may be the choice of future research.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Tumor cells require a milieu of growth factors, secretory molecules, and crosstalk between different cells present in the tumor microenvironment for their growth and proliferation. An increasing amount of evidence suggests that Wnt signaling acts as bridge between tumor cells and the tumor microenvironment for their preferential growth and progression. Stromal cells and inflammatory cells present in the extracellular matrix of the tumor microenvironment secrete Wnt ligands that promote tumor invasion, metastasis, and tolerance. Although Wnt signaling is important for hematopoiesis and immune cell development and proliferation, the larger picture suggest that a context-dependent regulation of Wnt signaling in the tumor microenvironment renders immune cells tolerance toward immune escape by inhibiting tumor antigen presentation. Various reports unanimously support the hypothesis that both the canonical and non-canonical Wnt pathway in the tumor microenvironment induce a signaling cascade, leading to EMT, metastasis, and cancer stem cell maintenance. With the advent of new high throughput technologies, we are in a better state of understanding of the mechanism of both the canonical and non-canonical Wnt signaling pathway. This understanding is the basis of translational research targeting the important molecules of this pathway and their downstream effectors in cancer and leveraging this knowledge toward designing personalized medicine. Several Wnt/β-catenin inhibitors that have the potential for use in anticancer therapies have been recognized. Clinical trials for these drugs are in various stages, and some of them are showing immense potential for future anticancer therapy. Further, these inhibitors, in combination with existing immune therapy or using natural compounds, can do wonders in eliminating higher grades of cancer and metastatic tumors. However, there remains a scope for further study to investigate and counter the side effects of harnessing the Wnt/β-catenin signaling, as it is important for cellular homeostasis.

AUTHOR CONTRIBUTIONS

Conception of idea was done by SC, AA, and SP. Manuscript writing and editing was done by all the authors.

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REVIEW
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WNT Signaling in Tumors: The Way to Evade Drugs and Immunity

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WNT/β-catenin signaling is involved in many physiological processes. Its implication in embryonic development, cell migration, and polarization has been shown. Nevertheless, alterations in this signaling have also been related with pathological events such as sustaining and proliferating the cancer stem cell (CSC) subset present in the tumor bulk. Related with this, WNT signaling has been associated with the maintenance, expansion, and epithelial-mesenchymal transition of stem cells, and furthermore with two distinctive features of this tumor population: therapeutic resistance (MDR, multidrug resistance) and immune escape. These mechanisms are developed and maintained by WNT activation through the transcriptional control of the genes involved in such processes. This review focuses on the description of the best known WNT pathways and the molecules involved in them. Special attention is given to the WNT cascade proteins deregulated in tumors, which have a decisive role in tumor survival. Some of these proteins function as extrusion pumps that, in the course of chemotherapy, expel the drugs from the cells; others help the tumoral cells hide from the immune effector mechanisms. Among the WNT targets involved in drug resistance, the drug extrusion pump MDR-1 (P-GP, ABCB1) and the cell adhesion molecules from the CD44 family are highlighted. The chemokine CCL4 and the immune checkpoint proteins CD47 and PD-L1 are included in the list of WNT target molecules with a role in immunity escape. This pathway should be a main target in cancer therapy as WNT signaling activation is essential for tumor progression and survival, even in the presence of the anti-tumoral immune response and/or antineoplastic drugs. The appropriate design and combination of anti-tumoral strategies, based on the modulation of WNT mediators and/or protein targets, could negatively affect the growth of tumoral cells, improving the efficacy of these types of therapies.

Keywords: WNT, β-catenin, ABCB1, PD-L1, CD47, multidrug resistance (MDR), immunity escape, cancer

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INTRODUCTION

Tumors consist of a heterogeneous mix of cellular populations composed of a small number of cancer stem cells, stromal cells, and tumor infiltrating immune cells, among others. Supporting this idea, several observations have indicated that each specific tumor contains subclones with a wide assortment of gene mutations and promoter hypermethylation in all the cells that constitute the tumor and its microenvironment (1–3).

Currently, two proposals seek to explain tumor heterogeneity. The clonal evolution model proposes that arbitrary mutations in each tumor could generate clones with acquired advantages under adverse selection requirements such as oxygen and/or nutrients. Such clones will expand while the others, less adapted to these conditions, will disappear. Different requirements for tumor growth may be present in different areas of the tumor or at different times or, furthermore, clones with better resistance mechanisms could became advantageous after therapy application. With this proposal, all the cells within a tumor could regenerate the tumor in a different location. The cancer stem cell (CSC) model assumes that only the subset of cancer stem cells on each tumor possesses the capacity to initiate and maintain tumor growth (4). Thus, CSCs are responsible for tumor heterogeneity by generating a variety of cell types that can be reverted, since the terminally differentiated cells can go back and gain CSCs properties under specific conditions. This concept of cellular plasticity unifies the two current models proposed (5–10).

Normal stem or differentiated cells could originate CSCs through a process of consecutive mutations that lead to the acquisition of characteristic cancer stem cell properties of self-renewal, pluripotency, tumor reconstitution capacity, chemo-resistance, low immunogenicity, and/or immune escape capacity. Drug resistance could be mediated by several mechanisms such as the acquisition of quiescence, improved DNA repair, drug efflux capacity, decreased apoptosis, and interaction with the microenvironment (11). Low immunogenicity and/or immunity evasion could be acquired by different strategies such as the production of immunosuppressive molecules; the recruitment of regulatory cells of the immune system; the expression of inhibitory molecules of the anti-tumoral immune response; the loss of tumor antigen expression, the downregulation of major histocompatibility complex I and II (MHC-I and MHC-II) expression, and/or the inhibition of co-stimulatory molecules on antigen presenting cells. These characteristics, mean that the CSCs are the main players in tumor initiation, progression, invasion, metastasis, drug-resistance, and recurrence (11).

The oncogenic properties of CSCs require a number of developmental pathways previously associated to the regulation of normal stem cells (12). Among them, the WNT pathway is deregulated in epithelial-mesenchymal transition (EMT) and CSCs (13). Mutations in WNT signaling components, such as APC, AXIN, β-catenin, and WNT ligands were first observed in colorectal cancers, but have also been reported in other solid tumors (14–21) and hematological malignancies, including leukemia and Multiple Myeloma (MM) (22–24).

There are different WNT pathways interconnected with each other which have been classified as canonical (β-catenin-dependent) and non-canonical signaling pathways. In general, it is assumed that the canonical WNT cascade is responsible for self-renewal, proliferation, or differentiation of progenitor cells, and that the non-canonical cascade participates in the maintenance of stem cells, cell movement, or inhibition of the canonical pathway (25).

WNT SIGNALING COMPONENTS AND PATHWAYS

WNT Ligands, Receptors, and Co-receptors

The WNT ligands family is constituted by 19 secreted glycoproteins that can participate in one or several WNT signaling pathways in a manner that can be autocrine or paracrine. They exhibit specific expression patterns and functions and are highly conserved from invertebrates to mammals (26, 27). All WNT ligands become glycosylated in the endoplasmic reticulum (ER); and also acylated by the O-acetyltransferase Porcupine (PRCN) (28–31). Next, lipid-modified WNT ligands bind to the transmembrane protein Evenness interrupted WNTless (Evi/WIs) and are transported to the plasma membrane *via* the Golgi apparatus with the assistance of the p24 proteins (32–34). Finally, the transportation of WNT ligands on the extracellular space occurs in membrane enclosed vesicles such as exosomes (28, 31, 35).

The family of Frizzled (FZD) receptors interacts with WNT ligands and with the co-receptor's low-density lipoprotein receptor-related proteins 5,6 (LRP5/6). While the complex consisting of WNT, FZD, and LRP proteins activates the canonical WNT/β-catenin signaling cascade, the complex formed by FZD and/or ROR1/ROR2/RYK (Receptor tyrosine kinase-like orphan receptor) receptors activates non-canonical WNT signaling cascades (WNT/PCP or planar cell polarity and the WNT/Ca2+ signaling cascades). The complex WNT-FZD-LRP also activates the WNT/STOP (stabilization of proteins) route which is a subtype of the non-canonical WNT signaling pathway which decelerates protein degradation when cells prepare to divide during mitosis (36–38).

WNT Canonical Pathway: On and Off

The central point of this pathway is the activation of the protein β-catenin, which can be found in the cell in different forms and locations. Thus, at the cytoplasmic membrane, β-catenin remains associated with E-cadherin and, through α-catenin, connects actin filaments to form the cytoskeleton (**Figure 1A**, left panel); in the cytoplasm, β-catenin levels are strictly controlled; and in the nucleus this protein regulates transcriptional activation and chromatin remodeling.

In the absence of ligands, the WNT pathway is inactive (**Figure 1A**, left panel) and β-catenin is continuously synthesized, ubiquitinated, and degraded in the cytosol by a destruction complex constituted by the two scaffold proteins, adenomatous polyposis coli (APC) and axis inhibition protein 1 (AXIN1), Ser/Thr kinases such as casein kinase 1 (CK1α, ε γ δ) and glycogen synthase kinase 3β (GSK3β), and two transcriptional regulators (YAP/TAZ; Yes-associated protein/transcriptional co-activator with a PDZ-binding domain) of the Hippo pathway (37–39). Thus, β-catenin will not be available for nuclear transport and transcriptional regulation.

On the contrary, when WNT binds to the FZD receptor, the canonical WNT pathway becomes active (**Figure 1A**, right panel)

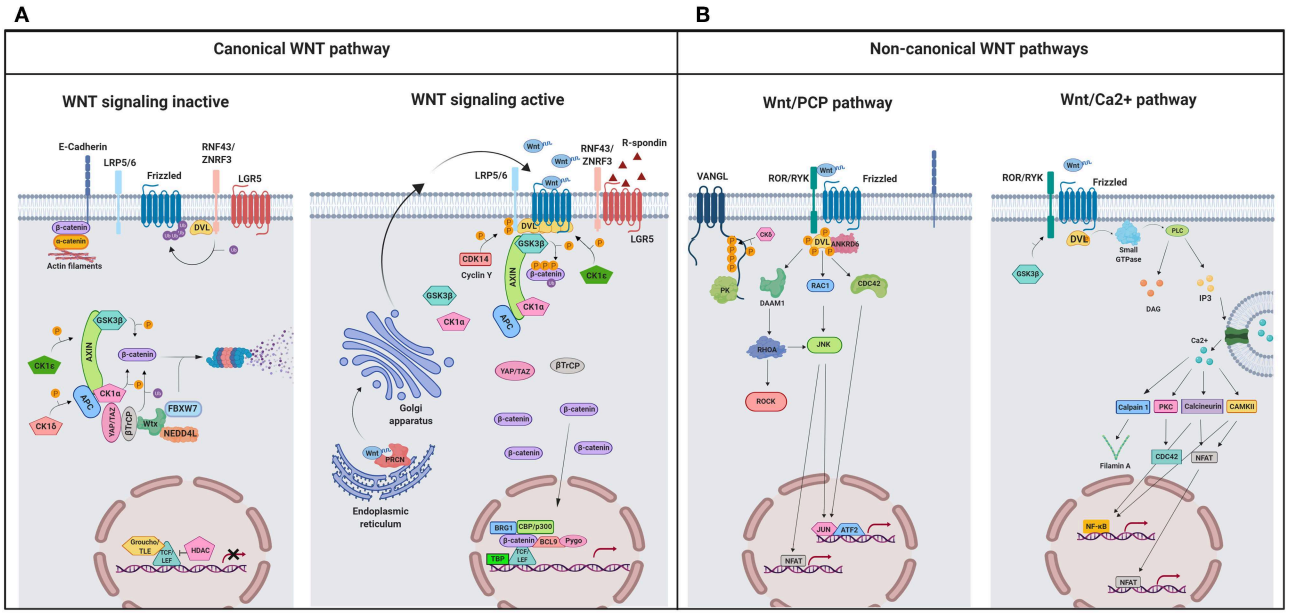

FIGURE 1 | A schematic illustration representing different WNT signaling pathways. **(A)** Canonical WNT signaling. Left panel shows inactive pathway. In the absence of WNT ligands, β-catenin is phosphorylated by the destruction complex, constituted by the scaffolding proteins APC and AXIN and the kinases GSK3β and CK1α. Then, β-catenin is ubiquitinated and targeted for proteasomal degradation by the complex containing β-TrCP, FBXW7, NEDDL4, and WTX proteins. Thus, β-catenin degradation prevents its presence in the nucleus where a complex formed by TCF/LEF and TLE/Groucho binds HDACs to inhibit transcription of target genes. Right panel shows canonical WNT signaling active. The binding of WNT ligands to FZD receptors and LRP co-receptors activates WNT signaling. LRP receptors are phosphorylated by CK1α and GSK3β. Then, DVL proteins polymerize and are activated at the plasma membrane inhibiting the destruction complex. This results in stabilization and accumulation of β-catenin in the cytosol and its subsequent translocation into the nucleus where it displaces TLE/Groucho repressors forming an active complex with TCF/LEF proteins that bind co-activators such as CBP/p300, BRG1, BCL9, and PYGO. An alternative way of β-catenin signaling includes the disruption of epithelial E-cadherin interactions, which breaks the binding of β-catenin to the cytoplasmic domain of cadherin and leads to the accumulation of β-catenin first in the cytosol, and later in the nucleus. **(B)** Schematic illustration representing the main non-canonical WNT pathways. Left panel shows the WNT/PCP pathway. WNT ligands bind to the FZD receptor and the co-receptors ROR 1/2 (or RYK). Then, DVL is recruited and activated followed by VANGL activation. Then DVL binds to the small GTPase RHO A with the collaboration of the cytoplasmic protein DAAM1. The small GTPases RAC1 and RHO activate ROCK and JNK. This leads to rearrangements of the cytoskeleton and/or transcriptional responses via for example, ATF2 and/or NFAT. Right panel shows the WNT/Ca2+ pathway. The signaling is initiated when WNT ligands bind to the FZD receptor and the co-receptor ROR 1/2 (or RYK). Then, DVL is recruited and activated and binds to the small GTPase which activates phospholipase C leading to intracellular calcium fluxes and downstream calcium dependent cytoskeletal and/or transcriptional responses. APC, adenomatous polyposis coli; BCL9, B-cell CLL/lymphoma 9 protein; β-TrCP, β-Transducin repeat-containing protein; BRG1, Brahma related gene 1; CAMKII, calmodulin-dependent protein kinase II; CBP, CREB-binding protein; CDC42, cell division control protein 42; CELSR, cadherin EGF LAG seven-pass G-type receptor; CK1α,ε,δ, casein kinase 1α,ε,δ; DAAM1, DVL associated activator of morphogenesis; DAG, diacylglycerol; DVL, disheveled; FBXW7, F box/WD repeat-containing protein 7; FZD, Frizzled; GSK3β, glycogen synthase kinase 3β; IP3, inositol 1,4,5 triphosphate; JNK, JUN kinase; LGR5, Leucine-rich repeat-containing G-protein-coupled receptor 5; LRP5/6, low-density lipoprotein receptor-related protein 5/6; NEDD4L, neural precursor cell expressed, developmentally downregulated 4-like; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; PK, Prickle; PKC, protein kinase C; PLC, Phospholipase C; p300, E1A Binding Protein p300; RAC, Ras-related C3 botulinum toxin substrate; RHOA, Ras homolog gene family member A; ROCK, Rho kinase; ROR1/2, bind tyrosine kinase-like orphan receptor 1 or 2; RYK, receptor-like tyrosine kinase; TBP, TATA-binding protein; PRCN, Porcupine; PYGO, Pygopus; RNF43, Ring finger protein 43; RSPO, R-spondin; TCF/LEF, T-cell factor/lymphoid enhancer factor; TLE, Transducin-Like Enhancer of Split proteins; VANGL, Van Gogh-like; WTX, Wilms tumor suppressor protein complex; YAP/TAZ, Yes-associated protein/Transcriptional co-activator with a PDZ-binding domain; ZNRF3, Zinc and Ring Finger 3. Created with BioRender.com.

and the recruitment of co-receptors LRP5/6 changes AXIN conformation, impairing its interaction with β-catenin and preventing the phosphorylation of this protein (40, 41). Alternatively, Li et al. (42) propose a model in which the destruction complex can bind and phosphorylate β-catenin, but the absence of β-TrCP within the complex prevents ubiquitination and degradation of the protein (38, 43), so that β-catenin accumulates in the cytosol and travels to the nucleus, where it induces the transcription of specific genes (**Figure 1A**, right panel) (40, 43–46) (listed in http://www.stanford.edu/Brnusse/WNTwindow.html).

Non-canonical or Alternative WNT Signaling Pathways

Non-canonical WNT pathways do not require β-catenin stabilization and the signal initiates through the binding of WNT to the FZD receptor without LRP co-receptor participation. Although several non-canonical WNT pathways have been identified, the best known are the WNT/PCP and the WNT/Ca2+ pathways (**Figure 1B**).

WNT/PCP signaling is implicated in the establishment of cell polarity and cell migration (31). In this pathway (**Figure 1B**, left panel), the binding of the WNT ligand initiates a cascade in which

ROR/RYK helps to transmit the signal to VANGL2 and induces its phosphorylation (47–50). Additionally, RHOA and RAC activate JUN kinase (JNK), which can induce gene transcription through the activation of AP1 (jun-ATF-2) and the nuclear factor of activated T cells (NFAT) (**Figure 1B**, left panel). A crosstalk exists between WNT/PCP and the canonical WNT pathway as it has been shown that VANG-1/VANGL negatively regulates the WNT/β-catenin signaling by a mechanism dependent on DVL (51).

The WNT/Ca2+ signaling pathway (**Figure 1B**, right panel) is activated by the binding of the WNT ligands to the FZD family of proteins, which in turn activates phospholipase C (PLC). The secondary messengers induce the release of intracellular calcium and then calcium dependent kinases such as calpain-1 and calcineurin (Cn) are activated. These kinases activate the expression of transcription factors such as NFAT and nuclear factor kappa B (NFκB). This pathway is fundamental in the regulation of cell adhesion, cell migration, and embryonic development (25).

Modulators of WNT Signaling: RNF43/ZNRF3, RSPO, YAP/TAZ, and VANGL

RNF43 (Ring finger protein 43) and its homolog ZNRF3 (Zinc and Ring Finger 3) promote poly-ubiquitination of lysines in the cytoplasmic sequence of FZDs proteins (52, 53) inducing endocytosis and the destruction of these receptors at the lysosome (**Figure 1A**) (28).

Another WNT regulator is Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), a protein that binds to the R-spondin ligand family members (RSPO) and maintains WNT signaling through the neutralization of the RNF43/ZNF3 ligases (**Figure 1A**) (36, 37, 54, 55). YAP/TAZ are also WNT regulators and compete with LRP5/6 for the same binding domain of AXIN. Thus, the association of AXIN to YAP/TAZ is incompatible with its association to LRP5/6 (**Figure 1A**, right panel) (56). Finally, Van Gogh-like (VANGL) and Prickle (PK) are two crucial components of the planar cell polarity pathway and antagonists to the canonical WNT/β-catenin pathway (**Figure 1B**, left panel). Therefore, the absence of VANGL1/2 decreases the response threshold to WNT3a, without initiating activation of canonical WNT signaling however (51).

WNT SIGNALING ACTIVATION IN HEALTHY IMMUNE RESPONSE AND IN TUMOR CELL SURVIVAL

WNT signaling should be considered a multifactorial pathway which regulates the transcription of genes that participate in most of the mechanisms used by healthy cells to differentiate and survive. Supporting this, WNT signaling plays a main role in cellular homeostasis, regulating immune cell development and function. Thus, the non-canonical WNT pathway inhibits the differentiation of quiescent HSCs by controlling β-catenin activation (57, 58). Conversely, canonical WNT signaling promotes T cell lymphopoiesis (5, 59–61). The role of WNT signaling in immunity also includes the regulation of peripheral T cell activation and differentiation. As evidence of this, TCF activity has been shown to play crucial roles in the differentiation of memory CD8+ T cells as well as in inducing specific TH subset responses (59, 61). In this sense, the non-canonical WNT signaling by WNT5A induces the secretion of IL-12, by dendritic cells favoring TH1 responses (59, 61–63). However, activation of the canonical pathway promotes TH2 polarization. Specifically, TCF1 inhibits differentiation of naive CD4+ T cells into TH1 and TH17 cells and promotes differentiation into the TH2 and TFH (T follicular helper cells) subsets. Regulatory T (Tr) cell survival is also promoted by the canonical WNT pathway, whilst the effector function and development of TH17 cells are inhibited. Nevertheless, TCF1 also limits the suppressive activity exerted by regulatory T cells in the effector T cell population. Finally, TCF1 is essential for the development of innate lymphoid cells (ILCs) natural killer (NK) cells and DC differentiation (**Figure 2**) (59, 61, 64, 65).

Since WNT signaling is crucial to immune homeostasis, alterations of this pathway in pathologies such as cancer can also be associated with the profound dysregulation of protective anti-tumoral immune responses [reviewed in (45, 66, 67)]. In fact, WNT signaling is associated with the onset of many types of solid and liquid tumors. As an example, mutations involved in the WNT signaling pathway were found in more than 90% of carcinomas of the colon and rectum [reviewed in (68)]. Related to that, it is broadly accepted that the first step in the progression to CRC frequently consists of APC gene mutations associated with the activation of the canonical WNT pathway. Supporting this, adenoma regression was observed in a murine model when APC function was recovered. Additionally, activating mutations in β-catenin, mediated or not by APC mutations, were observed in 80% of cases of CRC (69). RNF43 mutations causing WNT signaling activation were found in over 18% of colorectal and endometrial cancers (70, 71) which were extremely dependent on the WNT pathway. Since RNF43 mutations make the PDAC cell lines sensitive to treatment with Porcupine inhibitors, the growth of these tumors also relies on WNT ligand secretion. Conversely, induction of DKK1, a WNT antagonist, or treatment with an FZD-specific antibody delays PDAC development (72). Additionally, RSPO translocations are described in 4–18% of patients with gastric, ovarian, and endometrial cancer and about 9% of colorectal cancers (70). Recently, ROR1 and ROR2 co-receptor upregulation was associated to the two different melanoma states: proliferative and invasive. These phenotypes are in part the result of the balance between canonical and non-canonical WNT activation. The treatment of proliferative melanoma cells, expressing ROR1, with WNT5a induced ROR1 degradation, increased ROR2 expression and high invasiveness *in vivo*. (65). WNT signaling activation is also observed in ∼50% of patients with breast cancer and is associated with a decrease in overall survival. Although only a small percentage of tumors carry mutations of the main WNT pathway regulators, canonical WNT ligands and receptors are frequently overexpressed, whereas antagonists are downregulated. Among all the WNT regulators, high LGR4 expression was correlated with low patient survival. In Lgr4-deficient mouse models,

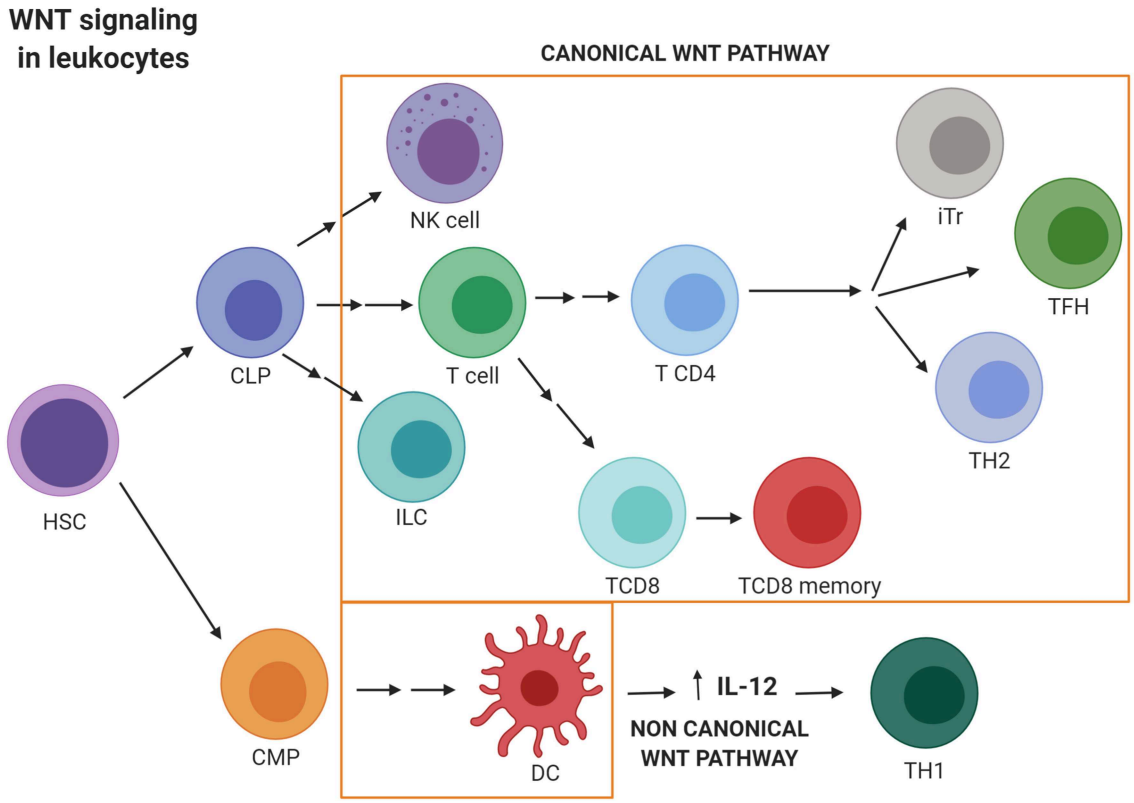

FIGURE 2 | WNT signaling activation in leukocytes. Canonical WNT signaling promotes T cell lymphopoiesis and regulates peripheral T cell activation and differentiation. Thus, canonical WNT activity promotes differentiation into the TH2 and TFH subsets. Regulatory T (Tr) cells survival is also promoted by the canonical WNT pathway. Nevertheless, TCF1 limits the suppressive activity exerted by Tr in the effector T cell population. Finally, WNT/β-catenin activation is essential for the development of innate lymphoid cells (ILCs) natural killer (NK) cells and DC differentiation. Conversely, non-canonical WNT signaling induces the secretion of IL-12 by dendritic cells favoring TH1 responses. CLP, Common lymphoid progenitor; CMP, Common myeloid progenitor; DC, Dendritic cell; ILC, Innate lymphoid cell; iTr, induced regulatory T cell; HSC, hematopoietic stem cell; NK, Natural killer; TFH, T follicular helper cell; TH, T helper. Created with BioRender.com.

researchers found a significant delay in mammary tumor development, proliferation, and the occurrence of metastasis. Further molecular analysis demonstrated that LGR4 knockdown inhibited WNT/β-catenin signaling, and the expression of epithelial–mesenchymal transition (EMT) mediators. Finally, there was a 90% decline in the number of CSCs in human breast-cancer cells and in the mouse mammary tumor virus (MMTV)-WNT1 transgenic mouse model. Thus, WNT signaling is implicated in the maintenance of mammary stem cells and breast-cancer stem cells (73–76). Supporting these results, in non-small cell lung cancer it has been shown that maintaining the lung cancer stem cell population requires the degradation of the WNT negative regulators, increasing β-catenin mediated WNT activity (77). Furthermore, WNT signaling plays a critical role in the maintenance and propagation of ovarian cancer stem cells (78); it is critical in desmoid tumor formation (79), and in head and neck squamous carcinomas (HNSCC), where researchers have shown CSC elimination after WNT/β-catenin signaling was downregulated.

WNT signaling deregulation also plays an important role in the development of hematological tumors. WNT ligands and receptors are expressed in the hematopoietic stem cells (HSC) and are present in the bone marrow (59). Thus, the WNT/β-catenin pathway is highly activated in AML (80–82), CML (83, 84), Multiple myeloma (MM), and other types of leukemia. In fact, in mouse models of AML, the WNT/β-catenin signaling pathway is required for leukemia stem cells (LSCs) self-renewal (85). Similarly, a high expression of β-catenin and WNT pathway associated genes has been observed in LSCs in CML (86). Deregulation of the WNT pathway is also associated with abnormal expression of LGR4 in cells isolated from MM patients (87, 88).

WNT signaling has been involved not only in tumor development but also in the capacity of tumoral cells to escape different types of cellular stresses, such as drug treatments and the host immune response. In this sense, many attempts have been made to find the connection between drug resistance and immune evasion (89), and common pathways controlling both processes have been explored. Within the WNT signaling pathways, many molecules have been described as being the main drivers of the transcriptional activation of the genes involved in the two mechanisms (90–94).

WNT Signaling in Tumors Drug Resistance

Cancer stem cells develop several resistance mechanisms, which protect the cells from drug damage and make them resistant to chemotherapeutic drugs (11, 12). In parallel with the models explaining tumor heterogeneity, two different proposals account for the existence of cancer drug resistance. According to the clonal evolution model, a population of tumor cells can acquire drug resistance by sequential genetic modifications. After chemotherapy, only the drug-resistant cells within the tumor survive and proliferate, regenerating the tumor bulk made up of the drug-resistant cells progeny. Thus, all tumoral cells became drug resistant. By contrast, the CSC model postulates that after drug exposure, only CSCs (which harbor intrinsic resistance mechanisms) survive. These stem cells then divide and regenerate the tumor mass that will be constituted by stem and differentiated cells, reestablishing the tumoral heterogeneity. The acquisition of drug resistance seems to have features from both models.

Generally, cancer drug resistance involves the participation of a variety of cellular mechanisms, including: drug target mutations; oncogene/onco-suppressor deregulations; activation of pathways blocking the drug action; increased DNA damage repair; overexpression of drug efflux pumps (ABC-transporters); and the induction of cell adhesion-mediated drug resistance, originating due to the crosstalk between tumor and stromal cells. Some of these resistance mechanisms, specifically the over-expression of ABCB1 and CD44 molecules among others, develop because of WNT signaling pathway deregulation in the cancer stem cell subset (**Table 1**) (104).

WNT Signaling and Extrusion of Chemotherapeutic Drugs: ABCB1

The superfamily of ABC transporters mediates the efflux or uptake of specific substrates through cellular membranes (plasma membrane, endoplasmic reticulum, Golgi apparatus, peroxisomes, and mitochondria). The number and nature of the substrates is varied and includes physiological and xenobiotic molecules. These transporters are highly conserved and present in prokaryotes and eukaryotes. The ABC transporters are characterized by two transmembrane (TMs) and two nucleotide-binding domains (NBs) or ATP binding cassettes. Human ABC transporters include 48 functional genes classified into seven subfamilies (from A to G) based on different characteristics: similarity in gene structure, order, and sequence homology in the NB and TM domains.

ABCB1 (ATP-binding cassette B1, also known as MDR-1 or P-GP) was the first cloned human ABC transporter. Physiologically, this protein, and other members of the family, transports hydrophobic, and hydrophilic compounds across the placenta, intestine, and other locations and contribute to the blood-brain barrier. Furthermore, this and other transporters actively efflux xenobiotics to protect the cells from cytotoxic agents (11). This includes the capacity to expel a variety of drugs outside the cancer cells, thus inducing chemoresistance in numerous solid tumors and hematological malignancies (13, 109, 110). In that way, ABCB1 contributes to the acquisition of a multidrug resistant (MDR) phenotype since it can bind and extrude a huge repertoire of drugs, thus leading to treatment failure and tumor relapse. Other roles attributed to these proteins include the transport of ABC proteins to intracellular (e.g., cytoplasmic vesicles) or extracellular (e.g., exosomes) compartments to improve drug sequestration (111, 112); tumor cell proliferation, invasion, and deregulation of the pathways involved in apoptosis or complement-mediated cytotoxicity (109, 110, 113–116).

Stem cells, including CSC, exhibit high expression levels of the two main MDR genes, ABCB1 and ABCG2 (ATP-binding cassette G2) (11). In addition, since the promoter of the ABCB1 gene in humans contains several TCF4/LEF binding motifs, this protein is a target gene of the β-catenin/TCF4 transcriptional regulators. In fact, activation of β-catenin augments ABCB1 expression, which confirms the direct connection between the WNT/β-catenin pathway and chemoresistance. In fact, several studies have demonstrated the existence of this connection in tumor cells which have, in this way, acquired a multidrug resistant phenotype (**Figure 3**) (117, 118).

Frizzled receptors and ABCB1-mediated multidrug resistance

FZD1 was reported to upregulate ABCB1 expression through WNT/β-catenin signaling in solid tumors and hematological malignancies. Both FZD1 and ABCB1 locate in the same region within the 7q21 chromosome. Knockdown of FZD1 induces a significant decrease of ABCB1 protein expression in a human myelogenous leukemia cell line and the recovery of chemosensitivity to antineoplastic drugs by those cells. Either way, the knockdown of ABCB1 using antisense oligonucleotides restored drug sensitivity in the previously resistant cell line (95). Downregulation of FZD1 also resulted in β-catenin degradation and therefore the absence of this protein in the nucleus. Moreover, it has been shown that the knockdown of FZD1 triggers also a decrease in the proliferative capacity of MDR leukemic cells through the WNT/β-catenin cascade. In fact, in cells from patients with acute myeloid leukemia (AML), elevated FZD1 expression at diagnosis was associated with more problems in achieving remission, and a tendency to recurrence. Thus, in patients with relapse, increased FZD1, rather than ABCB1 expression, induced high proliferation and chemoresistance in leukemic cells (96).

WNT/β-catenin signaling deregulation, specifically FZD1 overexpression, has also been observed in neuroblastoma multidrug resistant cell lines. After silencing FZD1 in MDR cells there was inhibition of β-catenin translocation to the nucleus. Consequently, ABCB1 expression and efflux activity were reduced, causing the reversal of the MDR phenotype in those cancer cells (97, 98). Another study explored the role of miR-27a in the modulation of ABCB1-mediated MDR in hepatocellular carcinoma (HCC) using the sensitive cell line BEL-7402 and its resistant counterpart, BEL-7402/5-fluorouracil. The increase in miR-27a levels was associated with enhanced sensitivity of these cells to 5-fluorouracil (5-FU), higher 5-FU-induced apoptosis and downregulation of MDR1/P-glycoprotein expression. That study showed that miR-27a reverses the ABCB1-mediated MDR by downregulation of FZD7 and the inhibition of the WNT/β-catenin pathway (99).

Furthermore, methylation of SFRPs (secreted frizzle-related protein), which inhibits WNT/β-catenin signaling,

TABLE 1 | Role of WNT components in drug resistance.

WNT component	Role in drug resistance	Type of cancer	References
FZD1 increase	Increased ABCB1 expression	Solid and hematological tumors Human myelogenous leukemia	(95, 96)
FZD1 increase	Increased ABCB1 expression and efflux activity	Neuroblastoma	(97, 98)
FZD7 increase	Increased ABCB1 expression	Hepatocellular carcinoma	(99)
SFRPs methylation	Increased ABCB1 expression	Several types of leukemia Ovarian cancer Cervical cancer Breast cancer	(100)
LGR5 increase	Increased ABCB1 expression	Colorectal cancer	(101)
YAP/TAZ increase	Increased drug resistance	Breast cancer Melanoma	(102, 103)
PYGO2 overexpression	Increased ABCB1 expression	Breast cancer	(104)
APC mutations	Increased CD44v6 expression	Colorectal cancer	(105)
CK1a and GSK3a/b inactivation	Increased drug resistance	Multiple myeloma	(106, 107)
Undefined WNT mediators	Increased CD44 expression	Multiple myeloma	(108)

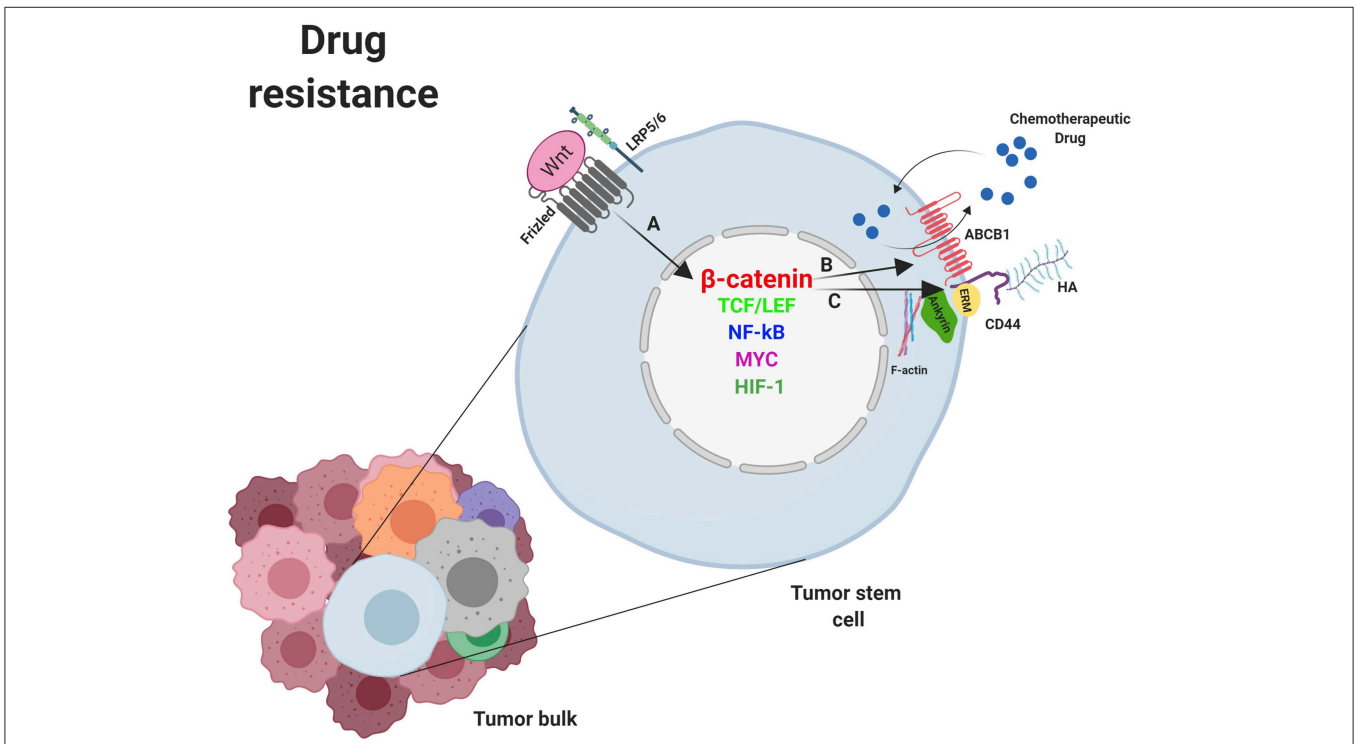

FIGURE 3 | Mechanisms of drug resistance induced by WNT/β-catenin signaling in cancer stem cells. **(A)** Nuclear β-catenin, by means of MYC, TCF/LEF, NF-κB, SNAI1, HIF-1, and other transcription factors, promotes the transcription of genes involved in mechanisms supporting tumor cells survival such as deregulation of molecules involved in drug resistance. **(B)** ABCB-1 and **(C)** CD44. Overexpression of ABCB1 and/or CD44 favor the extrusion of chemotherapeutic drugs out of the cell. ABCB1, ATP-binding cassette B1; ERM, Ezrin, Radixin, Moesin; HA, Hyaluronan; HIF-1, Hypoxia inducible factor 1; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; NF-κB, nuclear factor kappa B; MYC, Myelocytomatosis oncogene cellular homolog; SNAIL, Zinc finger protein SNAI1; TCF/LEF, T-cell factor/lymphoid enhancer factor. Created with BioRender.com.

was reported in several types of leukemia, and associated with resistance to cisplatin in ovarian cancer and with a poor prognosis in cervical and breast cancers. In fact, overexpression of β-catenin was observed in leukemia cells from patients with methylated *SFRP5*, and a demethylation compound recovered SFRP5 protein expression and reduced ABCB1 protein levels. Thus, altered *SFRP5* gene methylation may participate in the development and/or progression of different types of cancers through WNT signaling pathway activation (100).

LGR5 and ABCB1-mediated drug resistance

LGR5 is another target gene of the WNT pathway as well as a marker of colorectal carcinoma CSCs. Several studies have associated higher LGR5 levels with a poor response of colorectal cancer patients to 5-fluoracil-based treatment. In addition, LGR5 positively modulates the ABCB1 expression in CRC cells. That study, as well as others, demonstrated that in cancer cells, LGR5 promotes stem cell properties such as chemoresistance through the positive regulation of the extrusion pump ABCB1 (101).

YAP/TAZ and ABCB1-mediated drug resistance

Like other WNT regulators, the activation of YAP/TAZ supports the survival of CSCs treated with conventional chemotherapy, protecting cancer cells against DNA damage. Thus, YAP/TAZ activation protects breast CSCs from paclitaxel, doxorubicin, cisplatin, and radiations and favors resistance to therapies targeting certain molecules in tumor cells with specific oncogenic alterations. Lin et al. reported that YAP confers resistance to inhibitors of RAF and MEK signaling pathways in tumoral cell lines containing activating mutations in *BRAF*, *KRAS* or *NRAS* genes (102). Conversely, the decrease of YAP enhanced RAF and MEK inhibitor efficacy in mutant cells resistant to monotherapy with those inhibitors. Furthermore, the YAP mechano-transduction pathway is involved in resistance in melanoma and breast cancer (103).

Pygopus and ABCB1-mediated drug resistance

PYGO2 is overexpressed in several types of cancer (breast, ovarian, lung, glioma, and esophageal squamous cell carcinoma), and plays a decisive role in the carcinogenesis of these tumors. In fact, PYGO2 was the most upregulated gene in chemo-resistant breast cancer cells. Experimental results indicated that PYGO2 upregulated ABCB1 expression in resistant cells through the WNT/β-catenin cascade. As expected, PYGO2 inhibition restored drug sensitivity in MDR cells by decreasing ABCB1 expression, reducing the breast cancer stem cell subset following chemotherapy. Furthermore, RNA samples from tumors extracted by surgery from 64 paired patients significantly increased PYGO2 and/or ABCB1 expression after chemotherapy, thus underlining a crucial role for the WNT/β-catenin pathway mediated by PYGO2 in the clinical chemoresistance of breast cancer (104).

WNT and Activation of Cell Adhesion-Mediated Drug Resistance (CAM-DR): CD44

The constant interaction between the tumor and the surrounding microenvironment influences the cellular fate of CSCs. The protein CD44 is especially important in this communication process since it constitutes a platform for signaling that incorporates cellular microenvironmental information. This molecule integrates signals from growth factors, cytokines and others, and sends them to cytoskeletal proteins associated to the membrane or the nucleus where gene expression is regulated. CD44 upregulates the expression of cell cycle proteins inhibitors, anti-apoptotic proteins, and ABC transporters, and others, thus inducing a form of drug resistance known as cell adhesion-mediated drug resistance (CAM-DR).

The CD44 family is constituted by single-pass, glycosylated class-I transmembrane proteins of 85–90 kDa. The extracellular N-terminal portion of CD44 proteins binds to the glycosaminoglycan hyaluronan (HA), among other ligands, while the C-terminal cytoplasmic tail binds the cytoskeletal linker proteins ezrin, radixin, moesin (ERM), and ankyrin (119), and F-actin (120–122).

The different members of the CD44 family are originated by the alternative splicing of 10 exons which codified for the extracellular domain of the protein. This domain can be completely deleted, generating CD44s or giving rise to various combinations encoding CD44 variant members (CD44v). Although CD44 is found in many normal cell types, it is used as a surface marker for CSCs from several types of tumors (11).

CD44 is a positive regulator of the WNT/β-catenin signaling pathway through the control of LRP5/6 phosphorylation and its location at the plasma membrane (105). Therefore, the ability of CD44 to bind to the cytoskeleton might provide a platform necessary for the interaction between LRP5/6 and kinases such as GSK3β and CK1. Furthermore, the shuttle of LRP5/6-charged vesicles from the Golgi to the membrane might use the F-actin tracks anchored to the plasma membrane through the complex CD44-ERM (123).

Several articles have been published supporting a role for CD44 in CAM-DR. Thus, overexpression of CD44v6 associates with colorectal cancer in advanced stages and is characterized by mutations in the WNT pathway (e.g., APC mutations) (105). Additionally, it was reported that the degree of cell adhesion in MM showed a negative correlation with the sensitivity of these cells to doxorubicin (106). Furthermore, long term exposure of human multiple myeloma cell lines (HMCLs) to lenalidomide increased the level of resistance to this molecule (107) and the expression and activity of β-catenin with enhanced transcription of the WNT target genes cyclin-D1 and myelocytomatosis oncogene cellular homolog (*MYC*). These effects were the consequence of CK1α suppression, and GSK3α/β inactivation, since an increase in the phosphorylation of the inhibitory residues of this protein was observed. In another study in MM, CD44 was identified as the main effector molecule of lenalidomide resistance mediated by the WNT cascade. Overexpression of this protein was observed in lenalidomide-resistant human multiple myeloma cell lines (HMCLs), and in correlation with that, increased adhesion to bone marrow (BM) stromal cells was detected, indicative of CAM-DR. Inhibition of CD44 reduced the adhesion of MM cells and reversed the resistance to lenalidomide (108).

CD44 associates with ABC pumps (124, 125) such as the ABCB1 protein and regulates its gene expression (**Figure 3**). The process requires the activation of CD44 by HA binding, which promotes PI3K activation and stimulates HA production and ABCB1 expression (126). Alternatively, the binding of HA to CD44 induces the expression and activation of the transcription factor p300 which, together with β-catenin and NFκB-p65, sustains *ABCB1* transcription (127). Engagement of CD44 and

activation of specific transcription factors in EMT can also induce apoptosis resistance. Thus, it was demonstrated that the CD44–HA interaction to PKCε induced transport of Nanog to the nucleus, leading to miR-21 synthesis and upregulation of ABCB1 and apoptosis inhibitors (128, 129).

The association between ABCB1/P-GP and CD44 has also been demonstrated by experiments of co-immunoprecipitation and co-localization within the plasma membrane, and further confirmed in a yeast two-hybrid system. In another study, the examination of primary and metastatic tumor samples from osteosarcoma patients showed that a high expression of CD44 was associated with osteosarcoma metastasis and recurrence and CD44 was considered as a solid predictor for chemotherapy response and overall survival in osteosarcoma patients. The results in osteosarcoma cell lines with constitutive knockout of *CD44* gene by CRISPR/Cas9 system verified that CD44 mediates migration, invasion, proliferation and drug resistance to doxorubicin in osteosarcoma cells. In another study, the downregulation of CD44 protein by siRNA in cancer cells from patients with ovarian carcinoma confirmed that the mRNA levels of CD44 and ABCB1 correlate positively. In summary, numerous studies support the hypothesis that CD44 may contribute to tumor drug resistance by regulating ABCB1 expression (130).

WNT Signaling in Immune Escape by Tumors

The host immune system constitutes a wall against tumor formation through the innate and the adaptive immune response. The primary role of immune effectors such as macrophages, dendritic cells (DCs), and T cells is to discriminate healthy cells from pathogens or tumoral cells through receptors on their cell surface. These molecules integrate all the signals received from microorganisms and/or cells and switch the balance to activation or inhibition of the immune response. Nevertheless, cancer cells can evade detection by the immune cells through the expression of surface molecules that mimic the signals released by healthy cells. Thus, tumoral cells prevent the arrival of immune effectors to the tumor area or, in the event that effector cells infiltrate the tumor, induce their inactivation and death. These molecules expressed by healthy or tumoral cells to keep the immune system under control and their receptors are collectively called checkpoint proteins. These "immune checkpoints" normally function to control excessive immune activation but are also used by tumors to evade the immune system. Because of this, tumors can be classified based on the existence or lack of a T-cell-inflamed tumor microenvironment, and this phenotype correlates with the response to an immune-checkpoint blockade. An inverse correlation has been observed between WNT/β-catenin pathway activation and T-cell infiltration across most human cancers (131, 132). The connection between immune exclusion and the WNT/β-catenin pathway was identified for the first time in metastatic melanoma. When comparing samples categorized as either T cell–inflamed or non-T cell–inflamed it was observed that almost half of the non-T cell–inflamed tumor subset showed increased activation of the WNT/β-catenin signaling pathway. Mechanistic studies using genetically engineered mouse models confirmed that melanomas with increased WNT/β-catenin activation lacked tumor-infiltrating T cells, mimicking the non-inflamed phenotype observed in patients with melanoma. This effect is due to failed recruitment of specific dendritic cells into tumors, leading to impaired recruitment of T cells to the tumor microenvironment (131, 132). Some of the mechanisms involved in the escape of immunity develop after the activation of WNT in tumor cells. Among them, decrease of secretion by tumoral and/or stromal cells of immune cell-attracting chemokines, such as CC-chemokine ligand 4 (CCL4), should be highlighted (61). Other escape mechanisms involve the expression of checkpoint inhibitors such as programmed death ligand 1 (PD-L1) and CD47 which mainly control the activity of tumor-specific T cells and macrophages (61, 133, 134). PD-L1 and CD47 transcription is controlled by MYC, a proto-oncogene identified as a WNT target gene. Thus, mutations in components of the WNT/β-catenin signaling pathway induce aberrant MYC expression and, because of that, increased expression of the immune checkpoint proteins PD-L1 and CD47 and a non-T cell-inflamed tumor phenotype. Conversely, MYC inactivation led to a decrease in the expression of the above proteins, favoring the accumulation of tumor-associated T cells and macrophages (133–135).

Impaired DCs Recruitment: CCL4 Inhibition

Anti-tumoral immune cells such as DCs and T cells need to reach the tumor bulk to perform their anti-tumoral activity. Nevertheless, tumors develop mechanisms to avoid immunity by disrupting chemokine secretion. It has been observed that the secretion of chemokines implicated in effector T-cell recruitment is significantly reduced in several types of tumors lacking dendritic cells and CD8+ T-cell infiltrate. Among the chemokines that act on the recruitment of DCs and whose secretion is reduced in many non-T cell inflamed tumors, CC-chemokine ligand 4 (CCL4/MIP-1beta) should be highlighted (61, 136). As an example, immune exclusion mediated by activation of the WNT–β-catenin pathway was observed in melanoma when expression of CCL4 was inhibited and DCs were no longer recruited into the tumor. Consequently, no T cell priming occurs, and effector T cells were not present at the tumor area (137). Supporting the previous results, a study involving 266 cases of metastatic melanoma found that approximately one third of the melanoma metastatic lesions were non-T cell inflamed and half of them showed activation of WNT–β-catenin signaling in tumor cells. Further support that activation of β-catenin in tumors was related to T cell exclusion was obtained in a genetically engineered mouse model of melanoma which conditionally expressed a dominant stable form of β-catenin. Although all the mice developed melanoma, β-catenin-positive tumors had minimal T cell infiltration and were resistant to therapy based on checkpoint blockade. In β-catenin-positive melanoma cells, secretion of CCL4 and other chemokines was reduced, with insufficient recruitment of specific DCs into the tumor area and defective host priming of antigen-specific T cells (138, 139). Another study in bladder cancer demonstrated the WNT7/β-catenin pathway activation in non-T cell-inflamed tumors (140). Studies in melanoma cells also showed that the

CCL4 chemokine and the BATF3 transcription factor were linked to the recruitment of the dendritic cells necessary for T-cell activation (141). Additionally, gene expression of WNT7B was explored in urothelial bladder cancer and an inverse correlation with the presence of CD8 cells was found, further supporting a link between the absence of intratumoral T cells and the activation of WNT signaling. This result was also confirmed by immunohistochemistry. Indeed, detection of CD8 transcripts and BATF3 and CCL4 expression within the tumor area inversely correlated with WNT7B expression (**Figure 4**) (140).

Inhibition of Tumor-Associate Macrophages (TAM) and DCs Phagocytosis Through "Don't Eat Me" Signals: CD47

CD47 (Integrin-Associated Protein, IAP) is a transmembrane glycoprotein, member of the Ig superfamily, with an IgV-like extracellular domain and a short cytoplasmic tail. CD47 expression is ubiquitous in human cells where it is a "marker of self" functioning as a "don't eat me" signal. CD47 has been shown to interact in cis with αvβ3, αIIbβ3, and α2β1 integrins and in trans with thrombospondins (TSPs) and with regulatory molecules belonging to the signal regulatory protein (SIRP) family. SIRPα and SIRPγ are ligands for CD47. SIRPα is highly expressed on myeloid cells (dendritic cells, macrophages, and neutrophils) and smooth muscle cells and discretely expressed by cultured murine and human endothelium. SIRPγ is expressed by T-cells, NK cells, and some B-cells (142).

Interaction of CD47 to SIRP-α promotes the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) on SIRP-α, and the recruitment to the membrane of Src homology region 2 domain-containing phosphatases (SHP-1 and SHP-2), with the subsequent inhibition of myosin-IIA accumulation at the phagocytic area, blocking phagocytosis (143, 144).

CD47 can be expressed on many healthy cells in mice and humans and is highly expressed on a variety of CSCs, including both hematopoietic and solid tumors (145). Expression of CD47 in tumor cells avoid their recognition and elimination by macrophages, dendritic cells, and T cells and induces epithelial-mesenchymal transition (EMT) through modulation of N-cadherin and E-cadherin (146). In fact, CD47 protein expression was significantly high in ovarian cancer and associated with patient stage, chemotherapy resistance, and prognosis. Similar results have been observed in glioma cells, breast cancer cells (147), not small-cells lung carcinoma (NSCLC), PDAC, and others (147–150). The presence of CD47 in AML was also associated with a high self-renewal potential of cancer stem cells and with low patient survival (151, 152). CD47 overexpression also correlates with poor prognosis in head and neck squamous cell carcinoma, melanoma, and osteosarcoma (153–156).

Various transcription factors have been proposed to bind to the promoter of CD47 and explain its upregulated expression in different tumors. Some of them like NF-κB, MYC, SNAI1, ZEB1, HIF-1 and the PKM2–β-catenin–BRG1-TCF4 complex (134, 148, 157, 158) have been implicated in CD47 expression, which strongly suggests that the WNT/β-catenin pathway is involved (**Figure 4**). Supporting this idea, it has been shown that a constant activation of β-catenin is needed for glioma progression (159), and increased levels of its target genes, such as CD47, have been associated with high-grade GBM (16). Conversely, the inhibition of β-catenin in mutant glioma cells abrogated CD47 expression as well as the interaction between β-catenin and TCF4. The reverse effect was observed in the same cells upon the pharmacological elevation of nuclear β-catenin levels. As expected, the CD47 transcriptional downregulation negatively affected the phagocytosis of cancer cells by microglia (158).

In addition, miRNAs have been described as regulators of stem cells and related with the overexpression of CD47 in cancers. miR-133a acts as a tumor suppressor gene, and is downregulated in many types of tumors (160). miR-133a also regulates the transcription factor TCF7, which is essential in the activation of canonical WNT signaling (161).

In another study, miR-708 induced repression of the WNT/β-catenin signaling pathway in BCSCs, causing inhibition of self-renewal and chemoresistance in these cells. miR-708 was shown to directly bind the CD47 gene regulating the expression of this molecule and the tumor-associated macrophage-mediated phagocytosis (162).

Finally, a role for CD47 in drug resistance has been described. In hepatocellular carcinoma cells (HCC), it was found that clones resistant to sorafenib exhibited increased cancer stem cell characteristics, such as tumorigenicity, self-renewal, and invasiveness. Moreover, an increase in CD47 expression, dependent on nuclear factor kappa B (NF-κB) activation, was found. The knockdown or blocking of CD47 in sorafenib-resistant HCC cells consistently demonstrated an increased sensitization to sorafenib by these cells, suggesting that CD47 signaling might be involved in the sensitization to this drug. Therefore, CD47 may have a role through many as yet unknown pathways in drug resistance (163).

Elimination of Activated T Cells Through "Don't Find Me" Signals: PDL-1

PD-L1 is a type I single-pass transmembrane protein, member of the B7 family, organized in an IgV-like domain, an IgC-like domain, and a cytoplasmic tail (163, 164). PD-L1 interacts with the receptor, programmed cell death 1 (PD-1), on activated cytotoxic T cells through the IgV domain. Next, PD-1 forms aggregates with TCR and costimulatory receptor CD28 and recruits the SHP2 phosphatase, leading to its dephosphorylation and inactivation (165, 166). Last, effector T cells become exhausted by the decreased phosphorylation of crucial signaling molecules which regulate activation and proliferation mediated by NFAT (165–167). PD-L1 is expressed in many cell types including macrophages and dendritic cells (164), and tissues such as heart, lung, and placenta (168), and is also overexpressed with immune activation (169). The PD-L1/PD-1 interaction keeps the balance between tolerance and autoimmunity and its deficient or excess functioning can trigger several diseases, including auto-immune diseases such as arthritis and lupus (170). PD-L1 expression has been found to be positive in 5–40% tumor cells such as lung, colon, melanoma, bladder and renal and hepatocellular carcinomas, head and neck cancers, ovarian cancer, and hematologic malignancies (171), inducing

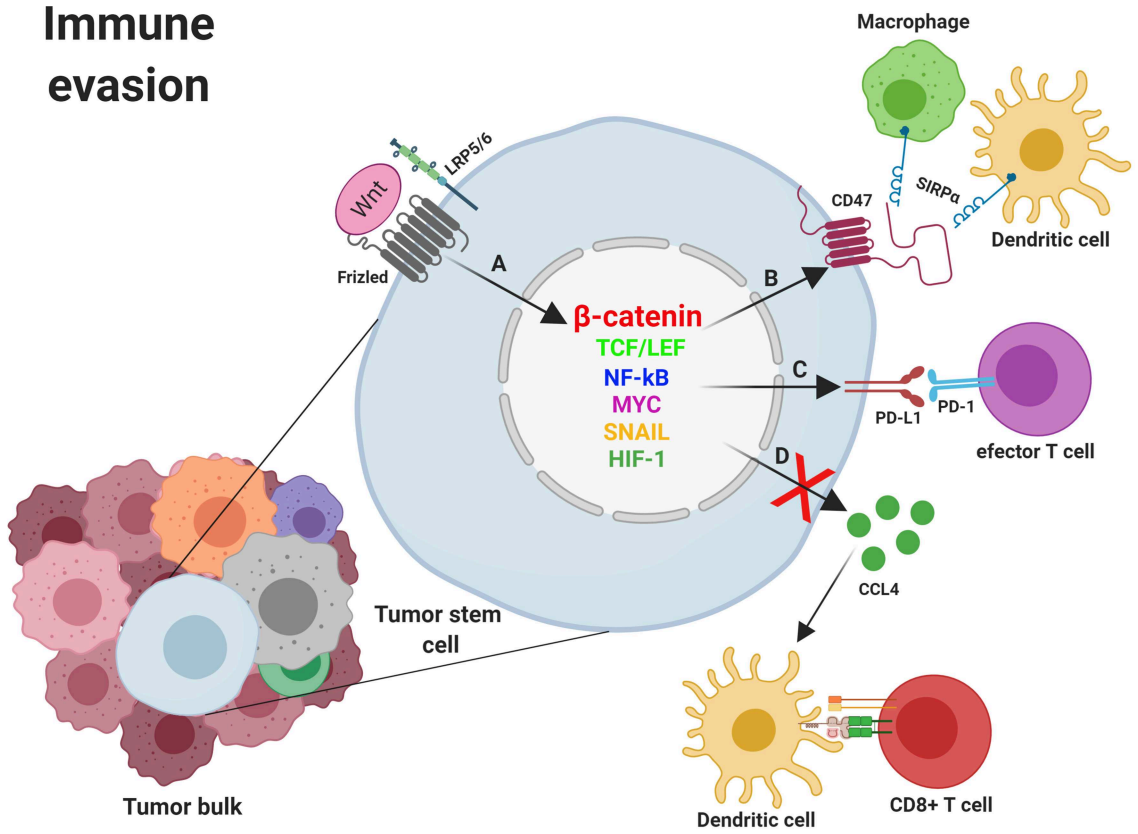

FIGURE 4 | Mechanisms of immune evasion induced by WNT/β-catenin signaling in cancer stem cells. **(A)** Nuclear β-catenin, by means of MYC, TCF/LEF, NF-κB, SNAI1, and other transcription factors, promotes the transcription of genes involved in mechanisms supporting tumor cells survival such as the deregulation of molecules involved in immune evasion (CCL4, CD47, and PD-L1). **(B)** The binding of CD47 to SIRPα might prevent phagocytosis of tumor cells (and/or fragments derived from them) by macrophages and dendritic cells with the subsequent absence of T cell activation. **(C)** Expression of PD-L1 might directly impair effector T cells function within the tumor microenvironment. **(D)** Inhibition of CCL4 secretion by tumor cells avoids Dendritic cells recruitment to the tumor microenvironment and the subsequent T cell priming and activation. CCL4, CC-chemokine ligand 4; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; NF-κB, Nuclear factor kappa B; MYC, Myelocytomatosis oncogene cellular homolog; PD-1, Programmed cell death 1; PD-L1, programmed death ligand 1; SIRPα, Signal regulatory protein Alpha; SNAIL, Zinc finger protein SNAI1; TCF/LEF, T-cell factor/lymphoid enhancer factor. Created with BioRender.com.

elimination of effector cells through interaction of PD-L1 on the surface of cancer cells with PD-1 on the T cells plasma membrane (171–174). It has also been shown that PD-L1 expression is modulated by the WNT pathway (**Figure 4**). Thus, Triple Negative Breast Cancer Stem Cells (TNBCSCs) exhibit PD-L1 overexpression through the WNT cascade and the upregulation or downregulation of this cascade significantly affects the expression of this molecule (175). In testicular germ tumors β-catenin was described as a marker of poor outcome and a positive correlation with PD-L1 expression was observed, as was a decrease in immune infiltration (176). In melanoma cells, the activation of WNT/β-catenin results in the absence of T cell infiltration in the tumor microenvironment. This effect appears to be a consequence of the action of the negative regulatory pathway PD-L1-PD-1 (141). Finally, an increase in PD-L1 in the population of breast mesenchymal-like cancer cells, but specially in breast CSCs, has been observed during EMT by the EMT/β-catenin/STT3/PD-L1 signaling axis. Specifically, it has been shown that the ER-associated N-glycosyltransferases STT3A and STT3B are required for PD-L1 induction through regulating its glycosylation. This produces PD-L1 stabilization by antagonizing β-TrCP-dependent proteasome degradation of this molecule. In this study, the authors also identified that etoposide suppressed the EMT/β-catenin/STT3/PD-L1 axis through TOP2B degradation-dependent nuclear β-catenin reduction, leading to PD-L1 downregulation of CSCs and non-CSCs, and sensitization of cancer cells to anti-Tim-3 therapy. (177). In summary, several studies support an association between WNT/β-catenin signaling and immune evasion through the regulation of the "don't find me signal" PD-L1 and other molecules involved in immune control.

NEW STRATEGIES FOR EFFECTIVE ANTITUMORAL THERAPY

Most of the anticancer chemotherapies currently being used work by killing highly proliferating cells, which, in many tumors, are

mostly non-CSCs, thus decreasing the tumor size. However, the small CSC population present in the tumor bulk is constituted by relatively slow cycling quiescent cells with a higher repair mechanism and are innately resistant to therapy. Furthermore, radiation and chemotherapy may trigger cellular stress response mechanisms enhancing stemness characteristics in non-CSCs and thereby increasing their capacity for adaptation and survival. Thus, conventional chemotherapy can increase the fraction of CSCs within a cancer, making them resistant to treatment, and re-establishing tumor growth in the same or in a distant location from the primary tumor, leading to the formation of metastases which are usually far more resistant to chemotherapy than primary tumors. This has been shown following the treatment of different tumor types, including brain, head and neck, lung, breast, and liver, amongst others (178). Therefore, in contrast to previous therapeutic approaches, new treatments need to consider strategic combinations that could kill CSCs and non-CSCs at once as well as preventing the transition from non-CSCs to CSCs.

Several anti-neoplastic drugs targeting specific molecules are currently in clinical use. One type includes inhibitors of signaling molecules such as inhibitors of tyrosine kinases, Raf, MEK, PI3K, mTOR (mammalian target of rapamycin), and WNT/β-catenin signaling pathways, which are essential for the proliferation of tumoral cells. Another type of therapy is based on obtaining and using monoclonal antibodies (mAbs) to surface proteins highly expressed in cancer cells, such as CD20 in lymphoma (rituximab), HER2 in breast cancer (trastuzumab), and epidermal growth factor receptor (EGFR) in colon cancer (cetuximab). Another type of therapy includes compounds and/or mAbs targeting molecules with a high expression in CSCs and with an essential role in drug resistance and/or tumor immune escape. Inhibitors and antibodies (Abs) specific for ABCB1, CD44, CD47, and/or PD-L1 belong to this group. The great heterogeneity of tumor cellular composition and the complex mechanisms involved in the tumorgenicity in CSCs and non-CSCs subsets requires the use of many different therapeutic approaches and targets, based on the characteristics of the tumor and the patient. Many of these approaches developed to eliminate tumor bulk are used in combination and are currently under clinical evaluation.

Targeting Molecules Involved in WNT Pathway

Targeting the WNT signaling pathway in CSCs is an interesting and promising approach for anti-tumoral therapy. Although this signaling pathway is expressed in normal cells, deregulation is found in CSCs. Compounds with WNT inhibitory properties can target the CSCs but also normal cells, inducing adverse effects. In this regard, therapeutic molecules should be modified or combined with other therapies to improve their specificity and efficiency. Thus, current chemotherapeutic strategies focus on targeting WNT signaling in specific tumor subclasses or with specific mutational characteristics. Ideally, this tumor-targeted therapy should have at least, three main effects: first, recruitment of dendritic cells, macrophages, and effector T cells in the tumor area; second, reversal of resistance to tumor drugs; and third, inhibition of tumor evasion. Since canonical WNT signaling promotes not only T cell lymphopoiesis but also regulation of peripheral immune cells activation and differentiation (61, 72), ongoing therapy should also have a minimal incidence on immune cells, specifically those infiltrated in the tumor area. Otherwise, mechanisms of anti-tumoral immunity will not function properly and the effectiveness of the immune cells fighting the tumor will be seriously compromised. Finally, given the crucial role of WNT signaling in the maintenance of stem cells and the regeneration of tissues and organs in homeostasis, unwanted side effects should be carefully evaluated. In fact, although diverse types of WNT/β-catenin pathway inhibitors are under development as anti-neoplastic therapies in many hematologic and solid malignancies, none have been approved for clinical use (**Figure 5A**) (36).

Targeting WNT Ligands

Monoclonal antibodies, recombinant proteins, and other inhibitors that neutralize WNT ligands or agonists of these molecules are under clinical investigation and have demonstrated WNT inhibition and tumor reduction in melanoma, sarcoma, colorectal cancers, non-small cell lung carcinoma, and mesothelioma. For example, a hexapeptide has been synthesized which can imitate the characteristics of the WNT5a ligand and inhibit tumor cell migration *in vitro*. That compound is being tested in a phase 1 study of patients with breast, colon, and prostate metastatic cancers (36, 37). Moreover, a WNT inhibitor which binds and sequesters WNT, blocking the signaling cascade, is in Phase 1b clinical trials (clinicaltrials.gov). Additionally, specific inhibitors have been found that neutralize Porcupine, preventing acylation, and WNT secretion (179). These inhibitors have been tested in phase I trials in melanoma, breast cancer, and pancreatic cancer, metastatic colorectal and head and neck cancers. Promising therapeutic results have also been obtained in pre-clinical models of CRC with another treatment, based on the use of a blocking antibody targeting RSPO3 which produces a loss of stemness and differentiation.

Targeting Frizzled Receptors and Co-receptors

Several trials testing Abs specific for receptors and co-receptors of the WNT signaling pathway are currently in process. For example, an anti-Fz10 radiolabeled mAb is being evaluated for the treatment of synovial sarcoma in a phase I clinical trial. Also, a monoclonal antibody specific to five human FZD receptors inhibits the growth of human tumor xenografts in mouse models. This antibody is currently included in phase I clinical trials used as monotherapy or in combination with taxanes in breast and pancreatic cancer and non-small cell lung cancer. It is also being used in combination with conventional chemotherapy in phase 1b clinical trials with breast, liver, ovarian, pancreatic, and lung cancer patients (180). Additionally, the use of Abs conjugated to cytotoxic drugs targeting LGR5 has demonstrated therapeutic effectiveness in xenografts and in genetic mouse models of CRC.

Disheveled Inhibitors

A non-steroidal anti-inflammatory drug (NSAID) which targets DVL is currently undergoing testing in phase II trials,

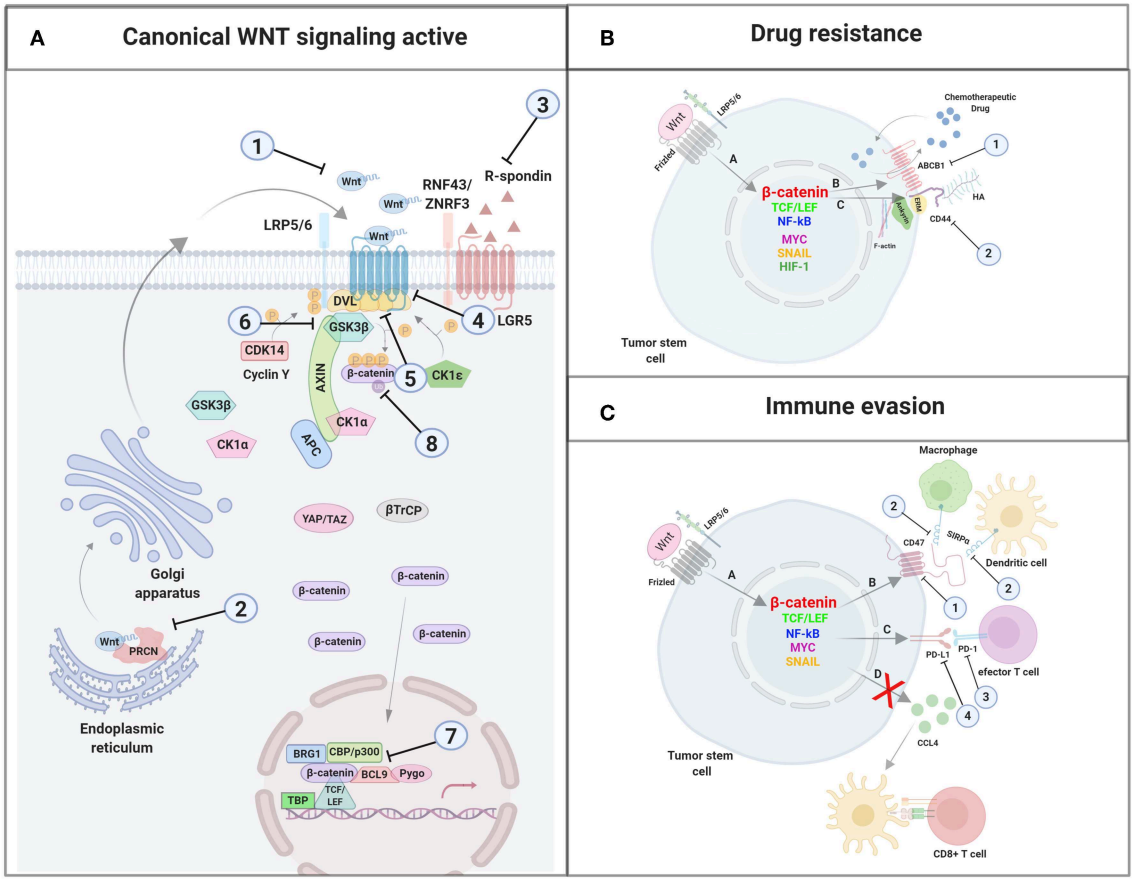

FIGURE 5 | Inhibitors used for antitumoral therapy. **(A)** Agents targeting molecules of the WNT canonical pathway: (1) Targeting WNT ligands or agonists; (2) Porcupine specific inhibitors; (3) Blocking antibody targeting RSPO3; (4) Frizzled blocking agents; (5) Agents targeting LGR5; (6) GSK3β inhibitors; (7) Transcription complex inhibitors; (8) Agents targeting β-catenin expression. **(B)** Agents targeting molecules involved in drug resistance: (1) Inhibitors of the extrusion pump ABCB1; (2) Blocking antibodies targeting CD44. **(C)** Agents targeting molecules involved in immune evasion: (1) CD47 blocking agents; (2) Agents targeting SIRPα; (3) Agents targeting PD-1; (4) Agents targeting PD-L1. Created with BioRender.com.

although multiple non-NSAID inhibitors of DVL have also been developed. Specifically, these agents block the binding between FZD and DVL at the membrane, inhibiting DVL activation. As a consequence of this, the destruction complex is stabilized, thus promoting β-catenin degradation (12, 151, 181).

GSK3β Inhibitors

The effect of an inductor of GSK3β degradation was tested in combination with chemotherapy on cholangiocarcinoma (CCA) cells and was shown to enhance the effect of classical chemotherapy. This agent downregulated ABCB1 expression in a GSK3β-dependent manner (182). In addition, lawsone derivatives are compounds which induce collateral sensitivity in MDR ABCB1-overexpressing cells. These compounds decrease β-catenin activity in a reporter cell line and downregulate c-MYC, ABCB1/P-GP, and FZD7 protein expression. Furthermore, WNT/β-catenin signaling was selectively inhibited in resistant cell lines (108).

TCF/CBP/β-Catenin Transcription Complex Inhibitors

Another inhibitor of WNT/β-catenin signaling that has been synthesized specifically binds to the N-terminus of CBP and not p300, blocking only the interaction between CBP and β-catenin. The compound leads to differentiation and loss of self-renewal capacity in pre-B ALL cells. Also, this drug downregulated survivin, an inhibitor-of-apoptosis protein, (IAP) in primary ALL and, in combination with conventional therapy and independently of the mutational status of CBP and chromosomal aberration, eradicates drug-resistant primary leukemia *in vitro* and prolongs the survival of NOD/SCID mice inoculated with primary ALL (183–185).

Additionally, the use of RNA interference technology to knock down the expression of β-catenin results in the reduction of many CSC properties, such as proliferation, migration, drug resistance, and expression of transcription factors such as OCT-4, amongst others (77).

Also, CRISPR/Cas9 genome editing technology has been used to delete β-catenin. Thus, results have shown reduced

tumorigenesis in mixed lineage leukemia and regression of epidermal tumors by depleting CSCs.

Another experimental strategy involves the activation of quiescent CSCs subsets which increases their sensitivity to chemotherapeutic drugs. Quiescence has been associated with the activity of the ubiquitin ligase FBXW7, which downregulates MYC levels. Thus, in experimental models of chronic myeloid leukemia (CML), genetic ablation of *FBXW7* in quiescent leukemic stem cells reprograms them to re-enter the cell cycle and the cells become susceptible to imatinib (186).

Targeting Molecules Involved in Drug Resistance: ABCB1 and CD44

Targeting ABCB1

Several attempts have been made to efficiently counteract the action of the extrusion pump ABCB1/P-GP and although many pump extrusion inhibitors have been assayed in clinical trials, none of them have been approved for use in patients. Currently, the third and fourth generations of ABCB1/P-GP inhibitors are being designed and/or tested. Third generation inhibitors use nanomolar concentrations to increase their effectiveness at reversing MDR compared to first- and second-generation compounds. Thus, elacridar and zosuquidar significantly inhibited ABCB1 with low toxicity in phase I clinical trials. More specifically, zosuquidar, an oral ABCB1 inhibitor, has been used in the treatment of acute myeloid leukemia, and improves the uptake of daunorubicin, idarubicin, and mitoxantrone. Another inhibitor, tariquidar, has elevated ABCB1 affinity and reduces the ATPase activity of the extrusion pump. Nevertheless, phase III assays of tariquidar with carboplatin/paclitaxel or with vinorelbine were closed due to toxicity issues. Tetrandrine, an alkaloid isolated from *Stephania tetrandra,* modulates the activity of ABCB1 and has also been used with doxorubicin in phase I clinical trials (www.clinicaltrials.gov) in the treatment of multidrug resistant cancers (187).

More recently, researchers have been working on the fourth generation of ABCB1 inhibitors, which are natural compounds derived from plant extracts, marine organisms, fungi and other sources, and exhibit modulatory properties on ABCB1, less cytotoxicity, and better oral bioavailability. The catalog of these compounds includes alkaloids, coumarins, flavonoids, and terpenoids, many of which are ABCB1 inhibitors. Thus, trabectedin, cytarabine, and Halaven have been approved for use with patients due to their powerful ABC drug transport reversal activity (188). Other phytochemicals such as curcumin and quercetin prevent ABCB1 function reversing MDR in human cancer cell lines (189, 190). Another example is piperine, an alkaloid and major component of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). This compound exhibits P-GP inhibitory activity, and also antitumoral, antioxidant, antimicrobial, and hepatoprotective functions (191). Other flavonoids such as luteolin and casticin have been shown to be active on glioma stem-like cells (192, 193). 8-Bromo-7-methoxychrysin (BrMC) induces apoptotic cell death on hepatocellular carcinoma (Hep-G2 cell line) by generating reactive oxygen species (ROS) (194). Additionally, LY294002 is a compound with a PI3K inhibitory activity that induces apoptosis in osteosarcoma CSCs by blocking the cell cycle. This compound also inhibits ABCG2/BCRP, ABCB1/MDR1/P-GP, and ABCC1/MRP1, three transporters with a high expression in stem cells. In addition, salinomycin, an antibiotic isolated from *Streptomyces albus*, interferes with ABC transporters and CSC pathways in many solid tumors and hematological cancers (187, 195).

Thus, novel compounds are being considered as new extrusion pumps inhibitors. Nevertheless, many adverse effects must be considered. Inhibition of efflux pumps can cause adverse effects in normal stem cells, since they exhibit high expression of several extrusion pumps which they need for their physiological function. In addition, ABCB1, and other pumps, play an essential role in keeping the integrity of the blood brain barrier, and interfering with their normal function could have negative consequences for the patient's health (**Figure 5B**) (187, 196, 197).

Targeting CD44

Another promising approach to blocking essential CSC signaling pathways is the use of therapy based on Abs. Thus, several anti-CD44 monoclonal Abs have been obtained with very promising results, since some of them selectively eliminate the self-renewal properties of CSCs in several malignancies and cancer cell lines. Specifically, the antibody H90 can discern between the stem cells from conventional hematopoietic progenitor cells and from AML cells. Also, the antibody P245 inhibits estrogen and progesterone receptor and HER2 in TNBCC and H4C4 blocks tumor sphere formation in human pancreatic carcinoma cells and inhibits tumorigenesis in a murine xenograft model. Notably, another recombinant and humanized anti-CD44 monoclonal antibody (RO5429083/RG7356) blocks tumorigenesis in head-and-neck carcinoma cells in mice by the cytolytic action of natural killer (NK) cells. Moreover, this antibody can eliminate triple-negative MDA-MB-231 breast cancer and CLL cells (**Figure 5B**) (198).

Targeting Molecules Involved in Immune Evasion: CD47 and PD-L1

Targeting CD47

The interaction CD47-SIRPα has been intensively investigated as potential cancer therapy (199–201). Several humanized monoclonal Abs have been obtained with encouraging therapeutic results. For example, Hu5F9-G4, the first humanized mAb to human CD47 increases the phagocytosis of tumor cells by macrophages *in vitro* and eliminates tumors in xenograft mouse models. The humanized mAb to human CD47, Hu5F9-G4, and CC-90002, are being tested in phase I or I/II clinical trials for solid tumors and hematological malignancies (202). Treatment with another anti-CD47 antibody, B6H12.2, has been shown to improve CD8+ T-cell cytotoxicity and macrophages phagocytosis and decrease tumorigenesis in AML CSCs in animals. Additionally, promising results have been obtained in many types of cancer cells. Therapy based on the use of B6H12 also decreased tumor formation in a leiomyosarcoma mouse model and the reduction of CSCs in pediatric brain carcinomas (151).

Moreover, another therapeutic approach is the design and synthesis of engineered recombinant SIRPα proteins with improved affinity for the CD47 molecule blocking endogenous CD47-SIRPα interaction. Specifically, TTI-621 is formed by the Ig-V-like domain of human SIRPα linked to the Fc region of human IgG1 (203). Similarly, the recombinant protein ALX148 is constituted by a variant of the Ig-V-like domain of human SIRPα anchored to an inactive Fc domain (203, 204). Both treatments are being used in hematological malignancies or solid tumors; as single medication or with Abs specific for tumoral antigens, with radiotherapy or with immune checkpoint inhibitors. CD47 blockade promotes antibody-dependent cellular phagocytosis (ADCP) of tumor cells by macrophages and tumor killing by cytotoxic T lymphocytes. In contrast, since many cell types express CD47, therapy based on targeting that molecule could induce adverse effects and, in fact, treatment with anti-CD47 Abs induced the appearance of anemia in monkeys (**Figure 5C**) (204).

Targeting SIRPα

Another potentially successful strategy for cancer therapy is the combination of a molecule that blocks SIRPα with Abs specific for tumoral antigens. In fact, the combined use of anti-SIRPα murine Abs and rituximab, eliminated human Raji cells grafted into non-obese diabetic (NOD)/SCID mice (205). Moreover, blocking CD47-SIRPα interaction with an antibody specific for human SIRPα enhanced killing *in vitro* by macrophages of HER2+ breast cancer cells previously opsonized with the anti-HER2 monoclonal antibody, trastuzumab (206).

The therapeutic success of anti-SIRPα blocking Abs in tumors SIRPα-positives, might be based on the activation of the ADCP mechanism against tumor cells by macrophages together with the elimination of the phagocytosis blockade exerted by the CD47-SIRPα interaction. In fact, the use of a murine Ab anti-SIRPα reduced tumorigenesis in mice inoculated with cells from renal cell carcinoma or melanoma (205). Cytotoxic T cells and NK cells could also participate in the antitumor mechanisms triggered by the anti-SIRPα Abs (205). Such Abs could favor activation of cytotoxic T lymphocytes by the macrophages and DCs infiltrated in the tumor area and induce antibody-dependent cellular cytotoxicity (ADCC) by NK cells toward tumor cells (206–208). It is of great importance that this type of treatment has thus far not induced adverse effects such as anemia or neurotoxicity in mice (206). In addition, recombinant CD47 proteins blocking the interaction between CD47 and SIRPα may also contribute to the elimination of tumor cells. Indeed, a variant of CD47 with improved affinity for SIRPα had a synergistic effect when used in combination with tumor-specific monoclonal Abs to ameliorate phagocytosis of tumor cells *in vitro* (209), although its antitumoral effects *in vivo* have not been evaluated (**Figure 5C**).

Targeting PD-L1

Therapies based on anti-PD-1 and anti-PD-L1 Abs have been designed to augment the cytotoxic T-cell attack to tumor cells. However, these treatments sometimes fail because of the emergence of resistance in patients. Furthermore, these Abs sometimes do not target all the CSCs present in the tumor bulk or induce insufficient signaling responses. To solve these problems, researchers have obtained multi-specific Abs with the capacity to bind more than one membrane receptor. These chimeric Abs are very efficient at blocking ligand binding and inhibiting downstream signaling. Several inhibitors of PD-L1 have been obtained such as atezolizumab, durvalumab, avelumab, and inhibitors of PD-1 include nivolumab and pembrolizumab (168). Treatment of patients with PD-1 and PD-L1 inhibitors, alone or in combination with standard chemotherapy, has demonstrated preliminary positive results in advanced TNBC (151, 175).

Finally, CRISPR/Cas9 is another method to cleave DNA, allowing edition in any cell. A clinical trial based on this technology was initiated to combat non-small-cell lung cancer by deleting the PD-1 receptor (**Figure 5C**) (151).

Combination of Treatments to Target Several Immune Checkpoint Inhibitors

Combinations of therapies based on targeting immune molecules with standard chemotherapeutic drugs and novel therapeutic agents could improve the efficacy of immune checkpoint inhibition in unresponsive patients. The strategy is based on the joint use of Abs and/or recombinant proteins targeting checkpoint inhibitors, chemotherapeutic drugs, cancer vaccines, and immune-stimulatory molecules. These combination therapies are currently being tested in pre-clinical models and patients. The joint blockade of the CD47-SIRPα and PD-1-PD-L1 interactions might have a synergistic effect in the elimination of tumor cells. Indeed, an anti-CD47 nanobody that inhibits the CD47-SIRPα interaction synergized with a PD-L1 antagonist significantly reducing the growth of tumors in mice previously injected with melanoma cells (210, 211). The efficacy of therapy based on the combined use of anti-PD-1 and anti-CTLA-4 antibodies was enhanced by using anti-CD47 blocking Abs in a murine model of esophageal squamous cell cancer (210). Moreover, combined therapy with SIRPα and PD-1 blocking agents had a synergistic antitumoral effect in a murine model of colon cancer (205). The combination of agents with capacity to inhibit the CD47-SIRPα interaction with Abs that block the PD-1-PD-L1 binding is a promising therapeutic approach for the treatment of a broad range of cancers (144, 210, 211).

CONCLUSIONS

WNT/β-catenin signaling is a highly conserved pathway involved in multiple essential homeostatic functions. Nevertheless, deregulation of this pathway is involved in cancer progression, drug resistance, and immunity escape. Thus, at the same time the WNT pathway is activated in CSCs, a complex transcriptional program is initiated, resulting in the expression of genes such as *ABCB1* and *CD44*, which are involved in drug resistance, and *PD-L1* and *CD47*, which are well-known in immunity. This increased WNT activation in tumor cells is responsible for the start of different survival programs which have made tumor cells highly resistant to the anti-tumoral response and/or the drug treatment. For a long time, inhibitors and/or blocking Abs have been used to counteract the action of ABCB1 as an extrusion pump or to block PD-L1 and/or CD47, and

combined therapy has been relatively successful. Additionally, many components of the WNT pathway have been the focus of the scientific community as promising therapeutic targets to eliminate tumor cells. Nevertheless, although the combination of several therapeutic approaches has been promising, many challenges remain to be solved in order to design an effective anti-tumoral treatment which targets tumoral cells, including the small population of CSCs that constitutes a critical subset of the tumor bulk.

AUTHOR CONTRIBUTIONS

EM-O wrote and revised the manuscript and supervised the figures and references. AS-F assisted in the elaboration of the figures and references list. IO-P organized the references list. All authors assisted in the conception of this review, acquisition of relevant literature, and gave their approval of the last version to be published.

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