Teaser WNT signalling is a relevant, yet underappreciated pathway in asthma. Recent insights into the pathology of asthma have highlighted this pathway as a potential novel therapeutic point of intervention. With this in mind, we attempt to answer the question: is WNT signalling a valid target for asthma therapy?

Revisiting asthma therapeutics: focus on WNT signal transduction

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Asthma is a complex disease of the airways that develops as a consequence of both genetic and environmental factors. This interaction has highlighted genes important in early life, particularly those that control lung development, such as the Wingless/Integrase-1 (WNT) signalling pathway. Although aberrant WNT signalling is involved with an array of human conditions, it has received little attention within the context of asthma. Yet it is highly relevant, driving events involved with inflammation, airway remodelling, and airway hyper-responsiveness (AHR). In this review, we revisit asthma therapeutics by examining whether WNT signalling is a valid therapeutic target for asthma.

Introduction

Asthma is a heterogeneous chronic inflammatory disease of the large and small airways. Over the past couple of decades, we have come to consider asthma not as a single disease entity, but rather as a collection of different conditions with overlapping symptomatology, but diverse aetiologies [1]. In most parts of the world, asthma prevalence is continuing to increase or remains stable and is considered one of the most common chronic disorders worldwide [2]. Asthma affects approximately 300 million people across the world and is a huge burden on healthcare expenditure [3,4]. A hallmark feature of asthma is AHR, defined as the exaggerated bronchoconstriction response to specific and nonspecific stimuli. AHR results from a variable and persistent component, driven by either chronic inflammation or the progressive development of structural changes, respectively [5]. Structural changes, termed 'airway remodelling', encompass increased airway smooth muscle (ASM) mass, mucous gland hypertrophy, bronchial microvascular remodelling, subepithelial fibrosis, and epithelial changes, including cell detachment and goblet cell hyperplasia [6]. Although the mortality rate has reduced significantly over the years with the regular use of inhaled glucocorticosteroids, 250 000 people still die from asthma annually and the global impact of asthma remains high [7,8]. The prevalent mortality and morbidity is in part because of both poor adherence [9] and response to corticosteroids in severe asthmatics and asthmatics who smoke [10] and, in some cases, patients experience no clinical effect at all [11]. In addition, the effects of corticosteroids on airway remodelling remain controversial, and are rarely clinically

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significant for low doses [12–15]. Bronchial thermoplasty has shown promise in decreasing smooth muscle mass in severe asthmatics for up to at least 2 years [16], and is associated with improved quality of life, and reduced symptoms and number of exacerbations [17]. However, the procedure is invasive and not without complications [18] and, in some cases, is without clinical benefit [19]. Thus, there is a clear need for new therapies for asthma that overcome the shortcomings of those that are currently available. In this review, we discuss the evidence that supports the involvement of WNT signalling in asthma and we evaluate the WNT pathway as a potential therapeutic target.

WNT signalling

WNT signalling is an ancient pathway that dates back to the earliest metazoans that started to develop a patterned body axis, and expanded dramatically as animals evolved into more complex organisms [20]. In mammals, there are 19 different WNT family members. They are critically involved in regulating embryogenesis and control diverse processes later in life, including cell proliferation, survival, migration, polarity, specification of cell fate, and self-renewal in stem cells [21]. It is of no surprise that perturbation of the levels of WNT ligands, or altered activity of its downstream effectors, results in developmental defects and contributes to disease aetiology. Given the large diversity of WNT signalling components, researchers have attempted to group individual WNT proteins into classes based on their intrinsic capabilities to activate the transcriptional regulator β-catenin [22]. This resulted in WNTs being categorised as either canonical (β-catenin dependent), or noncanonical (β-catenin independent). However, the intrinsic properties of WNT ligands only cover part of the story and, in view of the increasing complexity of WNT signalling networks, it seems incongruous to refer to individual WNTs using this nomenclature. Throughout this review, we view WNTs within the context of the pathway that they are part of and use the terms 'WNT/β-catenin' and 'β-catenin-independent' signalling accordingly.

WNT ligands are secreted proteins that are covalently modified by glycosylation and palmitoylation before entering the extracellular space. Palmitoylations render them hydrophobic and tether them to cell membranes or their cognate receptors, known as Frizzled (FZD) receptors. They signal in an auto- and paracrine fashion, mostly through a cell-bound manner [23,24]. In the case of β-catenin-dependent signalling, once secreted from their host cell, WNT ligands engage their cognate FZD receptors and the LRP5/6 transmembrane co-receptor, inducing complex formation between the two (Fig. 1). This results in a conformational change and enables phosphorylation of the cytoplasmic LRP tail, which inhibits glycogen synthase kinase 3 (GSK-3) [25] and allows binding of the scaffold protein Axin. Conversely, when WNT ligands are absent, Axin forms a complex together with adenomatous polyposis coli (APC) and the constitutively active serine-threonine kinases Casein kinase (CK)-Iα and GSK-3. This so-called 'destruction complex' captures β-catenin and subjects it to sequential phosphorylation at serine 45 by CK-Iα, followed by phosphorylation at positions 41, 37, and 33 by GSK-3 at the N terminus, leading to its proteosomal degradation [26,27]. WNT pathway activation results in recruitment of Axin to the phosphorylated tail of LRP. As a result, the destruction complex, while remaining intact, becomes saturated with the phosphorylated form of β-catenin. This results in newly synthesised β-catenin accumulating and translocating to the nucleus independently of transporter receptors [28] to facilitate gene transcription [29]. Nuclear β-catenin governs transcriptional programs through association with an array of transcription factors, including the T cell factor/Lymphoid enhancer-binding factor 1 (TCF/LEF1) family [30].

The β-catenin-independent pathways are more diverse in their intermediate effectors and final biological outcomes, including orientation of cell division, planar cell polarity, and convergent extension, and can include both transcriptional and nontranscriptional responses in the cell (Fig. 1) [31]. The best-characterised β-catenin-independent WNT pathways are the planar cell polarity (PCP) pathway and the WNT/calcium pathway. Activation of PCP results in downstream events that involve activation of the small GTPases Rac-1, RhoA, and Jun-N-terminal kinase (JNK). Activation of these effectors can lead to changes in cytoskeletal structure or cell polarity, either directly or through transcriptional activation [32]. PCP signalling generally does not require the presence of LRP5/6, but instead utilises the co-receptors RAR-related orphan receptor (ROR), related to receptor tyrosine kinase (Ryk) and tyrosine-protein kinase-like 7 (PTK7) [33]. WNT/calcium signalling involves the FZD-mediated activation of phospholipase C (PLC), which stimulates the production of diacylglycerol and inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$) [34]. $Ins(1,4,5)P_3$ triggers calcium release from intracellular stores and subsequent activation of calcium-dependent factors, such as calmodulin-dependent kinase II (CAMKII), calcineurin, and certain isoforms of protein kinase C (PKC). These in turn act on the transcriptional regulator nuclear factor associated with T cells (NFAT) to promote gene transcription.

Asthma genetics and epigenetics

Indications from GWA studies

Asthma frequently expresses itself in early life and has a substantial heritable component [35,36], indicating a strong genetic contribution to disease susceptibility. Furthermore, suboptimal foetal growth, maternal micronutrient deficiencies (e.g., vitamin E or vitamin D), and maternal smoking are associated with impaired infant lung function and subsequent predisposition to develop asthma later in life [37–39], suggesting that asthma develops as a consequence of the interaction of multiple environmental and genetic factors. Pre- or perinatal exposures can also drive remodelling upon birth. For example, maternal smoking during pregnancy induces airway remodelling in mouse offspring [40], and these changes are associated with the differential expression of WNT pathway genes in neonates [41]. This is in accordance with the observation that, in many asthmatics, airway remodelling develops in early life, even before asthma is officially diagnosed [42–49]. Despite the large number of studies aimed at identifying susceptibility loci, genome-wide association studies (GWAS) of asthma have only yielded a few targets as strong asthma susceptibility genes [50] that only explain a small proportion of asthma heritability, with limited ability to predict overall disease risk. GWA studies are generally restricted to common single-nucleotide polymorphisms (SNPs), but not rare or copy number variants, and positive hits require exceedingly small *P* values to declare

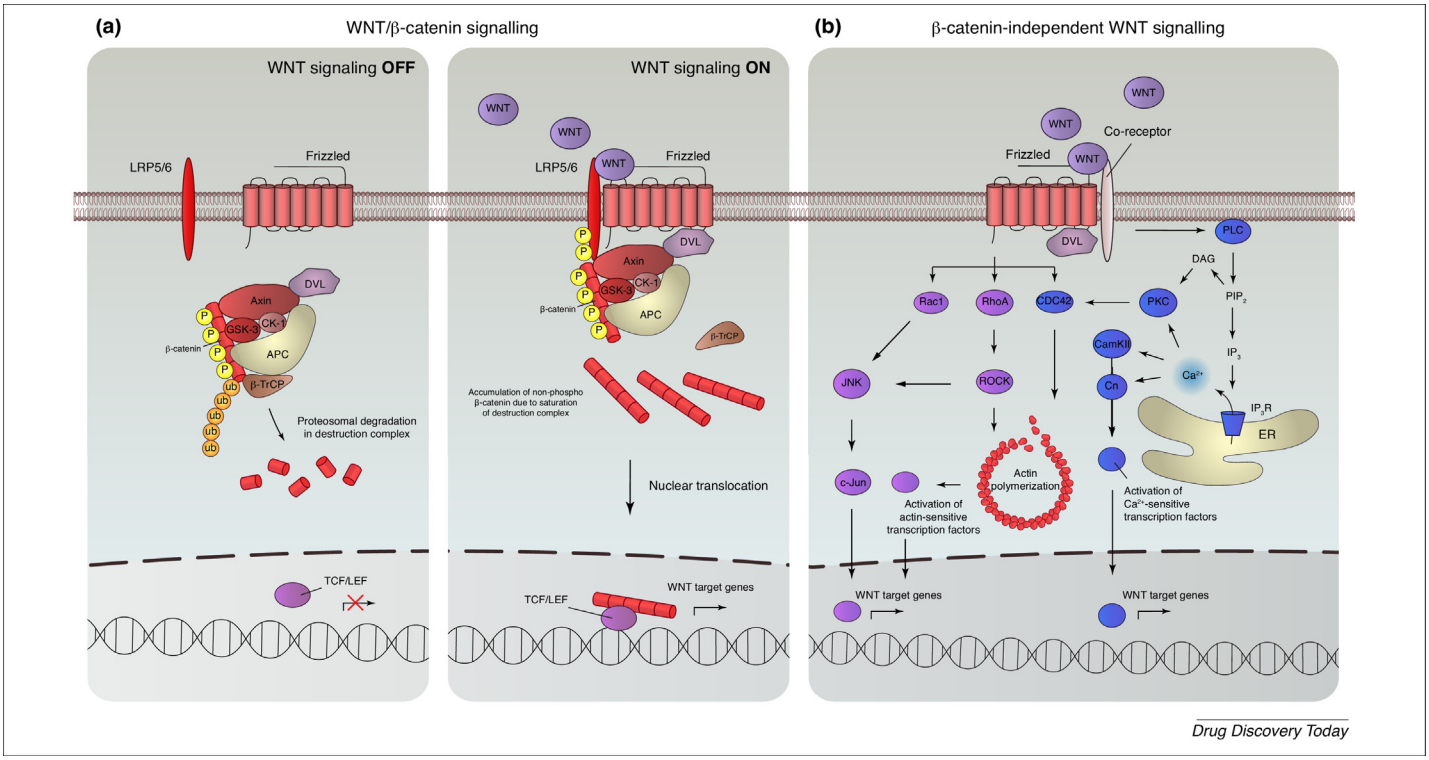

FIGURE 1

Wingless/Integrase-1 (WNT) signalling pathways. Simplified scheme showing the main WNT pathways. (a) WNT/β-catenin signalling. Under steady-state conditions and in the absence of WNT ligands, glycogen synthase kinase 3 (GSK-3) phosphorylates β-catenin, which triggers its degradation. In the presence of extracellular WNT ligands, the destruction complex [comprising GSK-3, casein kinase-Iα (CK-Iα), Axin and adenomatosis polyposis coli (APC)] is recruited to the WNT–receptor complex and inactivated. This saturates the destruction complex and allows newly formed β-catenin to accumulate and translocate to the nucleus, where it activates the transcription of target genes under the control of T cell factor (TCF), among others. (b) β-catenin-independent signalling with purple- and blue-labelled components depicting planar cell polarity (PCP) and WNT/Ca^{2+} signalling, respectively. PCP signalling triggers activation of the small GTPases RhoA and Rac-1, which in turn activate Rho kinase (ROCK) and Jun-N-terminal kinase (JNK), leading to actin polymerisation. This pathway is prominently involved in the regulation of cell polarity, cell motility, and airway smooth muscle contraction. The WNT/Ca^{2+} pathway activates Ca^{2+}- and calmodulin-dependent kinase II (CamKII), protein kinase C (PKC), and calcineurin (Cn). Calcineurin activates Ca^{2+}-sensitive transcription factors, including nuclear factor of activated T cells (NFAT), which regulates the transcription of genes controlling cell fate and cell migration. Abbreviations: β-TrCP, beta-transducin repeat-containing E3 ubiquitin protein ligase; DVL, Dishevelled; LRP5/6, low-density lipoprotein receptor-related protein 5/6; ub, ubiquitin.

significance, thus filtering out many potential true associations. In addition, the statistical models used in GWAS are simplistic and do not take into account models of interactions, such as gene×environment, which is highly relevant for asthma. Therefore, a complex disease such as asthma might require a more sophisticated approach. Indeed, when incorporating gene interplay, WNT signalling was found to strongly associate with asthma risk, in particular *FZD3* and *FZD6* [51]. The importance of genotype-specific responses to environmental exposures suggests that genes that control lung development are especially relevant for asthma risk. Three large meta-analyses of GWAS from individuals of European decent were recently published, and identified 28 loci that were associated with lung function [52–55]. These studies prompted the question whether the same set of genes were implicated in chronic lung disease, such as asthma or chronic obstructive pulmonary disease (COPD). Two follow-up meta-analyses studies were performed by a single group to determine specifically whether the identified loci from these studies, associated with lung function in the general population, also determined lung function in individuals with asthma. They found that genetic variants related to the gene encoding Family With Sequence Similarity 13 Member A (*FAM13A*) associated with both lung function [52–56] and asthma [57–59]. Interestingly, *FAM13A* has also consistently been linked with COPD [60–70], even in those who have never smoked [71]. Importantly, FAM13A was recently found to regulate β-catenin stability, highlighting WNT signalling in asthma [72]. Although the function of FAM13A remains to be further investigated (Box 1) [72,73], two splice variants have been identified in humans [FAM13A isoform 1 (long variant) and isoform 2 (short variant) [74]], expressed in mucosal cells, club cells, airway epithelial cells, alveolar cells, and alveolar macrophages [72]. Further evidence in support of this view has come from several studies. In one study, of five selected WNT signalling pathway genes that were differentially expressed in human foetal pseudoglandular and canalicular-stage lung tissue samples, two genes, encoding WNT-1-inducible-signaling pathway protein-1 (*WISP-1*) and WNT inhibitory factor-1 (*WIF-1*), harboured polymorphisms in children diagnosed with mild to moderate persistent asthma (Box 1) [75]. This was later confirmed in asthmatics of Chinese decent [76].

BOX 1

Asthma susceptibility genes

Several susceptibility genes have been associated with asthma risk, but for some it is not always clear how they affect cell behaviour. The function of *FAM13A* is not entirely clear, but studies have suggested a role in stabilising levels of β-catenin through interaction with PP2A. In HEK293T and A549 cells, FAM13A is phosphorylated at Ser 322 by Akt, which increases its binding affinity with 14-3-3, leading to cytoplasmic sequestration of FAM13A [73]. FAM13A bound to the B56 regulatory subunit of PP2A leads to dephosphorylation at Ser 322, and promotes nuclear localisation. FAM13A also interacts with Axin, but not GSK-3,and it has been suggested that FAM13A regulates post-translational modification(s) of Axin in the nucleus, leading to increased Axin turnover, which indirectly increases β-catenin stability [73]. Another study showed that, in 16HBE cells, overexpression of FAM13A resulted in increased phosphorylation (Ser 33 and 37, and Thr 41) and reduced levels of β-catenin [72]. Similarly, depletion of FAM13A increased β-catenin stability and TOPFlash reporter activity. These different findings warrant further investigation.

In light of these results, it is worth noting that FAM13A contains a putative nuclear export signal (NES) sequence [73], as well as a bipartite nuclear localisation signal (NLS) and two shorter Pat7 sequence motifs, which suggest the nuclear presence and function of FAM13A [74]. Isoform two is also associated with a RhoGAP domain, known to affect Rho family GTPases [74]. Belonging to the family of secreted matricellular CCN proteins, WISP-1, along with other CCN family members, can interact with various receptors, including LRPs, as part of WNT/β-catenin signalling [215]. However, its precise function remains poorly described. WISP-1 can interact with integrins through several integrin recognition sites [216–218]. Thus, it could serve as a mediator of cell–matrix adhesion in a pleiotropic, cell-specific manner, with potential distinct functions depending on different cell surface receptors in different cell types [219]. Functionally, WISP-1 has been shown to drive proliferative and EMT responses in alveolar epithelial cells and increase the synthesis of ECM components in fibroblasts. Antibody-mediated inhibition of WISP-1 improved lung function in the bleomycin mouse model for pulmonary fibrosis [220]. Both DKK-3 and WIF-1 are secreted negative regulators of WNT signal transduction [221]. WIF-1 can directly bind and antagonise some WNT ligands. In addition, it contains a heparin sulfate-binding site (membrane-bound glycosaminoglycans, commonly covalently linked to heparin sulfate proteoglycans, thought to mediate localisation of WNTs near the target cell surface [222]), which is not necessary for, but greatly facilitates, WNT inhibition [221]. Generally, inhibition of WIF-1 exacerbates WNT/β-catenin signalling, and its expression is commonly silenced in human lung cancer [223,224]. By contrast, DKK inhibits WNT signalling by preventing WNT binding with LRP5/6 [225]. Interestingly, whereas WNT ligands typically bind to only one or two distinct structural domains within LRP5/6, DKK binds several, and, therefore, can potentially antagonise different WNT proteins simultaneously [226]. Similar to WIF-1, inhibition of DKK generally results in activation of WNT/β-catenin signalling, and its decreased expression is relevant in lung cancer. Its functional significance in relation to asthma is described in more detail in the main text.

Indications from epigenetic studies

GWAS alone is unable to address whether SNPs are protective or whether they accelerate disease development, or even if the predicted gene is the key gene at that GWAS locus. Thus, focussing on epigenetic markers is a highly valuable tool to complement GWAS data. In one study, the β-catenin-dependent gene encoding low-density lipoprotein receptor-related protein 5 (*LRP5*), as well as *WNT2*, *APC* and several other WNT genes were differentially methylated specifically in blood monocytes of patients with neutrophilic asthma, but not eosinophilic asthma [77]. Another study showed that differentially methylated regions corresponding to elevated expression of the *CTNNB1* (encoding β-catenin) and *AXIN2* (a β-catenin target gene) genes in whole-blood samples from children at the time of birth, were associated with the increased risk of the child developing late or persistent wheeze later in life [78], which increased when mothers were exposed to high levels of stress. By contrast, at 4 years of age, this association no longer remained, suggesting that early exposures are critical in disease development.

Indications from lung development

The importance of β-catenin in driving lung developmental pathways has been demonstrated in numerous studies. Mice with β-catenin knocked out at embryonic day (E)14.5 in pulmonary epithelial cells (giving rise to airway and alveolar epithelial cells after birth) develop proximal lung tubules that differentiate normally. However, lungs fail to form peripheral airways and instead develop into proximal tubules, resulting in early death after birth [79]. By contrast, overexpression of β-catenin in CCSP-expressing Clara cells (which start to express CCSP approximately at E14.5) perturbs epithelial cell differentiation and causes goblet cell hyperplasia and air space enlargement [80]. In addition, constitutive expression of stabilised β-catenin prevents differentiation into secretory Clara cells and terminally differentiated ciliated cells, which is accompanied by a corresponding increase in functionally immature epithelial cells [81]. β-catenin is also important in the mesenchymal lineage. Mesenchymal deletion of β-catenin impairs the amplification, but not differentiation, of parabronchial smooth muscle progenitor cells as well differentiation into mature endothelial cells [82], and several WNT ligands [83–85] are essential for smooth muscle cell development in the airways.

An important area of study will be to further characterise the functional significance of genetic variants associated with WNT signalling and asthma risk, where genetic and environmental interactions are key to furthering our understanding of asthma. Although large-scale GWA studies incorporating interactions could prove challenging, a more flexible alternative to studying global transcriptional and epigenetic responses to key exposures relevant for asthma could include *in vivo* and *in vitro* models. Of particular interest here is the FAM13A locus. How FAM13A regulates β-catenin is an important question to answer, not only in adult life, but also during lung development. This will also help us understand how different SNPs within the FAM13A region relate to different diseases, such as asthma and COPD, which have both been associated with SNPs linked to th *FAM13A* [60–70].

WNT signalling in asthma: evidence from animal models

Animal models, although lacking the genetic background that asthmatic individuals have, nonetheless provide a valuable tool to observe how disease development might occur, and to disentangle which factors are a cause or determinant of the disease. Allergic asthma in mice is typically modelled by exposure to

ovalbumin (OVA) in combination with aluminium hydroxide as an adjuvant to facilitate the early-phase allergic response and skew inflammatory events in favour of T-helper type 2 (Th2) cells. Alternative allergens that are used include extracts of purified proteins from house dust mites, cockroaches, ragweed, or fungi [86]. In addition, occupational asthma can also be modelled and is usually accomplished by exposure to di-isocyanates, the most commonly identified cause of occupational asthma. Protocols differ, but generally include subcutaneous injection with liquid toluene di-isocyanate (TDI) (sensitisation), followed by inhalation with TDI vapours (challenge). Although substantial differences have been noted, many features of di-isocyanate asthma are similar to atopic asthma, including airway inflammation characterised by activated $CD4^+$ T cells, eosinophils, and mast cells, airway remodelling, and increased levels of interleukin (IL)-4 and IL-5 [87].

Allergic asthma models have frequently been associated with a change in WNT signalling, although the direction appears to depend on the duration of the protocol and route of administration of the allergen. In acute OVA models (up to 3 days of challenge), β-catenin expression is generally reduced compared with control lungs [88–91], whereas in chronic OVA models (10 weeks or more), β-catenin expression is generally higher [89,90]. For occupational asthma models, the results are less clear. Balb/c mice sensitised to TDI for 3 weeks and then challenged for 1 week showed either reduced [83,92–95] or mildly increased [93] levels of total β-catenin, concomitant with increased levels of the non-phosphorylated form of β-catenin [93,94]. Alternative, but less-frequently used asthma models are also associated with changes in WNT/β-catenin signalling. Mice exposed to a mixture of benzene, toluene, xylene (collectively called BTX), and formaldehyde (FA) showed differential expression of several WNT-related miRNAs [95], and *Aspergillus fumigatus*-exposed mice exhibited elevated levels of Axin-2 in the ASM and epithelial layers [83]. The initial reduction in β-catenin activity in the acute allergen model might reflect a physiological response to protect the host from excessive amounts of β-catenin. As ovalbumin exposure increases over time, this response might eventually lose ground as airway remodelling starts to develop, accompanied by increased activation of β-catenin. *CTNNB1* is a pleiotropic gene and its activation requires tight regulation to coordinate cell behaviour. This translates into transient periods of activation, where both activation and diminution act in quick succession. As such, it is possible that *CTNNB1* is activated in a wave pattern in response to allergens, and failure to detect differences in *CTNNB 1*expression could be a result of 'missing the wave'. In addition, some of the measured variables are not restricted to WNT signalling. For example, inactivation of GSK-3 through phosphorylation and the corresponding increase in β-catenin stability is achieved through WNT-independent factors, such as PKB/Akt [96,97], phospholipase C [98], or PKA [99]. Therefore, these findings might not reflect WNT-pathway activation. Finally, WNT pathway activation might not always be best determined by its expression. For example, studies with both animal models [92,93] and biopsies from patients with asthma [100] have shown decreased expression of the membrane-bound protein E-cadherin, resulting in disruption of barrier function. This observed reduction was paralleled by a decrease in junctional β-catenin, which might become active as it diffuses into the cytosol. These changes are maintained when epithelial cells are isolated and cultured in air liquid interface (ALI), suggesting that they are intrinsic in nature.

WNT signalling and inflammation in asthma

Asthma is primarily considered a disease associated with activation of the adaptive immune response, most notably the Th2 cell-dependent promotion of immunoglobulin (Ig)E production and recruitment of mast cells. However, asthma is also characterised by innate immune responses that influence the activation and trafficking of dendritic cells (DCs), production of innate immune cytokines, and priming of lymphoid cells [101]. Both of these axes involve WNT signalling (Fig. 2).

Evidence for β-catenin-independent WNT signalling

Evidence suggests a strong link between β-catenin-independent WNT signalling and allergic inflammation. WNT-5A was recently implicated in asthma in peripheral blood mononuclear cells (PBMCs). PBMCs isolated from healthy individuals, treated with either IL-4 or IL-13 for 24 h, and then processed for microarray analyses, showed increased expression of WNT-5A for both IL-4 and IL-13 [102]. Accordingly, WNT-5A expression could be completely prevented by anti-IL-13 mAb. These findings were extended in another study towards patients with asthma, where endobronchial biopsies from patients with mild-to-moderate asthma, stratified into 'Th2-high' and Th2-low' subphenotypes on the basis of a signature of three IL-13-inducible genes, were analysed by whole-genome microarray analyses. The authors reported that multiple WNT genes were positively correlated with the Th2-high signature [103]. Moreover, *WNT5A* was found to be increasingly expressed in PBMCs from asthmatics of Korean decent [104]. These findings suggest a link between β-catenin-independent WNT signalling and Th2-high asthma, or possibly between WNTs and allergy, which is generally considered to be a Th2-predominant response. A more recent paper has substantiated this idea, in which bronchial airway epithelial brushings were screened for differentially expressed genes and then correlated to fractional exhaled nitric oxide (FeNO) [105]. The authors then used *k*-means clustering to partition the subset of genes that correlated with FeNO into five different asthma phenotypes, or subject clusters. One cluster was enriched with WNT pathway genes, including *WIF1*, *WNT5B*, and *DKK3*. Of note, all of the patients in this cluster were atopic and had a normal FeNO, but the earliest age of asthma onset, longest disease duration, and a high disease severity and percentage of bronchoalveolar lavage (BAL) lymphocytes. Moreover, this cluster showed elevated levels of tumour necrosis factor (TNF)-α signalling, which is known to drive expression of noncanonical WNT mediators [106]. ASM cells from patients with mild-to-moderate asthma have also been shown to contain elevated levels of WNT-5A compared with healthy ASM [107]. Apart from its role in regulating bronchomotor tone, ASM is intimately involved in modulating airway inflammation [108]. Collectively, these results imply a role for β-catenin-independent WNT signalling and inflammation, in particular allergic responses.

Evidence for WNT/β-catenin signalling

In blood samples, polymorphisms within the promoter region of *CTNNB1* have been associated with either an increased or

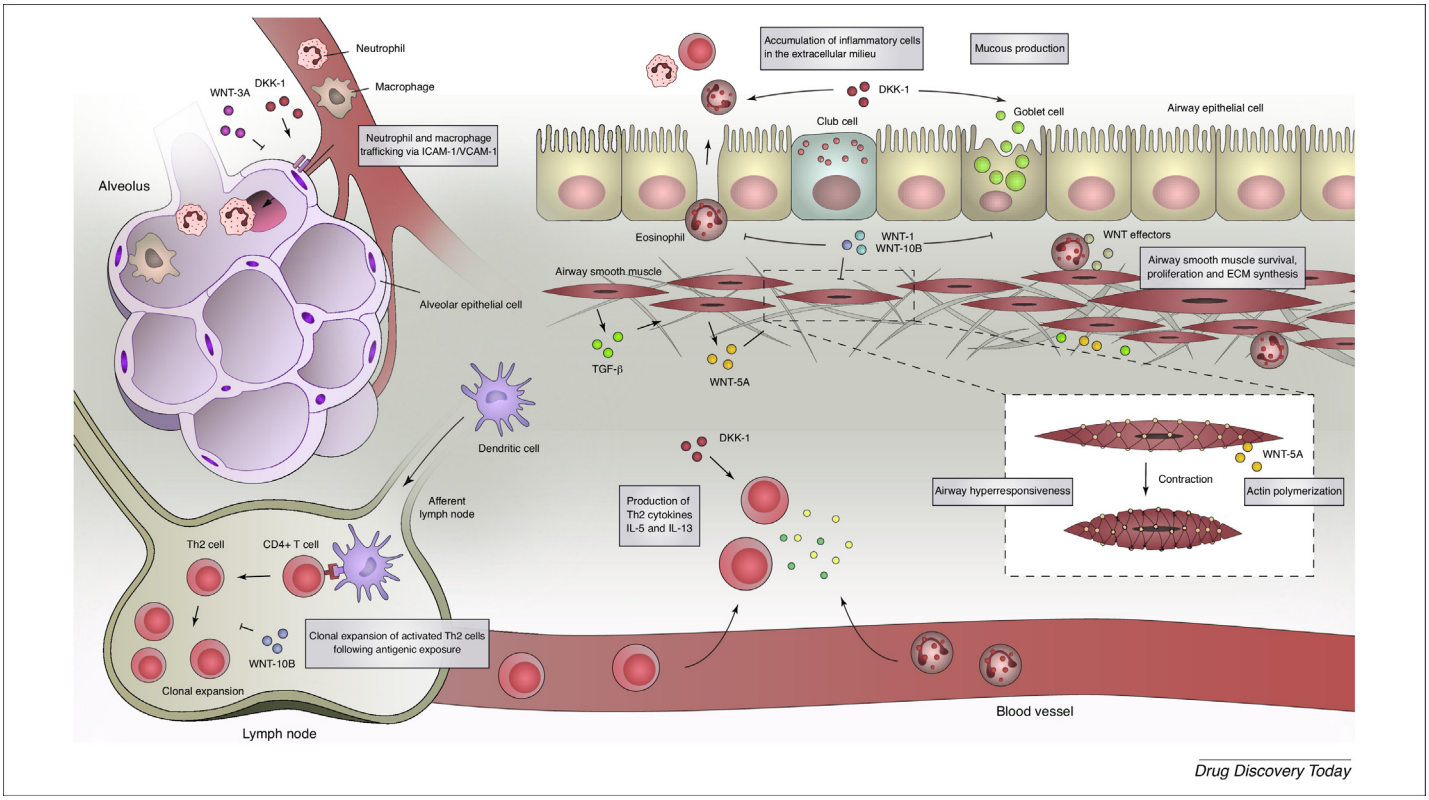

FIGURE 2

Involvement of Wingless/Integrase-1 (WNT) signalling in asthmatic responses. Release of WNT ligands that engage in WNT/β-catenin signalling generally suppresses adaptive immune responses at various levels. Trafficking of inflammatory cells into the alveolar space because of upregulation of adhesion molecules, proliferation of activated T helper 2 (Th2) cells following antigenic exposure, and expression of Th2 cytokines are all inhibited upon activation of WNT/β-catenin signalling. Conversely, secreted negative regulators of WNT signalling [e.g., Dickkopf-1 (DKK-1)] can undo this inhibition. Suppression of β-catenin also signalling attenuates airway remodelling, examples including airway smooth muscle growth and synthesis of extracellular matrix proteins. β-catenin-independent WNT signalling exerts diverse effects that, in general, are poorly described. Examples are modulation of airway smooth muscle contraction and activation of inflammatory responses. There is also a substantial amount of cross-regulation between β-catenin-independent WNT signalling and other pathways, such as transforming growth factor (TGF)-β signalling, which collectively drives airway remodelling.

decreased risk of developing asthma, depending on whether these variants increased or decreased the expression of β-catenin, respectively [109]. Furthermore, endobronchial biopsies from patients with mild-to-moderate asthma showed that *WNT3A* and *WNT10A* associated with Th2-high asthma [103]. In support of this, the β-catenin destruction effector genes *Axin1*, *APC* and *GSK3b* were all found to be decreased in PBMCs from Korean patients with asthma [104].

Collectively, these results support the view that both axes of WNT signalling are elevated in asthmatic tissues and link to Th2-specifc inflammation. These results are largely backed up by mechanistic and translational studies in animal models, although some discrepancies exist, which are further outlined below.

Evidence from mechanistic studies on adaptive immunity

WNT/β-catenin signalling is critically involved in T cell development in the thymus [110,111], primarily through interaction of β-catenin with the transcription factor special AT-rich-binding protein 1 (SATB1) [112], which was recently also shown to be associated with mucous hypersecretion [113]. However, WNT/β-catenin has also been implicated in the Th2-mediated response that occurs after maturation in the thymus, specifically within the context of allergy. Transgenic mice producing WNT-1 in a tetracycline-based (tet-ON) manner, under control of the Clara cell secretory protein (CCSP) promoter, specific for Clara cells, and subjected to OVA exposure to drive allergic asthma-like changes, showed attenuated AHR, BAL eosinophilia, and a reduction in mucus production [114]. Overexpressed WNT-1 had no effect on systemic sensitisation, as evidenced by unchanged OVA-specific IgE, IgG1, and IgG2b levels in serum. Treatment with the nonselective GSK-3 inhibitor lithium chloride could mimic these results, highlighting the role of β-catenin signalling in this response. In line with this, mice with a homozygous hypomorphic mutation at the Dickkopf-1 (DKK-1) allele, in which DKK-1 expression is reduced by approximately 90%, showed amplified WNT/β-catenin signalling, accompanied by reduced levels of neutrophils, eosinophils, and $CD4^+$ T cells in BAL fluid in response to allergen challenge with house dust mites [115]. In another study, suppression of DKK-1 by a neutralising antibody, or administration of WNT-3A, reduced neutrophil trafficking during acute inflammation [116]. Moreover, inhibition of DKK-1 reduced the production of IL-4, IL-5, IL-10, and IL-13 in $CD4^+$ T cells, and suppressed interferon (IFN)-γ expression under Th1-cell polarisation conditions [112,117]. WNT–10 B was also recently implicated in Th2 activation [118]. WNT-10 is expressed in airway epithelium as well

as in T cells. Full-body ablation of WNT-10 resulted in an increased Th2 predominant inflammatory response in an acute house dust mite mouse model. BAL fluid eosinophils were elevated as well as whole-lung homogenate expression of IL-4 and IL-13 and infiltration of antigen-specific effector cells in the lungs, although there was no difference in the proportion of infiltrated T cells within the lungs of WNT-10B$^{-/-}$ mice. However, among the infiltrated cells was a higher number of effector cells, characterised as CD44^{high} CD62L^{low}, suggesting that antigen exposure is a requisite for the WNT-10B$^{-/-}$ state to take effect [119]. In line with this, sorted T cells from WNT-10B$^{-/-}$ mice exposed to IL-4 to drive Th2 polarisation exhibited increased GATA-3 and IL-4 expression. Of note, these changes were absent under baseline conditions, and no differences were found in expression of T-box transcription factor, (T-bet; expressed in CD4^{+} T cells committed to Th1 T cell development). Moreover, WNT-10B$^{-/-}$ T cells exposed to CD3/CD28 to drive clonal T cell expansion through ligation of the T cell receptor (TCR), showed increased proliferation. Other immune cells have also been linked with WNT signalling, although not all of these studies have been tested within the context of an asthma or allergic inflammatory model. Isolated DCs exposed to curcumin, a natural substance that increases β-catenin activity in these cells, prevented upregulation of the activation markers CD40 and CD68 induced by lipopolysaccharide (LPS). Curcumin also prevented lymphocyte proliferation following exposure to LPS in a mixed lymphocyte reaction assay, and reduced OVA-induced accumulation of inflammatory cells in the BAL fluid of mice [120]. Furthermore, intestinal DCs deficient in β-catenin are compromised in their ability to produce retinaldehyde dehydrogenases (RALDH) [121], an enzyme that is part of the conversion of vitamin A to retinoic acid. Failure to mount a RALDH response subsequently shifts Th polarisation in favour of Th1 cells [122]. Although retinoic acid production by DCs has been considered to be limited to gut-resident DCs only, other DC populations have recently been shown to also express RALDH, particularly lung-resident DCs, which express RALDH-2 [123,124]. Survival of eosinophils has also been reported to require the nuclear presence of β-catenin, which can be triggered via IL-5 in a WNT-independent manner [125]. Moreover, eosinophils from patients with asthma can modulate the WNT secretory profile of cultured ASM cells when adhered to [126,127]. These changes can subsequently affect how smooth muscle cells proliferate and maintain their extracellular matrix (ECM) surroundings. Other cell types, such as mast cells or B cells, have thus far not been researched in an asthmatic or allergic setting, although active WNT signalling is required for their proper differentiation [128,129].

Evidence from mechanistic studies on innate immunity

WNT/β-catenin signalling has also been demonstrated to regulate innate immune responses, primarily through interaction with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a transcription factor that drives the expression of multiple cytokines, chemokines, and cell adhesion molecules that are involved in asthma pathophysiology. Its activation occurs mainly through ILs or TNF, or is elicited by the activation of Toll-like receptors (TLRs) during a bacterial or viral exacerbation. The usefulness of targeting NF-κB in asthma has already been demonstrated by the efficacy of glucocorticosteroids, which can be contributed in part to the inhibition of NF-κB [130]. β-catenin has been shown to interact with both the p65 [131–133] and p50 [131,132,134–136] subunit of NF-κB in various cell types, generally resulting in impaired DNA binding, transactivation activity, and target gene expression mediated by NF-κB. GSK-3 is also required for NF-κB activation via degradation of β-catenin [137–139], although direct phosphorylation of NF-κB p65 by GSK-3 has also been proposed [140,141]. Interestingly, another line of research has proposed a dependency of NF-κB on β-catenin. Increased β-catenin signalling in alveolar epithelial cells enhanced NF-κB signalling and transcriptional output *in vitro* [142]. The nuclear cofactors CREB-binding protein (CBP) and E1A binding protein p300 (p300) have been shown to be required for β-catenin and NF-κB interactions [143,144]. It was recently shown that, in ASM, inhibition of the β-catenin–CBP interaction could amplify NF-κB-mediated inflammation, whereas inhibition of the β-catenin–p300 interaction could attenuate it (authors' unpublished findings, 2017). Although detailed molecular events remain to be determined, these results suggest a molecular switch that directly controls NF-κB output, requiring the presence of β-catenin (Fig. 3).

The interconnected nature of WNT/β-catenin signalling with both adaptive and innate immune responses complicates the interpretation of genetic screening studies that have implicated β-catenin signalling in asthma. In addition, both inflammatory cascades interact with each other. Innate immune mechanisms are required for DC priming [145] and can amplify a Th2 response to inhaled ovalbumin [146,147]. Furthermore, commercially available OVA is known to contain traces of LPS [148], which facilitate the priming of Th cells to inhaled OVA. The degree of LPS exposure in conjunction with OVA also determines the type of T cell response that is elicited (e.g., Th1 versus Th2) [146,149–152]. NF-κB p50$^{-/-}$ mice were completely devoid of airway inflammation when challenged with inhaled allergen in a murine model of asthma [153], and it has been shown that NF-κB is critical for Th2 differentiation through expression of GATA-3 [154]. To help facilitate the drug development process, future efforts aimed at identifying new WNT targets should extricate innate from adaptive immunity.

WNT signalling and airway remodelling in asthma

Evidence for β-catenin-independent WNT signalling

WNT-5A is increasingly expressed in ASM cells of patients with mild to moderate asthma compared with healthy individuals [107]. A recent study implicated WNT-5A with ASM contraction, where WNT-5A acts via autocrine signalling to promote actin polymerisation in ASM cells [155]. The increased presence of actin filaments increased maximum force generation in ASM cells without affecting sensitivity to histamine. Increased activity of WNT pathway activation and modulation of the actin cytoskeletal network could serve as an alternative model to explain AHR in asthma [155]. In addition, WNT-5A is responsible for some of the actions mediated by transforming growth factor (TGF)-β. In human ASM, fibronectin and collagen synthesis following stimulation with TGF-β requires *de novo* synthesis of WNT-5A and subsequent activation of TGF-β-activated kinase 1 (TAK1) and specificity protein-1 (SP-1) [107,156]. It was further shown that some of the effects of WNT-5A initiated by TGF-β require the

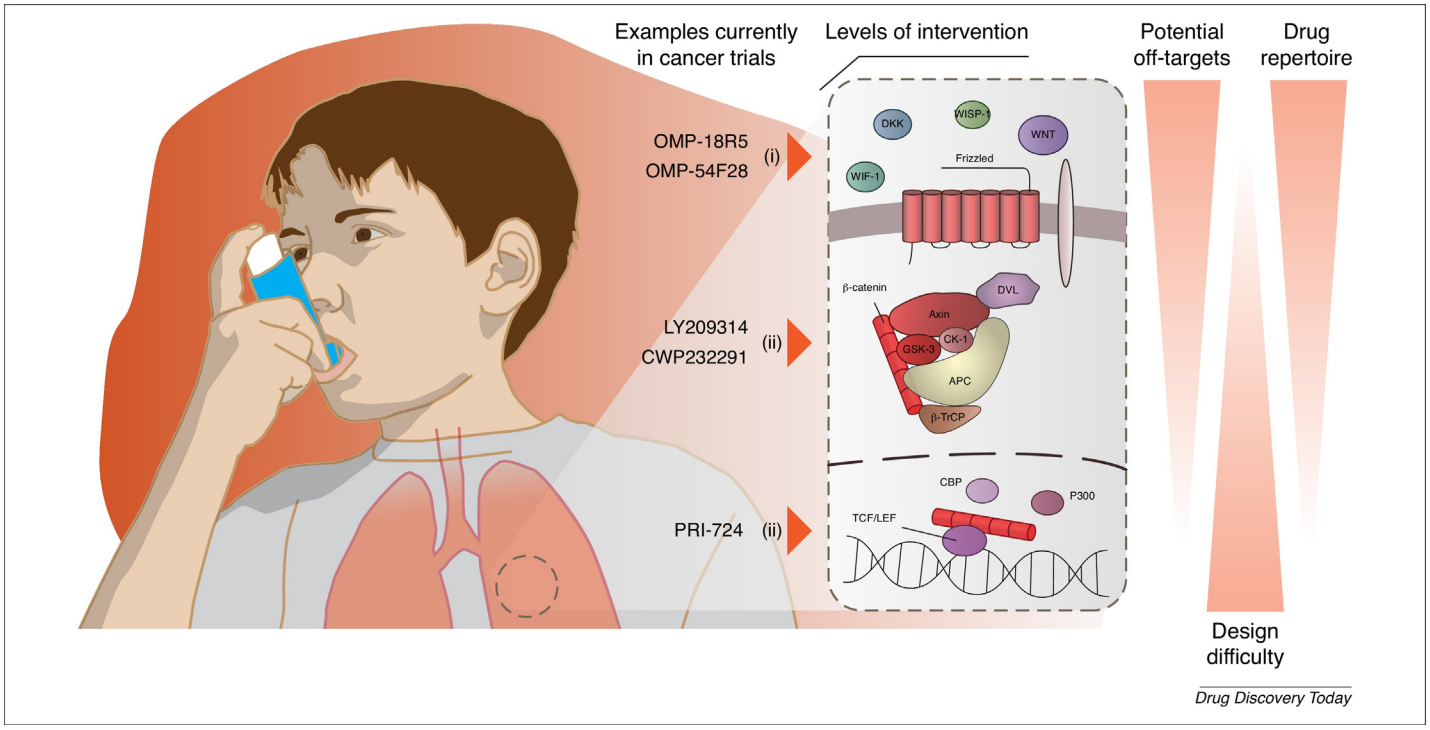

FIGURE 3

Targeting Wingless/Integrase-1 (WNT) signalling in asthma. When WNT pathway inhibition is to be achieved to treat asthma, it is important to consider the different levels of intervention, which can profoundly impact the final outcome of the treatment. Here, we categorise these levels in three compartments based on cellular architecture: (i) the extracellular environment; (ii) the cytosolic environment; and (iii) the nuclear compartment. Generally, designing compounds that act upstream can result in a lack of specificity, because of the interconnected nature of cell signalling and its numerous feedback loops. In this case, potential off-target effects can be expected. At the same time, this approach allows for a broader therapeutic reach, because upstream effectors (e.g., WNT ligands) are more likely to be shared by different cell types compared with downstream effectors. It also presents a more diverse platform for drug development; because cell permeability is not required, small molecules, monoclonal antibodies, recombinant proteins, and receptor constructs (e.g., fusion proteins) are all part of the drug repertoire. Conversely, designing compounds that act downstream in the cell allows for the inhibition of specific signalling events, thus minimising off-target effects. Maximum specificity can be achieved by inhibiting only a specific subset of protein–protein interactions, for example the interaction of β-catenin with CREB-binding protein (CBP), but not E1A binding protein p300 (p300). However, targeted therapy in the nucleus presents more difficulties in the drug designing phase, because compounds have to cross several barriers before reaching their designated site, requiring them to be soluble and cell permeable, or encapsulated by a delivery vehicle. Additionally, the delivery and retainment of drugs inside the cell can be highly dependent on the presence and activity of ABC transporters that are expressed in the lungs and that might use pulmonary drugs as substrates.

release of actin-binding proteins following formation of actin filaments. Of particular interest here is myocardin-related transcription factor A, which is released upon WNT stimulation and can drive expression of TGF-β target genes [157]. Individually, WNT-5A is unable to achieve the effects mediated by TGF-β, indicating that cooperative signalling is a requisite for this effect [155]. The effects of TGF-β–WNT–MRTF-A are also relevant for other aspects of airway remodelling. For example, MRTF-A is critically involved in the induction of TGF-β-mediated epithelial–mesenchymal-transition (EMT) [158,159] and the epithelial–myofibroblast transition [160]. Myofibroblasts are a rich source of ECM proteins, and MRTF-A is an important mediator of myofibroblast activation and expression of ECM proteins [161]. Inhibition of mechanotransduction by blocking the RhoA–MRTF-A axis attenuated experimental pulmonary fibrosis in mice [162]. In asthma, cross-regulation between TGF-β and WNT signalling could allow for the development of treatment strategies that can overcome the shortcomings of drugs that target TGF-β signalling more directly (which are associated with severe adverse effects). Going forward, it is essential that we study this level of integration in more detail, because the nature of this crosstalk can be overwhelmingly complex and context dependent [163]. Failure to recognise this level of integration will confound the development of effective therapeutic interventions in a complex disease such as asthma.

Evidence for WNT/β-catenin signalling

β-catenin is a critical regulator of airway remodelling, particularly in ASM and fibroblasts. Both cells require active β-catenin signalling to promote cell growth [90,164] and production of ECM proteins [165–167]. Although the nature of WNT/β-catenin expression in animal models for asthma is controversial (see above), targeting this pathway could still be beneficial in a therapeutic setting. OVA-exposed Balb/c mice treated with small interfering (si)RNA targeted against β-catenin showed considerably reduced parameters of airway remodelling. Both deposition of newly synthesised collagen and expression of alpha smooth muscle actin (α-SMA) were attenuated following inhibition of β-catenin [89]. Similarly, inhibition of the β-catenin–CBP interaction with the small-molecule ICG-001 was able to prevent ASM thickness after repeated OVA challenge and showed a trend towards a decline in peribronchial collagen deposition [90]. These results have been

corroborated in a mouse model for occupational asthma [94]. Moreover, inhibition of WISP-1 (an inducer of WNT/β-catenin signalling) by a neutralising antibody attenuated OVA-induced ASM thickening in rats [168].

Current therapies and WNT signalling

According to the Global Initiative for Asthma (GINA), key points in asthma management are to achieve good symptom control and to minimise future risk of exacerbations, fixed airflow limitation, and adverse effects of treatment [169], highlighting our lack of understanding of asthma aetiology and the focus on symptomatic rather than curative treatment. To date, there have been no clinical trials for asthma involving the modulation of the WNT signalling pathway. Current therapy is mainly based on (combinations of) inhaled corticosteroids, β_2-adrenergic receptor agonists, and leukotriene inhibitors [169,170]. Some of these, most notably glucocorticoids, have been reported to elicit secondary effects on WNT signal transduction, mainly in off-target tissues. Mesenchymal cell commitment towards osteoblastic differentiation to promote bone formation requires endogenous glucocorticoids that signal through WNT pathways downstream [171]. In line with this, osteoporosis, one of the most frequent adverse effects of long-term glucocorticoid therapy [172], is accompanied by inhibition of WNT/β-catenin signalling in osteoblasts. Glucocorticoids activate GSK-3 [173], inhibit TCF/LEF [174], and increase the expression of WNT pathway inhibitors, such as DKK-1 [175–177] and soluble Frizzled-related protein-1 (sFRP-1) [178]. GSK-3 can also phosphorylate the glucocorticoid receptor (GR), which facilitates its response to glucocorticoids [179–181]. It would be interesting to assess the effects of glucocorticoids in different tissues that are more relevant for asthma pathophysiology, and to evaluate whether potential effects on WNT signalling activation are clinically significant. At the moment, there is no evidence that both short-acting and long-acting β_2-adrenergic receptor agonists can modulate WNT signalling in the lung. One study addressed the interaction of fenoterol with WNT pathway components in human bronchial rings [182], but these findings have thus far not been corroborated and require additional verification. Although it has been shown that cysteinyl leukotrienes can activate β-catenin signalling [183,184], primarily in a WNT-independent manner through activation of phosphatidylinositol 3-kinase (PI3K) [185], there are no studies that have shown WNT pathway modulation by any of the currently available cysteinyl leukotriene-receptor antagonists (montelukast, zafirlukast, and pranlukast). The same holds true for most other available treatment strategies, examples being IgE inhibition with omalizumab or cholinergic pathway inhibition with tiotropium. Of note, asthma treatment is moving towards personalised medicine and a focus on asthma pheno- and endotypes [186–188]. Drug therapies previously deemed ineffective have gained renewed interest in light of these developments, one example being biologics targeted against Th2 cytokines [189]. Given the close involvement of WNT pathway components with innate and adaptive immunity, it would be interesting to re-evaluate these drugs based on their potential secondary and/or indirect effects on WNT signalling. In addition, several other drugs that are currently under trial might prove efficacious in terms of WNT signal modulation. For example, drugs that inhibit the prostaglandin (PG) D_2 receptor subtype DP2 [also known as the chemoattractant homologous receptor expressed on Th2 cells (CRTh2)], important in Th2 and type 2 innate lymphoid cell (ILC2) function [190,191], but possibly also in airway remodelling [190], are now in clinical development for asthma [192]. It is known that β-catenin functions downstream of the closely related eicosanoid PGE_2 in a cAMP-dependent manner in several malignant cell types [193,194], and it would be worthwhile to assess the effects of CRTh2 antagonists on β-catenin signalling.

Concluding remarks

More than 30 years after the discovery of what is possibly the oldest evolutionary conserved pathway in animals, and extensive research efforts to characterise this fundamental pathway, targeting WNT signalling in a clinical setting is still in its infancy. Despite intensive efforts to characterise this pathway in a disease setting, including asthma, unveiling a multitude of potential therapeutic points of intervention, there have been surprisingly few attempts to modulate WNT pathway components in clinical trials. This is not because of a lack of available reagents that target the WNT pathway [195], which are increasingly being discovered and developed. Some of these compounds are currently being tested in clinical trials, of which most are within the scope of cancer treatment, but most other fields have so far lagged behind, including asthma [196]. Most drugs tested in current trials target extracellular modulators of WNT signalling, including not only DKK-1, but also the WNT ligands themselves, as well as WNT receptors [196]. This is surprising, considering the widespread involvement of WNT signalling in almost every tissue within the human body, and is likely to have secondary effects in off-target tissues. Nonetheless, these studies will provide critical clues to the safety profile of these WNT modulators, and whether they can be efficacious in a therapeutic (cancer) setting. They will also provide important information about whether full inhibition or activation of WNT signalling is the right approach for therapy [197]. During drug-screening approaches, the most potent drugs are usually selected for and tested in a clinical setting. However, full reduction of aberrant WNT signalling might not necessarily be the right approach. Given that WNT signalling is intricately involved in tissue homeostasis, therapeutic targeting might require a more delicate approach, where WNT pathway activation needs to be brought back down to normal levels. In fact, the safety profile of WNT modulators currently in preclinical and clinical trials for cancer, including PRI-724 [198], LY209314 [199], CWP232291 [200], OMP-54F28 [201], and OMP-18R5 [202], show that many of these compounds share some of their adverse effects. Although the safety results from these studies showed good tolerability overall, most of these compounds associated with symptoms of nausea, diarrhoea, and vomiting. Although these adverse effects might raise concern over their general usability in the clinic, limiting systemic exposure by restricting the reach of these drugs to the lungs through inhalation could largely overcome these issues. Nonetheless, these results highlight the difficulty that resides in developing safe and effective therapeutic compounds targeting this complex pathway.

The increasing interest in characterising asthma phenotypes and endotypes, and the emerging concept that asthma might have a developmental basis, raises interesting thoughts in terms of future therapy. Identifying biomarkers for asthma development

and susceptibility in early life could pave the way for treatment strategies that could alter disease progression entirely, possibly even halting or reversing it. In light of the genetic and developmental aspects of asthma, targeting the WNT pathway would be a primary candidate in this regard. Of particular interest would be WNT-associated therapies that affect airway remodelling in early life, because remodelling may already develop before the onset of asthma symptoms. Targeting WNT signalling in patients who already have asthma might also be beneficial. Of special interest here are compounds that target the selective inhibition of the interaction between β-catenin and co-factors, such as CBP or p300, because they only disrupt a small subset of co-factor interactions, initiating a transcriptional program that potentially inhibits disease parameters, while leaving others intact. They also act significantly downstream in the cell, possibly preventing unwanted secondary effects. To improve the development of therapeutic targets downstream of WNT signalling, it is essential that we learn more about the nuclear actions of β-catenin, because this is currently an underappreciated topic. For example, CBP and p300 are paralogous genes that share a large degree of structural similarity, yet they are often ascribed opposing roles [203]. How CBP and p300 can exert these seemingly bimodal functions remains to be determined. It has been suggested that the interaction between β-catenin and CBP or p300 results from competition [203], but this model seems too much of an oversimplification, because CBP and p300 are known to facilitate transcriptional output through a plethora of additional transcription factors. Additionally, differential phosphorylation of CBP or p300 [203,204], or yet to be discovered binding partners, could govern selectivity and binding with regulatory components. Both the ability of CBP and/or p300 to modulate chromatin and acetylate β-catenin or other proteins through their histone acetyl-transferase (HAT) domain will be an important focus of study. Expanding our knowledge of these architectural elements will further our ability to design drug therapies that target a selective range of transcriptional events involved in disease, without interfering with the crucial role of WNT signalling in tissue homeostasis. Five clinical trials are currently underway, all in cancer, using the small-molecule inhibitor PRI-724 (an enantiomer of ICG-001), which selectively targets the β-catenin–CBP interaction, with no effect on p300 [205].

Type 2 inflammation can be efficiently suppressed in most patients with asthma with the regular use of inhaled glucocorticosteroids. Although Th2-high asthma is generally a corticosteroid-responsive endotype [206–208], a notable subgroup of patients with this endotype maintain symptoms and experience severe uncontrolled asthma despite regular use of steroids [209–213]. Novel drug treatment of this group of steroid-insensitive patients with severe asthma is warranted. Furthermore, some Th2-high asthmatics require high doses of inhaled steroids or oral steroids for maintenance therapy, and these patients are in need of alternatives to avoid excessive adverse effects. The advent of more specific inhibitors, such as biologicals targeted against type 2 inflammation, has raised hope that these drugs will provide similar benefits to patients with asthma, while displaying fewer adverse effects. However, compared with glucocorticoids, these compounds have a more limited effect on airway function and asthma control, even when stratified for different asthma pheno- or endotypes. Thus, they have so far not been able to replace steroid therapy and are adjunctive at best [214]. In addition, glucocorticoids have no noticeable effect on airway remodelling. This is where anti-WNT therapy could confer additional benefit, because of its combined effects on Th2 immunity, airway remodelling, and muscle biology. Most trials using anti-DKK-1 antibody therapy in cancer are now complete, and a positive outcome of these studies will be important in furthering our understanding towards the development of asthma therapy. Over the next couple of years, these results and others should shed new light on whether we can use the WNT pathway as a therapeutic target in asthma.

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