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The Wnt/β-Catenin Pathway Attenuates Experimental Allergic Airway Disease

Sebastian Reuter,* Helen Martin,* Hendrik Beckert,* Matthias Bros,† Evelyn Montermann,† Christina Belz,* Anke Heinz,* Svetlana Ohngemach,‡ Ugur Sahin,§ Michael Stassen,§ Roland Buhl,* Leonid Eshkind,‡,1 and Christian Taube*†,1

Signaling via the Wnt/β-catenin pathway plays crucial roles in embryogenesis and homeostasis of adult tissues. In the lung, the canonical Wnt/β-catenin pathway has been implicated in remodeling processes, development of emphysema, and fibrosis. However, its relevance for the modulation of allergic responses in the lung remains unclear. Using genetically modified mice with lung-specific inducible (doxycycline) Wnt-1 expression (CCSP-rtTA × tetO-Wnt1), the impact of Wnt on the development of allergic airway disease was analyzed. Overexpression of Wnt during the allergen challenge phase attenuated the development of airway inflammation in an acute model, as well as in a more therapeutic model of secondary challenge. These findings were further supported by treatment of allergen-sensitized mice with LiCl during challenge. Similar to Wnt, LiCl prevented the degradation of β-catenin and, thus, attenuated allergic airway inflammation and hyperresponsiveness. Migration studies revealed that lung-specific expression of Wnt reduced the migration of Ag-loaded dendritic cells (DCs) into the draining lymph nodes following allergen challenge. Administration of in vitro allergen-loaded DCs overcame Wnt-mediated suppression of airway inflammation. Furthermore, in vitro studies confirmed that DC-dependent T cell activation is impaired by blocking β-catenin degradation. These results demonstrate an important role for the canonical Wnt/β-catenin pathway in the DC-mediated regulation of allergic responses in the lung. The Journal of Immunology, 2014, 193: 485–495.

Over the last few decades, the incidence and prevalence of asthma, especially in Western countries, have increased dramatically. In recent years, it has become clear that asthma is a heterogeneous disorder based on multiple pathophysiological mechanisms that are thought to contribute to the different phenotypes of the disease (1). One prominent phenotype is allergic asthma, in which Th2 cells and Th2 cytokines, such as IL-4, IL-5, and IL-13, play a central role (2, 3). The mechanisms leading to asthma are affected by various interacting parameters, such as genetic predisposition, environmental factors, and exposure to allergens and infectious organisms. This can lead to sensitization against harmless Ags, resulting in allergic disease following repeated contact with the Ag/allergen. Dendritic cells (DCs) are the most effective cells for Ag presentation and represent sentinels of the immune system. When harmless self-Ag or environmental Ag is encountered, DCs induce tolerance or anergy (4, 5). However, in the presence of endogenous or exogenous danger signals, DCs become activated and induce an Ag-specific adaptive immune reaction, which leads to a memory response and sensitization. Via a spectrum of different mediators, DCs determine the type and strength of adaptive immune responses and play an important role in the development of the allergic response. Indeed, therapeutic modulation of DCs could be an efficient step to abrogate allergen-induced immune responses.

Recently, the Wnt/β-catenin pathway has been identified as important in developmental processes in embryogenesis (6). Wnt proteins are a group of 19 hydrophobic secreted glycoproteins. Wnt signals via binding to frizzled receptors that form complexes with the low-density lipoprotein receptor–related protein 5 or 6. Depending on the ligand/receptor combination, three signal cascades can be induced. First, activation of the canonical Wnt/β-catenin pathway prevents glycogen synthase kinase 3beta (GSK-3β)–mediated β-catenin degradation, leading to nuclear translocation of β-catenin and gene transcription (7). In contrast, noncanonical Wnt pathways do not use β-catenin signaling; they include the planar cell polarity pathway, which contributes to the regulation of tissue polarity and cell migration (8), and a Ca2+-mediated pathway, which inhibits β-catenin signaling and also induces migration (9). The canonical Wnt/β-catenin pathway is important for self-renewing processes in the course of development via the control of cell proliferation and terminal differentiation, as demonstrated in the gut (10, 11), hair (12), regulation of hematopoietic stem cells (13), and homeostasis of bone (14, 15). Moreover, Wnt ligands were shown to play an important role in developmental and homeostatic processes in the lung (16). Indeed,
Wnt-7b–deficient mice exhibit hypoplastic lungs (17), and a loss of Wnt-5a signaling induces increased cell proliferation, leading to a thickened interstitium and distal bronchial branching (18). Deregulation of Wnt molecules in the adult lung has been linked to formation of tumors (19), emphysema, and lung fibrosis (20, 21). In contrast, little is known about the involvement of Wnt in inflammatory and immunological responses in the lung, such as allergic airway inflammation.

Therefore, the aim of the current study was to analyze the function of Wnt-1/β-catenin signaling in the development and exacerbation of experimental allergic airway disease. In the current study, we observed that overexpression of Wnt-1 resulted in an attenuation of allergic airway disease, both in acute and therapeutic models.

Materials and Methods

Mice

C57BL/6J, B6 OTII, D011.10, and BALB/c mice were obtained from the Central Laboratory Animal Facility, University Medical Center, Johannes Gutenberg University Mainz. CCSP-rtTA and tetO-Wnt1 mice were generated as described in earlier reports (22, 23). Transgenic mice were maintained by crosses with wild-type FVB/N animals. CCSP-rtTA–transgenic mice were bred with tetO-Wnt1 animals to generate biteransgenic mice. Animals were genotyped using tail DNA. All mice used were aged 8–12 wk of age. Animal procedures were conducted in accordance with current federal, state, and institutional guidelines, and all experiments were approved by the local regulatory authorities.

Experimental protocols

To assess the effects of Wnt-1/β-catenin signaling, different experimental animal models were carried out as summarized in Fig. 1. For sensitization to the model Ag OVA, animals received an i.p. injection of 20 mg OVA (Sigma-Aldrich; Seelze, Germany), suspended in 2.25 mg aluminum hydroxide (Imject Alum; Pierce, Rockford, IL), in a total volume of 100 μl on days 0 and 14.

To analyze the effects of lung-specific overexpression of Wnt (Fig. 1A-1), Wnt/CCSP animals received doxycycline (DOX; 2 mg/ml sterile water; Sigma-Aldrich) (24) in drinking water starting from day 21 until the end of the experiment. Allergic airway disease was induced by challenge via the airways on days 28–30, whereby an OVA solution (1% in PBS) was nebulized daily for 20 min with an ultrasonic nebulizer [NE-U17; Omron, Hoofdorp, The Netherlands]. Readouts were performed 48 h following the last challenge. To analyze the effect of Wnt upregulation in animals with already established airway disease, a secondary challenge protocol was used (Fig. 1A-2) (25). In this protocol, animals were not exposed to the allergen for 6 wk following the first challenge phase; subsequently, they received a single secondary challenge via the airways by nebulization. To analyze the function of Wnt, CCSP-rtTA × tetO-Wnt1 animals received DOX treatment starting 7 d before the secondary challenge until the end of the experiment. Analysis of the animals was performed 48 h following the secondary challenge.

To assess whether upregulation of the Wnt metabolite β-catenin has a protective effect in wild-type animals, C57BL/6 mice were sensitized and challenged as described in Fig. 1A. Instead of inducing Wnt expression, animals received an i.p. injection of LiCl (dissolved in sterile water; Sigma-Aldrich; 200 mg/kg body weight/d) daily, starting 10 d before the first challenge (Fig. 1B).

To analyze the effects of a lung-specific upregulation of Wnt on DC migration, animals were anesthetized by i.p. injection of ketamine/rompun (Ketamin-ratiopharm; Ratiopharm, Ulm, Germany/Rompun 2%; Bayer Leverkusen, Germany) dissolved in 40 μl PBS (26, 27). At 12, 24, and 48 h following treatment, regional lymph nodes were collected and analyzed via FACS for OVA+ cells. Wnt overexpression was induced by DOX feeding of CCSP-rtTA × tetO-Wnt1 animals again starting 7 d before the i.n. challenge (Fig. 1C-1).

To further identify the role of DCs in Wnt-mediated events, sensitized animals received in vitro–generated and OVA-pulsed bone marrow–derived DCs (BMDCs) i.n. on day 28 (Fig. 1C-2); animals were analyzed on day 30.

Each experiment was performed at least twice, and at least three to five animals were used per group.

Assessment of allergic airway disease

Measurement of airway resistance and dynamic compliance were performed on anesthetized, intubated, and mechanically ventilated mice (flexiVent; SciQu, Montreal, QC, Canada), as previously described (28). Lungs were lavaged with 1 ml PBS. Cell count and viability assessment were determined via trypan blue exclusion. Differential cell counts for macrophages, lymphocytes, neutrophils, and eosinophils were performed on cytospun preparations stained with a Hemacolor Set (Merck).

Bronchoalveolar lavage fluid (BALF) supernatants were stored at −20°C. Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin, as previously described (28). Tissue sections were stained with H&E or periodic acid–Schiff (PAS). To assess airway inflammation, five randomly selected areas were scored for each slide by two experienced observers blinded to the experimental groups (29). Inflammation was scored on a scale from 0 to 4. PAS+ goblet cells were quantified per millimeter of bone marrow.

β-catenin staining was performed as recommended by the manufacturer. Slides were incubated with primary β-catenin Ab (R&D Systems, Minneapolis, MN) or isotype control in a final concentration of 2.5 μg/ml overnight at 4°C. Secondary Ab, as well as HRP conjugate, was incubated on slides, as described in the staining kit (Anto-Goat HRP-DAB; R&D Systems). Slides were analyzed and scored in a blinded fashion by

FIGURE 1. Treatment protocols. (A-1) Acute/prophylactic model: days 0 and 14 i.p. injection of OVA/alum; days 28–30 challenge by nebulization with 1% OVA solution; 7 d prior to challenge start daily DOX application by feeding; day 32 assay. (A-2) Secondary challenge/therapeutic model: days 0 and 14 i.p. injection of OVA/alum; days 28–30 challenge by nebulization with 1% OVA solution; 6-wk resting phase; secondary challenge by single nebulization with OVA; 7 d prior to secondary challenge start DOX feeding. (B) Lithium model: days 0 and 14 i.p. injection of OVA/alum; days 28–30 challenge by nebulization with 1% OVA solution; 6-wk resting phase; secondary challenge by single nebulization with OVA; 7 d prior to secondary challenge start DOX feeding. (C-1) DC migration model: days 0 and 14 i.p. injection of OVA/alum; day 28 i.n. application of fluorescence-labeled OVA; 7 d prior to challenge daily DOX application by feeding. (C-2) DC vaccination model: days 0 and 14 i.p. injection of OVA/alum; day 28 i.n. application of fluorescence-labeled OVA; 7 d prior to challenge daily DOX application by feeding.
at least four investigators. Scores ranged from 0 to 5, where 0 was no detectable brown staining and 5 was highly positive.

**FACS analysis**

FACS analysis was used to assess the migratory behavior of conventional DC, T cells, and regulatory T cell (Treg) populations in lung and draining lymph nodes (tLNs) ex vivo, as well as DC activation and T cell proliferation in vitro.

To analyze OVA+ DCs in the tLNs, single-cell preparations of tLNs and stained as described elsewhere (26). In short, tLNs were disrupted and transferred over a cell strainer into a 5-ml round-bottom tube (Becton Dickinson, Heidelberg, Germany). Cells were washed, and cell count was determined. Cell numbers were adjusted to $2 \times 10^7$ cells/ml FACS washing buffer, and $1 \times 10^6$ cells were used for each staining. Unspecific binding was blocked with FcR-blocking Abs (anti-CD16/CD32; BD Biosciences, Heidelberg, Germany). To identify DC populations, the cells were stained with FITC-labeled anti–MHC class II (eBioscience, San Diego, CA) and PE-labeled anti-mouse CD11c (BD Biosciences). To exclude B cells and further subdivide DC populations, cells were additionally stained with PerCP-Cy5.5-labeled anti-mouse B220 (BD Biosciences). Among the B220− cells, the CD11c+/MHCII+ proportion of OVA Alexa Fluor 647+ cells was determined. To further analyze DC subpopulations, additional staining was performed using PE-labeled anti-mouse CD103, PerCP-Cy5.5−labeled anti-mouse CD11b, PerC7−labeled anti-mouse CD11c, V450−labeled anti-mouse Ly6c, PE-labeled anti-mouse F4/80 (all from BD Biosciences) and Brilliant Violet−labeled anti-mouse Gr-1 (BioLegend). To analyze CD11b+ and CD103+ DCs, doublets were excluded, and CD11c+ MHCII+ cells were gated. Within these, OVA+ cells were analyzed further. Following exclusion of Ly6c+ cells, the proportions of CD103+ and CD11b+ cells were determined. The total number of OVA+ CD11c+ MHCII+ B220− CD103+ and OVA+ CD11c+ MHCII+ CD11b+ DCs was calculated by multiplying the fraction of positive cells by the total cell number (Supplemental Fig. 1). To determine the amount of plasmacytoid DCs (pDCs), OVA+ cells were analyzed following the exclusion of doublets. pDCs were characterized as B220+ CD11c− Gr-1− cells (30). Again, the total number of OVA+ CD11c− MHCII− B220− CD11c− Gr-1− DCs was calculated by multiplying the fraction of positive cells by the total cell number.

To analyze Tregs, adjusted single-cell suspensions of either lung or tLNs were stained with PerCP-Cy5.5-labeled anti-mouse CD3, PE-Cy7−conjugated anti-mouse CD4, and PE-labeled anti-mouse CD25 (all from BD Biosciences). Following incubation and washing, intracellular staining against Foxp3 was performed by using the Foxp3 Staining Buffer Set (eBioscience). After fixation and permeabilization, Foxp3 was stained by incubation with allophycocyanin-labeled anti-mouse Foxp3 Ab.

FACS measurements were performed on a FACS Canto II (BD Biosciences) using Diva software. Final analysis of FACS data and graphics was performed using FlowJo software (TreeStar, Ashland, OR).

**Ag stimulation of lung and lymph node single cells in vitro**

To assess in vitro Ag-specific cytokine production of lung and lymph node cells, single-cell suspensions of lung or lymph nodes were prepared under...
sterile conditions. Lungs were cut and transferred into a 50-ml vial. Collagenase type I (0.5 mg/ml; Sigma-Aldrich) was added and, following an incubation time of 45 min at 37°C in a shaking water bath, cells were resuspended at least three times through a cannula in a 10-ml syringe. Cells were transferred over a cell strainer (70 μm; BD Biosciences) into a new vial and were treated with Gey’s solution to eliminate RBCs. Subsequently, cells were washed twice, and cell count was determined. Lymph node single-cell preparations were made as described elsewhere in this section. Lung and lymph node single-cell preparations were adjusted to a concentration of 1 × 10^7 cells/ml test medium (TM; IMDM + 10% FCS [both from PAA], 1% Pen-Strep [Sigma-Aldrich]). Cells were incubated or not for 72 h with 250 μg/ml OV A (Sigma-Aldrich; Grade V) at 37°C. Following incubation, supernatants were harvested and stored at −20°C for further analysis.

**Adoptive transfer of Ag-pulsed BMDCs**

In short, following lysis and washing, bone marrow cells were incubated in culture medium (IMDM, 10% FCS, 50 μg/ml streptomycin, 100 U/ml penicillin supplemented with 5% GM-CSF) for 8 d to obtain BMDCs. On day 8, BMDCs were incubated with 100 μg/ml OVA overnight. As described in Fig. 1, 1 × 10^6 cells diluted in 80 μl PBS was administered i.n. in sensitized animals that were treated or not with DOX. Readouts were performed 48 h following DC administration. Unsensitized animals were used as a negative control.

**In vitro assays**

BMDCs were generated as described (32). On day 7, immature BMDCs were treated with OVA (5 μg/ml; Merck-Calbiochem; Darmstadt, Germany), and cells were incubated overnight. Subsequently, cells were exposed to various concentrations of recombinant human Wnt-1 (1, 10, 100 ng/ml; BioVision, Milpitas, CA), the GSK-3β-blocking agents LiCl (5, 10, 15 mM) or SB216763 (SB; 1, 5, 10 μM; Enzo Life Sciences, Lörach, Germany), or medium alone (control) for 24 h. To compare the effects of activated versus immature BMDCs, a part of each group was incubated with LPS for 60 min following treatment with the ligands. The TLR4 ligand LPS (1 μg/ml; Calbiochem/Merck, Darmstadt, Germany) was added to the cultures of the appropriate groups. After 24 h, DCs were stained for the expression of CD11c and MHC class II, as described, or stained with PE-conjugated anti-mouse CD80 (BD Biosciences) and anti-mouse CD86 (eBioscience), as well as with allophycocyanin-conjugated anti-mouse CD40 and anti-mouse CCR7 (both from BD Biosciences). Expression patterns were assessed by FACS and analyzed via FlowJo software.

To analyze effects on T cell proliferation, OVA-loaded BMDCs were generated as described before and treated with ligands. Furthermore, OVA-BMDC cultures were split in half and activated by adding LPS or by incubating in medium alone. Twenty-four hours following treatment, DCs were cocultured for 72 h with CD4+ CFSE-labeled OVA-transgenic T cells. Splenocytes collected from spleens of D010.11 animals were purified using MACS Separator–LS columns (Miltenyi Biotech, Bergisch Gladbach, Germany), biotinylated anti-mouse CD4 (clone H129.19; Institute for Immunology, Muinz, Germany), and streptavidin-coated beads to obtain a CD4+ cell population. Purifications were performed according to the manufacturer’s instructions. Cells were adjusted to 1 × 10^6 cells/ml, and carboxyfluorescein diacetate succinimidyl ester (Invitrogen Life Technol-
Fig. 4. Blockade of b-catenin degradation by application of LiCl during challenge prevents the development of allergic airway disease in wild-type C57BL/6 mice. To assess the effects of b-catenin degradation blockade in wild-type C57BL/6 mice, they were sensitized and challenged with OVA. Parameters of allergic airway disease were compared among animals sensitized and challenged (sens), those additionally treated with LiCl before challenge (sens + LiCl), and appropriate controls (unsens/unsens + LiCl). Treatment of sensitized and challenged animals with LiCl reduced all features of the asthmatic phenotype. (A) AHR of sens animals, sens + LiCl animals, unsens animals, and unsens + LiCl animals. (B) Composition of BALF. Inflammatory score (C), mucus-producing PAS+ cells per millimeter of basal membrane (D), and titers of OVA-specific IgE, IgG1, and IgG2b (E) in unsensitized (sens ), sensitized but not challenged (sens + chall ), sensitized and challenged (sens + chall +) and sensitized and challenged treated with LiCl (sens + chall + LiCl +) animals. Data are mean ± SEM (n = 15 mice/group from three independent experiments). *p ≤ 0.01, ***p ≤ 0.001, ANOVA. ns, not significant.
sensitized and challenged wild-type controls left untreated (Supplemental Fig. 2). Wnt-1 overexpression in the lung and the associated reduction in allergen-induced inflammation did not affect systemic sensitization, as evidenced by unchanged OVA-specific IgE, IgG1, and IgG2b levels in sera, irrespective of DOX treatment (Fig. 2E).

Increased Wnt-1 expression decreases airway inflammation in a secondary allergen challenge model

Overexpression of Wnt-1 reduced allergic airway disease in a primary, prophylactic model of allergic airway disease. The effect of Wnt-1 overexpression also was assessed in a more therapeutic model of secondary allergen challenge (Fig. 1A-2). To this end, CCSP-rtTA × tetO-Wnt1 mice were sensitized and then challenged with nebulized OVA to induce allergic airway disease. Five weeks later, CCSP-rtTA × tetO-Wnt1 mice received either DOX to induce Wnt-1 expression or PBS as a control. After another week, one secondary challenge was performed. As expected, secondary airway challenge induced AHR, airway inflammation, and mucus cell metaplasia in CCSP-rtTA × tetO-Wnt1 mice that received PBS. Similar to the results in the primary airway challenge model, application of DOX reduced AHR, airway inflammation, and mucus cell numbers (Fig. 3A–D). Again, systemic levels of OVA-specific Igs were unaffected by the administration of DOX, because OVA-specific IgE, IgG1, and IgG2b titers were comparable in all sensitized animals (Fig. 3E).

Treatment of wild-type mice with LiCl protects them from development of allergic airway disease

LiCl is known to prevent β-catenin degradation and, therefore, mimics canonical Wnt signaling (34). To analyze whether the Wnt-1–induced regulatory effects in CCSP-rtTA × tetO-Wnt1 mice are achievable in wild-type animals, C57BL/6 animals were treated with LiCl (Fig. 1B). Sensitized mice were exposed to LiCl 10 d prior to the challenge, as described in Materials and Methods. AHR and airway inflammation were assessed 48 h following the last allergen exposure (Fig. 1C). Application of LiCl resulted in a reduced AHR in comparison with untreated mice (Fig. 4A). Furthermore, numbers of eosinophils in the BALF, influx of inflammatory cells in the lung tissue, and goblet cell metaplasia also were significantly decreased in the LiCl-treated animals (Fig. 4B–D). However, treatment with LiCl had basically no effect on sensitization because serum levels of OVA-specific Igs remained unaffected (Fig. 4E). Yet, LiCl treatment reduced concentrations of Th2 cytokines, such as IL-4 and IL-5, in BALF, as well as in supernatants of unstimulated and restimulated cells isolated from regional lymph nodes compared with levels of the respective control group (Supplemental Fig. 3A, 3B). Treatment with LiCl had no effect on the frequency of Tregs (defined as CD4+ CD25+ Foxp3+ cells) (Supplemental Fig. 3C, 3D).

Wnt overexpression does not affect induction of Tregs but does affect the migratory behavior of Ag-loaded DCs

Stabilization of β-catenin induced by Wnt-1 has been associated with increased survival of Tregs (35). Therefore, the number of Tregs (defined as CD4+ CD25+ Foxp3+ cells) was assessed in lungs and tLNs of animals following secondary airway challenge. However, there were no significant differences in the frequencies and absolute numbers of Tregs in either the lungs or lymph nodes of sensitized and challenged CCSP-rtTA × tetO-Wnt1 mice that did or did not receive DOX (Fig. 5, data not shown). This suggests that significant expansion of Tregs does not occur following Wnt overexpression.

To further assess the mechanisms underlying the suppressive effect of Wnt on the development of allergic airway disease, DC migration was investigated following allergen challenge. To this

FIGURE 5. Frequencies of Tregs are unaffected in mice overexpressing Wnt-1 in lung. Lungs and tLNs were analyzed for Tregs (characterized as CD4+ CD25+ Foxp3+ cells by FACS). Percentage of CD25+ Foxp3+ Tregs within CD4+ T cell population in tLNs (A) or lungs (B). Dot plots show Foxp3 versus CD25 staining among CD4+ cells. Data are mean ± SEM (n = 4–6 mice/group for tLN analysis and n = 6 mice/group for lung analysis from two independent experiments). ns, not significant.
end, sensitized CCSP-rtTA × tetO-Wnt1 mice were exposed to a single i.n. application of the fluorescent OVA derivative OVA–Alexa Fluor 647 (Fig. 1C-1). At 12, 24, and 48 h following application, tLNs were isolated and analyzed by FACS for immigration of OVA+ B220− CD11c+ MHCII+ DCs (Fig. 6A, Supplemental Fig. 1). Similar to previous results (26), the numbers of fluorescent DCs peaked at 24 h after the application of labeled Ag (Fig. 6B, 6C). Interestingly, pretreatment of mice with DOX resulted in a significant reduction in fluorescent cells in regional lymph nodes, comparable to the numbers found in nonsensitized animals. Further characterization of the migratory DCs showed that MHCII+ CD11c+ CD11b+ DCs and MHCII+ CD11c+ CD103+ DCs were decreased in tLNs in DOX-exposed animals following allergen exposure compared with sensitized and challenged animals that did not receive DOX (Fig. 6D, Supplemental Fig. 1). However, this decrease was only statistically significant for MHCII+ CD11c+ CD11b+ DCs. The numbers of MHCII+ CD11c+ B220+ GR1+ DCs were low and were not statistically different among all experimental groups (Fig. 6D).

Transfer of OVA-loaded DCs abrogates Wnt-induced regulatory effects

To further analyze whether the regulatory effect of Wnt is mediated by DCs, ex vivo–generated wild-type DCs were loaded with Ag and applied prior to airway challenge to DOX-exposed animals. Following systemic sensitization, CCSP-rtTA × tetO-Wnt1 animals received DOX daily to induce Wnt expression, starting 7 d prior to the application of Ag-pulsed DCs (Fig. 1C-2). Indeed, a single i.n. application of Ag-pulsed DCs caused increased numbers of eosinophils in sensitized animals but not in nonsensitized controls (Fig. 7). Interestingly, no significant differences in eosinophil numbers could be detected in sensitized mice that received DOX compared with mice that did not receive DOX (Fig. 7A). Similarly, there also was no difference in tissue inflammation or cytokine production in supernatants of unstimulated cells or stimulated cells from lung and lymph nodes following application of Ag-pulsed DCs in sensitized animals treated or not with DOX (Fig. 7B–D). These data suggest that allergen-pulsed DCs can override the suppressive effect of Wnt-1 in this model.

In vitro effects of Wnt-1/β-catenin signaling on DC/T cell interaction

In vitro assays were performed to analyze the Wnt-1–mediated effects on DCs and their interplay with T cells more closely. Unstimulated and LPS-activated OVA-loaded BMDCs were cocultured with CFSE-labeled OVA peptide–specific CD4+ T cells. To assess the effects of Wnt-1, this factor was added in various concentrations during DC activation (only DCs) or during the complete DC/T cell coculture period (total). Addition of Wnt did not affect the expression of co-
stimulatory molecules on DCs (Supplemental Fig. 4A). Interestingly, OVA-specific T cells incubated with OVA-loaded DCs proliferated strongly, whereas T cells that were incubated with Wnt-exposed DCs proliferated significantly less. This was true when Wnt was added only to the DC culture or to the DC/T cell coculture (Fig. 8A). In addition, expression of activation markers on T cells and cytokine production were much lower in T cells that were exposed to Wnt-treated DCs compared with untreated DCs (Fig. 8B, 8C).

In agreement with these data, a protective effect of b-catenin accumulation following blockade of GSK-3b also was detected. Activated DCs incubated with LiCl or the highly specific GSK-3b inhibitor SB prevented T cell proliferation and IFN-γ secretion (Supplemental Fig. 4B, 4C).

**Discussion**

The present study demonstrated that increased expression of Wnt-1 in the lung abrogates the development of allergic airway disease. Induced Wnt-1 overexpression in allergen-sensitized animals before inhaled airway allergen challenge resulted in reduced numbers of eosinophils in the BALF, AHR, and mucus production. This effect was found in a more prophylactic primary challenge model, as well as in a chronic model in which an inflammatory lung disease already had been established. Similarly, in the latter model, Wnt-1 overexpression before and during secondary challenge reduced the development of AHR, eosinophilia in the BALF, and tissue inflammation, confirming the suppressive effect.

Wnt molecules have been ascribed important functions during lung development and different lung diseases. Wnt/b-catenin signaling is involved in tissue repair mechanisms in the lung, because reduced Wnt expression can contribute to increased development of emphysema (20). Additionally, overexpression of Wnt molecules induces proliferation and migration of fibroblasts and is involved in lung fibrosis (36, 37). Consequently, inhibition of the Wnt pathway represents a therapeutic option in bleomycin-induced...
induced fibrosis (38). However, the role of the canonical Wnt pathway in the development and regulation of allergic airway disease is not well understood. Gene expression studies in patients with asthma revealed a correlation between Wnt expression and the Th2-specific phenotype of this disease. These studies demonstrated that increased mRNA expression of several Wnt cascade molecules positively correlated with a Th2 signature and impaired lung function in asthma patients (39, 40). Irrespective of these gene expression studies, the functional role of Wnt in allergic airway disease remains unexplained. In the current study, increased expression of Wnt-1 had a protective effect on the development of allergic airway disease. Wnt-1 overexpression did not affect levels of anti-OVA IgGs in sera of sensitized animals. This indicates that the protective effects are not due to a Wnt-1–dependent systemic modulation of the adaptive immune response but rather to local effects in the lung that abrogate the development of the disease. A nonspecific effect of DOX treatment on the reduction of airway inflammation was ruled out, because no reduction in allergic airway disease was detected in sensitized and challenged wild-type animals exposed to DOX. The relevance of these findings was further elucidated in a rechallenge model. This more chronic model confirmed the decrease in airway inflammation, AHR, and mucus cell numbers and demonstrated again that increased Wnt-1 expression suppressed allergic airway disease, even in a therapeutic model.

To further support our findings of β-catenin–dependent suppression of allergic disease, pharmacological agents known to activate β-catenin were applied. Application of the GSK-3β in-

**FIGURE 8.** Wnt-1 affects DC functions in vitro. Immature and mature BMDCs were incubated with OVA; aliquots were treated with different doses of Wnt-1 and subsequently incubated with OVA peptide–specific T cells. T cell activation was analyzed. (A) OVA-loaded immature or mature DCs were cocultured with CFSE-labeled CD4+ T cells expressing an OVA peptide transgenic TCR. After 72 h, proliferation of T cells was analyzed by measuring CFSE fluorescence reduction via FACS. Dot plots depict CD4+ versus CFSE (left panels). Bar graph depicts CFSE fluorescence in the CD4+ T cell population (right panel). Shaded graph represents T cells cocultured with untreated OVA-loaded immature DCs. The green lines depict T cells cocultured with LPS-stimulated OVA-loaded mature DCs. The red lines represent proliferation of Wnt-1–treated T cells. Wnt-1 was supplemented during the entire incubation period. Bar graph shows the percentage inhibition of T cell proliferation from T cell cocultures with differentially Wnt-1–pretreated BMDCs in comparison with the positive control (OVA-loaded mature DC + T cells). Wnt-1 was applied only during BMDC culture (only DC) or during DC/T cell coculture (total). (B) Mean fluorescence intensity (MFI) of CD44 (left panel) or CD25 (right panel) on the surface of CD4+ CFSE+ T cells cocultured with either BMDC population and pretreated as indicated. (C) Cytokines were detected in the supernatants of the BMDC/T cell cocultures after 72 h of incubation. Bar graph represents the percentage inhibition of IFN-γ secretion in the described groups. Two experiments were performed; graphs and dot plots depict results from one of two independent experiments (n = 2). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 versus positive control, t test.
hibitor LiCl to mimic the effect of canonical Wnt activation (34) caused similar effects as did DOX-induced Wnt-1 overexpression in transgenic mice. Comparable with forced overexpression of Wnt-1, application of LiCl during challenge also led to reduced development of allergic airway disease in sensitized animals. Development of AHR, eosinophilia in BALF, airway inflammation, and goblet cell metaplasia were abrogated in animals treated with LiCl. The conclusions from the present results are in line with findings in other models of lung disease. The GSK-3β-inhibitory effect of LiCl was investigated in a mouse model of emphysema; it caused attenuation of the disease (20).

Immunological inflammatory responses are controlled by the induction of Foxp3+ /CD25+ Tregs. In vitro studies demonstrated that β-catenin supports Treg survival (35). Surprisingly, neither Wnt-1 overexpression nor LiCl treatment resulted in increased frequencies of Tregs in the lungs and these animals showed concentrations of the regulatory cytokine IL-10 in BALF and restimulated lung and lymph node cell suspensions that were comparable to sensitized and challenged animals. These findings suggest that Tregs are not pivotally responsible for the observed reduction in the allergic inflammatory response. DCs are also critically involved in shaping immune responses; depending on their state of activation, they induce or suppress Ag-specific immune responses. DC activation is modulated by exogenous and endogenous signals, and the composition of their microenvironment determines the outcome of the immune response. It was shown that DCs are required to induce allergic immune responses in the lungs (5, 41). Ag uptake, processing, and migration to the tLNs are essential steps for the induction of an adequate T cell response. The activation state of DCs determines whether Ag-specific tolerance or immunity is initiated. In our study, Wnt-1 overexpression in the lung resulted in reduced migration of Ag-loaded DCs from the lung to the tLNs in sensitized animals following inhaled allergen exposure. These findings suggest that Wnt-1 suppressed DC activation following allergen challenge and, thereby, inhibited the development of the allergic response in the lung. This assumption is further supported by the finding that application of in vitro Ag-loaded BMDCs overcame the effects of Wnt-1 overexpression in the lung. When BMDCs were instilled i.n. to sensitized and challenged animals, airway inflammation, AHR, and cytokine production developed, irrespective of Wnt-1 overexpression. These observations support the hypothesis that the Wnt-1/β-catenin pathway suppresses DC activation in the lung. Studies in the intestine corroborate these findings. Manicassamy et al. (42) demonstrated that ablation of β-catenin in DCs led to an enhanced inflammatory phenotype in a mouse model of inflammatory bowel disease. Furthermore they showed that β-catenin was important to sustain a tolerance-inducing DC phenotype, thereby maintaining equilibrium between anergy and immune response. Moreover, a study demonstrated that disruption of E-cadherin on BMDCs, which also results in activation of the β-catenin pathway, led to tolerogenic DCs that were able to suppress experimental autoimmune encephalomyelitis (43). However, an effect of Wnt molecules directly on T cell migration and function cannot be ruled out. Indeed, Wnt molecules can upregulate matrix metalloproteinases that facilitate the transmigration of T cells into the tissue (44).

An in vitro model was established to further analyze the effect of the Wnt-1/β-catenin pathway on DC activation and allergen-specific T cell cross-talk. Interestingly, application of Wnt-1 to unstimulated or LPS-activated OVA-loaded BMDCs had no effect on the expression patterns of the costimulatory molecules CD86, CD80, and CD40. However, BMDCs treated with Wnt-1 had an impaired ability to induce a T cell response, because T cell proliferation, surface expression of activation markers, and cytokine secretion were all impaired. This suppressive effect of Wnt-1 on DCs seems to be comparable to the effect of other Wnt molecules. Wnt-5a suppressed the production of IL-12 and IFN-γ in human PBMCs exposed to microbial compounds (45). Similar to Wnt-1, inhibition of GSK-3β by LiCl or the highly specific inhibitor SB resulted in modulation of DC/T cell interactions. SB treatment caused a suppression of T cell proliferation, but this was only apparent when the inhibitor was constantly present during the coculture. Blockade of β-catenin degradation during the DC activation period alone was insufficient to suppress DC-mediated T cell proliferation. Nevertheless, induction of tolerance is not necessarily accompanied by a reduction in T cell proliferation. Also, Ag delivery from DCs under steady-state conditions induced T cell proliferation, without generation of T cell subtypes and prolonged activation (46). Moreover, OVA-pulsed immature DCs induced T cell proliferation, but only mature OVA-pulsed DCs were able to initiate the production of IFN-γ (47). In the current study, treatment of BMDCs with LiCl or SB was able to prevent cytokine production of T cells. This phenomenon was observed, irrespective of whether LiCl or SB was present during the coculture of DCs and T cells or whether DCs were treated before the onset of the cocultures. These results suggest that Wnt-1, via GSK-3β blockade, mediated accumulation of β-catenin, which, in turn, suppressed BMDC activation.

In summary, the present experiments demonstrate in an acute, as well as in a therapeutic, model that activation of the canonical Wnt-1/β-catenin pathway ameliorates the development of allergic airway disease. This effect seems to be linked to the suppression of DC activation, because overexpression of Wnt-1 decreased migration of DCs to the tLNs and the induction of appropriate T cell responses. The present data demonstrate that the Wnt-1/β-catenin pathway represents a novel interesting target to modulate allergic responses.

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Disclosures

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References


