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*J Immunol* published online 13 June 2014
http://www.jimmunol.org/content/early/2014/06/13/jimmunol.1400013

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/06/13/jimmunol.1400013.DCSupplemental

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

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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: 000–000.

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-β-catenin attenuates asthma

Materials and Methods

Mice

C57BL/6j, B6 OTI, DO11.10, 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 bimatagenic 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-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 function of Wnt-1/β-catenin signaling in the development and exacerbation of experimental allergic airway disease, the current study, we observed that overexpression of Wnt-1 resulted in an attenuation of allergic airway disease, both in acute and therapeutic models.

Assessment of allergic airway disease

Measurement of airway resistance and dynamic compliance were performed on anesthetized, intubated, and mechanically ventilated mice (flexiVent; Scriq, 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 cytocentrifuged 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 (Anti-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; 7 d prior to challenge start daily DOX application by feeding; day 32 assay. (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 start daily DOX application by feeding; day 32 assay. (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 start daily DOX application by feeding.
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 × 10^7 cells/ml FACS washing buffer, and 1 × 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, PeCy7-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−MHCI+ 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+ MHCI− B220− CD103+ and OVA+ CD11c− MHCI− 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− MHCI− 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, PeCy7–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 FACSCanto 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 OVA (Sigma-Aldrich; Grade V) at 37°C. Following incubation, supernatants were harvested and stored at −20°C for further analysis.

Ag-specific and cytokine ELISA

Serum was obtained 48 h after the last challenge. OVA-specific IgG1 and IgG2b titers were determined by ELISA (BD Pharmingen), following the manufacturer’s protocol, as described (28). All Abs were used in concentrations recommended by the manufacturer. OVA-specific IgE titers were analyzed following the protocol, as described (31). The Ab titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear-regression analysis.

To determine cytokine concentrations in BALF and supernatants of restimulated lymph node and lung cells, cytokine levels were analyzed according to the manufacturer’s instructions for IL-4, IL-5, IFN-γ, IP-10 (all from BD Pharmingen), and IL-13 (R&D Systems).

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 × 10^7 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, Lörrach, Germany) for 48 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 DO10.11 animals were purified using MACS Separator–LS columns (Miltenyi Biotec, 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 × 10^7 cells/ml and carboxyfluorescein diacetate succinimidyl ester (Invitrogen Life Technol-
flammation or AHR following allergen exposure compared with Wnt was used (Fig. 1A-1). Wnt-1 signaling prevents the cytoplasmic degradation of β-catenin. To analyze whether DOX application leads to efficient accumulation of β-catenin in the lungs of CCSP-
rtTA × tetO-Wnt1 animals, lung slices were analyzed for β-catenin expression. Indeed, CCSP-rTα × tetO-Wnt1 mice treated with DOX showed an increased expression of β-catenin mainly in the airway epithelium in comparison with mice that were sensitized but not treated with DOX (Supplemental Fig. 1).

Systemic sensitization of CCSP-rTα × tetO-Wnt1 mice with the model allergen OVA and subsequent aerosol exposure resulted in several characteristic features of allergic airway disease, including airway and lung inflammation, airway hyperresponsiveness (AHR), mucus cell metaplasia, and increased levels of OVA-specific IgE and IgG1 (Fig. 2). Interestingly, treatment of sensitized CCSP-rTα × tetO-Wnt1 mice with DOX, starting 7 d prior to the first challenge (sens + DOX), resulted in an attenuation of AHR, eosinophilia in the BALF, and a reduction in mucus cell numbers (Fig. 2). Lung tissue inflammation was reduced slightly in CCSP-rTα × tetO-Wnt1 mice with DOX; however the difference compared with CCSP-rTα × tetO-Wnt1 mice without DOX did not reach statistical significance. To assess whether DOX treatment has any effect on the allergic airway disease, sensitized and challenged wild-type animals treated or not with DOX were analyzed. No direct effect of DOX on the development of allergic airway disease was observed. Sensitized wild-type animals treated with DOX showed no significant differences in the degree of airway inflammation or AHR following allergen exposure compared with

**Statistical analysis**

ANOVA was used to determine the levels of difference among all groups. Comparisons for all pairs were performed using the unpaired Student t test. The level of significance for p values was set at 0.05. Values for all measurements are expressed as mean ± SEM.

**Results**

Expression of Wnt suppresses the development of allergic airway disease

To analyze the role of Wnt during the development of allergic airway disease, a model of lung-specific DOX-inducible overexpression of β-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 \times tetO-Wnt1 mice were sensitized and then challenged with nebulized OVA to induce allergic airway disease. Five weeks later, CCSP-rtTA \times 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 \times 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 IgGs 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 \times 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 IgGs 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 \times 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 \times 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 \times 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 was also 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-

**FIGURE 6.** Wnt-1 expression influences the migration of Ag-loaded DCs. To assess DC migration, animals were challenged by i.n. application of OVA–Alexa Fluor 647 (OVA-Alexa647). DCs were characterized as CD11c\(^+\) MHCII\(^+\) B220\(^-\) cells by FACS. Among this population, the number of OVA-Alexa647\(^-\) cells was determined. (A) FACS plots show CD11c\(^+\) and MHCII\(^+\) cells among the population of B220\(^-\) cells in the tLN at 24 h following i.n. treatment (upper panels). Graphs depict OVA-Alexa647\(^-\) cells within the CD11c\(^+\) MHCII\(^+\) DC population (lower panels). White areas represent the respective CCSP\(^+\)Wnt-1 unsens + DOX, CCSP\(^+\)Wnt-1 sens – DOX, and CCSP\(^+\)Wnt-1 sens + DOX groups, whereas the light grey areas denote the OVA-Alexa647\(^-\) control group. (B) Migration kinetics of OVA-Alexa647\(^-\) DCs were analyzed by calculating the total numbers of OVA-Alexa647\(^-\) DCs at 12, 24, and 48 h following i.n. application of OVA-Alexa647. Sensitized and challenged animals without DOX treatment are denoted by filled circles; animals also treated with DOX are denoted by open circles. (C) Total number of OVA-Alexa647\(^-\) DCs in the tLNs 24 h following application of Alexa Fluor 647. For (B) and (C), data are mean ± SEM (n = 8 mice/group from two independent experiments for the 24-h time point, and n = 4 animals/group from one experiment for the 12- and 48-h time points). (D) Total number of OVA\(^+\) DC subpopulations in the tLN. The total number of OVA\(^+\) CD11c\(^+\) MHCII\(^+\) Ly6c\(^-\) CD11b\(^+\) DCs, OVA\(^+\) CD11c\(^+\) MHCII\(^+\) Ly6c\(^+\) CD103\(^+\) DCs, and OVA\(^+\) B220\(^-\) CD11c\(^+\) Gr1\(^-\) pDCs. Data represent cell numbers for unsensitized WT (white bars), sensitized WT (diagonal striped bar), and unsensitized (black bar) and sensitized (horizontal striped bar) CCSP-rtTA \times tetO-Wnt1 mice that received DOX. Mean ± SEM are given. *p ≤ 0.05, ANOVA.
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 β-catenin accumulation following blockade of GSK-3β also was detected. Activated DCs incubated with LiCl or the highly specific GSK-3β 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/β-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 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-
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 tLN 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 tLN 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 tLN 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.

Acknowledgments

We thank Julia Altmeyer and the FACS Core Facility of the Institute of Toxicology (University Medical Center Mainz) for technical support and Pieter Hiemstra and Merete Long (Leiden University Medical Center) for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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