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Canonical and Noncanonical Wnt Proteins Program
Dendritic Cell Responses for Tolerance

Cecilia Oderup,*† Melissa LaJevic,*† and Eugene C. Butcher*†,‡

Ag-presenting dendritic cells (DCs) interpret environmental signals to orchestrate local and systemic immune responses. They govern the balance between tolerance and inflammation at epithelial surfaces, where the immune system must provide robust pathogen responses while maintaining tolerance to commensal flora and food Ags. The Wnt family of secreted proteins, which control epithelial and hematopoietic development and homeostasis, is emerging as an important regulator of inflammation. In this study, we show that canonical and noncanonical Wnts directly stimulate murine DC production of anti-inflammatory cytokines. Wnt3A triggers canonical β-catenin signaling and preferentially induces DC TGF-β and VEGF production, whereas Wnt5A induces IL-10 through alternative pathways. The Wnts also alter DC responses to microbe- or pathogen-associated molecular patterns, inhibiting proinflammatory cytokine induction in response to TLR ligands and promoting DC generation of Foxp3+ regulatory T cells. Moreover, although both Wnts suppress proinflammatory responses to bacterial endotoxin and to TLR1/2, TLR7, and TLR9 ligands, Wnt5A, but not Wnt3A, inhibits IL-6 production in response to the viral mimic, polyinosinic:polycytidylic acid. Thus, Wnt family members directly and differentially regulate DC functions, an ability that may contribute to the balance between tolerance and inflammation at epithelial sites of exposure to microbes and environmental Ags. The Journal of Immunology, 2013, 190: 000–000.

Dendritic cells (DCs) process Ags and microenvironmental signals to control innate and adaptive immunity. They detect microbe and pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, including the TLRs. PAMPs trigger increased surface expression of MHC–Ag complexes and costimulatory molecules essential for the initiation of adaptive immune responses. TLR activation also induces cytokines and other immune stimulatory factors that regulate the recruitment and activation of innate immune cells and shape the adaptive immune response (1). However, DCs are also critical in maintaining tissue homeostasis by presenting self- and food Ags, as well as commensal microbe-derived products, in a tolerance-promoting context that depends, in part, on DC secretion of immune suppressive cytokines and on the generation of regulatory T cells (Tregs) (2). Dysregulated DC control of the balance between inflammatory and tolerogenic/homeostatic immune responses contributes to immunopathology in autoimmune disease and to mechanisms of immune diversion or escape in cancer. In this context, significant attention recently has focused on identification of microenvironmental factors that regulate the balance between proinflammatory and tolerogenic functions of DCs.

The Wnt family of proteins regulates cell proliferation and differentiation in normal tissues and in cancer. Wnts are expressed in the intestine, lung, and skin (3, 4), where they are crucial for regulation of epithelial cell turnover, as well as in the bone marrow and thymus, where they regulate the development of cells of hematopoietic origin, including DCs (5). Wnts regulate the development of conventional DCs (cDCs) from monocytes or bone marrow precursors in vitro (6) and were reported to have both proinflammatory and anti-inflammatory effects on monocytes and macrophages (7–10). Studies based on the modulation of downstream Wnt-signaling molecules suggest a potential role for Wnts in DC function, as well: β-catenin, a multifunctional adaptor protein and transcriptional coactivator, is stabilized by canonical Wnt signaling and directs transcriptional activation of many Wnt-responsive genes. DC-specific deletion of β-catenin increased proinflammatory cytokine production and intestinal inflammation in mice (11). However, β-catenin activity is also regulated by cell adhesion and other Wnt-independent pathways: disruption of cell–cell contacts or mechanical agitation of DCs induces a tolerogenic DC phenotype dependent on signaling by β-catenin (12, 13). Moreover, Wnts can also activate β-catenin–independent “noncanonical” signaling mechanisms with both pro- and anti-inflammatory effects, depending on the cell context and experimental model (7, 9, 10, 14). Interestingly, a noncanonical Wnt controls a pri-mordial mechanism for tolerance and resolution of inflammation in Drosophila (15). In this study, we assessed directly the effects of Wnts on the biology and functional responses of differentiated DCs.

We report that Wnt3A and Wnt5A directly induce immunoregulatory cytokine production by differentiated DCs. Moreover, they redirect the DC response to PAMPs, suppressing DC activation...
and inflammatory cytokines while promoting Treg generation. Consistent with their signaling in other cell systems, Wnt3α activates canonical β-catenin signaling in DCs, whereas Wnt5α triggers noncanonical signaling cascades. Although both Wnts support a tolerogenic DC phenotype, they induce distinct patterns of tolerogenic cytokine production and differential DC responses to TLRs. The results suggest an important role for the Wnt family in governing DC function and immune responses.

Materials and Methods

Mice
C57BL/6 and B6.SJL/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the Veterinary Medical Unit facility of the Veterans Affairs Palo Alto Health Care Systems. All animal studies were approved by the Institutional Animal Use and Care Committee, and experimentation was conducted in accordance with American Association for the Accreditation of Laboratory Animal Care guidelines.

Cell isolation
DCs were isolated from mesenteric lymph nodes (MLNs) and peripheral lymph nodes (PLNs) of 4–10-wk-old female or male C57BL/6 or B6.SJL/J mice by Collagenase IV digestion (250 U/ml; Worthington Biochemical, Lakewood, NJ) for 30 min at 37˚C, followed by positive selection of CD11c+ cells using CD11c MicroBeads (Milteny Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. In some experiments, we took advantage of the ability of the Flt3 ligand (Flt3L) to expand subpopulations with minimal alterations in their phenotypic or functional capabilities (16). C57BL/6 or B6.SJL/J mice were injected s.c. with 5 × 10^6 Flt3L-secreting B16 melanoma cells (hereafter termed “Flt3L-treated mice”) (17). After 14 d, MLNs and PLNs were harvested, and DCs were isolated by CD11c MicroBeads (Milteny Biotec). For some studies, CD11c+ DCs were further separated into total cDCs as Lin-(CD3, CD19, NK1.1)+, CD11c+B220−or CD103−cDCs, Lin−CD11c+B220+, CD103−cDCs, and plasmacytoid DCs (pDCs) as Lin−CD11c+B220−CCR9−using a FACSaria. Spleen CD4+ T cells were isolated by positive selection using CD4 MicroBeads (Milteny Biotec).

Cell culture and CFSE labeling
DCs (1–5 × 10^6 cells/ml) were cultured in complete RPMI 1640 supplemented with 10% FCS alone or in the presence of R837 (0.5 μg/ml), LPS (5 μg/ml), Pam3CSK4 (5 μg/ml), CpG-ODN1585 (5 μg/ml), polyinosinic-polycytidylic acid [poly(I:C); 5 μg/ml] (all from InvivoGen, San Diego, CA), recombinant murine Wnt3a (PeproTech or R&D Systems, Minneapolis, MN), or recombinant human/mouse Wnt5α (R&D Systems). Cytokine secretion was determined at 20 h of culture by ELISA or Luminescence analysis of culture supernatants. Expression of MHC class II (MHCII), CD80, CD86, and CCR7 was determined by FACS at 6 h of culture. For coculture experiments, DCs (4 × 10^5/ml) were pretreated for 5 h with medium alone, CpG A alone, or CpG A+B (10 μg/ml each) or recombinant murine Wnt3α (5 μg/ml) alone. Thereafter, cells were collected and washed extensively in complete RPMI 1640 prior to coculture with T cells. CD4+ T cells were labeled with CFSE (1 μM; R&D Systems) for 5 min at 37˚C, followed by extensive washing, prior to culture. DCs (5 × 10^5/ml) and CD4+ T cells (1.25 × 10^5/ml) were cultured in the presence of anti-CD3 (0.5 μg/ml) alone or with anti-CD3 in combination with recombinant human TGF-β (3 μg/ml; R&D Systems). After 24 h, cell suspensions were loaded with CFSE by 30 min with 3 μM CFSE, washed twice, and resuspended in streptavidin-PE. After incubation for 40 min at room temperature, two additional vacuum washes were performed, and the samples were resuspended in reading buffer. Plates were read using a LumineX 200 instrument with a lower bound of 100 beads/sample/cytokine.

Western blot analysis
Nuclear and cytoplasmic protein fractions were prepared using the Nuclear Extract Kit (Active Motif), according to the manufacturer’s instructions, from Flt3L-treated mouse lymph node CD11c+ DCs (5 × 10^6 cells/ml) cultured in the presence or absence of Wnt3α or Wnt5α (0.3 and 3 μg/ml) for 30 min and 2 h. Protein concentration was determined with the BCA protein assay kit (Pierce), and 10 μg protein was resolved on 4–12% NuPAGE Bis-Tris Mini Gels (Life Technologies) and transferred to Immobilon-FL transfer membrane (Millipore). Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences) and probed with Abs for B-catenin (1:1000), tubulin (1:2000), and lamin B (1:2000), and proteins were detected using the Odyssey CLx imaging system (LI-COR Biosciences). Signal intensity was analyzed with Image Studio software.

Kinex protein microarray
Preparation of protein samples was completed according to standard Kinexus recommendations (http://www.kinexus.ca/services). Samples were prepared from lymph node DCs of Flt3L-treated mice cultured at 5 × 10^6 cell/ml for 30 min with 5 μg/ml Wnt3α or with control media. Analysis of the data was carried out by C. Laudanna and Simone Zorzan (Università di Verona, Verona, Italy) in a modification of the standard Kinexus analytics and of applications of Z transformation to microarray studies (18). Z scores (a measure of the amount of change of the signal in relation with its SD) were calculated from the Kinexus logarithmic “log2” read values on the basis of the SD of the replicates relative to Abs with good read (flag = 0 in both the physical replicates). Abs with poor reads (flag = 1) were excluded. Z ratios are calculated as differences between Z scores on control and Wnt5α-treated samples. A percentage error value was obtained as percentage ratio between average and SD on the two good physical replicas of each probe. Proteins that met the following criteria were highlighted as candidate proteins changed by Wnt5α signaling: Z ratios ≥1.7 or ≤−1.7, maximum percentage error between the two physical replicates of the probe (as calculated above) <19%, and >2-fold change in mean value induced by Wnt5α treatment.

Statistics
Data are presented as mean values ± SEM, unless otherwise indicated. Statistical significance between sets of data was assessed with the two-tailed unpaired Student’s t test.

Results
Wnts stimulate anti-inflammatory cytokine production in DCs
DCs express genes encoding several Wnt receptors and downstream Wnt effector molecules (11, 19) (http://www.imgen.org), suggesting that DCs have the machinery to respond to Wnts. To
determine whether Wnts directly affect differentiated DCs, we assessed the ability of Wnt3A and Wnt5A to induce cytokine production in freshly isolated CD11c\(^+\) DCs from mouse lymph nodes. Overnight stimulation of DCs with recombinant Wnt3A or Wnt5A stimulated secretion of the immune regulatory cytokine TGF-\(\beta\). The levels induced by Wnt3A were higher than those induced by Wnt5A (Fig. 1A). Interestingly, Wnt5A (but not Wnt3A) also induced IL-10, whereas Wnt3A (but not Wnt5A) strongly induced VEGF-A, an angiogenic growth factor that was recently implicated in immune regulation, as well (20–22) (Fig. 1A). Not all cytokines were modulated: constitutive low or undetectable expression of MCP-1 (CCL2), MCP-3 (CCL7), IP-10 (CXCL10), G-CSF, GM-CSF, IFN-\(\gamma\), IL-1\(\alpha\), IL-1\(\beta\), IL-12p70, IL-5, and IL-13 was not affected by the Wnts (data not shown). Thus, Wnts directly and selectively promote DC production of immune regulatory cytokines.

**Wnts reprogram DC responses to PAMPs**

TLRs activate DCs to produce proinflammatory cytokines and chemokines. We assessed the effects of the Wnts on TLR responses of DCs from normal mouse lymph nodes and from lymph nodes of mice in which DC populations were expanded with Flt3L, as previously described (17). DCs were stimulated overnight with TLR ligands in the presence or absence of recombinant Wnt3A or Wnt5A. Both Wnts effectively suppressed LPS-induced IL-6 and MIP-1\(\alpha\) (CCL3) production by normal lymph node DCs and reduced TNF-\(\alpha\) (Fig. 2A). IL-12p40 production was reduced, as well, in DCs from normal mice (although not in in vivo Flt3L-expanded DCs, which otherwise behaved similarly to DCs from untreated mice; see below). In contrast, Wnt3A induced secretion of VEGF, and both Wnts induced secretion of TGF-\(\beta\) in the presence of LPS (Fig. 2A). There was little or no effect on LPS-induced secretion of RANTES (CCL5), IFN-\(\gamma\), IL-1\(\beta\), IL-1\(\alpha\), CCL2, CCL7, CXCL1, GM-CSF, IL-12p70, and IL-13 were not significantly induced by TLRs or Wnts (data not shown). Moreover, Wnt inhibition of TLR-stimulated IL-6 was not limited to LPS responses: both Wnts also suppressed induction of IL-6 by total DCs in response to the TLR9 ligand CpGA (Fig. 2B).

In studies with FACS-purified cDCs (CD11c\(^{high}\) B220\(^{low}\)) from Flt3L-treated mice, both Wnts suppressed IL-6 secretion in response to LPS, CpGA, R837 (imiquimod), and the TLR1/2 ligand Pam3CSK4 (Fig. 2C). TLR stimulation of IL-12p40 production was unaffected. Under the same culture conditions, Wnt5A induced IL-10 secretion similarly in the presence or absence of TLR stimulation (Fig. 2C). Wnt inhibition of IL-6 and induction of IL-10 were dose dependent. Both Wnts also inhibited IFN-\(\alpha\) secretion induced by CpGA stimulation (Fig. 2C).

We also assessed the effects of Wnts on the responses of CD103\(^+\) cDCs (CD11c\(^{high}\) B220\(^{low}\) CD103\(^+\) cells), a subset implicated in mucosal immunity and immune regulation (23). The Wnts suppressed TLR-induced IL-6 and increased IL-10 production by CD103\(^+\) cDCs to a similar extent as observed for total cDCs (Fig. 2C, insets). Both Wnts also inhibited secretion of IL-6 and TNF-\(\alpha\) by sorted CD11c\(^{high}\) B220\(^{low}\)CCR9\(^{+}\) pDCs stimulated with the pDC-selective TLR7 ligand R837 (data not shown; IL-10 was not analyzed in pDCs). Although we cannot exclude other subset- or Wnt protein–specific effects, the results suggest that inhibition of TLR-induced proinflammatory cytokines and induction of suppressive cytokines may be a common feature of Wnt–DC interactions.

Interestingly, the two Wnts displayed distinct effects on DC responses to the viral RNA mimic poly(I:C): Wnt5A efficiently suppresses poly(I:C)-stimulated IL-6 secretion, whereas Wnt3A does not (Fig. 3), indicating a unique ability of Wnt3A to differentiate between PAMPs.

**Wnts do not prevent DC maturation**

Whether activated by PAMPs or by tolerogenic stimuli, such as “cluster disruption” or mechanical manipulation, mature DCs are characterized by increased surface display of MHCII–peptide complexes and costimulatory molecules (i.e., CD86, CD80), as well as expression of the chemokine receptor CCR7 that allows migrating DCs to exit tissue sites of Ag uptake and migrate to draining lymph nodes. Together, these features are required for efficient presentation of Ags to T cells in the draining lymph node, whether for tolerance or immune stimulation. In our experiments, we isolated DCs from lymph nodes of Flt3L-treated mice using positive selection with CD11c-conjugated beads, a process that initiates in vitro maturation without inducing inflammatory cytokines (12, 13). Isolated DCs were cultured in medium alone or in the presence of TLR9 ligand CpGA, with or without Wnt3A or Wnt5A, as above. Cultured DCs rapidly upregulated MHCII, CD80, CD86, and CCR7 in medium alone and further in response to CpGA. Importantly, Wnts had no effect on MHCII and costimulatory molecule induction, and it only minimally reduced CCR7 upregulation (Fig. 4). Thus, Wnts upregulate immune regulatory cytokines without inhibiting DC maturation, allowing development of a mature tolerogenic DC phenotype.

**Wnts reprogram DC responses to TLR ligands to promote Foxp3\(^+\) Treg generation**

To determine whether Wnt preconditioning alters the effects of DCs on T cells, we stimulated DCs with TLR ligand, either alone or in combination with Wnts, as before. After extensive washing, the conditioned DCs were used as stimulators in secondary cultures with CD4\(^+\) T cells and anti-CD3. Wnt+CpG-preconditioned DCs generated a 50–75% decreased frequency of IFN-\(\gamma\)-producing T cells, whereas the generation of TNF-\(\alpha\)-producing cells was only marginally reduced by Wnt5A, and overall T cell expansion was unaffected compared with control DCs preconditioned with TLR ligand alone (Fig. 5A). Pretreatment of DCs with Wnt alone, in the absence of TLR ligand, did not alter their ability to promote basal levels of T cell proliferation and showed only slight and variable (nonsignificant) inhibitory effects on IFN-\(\gamma\) production compared with medium control–treated DCs (Supplemental Fig. 1,
FIGURE 2. Wnts suppress TLR-induced proinflammatory cytokine production. (A) Effect of Wnts on LPS responses of DCs from normal mouse lymph nodes. CD11c+ DCs were stimulated in vitro with LPS (5 \(\mu\)g/ml) in combination with Wnt3A (3 \(\mu\)g/ml) or Wnt5A (3 \(\mu\)g/ml) for 20 h. Cytokine secretion was determined by multiplex Luminex analysis of culture supernatants. Data represent cytokine concentrations from one of two experiments performed with similar results. (B) Wnts inhibit CpG, as well as LPS, stimulation of IL-6 in DCs. CD11c+ DCs isolated from pooled lymph nodes from normal mice were stimulated in vitro with LPS (5 \(\mu\)g/ml) or CpG (5 \(\mu\)g/ml) in combination with Wnt3A or Wnt5A (3 \(\mu\)g/ml) for 20 h. Cytokine secretion was determined by ELISA. Data are mean (± SEM) cytokine concentrations from three to five experiments. Symbols represent individual experiments. (C) Dose-dependent Wnt effects on TLR responses. Total FACS-sorted cDCs (CD11c\(^{hi}\), B220\(^{-}\)) or CD103\(^{+}\) cDCs (insets) isolated from MLNs (IL-6, IL-12p40, and IL-10 graphs) or total CD11c+ DCs isolated from pooled lymph nodes (VEGF-A and IFN-\(\alpha\) graphs) of FLT3L-treated mice were stimulated in vitro with LPS (5 \(\mu\)g/ml), CpG (5 \(\mu\)g/ml), or Pam3CSK4 (5 \(\mu\)g/ml) in the presence of various doses of Wnt3A or Wnt5A for 20 h. Cytokine secretion was determined by ELISA of culture supernatants. For IL-6, IL-12p40, and IFN-\(\alpha\), data are presented as the percentage of cytokine concentration in the presence of TLR alone, without Wnt. (In the absence of Wnts, values [\(\mu\)g/ml] were as follows: IL-6 [for Wnt3A] LPS 820, CpG 3,400, Pam3CSK4 730; IL-6 [for Wnt5A] LPS 670, CpG 4,150, Pam3CSK4 660; IL-12p40 [for Wnt3A] LPS 18,310, CpG 25,220, Pam3CSK4 18,900; IL-12p40 [for Wnt5A] LPS 16,550, CpG 32,140, Pam3CSK4 13,530; and IFN-\(\alpha\) in units/ml [for Wnt3A and Wnt5A] 1650.) For IL-10 and VEGF-A induction, data are the percentage of maximum cytokine concentration induced by each TLR ligand or control. (Maximum values [\(\mu\)g/ml] were as follows: (Figure legend continues)
DCs with TGF-β, there was a clear synergy of Wnt+TLR ligand–preconditioned expansion of Tregs under the in vitro conditions used in this study, produced expansion of Foxp3+ Tregs under the in vitro conditions used in this study, produced expansion of Foxp3+ Tregs under the in vitro conditions used in this study, produced expansion of Foxp3+ Tregs under the in vitro conditions used in this study, produced expansion of Foxp3+ Tregs under the in vitro conditions used in this study, produced expansion of Foxp3+ Tregs under the in vitro conditions used in this study. Thus, although the levels of exogenous TGF-β stimulated DC induction of Tregs alone, without Wnt. (Cytokine concentration in the absence of Wnt: IL-6 [for Wnt3A] 1,100 pg/ml; IL-6 [for Wnt5A] 1,000 pg/ml; IL-12p40 [for Wnt3A] 19,160 pg/ml; IL-12p40 [for Wnt5A] 12,800 pg/ml.) Results are presented as mean ± SEM (n = 3 for IL-6) and mean ± SD (n = 2 for IL-12p40).

FIGURE 3. Differential effects of Wnt3A and Wnt5A on poly(I:C)-induced IL-6 production. cDCs (CD11c+bright, B220−) FACS sorted from MLNs of Flt3-treated mice were stimulated in vitro with poly(I:C) (5 μg/ml) in the presence of Wnt3A or Wnt5A. Supernatants were collected at 20 h of culture and analyzed by ELISA to determine the concentration of secreted cytokines, which is presented as the percentage of cytokine concentration in the presence of TLR alone, without Wnt. CD103+ DC responses (data not shown). To examine the effects of Wnts on TLR-stimulated DC induction of Tregs, we included TGF-β in the secondary cocultures. Wnt+CpG-pretreated DCs generated a much higher frequency of Foxp3+ Tregs compared with control DCs preconditioned with TLR alone (Fig. 5B). Consistent with the known Treg-promoting effect of TGF-β, there was minimal generation of Tregs by CpGA- or medium control–stimulated DCs in the absence of exogenous TGF-β, and this was not enhanced significantly by Wnts (Fig. 5B, Supplemental Fig. 1). Thus, although the levels of TGF-β induced by Wnts in DCs were insufficient to promote expansion of Tregs under the in vitro conditions used in this study, there was a clear synergy of Wnt+TLR ligand–preconditioned DCs with TGF-β, resulting in increased Treg generation and reduced expansion of Foxp3+CD4+ T cells (Fig. 5B, Supplemental Fig. 1). Addition of neutralizing anti-Wnt5A Ab (10 μg/ml) to the secondary cocultures had no effect on Foxp3+ T cell generation by Wnt5A+CpG-preconditioned DCs, ruling out a direct effect of residual Wnt on T cells (data not shown).

Wnt3A triggers β-catenin activation and Wnt5A noncanonical signaling in DCs
Wnt3A treatment of DCs induced accumulation and translocation of β-catenin from the cytoplasm to the nucleus (Fig. 6A), consistent with its well-characterized activation of canonical Wnt signaling in other cell systems (24). In contrast, Wnt5A failed to alter β-catenin levels or affect its nuclear translocation in isolated DCs. To confirm an effect of Wnt5A on DC intracellular signaling, analysis of proteins affected by Wnt5A was carried out using Kinex protein microarrays that interrogate >800 proteins and phosphoproteins (Fig. 6B, Supplemental Table I). Comparison of control untreated and Wnt5A-treated CD11c+ DCs revealed effects on multiple signaling molecules, including components of calcium, MAPK, growth factor, NF-κB, PKC, and cytokine STAT pathways (Fig. 6B). Interestingly, several of the modulated proteins have been implicated in IL-10 regulation, with the potential to contribute to Wnt5A induction of IL-10 in DCs (see Discussion), and others are known moderators of proinflammatory NF-κB signaling. We conclude that Wnt5A triggers significant alterations in intracellular signaling molecules in DCs.

In hematopoietic stem cell differentiation and in colon cancer cells, noncanonical signaling triggered by Wnt5A counteracts and inhibits β-catenin activation by canonical Wnts (25, 26). However, in DCs, Wnt5A failed to inhibit or alter Wnt3A induction of VEGF, and it did not inhibit Wnt3A-induced β-catenin translocation (Fig. 7). Moreover, excess Wnt3A had no effect on Wnt5A-induced IL-10 secretion (Fig. 7). These findings underscore the specialized nature of DC responses to Wnt signaling.

Discussion
We showed that Wnt3A and Wnt5A, two Wnts with opposing effects in many cellular systems, differentially induce immune regulatory cytokines but share a common ability to inhibit proinflammatory DC responses. Brief pretreatment with Wnts during TLR stimulation further altered DC function to promote Foxp3+ Tregs while limiting generation of IFN-γ–producing effector T cells, suggesting that Wnt reprogramming of DCs can be translated into tolerogenic effects on the adaptive immune response.

For our studies, we chose to examine Wnt3A as a representative Wnt that characteristically activates the canonical β-catenin pathway and Wnt5A, which, in most cell systems, mediates noncanonical signaling (24). Both Wnts induced tolerogenic changes in DCs, but the responses that they produced were distinct. In
studies of primary or in vivo Flt3L-expanded lymphoid tissue–derived DCs, both Wnts stimulated immune regulatory cytokines, but Wnt3A preferentially induced TGF-β, whereas Wnt5A was a potent stimulus for IL-10 production. Wnts also induce TGF-β in fibroblasts (27), and although Wnt induction of IL-10 has not been reported, to our knowledge, Gsk3β inhibition or β-catenin signaling stimulates IL-10 production in TLR-activated human monocytes (28) and T cells (29). TGF-β and IL-10 are critical participants in the maintenance of tolerance and immune homeostasis, acting on innate immune and stromal cells to suppress production of proinflammatory cytokines/chemokines and modulate downstream adaptive immune responses (30). They also have important functions in protection at mucosal surfaces: IL-10 acts on innate immune and stromal cells to suppress production of proinflammatory cytokines/chemokines and modulate downstream adaptive immune responses (30). They also have important functions in protection at mucosal surfaces: IL-10 acts on innate immune and stromal cells to suppress production of proinflammatory cytokines/chemokines and modulate downstream adaptive immune responses (30).

Interestingly, Wnt3A, but not Wnt5A, strongly stimulated DC production of VEGF-A, as well. Wnt3A was reported to upregulate VEGF in osteoblasts (36) and retinal epithelial cells (37) via the canonical β-catenin–dependent pathway. In addition to its established role in angiogenesis, VEGF has recently emerged as an immune regulatory cytokine. VEGF produced in the tumor microenvironment inhibited the proinflammatory maturation of DCs (20) and generated an increased frequency of Tregs (22), and it can directly suppress T cell activation through VEGFR-2 (21). Thus VEGF expression by Wnt-stimulated DCs may play an immune regulatory role complementing those of IL-10 and TGF-β.

In addition to these effects on basal DC activity, the Wnts effectively reprogram DC responses to PAMPs. Both Wnt3A and Wnt5A inhibit the induction of the proinflammatory cytokines IL-6, MIP-1α (CCL3),TNF-α, and IFN-α in response to TLR ligands. Interestingly however, Wnt5A suppresses IL-6 secretion in response to the viral mimic poly(I:C), just as to other TLR ligands, but Wnt3A does not. Thus, Wnt3A can differentiate between PAMPs, allowing DC proinflammatory responses to poly(I:C) while
suppressing responses to other TLR ligands. Poly(I:C) is a synthetic dsRNA that mimics RNA virus infections and initiates cellular responses through TLR3, Rig-1, and MDA-5. The selective ability of Wnt3A-treated DCs to produce proinflammatory IL-6 in response to poly(I:C) may allow a particularly aggressive immune response to RNA viruses in environments containing Wnt3A or related Wnt family members that signal via the canonical pathway (see below). Wnt3, a close structural and functional paralog of Wnt3a, is selectively expressed by Paneth cells of the crypt epithelium in the intestines where epithelial stem cells reside (38, 39). Wnt3 may allow rapid responses of crypt-associated DCs to RNA viruses infecting the proliferating epithelial cell compartment, while still inhibiting responses to non-PAMP molecules. In contrast, Wnt5A is expressed by unidentified mesenchymal cells in the intestinal lamina propria, especially in the apical region of the lamina propria (39), and by fibroblastic reticular cells in lymphoid tissues (19). Together, the results suggest the potential for sophisticated control of DCs by Wnts as a function of their microenvironmental localization.

In contrast to their inhibition of proinflammatory cytokine production, the Wnts did not inhibit culture-induced or TLR-stimulated DC upregulation of costimulatory molecules required for efficient T cell interactions. Moreover, they only had a minor inhibitory effect on upregulation of CCR7, which mediates DC migration from tissues into draining lymph nodes. These hallmarks of DC maturation are required for DC Ag presentation in vivo whether for tolerance or immune activation. Thus, the Wnts permit efficient DC maturation while reprogramming their basal and TLR-induced cytokine responses. In accordance with their tolerogenic phenotype, both Wnt3A- and Wnt5A-preconditioned DCs supported enhanced generation of Tregs while inhibiting the generation of IFN-γ-producing Th1 cells during in vitro T cell coculture. This effect is seen in the context of TLR stimulation and, in our in vitro coculture setting, required exogenous TGF-β, revealing a synergistic effect of Wnts and TGF-β in DC generation of Tregs. IL-6 is known to suppress TGF-β-induced Treg expansion while favoring inflammatory effector responses in the presence of TGF-β (40). Both Wnt3A and Wnt5A potently reduce TLR-induced IL-6 production, and it is likely that their stimulation of the Treg response reflects this suppression of DC IL-6 secretion.

Consistent with its well-established role as a canonical Wnt in other cell systems, Wnt5A induced the accumulation and nuclear translocation of β-catenin in DCs. As outlined above, β-catenin signaling is now well established as a mediator of tolerogenic DC maturation (11–13). Although Wnt5A failed to stimulate β-catenin, protein array analysis was consistent with effects on multiple signaling pathways in Wnt5a-treated cells. Alterations were observed in calcium- (e.g., CAMK4, DGKz), PI3K- (PTEN and AKT), MAPK-, and NF-κB–signaling components. Several of the changes observed in Wnt5A-treated DCs have been associated with anti-inflammatory responses in other cell types or settings. For example, mediators of inflammatory gene regulation, including STAT1a/b, STAT3, PTEN, and TRAIL, were downregulated, as were proteins associated with activation of NF-κB (NFKBp50 and IKKB), a key mediator of inflammatory responses (41). In contrast, several upregulated proteins have been implicated in anti-inflammatory responses [e.g., heat shock protein-ab-crystallin, protein tyrosine phosphatase Shp1 (42), and phospho-Dok2 (43)]. Several Wnt5A-upregulated or -activated proteins have been implicated in the positive regulation of IL-10, as well, including Shp1 (42), AKT (44), Erk1/2 (45), the atypical PKCs (46), and phospho-CREB, which can bind the IL-10 promoter and positively regulate its transcription in response to Erk and AKT signaling (45). In addition to immune-responsive proteins, changes were observed in several signaling molecules involved in growth/survival; EGFR, ErbB2, phospho-KIT, AKT, and Erk1/2 were upregulated, whereas proapoptotic TRAIL was downregulated. Although these studies were undertaken primarily to confirm an impact of Wnt5A on intracellular signaling, the diverse modifications observed suggest the possibility that Wnt5A induces extensive reprogramming of DC-signaling networks. Future studies will be required to define specific pathways involved in the unique DC responses (e.g., IL-10 induction, versus suppression of TLR-mediated activation) induced by Wnt5A.

It was perhaps surprising that Wnt3A and Wnt5A both condition DCs for tolerogenesis. In most cellular systems, as in DCs, Wnt3A acts through the canonical pathway, inhibiting Gsk3β and inducing downstream β-catenin accumulation, whereas Wnt5A activates noncanonical signaling and can even antagonize the canonical pathway, such that the two Wnts frequently have opposing functions (24). Indeed, although Wnt3A acting through β-catenin is anti-inflammatory for macrophages (8), Wnt5A can be either proinflammatory (9) or anti-inflammatory (10). Similarly, Wnt5A inhibits, whereas Wnt3A promotes, CDC development from mouse and human bone marrow (6), and Wnt5A and Wnt5A often display opposing roles in developing T and B cells (5). Although the two Wnts trigger distinct responses in differentiated DCs as well, they nonetheless both induce immune regulatory phenotypic features.
and suppress the proinflammatory DC response to the majority of TLRs. These shared immune suppressive effects on DCs suggests a unique importance for tolerogenic immune regulation through Wnt–DC interactions. This concept is further supported by the fact that Wnt5A, which in other cell systems counteracts Wnt3A/canonical Wnt signaling, had no effect on Wnt3A-induced β-catenin translocation on Wnt3A stimulation of VEGF in DCs.

Consistent with our findings, Hack et al. (47) recently reported that Wnt5A suppresses human blood pDC activation by CpG. Together with this recent report, our findings demonstrate a direct effect of Wnt proteins on differentiated tissue DCs, confirming the hypothesized role for canonical Wnts in inducing DC tolerogenesis but also contrasting DC responses to canonical versus noncanonical Wnts. Previous studies of β-catenin–dependent mechanisms in immune tolerance and of Wnt effects on in vitro development of DCs from monocytes provided the rationale for assessing the role of Wnts in DC function. Two studies showed that disruption of E-cadherin–dependent cell clustering of immature in vitro bone marrow–derived DCs resulted in a mature (costimulatory molecule^{high} MHCII^{high} CCR7^{+}) tolerogenic phenotype, the induction of which involved β-catenin (12, 13). Cluster disruption–induced DCs failed to secrete inflammatory cytokines (IL-6, TNF-β), and a deficiency in DC IL-10 and TGF-β production. In combination, these findings strongly support the concept that Wnt signaling at the early monocyte stage, rather than at a later DC stage, was responsible for Wnt-mediated modulation in this study. Therefore, the difference in the induced DC phenotypes and Wnt3A/Wnt5A responses reported in this article compared with those reported by Valencia et al. (48) could reflect differential and cell type–specific Wnt signaling/Wnt effects in monocytes versus DCs. Indeed, Wnt5A displayed proinflammatory effects in the differentiating monocyte system: supernatants of Wnt5A-treated monocytes contained high levels of IL-6 compared with control cultures (48), which is in line with previous reports of Wnt5A’s effects on macrophages (9) but is in clear contrast to our observation of its anti-inflammatory effects on differentiated tissue DCs.

Finally, elegant studies by Manicassamy et al. (11) showed that mice with a selective DC deficiency in β-catenin display enhanced sensitivity to chemically induced colitis (dextran sulfate sodium), associated with increased production of inflammatory cytokines and a deficiency in DC IL-10 and TGF-β production. In combination with our finding that Wnt3A induces β-catenin activation and TGF-β secretion in DCs, the demonstrated importance of DC-intrinsic β-catenin signaling to immune tolerance in vivo provides strong support for the proposed role of Wnts in regulating DC responses and maintaining tolerance under homeostatic conditions.

Interestingly, Wnt expression is increased during infection and inflammation: Wnt5A is upregulated in sepsis (9), in granulomas of Mycobacterium tuberculosis patients (7, 8), and in bacterial periodontitis (49), and Wnts are increased or abnormally expressed in arthritis synovial tissue (50), atherosclerotic plaques (51), psoriatic or wounded skin (3, 52), and in inflammatory bowel disease (53). Our results suggest that induced Wnt proteins may modulate DC responses to limit inflammation after trauma or infection. In this context, it is interesting that WntD, a Drosophila Wnt protein that signals through noncanonical pathways, plays a critical role in resolving inflammatory responses in the fly (15), raising the possibility that Wnt regulation of immune response is a primordial mechanism for achieving immune homeostasis. However, Wnt5A is also upregulated in granulomas (7, 8), and Wnt–DC interactions could play a negative role in the persistence of chronic infections (e.g., tuberculosis). Finally, although tolerance to innocuous Ags and commensal organisms is advantageous, suppression of the immune response is undesirable in the context of tumor immunity. Wnts are frequently overexpressed in cancers (4), and their immune suppressive and tolerogenic effects on DCs could contribute to tumor immune evasion and cancer progression.

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Disclosures

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