Wnt5a Skews Dendritic Cell Differentiation to an Unconventional Phenotype with Tolerogenic Features

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Dendritic cells (DCs) are critical regulators of immune responses that integrate signals from the innate and adaptive immune system and orchestrate T cell responses toward either immunity or tolerance. Growing evidence points to the Wnt signaling pathway as a pivotal piece in the immune balance and focuses on DCs as a direct target for their immunoregulatory role. Our results show that the increase in Wnt5a signaling during the differentiation of human DCs from monocytes alters their phenotype and compromises their subsequent capacity to mature in response to TLR-dependent stimuli. These Wnt5a-DCs produce scant amounts of IL-12p70 and TNF-α but increased levels of IL-10. Consequently, these Wnt5a-DCs have a reduced capacity to induce Th1 responses that promote IL-10 secretion by CD4 T cells. Changes in the transcriptional profile of Wnt5a-DCs correlate with their unconventional phenotype caused presumably by increased IL-6/IL-10 signaling during the process of DC differentiation. The effect of Wnt5a is not a consequence of β-catenin accumulation but is dependent on noncanonical Ca^{2+}/calmodulin-dependent protein kinase II/NF-κB signaling. Our results therefore suggest that under high levels of Wnt5a, typical of the inflammatory state and sepsis, monocytes could differentiate into unconventional DCs with tolerogenic features. ***The Journal of Immunology*, 2011, 187: 4129–4139.**

Dendritic cells (DCs) play a central role orchestrating adaptive immune responses against pathogens or cancer while maintaining tolerance to self Ags and innocuous micro-organisms or molecules. These dual-antagonistic functions are balanced by a microenvironment that affects developing DCs or signals received during DC activation. This milieu determines DC functional diversity and allows the organization of discrete DC subpopulations into different anatomical compartments, which present specific immunological requirements, and adaptation of their activities to the organism's state (1–3). Recent growing evidence points to Wnt proteins and Wnt signaling components as important modulators of the immune system that participate in the balance between tolerance and immunity and reveal DCs as one of their direct targets (4–6).

Wnt proteins are a large family of secreted proteins widely expressed during development when they are implicated in an enormous array of processes (7). In adults, Wnt proteins regulate stem cell renewal and differentiation and modulate bone remodeling (8), adipose (9), and tissue homeostasis and hematopoiesis (10), and Wnt signaling deregulation is associated with cancer (11). Wnt proteins also have been proposed as endocrine factors involved in aging (12, 13) and septicemia (14).

To trigger a cellular response, a single Wnt ligand can simultaneously activate distinct signaling pathways (15, 16). Ultimate Wnt signaling output and biological consequences depend on both the Wnt ligand and the receptor context. Canonical signaling is initiated through a multimeric cluster of low-density receptor-related protein 5/6 (LRP5/6) and Frizzled (Fzd) receptors that recruit the cytoplasmic proteins dishevelled, axin, and glycogen synthase kinase 3 (GSK3) to the membrane. This results in LRP phosphorylation and disassembly of the β-catenin destruction complex, thus preventing constitutive phosphorylation of β-catenin by GSK3 and its subsequent proteolytic destruction. Ultimately, β-catenin accumulates in the nucleus and activates the transcription of Wnt target genes. In parallel to β-catenin transcriptional activity, canonical signaling also may influence a wider range of metabolic and signaling pathways through its inhibitory effect on GSK3 activity (17). Noncanonical signaling occurs independently of β-catenin stabilization. Fzd-activated dishevelled can associate with small GTPases, including Rac, Cdc42, and Rho. They in turn activate ROCK kinase and JNK, which remodel the cytoskeleton and promote c-Jun–regulated cell expression. Fzd–LRP receptor complexes also can signal via G proteins, which induce Ca^{2+} flux that activates calcium/calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC), and subsequently NF-AT. CaMKII can lead to the activation of a nemo-like kinase, which may interfere with β-catenin transcriptional activity (15, 16). Different components of the Wnt pathway have immunomodulatory properties and are possible targets for therapeutic intervention. GSK3 inhibition has a global anti-inflammatory effect and negatively affects innate and adaptive immune responses (18, 19). Endothelial cell-derived Wnt proteins activate the β-catenin–dependent pathway necessary for optimal effector T cell transmigration (20), while inflammation makes macrophages sensitive

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Abbreviations used in this article: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CBA, Cytometric Bead Array; DC, dendritic cell; Fzd, Frizzled; GSK3, glycogen synthase kinase 3; ID, inhibitor of DNA binding; iDC, immature dendritic cell; IRF, IFN regulatory factor; LRP, low-density receptor-related protein; MFI, mean fluorescence intensity; PDL, programmed cell death ligand; PKC, protein kinase C; poly I:C, polyinosinic-polycytidylic acid; SOCS, suppressors of cytokine signaling.

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to Wnt3a, which in turn reduces their proinflammatory effector mechanisms (21). However, Wnt5a, considered a prototypical noncanonical Wnt protein, has been proposed as a proinflammatory signal involved in macrophage (14, 22) and endothelial (23) activation, implicated in rheumatoid arthritis pathogenesis (24), and detected in other human inflammatory diseases (22, 23).

Several studies have revealed that DCs are important targets for the immunomodulatory activity of Wnt signaling. Cooperation of the canonical Wnt pathway with Notch signal is required for the differentiation of hematopoietic progenitor cells into DCs (5). It also has been demonstrated that GSK3 activity profoundly influences DC differentiation and maturation. GSK3 inhibits the differentiation of monocytes into macrophages, thus allowing DC differentiation. In immature monocyte-derived DCs, this enzyme is constitutively active suppressing spontaneous maturation and enhancing cytokine production after CD40L- or *Escherichia coli*-dependent activation (25). Consistent with these observations, inhibition of GSK3 activity or activation of the β-catenin pathway through the disruption of E-cadherin–mediated adhesion in immature DCs (iDCs) induces a semimature tolerogenic phenotype that could correlate with the functional characteristics of the migratory DCs that reach lymph nodes in the steady state (4). More recently, Manicassamy et al. (6) revealed a role for β-catenin in the balance between immunity and tolerance in murine intestinal mucosa. Intestinal lamina propria DCs expressed Wnt ligands and Fzd receptors, and Wnt-mediated activation of β-catenin programs them to induce regulatory T cells (6).

In the present study, we show that Wnt5a impairs human monocyte-derived DC differentiation. DCs that differentiate in the presence of exogenous Wnt5a (Wnt5a-DCs) show basic phenotypic features of DCs but upon TLR stimulation produce scant amounts of IL-12 and TNF-α but higher amounts of IL-10. Consequently, these Wnt5a-DCs have a reduced capacity to induce Th1 responses that promote IL-10 secretion. We also observed that Wnt5a-DCs showed an altered transcription profile suggestive of their unconventional phenotype, focusing our interest on the signaling mechanisms by which Wnt5a alters DC differentiation.

Materials and Methods

Human monocyte differentiation

Human $CD14^+$ monocytes were isolated from buffy coats (Centro de Transfusión de la Comunidad de Madrid, Madrid, Spain) by density gradient centrifugation and immunomagnetic cell separation using anti-CD14–conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (>95%) were cultured in 48-well plates at a density of 1×10^6 cells per milliliter in AIMV medium supplemented with 2 mM L-glutamine (Sigma-Spain), 20 ng/ml GM-CSF, and 20 ng/ml IL-4 (both from Invitrogen, Carlsbad, CA), in the presence or absence of 100 ng/ml Wnt3a or Wnt5a (both from Chemicon International, Temecula, CA) for 6 d. One half of the medium was replaced every 2 d. In some experiments, GM-CSF/IL-4 monocyte cultures were treated with Wnt5a alone or Wnt5a plus specific signaling inhibitor molecules only during the first 24 h of culture, when total medium from the cultures was substituted for 24 h GM-CSF/IL-4 monocyte-conditioned medium. In the Wnt signaling inhibition assays, cells were pretreated with either Go6983 (5 mM) purchased from Tocris Bioscience (Bristol, U.K.), CK59 (1–50 μM), SP600125 (20 μM), or JSH-23 (5–10 μM) (from Merck KGaA, Darmstadt, Germany) for 15 min before adding Wnt5a and GM-CSF/IL-4 mixture.

FACS analysis

The following mAbs conjugated with FITC, PE, PE-Cy5, or allophycocyanin were used for flow cytometric analysis, as described previously (26): CD14 (M5E2) (Inmunostep, Salamanca, Spain), CD1a (HI149), HLA-DR (G46-6), CD40 (5C3),CD80 (L307.4) (all from BD Biosciences, San Jose, CA), CD83 (HB15e), CD86 (IT2.2), CD197/CCR7 (TG8), CD205/DEC-205 (MG38), CD206/MMR (15-2), CD209/DC-SIGN(9E9A8), CD273/programmed cell death ligand 2 (PDL2) (24F.10C12), CD274/PDL1 (29E.2A3), CD324/E-cadherin (67A4) (BioLegend, San Diego, CA). For intracellular staining of phosphorylated STAT5, STAT6, and CaMKII, cells were treated with CellFIX BD Phosflow Perm Buffer III (BD Biosciences) and stained with PE-conjugated anti-phospho-STAT5 (Tyr^{694}) and with PE-conjugated anti-phopho-STAT6 (Tyr^{641}) (both from BD Biosciences) or with anti-phospho-CaMKIIα (Thr^{286}) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by allophycocyanin-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories). For the intracellular staining of IFN regulatory factor 1 (IRF1), IRF8, inhibitor of DNA binding 3 (ID3), suppressors of cytokine signaling 3 (SOCS3), and total β-catenin, cells were fixed and permeabilized using Cytofix/Cytoperm solution, washed with Perm/Wash buffer (BD Biosciences), and stained with specific rabbit polyclonal Abs for IRF1, IRF8, ID3, and SOCS3 (all from Santa Cruz Biotechnology) followed by allophycocyanin-conjugated donkey anti-rabbit IgG or with FITC-conjugated anti-β-catenin mAb (BD Biosciences) (14). Analyses were conducted with a FACSCalibur flow cytometer (BD Biosciences) from the Centro de Citometría y Microscopía de Fluorescencia (Complutense University, Madrid, Spain)

Immunofluorescence analysis

For immunofluorescence analysis, monocytes were differentiated in DCs as described above but cultured in 0.8 cm^2 per well chamber slides (Thermo Fisher Scientific). The attached cells were fixed in situ and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) and stained with mouse anti-human HLA-DR (BD Biosciences) followed by Alexa Fluor 488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) and Alexa Fluor 633-conjugated phalloidin (Invitrogen). Hoechst 33258 was used as a nuclear stain (Invitrogen). Slides were mounted with Prolong Gold (Invitrogen) and examined on an Axioplan-2 microscope (Zeiss, Oberkochen, Germany) from the Centro de Microscopía y Citometría.

DC stimulation

At day 6 of culture, iDCs were washed to remove Wnt3a/ Wnt5a and cytokines and stimulated for 24–48 h with different TLR agonists: LPS (100 ng/ml), polyinosinic-polycytidylic acid (poly I:C) (12.5 μg/ml), FSL1 (100 ng/ml), ssRNA40 (1 μg/ml), Pam3CSK4 (250 ng/ml), or flagellin (500 ng/ml) (all from Invivogen, San Diego, CA).

Mixed lymphocyte reaction

Different numbers of TLR-DCs were used as stimulators for allogeneic naive CD4 T cells (1.5×10^5) isolated from human peripheral blood. $CD45RO^-$ CD4 T cells were labeled previously with 5 μM CFSE (Sigma-Spain). The cocultures were performed in 96-well flat-bottom culture plates, using RPMI 1640 supplemented with 10% FCS (Invitrogen). Lymphocyte proliferation was assessed by the CFSE dilution method in $CD3^+$ cells from day 7 cocultures.

Cytokine measurements

Culture supernatants of TLR-DCs and DC/T cell cocultures were harvested, and cytokine secretion was measured. Production of IL-12p70, IFN-γ, IL-10, IL-4, TNF-α, and IL-8 and IL-1β, IL-6, IL-17a, and TGF-β were analyzed using ELISA MAX (BioLegend) and Cytometric Bead Array (CBA) assays (BD Biosciences), respectively, according to the manufacturers' instructions.

Internalization assays

Nonspecific pinocytosis or mannose receptor-mediated endocytosis was measured by incubating day 6 DCs at 37°C for 45 min with 1 mg/ml Lucifer yellow (Sigma-Aldrich) or 40-kDa FITC-dextran (100–500 μg/ml), respectively. Control samples were treated similarly at 0°C. After incubation with the exogenous materials, fluorescence intensity was measured by flow cytometry.

Real-time quantitative RT-PCR

RNA isolation and total cDNA synthesis were performed as described previously (27) for assays for ID2, ID3, PU.1, ETS1, IRF1, IRF2, IRF3, IRF4, IRF8, SOCS1, SOCS3, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR8 (all from Applied Biosystems, Foster City, CA). GAPDH (predeveloped TaqMan) was used as an endogenous control. All of the PCR reactions were set in duplicate using the TaqMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's instructions. Amplifications, detections, and analyses were performed in a 7900HT Fast Real-Time PCR System (Centro de Genómica, Complutense University, Madrid, Spain). The ΔCt method was used for normalization to GAPDH mRNA.

Statistical analysis

Unpaired two-tailed Student *t* tests were used to compare differences. Values of $*p \le 0.05$, $**p \le 0.005$, and $p \le 0.0005$ were considered to be statistically significant.

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Results

Wnt proteins interfere with the differentiation of iDCs from human monocytes

We analyzed the differentiation of peripheral blood CD14 monocytes into iDCs when cultured in serum-free medium supplemented with GM-CSF/IL-4 containing either Wnt3a or Wnt5a, typically considered canonical and noncanonical Wnt ligands, respectively.

The addition of Wnt proteins to the GM-CSF/IL-4 cytokine mixture did not change total cell yield or cell survival along the culture period but triggered important modifications of the DC phenotype. These effects were more profound in cells generated under the influence of exogenous Wnt5a. The addition of Wnt5a inhibited to some extent the downregulation of CD14 expression but increased, like Wnt3a, the percentage of $CD1a^+$ cells in each individual donor (Fig. 1*A*, 1*B*). Thus, day 6 Wnt5a-treated cultures yielded 10–40% of $CD14^{lo}$ cells that expressed mostly CD1a. Further phenotypic characterization of immature Wnt5a-DCs revealed a boost in DC-SIGN, PDL1, and PDL2 levels, a slight increase in CD40 levels, and a reduction in HLA-DR, CD86, and MMR expression. E-cadherin, CD80, and CD83 levels were hardly affected. Comparatively, Wnt3a treatment exerted a lower impact on DC phenotype than Wnt5a (Fig. 1*C*, Supplemental Table I)

Morphologically, we did not find differences in the forward side and scatter characteristics of cells detached from cultures (data not shown). However, in situ microscopic observation of HLA-DR immunofluorescence staining showed that, whereas control iDCs were mostly round cells, rich in lamellipodium extensions, Wnt-treated cells formed a network of adherent elongated cells, rich in filopodia extensions, a feature more evident in Wnt5a-treated cultures (Supplemental Fig. 1).

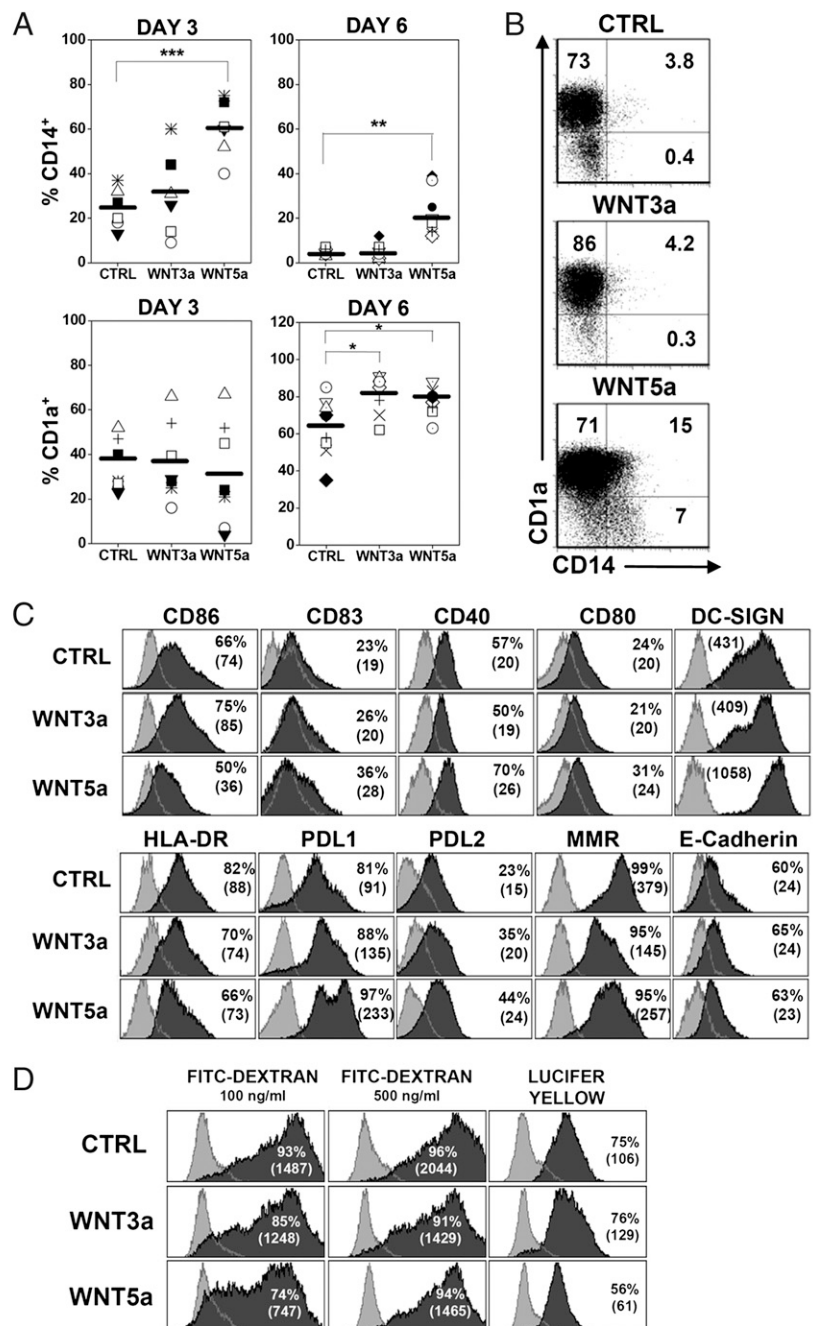

FIGURE 1. Wnt proteins alter DC differentiation from human monocytes. Human $CD14^+$ monocytes were cultured for 6 d with GM-CSF/ IL-4 in either the presence or the absence of Wnt3a (100 ng/ml) or Wnt5a (100 ng/ml). *A*, The scatter plots show the percentage of $CD14^+$ or $CD1a^+$ cells recovered after 3 or 6 d from either control or Wnt-treated cultures (n = 6–8). Each symbol represents the result of a single donor, and average values are indicated with horizontal bars. *B* and *C*, Phenotypic characterization of day 6 iDCs. Dot plots show CD14 versus CD1a expression (*B*), and histograms represent expression of surface Ags (black) compared with background fluorescence (light gray) (*C*). The percentage of positive cells and the total mean fluorescence intensity (MFI) (in parentheses) are shown in each histogram. Data are representative of nine independent experiments. *D*, Uptake of soluble material by iDCs. iDCs at day 6 of culture were incubated in the presence of FITC-dextran or Lucifer yellow at 37˚C, and after 45 min the internalization was measured by flow cytometry (black histograms). Control samples were treated likewise at 4˚C (light gray histograms). Data are representative of three independent experiments. $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$, by unpaired t test.

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Immature DCs have the ability to internalize Ags avidly through a variety of mechanisms, including specific receptor-mediated endocytosis and nonspecific uptake by constitutive macropinocytosis. Wnt-DCs were less efficient in specific dextran uptake, which correlated with their lower expression of MMR. We also found a decrease in nonspecific fluid phase endocytosis by Wnt5a-DCs (measured as uptake of Lucifer yellow) (Fig. 1*D*, Supplemental Table I)

Wnt-DCs show an altered response to TLR ligands

Cells differentiated in the presence of Wnt proteins were analyzed for their phenotypic maturation and cytokine profile after activation by TLR stimuli. These experiments were performed in the absence of exogenous Wnt proteins. Our results showed that the capacity of Wnt-DCs to maturate after TLR ligation was altered. In general terms, Wnt5a-DCs were more affected than Wnt3a-DCs. Upon TLR4–LPS stimulation, Wnt5a-DCs did not properly upregulate CD83, HLA-DR, CD40, CD86, CD80, and CCR7 molecules. In contrast, in response to LPS, Wnt5a-DCs increased the expression of the inhibitory ligands PDL1 and PDL2 like control DCs did, which could promote inhibitory output during their interaction with T cells (Fig. 2*A*, Supplemental Table II). The phenotypic maturation of Wnt5a-DCs triggered by other bacterial TLR ligands, TLR2/1–Pam3CSK4, and TLR5–flagellin stimuli were

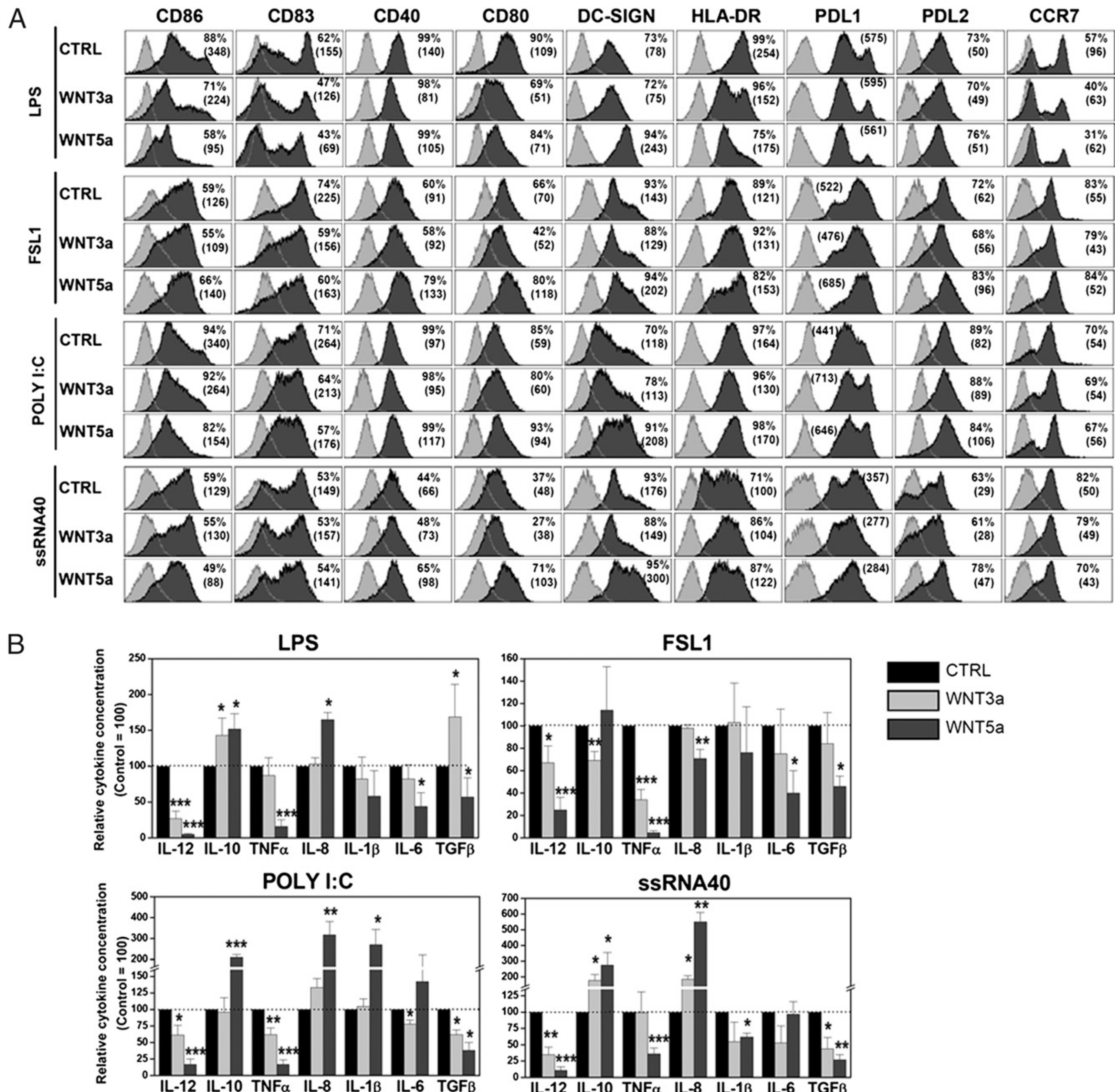

FIGURE 2. Response of Wnt-DCs to TLR ligands. Day 6 iDCs differentiated in the presence or absence of Wnt proteins were stimulated for 24–48 h with different TLR agonists (LPS, FSL1, poly I:C, and ssRNA40). Culture medium was replaced gently before adding the stimuli to avoid the influence of Wnt treatment. *A*, Histograms show the expression of surface molecules (black) analyzed by flow cytometry after 24 h of TLR stimulation. The percentage of positive cells, determined according to background staining (light gray), and the total MFI (in parentheses) are shown in each histogram. Data are representative of five independent experiments. *B*, Culture supernatants of TLR DCs were collected 48 h after stimulation. The supernatants were analyzed for IL-12p70, IL-10, TNF-α, and IL-8 by ELISA and IL-1β, IL-6, and TGF-β by CBA assays. Note the *y*-axis break and scale change at break. Data represent the mean (±SD) of five to nine independent experiments, expressed relative to individual controls (black bars, referred to as 100%). $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$, by unpaired *t* test.

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affected like the TLR4 response (data not shown). The response of Wnt-DCs to the synthetic lipoprotein FSL1, which signals through TLR2/6 dimers, or to viral TLR ligands, such as poly I:C/TLR3 and ssRNA40/TLR8, also was assayed, and the effects observed were less remarkable (Fig. 2*A*, Supplemental Table II). Nevertheless, as compared with their control counterparts, only FSL1-stimulated Wnt5a-DCs properly upregulated CD86 expression, and PDL1 or PDL2 expression was increased in Wnt5a-DCs after these stimuli.

Because cytokine production by activated DCs plays a critical role in the differentiation of primed T cells, we assessed by protein ELISA or CBA assays the capacity of Wnt-DCs to secrete diverse cytokines. Wnt-DCs produced significantly lower levels of pro-Th1 cytokines IL-12p70 and TNF-α than control DCs while the secretion of IL-10 regulatory cytokines was increased, the latter after all of the stimuli with the exception of FSL1 stimulation. Accordingly, the IL-10/IL-12 ratio increased, which could impair the capacity of Wnt-DCs to polarize naive T cells into Th1 clones. We also found differences in the capacity of Wnt-DCs to secrete other cytokines such as IL-8, IL-6, IL-1β, and TGF-β, but the effects were dependent on TLR stimulus and/or Wnt ligand treatment (Fig. 2*B*).

Wnt5a-DCs stimulated through TLR are poor allostimulators that promote IL-10 secretion by naive T cells

DCs stimulated with TLR agonists were cultured in MLR with naive $CD4^+$ T cells to analyze their allostimulatory capacity. Apart from DCs maturated by poly I:C, TLR-stimulated Wnt-DCs induced a lower proliferative response of T cells than their control counterparts. The ability of DCs to polarize naive T cells was determined by analyzing cytokine levels in day 7 MLR supernatants. Control DCs were able to efficiently polarize Th1 responses but failed to induce Th2 or Th17 responses, thus leading to consistent levels of IFN-γ detected in MLR supernatants and low and undetectable levels of IL-4 and of IL-17α, respectively. Naive $CD4^+$ T cells primed with TLR-stimulated Wnt5a-DCs produced lower amounts of IFN-γ and TNF-α but increased the secretion of IL-10, IL-8, and IL-6, the latter two only after some of the TLR stimuli. No differences were found in the secretion of IL-4 or IL-17α (data not shown). With respect to Wnt3a-DCs, cytokine levels detected in these MLR supernatants were not significantly different from those found in their individual controls (Fig. 3).

Changes in the transcription profile account for the impaired function of Wnt5a-DCs

Downregulation of TLR expression might account for the impaired response of Wnt-DCs to TLR stimuli. Comparative real-time PCR analysis in immature control and Wnt-DCs showed that TLR3, TLR4, and TLR5 genes were transcribed in Wnt-DCs at significantly lower levels than those in their individual controls, which agrees with their inadequate response upon stimulation with poly I:C, LPS, and flagellin, respectively. However, although the response of Wnt5a-DCs to Pam3CSK4, FSL1, or ssRNA40 also was affected, we did not find significant changes in TLR2, TLR6, or TLR8 transcript levels, and we observed an increase of TLR1

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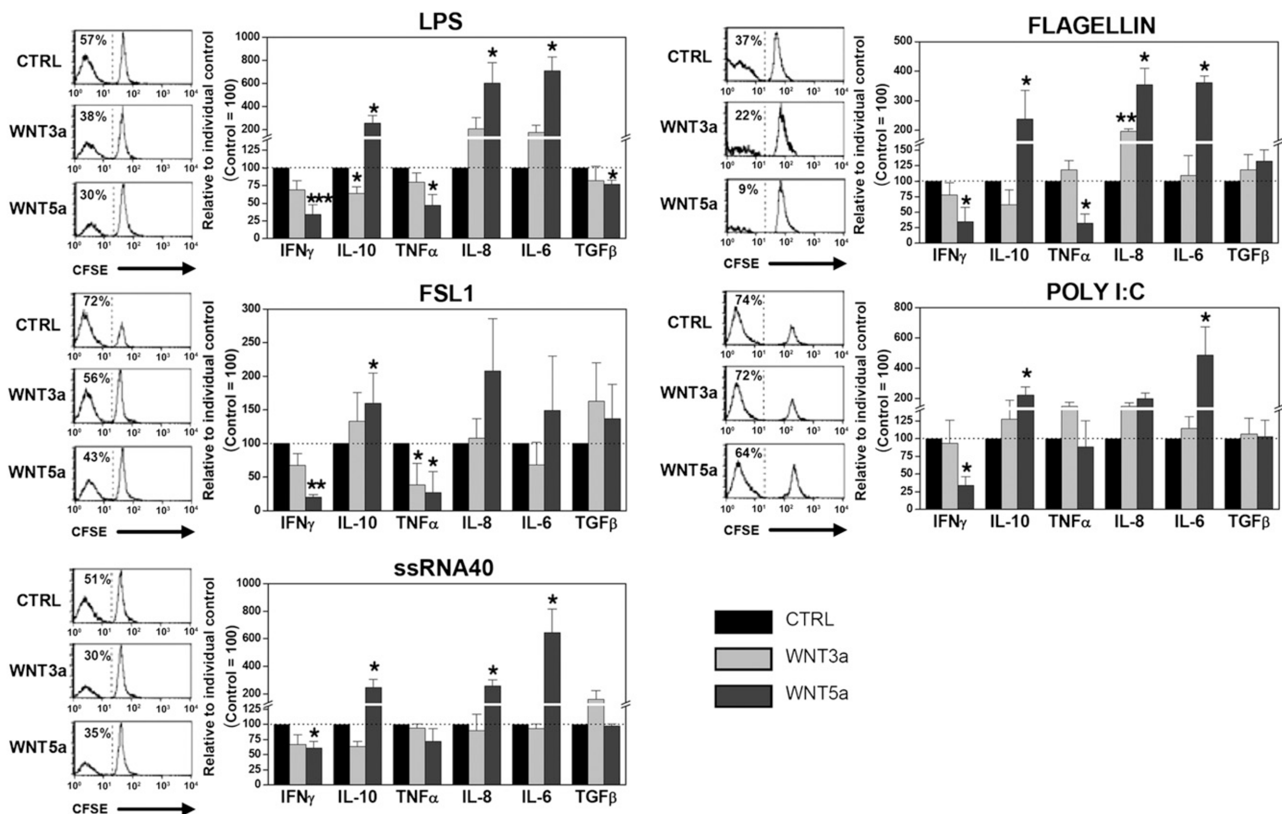

FIGURE 3. TLR-stimulated Wnt5a-DCs show lower allostimulatory capacity and promote IL-10 secretion by naive T cells. DCs stimulated with TLR agonists were cultured in MLR with CFSE-labeled allogeneic naive $CD4^+$ T cells at a 1:10 DC/T cell ratio. After 7 d, lymphocytes were harvested, and the percentage of proliferating cells was calculated by the CFSE dilution method (gated on the $CD3^+$ cell population). Histograms show the percentage of cells with ≥1 cycle of division (using T cells cultured in the absence of stimulation as controls). Data are representative of at least three independent experiments. Supernatants of DC/T cell cocultures were harvested at day 7, and the amounts of IFN-γ, IL-10, TNF-α, IL-8, IL-6, and TGF-β were tested. Data represent the mean (±SD) of six to nine independent experiments, expressed relative to individual controls (black bars; referred to as 100%). $*p \le 0.05$, $**p \le 0.005$, $***p \le 0.0005$, by unpaired *t* test.

transcription in Wnt5a-DCs (Fig. 4*A*). We therefore investigated whether other mechanisms irrespective of TLR expression also could determine the functional characteristics of Wnt-DCs.

An analysis of mRNA levels for several transcriptional regulators revealed that immature Wnt5a-DCs expressed lower levels of ID2 and IRF8, an essential regulator of IL-12 expression, and showed higher amounts of IRF1 and IRF2 transcripts. IRF1 and IRF8 are critical for IL-12 transcription (28), and consequently their transcription was induced in control DCs after LPS stimulus (6- to 14-fold increase). Wnt5a-DCs failed to properly upregulate the expression of both genes after LPS stimulation (Fig. 4*C*), which led to lower IRF8 and IRF1 protein levels (Fig. 4*D*). Interestingly, Wnt-DCs showed higher levels of ID3 and SOCS3 expression (Fig. 4*B*, 4*E*).

Wnt5a diminishes the response of monocytes to the GM-CSF/IL-4 cytokine mixture and alters the DC differentiation genetic program

Because the outcome of in vitro monocyte differentiation into DCs ultimately depends on efficient GM-CSF and IL-4 signaling, we examined the phosphorylation of STAT5 and STAT6 in monocytes treated with Wnt5a or Wnt3a. Treatment of monocytes with Wnt proteins resulted in significant but partial inhibition of both STAT5 and STAT6 phosphorylation. These inhibitory effects became evident within the time frame between 2 and 20 h of cell culture, which suggests that they are a secondary effect of Wnt signaling (Fig. 5*A*). We found that Wnt treatment induced SOCS3, but not SOCS1, expression during the first hours of culture (Fig. 5*B*). SOCS3 showed the highest transcription in Wnt5a-treated cultures at 3 h (Fig. 5*B*) but as mentioned above remained high in day 6 iDCs (Fig. 4*B*).

During the first hours of monocyte culture, GM-CSF/IL-4 signaling triggers a genetic program that leads to DC development, inducing drastic changes in the expression of several regulatory proteins and transcription factors critical for DC differentiation (Fig. 5*C*). In agreement with a modulatory, but not blocking, effect of Wnt5a in DC differentiation, we found normal regulation of most of these genes during the first hours of culture. Nevertheless, Wnt5a treatment blocked the important reduction of ID2 transcription observed in control monocytes during the first 3 h of culture and partially inhibited the upregulation of IRF3 and PU.1 gene expression that occurred in the following 17 h. During this period, Wnt5a treatment also induced a decrease in IRF8 transcription, which, as described above, remained lower than that in controls in day 6 iDCs (Fig. 4*B*).

The foregoing results are consistent with an early effect of Wnt5a signaling on DC differentiation. Consequently, we observed that the addition of Wnt5a only during the first 24 h of culture was sufficient to exert most of its effects (Fig. 5*D*–*F*). Monocytes that begin to differentiate into DCs in an environment rich in Wnt5a therefore would differentiate into functionally impaired DCs independently of later Wnt5a signaling.

Wnt signaling induces the secretion of IL-6, IL-10, and TNF-α by monocytes

Monocytes could respond to Wnt5a by secreting cytokines such as IL-6 or IL-10 that can interfere with DC differentiation. Supernatants from Wnt5a-treated cultures contained extremely high amounts of IL-6 and IL-10 cytokines compared with those of their control counterparts (Fig. 6*A*). Again, the effects of Wnt5a were significantly higher than those of Wnt3a. The induction of IL-6 secretion by Wnt5a was evident after 3 h of culture and peaked at 24 h (Fig. 6*B*). IL-10 secretion began later than that of IL-6 and was evident only after the overnight culture period. After this initial boost of IL-6 and IL-10 secretion in Wnt-treated cultures, cytokine levels remained higher until day 6 (data not shown). In the absence of the GM-CSF/IL-4 mixture, monocytes treated with Wnt5a doubled IL-6 and IL-10 secretion (data not shown). This

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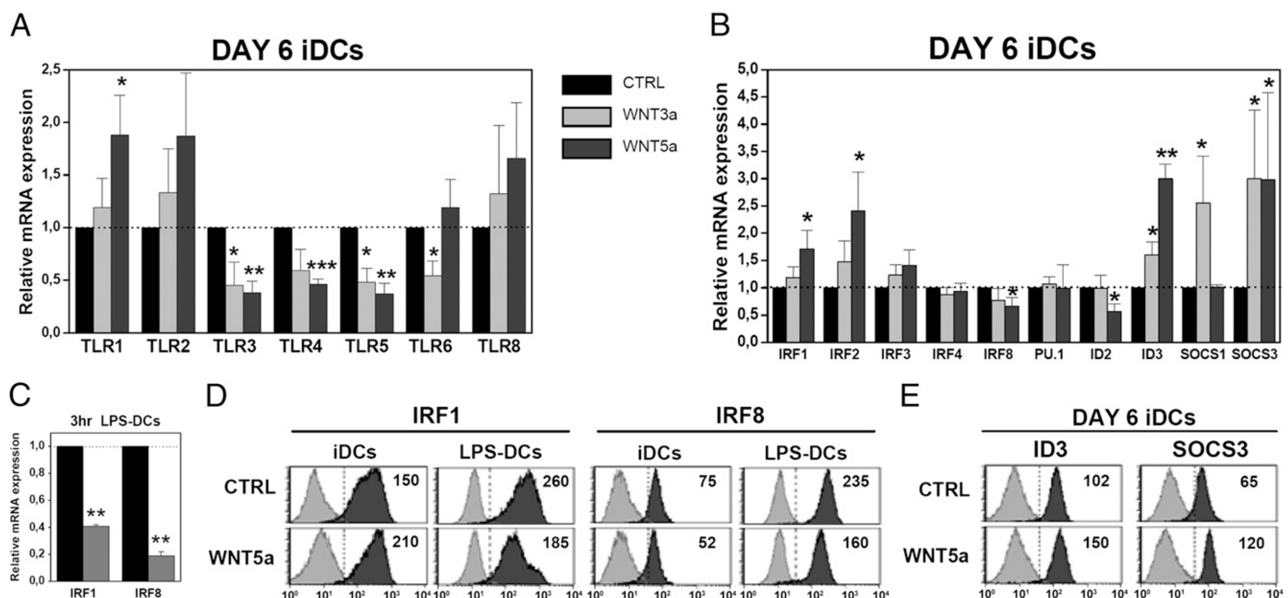

FIGURE 4. Changes in the transcription profile of Wnt5a-DCs correlate with their unconventional phenotype. *A* and *B*, Real-time PCR quantification of mRNA levels in day 6 iDCs differentiated from monocytes in the presence or absence of Wnt proteins. mRNA expression of different TLRs (*A*) and transcriptional factors and regulatory proteins (*B*) in Wnt-DCs are expressed relative to individual control values (black bars; referred to as 1). *C*, Control and Wnt5a-DCs were stimulated with LPS for 3 h, and IRF1 and IRF8 mRNA expression was analyzed. Variations between samples were corrected by normalizing against the expression of GAPDH, which was used as an endogenous control. Data represent the mean (±SD) of three to five independent experiments. *D* and *E*, Intracellular IRF1, IRF8 (*D*), ID3, and SOCS3 (*E*) protein levels were analyzed by flow cytometry in control and Wnt5a day 6 iDCs or after 20 h of LPS stimulation. The total MFI (in parentheses) is shown in each histogram. Data are representative of three independent experiments. $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$, by unpaired *t* test.

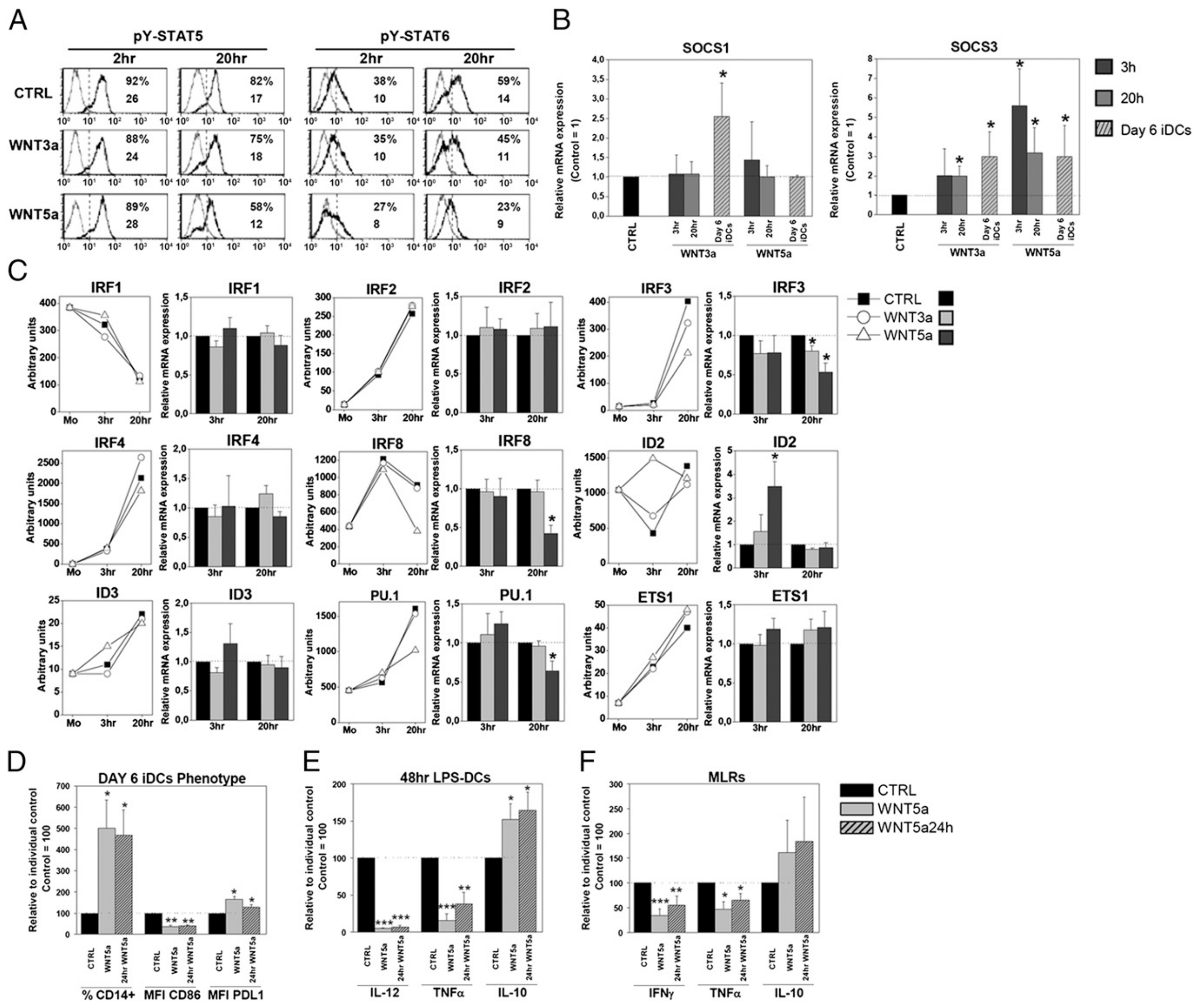

FIGURE 5. Wnt5a interferes with GM-CSF/IL-4 cytokine signaling and modified the early DC differentiation program. Human $CD14^+$ monocytes were cultured with GM-CSF/IL-4 in either the presence or the absence of Wnt3a or Wnt5a. *A*, After 2 or 20 h of culture, intracellular STAT5 and STAT6 tyrosine phosphorylation levels were analyzed by flow cytometry. The percentage of positive cells, determined according to background staining (gray histograms), and the total MFI are show in each histogram. Data are representative of four independent experiments. *B*, SOCS1 and SOCS3 expression was analyzed by quantitative RT-PCR analysis during Wnt-DC differentiation (3 and 20 h of culture and day 6 iDCs). Data shown are relative to individual control values (black; set as 1). *C*, Monocytes were cultured as indicated for 0 (monocyte, Mo), 3, or 20 h, and the expression of several transcription factors and regulatory proteins were analyzed by quantitative RT-PCR. Line graphs represent the kinetics of mRNA expression in control (black squares), Wnt3a (open circles), or Wnt5a (open triangles) cultures; data are representative of three independent experiments. Bar graphs show transcription levels relative to individual control values (black bar; referred to as 1). Data represent the mean (±SD) of four independent experiments. Variations between samples were corrected by normalizing against the expression of GAPDH, which was used as an endogenous control. *D–F*. The first 24 h of culture are sufficient for inducing Wnt5a effects in DC differentiation. Human $CD14^+$ monocytes were cultured in the GM-CSF/IL-4 cytokine mixture as described above. Wnt5a was added to the DC differentiation culture (Wnt5a-DCs) or during just the first 24 h (24 h-Wnt5a-DCs). To remove Wnt5a, culture medium was replaced with 24 h GM-CSF/IL-4 monocyte-conditioned medium. Both control and Wnt5a cultures underwent an equivalent process. *D*, Flow cytometry analysis of day 6 iDCs. The percentage of $CD14^+$ cells and the mean fluorescence values of CD86 and PDL1 are shown for Wnt5a and 24 h-Wnt5a-DCs relative to those of control iDCs cultures. *E*, Control, Wnt5a, and 24 h-Wnt5a-DCs were stimulated with LPS for 48 h. Production of IL-12p70, TNF-α, and IL-10 in culture supernatants was quantified by ELISA. *F*, Cytokine production of primed T cells. DCs stimulated with LPS were cultured in MLR with naive $CD4^+$ T cells at a 1:10 DC/T cell ratio. Supernatants of DC/T cell cocultures were harvested at day 7, and the levels of IFN-γ, TNF-α, and IL-10 were tested by ELISA. Data represent the mean (±SD) of four to five independent experiments, expressed relative to individual controls (black bars; referred to as 100%). $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$, by unpaired *t* test.

suggests that signals inducing DC differentiation could inhibit monocyte response to Wnt5a. Remarkably, iDCs and cord blood-derived $CD14^+$ cells did not secrete IL-6 or IL-10 in response to the same dose of Wnt5a, thus suggesting that this response was specific to monocytes (J. Valencia and R. Sacedón, unpublished observations).

In contrast with the effects observed after Wnt5a treatment, the addition of high doses of IL-6 (100 ng/ml) to GM-CSF/IL-4 monocyte cultures have been proven to skew monocyte differentiation to macrophages that maintain CD14 expression but lack CD1a (29). However, simultaneous addition of TNF-α may overcome IL-6–driven macrophage differentiation by inhibiting

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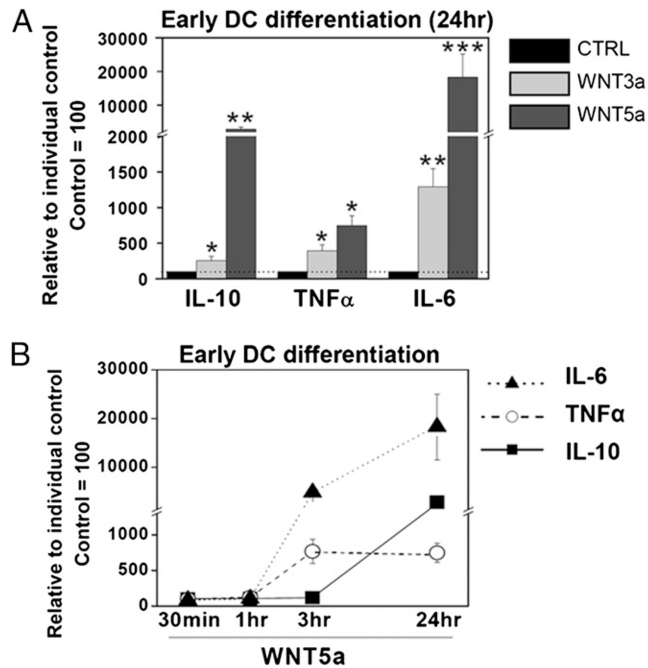

FIGURE 6. Wnt signaling induces IL-6, IL-10, and TNF-α secretion during early DC differentiation. Human $CD14^+$ monocytes were cultured with GM-CSF/IL-4 in the presence or absence of Wnt3a or Wnt5a. *A*, After 24 h of culture, supernatants were harvested and analyzed for IL-6, IL-10, and TNF-α. Absolute cytokine levels of control cultures: IL-6, 77.4 ± 22 pg/ml; IL-10, 9 ± 4 pg/ml; TNF-α, 117 ± 41 pg/ml. Data represent the mean (±SD) of four independent experiments, expressed relative to individual control (black bars; referred to as 100%). *B*, Kinetic cytokine production. Supernatants of Wnt5a-DC cultures were harvested at 30 min, 1, 3, and 24 h, and the levels of IL-6, IL-10, and TNF-α were tested. Data represent the mean (±SD) of three independent experiments, expressed relative to individual control (referred to as 100%). Note the *y*-axis break and scale change at break. $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$, by unpaired *t* test.

M-CSF signaling in monocytes (30). We therefore analyze TNF-α in DC differentiating cultures and observe that Wnt proteins also promoted TNF-α secretion, which may redirect DC differentiation (Fig. 6*A*, 6*B*).

Noncanonical signaling mediates Wnt5a response in monocytes

We next analyzed the signaling mechanisms by which Wnt5a altered DC differentiation. Central to the Wnt canonical pathway is the stabilization of β-catenin protein through the inhibition of GSK3. Using flow cytometry, we observed that the levels of β-catenin increased in human monocytes after 3 h of DC differentiation culture but decreased during the following 20 h. The addition of Wnt5a to monocyte cultures interfered with β-catenin accumulation and reduced its levels with respect to their individual controls (Fig. 7*A*), thus suggesting that Wnt5a effects could be independent of β-catenin transcriptional activities. In support of this possibility, stabilization of β-catenin by LiCl treatment of GM-CSF/IL-4 monocyte cultures did not mimic Wnt5a effects (25) (Supplemental Fig. 2).

Alternatively, through a noncanonical pathway, Wnt5a can stimulate intracellular Ca^{2+} mobilization, which acts as a secondary messenger and can trigger CaMKII activation (31). Supporting this option, we demonstrated by flow cytometry that, although control cultures showed a basal level of CaMKII activation, Wnt5a induced a higher percentage and intensity mean values for phospho-CaMKIIα (Thr^{286}) staining (Fig. 7*B*). We next checked whether the specific inhibition of CaMKII by CK59 could rescue DC differentiation and function after Wnt5a treatment. To this purpose, we restricted the treatments (Wnt5a alone or with the specific inhibitor) to the first 24 h of differentiation cultures, thus avoiding the possible toxic effects of a longer treatment with the CaMKII inhibitor. We followed this procedure with all of the inhibitors used. We found that, in a dose-dependent manner, CK59 almost blocked monocyte cytokine secretion induced by Wnt5a during the first hours of the GM-CSF/IL-4 cultures (Fig. 7*C*) and restored several of the phenotypic (data not shown) and functional (Fig. 7*E*) alterations found in Wnt5a-DCs. However, CaMKII inhibition did not recover IL-12/TNF-α production by Wnt5a-DCs after LPS stimulation (Fig. 7*D*). This observation suggests that Wnt5a signaling also may activate alternative pathways.

As a consequence of intracellular Ca^{2+} mobilization, concomitantly with CaMKII activation, Wnt5a signaling can switch on PKC and, independently of Ca^{2+}, can signal via JNK (15, 16). A JNK-specific inhibitor (SP600125) did not reduce the amount of IL-6 secreted by monocytes in response to Wnt5a but increased that of IL-10 (Fig. 7*F*). The inhibition of PKC (Go6983) was able to reduce IL-6 in supernatants from monocytes treated with Wnt5a but drastically increased IL-10 secretion (Fig. 7*F*). Accordingly, these inhibitors did not recover IL-12 and TNF-α secretion by Wnt5a-DCs (data not shown). Wnt5a also could activate NF-κB activity (23, 32) through an incompletely understood mechanism. Inhibition of NF-κB significantly reduced the secretion of cytokines by monocytes in response to Wnt5a and restored IL-12 secretion by Wnt5a-DCs, thus demonstrating a role for NF-κB in the effect of Wnt5a (Fig. 7*F*, 7*G*).

Discussion

In this report, we demonstrate that the increase in Wnt5a signaling during the differentiation of DCs compromises their functional capabilities. Wnt5a does not blockade the generation of DCs from monocytes but induces irreversible changes in their genetic differentiation program, thus leading to phenotypically altered DCs that present a lower capacity to uptake Ags and show an altered response to TLR ligands. Wnt5a-DCs stimulated via TLR produce scant amounts of IL-12p70 and TNF-α but increased amounts of IL-10. They have a reduced allostimulatory capacity and lead to less IFN-γ production but higher IL-10 secretion in MLR assays. The transcriptional profile of Wnt5a-DCs reveals changes that could account for their functional failures, presumably related in part with the high autocrine IL-6 and IL-10 signaling during the process of DC differentiation. The effect of Wnt5a is not dependent on β-catenin accumulation but is driven by Ca^{2+}-dependent noncanonical signaling, and inhibition of CaMKII and NF-κB activation restores normal DC differentiation despite Wnt5a treatment.

The effects of canonical Wnt3a on DC differentiation are not opposite to those exerted by Wnt5a, a prototypical Wnt for noncanonical signaling. On the contrary, there are some concordances between both treatments, although Wnt3a is not able to induce most of the changes found after Wnt5a treatment or its effects are less strong. This suggests a low level of signaling potency for Wnt3a on human monocytes. Wnt signaling output is dependent on both Wnt ligand and receptor context, and the same receptor complex, through dishevelled activation, can activate two (or more) routes in parallel (15, 16). Normally, a cell population expresses a mixture of Wnt receptors, and it is difficult to forecast ultimate Wnt signaling output. Human monocytes express LRP6 (33) and several Fzd (Fzd1, Fzd4, Fzd5, and Fzd7) (34) receptors. Monocyte culture induces an initial β-catenin stabilization, probably induced

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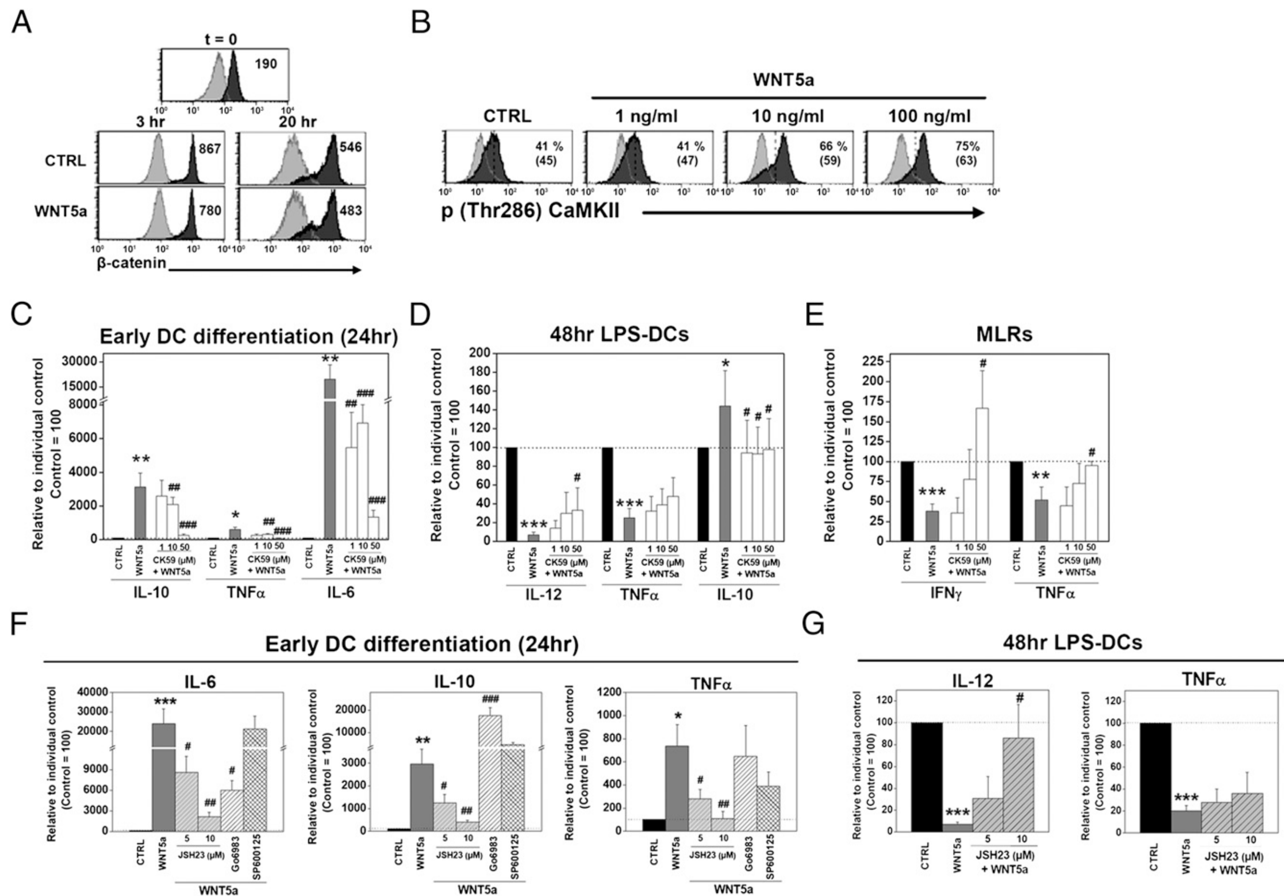

FIGURE 7. Wnt5a signals in monocytes through a noncanonical pathway. Human CD14$^+$ monocytes were cultured in the GM-CSF/IL-4 cytokine mixture as described above. *A*, Intracellular levels of β-catenin were determined by flow cytometry in monocytes and after 3 or 20 h of culture with or without Wnt5a. Total MFI is shown in each histogram. Data are representative of four independent experiments. *B*, Intracellular phospho-CaMKIIα (Thr^{286}) levels were analyzed by flow cytometry after 1 h under control conditions or in the presence of increasing doses of Wnt5a (1, 10, or 100 ng/ml). The percentage of positive cells, determined according to background staining (light gray), and the total MFI (in parentheses) are shown in each histogram. Data are representative of three independent experiments. *C–G*, Human CD14$^+$ monocytes were differentiated into DCs in the presence of Wnt5a plus different doses of specific inhibitors for CaMKII (CK59; 1–50 μM), NF-κB (JSH-23; 5–10 μM), PKC (Go6983; 5 μM), or JNK (SP600125; 20 μM). After 24 h of GM-CSF/IL-4 culture, treatments were removed, and culture medium was replaced by 24 h GM-CSF/IL-4 monocyte-conditioned medium. Both control and treated cultures underwent an equivalent process. Supernatants from 24 h GM-CSF/IL-4 cultured monocytes (*C*, *F*), 48 h LPS-stimulated DCs (*D*, *G*), and MLR cultures established with LPS DCs (as described above) (*E*) were harvested, and the amounts of different cytokines were analyzed. Data represent the mean (±SD) of three to four independent experiments, expressed relative to individual control (black bars; referred to as 100%). $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$, significance relative to control nontreated cultures by unpaired *t* test. $\#p \leq 0.05$, $\#\#p \leq 0.005$, $\#\#\#p \leq 0.0005$, significance relative to Wnt5a-DCs by unpaired *t* test.

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by the adhesion process (35), although later, presumably as a response to DC differentiation signals, the levels of β-catenin decrease. In fact, GSK3 activity is crucial for the differentiation of monocytes into DCs and prevents their deviation to macrophage lineage (25) (results reported in this article), and the GM-CSF/IL-4 cytokine mixture should induce its activation. In our cultures, Wnt5a does not increase β-catenin over control values; rather, on the contrary, we observed that it causes a slight decrease. Indeed, Wnt5a has been demonstrated to antagonize the canonical Wnt pathway and promote β-catenin degradation independently of GSK3 activity in fibroblasts, hematopoietic stem cells, and cancer cells (36, 37). Alternatively to the Wnt/β-catenin pathway, our current results indicate that monocytes respond to Wnt5a through a noncanonical Ca^{2+}-dependent pathway that increases CaMKII activity and NF-κB, a signaling route that shares common features with TLR-mediated activation (38, 39), which has been demonstrated to block DC differentiation (40, 41). Wnt5a also activates this noncanonical pathway in macrophages and endothelium and triggers a proinflammatory response (14, 23). Similarly, our results show that monocytes are stimulated by Wnt5a to secrete proinflammatory cytokines such as IL-6 and TNF-α but also IL-10, which, in part, could mediate Wnt5a effects on DC differentiation.

DCs play a dual role in governing T cell responses. Depending on their functional capabilities, DCs can stimulate or suppress immune reactions. Their ability to acquire immunostimulatory features in response to exogenous stimuli, such as TLR ligation, is crucial to mediate the interplay between innate and adaptive immune responses against pathogens, because immature or partially mature DCs may exert a tolerogenic or immunosuppressive effect. Accordingly, tolerogenic DCs generated in vitro do not mature properly in response to TLR stimuli and are characterized by lower IL-12 secretion and increased IL-10 production like Wnt5a-DCs (42, 43). Hyporesponsiveness of Wnt5a-DCs to stimuli via TLR could underlie part of their functional defects. However, although Wnt5a-DCs show lower expression of TLR3, TLR4, and TLR5 than control DCs, we found higher or normal levels of TLR1, TLR2, TLR6, and TLR8 transcripts. The higher expression of SOCS3 observed in Wnt5a-DCs also can interfere with their stim-

ulation, because it abrogates the cytokine Jak-Stat signal transduction pathway (44). Analogous with our results, SOCS3 overexpressing DCs secrete low amounts of IL-12 but high levels of IL-10 and show a tolerogenic phenotype (45). Expression of SOCS3 has been proven to be induced by different cytokines, including IL-6 and IL-10 (44), which were increased during DC differentiation in the presence of Wnt5a. High IL-10 signaling during DC differentiation also could be one of the causes for increased ID3 expression in Wnt5a-DCs, which in turn would account for the higher capacity of Wnt5a-DCs to secrete IL-10. In support of this hypothesis, IL-10 signaling increases ID3 expression, and ID3 overexpression enhanced IL-10 production in $CD14^+$ monocytes, thus establishing a positive feedback in the regulation of these genes (46). In agreement, high ID3 expression is characteristic of TGF-β/IL-4 activated M2 macrophages that express increased levels of IL-10 (47). Another feature of Wnt5a-DCs previously reported in tolerogenic DCs is their reduced expression of ID2 (42), although its functional significance remains elusive. In any case, the most remarkable characteristic of Wnt5a-DCs is their failure to secrete IL-12 in response to all of the TLR stimuli assayed, even those that lead to normal CD83 expression. Higher SOCS3 expression or the IL-10/ID3 signaling loop may interfere with IL-12 expression. Indeed, Wnt5a-DCs failed to properly increase IRF1 and IRF8 transcription after stimulation, two positive and essential factors for priming IL-12 secretion (28).

Recent reports reveal that the Wnt signaling pathway components have a role during the differentiation of DCs and shape their functional features. However, their involvement seems to be complex and dependent on developmental stage and/or DC lineage. In support of a positive effect for canonical Wnt signaling on DC differentiation and function, Zhou et al. (5) show that the addition of Wnt3a or the inhibition of GSK3 increase the proportion of DCs recovered from hematopoietic progenitor cell cultures and promote their allostimulatory capacity, while β-catenin deletion has the opposite effect. Nevertheless, GSK3 inhibition blocks the differentiation of monocytes into DCs, (25) and transient β-catenin stabilization reduces the capacity of human thymic progenitors to yield DCs (27). However, GSK3 activity is necessary to avoid spontaneous but abnormal maturation of DCs (4, 25), and specific knockout of the β-catenin gene after CD11c expression in DCs disrupts intestinal immunotolerance, enhancing the onset of inflammatory bowel diseases (6).

In vivo Wnt5a levels increase under numerous proinflammatory situations. Monocytes and macrophages upregulate Wnt5a secretion in response to different bacterial stimuli, thus increasing Wnt5a levels in the sera from patients with sepsis (14). Wnt5a also is detected in human inflammatory diseases such as granulomatous lesions from patients infected with *Mycobacterium tuberculosis* (22), atherosclerotic plaques, and rheumatoid arthritis but not in normal tissue (23). Under these conditions, monocytes could respond to Wnt5a, and this could interfere with their differentiation into DCs, resulting in an in vivo effect similar to that shown in this article. Accordingly, Wnt5a might contribute to the attenuation of inflammatory responses or to chronic bacterial infection that negatively affect the function of DCs that differentiate after the onset of the inflammatory process. Likewise, Wnt5a also could be a factor that contributes to the observed dysfunction of DCs that develops during polymicrobial sepsis that impairs the resolution of secondary infections (48). Nevertheless, the local concentrations of soluble inhibitors for Wnt5a or additional and unknown signals produced during the inflammatory process could override Wnt5a-negative effects, allowing normal DC development. Indeed, monocyte-derived DCs develop under inflammatory conditions (49, 50), and our results indicate that the GM-CSF/IL-4 cytokine mixture attenuates the response of monocytes to Wnt5a.

In conclusion, Wnt5a is produced under inflammatory conditions and exerts a proinflammatory role acting on macrophages or endothelium (14, 22, 23). However, our results suggest that this Wnt protein also could activate monocytes and that this would interfere with the DC differentiation program, thus leading to unconventional DCs with tolerogenic features. Further research is necessary to determine in vivo factors that drive monocyte-derived DC differentiation under high concentrations of Wnt5a that are typical of the inflammatory state or bacterial infection. The identification of such a signaling milieu could upgrade the design of more effective immunotherapeutic strategies for the treatment of infection and sepsis.

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Disclosures

The authors have no financial conflicts of interest.

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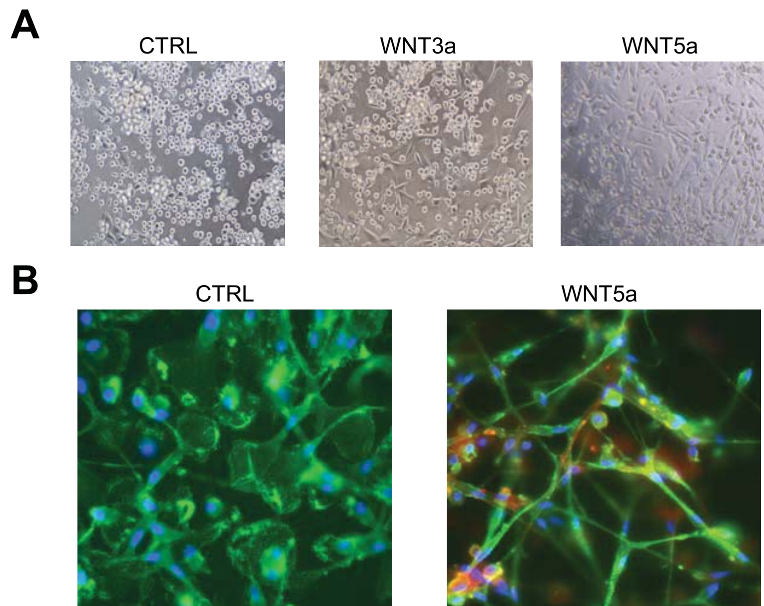

S1. Morphologycal analysis of Control and Wnt5a-DCs. Light microscopic images (A) and immunofluorescence analysis (B) of day 6 iDCs differentiated from monocytes in the presence or not of Wnt5a protein. DCs were stained for HLA-DR (green fluorescence) and Hoechst 33258 was used as nuclear staining (blue fluorescence). Results shown are representative of three experiments. Note the round shaped cells present in the control cultures whereas adherent, elongated cells form a network in the Wnt5a-treated ones.

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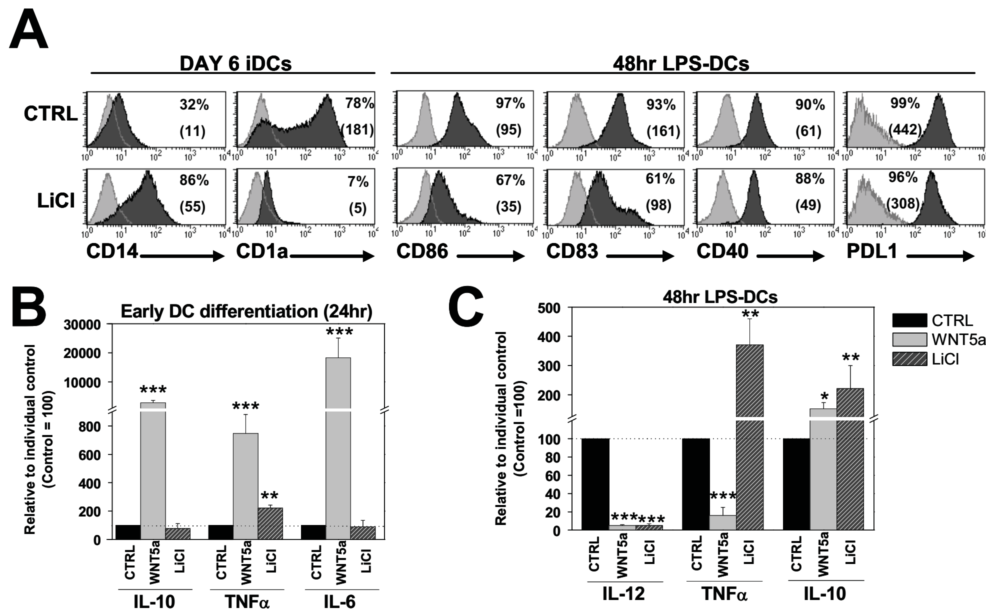

S2. LiCl treatment did not mimic Wnt5a effects in DC differentiation. Human CD14+ monocytes were cultured with GM-CSF and IL-4 in the presence or absence of Wnt5a or LiCl (10μM). (A) Flow cytometry analysis of day 6 iDCs and 48hr LPS-DCs. Notice LiCl treatment of monocytes during their DC differentiation induced an important accumulation of CD14+ cells while blocked the acquisition of CD1a expression. This phenotype is consistent with monocyte differentiation into macrophage like-cells. After LPS stimulation these cells showed significant lower expression of CD83 and CD86, while PDL1 and CD40 levels were not affected. Data are representative of three independent experiments. (B) After 24 hours of culture, supernatants were harvested and analyzed for IL-6, IL-10 and TNFα. LiCl treatment did not increase IL-6 or IL-10 production during the first hours of monocyte culture like Wnt5a does. (C) Immature DC differentiated in the presence or not of Wnt5a or LiCl, was stimulated with LPS for 48 hours. Production of IL-12p70, TNFα and IL-10 in culture supernatants was quantified by ELISA. Notice that LiCl-DCs responded to LPS producing almost null levels of IL-12p70 but higher amounts of TNFα than control DCs. Data represent the mean (± SD) of three to four independent experiments, expressed relative to individual controls (black bars; referred as 100%). **, $P \leq 0.005$; ***, $P \leq 0.0005$, by unpaired *t*-test. Note Y-axis break and scale change at break.

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Table S1. Phenotypic changes in day 6 immature Wnt-DCs

	WNT3a-DCs	WNT5a-DCs
CD86	62±11*	51 ± 17***
CD83	113±38	125±43
CD40	90±14	126±30
CD80	93±12	138±20
DC-SIGN	89±23	228±62*
HLA-DR	75±15*	8 ±13*
PDL1	143±19*	259±43*
PDL2	127± 9	165±21*
MMR	41±4**	63±17*
ECadherin	110±31	110±45
DEXTRAN (100 ng/ml)	57±23*	59±17*
DEXTRAN (500 ng/ml)	72±15*	55±15*
LUCIFER YELLOW	110±16	52±8*

Day 6 iDCs differentiated in the presence or not of Wnt proteins were analyzed by flow cytometry as described in Material and Methods. Results shown are MFI numbers in Wnt-DCs normalized to control conditions (control-DCs =100). Representative histograms are shown in Figure 1. Data represent the mean (± SD) of five independent experiments. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$, by unpaired *t*-test.

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Table S2. Phenotypic changes in Wnt-DCs after TLR stimulation

	LPS		FSL1		POLY I:C		ssRNA40	
	WNT3a-DCs	WNT5a-DCs	WNT3a-DCs	WNT5a-DCs	WNT3a-DCs	WNT5a-DCs	WNT3a-DCs	WNT5a-DCs
CD86	64± 6*	35±20 **	115±41	105±29	77±10*	62±20*	97±6	65±12*
CD83	82±19	50±15***	66±4*	65±19*	96±23	63±23*	89±20	97±8
CD40	68±5*	60±18	100±10	146±56	99±19	119±11	101±23	129±22
CD80	56±8*	52±18*	72±12*	142±48	97±7	153±38	84±11	145±59
DC-SIGN	104±12	246±90*	75 ± 32	128±26	90±19	164±33*	70±27	149±38*
HLA-DR	75±20*	63±24*	110±12	104±34	80±15*	112±34	109±26	99±34
PDL1	123±31	90±28	88±4	126±12*	153±20	152±17*	85±11	93±28
PDL2	99±22	89 ± 20	90±8	149±7*	103±9	130±8*	103±23	159±27*
CCR7	68±3**	70 ± 9*	97±23	98±10	101±7	82±29	98±43	92±14

Day 6 iDCs differentiated in the presence or not of Wnt proteins were stimulated with different TLR agonist (LPS, FSL1, poly I:C and ssRNA40) and their maturation phenotype was analyzed by flow cytometry. Results shown are MFI numbers in Wnt-DCs normalized to control conditions (control-DCs =100). Representative histograms are shown in Figure 2. Data represent the mean (± SD) of five independent experiments. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$, by unpaired *t*-test.

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