CHAPTER 3

Transcriptionally active β-catenin is involved in monocyte to DC maturation and lacking in Langerhans cell histiocytosis

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Abstract

Langerhans cell histiocytosis (LCH) is characterized by an accumulation of pathological dendritic cells (DC). Recent findings indicate that BRAF activation is important in LCH etiology, but this does not provide a full explanation of clinical appearance of the disease. LCH cells have an endogenous maturation block, since they have an immature phenotype and lack antigen-presenting function although their inflammatory environment would activate DC under normal conditions. Beta-catenin is, as component of the Wnt-pathway, involved in the differentiation DC. To investigate whether a defect in the β-catenin pathway might be involved in the pathology of LCH, an in vitro model of human monocyte-derived DC (moDC) differentiation as well as sixty-nine LCH samples were used. We demonstrate that transcriptionally active β-catenin is increasingly expressed during DC differentiation of monocytes and that inhibition of the β-catenin/TCF transcriptional complex blocks monocyte differentiation towards moDC. Furthermore, we show that transcriptionally active β-catenin is essentially absent in the nucleus of LCH cells. Comparison of the gene expression profile of LCH cells with a list of β-catenin target genes confirms that LCH cells have down-regulated β-catenin signaling. These results suggest that the lack of transcriptionally active β-catenin might contribute to the pathology of LCH.

Introduction

Langerhans cell histiocytosis (LCH) is the most common proliferative disorder of the mononuclear phagocyte system (reviewed in (*1*)). This rare disease is characterized by granulomatous lesions, which can develop in various organs and consists of an accumulation of immature, activated pathological Langerhans cells (LC), called LCH cells. In addition, normal inflammatory cells, such as T cells, macrophages, multinucleated giant cells and eosinophils contribute to the lesions in different frequencies. A recent breakthrough in the field has indicated that an activating V600E mutation in the *BRAF* gene is present in about half of the LCH cases in the absence of cytogenetic abnormalities or other protein genome rearrangement mutations, while the associated RAS-MAPK pathway is activated in all cases regardless of mutation status (*2-4*). This strongly supports a neoplastic nature of LCH. However, up to now no clear relationship between clinical phenotype and LCH cell characteristics has been established, and therefore these findings provide only a partial elucidation of LCH etiology.

LCH cells seemingly have an endogenous maturation block, since they have an immature phenotype and lack antigen-presenting function even though the lesional environment contains an excessive amount of inflammatory cytokines that would activate LC under normal conditions (*5*). These observations suggest that the LCH cells have an intrinsic defect, either or not related to the activated RAF-MAPK route, that disables them to obtain a fully mature phenotype and function, while maintaining proliferative capacity. On the basis of similarities with the role of β -catenin in proliferative and adhesive deviations in epithelial tissues, we hypothesized before that a defect might be harbored in the E-cadherin/β-catenin pathway, leading to the cellular deviations observed in LCH (*6*).

Beta-catenin is part of the canonical Wnt-signaling pathway (reviewed in (*7*)). In addition, it has an alternative role in cell-cell contacts as the protein can also attach to E-cadherin at the cell membrane. Upon dissociation from E-cadherin, β -catenin accumulates in the cytoplasm and may assume a role as transcriptional co-activator. In the absence of Wnt proteins, cytoplasmic β-catenin is phosphorylated at N-terminal serine/threonine residues by a destruction complex, which consists of adenomatous polyposis coli (APC), axis inhibition protein (Axin), casein kinase 1, and glycogen synthase kinase 3β (GSK3β), and is subsequently degraded in the

proteasome. In the presence of Wnt signaling the destruction complex is inactivated, enabling β-catenin to translocate to the nucleus in its N-terminally non-phosphorylated form, where it displaces groucho-related repressor from the transcription factor T cell factor (TCF)-lymphoid enhancer factor (LEF). TCF-LEF is then able to mediate expression of Wnt target genes which are involved in differentiation, proliferation, and maturation.

A few studies have investigated the role of E-cadherin and β-catenin in the development of dendritic cells (DC) and LC. Riedl et al. showed that disruption of human LC clusters, resulting in breakage of E-cadherin-mediated cell-cell adhesion, caused the induction of a mature LC phenotype (8). Similarly, Jiang et al. reported that disruption of E-cadherin-mediated cell-cell contacts activated the β-catenin pathway in mouse bone marrow-derived DC (BMDC) (9). This resulted in phenotypically mature DC with a tolerogenic phenotype as they did not produce pro-inflammatory cytokines but stimulated regulatory T cells. Recent in vivo studies used CD11c-specific ablation of β-catenin and confirmed the contribution of DC β-catenin expression in maintaining the balance between tolerogenic and immunogenic responses in the gut (*10*). Investigating early steps in DC development, Zhou et al. described that β-catenin activity in human and mouse hematopoietic progenitor cells (HPCs) is required for their differentiation towards DC, and that this is realized by cooperation between Notch and Wnt signalling (*11*).

In a pilot study in LCH patient samples, we found that the transcriptionally active, non-phosphorylated form of β-catenin is not present in LCH cells. Furthermore, we could also confirm earlier findings that most LCH cells are also E-cadherin-negative (*12*). In order to confirm and interpret these observations, the present study was set up to investigate a putative role of β-catenin in the differentiation and maturation of human monocyte-derived DC (moDC), as these cells are now recognized as one of the possible origins of LCH cells (*1*). Our data demonstrate that transcriptionally active β-catenin is required during the early phase of monocyte differentiation towards moDC, thus complementing findings of β-catenin involvement in both early DC development and final maturation. Remarkably, the transcriptionally active form of β-catenin is typically absent in the nucleus of LCH cells. These findings suggest that β-catenin activity is possibly aberrantly down-regulated in LCH cells, thereby preventing these cells from acquiring a mature developmental stage.

Materials and Methods

ANTIBODIES

CD1a (T6-RPE/O10; Beckman Coulter/Dako), CD14 (MΦP9-PE/APC; BD Biosciences), CD40 (LOB7/6-FITC; Serotec), CD80 (L307.4-PE; BD Biosciences), CD86 (2331(FUN-1)-FITC; BD Biosciences), HLA-DR (L234-FITC; BD Biosciences), DC-SIGN (120507-PE; R&D Systems), β-catenin (14-FITC; BD Transduction Laboratories), (non-phosphorylated) β-catenin (polyclonal; Millipore, cat.no. 06-734) (*13*), and Langerin (929F3.01; Dendritics) were used as primary antibodies. Goat anti-mouse-FITC (Sanquin), goat anti-mouse-TRITC (Jackson Immuno Research), and swine anti-rabbit-FITC (DAKO) were used as secondary antibodies. Corresponding isotype controls were purchased from either BD Biosciences or eBioscience.

MONOCYTE ISOLATION AND DC CULTURE

Monocytes were isolated from fresh buffy coats obtained from the local blood bank (Sanquin) after informed consent was given by donors. First, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus (GE Healthcare, Chalfont St. Giles, United Kingdom) and density centrifugation as described previously (*14*). PBMC were then incubated with paramagnetic beads labeled with CD14 antibody (Miltenyi, Auburn, CA, USA) for 20 minutes at 4^{o} C according to manufacturer's instructions. $CD14^{+}$ monocytes were subsequently isolated from magnetic columns by positive selection.

$CD14^{+}$ monocytes were cultured for 7 days in RPMI-1640 with Ultraglutamine, supplemented with 10% fetal calf serum, 0.1% penicillin (100 U/ml)/streptomycin (100 mg/ml), all obtained from Bio Whittaker Europe, Verviers, Belgium, 100 ng/ml IL-4, and 80 ng/ml GM-CSF (Peprotech, Rockyhill, NJ, USA). LPS serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA) was added after 6 days to induce final maturation of the moDC in the subsequent 18-24 hours. To determine the functional involvement of β-catenin in the differentiation of monocytes into moDC, PKF115-584 (generously provided by Novartis, Basel, Switzerland), which is an inhibitor of the interaction between β-catenin and Tcf-Lef, was applied at optimal concentration (25 nM) at different time points. The solvent DMSO (Merck, Whitehouse Station, NJ, USA) was used as control.

FLOWCYTOMETRY

For cell surface marker staining, cells were incubated with primary antibodies for 30 minutes on ice. Cells were then washed with and resuspended in PBS/1% BSA buffer and stored at 4^{o} C in the dark until analysis. For β-catenin staining cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with Saponin buffer (0.5% Saponin in PBS/0.5% BSA). Cells were then pre-incubated with normal mouse or normal swine serum for 1 hour (when staining for total or non-phosphorylated β-catenin, respectively), washed with Saponin buffer and subsequently incubated with anti-β catenin-FITC antibody (total β-catenin) or polyclonal anti-β catenin antibody (non-phosphorylated β-catenin) for 1 hour on ice. Cells were washed again and for non-phosphorylated β-catenin staining incubated with FITC-conjugated swine anti-rabbit secondary antibody for 1 hour. For dead cell exclusion in unfixed samples, 7-AAD (BD Biosciences) was added to cells according to manufacturer's instructions. Cells were analyzed using a FACS Calibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Diego, CA, USA). Quantification of antibody binding was performed by interpolation of cellular fluorescence values on a standard curve prepared using microspheres of known fluorescence (Sphero Calibration Particles, Spherotech, Inc., Lake Forest, IL, USA).

IMMUNOFLUORESCENCE ANALYSIS OF IN VITRO DERIVED MODC

Cultured moDC were harvested and allowed to adhere to poly-L-lysine-coated microwell slides for 1 hour at 37^{o} C. All further steps were performed at room temperature (RT). Cells were incubated with cell surface marker antibodies (CD14 or CD1a) for 1 hour, washed with PBS and incubated with goat anti-mouse TRITC for 1 hour at RT. Cells were washed again, fixed with 4% paraformaldehyde for 15 minutes, and further processed for β-catenin staining as described above. After staining, preparations were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Images were acquired with a laser scanning confocal microscope (LSM510; Zeiss, Oberkochen, Germany).

IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMICAL ANALYSIS OF LCH PATIENT SAMPLES

All LCH samples were obtained from Erasmus University Medical Center and Leiden University Medical Center and were handled in a coded fashion according to the ethical guidelines as described in the Code for Proper Secondary Use of Human Tissue in the Netherlands of the Dutch Federation of Medical Scientific Societies. Paraffin-embedded sections (4 µm) were cut and stained as described previously (*15*). Non-specific binding of primary antibodies was prevented with normal goat serum for 30 minutes. Anti-CD1a, monoclonal anti-β catenin-FITC or polyclonal anti-β-catenin were used as primary antibodies. Results were studied by confocal microscopy as described above. For immunohistochemical analysis anti-Langerin, anti-β-catenin-FITC, and polyclonal anti-β-catenin were used as primary antibodies. Binding was detected by Dako ChemMate Envision Kit/HRP (Dako, Glostrup, Denmark) according to manufacturer's instructions. Counterstaining was done with Harris haematoxilin. Sections were dehydrated and covered with Pertex. Images were acquired with an Axioskop microscope (Zeiss).

REAL-TIME QUANTITATIVE PCR

RNA was isolated from the cultured moDC using minipreparation columns (RNeasy, Qiagen, Venlo, the Netherlands) according to manufacturer's protocol. After extraction, the concentration was determined and RNA was stored at -80 °C until use. cDNA was obtained by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. Quantitative PCR was performed with *Taq*Man Universal PCR Master Mix and two different *Taq*Man gene expression assays for *Axin2*: Hs01063170 and Hs00610342 (Applied Biosystems, Foster City, CA, USA). PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. PCR amplification of the housekeeping gene *ABL* was performed as a control. Data are expressed as cycle threshold (CT) values relative to *ABL* (ΔCT values).

Results

Expression of transcriptionally active β-catenin increases during DC differentiation of monocytes

Beta-catenin signaling has been shown to promote early differentiation of human $CD34^+$ HPC and mouse myeloid progenitor cells towards DC (*11*), as well as to stimulate final maturation of mouse BMDC (*9*). To address whether β-catenin is also involved in the intermediate step of human monocyte to moDC differentiation, we determined the expression levels and cellular location of total and transcriptionally active, non-phosphorylated β-catenin in human monocytes cultured in the presence of GM-CSF and IL-4 for 7 days. LPS was added to half of the samples after 6 days of culture to induce final maturation. DC maturation was monitored by decreasing CD14 expression and increasing expression of DC maturation markers (Fig. 1A). In freshly isolated monocytes β-catenin was predominantly associated with the cell membrane, while non-phosphorylated β-catenin was hardly detectable in any compartment at this stage (Fig. 1B). After 3 days of cell culture, total β-catenin expression appeared to have translocated from the cell membrane to the cytoplasm and was also visible in some nuclei. Non-phosphorylated β-catenin was also present in the cytoplasm and a few nuclei. After 7 days, total as well as non-phosphorylated β-catenin were abundantly present in both intracellular compartments. Also, cells treated overnight with LPS, to induce final maturation, showed abundant staining for total as well as for non-phophorylated β-catenin, comparable to moDC not treated with LPS. Quantitative flowcytometry showed that the expression of both total- and non-phosphorylated β-catenin increased approximately 6-fold during the differentiation process (Figure 1C-E). The difference in absolute MESF between total- and non-phosphorylated β-catenin (Fig. 1E) can be explained by the amplified fluorescence signal caused by the secondary conjugated antibody used for the detection of non-phosphorylated β-catenin. Induction of final maturation with LPS on day 6 caused no significant change in the level of non-phosphorylated β-catenin expression, compared to spontaneously matured cells.

To determine whether the non-phosphorylated β-catenin was transcriptionally active, expression of the β-catenin target gene *Axin2* was evaluated by quantitative PCR. Figure 2 shows that *Axin2* mRNA was already expressed at low levels by freshly isolated monocytes. During DC differentiation, transcription of the Axin2 gene was up-regulated with a peak on day 3. Interestingly, addition of LPS on day 6 increased *Axin2* transcription compared to the value for the untreated moDC, although this did not reach significance. This increase suggests that LPS stimulates β-catenin-dependent transcription in maturing moDC. Together, these observations demonstrate that both total and non-phosphorylated β-catenin increase during differentiation of monocytes towards moDC. The up-regulation of *Axin2* mRNA expression in combination with the acquisition of DC-specific phenotypic markers suggests that transcriptional activity of β-catenin is involved in the differentiation from monocytes to moDC.

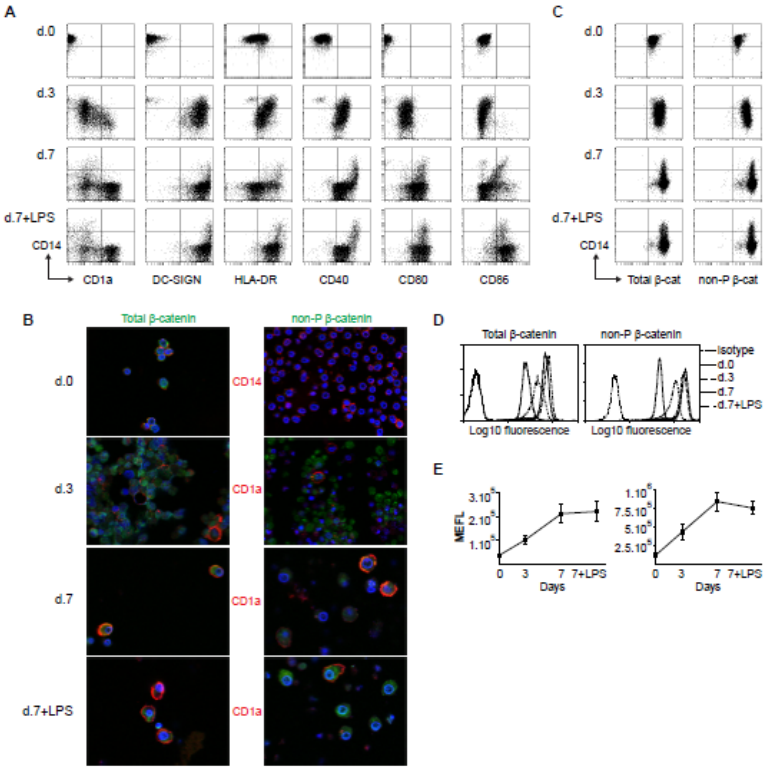

Figure 1. Non-phosphorylated β-catenin is increasingly expressed during monocyte differentiation towards moDC

DC maturation was induced in human peripheral blood monocytes by culture in the presence of GM-CSF and IL-4 for 7 days. In some samples LPS was added at day 6 to induce final maturation. (A) Membrane marker expression was assessed by flow cytometry and indicated the gradual loss of CD14 and acquisition of DC markers. Total as well as non-phosphorylated β-catenin expression was determined by immunofluorescence (A) and flowcytometry (B-D). From day 3 onwards, significant levels of both total (green) and non-phosphorylated β-catenin (red) were observed in the cytoplasm and nucleus of differentiating DC while blood monocytes only showed minimal levels of (A) non-phosphorylated β-catenin (red) at the cell membrane. Quantification of the expression of total- and non-phosphorylated β-catenin showed an increase of approximately 6-fold during the differentiation process (C-D). Addition of LPS on day 6 did not have a significant effect on the level of β-catenin expression (C-D). Data shown are representative of at least 3 experiments. Original magnification x 40 for panel B.

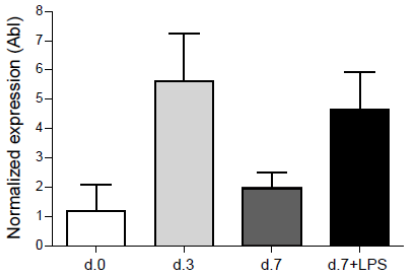

Figure 2. Beta-catenin is transcriptionally active during monocyte differentiation towards moDC

Beta-catenin transcriptional activity was measured by determination of the mRNA expression levels of the β-catenin target gene *Axin2* by RQ-PCR and normalized to Abl expression. *Axin2* was already present in monocytes and showed a peak level expression on day 3, indicating that β-catenin is transcriptionally active in monocytes differentiating towards moDC. Mature DC showed a significantly higher expression of *Axin2* compared to monocytes. Shown are the means ± SEM; n = 4.

INHIBITION OF THE B-CATENIN/TCF TRANSCRIPTIONAL COMPLEX BLOCKS MONOCYTE DIFFERENTIATION TOWARDS MODC

Non-phosphorylated β-catenin needs to associate with TCF/LEF in the nucleus in order to function as a transcriptional co-activator. To investigate a causal relationship between DC maturation and β-catenin activity, we evaluated the effect of the β-catenin/TCF complex-specific inhibitor PKF115-584 on the monocyte differentiation process induced by GM-CSF and IL-4. Notably, addition of 25 nM PKF115-584 to monocytes from day 0 significantly inhibited the acquisition of DC maturation markers, in particular CD80, HLA-DR, DC-SIGN, and CD1a (Fig. 3A). Addition of LPS on day 6 did not result in increased maturation marker expression, indicating that the blocked maturation could not be overcome by TLR signaling. In contrast to the previous findings, application of PKF115-584 at a later time point, i.e. from day 3 (data not shown) or from day 6 (Fig. 3B), did not inhibit the DC maturation process. Together, these findings indicate that β-catenin transcriptional activity is essential early in the differentiation process from monocytes to moDC.

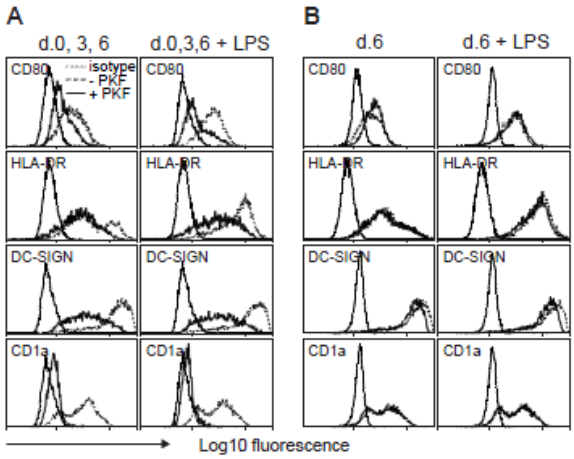

Figure 3. Inhibition of the β-catenin/Tcf transcriptional complex impedes human monocyte differentiation to moDC

Differentiating monocytes were treated with the β-catenin/TCF complex-specific inhibitor PKF115-584 (solid line) or DMSO as control (dashed line) on either day 0, 3, and 6 (A), or only on day 6 (B), and DC maturation marker expression was monitored on day 0, 3, and 7. Treatment with PKF115-584 from day 0 onwards inhibited the acquisition of DC maturation markers significantly. Addition of LPS on day 6 could not overcome this inhibition. In contrast, treatment on day 6 did not influence maturation marker expression, nor was the increase of HLA-DR expression after LPS treatment blocked. Data are representative of 3 experiments with similar results.

LCH CELLS DO NOT EXPRESS TRANSCRIPTIONALLY ACTIVE B-CATENIN IN THE NUCLEUS

LCH cells appear to be blocked in an immature, activated state of differentiation. In light of the finding that transcriptionally active β-catenin is involved in the differentiation and maturation of DC and that monocytes are putative precursors of pathological LCH cells (*1*), we determined the expression of total an non-phosphorylated β-catenin in LCH cells by immunohistochemistry and immunofluorenscence staining. In total, sixty-nine paraffin-embedded biopsies from patients with single system and multisystem LCH were used for this analysis (Table 1). Anti-CD1a and anti-Langerin antibodies were used to identify the pathological LC. Sixty-three samples were analyzed for the expression of total β-catenin and 56 samples (88.9%) were found to be positive (Fig. 4A, B). Additionally, 16 samples were analyzed for the presence of non-phosphorylated β -catenin. For the first 6 samples, a monoclonal antibody (clone 8E7) was used that binds specifically to a non-phosphorylated peptide of β-catenin, containing the GSK3β target residues Ser37 and Thr41 (*16*). A study by Hendriksen et al. described, however, that this antibody shows additional binding to an antigen located in the nucleus (*13*). We, therefore, decided to continue the staining for non-phosphorylated β -catenin with another, polyclonal, antibody which exclusively recognizes the consensus GSK3β phosphorylation site of human β-catenin (residues 29-49) (*13*). LCH cells in 11 out of 16 samples (68.8%) were negative for non-phosphorylated β -catenin (Fig. 4A, C), while blood vessels and stromal elements showed clear positivity. Five samples showed positive staining of LCH cells, but in four of these localization of non-phosphorylated β-catenin was confined to the cell membrane or cytoplasm, suggesting that β-catenin was not involved in transcription (Fig. 4D). Only a single sample out of 16 (6%) showed nuclear presence of non-phosphorylated β-catenin, as stained with the polyclonal antibody, in approximately 30% of the LCH cells.

Table 1. Total and non-phosphorylated β-catenin expression in LCH patient samples

Tissue of origin	Type of LCH (SS/MS/U)	Total β-catenin (pos/neg)	Non-P β-catenin (pos/neg)
Bone	48/6/1	45/6	2 (m), 1 (n, c)/6
Skin	6/2/0	7/1	2 (m)/1
Bone marrow	0/2/0	1/0	0/1
Lymph node	2/0/2	3/0	0/3

Since the specificity of 8E7 antibody for N-terminally non-phosphorylated β-catenin was convincingly shown, and only an additional, unknown nuclear component appeared to bind the antibody, we considered the negative results with 8E7 antibody a convincing indication of absence of non-phosphorylated β-catenin detection.
Abbreviations: SS: single-system LCH, MS: multi-system LCH, U: unknown, (n) nuclear, (c) cytoplasm), (m) membrane localization.

GENE EXPRESSION PROFILING SHOWS DOWN-REGULATED B-CATENIN SIGNALING IN LCH CELLS

To confirm inactivity of β-catenin-mediated transcription in LCH cells, we compared the results of the recently published gene expression profiling study in LCH (*17*) with the list known target genes of β-catenin signaling (http://www.stanford.edu/~rnusse/pathways/targets.html). The used version of this list (December 2009) comprises 107 mammalian genes known to be directly or indirectly regulated by β-catenin. From these, 87 genes are not differentially expressed between LCH cells and freshly isolated normal CD207-positive skin LC. Of the remaining 20 differentially expressed genes, 17 appear to be regulated according to their expected response ($p = 0.0006$ (Chi square test); Table 2), i.e. 16 are significantly down-regulated in LCH, which are known to be up-regulated by β-catenin signaling, while one (*CDKN2A*) is up-regulated and known to be down-

regulated by β-catenin. Only two genes (*MMP9* and *CDH1*/E-cadherin) are differentially regulated in the opposite direction of their expected regulation. One gene (*CD44*) is inconclusive due to unclear gene annotation in the LCH expression list. On the basis of these findings, we conclude that LCH cells indeed show a gene expression signature corresponding to significantly down-regulated β-catenin signaling without evident loss of the locus (*4*), in marked contrast to increasing β-catenin signaling already in early stages of normal DC development. A note of caution, however, is that the expression profile of LCH cells was determined relative to that of normal CD207-positive skin LC (*17*), while recent insights suggest that other myelomonocytic cells could also be progenitors of LCH cells (*1*), despite the overall similarity between LCH cells and skin LC.

Table 2. Gene expression profiling confirms down-regulated β-catenin signaling in LCH cells

Gene	alias	LCH cell profiling [1]	regulation by β-catenin [2]	regulated as predicted
CD44		?	↑	?
CDKN2A	p16	↑	↓	+
CLDN1	claudin-1	↓	↑	+
GJA-1	connexin43	↓	↑	+
CCND2	Cyclin D2	↓	↑	+
DKK3	Dickkopf	↓	↑	+
CDH1	E-cadherin	↓	↓	–
EGFR	EGF receptor	↓	↑	+
FOXN1		↓	↑	+
FZD7	Frizzled 7	↓	↑	+
IRX3		↓	↑	+
ISL1	Islet1	↓	↑	+
JAG1	Jagged1	↓	↑	+
KRTx	Keratin	↓	↑	+
MMP9		↑	↑	–
NRCAM		↓	↑	+
PITX2		↓	↑	+
SNAI2		↓	↑	+
TIAM1		↓	↑	+
TNFRSF19		↓	↑	+

[1] data as published in (*17*)

[2] β-catenin target genes as listed at:
http://www.stanford.edu/~rnusse/pathways/targets.html

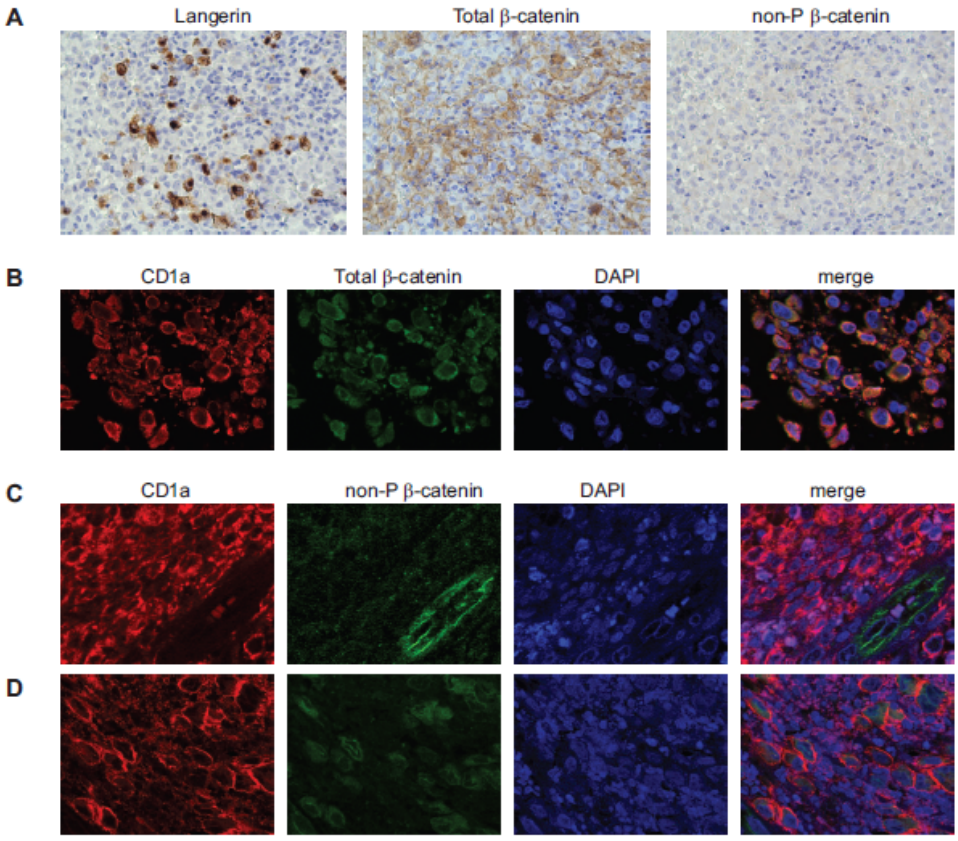

Figure 4. Non-phosphorylated β-catenin is not present in the nucleus of LCH cells.

LCH patient samples were stained by immunohistochemistry and immunofluorescence for total and non-phosphorylated β-catenin as well as for CD1a and Langerin to detect the LCH cells. The majority of lesional cells contained β-catenin but in virtually all cases this was not detectable as transcriptionally active, N-terminally non-phosphorylated β-catenin (A, B, C). The few samples that were positive for non-phosphorylated β-catenin showed mainly membranous and cytoplasmic staining while nuclear staining was not detected (D). Original magnification x 40 for panel A-D. See Table 1 for detailed quantitative findings.

Discussion

Elucidation of the etiology and the pathogenesis of LCH is crucial in order to develop a specific treatment for this disease. As LCH cells are characterized by an immature phenotype and significant pro-inflammatory cytokine production, we set out to investigate the status of transcriptionally active β-catenin in these pathological cells, since this form of β-catenin is known to be involved in the differentiation and maturation of DC at different levels (*18*) (*9*). We found that transcriptionally active β-catenin is rarely present in LCH cells (only 1 of 16 samples). To interpret our observation in comparison to a normal counterpart, we also determined the role of β-catenin in human peripheral blood monocytes developing into moDC, as monocytes are a putative origin of LCH cells (*1*). Beta-catenin transcriptional activity appeared to be indispensable in the early stages of human monocyte differentiation into moDC since inhibition of β-catenin activation blocked moDC development. This suggested to us that transcriptional activity of β-catenin is aberrantly regulated in LCH cells and that its absence could therefore be an important factor contributing to the pathogenesis of LCH. Since no consistent profound genomic aberrations have been described in LCH up to now (*4*), our results might be explained by abnormal regulation at transcriptional or post-transcriptional level.

Recent findings have indicated that the oncogenic BRAF V600E mutation is present in more than half of the investigated LCH specimens, strongly suggesting that LCH is a true neoplasm (*2, 3*). Independent of the BRAF mutational status, LCH cells stained positive for phospho-MEK and phospho-ERK, indicating activation of the RAS-RAF signaling cascade in all cases. Notably, activation of the RAS pathway has been shown to inhibit GSK3b activity via PI-3 kinase and AKT activation *(19*), and thus activation of β-catenin signaling, rather than the observed inhibition, would have been expected in LCH cells. Neoplastic transformation of cells typically requires multiple pathways to be affected, and it is tempting to speculate that absence of β-catenin signaling is an additional contributing factor to cellular dysregulation in LCH, given its importance in DC differentiation. The finding that molecular aberrancies in the RAS-RAF and β-catenin pathways often coincide in cancers such as colon carcinoma (*20*) supports the notion that they have distinct but additive roles in cell transformation. However, in contrast to an activation of the β-catenin pathway as found in many carcinomas, in LCH we find a consistent down-regulation in virtually all

LCH cases. This is not unprecedented in tumorigenesis, since recently a similar inactivity of the Wnt/catenin cascade was observed in high-grade osteosarcoma (*21*). The absence of Wnt/β-catenin signaling in LCH cells could potentially be due to an intrinsic defect, although up to now no consistent genetic or chromosomal abnormalities have been reported (*4*) other than the BRAF mutation. Another possibility would be that micro-environmental factors are responsible for the immature DC phenotype and lack of transcriptionally active β-catenin in these pathological LCs. $CD14^+$ monocytes could differentiate into LC upon stimulation with the Notch ligand Delta-1 (*22*), and in DC Delta-1 up-regulated Frizzled receptors on the cell surface (*11*). Through these receptors environmental Wnt proteins are able to activate the Wnt pathway, thereby increasing the levels of transcriptionally active β-catenin. The apparent link between the Notch and Wnt/β-catenin pathways in DC/LC differentiation and maturation tempts to speculate that dysregulated Notch signaling in the LCH lesion might contribute to the accumulation of LCH cells with an immature phenotype.

Alternative environmental factors that could explain the down-regulation of β-catenin signaling include members of the Dickkopf family, such as DKK-1 and -4. These antagonize Wnt signaling by blocking subunits of the Wnt receptor complex (*23*). Interestingly, expression of DKK-1 is induced by p53 (*24*), and LCH cells are known to express significant levels of p53 protein (*25*). This is likely related to their neoplastic state, in which further maturation of the cells *in situ* is blocked.

A functional role for β-catenin in differentiation and maturation processes within the DC lineage has been described by other groups (recently reviewed in (*18, 26*)). Zhou et al. reported that β-catenin is required for the early differentiation of mouse and human $CD34^+$ HPC into conventional DC (*11*). Furthermore, they showed that over-expression of β-catenin leads to an increased number of terminally matured $CD11c^+$ DC in HPC cultures as indicated by the significant expression of CD86 and MHC class II. In line with these findings, Jiang et al. earlier described that the activation of β-catenin/TCF-regulated transcription by disruption of the E-cadherin-mediated cell-cell adhesion in mouse BMDC induces final maturation of these cells (*9*). These β-catenin-matured DC exert tolerogenic functions, indicated by the induction of regulatory T cells and the absence of inflammatory cytokine production. Together, these studies indicate a role for β-catenin in the early lineage decision-making phase as well as in the terminally differentiated phase of DC development, respectively. Our

data complement these findings by showing a relevant role for β-catenin in the intermediate differentiation steps from monocytes to iDC.

In a recent study, specific deletion of β-catenin in CD11c-expressing cells was used to confirm the role of β-catenin in the regulation of the tolerogenic function of mouse DC (*10*). However, a question that remains to be answered is whether β-catenin activation also has a function in the development of immunogenic DC. Jiang et al. compared mouse BMDC matured through TLR4 activation by LPS stimulation with DC matured by cluster disruption and describe the up-regulation of non-phosphorylated β-catenin in concurrence with the development of tolerogenic DC after cluster disruption (*9*). Transduction with different reporter systems to monitor TCF-dependent transcription showed that cluster disruption, but not LPS stimulation, leads to TCF activation in BMDC, suggesting that β-catenin is not activated to function at transcriptional level through LPS signaling in these cells. In contrast, other reports describe an active role for β-catenin in the generation of pro-inflammatory monocyte-derived cells. For instance, Rodionova et al. reported that stimulation of human moDC with *E.coli* leads to inhibition of GSK3β and increased levels of transcriptionally active β-catenin (*27*). Similarly, human monocytes or alveolar macrophages exposed to LPS showed an increased expression of transcriptionally active β-catenin (*28, 29*). Together, these data suggest that β-catenin signaling can be activated in both tolerogenic and immunogenic DC as well as in other mononuclear phagocytes after TLR signaling.

What could be the role of β-catenin signaling in tolerogenic vs. immunogenic DC? LPS induces in DC the release of various cytokines, including IL-1a, IL-6, TNF-a, and IL-12p40, while DC matured by cluster disruption, and thus by β-catenin signaling alone, do not secrete these pro-inflammatory cytokines (*9*). Interestingly, the secretion of pro-inflammatory cytokines by monocytes and moDC stimulated with E.coli was significantly reduced when the cells were treated with a GSK3β inhibitor, thus allowing β-catenin activation (*29*). This suggests that β-catenin signaling under inflammatory conditions may serve as a negative regulator of pro-inflammatory cytokine production. This is in line with the recent observation in intestinal DC that β-catenin is required for the expression of anti-inflammatory mediators such as IL-10 and TGF-β (*10*). Applying this to LCH, the lack of β-catenin signaling could contribute to a preponderance of pro-inflammatory cytokines and chemokines as observed in LCH cells (summarized in (*1*)).

Together, we have shown that transcriptionally active β-catenin is not present in LCH cells, while it is indispensable in monocyte to moDC differentiation. Several lines of evidence suggest that this relates to the immature and pro-inflammatory state of LCH cells. In addition, absence of β-catenin signaling may contribute to the neoplastic nature of the cells, initiated by the activation of the RAS-RAF pathway, since both signaling cascades are largely independent entities known to be involved in malignant transformation of other cells. Future research in this direction is clearly warranted and might focus on microenvironmental factors influencing β-catenin signaling as well as on the putative therapeutic potential to modulate the LCH cell neoplastic and inflammatory state via stimulation of the β-catenin pathway.

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Authorship contributions

G.I.B., B.J.H.B. and F.F.N. performed and evaluated experiments; F.F.N., R.R.K. and P.W.H. obtained tissue specimens and evaluated experimental results, A.M.C.-J. evaluated experimental results; R.M.E. and P.J.M.L. conceived and coordinated this study. G.I.B. and P.J.M.L. wrote the manuscript, which was corrected and approved by the other authors.

Conflict of interest diclosure

The authors declare no competing financial interests.

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