Protein complexes associated with β-catenin differentially influence the differentiation profile of neonatal and adult CD8+ T cells

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Abstract
The canonical Wnt signaling pathway is a master cell regulator involved in CD8+ T cell proliferation and differentiation. In human CD8+ T cells, this pathway induces differentiation into memory cells or a “stem cell memory like” population, which is preferentially present in cord blood. To better understand the role of canonical Wnt signals in neonatal or adult blood, we compared the proteins associated with β-catenin, in nonstimulated and Wnt3a-stimulated human neonatal and adult naive CD8+ T cells. Differentially recruited proteins established different complexes in adult and neonatal cells. In the former, β-catenin-associated proteins were linked to cell signaling and immunological functions, whereas those of neonates were linked to proliferation and metabolism. Wnt3a stimulation led to the recruitment and overexpression of Wnt11 in adult cells and Wnt5a in neonatal cells, suggesting a differential connexion with planar polarity and Wnt/Ca2+ noncanonical pathways, respectively. The chromatin immunoprecipitation polymerase chain reaction β-catenin was recruited to a higher level on the promoters of cell renewal genes in neonatal cells and of differentiation genes in those of adults. We found a preferential association of β-catenin with CBP in neonatal cells and with p300 in the adult samples, which could be involved in a higher self-renewal capacity of the neonatal cells and memory commitment in those of adults. Altogether, our results show that different proteins associated with β-catenin during Wnt3a activation mediate a differential response of neonatal and adult human CD8+ T cells.

Keywords
β-catenin, CBP/p300, CD8+ T cells, neonate immunity, proteomics
1 | INTRODUCTION

Human neonates are highly susceptible to infections by intracellular pathogens, which are a major cause of infant morbidity and mortality (World Health Organization, 2017). The function of neonatal T cells is considered to be immature, skewed, and tolerant. Under very strong stimulation, however, neonatal cells are capable of adult-level T-cell responses, indicating their high activation threshold (Adkins, Leclerc, & Marshall-Clarke, 2004).

The Wnt signaling pathways are master regulators of development and multiple biological functions, including antagonist roles in undifferentiated cell renewal and differentiation (Teo & Kahn, 2010). There are 19 mammalian Wnt proteins that can be recognized by 10 frizzled (Fzd) receptors and different coreceptors, the combination of which determine the signaling pathway and outcome of the cell. The canonical pathway can be activated by Wnt1, Wnt3a, and Wnt8 and depends on the engagement of Fzd and low-density lipoprotein-receptor-related proteins (LRP) 5/6. It relies on the stabilization of β-catenin, through the disassembly of the β-catenin degradation complex, formed by the scaffold protein axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK3β), and casein kinase 1 (CK1). In the absence of Wnt signals, β-catenin is phosphorylated by GSK3β, a mark that provokes its ubiquitination and degradation. Once the Wnt signal is received, β-catenin is stabilized in the cytosol and forms protein complexes that mediate its import to the nucleus and activation of gene expression, which is mediated by the T cell factor (TCF) and Lymphoid enhancer-binding factor (LEF) family of transcription factors (MacDonald, Tamai, & He, 2009). The noncanonical Wnt-dependent signaling pathways are broadly grouped into the Wnt/Ca²⁺ signaling pathway and the planar polarity pathway. Wnt5a, with coreceptors Ror1/2, has been associated with the activation of the Wnt/Ca²⁺ pathway and Wnt11 with the planar polarity pathway.

The protein complexes associated with the β-catenin translocate to the nucleus and associate with the transcription factors TCF and LEF to displace the GROUCHO family of proteins, to allow the transcription of the target genes (Gao, Xiao, & Hu, 2014). The later are inhibitors of gene transcription that keep the Wnt transcription of the target genes (Gao, Xiao, & Hu, 2014). The later LEF to displace the GROUCHO family of proteins, to allow the translocation to the nucleus and associate with the transcription factors TCF and LEF.

β-catenin is essential for the expression of genes involved in self-renewal of stem cells, whereas p300-containing complexes mediate transcription of differentiation-related genes (Teo & Kahn, 2010). A dual role for CBP and p300 was also described during myocytes, differentiation. Using mutant forms of CBP and p300, it was shown that p300, but not CBP, is important for cell differentiation in vivo (Roth et al., 2003). Again, in colorectal cancer, in which a role of Wnt signaling has been clearly established in cell transformation, it was found that CBP promotes proliferation and is a marker of bad prognosis, whereas p300 is important for differentiation of these cells (Bordonaro & Lazarova, 2015). These reports suggest that p300 and CBP associated with β-catenin act as a switch that promotes either differentiation or cell renewal, respectively.

The involvement of the Wnt-β-catenin pathway in CD8⁺ T cell differentiation into memory precursor effector cells (MPEC), but not into short lived effector precursor cells (SLEC), was demonstrated in adult mice models in vivo. It was clearly shown that the pathway is active in naïve, MPECs, and memory cells, but is silenced in SLECs and effector cells (Boudousque et al., 2014; Gattinoni et al., 2009). In addition, the activation of the canonical Wnt signaling pathway also induces a phenotype that has been called "stem cell memory like (TSCM)". These are self-renewing multipotent CD8⁺ T cells that have a mixed phenotype between memory and stem cells, which could be useful for bone marrow transplantation purposes and immune-cell-mediated cancer treatments (Forget et al., 2012; Li et al., 2014; Scholz et al., 2016). The TSCM phenotype is enriched in neonatal as compared with adult blood (Xu et al., 2016).

We have previously shown that human neonatal CD8⁺ T cells have a distinctive transcriptomic and epigenetic landscape, characterized by the expression of genes involved in cell cycle and innate immunity. We also found that neonatal cells have an increased homeostatic proliferation, but reduced clonal expansion and effector functions. Several transcription factors were found differentially expressed between adult and neonatal cells, including the β-catenin-dependent TCF4 and LEF1 factors (Galindo-Albarran et al., 2016).

Our aim in this study was to understand the potential dual role of β-catenin-mediated signaling in the maintenance of cell renewal capacity and differentiation of neonatal and adult human CD8⁺ T cells. For this, we made a proteomic identification of the proteins that associate with β-catenin in human neonatal and adult CD8⁺ T cells, before and after stimulation with Wnt3a. We found common and differentially recruited proteins in the neonatal and adult cells that were involved in the formation of complexes with different biological activities. In the adult cells, the complexes formed were associated with the control of immunological function and cell signaling, whereas in those of neonates with proliferation and metabolism. A notable difference was the Wnt3a-induced association of β-catenin with Wnt11 in the adult cells complexes and Wnt5a in those of neonates, which were accompanied by a differential overexpression of these proteins. This suggests an alternative connexion of Wnt3a-activated cells with the cell polarity pathway in adult cells and Wnt/Ca²⁺ in the neonatal samples. Evaluation of β-catenin recruitment to the promoters of target genes showed a
higher enrichment of β-catenin to the promoters of proliferation genes in the neonatal cells. Finally, we found that β-catenin preferentially recruits CBP in neonatal cells and p300 in those of adults. Altogether our results suggest that a cellular switch, mediated by β-catenin-associated protein complexes, could participate in the control self-renewal or differentiation of human CD8+ T cells. This clarifies the role of the canonical Wnt signaling pathway in CD8+ T cell populations, which are considered for immunological cancer treatments and cell replenishment in transplantation procedures.

2 | MATERIALS AND METHODS

2.1 | Cell preparation

Adult CD8+ T cells were obtained from leukocyte concentrates of healthy adult (Walham, MA) donors from Centro Estatal de la Transfusión Sanguínea, Cuernavaca, Mexico. Neonatal blood was collected from the cord vein of full-term vaginal deliveries of healthy babies at Hospital General Parres, with informed consent of mothers. Blood was collected just after the deliveries and before placental expulsion. The Ethical Committee of Hospital Parres approved this procedure. Total blood was separated with ficoll gradient: 3 mL of cultured erythrocytes from the same donor and depleted the unwanted cells with RossetteSep™ CD8+ T cell enrichment cocktail (15063; StemCell Technologies, Canada). The cells were further purified by depletion of CD45RO+, CD44+, and B220+ cells with Protein A/G magnetic beads (8803; Pierce; Thermo Fisher Scientific, Bremen, Germany), ligated to the corresponding antibodies (CD45RO: UCHL1; CD44: IM7 and B220: RA3-6B2; eBiosciences, San Diego, CA). This method allowed us to have more than 94% naive CD3+CD8+ T cells (Figure S1). The cells were maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 1% L-glutamine, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 5% fetal calf serum under 5% CO2 at 37°C for 2 hr, followed by incubation overnight with protein A/G magnetic beads (8803; Pierce; Thermo Fisher Scientific, Waltham, MA). Beads were washed three times with PBS, using a magnetic separation rack to retain marked cells, after which samples were boiled in sodium dodecyl sulfate (SDS) sample buffer (SDS 12%, Tris base 0.6 M pH 6.8, bromophenol blue 1.2 μg/mL, and β-mercaptoethanol 5%). An example of the total proteins and immunoprecipitated proteins is shown in Figure S2.

2.2 | Flow cytometry

Antibodies recognizing the following molecules coupled to the indicated fluorochromes were used to assess the purity of the cell preparations: PE anti-human CD8α (Hit8aOKT8; 50-0089-T100; Tonbo Biosciences), FITC anti-human CD3 (OKT3; 35-0037-T100; Tonbo Biosciences), APC anti-human CD45RO (UCHL1; 20-0457-T100; Tonbo Biosciences). The cells were subsequently washed twice in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and resuspended for fluorescence-activated cell sorting (FACS) analysis. Flow cytometry was performed on the FACSCanto cytometer using the BD FACSDiva software. Data analysis was performed using the FlowJo software (FlowJo, LLC).

2.3 | Immunoprecipitation

The cells were left untreated or incubated in 24-well plates with Wnt3a for 12 hr. After washing twice with PBS, cells were lysed in lysis buffer (137 mM NaCl, 20 mM Tris pH 8, 1% NP-40, 10% glycerol, 50 mM NaF, 2 mM ethylenediaminetetraacetic acid and 4% protease inhibitor cocktail [P8340; Sigma-Aldrich®, St. Louis, MO]). After clearance by centrifugation at 16,000g for 30 min at 4°C, lysates were incubated with 2 μg of anti-β-catenin antibody (E-5; sc-7963; Santa Cruz Biotechnology, Dallas, TX) at 4°C for 2 hr, followed by incubation overnight with protein A/G magnetic beads (8803; Pierce; Thermo Fisher Scientific, Waltham, MA). Beads were washed three times with PBS, using a magnetic separation rack to retain marked cells, after which samples were boiled in sodium dodecyl sulfate (SDS) sample buffer (SDS 12%, Tris base 0.6 M pH 6.8, bromophenol blue 1.2 μg/mL, and β-mercaptoethanol 5%). An example of the total proteins and immunoprecipitated proteins is shown in Figure S2.

2.4 | Mass spectrometry analysis

The β-catenin immunoprecipitates were concentrated in SDS-polyacrylamide gel electrophoresis (PAGE) pre-gel. The bands were then cut, and the gel fragments were washed sequentially with 50% (v/v) acetonitrile in water (acetonitrile mixed with 100 mM ammonium bicarbonate [1:1] and 100% acetonitrile as previously described; Lino, Carrillo-Rayas, Chagolla, Gonzalez, & de la Vara, 2006). The proteins in the gel fragments were then reduced, carbamidomethylated, and digested with trypsin, and the resulting peptides were extracted from the gel, as previously described (Shevchenko, Wilm, Vorm, & Mann, 1996).

These peptides were analyzed by mass spectrometry as described (Garcia Montes de Oca et al., 2016) and separated in a nanoAcuity Nanoflow LC System (Waters Corp., Milford, MA). The peptides were first trapped in a precolumn (Symmetry® C18, 5 μm, 180 μm × 20 mm; Waters) and then were separated in a 10-cm capillary ultra high performance liquid chromatography (UPLC) column (100-μm ID BEH C18 1.7 μm particle size) that was run at 35°C with the following gradient: 3–50% Solvent B (0.1% formic acid in acetonitrile) in Solvent A (0.1% formic acid in water) for 30 min, 50–85% B (1 min), 85% B (7 min), and 3% B (22 min). The separated peptides were directly electrosprayed into a linear ion trap LTQ Velos mass spectrometer (Thermo Fisher Scientific) that was equipped with a nanoelectrospray ion source and controlled by the Xcalibur 2.1 software. This spectrometer was operated in data-dependent acquisition mode, automatically alternating a full-scan (m/z 400–2,000) spectrum with a collision-induced dissociation spectrum of the most abundant peptide ions.

The data were analyzed using the X!Tandem search engine on a LabKey or a Trans Proteome Pipeline (Deutsch et al., 2010) platform, and the results were validated with the PeptideProphet and ProteinProphet software. The fragmentation spectra were compared with theoretical two H. Sapiens Refseq databases that were obtained from NCBI: one with lymphocyte-related protein sequences and the other with proteins involved in the Wnt signaling pathway. The following search parameters were used: constant modifications,
carbamidomethylated C, variable modifications, and oxidized M. The ion-mass tolerances were 4 Da for precursor ions and 2 Da for fragmentation products. Two separate experiments with an equivalent amount of identified proteins were analyzed. To include proteins for further analysis, we selected those in which the ProteinProphet probability was >0.95, with at least four unique identifying peptides per protein and that were identified in the two experimental samples. All identified proteins are presented in Tables S1 and S2.

2.5 | Protein analysis

We performed functional analysis to the differentially β-catenin-associated proteins under nonstimulated or Wnt3a-stimulated conditions from neonate or adult CD8+ T cells. For this, we used the Database for Annotation, Visualization and Integrated Discovery software in connection with the Kyoto Encyclopedia of Genes and Genomes PATHWAY, UP KEYWORDS (Uniprot), and Gene Ontology Consortium: Biological Process and InterPro. We took Benjamini corrected p values for significant differences between neonatal and adult CD8+ T cell samples. For interactome analysis, we used String 10.5 to predict protein interactions and visualized protein complexes with the Cytoscape 3.5.1 software.

2.6 | Chromatin immunoprecipitation

After stimulation, the cells were washed with PBS and cross-linked in 1% formaldehyde for 20 min. The reaction was stopped with 0.22 M glycine. The nuclei were isolated and sonicated for 20 min with pulse 10 s and 60% amplitude to yield fragments of 300–500 base pairs. After purification by centrifugation with phenol:chloroform:isoamyl alcohol (25:24:1; pH 8), cross-linked protein/DNA complexes were immunoprecipitated with 2 μg of anti-β-catenin antibody (E-5; sc-7963; Santa Cruz Biotechnology). Total chromatin was used as a control (input). Both input and immunoprecipitated chromatin was reversed cross-linked by heating at 50°C for 12 hr, and DNA was purified with mini columns (Expin PCR SV; Genall) and quantitated with a high sensitivity dsDNA kit in the Qubit fluorometer (Thermo Fisher Scientific).

2.7 | Real-time polymerase chain reaction

For gene expression evaluation, cells were centrifuged, washed, and placed in Trizol (15596-018; Invitrogen; Thermo Fisher Scientific) before freezing. RNA was extracted according to the manufacturer’s instructions. The integrity of the RNA was determined by electrophoresis and quantified with Qubit RNA HS kit in a Qubit fluorometer. Complementary DNA was synthesized from total RNA using RevertAid Reverse Transcriptase (SO131; Thermo Fisher Scientific). Quantitative polymerase chain reactions (qPCRs) were performed using SYBR Green PCR Master Mix (4309155; Life Technologies; Thermo Fisher Scientific). The messenger RNA (mRNA) levels were calculated with standard curves for each gene and were reported relative to those of GAPDH gene as standard.

Specific oligonucleotides were designed to amplify regions corresponding to the mRNA of the genes (Table S3). The mRNA sequence of the specific isoforms of the aforementioned genes was obtained from the NCBI database. Subsequently, these sequences were entered into the Primer-BLAST online program to ensure specificity of the amplicon.

For evaluation of the β-catenin-associated with specific gene promoters, chromatin immunoprecipitation analysis was performed, followed by qPCR. Specific primers in the gene promoters, containing TCF/LEF binding motifs, were selected using the “Eukaryotic Promoter Database” (http://epd.vital-it.ch/) to find promoters from the position −1,000 to +100 of the gene. “MatInspector” analysis available at www.genomatix.de allowed us to find the TCF-1/LEF1 binding motifs. Regions with more than one motif were chosen. Oligos were obtained as described above, from a region −250 to +250 of the motifs. Sequences are shown in Table S3.

All amplicons produced a single peak after denaturation, indicating a single amplicon.

2.8 | Immunoblots

Protein extracts or β-catenin immunoprecipitates were boiled with sample buffer for 5 min at 100°C and separated on a PAGE 4–12% mini-gel (mini-PROTEAN®). Proteins were transferred to a polyvinylidene fluoride membrane in a wet chamber (Bio-Rad) at 400 mA for 4 hr. The membrane was blocked with TBST (TBS; Tris base 19 mM, NaCl 137 mM, pH = 7.8 and TBST: Tris Based Saline containing+Twee 20), 1% fat free milk for 1 hr at room temperature and subsequently incubated with anti-β-catenin (E-5; Santa Cruz Biotechnology), anti-CBP (C-1; Santa Cruz Biotechnology), anti-P300 (C-20; Santa Cruz Biotechnology), anti-wnt5a (ab174963; Abcam, UK), anti-wnt11 (Ab31962; Abcam) or anti-β-actin antibodies (rabbit polyclonal immunoglobulin G [IgG]; Santa Cruz Biotechnology). The membrane was washed with TBST three times and subsequently incubated with anti-mouse IgG-HRP (DO80; Santa Cruz Biotechnology) anti-rabbit IgG-HRP (LO204; sc-2301; Santa Cruz Biotechnology) for 2 hr at room temperature. After washing the membrane with TBST and TBS, membranes were treated with enhanced chemiluminescence western blotting detection reagent (PE191121; 32132; Pierce® ECL Plus, Appleton, WI) and bands were revealed with radiography films Medical X-ray General Purpose Blue (825 024; Ref 604 0331; Kodak, Rochester, NY). Densitometry analysis was performed with ImageJ (http://rsbweb.nih.gov/ij/).

3 | RESULTS

3.1 | Differences in β-catenin-associated proteins in neonatal and adult CD8+ T cells

The canonical Wnt signaling pathway controls the antagonist responses of cell renewal and differentiation. To get insights into the proteins involved in neonatal and adult CD8+ T cells responses,
we purified naive CD8+ T cells (Figure S1) and immunoprecipitated β-catenin from protein extracts of untreated or Wnt3a-stimulated cells. Proteins were identified by mass spectrometry analysis from two independent experiments of each cell population (Tables S1 and S2). Under the untreated conditions, a total of 101 and 96 proteins were identified in the adult and neonatal samples, respectively, with 40 common proteins (Figure 1a). After stimulation with Wnt3a, the number of recruited proteins increased about 4-fold, to 455 and 451 in adult and neonatal cells, respectively, with 402 common proteins (Figure 1c).

The functional analysis of the cell-type specific β-catenin-recruited proteins showed conserved functions and also differences between neonatal and adult cells (Figure 1b). Among the differential functions, we found that in the nonstimulated adult samples, β-catenin-associated proteins were involved in a cell polarity pathway and were rich in kinase-like domains, armadillo fold, and shisa family of proteins. Armadillo is a common fold domain present in β-catenin and Wnt signaling transduction proteins, whereas the Shisa family refers to proteins that control the maturation and trafficking of Fzd receptors to the cell membrane. In the neonatal cells, the β-catenin-associated proteins were enriched in the melanogenesis pathway and in neural differentiation and Fzd-binding GOterms. Fzd receptors associated with β-catenin under the nonstimulated conditions were only found in the neonatal cells. The enrichment in the melanogenesis pathway could relate to the high reactive oxygen species production of the neonatal cells (Galindo-Albarran et al., 2016). The neural differentiation GOterm could refer to the recruitment of presenilin1 and associated proteins in the neonatal immunoprecipitates, which suggests a connection with the Notch Pathway in these cells.

The proteins identified after Wnt3a stimulation in adult cells (Figure 1d) were associated with a higher activity of the Wnt signaling pathway and were rich in kringle, shisa, and kinase proteins. The recruitment of kinases is in agreement with a higher expression of signaling proteins in adult CD8+ T cells (Galindo-Albarran et al., 2016). The proteins with kringle domains are binding mediators and regulators of proteolytic activity. In the neonatal cells, the recruited proteins were associated with proliferation, stemness, and hippo signaling pathway that regulates cell proliferation, apoptosis, and stress responses. Altogether, these results suggest a mature, highly controlled, Wnt signaling pathway in the adult CD8+ T cells. In the neonatal cells, however, our results suggest the involvement of this pathway in stemness, cell cycle, and developmental processes.

Next, we focused our analysis on the differences of β-catenin-immunoprecipitated proteins between neonatal and adult CD8+ T cells within the Wnt signaling pathway (Figure 2). Orange color refers to common proteins, red to adult-specific proteins, and blue to neonatal-specific proteins. In the nonstimulated neonatal cells (Figure 2a), we found Wnt5b and Wnt8a, whereas in the adult cells, Wnt9b was recruited. Wnt5b is related to cell migration in cancer cells and estrogenic differentiation (Harada et al., 2017). Little is known about Wnt8a and Wnt9, except that they participate in development. Only in the adult’s samples, the association of LEF and TCF with β-catenin complexes was found under basal conditions, and only in the neonatal cell samples, Fzd receptors were found to be associated with β-catenin.

**FIGURE 1**  Functional analysis of β-catenin immunoprecipitates. (a) and (b) Proteins from nonstimulated or (c) and (d) Wnt3a-stimulated CD8+ T cells. (a) and (c) Venn diagrams indicating the adult and neonatal cells exclusive and common proteins. (b) and (d) Functional analyses of neonatal (blue) and adult (red) specifically recruited proteins [Color figure can be viewed at wileyonlinelibrary.com]
In the Wnt3a-stimulated cells (Figure 2b), the number of proteins associated with β-catenin was increased, as expected. Under these conditions, recruitment of Fzd receptors was found both in neonatal and adult cells as well as in all the proteins of the β-catenin degradation complex and transcription factors TCF and LEF. Only in adult cells, MAP3K7 transforming growth factor beta-activated kinase (Tak1), frequently rearranged in advanced T cell lymphomas (FRAT1), and CXXC4 were recruited, indicating a connection with mitogen-activated protein kinase (MAPK) pathway, ATM-dependent DNA damage control, and control of Wnt signaling, respectively. Although in the stimulated cells, Axin2 was found in both cell populations, Axin1 was only recruited in the neonatal cells and in untreated and Wnt3a-treated samples. 

**FIGURE 2** β-Catenin-associated proteins in Wnt pathways. Proteins from (a) nonstimulated and (b) Wnt3a-stimulated CD8+ T cells. Proteins only found in neonatal cells are depicted in blue, in adult cells in red, and in both cells in orange. The diagram was created in Cell Designer® [Color figure can be viewed at wileyonlinelibrary.com]
differential role of Axin1 and Axin2 has been reported; the latter is more associated with the destruction complex activity (Thorvaldsen, Pedersen, Wenzel, & Stenmark, 2017). Differences in the recruitment of Wnt proteins were also found in the Wnt3a-stimulated cells. Wnt5a, Wnt10A, and Wnt16 were recruited only in neonatal cells, and Wnt11 only in the adult samples, suggesting the connexion with two independent branches of the noncanonical Wnt pathways in the cells from the two different age populations. Wnt11 induces the cell polarity pathway that is more strongly related to cell differentiation (Cohen, Tian, & Morrissey, 2008; Lange, Mix, Rateitschak, & Rolfs, 2006; Terami, Hidaka, Katsumata, Iio, & Morisaki, 2004) and Wnt5a with calcium signaling. Higher calcium waves have been reported in neonatal T cells (Palin, Ramachandran, Acharya, & Lewis, 2013).

In neonatal cells, we also found presenilin1 in the β-catenin complexes. This protein is the catalytic subunit of the γ-secretase complex (PS-1) and is probably related to a connexion with the Notch signaling pathway. On the contrary, only in adult cells, the MAPKKK Tak 1 and the nucleic acid binding protein Sbp1 were recruited in the β-catenin complexed proteins, suggesting a link with the MAPK and p53 signaling pathways, respectively. Differences were also found in members of Dkk, DVL, and axin family proteins, all of which are controlling proteins of the canonical pathway. Altogether, our results show that the differences in protein complexes recruited by the β-catenin immunoprecipitates from neonatal and adult CD8+ T cells could be biologically significant.

3.2 | Interactome analysis of the β-catenin-immunoprecipitated proteins

To identify the protein complexes formed by the specific β-catenin-recruited proteins in neonatal and adult cells, we analyzed the interactome (Figure 3). The protein complexes from neonatal cells were mostly involved in positive regulation of cell cycle, chromatin remodeling, calcium pathways, and metabolism. Three proteins involved in several of the neonatal complexes were Wnt5, Axin1, and presenilin1. Wnt5a was involved in complexes associated with calcium metabolism and proliferation, in association with the replication-dependent histone Hist1H4H. Axin1 was involved in complexes related to proliferation, microtubule organization, and ubiquitination. Presenilin1 is a protease that cuts the Notch receptor during the Notch pathway activation. A role of the Notch signaling pathway, in association with the Wnt pathway, has been described during CD8+ T cells differentiation into stem-cell-memory-like populations (Kondo et al., 2017).

The preferentially recruited proteins in adult cells were more associated with the regulation of immune system processes, kinases activities, and cell signaling. The protein SFRP1, member of the soluble frizzled-related proteins, was present in adult cell complexes. This protein is a negative modulator of Wnt signaling, leading to a reduction in β-catenin levels and to an inhibition of proliferation in vascular cells (Ezan et al., 2004). Also central in adult cells complex’s formation were MAP3K7 (Tak) and Wnt11.

The specific complexes formation in adult and neonatal cells could be the result of increased gene expression of the population-specific complexes or posttranslational modifications. We thus evaluated the expression of six of the genes involved in the formation of the specific complexes in both cell’s populations. As shown in Figure 4a, wnt5A was only detected in the neonatal cells, both untreated and Wnt3a stimulated. psen-1 and axin2 expression did not change as compared with adult cells or as a result of stimulation. For the adult genes, wnt11 and sfrp1 were overexpressed in adult cells, the later only under Wnt3a-stimulated conditions. The overexpression of Wnt5A in neonatal cells and of Wnt11 in the adult samples was also found at the protein level (Figure 4b).

Altogether these experiments show that some the differentially recruited proteins could be the result of different gene expression and corroborate that different protein complexes in neonatal and adult CD8+ T cells could be responsible for the enriched presence of T<sub>SCM</sub> phenotype in cord blood cells.

3.3 | Association of β-catenin with gene promoters differs in neonatal and adult cells

The differences in protein complexes recruited by β-catenin in neonatal and adult CD8+ T cells prompted us to investigate whether there is a differential recruitment of β-catenin to gene promoters in the neonatal and adult cells. For this, we performed experiments of chromatin immunoprecipitation followed by qPCR (ChiP–qPCR) of gene promoters associated with proliferation (birc5, ccd1, pou5f1, and s100a4), cell signaling (cjun, fra1, fos, and axin2), and differentiation (gzm, pdrm, and nkd1). As shown in Figure 5, enrichment of β-catenin was higher for birc5, ccd1, and pou5f1 in the neonatal cells. At basal levels, axin2 promoter was more enriched in β-catenin in the neonatal cells, but after Wnt3a stimulus, adult cells showed enrichment in β-catenin recruitment to the neonatal cell levels. In the adult cells, a higher enrichment in β-catenin recruitment was observed for fos promoter, both in nonstimulated and Wnt3a-treated cells. Recruitment of β-catenin to the jun promoter only increased in Wnt3a-stimulated adult cells. This is in agreement with the bigger involvement of the MAPK pathways in the adult cells. For gzm and nkd1, associated with cell differentiation, there was a significant increase in the β-catenin recruitment in the adult cells as compared with those of neonates.

Our gene expression experiments showed that birc5 was overexpressed in the neonatal cells under basal conditions and was not further stimulated by Wnt3a treatment; pou5f1 was only overexpressed in response to Wnt3a in the neonatal cells, and axin2 expression was induced by Wnt3a in the adult cells, to the neonatal cells’ basal levels (Figure 5). Only for these three genes, there was a correspondence between β-catenin recruitment and gene expression. Although β-catenin could have a permissive role in the expression of the other genes, their expression is also controlled by other transcription factors, such as the T cells receptor (TCR) inducible factors activator protein 1 (AP-1), nuclear factor kappa-B (NFκB), nuclear factor of activated T cells (NFAT), and cAMP response element (CREB).
Next, we evaluated specifically the association of CBP or p300 with β-catenin in neonatal and adult cells. It has been proposed that the association of β-catenin with either CBP or p300 is a molecular switch that flips the balance toward proliferation or differentiation of the cells, respectively. CD8⁺ T cells from adult or neonate donors differ in their proliferation versus differentiation profile. Neonatal cells have a higher stemness capacity and adult cells differentiate preferentially into memory cells and its precursors in response to canonical Wnt signals (Boudousquie et al., 2014; Galindo-Albarran et al., 2016; Gattinoni et al., 2009; Xu, Zhang, Luo, & Li, 2015). In agreement with this notion, our results from the proteomic characterization of the complexes associated with β-catenin in adult cells complexes are balanced toward differentiation and in neonatal cells toward proliferation. To specifically evaluate the association of

**FIGURE 3** Interactome showing a representation of protein complexes recruited by β-catenin. Complexes formed in (a) adult or (b) neonatal CD8⁺ T cells. Red circles represent proteins only recruited in immunoprecipitates from adult cells, blue circles from neonatal cells, and orange circles in both samples. Image created using Cytoscape 3.5.1 [Color figure can be viewed at wileyonlinelibrary.com]
CBP or p300 with β-catenin in neonatal and adult CD8\(^+\) T cells, we performed β-catenin immunoprecipitation experiments followed by β-catenin, CBP, and p300 immunoblots. As shown in Figure 6, in neonatal but not adult cells, β-catenin was strongly associated with CBP after Wnt3a stimulation. On the contrary, only adult cells significantly increased their association with p300 after Wnt3a stimulation. Altogether, these results suggest that the preferential association of CBP with β-catenin in neonatal CD8\(^+\) T cells could be contributing to their stemness phenotype after Wnt3a stimulation. On the contrary, the association of p300 with β-catenin in adult CD8\(^+\) T cells could predispose their differentiation into memory cells and its precursors.

4 | DISCUSSION

In a previous work, we described that neonatal CD8\(^+\) T cells have a distinct transcriptomic and epigenetic programming, characterized by an inflammatory innate response, a high homeostatic proliferation rate, and low signaling and cytotoxic functions (Galindo-Albarran et al., 2016). Among the transcription factors that we found enriched in the neonatal cells were Tcf4 and Lef1, which are the responsive factors of the Wnt signaling pathway. Both neonatal and adult CD8\(^+\) T cells responded to Wnt3a treatment with an over fourfold increase in the amount of proteins that associated with β-catenin. Although 78% of those proteins were shared between adult and neonatal cells (402 proteins), the specifically recruited proteins (102 proteins) could be determinant for the specific neonatal or adult cells’ responses. Adult-specific proteins were associated with phosphorylation, cell polarity, MAPK, and differentiation, whereas those specifically recruited in the neonatal cells were involved in proliferation, metabolism, and development.

In the adult cells, under basal conditions, the association of β-catenin with the transcription factors TCF/LEF and NFAT5 was observed, suggesting the presence of β-catenin already in the nucleus. The pathway regulators APC and FRAT1 were also found...
β-Catenin bound to gene promoters and gene expression. Primers were designed for the selection of TCF/LEF target genes with self-renewal, signaling or differentiation roles. Upper panels: Enrichment of β-catenin to the promoters of these genes (ChIP/qPCR). Lower panels: gene expression (RT-qPCR). Experiments were performed from at least three independent samples of neonatal or adult cells. *p < 0.05. ChIP: chromatin immunoprecipitation; RT-qPCR: quantitative reverse transcription polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]
in the adult cells immunoprecipitates. This suggests a longer experience with the activity of the pathway in the adult cells, which could be related to the long life of human naive T cells (Vrisekoop et al., 2008). After stimulation, as expected, a bigger proportion of proteins within the Wnt-mediated signaling pathways were found. Among the proteins differentially associated with β-catenin, we retrieved differences in Wnt proteins themselves. In the neonatal cells, Wnt5a was found overexpressed as compared with adult cells, suggesting a link of Wnt3a activation with the activation of the Wnt/Ca²⁺ signaling pathway. Wnt5a is also related to autophagy induction in response to bacterial infections (Jati et al., 2018), which could be part of an innate-immunity-like response we previously found in neonatal CD8⁺ T cells. In the adult cells, Wnt11 was overexpressed. This protein induces the Wnt cell polarity pathway and signaling through protein kinase C. It is characterized by cytoskeleton reorganization and changes in cell polarity leading to differentiation in several cell types (Terami et al., 2004; Uysal-Onganer et al., 2010).

**FIGURE 6** β-Catenin, CBP, and p300 immunoblots from β-catenin immunoprecipitates. Protein extracts from nonstimulated (NS) of Wnt3a-stimulated neonatal (right panels) and adult (left panels) CD8⁺T cells were immunoprecipitated with β-catenin antibodies. Representative immunoblots from (a) adult cells and (b) neonatal cells. (c)–(h) Densitometric analysis of immunoblots, results are reported as means±SD of three independent experiments and expressed as optical density value calculated with ImageJ; *p < 0.05; (b) and (d) β-catenin blots; (e) and (f) CBP blots; (g) and (h) p300 blots. Experiments were performed from at least three independent samples of neonatal or adult cells. *p < 0.05. SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]
Interactome analysis of the specifically recruited proteins showed that the complexes from neonatal cells were mostly involved in the positive regulation of cell cycle, chromatin remodeling, calcium pathways, and metabolism. Wnt5a was found central to calcium metabolism and proliferation, which was also associated with the recruitment of the replication-dependent histone Hist1H4H. Others and we have reported an increased homeostatic proliferation in neonatal CD8\(^+\) T cells (Galindo-Albarran et al., 2016; Marchant & Goldman, 2005; Schonland et al., 2003). The potential involvement of the \(\beta\)-catenin-dependent Wnt signaling pathway in the neonatal cells’ proliferation was made evident by the formation of complexes associated with proliferation, the enrichment of \(\beta\)-catenin on the promoters of cell renewal genes, and the induction of these same genes’ transcription by Wnt3a signaling. Oct4 (pou5f1) was found overexpressed in the neonatal cells, as well as the apoptosis inhibitor survivin (birc5), which could be involved in self-renewal.

Also important to the neonatal cells complexes formation was presenilin1, a protease that is involved severing Notch receptor during the pathway activation. A role of the Notch signaling pathway, complementary to the Wnt pathway, has been described for differentiation into stem-cell-memory-like populations (Kondo et al., 2017) and also in Alzheimer disease, associated with a PS-1 mutant, in which the blockage of CBP re-established neuronal differentiation (Teo, Ma, Nguyen, Lam, & Kahn, 2005). The preferentially recruited proteins in adult cells were more associated with immune system processes, regulation of kinase activities, and cell signaling. The protein SFRP1, member of the soluble frizzled-related complexes was central to several of the adult cell complexes. The sfrp1 gene was found overexpressed in adult Wnt-3a-stimulated cells. These proteins are negative modulators of Wnt signaling, leading to a reduction in \(\beta\)-catenin levels and to an inhibition of proliferation in vascular cells (Ezan et al., 2004). The product of the gene map3k7 was also only recruited in adult cells. This gene codes for TAK1, an important regulator of cell death, through NFkB activation and also through NFkB-independent pathways, such as oxidative stress and receptor-interacting protein-kinase-1-dependent pathway (Lui et al., 2011; Mihaly, Ninomiya-Tsuji, & Morioka, 2014). The nuleic acid binding protein Sbp1 was also recruited to the \(\beta\)-catenin complexed proteins from adult cells, establishing a connexion with the p53 signaling pathway.

To evaluate whether the changes in protein complexes lead to differential recruitment of \(\beta\)-catenin to the promoters of genes associated with self-renewal, signaling, and differentiation, we performed ChiP/qPCR experiments. Enrichment of \(\beta\)-catenin over the input was observed for most of the genes, in both cell populations; however, significant differences between neonatal and adult cells were found. Enrichment of \(\beta\)-catenin was higher in self-renewal associated genes in the neonatal cells. On the contrary, differentiation-associated genes recruited a higher proportion of \(\beta\)-catenin to their promoters in adult cells. This reminded us of the dual role of the Wnt signaling pathway reported for other cell types. It has been reported in stem cells, myocytes, neurons, and cancer cells that the association of CBP or p300 with \(\beta\)-catenin have opposite roles in the control of self-renewal and differentiation (Bordonaro & Lazarova, 2015; Roth et al., 2003; Teo & Kahn, 2010; Teo et al., 2005). Because of the high homology of CBP and p300, the distinction between both proteins could be clouded in proteomic analysis, in which we only identified p300 associated with both neonatal and adult cells in Wnt3a-treated cells. We thus performed immunoblot to specifically evaluate the interaction of \(\beta\)-catenin with either p300 or CBP, using specific antibodies that distinguish between both proteins. Our results show that in neonatal cells, Wnt3a treatment significantly induced the association of CBP with \(\beta\)-catenin, whereas in adult cells, the same stimulus-induced p300 recruitment. The N-terminal 110 amino acids of CBP or p300 are responsible for the association of these proteins with \(\beta\)-catenin. The affinity of this interaction is meditated by MAPK phosphorylation, in the case of CBP in Ser92 and for p300 by Ser12. Using specific inhibitors, it was determined that Erk could be responsible for the Ser92 phosphorylation of CBP and p38 for Ser12 in p300 (Ma, Guo, Shan, & Xia, 2012; Xia, Guo, & Ma, 2011). Differential activation of Erk or p38 MAPK could thus mediate the differential recruitment of CBP or p300 in neonatal and adult CD8\(^+\) T cells. A different metabolism in neonatal and adult cells could also explain the differences (Scholz et al., 2016). Our results suggest that a cell switch between differentiation and self-renewal, mediated by p300 versus CBP association with \(\beta\)-catenin, could participate in the higher renewal capacity of neonatal CD8\(^+\) T differentiation of adult cells into memory cells.

Altogether, our data show that both neonatal and adult CD8\(^+\) T cells respond to Wnt3a with a set of cell population-specific proteins that result in a differential recruitment of \(\beta\)-catenin to the promoters of self-renewal or differentiation genes. These proteins may form complexes that could be responsible for the preferential differentiation of adult cells into memory cells and of neonatal cells into T\(_{SCM}\) populations. Our results highlight important differences in protein complexes formed in neonatal and adult cell responses to the canonical Wnt signaling, which could be important considerations in managing the neonatal cell responses and cord blood transplantation. In addition, the identification of the complex-forming proteins and potential mechanisms shaping the \(\beta\)-catenin nucleated complexes could be useful for a better control of this important pathway for cancer treatments.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

G. N. H.-A. performed a large part of the experiments and wrote manuscript; O. H. L.-P., D. Y. G. R., E. C. F., L. A. K.-C., R. G. L. C., O. B. A.-L., and O. R.-P. performed experimental work; S. S. contributed with the design, discussion, and financing of the study; B. L.-A., A. C.-L., and L. E. G.-V. contributed with proteomics and financing of the study; M. A. S. C. conceived the study, wrote the manuscript, and financed the study.

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