

Dectin-1 activation unlocks *IL12A* expression and reveals the T_H1 potency of neonatal dendritic cells

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Background: Early life is characterized by a high susceptibility to infection and a T_H2 -biased CD4 T-cell response to vaccines. Toll-like receptor (TLR) agonists are currently being implemented as new vaccine adjuvants for T_H1 activation, but their translation to the field of pediatric vaccines is facing the impairment of neonatal innate TLR responses.

Objective: We sought to analyze C-type lectin receptor pathways as an alternative or a coactivator to TLRs for neonatal dendritic cell activation for T_H1 polarization.

Methods: Neonatal monocyte-derived dendritic cells (moDCs) were exposed to various combinations of TLR agonists with or without Dectin-1 agonist. IL-12 and IL-23 responses were analyzed at the transcriptional and protein levels after stimulation. The intracellular pathways triggered by combined TLR plus Dectin-1 stimulation was determined by using pharmacologic inhibitors. The capacity of neonatal moDCs to differentiate naive CD4 T_H cells was evaluated in cocultures with heterologous neonatal naive T cells. Curdlan was finally tested as an adjuvant within a subunit tuberculosis vaccine in neonatal mice.

Results: Simultaneous coactivation through Dectin-1 and TLRs induced robust secretion of IL-12p70 by neonatal moDCs by unlocking transcriptional control on the p35 subunit of IL-12.

Both the spleen tyrosine kinase and Raf-1 pathways were involved in this process, allowing differentiation of neonatal naive T cells toward IFN- γ -producing T_H1 cells. *In vivo* a Dectin-1 agonist as adjuvant was sufficient to induce T_H1 responses after vaccination of neonatal mice.

Conclusion: Coactivation of neonatal moDCs through Dectin-1 allows TLR-mediated IL-12p70 secretion and T_H1 polarization of neonatal T cells. Dectin-1 agonists represent a promising T_H1 adjuvant for pediatric vaccination. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Newborn, dendritic cells, T_H1 , adjuvant, C-type lectin receptor, Toll-like receptor, innate immunity

Infectious diseases are responsible for millions of infant deaths worldwide, despite the availability of vaccines. The challenge of early-life vaccination arises from the particularities of the neonatal immune system because most vaccines have limited efficacy for children less than 6 months of age.^{1,2} The efficiency of a vaccine strongly relies on the adjuvant used to activate the innate immune system and to deliver antigen to dendritic cells (DCs) under appropriate inflammatory conditions. The DC population is a heterogeneous population in which DC subtypes differ in function, localization, and phenotype. Protection against intracellular pathogens needs the generation of a T_H1 -type immune response driven by secretion of IL-12p70 by DCs. In human subjects subsets of DCs include Langerhans cells, CD14⁺ interstitial DCs, and CD11b⁺ dermal DCs in tissues and BDCA1⁺ myeloid dendritic cells (mDCs), BDCA3⁺ mDCs, and BDCA2/4⁺ plasmacytoid dendritic cells (pDCs) in peripheral blood.³ Human inflammatory DCs have recently been described in inflammatory synovial fluids and tumor ascites.⁴ All subsets of mDCs express Toll-like receptor (TLR) 1 to TLR10, with the exception of TLR9.⁵ MoDCs express a TLR pattern similar to that of inflammatory DCs⁴ and produce IL-12p70 on TLR stimulation.⁶ Synthetic TLR agonists have been developed and recently introduced successfully as adjuvants in adult therapeutic and prophylactic vaccination. Monophosphoryl lipid A (MPLA), a detoxified form of LPS and an agonist of TLR4, is currently approved and has been introduced in combination with alum in such vaccines as Fendrix (GlaxoSmithKline, Uxbridge, United Kingdom) and Cervarix (GlaxoSmithKline) against hepatitis B virus (HBV) and human papillomavirus, respectively.

If the use of TLR agonists in adult vaccination is a promising field, the picture in neonates and infants is very different. The only trial to date involving a TLR agonist as an adjuvant for pediatric immunization is the RTS,S/AS01 vaccine against malaria (GlaxoSmithKline). This vaccine candidate is a liposomal formulation including MPLA and the saponin-containing QS-21

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Abbreviations used

CARD9:	Caspase recruitment domain-containing protein 9
CFU:	Colony-forming units
CLR:	C-type lectin receptor
Ct:	Cycle threshold
DC:	Dendritic cell
DZN:	Depleted zymosan
FACS:	Fluorescence-activated cell sorting
HBV:	Hepatitis B virus
IFA:	Incomplete Freund adjuvant
ITAM:	Immunoreceptor tyrosine-based activation motif
mDC:	Myeloid dendritic cell
moDC:	Monocyte-derived dendritic cell
MPLA:	Monophosphoryl lipid A
OVA:	Ovalbumin
pDC:	Plasmacytoid dendritic cell
PIC:	Polyinosinic:polycytidylic acid
qPCR:	Quantitative PCR
Syk:	Spleen tyrosine kinase
TLR:	Toll-like receptor
WGP:	Whole glucan particle

immunostimulant. The completed phase III trial showed only modest efficacy, with an overall 30% protection against severe malaria.⁷

Past studies emphasized the gradual acquisition of TLR responsiveness in newborns and infants in whole blood and PBMCs,^{8,9} with an age-dependent maturation of IL-12p70 secretion capability throughout childhood.¹⁰ These studies also show that neonatal immune cells seem to produce more IL-10 and T_H17-polarizing cytokines (IL-6, IL-1B, and IL-23) after TLR stimulation. Interestingly, TLR7/8 agonists seem more potent for activating neonatal cells,¹¹⁻¹³ although they do not induce IL-12p70 in neonatal DCs.¹⁴ A defect in nucleosome remodeling prevents transcription of the p35 subunit of IL-12p70 in neonatal DCs,¹⁵ which therefore produce more IL-23 in response to TLR4 or TLR8 stimulation.¹⁶ Neonatal monocyte-derived dendritic cells (moDCs) also have a defect in association of the transcription activator CREB-binding protein and interferon regulatory factor 3,¹⁷ a transcription factor necessary for transcription of IL-12p35.¹⁸ Altogether, these data emphasize a highly regulated TLR response in newborns that makes TLR agonists poor candidates for neonatal adjuvantation.

Mycobacterium bovis BCG is a widely used live vaccine that protects against tuberculous meningitis in children and prevents miliary/disseminated tuberculosis. BCG is administered successfully at birth, and PBMCs from vaccinated infants mount a successful T_H1 response to purified protein derivative.¹⁹ Interestingly, BCG vaccination in early life also significantly enhances T- and B-cell responses to unrelated vaccine antigens. Coadministration of BCG in human infants enhanced both T_H1 and T_H2 cytokine responses to HBV vaccination and antibody responses to both HBV and oral polio vaccines.²⁰ BCG is a live attenuated vaccine and is thus self-adjuvanted, with many microbial patterns. Despite current findings regarding the receptors involved in recognition of mycobacteria, the mechanisms allowing BCG to trigger a T_H1 response in newborns remain poorly understood. BCG is able to activate DCs²¹ and mobilize innate pathways, although through different mechanisms and outcomes, depending on the population.²² Various pathogen recognition receptors are involved in BCG sensing,

including TLR2, TLR4, and TLR9, but also through another family, the C-type lectin receptors (CLRs), including the mannose receptor and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin.²³

CLRs are a class of pathogen-associated receptors expressed widely in the myeloid lineage in which they trigger various functions from gene transcription to endocytosis and antimicrobial activity.²⁴ Among these CLRs, Dectin-1 is a receptor originally described on DCs both in mice and human subjects²⁵ that recognizes β -1,3- and β -1,6-linked glucans present in the cell walls of fungi and some bacteria.²⁶ After stimulation with curdlan (a high-molecular-weight linear polymer of β -1,3-linked glucose), Dectin-1 can directly induce cell activation through an immunoreceptor tyrosine-based activation motif (ITAM) domain in its cytoplasmic tail that recruits the spleen tyrosine kinase (Syk)/Caspase recruitment domain-containing protein 9 (CARD9) pathway to induce secretion of a vast array of cytokines.²⁷⁻²⁹ Importantly, CLRs are also involved in recognition of and response to *Mycobacterium tuberculosis*. In addition to the mannose receptor and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin, Mincle and macrophage C type lectin receptors have been recently described to recognize cord factor expressed on the surface of *M tuberculosis*.^{30,31} Dectin-1 has also been reported to be involved in macrophage and DC recognition of and response to *M tuberculosis*, although the ligand involved in this interaction remains to be identified.^{32,33}

In this study we sought to identify an innate pathway for DC activation that can promote a T_H1 response in newborns. We demonstrated that activation of Dectin-1 by curdlan on neonatal moDCs can overcome the transcriptional control of the IL-12p35 subunit and allows TLR-induced IL-12p70 secretion by neonatal DCs and further T_H1 polarization of neonatal T cells.

METHODS

Blood and cell isolation

Buffly coats were obtained from adult donors by Etablissements Français du Sang (Paris, France). Heparinized cord blood samples were collected in 2013 from healthy neonates from the Maternity Port Royal (Paris, France) or Hôpital Saint-Louis (Paris, France). Written consent was obtained from the mothers. This study was carried out with the approval of the Ethics Committee of the Pasteur Institute (Rbm 2010-09 and Rbm 2014-01) and the French regional committee CPP IDF I and IV in agreement with the principles of the Declaration of Helsinki.

Adult and cord blood were layered onto Ficoll-Hypaque and centrifuged. Mononuclear cells were recovered, and specific cell subsets were then enriched with MACS enrichment kits (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Monocytes were enriched with CD14 microbeads, naive T cells were enriched with CD4 microbeads, and red cells were enriched with CD235a microbeads. Cells were further sorted with a FACSaria III cell sorter (BD Biosciences, San Jose, Calif). Monocytes were sorted as CD11c⁺CD14⁺ cells, naive T cells were sorted as CD3⁺CD4⁺CD25⁻CD45RO⁻, and CD71⁺ red cells were sorted as CD235a⁺CD71⁺ cells. Purity was routinely greater than 98%. For a list of antibodies, see Table E1 in this article's Online Repository at www.jacionline.org.

Reagents

Polyinosinic:polycytidylic acid (PIC; HNV, TLR3 agonist), R848 (TLR7/8 agonist), LPS (LPS-EK standard, TLR4 agonist), depleted zymosan (DZN; Dectin-1 agonist), dispersible whole glucan particles

(WGPs; Dectin-1 agonist), piceatannol (Syk inhibitor), LY294002 (phosphoinositide 3-kinase inhibitor), U0126 (MEK1/2 [mitogen-activated protein kinase/extracellular signal-regulated kinase] inhibitor), and SB202190 (p38 inhibitor) were purchased from InvivoGen (Carlsbad, Calif). Curdlan (Dectin-1 agonist) was purchased from Wako (Osaka, Japan). Raf-1 kinase inhibitor I was purchased from Calbiochem (Nottingham, United Kingdom).

Fluorescence-activated cell sorting analysis of Dectin-1 expression on leukocytes

Dectin-1 expression was analyzed by means of fluorescence-activated cell sorting (FACS) on PBMCs or cord blood mononuclear cells. T cells were defined as CD3⁺ cells, B cells as CD20⁺ cells, natural killer cells as CD3⁺CD56⁺ cells, pDCs as CD123^{hi}BDCA4⁺ cells, mDCs as CD20⁺CD11c⁺CD1c⁺CD14⁺ cells, CD141 DCs as CD11c⁺CD141⁺ cells, and monocytes as CD11c⁺CD14⁺ cells.

MoDCs

Human CD11c⁺CD14⁺ monocytes isolated from adult or cord blood were cultured with 100 ng/mL (500 UI/mL) GM-CSF plus 50 ng/mL (250 UI/mL) IL-4 (Miltenyi Biotec) at 10⁶ cells/mL in complete RPMI medium supplemented with 10% FCS (ICN Biomedicals, Irvine, Calif), 2-mercaptoethanol (5 × 10⁻⁵ mol/L; Sigma, St Louis, Mo), and antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL; Gibco, Life Technologies, Carlsbad, Calif). At day 6, moDCs used for stimulation corresponded to CD14⁺CD11c⁺CD11b⁺CD1c⁺ HLA-DR⁺CD1a⁺ cells, and purity was greater than 95%. MoDCs were cultured at 10⁶ cells/mL, as indicated, in the presence of various stimuli: 25 µg/mL PIC, 10 µg/mL LPS-EK or 1 µg/mL R848, 100 µg/mL curdlan, 100 µg/mL DZN, or 100 µg/mL dispersible WGPs. For combined TLR stimulation, LPS was used at 1 µg/mL. Twenty-four- to 72-hour culture supernatants were used to test cytokines by means of ELISA or to culture naive T cells for T_H polarization. In some experiments pharmacologic inhibitors were used as follows: 10 µmol/L piceatannol, 100 µmol/L LY294002, 20 µmol/L SB202190, 20 µmol/L Raf-1 kinase inhibitor I, or 25 µmol/L U0126.

Naive T-cell differentiation

MoDCs were cultured at 2 × 10⁴ cells/mL in U-bottom 96-well plates in RPMI 1640 complete medium with 100 ng/mL GM-CSF and 50 ng/mL IL-4 with 25 µg/mL PIC, 10 µg/mL LPS-EK, 1 µg/mL R848, or combined TLR stimuli with or without 100 µg/mL curdlan. After 24 hours, 5 × 10⁴ CD3⁺CD4⁺CD25⁻CD45RO⁻ heterologous naive T cells were added to the culture. Five days later, cell-culture supernatants were harvested and stocked at -20°C until further use.

Naive T cells were also cultured with cell-free supernatant from moDC cultures for 5 days and then boosted with 1 µg/mL ionomycin plus 50 ng/mL phorbol 12-myristate 13-acetate with GolgiPlug (BD Biosciences) for 6 hours. Cytokine production was later analyzed by means of FACS.

Detection of mRNA by means of FACS

Detection of mRNA for IL-12p40 and IL-12p35 was realized by using Quantigene FlowRNA technology (Affymetrix, eBioscience, Santa Clara, Calif), according to the manufacturer's instructions. Briefly, moDCs were activated for 12 hours with PIC, plus R848 or LPS plus R848 with or without curdlan, stained for CD11b, and then fixed and permeabilized for 1 hour in 4% paraformaldehyde, 10 minutes in ice-cold methanol, and then 30 minutes again in 4% paraformaldehyde. Specific target probes for IL-12p40 and IL-12p35 mRNA were added and hybridized for 2 hours at 40°C. mRNAs then followed a 2-step amplification (1.5 hours before amplification, followed by 1.5 hours of amplification) before hybridization with fluorochrome-labeled probes. Fluorescence was measured on an LSRFortessa (BD Biosciences). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a positive control.

RT-PCR and quantitative PCR

Total RNA was extracted with the RNeasy Mini plus Kit (Qiagen, Courtaboeuf, France). RNA samples were converted to cDNA by using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Marnes-la-Coquette, France). Primers for p19, p35, P40, and the housekeeping gene were designed (see Table E2 in this article's Online Repository at www.jacionline.org). Quantitative PCR (qPCR) was performed on the CFX96 (Bio-Rad laboratories) using SsoAdvanced SYBR green Supermix (Bio-Rad Laboratories). At the end of 40 amplification cycles, quantification was determined based on the cycle threshold (Ct), and specific amplification was confirmed by means of analysis of the melting curve. The relative quantification of gene expression was calculated by using the comparative Ct method (2^{-ΔΔCt}), where ΔΔCt = (Ct of target gene - Ct of reference gene) of the treated condition normalized by ΔΔCt of the control condition.

ELISAs

IL-12p40, IL-12p70, IL-23p19, IFN-γ, IL-13, IL-17, IL-10, and TNF-α levels were measured in culture supernatants by using ELISA kits (eBioscience), according to the manufacturer's instructions.

Evaluation of TLR2/4 activation

Bone marrow-derived DCs generated from bone marrow from C57BL/6 or TLR2/4-KO mice were stimulated with 100 µg/mL curdlan, 100 µg/mL DZN (InvivoGen, San Diego, Calif), or 100 µg/mL dispersible WGPs (InvivoGen). After 48 hours, cell-culture supernatants were harvested and stored at -20°C until further use. The potency of curdlan to activate TLR4 was also assessed with the HEK-Blue hTLR4 cell line. Briefly, HEK-Blue hTLR4 or HEK-null2 cells (InvivoGen) were stimulated for 24 hours with a range of concentrations of LPS or curdlan. OD at 650 nm was then measured on a Multiskan Spectrum device (Thermo Scientific, Waltham, Mass).

In vivo experiments

C57BL/6 and BALB/c mice (Charles River Laboratories, Wilmington, Mass) were mated and housed on site. Seven-day-old mice were vaccinated subcutaneously with ovalbumin (OVA) in incomplete Freund adjuvant (IFA) supplemented or not with 2 mg of curdlan, 20 µg of LPS, or both to analyze adaptive immune responses. Alternatively, mice received TB10.4 supplemented with curdlan or DMT containing MPLA. Lymph node cells were restimulated with OVA or TB10.4, and 96-hour supernatants were tested for IFN-γ, IL-5, and IL-17 by means of ELISA (eBioscience). For TB10.4 vaccination, spleen cells were also restimulated with TB10.4, and IFN-γ and IL-17 responses were assessed in individual mice by using ELISPOT. Animal studies were approved by the Institut Pasteur Safety Committee in accordance with French and European guidelines.

M tuberculosis protection experiments

Virulent *M tuberculosis* H37Rv strain was grown at 37°C in Dubos broth (Becton Dickinson, Sparks, MD) complemented with albumin, dextrose, and catalase (Difco, Becton Dickinson). Experiments done with *M tuberculosis* H37Rv were all performed in an L3 laboratory in accordance with the hygiene and security recommendations of Institut Pasteur. Vaccinated mice (n = 6 per group) were challenged through the aerosol route with H37Rv strain by using a homemade nebulizer. To do so, 5 mL of a suspension at 5 × 10⁶ colony-forming units (CFU)/mL were aerosolized to obtain an inhaled dose of approximately 200 CFU per mouse. Infected mice were housed in isolators in L3 animal facilities. One month after infection, mycobacterial burdens in the lungs were determined subsequent to homogenization by using 2.5-mm-diameter glass beads and an MM300 homogenizer (Qiagen). Serial dilutions of homogenates were seeded on 7H11 Agar complemented with OADC (Difco, Becton Dickinson), and CFU were counted after 20 days of incubation at 37°C. Experiments were conducted under agreement number 2012-0005.

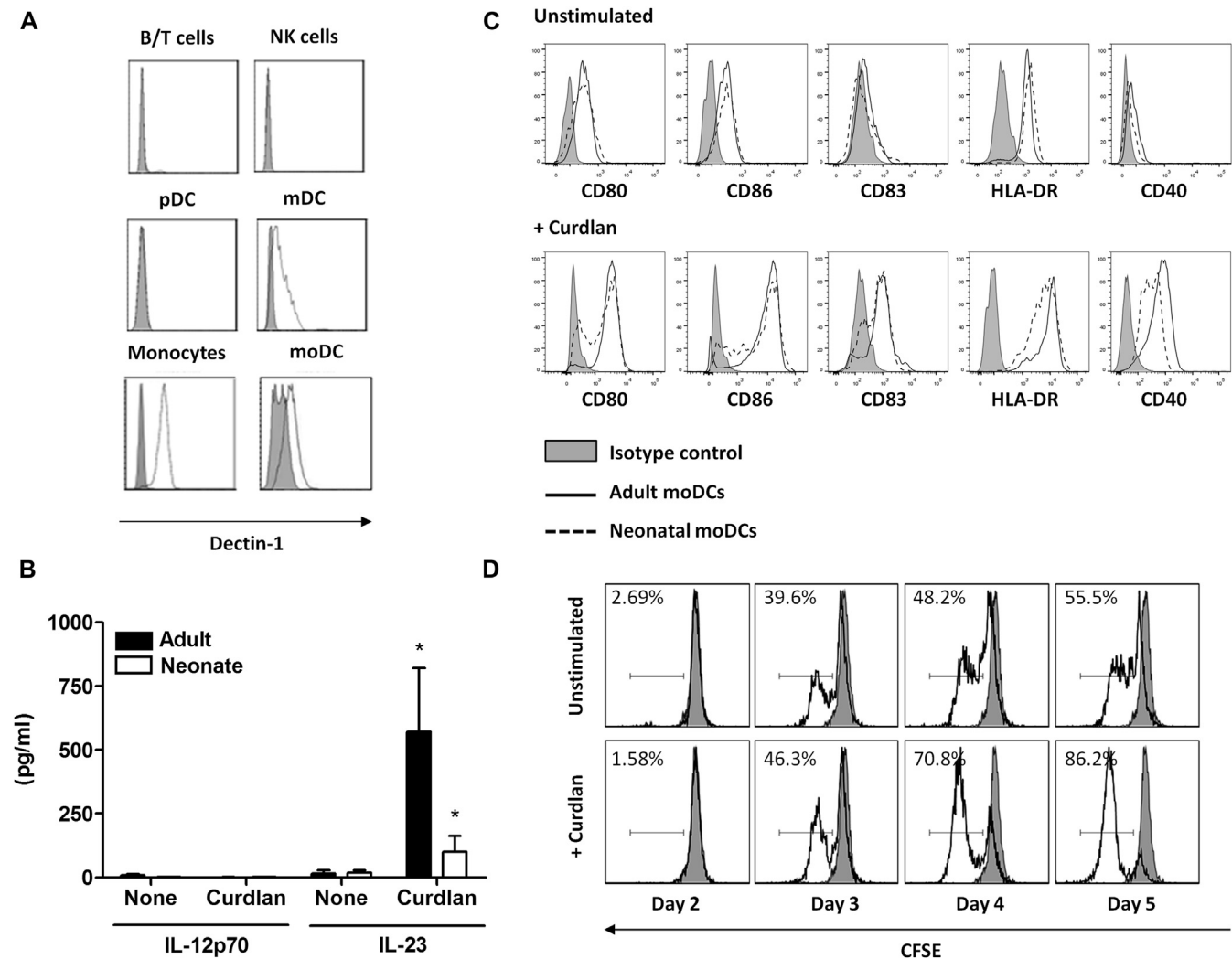


FIG 1. MoDC maturation after Dectin-1 triggering. **A**, Expression of Dectin-1 on cord blood leukocytes and moDCs. **B**, Neonatal ($n = 9$) or adult ($n = 6$) moDCs stimulated with 100 $\mu\text{g/mL}$ curdlan. IL-12p70 and IL-23 levels were measured by means of ELISA in supernatants. Results are expressed as means $\pm$ SDs. $*P < .05$ compared with unstimulated cells. **C**, Expression of costimulatory molecules was evaluated on curdlan-stimulated adult or neonatal moDCs. **D**, Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled naive T cells cultured with resting or curdlan-activated heterologous neonatal moDCs.

Statistical analysis

Statistical analyses were performed with Prism 4 software (GraphPad Software, La Jolla, Calif) using the unpaired Student t test. P values of less than .05 were considered statistically significant.

RESULTS

Neonatal and adult response to Dectin-1 stimulation

Most studies have determined Dectin-1 expression based on mRNA expression and not surface protein expression. Using FACS, we investigated expression of Dectin-1 in moDCs and various leukocyte populations in cord or adult blood. MoDCs were generated from neonatal or adult monocytes (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). Dectin-1 was expressed by moDCs from both cord (Fig 1, A) and adult (see Fig E2) blood at similar levels. Dectin-1 was not expressed by any lymphoid cells (B cells, T cells, and natural

killer cells) or on pDCs. However, Dectin-1 was present on BDCA1⁺ DCs (Fig 1, A). Dectin-1 was also found to be highly expressed on monocytes (Fig 1, A, and see Fig E3 in this article's Online Repository at www.jacionline.org).

Among the 3 Dectin-1 agonists tested, curdlan was the strongest IL-12p40 inducer by both adult and newborn DCs and thus was selected for the study (see Fig E4 in this article's Online Repository at www.jacionline.org). We could not detect any significant secretion of IL-12p70 in adult or neonatal moDCs stimulated by curdlan (Fig 1, B). However, IL-23 was readily induced, particularly in adult moDCs. Curdlan was also able to stimulate primary cells, as demonstrated by the secretion of IL-6, IL-10, IL-12p40, and TNF- α after whole blood activation (see Fig E5 in this article's Online Repository at www.jacionline.org). Curdlan-stimulated DCs upregulated the costimulatory molecules CD80, CD86, and CD40, as well as CD83 and HLA-DR (Fig 1, C). Curdlan-stimulated DCs were also able to increase T-cell proliferation in an MLR assay (Fig 1, D). Curdlan did not

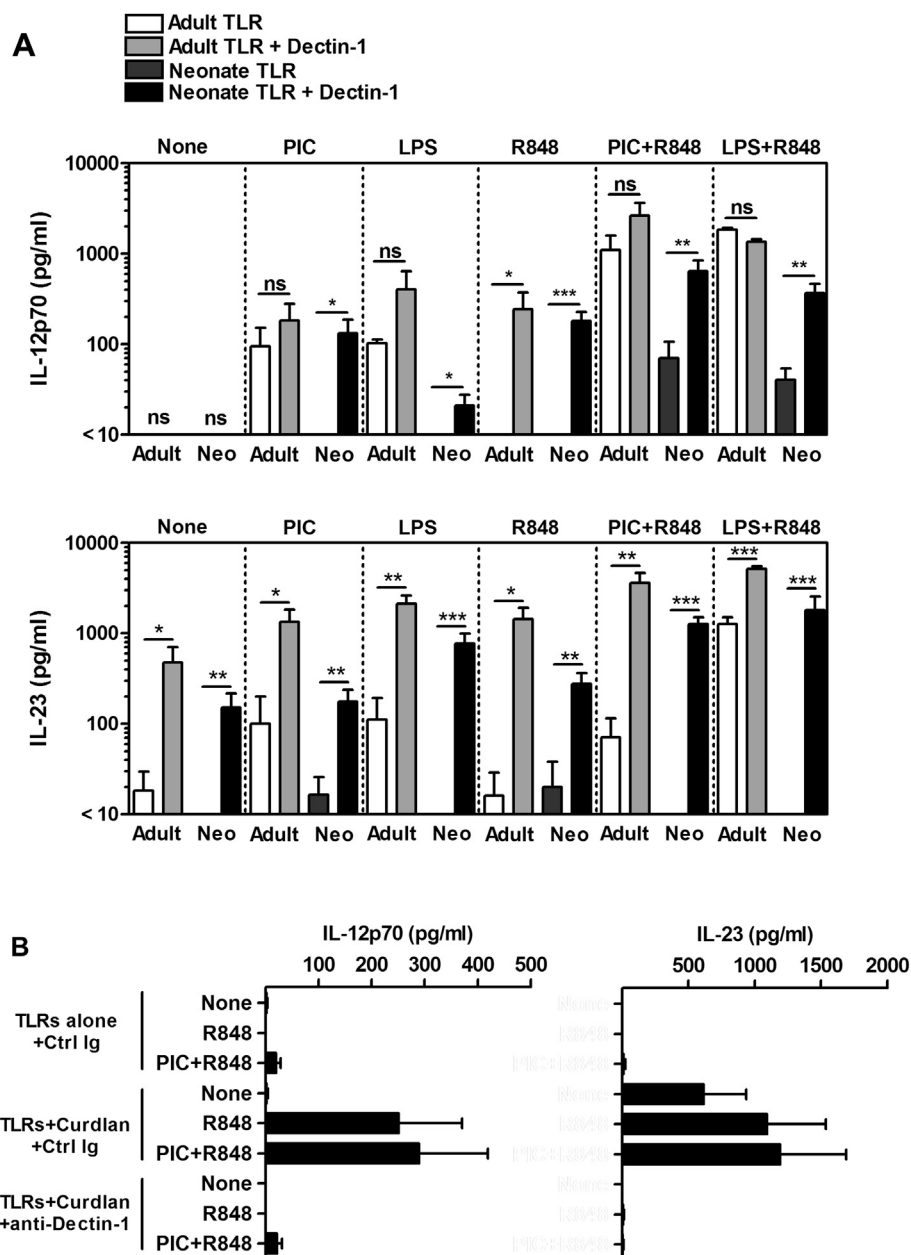


FIG 2. Neonatal and adult response to combined Dectin-1/TLR stimulation. **A**, Adult ($n = 7$) or neonatal ($n = 18$) moDCs were stimulated, as indicated. After 72 hours, supernatants were tested for IL-12p70 and IL-23 by means of ELISA. Results are expressed as means $\pm$ SDs. *ns*, Not significant. $*P < .05$, $**P < .01$, and $***P < .001$. **B**, Neonatal moDCs ($n = 2$) were stimulated as indicated in the presence of control immunoglobulin or anti-Dectin-1-neutralizing mAb. After 72 hours, supernatants were tested for IL-12p70 and IL-23 by means of ELISA.

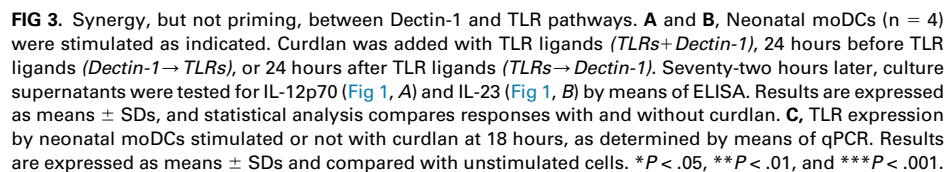
induce TLR2 and TLR4 activation, demonstrating the lack of endotoxin contaminants (see Figs E6 and E7 in this article's Online Repository at www.jacionline.org). Altogether, our results show that β -glucans alone can activate moDCs efficiently but are not sufficient to promote IL-12p70 secretion.

Dectin-1 signaling pathway synergizes with the TLR signaling pathway

We then evaluated whether Dectin-1 triggering with curdlan would affect the TLR response. Single stimulation of TLR4

induced low levels of IL-12p70 secretion in adult moDCs but not in neonatal moDCs (Fig 2, A). Combined TLR stimulation was much more efficient in inducing IL-12p70 in adult moDCs but only weakly induced secretion in cord blood-derived moDCs. IL-23 shares the p40 subunit with IL-12p70. Similarly, we were not able to detect any IL-23 by neonatal moDCs; this is in contrast to adult moDCs, which readily secrete IL-23 on TLR4 plus TLR7/8 stimulation. These results emphasize the TLR response deficiency of newborns at the moDC level.

Adult moDCs did not have significantly increased IL-12p70 secretion on Dectin-1 stimulation (with the notable exception of



and neonatal mDCs, and the induction of IL-6 was higher in neonates (see [Fig E10](#)). We also analyzed whether curdlan was able to activate primary cells. Blood-derived mDCs are the main producers of IL-12p70, whereas monocytes contribute poorly to IL-12p70 production.^{12,34} The scarcity of neonatal mDCs (see [Fig E11, A](#), in this article's Online Repository at www.jacionline.org) and the limitation in cord blood volume precluded their purification and study. When curdlan alone or in combination with TLR agonists was used to stimulate primary monocytes from adult and cord blood, IL-6, but not IL-12p70, was induced in these conditions (see [Fig E11, B](#)). Priming of neonatal mDCs through Dectin-1 before TLR activation did not induce IL-12p70 nor did it modify IL-23 secretion ([Fig 3](#)). On the contrary,

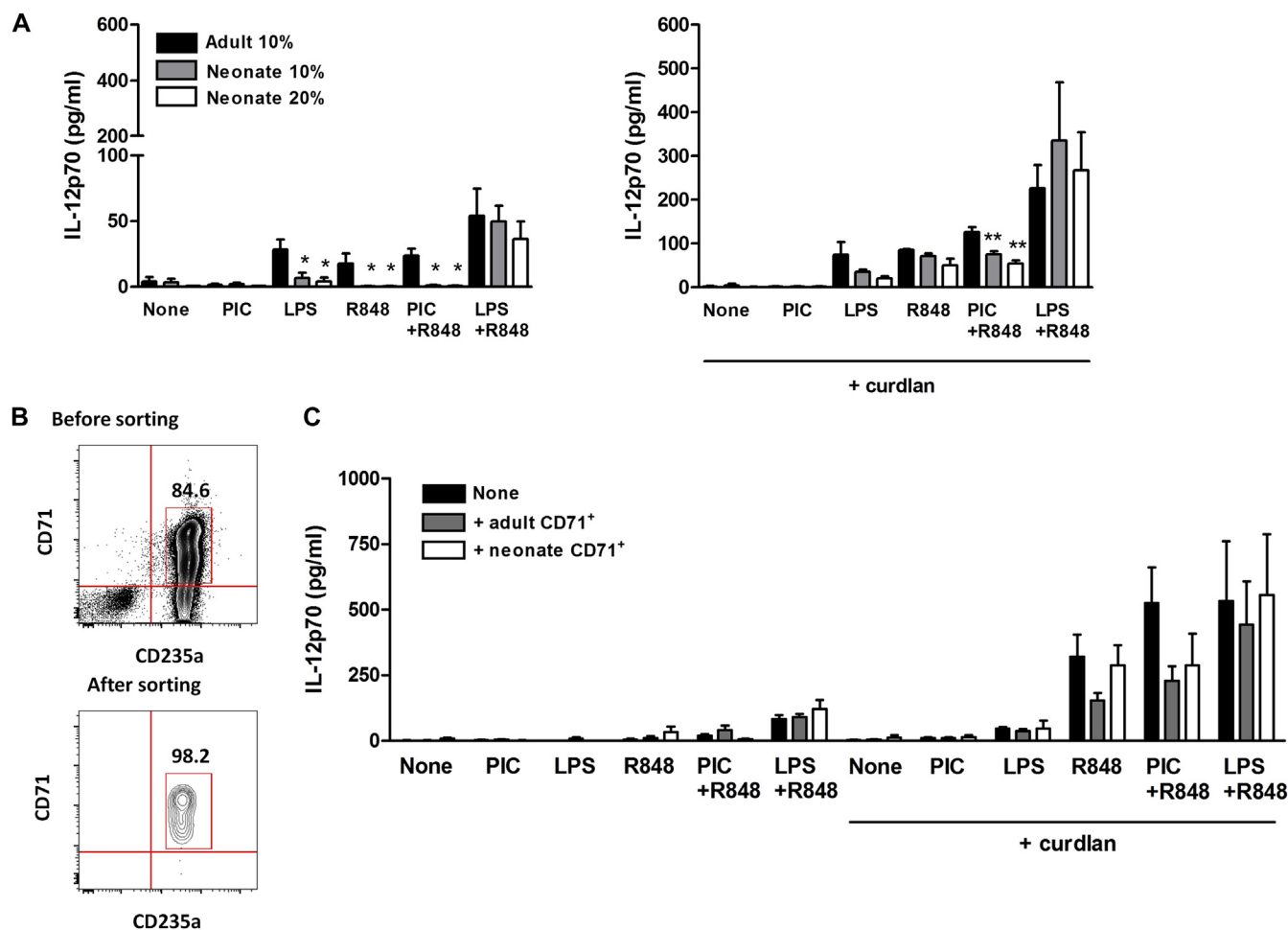


FIG 4. Neonatal suppressive factors do not impair IL-12p70 rescue in neonatal moDCs. Neonatal moDCs ($n = 4$) were stimulated as indicated in the presence of 10% to 20% cord blood serum. **A**, Adult serum was used as a control. IL-12p70 and IL-23 levels were measured by means of ELISA. Results are expressed as means $\pm$ SDs, and statistical analysis compares neonatal serum with 10% adult serum. **B**, Neonatal moDCs ($n = 4$) were cocultured with FACS-sorted adult or neonatal CD71⁺ erythroid cells and stimulated as indicated. **C**, IL-12p70 responses are expressed as means $\pm$ SDs, and statistical analysis compares the response with and without erythroid cells in the culture. * $P < .05$ and ** $P < .01$.

stimulation with curdlan after TLR activation increased significantly the secretion of IL-23 for PIC, R848, and PIC plus R848 stimulation (Fig 3, B). Dectin-1 signaling was also able to trigger IL-23 production by neonatal moDCs, although at lower levels than in adults. At the transcriptional level, curdlan stimulation of neonatal moDCs decreased the expression of both *TLR3* and *TLR4* while increasing the expression of *TLR7* and *TLR8* (Fig 3, C). These results provide a rationale for the stronger induction of IL-12p70 after stimulation of neonatal moDCs with curdlan plus the TLR7/8 agonist R848 but do not fully explain the negative effect of the sequential stimulation on the secretion of IL-12p70.

Cord blood CD71⁺ erythroid cells³⁵ and neonatal plasma³⁶ are endowed with suppressive properties and inhibit cytokine production. The suppressive effect of neonatal plasma can be observed with a very low level of IL-12p70 produced by neonatal moDCs after TLR stimulation. Importantly, Dectin-1/TLR stimulation still increased IL-12p70 secretion by neonatal moDCs in the presence of neonatal plasma for all combinations, except PIC plus R848 plus curdlan-stimulated moDCs, for which a slight reduction can be seen (Fig 4, A). We did not observe any significant

effect of CD71⁺ erythrocytes on the secretion of IL-12p70 by neonatal moDCs (Fig 4, B and C). Overall, these results show that Dectin-1 stimulation of neonatal moDCs rescues their ability to secrete IL-12p70 on TLR stimulation in the presence of potential neonatal suppressive factors.

Dectin-1 signaling pathway synergizes with the TLR signaling pathway through multiple pathways

Dectin-1 induces cell activation through the canonical Syk/CARD9 pathway and the alternative Raf-1 pathway. We analyzed the role of these pathways on the secretion of IL-12p70, IL-23, and IL-6 by neonatal moDCs after curdlan plus R848 and curdlan plus PIC plus R848 stimulation using pharmacologic inhibitors (Fig 5, A). Inhibition of Syk totally abrogated secretion of IL-12p70 by neonatal moDCs and partially abrogated secretion of IL-6 and IL-23. However, inhibition of Raf-1 affected specifically IL-12p70 but not IL-23 and only weakly affected IL-6 secretion. Inhibition of IL-12p70 by Raf-1 kinase inhibitor I was dose dependent (Fig 5, B).

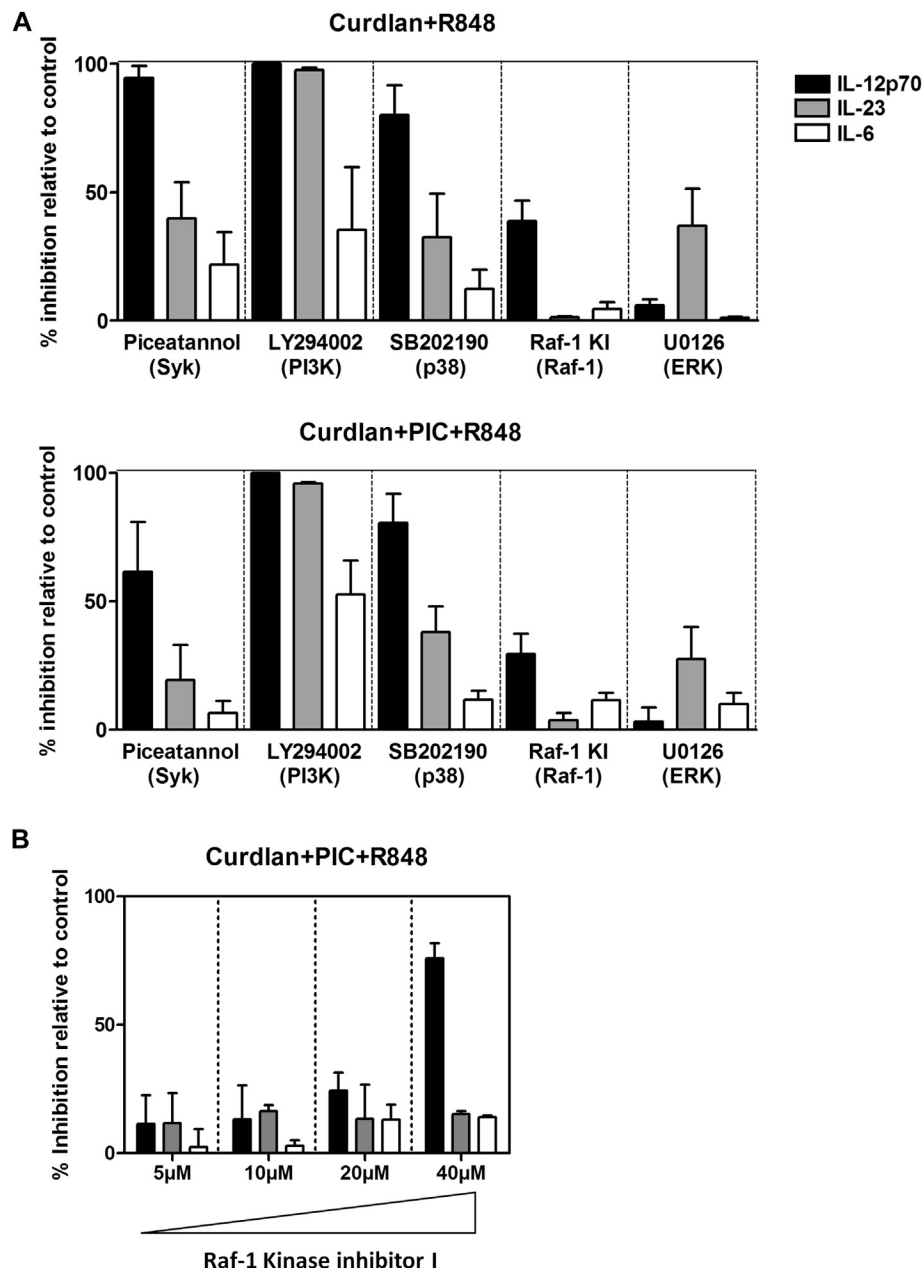


FIG 5. Intracellular pathways involved in TLR/Dectin-1 synergy. Neonatal moDCs ($n = 3$) were treated with indicated inhibitors and then stimulated for 72 hours with either 1 $\mu\text{g/mL}$ R848, 25 $\mu\text{g/mL}$ PIC, 100 $\mu\text{g/mL}$ curdlan, or combined stimuli. **A**, Cell-culture supernatants were tested for IL-12p70, IL-23, and IL-6 by means of ELISA. Results are shown as the percentage of inhibition of cytokine secretion compared with culture of moDCs without inhibitors and are expressed as means $\pm$ SDs of 2 experiments. **B**, Dose effect of Raf-1 kinase inhibitor I on secretion of IL-12p70, IL-23, and IL-6.

Interestingly, inhibition of phosphoinositide 3-kinase or p38 pathways also totally abrogated secretion of IL-12p70 by neonatal moDCs. Similar to what we observed with inhibition of Raf-1 and IL-12p70, inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by U0126 specifically inhibited IL-23 secretion. These results suggest that restoration of cytokine secretion of neonatal moDCs after Dectin-1/TLRs combined stimulation involves multiple pathways, with the Raf-1 pathway being specifically involved in IL-12p70 secretion.

Dectin-1 signaling allows neonatal DCs to overcome transcriptional control of the p35 subunit

The p35 subunit of IL-12p70 has been shown to be negatively regulated in neonatal moDCs at the transcriptional level.¹⁵ We investigated whether Dectin-1 stimulation could remove this transcriptional inhibition. mRNA expression for the p40 (*IL12B*) and p35 (*IL12A*) subunit of IL-12 and the p19 (*IL23A*) subunit of IL-23 was assessed on activated neonatal moDCs by using qPCR. Stimulation with curdlan strongly induced expression of both *IL12B* and *IL23A* mRNA but not *IL12A* (Fig 6, A). However, expression of

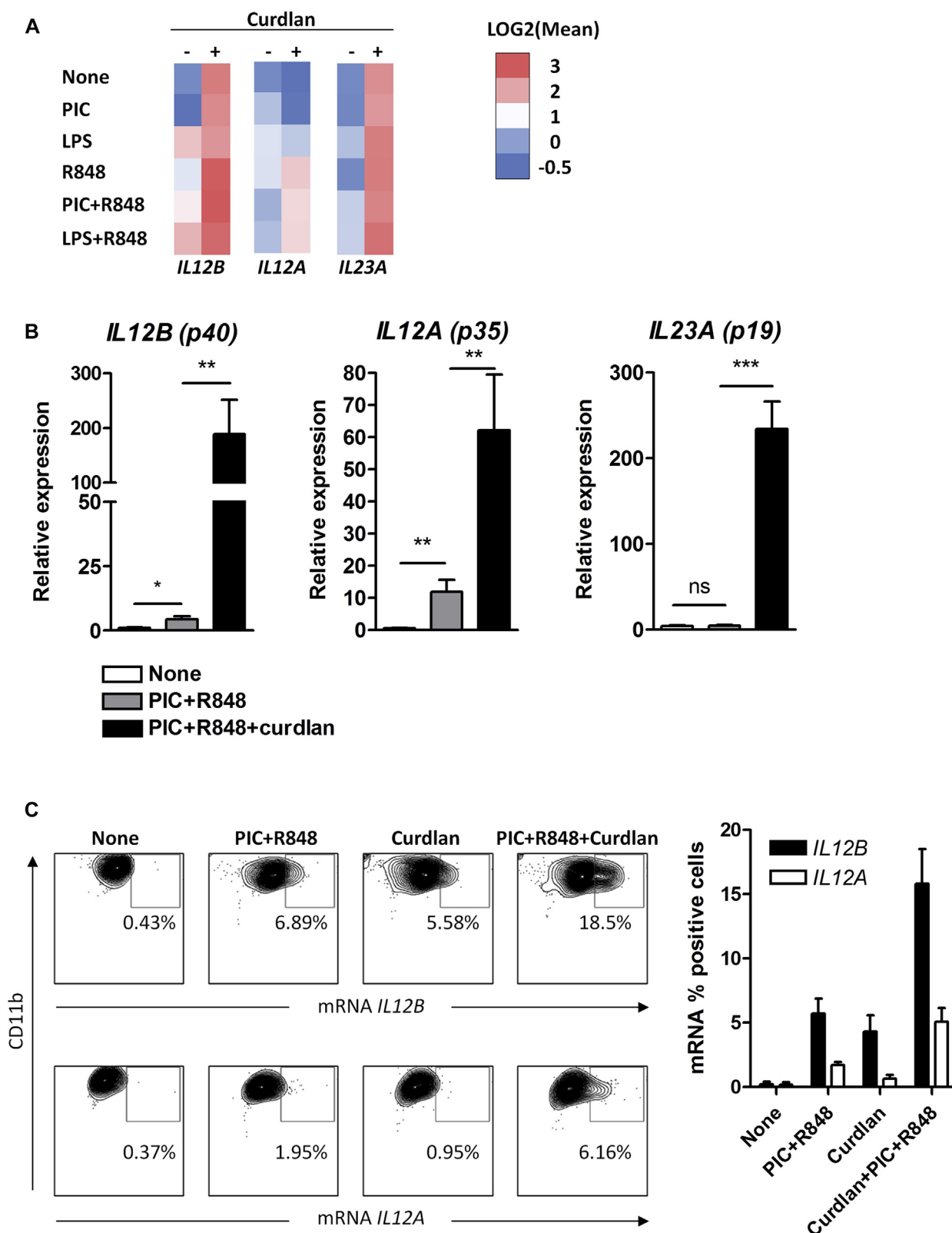


FIG 6. Restoration of *IL12A* transcription by TLR/Dectin-1 combined stimulation. Neonatal moDCs ($n = 4$) were stimulated for 12 hours as indicated. mRNA levels of *IL12A*, *IL12B*, and *IL23A* were determined by means of qPCR (**A** and **B**) or FACS with Quantigene FlowRNA after staining with anti-CD11b antibody and indicated fluorescent primers (**C**). Fig 6, **A**, Results are expressed as a heat map of the $\log_2$ of mean relative expression. Fig 6, **B**, Relative expression of mRNA normalized on glyceraldehyde-3-phosphate dehydrogenase expression, and results are expressed as means $\pm$ SDs. ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$. Fig 6, **C**, Representative experiment (*left*) and pooled results (*right*, means $\pm$ SDs) of measurement of *IL12A* and *IL12B* mRNA expression by using FACS.

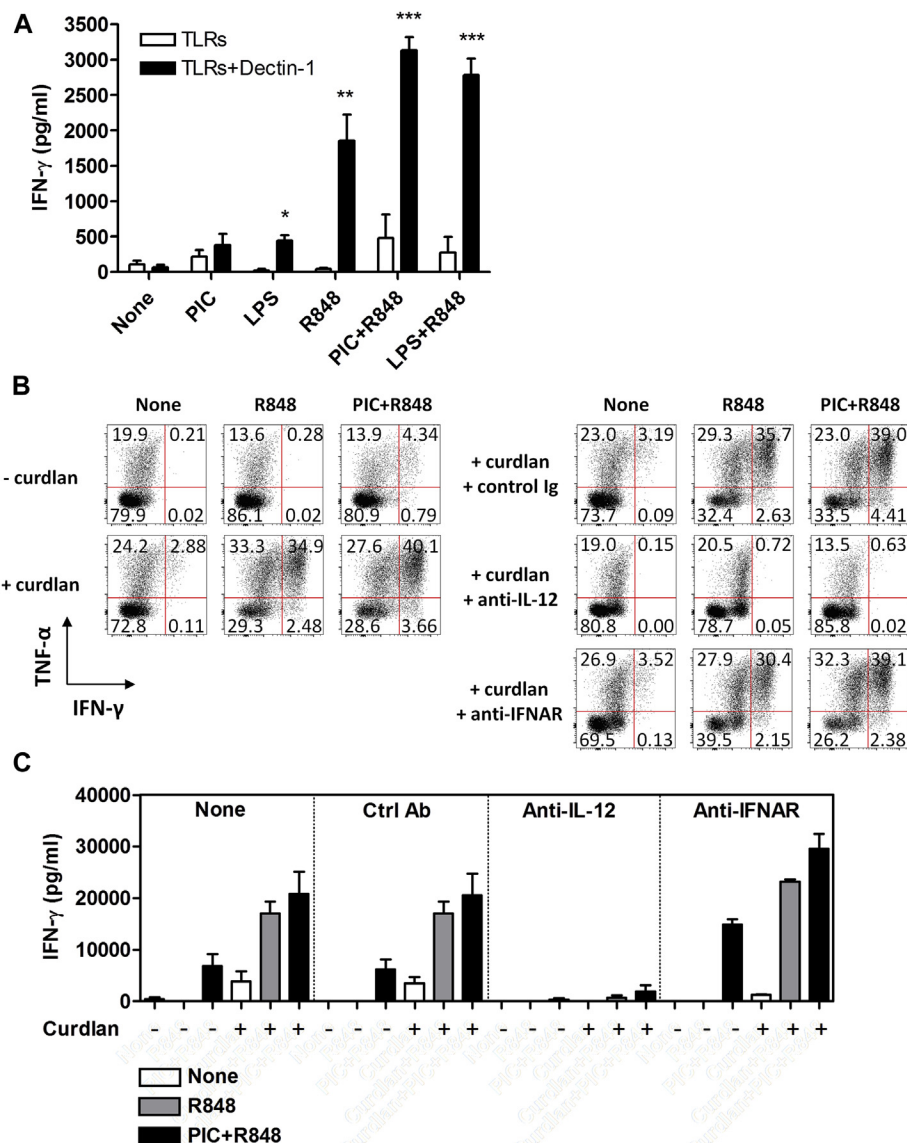


FIG 7. Induction of IFN- γ -producing neonatal T_H1 T cells by TLR/Dectin-1-stimulated neonatal moDCs. Neonatal moDCs ($n = 3$) were stimulated with indicated stimuli for 24 hours and then cultured with neonatal heterologous naive T cells ($CD3^+CD4^+CD56^-CD25^-CD45RO^-$ cells). **A**, Supernatants were harvested and tested for IFN- γ by means of ELISA. Results are expressed as means $\pm$ SDs. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with TLR-only activated cocultures. **B**, Neonatal naive T cells were cultured with 25% supernatant of activated neonatal moDCs for 5 days on plate-bound anti-CD3 plus anti-CD28. Cells were analyzed for intracellular IFN- γ and TNF- α by means of FACS. Data are representative of 2 experiments. **C**, Supernatants were harvested and tested for IFN- γ , IL-13, and IL-17 by means of ELISA. Results are expressed as means $\pm$ SDs of 2 experiments.

IL12A mRNA was increased when neonatal moDCs were costimulated with curdlan plus R848, curdlan plus PIC plus R848, or curdlan plus LPS plus R848 (Fig 6, A and B). We also checked the expression of *IL12B* and *IL12A* mRNA using FACS. Although *IL12B* mRNA could be detected after PIC plus R848, curdlan, and PIC plus R848 plus curdlan stimulation, mRNA for the *IL12A* subunit was only slightly expressed on PIC plus R848 stimulation but was strongly increased with the addition of curdlan (Fig 6, C), which is consistent with our qPCR results. These results confirm that Dectin-1 signaling is able to overcome the inhibition that affects the transcription of the p35 subunit of IL-12 in neonatal moDCs, allowing TLR-mediated induction of IL-12p70 secretion in neonatal DCs.

Dectin-1 signaling restores the capability of neonatal moDCs to induce the generation of IFN- γ -producing T_H1 T cells

Neonatal naive T cells were sorted by means of FACS (see Fig E12 in this article's Online Repository at www.jacionline.org) and cultured for 5 days with heterologous moDCs stimulated for 24 hours with TLRs with or without curdlan to assess T-cell differentiation. We did not detect significant IFN- γ secretion by T cells in the presence of TLR-activated neonatal moDCs (Fig 7, A), whereas adult moDCs did produce such secretion (see Fig E13, A, in this article's Online Repository at www.jacionline.org). IFN- γ production was induced in T-cell cultures

primed by neonatal moDCs stimulated in the presence of curdlan (Fig 7, A and B). Neither IL-13 and IL-17 (see Fig E13, B) nor IL-10 (see Fig E13, C) were detected under these conditions. Induction of T_H1 cells with PIC plus R848-activated adult moDCs was not increased after costimulation with curdlan (see Fig E13, A). Neonatal T_H1 cells can be differentiated by neonatal pDCs in an IFN- α manner.³⁷ To assess the requirement of IL-12p70, but not type 1 interferon, for the T_H1 polarization, we used cell-free supernatant from cultures of activated neonatal moDCs with blocking antibodies. An anti-IL-12 blocking antibody, but not anti-type 1 interferons receptor antibody, abrogated the induction of IFN- γ -producing T cells (Fig 7, C). Altogether, these results show that Dectin-1 signaling not only restores the potency of neonatal moDCs to secrete IL-12p70 after TLR stimulation but also restores their ability to polarize naive T cells toward T_H1 differentiation.

Implementation of neonatal vaccination with adjuvant targeting Dectin-1 promotes the T_H1 response *in vivo*

To assess *in vivo* the capacity of Dectin-1 targeting to promote T_H1 responses, we used a neonatal mouse model with 7-day-old mice.^{2,38} We used OVA antigen with curdlan emulsified with IFA, and control neonatal mice received OVA in IFA alone. Under these conditions, curdlan-vaccinated mice compared with those receiving IFA alone had a strong T-cell response with high IFN- γ and IL-17 levels and reduced IL-5 levels (Fig 8, A). OVA mixed with LPS induced much lower levels of IFN- γ , and LPS plus curdlan produced similar levels of IFN- γ than curdlan alone. The capacity of curdlan to induce a T_H1 response *in vivo* in the absence of TLR4 activation will need further investigation. In addition, curdlan induced T_H17 cells *in vivo* that were not induced by neonatal moDCs *in vitro*. One likely explanation would be the contribution of accessory immune or stromal cells able to provide the additional signals. To fully assess the potency of curdlan, we evaluated its adjuvant properties in the *M tuberculosis* model. We used mycobacterial TB10.4 antigen, which was previously shown to induce protective responses against *M tuberculosis* H37Rv challenge when adjuvanted with the DMT adjuvant containing the TLR4 agonist MPLA.³⁹ Curdlan induced stronger neonatal T_H1 and T_H17 responses than the MPLA-based adjuvant (Fig 8, B). Accordingly, this immune response to TB10.4/curdlan vaccination, but not the one induced by TB10.4/DMT, afforded protection against *M tuberculosis* H37Rv challenge (Fig 8, C). In conclusion, Dectin-1 represents a promising target for pediatric adjuvant setting.

DISCUSSION

In this study we characterized the response of neonatal DCs to the CLR Dectin-1. We showed that Dectin-1 stimulation by curdlan alleviates inhibition of the p35 subunit of IL-12, leading to secretion of bioactive IL-12p70 by neonatal moDCs and subsequent differentiation of T_H1 responses.

Effector memory IFN- γ -producing T_H1-like cells develop during fetal life.⁴⁰ Nevertheless, neonatal T-cell responses in human subjects are mainly T_H1 deficient and T_H2 biased.² Naive fetal and neonatal CD4 T cells tend to develop into T_H2 cells⁴¹ as a result of epigenetic regulation at the IL-4 locus.⁴² However, differentiation of mature T_H2 cells developed from neonatal and infant naive CD4⁺ T cells is abrogated by inflammatory cytokines, including

IL-6, IL-1 β , and IL-12.⁴³ Accordingly, we did not detect any T_H2 and T_H17 responses under Dectin-1/TLR coactivation. Neonatal moDCs stimulated with curdlan and TLR agonists secreted IL-6 and IL-23, but we did not induce a detectable amount of IL-1 β secretion under any condition, which most likely explains the lack of T_H17 differentiation. Neonatal moDCs were proposed to be more pro-T_H17 than adult moDCs because of a higher capacity to produce IL-23 in response to LPS and R848 but not to PIC.¹⁶ Combined curdlan and TLR agonist stimulation induced significantly higher IL-23 secretion by adult moDCs compared with that seen in neonatal moDCs. Curdlan unlocked the IL-12 response in neonatal moDCs, but we did not observe any significant difference in adult moDCs. This IL-12p70 response can antagonize the generation of T_H17 cells by decreasing both expression of the master transcription factor retinoic acid–related orphan receptor γ t and production of IL-17 in human subjects.⁴⁴

In summary, combined TLR/Dectin-1 stimulation of neonatal moDCs allows IL-12p70-mediated T_H1 differentiation of naive neonatal T cells and restricts the differentiation of naive T cells to this path, thus correcting the T_H2/T_H17 bias originally observed in newborns. Antigen coupling to Dectin-1 agonists is efficient not only to trigger T_H1 immunity but also to reduce potentially detrimental T_H2 response.⁴⁵ The capacity of curdlan to rescue the TLR-mediated T_H1 response in neonates highlights the potency of Dectin-1 agonists as potential T_H1 adjuvants for pediatric vaccination.

CLRs are a vast family of receptors that represent sensors of both danger and microbial signals. CLR ligands include carbohydrate, protein, and lipid components of both microbes and self, inducing phagocytic, proinflammatory, or anti-inflammatory reactions.²⁴ Dectin-1 is part of the CLR group V that uses a hemITAM pattern to mediate signaling through Syk.²⁷ Other CLRs signal directly with their own ITAM domain (group V) or through association with FcR γ or DAP12 molecules (group II and group V), all in a Syk/CARD9-dependent manner.²⁴ Mincle and macrophage C type lectin, which both use FcR γ , recognize mycobacterium-derived trehalose dimycolate and its synthetic derivative, trehalose dibehenate, to activate myeloid cells.³ The CAF01 adjuvant developed by the Staten Serum Institute is based on trehalose dibehenate and showed promising results in preclinical studies with the *M tuberculosis* antigen Ag85B-ESAT-6.^{46,47} CAF01 adjuvant is now implemented in *M tuberculosis* and HIV candidate vaccines in clinical trials (clinical trial NCT00922363 and NCT01141205). Altogether, this strongly supports the development of CLR-based adjuvants for pediatric vaccination.

Collaboration between pathogen recognition receptors best mimics encounter of the innate immune system with microorganisms. Coactivation of TLRs and CLRs can lead to contrasting results and needs to be carefully designed because the CLR family is very heterogeneous and includes both activators and inhibitory receptors²⁴ and the consequences are cell specific. We previously demonstrated that combined CLR-TLR activation engaged an anti-inflammatory program in murine neutrophils, leading to release of IL-10 in response to mycobacteria.⁴⁸ Engagement of several CLRs can impair IL-12-dependent T_H1 responses and promotion of T_H2 polarization, as shown in *Fonsecaea monophora* infection.⁴⁹ In conclusion, integration of signals from TLR and CLR innate pathways requires fine tuning for appropriate adjuvant design.

Dectin-1 triggering induces an ITAM-mediated activation of the Syk/CARD9 pathway, which ultimately leads to

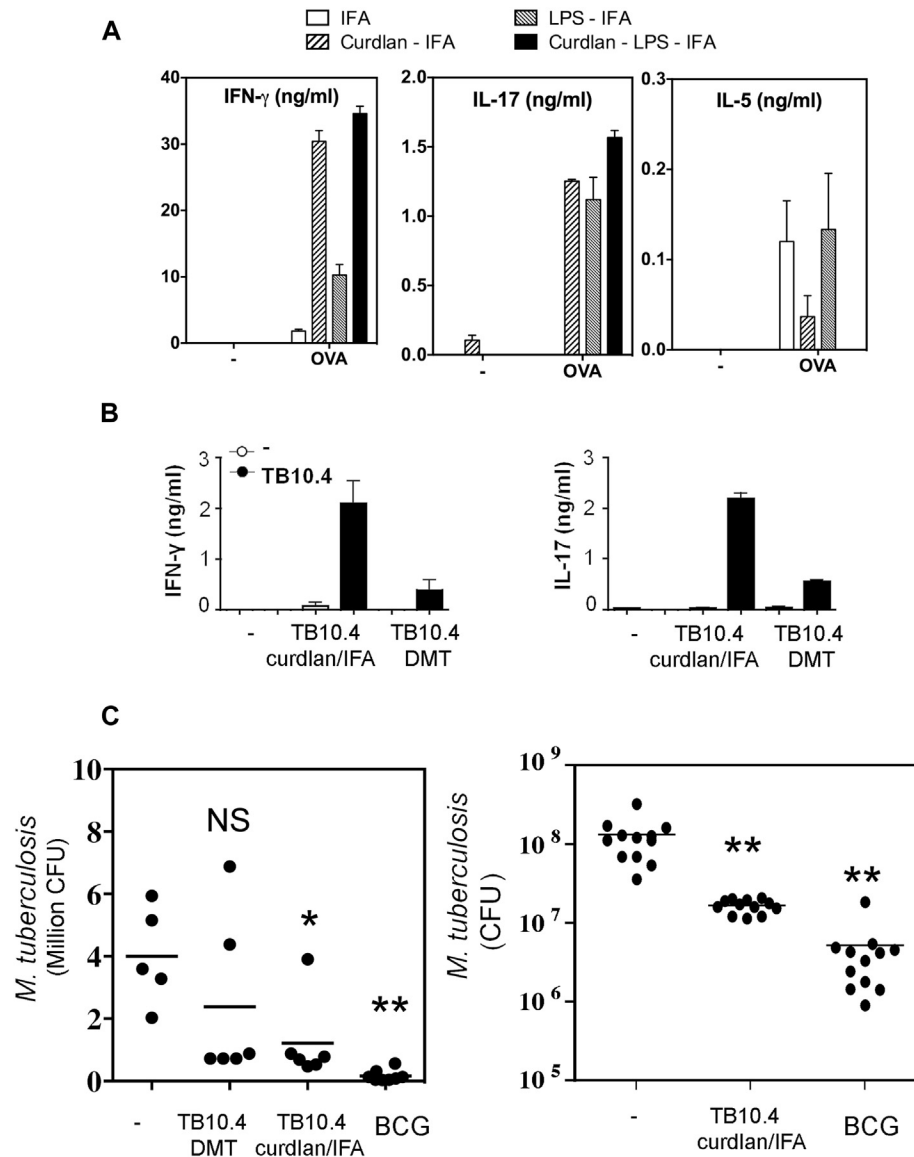


FIG 8. Neonatal mice have T_H1/T_H17 -mediated protection against tuberculosis after *in vivo* targeting of Dectin-1. **A**, Neonatal B6 mice were vaccinated subcutaneously with 100 μ g of OVA emulsified in IFA alone, 2 mg of curdlan, or 20 μ g of LPS or LPS plus curdlan. Two weeks later, draining lymph node cells were restimulated, and OVA culture supernatants were tested for IFN- γ , IL-5, and IL-17. **B**, Neonatal BALB/c mice ($n = 4$) were left untreated or vaccinated subcutaneously with 100 μ g of TB10.4 emulsified in IFA with 2 mg of curdlan or in MPLA-based DMT adjuvant. Two weeks later, draining lymph node cells were restimulated with TB10.4, and supernatants were tested for IFN- γ and IL-17. Results are expressed as means $\pm$ SDs of triplicates. **C**, Neonatal mice were left untreated, vaccinated subcutaneously with BCG or 100 μ g of TB10.4 in DMT, or emulsified in IFA plus curdlan. Three weeks later, mice were challenged by means of aerosol exposure to 100 CFU of H37rv *M. tuberculosis*. Values in CFU were determined in the lungs 1 month after infection. Results are expressed as means $\pm$ SDs of individual mice (left panel, $n = 6$ mice per group; right panel, $n = 12$ per group). NS, Not significant. * $P < .05$ and ** $P < .01$ compared with the untreated infected group.

translocation of nuclear factor κ B in the nucleus.²⁹ Noncanonical pathways of Dectin-1 activation have also been described through Raf-1.⁵⁰ We showed that the synergy between Dectin-1 and TLRs relies on multiple pathways. Both Syk and p38 mitogen-activated protein kinase pathways were fully required for IL-12p70 secretion by neonatal moDCs but only affected partially the secretion of IL-23 and IL-6. Interestingly, we found that inhibition of the Raf-1 and MEF/extracellular signal-regulated kinase pathways specifically inhibits IL-12p70 and

IL-23, respectively. *Candida albicans* was shown to enhance TLR activation through Raf-1.⁵¹ In another study Dectin-1/Raf-1 promotes acetylation of p65 and enhances transcription of several genes, including the *IL12A* gene.⁵⁰ The acetylation of p65 increases its DNA-binding affinity and prolongs its nuclear localization. Our results indicate that such a Raf-1-dependent mechanism allows neonatal moDCs to overcome the initial nucleosome remodeling defect affecting transcription of *IL12A*.¹⁵

Although *in vitro* monocyte-derived DCs correspond to the closest relative to inflammatory DCs,⁴ it will be of great interest to investigate the effect of the combination of several pattern recognition receptors in blood mDCs. The paucity of these cells and the limited availability of cord blood most likely explain the relative absence of data in the literature about the functionality of cord blood mDCs. To our knowledge, the only study analyzing neonatal blood mDCs was based on intracellular cytokine staining on bulk mononuclear cells or whole blood without assessing IL-12p70 production.⁵² Analysis of mixed cell culture impairs accurate characterization of what results from the intrinsic features of mDCs or from cofactors enhancing or dampening their activities.

Taken together, our data demonstrate the potency of the Dectin-1 pathway to restore TLR-induced IL-12p70 secretion and T_H1 polarization by neonatal DCs. Our results clearly show that innate pathways that alternate to TLRs can efficiently mobilize neonatal DC activity and that targeting CLR s thus represents a new strategy to design novel adjuvants for pediatric vaccination.

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Key messages

- Dectin-1 coactivation of neonatal mDCs allows TLR-mediated IL-12p70 secretion and T_H1 polarization of neonatal naive T cells.
- Dectin-1 signaling unlocks transcriptional control on the p35 unit of IL-12.
- Dectin-1 agonist can be used efficiently as an adjuvant to induce T_H1 responses and protect neonatal mice against tuberculosis challenge.

REFERENCES

- PrabhuDas M, Adkins B, Gans H, King C, Levy O, Ramilo O, et al. Challenges in infant immunity: implications for responses to infection and vaccines. *Nat Immunol* 2011;12:189-94.
- Siegrist CA. Neonatal and early life vaccinology. *Vaccine* 2001;19:3331-46.
- Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology* 2013;140:22-30.
- Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, et al. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* 2013;38:336-48.
- Schreibelt G, Tel J, Sliepen KH, Benitez-Ribas D, Figdor CG, Adema GJ, et al. Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. *Cancer Immunol Immunother* 2010;59:1573-82.
- Heuffer C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, et al. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol* 1996;26:659-68.
- Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med* 2012;367:2284-95.
- Reikie BA, Adams RC, Ruck CE, Ho K, Lelgadowicz A, Pillay S, et al. Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life. *PLoS One* 2012;7:e44763.
- Smolen KK, Ruck CE, Fortuno ES III, Ho K, Dimitriu P, Mohn WW, et al. Pattern recognition receptor-mediated cytokine response in infants across 4 continents. *J Allergy Clin Immunol* 2014;133:818-26.e4.
- Upham JW, Lee PT, Holt BJ, Heaton T, Prescott SL, Sharp MJ, et al. Development of interleukin-12-producing capacity throughout childhood. *Infect Immunol* 2002;70:6583-8.
- Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. *Blood* 2006;108:1284-90.
- Philbin VJ, Dowling DJ, Gallington LC, Cortes G, Tan Z, Suter EE, et al. Imidazoquinoline Toll-like receptor 8 agonists activate human newborn monocytes and dendritic cells through adenosine-refractory and caspase-1-dependent pathways. *J Allergy Clin Immunol* 2012;130:195-204.e9.
- Zhang X, Casatelli N, Lemoine S, Mozeleski B, Azria E, Le Ray C, et al. Plasmacytoid DC engagement by Flu vaccine as a surrogate strategy for driving Th1 responses in human neonatal settings. *J Infect Dis* 2014;210:424-34.
- Krumbiegel D, Zepp F, Meyer CU. Combined Toll-like receptor agonists synergistically increase production of inflammatory cytokines in human neonatal dendritic cells. *Hum Immunol* 2007;68:813-22.
- Goriely S, Van Lint C, Dadkhah R, Libin M, De Wit D, Demonte D, et al. A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. *J Exp Med* 2004;199:1011-6.
- Vanden Eijnden S, Goriely S, De Wit D, Goldman M, Willems F. Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns. *Eur J Immunol* 2006;36:21-6.
- Aksoy E, Albarani V, Nguyen M, Laes JF, Ruelle JL, De Wit D, et al. Interferon regulatory factor 3-dependent responses to lipopolysaccharide are selectively blunted in cord blood cells. *Blood* 2007;109:2887-93.
- Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, Lin R, et al. Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. *Blood* 2006;107:1078-84.
- Vekemans J, Amedei A, Ota MO, D'Elios MM, Goetghebuer T, Ismaili J, et al. Neonatal bacillus Calmette-Guerin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. *Eur J Immunol* 2001;31:1531-5.
- Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, et al. Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol* 2002;168:919-25.
- Jiao X, Lo-Man R, Guernonprez P, Fiette L, Deriaud E, Burgaud S, et al. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J Immunol* 2002;168:1294-301.
- van den Biggelaar AH, Prescott SL, Roponen M, Nadal-Sims MA, Devitt CJ, Phuanukoonnon S, et al. Neonatal innate cytokine responses to BCG controlling T-cell development vary between populations. *J Allergy Clin Immunol* 2009;124:544-50, e1-2.
- O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol* 2013;31:475-527.
- Sancho D, Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol* 2012;30:491-529.
- Ariizumi K, Shen GL, Shikano S, Xu S, Ritter R III, Kumamoto T, et al. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J Biol Chem* 2000;275:20157-67.
- Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 2007;8:31-8.
- Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, et al. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 2005;22:507-17.
- Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* 2005;106:2543-50.
- Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, et al. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 2006;442:651-6.
- Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med* 2009;206:2879-88.
- Miyake Y, Toyonaga K, Mori D, Kakuta S, Hoshino Y, Oyamada A, et al. C-type lectin MCL is an FcRgamma-coupled receptor that mediates the adjuvant activity of mycobacterial cord factor. *Immunity* 2013;38:1050-62.
- Rothfuchs AG, Bafica A, Feng CG, Egen JG, Williams DL, Brown GD, et al. Dectin-1 interaction with *Mycobacterium tuberculosis* leads to enhanced IL-12p40 production by splenic dendritic cells. *J Immunol* 2007;179:3463-71.
- Yadav M, Schorey JS. The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* 2006;108:3168-75.
- Bekeredjian-Ding I, Roth SI, Gilles S, Giese T, Ablasser A, Hornung V, et al. T cell-independent, TLR-induced IL-12p70 production in primary human monocytes. *J Immunol* 2006;176:7438-46.
- Elahi S, Ertelt JM, Kinder JM, Jiang TT, Zhang X, Xin L, et al. Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. *Nature* 2013;504:158-62.

36. Levy O, Zarembek KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF- α induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J Immunol* 2004;173:4627-34.
37. Zhang X, Casartelli N, Lemoine S, Mozeleski B, Azria E, Le Ray C, et al. Plasmacytoid dendritic cells engagement by influenza vaccine as a surrogate strategy for driving T-helper type 1 responses in human neonatal settings. *J Infect Dis* 2014;210:424-34.
38. Sun C-M, Deriaud E, Leclerc C, Lo-Man R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* 2005;22:467-77.
39. Hervas-Stubbs S, Majlessi L, Simsova M, Morova J, Rojas MJ, Nouze C, et al. High frequency of CD4+ T cells specific for the TB10.4 protein correlates with protection against *Mycobacterium tuberculosis* infection. *Infect Immun* 2006;74:3396-407.
40. Zhang X, Mozeleski B, Lemoine S, Deriaud E, Lim A, Zhivaki D, et al. CD4 T cells with effector memory phenotype and function develop in the sterile environment of the fetus. *Sci Transl Med* 2014;6:238ra72.
41. Rose S, Lichtenheld M, Foote MR, Adkins B. Murine neonatal CD4+ cells are poised for rapid Th2 effector-like function. *J Immunol* 2007;178:2667-78.
42. Yoshimoto M, Yoder MC, Guevara P, Adkins B. The murine Th2 locus undergoes epigenetic modification in the thymus during fetal and postnatal ontogeny. *PLoS One* 2013;8:e51587.
43. Hebel K, Weinert S, Kuropka B, Knolle J, Kosak B, Jorch G, et al. CD4+ T cells from human neonates and infants are poised spontaneously to run a nonclassical IL-4 program. *J Immunol* 2014;192:5160-70.
44. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007;204:1849-61.
45. Mintz-Cole RA, Gibson AM, Bass SA, Budelsky AL, Reponen T, Hershey GK. Dectin-1 and IL-17A suppress murine asthma induced by *Aspergillus versicolor* but not *Cladosporium cladosporioides* due to differences in beta-glucan surface exposure. *J Immunol* 2012;189:3609-17.
46. Kamath AT, Rochat AF, Christensen D, Agger EM, Andersen P, Lambert PH, et al. A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. *PLoS One* 2009;4:e5771.
47. Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J Immunol* 2009;182:8047-55.
48. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* 2009;31:761-71.
49. Wevers BA, Kaptein TM, Zijlstra-Willems EM, Theelen B, Boekhout T, Geijtenbeek TB, et al. Fungal engagement of the C-type lectin muncle suppresses dectin-1-induced antifungal immunity. *Cell Host Microbe* 2014;15:494-505.
50. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC, et al. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat Immunol* 2009;10:203-13.
51. Ifrim DC, Joosten LA, Kullberg BJ, Jacobs L, Jansen T, Williams DL, et al. *Candida albicans* primes TLR cytokine responses through a Dectin-1/Raf-1-mediated pathway. *J Immunol* 2013;190:4129-35.
52. Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. *J Immunol* 2009;183:7150-60.

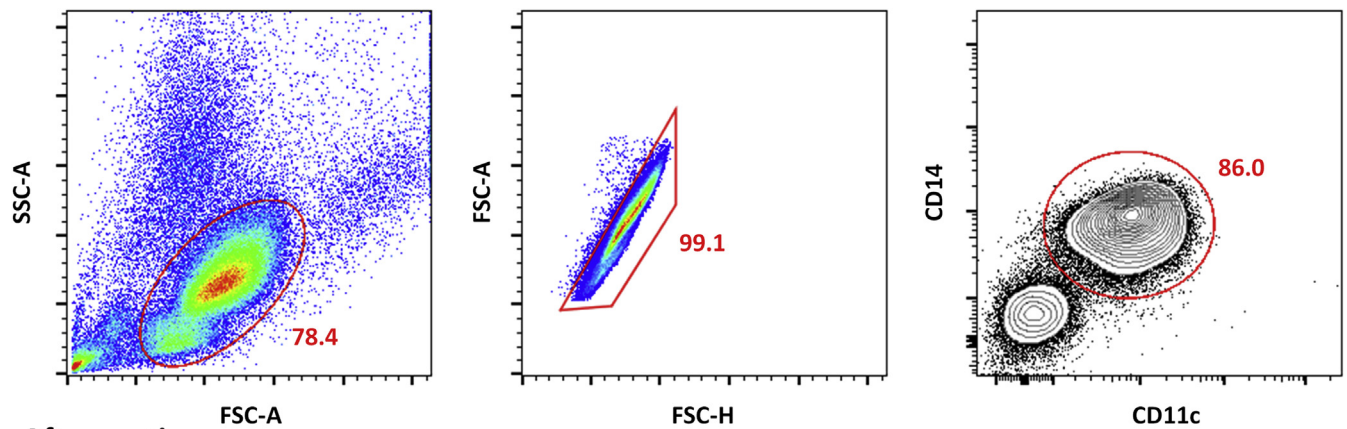
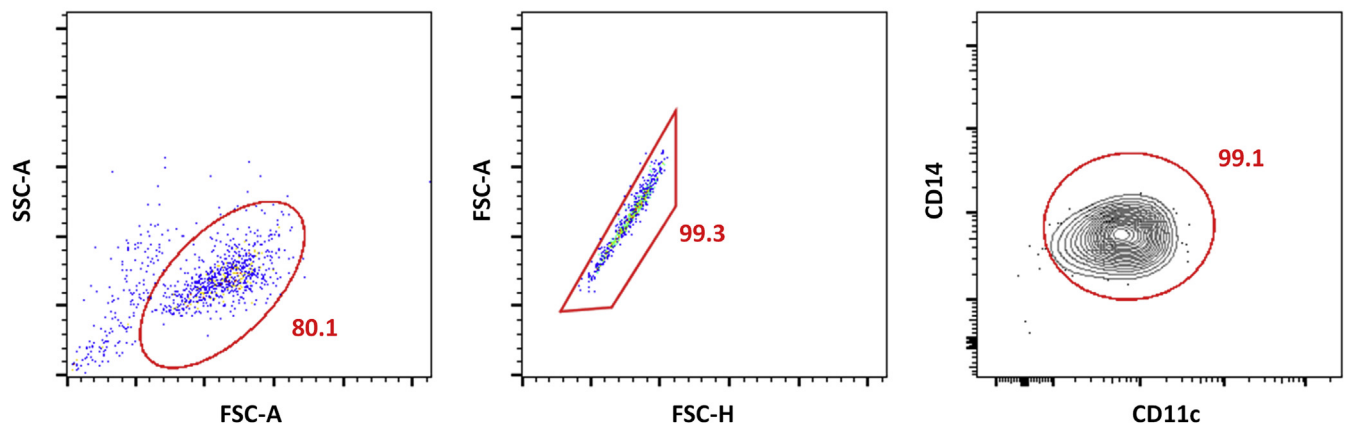
Before sorting**After sorting**

FIG E1. Gating strategy for purification of monocytes. CD11c⁺CD14⁺ monocytes from cord blood were FACS sorted from CD14⁺ enriched mononuclear cell suspension. *FSC*, Forward scatter; *SSC*, side scatter.

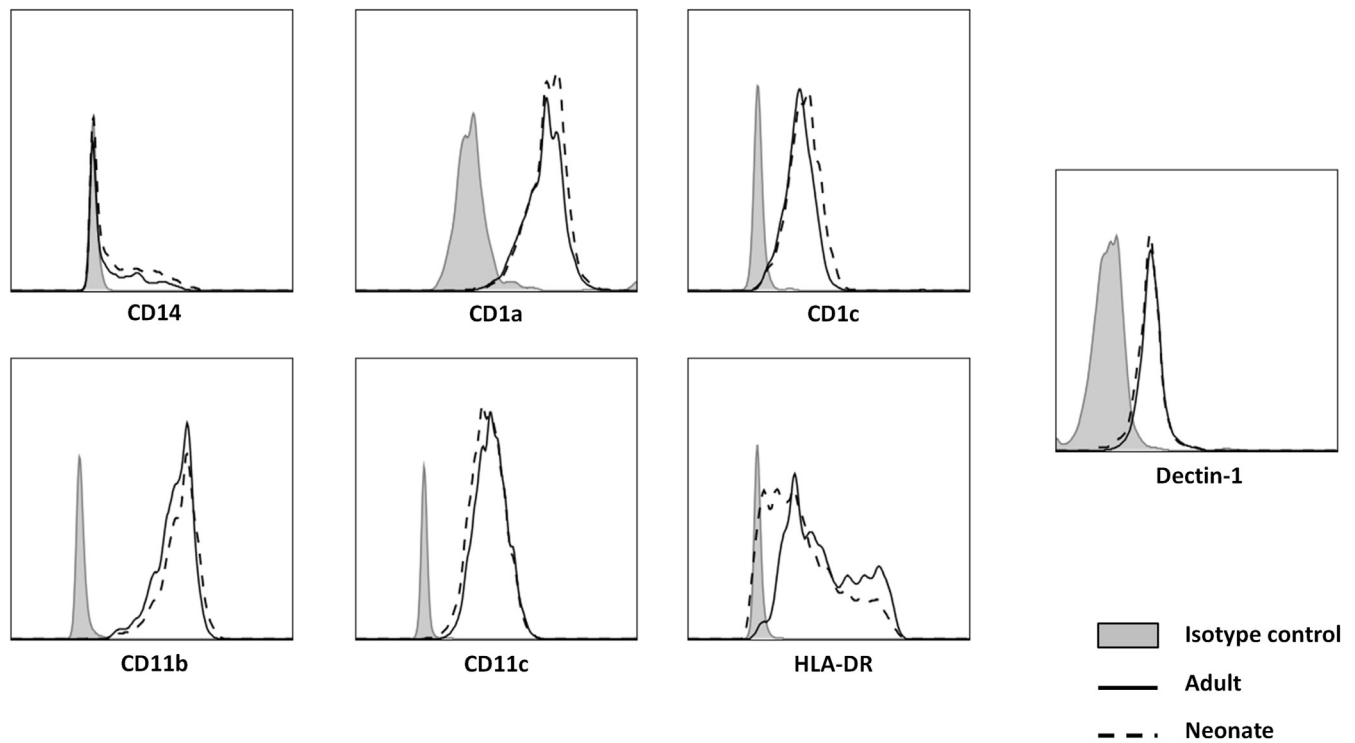


FIG E2. Phenotype of moDCs. Monocytes from peripheral or cord blood were purified as CD14⁺CD11c⁺ cells by using FACS, as in [Fig E1](#), and then cultured for 6 days with 100 ng/mL GM-CSF and 50 ng/mL IL-4. Expression of CD14, CD1a, CD1c, CD11b, CD11c, HLA-DR, and Dectin-1 was evaluated by using FACS. No difference was observed between cord and adult moDCs.

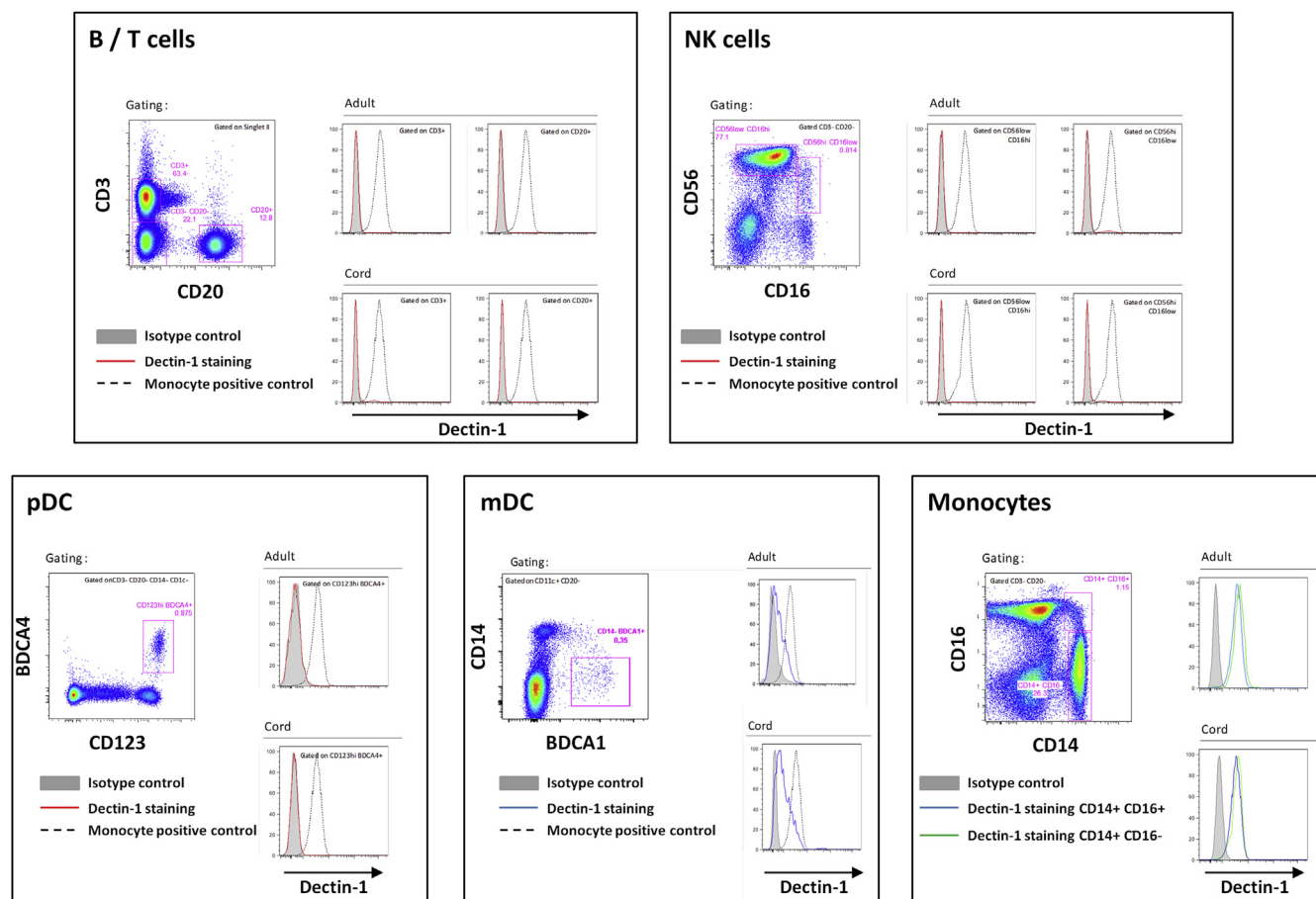


FIG E3. Expression of Dectin-1 in mononuclear cells. Dectin-1 expression was evaluated by means of FACS on PBMCs or cord blood mononuclear cells (CBMCs). No difference of expression was observed between PBMCs and CBMCs. T cells were defined as CD3⁺ cells, B cells as CD20⁺ cells, natural killer (NK) cells as CD3⁺CD56⁺ cells, pDCs as CD123^{hi}BDCA4⁺ cells, mDCs as CD20⁺CD11c⁺BDCA1⁺ cells, BDCA3 DCs as CD11c⁺BDCA3⁺ cells, and monocytes as CD14⁺ cells.

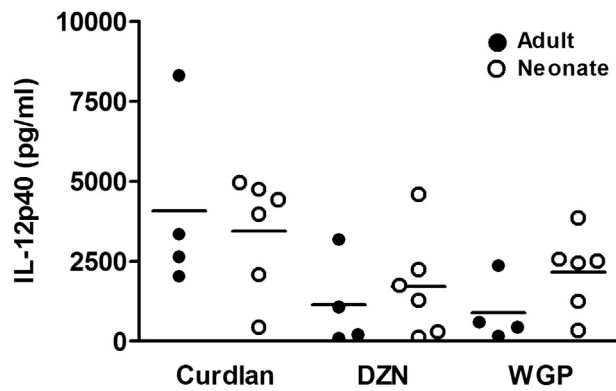


FIG E4. Comparison of Dectin-1 agonists. MoDCs were generated from adult ($n = 4$) or neonatal ($n = 6$) monocytes and stimulated for 72 hours with either 100 $\mu\text{g/mL}$ curdlan, DZN, or WGP. IL-12p40 levels were measured by means of ELISA in culture supernatants.

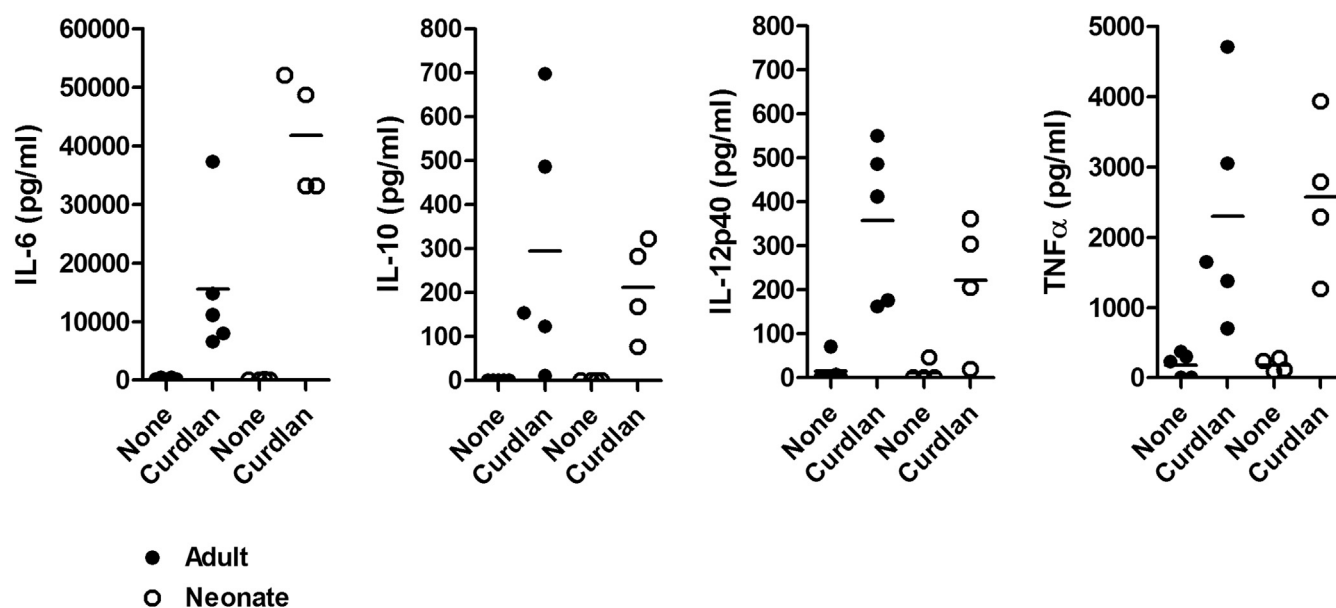


FIG E5. Secretion of cytokines after whole blood stimulation with curdlan. Two hundred fifty microliters of whole peripheral blood ($n = 5$) or cord blood ($n = 4$) was cultured for 24 hours with 100 $\mu\text{g/mL}$ curdlan. Plasma was collected and tested by using ELISA for IL-6, IL-10, IL-12p40, and TNF- α .

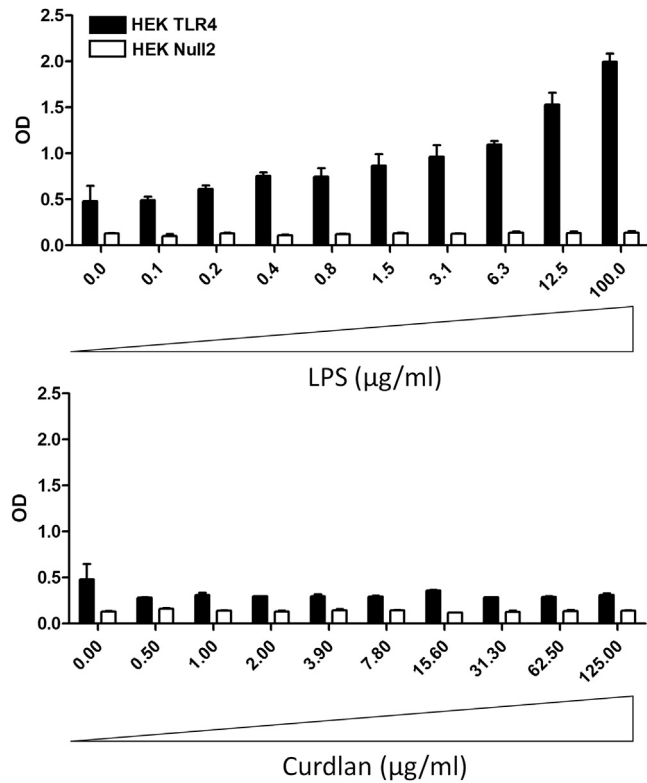


FIG E6. Evaluation of the capacity of curdlan to trigger TLR4 signaling. HEK-Blue hTLR4 or HEK Null2 cells were stimulated with either LPS or curdlan at different concentrations for 24 hours. Cell activation was quantified by measuring the change in OD at 650 nm.

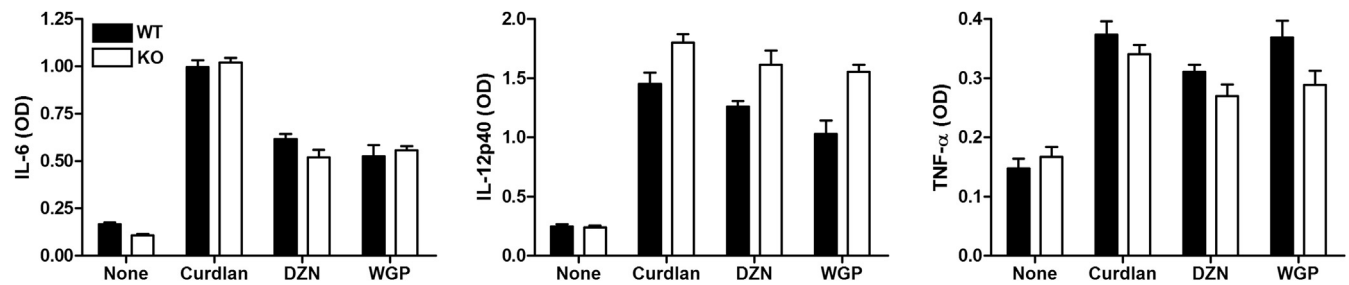


FIG E7. Evaluation of TLR2/TLR4 ligand contamination in curdlan-stimulated cells. Bone marrow–derived DCs were generated from bone marrow from C57BL/6 mice or TLR2/4-KO mice and stimulated for 48 hours with 100 μ g/mL curdlan, 100 μ g/mL DZN, or 100 μ g/mL WGP. Cell-culture supernatants were harvested and tested for IL-6, IL-12p40, and TNF- α by means of ELISA.

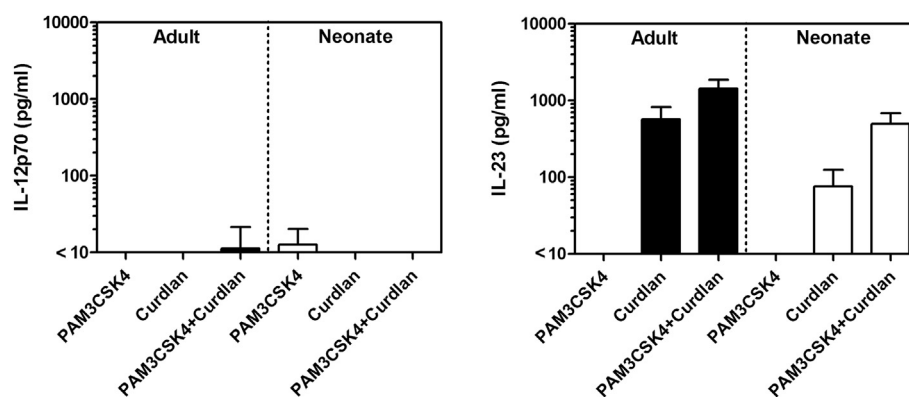


FIG E8. Secretion of IL-12p70 and IL-23 by TLR2/Dectin-1-stimulated moDCs. Adult ($n = 4$) or neonatal ($n = 8$) moDCs were stimulated with either $1 \mu\text{g/mL}$ PAM3CSK4, $100 \mu\text{g/mL}$ curdlan, or combined stimuli. After 72 hours, the cell-culture supernatant was harvested, and IL-12p70 and IL-23 levels were measured by means of ELISA. Results are expressed as means $\pm$ SDs.

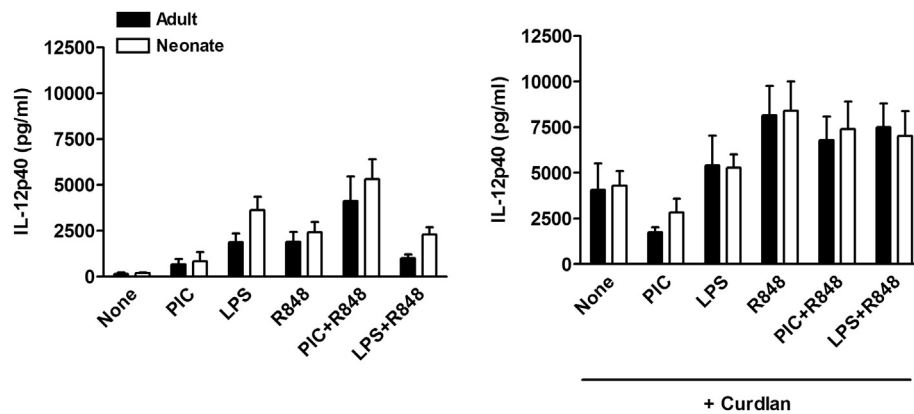


FIG E9. Secretion of IL-12p40 by TLRs or TLR/Dectin-1–stimulated moDCs. Adult ($n = 4$) or neonatal ($n = 4$) moDCs were stimulated with either 25 $\mu\text{g/mL}$ PIC, 1 $\mu\text{g/mL}$ R848, 10 $\mu\text{g/mL}$ LPS, 100 $\mu\text{g/mL}$ curdlan, or combined stimuli. After 72 hours, the cell-culture supernatant was harvested, and IL-12p40 levels were measured by means of ELISA. Results are expressed as means $\pm$ SDs.

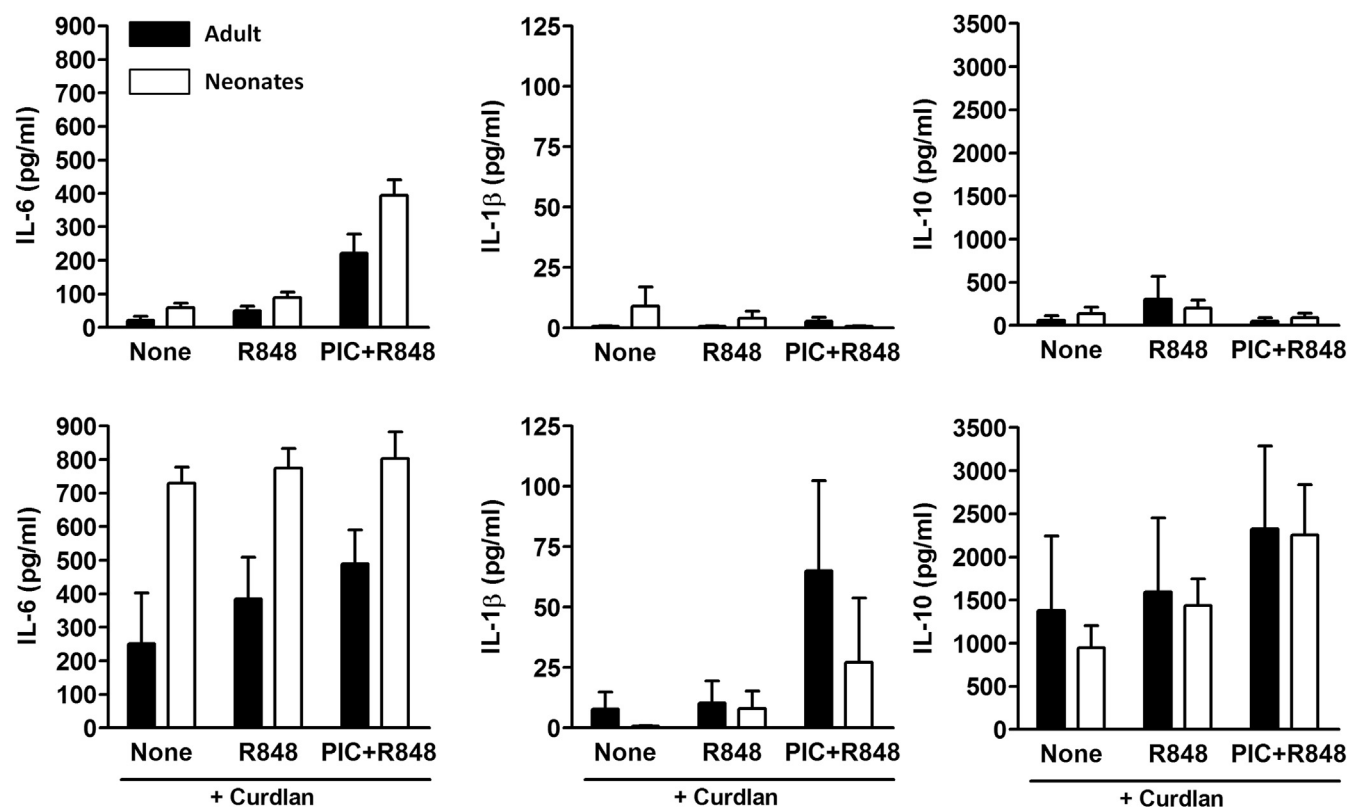


FIG E10. Secretion of IL-6, IL-1 β , and IL-10 by TLRs or TLR/Dectin-1-activated moDCs. Adult ($n = 4$) or neonatal ($n = 4$) moDCs were stimulated with either 1 μ g/mL R848, 25 μ g/mL PIC plus 1 μ g/mL R848, 100 μ g/mL curdlan, or combined stimuli. After 72 hours, the cell-culture supernatant was harvested, and IL-6, IL-1 β , and IL-10 levels were measured by means of ELISA. Results are expressed as means $\pm$ SDs.

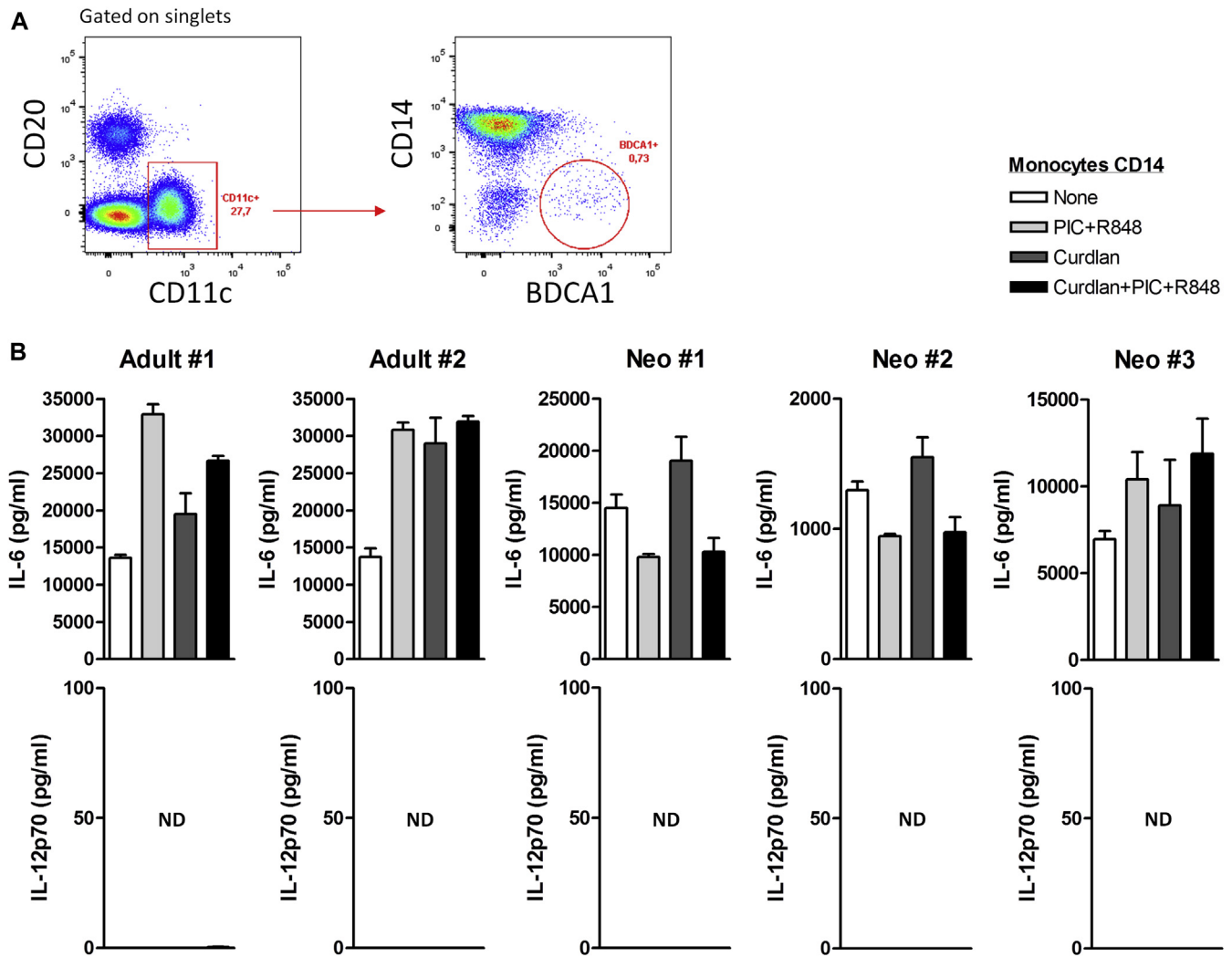


FIG E11. Secretion of IL-6 and IL-12p70 by TLRs or TLR/Dectin-1-stimulated monocytes. **A**, Identification of cord blood-derived mDCs. mDCs were characterized as CD11c⁺CD20[−]CD14[−]BDCA1⁺ cells. **B**, Adult ($n = 2$) or neonatal ($n = 3$) monocytes were sorted by means of FACS and stimulated with 25 μ g/mL PIC plus 1 μ g/mL R848, 100 μ g/mL curdlan, or combined stimuli. After 48 hours, the cell-culture supernatant was harvested, and IL-6 and IL-12p70 levels were measured by means of ELISA. Results are expressed as means $\pm$ SDs. ND, Not determined.

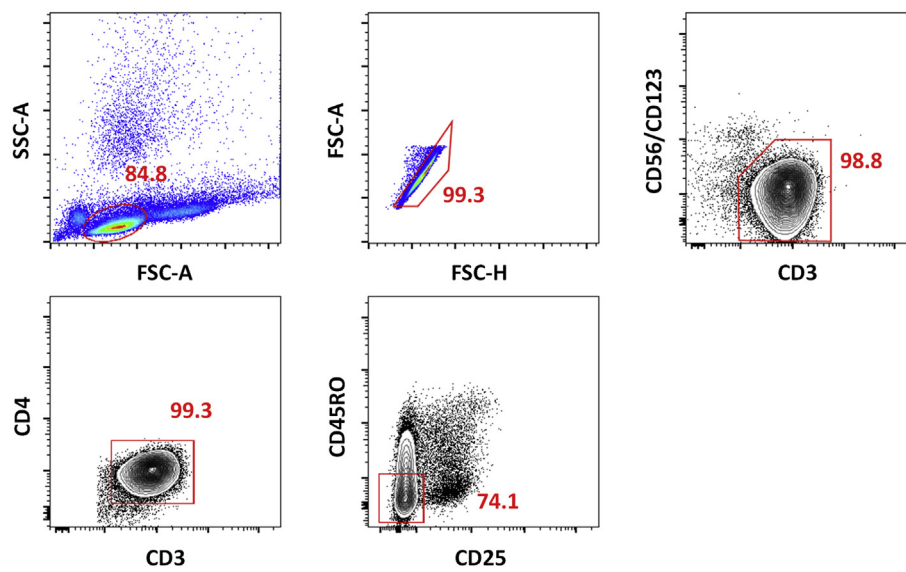
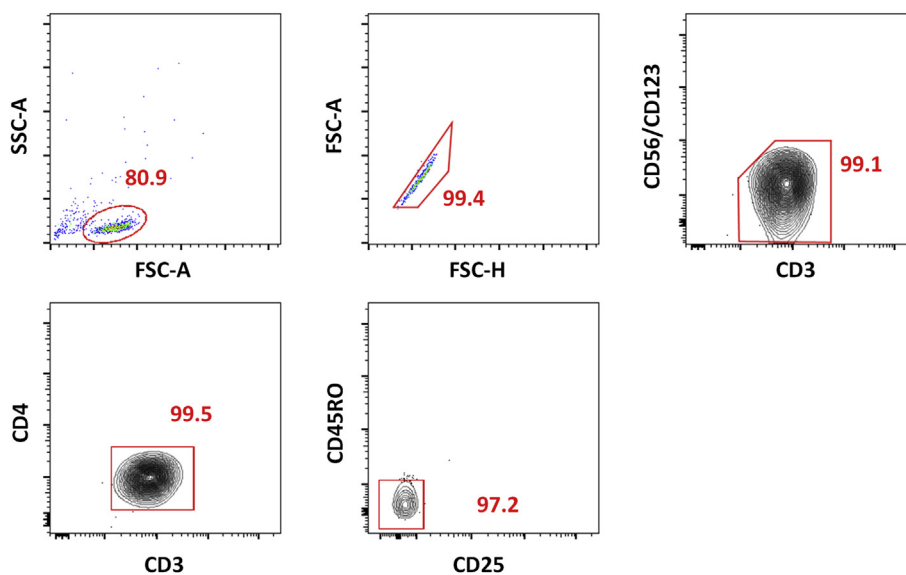
Before sorting**After sorting**

FIG E12. Gating strategy for purification of naive T cells. Cord blood $CD3^+CD4^+CD123^-CD56^-CD25^-CD45RO^-$ naive T cells were FACS sorted from a $CD4^+$ enriched mononuclear cell suspension. *FSC*, Forward scatter; *SSC*, side scatter.

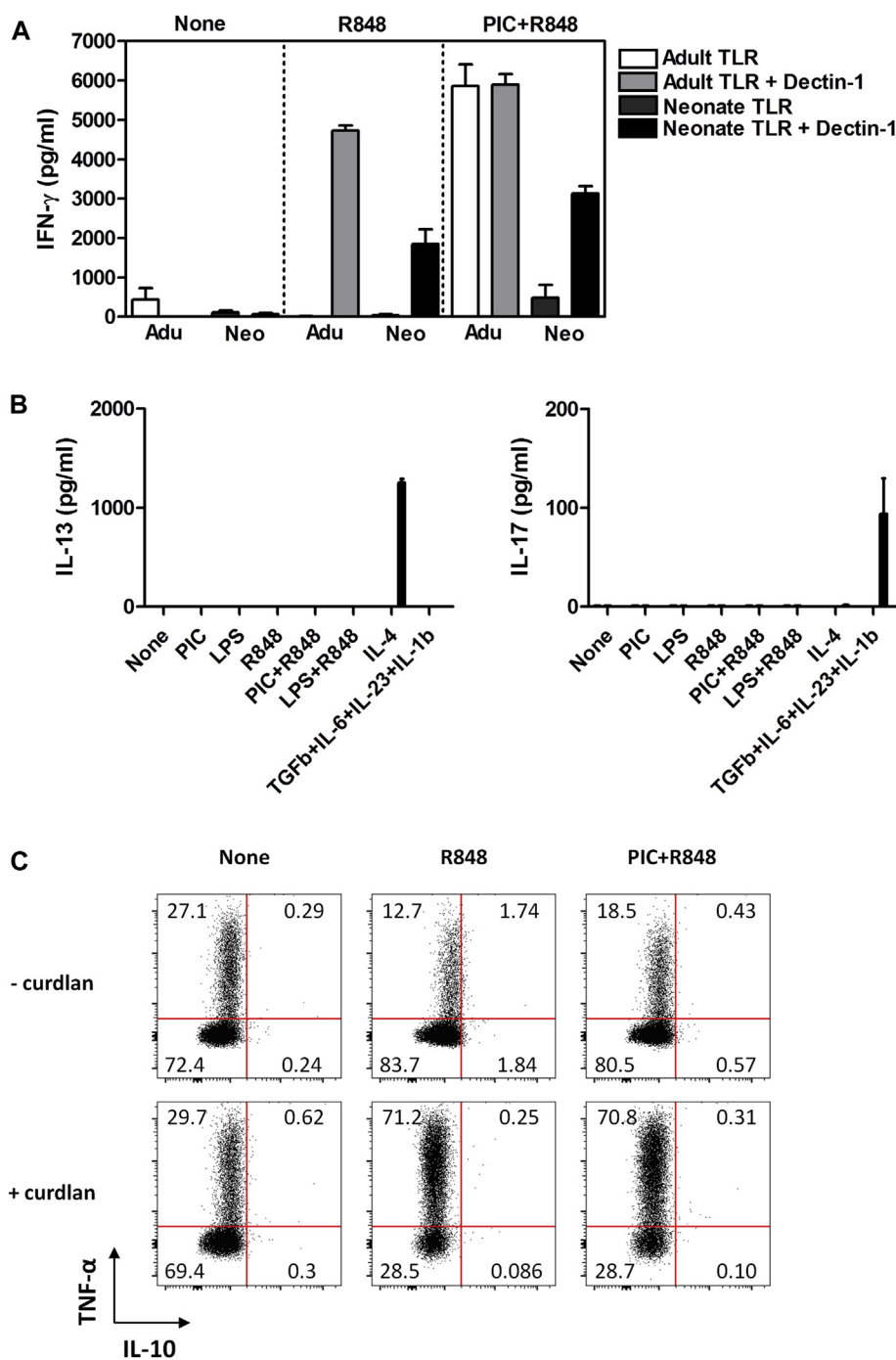


FIG E13. Differentiation of neonatal naive T cells cultured with neonatal or adult moDCs. Neonatal ($n = 3$) or adult ($n = 2$) moDCs were stimulated with either 25 $\mu\text{g/mL}$ PIC, 1 $\mu\text{g/mL}$ R848, 100 $\mu\text{g/mL}$ curdlan, or combined stimuli for 24 hours, and then neonatal heterologous naive T cells ($\text{CD}3^+ \text{CD}4^+ \text{CD}56^- \text{CD}25^- \text{CD}45\text{RO}^-$ cells) were added to the culture for 5 more days. **A** and **B**, $\text{IFN-}\gamma$ (Fig E13, A) and IL-13 and IL-17 (Fig E13, B) were tested by means of ELISA in the cell-culture supernatant. Neonatal T cells stimulated with plate-bound anti-CD3 and anti-CD28 with 10 ng/mL IL-4 or 10 ng/mL TGF- β plus 25 ng/mL IL-1 β plus 10 ng/mL IL-6 plus 10 ng/mL IL-23 were used as $\text{T}_\text{H}2$ and $\text{T}_\text{H}17$ controls, respectively. **C**, Neonatal naive T cells were cultured with 25% supernatant of activated neonatal moDCs for 5 days on plate-bound anti-CD3 plus anti-CD28. The cells were analyzed for intracellular IL-10 by means of FACS.

TABLE E1. Antibodies used in the study

Target	Clone	Isotype	Company
CD14	61D3	IgG ₁ ,k	eBioscience, San Diego, Calif
CD11c	3.9	IgG ₁ ,k	eBioscience
CD3	OKT3	IgG _{2a} ,k	eBioscience
CD3	OKT3	IgG _{2a} ,k	BioLegend, San Diego, Calif
CD28	CD28.2	IgG ₁ ,k	BioLegend
CD4	SK3	IgG ₁ ,k	eBioscience
CD123	6H6	IgG ₁ ,k	eBioscience
CD56	MEM188	IgG _{2a} ,k	eBioscience
CD25	BC96	IgG ₁ ,k	eBioscience
CD45RO	UCHL1	IgG _{2a} ,k	eBioscience
CD20	2H7	IgG _{2b} ,k	eBioscience
CD16	3G8	IgG ₁ ,k	BD, San Jose, Calif
BDCA4	AD5-17F6	IgG ₁	Miltenyi Biotec, Bergisch Gladbach, Germany
BDCA1	L161	IgG ₁	eBioscience
Dectin-1	259931	IgG _{2b}	R&D Systems, Minneapolis, Minn
HLA-DR	LN3	IgG _{2b} ,k	eBioscience
CD1a	HI149	IgG ₁ ,k	BD
IFN- γ	4S.B3	IgG ₁ ,k	eBioscience
IL-13	JES10-5A2	IgG ₁ ,k	BioLegend
IL-17	eBio64DEC17	IgG ₁ ,k	eBioscience
TNF- α	MAb11	IgG ₁ ,k	eBioscience
IL-12 blocking	C8.6	IgG ₁ ,k	BioLegend
IFNAR blocking	MMHAR-2	IgG _{2a}	PBL Assay Science, Piscataway Township, NJ
Dectin-1 blocking	22H8	IgG ₁	InvivoGen, San Diego, Calif

IFNAR, Type 1 interferons receptor.

TABLE E2. List of primers for qPCR experiments

P19	Forward	ACAACTGAGGGAACCAAACCA
	Reverse	TTCCACTTGCTTTGAGCCTGAT
P35	Forward	GCCTTCACCACTCCCAAAAC
	Reverse	TGTCTGGCCTTCTGGAGCAT
P40	Forward	TGGAGATGCTGGCCAGTACA
	Reverse	AGCAGGAGCGAATGGCTTAG
GADPH	Forward	GACCTGACCTGCCGTCTAGAAA
	Reverse	CGCCTGCTTCACCACCTT
TLR3	Forward	ACAAGTACCTGCAGCTGACT
	Reverse	CTTCGGAGCATCAGTCGTTG
TLR4	Forward	ATGGATCAAGGACCAGAGGC
	Reverse	AGGACCGACACACCAATGAT
TLR7	Forward	AGTCCAAGTTCCTCCAGCTC
	Reverse	GAAGTATGGGTGAGCTTGCG
TLR8	Forward	ACGGAAACCCCTTTGAATGC
	Reverse	TTCCCTCTTTGATCCCCAGG