

Enhanced expression of IL-27 mRNA in human newborns

Krumbiegel D, Anthogalidis-Voss C, Markus H, Zepp F, Meyer CU. Enhanced expression of IL-27 mRNA in human newborns. *Pediatr Allergy Immunol* 2007.

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Interleukin (IL)-27, a heterodimer composed of Epstein–Barr virus-induced gene 3 (EBI3) and p28, is an early product of activated dendritic cells (DC). Binding of IL-27 to the WSX-1 receptor initiates Th1 (Thelper 1) responses in naïve T cells. In order to assess the Th1 responses in human neonates with high susceptibility to infectious diseases, expression of EBI3-, p28- and WSX-1-mRNA in response to Toll-like receptor ligands was compared in neonate and adult monocyte-derived (m)DC. Only the combined addition of ligands induced expression of IL-27p28 mRNA. Surprisingly, neonatal mDC produced significantly more IL-27p28 mRNA than that obtained from adults. Furthermore, there was enhanced expression of EBI3 mRNA in cord blood as compared with adult blood. In addition, the secretion of WSX-1 mRNA in neonatal mDC and T cells was also significantly increased. Taken together, these findings indicate that the restricted Th1 responses in human newborns owing to deficient IL-12 production may be compensated for, in part, by enhanced IL-27 secretion.

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Key words: cord blood; dendritic cells; Epstein–Barr virus-induced gene 3; neonatal; WSX-1

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Accepted 15 October 2007

The increased susceptibility to infections which occurs in human newborns (1) is thought to be the result of the immaturity of neonatal dendritic cells (DC). It is well accepted that the expression of the key Th1-trophic cytokine, interleukin (IL)-12, on neonatal DC is diminished (2). Other studies have indicated that IL-27, a cytokine that is closely related to IL-12, also regulates Th1 immune responses (3). IL-27 is a heterodimer composed of Epstein–Barr virus-induced gene 3 (EBI-) and IL-27p28 (4). The cytokine receptor, WSX-1 constitutes with gp130, a functional receptor for IL-27 (5). IL-27 induces proliferation in naïve CD4⁺ T cells and the expression of T-bet, a master transcriptional regulator for Th1 differentiation (4). On the other hand, IL-27 suppresses the secretion of proinflammatory cytokines, including IL-23-induced IL-17 on activated T cells (6). The production of cytokines of the IL-12 family by DC is triggered when TLR are activated by pathogen-associated molecular patterns present on bacterial, fungal, and viral

microbes (7). The characteristics of IL-27 expression in neonatal cells are currently unknown. In order to better elucidate the role of IL-27 in the regulation of the neonatal immune response with respect to the high susceptibility to infectious diseases, we examined the synthesis of IL-27p28 mRNA and EBI-3 mRNA, and the expression of WSX-1 mRNA of TLR-activated, monocyte-derived DC (mDC) and isolated CD4⁺ T cells from human newborns and compared the findings with the respective expression patterns in adult cells.

Materials and methods

Neonatal and adult CD4⁺ and CD14⁺ cell isolation

Umbilical cord blood samples were collected from healthy, full-term infants, delivered *via* caesarean section. Leukocytes from adults were enriched *via* leukapheresis. After density centrifugation through Ficoll-Hypaque (PAA,

Pasching, Austria), the mononuclear cell fraction was washed and magnetically labeled with anti-CD4 beads and anti-CD14 beads (MiltenyiBiotec, Bergisch-Gladbach, Germany). Labeled cells were enriched in a magnetic field according to the manufacturer's instructions (VarioMACS; MiltenyiBiotec). Using this approach, a yield of 33–70% CD4⁺ cells and 5–27% CD14⁺ cells of the total mononuclear cell fraction was obtained. The purity of the cells after separation was 92% ($\pm 6\%$) for CD4⁺ cells and 95% ($\pm 4\%$) for CD14⁺ cells.

Generation of DC from monocytes

Immature DC (imDC) were generated in cultures of CD14⁺ cells (1×10^6 cells per well) in 24-well flat-bottom plates (Nunc, Wiesbaden, Germany) in RPMI 1640, containing 2 mM l-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin (Invitrogen, Karlsruhe, Germany), 1 mM sodium pyruvate, $1 \times$ MEM (minimal essential medium) amino acids (Biochrom, Berlin, Germany), 0.05 mM β -mercaptoethanol (Sigma-Aldrich, Munich, Germany), 5% ES-FCS (fetal calf serum for embryonic stem cells) (Biochrom), 100 ng/ml rhGM-CSF (recombinant human granulocyte-macrophage colony-stimulating factor), and 20 ng/ml rhIL-4 (Strathmann, Hamburg, Germany). After 3 days, the cultures were supplemented for 48 h with different mixtures of Toll-like receptor (TLR) ligands and mediators in fresh medium to generate mature (ma)DC: LPS (lipopolysaccharide) (10 μ g/ml; InvivoGen, San Diego, CA, USA), ssRNA40 (5 μ g/ml; InvivoGen), and IFN γ (interferon) (50 ng/ml) (Strathmann). Phenotype of imDC (treated with GM-CSF/IL-4 for 3 days) were analyzed by flow cytometry with anti-CD209 antibodies ($92.4\% \pm 5.5\%$) and phenotype of maDC (treated with TLR ligands plus/minus IFN γ for 48 h) were analyzed with anti-CD86 antibodies ($86.8\% \pm 6.7\%$) (all BDTM Biosciences, San Diego, CA, USA) (data not shown).

Real-time PCR of IL-27p28, EBI-3 and WSX-1 mRNA

Purified T cells, monocytes, imDC, and maDC were washed with phosphate-buffered saline (PBS), and the total RNA was isolated (High Pure RNA-Isolation Kit including Dnase; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized, using Random Primers (Promega, Mannheim, Germany), anti-Rnase (Ambion, Austin, TX, USA) and an Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany).

Quantification of IL-27p28, EBI-3 and WSX-1 transcripts was performed by real-time quantitative PCR using a LightCyclerTM (Roche) based on general SYBR green (LightCyclerTM FastStart DNA Master SYBR Green I; Roche) fluorescence detection. Normalization to house-keeping gene (β -actin) was performed for each sample: β -actin mRNA remains stable during DC stimulation. Standard curves were generated for β -actin, p28, EBI-3 resp. WSX-1 mRNA in each sample. Then, the concentration of the target in each sample was divided by the concentration of β -actin in the same sample. Primer sequences: β -actin: f, gga ctt cga gca aga gat gg; r, agc act gtg ttg gcg tac ag (234-bp product); p28: f, gag cag ctc cct gat gtt tc; r, agc tgc atc ctc tcc atg tt (236-bp product); EBI-3: f, tgt tct cca tgg ctc cct ac; r, gct ccc tga cgc ttg taa c (158-bp product); and WSX-1: f, cca tac ccc tga ccc ctg ttg aga t; r, cag agg ttc cct gat acc cac aca t (175-bp product).

Results and discussion

Considerably enhanced IL-27 mRNA expression in neonatal DC

Monocyte-derived DC from neonatal and adult blood were stimulated with TLR4 and TLR8 ligands and analyzed for IL-27p28 and EBI-3 mRNA expression. As shown in Fig. 1a, only the combination of LPS and IFN γ induced the expression of IL-27p28 mRNA in neonatal maDC. LPS combined with ssRNA induced a slight expression of p28 and EBI-3 mRNA in adult maDC. Interestingly, neonatal maDC produced significantly more IL-27p28 mRNA in comparison with adult maDC in response to LPS and IFN γ .

Combining LPS and IFN γ also induced increased expression of EBI-3 mRNA in neonatal and adult maDC (Fig. 1b), indicating that IFN γ is required for bioactive IL-27 production that consists of p28 and EBI-3 (4). Our results are consistent with previous reports which have demonstrated that IFN γ is a major enhancing signal of all IL-12 family members (4). As with IL-27p28, EBI-3 mRNA expression was significantly increased in neonatal maDC.

Upon interaction with TLR, it has been published that neonatal DC are able to produce similar or even higher levels of IL-23 than adults (8), although neonatal DC are deficient in the secretion of bioactive IL-12 (2). For adults, the synergistic effects of TLR4 and TLR8 ligands were shown to increase the production of IL-12 and IL-23 with Th1-sustaining capacity (9). Regarding IL-27, we also observed this synergis-

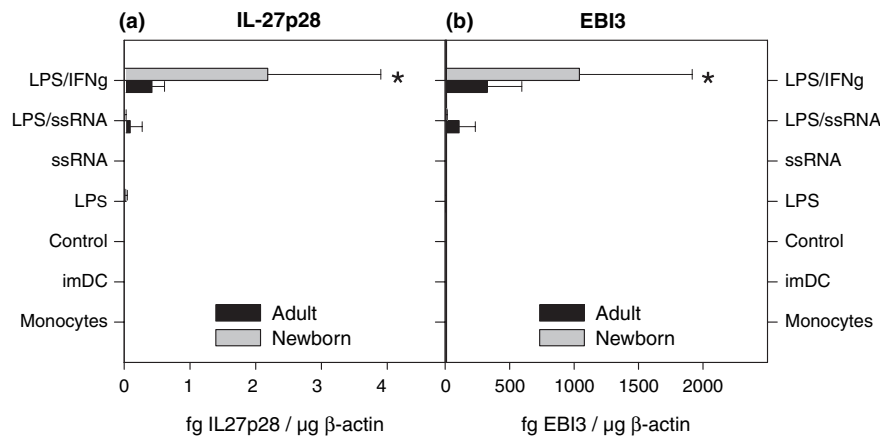


Fig. 1. Expression of IL-27p28 (a) and EBI-3 (b) mRNA in adult and neonatal mDC. Adult and neonatal mDC were stimulated with different mixtures of TLR ligands and mediators for 48 h. Unstimulated mDC acted as control. RNA was isolated, cDNA synthesized and quantification of p28 and EBI-3 transcripts was performed by real-time PCR. mRNA levels were normalized against β -actin levels. The figures show medians of 12 samples each. Comparison of neonatal vs. adult * $p < 0.05$ (Student's t -test).

tic effect. Studies in mice have shown that IL-27 may be required earlier in the generation of antimicrobial responses than IL-12, suggesting that IL-27 plays a major role in the early induction of Th1 differentiation until sufficient IL-12 is produced (3). Our results indicate that in human newborns, IL-27 clearly seems to be available to compensate for neonatal IL-12 production.

Increased expression of IL-27 receptor WSX-1 in neonatal DC and T cells

Furthermore, we analyzed the expression of the IL-27 receptor WSX-1 mRNA of stimulated neonatal and adult DC (Fig. 2a). Independent

of the stimulus, neonatal monocytes, imDC and maDC produced greater WSX-1 mRNA than adult DC. Although the production of IL-27 has only been reported in endothelial cells, DC, and monocytes, expression of WSX-1 was detected in a variety of immune cells. Even though IL-27 is the only known ligand for WSX-1, the effects of WSX-1 binding and signaling may be influenced by its interaction with other pro- and anti-inflammatory cytokines which have not been identified. Moreover, the enhanced expression of WSX-1 mRNA in neonatal DC may represent the sensitivity for IL-27 and its pro- or anti-inflammatory properties, as for monocytes, for which it is known that IL-27 can stimulate the production of inflammatory

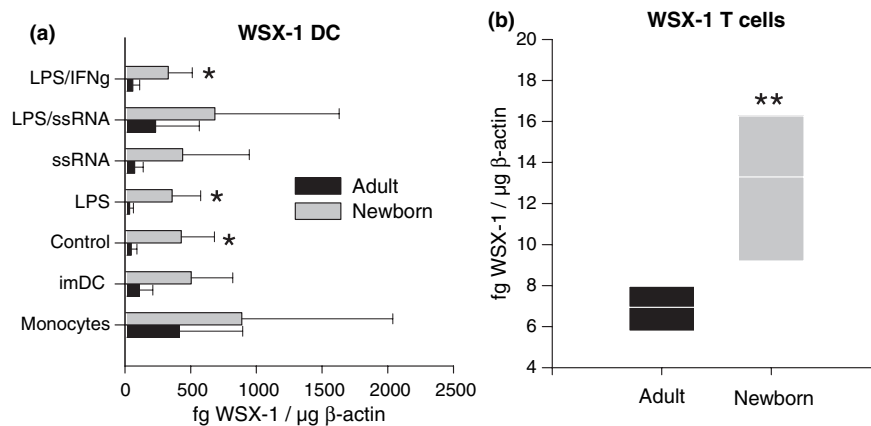


Fig. 2. Expression of WSX-1 mRNA in adult and neonatal DC (a) and T cells (b). Adult and neonatal DC were stimulated with different mixtures of TLR ligands and mediators for 48 h. Adult and neonatal T cells were separated by MACS technology. Unstimulated mDC acted as control. RNA was isolated, cDNA synthesized and quantification of WSX-1 transcripts was performed by real-time PCR. mRNA levels were normalized against β -actin levels. Fig. 2(a) shows median of six samples each, Fig. 2(b) box plots with median and statistical spread of six samples each. Comparison of neonatal vs. adult * $p < 0.05$; ** $p < 0.01$ (Student's t -test).

cytokines in an autocrine manner (5). This is consistent with our observation that monocytes expressed more WSX-1 mRNA than adult and neonatal DC.

Additionally, we demonstrated that the production of IL-27 receptor WSX-1 mRNA in neonatal T cells was obviously higher than in adult T cells (Fig. 2b). With the expression of WSX-1, neonatal T cells are able to respond to IL-27, either by producing IFN γ and thus supporting Th1 immune responses (10) or by inhibiting T cell proliferation (6). However, our results indicate that IL-27 plays an important role in neonatal immunity along with IL-23, which also is an inducer of IFN γ by cord blood T cells (11). It will be interesting to clarify the differential requirements of IL-23 and IL-27 during the time course of Th1 development in human newborns.

In conclusion, with an enhanced expression of WSX-1, neonatal T cells may also be more susceptible to the inhibitory effects of IL-27, resulting in deficient Th1 differentiation. The high capacity of neonatal mDC to produce IL-27 and the potentially high capacity of neonatal DC and T cells to respond to IL-27, however, may compensate the reduced production of IL-12p70 in early life. These findings warrant further investigation concerning the role of IL-27 in neonatal high susceptibility to infectious diseases.

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