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ORIGINAL ARTICLE

Vitamin D_3 induces expression of human cathelicidin antimicrobial peptide 18 in newborns

Yuka Misawa · Atsushi Baba · Susumu Ito · Miyuki Tanaka · Masaaki Shiohara

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Abstract Bactericidal activities of neutrophils occur by two distinctive mechanisms that are oxygen-dependent and -independent. Human cathelicidin antimicrobial peptide 18 (hCAP18), also known as LL-37/FALL-39, is a neutrophil-specific granule protein. We compared the content of hCAP18 and neutrophil gelatinase-associated lipocalin (NGAL), another neutrophil-specific granule protein, in neutrophils of both neonates and adults by flow cytometry. The percentage as well as fluorescence intensity ratio of hCAP18 and NGAL expression in neonate neutrophils were significantly lower than in adults. Expression of hCAP18 in monocytes, however, was not significantly different between neonates and adults. Both hCAP18 and NGAL expression increased in an age-dependent fashion. Plasma concentration of these peptides measured by enzyme-linked immunosorbent assay was not significantly different between neonates and adults. Oral intake of 1α hydroxy vitamin D_3 ($1\alpha(OH)D_3$) in rickets patients for 4 weeks significantly increased hCAP18 expression in neutrophils compared to age-matched healthy controls without $1\alpha(OH)D_3$, indicating the potential of vitamin D_3 as a regulator of the innate immune response of neonates.

Keywords Human · Neutrophils · Phagocytosis

Y. Misawa · A. Baba · M. Tanaka · M. Shiohara (✉)
Department of Pediatrics, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390-8621, Japan
e-mail: shiohara@shinshu-u.ac.jp

S. Ito
Research Center for Human and Environmental Sciences, Division of Instrumental Analysis, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390-8621, Japan

1 Introduction

Bacterial infections are important causes of neonatal morbidity and mortality [1, 2]. Both preterm and term neonates are immunocompromised after birth compared with older children and adults. Moreover, the proportion of low-birth-weight (LBW) and very low-birth-weight (VLBW) infants was 4.2 and 0.1% in 1980, but increased to 8.3 and 0.6% in 2000 in Japan [3]. LBW infants have improved survival, but have more chance of being exposed to a continuous risk of bacterial infections. Neonates are susceptible to systemic infections, including *group B streptococcus* (GBS), *Escherichia coli*, or *Listeria monocytogenes* as early onset bacteremia [4, 5]. Moreover, *Enterococci* and *Staphylococcus aureus* cause nosocomial, late-onset infection in neonates [2]. The mortality rate of late-onset sepsis caused by Gram-negative bacteria in VLBW infants is up to 36% [6]. It is an important and urgent problem to reduce or prevent severe bacterial infection among neonates.

Neutrophils play an important role in innate immune defense against microorganisms. Neutrophils migrate from peripheral blood into infection sites, recognize microorganisms, and initiate antimicrobial activities. In addition to oxygen-dependent bactericidal systems, neutrophils have oxygen-independent antimicrobial proteins or peptides (AMP) [7]. These include primary, secondary (specific) or tertiary granule AMP [8]. The combination of AMP, including primary AMP α- or β-defensin and secondary AMP cathelicidin, is one of the major components of this defense system against microbial infection in humans [7–9].

Human cathelicidin antimicrobial peptide of 18 kDa (hCAP18), also called LL-37 or FALL-39 [10–12], is the only known human cathelicidin [13]. hCAP18 is transcribed and translated in promyelocyte to myelocyte stages

of myeloid differentiation in bone marrow (BM). The precursor of cathelicidin consists of an N-terminal signal peptide, cathelin domain, and C-terminal antimicrobial peptide (LL-37), which is activated by cleavage from the cathelin portion [12, 14], and is stored in secondary or specific granules of neutrophils [15]. hCAP18 is also expressed in the squamous epithelia [16], epididymis [17] and lungs [18]. Expression of hCAP18 in epithelial cells is inducible by infection and inflammation [19]. Neutrophil gelatinase-associated lipocalin (NGAL) is also a specific granule protein [8, 9]. Cationic AMP, including hCAP18, bind to the microbial cell membrane, change its permeability, disrupt the metabolism of bacteria and fungi, and kill them [20]. NGAL exerts its antimicrobial activities by binding bacterial catecholate-type ferric siderophores, leading to iron depletion [21, 22]. hCAP18 shows antimicrobial activities against herpes simplex virus and human immunodeficiency virus (HIV) [23, 24]. AMP quickly kill microbes and are supposed to be effective in killing antibiotic-resistant bacteria. In addition to antimicrobial actions, hCAP18 has been shown to regulate inflammatory and immune responses. Some of these activities include neutralization of lipopolysaccharide [10], chemoattraction of neutrophils, T cells [25] or mast cells [26], induction of differentiation of dendritic cells [27] and suppression of neutrophil apoptosis [28]. Specific-granule deficiency (SGD), which showed inactivation mutations of myeloid-specific transcription factor, CCAAT/enhancer binding protein (C/EBP) ε, and was deficient in neutrophil granular proteins, including hCAP18, showed immunodeficiency against bacteria and fungi from infancy [29, 30].

One reason why neonates are at high risk for bacterial infections is that they show developmental delay in maturation of the innate immune system, including chemotaxis and killing activities of neutrophils [31, 32]. From the aspect of killing activities, reactive oxygen species (ROS) production by nicotinamide adenine dinucleotide phosphate oxidase is the main oxygen-dependent bactericidal action and plays an important role, but the ability of ROS production of neutrophils in neonates is reported to be a similar level to adults [33]. Reports on oxygen-independent antimicrobial activities in neutrophils of neonates are limited. In this study, we focused on neutrophil secondary granule peptide, hCAP18 expression, in neonates, which plays an important role in oxygen-independent antimicrobial activities.

2 Materials and methods

2.1 Samples and cell culture

This study was approved by the Committee for Medical Ethics of Shinshu University School of Medicine.

Samples from normal healthy volunteers were obtained after receiving informed consent from them or their parents. Twenty-five neonates and adults were studied. Neonate samples were obtained from 0 to 5 days after birth (median 2 days, male $n = 13$, female $n = 12$), and adult samples were from 21- to 46-year-olds (median 28 years old, male $n = 12$, female $n = 13$).

Human cord blood cells (CB) were obtained following informed consent, and incubated in α-minimum essential medium (α-MEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (FCS; HyClone, Logan, Utah, USA).

Human myeloid leukemia cell line U937 was cultured in α-MEM containing 10% FCS either with or without 10^{-7} M of 1α,25 dihydroxy vitamin D_3 (VD) or its derivative 1α,25 dihydroxy-22-oxacalcitriol (OCT) (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) for 24 or 48 h.

2.2 Flow cytometric analysis and cell sorting

Monoclonal antibodies (mAb) for fluorescein isothiocyanate (FITC)-CD13, phycoerythrin (PE)-CD11b, peridinin chlorophyll protein (PerCP)-CD45, and hCAP18, NGAL were purchased from BD immunocytometry systems (Mountain View, CA, USA) and HyCult Biotechnology (Uden, The Netherlands), respectively. For the analysis of cytoplasmic hCAP18 or NGAL expression in peripheral blood (PB) neutrophils, CB, or cell line, 1–2 $\times$ 10^6 whole blood cells or U937 cells were collected in polystyrene tubes and incubated with appropriately diluted PerCP-CD45, FITC-conjugated goat anti-mouse IgG and hCAP18 or NGAL mAbs, in the same method as described previously [34]. The cells were washed twice and analyzed with a FACScan flow cytometer, using the Lysis II software program (BD Immunocytometry Systems). Viable cells were gated according to their forward light-scatter characteristics (FSC) and side-scatter characteristics (SSC). For cell sorting, BM, and CB mononuclear cell were stained with FITC-CD13 and PE-CD11b. $CD13^+CD11b^-$ population was sorted by FACSVantage (BD Immunocytometry Systems).

2.3 Analysis of protein expression

Western blot analysis was performed essentially as described previously [35]. Total cell lysates were electrophoresed through 12% polyacrylamide–sodium dodecyl sulfate (SDS) gels. hCAP18 (HyCult Biotechnology), C/EBPε (C-22; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (C-2; Santa Cruz Biotechnology) antibodies were used at 0.2 μg/ml.

2.4 Measurement of plasma concentration of LL-37 and NGAL by enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of LL-37 and NGAL were analyzed using the Human hCAP18/LL-37 ELISA test kit (Hycult Biotechnology) and the NGAL ELISA kit (Antibodyshop, Gentofte, Denmark), respectively. We followed the assay procedures recommended by each manufacturer.

2.5 Real-time reverse-transcribed (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from CB cells treated with 10^{-7} M of VD for 48 h, or BM and CB $CD13^{+}$, $CD11b^{-}$ cells using Isogen reagent (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. RNA (200 ng) was reverse-transcribed using random-hexamer primers and avian myeloblastosis virus (AMV)-reverse transcriptase (Takara, Tokyo, Japan). The quantitation of mRNA levels used a real-time fluorescence detection method according to the manufacturer's protocol. The specific primer pair and probe for hCAP18, C/EBPε, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes, respectively, were from the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). Real-time PCR was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using the primer pair and an oligonucleotide probe with a 5′ fluorescein reporter dye (FAM), and a 3′ non-fluorescent quencher conjugated with a 3′ minor groove binder (MGB). In each experiment, duplicates of cDNA from samples in a 50-μl reaction mixture containing 1× Universal PCR Master Mix (Applied Biosystems), 900 nM of the primer pair, 250 nM of an oligonucleotide probe with a 5′ fluorescent reporter dye, and a 3′ quencher dye, were used. Relative gene expression was determined based on the threshold cycles of the genes for C/EBPε, hCAP18, and GAPDH. Comparative Ct method was applied for VD experiment and CB cells without VD treatment were used as the standard. The assays were performed in triplicate, and mean values of the three experiments are given.

2.6 $1\alpha(OH)D_3$ administration in patients with rickets of prematurity

Neonates with roentgenographic findings with flaring and cupping in the distal region of the radius and ulnal bones were diagnosed with rickets, and oral administration of 0.1 μg/kg/day of $1\alpha(OH)D_3$ (alfacalcidol; Chugai Pharmaceutical Co., Ltd) was started after diagnosis. PB was drawn from patients before and after 4-week $1\alpha(OH)D_3$ administration. Simultaneously, PB from control babies was drawn at 4-week intervals, and hCAP18 expression was analyzed by flow cytometry.

2.7 Statistical analysis

A non-parametric test using the Bonferroni/Dunn method was used to evaluate the age-dependent expression of AMP. The Mann–Whitney *U* test was applied for other experiments using StatView software (version 5.0; Cary, NC).

3 Results

3.1 Comparison of hCAP18 expression in neutrophils of neonates and adults

We compared the percentages of hCAP18 expression in peripheral blood (PB) neutrophils of neonates and adults by flow cytometry. Whole PB was stained with PerCP-conjugated CD45 Ab and FITC-conjugated hCAP18 Ab. Stained cells were developed according to side-scattered characteristics (SSC) and fluorescence intensities of PerCP, and the cell population of neutrophils was gated and analyzed for hCAP18 expression. These cell populations were positive for CD16, which is a low-affinity Fc gamma receptor (FcγRIII) [36] (data not shown). Figure 1a and b show typical analysis results. In neonates, neutrophils stained with isotype-matched IgG control Ab or hCAP18 Ab were positive for hCAP18 expression in 1.3 and 6.4%, respectively (Fig. 1a). In adults, IgG control or hCAP18 Ab-stained neutrophils were positive for hCAP18 expression in 1.2 and 99.3%, respectively (Fig. 1b). Figure 1c shows the results of 25 samples of PB from neonates and adults, respectively. The mean percentage of neonates was 37.1% [95% confidence interval (CI) 30.1–44.2] with a median of 35.4% [interquartile range (IQR) 21.6–43.7]. The mean percentage of adults was 85.2% (95% CI: 79.5–90.9) with a median of 89.9% (IQR 82.4–94.4) ($P < 0.0001$). We also compared the expression of another neutrophil secondary granule protein, NGAL [21, 37], in the same manner (Fig. 1c, right panel). The mean percentage of neonates was 49.9% (95% CI 40.8–59.1) with a median of 45.8% (IQR 29.9–58.8). In adults, the mean percentage was 93.0% (95% CI 90.4–95.5) with a median of 96.1% (IQR 89.4–97.2) ($P < 0.0001$). These data showed that the expression of neutrophil secondary granule proteins hCAP18 and NGAL was significantly reduced in neonates compared to adults.

Next, we compared the expression of hCAP18 and NGAL in neutrophils using the mean fluorescence intensity (MFI) ratio (MFI of each granular protein divided by that of IgG control) in 25 samples of neonates and adults,

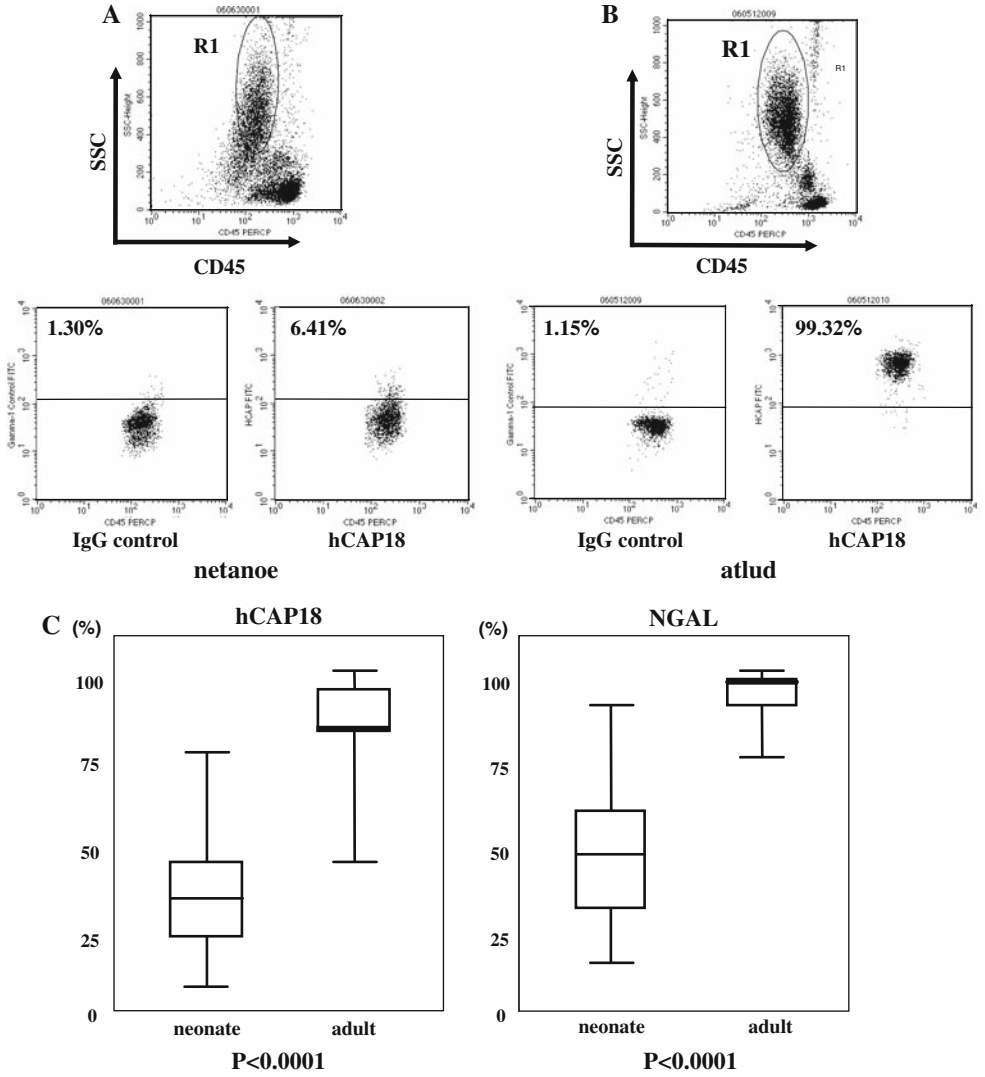

Fig. 1 Comparison of percentages of hCAP18 and NGAL expression in neutrophils derived from 25 neonates and adults, respectively. PB cells derived from neonates (**a**) or adults (**b**) were stained with CD45-PerCP and FITC-conjugated IgG control Ab or hCAP18 Ab. Flow cytometric analysis was then performed. *Upper panels* show *dot* blots of total PB cells developed by intensities of CD45-PerCP and SSC. Neutrophil population was gated in circles as indicated by R1. *Lower panels* show *dot* blot analysis of cells gated as R1 stained with IgG control Ab (*left*) or hCAP18 Ab (*right*), respectively. *Figures* show representative results of flow cytometric analysis. Percentages of positive cells are indicated in *upper left* of each panel. **c** Percentages of hCAP18 or NGAL expression in neutrophils derived from 25 neonates and adults revealed by flow cytometric analysis are shown by *box* and *whisker plot*. Values are expressed as the median (*horizontal line in each box*), with the quartiles (*top and bottom of the box*) and range (I bar). Statistical analysis was performed and *P* values indicated at the bottom of each figure

respectively. MFI showed relative levels of hCAP18 content per neutrophil. Figure 2a shows typical analysis results. The mean MFI ratio in hCAP18 expression of neonates was 3.33 (95% CI 2.82–3.83) with a median of 3.20 (IQR 2.45–4.13). In adults, the mean MFI ratio was 6.63 (95% CI 5.67–7.59) with a median of 6.11 (IQR 3.01–7.54) ($P < 0.0001$) (Fig. 2b). The mean MFI ratio in NGAL expression of neonates was 8.88 (95% CI 6.73–11.04) with a median of 8.11 (IQR 5.09–10.78). In adults, the mean MFI ratio was 38.52 (95% CI 33.21–43.83) with a median of 36.45 (IQR 28.79–45.67) ($P < 0.0001$) (Fig. 2b). These results also showed reduced expressions of hCAP18 and NGAL in neutrophils of neonates compared with adults. In contrast, the MFI ratio of neutrophil primary granule protein, myeloperoxidase (MPO), was not significantly different between neonates and adults. The mean MFI ratio of neonates was 6.20 (95% CI 5.57–6.80) with a median of 6.08 (IQR 5.15–6.95). In adults, the mean MFI ratio was 7.18 (95% CI 6.42–7.94) with a median of 7.06 (IQR 5.98–7.94) ($P = 0.0523$) (Fig. 2b, right panel).

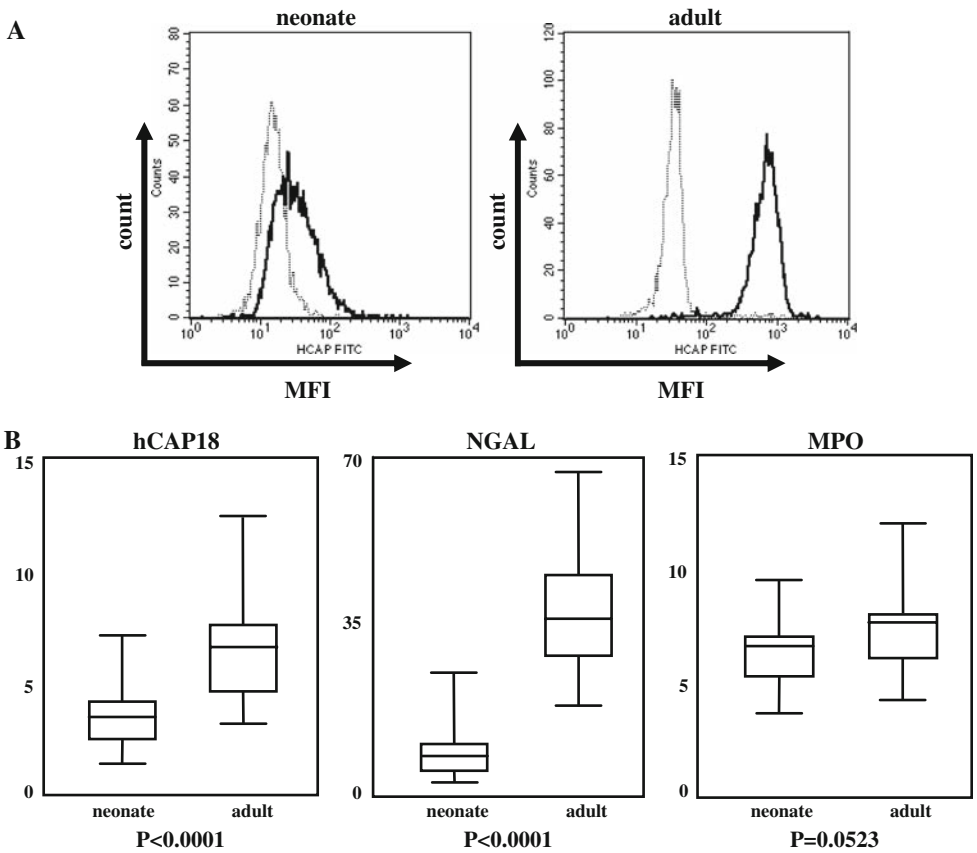

Fig. 2 Comparison of mean fluorescence intensity (*MFI*) ratio of hCAP18, NGAL and MPO in 25 neonates and adults, respectively. Neutrophils were stained with anti-hCAP18 Ab and secondary FITC-conjugated anti-mouse IgG, and flow cytometric analysis was performed. *Upper panels* show histograms of mean fluorescence intensities of hCAP18 expression in neonates (*left panel*) and adults (*right panel*) (**a**). *Solid line* and *bold line* indicate control IgG- and anti-hCAP18 Abs-treated cells, respectively. Figures showed representative result of flow cytometric analysis. *Lower panels* show the ratios between MFI derived from hCAP18, NGAL or MPO Abs-stained cells, and IgG control Ab-stained cells of neonates and adults, respectively, are shown by *box* and *whisker plot* in each panel (**b**). Statistical analysis was performed and *P* values indicated at the bottom of each figure

3.2 Comparison of hCAP18 expression in monocytes of neonates and adults

hCAP18 is also expressed in PB monocytes as well as in neutrophils [38]. Next, we examined the expression of hCAP18 in monocytes of neonates and adults of the same individuals used in Figs. 1 and 2 (Fig. 3). The mean percentage of neonates was 19.0% (95% CI 14.5–23.4) with a median of 17.5% (IQR 9.8–25.4). In adults, the mean percentage was 22.6% (95% CI 17.6–27.7) with a median of 20.9% (IQR 15.5–30.4) ($P = 0.2646$). The mean MFI ratio of neonates was 1.41 (95% CI 1.25–1.57) with a median of 1.32 (IQR 1.13–1.68). In adults, the mean MFI ratio was 1.37 (95% CI 1.12–1.62) with a median of 1.40 (IQR 0.89–1.69) ($P = 0.5936$). These results showed that hCAP18 was expressed at the same level in PB monocytes of neonates and adults.

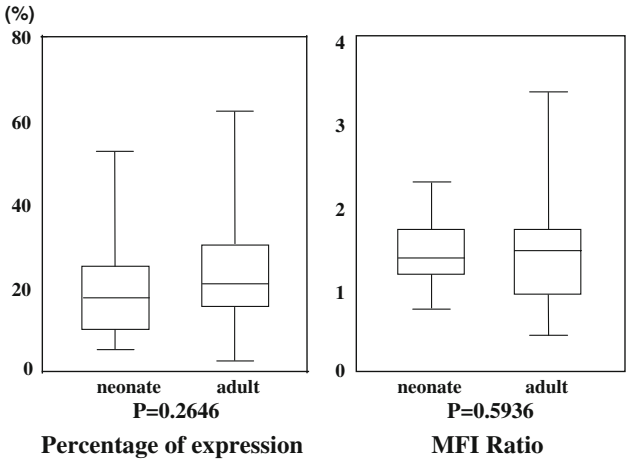

Fig. 3 Comparison of percentages and MFI ratio of hCAP18 and NGAL expression in PB monocytes derived from neonates and adults, respectively. Percentage and MFI ratios of hCAP18 expression in monocytes revealed by flow cytometric analysis are showed by *box* and *whisker plot*. Statistical analysis was performed and *P* values indicated at the *bottom* of each figure

3.3 Age-dependent expression of hCAP18 and NGAL

The results that hCAP18 and NGAL expressions in adults were more abundant than in neonates suggested the age-dependent increase of these peptide expressions. We therefore compared hCAP18 and NGAL expressions in neutrophils according to age (Fig. 4a, b). PB from five generations was separated by age as follows: (1) days 0–7 after birth, (2) 1 week to 1 month, (3) 1 month to 1 year, (4) 1 year to 15 years, and (5) over 15 years, and examined these peptide expressions, and expression levels were compared with (1) days 0–7 after birth. Mean percentages of hCAP18 expression in each generation were 37.1, 48.6

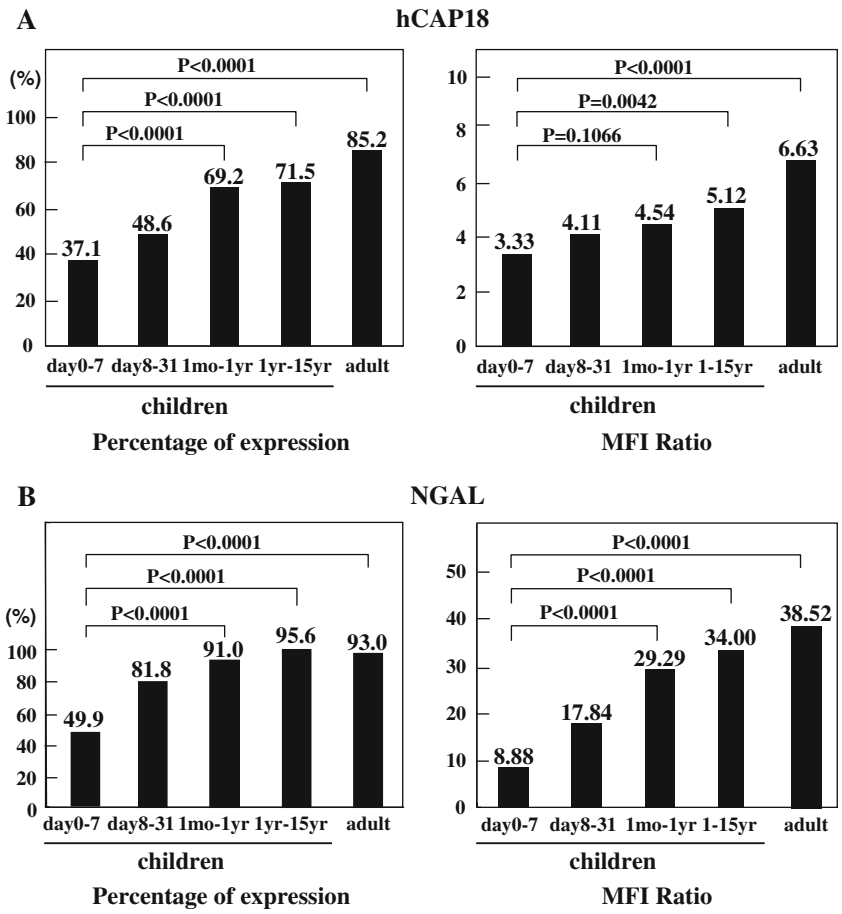

Fig. 4 Age dependence of hCAP18 and NGAL expression in neutrophils. Neutrophils derived from each generation of (1) days 0–7 after birth, (2) days 8–31, (3) 1 month to 1 year, (4) 1–15 years, and (5) 15–48 years old, were analyzed for the percentage (*left panel*) and MFI ratio (*right panel*) of hCAP18 (**a**) and NGAL (**b**) expression. Statistical analysis was performed and *P* values indicated in each panel

($P = 0.1462$), 69.2 ($P < 0.0001$), 71.5 ($P < 0.0001$), and 85.2% ($P < 0.0001$), respectively. Mean MFI ratios in each generation were 3.33, 4.11 ($P = 0.3215$), 4.54 ($P = 0.1066$), 5.12 ($P = 0.0042$), and 6.63 ($P < 0.0001$), respectively. hCAP18 expressions in each generation of (3) 1 month to 1 year and (4) 1 year to 15 years, in addition to (5) over 15 years, were significantly more abundant than in (1) days 0–7 after birth, revealed by both percentage and MFI ratio analysis. The expression of NGAL was also shown to be an age-dependent increase, as observed in hCAP18 (Fig. 4b). These results revealed that expressions of both hCAP18 and NGAL in neutrophils had increased significantly by 1 year after birth.

3.4 Plasma concentration of LL-37 and NGAL

hCAP18 and NGAL are known to be expressed in epithelial cells as well as in neutrophils and excreted in plasma, urine, or salivary glands [39–41]. We measured plasma concentrations of LL-37, an active form of hCAP18, and NGAL in neonates and adults by ELISA (Fig. 5). Mean concentrations of LL-37 in five neonates or adults were 1.08 and 1.38 μg/ml, respectively ($P = 0.7511$). Mean concentrations of NGAL in five neonates or adults were 93.55 and 94.05 ng/ml, respectively ($P = 0.4647$). These results showed that plasma concentrations of LL-37 and NGAL were not significantly different between neonates and adults. There was no obvious correlation between plasma concentration and neutrophil expression of both AMP.

3.5 Oral 1α(OH)D_3 induced hCAP18 expression in neonatal neutrophils

VD has been shown to induce hCAP18 expression in acute myeloid cell line HL-60 as well as in bone marrow (BM)-derived macrophages and fresh BM cells independent of C/EBPε [42–45]. We cultured a leukemic cell line, U937 cells, with 10^{-7} M of VD or a derivative of VD, 1,25$(OH)_2$-22-oxacalcitriol (OCT), for 48 h, and analyzed the expression of hCAP18 by flow cytometry. hCAP18 expression in U937 cells cultured with VD or OCT for 48 h increased 2.2 and 2.1 times, respectively, compared to those cultured without these reagents (Fig. 6a). Western blot analysis of hCAP18 expression in U937 cells cultured with VD or OCT for 24 and 48 h revealed the time-dependent increase of hCAP18 expression compared to before adding these reagents, in the same manner as

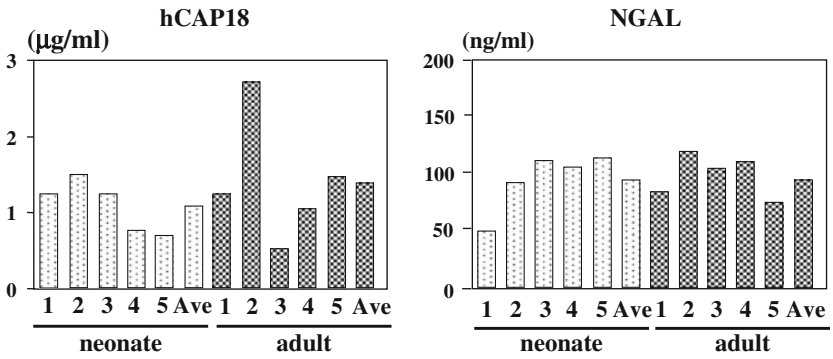

Fig. 5 Comparison of plasma concentrations of LL-37 and NGAL. Plasma concentrations of LL-37 and NGAL in five neonates and adults, respectively, were measured by ELISA, and shown by *bars* in each panel. Ave, average levels of LL-37 and NGAL concentrations in five samples of either neonates or adults

observed by flow cytometric analysis (Fig. 6b). Expression of C/EBPε was also observed in U937 cells cultured with VD or OCT for 24 and 48 h. Expression of hCAP18 mRNA in five CB samples was revealed to be up-regulated 16.8-fold by culture with 10^{-7} M of VD for 48 h (Fig. 6c). These results led us to examine whether $1\alpha(OH)D_3$ (alfacalcidol) administration could induce hCAP18 expression in neonatal neutrophils. Neutrophils of four patients with premature rickets before and after treatment for 4 weeks with 0.1 μg/kg/day of $1\alpha(OH)D_3$ were analyzed for hCAP18 expression by flow cytometry (Fig. 6d). As a control, neutrophils from healthy babies without $1\alpha(OH)D_3$ treatment were used. The percentages of hCAP18 expression in control or rickets babies before $1\alpha(OH)D_3$ administration were 21.8 and 24.2%, respectively ($P = 0.807$). hCAP18 expression in control neutrophils from healthy babies after 4 weeks was 43.5%. On the other hand, that in rickets patients treated with $1\alpha(OH)D_3$ increased to 83.8%. hCAP18 expression was significantly increased in babies treated with $1\alpha(OH)D_3$ compared to control babies without $1\alpha(OH)D_3$ treatment ($P = 0.014$).

4 Discussion

In this study, we showed that the expression of neutrophil-specific granule protein hCAP18 as well as NGAL was impaired in neonates compared to in adults, and that $1\alpha(OH)D_3$ could induce hCAP18 expression in neonates. Relative levels of hCAP18 or NGAL-containing neutrophils in neonates were both about 44 and 54%, respectively, compared to those in adults. Studies of neutrophil function in neonates have focused on chemotaxis, adherence, or phagocytosis. Neutrophil adherence and chemotaxis were significantly impaired in neonates compared to adults [31, 32]. Neutrophil phagocytosis in neonates was almost equal to that in adults, but bactericidal ability decreased with the amount of lactoferrin, which was associated with the production of $OH^{\cdot}$ [33, 46]; however, studies on oxygen-independent bactericidal mechanisms of neonatal neutrophils are limited. One report on antimicrobial peptide stated that the content of bactericidal/permeability-increasing protein (BPI), which is a primary granule peptide, is reduced in neutrophils of neonates [47]. hCAP18 and NGAL are synthesized and stored in secondary or specific granules of neutrophils and secreted into phagosomes in which other bactericidal peptides, or ROS are released against environmental microbes. Neutrophils in neonates with less cellular hCAP18 and NGAL content compared to those in adults are suggested to have disadvantages at inflammation sites and are predicted to be associated with decreased activity against pathogens and susceptibility to bacterial infection. Plasma concentrations of LL-37 and NGAL, however, were not significantly different in neonates and adults. One reason for the same level of these proteins in plasma of neonates and adults despite different peptide expression levels in neutrophils might be that the neutrophil count in neonates was approximately three times greater than in adults. Another possible reason is that the expression and excretion of these peptides in epithelial cells are not different between neonates and adults. Further studies are needed to ascertain the differences in plasma levels of these AMP, using more samples. Neutrophils function in innate immunity to bacterial infection, not in blood flow, but mainly in inflamed tissues. Concentrations of antimicrobial peptide at inflammatory sites are more elevated than at non-inflammatory sites [48]. It is suggested that more neutrophils are required in neonates than in adults at inflammation sites to reach sufficient hCAP18 or NGAL concentration to have antimicrobial activity. Interestingly, the level of hCAP18 expression in neonatal neutrophils varied. Three of 25 neonates had an equal amount of hCAP18 compared to adults; however, 24% of neonate neutrophils were markedly deficient in hCAP18 expression (<20%). Furthermore, hCAP18 expression in two of these six samples was less than 10%. These neonates also had a low percentage of NGAL expression. It is possible that neonates with a low content of these antimicrobial peptides are at high risk for severe bacterial infection.

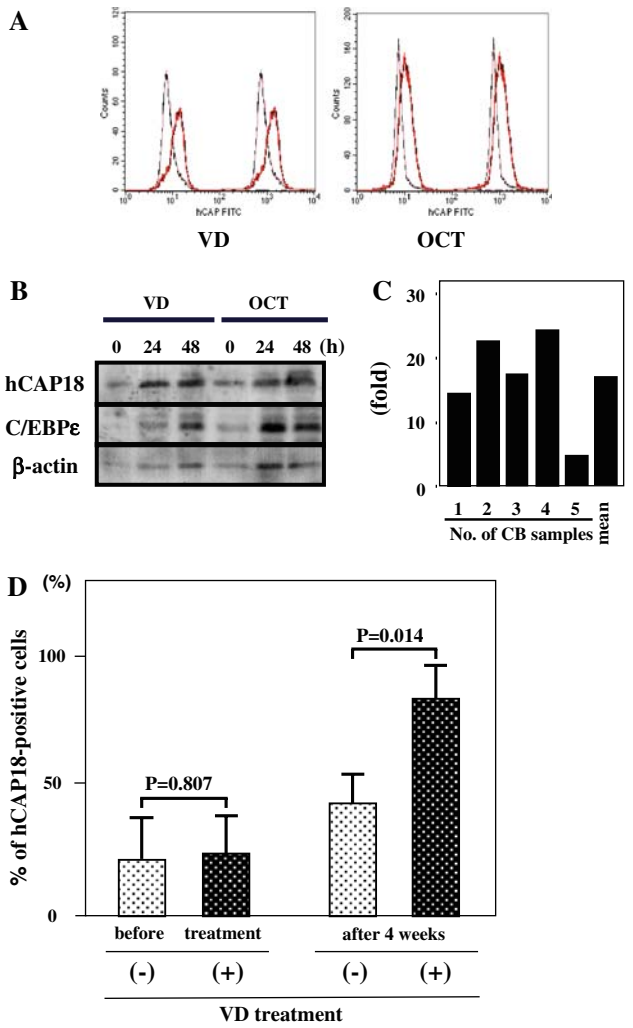

Fig. 6 Effects of vitamin D on the expression of hCAP18. **a** U937 cells were treated with 1×10^{-7} M of 1α,25 dihydroxyvitamin D_3 (*VD*) or its derivative 1α,25 dihydroxy-22-oxacalcitriol (*OCT*) for 48 h. Cells were stained with anti-hCAP18 Ab and secondary FITC-conjugated anti-mouse IgG, and flow cytometric analysis was performed. Solid line and bold line indicate untreated and VD- or OCT-treated cells, respectively. **b** Total cell lysates of U937 cells treated with 1×10^{-7} M of VD or OCT for 24 or 48 h were analyzed by Western blotting for hCAP18 and C/EBPε expressions. Subsequent probing of the same blot for β-actin demonstrated equivalent loading of protein in each lane. **c** CB cells (1×10^7 cells per sample) from five individuals were untreated or treated with 1×10^{-7} M of VD for 48 h. Real-time PT-PCR was performed using primers and probe for hCAP18 and GAPDH. Each bar represented fold increase of relative expression of hCAP18 in each cell treated with VD compared to untreated cells. Bar on right side shows the mean and SD values of fold increase derived from five CB samples. **d** Effects of 1α(OH)D_3 treatment of rickets patients on hCAP18 expression in PB neutrophils were analyzed as described in "Materials and methods". Percentage of CAMP expression between rickets patients before or after treatment with 0.01 μg/kg/day of 1α(OH)D_3 for 4 weeks and age-matched control healthy neonates without treatment was compared by flow cytometric analysis. Statistical analysis was performed and *P* values indicated in the panel

hCAP18 and NGAL expressions were age-dependent. The percentage of hCAP18 expression was low within 31 days after birth, but increased to the adult level after 1 month of age. Neutrophil primary granule protein, BPI, or specific granule protein lactoferrin, are also reported to be deficient in neonate neutrophils [46, 47]. These findings suggest that the final differentiation, but not proliferation, of myeloid cells in bone marrow is not completed in neonates. Neutrophil granules are reported to be formed sequentially along with myeloid differentiation. Primary granules formed at the promyelocyte stage have a high content of MPO. Secondary and tertiary granule formation, which is peroxidase-negative, starts at myelocyte to metamyelocyte stages and bands to segmented neutrophils, respectively, in myelopoiesis. Bud and transport of vesicles containing hCAP18 from Golgi might be incomplete in the bone marrow of neonates. As shown in prior studies [30, 49], myeloid-specific transcription factor C/EBPε is closely related to the induction of hCAP18 and NGAL expression in human neutrophils. Moreover, BPI and lactoferrin, the expressions of which are regulated by C/EBPε, are also reduced in neonates [34, 50]. On the other hand, primary granule protein MPO, which is regulated independently of C/EBPε, was not reduced in neonates. These findings suggested that differences in the expression of C/EBPε in myeloid progenitors between neonates and adults might cause different levels of antimicrobial peptides, but expression levels of C/EBPε in $CD13^+CD11b^-$ cells, which were promyelocyte to myelocyte stages of myeloid differentiation in morphology, were not different between CB and BM, as revealed by real-time RT-PCR (data not shown). Translational levels of C/EBPε might therefore be different between CB and BM. Further studies are needed to reveal the mechanism of differences in AMP expression between neonates and adults.

Plasma concentration of hCAP18 in SGD was extremely low, even in sepsis, as well as in non-inflammatory conditions (data not shown). Plasma concentration of NGAL in SGD under normal conditions was also low but increased to 500 ng/ml in sepsis, the concentration which is usually observed in inflammatory states of normal controls (data not shown). These observations suggest that the regulatory mechanism in the induction of hCAP18 and NGAL was not identical and that alternative mechanisms other than C/EBPε would induce NGAL expression in inflammatory conditions.

Induction of hCAP18 expression in neutrophils of rickets patients by 1α(OH)D_3 administration is a direct effect of immature myeloid progenitor cells in vivo. The etiology of prematurity in rickets is mainly calcium and phosphorus deficiency, but not VD deficiency [51]. Serum VD concentration in rickets at onset was not significantly different from

that of control babies (data not shown). It is supposed that the pharmaceutical, but not physiological, concentration of VD worked on myeloid progenitors. The percentage of hCAP18-positive neutrophils in rickets patients who had more than 50% of neutrophils with hCAP18 was not significantly changed by $1\alpha(OH)D_3$ administration (data not shown). These data suggest that VD worked more efficiently on neonates with immature neutrophils.

Neonates are susceptible to bacterial infections, including *Streptococcus*, *Escherichia coli,* and *Listeria* [1, 2], which can cause pneumonia, meningitis, and sepsis, leading to life-threatening complications. Moreover, antibiotic-resistant bacteria, such as *Staphylococcus aureus* and *Klebsiella pneumonia* have caused serious and life-threatening infections in immunocompromised hosts, including neonates [52, 53]. The development of novel therapeutic approaches against drug-resistant bacterial infection is an urgent problem. The innate immune system of the human body is a natural defense mechanism against invading microbes. Cationic antimicrobial peptides, including α-defensin, cathelicidin, and NGAL, play important roles in this system. Some of these peptides have been commercially developed as natural antibiotics [54, 55]. External administration or up-regulation of endogenous hCAP18 expression by VD in neonates is a potential prophylactic or therapeutic approach, contributing to the decrease of severe bacterial infection among neonates. Other compounds together with VD are reported to show cooperative activities with hCAP18 expression; for example, sodium butyrate, a histone deacetylase inhibitor, showed an additive effect of hCAP18 expression by VD alone in U937 cells [44]. Using a reagent acting cooperatively with VD will be beneficial for the effective induction of hCAP18. Screening babies for a lower expression of hCAP18 will be beneficial to identify high risks of infection and candidates for therapy with natural antibiotics.

Conflict of interest statement The authors declare no competing financial interests.

References

1. Polin RA, Parravicini E, Regan JA, Taeusch HW. Bacterial sepsis and meningitis. In: Taeusch HW, Ballard RA, Gleason CA, editors. Avery's diseases of the newborn. 8th ed. Philadelphia: Elsevier Saunders; 2005. p. 551–77.
2. Stoll BJ. Infections of the neonatal infant. In: Kliegman RM, Jenson HB, Behrman RE, Stanton BF, editors. Textbook of pediatrics. 18th ed. Philadelphia: Elsevier Saunders; 2007. p. 794–811.
3. Takimoto H, Yokoyama T, Yoshiike N, Fukuoka H. Increase in low-birth-weight infants in Japan and associated risk factors, 1980–2000. J Obstet Gynaecol Res. 2005;31:314–22.
4. Baltimore RS. Perinatal bacterial and fungal infections. In: Jenson HB, Baltimore RS, editors. Pediatric infectious diseases. 2nd ed. Philadelphia: W.B. Saunders; 2002. p. 1119–34.
5. Gaynes RP, Edwards JR, Jarvis WR, Culver DH, Tolson JS, Martone WJ. Nosocomial infections among neonates in high-risk nurseries in the United States. National nosocomial infections surveillance system. Pediatrics. 1996;98:357–61.
6. Lewis DB, Wilson CB. Developmental immunology and role of host defences in neonatal susceptibility to infection. In: Remington JS, Klein JO, editors. Infectious diseases of the fetus and newborn infant. 4th ed. Philadelphia: Saunders; 2000. p. 20–99.
7. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood. 1997;89:3503–21.
8. Gullberg U, Andersson E, Garwicz D, Lindmark A, Olsson I. Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development. Eur J Haematol. 1997;58:137–53.
9. Borregaard N, Theilgaard-Monch K, Sorensen OE, Cowland JB. Regulation of human neutrophil granule protein expression. Curr Opin Hematol. 2001;8:23–7.
10. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect Immun. 1995;63:1291–7.
11. Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG, Gudmundsson GH. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. Proc Natl Acad Sci USA. 1995;92:195–9.
12. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood. 2001;97:3951–9.
13. Durr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta. 2006;1758:1408–25.
14. Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. 1995;374:1–5.
15. Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood. 1997;90:2796–803.
16. Frohm-Nilsson M, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M. The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect Immun. 1999;67:2561–6.
17. Malm J, Sorensen O, Persson T, Frohm-Nilsson M, Johansson B, Bjartell A, et al. The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect Immun. 2000;68:4297–302.
18. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci USA. 1998;95:9541–6.
19. Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, et al. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem. 1997;272:15258–63.
20. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria ? Nat Rev Microbiol. 2005;3:238–50.
21. Kjeldsen L, Bainton DF, Sengelov H, Borregaard N. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood. 1994;83:799–807.
22. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002;10:1033–43.

23. Gordon YJ, Huang LC, Romanowski EG, Yates KA, Proske RJ, Dermott AM. Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. Curr Eye Res. 2005; 30:385–94.
24. Bergman P, Walter-Jallow L, Broliden K, Agerberth B, Soderlund J. The antimicrobial peptide LL-37 inhibits HIV-1 replication. Curr HIV Res. 2007;5:410–5.
25. Yang D, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med. 2000;192:1069–74.
26. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. Immunology. 2002;106:20–6.
27. Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J Immunol. 2004;172:1146–56.
28. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. J Immunol. 2006;176:3044–52.
29. Lekstrom-Himes JA, Dorman SE, Kopar P, Holland SM, Gallin JI. Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein ε. J Exp Med. 1999;189:1847–52.
30. Gombart AF, Shiohara M, Kwok SH, Agematsu K, Komiyama A, Koeffler HP. Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein-ε. Blood. 2001;97:2561–7.
31. Klein RB, Fischer TJ, Gard SE, Biberstein M, Rich KC, Stiehm ER. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics. 1977;60:467–72.
32. Krause PJ, Herson VC, Boutin-Lebowitz J, Eisenfeld L, Block C, Lobello T, et al. Polymorphonuclear leukocyte adherence and chemotaxis in stressed and healthy neonates. Pediatr Res. 1986;20:296–300.
33. Ambruso DR, Stork LC, Gibson BE, Thurman GW. Increased activity of the respiratory burst in cord blood neutrophils: kinetics of the NADPH oxidase enzyme system in subcellular fractions. Pediatr Res. 1987;21:205–10.
34. Tanaka M, Gombart AF, Koeffler HP, Shiohara M. Expression of bactericidal/permeability-increasing protein requires C/EBPε. Int J Hematol. 2007;85:304–11.
35. Shiohara M, Taniguchi S, Masumoto J, Yasui K, Koike K, Komiyama A, et al. ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. Biochem Biophys Res Commun. 2002;293:1314–8.
36. Gessner JE, Grussenmeyer T, Kolanus W, Schmidt RE. The human low affinity immunoglobulin G Fc receptor III-A and III-B genes. Molecular characterization of the promoter regions. J Biol Chem. 1995;270:1350–61.
37. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993;268:10425–32.
38. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, et al. The human antimicrobial and chemotactic peptides LL-37 and alfa-defensins are expressed by specific lymphocyte and monocyte populations. Blood. 2000;96:3086–93.
39. Sorensen O, Bratt T, Johnsen AH, Madsen MT, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma. J Biol Chem. 1999;274:22445–51.
40. Nielsen BS, Borregaard N, Bundgaard JR, Timshel S, Sehested M, Kjeldsen L. Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut. 1996;38:414–20.
41. Woo JS, Kim KM, Kang JS, Zodpe P, Chae SW, Hwang SJ, et al. Expression of neutrophil gelatinase-associated lipocalin in human salivary glands. Ann Otol Rhinol Laryngol. 2007;116:599–603.
42. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. FASEB J. 2005;19:1067–77.
43. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science. 2006;311:1770–3.
44. Gombart AF, O'Kelly J, Saito T, Koeffler HP. Regulation of the CAMP gene by 1, 25(OH)2D3 in various tissues. J Steroid Biochem Mol Biol. 2007;103:552–7.
45. Liu PT, Stenger S, Tang DH, Modlin RL. Vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. J Immunol. 2007;179:2060–3.
46. Ambruso DR, Bentwood B, Henson PM, Jr. Johnston RB. Oxidative metabolism of cord blood neutrophils: relationship to content and degranulation of cytoplasmic granules. Pediatr Res. 1984;18:1148–53.
47. Levy O, Martin S, Eichenwald E, Ganz T, Valore E, Carroll SF, et al. Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein. Pediatrics. 1999;104:1327–33.
48. Bowdish DM, Davidson DJ, Hancock RE. A re-evaluation of the role of host defence peptides in mammalian immunity. Curr Protein Pept Sci. 2005;6:35–51.
49. Gombart AF, Kwok SH, Anderson KL, Yamaguchi Y, Torbett BE, Koeffler HP. Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBPε and PU.1. Blood. 2003;101:3265–73.
50. Khanna-Gupta A, Zibello T, Sun H, Gaines P, Berliner N. Chromatin immunoprecipitation (ChIP) studies indicate a role for CCAAT enhancer binding proteins alpha and epsilon (C/EBPα and C/EBPε) and CDP/cut in myeloid maturation-induced lactoferrin gene expression. Bood. 2003;101:3460–8.
51. Greenbaum LA. Rickets and hypervitaminosis. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF, editors. Textbook of pediatrics. 18th ed. ed. Philadelphia: Elsevier Saundars; 2007. p. 253–63.
52. Chuang YY, Huang YC, Lee CY, Lin TY, Lien R, Chou YH. Methicillin-resistant Staphylococcus aureus bacteraemia in neonatal intensive care units: an analysis of 90 episodes. Acta Paediatr. 2004;93:786–90.
53. Crivaro V, Bagattini M, Salza MF, Raimondi F, Rossano F, Triassi M, et al. Risk factors for extended-spectrum beta-lactamase-producing Serratia marcescens and Klebsiella pneumoniae acquisition in a neonatal intensive care unit. J Hosp Infect. 2007;67:135–41.
54. Hancock RE, Patrzykat A. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr Drug Targets Infect Disord. 2002;2:79–83.
55. Zasloff M. Antimicrobial peptides in health and disease. N Engl J Med. 2002;347:1199–200.