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# Cell surface antigens of neonatal monocytes are selectively impaired in basal expression, but hyperresponsive to lipopolysaccharide and zymosan

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ABSTRACT

Toll-like receptors (TLRs) are important components of the innate immune system, but how neonatal TLR-mediated immune responses differ from those of adults is unknown. We aimed to clarify the TLR-mediated expression profiles of cell surface antigens related to antigen presentation in neonates. CD14-positive monocytes were isolated from human cord blood and adult peripheral blood and then stimulated with lipopolysaccharide (LPS; TLR4 agonist) or zymosan (TLR2/6 agonist) or left unstimulated. Expression levels of the surface antigens major histocompatibility (MHC)-class II, CD80, CD86, CD11b, CD11c, CD14, and CD16 were then evaluated by flow cytometry. Cord blood $CD14^{+}CD16^{high}$ monocytes (CBM) showed significantly lower basal levels of MHC-class II, CD80, and CD11b than adult blood $CD14^{+}CD16^{intermediate}$ monocytes (ABM) ($P < 0.01$, $P < 0.001$, $P < 0.001$, respectively). LPS stimulation enhanced expression of MHC class II, CD80, and CD11b significantly more in CBM than in ABM ($P < 0.001$, $P < 0.01$, $P < 0.01$, respectively), resulting in levels that did not differ between CBM and ABM. Zymosan stimulation also enhanced expression of MHC class II, CD86, CD11b, and CD11c significantly more in CBM than in ABM ($P < 0.001$, $P < 0.01$, $P < 0.001$, $P < 0.01$, respectively), resulting in levels of CD86 and CD11c that did not differ in CBM and ABM. However, MHC class II, CD80, and CD11b remained significantly higher in ABM than in CBM ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively). These data indicate that CBM and ABM have distinct phenotypes and responses to stimulation.

## 1. Introduction

Gastrointestinal and respiratory infections cause over 40% of deaths in children younger than 5 years across the world (Liu et al., 2015; Tokuhara, 2018a). The ability of neonates and infants to acquire protective immunity after infection or immunization is poor; infection leading to severe outcomes (e.g., sepsis and pneumonia) is a major cause of neonatal death (˜20%) (Liu et al., 2015). Protective humoral immunity, represented as pathogen-specific systemic IgG and mucosal (e.g., respiratory and intestinal) IgA (Tokuhara, 2018a; Tokuhara et al., 2018b, 2010), is induced by maturation and class-switching of B cells and mediated via T cell–dependent and/or –independent pathways. As an initial step in both pathways, antigen-presenting cells (APCs), such as dendritic cells (DCs), recognize pathogen via toll-like receptors (TLRs); the APCs then process the pathogen-specific peptide, complex it with MHC class II antigen associated with co-stimulatory molecules (CD80 and CD86), and present it to T and B cells (Guermonprez et al., 2002). Upon stimulation by pathogen, APCs also produce cytokines (e.g., IL-6), which activate B cell maturation (Tokuhara et al., 2018b). Therefore, elucidation of immune responses related to antigen presentation by APCs in neonates and infants may provide keys to understanding the reasons for high susceptibility to infection in these populations and facilitate development of effective vaccines.

Previous immunological studies focusing on TLR-mediated cytokine profiles of neonatal APCs have used cord blood, which can be collected non-invasively from the placenta after birth and reflects neonatal immune system (Kollmann et al., 2009; Schüller et al., 2013; Levy et al., 2004; Nohmi et al., 2015; Yanai et al., 2016; Nguyen et al., 2010; De Wit et al., 2003). All of these studies indicated that TLR-mediated cytokine responses are not completely impaired in neonates but rather are

*Abbreviations:* APC, antigen presenting cell; DC, dendritic cell; mAb, monoclonal antibody; TLR, toll-like receptor

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selectively reduced or altered depending on gestational age, the types of APCs or TLRs, and plasma or maternal factors.

A previous study reported that neonatal monocytes show low basal expression of MHC class II compared with adult monocytes and smaller TLR 4-and TLR 9–mediated increases of MHC class II and CD80 expression (Nguyen et al., 2010), whereas another study reported no differences in basal expression of MHC class II, CD80, CD86, and CD40 between neonatal and adult DCs and smaller TLR-mediated increases in CD40 and CD80 expression in neonatal DCs than adult DCs (De Wit et al., 2003). These two studies indicate that basal and TLR-stimulated expression of cell surface molecules are selectively impaired in neonates, but further studies are necessary to confirm these issues. Therefore, we aimed to use purified cord blood monocytes to clarify the expressions of cell surface molecules related to antigen presentation in neonatal immune cells at rest and when stimulated. Our results show that neonatal monocytes ($CD14^+CD16^{high}$) have a phenotype distinct from that of adult monocytes ($CD14^+CD16^{intermediate}$). We also demonstrated low basal expression of MHC class II, CD80, and CD11b on neonatal monocytes; and we clarified the degree to which TLR4- and TLR2/6-stimulation enhanced expression of neonatal monocyte surface antigens compared with enhancement of these antigens in adult cells. These results contribute to our understanding of the neonatal innate immune system and could be the foundation for developing vaccine adjuvants for use in children.

## 2. Materials and methods

### 2.1. Blood samples

Heparinized cord blood (30 to 50 ml) was collected during elective caesarean sections in women with healthy, full-term pregnancies (37.8 ± 0.4 weeks gestational age, mean ± 1 SD, n = 15) and without premature rupture of membranes of infection. Heparinized peripheral blood (50 to 75 ml) was collected from healthy adults without infection (26.7 ± 3.0 years, mean ± 1 SD, n = 19, male/female = 12/7). Mononuclear cells were separated from the cord or adult peripheral blood within 2 h after blood collection. The study protocol conformed to the principles of the Declaration of Helsinki. The ethics committee of our institution approved the study protocol (No. 3598), and written informed consent was obtained before the procedures commenced.

### 2.2. Monocyte separation

Heparinized cord or adult blood was layered onto Lymphocyte Separation Medium (126-04871; Wako Pure Chemical Industries Ltd., Osaka, Japan) followed by centrifugation, and the mononuclear cell layer was collected. Monocytes were isolated from mononuclear cells by positive selection using magnetic microbeads coupled to an anti-CD14 monoclonal antibody (mAb) (130-050-201; Miltenyi Biotec, Auburn, CA, USA) in accordance with the manufacturer's instructions (Nohmi et al., 2015; Yanai et al., 2016). Approximately $1 \times 10^7$ CD14-positive monocytes were separated from 50 ml of cord blood. The purity of isolated monocytes (> 97% CD14+; > 99% cell viability) was confirmed by fluorescence-activated cell sorting (FACS; LSR II, BD Biosciences) using APC-conjugated anti-human CD14 mAb (BD Pharmingen Catalog No: 555399) (Nohmi et al., 2015).

### 2.3. TLR stimulations

Cord blood monocytes (CBM) and adult blood monocytes (ABM) were separately transferred to 24-well plates ($10^6$ cells/well) and incubated with the TLR4 ligand lipopolydsaccharide (LPS; 100 ng/ml; derived from *Escherichia coli* serotype O111 B4; Sigma) or the TLR2/6 ligand zymosan (10 μg/ml; IMG-2212, Imgenex) for 6 h, or incubated without treatment (control) at 37 °C in humidified air containing 5% $CO_2$ as previous described (Nohmi et al., 2015). The concentrations of TLR ligands used were determined in preliminary experiments.

### 2.4. Flow cytometry

All antibodies used for flow cytometry were obtained from BD Pharmingen. To characterize their phenotype, monocytes isolated from cord blood and adult peripheral blood were stained with an APC-conjugated anti-human CD14 mAb (described in *Monocyte separation* section) and a PE-labeled anti-human CD16 mAb (Catalog No: 555407) prior to TLR stimulation. To characterize the expression of cell surface antigens related to antigen presentation, cells were collected after TLR stimulation and incubated with an Ab cocktail consisting of an FITC-conjugated anti-human HLA-DP, DQ, DR mAb (MHC class II; Catalog No: 555558), PE-labeled anti-human CD11b mAb (Catalog No: 555388), APC-conjugated anti-human CD11c mAb (Catalog No: 559877); or PE-labeled anti-human CD80 mAb (Catalog No: 557227) and APC-conjugated anti-human CD86 mAb (Catalog No: 555660). These samples underwent FACS (LSR II, BD Biosciences), and the mean fluorescent intensity (MFI) scores or the percentage positive cells were calculated by Flowjo software (version 10.4.2). TLR-mediated expression of cell surface antigens with and without TLR stimulation are reported as MFI. In addition, enhancement ratios were calculated as [MFI after TLR stimulation] divided by [MFI without TLR stimulation].

### 2.5. Cytokine assay

The concentrations of human IL-8, IL-1β, IL-6, and tumor necrosis factor (TNF)-α in the cell-culture supernatants and serum from cord and adult blood were measured by using a human inflammatory cytokine cytometric bead assay kit (catalog no. 551811, BD Pharmingen, San Jose, CA, USA) in accordance with the manufacturer's instructions (Nohmi et al., 2015; Yanai et al., 2016). Briefly, 50 μL of supernatant was mixed with 50 μL of mixed capture beads. After 3 h of incubation at room temperature, the samples were washed, suspended in phosphate-buffered saline, and then analyzed by flow cytometry (FACS LSR II, Becton Dickinson, Franklin Lakes, NJ, USA) and CBA Analysis Software (BD Biosciences).

### 2.6. Statistics

The differences in expression levels of surface molecules between CBM and ABM were analyzed by using the Mann–Whitney *U* test. Values of $P < 0.05$ were considered statistically significant. Statistical significance is expressed in the Figures and Tables through the use of asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

## 3. Results

### 3.1. Characteristics of neonates and cord blood

All newborns were healthy, with an Apgar score (mean ± 1 SD) of 8.7 ± 0.5 at 5 min. None of the newborns had macrosomia, cardiac anomaly, respiratory distress syndrome, or infectious diseases. Their cord blood IgM (mean ± 1 SD) was 8.7 ± 2.8 mg/dL (range, 5 to 15; normal upper limit, 20), indicating a lack of intrauterine or peripheral infection. The serum from the cord blood showed no excessive elevation of inflammatory cytokines (IL8, TNF-α, IL-1β, IL-6), and the cytokine concentrations were not statistically different from those in adult serum (Supplementary Table 1). These results indicate that CBM used in this study were not activated by occult infection or premature rupture of the membrane.

### 3.2. MHC class II, CD80, and CD11b basal expression levels were low in CBM

The basal expression levels of MHC class II antigen, CD80, and

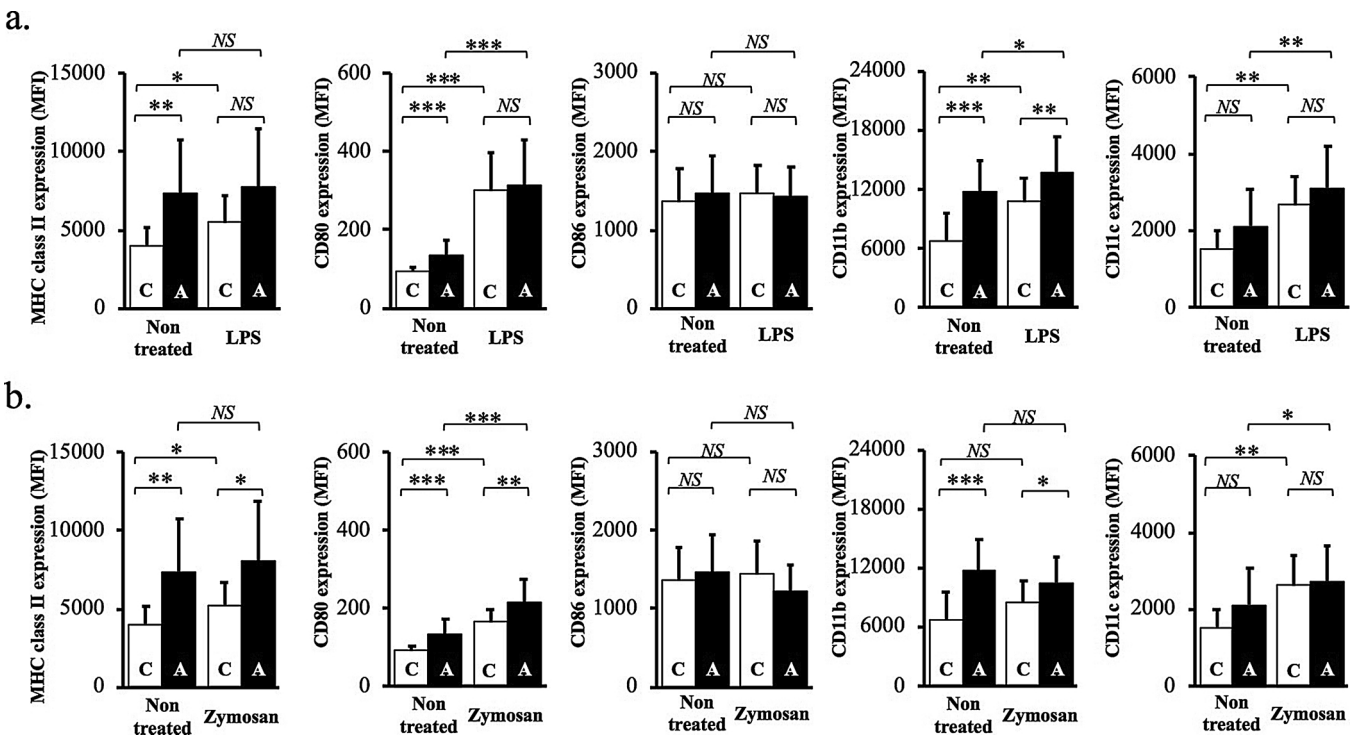


**Fig. 1.** Cell surface antigen profiles after stimulation with LPS (TLR4 agonist) or zymosan (TLR2/6 agonist).
a. Expression of cell surface antigens (MHC class II, CD80, CD86, CD11b, and CD11c) on cord blood monocytes and adult blood monocytes after 6 h of stimulation with LPS (TLR4 agonist) or no stimulation (Non treated).
b. Expression of cell surface antigens (MHC class II, CD80, CD86, CD11b, and CD11c) in cord blood monocytes and adult blood monocytes after 6 h of stimulation with zymosan (TLR2/6 agonist) or no stimulation (Non treated).
Data are means ± SD of mean fluorescence intensity (MFI; n = 15 and 19 for cord blood and adult blood, respectively). *P < 0.05, **P < 0.01, ***P < 0.001, *NS*, not significant, P values are from the Mann–Whitney *U* test. LPS, lipopolysaccharide; C, cord blood monocytes; A, adult blood monocytes.

**Table 1**
Summary of the expression of cell surface antigens.

| | MFI | | | | |
|---|---|---|---|---|---|
| | MHC | CD80 | CD86 | CD11b | CD11c |
| Non-treated | CBM < ABM | CBM < ABM | NS | CBM < ABM | NS |
| LPS-stimulated | NS | NS | NS | CBM < ABM | NS |
| Zymosan-stimulated | CBM < ABM | CBM < ABM | NS | CBM < ABM | NS |
| | Enhancement Ratios | | | | |
| LPS/non-treated | CBM > ABM | CBM > ABM | NS | CBM > ABM | NS |
| Zymosan/non-treated | CBM > ABM | NS | CBM > ABM | CBM > ABM | CBM > ABM |

MFI, mean fluorescence intensity; ABM, adult blood monocytes; CBM, cord blood monocytes; LPS, lipopolysaccharide; NS, not significant.

CD11b were significantly lower in CBM than in ABM (P < 0.01, P < 0.001, P < 0.001, respectively), whereas there was no significant difference in basal levels of CD86 and CD11c between the two types of monocytes (Fig. 1 and summarized in Table 1).

### *3.3. LPS or zymosan stimulation selectively enhanced the surface molecule expression in CBM and ABM*

Compared with the non-treated samples, LPS stimulation significantly enhanced the expression levels of the surface antigens MHC class II antigen, CD80, CD11b, and CD11c (P < 0.05, P < 0.001, P < 0.01, P < 0.01, respectively), but not CD86 (P > 0.05), in CBM, whereas it significantly enhanced the expression levels of CD80, CD11b, and CD11c (P < 0.001, P < 0.05, P < 0.01, respectively) but not MHC class II antigen or CD86 (P > 0.05), in ABM (Fig. 1a).

To determine how much LPS enhances the surface molecule expression compared with the basal levels, we calculated enhancement ratios (Table 1 and Supplementary Fig. 1). CBMs showed significantly higher enhancement ratios than ABMs for expression of MHC class II antigen, CD80, and CD11b (P < 0.001, P < 0.01, P < 0.01, respectively) but not for CD86 and CD11c (P > 0.05) (Supplementary Fig. 1). These results demonstrated that LPS enhanced the expression of antigen-presentation-related surface antigens more in CBM than in ABM.

In contrast, the expression levels after LPS stimulation were not statistically different between CBM and ABM for MHC class II, CD80, CD86, and CD11c and were higher in ABM than in CBM for CD11b (P < 0.01) (Fig. 1). Thus, despite the strong enhancement ratios in CBM, the low basal expression of these surface antigens meant that their expression in CBM after stimulation was not remarkably high.

Zymosan stimulation provided results similar to those of LPS. Zymosan stimulation significantly enhanced the expression levels of MHC class II, CD80, and CD11c in CBM (P < 0.05, P < 0.001, P < 0.01, respectively) but not CD86 or CD11b (P > 0.05) (Fig. 1b). In ABM, zymosan significantly enhanced the expression levels of CD80 and CD11c (P < 0.001 and P < 0.05, respectively) but not MHC class II antigen, CD86, or CD11c (P > 0.05) (Fig. 1b).

In response to zymosan stimulation, CBM showed significantly higher enhancement ratios than ABM for expression of MHC class II antigen, CD86, CD11b, and CD11c ($P < 0.001$, $P < 0.01$, $P < 0.001$, $P < 0.01$, respectively) but not for CD80 ($P > 0.05$) (Supplementary Fig. 1 and Table 1). These results demonstrated that zymosan enhanced antigen-presentation-related surface antigens more in CBM than in ABM.

In contrast to the enhancement ratios, the expression levels after zymosan stimulation were not statistically not different between CBM and ABM for CD86 and CD11c, and were higher in ABM than CBM for MHC class II, CD80, and CD11b ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively) (Fig. 1b). Together with the results of the LPS stimulation, these results suggest that, despite the strong enhancement ratios in CBM, the low basal expression of these surface antigens in CBM meant that their expression after stimulation was not remarkably high.

### 3.4. CD16 expression is different between CBM and ABM

Because the basal levels and degrees of stimulation of various surface antigens related to antigen presentation were different between CBM and ABM, we used FACS to examine whether the phenotype (CD14 and CD16) differed between these two types of monocytes. CBM showed a $CD14^{high}CD16^{high}$ phenotype, whereas ABM consisted of $CD14^{high}CD16^{intermediate}$ cells before TLR stimulation (Fig. 2). After TLR stimulation by LPS or zymosan, CD16 expression on the $CD14^{+}$ CBM was reduced to a level (CD16 intermediate) statistically similar to that on $CD14^{+}$ ABM.

### 3.5. Cytokine profiles after TLR stimulation

Inflammatory cytokines (IL-8, IL-6, IL-1β, and TNF-α) were remarkably elevated after 6 h of LPS or zymosan stimulation in both CBM and ABM (Fig. 3). There were no significant differences in cytokine concentration between CBM and ABM, except that the concentration of TNF-α was higher in ABM than in CBM after zymosan stimulation ($P < 0.05$). These results suggest that the difference in innate immunity between neonates and adults is due to the expression of surface antigens rather than to cytokine profiles after 6 h of TLR stimulation.

## 4. Discussion

Innate immune responses mediated by APCs are an important initial step in inducing protective immunity against pathogens, but the TLR-mediated expression levels of cell surface antigens on neonatal APCs are not fully understood. Here, by using cord blood, we demonstrated that neonatal monocytes have a phenotype and response to stimulation distinct from those of adult monocytes: basal expression levels of a subset of cell surface antigens related to antigen presentation were low in neonatal $CD14^{+}CD16^{high}$ monocytes but responded more to TLR stimulation than they did in adult $CD14^{+}CD16^{intermediate}$ monocytes, which had higher basal levels of expression, resulting in similar levels after stimulation.

We observed low basal expression of the surface antigens MHC class II antigen, CD80, and CD11b—but not CD11c and CD86—on neonatal monocytes. Previously, Nguyen et al. reported that basal expression of MHC class II antigen—but not CD80—is low on neonatal monocytes and DCs (Nguyen et al., 2010), whereas De Wit et al. reported comparable basal expression of MHC class II antigen, CD80, and CD86 in neonatal and adult DCs (De Wit et al., 2003). Taken together, these findings suggest that not all neonatal surface antigens related to the antigen presentation are impaired, but rather some antigens, especially MHC class II antigen, are selectively low. Comparison of basal CD11b expression on monocytes or DCs between neonates and adults has not been described previously. Our observation of low expression of CD11b on neonatal monocytes indicates the immaturity of these monocytes; such immaturity may also explain the observed selective impairment of MHC class II antigen and CD80.

Neonates are susceptible to infections and respond weakly to vaccines. We hypothesized that low basal expression of surface antigens (e.g., MHC class II) are involved in the underlying mechanisms. MHC class II antigen and CD80 are key molecules for providing peptides from pathogens or vaccine antigens to B and T cells, which is necessary for the initiation of acquired immunity. The vaccine-mediated acquisition of protective immunity is impaired in neonates compared with that in infants when the vaccine is administered without adjuvant (Lieberman et al., 1995; Kurikka et al., 1995), and multiple booster shots are needed to sustain immune responses and maintain immunological memory in this population (PrabhuDas et al., 2011). Although the transfer of maternal antibodies is believed to influence the induction of

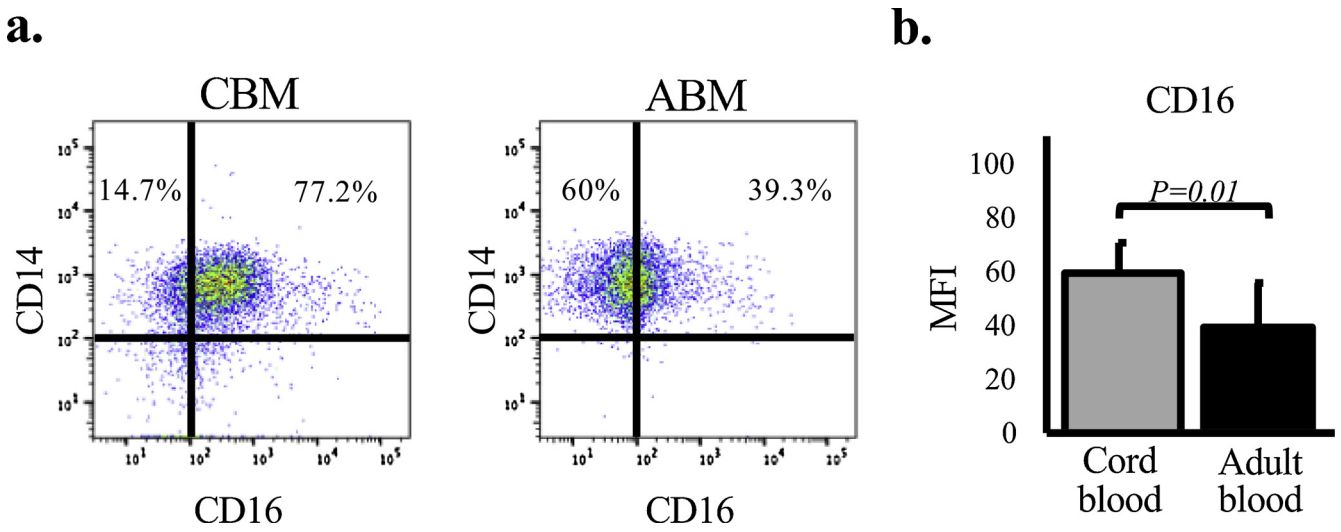


**Fig. 2.** CD14 and CD16 profiles in CBM and ABM.
a. CD14 and CD16 profiles in cord blood monocytes (CBM) and adult blood monocytes (ABM) isolated by using anti-CD14 magnetic beads. Percentages in the right upper quadrant represent the densities of CD14 and CD16 double-positive cells. Percentages in the left upper quadrant represent the densities of CD14-positive and CD16-negative cells.
b. Mean fluorescence intensity (MFI) of CD16 was compared between cord and adult blood CD14+ monocytes. Data are means ± 1 SD (n = 9). The P value is from the Mann–Whitney *U* test.

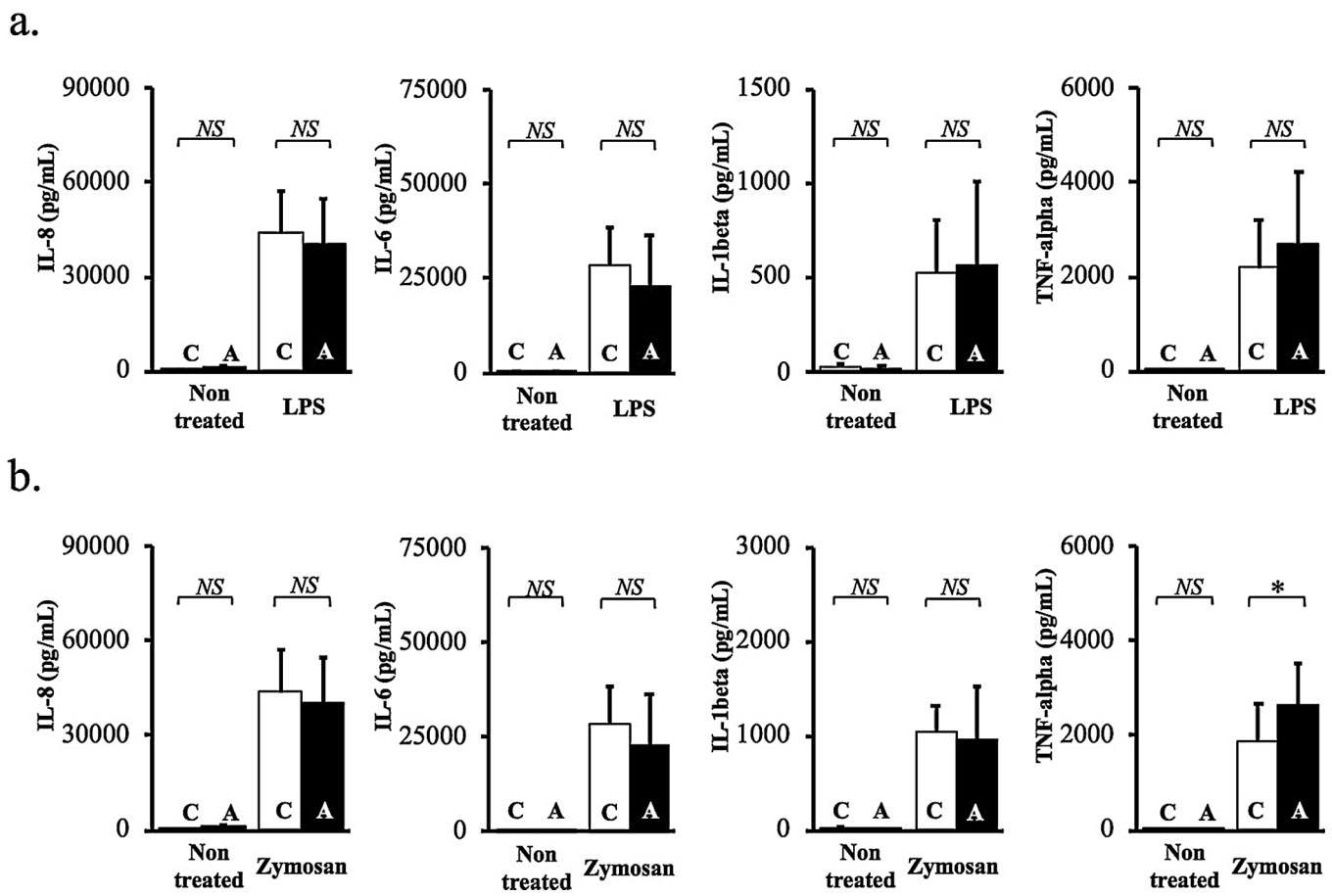


**Fig. 3.** Cytokine profiles after stimulation with LPS (TLR4 agonist) or zymosan (TLR2/6 agonist).
Concentrations of cytokines (IL-8, IL-1β, IL-6, and TNF-α) in the cell-culture supernatants were determined after 6 h of LPS (a) or zymosan (b) stimulation. Data are expressed as means ± 1 SD. *P < 0.05, *NS*, not significant, P values are from the Mann–Whitney *U* test. IL, interleukin; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; C, cord blood monocytes; A, adult blood monocytes.

vaccine-induced specific immunity, our results suggest that selective impairment in basal expression of MHC class II antigen and CD80 are also involved in the poor recognition of vaccine antigen by APCs in neonates, leading to the insufficient induction of specific immunity.

Low MHC class II antigen expression may have clinical significance. Low expression of MHC class II antigen on monocytes is closely related to the frequency of severe respiratory syncytial virus infection (Ahout et al., 2016). Reduced MHC class II antigen expression is also characteristic of MHC-class II deficiency (bare lymphocyte syndrome), which is associated with increased susceptibility to infections and intractable diarrhea (Kuo et al., 2013). Because the primary function of MHC class II antigen is to present peptide antigen to T cells, low MHC class II antigen expression would be expected to result in insufficient induction of protective immunity. The low MHC class II antigen expression observed here in the neonatal period is possibly a transient phenomenon, and expression may be upregulated with maturation during the first year of life to levels comparable to those in adults (De Wit et al., 2003). However, we propose that the low MHC class II antigen expression during the neonatal period likely explains the high susceptibility to infection and the high risk of developing severe outcomes in neonates and infants (Liu et al., 2015). Low MHC class II antigen expression could also be the mechanism underlying the weak induction of protective immunity by vaccination and the need for repeated vaccinations in infants (Tokuhara, 2018a; Mendelman et al., 2001; Hill et al., 2006).

The hyperresponsiveness of CBM to TLR stimulation may have a beneficial aspect in the application of TLR as a vaccine adjuvant. Current strategies for developing safe vaccines tend to use an isolated protein of the pathogen (e.g., a virus-like particle) that is less antigenic than the whole pathogen (Tokuhara, 2018a); such vaccines need an effective adjuvant to provoke an innate immune response and enable APCs to recognize vaccine antigen in neonates and infants. Our finding that the degree of responsiveness to the TLR4 and TLR2/6 agonists LPS and zymosan was significantly higher in neonates than in adults suggests that TLR agonists could be used as vaccine adjuvants to complement the weak antigen presentation capacity of neonatal and infantile APCs, thereby providing effective antigen presentation and inducing acquired immunity. In contrast to their effect in neonates, the TLR4 and TLR2/6 agonists did not enhance MHC class II expression in adult monocytes; thus, a strategy of using TLR agonists as vaccine adjuvants may not be effective in adults.

TLR-mediated hyperresponsiveness of surface antigens may also have the harmful effect of exacerbating inflammation in immune diseases or multiple organ injury during sepsis. Sepsis has been associated with an increase in the expression of CD80 not but of CD86 (Nolan et al., 2008). Another study found that CD11b density increased before a blood culture confirmed sepsis in extremely-low-birth-weight infants, indicating that CD11b density can be used for earlier diagnosis of late-onset infection in this population (Turunen et al., 2005). Infections with gram-negative bacteria and fungi are important contributors to the high mortality rate in the neonatal period. In our study, LPS and zymosan, cell wall components of gram-negative bacteria and fungi, respectively, significantly enhanced the expression of CD11b and/or CD80 in CBM compared with the expression in ABM. Those phenomena may reflect that neonates are prone to develop sepsis associated with immunological hyperresponsiveness mediated by pathogen-TLR

interaction.

Monocytes are classically defined as $CD14^+$ cells but can also be characterized by CD16 expression (Gordon and Taylor, 2005; Ziegler-Heitbrock, 2007). Here, we demonstrated that CD16 expression was significantly higher on neonatal CD14+ monocytes than on adult CD14+ monocytes. Adult monocytes with the phenotype $CD14^+CD16^{low-to-intermediate}$ are considered to be classical monocytes (Gordon and Taylor, 2005; Ziegler-Heitbrock, 2007), but the neonatal monocytes in the current study showed the distinct phenotype of $CD14^+CD16^{high}$ (Fig. 3). In adults, another type of monocyte ($CD14^{low}CD16^{high}$), which was not found in our study, shows higher antigen-presentation capacity (e.g., high MHC class II expression) than classical monocytes (Ziegler-Heitbrock, 2007). Despite the higher CD16 expression on neonatal monocytes compared with adult monocytes, basal MHC class II expression was significantly lower in neonatal monocytes ($CD14^+CD16^{high}$), as mentioned above. Furthermore, TLR stimulation reduced CD16 expression in CBM to the same level (low to intermediate) as in ABM. Therefore, the initial phenotype ($CD14^+CD16^{high}$) of neonatal monocytes may reflect their immature status compared with $CD14^+CD16^{low-to-intermediate}$ adult monocytes.

The current study has some limitations in terms of evaluated immune cells and TLRs. There are ten types of TLRs in humans (Kawai and Akira, 2010), but because of limited resources, we chose to focus on TLR4 and TLR2/6, which recognize *E. coli* and group B streptococcus (Henneke et al., 2008; Kaper et al., 2004), respectively, important pathogens causing meningitis in neonates and infants (Stoll et al., 2011). In addition, our study evaluated monocytes but not DCs because of the abundance of monocytes and extremely low numbers of DCs in cord and adult blood. Elucidation of innate immune responses in neonatal DCs and via other TLRs will be achieved in future studies.

In conclusion, we demonstrated that neonatal CD14+ monocytes have a distinct phenotype and response compared with those of adults. Cell surface antigens of neonatal $CD14^+CD16^{high}$ monocytes have lower basal expression of MHC class II, CD80, and CD11b but are hyperresponsive to LPS and zymosan compared with adult $CD14^+CD16^{intermediate}$ monocytes. These results improve our understanding of neonatal and infantile immature innate immunity and raise the possibility of using TLR agonists as vaccine adjuvants in children to increase the induction of protective immunity.

## Declaration of Competing Interest

None of the authors has any conflict of interest related to this manuscript.

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