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Granulocytic myeloid-derived suppressor cells (GR-MDSC) accumulate in cord blood of preterm infants and remain elevated during the neonatal period

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SUMMARY

Preterm delivery is the leading cause of perinatal morbidity and mortality. Among the most important complications in preterm infants are peri- or postnatal infections. Myeloid-derived suppressor cells (MDSC) are myeloid cells with suppressive activity on other immune cells. Emerging evidence suggests that granulocytic MDSC (GR-MDSC) play a pivotal role in mediating maternal–fetal tolerance. The role of MDSC for postnatal immune-regulation in neonates is incompletely understood. Until the present time, nothing was known about expression of MDSC in preterm infants. In the present pilot study, we quantified GR-MDSC counts in cord blood and peripheral blood of preterm infants born between 23 + 0 and 36 + 6 weeks of gestation (WOG) during the first 3 months of life and analysed the effect of perinatal infections. We show that GR-MDSC are increased in cord blood independent of gestational age and remain elevated in peripheral blood of preterm infants during the neonatal period. After day 28 they drop to nearly adult levels. In case of perinatal or postnatal infection, GR-MDSC accumulate further and correlate with inflammatory markers C-reactive protein (CRP) and white blood cell counts (WBC). Our results point towards a role of GR-MDSC for immune-regulation in preterm infants and render them as a potential target for cell-based therapy of infections in these patients.

Keywords: intra-amniotic infection, infection, MDSC, preterm infants, sepsis

Introduction

Preterm delivery is the leading cause of perinatal morbidity and mortality [1,2]. In Germany, approximately one of ten children is born before 37 weeks of gestation (WOG) [3] and mortality rises with decreasing gestational age to up to 40% [4]. Besides respiratory complications, infections are a main reason for death in preterm infants [5], affecting every third very low birth weight (VLBW) infant. The high susceptibility to infections in neonates, and especially in preterm infants, is attributed to their altered immune response to pathogens [6–8]. During pregnancy, the immune system predominantly exhibits tolerogenic features that protect the fetus from maternal rejection; postnatally, however, these features may predispose to infections [6,9]. Potential mechanisms of postnatal immunosuppression are a bias of T helper (Th) cell responses towards Th2 and an accumulation of immune-modulatory cells such as regulatory T cells (T_{regs}), regulatory B cells (B_{regs}) and CD71 positive erythroid cells [7,10,11]. Recently, we and others have described myeloid-derived suppressor cells (MDSC) as potential immune regulators during pregnancy [12–15].

MDSC are myeloid cells that suppress innate and adaptive immune responses [16]. In humans, MDSC can be divided into two subgroups: granulocytic MDSC (GR-MDSC) expressing granulocytic lineage markers CD15 and/or CD66b, and monocytic MDSC (MO-MDSC) expressing the monocytic antigen CD14. Both subsets express CD33, but lack expression of the human leucocyte antigen, D-related (HLA-DR) [17,18]. A phenotypical difference between GR-MDSC and mature granulocytes is the

lower density of GR-MDSC, sedimenting with mononuclear cells (MNC) after density gradient centrifugation. GR-MDSC, but not MO-MDSC, accumulate during pregnancy in both mother and fetus [12–15,19], and are supposed to play a role in mediating maternal–fetal tolerance. Recently, we demonstrated in cord blood of healthy term neonates that GR-MDSC modulate Th cells towards a tolerogenic phenotype with predominance of Th2 and induction of T_{regs} [20]. This finding indicates clearly the interactions between the known immune-modulatory cells, ensuring tolerance.

While the current concept of postnatal immune adaptation supposes an attenuation of tolerogenic effector mechanisms, the role of fetal/neonatal GR-MDSC in postnatal life is as yet unclear, particularly in preterm infants – a highly vulnerable cohort for infection.

Besides their role during pregnancy, MDSC have been described frequently in adults to accumulate during inflammatory processes such as trauma or sepsis [21–24]. It is unclear, however, if severe infection also induces an expansion of MDSC during the unique period of neonatal life.

In the present pilot study, we quantified GR-MDSC counts in cord and peripheral blood of preterm infants born between 23 + 0 and 36 + 6 WOG during the first 3 months of life and analysed the effect of perinatal infections. We show that accumulation of GR-MDSC in cord blood was independent of gestational age and that GR-MDSC counts remained elevated in peripheral blood of healthy preterm neonates. Furthermore, we show that despite the already elevated GR-MDSC levels in preterm neonates, a further expansion occurred in those born from intra-amniotic infection (IAI) or suffering from postnatal sepsis. GR-MDSC levels correlated with the number of white blood cell (WBC) and C-reactive protein (CRP) levels. These results point again towards a role of GR-MDSC in regulating the neonatal immune response, and emphasize the current concept of neonatal immune adaptation by a gradual decrease in tolerogenic effector mechanisms.

Methods

Patients

The local ethics committee approved this study, and all parents gave written informed consent (protocol number 178/2011BO1 and 682/2016BO1). From October 2012 to October 2013, cord blood from preterm infants (born between 23 + 0 WOG and 36 + 6 WOG, $n = 71$) and term-born infants (born after 37 + 0 WOG, $n = 47$) who were born in the Department of Obstetrics and Gynaecology at Tuebingen University Hospital was collected immediately after caesarean section. From October 2012 to October 2013 and from October 2016 to February 2017 postnatal peripheral blood samples were obtained from preterm infants (born between 23 + 0 WOG and 36 + 6 WOG), $n = 61$, 51 infants without severe complications (exclusion criteria: neonatal sepsis, connatal infections, severe respiratory distress syndrome, treatment with dexamethasone for chronic lung disease, focal intestinal perforation, necrotizing enterocolitis, surgery during the last 7 days before blood withdrawal, immune deficiency disorders) at the time-point of sample collection and 10 infants with postnatal sepsis admitted to the Department of Neonatology at Tuebingen University Children's Hospital. The blood was collected during routine blood sampling. Healthy adult blood donors served as controls.

Definitions

Cause of preterm delivery. Cause of preterm delivery was determined by the attending obstetrician. Preterm labour was defined as labour refractory to tocolysis or complete cervical dilatation. Suspected IAI was defined as increased maternal inflammatory markers (WBC counts > 15 000/µl or CRP > 1 mg/dl) and at least one clinical sign of IAI (foetid amniotic fluid, maternal fever, fetal tachycardia > 160/min). Pre-eclampsia/eclampsia was defined according to the guidelines of the German Society of Obstetrics and Gynecology (DGGG) (arterial hypertension and proteinuria first diagnosed after 20 WOG). Pathological Doppler was defined according to the guidelines of the DGGG (increased resistance index (RI) above the 95th percentile of the umbilical artery or reduced RI below 5th percentile of the medial cerebral artery) [25]. Other reasons for preterm delivery included placental abruption, maternal exhaustion due to multiple pregnancies, fetal abnormalities, etc.

Gestational age. Gestational age was calculated based on early prenatal ultrasound and obstetric examination. Small for gestational (SGA) was defined as birth weight below the 10th centile for gestational age according to gender-specific German birth weight standards [26], with appropriate for gestational age (AGA) being defined as birth weight between the 10th and 90th centile in these standards.

Neonatal sepsis. Neonatal sepsis was defined as systemic inflammatory response syndrome (SIRS; at least two of the following clinical signs, one of which must be abnormal temperature or leucocyte count: temperature > 38·0°C or < 36·5°C, tachycardia or bradycardia, increased respiratory rate or need for mechanical ventilation for an acute process and elevated or depressed leucocyte counts) [27] and increased inflammatory markers: interleukin (IL)-6 > 100 ng/dl at the time-point of first clinical signs and CRP > 1·0 mg/dl after 24 h [28] that resulted in antibiotic treatment for more than 48 h by the attending neonatologist. Divergent to the definition of neonatal sepsis in the German Neonatal Nosocomial Infection Surveillance System (NEO-KISS), we regarded episodes requiring

antibiotic therapy for more than 48 h as clinically relevant. As CRP-guided therapy is the standard of care in our unit [29], the NEO-KISS definition was not appropriate. Early-onset sepsis (EOS) was defined as neonatal sepsis occurring postnatally within the first 72 h and late-onset sepsis (LOS) as occurring after 72 h [30]. Inclusion into the sepsis group was based on clinical criteria. Blood culture was positive in two of the 10 sepsis cases. None of the children included in the sepsis group received supportive corticosteroids. As we used residuals of routine blood withdrawals, the time-point of GR-MDSC quantification during sepsis was not standardized and was between days 0 and 3 after sepsis onset.

IAI. IAI was defined as increased maternal inflammatory markers (WBC counts $>$ 15 000/μl or CRP $>$ 1 mg/dl) and at least one clinical sign of IAI (foetid amniotic fluid, maternal fever, fetal tachycardia) and postnatal signs of neonatal infection (temperature $>$ 38·0°C or $<$ 36·5°C, tachypnoea, tachycardia $>$ 200/min, reduced microcirculation and at least one laboratory sign: CRP $>$ 1·0 mg/dl, WBC count $<$ 5000/μl, platelets $<$ 1000/μl).

Cell isolation and flow cytometry

Blood samples were processed within 24 h after withdrawal. For the analysis of GR-MDSC in cord blood, cord blood MNC were isolated by density gradient centrifugation according to previously described protocols [14,19]. For the analysis of GR-MDSC in peripheral blood of preterm infants, we used the remains from routine blood withdrawals that we retrieved from our main laboratory. The range of whole blood that we worked with was between 50 and 200 μl. MNC were prepared from ethylenediamine tertraacetic acid (EDTA) or heparinized blood samples by Ficoll density gradient centrifugation. Whole blood was diluted in phosphate-buffered saline (PBS) to a total volume of 1 ml and added carefully onto 500 μl lymphocyte separation solution (Bicochrom GmbH, Berlin, Germany) in a 1·5-ml Eppendorf vial. Cells were centrifuged and the MNC layer was collected. Cell count was determined and cells were diluted in PBS at a concentration of 1×10^6 cells/ml for extracellular staining. GR-MDSC were characterized as $CD66b^+CD33^+CD14^-HLA\text{-}DR^{low/-}$ cells, according to previously established human MDSC characterization methods [14,19]. Gating strategy is depicted in Supporting information, Fig. S1. Antibodies used for extracellular staining were anti-CD66b-fluorescein isothiocyanate (FITC) (clone G10F, concentration 1 μl/1 $\times$ 10^5 cells), anti-CD33-phycoerythrin (PE) (clone WM53, concentration 1 μl/1 $\times$ 10^5 cells), anti-HLA-DR-perididin chlorophyll-cyanin 5.5 (PerCP-Cy5.5) (clone G46-6, concentration 0·5 μl/1 $\times$ 10^5 cells) and CD14-allophycocyanin (APC) (clone MφP9, concentration 50 ng at 1×10^5 cells) (all purchased from BD Biosciences, Heidelberg, Germany). Antibodies were tested for their specificity by isotype control staining when introduced into our laboratory. Viability stain of single samples after density gradient centrifugation revealed approximately 10% dead cells. Data acquisition was performed with a fluorescence activated cell sorter (FACS)Calibur flow cytometer (BD Biosciences) and data were analysed via CellQuest Pro Software (BD Biosciences).

WBC, immature information and CRP

WBC counts, immature information (IMI) and CRP were assessed as part of the clinical routine laboratory evaluation from EDTA or heparinized blood samples by the central laboratory of Tuebingen University hospital. Haematological parameters were determined using the Sysmex XE-2100 analyzer (Sysmex GmbH, Norderstedt, Germany). Plasma concentrations of CRP and IL-6 were measured using the wide-range CRP assay on the ADVIA 1200 clinical chemistry analyser and the IMMULITE XPI solid phase immunoassay system, respectively (both from Siemens Healthcare Diagnostics, Eschborn, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Data were analysed for Gaussian distribution using the D'Agostino-Pearson omnibus normality test. As data were not distributed normally, differences between two groups were evaluated using the Mann–Whitney test. Differences between more than two groups were evaluated by Kruskal–Wallis test and Dunn's multiple comparisons test. Correlations were analysed by Spearman's correlation. For correlation analyses (CRP, IMI and WBC), repeated measurements of the same infant were included. A *P*-value $<$ 0·05 was considered statistically significant.

Results

Study cohort

During the observational period, 197 individuals were included into this pilot study: 122 preterm infants, 48 term infants and 13 adults. Cord blood only was collected from 71 preterm infants and all term infants, while in 51 preterm infants postnatal peripheral blood was collected during routine blood withdrawals. From 15 infants we obtained blood at age $>$ 28 days, from 17 infants at age between 8 and 28 days and from 19 infants at age between 1 and 7 days.

GR-MDSC are elevated in cord blood of preterm infants independent of gestational age

We first analysed GR-MDSC counts in cord blood of preterm infants in comparison to term neonates and adults. Supporting information, Fig. S1 shows the gating strategy for GR-MDSC in cord blood of preterm infants, term

Table 1. Clinical characteristics of infants included in cord blood analysis

	All preterm infants	23 + 0–28 + 6 weeks	29 + 0–32 + 6 weeks	33 + 0–36 + 6 weeks	Term infants
No.	71	24	26	21	48
Gestational age (weeks)	30 + 2	26 + 1	30 + 5	34 + 5	39 + 2
Birth weight (g)	1436·5	806·5	1469·2	2116·0	3286·2
Birth percentile	35·2	33·9	43·0	27·2	38·8
SGA (%)	16·9	16·7	11·5	23·8	14·6
Gender (% male)	40·8	44·0	30·8	50·0	68·8
Multiple pregnancies (%)	50·7	37·5	61·5	52·4	10·0
Mode of delivery (%)					
spontaneous	12·7	4·2	7·7	27·3	43·8
Elective C/S	42·3	41·7	46·2	38·1	25·0
Emergency C/S	45·1	54·2	46·2	33·3	31·3
Reason for delivery (%)					
Preterm labour/suspected IAI	52·1	50·0	57·7	47·6	–
Pre-eclampsia/eclampsia	11·3	4·2	15·4	14·3	–
Pathological Doppler	18·3	12·5	23·1	14·3	–
Others	18·3	33·3	3·8	23·8	

SGA = small for gestational age (birth weight <10th percentile); C/S = caesarean section; IAI = intra-amniotic infection (maternal inflammatory markers (white blood cell counts > 15 000/μl or CRP > 1 mg/dl) and at least one clinical sign for IAI (fetid amniotic fluid, maternal fever, fetal tachycardia) and postnatal signs for infection of the neonate (temperature > 38·0°C or < 36·5°C, tachypnoe, tachycardia > 200/min, reduced microcirculation and one laboratory sign: CRP > 1·0 mg/dl, white blood cell count < 5000/μl, platelets < 100/μl).

infants and in peripheral blood of preterm infants. Preterm infants were divided into three gestational age groups (group 1: 23 + 0–28 + 6 WOG; group 2: 29 + 0–32 + 6 WOG; and group 3: 33 + 0–36 + 6 WOG). Table 1 shows clinical characteristics of the study cohort. We found that percentages of GR-MDSC were elevated in cord blood of preterm infants of all gestational ages compared to adults (median 1·3% for group 1; 1·4% for group 2; 1·8% for group 3; and 1·9% for term neonates *versus* 0·2% for adults, $P < 0{\cdot}01$, $n = 13$–47). There were no differences between gestational age groups, either among the preterm groups or in comparison to term neonates (Fig. 1). Subgroup analyses revealed that infants born spontaneously had slightly higher GR-MDSC levels than infants born by caesarean section (Supporting information, Fig. S2a). There were no differences in percentages of GR-MDSC between singleton in comparison to multiple pregnancies (Supporting information, Fig. S2b), SGA and AGA infants (Supporting information, Fig. S2c) and male and female infants (Supporting information, Fig. S2d), as well as between infants that were exposed to prenatal magnesium (Supporting information, Fig. S2e) and infants exposed to prenatal corticosteroids (Supporting information, Fig. S2f), influencing factors that have been described for T_{reg} counts in preterm infants [31].

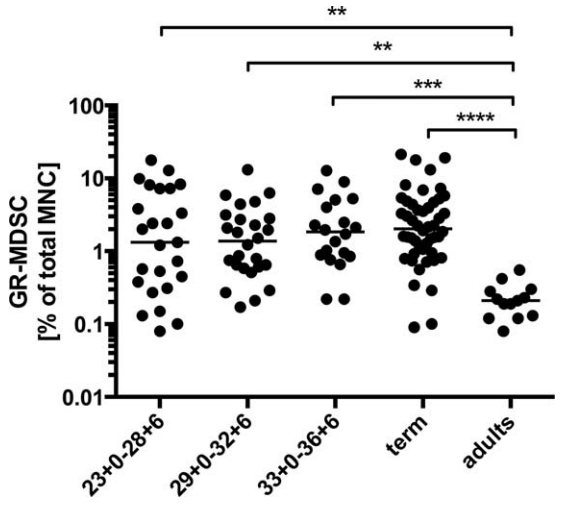

Fig. 1. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in cord blood of preterm infants. Mononuclear cells (MNC) were isolated from cord blood (CBMC) of preterm infants, term infants and adults (PBMC). Scatter diagram showing the percentage of GR-MDSC from total CBMC of preterm and term infants and from total PBMC of adult controls. $n = 13$–48, $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$; Kruskal–Wallis test and Dunn's multiple comparison test.

GR-MDSC remain elevated during the neonatal period and drop after day 28 of life

Next, we quantified GR-MDSC in peripheral blood samples of preterm infants without severe complications at the time of sample collection during their first postnatal weeks. Infants were subdivided into three groups (group 1: perinatal, days 1–7; group 2: neonatal, days 8–28; group 3: post-neonatal, > day 28). Table 2 shows clinical characteristics of infants included in the analysis. We found that GR-MDSC remained elevated during the perinatal (median 2·0%) and neonatal periods (median 2·1%) compared to the post-neonatal period (median 0·5%) and to adult controls (median 0·2%, $P < 0{\cdot}0001$, $n = 13$–19). Levels found after day 28, however, were not significantly different from

Table 2. Clinical characteristics of infants included in postnatal blood analysis

	All preterm infants	Days 1–7	Days 8–28	Day >28
No.	51	19	17	15
Gestational age (weeks)	31 + 3	33 + 3	30 + 2	30 + 1
Postnatal age (days)	20·8	1·5	15·8	50·9
Birth weight (g)	1584·4	2002··9	1300·3	1376·3
Birth percentile	35·6	35·8	31·5	39·7
SGA (%)	17·6	10·5	18·8	26·7
Gender (% male)	51·0	57·9	41·2	53·3
Multiple pregnancies (%)	23·5	10·5	47·1	13·3
Mode of delivery (%)				
Spontanous	13·7	26·3	0·0	13·3
Elective C/S	49·0	47·4	29·4	53·3
Emergency C/S	37·3	26·3	70·6	33·3
Reason for delivery (%)				
Preterm labour/suspected IAI	47·1	47·4	52·9	40·0
Pre-eclampsia/eclampsia	11·8	21·1	11·8	0·0
Pathological Doppler	11·8	10·5	17·6	6·7
Others	29·3	21·0	17·7	53·3

SGA = small for gestational age (birth weight <10th percentile); C/S = caesarean section; IAI = intra-amniotic infection (maternal inflammatory markers (white blood cell counts > 15 000/μl or CRP > 1 mg/dl) and at least one clinical sign for IAI (fetid amniotic fluid, maternal fever, fetal tachycardia) and postnatal signs for infection of the neonate (temperature > 38·0°C or < 36·5°C, tachypnoe, tachycardia > 200/min, reduced microcirculation and one laboratory sign: CRP > 1·0 mg/dl, white blood cell count < 5000/μl, platelets < 100/μl).

adult controls ($P > 0{\cdot}05$, $n = 13$–15) (Fig. 2a). Correlation analysis showed that postnatal age correlated significantly with GR-MDSC counts in peripheral blood ($P < 0{\cdot}0001$, $r = -0{\cdot}57$, $n = 51$) (Fig. 2b). Analysis of other immune cell populations showed a decrease in neutrophils similar to the decrease observed in GR-MDSC, while lymphocytes increased during the neonatal period and monocytes remained unchanged (Supporting information, Fig. S3a–f).

GR-MDSC are elevated in infants with IAI or neonatal sepsis

To evaluate whether GR-MDSC may expand further during perinatal infections, we compared GR-MDSC levels in cord blood of preterm infants born from IAI with those born from mothers without any signs of infection. As shown in Fig. 3, infants born from IAI had significantly higher GR-MDSC counts than controls (median 5·3% *versus* 1·0%, $n = 12/59$, $P = 0{\cdot}002$, Fig. 3a). Similar results were obtained

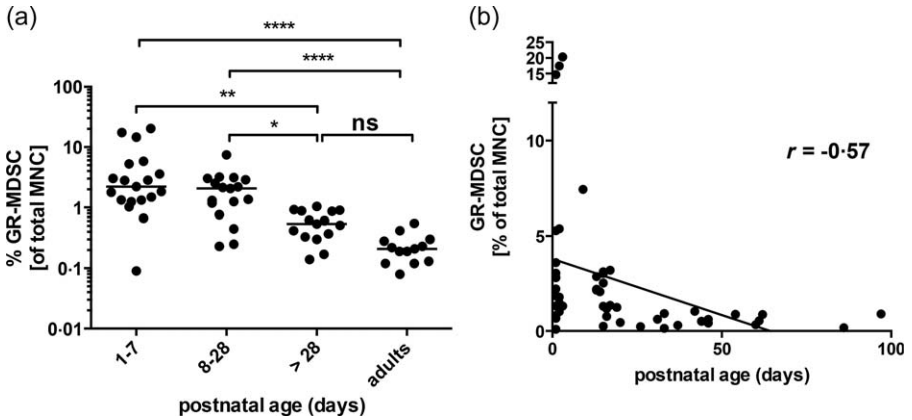

Fig. 2. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in peripheral blood of preterm infants. Mononuclear cells (MNC) were isolated from peripheral blood of preterm infants during the first 3 months of life and from adults. (a) Scatter diagram showing the percentage of GR-MDSC from total peripheral blood mononuclear cells (PBMC) of preterm infants in the perinatal period days 1–7, the neonatal period days 8–28, the period beyond day 28 and adult controls. $n = 13$–19, $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$; n.s. = not significant; Kruskal–Wallis test and Dunn's multiple comparison test. (b) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants depending on postnatal age. Regression line shows correlation between percentages of GR-MDSC and postnatal age. $n = 51$; $^{****}P < 0{\cdot}0001$; Spearman's correlation.

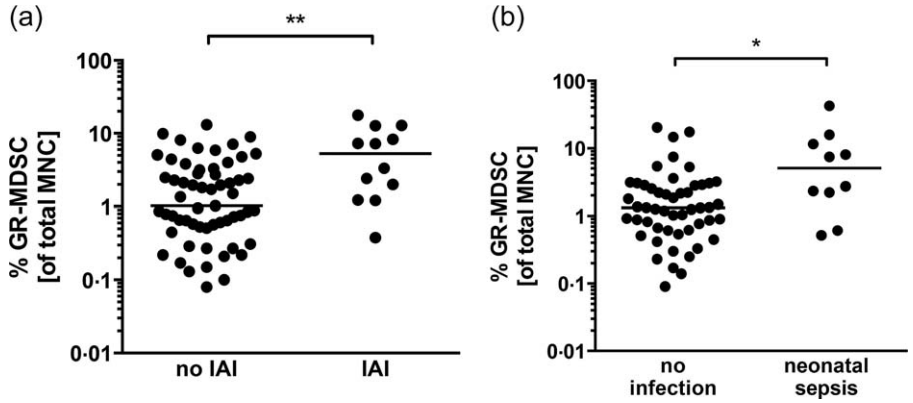

Fig. 3. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in preterm infants with intra-amniotic infection (IAI) or neonatal sepsis. Mononuclear cells (MNC) were isolated from cord (CBMC) or peripheral blood (PBMC) of preterm infants. (a) Scatter diagram showing the percentage of GR-MDSC from total CBMC of preterm infants without IAI (no IAI) or with IAI (IAI). n = 12–59; $^{**}P < 0{\cdot}01$; Mann–Whitney test. (b) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants without infection (no infection) or with postnatal sepsis (neonatal sepsis). n = 10–51; *** $P < 0{\cdot}001$; Mann–Whitney test.

when preterm infants with neonatal sepsis were compared with non-infected controls (median 5·1% *versus* 1·3%, $n = 10/51$, $P < 0{\cdot}05$, Fig. 3b). Supporting information, Fig. S4 shows GR-MDSC counts in infants with or without sepsis in the three postnatal age groups. Table 3 shows clinical characteristics of infants with sepsis.

Table 3. Clinical characteristics of infants with neonatal sepsis

	Infants with neonatal sepsis
No.	10
Gestational age (weeks)	25 + 3
Postnatal age (days)	22·9
Birth weight (g)	674·6
Birth percentile	24·0
SGA (%)	30·0
Gender (% male)	60·0
Multiple pregnancies (%)	50·0
Mode of delivery (%)	
Spontaneous	10·0
Elective C/S	70·0
Emergency C/S	20·0
Reason for delivery (%)	
Preterm labour/suspected AIS	30·0
Pre-eclampsia/eclampsia	30·0
Pathological Doppler	0·0
Others	40·0
Type of sepsis	
EOS	30·0
LOS	70·0
Blood-culture positive	20·0

SGA = small for gestational age (birth weight < 10th percentile); C/S, caesarean section; IAI = intra-amniotic infection (maternal inflammatory markers (white blood cell counts > 15 000/μl or CRP > 1 mg/dl) and at least one clinical sign for IAI (foetid amniotic fluid, maternal fever, fetal tachycardia) and postnatal signs for infection of the neonate (temperature > 38·0°C or < 36·5°C, tachypnoea, tachycardia > 200/min, reduced microcirculation and one laboratory sign: CRP > 1·0 mg/dl, white blood cell count < 5000/μl, platelets < 100/μl).

GR-MDSC counts correlate with CRP and WBC counts

Lastly, we asked whether there is a correlation between GR-MDSC counts and inflammatory markers in preterm infants. Therefore, we compared percentages of GR-MDSC in peripheral blood of infants with normal (< 1·0 mg/dl) and elevated CRP (> 1·0 mg/dl). We found that GR-MDSC levels were fourfold higher in infants with elevated CRP than in controls (median 8·8% *versus* 2·2%, $n = 36/89$, $P = 0{\cdot}0002$, Fig. 4a). GR-MDSC levels correlated positively with CRP ($n = 87$, $P = 0{\cdot}02$, $r^2 = 0{\cdot}06$, Supporting information, Fig. S4). Furthermore, we found a positive correlation of WBC counts with percentages of GR-MDSC ($n = 206$, $P < 0{\cdot}0001$, $r = 0{\cdot}52$, Fig. 4b) as well as IMI channel and percentages of GR-MDSC ($n = 174$, $P < 0{\cdot}0001$, $r = 0{\cdot}61$, Supporting Information, Fig. S5b).

Discussion

MDSC have been described widely to play an important role for immune regulation during pathological processes such as tumours, infections or transplant reactions [16,17,21,24,32,33]; recently, however, increasing evidence has emerged revealing that they also control immune adaptation to pregnancy and contribute to maternal–fetal tolerance both maternally and fetally [12–15,19,34,35]. Until now, little is known about their impact on immune regulation in neonates, and especially in preterm infants. We thus analysed the expression of MDSC in cord blood and peripheral blood of preterm infants, focusing on GR-MDSC, as previous studies showed an accumulation of GR-MDSC, but not MO-MDSC, in cord blood of healthy

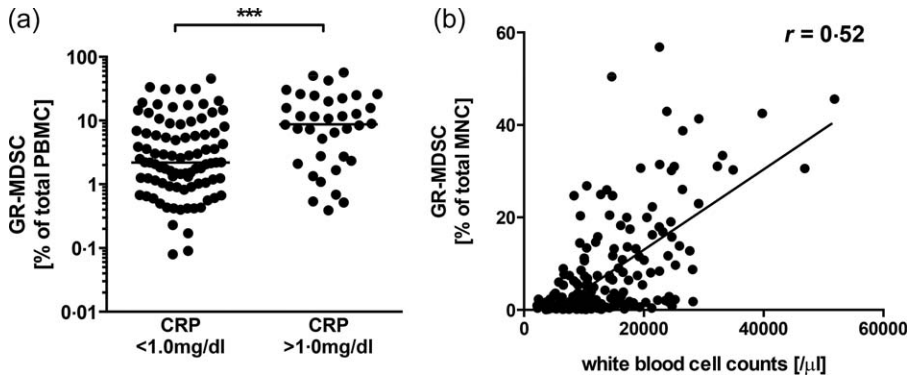

Fig. 4. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in dependence of inflammatory markers C-reactive protein (CRP) and white blood cell counts (WBC). Mononuclear cells (MNC) were isolated from peripheral blood (PBMC) of preterm infants and CRP and white blood cell counts were measured as part of routine laboratory evaluation. (a) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants with normal (CRP $<$ 1·0 mg/dl) and elevated CRP (CRP $>$ 1·0 mg/dl). n = 36–89; $^{***}P < 0{\cdot}001$. Mann–Whitney test. (b) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants depending on white blood cell count. Regression line shows correlation between percentages of GR-MDSC and white blood cell count. n = 206; $^{****}P < 0{\cdot}0001$, Spearman's correlation.

term neonates [14]. We found that (i) GR-MDSC were increased in cord blood of preterm infants independent of gestational age and that (ii) GR-MDSC counts remained elevated during the neonatal period and decreased after day 28. Furthermore, we found that (iii) infants with IAI or postnatal sepsis had significantly higher GR-MDSC counts than non-infected infants and that (iv) GR-MDSC counts correlated with the inflammatory markers CRP and WBC counts.

First, we found that GR-MDSC were increased similarly in cord blood of term and preterm infants of all gestational age groups. This is in line with our previous results in pregnant women, where we found increased numbers of GR-MDSC during the entire pregnancy, but also no differences between different pregnancy stages [19]. Only few data exist regarding other immune-regulatory cell populations in preterm infants. Some groups showed a negative correlation of gestational age and T_{reg} numbers in cord blood, respectively, in peripheral blood of preterm infants collected during the first days of life [10,31,36]. However, it should be mentioned that T_{regs} in preterm infants were analysed only with regard to quantitative changes in cell counts, but not concerning their functional activity. Nothing is known about the distribution of $CD71^+$ erythroid cells in preterm infants, which have been described recently to mediate immunosuppression after birth in term-born infants and mice [11]. As innate immune cells, GR-MDSC may act more non-specifically than T_{regs}, generally suppress immune rejection between mother and fetus and thus remain elevated constantly during the entire course of pregnancy.

Next, we found that GR-MDSC levels remained elevated during the whole neonatal period until postnatal day 28 and correlated negatively with postnatal age. This is again in line with GR-MDSC counts in maternal blood after birth [19]. Regarding other immune cell populations in preterm infant blood, we found a decrease in neutrophils parallel to the decrease in GR-MDSC and unchanged monocyte levels, but an increase in lymphocytes during the neonatal period. As lymphocytes are the main target population of GR-MDSC, the ratio between effector cells and target cells increases strongly during the neonatal period, supporting the hypothesis that changes in GR-MDSC levels may be relevant for impaired immune responses in neonates immediately after birth and for immune adaptation during the first weeks of life.

Taken together, our data may hint towards a differential role of GR-MDSC in pre- and postnatal life. Before birth, GR-MDSC may play a critical role in maintaining maternal–fetal tolerance [12,13,34,35], being beneficial for fetal life, whereas postnatally elevated GR-MDSC numbers may contribute to postnatal immunosuppression and susceptibility to infections. The functional role of elevated GR-MDSC levels during neonatal life will be the content of further studies in mice.

Results on the role of MDSC during sepsis are conflicting [21,24]. We now show that, despite already elevated levels in the neonatal period, a further accumulation of GR-MDSC occurs during sepsis in preterm infants. Delano *et al.* [21] first showed that during polymicrobial sepsis an accumulation of MDSC occurred, and that this led to a T cell suppression and Th2 polarization in mice. Adoptive transfer of MDSC in sepsis-prone mice resulted in reduced mortality, pointing towards a protective role of MDSC in sepsis [23]. A more detailed analysis revealed that, during sepsis, a differentiation of MDSC from a pro- to an anti-inflammatory phenotype occurred, which was accompanied by an initially increased, then reduced, mortality [24].

Recently, we could show that GR-MDSC from cord blood polarized T cells towards a Th2 response and induced T_{regs} [20], similar to the results of Delano *et al.* [21] during sepsis. Given the observations of Brudecki *et al.*, one could hypothesize that a sepsis-induced proinflammatory and detrimental MDSC population encounters an already suppressed immune system, leading to uncontrolled inflammation and contributing to the high mortality observed in neonatal sepsis. It has been shown further that high levels of GR-MDSC in the initial phase of sepsis predicted the risk for secondary infections [37]. A GR-MDSC-induced, prolonged alteration of immune responses may be an explanation for the occurrence of post-inflammatory diseases such as bronchopulmonary dysplasia (BPD) and periventricular leucomalacia (PVL), seen predominantly in preterm infants [38,39].

Lastly, we found that percentages of GR-MDSC in peripheral blood correlated positively with the inflammatory markers CRP and WBC counts. Similar results were obtained in thyroid cancer patients, where high MDSC numbers were accompanied by elevated CRP levels [40]. As an acute-phase protein (APP), CRP is synthesized in the liver mediated by the proinflammatory cytokine IL-6 [41], which has also been described to induce MDSC [42]. Furthermore, abrogation of APP production was shown to disturb mobilization and accumulation of MDSC in septic mice [23], so it seems obvious that a direct relation between MDSC and APPs exists. Further, we found a positive correlation between percentages of GR-MDSC and IMI in peripheral blood of preterm infants. As GR-MDSC have cellular properties of immature myeloid cells with lower density, lower granule content and less segmented nucleus than mature granulocytes [17], this correlation seems not surprising. In one study, IMI has been shown to be a sensitive and specific marker for diagnosis of neonatal sepsis [43]; whether or not GR-MDSC could be used as a complementary inflammatory marker in neonates should be addressed in prospective studies.

There are some limitations of our study. First, due to the low sample sizes obtained from preterm infants, we were unable to perform functional analyses of GR-MDSC, neither in uninfected controls nor infected patients, to confirm that they indeed exhibit suppressive properties. However, using the same isolation technique, previous data from cord blood showed immunosuppressive properties of these cells [20]. Secondly, blood samples for quantification of postnatal GR-MDSC came from routine withdrawals to avoid taking additional blood volumes from these very small infants. Thus, longer storage times than in our previous studies had to be accepted. However, kinetic analyses revealed that during up to 24 h of storage time no significant change in the percentages of GR-MDSC occurred. Also, due to the dependency on the remains of routine blood withdrawals, it was not possible to analyse samples at a standardized time-point of sepsis. Sepsis samples included in our study were taken between days 0 and 3 after onset of sepsis – a fact that could have influenced our results. Thirdly, we measured lower overall GR-MDSC levels compared to our previous study [14]. We assume that this is due to changed antibodies and modified purification methods adapted to the small blood volumes. Fourthly, our definition of IAI was based on clinical signs without histological validation of chorioamnionitis, making it difficult to distinguish from fetal inflammatory response syndrome without infection.

Taken together, we analysed expression of GR-MDSC in preterm infants showing that they do not drop immediately after birth, but remain elevated during the neonatal period. Infectious diseases led to a further expansion of GR-MDSC in preterm infants, as has been shown previously for adult patients. These results point again towards an important role of GR-MDSC not only for immune tolerance during fetal life, but also for postnatal immune regulation and altered host defence in preterm infants. Targeting GR-MDSC could be a new strategy for supportive treatment of neonatal sepsis.

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Disclosure

There are no financial and commercial conflicts of interest to disclose.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Gating strategy for granulocytic-derived suppressor cells (GR-MDSC). Mononuclear cells (MNC) were isolated from cord blood (CBMC) of preterm infants or term infants and from peripheral blood mononuclear cells (PBMC) of preterm infants. Density plots for forward-scatter (FSC) *versus* side-scatter (SSC) and for CD66b *versus* CD33, CD14 and human leucocyte antigen D-related (HLA-DR) show phenotype of $CD66b^{+}CD33^{+}CD14^{-}$ $HLA\text{-}Dr^{low/neg}$ GR-MDSC in preterm cord blood (left), term cord blood (middle) and preterm peripheral blood (right).

Fig. S2. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in cord blood of preterm infants. Mononuclear cells (MNC) were isolated from cord blood (CBMC) of preterm infants. (a–f) Scatter diagrams showing the percentage of GR-MDSC from total CBMC of preterm infants born by elective caesarean section (elective C/S) or spontaneously (spontaneous). $n = 42/29$, $^{*}P < 0{\cdot}05$. Mann–Whitney *U*-test (a) of singleton pregnancies or multiple pregnancies. $n = 37/34$, n.s. = not significant. Mann–Whitney test (b) of preterm infants with birth weight appropriate for gestational age (AGA) or small for gestational age (SGA). $n = 55/13$, n.s. = not significant. Mann–Whitney test (c) of male and female preterm infants. $n = 29/42$, n.s. = not significant. Mann–Whitney test (d) of preterm infants exposed to prenatal magnesium. $n = 23/43$, n.s. = not significant, Mann–Whitney test (e) and of preterm infants exposed to prenatal corticosteroids. $n = 9/58$, n.s. = not significant, Mann–Whitney test.

Fig. S3. Percentages and absolute numbers of neutrophilic cells, lymphocytes and monocytes in peripheral blood of preterm infants. Percentages of neutrophilic cells, lymphocytes and monocytes as well as absolute cell numbers were measured as part of routine laboratory evaluation. (a–f) Scatter diagram showing the percentage of neutrophilic cells (a), lymphocytes (c) and monocytes (e) from total white blood cells (WBC) and absolute cell counts of neutrophilic cells (b), lymphocytes (d) and monocytes (f) in peripheral blood of preterm infants in the perinatal period days 1–7, the neonatal period days 8–28 and the period beyond day 28. $n = 13$–18; $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; n.s. = not significant; Kruskal–Wallis test and Dunn's multiple comparison test.

Fig. S4. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in cord blood of preterm infants with postnatal infection. Mononuclear cells (MNC) were isolated from peripheral blood (PBMC) of preterm infants. Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants without infection (ctrl) or postnatal infection (infection) infants in the perinatal period days 1–7, the neonatal period days 8–28 and the period beyond day 28. $n = 2$–20.

Fig. S5. Quantification of granulocytic myeloid-derived suppressor cells (GR-MDSC) in dependence of C-reactive protein (CRP) and immature information (IMI). Mononuclear cells (MNC) were isolated from peripheral blood mononuclear cells (PBMC) of preterm infants and CRP and IMI were measured as part of routine laboratory evaluation. (a) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants with elevated CRP-level ($CRP > 1{\cdot}0$ mg/dl) depending on CRP. Regression line shows correlation between percentages of GR-MDSC and CRP. $n = 87$; $^{****}P < 0{\cdot}0001$; Spearman's correlation. (b) Scatter diagram showing the percentage of GR-MDSC from total PBMC of preterm infants depending on IMI. Regression line shows correlation between percentages of GR-MDSC and IMI. $n = 174$; $^{****}P < 0{\cdot}0001$; Spearman's correlation.

