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Neutrophil and endothelial adhesive function during human fetal ontogeny

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ABSTRACT

Attenuation of the immune response contributes to the high rate of neonatal infections, particularly in premature infants. Whereas our knowledge of innate immune functions in mature neonates is growing, little is known about the ontogeny of neutrophil recruitment. We investigated neutrophils and ECs in the course of gestation with respect to rolling and adhesive functions. With the use of microflow chambers, we demonstrate that the neutrophil's ability to roll and adhere directly correlates with gestational age. These adhesion-related abilities are very rare in extremely premature infants (<30 weeks of gestation), which may correlate with our observation of markedly reduced expression of PSGL-1 and Mac-1 on neutrophils in preterm infants. In parallel, the capacity of HUVECs to mediate neutrophil adhesion under flow increases with gestational age. In addition, HUVECs from extremely premature infants exerting the lowest ability to recruit adult neutrophils show a diminished up-regulation of E-selectin and ICAM-1. Finally, by following neutrophil function postnatally, we show that maturation of PMN recruitment proceeds equivalently during extra- and intrauterine development. Thus, PMN recruitment and EC adhesion-related functions are ontogenetically regulated in the fetus, which might contribute significantly to the high risk of life-threatening infections in premature infants. *J. Leukoc. Biol.* 93: 175–184; 2013.

Introduction

Severe infections remain a leading cause of neonatal morbidity and mortality, despite advances in intensive care medicine and the early use of antibiotics. It is estimated that worldwide, over 1 million neonates die annually due to overwhelming infections [1]. The incidence of sepsis correlates inversely with the gestational age of the infants, and premature infants, in particular, show a unique susceptibility to (bacterial) pathogens with secondary sepsis rates ranging from 22% to 36% in very low birth-weight neonates and up to 62% in extremely premature infants [2–4]. In addition to extrinsic factors, such as invasive procedures and long hospital stays, an immaturity of the immune system is thought to account for the increased rate of neonatal infections [5].

During infection, PMNs play a crucial role in the early innate immune response [6]. The importance of these cells for host defense against invading microorganisms is underscored by the high risk of infections in patients with neutropenia or neutrophil disorders [7, 8]. This is particularly relevant for neonates, as it has been shown that they largely depend on the innate immune system for protection against sepsis, whereas the adaptive immune system initially has a minor role [9]. To fight pathogens, neutrophils are recruited from the vasculature into infected tissue in a multistep adhesion process involving a complex interplay of adhesion molecules with their respective ligands located on the neutrophil membrane and on ECs [10]. First, circulating PMNs are captured and begin to roll along the vessel wall through selectins (P-, E-, and L-selectin), binding to selectin-ligands, such as PSGL-1 [11, 12]. During rolling, neutrophils come in close contact with chemokines presented on inflamed endothelium, which bind to specific chemokine receptors on the neutrophil surface, leading to a conformational change and activation of neutrophil-expressed β-2 integrins (inside-out signaling). This results in firm neutrophil arrest, a prerequisite for subsequent transmigration of the cells into tissue [10]. At present, our detailed understanding of the PMN recruitment cascade is based primarily on studies performed on adult organisms [10]. It has long been known that there are distinct differences between neonates and adults in neutrophil adhesiveness and migratory behavior [13]. However, our knowledge about the ontogeny of neutrophil recruitment in

Abbreviations: EC=endothelial cell, FOV=field of view, h=human, Mac-1=macrophage-1 antigen, MFI=mean fluorescence intensity, PSGL-1=P-selectin glycoprotein ligand-1, VE=vascular endothelial, vWF=von Willebrand factor

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the developing fetus remains incomplete. Animal experiments addressing this matter are scarce, have been performed primarily in nonmammalian organisms, but indicate an impaired response of the innate immune system during fetal development [14, 15]. These findings parallel the clinical observation of an inverse relationship between sepsis incidence and gestational age, with highest infection rates found in extremely premature infants. However, so far, it is still unclear whether PMN recruitment in human fetuses is subjected to a similar maturation process of the immune system during gestation and whether this maturation process can be accelerated following premature delivery.

In the present study, we investigated the recruitment of PMN from premature and mature human neonates. Our experiments reveal that the neutrophil's ability to roll, adhere, and express adhesion molecules increases significantly with gestational age. Intriguingly, the maturation of PMN recruitment during fetal development is not dependent on intrauterine factors, as comparisons of PMN function between premature infants with the same postconceptional but a different postnatal age demonstrated. In addition, we observed a corresponding reduced capacity of ECs from premature infants to up-regulate adhesion molecules and to induce PMN recruitment upon LPS stimulation, thus identifying another neonatal deficiency potentially contributing to the pathogenesis of severe infections in premature infants.

MATERIALS AND METHODS

Sample collection and study population

All neonates included in the study were delivered by Caesarean section. Umbilical cord blood and a piece of the umbilical cord were collected immediately after delivery. Exclusion criteria were maternal HIV infection, congenital malformations, or suspected syndromes, vaginal delivery, and familial immune diseases. Peripheral venous blood from infants and healthy adult volunteers was drawn by venipuncture. Standard blood collection tubes (S-Monovette, Sarstedt, Nümbrecht, Germany) containing trisodium citrate were used for anticoagulation of blood samples.

Based on their gestational age, as estimated by the date of the last menstrual period and by ultrasound measurements, infants were grouped into extremely premature infants (≤30 completed weeks of gestation), moderately premature infants (30+1–36+6 weeks of gestation), and mature neonates (>37 completed weeks of gestation). Because of small sample volumes, especially in the extremely premature infant group, we were unable to perform all measurements on each individual. The exact number of subjects for each experiment is given in the figure legends. We recorded baseline characteristics, including gender and birth weight; clinical data, such as antenatal steroid treatment; and the reason for prematurity or Caesarean section, as well as laboratory values (cord blood pH, differential blood-cell count, C-reactive protein).

Isolation of PMNs

As leukocyte and differential counts vary largely among different neonatal age groups, we isolated PMNs from the umbilical cord or peripheral venous blood for standardization of experimental conditions. Whole blood was layered onto a Ficoll density gradient (LSM 1077; PAA Laboratories GmbH, Coelbe, Germany) and centrifuged (1200 *g*, 20 min, room temperature). The resulting erythrocyte-granulocyte pellet was washed twice in Dulbecco's PBS (1×, without Ca^{2+} and Mg^{2+}; Invitrogen GmbH, Darmstadt, Germany) before lysis of erythrocytes by addition of hypotonic buffer (0.15 M NH_4Cl, 0.01 M $NaHCO_3^-$, 0.001 M EDTA, in Aqua ad injetabilia) for 7 min. The remaining granulocytes were washed twice, resuspended in 1 ml PBS, and counted in a Neubauer chamber using Turks solution (Merck, Darmstadt, Germany). By this method, we achieved 76% (premature infants) to 96% (adults) purity and >90% viability of cells. For some experiments, whole blood from adult donors was pretreated with bethamethasone (0.02 μg/ml; Celestan, Essex Pharma GmbH, Munich, Germany) for 1 h before isolation of PMN.

Isolation and culture of HUVECs

ECs were isolated from the umbilical cord vein using collagenase A (1 mg/ml; Roche Diagnostics GmbH, Mannheim, Germany), as reported previously [16]. The cells were resuspended in EC growth medium, supplemented with 10% FCS, basic fibroblast growth factor (1.0 ng/ml), EC growth supplement/heparin (0.004 ml/ml), endothelial growth factor (0.1 ng/ml), hydrocortisone (1.0 μg/ml), and phenol red (0.62 ng/ml; all PromoCell GmbH, Heidelberg, Germany) and 1% penicillin/streptomycin (10,000 U/10 mg/ml; PAA Laboratories GmbH), and grown in standard cell culture dishes. When confluent, cells were harvested using trypsin/EDTA (0.5 mg/0.22 mg/ml; PAA Laboratories GmbH) and cultured further in Medium 199 (Invitrogen GmbH) containing 10% FCS. For flow cytometry, cells of Passage II were collected by use of EDTA (5 mM; Merck) alone. For flow chamber assays, cells of Passage II were transferred into microflow chambers (μ-Slide VI ibiTreat; ibidi, Martinsried, Germany) and grown to confluence overnight. In some cases, freshly isolated HUVECs were incubated with betamethasone (0.02 μg/ml; Celestan, Essex Pharma GmbH) for 12 h and processed further as described above.

Flow chamber experiments

To analyze PMN recruitment, we used an in vitro flow chamber assay. For this purpose, glass capillaries (2×0.2 mm; VitroCom, Mountain Lakes, NJ, USA) were assembled into microflow chambers, as described previously [17]. To assess PMN rolling, the flow chambers were coated with rhP-selectin (CD62-P, 5 μg/ml; R&D Systems, Wiesbaden-Nordensteadt, Germany) or rhE-selectin (CD62-E, 5 μg/ml) in PBS with 0.1% BSA (PAA Laboratories GmbH). For adhesion studies, a combination of selectin (rhP-selectin 10 mg/ml or rhE-selectin 4 mg/ml), chemokine rhCXCL8 (IL-8, 10 μg/ml; R&D Systems), and integrin-ligand rhICAM-1 (CD54, 4 μg/ml; R&D Systems) was used. After overnight incubation, the flow chambers were blocked with 5% casein (from bovine milk; Sigma-Aldrich, Munich, Germany) in PBS for 2 h at room temperature and flushed with PBS. For negative controls, we used four approaches: chemokine or selectin omission, addition of EDTA to the cell suspension, and coating with PBS/0.1% BSA alone. Before the experiments, the isolated PMNs were resuspended in DMEM (low glucose without glutamine; Invitrogen GmbH), containing 1% BSA, $CaCl_2$ (1 mM), and $MgCl_2$ (1 mM), to a calculated concentration of 250,000 cells/ml (rolling assay) or 500,000 cells/ml (adhesion assay). The cell suspension was flushed through the flow chamber using a high-precision syringe pump (Harvard Apparatus, Holiston, MA, USA) at a flow rate of 0.115 ml/min, resulting in a shear stress of ~1 dyne/cm^2 [17].

In other experiments investigating the contribution of neonatal ECs to PMN recruitment, we stimulated HUVECs grown in ibidi flow chambers (3.8×4 mm, μ-Slide VI) with LPS (10 μg/ml, LPS from *Escherichia coli* 0111:B4; Sigma-Aldrich) for 4 h. After washing with HBSS (Pharmacy of the University Hospital Munich, Germany), the chambers were connected to a PE 50 tube (intramedic polyethylene tubing, inner diameter 0.58 mm; outer diameter 0.965 mm; Becton Dickinson, Franklin Lakes, NJ, USA) and perfused with PMNs from a healthy adult donor at a concentration of 250,000 cells/ml and a rate of 0.91 ml/min, resulting in a shear stress of ~1.6 dyne/cm^2 based on the manufacturer's description (ibidi Application Note #11; www.ibidi.com).

Microscopy and data analysis

The flow chamber studies were performed on an upright microscope (BX51; Olympus, Hamburg Germany) with a saline immersion objective (XLUMPlanFl, 20×, 0.95 NA) or on an inverse microscope (IM35, Nikon Fluor 20×, 0.75 NA; Zeiss MicroImaging GmbH, Munich, Germany) for

observation of ibidi chambers. Experiments were recorded for 10 min on a Super VHS recorder via a charge-coupled device camera (Model CF8/1; Kappa optronics GmbH, Germany) for later, off-line analysis. PMN rolling was quantified by counting the number of cells/FOV (740 mm×565 μm), moving at a velocity significantly lower than the center-line velocity for >30 s. Adherent PMNs were defined as cells that remained stationary for >30 s.

Flow cytometric analysis of adhesion molecule expression

Surface expression of adhesion molecules on PMNs and ECs was measured by flow cytometry. Isolated PMNs were incubated with primary mAb (all IgG1 mouse anti-human; BD PharMingen, Heidelberg, Germany) to LFA-1 (CD11a), Mac-1 (CD11b), PSGL-1 (CD162), or CXCR2 (CD182) for 45 min on ice. A PE-labeled goat anti-mouse antibody was used as a secondary antibody. Unstimulated and LPS-stimulated (4 h, 10 μg/ml) HUVECs of Passage II were stained with PE-labeled mAb (all IgG1 mouse anti-human; BD PharMingen) to E-selectin (CD62-E) or ICAM-1 (CD54) for 45 min on ice. For EC characterization, unstimulated HUVECs were treated with PE-labeled antibody to PECAM (CD31, IgG1; BD PharMingen) or with unlabeled primary antibody to vWf (IgG1; Dako, Eching, Germany) with PE-labeled goat anti-mouse secondary antibody. Measurements were performed on a FACSorter flow cytometer (Becton Dickinson) using the CellQuest Pro software (Becton Dickinson). The MFI for 10,000 cells/sample was obtained using log detection settings. All antibodies were normalized against isotype-matched controls.

Immunofluorescence staining

HUVECs from premature and mature neonates (for isolation procedure, see above) were grown to confluence in μ-Slides (ibidi) and fixed by addition of 3.7% paraformaldehyde (Applichem, Darmstadt, Germany) in PBS (Invitrogen GmbH) for 10 min at room temperature. After rinsing with PBS, cells were blocked with 1% BSA (PAA Laboratories GmbH) in PBS for 30 min at room temperature. Cells were incubated with primary monoclonal mouse anti-human VE-cadherin (CD144, IgG1; eBioscience, Frankfurt, Germany) for 2 h at room temperature, washed, and incubated with a secondary Alexa 546-labeled goat anti-mouse antibody (Invitrogen GmbH) for 2 h at room temperature. After repeated washing steps to remove unbound antibody, mounting medium (Permafluor; Beckman Coulter, Fullerton, CA, USA) was added to the channels, and VE-cadherin expression was detected by confocal laser-scanning microscopy (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany) using a 40× oil immersion objective (HCX PLAPO CS 40×/1.25–0.75 oil) and Leica Application Suite software.

Statistical analysis

For multiple comparisons between groups, a Kruskal-Wallis ANOVA on ranks was used, followed by Student-Newman-Keuls or Dunn's post hoc test, as appropriate. To test for nonlinear correlation, Spearman's rank correlation coefficient was determined. A P value <0.05 was considered statistically significant. All statistical analyses were carried out with SigmaStat 3.5 (Systat Software GmbH, Germany). Data are presented as mean ± SEM.

Study approval

The study was approved by the local Medical Ethical Committee of the Ludwig-Maximilians-Universität (Project 249-08), and informed, written consent was obtained from the mothers of all participants during pregnancy prior to inclusion into the study.

RESULTS

Study population

From 10/2008 to 12/2009, a total of 58 newborns were included in this study. Infants were classified according to their gestational age into extremely premature infants (n=20), moderately premature infants (n=18), and mature neonates (n=19). Reasons for prematurity were placental insufficiency, premature labor and/or premature rupture of membranes, pre-eclampsia, HELLP syndrome, pathologic Doppler flow, suspected amnion infection, and twin pregnancy. Baseline characteristics and laboratory data of the patient groups are given in **Table 1**. As children were included consecutively, the gender distribution among the experimental groups shows some variation. As expected, premature neonates had a significantly lower birth weight than mature neonates. The white blood-cell count and the number of PMNs in whole blood, as well as the arterial pH value, also differed significantly among the groups.

Rolling and adhesion of neonatal PMNs in relation to gestational age

To assess whether gestational age influences PMN recruitment under dynamic conditions, we quantified rolling and adhesion of neutrophils obtained from umbilical cord blood of premature and mature infants in a microflow chamber system at a shear stress of ~1 dyne/cm^2, resembling a physiological shear stress found in postcapillary venules in vivo [18]. When looking at PMNs from adult healthy controls, we observed a continuous increase in the number of rolling cells in P-selectin (**Fig. 1A**)- and

TABLE 1. Patient Characteristics and Laboratory Values of Umbilical Cord Blood

Data	Extremely premature infants	Moderately premature infants	Mature neonates	P value (ANOVA)
Clinical	n = 20	n = 18	n = 19	
Gestational age (weeks)	27 3/7 ± 1 6/7	32 4/7 ± 1 5/7	38 4/7 ± 1 0/7	n.a.
Birth weight (g)	1043 ± 240	1825 ± 382	3378 ± 448	<0.001
Female/male	6/14	6/12	9/10	n.a.
Laboratory	n = 17	n = 15	n = 16	
WBC (G/l)	7.7 ± 4.9	7.5 ± 3.3	15.5 ± 6.9	<0.001
PMN (%)	31 ± 22	30 ± 12	50 ± 12	0.018
Hct (%)	40 ± 7	43 ± 6	47 ± 6	n.s.
Platelet count (G/l)	228 ± 95	217 ± 46	269 ± 35	n.s.
CrP (mg/dl)	<0.5	<0.5	<0.5	n.a.
Arterial pH	7.27 ± 0.09	7.35 ± 0.04	7.32 ± 0.05	0.029

Please note that laboratory data could not be obtained from each child as a result of small sample volume or sampling errors. Data are given as mean ± SEM. n.a., Not applicable; WBC, white blood-cell count; Hct, hematocrit; CrP, C-reactive protein.

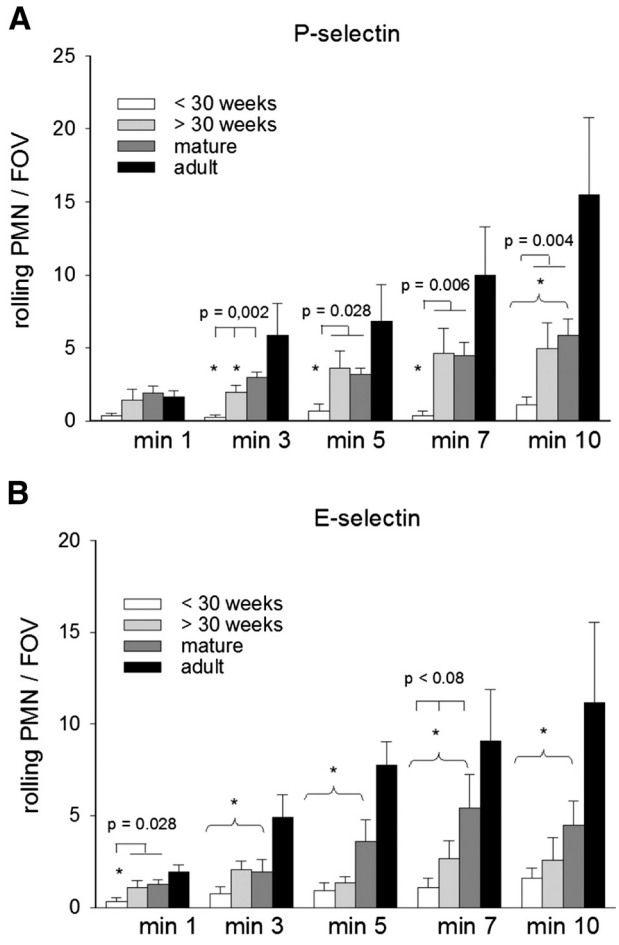

Figure 1. Rolling of neonatal and adult neutrophils under flow. PMNs, isolated from umbilical cord or peripheral venous blood (250×10^3/ml), were perfused through microflow chambers coated with (A) rhP-selectin (5 μg/ml) or (B) rhE-selectin (5 μg/ml) for 10 min at ~1 dyne/cm^2, and the number of rolling cells was quantified. Data are given as mean ± SEM (n=6/group). *Significance versus adult control; squared brackets indicate significance between experimental groups (P<0.05, ANOVA on ranks).

E-selectin (Fig. 1B)-coated flow chambers during the 10-min observation period. Compared with adult controls, the number of rolling PMNs was significantly lower in mature neonates and even further reduced in premature infants (Fig. 1A and B). Neutrophils from extremely premature infants showed almost no rolling on immobilized P- or E-selectin, suggesting that neutrophil rolling is developmentally regulated during fetal life.

Next, we studied PMN adhesion in flow chambers coated with a combination of P- or E-selectin, the CXCR2 chemokine IL-8, and the integrin ligand ICAM-1. We observed that the ability of neutrophils to adhere under conditions of flow strongly and significantly depends on gestational age. Whereas PMNs from extremely premature infants rarely showed adhesion (**Fig. 2A** and **B**), there were some adherent cells in the moderately premature infant group. A further increase in adhesion was seen when PMNs from mature neonates were perfused through the chambers. However, even mature neonates did not reach the levels seen in the adult control group (Fig. 2A and B). When plotting the number of adherent cells at 10 min against the gestational age of each individual, we found a highly significant correlation (Fig. 2C). To test whether PMN adhesion is mediated specifically by the immobilized molecules used in the experiment, we performed control microflow chamber experiments, where the selectin or the chemokine was omitted during coating, chambers were coated with PBS/BSA alone, or EDTA was added to the perfusion medium to eliminate divalent cations necessary for specific binding. We did not observe significant numbers of adherent cells from adult healthy donors in any of the control chambers (Fig. 2D), ruling out nonspecific PMN adhesion in our assay.

Recruitment of adult PMNs on HUVECs from premature and mature neonates

For the successful recruitment of PMNs in vivo, neutrophils need to interact with adhesion molecules expressed on ECs [10]. It has been shown that age-dependent variations in neutrophil recruitment to fetal porcine ECs exist, with a relative inability of ECs from midgestation fetuses to interact with neutrophils under conditions of flow [19]. We assessed whether ECs isolated from the umbilical cord of premature and mature infants are capable of supporting recruitment of PMNs isolated from adult healthy controls using the microflow chamber assay. As expected, we did not observe significant rolling or adhesion of adult PMNs on unstimulated HUVECs in any group (**Fig. 3A** and **B**). Upon EC stimulation with LPS for 4 h, PMNs started to roll and adhere on the HUVEC layer in all experimental groups. However, the extent of PMN rolling and adhesion differed significantly between the groups and depended on the gestational age of the neonates (Fig. 3C and D). After 10 min, the number of cells rolling on HUVECs from mature infants was nearly fivefold above the numbers found on HUVECs from extremely premature infants (Fig. 3A and C), and the number of adherent PMNs was nearly threefold higher (Fig. 3B and D).

Age-dependent expression of adhesion molecules on fetal PMNs and umbilical cord ECs

As a result of the age-dependent differences observed in PMN recruitment, we characterized the cells isolated from the umbilical vein of premature and mature neonates and quantified adhesion molecule expression by flow cytometry and confocal laser-scanning microscopy. Unstimulated cells expressed similar levels of CD31 and vWF, as well as VE-cadherin, demonstrating their endothelial nature (**Fig. 4A** and **B**). We then quantitated by flow cytometry the adhesion molecule expression on umbilical vein ECs from premature and mature neonates. ICAM-1 was expressed constitutively at low levels on untreated cells, whereas E- and P-selectin were not detected (Fig. 4C). After stimulation with LPS, expression of E-selectin increased significantly in all groups. However, the amount of up-regulation was lowest in extreme premature infants. Comparably, we found a significant LPS-stimulated up-regulation of ICAM-1 expression in term neonates, whereas ICAM-1 expression was less inducible by LPS in premature infants (Fig. 4C). In line with prior reports showing that LPS does not induce

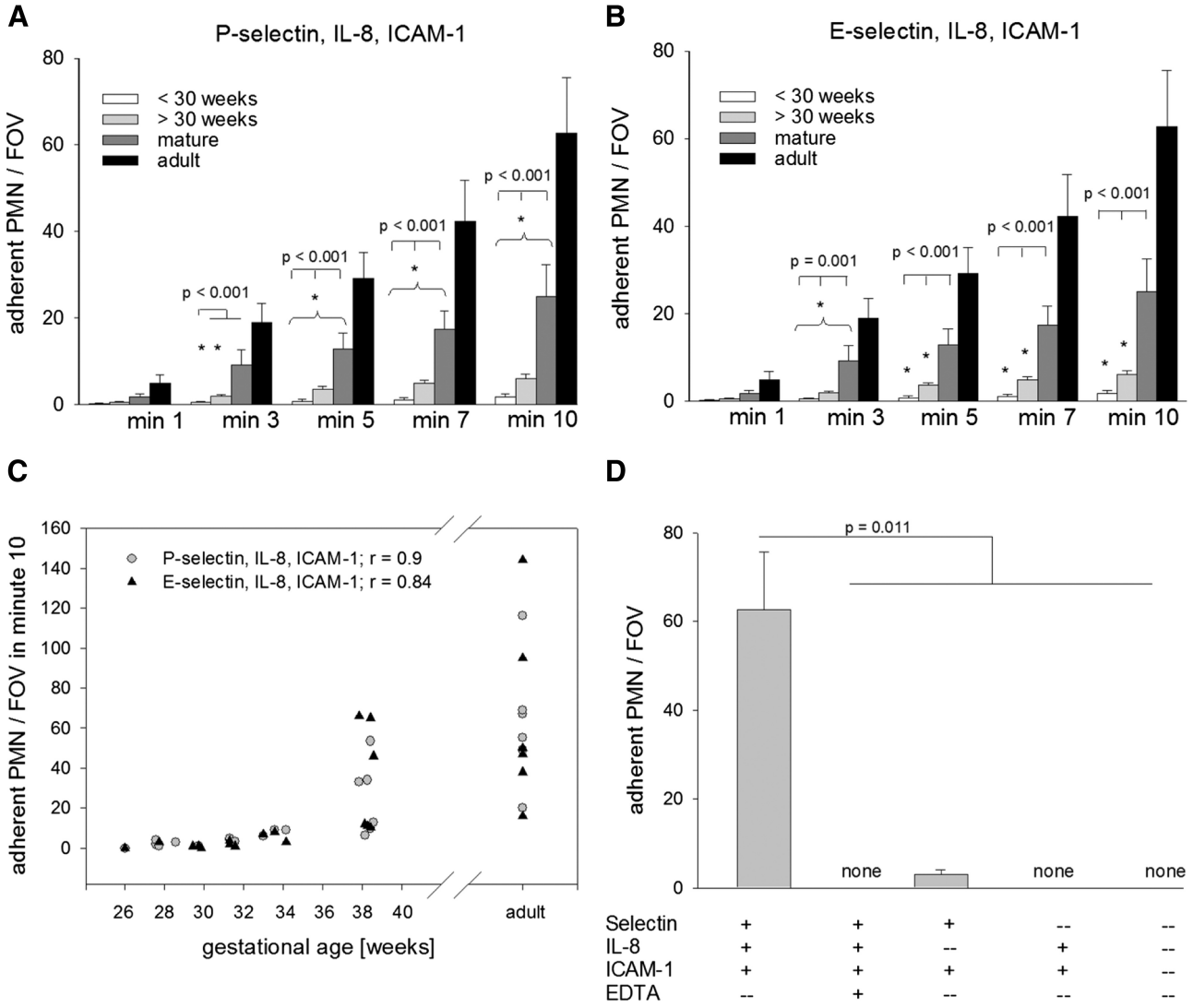

Figure 2. Adhesion of neonatal and adult neutrophils under flow. PMNs isolated from umbilical cord or peripheral venous blood (500×10^3/ml) were perfused through microflow chambers coated with (A) P-selectin (10 μg/ml), IL-8 (10 μg/ml), and ICAM-1 (4 μg/ml) or (B) E-selectin (4 μg/ml), IL-8 (10 μg/ml), and ICAM-1 (4 μg/ml) for 10 min at ~1 dyne/cm^2, and the number of adherent cells was quantified. Data are given as mean ± SEM (n=6/group). *Significance versus adult control; squared brackets indicate significance between experimental groups (P<0.05, ANOVA on ranks). (C) Correlation of the number of adherent cells in Minute 10 with the gestational age of the neonates (P<0.01, Spearman rank-order correlation). For comparison, adhesion of adult cells in Minute 10 is plotted. (D) For negative controls, different approaches were used: coating with PBS/BSA alone, omission of the selectin or the chemokine, and chelation of divalent cations by addition of EDTA (5 mM) to the perfusion medium. Adhesion of PMNs from adult healthy controls at Minute 10 is depicted (left bar in D is identical to black bar, min 10, in A; n=2–3/group).

P-selectin surface expression in HUVECs [20], we were not able to detect any P-selectin on LPS-stimulated HUVECs by means of flow cytometric analysis (not shown).

Next, we measured the surface expression of adhesion molecules relevant for rolling and adhesion on neonatal and adult neutrophils. For this purpose, PMNs were quantified for the selectin-ligand PSGL-1, the chemokine receptor CXCR2, and the β-2 integrins LFA-1 and Mac-1 using flow cytometry (Fig. 4D). Whereas expression levels of CXCR2 and LFA-1 were identical among our experimental groups, we found that PSGL-1 and Mac-1 surface expression was reduced significantly in extremely premature infants compared with adult controls. The expression of these molecules subsequently increased with advancing gestational age (Fig. 4D), suggesting that reduced expression of PSGL-1 and Mac-1 contributes to reduced neutrophil rolling and adhesion during fetal development.

Influence of antenatal steroid treatment on PMN and HUVEC functions

Acceleration of fetal lung maturation by betamethasone treatment of pregnant women with impending preterm delivery is nowadays standard clinical practice [21, 22]. In our patient cohort, 89% of extremely premature infants and 72% of moderately premature infants were exposed to antenatal maternal

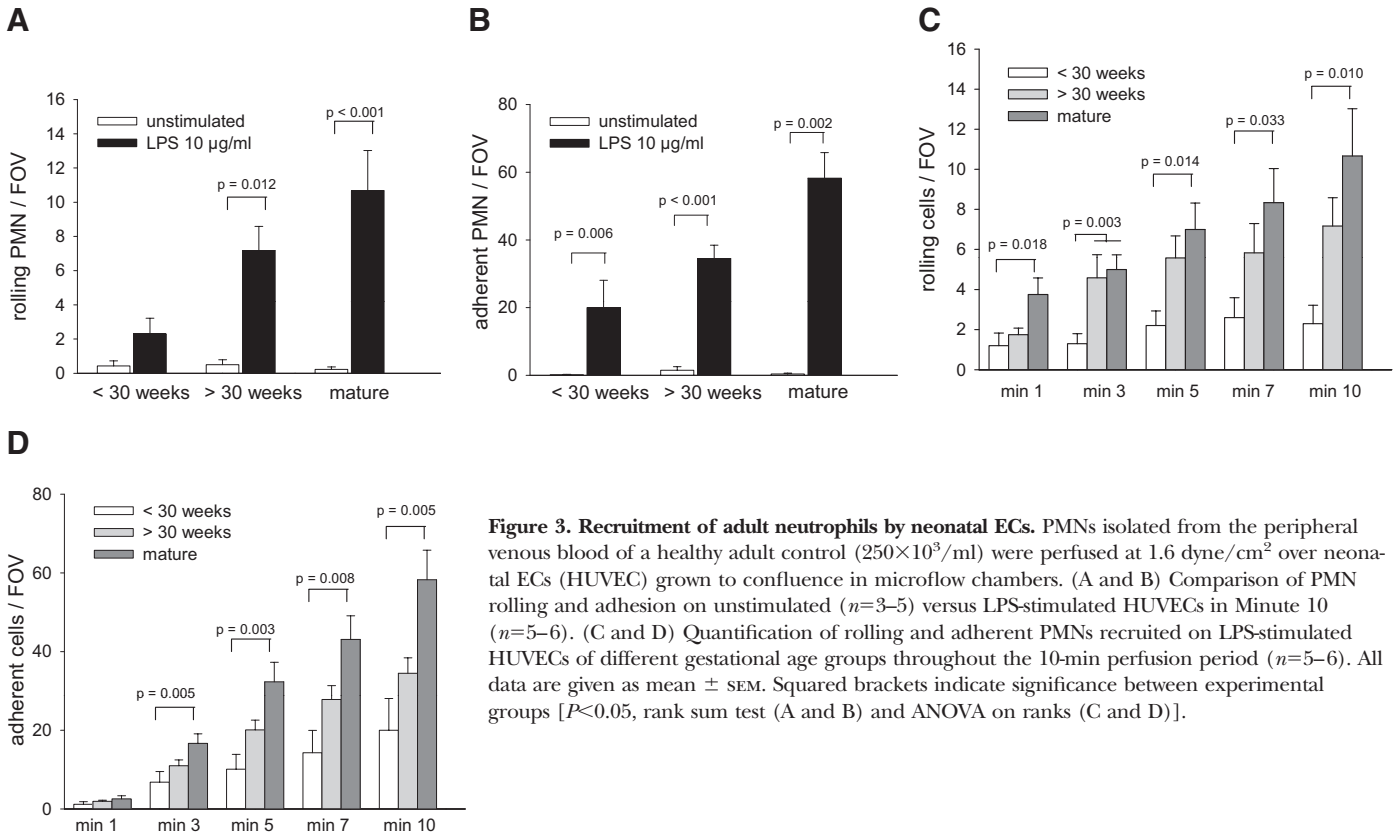

Figure 3. Recruitment of adult neutrophils by neonatal ECs. PMNs isolated from the peripheral venous blood of a healthy adult control (250×10^3/ml) were perfused at 1.6 dyne/cm^2 over neonatal ECs (HUVEC) grown to confluence in microflow chambers. (A and B) Comparison of PMN rolling and adhesion on unstimulated (n=3–5) versus LPS-stimulated HUVECs in Minute 10 (n=5–6). (C and D) Quantification of rolling and adherent PMNs recruited on LPS-stimulated HUVECs of different gestational age groups throughout the 10-min perfusion period (n=5–6). All data are given as mean ± SEM. Squared brackets indicate significance between experimental groups [P<0.05, rank sum test (A and B) and ANOVA on ranks (C and D)].

steroid treatment. To exclude the possibility that such exposure is responsible for the reduction of PMN recruitment seen in premature neonates, we performed microflow chamber experiments using adult donor PMNs that were pretreated (1 h) with betamethasone at a concentration found in the umbilical cord blood of neonates [23]. Adhesion of betamethasone-pretreated adult PMNs in flow chambers coated with P-selectin, IL-8, and ICAM-1 was similar to adhesion observed for untreated adult control PMNs (**Fig. 5A**). In addition, we incubated freshly isolated HUVECs with betamethasone (12 h) before seeding into flow chambers. HUVECs pretreated with betamethasone supported LPS-induced adhesion of adult PMNs to the same extent as HUVECs without prior betamethasone incubation (Fig. 5B). Furthermore, flow cytometric analysis of LPS-stimulated HUVECs (Passage II) demonstrated that the LPS-induced up-regulation of E-selectin and ICAM-1 was independent of prior betamethasone treatment (Fig. 5C), suggesting that betamethasone does not interfere with neutrophil recruitment in our setup.

Postnatal development of PMN recruitment

Recent reports indicated that neutrophils are subjected to a postnatal maturation process [24–26]. This prompted us to investigate the development of PMN recruitment postnatally and evaluate whether exposure of infants to extrauterine factors changes the development of PMN function when compared with the situation in utero. To address this, we compared adhesion of isolated neutrophils from premature infants born at <30 weeks of gestation directly after birth and 4–6 weeks later using P-selectin-, ICAM-1-, and IL-8-coated flow chambers. Compared with the adhesion observed for PMNs isolated directly after birth, PMN adhesion increased significantly with advancing postnatal age of the infants (**Fig. 6A**). However, this increase in adhesion (extrauterine development) was similar to the adhesion observed for PMNs obtained from umbilical cord blood of premature infants born >30 weeks of gestation (intrauterine development), which were matched for the same postconceptional age (Fig. 6B). These findings indicate that extrauterine factors do not seem to have a relevant influence on the development of neutrophil-adhesive capacity, implying that neutrophil maturation during development follows an intrinsic program rather than depending on postnatal factors.

DISCUSSION

The recruitment of neutrophils to sites of infection poses a key event in innate host defense against invading microorganisms. Whereas numerous studies investigating PMN functions in adult organisms have broadened the general understanding of this process, our knowledge of the developing innate immune system during gestation remains limited. In the present study, we analyzed PMN recruitment during human fetal development at different time-points of gestation.

Earlier reports have shown that neutrophil rolling and adhesion can be studied under defined conditions in microflow

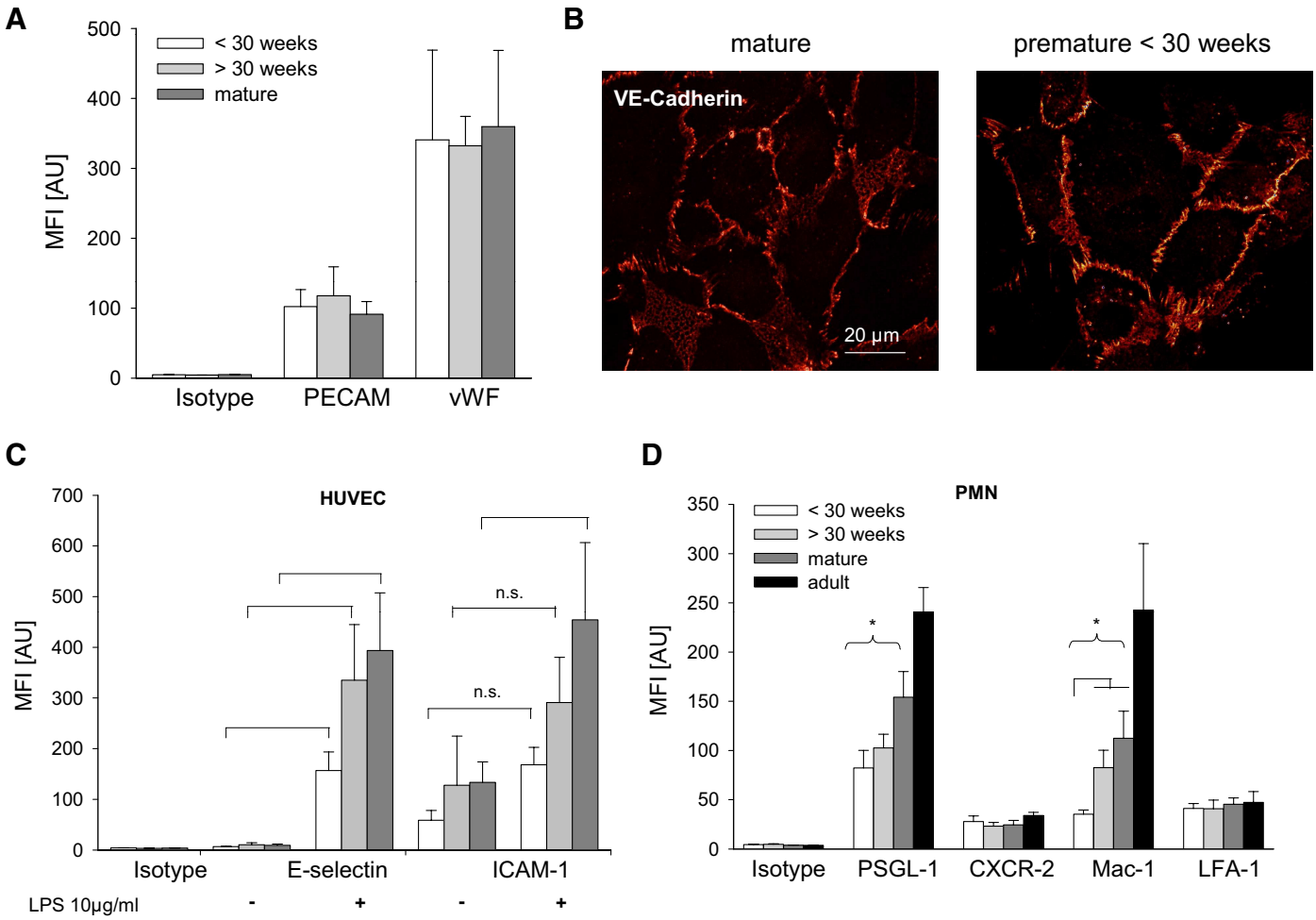

Figure 4. Expression of adhesion molecules on neutrophils and HUVECs. Cells isolated from the umbilical vein of premature and mature neonates were characterized as ECs by their expression of (A) PECAM (CD31) and vWF, as measured by flow cytometry, and (B) VE-cadherin (CD 144, Alexa 546), as seen in confocal laser-scanning microscopy (40× objective). Data are given as mean ± SEM (n=3–5/group). (C) Flow cytometric quantification of E-selectin and ICAM-1 surface expression on unstimulated (n=3) and LPS-stimulated HUVECs (n=6). (D) Flow cytometric quantification of PSGL-1, CXCR2, and Mac-1 (n=5–6) and LFA-1 (n=3–5) surface expression on isolated neonatal and adult PMNs. All data are given as mean ± SEM. *Significance versus adult control; squared brackets indicate significance between experimental groups (P<0.05, ANOVA on ranks).

chambers, guaranteeing reproducibility of experiments and comparability among different experimental groups [17]. With the use of this model, we demonstrate that recruitment of neutrophils under shear stress is almost absent in extremely premature infants. With advancing fetal development, the capability to sufficiently support rolling and adhesion of neutrophils increased gradually; however, even mature neonates did not reach the level of adult controls. This observation is in line with previous reports demonstrating decreased adhesion of human neonatal neutrophils under conditions of flow compared with adult neutrophils [27, 28]. Of importance, these earlier studies only included mature infants and did not take into account possible ontogenetic differences. Previous reports addressing neutrophil adhesion and migration in preterm infants demonstrated a reduction of PMN adhesion and chemotaxis compared with mature neonates or adults. Yet, the data are controversial in regard to age-dependent differences between premature and mature neonates [29–31] and have to be interpreted carefully, as experiments were performed under static conditions, altering adhesion and migration properties [32].

Here, we analyzed neutrophil rolling and adhesion under in vitro settings resembling venous blood flow. Capture and rolling of leukocytes in this setting are mediated by selectin–selectin ligand interactions. For mature neonates, it was described previously that neutrophil rolling on P- or E-selectin-presenting monolayers or on histamine- or IL-1-stimulated HUVECs, respectively, is impaired compared with adult controls [27, 33]. By analyzing neutrophils isolated from premature infants, we could expand those earlier findings and demonstrate that the ability of neutrophils to roll on immobilized P- or E-selectin is even reduced further in extremely premature neonates and increases progressively with increasing maturity of the infants. The severe reduction of capture and rolling events observed for extremely premature infants is very likely to contrib-

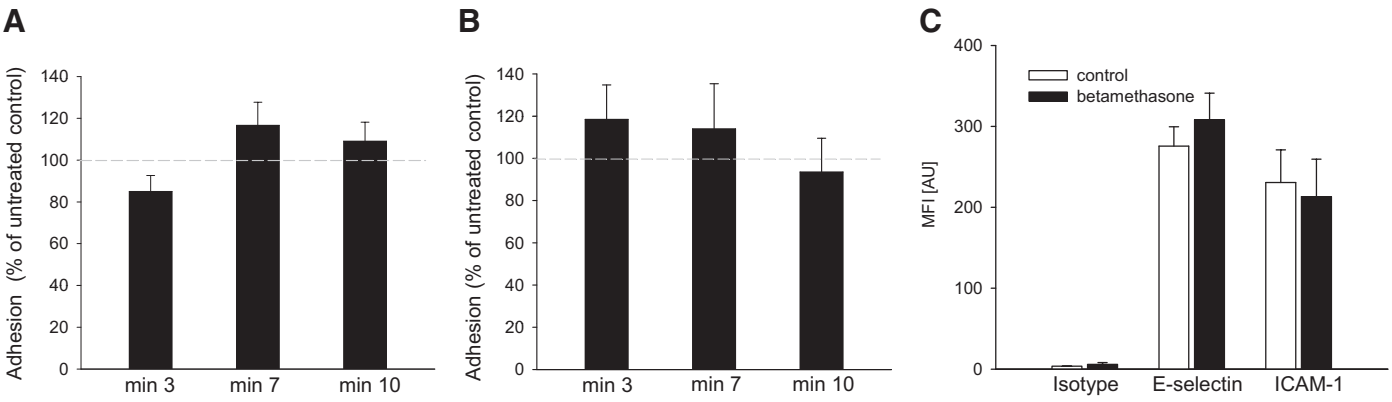

Figure 5. Influence of betamethasone treatment on experimental PMN recruitment and adhesion molecule expression. (A) Peripheral blood of adult controls was pretreated with betamethasone (0.02 μg/ml) for 1 h, and adhesion of isolated PMNs in flow chambers coated with P-selectin (10 μg/ml), IL-8 (10 μg/ml), and ICAM-1 (4 μg/ml) was compared with untreated control (=100%, dashed line; n=3/group). (B) Freshly isolated HUVEC from mature neonates (n=2) were pretreated with betamethasone (0.02 μg/ml), and LPS-stimulated recruitment of adult PMNs on HUVECs was compared with untreated control (=100%, dashed line; four chambers/group). (C) LPS-stimulated expression of adhesion molecules on HUVECs pretreated with betamethasone was measured by flow cytometry and compared with untreated control (n=3/group). All data are given as mean ± SEM.

ute to the very low amount of adhesion found in this age group, as capture and rolling are prerequisites for firm adhesion of cells to the endothelium [34].

In addition, quantitative and qualitative differences in neutrophil integrins and selectins are proposed to be responsible for the reduced recruitment of neonatal neutrophils compared with adults. This includes a decreased basal surface expression of L-selectin and a defective up-regulation of the β-2 integrin Mac-1 (CD11b/CD18) [27, 31, 35, 36]. Likewise, we observed a clear correlation of Mac-1 surface expression with advancing gestational age, possibly contributing to the observed correlation of PMN adhesion with gestational age. By contrast, LFA-1 was already expressed early during gestation and did not change with advancing maturation of the fetuses. Despite being reported consistently, the nature of this difference remains elusive [24, 31, 35]. It has been known for a long time that integrin expression is regulated differentially in a cell- and development-specific manner [37]. Whereas LFA-1 expression is controlled mainly through transcriptional activity, Mac-1 can also be mobilized from intracellular storage pools. In addition, CD18 and CD11a are widely expressed early during leukocyte differentiation, whereas CD11b expression is limited to the myeloid linage and occurs only at later time-points during myeloid cell differentiation. As LFA-1 and Mac-1 share the common β-subunit (CD18), it was suggested that a deficient production of the specific α-subunit (CD11b) may account for the reduced total cell content of Mac-1 observed in neonatal neutrophils [35]. This, in turn, may also explain the impaired basal Mac-1 expression on fetal and neonatal PMNs and its impaired up-regulation upon stimulation, described in earlier reports. In addition, it might also be possible that defective storage and/or mobilization from intracellular storage pools account for the decreased Mac-1 surface expression on fetal and neonatal neutrophils.

Furthermore, we were able to show that the expression of PSGL-1, the major selectin ligand on neutrophils, is also dependent on fetal maturity, as its expression increases during gestation. As suggested by Tcharmtchi et al. [38], reduced PSGL-1 expression may contribute to the diminished rolling capacity of neonatal neutrophils. However, the severity of impairment of fetal PMN recruitment observed in this study points toward other contributing factors. It is possible that our findings may also be related to a diminished response of neonatal neutrophils to chemokine stimulation [39]. Although we did not observe differences in the expression of the chemokine receptor CXCR2, which mediates intravascular neutrophil activation by the CXCL chemokine IL-8 [40], we cannot exclude the possible alterations in intracellular signaling pathways that may affect neutrophil activation. In this context, it has been reported that downstream signals involved in LPS-mediated neutrophil activation are attenuated in newborns [41].

As neutrophil recruitment also involves EC adhesive functions, we further analyzed the adhesive properties of neonatal ECs in the time course of gestation. Our findings clearly demonstrated that EC adhesion-related function depends on gestational age of the infants and is affected significantly in extremely premature infants, leading to reduced neutrophil recruitment as compared with mature infants. Our findings are supported by a comparable study investigating recruitment of adult neutrophils by fetal porcine ECs isolated at different time-points of gestation. This study revealed a similar age dependence of neutrophil-endothelial interactions with very little adhesion of neutrophils to early-gestation fetal EC [19]. This diminished interaction might, in part, be a result of alterations in the expression of inflammatory adhesion molecules on the EC. In this context, it has been reported that the expression of P-selectin on EC is reduced in premature human neonates compared with mature newborns and depends on the degree of maturity of the infants [42]. We now show that up-regulation of E-selectin and ICAM-1 upon stimulation with LPS is also reduced in ECs from premature neonates compared with mature infants. Steroids are known

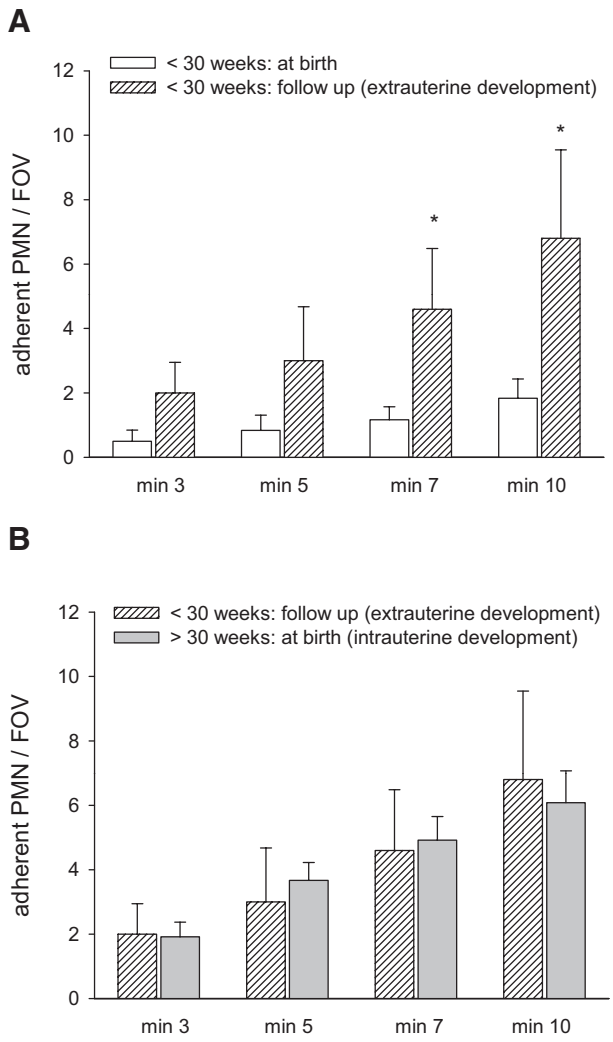

Figure 6. Postnatal maturation of PMN recruitment. Postnatal development of PMN adhesion in flow chambers coated with P-selectin (10 μg/ml), IL-8 (10 μg/ml), and ICAM-1 (4 μg/ml) was evaluated in the group of extreme premature infants (born <30 weeks of gestation) in a follow-up experiment, 4–6 weeks after birth, when the infants had reached a corrected postnatal age of >30 weeks of gestation ("extrauterine development"; hatched bars in (A) and (B)). Results were compared with (A) adhesion of PMN from those children at birth (white bars; data taken from Fig. 1) and (B) adhesion of PMN from moderate premature infants (>30 weeks of gestation) at birth ("intrauterine development"; gray bars; data taken from Fig. 1). Data are given as mean ± SEM (n=5–6). *Significance versus <30 weeks at birth (P<0.05, rank sum test).

to influence endothelial adhesion molecule expression [43, 44] and alter neonatal neutrophil function [45, 46]. Therefore, we performed separate experiments to analyze the impact of antenatal betamethasone treatment on neutrophils and ECs. In our experimental setting, pretreatment with betamethasone had no negative influence on neutrophil recruitment, thus supporting the concept of a true inherent immaturity of the innate immune response rather than a simple iatrogenic-induced effect.

The maturation of the PMN-recruitment process observed in the present study is likely to reflect a physiological adaptation to changing demands during fetal development [13]. As the intrauterine environment is usually sterile, the developing fetus does not need to mount a strong immune response. In contrast, a strong inflammatory reaction with production of TH_1-polarizing cytokines at the feto-maternal interface might even lead to the abortion of the fetus [47]. Toward the end of gestation, however, functional defenses against extrauterine pathogens become increasingly important. Interestingly, Zhao and coworkers [48] demonstrated recently in this context that newborn mice are capable of mounting a hyper-innate immune response compared with adult animals, resulting in what was termed "cytokine storm". All the more, we were surprised to find that the adhesive properties of fetal neutrophils in extreme premature newborns were not altered postnatally by exposure to extrauterine factors (i.e., bacterial colonization of the gut) but showed the same maturation as PMNs from infants that developed in utero, suggesting that intra- und extrauterine maturation of leukocyte recruitment are synchronized and regulated mostly by yet-unknown intrinsic factors that deserve future investigation.

Over the past decades, advances in the medical care of newborns have enabled the survival of ever-younger premature infants. This achievement is paid for with an increasing risk of severe infections, especially in extremely premature neonates. The successful prevention and treatment of neonatal infections are ongoing clinical challenges, demanding new therapeutic strategies. Further insight into the developmental regulation of fetal innate immune processes is crucial for a better understanding of the events taking place during neonatal infection. In conclusion, our experiments reveal that PMN recruitment in the fetus is subjected to a maturation process that involves neutrophil and EC functions. In this context, the inability of extremely premature neonates to sufficiently recruit PMNs is likely to contribute to their increased susceptibility to life-threatening infections. Future research is necessary and should be aimed at unraveling the precise mechanism governing the ontogeny of fetal and neonatal innate immune responses.

AUTHORSHIP

C.N. designed the project, performed and analyzed experiments, and wrote the manuscript. A.G. planned and performed experiments and analyzed data. M.P. performed flow cytometric experiments. S.B. performed flow chamber experiments. D.F., O.G-B., E.Q., and U.H.v.A. provided suggestions on the project and revised the manuscript. M.S. designed and supervised the project and wrote the manuscript.

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KEY WORDS:
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