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Neonatal exchange blood is a rich source of hematopoietic stem and progenitor cells for transplantation

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CONTRIBUTIONS

Y.L. performed animal experiments, flow cytometric analysis, CFU and MLR assays, analyzed data and helped with writing the manuscript; E.D. recruited the participants and arranged the collection of clinical samples; P.G performed experiments at the

stem cell laboratory and was key in processing of the samples. P.C.W. and S.d.H. assisted in flow cytometric analysis of bone marrow and blood from transplanted mice. T.W., A.W., J.T., P.H. assisted in sample processing, experiments and manuscript writing. R.F. conceived the study together with E.D, JT and assisted in the experiments as well as handled the overall logistics of the project.

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MAIN TEXT

The success of umbilical cord blood (UCB) transplantation is closely linked to the infused $CD34^+$ cell dose, with higher doses per kilogram associated with more favorable outcomes[1]. The clinical demand for high-dose UCB grafts is underscored by commercial efforts to develop ex vivo-expanded UCB products, and blood derived from neonatal exchange transfusions may provide yet another source of high-dose $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) with characteristics similar to UCB. Neonates with hemolytic disease of the newborn (HDN) undergo exchange transfusion after birth to halt hemolysis[2], and this procedure yields large volumes of blood (mean 378 mL) that are routinely discarded. We hypothesized that neonatal exchange blood (NEB) is rich in $CD34^+$ HSPCs and observed that NEB units (n = 12) from our neonatal intensive care unit (NICU) contained, on average, almost four times as many $CD34^+$ HSPCs (16.6×10^6) as banked UCB units (4.4×10^6)[3]. Furthermore, NEB-derived $CD34^+$ HSPCs supported multilineage engraftment comparable to UCB in murine xenotransplantation assays. These findings suggest that NEB, which is routinely collected under sterile conditions in NICUs, could be explored as an additional source of $CD34^+$ HSPCs for banking, pending formal clinical evaluation of safety and efficacy.

UCB accounts for approximately 3.8% of all stem cell transplantations in Europe[4] and remains a significant source in Japan, representing around 24% of transplants in contemporary surveys[5]. UCB transplantation offers unique clinical advantages, including rapid availability of cryopreserved units, greater tolerance for HLA mismatching[6], and a lower incidence of chronic graft-versus-host disease (GvHD)[7, 8], while maintaining graft-versus-leukemia (GvL) activity[9,10]. For pediatric

myeloid malignancies, UCB transplantation has been shown to provide superior relapse-free survival compared with other stem cell sources[10].

The principal limitation of UCB is its relatively low content of $CD34^+$ hematopoietic stem and progenitor cells (HSPCs), leading to delayed engraftment and a higher risk of transplant-related complications[11]. Widespread adoption of delayed cord clamping to allow the newborn to reclaim much of the placental blood volume has further reduced cell yields from UCB collection. In a study of 1,204 UCB collections, the probability of obtaining a clinically useful graft fell from 22.1% with immediate (0–30 s) clamping to 2.4% when clamping was delayed beyond 120 s[12]. Many national guidelines now recommend delaying cord clamping for at least 180 s, supported by data from a large randomized controlled trial showing that delaying clamping for 180 s improved children's fine motor and social skills at 4 years of age[13]. Given these challenges, alternative sources of $CD34^+$ HSPCs are needed for UCB banking. We propose that NEB, which is routinely collected in NICUs during exchange transfusions for HDN in newborns, could be used to supplement these efforts. HDN results from maternal alloimmunization against paternally derived antigens on fetal red blood cells and is therefore not expected to harbor disease-causing germline mutations (Figure 1A). The textbook example is the production of anti-D in RhD-negative mothers carrying RhD-positive fetuses. Antibody-mediated hemolysis after birth leads to rapid bilirubin accumulation and, if untreated, can cause kernicterus; exchange transfusion prevents further hemolysis by replacing the neonate's red cells with antigen-negative donor cells and generates substantial volumes of NEB (Figure 1B).

To assess the clinical potential of NEB, we collected 12 units of NEB from neonates undergoing exchange transfusion within 72 hours of birth at Karolinska

University Hospital. We collected an additional six units of UCB from elective C-sections as control material; four NEB units were cryopreserved for *in vivo* studies, informed consent was obtained from both parents, and the study was approved by the Swedish Ethical Review Board (Dnr 2021-03715) in accordance with the Helsinki Declaration. TBNK and CD34 counts in NEB and UCB units were performed at our JACIE-accredited stem cell laboratory following ISHAGE guidelines. $CD34^+$ subsets and human engraftment in mice were analyzed by multicolor flow cytometry using BD Symphony A5 for acquisition and FlowJo for analysis (Table S1). All flow cytometry analyses included live/dead discrimination and doublet exclusion. For colony-forming assay, we used 400 $CD34^+$ cells per sample plated in MethoCult™ H4434 with cytokines according to the manufacturer's instructions. Under approval from the Swedish Agricultural Authority (Dnr 14530-2023), immunodeficient NSG mice (NOD.Cg-Prkdc scid Il2rg tm1Wjl /SzJ) received 3,000–70,000 human $CD34^+$ cells intravenously after 200 cGy sublethal irradiation, with blood and bone marrow analyzed 16 weeks later and secondary recipients given 100,000 $CD34^+$ cells and assessed at 18 weeks. Differences between NEB and UCB were evaluated in an exploratory manner with Student's t-test or one-way ANOVA (Shapiro–Wilk test for normality; $p < 0.05$ was considered statistically significant).

The twelve ETs yielded a mean NEB volume of 374 mL (Figure 2A). NEB yield of $CD34^+$ HSPCs significantly exceeded UCB controls. The mean $CD34^+$ count was 16.6×10^6 (range $4.0 - 54.2 \times 10^6$; Figure 2B). Phenotypic analysis by multiparameter flow cytometry showed that there was a high content of $CD34^+CD38^-$ (HSCs) and $CD34^+CD38^-CD90^+CD45RA^-$ stem cells (LT-HSCs) in NEB (Figure 2C-E). However, the percentage of HSCs and LT-HSCs within the $CD34^+$ populations was lower in NEB compared to UCB (Figure 2D, E), which might result from the

higher fraction of $CD34^+$ cells in NEB. Further functional assay with colony-forming unit in culture (CFU-C) showed that all types of CFU-Cs representing HSPCs are present in NEB (Figure 2F). Notably, the formation of colonies with mixed lineages (CFU-GEMM) confirmed enrichment of HSCs in NEB.

The average total nucleated cell (TNC) count was 5.4 x 10^9 in NEB compared to 0.95 x 10^9 in UCB (Figure 2G) and 1.27 x 10^9 in banked UCBs[3]. Accessory immune cells are critical for immune reconstitution and GvL effects after stem cell transplantation[14]. While T and B cells were significantly more numerous in NEB (Figure 2I, J), NK cells represented a lower percentage of the TNCs in NEB; however, their absolute numbers were comparable to those in UCB units (Figure 2K). Despite the clinical condition necessitating the exchange procedure, the phenotype of NEB-derived T cells paralleled that of UCB in terms of CD4:CD8 ratio, activation (%$CD69^+$), T_{reg} and γδ T cell content (Figure S1H-K; Figure S2D, S2E). Furthermore, their functional behavior in mixed lymphocyte reaction was comparable to UCB with respect to proliferation and cytokine release (Figure S1L-P; Figure S2F). Although these preclinical data are promising, the net clinical impact of the high TNC on GvHD and GvL requires further investigation in future clinical studies.

In NSG mice, NEB- and UCB-derived $CD34^+$ HSPCs showed similar dose-dependent, multilineage reconstitution of human $CD45^+$ cells in peripheral blood (PB) and bone marrow (BM) at 16 weeks (Figure 3A, F). In PB, human myeloid and lymphoid cells were readily detected (Figure 3B–E; Figure S2A), and in BM we observed human-derived myeloid, lymphoid, megakaryocytic, and erythroid lineages (Figure 3G–I; Figure S2B, S2C), together with a sustained presence of human $CD34^+$ cells (Figure 3J). Secondary transplantation confirmed durable

repopulating activity in both UCB and NEB grafts (Figure S1Q–W), indicating that NEB-derived HSPCs possess primitive, multipotent characteristics comparable to those of UCB. Notably, a case report by Li *et al.* in *The Lancet* described successful use of NEB and UCB in an HLA-identical sibling transplantation[15] providing early clinical evidence for the feasibility of NEB transplantation. Taken together, these data support NEB as a feasible transplant graft that can supplement existing stem cell sources for both pediatric and adult patients.

Given the continued clinical demand for high-dose UCB units and ongoing challenges of low cell yield from UCB collections due to delayed cord clamping practices at maternity wards[12], NEB collection offers an enticing alternative pathway to supplement UCB banks. While global ET statistics are limited, a large Californian study estimated that 1.2 per 1,000 newborns meet criteria for undergoing ET set by the American Academy of Pediatrics (AAP)[2]. Based on current birth rates, this translates to tens of thousands of newborns per year across the world, presenting an ample opportunity for systematic banking. Moreover, banking NEB is a readily implementable strategy because it is already collected under aseptic conditions in neonatal intensive care units (NICUs) as part of routine care, eliminating the need to hire additional collection staff. Instead of discarding NEB after neonatal ETs, it could be transferred to local UCB banks with relatively modest logistical adaptations to existing workflows, providing a straightforward means to expand existing inventories with high-dose $CD34^+$ units. Nonetheless, integration of NEB collection into routine NICU practice will require careful attention to consent procedures and coordination with UCB banks.

In summary, neonatal exchange transfusions generate large volumes of clinically valuable blood that are highly enriched in accessory immune cells and

$CD34^+$ HSPCs, capable of robust engraftment and multilineage reconstitution in NSG mice. Systematic collection of this material from NICUs and routing to local UCB banks is logistically feasible and has the potential to augment conventional UCB inventories. However, these findings are based on a small, single-center cohort and should be interpreted as proof-of-concept. Prospective clinical studies are warranted to define the characteristics of NEB-derived grafts in human transplantation, particularly in light of the much higher number of T cells, before NEB can be considered for broader implementation in stem cell banking and transplantation protocols.

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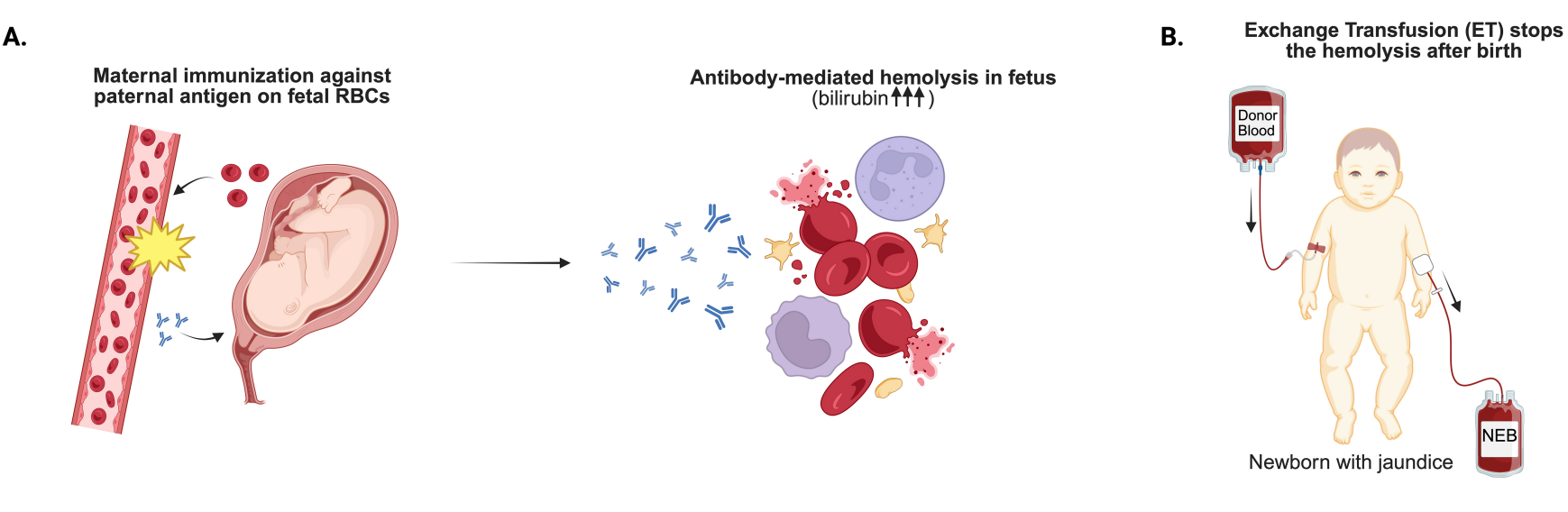
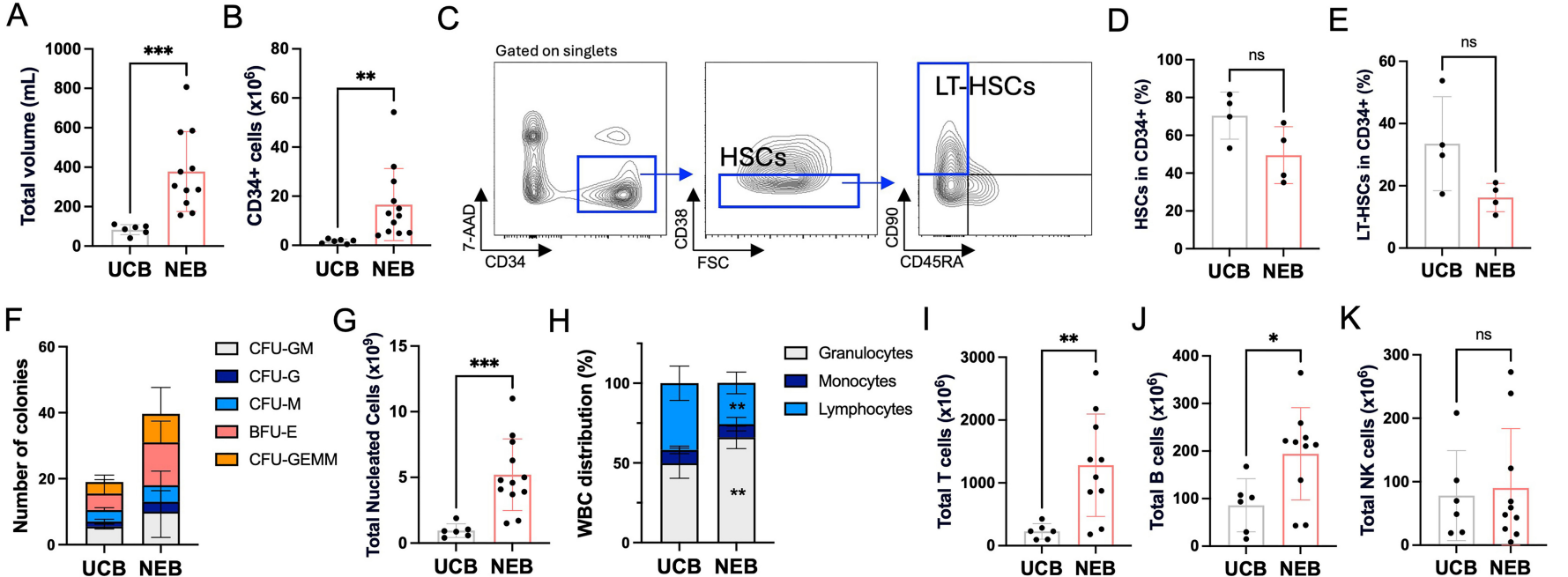
FIGURE LEGENDS

Figure 1. Schematic of exchange transfusion (ET) in hemolytic disease of the newborn (HDN). (A) Maternal alloimmunization against paternally inherited fetal red blood cell antigens (e.g., RhD) leads to production of IgG antibodies that cross the placenta and cause immune-mediated hemolysis in the fetus. (B) After birth, ongoing hemolysis and loss of maternal hepatic clearance rapidly overwhelm the neonatal liver, resulting in severe hyperbilirubinemia; exchange transfusion stops the hemolysis by replacing the infant's erythrocytes with antigen-negative erythrocytes from a donor. This procedure produces large amounts of neonatal exchange blood (NEB). Image created in BioRender.com.

Figure 2. Neonatal exchange blood is a rich source of hematopoietic stem and progenitor cells. (A) Total volume of collected umbilical cord blood (UCB) and neonatal exchange blood (NEB) samples. (B) Total number of $CD34^+$ cells. (C) Gating strategy for flow cytometric analyses of $CD34^+$ HSPCs. (D) The percentage of HSCs ($CD34^+CD38^-$) and (E) LT-HSCs ($CD34^+CD38^-CD45RA^-CD90^+$) within the $CD34^+$ populations of UCB and NEB. (F) The number of colonies for primitive erythroid progenitor cells (BFU-E), granulocyte-macrophage progenitor cells (CFU-G, CFU-M, CFU-GM) and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitor cells (CFU-GEMM) formed by $CD34^+$ stem cells from CB or NEB. (G) Total number of nucleated cells in collected UCB and NEB samples. (H) Comparison of Granulocytes, Monocytes, and Lymphocytes among white blood cells in UCB and NEB. (I-K) Total number of B cells (I), T cells (J) and NK cells (K) in UCB and NEB. (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ by student's t-test, ns: no significant difference).

Figure 3. CD34$^+$ cells from neonatal exchange blood are capable of engraftment and multilineage differentiation in vivo. (A) The percentage of hCD45 in the peripheral blood (PB) at 16 weeks after transplantation in mice transplanted with 3,000 to 70,000 CD34$^+$ cells. (B-E) The percentage of CD3$^+$ T cells (B), CD19$^+$ B cells (C), CD33$^+$ myeloid cells (D) and CD56$^+$ NK cells (E) in hCD45$^+$ in the PB. (F) The percentage of hCD45 in the bone marrow (BM) at 16 weeks after transplantation in mice transplanted with 3,000 to 70,000 CD34$^+$ cells. (G-I) The percentage of hCD11b$^+$ myeloid cells (G) hCD41$^+$ megakaryocytes (H), and hCD71$^+$ erythroid progenitor cells (I) in hCD45$^+$ in BM at 16 weeks. (J) The percentage of CD34$^+$ cells in hCD45$^+$ populations in the BM at 16 weeks post-transplantation. (*$p<0.05$ by student's t-test, ns: no significant difference).


A.
Maternal immunization against paternal antigen on fetal RBCs
Antibody-mediated hemolysis in fetus
(bilirubin↑↑↑↑)
B.
Exchange Transfusion (ET) stops the hemolysis after birth
Donor Blood
NEB
Newborn with jaundice


A
Total volume (mL)
1000
800
600
400
200
0
***
UCB NEB
B
CD34+ cells (x10^6)
80
60
40
20
0
**
UCB NEB
C
Gated on singlets
7-AAD
CD34
HSCs
CD38
FSC
LT-HSCs
CD90
CD45RA
D
HSCs in CD34+ (%)
100
80
60
40
20
0
ns
UCB NEB
E
LT-HSCs in CD34+ (%)
60
40
20
0
ns
UCB NEB
F
Number of colonies
60
40
20
0
CFU-GM
CFU-G
CFU-M
BFU-E
CFU-GEMM
UCB NEB
G
Total Nucleated Cells (x10^9)
15
10
5
0
***
UCB NEB
H
WBC distribution (%)
100
50
0
Granulocytes
Monocytes
Lymphocytes
**
**
UCB NEB
I
Total T cells (x10^6)
3000
2000
1000
0
**
UCB NEB
J
Total B cells (x10^6)
400
300
200
100
0
*
UCB NEB
K
Total NK cells (x10^6)
300
200
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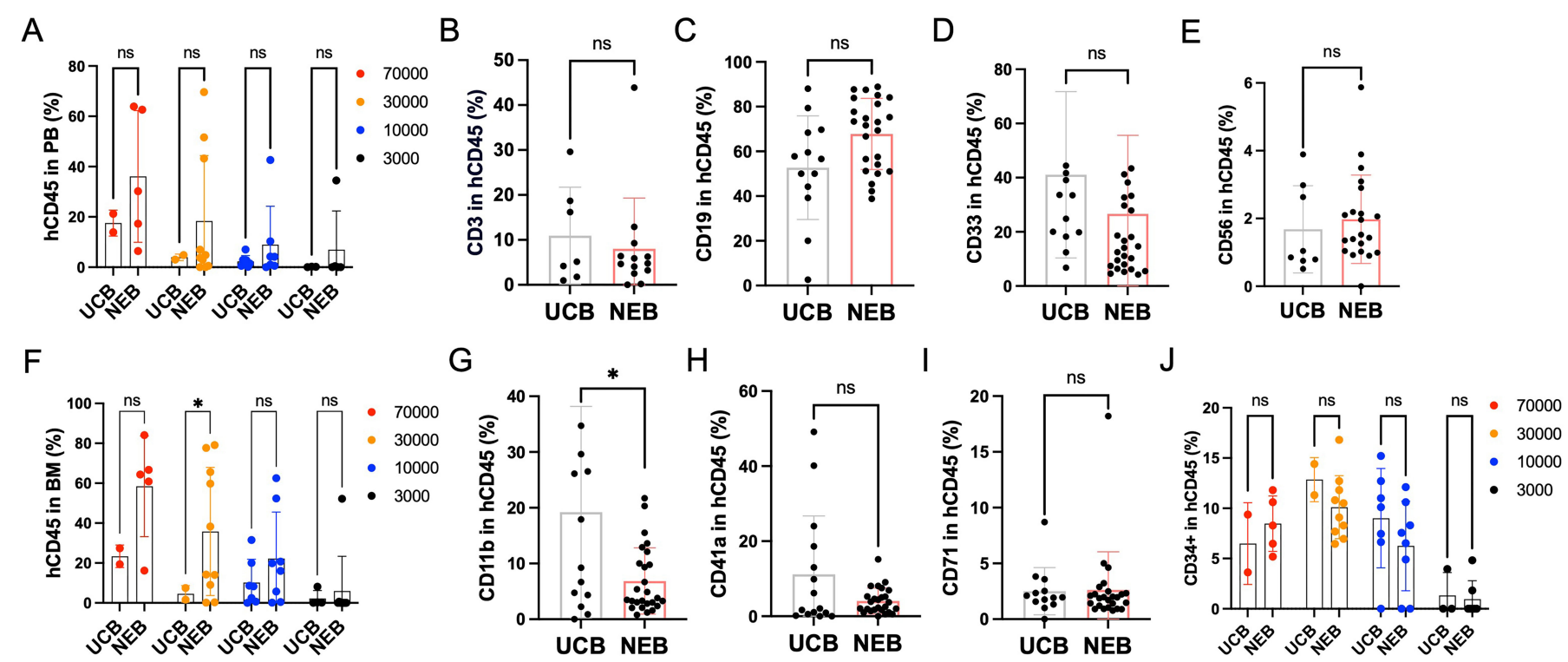
0
ns
UCB NEB


A
hCD45 in PB (%)
ns
70000
30000
10000
3000
UCB
NEB
B
CD3 in hCD45 (%)
C
CD19 in hCD45 (%)
D
CD33 in hCD45 (%)
E
CD56 in hCD45 (%)
F
hCD45 in BM (%)
*
G
CD11b in hCD45 (%)
H
CD41a in hCD45 (%)
I
CD71 in hCD45 (%)
J
CD34+ in hCD45 (%)