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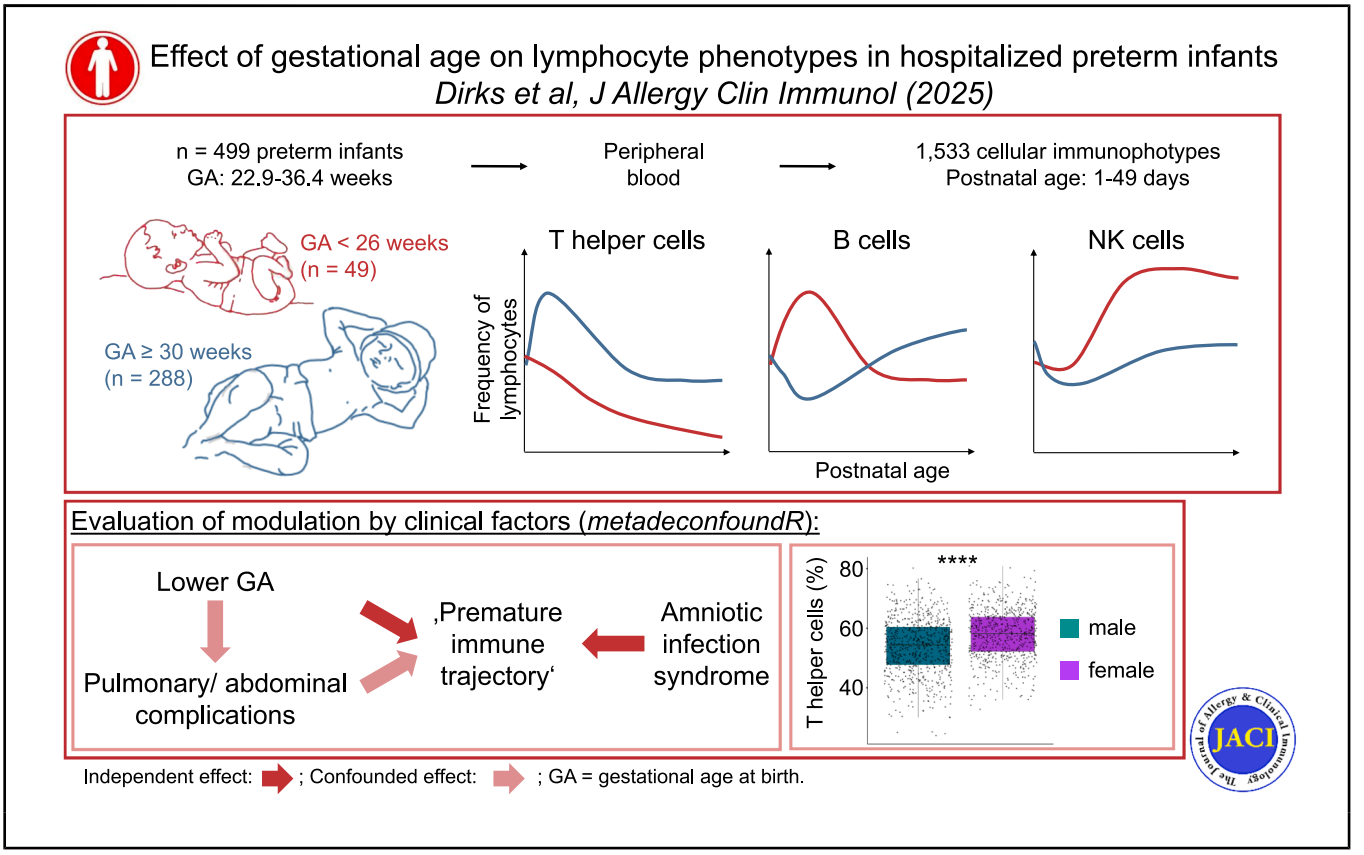
GRAPHICAL ABSTRACT



Capsule summary: This large study defines postnatal profiles of the adaptive immune system in hospitalized preterm infants, highlighting the influence of gestational age at birth. Furthermore, it emphasizes the impact of amniotic infection syndrome and sex on immune development.

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Effect of gestational age on lymphocyte phenotypes in hospitalized preterm infants

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Background: Preterm infants exhibit an increased susceptibility to infections. To assess the contribution of adaptive immunity to this vulnerability, it is crucial to study its postnatal development.

Objectives: We sought to define profiles of adaptive immune-cell subsets in large cohorts of preterm infants, investigating the influence of gestational age (GA) and perinatal factors on their development.

Methods: Two German tertiary care neonatal intensive care unit cohorts (cohort 1: n = 499; cohort 2: n = 78) of hospitalized preterm infants (GA, 22.9-36.4 weeks) underwent flow cytometric phenotyping of peripheral blood lymphocyte subsets within the first 49 days of life. MetadeconfoundR package was used to evaluate (confounding) effects of clinical conditions on lymphocyte profiles.

Results: GA at birth was a primary determinant of profiles of lymphocyte subsets. The most premature infants displayed persistently lower $CD4^+$ T_H-cell frequencies, an early transient increase in B cells, and a later expansion of natural killer cells (*T_H-low B-high* natural killer–*high* phenotype). Detailed analysis revealed a less naive but more effector and regulatory $CD4^+$ T-cell phenotype in preterm infants with lower GA at birth. Amniotic infection syndrome further accentuated this "premature" immune profile, which was also more prevalent in infants with typical complications of prematurity. In contrast, female sex was associated with higher $CD4^+$ T_H-cell frequencies.

Conclusions: This study provides a comprehensive characterization of adaptive immune development in hospitalized preterm infants during the first weeks of life, demonstrating a strong dependence on GA and modulation by perinatal factors. The identified distinct developmental profiles offer a valuable reference framework for interpreting immune phenotyping data and highlight potential associations between immunologic immaturity and clinical outcomes in this vulnerable population. (J Allergy Clin Immunol 2026;157:506-16.)

Key words: *Adaptive immunity, T_H cells, B cells, amniotic infection syndrome, sex, preterm infants*

Preterm infants constitute a distinct population with an increased susceptibility to infections early in life. In addition to immature physical barriers and inappropriate innate immune responses, this vulnerability has been attributed to an immature adaptive immune system.[1-5] The adaptive immune system undergoes dynamic maturation throughout fetal and neonatal development, shaped by gestational age (GA), postnatal age, and environmental influences. However, studying immune development in extremely preterm infants remains challenging, owing to clinical heterogeneity and the limitations posed by small blood sample volumes.

Previous research has highlighted that preterm infants exhibit marked differences in the development of the adaptive immune system compared with term infants. These differences include lower T_H-cell counts, altered T-cell differentiation, and increased regulatory T (Treg)-cell frequencies.[6-12] Despite these insights, many existing studies are limited by small sample sizes, limited longitudinal data, or cohort bias, such as reliance on infants flagged by abnormal severe combined immunodeficiency screening.[13] In addition, although cord blood studies provide valuable insights, they do not reflect the dynamic postnatal changes in immune-cell populations and have been shown to poorly represent the peripheral blood immune phenotype after birth.[4,14] Our understanding of the dynamic interplay between extrinsic factors—such as environmental exposures in neonatal intensive care units—and intrinsic determinants of immune system ontogeny remains incomplete. This underscores the need for longitudinal studies using large, unbiased cohorts to define developmental trajectories of the adaptive immune system and identify modifying factors.

To address these gaps, this study leveraged the routine implementation of detailed flow cytometric immune profiling in 2 large cohorts of hospitalized preterm infants, with measurements spanning the first 49 days of life. Integrating clinical metadata into the analysis, this study reveals distinct developmental profiles of adaptive immune cells during the early

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Abbreviations used
AIS: Amniotic infection syndrome
BPD: Bronchopulmonary dysplasia
FIP: Focal intestinal perforation
GA: Gestational age
IRON: Immunoregulation of the Newborn
LOS: Late-onset sepsis
NK: Natural killer
Treg: Regulatory T

postnatal period, underscores GA as a key determinant of adaptive immune-cell dynamics, and lays the foundation for future studies and clinical translation. In light of the rapid postnatal shifts in lymphocyte composition, the data also provide an unbiased reference framework for interpreting immune phenotyping data in hospitalized preterm infants.

METHODS

Hospitalized preterm infants were enrolled in the IRoN (Immunoregulation of the Newborn) study in 2 tertiary care neonatal intensive care units in Germany (*Immune Profiling cohort*: n = 499, University Hospital of Lübeck; *Extended Phenotyping cohort*: n = 78, University Hospital of Würzburg) for phenotyping of the adaptive immune-cell subsets from peripheral blood in the first 49 days of life. Written informed consent was obtained from parents or legal representatives. The study protocols were approved by the local ethics committees at the University of Lübeck (IRON AZ 15-304) and the University Hospital of Würzburg (IRON Würzburg 11/21 me). Detailed inclusion criteria and outcome definitions are described in this article's Methods section in the Online Repository at www.jacionline.org. Baseline characteristics of both study populations and major in-hospital complications related to prematurity are detailed in Table I.

Blood samples were obtained exclusively as part of routine clinical procedures. In most cases, these were performed for monitoring iron and bone metabolism or for laboratory controls during (partial) parenteral nutrition. Importantly, clinically suspected infections were not considered a routine clinical indication for blood sampling. Lymphocyte subsets were analyzed by flow cytometry after surface staining in whole blood as part of routine diagnostic workflows of specialized local laboratories. Standard R packages (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) were used for statistical analysis and visualization. Group differences were assessed using 1-way ANOVA or Kruskal-Wallis test (for nonnormally distributed variables). Pairwise subgroup comparisons were conducted using the Wilcoxon rank-sum test with Benjamini-Hochberg correction for multiple testing. The MetadeconfoundR package (https://github.com/TillBirkner/metadeconfoundR) was used to evaluate independent and potentially confounding effects of clinical factors on lymphocyte subset frequencies.[15] The tool uses univariate statistics to identify associations between lymphocyte subsets and corresponding clinical information, subsequently applying nested linear model comparisons to account for confounding effects. Details of flow cytometry and computational analysis strategies are explained in this article's Methods section in the Online Repository.

TABLE I. Characteristics at birth and major in-hospital complications attributed to prematurity

Characteristics	Immune *Profiling* cohort	*Extended Phenotyping* cohort
n	499	78
GA at birth (wk), median (range)	30.6 (22.9-36.4)	28.1 (23.9-31.9)
Birth weight (g), median (range)	1419 (305-2820)	958 (315-1490)
Female, n (%)	255 (51.1)	39 (50.0)
Delivery by C-section, n (%)	446 (89.4)	67 (85.9)
Multiples, n (%)	153 (30.7)	26 (33.3)
IVH, n (%)	35 (7.0)	5 (6.4)
Grade 1/2/3/4	14/14/3/4	4/0/1/0
FIP, n (%)	10 (2.0)	0
NEC, n (%)	10 (2.0)	1 (1.3)
BPD, n (%)	89 (17.8)	7 (9.0)
AIS, n (%)	187 (37.9)	29 (37.2)
Suspected/proven/NA	136/51/5	11/18/0
Sepsis (blood culture proven), n (%)		
EOS	13 (2.6)	2 (2.6)
LOS	31 (6.2)	8 (10.3)

Numbers indicate absolute counts when not stated otherwise.
EOS, Early-onset sepsis; *GA*, gestational age; *IVH*, intraventricular hemorrhage; *NA*, not available/applicable; *NEC*, necrotizing enterocolitis.

RESULTS

Adaptive immune-cell profiles depend on GA at birth

To evaluate the postnatal development of the adaptive immune system in preterm infants, we analyzed peripheral blood lymphocyte subset distributions from 499 hospitalized preterm infants in the *Immune Profiling cohort*. Seventy-three percent of the infants provided at least 2 longitudinal measurements (median, 3 time points; range, 1-8), contributing 1533 measurements within the first 49 days of life. Baseline characteristics and major in-hospital complications related to prematurity are detailed in Table I.

We hypothesized that GA at birth is a major discriminator of postnatal profiles of the adaptive immune system. To follow this hypothesis in an unbiased way, the study population was divided into 6 subgroups on the basis of GA at birth (see Table E1 in this article's Online Repository at www.jacionline.org) and immune-cell frequencies were assessed using principal-component analysis. Despite the fact that we observed relevant dynamics of the cellular immunophenotype due to postnatal age in all GA groups, the groups remained separated along principal component 1 during the study time (Fig 1; see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Infants with lower GA at birth exhibited lower principal component 1 values, indicating reduced T- and T_H-cell frequencies alongside increased B-cell proportions, which was also reflected when analyzing absolute cell counts (see Fig E2, *B*, and Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, the cellular pattern in the different GA groups did not converge with increasing postmenstrual age during the analyzed time frame (Fig E2, *C*). Therefore, this analysis suggests that the postnatal cellular profiles of the adaptive immune system are mainly determined by GA at birth.

T_H-low B-high natural killer–high phenotype in more premature infants

To deconstruct these system-level observations, we compared the distribution of lymphocyte subsets between GA groups across

FIG 1. Adaptive immune-cell profiles depend on GA at birth. **A,** PCA representations of cellular immunophenotyping in preterm infants in the first 49 days of life grouped for GA at birth (weeks). Colors indicate the postnatal age (days). Each dot represents an individual sample. **B,** PCA representations of the same measurements as in Fig 1, *A*, grouped for postnatal age at blood draw (days) and colored due to GA at birth. Bigger circles indicate geometric mean of respective groups, when more than 10 individual measurements were available in a GA group at the defined postnatal age. *PC*, Principal-component; *PCA*, principal-component analysis.

defined postnatal time intervals in more detail. Time intervals were adapted to the very dynamic absolute and relative changes in the first week of life and to the less pronounced changes at later time points (see Fig E4 in this article's Online Repository at www.jacionline.org). Significantly lower proportions of $CD3^+$ T cells were observed in more premature infants during the early postnatal weeks when compared with less premature neonates, primarily due to reduced frequencies of $CD4^+$ T_H cells (Fig 2, *A*, and Fig E5, *A*, in this article's Online Repository at www.jacionline.org). In contrast, B-cell frequencies during the first 3 weeks of life were higher with decreasing GA at birth (Fig 2, *C*). Although $CD4^+$ T_H-cell frequencies remained consistently reduced in these infants throughout the study period, B-cell frequencies declined and natural killer (NK)-cell frequencies increased significantly with advancing postnatal age (Fig 2, *A*, *C*, and *D*). Changes in $CD8^+$ cytotoxic T-cell frequencies were less prominent and no significant differences in CD4/CD8 ratios were detected between GA groups (Fig 2, *B*; see Fig E5, *B*). Concluding, the most premature infants showed a distinctive postnatal lymphocyte subset profile with a lasting reduction in $CD4^+$ T_H cells, an early increase in B cells, and a later expansion of NK cells (*T_H-low B-high NK-high* phenotype).

Perinatal inflammation and complications of prematurity are associated with the T_H-low B-high NK-high phenotype

To assess whether the *T_H-low B-high NK-high* phenotype could be attributed to perinatal factors beyond GA at birth, we applied the computational tool metadeconfoundR to the data set. Although causality cannot be inferred, this method enables the identification of significant associations and potential mutual confounding effects between clinical variables and immune-cell profiles. This approach confirmed that the distinct *T_H-low B-high NK-high* phenotype is strongly associated with prematurity (defined here as the number of weeks between the GA at birth and full-term gestation [40 weeks]; see Fig E6 in this article's Online Repository at www.jacionline.org). In addition, low birth weight and amniotic infection syndrome (AIS) were significantly associated with the *T_H-low B-high NK-high* phenotype and *metadeconfoundR* found these effects to be independent at most time points studied. Of note, when the effect of AIS was assessed using more traditional analytical approaches, the results showed consistent trends, but subgrouping and correction for multiple testing led to statistical significance in only some subgroups (see Fig E7 in this article's Online Repository at www.jacionline.org).

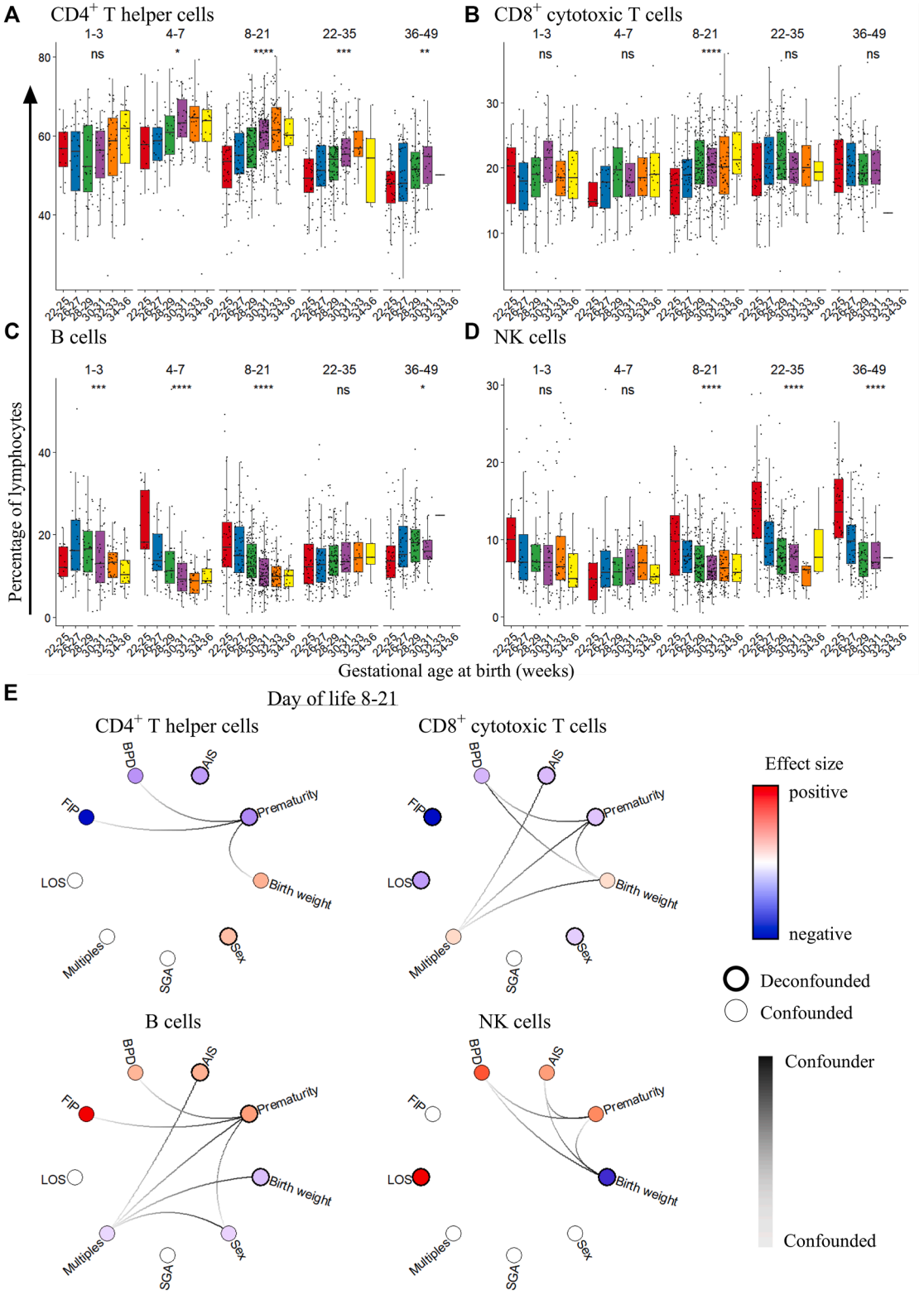

FIG 2. Lymphocyte subset profiles are shaped by GA at birth and perinatal factors. **A-D,** Boxplots of percentages of lymphocyte subsets grouped by GA at birth and postnatal time of sampling. Each point represents an individual sample. Significance was determined using 1-way ANOVA to compare subset frequencies between GA groups for a given range of time. *NS,* Not significant, * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. **E,** Circos representation of confounder analysis for selected cell subsets at day 8 to 21 of life. Unselected data are shown in Fig E6. Effect sizes for significant associations are marked with color-filled circles. Confounder status, and if present direction of confounding, is marked as shown in the legend. For example, greater prematurity, AIS, BPD, and FIP were significantly associated with lower $CD4^+$ T_H-cell frequencies, whereas higher birth weight and female sex are significantly associated with higher $CD4^+$ T_H-cell frequencies. For prematurity, AIS, and sex, these associations were found to be independent of the other variables and therefore marked as deconfounded. In contrast, the effects of birth weight, BPD, and LOS were marked as confounded by prematurity. *SGA,* Small for gestational age.

Perinatal complications more prevalent among extremely preterm infants— such as bronchopulmonary dysplasia (BPD), necrotizing enterocolitis, and focal intestinal perforation (FIP)—were also associated with a *T_H-low B-high NK-high* phenotype. These associations reached statistical significance especially for BPD and FIP from day 4 of life (Fig E6). For example, between postnatal days 8 and 21, low GA was significantly associated with lower frequencies of $CD4^+$ T_H and $CD8^+$ cytotoxic T cells, alongside higher frequencies of B and NK cells. Notably, low birth weight, AIS, BPD, and FIP were also associated with the *T_H-low B-high NK-high* phenotype. Although most associations involving BPD and FIP appeared confounded by GA, AIS exerted independent effects on T- and B-cell frequencies (Fig 2, *E*).

Interestingly, female sex was associated with consistently higher $CD4^+$ T_H-cell frequencies and elevated CD4/CD8 ratios across most of the study period. In contrast, early-onset sepsis was linked to a transient reduction in $CD8^+$ cytotoxic T- cell frequencies during the first 3 days of life and late-onset sepsis (LOS) was significantly associated with lower $CD8^+$ cytotoxic T-cell and higher NK-cell frequencies only from day 8 to 21 (Fig 2, *E*, and Fig E6).

Altogether, this integrative, data-driven approach confirmed the dominant role of GA in shaping early lymphocyte development and confirmed additional modulatory effects of perinatal inflammation (AIS) and, to a lesser extent, low birth weight —both contributing to a more "premature" immune phenotype. Likewise, pulmonary and abdominal complications were associated with similar immunologic signatures, although largely confounded by GA. Finally, the increased $CD4^+$ T_H-cell frequencies observed in female preterm infants highlight the limitations of more traditional analytical strategies (see Fig E8 in this article's Online Repository at www.jacionline.org) and emphasize the value of unbiased, metadata-integrated approaches.

In summary, our analysis of cell subsets of the adaptive immune system in hospitalized preterm infants from the *Immune Profiling cohort* revealed a significant and lasting reduction of $CD4^+$ T_H cells, an early increase in B cells, and a later expansion of NK cells across the postnatal period in the more premature infants. These findings provide a foundation for GA- and postnatal time-resolved immune phenotyping in preterm populations (Table II). Notably, MetadeconfoundR analysis indicated that perinatal inflammation, low birth weight, and complications typical of extreme prematurity contributed additional, partly independent effects to the "premature" immune phenotype beyond prematurity itself.

Comprehensive immune phenotyping of T- and B-cell subsets in the Extended Phenotyping cohort

Although the *Immune Profiling cohort* comprised a large number of clinically well-characterized preterm infants, enabling the identification of GA-dependent immune profiles, the depth of immunologic profiling was limited. To address this, we included preterm infants from a second cohort below 32 weeks of gestation at birth for comprehensive immune phenotyping (Table I; see Table E1). This allowed for a more detailed examination of potential mechanistic underpinnings of the divergent developmental profiles, including insights into functional states and polarization patterns of immune cells. Lymphocyte and lymphocyte subset counts in week 1 and at day 28 of life reproduced the results from the *Immune Profiling cohort* (different study center and laboratory, see Fig E9), underscoring the validity and comparability of both cohorts.

Persisting naive B-cell phenotype

Consistent with the findings in the *Immune Profiling cohort*, the most premature infants from the *Extended Phenotyping cohort* also had significantly higher total B-cell frequencies and counts in the first week of life (see Fig E10, *A*, in this article's Online Repository at www.jacionline.org). Although there was a trend toward higher NK-cell numbers and frequencies in these infants at day 28, this did not reach significance (see Fig E10, *B*). Analyzing B-cell subsets underlying the observed dynamics of total B cells showed a trend toward an increase in memory B-cell subsets (marginal zone like and switched memory B cells) in infants with the lowest B-cell counts (see Fig E10, *C*). Nevertheless, the B-cell phenotype especially in the very premature infants was predominantly naive and these findings persisted in the first month of life.

More premature infants show a less naive and increased effector and regulatory T_H-cell phenotype

To investigate the persistent reduction in $CD4^+$ T_H cells in very premature infants, we conducted an in-depth analysis of $CD4^+$ T_H-cell subsets. More premature infants showed a significant reduction in the proportion of naive cells, particularly recent thymic emigrants (Fig 3, *A-C*). Conversely, these very premature infants exhibited higher proportions of memory cells, particularly effector memory and terminal effector memory cells (Fig 3, *D-F*; see Fig E11, *A* and *B*, in this article's Online Repository at www.jacionline.org).

Next, we examined the frequency of Treg cells across GA groups. More premature infants exhibited reduced conventional T-cell and higher Treg-cell frequencies during the first week of life compared with more mature preterm infants, in terms of both total Treg cells and memory Treg cells (Fig 3, *G-I*). Notably, differences in memory Treg-cell frequencies persisted up to day 28 of life, whereas no significant differences were observed in the frequency of memory T cells within the conventional T-cell compartment (see Fig E12 in this article's Online Repository at www.jacionline.org). This further supports the hypothesis that more premature infants show a shift toward a memory phenotype among Treg cells but toward an effector phenotype among conventional T cells.

No significant differences were found in the distribution within $CD8^+$ cytotoxic T- cell subpopulations and the proportion of activated $CD4^+$ or $CD8^+$ T cells (indicated by expression of HLA-DR; see Fig E11, *C-E*). The frequency and absolute counts of TCRγδ-expressing T cells increased over the first month of life, with no significant differences between GA groups (Fig E11, *F*).

In summary, $CD4^+$ T_H-cell subsets in more premature infants are skewed toward an effector phenotype accompanied by a reduction in naive subsets and markers of thymic output. At the same time, there is a shift to a more regulatory phenotype. Importantly, these differences persist throughout the first month of life, suggesting that they depend on GA at birth.

TABLE II. Lymphocyte subset counts in preterm infants

subset	sampling (dol)	22-25	26-27	28-29	30-31	32-33	34-36	*P* value (ANOVA)
Lymphocytes*	1-3	3431 (1290-6130)	3248 (1178-4742)	3543 (1420-6800)	3343 (1164-5727)	4292 (1893-8489)	4598 (2659-7186)	.011
	4-7	3400 (1014-6205)	4079 (850-6472)	4026 (1292-7114)	4279 (2251-6695)	3655 (1960-6156)	3859 (2620-5290)	NS
	8-21	4603 (1880-8150)	5554 (2944-9388)	5411 (2737-8151)	5837 (3628-8698)	5717 (3081-7972)	6557 (4320-9612)	5.6×10^{-05}
	22-35	5235 (2087-8657)	4923 (2950-7440)	5541 (3355-8715)	5735 (3136-8188)	5293 (3456-8500)	—	NS
	36-49	4603 (1705-7720)	5069 (3010-7770)	5688 (3376-7994)	5083 (3634-6370)	—	—	.0046
T cells*	1-3	1698 (915-3426)	2305 (836-4465)	2536 (782-5163)	2406 (1244-4112)	3007 (1343-5426)	3369 (1826-5277)	9.8×10^{-06}
	4-7	1833 (682-3361)	2397 (998-3986)	2831 (1120-4717)	3141 (1550-5275)	2996 (1840-5390)	2991 (1640-4641)	.00069
	8-21	2826 (899-5494)	3646 (1917-6060)	4007 (2113-6128)	4267 (2671-5978)	4413 (2376-6740)	4753 (2934-7221)	3.2×10^{-13}
	22-35	3037 (1360-5379)	3441 (1989-5406)	4002 (2400-6003)	4094 (2254-6270)	3848 (2382-5492)	—	3.6×10^{-07}
	36-49	2915 (1203-5573)	3570 (2078-5645)	3809 (2347-5794)	3660 (2559-4720)	—	—	7.9×10^{-05}
$CD4^+$ T_H cells*	1-3	1243 (647-2353)	1743 (551-3474)	1902 (587-4170)	1777 (908-3214)	2253 (995-3975)	2544 (1280-4017)	6.6×10^{-06}
	4-7	1447 (556-2677)	1840 (831-3132)	2152 (852-3757)	2399 (1263-3762)	2331 (1339-3888)	2274 (1098-3798)	.0013
	8-21	2143 (649-3908)	2650 (1331-4050)	2959 (1337-4767)	3166 (1827-4402)	3309 (1546-4998)	3469 (1834-5245)	2.2×10^{-12}
	22-35	2163 (845-3762)	2399 (1342-3780)	2868 (1678-4345)	3021 (1656-4899)	2883 (1693-4065)	—	4.5×10^{-08}
	36-49	1974 (784-3919)	2490 (1424-4059)	2713 (1522-4421)	2665 (1787-3431)	—	—	8.3×10^{-06}
$CD8^+$ cytotoxic T cells*	1-3	471 (166-1030)	569 (251-1010)	645 (196-1163)	642 (282-1067)	766 (280-1812)	825 (373-1352)	.0044
	4-7	402 (126-715)	579 (131-1039)	700 (217-1198)	695 (313-1185)	673 (306-1241)	720 (363-1252)	.0063
	8-21	691 (184-1552)	930 (485-1657)	1035 (485-1715)	1056 (549-1613)	1030 (498-1686)	1200 (684-2077)	2.1×10^{-08}
	22-35	840 (290-1546)	964 (367-1494)	1091 (575-1818)	1038 (549- 1617)	984 (511-1757)	—	.0019
	36-49	876 (297-1541)	1012 (502-1566)	1050 (544-1707)	959 (509-1326)	—	—	.043
B cells*	1-3	306 (101-528)	619 (120-1496)	507 (158-1165)	477 (102-1281)	559 (149-1500)	493 (195-1218)	NS
	4-7	595 (163-1408)	509 (167-854)	453 (133-1109)	412 (124-841)	310 (104-586)	344 (163-567)	.0041
	8-21	733 (91-1635)	748 (273-1445)	690 (230-1433)	606 (129-1284)	573 (196-1031)	620 (164-1175)	.0053
	22-35	569 (167-1453)	674 (203-1284)	736 (302-1374)	748 (312-1442)	690 (304-938)	—	.039
	36-49	612 (108-1355)	836 (321-1439)	934 (462-1781)	883 (322-1470)	—	—	3.9×10^{-05}
NK cells*	1-3	231 (98-530)	293 (64-768)	263 (50-723)	309 (71-1033)	375 (71-1328)	318 (84-1161)	NS
	4-7	114 (15-211)	187 (68-371)	233 (42-507)	227 (71-411)	257 (77-560)	226 (104-483)	.028
	8-21	425 (27-1176)	426 (169-1011)	362 (116-760)	366 (108-771)	349 (131-689)	390 (218-619)	NS
	22-35	590 (215-1110)	462 (131-817)	417 (188-821)	419 (191-730)	332 (170-756)	—	2.6×10^{-05}
	36-49	561 (169-1122)	496 (208-884)	409 (165-902)	417 (179-926)	—	—	.0026
n†	1-3	18	28	39	41	52	34	
	4-7	16	24	35	44	37	30	
	8-21	39	44	91	85	68	28	
	22-35	37	47	91	52	21	5	
	36-49	41	46	64	23	1	0	

One-way ANOVA was used to determine *P* values for differences between GA groups at specific time points. *P* values < .05 were considered significant.
DOL, Day of life; *NS*, not significant.
*Cell counts are shown as medians (5%-95% percentiles) in cells per microliter in peripheral blood for each group at indicated postnatal ages. No values are shown for subgroups with <10 measurements.
†Number of individual patients contributing to the subgroup.

Predominant T_H1 and T_H2 phenotype in more preterm infants

We finally aimed to explore the functional and polarization state of $CD4^+$ T_H cells in the more premature infants. Conventional T_H and Treg cells with expression of the follicle-homing receptor CXCR5—as surrogate of circulating B-cell helping T follicular helper/regulatory cells—were found in very low frequencies in all GA groups and with only slight increases at day 28 (Fig 4, *A* and *B*). To explore the polarization status of memory T cells, we used chemokine receptor expression patterns as surrogate markers: More premature infants showed a trend toward higher frequencies of $CXCR3^+CCR4^-CCR6^-$ ("T_H1") and $CXCR3^-CCR4^+CCR6^-$ ("T_H2") among conventional T_H cells in the first week of life, which was more pronounced at day 28. On the contrary, the frequency of $CXCR3^-CCR4^+CCR6^+$ ("T_H17") cells was lower in these infants (Fig 4, *C*). Assessing T_H1-like, T_H2-like, and T_H17-like polarizations in Treg cells revealed reverse trends for T_H1- and T_H17-like Treg cells and a more T_H17-like predominance among Treg cells, which increased with postnatal age (Fig 4, *D*). Summing up, the above-observed enhanced effector and regulatory phenotype in more premature infants is linked to a predominance of T_H1 and T_H2 polarization among conventional T_H cells and of T_H17 polarization among Treg cells compared with less premature infants.

DISCUSSION

This study provides a comprehensive characterization of adaptive immune-cell profiles in hospitalized preterm infants in the first weeks of life, using the largest cohort published to date. By integrating data from 2 complementary cohorts, we elucidated the profound influence of GA at birth on the postnatal dynamics of T, B, and NK cells, with detailed insights into regulatory and memory compartments.

Consistent with previous reports, we observed a persistent reduction in T-cell and T_H-cell counts in preterm infants throughout the study period, a finding that did not converge with postmenstrual age. This corroborates observations from smaller cohorts.[4,12] However, studies assessing a broader immunophenotype report a convergence at later time points.[14] The

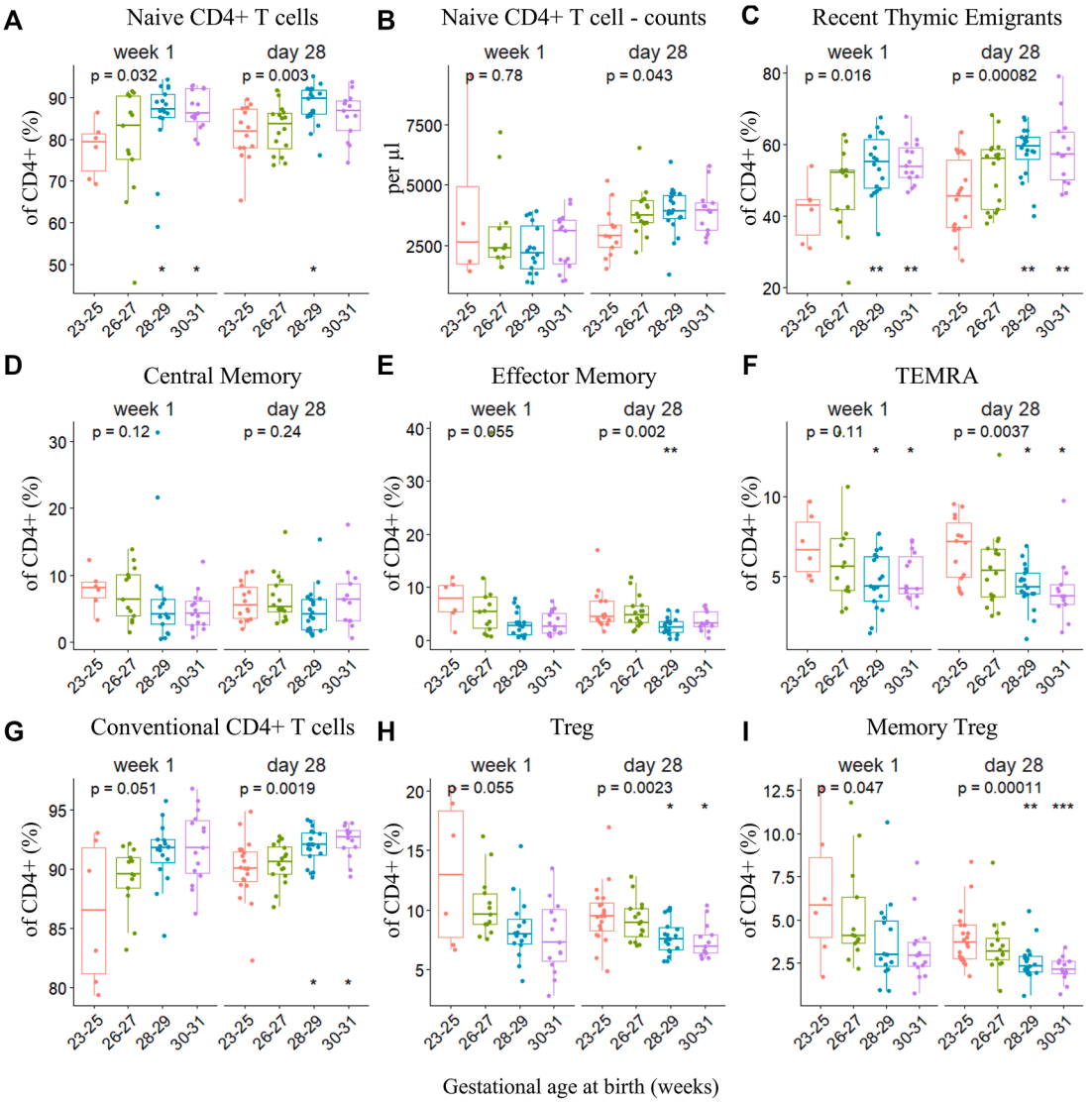

FIG 3. T_H-cell phenotype depends on GA at birth. **A-I,** Boxplots of percentages or counts of indicated T_H-cell subsets grouped by GA at birth and postnatal time of sampling. Each point represents an individual sample. *P* values indicate significances determined by Kruskal-Wallis test to compare subset frequencies between GA groups within sampling groups. *TEMRA*, Effector memory T cells reexpressing CD45RA. Asterisks mark significant differences to the GA group "23-25" at the given time point calculated using Wilcoxon rank-sum test adjusted with Benjamini-Hochberg correction for multiple testing; $*P < .05$, $**P < .01$, $***P < .001$.

persistent reduction in T_H cells observed may contribute to the heightened susceptibility to infections in extremely preterm infants. Furthermore, our analysis revealed that perinatal factors, such as AIS as cause of preterm delivery and postnatal abdominal or pulmonary complications, are associated with a more pronounced "prematurity of the immunophenotype." Although causality cannot be established in observational studies, this suggests a potential link between perinatal inflammation, immunologic immaturity, and the risk of complications.

Despite the well-established association between male sex and increased preterm morbidity and mortality, and the extensive literature on sex-related differences in (auto-)immunity in adults, studies addressing these questions in preterm infants remain limited.[4,16] Our study population, with its substantial sample size and rigorous control for confounding factors, allowed us to investigate this critical aspect. Notably, we found that female sex was associated with persistently elevated T_H-cell frequencies. This observation aligns with reports of higher T_H-cell frequencies and CD4/CD8 ratios in female adolescents and adults.[17] We hypothesize that this less immature immune phenotype in female preterm infants may contribute to their improved survival outcomes.

Our in-depth analysis of T_H-cell subset profiles in a second cohort confirmed previous findings of a less naive, but more effector and Treg-cell phenotype in more premature infants, accompanied by reduced thymic output.[4,10-12,18] Importantly, this phenotype persisted at 28 days of life, indicating a sustained effect of GA. Furthermore, we observed a predominance of T_H1,

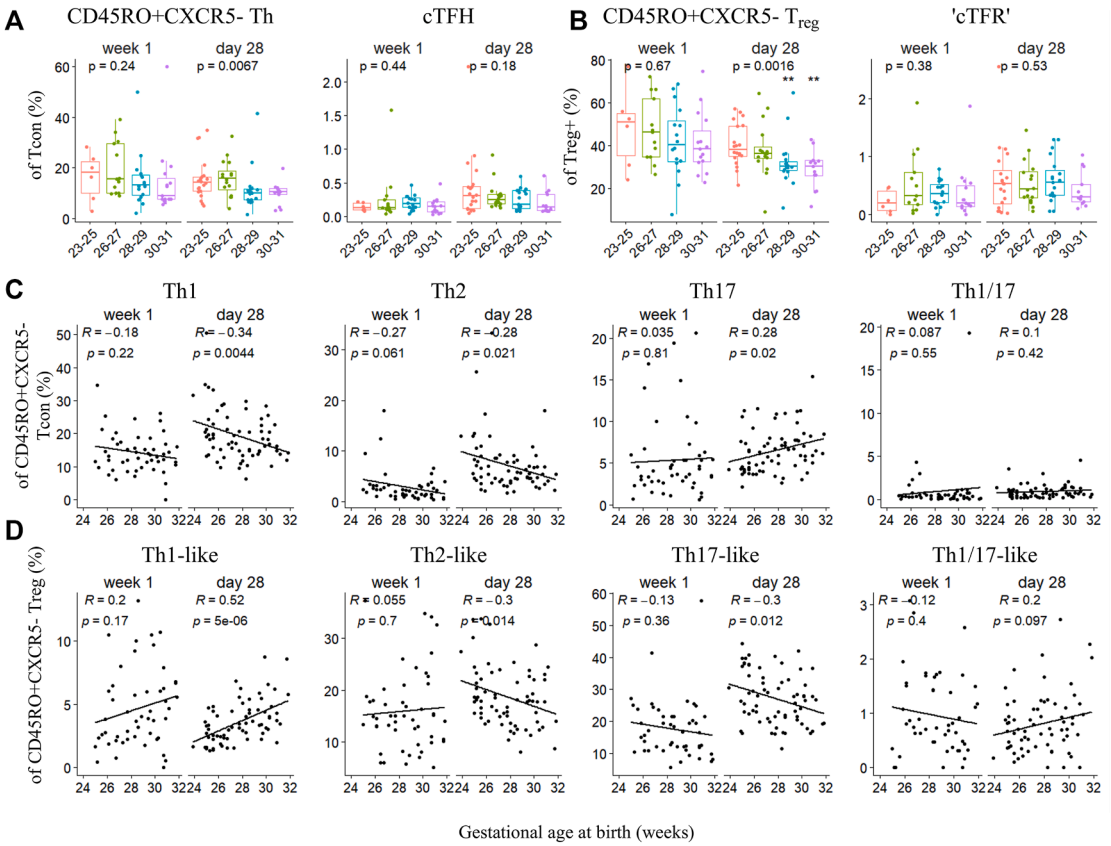

FIG 4. T_H1 phenotype predominance in more preterm infants. **A** and **B,** Boxplots of percentages of indicated conventional and regulatory $CD4^+$ T-cell subsets grouped by GA at birth and postnatal time of sampling. Each point represents an individual sample. *P* values indicate significances determined by Kruskal-Wallis test to compare subset frequencies between GA groups within sampling groups. Asterisks mark significant differences to the GA group "23-25" at the given time point calculated using Wilcoxon rank-sum test adjusted with Benjamini-Hochberg correction for multiple testing; $^{**}P < .01$. **C** and **D,** Scatterplots of polarization frequencies of Tcon and Treg cells as determined by *ex vivo* surface chemokine receptor expression patterns (for gating strategy, see Fig E1). Each point represents an individual sample. Correlation between GA at birth and polarization frequencies was determined using Pearson method. Pearson coefficient and *P* value are indicated for each time point. *cTFH,* Circulating T follicular helper cell; *cTFR,* circulating T follicular regulatory cell; *Tcon,* conventional T cell.

and to a lesser extent T_H2, memory T-cell polarization in more premature infants, even increasing by day 28. This contrasts with a report of a postnatal T_H2 skew using the flow cytometric assessment of lineage-specific transcription factors,[11] but aligns with single-cell RNA sequencing data demonstrating elevated T_H1-cell frequencies in very preterm infants.[4] These findings demonstrate significant and enduring alterations in T_H- and Treg-cell compartments, both in subset distribution and in polarization, influenced by GA. These changes may be further modulated by perinatal inflammation and potentially contribute to the development of complications. Because of clinical heterogeneity and limited sample sizes in the *Extended Phenotyping cohort*, larger studies are required to explore these associations.

The observed *T_H-low B-high NK-high* phenotype underscores that cellular profiles of the adaptive immune system in hospitalized preterm infants are not solely a consequence of dynamics of lymphocyte counts. We identified a previously undescribed, transient postnatal expansion of B cells in the most vulnerable premature infants, predominantly naive cells. Although the precise nature of this expansion—whether a developmental "wave" or a response to external stimuli—remains unclear, it may reflect the expansion of B cells observed in the fetal bone marrow during the second trimester.[19] Furthermore, we observed a significant increase in NK-cell frequencies in more premature infants and LOS was independently associated with higher NK-cell frequencies between day 8 and 21. Whether higher NK-cell frequencies are preceding or following LOS cannot be answered from this data set. Nevertheless, the observed decrease in $CD8^+$ cytotoxic T cells at this time point in infants with LOS has been described occurring 7 days after onset of LOS.[20]

As the largest data set phenotyping the adaptive immune system in preterm infants published to date, this study provides a valuable framework for evaluating cellular adaptive immunity in this population during the first 7 weeks of life. However, our data reflect the immunologic repertoire in a population-based cohort of hospitalized preterm infants with large clinical heterogeneity. Comparisons with published studies yielded similar ranges for T, T_H, and cytotoxic T cells.[6,8,9] In contrast, we observed relevant differences when comparing our data to reference values derived from preterm infants selected by abnormal T cell receptor excision

circles (TREC) screening and later categorized as healthy[13] (see Figs E13 and E14 in this article's Online Repository at www.jacionline.org). Especially the higher T_H-cell counts in our cohorts likely reflect the selection bias inherent in the TREC-screened cohort, which enriches for infants with reduced naive T-cell counts. Consequently, these reference values, although useful for excluding severe combined immunodeficiency, do not accurately represent cell trajectories of the adaptive immune system in the broader preterm population. Furthermore, they fail to capture the modulation of these profiles by GA and postnatal age. In conclusion, our longitudinal assessment of the cellular composition of the adaptive immune system in different GA groups provides a crucial context for interpreting immune development in preterm infants under specific clinical circumstances.

Despite its strengths, this study has limitations. The convenience sampling approach may introduce bias especially due to the nonstandardized sampling strategy, resulting in unequal representations of different individuals during the studied postnatal time. This limits the ability to track immune trajectories at the individual level. Because this study analyzed only the postnatal profiles in hospitalized preterm infants, its findings cannot be extended or compared with postnatal immune development in healthy term infants. Importantly, blood was obtained for routine clinical indications (eg, iron and bone metabolism monitoring and parenteral nutrition controls), not for suspected infections. Although a truly "healthy" preterm infant does not exist, these circumstances likely provide the closest feasible approximation of a baseline immune state in this population. Nevertheless, immune profiles may still have been influenced by subclinical infections or medications administered for clinical reasons. Another limitation is the heterogeneity of the underlying causes of preterm delivery, which often cannot be clearly distinguished as maternal or fetal in origin. Overlapping conditions—for example, placental insufficiency complicated by premature labor—further complicate classification. To avoid unsupported conclusions, we therefore restricted our analyses to the established Triple I definition of AIS and used small for GA status as a surrogate marker of placental insufficiency.

The smaller size of the *Extended Phenotyping cohort* limited statistical power for subset analyses, preventing the imputation of associations between clinical metadata and T-cell subsets. Furthermore, future studies with long-term follow-up are needed to assess the clinical consequences of these immune profiles, particularly in relation to infection risk, vaccine responses, and immune-mediated diseases. Expanding B-cell phenotyping with high-dimensional techniques, such as mass cytometry or single-cell RNA sequencing, will be crucial to further elucidate B-cell development and address potential artifacts in the low B-cell count samples. In addition, surface chemokine receptor expression patterns by flow cytometry as potential surrogate marker of T_H-cell polarization in preterm infants should be compared with functional and transcriptional data, which were outside the scope of this study.

Conclusion

This study provides a robust framework for understanding the development of the adaptive immune system in hospitalized preterm infants, highlighting the distinct and persistent effects of GA. The longitudinal assessment in a large study population enabled the computational weighting of associations with perinatal inflammation and complications, offering a substantial data set for generating future hypotheses regarding the clinical significance of the development of the adaptive immune system. By bridging immunology and neonatology, this work establishes a foundation for improving outcomes in this vulnerable population.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT version 4o to improve readability and English language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Clinical implications: Revealing how GA and perinatal factors shape the developing adaptive immune system, this study provides crucial insights for understanding the preterm vulnerability to infections.

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