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Exposure to chorioamnionitis alters the monocyte transcriptional response to the neonatal pathogen *Staphylococcus epidermidis*

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ABSTRACT

Preterm infants are uniquely susceptible to late-onset sepsis that is frequently caused by the skin commensal *Staphylococcus epidermidis*. Innate immune responses, particularly from monocytes, are a key protective mechanism. Impaired cytokine production by preterm infant monocytes is well described, but few studies have comprehensively assessed the corresponding monocyte transcriptional response. Innate immune responses in preterm infants may be modulated by inflammation such as prenatal exposure to histologic chorioamnionitis which complicates 40–70% of preterm pregnancies. Chorioamnionitis alters the risk of late-onset sepsis, but its effect on monocyte function is largely unknown. Here we aimed to determine the impact of exposure to chorioamnionitis on the proportions and phenotype of cord blood monocytes using flow cytometry, as well as their transcriptional response to live *S. epidermidis*. RNA-seq was performed on purified cord blood monocytes from very preterm infants (<32 weeks gestation, with and without chorioamnionitis-exposure) and term infants (37–40 weeks), pre- and post-challenge with live *S. epidermidis*. Preterm monocytes from infants without chorioamnionitis exposure did not exhibit an intrinsically deficient transcriptional response to *S. epidermidis* compared to term infants. In contrast, chorioamnionitis exposure was associated with hypo-responsive transcriptional phenotype regarding a subset of genes involved in antigen presentation and adaptive immunity. Overall, our findings suggest that prenatal exposure to inflammation may alter the risk of sepsis in preterm infants partly by modulation of monocyte responses to pathogens.

INTRODUCTION

Newborn infants face significant immunological challenges during their transition from intra- to extra-uterine life, as they encounter a wide range of microorganisms for the first time. These challenges are greatest for those born preterm, as the incidence of neonatal and early life infection is negatively associated with gestational age. Very preterm infants (born ≤ 32 weeks) are particularly susceptible, and *S. epidermidis* is the leading cause of late-onset sepsis in many preterm populations¹. Late-onset sepsis largely occurs between days 10 and 16 of life, affecting up to one third of very preterm infants². Late-onset sepsis is associated with potential mortality and considerable morbidity, including increased risk of chronic lung

disease, severe intraventricular hemorrhage, and adverse neurodevelopmental outcomes³. Understanding of the immunological mechanisms underlying the heightened susceptibility of preterm infants to sepsis is incomplete, but critical for developing effective preventative and therapeutic interventions. Importantly, 40–70% of preterm infants are exposed to histologic chorioamnionitis prior to birth⁴. Interestingly, preterm infants exposed to chorioamnionitis have a significantly reduced risk of developing late-onset sepsis⁵, suggesting that prenatal exposure to infection and inflammation may modulate the developing immune system.

Newborn infants depend on innate immune defences in the first weeks of life, prior to maturation of adaptive immune responses. Monocytes are central innate effector cells, and several phenotypic and functional characteristics of monocytes are impaired in preterm infants⁶. The primary monocyte deficit appears to be significantly reduced expression of inflammatory cytokines (TNF α , IL-1 β , IL-6 and IL-8) at the protein level following TLR stimulation. However, preterm infant monocytes express comparable levels of important immune cell-surface receptors (e.g. TLRs) and have no clear deficits in the phosphorylation cascades downstream of these receptors,⁷⁻⁹ suggesting a possible underlying transcriptional deficiency^{8, 10-13}. Therefore, a global transcriptional approach is well-suited to elucidate the mechanisms underlying the differences between preterm and term infant innate immune responses.

Here, we have used flow cytometry to evaluate frequencies of total monocytes and individual subsets within cord blood from term and preterm infants (with and without chorioamnionitis exposure), and RNA-seq to assess the transcriptome of purified infant monocytes pre- and post-challenge with live *S. epidermidis*. We aimed to identify deficiencies associated with preterm birth, and assess the impact of chorioamnionitis on monocyte function. This approach allows interrogation of the global, intrinsic monocyte transcriptional response following direct interactions with a common pathogen. Overall, we report minimal transcriptional differences between term and chorioamnionitis-unexposed preterm monocyte responses to *S. epidermidis*. In contrast, monocytes from chorioamnionitis-exposed preterm infants displayed a hypo-responsive transcriptional phenotype post-stimulation. These results indicate that prenatal exposure to infection and inflammation may alter the risk of infection at least in part via modulation of monocyte responses.

RESULTS

Both preterm birth and exposure to chorioamnionitis influence monocyte subset frequencies

Flow cytometry was used to compare the frequencies of cord blood monocyte subsets between term and preterm infants, as well as relative monocyte expression of CD14 and HLA-DR. Preterm infants without chorioamnionitis exposure had significantly reduced frequencies of total monocytes compared to both term and chorioamnionitis-exposed preterm infants (Figure 1A). All preterm infants (independent of chorioamnionitis) exhibited a significant reduction in the proportion of classical monocytes, and a significant increase in the proportion of intermediate monocytes compared to term infants (Figure 1B and 1C). The proportions of non-classical monocytes were not significantly different between groups (Figure 1D). With both classical and intermediate monocytes, a clear trend was observed where monocytes from preterm infant unexposed to chorioamnionitis showed the greatest relative difference to the term infant monocyte phenotype. Similarly, both preterm infants with and without chorioamnionitis exposure exhibited reduced CD14 surface expression on classical monocytes compared to term infants, with the greatest reduction observed in unexposed preterm infants (Figure 1E). A significant reduction in the frequency of total monocytes expressing HLA-DR (an MHC class II receptor involved in antigen presentation) was observed in chorioamnionitis-exposed preterm infants when compared to term infants (Figure 1F).

Distinct transcriptional profiles in monocytes stimulated with *S. epidermidis*

RNA-seq was performed on purified cord blood monocytes from chorioamnionitis-exposed or unexposed preterm infants, and term infants pre- and post-stimulation with live *S. epidermidis*. Principal component analysis was used to visualise the major contributors to transcriptional variation in our dataset. Within the first principle component, we observed complete separation between unstimulated, and *S. epidermidis* stimulated monocytes (Figure 2A). Unexpectedly, no clear separation between term and preterm infants (without

chorioamnionitis exposure) was observed within the first three principal components (together accounting for 71% of total variation). However, we did observe separation between chorioamnionitis-exposed preterm infants (except for one infant) and the remaining samples on principal component 3, indicating that chorioamnionitis exposure was the third largest source of variation within the data (5% of variation, Figure 2B).

Chorioamnionitis exposure is associated with reduced monocyte expression of key immune genes

Given the limitations of principal component analysis and its potential to obscure minor trends in the data, we next analysed the expression of selected key genes involved in key pathways that we hypothesised to be differentially regulated between preterm and term infants and/or additionally modulated by chorioamnionitis exposure. We compared expression of sixty-eight key genes in TLR, inflammatory, antigen presentation and apoptosis pathways between the infant groups (pre- and post-stimulation with *S. epidermidis*). Fifty-seven of these genes were significantly differentially expressed following monocyte stimulation with *S. epidermidis* across all three infant groups, with a comparable pattern of gene expression observed between groups for most genes. In general, *S. epidermidis*-stimulation resulted in monocyte down-regulation of Toll-like receptors (except TLR2) and genes involved in apoptosis, and up-regulation of cytokines, chemokines and several key TLR pathway genes (Figure 3A). Genes involved in antigen presentation were not collectively differentially regulated in *S. epidermidis*-stimulated monocytes compared to unstimulated monocytes.

We then performed differential expression testing for pairwise comparisons between the three infant groups (e.g. chorioamnionitis-exposed vs. unexposed preterm infants), for both unstimulated or *S. epidermidis*-stimulated monocytes, resulting in 6 comparisons. Among the sixty-eight key selected genes, no significant differences between term infant monocytes and chorioamnionitis-unexposed preterm infant monocytes were observed, in either the unstimulated or stimulated sample comparisons (data not shown). In contrast, chorioamnionitis-exposed preterm infant monocytes displayed significantly reduced expression of twelve genes compared to term infant monocytes, unexposed preterm infant monocytes, or both. *IRAK2* was identified in the unstimulated sample comparison; *TLR4*, *TLR5*, *TKB1*, *NFKBIA*, *CSF2*, *CSF3*, *NLRP3*, *CASP1* and *CD86* in the stimulated sample

comparisons, and *CD74* and *HLA-DRA* in both stimulated and unstimulated comparisons (Figure 3B).

Global analysis of monocyte transcriptome reveals hypo-responsiveness to *S. epidermidis* in infants exposed to chorioamnionitis

We next extended our analysis beyond the sixty-eight key genes, to identify all differentially expressed genes between infant groups in the unstimulated and stimulated samples (as above). The results are summarised in Table 2. Comparisons between chorioamnionitis-unexposed preterm infant monocytes and term infant monocytes yielded the lowest numbers of differentially expressed genes for both unstimulated (23 genes) and stimulated (2 genes) comparisons. In contrast, comparisons between chorioamnionitis-exposed preterm infant monocytes and both term (50 unstimulated; 765 stimulated) and unexposed preterm (169 unstimulated; 262 stimulated) infant monocytes resulted in larger numbers of differentially expressed genes, with the greatest dichotomy observed between the comparisons of *S. epidermidis*-stimulated monocytes (Supplementary figure 1). Across these comparisons, most differentially expressed genes were downregulated in chorioamnionitis-exposed preterm infant monocytes compared to the other groups, suggesting a pattern of immune hypo-responsiveness. The full lists of these differentially expressed genes are presented in Supplementary tables 1 and 2.

All genes identified as differentially regulated by chorioamnionitis-exposed preterm infant monocytes versus either infant group in unstimulated (202 genes) and stimulated (868 genes) comparisons were then analysed separately using Ingenuity Pathway Analysis (IPA) to identify over-represented biological pathways and predicted upstream transcriptional regulators. Both sets of genes were significantly associated with pathways involving activation of adaptive immunity, with the most significantly over-represented pathway for both gene sets being “Antigen Presentation” (Figure 4A-B). Similarly, many of the top predicted upstream transcriptional regulators are known drivers of T-cell activity (IL2, IFNG, IL27, CD38), antigen presentation (XBP1, CD2) or inflammation (TNF, IL1B), and were all predicted to be significantly inhibited (see Supplementary table 3 for all upstream regulators). We then looked at the patterns of expression for all genes within the “Antigen Presentation” pathway, or downstream of XBP1 (Figure 4C). We observed distinct clustering between unstimulated and *S. epidermidis*-stimulated samples across all infant

groups, with further clear subdivision between samples from chorioamnionitis-exposed infants and the other infant groups.

Comparable production of cytokines and chemokines in preterm and term infant monocytes at the protein level

As initial validation of the results of this study, we compared the expression of key effector cytokines at the protein level across infant groups and stimulation conditions. A panel of ten cytokines (IL-1 β , IL-6, IL-8, IL-10, IL-12p70, CXCL10, TNF α , CCL2, G-CSF and M-CSF) were measured in 2-hour culture supernatants (and 24-hour supernatants for a subset of infants with sufficient cells) using a multiplex bead-based immunoassay. IL-12p70 and M-CSF, were undetectable in all monocyte culture supernatants, and G-CSF was undetectable in most supernatants (95% of unstimulated, and 81% of *S. epidermidis*-stimulated samples). IL-1 β , IL-6, IL-8, IL-10, TNF α and CCL2 were significantly upregulated following stimulation with *S. epidermidis* (assessing all infant responses; Figure 5A-F). Many of these cytokines and chemokines were also detected as significantly upregulated within individual infant groups, and all were significantly upregulated across the infant groups at the gene level. In contrast, CXCL10 was significantly downregulated at the protein level upon *S. epidermidis* stimulation, but only upon analysis of pooled samples ($P = 0.015$, Figure 5G). In addition, CXCL10 expression was unchanged at the gene level in all infant groups ($P = 0.55$ – 0.93). Significant positive correlations between gene and protein levels were observed for all cytokines and chemokines upon assessment of pooled samples, although the trend was markedly weaker for IL-6 (Figure 5H). The only difference observed between infant groups was significantly reduced IL-1 β production in monocytes from chorioamnionitis-exposed preterm infants, compared to unexposed preterm infant monocytes. Importantly, assessment of protein production at a later time point (24 hours) also revealed no significant differences between infant groups, albeit with smaller sample sizes ($n=3$ – 4 , data not shown).

DISCUSSION

Here, we aimed to determine the impact of preterm birth and exposure to chorioamnionitis on the proportions and phenotype of cord blood monocytes and on monocyte transcriptional response to live *S. epidermidis*. We report decreased frequencies of classical monocytes in cord blood from preterm infants, with a compensatory increase in frequencies of CD16 $^{+}$ monocytes. We demonstrate that monocytes of preterm infants without chorioamnionitis exposure are transcriptionally similar to term infant monocytes. Using hypothesis-driven analyses, we show that overall gene expression of TLR, inflammatory, antigen presentation

and apoptosis pathway genes are similar between term and preterm chorioamnionitis-unexposed infants, indicating that preterm monocytes are not intrinsically transcriptionally deficient. This interpretation is supported by comparable protein levels of inflammatory cytokines and chemokines in culture supernatants from preterm and term infants (at both 2 and 24 hour time points). In contrast, monocytes of preterm infants with chorioamnionitis exposure displayed a significant hypo-responsive transcriptional phenotype in response to *S. epidermidis*, in a subset of genes involved in antigen presentation and activation of the adaptive immune system. These results highlight that prenatal exposure to infection and inflammation may alter the risk for sepsis, at least partly via modulation of monocyte responses.

The limited data on proportions of monocyte subsets in preterm and term infants are conflicting. In contrast to our findings, Sharma et al. report frequencies of CD16⁺ monocytes ranging between 5–80% of total cord blood monocytes with no differences between preterm and term infants¹⁴. In keeping with our findings, Wisgrill et al. report significantly increased frequencies of CD16⁺ intermediate monocytes in preterm infants and a smaller dynamic range of CD16⁺ monocytes in cord blood¹⁵. Differences between studies may arise from distinct flow cytometry gating strategies, particularly regarding the threshold for CD14⁺ staining on the non-classical monocyte subset. Unbiased analysis of flow cytometry data through clustering algorithms may circumvent the limitations inherent to subjective gating. Both studies however, report significantly higher proportions of CD16⁺ monocytes in neonates compared to adults, suggesting a developmental shift toward predominantly classical monocytes. Importantly, differences in the frequencies of monocyte subsets between preterm and term infants are unlikely to have obscured our transcriptional data, as we did not observe enrichment of monocyte subset-specific gene signatures in any infant group (data not shown).

The lack of deficiencies in overall gene expression or cytokine production in monocytes from preterm chorioamnionitis-unexposed infants compared to term infants was unexpected, as deficient monocyte responses to PRR-stimulation in preterm infants are widely described⁶. However, Lissner et al. also observed minimal transcriptional differences between purified monocytes from term infants and adults (following LPS or *Listeria monocytogenes*-stimulation)¹⁶, despite reports of impaired monocyte-dependent cytokine responses to LPS in neonates compared to adults^{17, 18}. One possible mechanism is regulation of monocyte

function through extrinsic factors either in cord plasma or from other mononuclear populations, which were removed in our experiments and those of Lissner et al. A variety of soluble mediators capable of modulating monocyte responses are present in plasma, including adenosine¹⁹, the antimicrobial peptide LL-37^{20, 21} and the alarmins S100A8/A9²². Ulas et al. compared LPS-induced monocyte responses between term infants and adults, and demonstrated that neonatal monocytes are not functionally impaired, but differentially programmed transcriptionally and epigenetically via activity of the endogenous TLR4 ligands S100A8/A9²². Changes in plasma composition associated with prematurity are described^{22, 23}. Therefore, differential regulation of monocyte responses by extrinsic plasma factors during gestation may explain discrepant findings; studies with whole blood stimulation demonstrate significant impairment of preterm monocytes to produce inflammatory cytokines^{11, 17}, whereas similar cytokine production between preterm and term infants when (as here) is observed when isolated monocytes are used²⁴⁻²⁷. This highlights a potential mechanism for differential immune regulation during gestation, which may contribute to preterm infant susceptibility to sepsis. Future studies should aim to assess the immunoregulatory properties of preterm infant peripheral whole blood plasma at the time of highest risk of neonatal sepsis (days 10–22 for late-onset sepsis). Media replacement studies to determine how persistent the effects of exposure to such factors are, would also be of interest.

In contrast to the above studies, a comparison of the basal transcriptional states between fetal (18–22 weeks GA) and adult bone marrow monocytes identified >2000 significantly differentially expressed genes, including several genes involved in pathogen recognition and inflammation²⁸. Together, these findings suggest that there are major transcriptional differences between fetal and adult monocytes, and relatively small transcriptional differences between preterm and term monocytes, indicating that continued maturation of innate immunity occurs in early life (<29 weeks GA) and correlates with the clinical risk of neonatal sepsis.

In the current study, chorioamnionitis-exposed preterm infants showed the most distinct transcriptional profile. It should be noted that differences observed here affect a relatively small (yet functionally important) subset of genes, and we have previously characterised the conserved infant monocyte response to *S. epidermidis*²⁹. Interestingly, most differentially expressed genes by chorioamnionitis-exposed infants were down-regulated or hypo-

responsive following *S. epidermidis*-stimulation compared to the other groups, indicating a potentially dampened immune response to infection. Responses to the Gram-negative pathogen *E. coli* were also assessed as part of previous work, however we observed a much smaller (and mostly overlapping) effect specific to chorioamnionitis-exposed monocytes (data not shown). Here, we also observed significantly reduced frequencies of HLA-DR+ monocytes in chorioamnionitis-exposed preterm infants. There are very limited data on how chorioamnionitis exposure impacts human monocyte responses. However, similar monocyte hypo-responsiveness occurs in sheep models of intrauterine infection and inflammation, where intra-amniotic exposure to LPS or *Ureaplasma parvum* is associated with decreased monocyte production of IL-6 and hydrogen peroxide, and lower surface expression of MHC class II and CD14 in preterm lambs³⁰⁻³³. The authors attribute these observations to endotoxin tolerance, and found that repeated exposure to intra-amniotic LPS had a cross-tolerance effect; attenuating the monocyte inflammatory response to TLR2, TLR5 and TLR9 agonists³³. Importantly, these sheep studies report a reversal of this phenotype toward hyper-responsiveness after 1–2 weeks. It would be of interest to determine monocyte responsiveness from preterm chorioamnionitis-exposed infants at later time points, especially as these infants are known to have a decreased risk of late-onset sepsis⁵. In contrast, a reduced capacity for antigen presentation is associated with sepsis in the first week of life, which may partly explain the heightened susceptibility for early-onset sepsis of chorioamnionitis-exposed neonates³⁴⁻³⁶. Azizia et al. reported similar frequencies of MHC class II cord blood monocytes between preterm and term infants, but significantly reduced frequencies in preterm infants exposed to chorioamnionitis³⁵. Similarly, preterm infants with fetal inflammatory response syndrome down-regulate several genes critical for antigen processing and presenting including CD74 and HLA-DRA³⁷. Finally, in line with our observation of reduced *CASP1* and *NLRP3* gene expression in preterm chorioamnionitis-exposed monocytes, others report a significant reduction in the frequencies of Caspase-1 positive monocytes (induced by LPS+ATP) in preterm infants exposed to chorioamnionitis¹⁴, suggesting that inflammasome activity may be negatively regulated by prenatal exposure to chorioamnionitis.

The only clinical parameter that differed between our preterm infants with or without chorioamnionitis-exposure was mode of delivery, which is inherent to the underlying inflammatory pathophysiology of spontaneous preterm birth. The impact of mode of delivery on immune development is incompletely understood. Furthermore, while mode of delivery

does impact absolute numbers of cord blood monocytes³⁸, differences in PRR-stimulated neonatal monocyte/mononuclear cells responses have not been attributable to mode of delivery in most studies^{13, 17, 18, 39}. Interestingly, the one infant with chorioamnionitis-exposure that did not cluster with the others within the principal component analysis, exhibited the lowest grade of chorioamnionitis, suggesting a potential dose effect on monocyte responses. Interpretation in this regard however warrants caution, due to the small sample size. We postulate that an intrinsic mechanism that influences transcription, such as epigenetic modification, may be differentially regulated in infants exposed to chorioamnionitis. Gestational age-dependent regulation of histone modifications relating to pro-inflammatory cytokine promoters in monocytes has been demonstrated, with the greatest disparity observed between cells of preterm infants <30 weeks GA and term infants⁴⁰. To date, no studies have investigated epigenetic changes associated with chorioamnionitis-exposure. This would be of interest as bacterial infection can induce a variety of epigenetic changes in the host including histone modifications and DNA methylation⁴¹, and chorioamnionitis exposure is associated with differential infection risk^{5, 42}.

We acknowledge some limitations. The sample sizes are relatively small and not matched for gender. In addition, we were not able to include term infants with chorioamnionitis exposure in this study, as routine screening is only performed in deliveries <37 weeks gestation. However, chorioamnionitis only occurs in a minority of term pregnancies (~3–5%)⁴³, and is generally less severe than that observed in preterm placentas⁴⁴. Furthermore, due to restraints on recruitment and sample volumes, we only included preterm infants of 29–32 weeks gestational age. Samples from extremely preterm infants (<28 weeks gestational age) would have been of particular interest, as these infants have the highest risk of late-onset sepsis, and consistently show the greatest differences in responses to term infants. The relatively mature gestational age of our cohort may have limited our ability to detect major transcriptional changes between preterm chorioamnionitis-unexposed and term infants, and larger studies resolving these limitations are warranted. Future studies should also aim to utilise fresh peripheral blood monocytes sampled during the period of greatest late-onset sepsis risk, a more physiologically relevant sample than cryopreserved cord blood monocytes. A major strength of our study was the use of live *S. epidermidis*; most studies have assessed preterm monocyte responses to purified TLR agonists which may be poorly representative of *in vivo* infection, and even killed *S. epidermidis* preparations induce significantly altered innate responses from mononuclear cells compared to live preparations⁴⁵.

369

370 CONCLUSIONS & FUTURE DIRECTIONS

371 We have demonstrated that the infant monocyte transcriptional response to *S. epidermidis* is
372 not globally altered by gestational age at birth, or prenatal exposure to chorioamnionitis.
373 Importantly however, exposure to chorioamnionitis skews the preterm infant monocyte
374 toward a hypo-responsive transcriptional response to *S. epidermidis* challenge, affecting a
375 subset of functionally important genes. These differentially expressed genes include well-
376 characterised immune response genes, together with those involved in antigen presentation
377 and activation of adaptive immunity. Given the high frequency of chorioamnionitis in
378 preterm birth, understanding the mechanisms responsible for this hypo-responsive
379 phenotype, including possible epigenetic modifications, may provide insight into the altered
380 risk of sepsis observed in the growing population of preterm infants.

381

382 AUTHOR CONTRIBUTIONS

383 Study conception and design by AC, TS, DH and CW. Sample recruitment and clinical
384 expertise by KS, PR and TS. Experimental work by EJ. Analysis and interpretation of data
385 by EJ, DH, CW, TS and AC. Drafting of manuscript by EJ. Critical revision by DH, CW,
386 DB, TS and AC.

387

388 METHODS

389 *Study population and sample collection*

390 The study was conducted at King Edward Memorial Hospital (KEMH; Perth, Australia). The
391 Human Research Ethics Committee approved the study (HREC ID: 814/EW) and written
392 informed consent was obtained from parents or guardians. Cord blood samples were selected
393 (based on sample availability) from a prospective study of newborn immunity. Infants were
394 classified as: preterm (29–32 weeks gestational age), either with or without chorioamnionitis,
395 and term infants (37–40 weeks). Chorioamnionitis was defined by analysing sections of the
396 chorioamniotic membranes, umbilical cord, chorionic plate and placenta using an adaptation
397 of a widely accepted semi-quantitative scoring system, as previously described⁵. Presence
398 and degree of maternal inflammation was defined by neutrophilic infiltration of the cellular
399 chorion, of the membranes or the chorionic plate. Incidence and severity of fetal
400 inflammation was defined by neutrophilic infiltration from the fetal vessels into the umbilical
401 vessels or the chorionic plate vessels. Demographic information for study participants is
402 presented in Table 1. Cord blood was collected into pre-heparinised syringes mixed 1:1 with

RPMI 1640 (Gibco). Cord blood mononuclear cells (CBMCs) were isolated using lymphoprep gradient centrifugation (Axis-Shield PoC) and cryopreserved using an established method preserving cell viability and function⁴⁶. RNA-seq data was obtained from a subset of these infants (n = 15, 11 preterm) who had adequate cell numbers for culture and sequencing. Preterm infants with and without chorioamnionitis exposure were matched by gestational age and birth weight.

Monocyte purification from CBMC

Cryopreserved CBMCs were thawed, washed in culture medium (RPMI 1640, 2mM glutamax, 10mM HEPES buffer, 1mM sodium pyruvate, and 0.05mM 2-Mercaptoethanol (Gibco)), and filtered through a 35 µm nylon filter (Corning). Cells were centrifuged and resuspended in PBS containing 2% w/v bovine serum albumin (Sigma-Aldrich), and 2% v/v heat-inactivated fetal calf serum (HI-FCS) at $\sim 5 \times 10^7$ cells/mL. CBMC were then stained with the following fluorescent monoclonal antibodies for 30 minutes at 4°C: CD14-PECy7 (clone M5E2), CD16-FITC (3G8) and CD3-APC (SK7) from BD Biosciences, CD19-AF647 (HIB19) from BioLegend and CD56-PE (N901) from Beckman Coulter. An aliquot of each stained CBMC sample ($\sim 1 \times 10^5$ cells) was transferred into a BD Trucount™ tube (BD Biosciences) for quantitation of total cells/mL, following acquisition of at least 2000 events on a BD FACSCanto™ II (BD Biosciences). All monocyte populations (classical: CD14⁺⁺CD16⁻, intermediate: CD14⁺⁺CD16⁺, and non-classical: CD14⁺CD16⁺) were collectively purified (median purity of 96.8%, range 91–97%) using a BD FACSARIA™ III, after exclusion of debris/doublets, B cells (CD19⁺), T cells (CD3⁺) and NK cells (CD56⁺), and collected into culture medium containing 30% filter-sterilised HI-FCS. Gating strategy is presented in Supplementary figure 2. For each sample, $\sim 2.5 \times 10^4$ sorted monocytes were analysed using a BD FACSCanto™ II (BD Biosciences) flow cytometer to determine purity, with an additional stain for HLA-DR (G46-6, BD Biosciences). All monocyte populations were used in downstream experiments.

Bacterial culture

Mid-log-phase cultures of *S. epidermidis* (WT-1457) were grown in Heart Infusion broth (Oxoid Thermo Fisher Scientific), and cryopreserved per an established in-house method⁴⁷. Frozen stocks were thawed, washed twice with sterile phosphate buffered saline (PBS; Gibco), and resuspended in PBS at a concentration of 5×10^7 CFU/mL. Bacterial viability was

confirmed by plating out ten-fold serial dilutions (10^{-1} to 10^{-6}) of each preparation onto blood agar (in triplicate) on the day of stimulation. Plates were incubated overnight (37°C, 5% CO₂), and CFU counted the following day.

Monocyte stimulation cultures

Purified monocytes were resuspended at 5×10^5 cells/mL in 500 μ L serum-free culture medium containing RPMI 1640, 2 mM glutamax, 10 mM HEPES buffer, 1 mM sodium pyruvate, and 0.05 mM 2-Mercaptoethanol (Gibco). A sample of monocytes was stained with 0.4% Trypan Blue, and counted using a Neubauer haemocytometer to determine total cell number and viability. 5×10^4 monocytes (at viability $\geq 98\%$) were stimulated for 2 hours at 37°C with 10 μ L of *S. epidermidis* (MOI of 10:1), or with 10 μ L of sterile PBS (negative control) in triplicate, in a 96-well polypropylene round-bottom plate (Corning). The final dose of *S. epidermidis* used in the study was chosen empirically based on maximising the inflammatory response at both gene and protein level (IL-6 and TNF α by PCR and Luminex) while minimising cell death (measured via LDH release and Annexin V/7-AAD staining by flow cytometry). Following stimulation triplicate cultures were pooled, supernatants harvested and stored at -80°C, and cells resuspended in 300 μ L RNeasy Protect Cell Reagent (Qiagen) and stored at -20°C before batch analysis. Each donor was processed on a separate day.

RNA isolation and RNA-seq

Total RNA was extracted from cultured monocytes using the AllPrep DNA/RNA Micro kit (Qiagen), with 1 U/ μ L of RNase inhibitor (SUPERase In, Ambion, Thermo Fisher Scientific) added to each sample. RNA quality was determined using a Bioanalyzer 2100 (Agilent Technologies) and cDNA libraries were constructed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina). Samples were normalised to the lowest weight sample (minimum 100ng). Each sample was indexed with a unique barcode adaptor sequence, allowing all samples to be pooled and sequenced over five lanes to reduce technical variability. RNA-seq (50 bp, single-end) was performed on an Illumina HiSeq 2000. Read libraries were mapped to the GRCh37 (hg19) Human genome using the R/STAR suite (v1.14.2) with default options⁴⁸, and summarised against Ensembl (v69) annotations, with a minimum Mapping Quality Score of thirty⁴⁹. The RNA-seq data have been deposited in

NCBI's Gene Expression Omnibus⁵⁰ and are accessible through GEO Series accession number GSE101880 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101880>).

Differential expression analysis

Raw aligned read counts were analysed using the R/limma Voom-normalised differential gene expression analysis method (R software v3.1.1, limma v3.20.9)^{51, 52}. Prior to Voom normalisation, read counts were filtered on the requirement that retained genes had at least five counts per million in at least four samples (the minimum group size in the experiment). Gene filtering was performed independently of the experimental variables to avoid the introduction of bias. Library sizes were scale-normalized using the Trimmed Mean of M-values method from the edgeR package to correct for differences in sequencing depth between libraries during Voom normalization⁵³. To calculate lists of differentially expressed genes between monocyte groups, limma differential expression analyses were performed using the Voom-normalised data as input. Empirical Bayes moderation of the standard errors in T-statistics was applied. As the experiment followed a block design with multiple samples from the same infant (unstimulated and stimulated), we accounted for "within infant correlation" in the linear models using blocking variables and the standard limma "duplicate correlation" function. Differential expression analysis tests were performed for both baseline (e.g. [chorioamnionitis-exposed preterm Control] vs. [term Control]) and stimulation-induced (e.g. [chorioamnionitis-exposed preterm *S. epidermidis*] vs. [term *S. epidermidis*]) differences.

Ingenuity® Pathway Analysis

Analysis of over-represented canonical pathways and identification of upstream transcriptional regulators was performed using Ingenuity Pathways Analysis (IPA; Qiagen) using right-tailed Fisher's exact tests and default settings for other options. For pathways analysis, Benjamini-Hochberg adjusted *P*-values <0.01 were considered significant. For analysis of upstream transcriptional regulators, results were filtered to remove chemicals, and *P*-values <0.01 were considered significant. Activation z-scores were also calculated to infer the activation state of predicted upstream regulators by comparing their known effect on downstream targets with observed changes in gene expression. Upstream regulators with activation z-scores ≥ 2 or ≤ -2 were considered "activated" or "inhibited", respectively.

Quantitative detection of cytokines/chemokines in culture supernatants

An in-house multiplex fluorescent-bead immunoassay was used to quantify levels of eleven cytokines, chemokines and growth factors in monocyte culture supernatants¹⁰. Primary antibodies directed against each cytokine/chemokine were washed to remove sodium azide using Vivaspin 500 centrifugal concentrators (Sigma-Aldrich), and covalently conjugated to carboxylated microspheres (Bio-Rad Laboratories Inc). Samples and protein standards were diluted in PBS containing 0.05% Tween 20 (Sigma-Aldrich) and 2% FCS, and incubated with antibody-conjugated microspheres in a 96-well Multiscreen Filter Plate (Merk Millipore) for 30 minutes with shaking (room temp, 500 rpm, protected from light). Biotinylated secondary antibodies were then added and the plate incubated for a further 30 minutes. The wells were washed (PBS, 1% BSA, 0.25% Tween 20, 0.001% sodium azide; Sigma-Aldrich) and 5 µg/mL streptavidin-PE conjugate (BD Biosciences) was added to develop for 15 minutes. Excess streptavidin-PE was washed off, and the samples acquired on a BioPlex® 200 System (Bio-Rad) to determine fluorescence in each specific bead region. Analyte concentrations were determined from a 5-PL standard curve of median fluorescence intensity, generated from recombinant protein standards using BioPlex Manager 5.0 software. Sample concentrations below the limit of detection were assigned a value equal to half that of the lowest standard.

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CONFLICT OF INTEREST

The authors declare there are no conflicting interests.

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FIGURE LEGENDS

Figure 1. Proportions and absolute counts of infant monocytes and monocyte subsets.

Flow cytometric analysis of monocyte subsets within CBMC. Frequencies of total monocytes, classical (CD14⁺⁺ CD16⁻), intermediate (CD14⁺⁺ CD16⁺) and non-classical (CD14⁺CD16⁺) monocyte subsets respectively, across infant groups (**a-d**). Median fluorescence intensity of CD14 on classical monocytes (**e**). The frequencies of total monocytes expressing HLA-DR across infant groups (**f**). Data are presented for individual donors (symbols, duplicate measurements for (**e**)) with bars representing the median (mean for (**e**)). N = 9 preterm, 7 preterm HCA⁺, and 6 term infants. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's multiple comparisons test or 1-way ANOVA with Holm-Sidak's multiple comparisons test for (**e**). **P* < 0.05, ***P* ≤ 0.01, *****P* ≤ 0.0001. CBMC, cord blood mononuclear cells; HCA, histologic chorioamnionitis; MFI, median fluorescence intensity.

Figure 2. Principal component analysis and hierarchical clustering.

A 3D scatterplot showing the first three principal components (**a**). Two distinct sample clusters are evident on principal component 1; unstimulated (●) and *S. epidermidis*-stimulated (▲) monocytes.

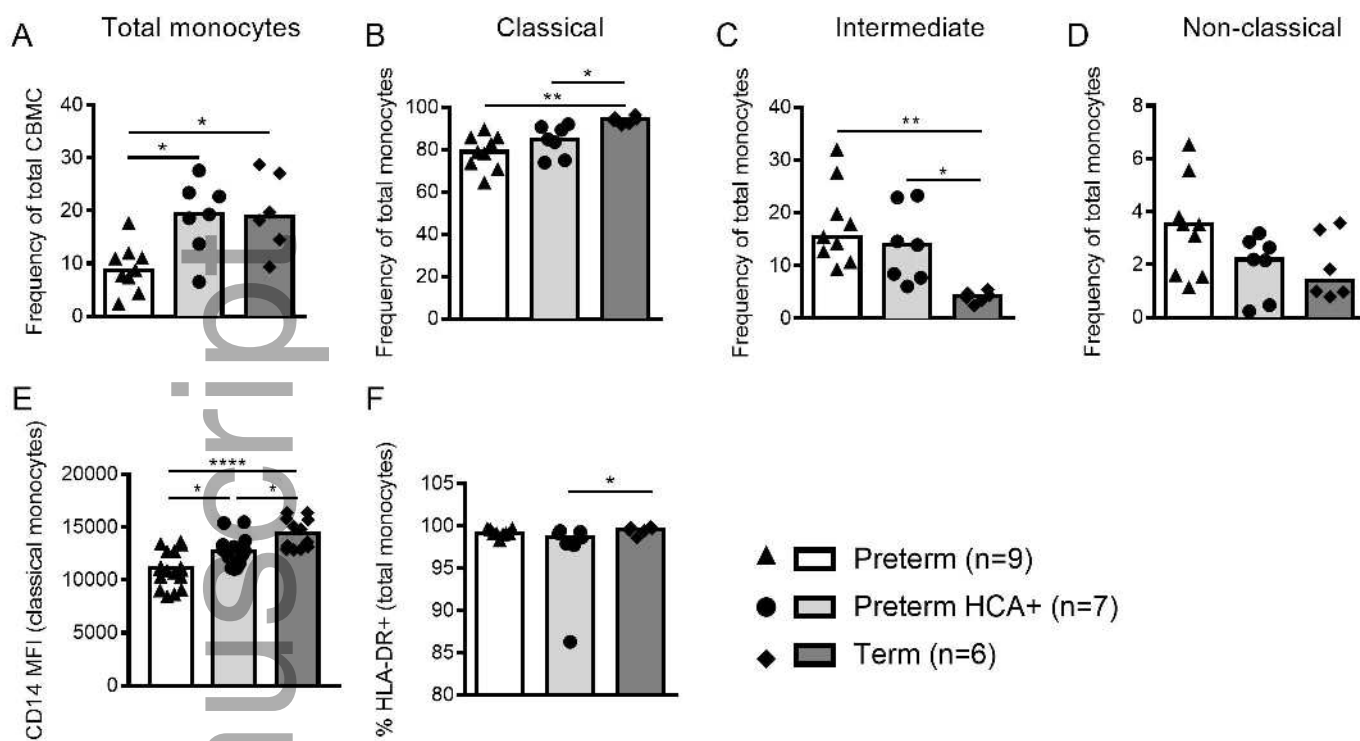
Within each of these clusters, four out of the five preterm chorioamnionitis-exposed infants cluster away from the other infant groups on principal component 3. Data points represent individual samples for term infants (dark grey, n=4), preterm infants (white, n=6) and preterm infants with chorioamnionitis (light grey, n=5). Scree plot showing the fraction of total variance in the data that is captured by each principal component (b).

Figure 3. Immune-specific genes differentially expressed by preterm chorioamnionitis-exposed infant monocytes. Heatmap visualising normalised log₂ gene expression of sixty-eight key genes in TLR, inflammatory, antigen presentation and apoptosis pathways across all infant groups and culture conditions (a). Genes and samples were ranked using unsupervised clustering. Red stars indicate genes differentially expressed by chorioamnionitis-exposed preterm infant monocytes, for which box and whisker plots are presented in (b). Expression values for unstimulated- and *S. epidermidis*-stimulated monocytes are shown. Statistical analysis was performed using limma for differential expression, with *P*-values corrected for multiple comparisons using the Benjamini-Hochberg method. **P* < 0.05, ***P* ≤ 0.01. UN, unstimulated; SE, *S. epidermidis*; HCA, histologic chorioamnionitis. N = 6 preterm, 5 preterm HCA+, and 4 term infants.

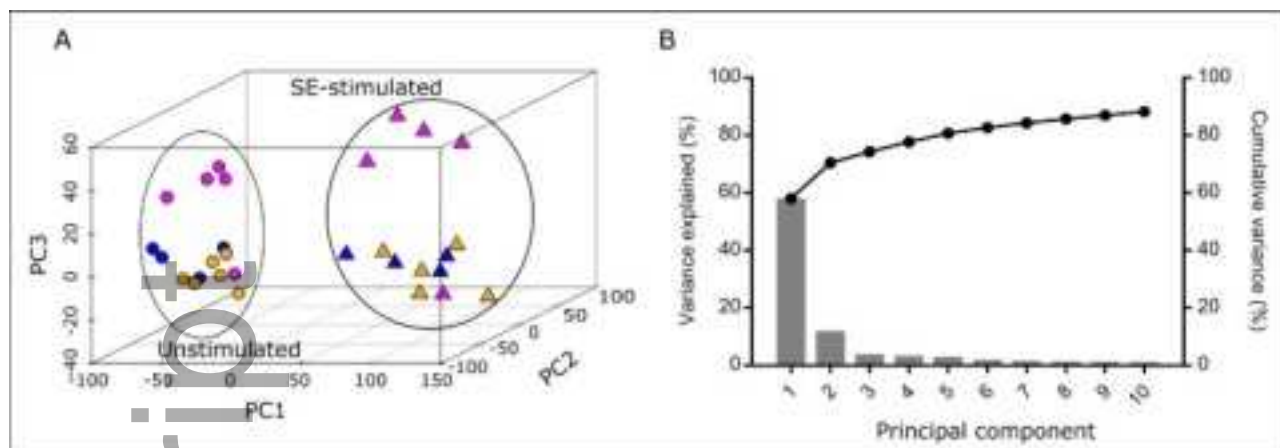
Figure 4. Preterm chorioamnionitis-exposed infant monocytes exhibit reduced expression of genes involved in antigen presentation or activation of adaptive immunity. Over-represented biological pathways associated with, and predicted upstream transcriptional regulators of the genes differentially expressed by chorioamnionitis-exposed preterm infant monocytes at baseline (a) and following stimulation with *S. epidermidis* (b). Dashed lines represent the threshold for significance (equivalent to a *P*-value < 0.01). The proportion of differentially expressed genes to total genes in each pathway is indicated. Transcriptional regulators are coloured red or blue, indicating predicted activation (z-score > 2) or inhibition (z-score < -2), respectively. (c) A heatmap of all genes within the “Antigen Presentation Pathway” or downstream of XBP1; red stars in (a) and (b). Genes and samples were ranked using unsupervised clustering. UN, unstimulated; SE, *S. epidermidis*; HCA, histologic chorioamnionitis. N = 6 preterm, 5 preterm HCA+, and 4 term infants.

Figure 5. Protein levels of inflammatory cytokines and chemokines in monocyte culture supernatants are significantly correlated with gene expression. Protein expression of IL-1β, IL-8, IL-10, TNFα, CCL2, CXCL10 and IL-6 were measured in unstimulated and *S.*

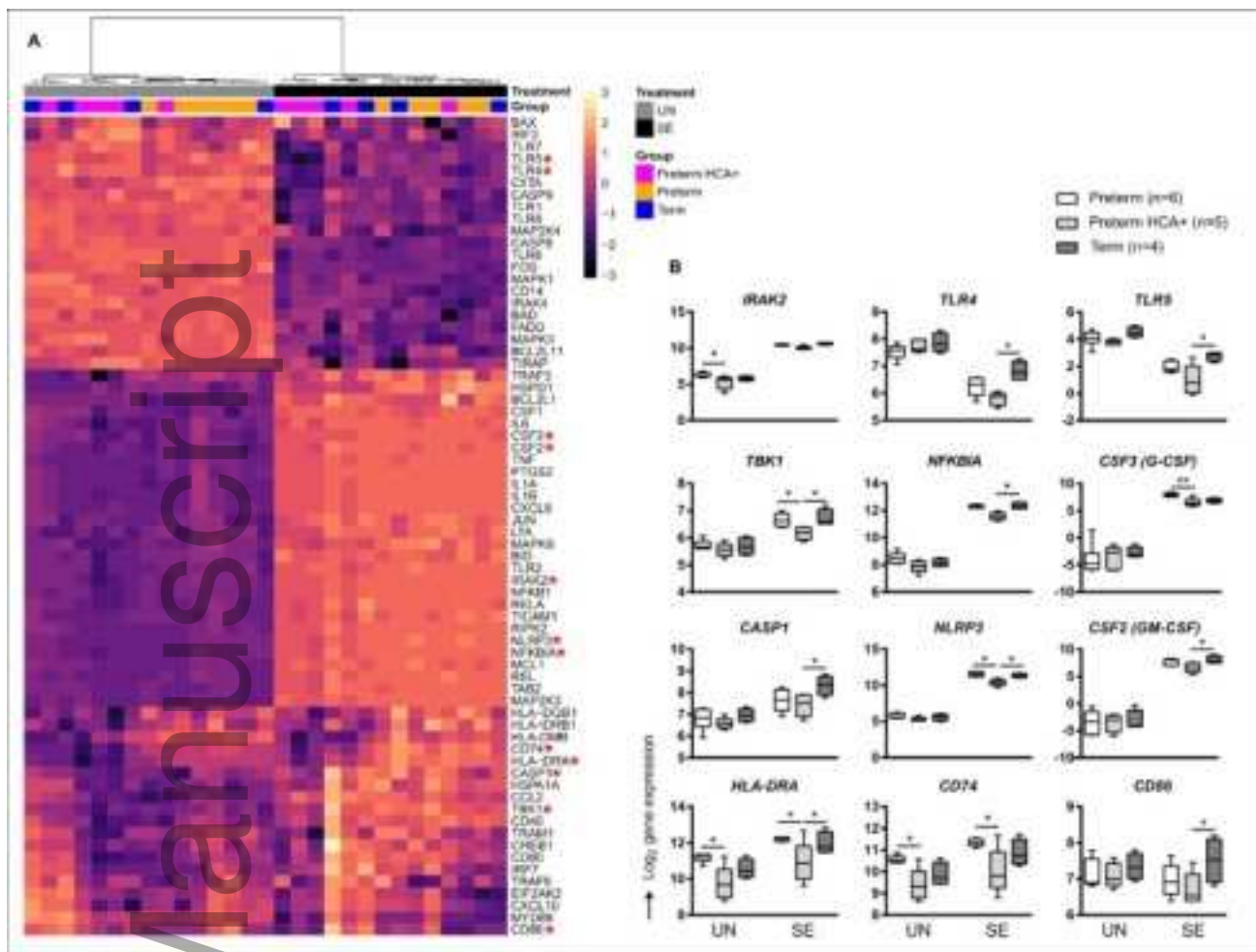
737 *epidermidis*-stimulated monocytes following 2 hours of culture (**a-g**). Data are presented as
738 box-whisker plots showing minimum, maximum, interquartile range and the median. N = 9
739 preterm, 7 preterm HCA+, and 5 term infants. Statistical analysis was performed using
740 Wilcoxon matched-pairs signed rank test for comparisons between culture supernatants
741 within individual groups; stars indicate significantly increased/decreased levels in *S.*
742 *epidermidis* stimulated supernatants compared to unstimulated supernatants. The Kruskal-
743 Wallis test with Dunn's multiple comparisons test between infant groups. * $P < 0.05$, ** P
744 ≤ 0.01 . There were significant positive correlations (Spearman correlation) between gene and
745 protein expression for all analytes (**h**). Gene expression was derived from normalised log₂
746 gene counts. P -values, correlation coefficients, and 95% confidence intervals are indicated
747 for each analyte. UN, unstimulated; SE, *S. epidermidis*; HCA, histologic chorioamnionitis.



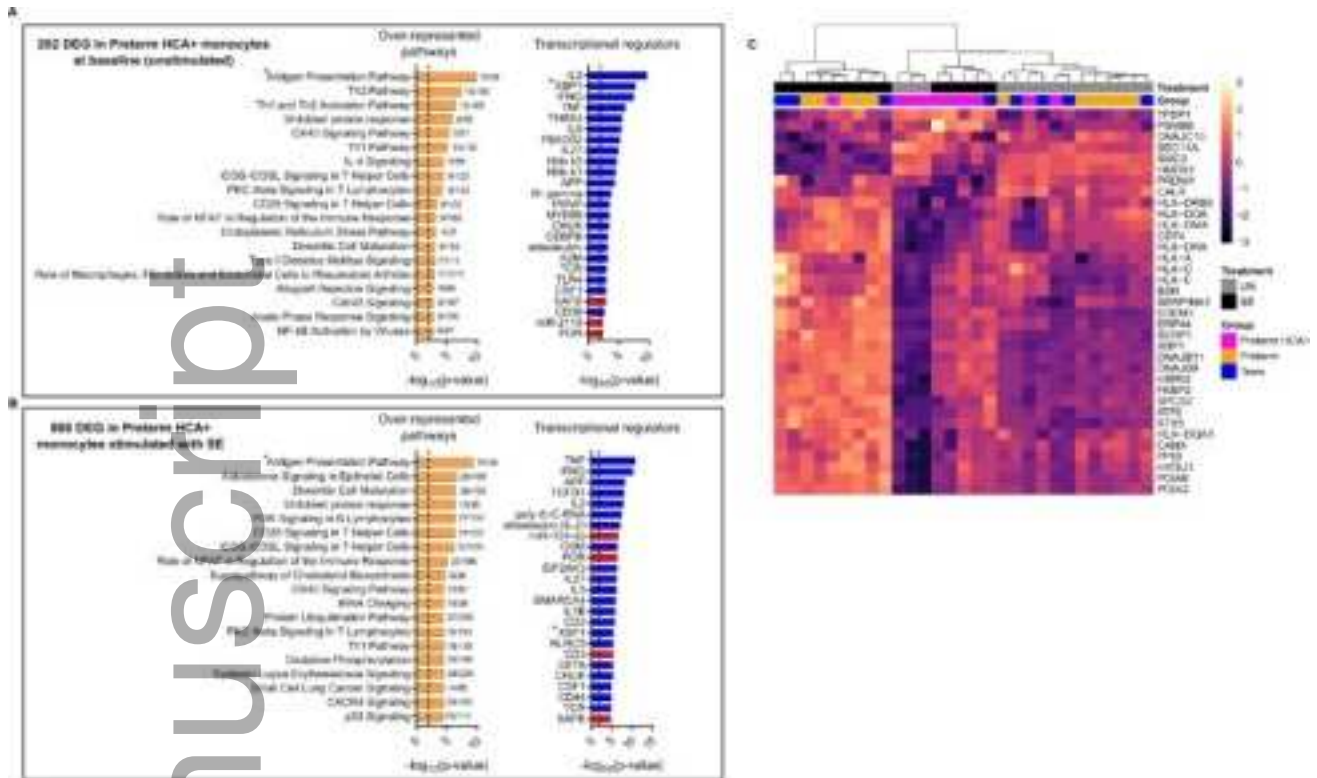
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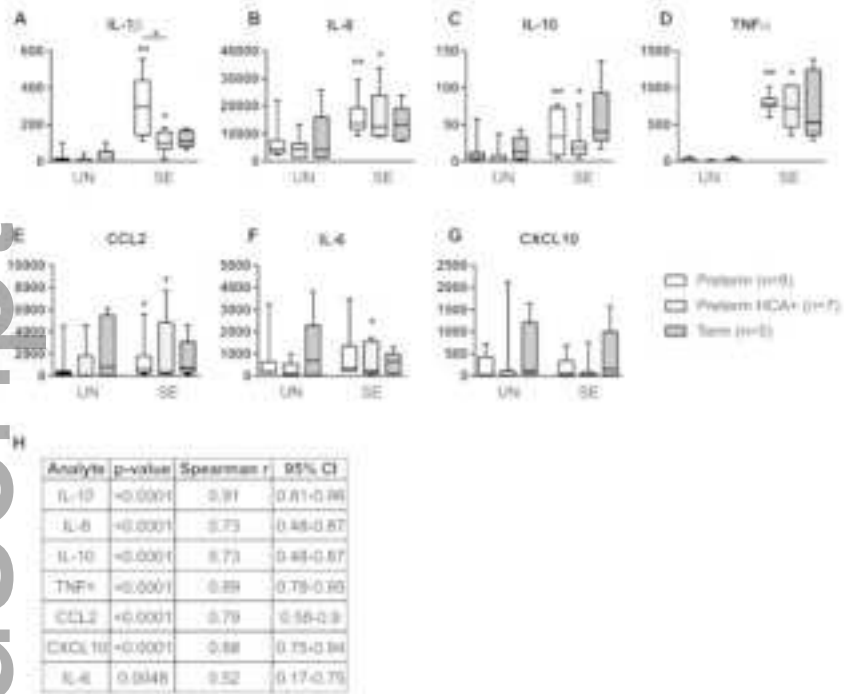
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