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Serum context shapes macrophage baseline state and LPS responsiveness in human monocyte-derived macrophages

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Background: Human monocyte-derived macrophages (MDMs) are central to translational immunology, yet most differentiation protocols rely on xenogeneic fetal calf serum (FCS), which may alter macrophage functional states. Autologous serum (AS) maintains the donor-specific immunometabolic milieu, but its impact on macrophage baseline programming and stimulus responsiveness has not been systematically evaluated.

Methods: Monocytes from 32 healthy donors were differentiated in parallel with 10% AS or FCS for five days with M-CSF, followed by 24-hour stimulation with LPS, IL-4/IL-10/TGF-β, or vehicle. Cytokine secretion was quantified by multiplex assay, activation markers by qPCR, and baseline transcriptional programs by bulk RNA sequencing.

Results: AS- versus FCS-differentiated MDMs showed significantly greater LPS-induced secretion of TNF-α, IL-6, IL-1β, IP-10, IL-23, TARC and IL-1RA together with higher *CD86* and *CCR7* expression, whereas these differences were not observed following IL-4/IL-10/TGF-β stimulation or in unstimulated cells. Transcriptomic analysis of unstimulated MDMs identified distinct serum-dependent baseline programs, with FCS associated with increased expression of inflammatory cytokines, chemokines, and innate immune activation genes, whereas AS was associated with genes involved in lipid metabolism, extracellular matrix remodeling, and immune surveillance.

Conclusions: Serum context is a major determinant of macrophage baseline state and inflammatory responsiveness. FCS is associated with a more inflammatory baseline and attenuated LPS-induced activation, while AS shows a metabolically and immunologically homeostatic baseline with greater LPS responsiveness. These findings demonstrate that serum composition shapes basal macrophage programs and differentially modulates responses to inflammatory versus alternative stimulation, supporting consideration of autologous serum when modeling donor-specific innate immune responses.

KEYWORDS
autologous serum, fetal calf serum, human monocyte-derived macrophages, innate immunity, LPS responsiveness, macrophage activation, macrophage plasticity, translational immunology

1 Introduction

Accurate modeling of patient-specific immune responses is central to translational research. Human monocyte-derived macrophages (MDMs) are widely deployed in translational and precision immunology to model innate immune responses across a broad range of disciplines, including oncology, cardiovascular biology, metabolism, dermatology, neurology, and regenerative medicine (1–5). Their utility stems from their accessibility, plasticity, and capacity to recapitulate disease-relevant macrophage phenotypes from patient-derived material. However, the reliability of conclusions drawn from MDM assays depends critically on the conditions under which these cells are generated (6). Chief among these is the choice of serum supplement used during differentiation, a variable that has received comparatively little systematic attention despite its profound biological relevance (7).

The large majority of MDM differentiation protocols rely on xenogeneic fetal calf serum (FCS) as the standard growth supplement (7). FCS provides growth factors, hormones, and nutrients required for cell survival and differentiation, and its widespread use reflects the practical advantages of batch-standardized, commercially available reagents (7, 8). However, as a xenogeneic product, FCS exposes human monocytes throughout the differentiation period to a complex mixture of bovine proteins, lipoproteins, immunoglobulins, and bioactive molecules that are absent from the human systemic milieu and may provide signals that differ from those encountered *in vivo* (7–9). This biological incongruence raises the concern that if FCS alters the functional state of differentiating macrophages, conclusions about macrophage inducibility, activation programs, and disease-specific immune phenotypes drawn from FCS-based assays may be systematically skewed and of limited translational relevance (7).

Autologous serum (AS) represents a donor-specific alternative to FCS, as it preserves the individual's immunometabolic milieu, including endogenous lipoproteins, growth factors, hormones, and immune mediators, under conditions that better recapitulate those of transition from monocytes to macrophages *in vivo* (10, 11). This is particularly relevant for personalized and disease-specific modeling, in which the goal is to capture donor-specific immune signatures rather than impose a standardized but non-physiological differentiation environment (4, 12). Given macrophages' well-established capacity to adopt highly context-dependent functional states driven by their local microenvironment (6, 12, 13), the disease- and tissue-specific signals present in AS may contribute to determining macrophage phenotype (12). Indeed, prior work has demonstrated that patient-derived macrophages differentiated in AS retain disease-relevant phenotypes, including altered TREM2 and APOE regulation in Alzheimer's disease patients and inflammatory polarization states in major depressive disorder (3, 5). A recent comparison of xeno-free human ABO serum with FCS similarly revealed significant differences in MDM surface marker expression and activation state (8). However, pooled human ABO serum, while xeno-free, cannot capture the full breadth of individual-specific physiological (dietary habits, micronutrient status, age-related changes in hormonal and inflammatory tone) and pathological variation, that collectively shape the immunometabolic milieu in which MDM differentiation occurs *in vivo* (14–17). While AS introduces greater inter-individual variability than standardized serum supplements, this variability reflects biologically meaningful donor-specific immune contexts and highlights the need for systematic comparison with FCS reference conditions.

Despite these considerations, a systematic, donor-matched, large-scale comparison of AS and FCS effects on primary human MDM baseline transcriptional state and stimulus responsiveness has not been performed. This gap is consequential for the field: if the two conditions systematically preconfigure macrophages into different functional states, the interpretation of FCS- and AS-based assays for translational immunology research would differ. Prior studies comparing serum conditions in primary human immune cell culture have been largely limited in scope. Comparisons of autologous or ABO-matched human serum with FCS have focused on non-macrophage cell types such as neutrophils or mesenchymal stem cells (18–20), assessed surface marker expression without parallel functional or transcriptomic readouts (8) or focused mainly on disease specific effects (1, 3, 5). Here, we address this gap through a donor-matched parallel comparison of AS and FCS effects on MDM differentiation in 32 healthy donors, integrating multiplex cytokine profiling and qPCR validation of activation markers under pro-inflammatory, anti-inflammatory, and resting conditions, and bulk RNA-sequencing of unstimulated MDMs.

2 Material and method

2.1 Participant recruitment and sample collection

Healthy controls (HC, n = 32) were included (mean age 44.1 ± 11.3 years; 21 females). Demographic characteristics of the healthy donors are found in Table 1. All participants provided written informed consent, underwent clinical and medical screening, and met predefined exclusion criteria, including acute or chronic inflammatory conditions, recent infection (<1 month) or vaccination (<3 months), chronic NSAID or corticosteroid use, heart failure, asthma or allergies, history of cancer or stroke, pregnancy or lactation, and poorly controlled diabetes or major cardiovascular, renal, hepatic, hematologic, endocrine, or neurologic disease. The study was approved by the local ethics committee (EA4/002/13) and conducted in accordance with the Declaration of Helsinki 1975, as revised in 2008.

TABLE 1 Demographic characteristics of study participants.

Characteristic	Healthy donors (n = 32)
Age, years	44,1 (11.3)
Female, No. (%)	21 (65.6 %)
BMI	24,1 (3.9)
Current smokers, No. (%)	7 (21.9 %)
Education, years	14.5 (2.1)
Non-Caucasian, No. (%)	4 (12.5 %)

Values are expressed as mean (SD) except when indicated; BMI, body mass index.

Fasting blood samples were collected between 8:00 and 12:00 a.m., and peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll™ density gradient centrifugation using established protocols (3, 5, 21). Serum was collected in vacuum tubes (BD Biosciences, Germany), allowed to clot at room temperature (RT) for up to 1.5 h, centrifuged at 1500 × g, and either used to prepare fresh RPMI medium containing 10% autologous serum (AS) and 1% penicillin/streptomycin (P/S, 10000 U/10 mg per ml; Biochrom) or stored at −80 °C until further use (3, 5, 22).

2.2 Monocyte enrichment, macrophage differentiation and stimulation

Monocytes were enriched by 24 h plastic adherence in 24-well plates (4.5×10^5 PBMCs/well) at 37 °C with 5% CO_2 in RPMI medium containing 1% P/S and 10 ng/ml human macrophage colony-stimulating factor (M-CSF, Miltenyi Biotec), with half of the wells supplemented with 10% FCS (Sigma-Aldrich®, Darmstadt, Germany, Product F7524, lot BCBT0730) and the other half with freshly prepared 10% AS. Cells from one healthy donor were plated on the one 24-well plate for a total of 6 days, with either FCS (12 wells) or AS (12 wells) – enriched RPMI media, and kept in the same conditions throughout the experiment. After a total of 5 days differentiation with M-CSF, macrophages were stimulated for 24 hours with LPS (50 ng/ml, Sigma, USA, Product Number L6529) or IL-4/IL-10/TGF-β (each 20 ng/ml, Peprotech, USA, Lot 110621, 091514, and 0506S354 respectively) according to established protocols (3, 5, 21) or left unstimulated (vehicle control, VEH). Cells and supernatants were collected 24 h later and stored at −80 °C until use.

2.3 RNA isolation, cDNA synthesis and Real-Time PCR

Total RNA was isolated from MDMs using the RNeasy Mini extraction kit (Qiagen, Hilden, Germany) as per manufacturer's instructions and stored at -80 °C until use. Total RNA was reverse-transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit™ (Thermo Fisher Scientific Inc., MA, USA) and stored at -20 °C until further measurement. To quantify expression levels, qPCR was performed with LightCycler™ 480 SYBR Green I Master (Roche, Mannheim, Germany) using 75 ng template cDNA and 500 nM standard primer concentrations, following manufacturer's instructions in an Applied Biosystems StepOne™ Real-Time PCR System (CA, USA). Melting curve analysis was performed to identify specific products with 1.2 °C increment from 65 to 95 °C. Relative quantification (ΔCt) and melting curve analysis were both carried out using the StepOne™ Real-Time PCR System software. All primers were designed and checked for their quality using the Primer-BLAST software. Sequences of primers are shown in Supplementary Table 1. *B2M* was used for data normalization (ΔCt). The expression fold change was calculated as $2^{-\Delta Ct}$ and further used for analysis.

2.4 RNA isolation and sequencing

Libraries were prepared from 150 ng total RNA utilizing the NEBNext rRNA Depletion Kit (Human) (NEB: E6310) and the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® Version 3.1_5/20 (NEB: E7765L). Indexing-PCR was performed with 11 cycles using the NEBNext Multiplex Oligos for Illumina (96 UDIs) (NEB: E6441). Final libraries were quantified by Qubit® dsDNA HS Assay Kit (ThermoFisher: Q32854) and the fragment distribution was checked by High Sensitivity D1000 ScreenTape assay (Agilent: 5067-5584) on a TapeStation 4200 system (Agilent). Libraries were pooled equimolarly and sequenced with 75 bp single-reads on a NextSeq 500 system (Illumina). RNA-seq was performed on a paired subset of 8 donors, in which AS- and FCS-differentiated MDMs were derived from the same individual and differentiated in parallel under identical conditions, with the sole variable being the serum supplement; this fully paired design controls for between-donor variability as a confounding factor and maximizes statistical power for detecting condition-specific transcriptional differences.

2.5 Assessment of cytokine levels

Levels of pro- and anti-inflammatory cytokines in supernatants and serum were measured using the LEGENDplex™ Human M1/M2 Macrophage Panel (10-plex; BioLegend, San Diego, USA). Samples were acquired on a BD FACSCanto™ II and analyzed with LEGENDplex™ Data Analysis Software v8.0 according to the manufacturer's instructions. The average intra-assay and inter-assay coefficients of variation were 10.4% and 6.8%, respectively.

2.6 Data analysis

Cytokine and qPCR data are presented as mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism (version 8.0.2). Cytokine values below the detection limit were set to half the lower limit of detection. To control for inter-assay variability in the multiplex assays, plate effects were normalized using z-score transformation. For each cytokine, z-scores were calculated separately within each plate/batch according to the formula: (measured value − plate mean) divided by the plate standard deviation. This approach standardized values across the six experimental plates while preserving within-plate variability. Importantly, all samples from a given donor, including AS- and FCS-differentiated MDMs across all stimulation conditions, as well as corresponding serum samples, were analyzed on the same plate/batch to avoid donor-specific batch effects. For cytokine analysis, because AS and FCS conditions were tested in parallel under the same conditions, using cells from the same donors, effects of serum type (FCS vs. AS) and stimulation condition (LPS, IL-4/IL-10/TGF-β, VEH) were assessed using two-way repeated-measures ANOVA with Bonferroni correction for multiple comparisons. Serum condition and stimulation were treated as within-donor factors, with donor as the repeated-measures subject. Sex-stratified analyses of LPS-induced individual cytokine responses were additionally assessed

using two-way mixed ANOVA with serum condition (AS vs. FCS) as a within-donor factor and sex as a between-donor factor. The sex × serum interaction was tested to determine whether the effect of serum condition differed between females and males. Bonferroni-adjusted within-sex comparisons were used for descriptive follow-up; these analyses should be regarded as exploratory given the unequal group sizes (female n=21, male n=11).

To assess the effect of AS vs. FCS on overall cytokine output, individual composite Z-scores were calculated for each participant by summing the Z-scores of pro- and anti-inflammatory cytokines into a respective pro- and anti-inflammatory score as described in detail by previously published protocols (5, 23, 24). In short, a composite Z-score was calculated for each participant by summing the Z-scores of pro- or anti-inflammatory markers resulted from the LPS or IL-4/IL-10/TGF-β stimulation into a pro- or anti-inflammatory individual Z-Score respectively: $Z_i = \sum_{k=1}^{i} Z_k^i$, where i = subject and k = marker. In each individual Zi -score for pro-inflammatory stimulation, the Z-scores of pro-inflammatory cytokines (IL-12p70, TNF-α, IL-6, IL-1β, IL-12p40, IL-23, IP-10; k =7) were summed. For anti-inflammatory score IL-10, TARC, IL-1RA (k =3) were included.

Moreover, Pearson correlation analysis was used to evaluate the relationships between individual cytokine levels in supernatant or serum and the respective Zi pro- or anti-inflammatory score across both serum conditions, where r represents the correlation coefficient.

As the qPCR data were not normally distributed, Friedman's ANOVA tests with Dunn's *post hoc* multiple comparison tests were applied. P-Values were adjusted for multiple testing. Statistical significance was set at $p< 0.05$.

Raw bulk RNA sequencing data were processed using *Salmon* (version 1.4.0) (25) against the GRCh38 reference transcriptome (GENCODE v38) for transcript-level quantification followed by downstream analyses in R (version 4.4.2). Gene-level quantifications were aggregated using *tximport* (26) based on Ensembl mapping. Differential gene expression was calculated using the DESeq2 package (version 1.46.0) (27) as previously described (28, 29). Transcriptional profiles of matched unstimulated AS- (n=8) and FCS-differentiated MDMs (n=8) were compared and gene expression changes with an adjusted p-value below 0.05 (Benjamini–Hochberg false discovery rate correction) and an absolute log2 fold change (log2FC) > 1.5 were considered differentially expressed. For visualization and downstream biological interpretation, a more stringent subset of differentially expressed genes (|log2FC| >1.5 and adjusted p-value<10−5) was selected. For analysis of individual genes of interest, DESeq2-normalized counts were extracted and visualized with *ggplot2*. Statistical testing was performed using a paired Wilcoxon signed-rank test. The code used for data analysis is available at https://github.com/liviuspenter/Macrophage_Cosma. Bulk RNA sequencing was restricted to the unstimulated condition because the transcriptomic assessment was exploratory and specifically designed to identify baseline transcriptional differences between AS- and FCS-differentiated MDMs that were not readily apparent from the functional measurements.

3 Results

3.1 Patient-specific macrophage culture

MDM cultures were differentiated from monocytes isolated from thirty-two healthy participants (Figure 1A). The demographic characteristics of the study cohort are summarized in Table 1. Following 24 h of stimulation with LPS, IL-10/IL-4/TGF-β, or vehicle, participant-derived MDMs displayed characteristic macrophage morphology, consistent with our previous observations under both FCS and AS conditions (3, 5, 21).

3.2 Autologous serum selectively enhances LPS-induced cytokine production in MDMs

We previously reported that stimulation of primary human MDMs with LPS or IL-10/IL-4/TGF-β induced the expression of pro- and anti-inflammatory cytokines respectively, both in AS and FCS condition (3, 5, 21). However, it remained unclear how the serum context in particular shaped macrophage inducibility. Therefore, we examined cytokine responses of LPS-, IL-10/IL-4/TGF-β-stimulated and unstimulated MDMs in AS- vs. FCS parallel culture conditions for all 32 healthy donors. Higher cytokine secretion was observed in AS-differentiated MDM cultures compared to those differentiated with FCS following LPS stimulation, including increased levels of TNF-α, IL-6, IL-1β, IL-1RA, IL-23, TARC, and IP-10 (Figures 1D–M; Table 2 for two-way RM ANOVA results). Moreover, this effect was significant at the individual cumulative Z-scores of both pro- ($p = 0.0001$) and anti- ($p = 0.0378$) inflammatory markers after LPS stimulation in AS vs. FCS (Figures 1B, C). Two-way repeated-measures ANOVA further demonstrated significant serum × stimulation interactions for IL-12p70, TNF-α, IL-6, IP-10, IL-1β, IL-23, and IL-12p40 (Table 2), indicating that the effect of stimulation differed between serum conditions for these pro-inflammatory cytokines. In contrast, no significant serum × stimulation interaction was observed for IL-10, IL-1RA, or TARC. To complement the relative Z-score analysis, Supplementary Figure 1 shows the ratios of stimulated to vehicle cytokine levels for all measured cytokines in AS- and FCS-differentiated MDMs (Supplementary Figure 1). Together, these findings indicate that serum context modifies macrophage responses to stimulation in a cytokine-specific manner, with the most consistent serum-dependent differences observed following LPS stimulation.

Interestingly, a trend toward higher cumulative Z-scores of anti-inflammatory markers were observed in unstimulated MDMs ($p = 0.0596$) differentiated in AS compared to FCS (Figure 1C), while cytokine secretion remained low in both unstimulated conditions when compared to stimulated cells. This prompted us to further investigate the unstimulated condition by bulk-RNA sequencing to assess whether this signal was accompanied by distinct baseline transcriptional programs not fully captured by the low cytokine secretion state.

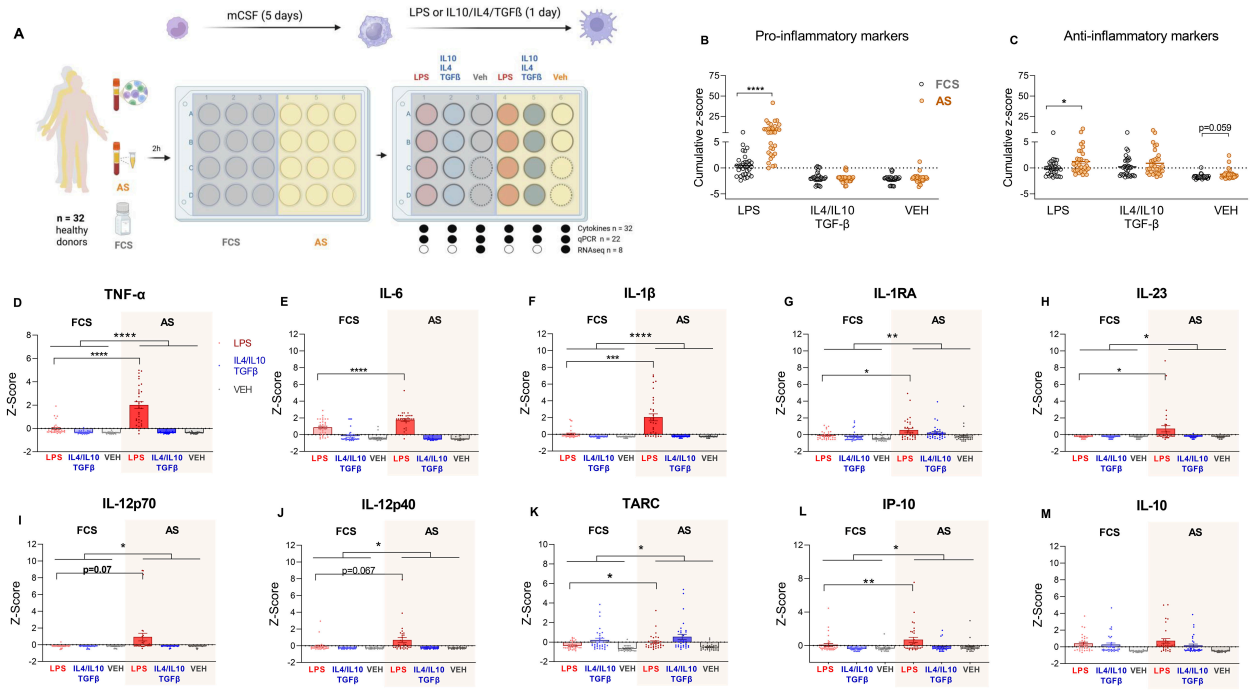


FIGURE 1
Serum effect on macrophage inducibility. **(A)** Experimental design of parallel differentiation of human monocyte-derived macrophages (MDMs) from healthy donors in autologous serum (AS) and fetal calf serum (FCS) for 5 days, followed by 24 hours stimulation with LPS, IL-4/IL-10/TGF-β, or vehicle (VEH). Created in BioRender. Cosma, (N) C. (2026) https://BioRender.com/r44tkan. **(B, C)** Cumulative Z-scores for pro- **(B)** and anti-inflammatory **(C)** cytokines demonstrate greater LPS responsiveness in AS conditions. Open respectively closed symbols represent values from FCS (gray)- and AS (brown)- enriched media. **(D–M)** Effects of AS- and FCS- enriched media on individual cytokine levels in MDM cultures 24 h after stimulation with LPS or IL-4/IL-10/TGF-β or VEH. Closed bars and symbols with (AS) and without (FCS) background represent LPS (red), IL-4/IL-10/TGF-β (blue) and VEH (gray), respectively. Each dot represents one donor (n = 32), with AS and FCS conditions analyzed in parallel. Data shown as mean ± SEM; Two-way repeated-measures ANOVA with Bonferroni's multiple-comparison test *p< 0.05, **p< 0.01, ***p< 0.001, **** p< 0.0001.

TABLE 2 **Effects of AS- and FCS- enriched media on individual cytokine levels in MDM cultures 24 h after stimulation with LPS or IL-4/IL-10/TGF-β or VEH.**

	IL12p70		Bonferroni's multiple-comparison test AS vs. FCS		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.001, 31.02) = 5.97	0.02	0.074	0.3899	0.3838
Serum condition	F (1,31) = 6.04	0.02			
Stimulation × Serum	F (1, 31) =5.33	0.03			
	TNF-α		Bonferroni's multiple-comparison test AS vs. FCS		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.006, 31.17) =69.94	<0.0001	<0.0001	0.8522	>0.999
Serum condition	F (1, 31) = 46.13	<0.0001			
Stimulation × Serum	F (1.007, 31.22) = 48.44	<0.0001			
	IL-6		Bonferroni's multiple-comparison test AS vs. FCS		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.289, 39.96) =127.3	<0.0001	<0.0001	0.0542	0.5880
Serum condition	F (1,31) =1.832	0.1856			
Stimulation × Serum	F (1.634, 50.64) = 32.61	<0.0001			

(Continued)

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TABLE 2 Continued

IP-10			Bonferroni's multiple-comparison test AS vs. FCS		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.040, 32.23) =8.324	0.0064	0.0088	0.2774	0.9727
Serum condition	F (1, 31) =7.102	0.0121			
Stimulation × Serum	F (1.23, 38.16) =5.901	0.0148			
IL-1β			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.001, 31.03) =37.84	<0.0001	0.0002	>0.9999	>0.9999
Serum condition	F (1, 31) =21.82	<0.0001			
Stimulation × Serum	F (1.001, 31.03) =21.22	<0.0001			
IL-23			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.001, 31.03) =6.509	0.0159	0.0445	0.8283	>0.9999
Serum condition	F (1, 31) =6.942	0.0130			
Stimulation × Serum	F (1.001, 31.03) =6.439	0.0164			
IL-12p40			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.001, 31.02) =14.7	0.0006	0.0671	0.9644	>0.9999
Serum condition	F (1,31) = 5.588	0.0245			
Stimulation × Serum	F (1.001, 31.04) =5.653	0.0214			
IL-1RA			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.473, 45.67) =14.85	<0.0001	0.0192	0.0694	0.1216
Serum condition	F (1, 31) =8.782	0.0058			
Stimulation × Serum	F (1.245, 38.6) =1.681	0.2042			
TARC			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.376, 42.66) = 17.57	<0.0001	0.0291	0.6131	0.1457
Serum condition	F (1, 31) = 6.516	0.0158			
Stimulation × Serum	F (1.209, 37.48) =0.5302	0.5041			
IL-10			**Bonferroni's multiple-comparison test AS vs. FCS**		
Effect	F (DFn, DFd)	p value	LPS	IL-4/IL-10/TGF-β	VEH
Stimulation	F (1.665, 51.62) =13.59	<0.0001	0.8598	>0.9999	>0.9999
Serum condition	F (1, 31) =0.3927	0.5355			
Stimulation × Serum	F (1.226, 38.01) =1.592	0.2178			

Two-way repeated-measures ANOVA followed by Bonferroni multiple-comparisons test. AS, autologous serum; FCS, fetal calf serum; LPS, lipopolysaccharide; VEH, vehicle/unstimulated macrophages.

3.3 Autologous serum differentially modulates pro-inflammatory gene expression in MDMs

To further characterize and validate the impact of serum context on macrophage inducibility, gene expression responses of MDMs to stimulation were assessed by qPCR in a subset of 22 healthy donors. Here, AS-differentiated MDMs exhibited a greater expression of the pro-inflammatory markers *CD86* and *CCR7* after LPS stimulation compared to FCS-differentiated cells (Figures 2A, B), whereas no difference was observed in the expression of the anti-inflammatory marker *CD206* between the AS and FCS conditions (Figure 2C). In contrast, no significant differences between AS and FCS conditions were detected in response to IL-10/IL-4/TGF-β stimulation (Figures 2A–C). These results suggest that serum context influences macrophage responsiveness, with greater induction of the pro-inflammatory candidate genes *CD86* and *CCR7* following LPS stimulation in AS.

3.4 Sex-dependent effects of autologous serum context on MDMs cytokine responses

Given the potential influence of biological sex in human serum composition (14, 16), we performed exploratory sex-stratified analyses to determine whether serum-dependent effects on macrophage inducibility were observable. AS differentiation was associated with higher LPS-induced secretion of TNF-α, IL-6, and IL-1β in both female and male donors, whereas IL-12p70 and IP-10 were significantly higher with AS only in female donors (Figures 3A–H; Supplementary Table 2). However, a significant sex × serum interaction was observed only for IP-10 (p=0.0044), indicating a sex-dependent difference in the magnitude of the AS–FCS effect for this cytokine (Supplementary Table 2). Although exploratory, these analyses indicate that the direction of the enhanced LPS responsiveness associated with AS differentiation was observed in both sexes, while significant AS–FCS differences for selected cytokines were observed only among female donors.

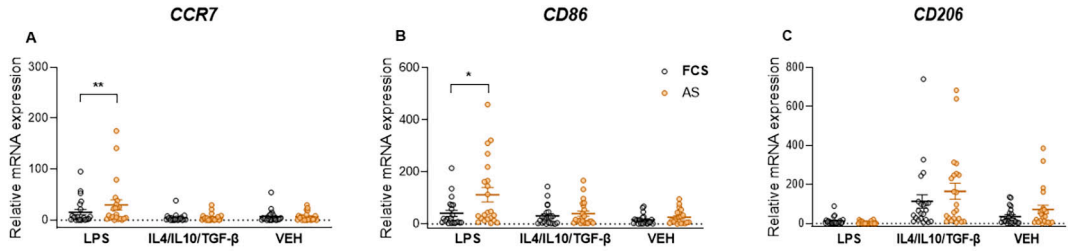


FIGURE 2
Autologous serum differentially regulates gene expression in human monocyte-derived macrophages (MDMs). Expression levels of **(A)** *CCR7*, **(B)** *CD86* and **(C)** *CD206* mRNA in LPS-differentiated MDMs in autologous serum (AS) and fetal calf serum (FCS)- enriched media. Each dot represents one donor (n = 22), with AS and FCS conditions analyzed in parallel. Open gray symbols represent FCS enriched medium; closed brown symbols indicate AS enriched medium. Data shown as mean ± SEM; Friedman's ANOVA with Dunn's *post-hoc* multiple comparison test, with p-values adjusted for multiple testing. *p< 0.05.

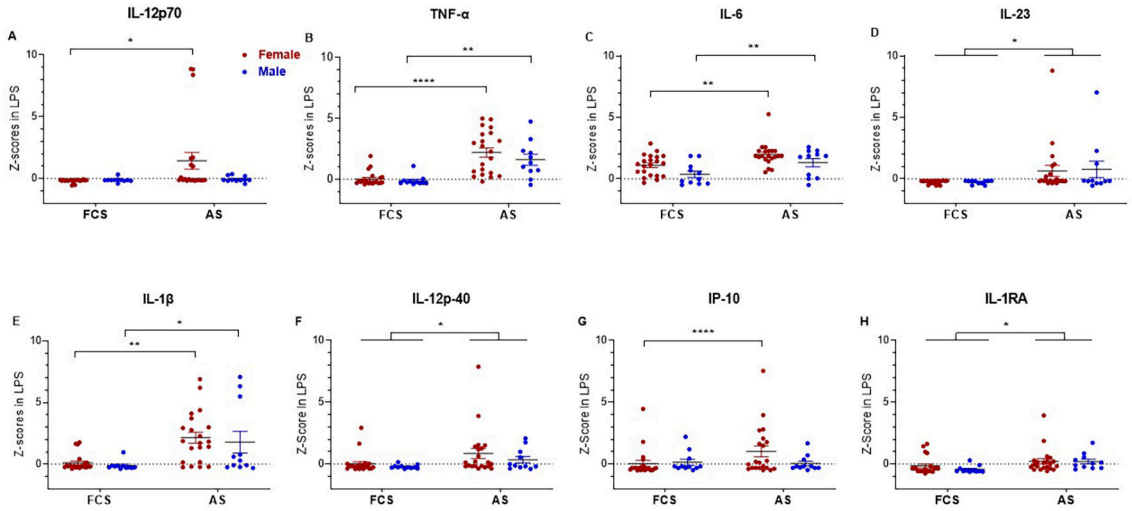


FIGURE 3
Sex-stratified analysis of LPS-induced cytokine production. **(A–H)** Sex effects on cytokine levels in MDM cultures 24 h after LPS-stimulation in in autologous serum (AS) and fetal calf serum (FCS)- enriched media. Each dot represents an individual healthy control subject (female n=21, male n=11). Closed symbols represent data from female (red)- and male (blue) participant-derived LPS-stimulated MDMs. Data shown as mean ± SEM. Two-way mixed ANOVA with serum condition (AS vs. FCS) as a within-donor factor and sex as a between-donor factor, followed by Bonferroni's multiple comparisons test *p< 0.05, **p< 0.01, ***p< 0.001, **** p< 0.0001.

3.5 Autologous serum stimulus-dependent cytokine responses are not explained by measured circulating cytokine levels

The increased absolute cytokine output in AS-differentiated MDMs (Figures 1B–M), prompted us to evaluate whether the serum-dependent effects were driven by inherent donor characteristics or by the differentiation environment. Although AS differentiation increased absolute cytokine output, individual cytokine responses showed similar patterns of association with the corresponding cumulative inflammatory scores in AS- and FCS-differentiated MDMs (Figures 4A–J). Importantly, measured circulating serum cytokine levels did not predict *in vitro* cytokine responses (Supplementary Tables 3-6), indicating that the measured circulating cytokines did not account for the observed inter-donor variation in macrophage responses. Collectively, these findings indicate that AS amplifies stimulus-dependent cytokine induction across donors, without evidence that this effect was explained by the measured circulating cytokine levels.

3.6 Autologous serum and fetal calf serum induce distinct baseline transcriptional programs in unstimulated MDMs

To uncover baseline transcriptional differences not evident under low-cytokine vehicle conditions, we performed exploratory bulk RNA-seq of unstimulated MDMs of both AS and FCS conditions from a total of n = 8 donors. We identified 109 high-confidence differentially expressed genes (absolute log2 fold change >1.5 and FDR corrected p-value< 10^{-5}), including 87 upregulated in AS and 22 upregulated in FCS (Figures 5A, B; gene-level effect estimates (log2FC) and adjusted P values in Supplementary Table 7). Functional annotation of these genes revealed distinct transcriptional programs associated with each serum condition (Supplementary Table 8). FCS- vs. AS- differentiated MDMs exhibited elevated expression of inflammatory cytokines and chemokines (*CCL2, CXCL8, CCL8, IL-1β*), activating innate immune receptors (*CD300E, S100A8*), cytoskeletal/migratory regulators (*PDPN, ADAM8*), oxidative/endoplasmic reticulum stress response genes (*HSPA1A, OSGIN1, NQO1*), and soluble innate immune effectors (*PTX3*), consistent with a more transcriptionally activated baseline state with enrichment of inflammatory and stress-associated programs (Figure 5B; Supplementary Tables 7, 8). In contrast, AS-differentiated MDMs preferentially expressed elevated levels of genes linked to lipid/mitochondrial metabolism (*PLIN2, FABP4, PDK4, DHRS9*), extracellular matrix interaction and tissue embedding (*FN1, SPON2, ADAMTS15, MMP12*), homeostatic immune surveillance (*C1QB, CLEC10A, CD300A, CXCL16*), and coordinated cell-cycle programs (*PLK1, CDK1, RRM2*), consistent with a comparatively quiescent baseline state characterized by metabolic, tissue-adaptive, and immune-surveillance programs (Figure 5B; Supplementary Tables 7, 8). To contrast these programs within shared pathways, we visualized representative gene pairs with reciprocal functions (Figures 5C–F); paired dot plots confirmed significant AS–FCS differences (Figures 5G–L). Importantly, the identified transcriptional differences were highly consistent across the eight donors, with strong concordance of the differentially expressed gene profiles between AS- and FCS-conditioned MDMs (Supplementary Figure 2), supporting the robustness of the observed transcriptional signature despite the limited RNA-seq cohort. Together, these data demonstrate that serum context preconfigures macrophage baseline transcriptional states prior to stimulation.

4 Discussion

We show for the first time in a large donor-matched cohort integrating functional and transcriptomic readouts that serum

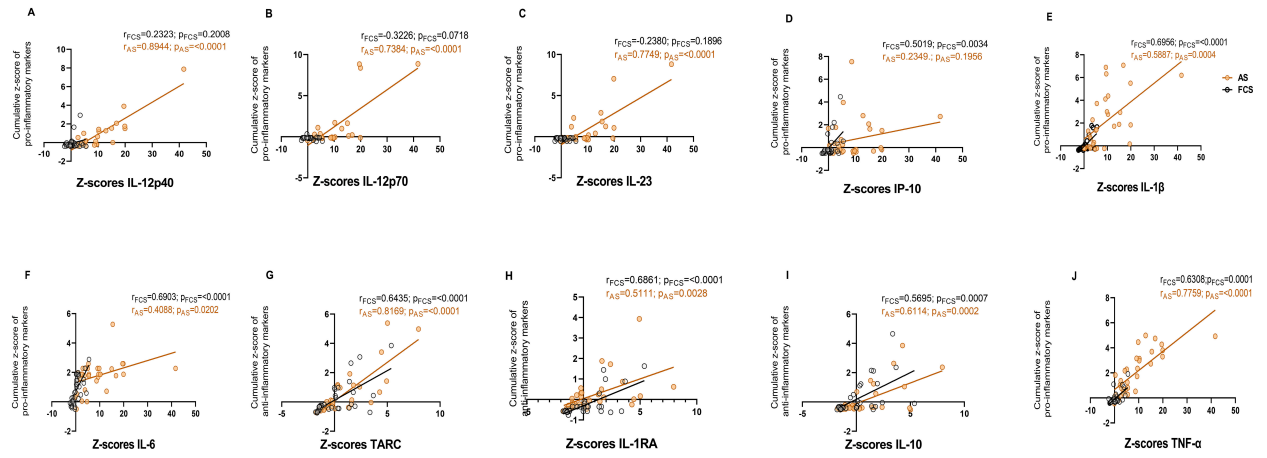


FIGURE 4
Correlations between cumulative Z-scores and individual cytokines in stimulated MDMs. **(A–J)** Significant correlations between cumulative Z-scores of pro-inflammatory markers and individual pro-inflammatory cytokine Z-scores in MDM cultures after LPS-stimulation, and between cumulative Z-scores of anti-inflammatory markers and individual anti-inflammatory cytokine Z-scores in MDM cultures after IL-4/IL-10/TGF-β-stimulation, in autologous serum (AS)- and fetal calf serum (FCS)- enriched media. Each dot represents an individual healthy control subject (n = 32 paired donors). Brown lines and symbols represent AS; black lines and symbols represent FCS. Pearson correlation analysis was used to test the relationships between individual cytokine levels across serum conditions where r represents the correlation coefficient.

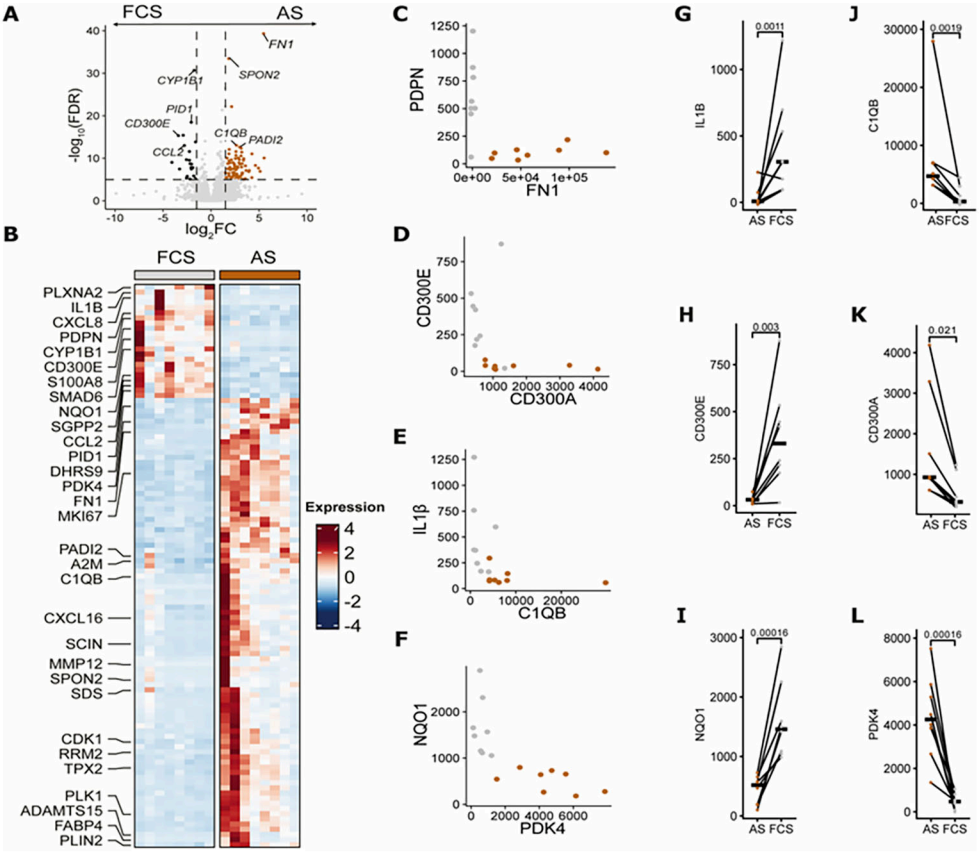


FIGURE 5
Autologous serum and fetal calf serum induce distinct baseline transcriptional programs in unstimulated human macrophages. **(A)** Volcano plot showing differential gene expression between unstimulated AS- and FCS-differentiated MDMs (n = 8 paired donors). Genes meeting the predefined significance threshold (absolute log2 fold change > 1.5; Benjamini–Hochberg false discovery rate corrected p-value< 10^{-5}) are highlighted. **(B)** Heatmap of differentially expressed genes demonstrating distinct baseline transcriptional programs under AS and FCS conditions. **(C–F)** Representative gene pairs with reciprocal biological functions illustrating the contrasting transcriptional programs in AS- and FCS-differentiated MDMs. **(G–L)** Paired dot plots of representative genes confirming significant differences between AS- and FCS-differentiated MDMs under unstimulated conditions. Statistical testing using Wilcoxon signed-rank test.

context is an important determinant of baseline transcriptional state and stimulus-dependent inducibility in primary human macrophages. While prior studies have reported phenotypic differences using human ABO serum or alternative supplements, these were largely limited to surface marker expression, small or diseased cohorts, and did not systematically assess paired functional or transcriptional states across stimulation conditions of in a large cohort of individualized human MDM cultures (3, 8, 21). In our parallel design across 32 healthy donors, FCS-differentiated MDMs adopted a pre-activated baseline, yet paradoxically showed reduced stimulus-dependent cytokine induction compared to AS-differentiated counterparts. AS-differentiated MDMs, by contrast, maintained a baseline enriched in lipid metabolism, extracellular matrix remodeling, and immune surveillance programs, while displaying significantly higher and proportionally coordinated cytokine responses upon LPS stimulation. These findings have direct implications for the design and interpretation of primary human macrophage assays across translational research contexts and support consideration of autologous serum as a donor-matched differentiation condition for personalized and disease-specific immune modeling.

The AS-associated transcriptional baseline we identify, encompassing higher expression of *FABP4, PLIN2, PDK4, DHRS9, FN1, SPON2, C1QB, CLEC10A*, and coordinated cell-cycle programs, shows features associated with metabolically active and structurally remodeling macrophage states, including programs related to lipid homeostasis, extracellular matrix interaction, and homeostatic immune surveillance (30, 31). *FABP4* and *PLIN2* are canonical markers of lipid-handling macrophages and are co-expressed in tissue-resident macrophage subsets with anti-inflammatory or lipid-associated functional states, including the TREM2-high lipid-associated macrophages identified in atherosclerotic lesions and the brain (12, 32, 33). *PDK4*, which suppresses glycolytic flux by inhibiting pyruvate dehydrogenase, is consistent with a metabolic profile oriented toward oxidative phosphorylation rather than the aerobic glycolysis that characterizes classically activated inflammatory macrophages (34). Together, these programs are consistent with a metabolically active, structurally remodeling, and immune surveillance-oriented macrophage state that retains the full dynamic range for stimulus-dependent activation. These transcriptional patterns should be interpreted as donor-matched evidence of coordinated baseline programs rather than as independently

validated molecular mechanisms, given the exploratory nature and limited size of the transcriptomic cohort.

The pre-activated baseline, we observe in FCS-differentiated MDMs, marked by elevated *IL-1β, CCL2, CXCL8, S100A8, CD300E*, and *PTX* expression alongside paradoxically reduced LPS inducibility, is consistent with a macrophage population that has integrated sustained activating signals during the five-day differentiation period and settled into an altered functional set point. While this pattern does not establish innate immune tolerance, it is compatible with a form of prior environmental conditioning in which sustained exposure to extracellular signals alters inflammatory gene programs and attenuates subsequent responses to stimulation (32, 35–37). The precise molecular initiators of this reprogramming by the xenogeneic FCS milieu were not the focus of this study and cannot be determined from our data. The present study was designed to compare physiologically relevant serum environments rather than identify individual serum components, including potential endotoxin contributions, responsible for the observed effects. Future work directly characterizing how specific components of the FCS environment affect chromatin accessibility in differentiating monocytes will be needed to resolve the mechanistic basis of these effects.

The cytokine profiling and qPCR data provide functional validation and pathway-specific context for the transcriptomic findings: the effect of AS vs. FCS on macrophage inducibility is not uniform but is most evident under LPS-induced pro-inflammatory stimulation. AS-differentiated MDMs showed significantly higher secretion of TNF-α, IL-6, IL-1β, IL-1RA, IL-23, and IP-10, as well as greater gene expression of the activation-associated markers *CD86* and *CCR7*, compared to FCS-differentiated cells following LPS stimulation. These findings were supported by cytokine protein measurements and qPCR analysis of selected activation-associated markers, together with significant differences in cumulative pro- and anti-inflammatory Z-scores. CD86 and CCR7 are commonly associated with inflammatory macrophage activation (8, 11, 38–40). Their selective upregulation under AS conditions following LPS-, but not IL-4/IL-10/TGF-β- stimulation, indicates that serum context influences the magnitude of macrophage responses in a stimulus-dependent manner rather than uniformly increasing responsiveness to all stimuli. Because LPS is primarily recognized through the TLR4 receptor complex and activates downstream inflammatory signaling networks including NF-κB and MAPK pathways, the enhanced LPS responsiveness observed in AS-differentiated MDMs is compatible with differences in one or more components of this inflammatory response axis (36, 37). However, TLR4 expression or function and downstream signaling events were not directly assessed in the present study. Thus, our data demonstrate a difference in the functional response to LPS but do not establish that altered TLR4 signaling is the underlying mechanism. Similarly, although the transcriptomic differences observed between serum conditions are consistent with distinct cellular states, we did not directly assess chromatin accessibility, epigenetic modifications, or other molecular mechanisms that could contribute to this phenotype. These mechanisms therefore remain potential explanations that require dedicated experimental investigation.

In contrast to the pronounced effects observed for LPS-stimulation, serum context showed comparatively limited effects on macrophage responses to the combined IL-4/IL-10/TGF-β stimulation. The absence of significant serum-dependent differences in *CD206* expression and in most cytokine responses under these conditions suggests that the response to this alternative stimulation was comparatively preserved across serum conditions (9, 31). Notably, although a trend toward higher cumulative anti-inflammatory Z-scores were observed in unstimulated AS-differentiated MDMs compared to FCS, cytokine secretion remained low under both resting conditions, suggesting that the observed differences reflect enhanced stimulus-dependent responsiveness rather than constitutive anti-inflammatory activity. Together, these findings indicate that serum context primarily influenced the magnitude of stimulus-induced inflammatory responses, with the most pronounced differences observed following LPS stimulation, while responses to the combined IL-4/IL-10/TGF-β stimulation were comparatively preserved (9, 37).

Beyond stimulus-dependent inducibility, our data also provide supportive evidence that AS is associated with a qualitatively distinct macrophage baseline state. Although cytokine secretion remained low under unstimulated conditions in both groups, AS-differentiated MDMs exhibited modestly higher albeit non-significant cumulative anti-inflammatory cytokine Z-scores. While these differences should not be interpreted as constitutive anti-inflammatory activity, they are directionally consistent with the transcriptomic findings, which demonstrated enrichment of genes associated with lipid metabolism, immune surveillance and extracellular matrix remodeling under AS conditions. Together, these complementary observations are consistent with AS-differentiated macrophages maintaining a metabolically and structurally active baseline state while retaining their capacity for robust inflammatory activation upon stimulation.

The exploratory sex-stratified analyses showing partly stronger LPS-induced cytokine responses in female donors under AS conditions are consistent with the well-established greater inflammatory responsiveness of female immune cells, which has been attributed to sex hormone effects on TLR signaling and transcriptional activation of inflammatory genes (5, 16). The greater separation between AS- and FCS-conditioned responses among female donors may indicate that the serum-dependent differences in inducibility interact with biological sex. However, these exploratory analyses require confirmation in larger cohorts specifically powered to assess sex-by-serum interactions.

The finding that individual serum cytokine levels did not predict *in vitro* cytokine responses under AS conditions, and that cytokines remained proportionally coordinated with cumulative inflammatory scores, indicates that AS does not simply import the donor's inflammatory tone into the cell culture system. Rather, it establishes a donor-matched differentiation environment in which macrophage functional responses are determined by cellular programming rather than passive reflection of circulating mediators. This dissociation between donor inflammatory status and macrophage inducibility is an important property for personalized and disease-specific assays, in which the goal is to assess

macrophage functional phenotypes while minimizing direct confounding by the donor's circulating inflammatory milieu.

Our results extend and contextualize earlier observations. Marek et al. recently reported that xeno-free human ABO serum significantly altered MDM surface marker expression compared to FCS, including upregulation of CD16 and CD163 under FCS and elevated CD86 under ABO serum (8). Our findings complement these observations by showing that serum-dependent differences extend beyond activation-associated marker expression to encompass distinct baseline transcriptional programs and stimulus-dependent cytokine responses in a larger, donor-matched cohort. As comprehensive surface-marker expression was not assessed in the present study, future studies using autologous serum should integrate flow-cytometric phenotyping with functional and transcriptional analyses to further characterize serum-dependent macrophage phenotypes. Another important question for future studies is whether the serum-dependent differences in macrophage state and stimulus responsiveness translate into altered macrophage-mediated interactions with other immune cells, including T-cell activation and polarization. Such experiments would extend the present findings from macrophage-intrinsic functional responses to downstream consequences for adaptive immune responses.

Several limitations warrant consideration. First, the present study does not include a direct comparison with ABO-matched allogeneic human serum. While AS optimally preserves the individual systemic milieu, its use in large-scale or multi-center studies is logistically demanding, and ABO-matched serum has been proposed as a practical compromise (7, 8). Whether the functional differences we observe between AS and FCS are fully recapitulated by ABO-matched serum, and whether allogeneic serum captures individual donor variation less precisely than AS, are important questions for future work. Second, the present study used a single FCS lot throughout all experiments. While the use of a standardized, single-lot protocol was a deliberate design choice to minimize within-study variability and ensure that any observed AS versus FCS differences were not attributable to lot-to-lot variation, it represents an important limitation on the generalizability of our findings. FCS composition is known to vary substantially between lots and manufacturers, with batch-dependent differences in eicosanoid content, growth factor concentrations, and bioactive lipid profiles shown to influence macrophage inflammatory mediator production (7, 8, 36). Whether the functional and transcriptional differences we observe between AS and FCS are consistent across FCS lots, or whether their magnitude is influenced by batch-specific composition, remains to be established in multi-lot comparisons. Our conclusions regarding FCS-associated macrophage states should therefore be interpreted as specific to the experimental conditions of this study rather than as a generalizable characterization of all FCS-based culture systems. Third, although the transcriptomic analyses were conducted in a subset of eight donors per condition, the fully paired design in which AS and FCS samples were derived from the same donors enabled donor-matched comparison of condition-specific transcriptional differences (37). Nevertheless, given the limited transcriptomic cohort, gene-level findings should be regarded as exploratory and interpreted primarily as evidence of coordinated transcriptional program differences between conditions rather than as independently validated molecular mechanisms. Replication in larger and independent transcriptomic cohorts will be required to confirm the identified gene-level signatures and further define their mechanistic basis. Together, these limitations define a clear agenda for subsequent studies while not diminishing the central finding that, within this carefully controlled, donor-matched parallel design, serum context is a determinant of macrophage baseline state and inducibility.

Our findings indicate that AS provides a physiologically relevant alternative to FCS for studying the functional repertoire of human macrophages *in vitro*. The central finding of this study is that AS and this specific FCS lot establish distinct macrophage states before stimulation: AS is associated with a metabolically and immunologically homeostatic transcriptional program and greater LPS-induced responsiveness, whereas FCS is associated with a more inflammatory baseline and attenuated LPS responses. These differences may be particularly relevant when FCS-differentiated MDMs are used to compare disease-specific immune phenotypes or stimulus-dependent cytokine responses, as the attenuated dynamic range under FCS could reduce sensitivity to biologically meaningful between-donor differences. The independence of AS-driven inducibility from donor inflammatory tone further argues that AS-based assays capture macrophage-intrinsic functional states rather than a passive reflection of systemic inflammation, a critical property for personalized and disease-specific immune modeling. Together, these findings support broader consideration of autologous or validated human serum-based culture systems in studies aiming to model donor-specific macrophage biology.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Charité – Universitätsmedizin Berlin, Berlin, Germany. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JH-R: Conceptualization, Supervision, Writing – review & editing, Resources. NC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original

draft, Writing – review & editing. LP: Data curation, Formal analysis, Writing – review & editing. NE: Data curation, Formal analysis, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2026.1981520/full#supplementary-material

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