# Proliferation is a driver of quorum sensing in an *in vitro* model of tissue resident macrophages.

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Y.D.J.P library preparation, data processing
P.W.A. analysis
S.M.G experimental resources
A.L.L. experimental resources
J.C. analysis
C.A.W. experimental design, analysis, writing, project funding

## Conflict of Interests

None

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## Abstract

Macrophages coordinate the initial host inflammatory response to tissue infection as well as mediating the reparative phase by producing growth factors that promote tissue repair. One model of this functional dichotomy is that peripherally recruited monocyte-derived macrophages drive acute inflammatory responses to infection, whereas tissue resident macrophages are responsible for tissue repair. Alternatively, inflammation and repair may be inter-dependent molecular programs, such that both recruited and resident cells have equivalent capacity to contribute. In this study, induced pluripotent stem cells or peripheral blood monocytes were used to generate models of tissue-resident and recruited macrophages, respectively. Using single-cell sequencing, we assessed whether lipopolysaccharide activation drives heterogeneous responses in these two macrophage models. We observe heterogeneity at population and gene expression level in the activation programs adopted by either macrophage population. We further identify proliferation to be a driver of quorum sensing in LPS responsiveness. Our findings highlight the usefulness of this model system to explore the differences between recruited and resident macrophages in response to tissue infection.

## Introduction

Macrophage (mϕ) heterogeneity has been described in the context of ontogeny and tissue-related phenotype and function. While distinct molecular phenotypes have been described for tissue resident and monocyte-derived mϕs (MDMs), phenotypic differences between these subsets in response to bacterial infection or tissue repair are less well understood. If distinct subsets of mϕs have predefined roles in response to infection – such as has been speculated as the divide between tissue resident and infiltrating mϕs – then heterogeneity might be explained by mixing of ontogenically and phenotypically distinct populations ((Gautiar et al., 2012; Xue et al., 2014) and reviewed by (Ginhoux and Guilliams, 2016)). Alternatively, if mϕ populations are intrinsically heterogeneous, then quorum-sensing will lead to distinct roles split randomly across a population, ensuring that the capacity for inflammation and repair is available regardless of the origin of the starting population (Muldoon et al., 2020).

Molecular descriptions of mϕ heterogeneity have contributed to perceptions of stochasticity versus predilection of mϕ responses to external signals. For example, previous descriptions of stochasticity in responses to polarising signals such as LPS have come from measuring a small number of genes across clones derived from cell lines. For example, *in vitro* culture studies have explored mϕ functional heterogeneity to a stimulus using murine RAW 264 cells (Muldoon et al., 2020; Ravasi et al., 2002). In the first study, 16 LPS-inducible genes derived from subclones of RAW 264 cells were measured using dot-blots (Ravasi et al., 2002). Clones expressed these inducible genes at varying levels after stimulation,

suggesting the coexistence of mϕ subsets that could be propagated as subpopulations that display heterogenous activation to the same signal. Similarly, (Muldoon et al., 2020) measured RelA recruitment to the TNF-promoter in RAW 264 cells to describe heterogenous activation of stimulated mϕs and observed a correlation between mCherry expression in populations of different densities after LPS stimulation. Despite the interesting observations in these studies, they have relied on a small number of genes to indicate heterogeneity of response, so it is not clear how representative these models are of functional heterogeneity.

Human induced pluripotent stem cells (iPSCs) provide opportunities to model tissue residency, and factors that shape human mϕ development and activation. Pluripotent stem cell-derived mϕs (PSCMs) are considered equivalent to tissue resident mϕs because these share ontogenic and transcriptional programs (Buchrieser et al. 2017; Calvanese et al. 2022) and are functionally equivalent to tissue resident mϕs in engraftment models in brain and lung (Takata et al. 2017). Through the construction of the Myeloid Atlas (Rajab et al. 2021), we further observed that PSCMs shared the transcriptional phenotype of tissue-resident mϕs. As PSCMs are representative of tissue-resident cells, they provide an experimental model to investigate sources of population heterogeneity in bacterial responses. However, while others (e.g., Alasoo et al. 2015) have shown that PSCMs respond to LPS, it is not known whether they can be tolerized on repeated exposure, which would more accurately mimic the regulatory program seen over the first few days of an infection than a single high dose pathogen exposure.

Here, we use single cell RNA-sequencing to investigate whether LPS activation and then re-stimulation drives heterogenous responses in two mϕ populations: PSCMs as a model of tissue residency, compared to MDMs as a model of peripheral derived mϕs recruited to the site of infection.

# Results

### MDMs and PSCMs share an acute inflammatory program.

Mϕs were derived from peripheral blood monocytes (MDM), or iPSC-cells (PSCM) and then maintained in identical conditions containing CSF-1. Using a pooled experimental design, mϕs from different donors or stem cell lines were cultured together (Figure 1A), and cells collected at each time point were barcoded for demultiplexing after single cell RNA-sequencing. We observed that even in the unstimulated time point, MDMs and PSCMs segregated into discrete clusters (Figure 1B). Benchmarking these onto the Stemformatics myeloid atlas confirmed that the PSCMs were transcriptionally similar to primary tissue-resident mϕs (Supplementary Figure 1A and B). Both PSCMs and MDMs show evidence of activation within 2 hours of stimulation with LPS (Figure 1B right). We observed LPS-tolerance in both cell populations, indicated by the clustering of cells recovering from the initial LPS challenge with the restimulated group, and shown in Figure 1B (right panel) by acute expression of *TNF*, a classic marker for measuring endotoxin tolerance.

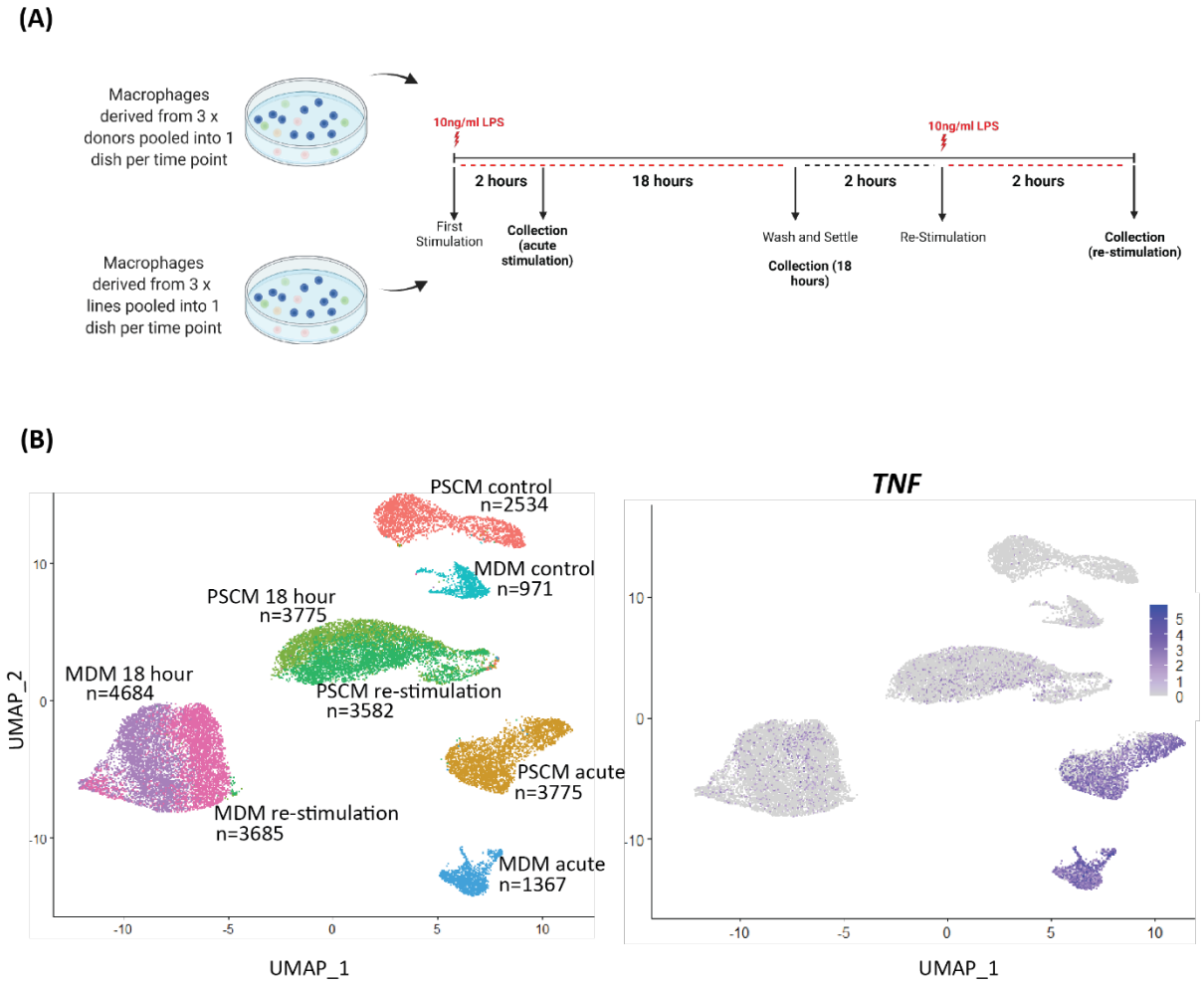



**Figure 1: MDMs and PSCMs share an acute inflammatory program.** **(A)** Experimental setup involved co-culture of peripheral blood monocyte-derived macrophages from 3 donors or pluripotent stem cell-derived macrophages from 3 iPSC lines. Cells were either acutely stimulated for 2 hours (acute stimulation), stimulated for 18 hours (18 hours), or were re-stimulated for 2 hours after an initial 18-hour stimulation and rest period (re-stimulation) prior to collection for 5' 10X Genomics® single cell preparation and sequencing. Sequencing was carried out at 20million reads per source/activation status. Counts were generated using Cellranger pipeline. Seurat was used for clustering and analysis. **(B) (left)** Uniform Manifold Approximation and Projection (UMAP) of peripheral blood monocyte-derived macrophages (MDM) (n=3) and human induced pluripotent stem cell-derived macrophages (PSCM) (n=3) consisting of unstimulated (control), acutely stimulated (acute), 18 hours stimulated (18hr) and re-stimulated (re-stimulation) samples. Number of cells (n) in each sample group shown. **(right)** Acute inflammatory gene Tumor Necrosis Factor (*TNF*) is expressed at 2 hours in LPS-stimulated MDMs and PSCMs. *See Supplementary Figure 1.*

**A shared transcriptional program of critical inflammatory mediators.** The other core genes with shared acute responses (Figure 2A, Table 1 and Supplementary Tables 1-2) included genes associated with negative regulators of cytokine signaling such as *SOCS3* and those associated with Nuclear Factor kappa B (NF-κB) pathway, and its regulation, including *NFKBIZ* and *RELB*. Amongst these core genes, we observed that both models expressed chemokines important for recruitment of peripheral leukocytes, including *CCL3*, *CCL4* and *CCL4L2*.

Both models appear to contribute to activation of inflammatory genes and display features that suggest both populations are involved in regulating this activity. The clearance of dead cells through phagocytosis assists in regulating inflammatory responses and forms an important part of its resolution We observed expression of genes associated with phagocytic activity, and its regulation, in both models through upregulation of *CD44* (Vachon *et al.* 2006) and *NRP2* (Roy *et al.* 2018).

Core activation, tolerance (downregulated upon re-stimulation) and training (upregulated upon re-stimulation) genes that were shared between MDMs and PSCMs were identified (Figure 2A, Table 1-2, Supplementary Tables 1-4). *TNF* is the archetypical inflammatory mediator and was induced and tolerized by both MDMs and PSCMs (Figure 1C, Figure 2A and Supplementary Table 3-4). Other tolerized genes included known inflammatory mediators such as *IL1A* and *IL1B.* Those that shared a similar training phenomenon included *LYN*, a Src-family tyrosine kinase known for its importance in regulating mϕ function in the context of phagocytosis and inflammation, and metallothionein *MT2A* (Figure 2A and Supplementary Table 3-4).

**Table 1: Core Acute Activation Program in MDMs and PSCMs.** MSigDB Hallmark pathways associated with the genes upregulated in a minimum 30% of cells in both MDMs and PSCMs after 2-hour stimulation with LPS ($Log_2FC > 0$, Adj. p value <0.05). *See Supplementary Table 1 and 2.*

| Hallmark pathway (K=200) | # Genes in Overlap (k) | k/K | FDR q-value | Members |
|---|---|---|---|---|
| TNFA SIGNALING VIA NFKB | 60 | 0.3 | 1.12E-106 | *ATP2B1 B4GALT1 BCL2A1 BTG1 BTG2 CCL20 CCL4 CCL5 CD44 CD83 CDKN1A CFLAR CXCL1 CXCL2 CXCL3 DRAM1 DUSP1 DUSP2 EHD1 EIF1 ETS2 GCH1 ICAM1 IER3 IFIT2 IL1A IL1B IL6 IRF1 JUNB KLF6 LITAF MAFF MAP2K3 MAP3K8 MCL1 NAMPT NFE2L2 NFKB1 NFKB2 NFKBIA NINJ1 PLAUR PLEK PNRC1 PPP1R15A REL RELB SAT1 SDC4 SOCS3 SOD2 TNF TNFAIP2 TNFAIP3 TNFAIP8 TNIP1 TRAF1 ZC3H12A ZFP36 CD40 HIF1A IFIT3 IFITM3 ISG15 LCP2 MT2A MX1 PELI1 PLSCR1 PTPN1 SLAMF7 CXCL8 IRAK2 LYN* |
| INTERFERON GAMMA RESPONSE | 27 | 0.135 | 8.22E-36 | |
| INFLAMMATORY RESPONSE | 22 | 0.11 | 4.00E-27 | |
| Uncategorized | 46 | - | - | *ABL2 ACSL1 ALCAM ARL8B BAZ1A C15orf48 CCL3 CCL3L1 CCL4L2 CLIC4 DDX5 DNAAF1 DNAJB6 EIF1B GLRX GPR84 GRAMD1A H3-3B HNRNPC IL1RN INSIG1 MALAT1 MBNL1 METRNL NCF1 NKBIZ NRP2 OTUD1 PIK3AP1 PIM3 PSTPIP2 RAP1B RILPL2 RIN2 SFPQ SMS SPAG9 TXN UBE2E1 VIM WTAP ZC3H12C* |

**Table 2: Core LPS Tolerance Program in MDMs and PSCMs.** MSigDB Hallmark pathways associated with the genes that were upregulated in minimum 30% of cells after 2-hour stimulation and then downregulated in minimum 30% of cells after re-stimulation with LPS in both MDMs and PSCMs ($Log_2FC. < 0$, Adj. p value <0.05). *See Supplementary Table 3 and 4.*

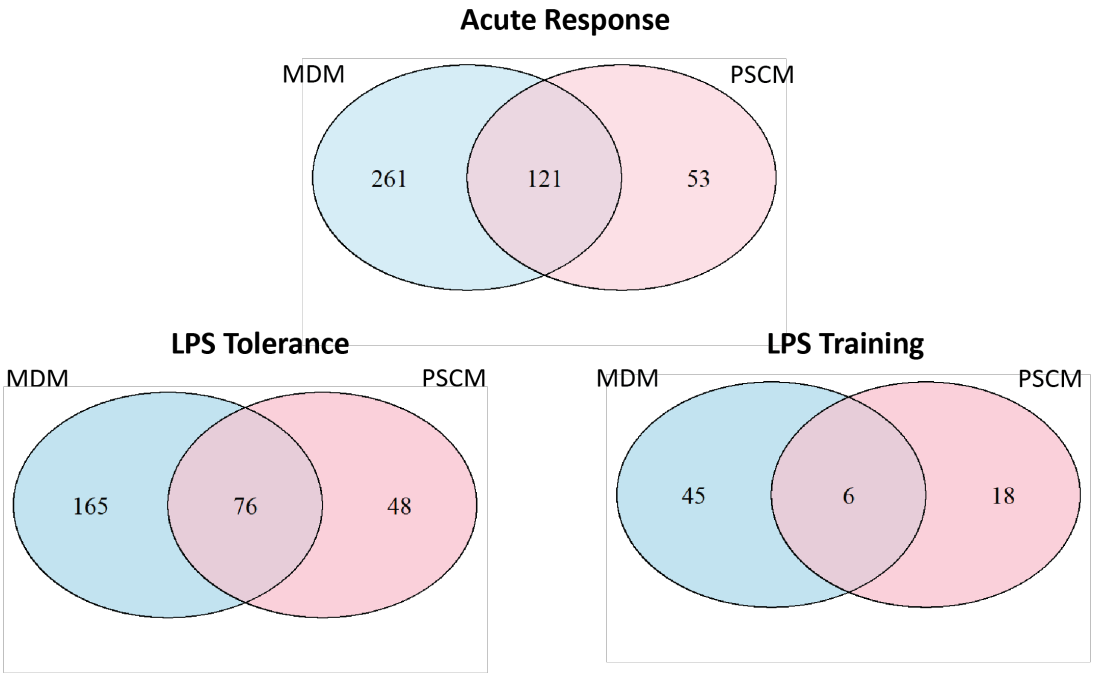
| Hallmark pathway (K=200) | # Genes in Overlap (k) | k/K | FDR q-value | Members |
|---|---|---|---|---|
| TNFA SIGNALING VIA NFKB | 41 | 0.205 | 6.48E-71 | *ATP2B1 BCL2A1 BTG2 CCL20 CCL4 CXCL1 CXCL2 CXCL3 DUSP1 DUSP2 EIF1 ETS2 ICAM1 IFIT2 IL1A IL1B IL6 IRF1 JUNB KDM6B KLF6 LITAF MAFF MAP2K3 MAP3K8 MCL1 NAMPT NFKB1 NFKBIA PHLDA1 PLEK PPP1R15A SDC4 SOCS3 TNF TNFAIP2 TNFAIP3 TNFAIP8 TNFSF9 TRAF1 ZFP36 HIF1A IRAK2 KIF1B LCP2 IFIT3 IRF8 OASL PELI1 PTPN1 SOCS3 ALCAM NFKBIZ XBP1* |
| INFLAMMATORY RESPONSE | 17 | 0.085 | 1.35E-21 | |
| INTERFERON GAMMA RESPONSE | 17 | 0.085 | 1.35E-21 | |
| IL2 STAT5 SIGNALING | 9 | 0.0452 | 3.24E-09 | |
| Uncategorised | 32 | - | - | *ABL2 ACSL1 BAZ1A CCL3 CCL3L1 CCL4L2 CLIC4 DDX5 DNAAF1 DNAJB6 EIF1B FNDC3B GLRX H3-3B HNRNPC INSIG1 MBNL1 METRNL OTUD1 PIM3 PLK3 PMAIP1 RAPGEF2 RIN2 SFPQ SPAG9 UBE2E1 VIM WTAP ZC3H12C* |

**A subset of genes escapes LPS-tolerance in PSCMs**.

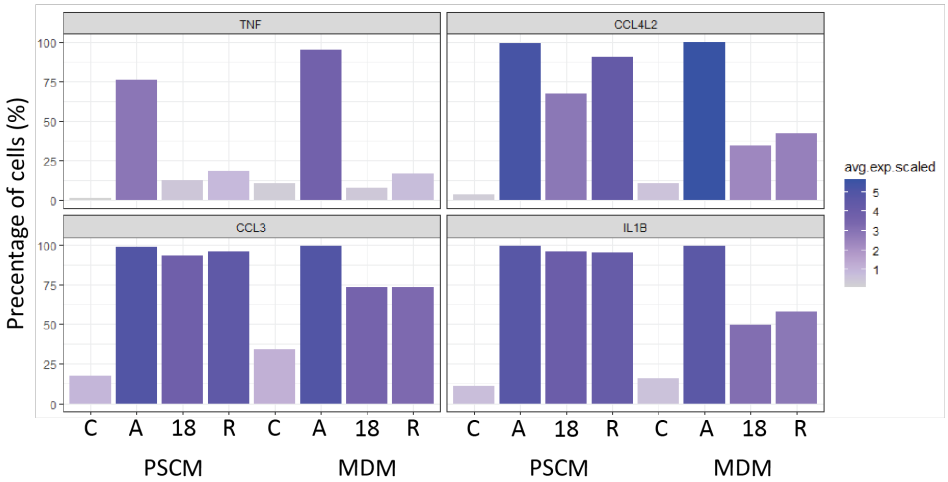
Greater than 50% of the genes uniquely expressed in acutely stimulated MDMs were genes that were also uniquely tolerized (Figure 2A). *TNF* was inducibly expressed by most cells in the MDM and PSCM acute time point, and LPS tolerance was demonstrated in both mϕ sources by low levels of *TNF* expression in relatively few cells after re-stimulation. (Figure 2B). In contrast, *CCL4L2* and *CCL3* (Figure 2B) were inducibly expressed in acutely stimulated mϕs from both models, but tolerised only in MDMs. Similarly, *IL1B* was observed in over 95% of PSCMs in the rested and restimulated timepoints, but less than 50% of MDM continued to express *IL1B* at these later timepoints, and at much lower levels of RNA abundance (Figure 2B). Ongoing expression of key cytokines and chemokines by PSCMs is consistent with a role for tissue-resident mϕs in recruitment of leukocytes in later stages of an infection.

Pathway enrichment analysis of the genes that were only upregulated in MDMs after acute stimulation and tolerized after re-stimulation were associated with IRF-activation and signaling. The Reactome Interferon signaling module scored highly in MDM groups overall (Figure 2C), and peaked in the PSCM rested, but not restimulated state. This suggests that MDMs preferentially engage the MyD88-independent arm of TLR-4 signaling early in the inflammatory process, while PSCM may be require further cytokine activation of the IFN pathways.

**(A)**


Acute Response
MDM
PSCM
261
121
53
LPS Tolerance
MDM
PSCM
165
76
48
LPS Training
MDM
PSCM
45
6
18

**(B)**


TNF
CCL4L2
CCL3
IL1B
Precentage of cells (%)
avg.exp.scaled
C A 18 R C A 18 R
PSCM
MDM
C A 18 R C A 18 R
PSCM
MDM

**(C)**

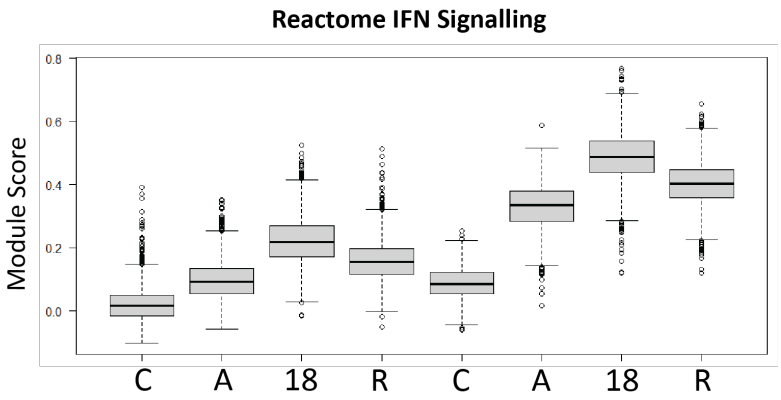

Reactome IFN Signalling
Module Score
C A 18 R C A 18 R

**Figure 2: A shared transcriptional program of critical inflammatory mediators.** (A) Venn diagrams of genes upregulated in min.pct 0.3 in both MDMs and PSCMs after 2-hour stimulation with LPS ($Log_2FC. > 0$, Adj. p value <0.05) (Acute Response), genes that were upregulated in minimum 30% of cells after 2-hour stimulation and then down regulated in minimum 30% of cells after re-stimulation with LPS in both MDMs and PSCMs ($Log_2FC. < 0$ Adj. p value <0.05) (LPS Tolerance), and genes that were upregulated in minimum 30% of cells after 2-hour stimulation and then further upregulated in minimum 30% of cells after re-stimulation with LPS in both MDMs and PSCMs ($Log_2FC. > 0$, Adj. p value <0.05) (LPS Training) (B) Plot showing percentage of cells in each sample group/stimulated groups expressing of inflammatory genes Tumor Necrosis Factor (*TNF*) C-C Motif Chemokine Ligand 4 Like 2 (*CCL4L2*), chemokine ligand 3 (*CCL3*). and interleukin-1β (*IL1B*). Bars coloured by low gene expression (grey) to high gene expression (dark purple) (C) Box plots of module score of genes from the Reactome IFN signalling pathway across PSCM and MDM groups.

## Proliferation drives quorum sensing in LPS responsiveness

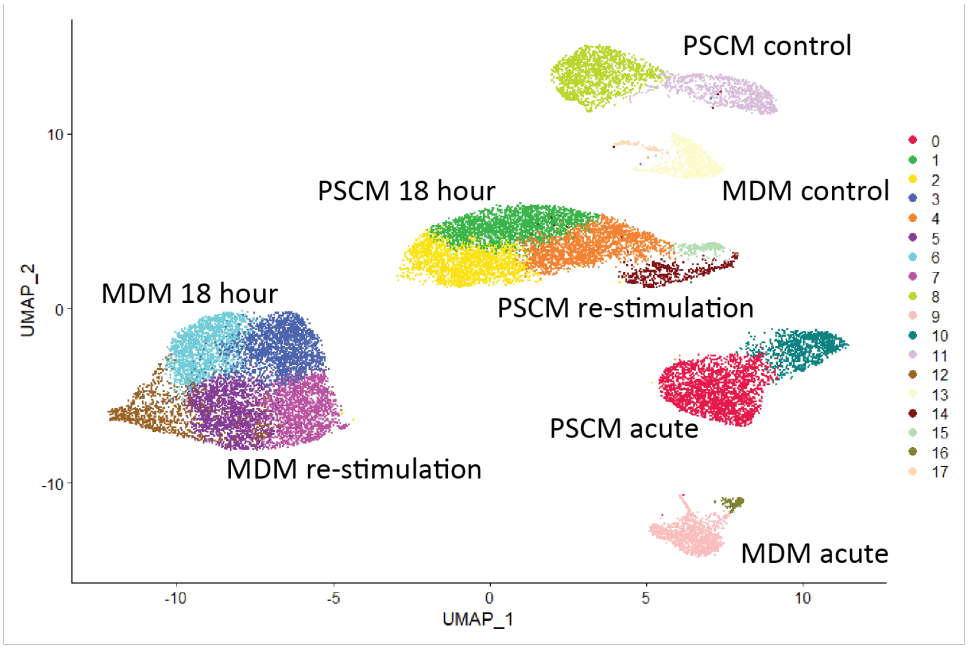
Cell density has been described to play a role in influencing the heterogeneity in activation states that arise within a mϕ population, termed quorum sensing (Muldoon *et al.* 2020). To explore heterogeneity in MDM and PSCMs clusters, we applied degrees of resolution to explore granularity in the clustering. We identified subsets across the clusters in both unstimulated and stimulated groups (Figure 3A and 3B) and observed obvious subsets that were associated with proliferation (Figure 3A; Supplementary Table 5 and 6). A proliferative subset was observed in mϕs from both sources, but whereas 50% of PSCMs were proliferative, only 10% of MDMs were. LPS stimulation did not change these proportions (Figure 3B).

To determine what genes were associated with the clusters identified across the populations (Figure 3A), we explored the differentially expressed genes for each of these subsets (Figure 3C and Supplementary Table 7). Markers associated with maturation including *LYZ* were observed in clusters 3 and 9 of MDM acute stimulation and MDM re-stimulation, respectively, and PSCM re-stimulation group

cluster 14. Proliferation markers including *STMN1* and *HIST1H4C* were found associated with the proliferative PSCM unstimulated and acutely stimulated subsets, clusters 11 and 10, respectively (Figure 3C). IFN-related genes, such as *IFIT2* and *IFNB1*, were identified markers of stimulated MDM groups (Figure 3C, Cluster 9 and 16). Population heterogeneity at 18-hour and re-stimulated groups, most notably in the MDMs, were associated with chemokine, *CCL19*, and chemokine receptor expression, *CCR7*. A re-stimulation MDM cluster overlapped with 18-hour simulation group had high expression of *CD52*, a glycoprotein described to play a role in suppressing TLR and NF-κB activation (Rashidi et al. 2018).

Core inflammatory mediators were expressed regardless of proliferating status. These were predominantly targets of post-transcriptional (AU-response element) regulation, which may be a mechanism that supports acute activation in a mϕ that is also dividing (Supplementary Tables 8-15). The genes upregulated in the non-proliferative subsets included transcription factors *IRF1*, *JUNB*, *KLF6* and *NFKB* family members. The OXPHOS pathway was represented in the non-proliferative PSCM clusters, which is consistent with metabolic remodeling of these cells.

**(A)**


PSCM control
MDM control
PSCM 18 hour
PSCM re-stimulation
MDM 18 hour
PSCM acute
MDM re-stimulation
MDM acute
UMAP_2
UMAP_1
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-10
-10
-5
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5
10
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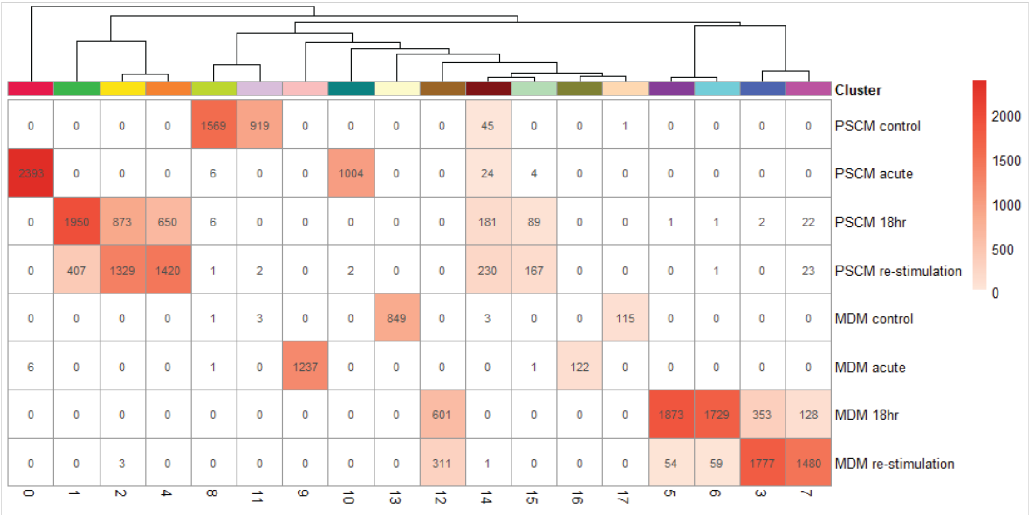
**(B)**


Cluster
PSCM control
PSCM acute
PSCM 18hr
PSCM re-stimulation
MDM control
MDM acute
MDM 18hr
MDM re-stimulation
2000
1500
1000
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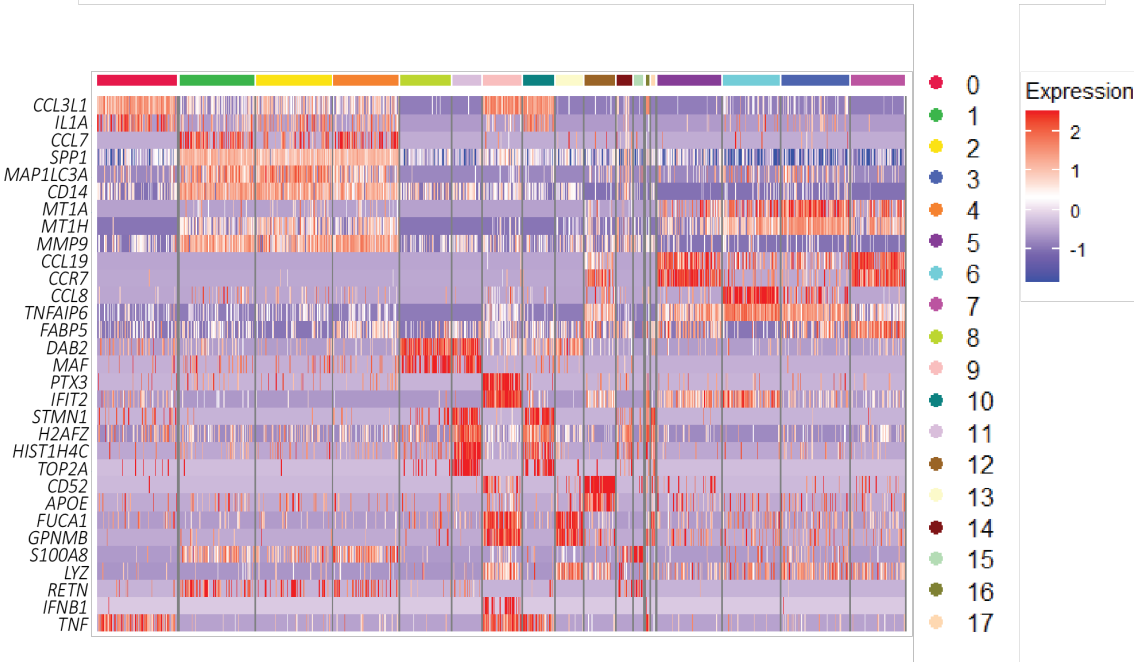
0
0 1 2 4 8 11 9 10 13 12 14 15 16 17 5 6 3 7

**(C)**


CCL3L1
IL1A
CCL7
SPP1
MAP1LC3A
CD14
MT1A
MT1H
MMP9
CCL19
CCR7
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FABP5
DAB2
MAF
PTX3
IFIT2
STMN1
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TOP2A
CD52
APOE
FUCA1
GPNMB
S100A8
LYZ
RETN
IFNB1
TNF
0
1
2
3
4
5
6
7
8
9
10
11
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16
17
Expression
2
1
0
-1

**Figure 3: Heterogeneity occurs at both population and gene expression level** (A) Uniform Manifold Approximation and Projection (UMAP) showing seurat clustering at resolution 0.9. (B) Number of cells per seurat cluster group in association with sample group shown. (C) Heat map of top genes (avg. $Log_2FC$) in each subcluster at resolution 0.9. See *Supplementary Tables 5-15.*

## Discussion

Mϕs play diverse roles in tissue defense, and repair, but whether this is due to predefined roles of functional mϕ subsets, or stochastic processes inherent to mϕ activation is less well understood. Two models of mϕ biology were tested here – the monocyte-derived mϕ (MDM) as a model of recruited cells, and iPSC-derived mϕs (PSCM) as a model of resident tissue biology. This is the first study showing that PSCM can be tolerized to repeated pathogenic challenge, illustrated by downregulation in expression of *NFKB1* and *TNF* upon re-stimulation with LPS. The inflammatory regulators that were shared in acutely stimulated MDM and PSCM were enriched for the post-transcriptional stabilization motif AU-rich elements, which appears to allow for high availability of these transcripts even in proliferating cells, where transcription may not be active at these loci. We propose that this may contribute to the different tolerance program adopted by PSCMs.

In both models, proliferation drove phenotypic heterogeneity in the acute phase of an LPS response, but this accounted for a minor subset of the MDM, whereas the proliferative subset represented about half of the PSCM population. Endotoxin tolerance in restimulated mϕs took two forms – in the degree of induction of an inflammatory gene on re-stimulation with LPS, or in the proportion of cells capable of expression of an inflammatory mediator. It was the PSCM rested and

restimulated group who continued to express cytokines, such as *IL1B,* and chemokines at a high level, possibly because these escaped tolerance in the cells proliferating on first exposure to LPS.

This observation might explain the heterogeneity that has been previously described in *in vitro* mϕ cultures, which used proliferating mouse myeloid cell lines. Genes previously shown by (Ravasi et al. 2002) and (Muldoon et al. 2020) to be stochastically expressed in mouse RAW264.7 cells included *IL6* and *TNF*, with observations that *IL6* was expressed by all RAW264 clones and *TNF* only expressed by 72% of them. Similarly, we observed that *TNF* was only expressed in about 75% of human PSCMs, but in 98% of MDMs after acute stimulation with LPS. *IL6* was inducible at very low levels in both *in vitro* human mϕ models. The density dependence of macrophage heterogeneity described by (Muldoon et al. 2020) is consistent with the model differences observed here. Given that most recruited MDM have exited the mitotic-cell cycle, we suggest that proliferative differences between recruited (blood-derived) mϕs and proliferative (resident-tissue derived) mϕs is consistent with a model of proliferative-dependent quorum sensing.

We hypothesize that early priming events shape blood monocytes to adopt a different activation program than tissue resident mϕ populations. The expression of IFN-related pathways in acutely activated MDM is consistent with MYD88-independent signaling in the 'recruited' rather than 'resident' macrophages. Activation of IRF-related pathways appears to be part of the monocyte-mϕ transition (Song et al. 2021). The genes that were highly expressed in acutely stimulated MDMs, including *IFNB1* and *OASL*, overlapped with the genes that were

also tolerized in MDMs, because there is a requirement for a gene to be expressed upon a first stimulation event in order to be tolerized upon re-exposure. Our model investigated the impact of high dose LPS challenge on acute and re-stimulation. However, LPS concentration in low-dose or supra-doses can have opposite effects on priming status of mϕs (Reviewed by Morris and Li, et al. 2012). The exposure of circulating stimuli on peripheral blood monocytes may be a driver for the different activation programs seen in MDMs and PSCMs.

In other words, the derivation of PSCMs in a sterile environment contrast with MDMs that have previously been exposed to a range of stimuli in circulation, *in vivo*, at a progenitor stage and therefore may be, arguably, inherently pre-primed. The data derived here suggests that this may set up differences in tissue resident and recruited phenotypes after continuous exposure or re-exposure to bacterial ligands. For example, the metabolic reprogramming evident in the PSCM non-proliferative clusters, particularly around OXPHOS metabolism, is consistent with a rewiring of resident mϕ phenotypes after bacterial infection. Exploring this phenomenon in mϕs cocultured with tissues, and for longer time frames, is necessary to understand the long-term consequences of such reprogramming in long-lived mϕs resident in tissues.

# Experimental Procedures

## Cell lines and ethics approvals

Stem cell work was carried out in accordance with The University of Melbourne ethics committee HREC (approval 1851831). Human pluripotent stem cells (hPSCs) used were: PB001.1 (Vlahos et al., 2019), obtained from the Stem Cell Core Facility at the Murdoch Children's Research Institute (hPSCreg: MCRIi001- A); HDF51 (Jones et al., 2013); RRID:CVCL_UF42) was kindly provided to Dr Andrew Laslett by Prof. Jeanne Loring (The Scripps Research Institute, CA, USA); KOLF2 (Wellcome Trust Sanger Institute; hPSCreg: WTSIi018-B) cells were kindly provided by Prof. Paul Hertzog (Hudson Institute of Medical Research).Monocytes were isolated from buffy coat, which was obtained from the Australian Red Cross Blood Service in accordance with The University of Melbourne ethics committee HREC (approval 1646608)

## Pluripotent Stem Cell Differentiation

Human pluripotent stem cells were differentiated into macrophages based on protocol described by (Joshi et al., 2019; Ng et al., 2008; Yanagimachi et al., 2013) with modifications. Modifications were as follows: embryoid bodies were kept in rotational cultures without transference to matrigel plates for adherence, and the collection of progenitors from week 2 were immediately re-suspended in RPMI-1640 containing L-Glutamine (Life Technologies), 10% Fetal Bovine Serum and 100ng/mL CSF1 for macrophage differentiation *(see macrophage differentiation).*

## Macrophage differentiation

Monocytes/progenitor cells were cultured in tissue-culture treated 6 well plates. Cells were cultured in RPMI-1640 medium containing L-Glutamine (Life Technologies) with 10% Fetal Bovine Serum and 100ng/ml recombinant Human M-CSF (R&D Systems; 216-MC) for 4 days.

## Stimulation assay

100,000 cells of HDF51-, PB001.1- and KOLF2-derived progenitors (or peripheral blood monocytes from 3 different donors) were seeded together into 4 wells (control, acute, 18 hour and re-stimulated/tolerance) of a Costar® 6 Well Clear Cell Culture Treated dish (Corning®; Cat. No. COR3516) in Gibco™ RPMI-1640 media (Thermo Fisher Scientific; Cat. No. 11875093) with 10% heat inactivated FBS (Thermo Fisher Scientific; Cat. No. 10082147 or 10099141) and 100ng/ml M-CSF (R&D Systems; Cat. No. 216-MC-500). On day 4, media was changed and cells left to settle for either 2 hours before 'tolerance' well was stimulated with 10ng/mL LPS, or 4 hours before '18 hour' well was stimulated with 10ng/mL LPS. After 18 hours, tolerance well was washed twice with Gibco™ PBS ($Ca^{2+}Mg^{2+}$ free; Thermo Fisher Scientific; Cat. No. 10010023) before fresh media was added and cells left to settle for 2 hours. After 2 hours, tolerance well was re-stimulated with 10ng/mL LPS for 2 hours. 'Acute' well was also stimulated with 10ng/mL LPS for 2 hours. After 2 hours, all wells were washed with Gibco™ PBS ($Ca^{2+}Mg^{2+}$ free; Thermo Fisher Scientific; Cat. No. 10010023) before cell release added. Cells were incubated with macrophage cell release for 5 minutes at 37$^{o}$C. Cells were then sprayed down and collected in media. Falcon (Corning®; Cat. No. CLS431470-500EA) with cells/PBS/Cell release/media was then centrifuged at 350g for 5 minutes. Cells were then resuspended and put through a Flowmi™ strainer (Flowmi; Cat. No. 64709-60)

before cell counts were carried out for sample preparation (*see 10x Illumina Single Cell Library Preparation and RNA Sequencing*)

## 10x Illumina Single Cell Library Preparation and RNA Sequencing

Single cell RNA-sequencing libraries were prepared using the Chromium Next GEM Single Cell 5' v2 (Dual Index) protocol as per manufacturer's instructions. Cell suspensions were prepared for a target recovery of 6000 cells per sample. Libraries were quality checked and quantified using the Agilent Tapestation D5000 High Sensitivity Tape. Two Sequencing runs were performed using performed using Novaseq6000 at University of Melbourne Centre for Cancer Research. Data processing and merging of sequencing runs were carried out through CellRanger v6.0.2 pipeline to generate files for analysis. Downstream analysis was carried out using Seurat package v4.0.5 (Hao, Y., Hao, S., et al. 2021)

## Gene Set Enrichment Analysis and ARE Motif Analysis

Au-rich element motifs were evaluated using the ARE motif database (https://brp.kfshrc.edu.sa/ared/) (Backeet, T. et al 2018) accessed 10/10/2022. The search space was restricted to 3'UTR motifs. Gene Set Enrichment Analysis (GSEA) was performed using the MSigDB Hallmark gene sets (Liberzon et al. 2015).

## Data and code availability

Single cell sequencing data are available through (GEO accession code pending). Code used for analysis is available at: https://github.com/wellslab/invitrosinglecell/blob/main/Analysiscode.R

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