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iMAC: An interactive atlas to explore phenotypic differences between *in vivo*, *ex vivo* and *in vitro*-derived myeloid cells in the Stemformatics platform.

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Summary:

Understanding the myriad of myeloid subsets and identifying specific and discrete phenotypes is challenging in a human setting. Laboratory models of human myeloid biology lack tissue context, but the differences between common models and *in vivo* phenotypes have not been systematically evaluated. Here, we assemble a large transcriptional atlas of human myeloid biology representing ~1000 samples, comparing freshly isolated, cultured, and stem-cell derived myeloid cell types. Common myeloid models including pluripotent stem cell-derived macrophages fail to recapitulate developmental or tissue contexts. A shared feature of pluripotent stem cell-derived macrophages is the atypical expression of collagen, in addition to being highly efferocytotic. The resulting iMAC atlas is available at the Stemformatics.org platform, where users can review gene expression as well as upload and benchmark their own samples for comparison against a library of human tissue-resident and laboratory models of macrophage or dendritic cell biology.

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1 **Introduction**

2 Macrophages are innate immune cells that are found resident in every tissue, with roles in
3 tissue homeostasis, and response to infection or injury. The distinct functional roles of tissue
4 macrophages are reflected in their transcriptional phenotypes: atlases of tissue-resident mouse
5 macrophages, for example, have given great insight into their complexity and heterogeneity
6 (Gautier et al., 2012; Gosselin et al., 2017). Individual transcriptome studies have played an
7 essential role in unravelling the importance of the environment on macrophage phenotype and
8 function (reviewed by (Huang and Wells, 2014)). These data evidence the shared molecular
9 pathways of myeloid cells responding to pathogenic challenge, and the receptiveness of
10 macrophages to environmental cues.

11
12 Much of our understanding of macrophage biology, including many of the molecular
13 mechanisms of innate immune signaling, have arisen from mouse gene knock-out studies.
14 However, cross-species comparisons of immune cells highlight differences between mouse and
15 man. These include the glycolytic switch associated with metabolic reprogramming in activated
16 mouse macrophages (Vijayan et al., 2019); divergent patterns of pathogen receptor expression
17 (Vijayan et al., 2012) and transcriptional responses to innate immune stimuli (Schroder et al.,
18 2012). Cross-species comparisons are further hampered by the absence of population level
19 immune-activation maps, with most mouse studies in macrophage biology conducted on a
20 limited number of inbred lines.

21
22 The need for improved molecular models of primary human cells is evident from the rising
23 popularity of single cell transcriptomic atlases, exemplified by the human cell atlas consortium
24 (Hay et al., 2018; Regev et al., 2017). However, unbiased profiling of cells also requires
25 computational predictions of cell identity, raising further questions about how best to accurately
26 identify immune cell populations resident in tissues, and discriminate these from circulating or
27 infiltrating peripheral blood cells. The isolation, and identification of tissue-resident myeloid cells
28 can be particularly fraught if populations are rare or hard to isolate using enzymatic or other
29 dissociation methods. These procedures can alter myeloid transcriptomes (Gosselin et al.,
30 2017), resulting in underrepresentation or phenotypic ambiguity of resident macrophages in
31 single cell maps of a tissue. It might be argued that human macrophages suffer from an identity
32 crisis, relying on equivalency to laboratory models of human macrophage biology such as ex-
33 vivo culture of monocyte-derived macrophages, which may not be appropriate as a benchmark
34 for specialized tissue functions.

35
36 The potential to model tissue residency, disease phenotypes and activation status of human
37 macrophages using pluripotent stem cells (PSC) is a growing area of interest (Reviewed (Lee et
38 al., 2018; Rajab et al., 2018)). However, the anatomical context or developmental ontogeny of
39 these cells is still not well understood, nor their capacity to model specialized behaviors of
40 myeloid cells including roles within a tissue niche. While databases such as BloodSpot (Bagger
41 et al., 2016) and Haematlas (Watkins et al., 2009) provide a useful snapshot of gene expression
42 of blood types, these lack depth with regard to tissue representation, common laboratory
43 models or activating stimuli. Here, we describe an integrated myeloid transcriptome atlas to
44 identify and benchmark human myeloid subpopulations from *ex vivo*, *in vivo* and *in vitro*

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45 sources. Our motivation was to construct a reference atlas of human myeloid biology that draws
46 on important studies already in the public domain, and that can be added to by the research
47 community as new cell models or new profiling platforms become available. The atlas is made
48 available as an interactive online resource <https://www.stemformatics.org/atlas/imac>.

49

50 **A reference atlas for human myeloid biology.**

51 We first compiled a reference transcriptional atlas (Figure 1A) from public and proprietary
52 transcriptomic data from 44 studies and ~1000 samples representing peripheral blood
53 monocytes, tissue-resident, *ex vivo* and *in vitro*-derived macrophages and dendritic cells.
54 Samples were curated with respect to phenotype, source and isolation method. Datasets were
55 processed through the Stemformatics pipeline which includes a stringent set of quality control
56 requirements for hosting on the Stemformatics.org portal (Choi et al., 2019a). We constructed
57 the atlas by implementing a two-step process (Angel et al., 2020). Firstly, transformation of
58 expression values from the original studies to percentile values to facilitate the comparison of
59 different experimental series was performed. Secondly, using a univariate estimation of their
60 platform dependence, genes whose expression values were significantly impacted by the way
61 that they were measured were removed from the atlas. This approach led to reproducible
62 clustering of distinct myeloid classes on a PCA (Figure 1B and Supplementary Figure 1).
63 Variables such as progenitor source (hematopoietic stem cell (HSC), monocyte-derived, or
64 pluripotent stem cell (PSC)-derived), culture status or activating condition contributed to
65 clustering of samples. The reproducibility of myeloid subsets including dendritic cells,
66 monocytes and neutrophils was validated by projecting an independent RNAseq dataset of well
67 annotated blood cell types from Haemopedia (Choi et al., 2019b) (Figure 1C).

68

69 We next assessed the usefulness of the atlas as an annotation reference tool for single cell
70 data. We took two datasets describing blood monocytes and dendritic cells (Villani et al., 2017)
71 and (Dutertre et al., 2019) which had previously not shown clear agreement on the identity of
72 dendritic cell (DC) subsets (Figure 1D). Using the iMAC atlas as the reference, the Villani
73 scRNA seq clusters clearly aligned to respective DC and monocyte groups, with a Capybara
74 similarity estimate (Figure 1E) predicting that the Villani data had identified 2 distinct
75 plasmacytoid DC (pDC) subsets, 2 distinct DC2 (CD1c+) subsets and an intermediate subset
76 that sat between the classical monocyte and DC2 clusters. The DC5 cluster in the original study
77 was described as an AXL+ SIGLEC6+ (AS-DC) subset that shared classical cell surface
78 markers with pDCs. AS-DCs were suggested to be the subset contaminating traditional pDC
79 isolation strategies with the capacity to stimulate T-cells. The Dutertre DC2 subsets aligned
80 closely with atlas DC2 cells. Thus, the iMAC atlas provides a useful annotation tool to
81 benchmark myeloid datasets that does not require identification of classical cell surface
82 markers, and that can predict novel or intermediate cell clusters.

83

84 **Monocytes acquire a 'Culture-Phenotype'**

85 Monocytes are post-mitotic blood cells derived from bone marrow progenitors that are short
86 lived in circulation and can repopulate macrophages in some tissue niches. The largest
87 population of circulating monocytes is marked by high levels of expression of the LPS co-
88 receptor CD14, which is typically used to isolate monocytes from blood. Intermediate and

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89 nonclassical subsets are marked by acquisition of the type III FcR γ , CD16 (Schmidl et al.,
90 2014) and are included in the atlas. Cultured monocytes have been previously described as
91 'activated', but while we observe a distinct culture phenotype (Figure 2), the transcriptome of
92 cultured cells mimics many of the features of a monocyte after extravasation into tissue. Figure
93 2B shows the grouping of peripheral blood monocytes, in a distinct cluster to monocytes that
94 have been exposed to tissue culture plastic and culture media. This culture phenotype is typified
95 here by a decrease in endothelial-adhesion proteins including the selectin SELL (Figure 2D).
96 Regulators of RAS/RAF signaling including SPRED2 (Wakioka et al., 2001) have an elevated
97 expression in cultured monocytes (Figure 2E), consistent with spreading and migration across
98 tissue culture plastics. The classical monocyte-derived macrophage (MDM) requires *in vitro*
99 differentiation of monocytes using several days exposure to growth factors such as
100 macrophage-colony stimulating factor (M-CSF; CSF-1) or granulocyte-macrophage colony-
101 stimulating factor (GM-CSF). These group distinctly from the cultured monocyte cluster,
102 spreading further upwards along the culture axis (Figure 2F).

103
104 One interactive feature of the atlas is that gene expression and cell source can be explored
105 together. The culture phenotype acquired by monocytes appears to be a prelude to activation,
106 which can be observed along an adjacent axis in Figure 2F and is exemplified by the expression
107 of IL6. Pathogen-activated phenotypes of cultured monocytes or MDM are typified by high
108 expression of this and other cytokines, but this is. Cultured monocytes express higher levels of
109 chemokines, such as CCL2, than peripheral monocytes (Figure 2G). Culture induces the
110 expression of SLAMF1 (Supplementary Table 1), which has shown to be necessary for TLR4
111 activation in human macrophages (Yurchenko et al., 2018). Cultured monocytes also express
112 higher levels of ITGB8 than circulating monocytes (Supplementary Table 1), which is necessary
113 for activating latent TGF- β (Kelly et al., 2018).

114
115 **Primary tissue-resident cells segregate into 'DC-like' and 'monocyte-like' communities.**

116 Circulating and tissue resident dendritic cells occupied a distinct transcriptional niche but share
117 the activation axis seen in monocytes and macrophages. *In vitro* differentiated dendritic cells,
118 expanded from cord blood isolated hematopoietic progenitor cells and monocytes, are
119 considerably removed from the *in vivo* cells (Figure 1 D and Figure 3A), including down-
120 regulation of receptors such as CX3CR1, IL18R1 and TLR7 (Figure 3B and Supplementary
121 Table 4). Other molecules, such as the cell-fusion protein DC-STAMP are gained in culture
122 (Figure 3C and Supplementary Table 4). In terms of proximity to other atlas cell types, these
123 appear high on the activation axis and are closely associated with monocyte-derived
124 macrophages. So, while providing useful models of myeloid biology, cord blood-derived
125 dendritic cells don't adequately capture key aspects of *in vivo* myeloid biology.

126
127 Isolation of primary tissue-resident macrophages is particularly difficult as this can result in
128 alterations in phenotype (Gosselin et al., 2017). The difficulty of isolating tissue-resident
129 populations from healthy human tissue is evident from the spread of tissue resident
130 macrophages in comparison to tissue-resident dendritic cells (Figure 3D), noting that several of
131 the macrophage datasets were obtained through surgical biopsies from patients with
132 inflammatory disease, and indeed mapped across the inflammatory axis. Tissue resident

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133 macrophages, including Kupffer cells, microglia, alveolar macrophages, gut and synovial
134 macrophages occupy a broad niche on the atlas between dendritic cells, peripheral blood
135 monocytes, and cultured monocytes (Figure 3D). Although there was no discrete partitioning of
136 macrophages from individual tissues, distinct classes of tissue resident macrophages were
137 observed in the atlas –(the alveolar, colon and macrophages isolated from tumour ascites
138 (TAM) (Figure 3D and Supplementary Figure 2A) grouped together between cultured
139 monocytes and CD1c+ dendritic cells, and a second spread containing synovial macrophages,
140 microglia and Kupffer cells aligned with the iPSC-derived cells.

141
142 Primary microglia included in the iMAC atlas include both *in vivo* isolated fetal and cultured *ex*
143 *vivo* fetal and adult microglia (Supplementary Figure 2A). The profiles of *in vivo* isolated fetal
144 microglia cluster apart from the spread of *ex vivo* cultured adult and fetal microglia. Cultured
145 tissue macrophages including cultured primary microglia or Kupffer cells shared a broad
146 transcriptional signature with monocyte-derived macrophages, and pluripotent-stem cell derived
147 myeloid cells.

148
149 **PSC-derived macrophages share features with tissue-resident macrophages despite**
150 **poor maturation**

151 Macrophages derived from human PSCs offer new opportunities to model *in vivo* macrophage
152 biology. When reviewing the studies contributing to this atlas, we noted that PSC-derived
153 macrophages are typically benchmarked against monocyte-derived macrophages (MDMs), or
154 cultured primary cells, using a suite of phenotyping techniques. Each experiment includes a
155 small number of samples for transcriptional profiling, with a few notable exceptions (Alasoo et
156 al., 2018). We argue that, given the spectrum of possible resident tissue macrophage
157 phenotypes, it would be more useful to compare PSC-derived cells against an atlas of possible
158 macrophage phenotypes. Whilst several groups reuse publicly available tissue macrophage
159 data, the opportunity to carry out large-scale comparisons to different primary myeloid cells has
160 been limited by the availability of relevant data on a compatible platform.

161
162 Microglia represent just over a third of PSC-directed myeloid differentiation studies in the atlas.
163 These do not resolve into a unique cluster but share transcriptional phenotypes with PSC-
164 derived and tissue-resident macrophages, which sit deep in the broad culture axis associated
165 with the expression of lipid-scavenging genes required for efferocytosis. The exception are
166 ‘cytokine-matured’ PSC-derived microglia samples from (Abud et al., 2017). These are closest
167 to the *in vivo* fetal microglia samples of the PSC-microglia but are also closely associated with
168 other primary tissue resident macrophages from lung, joint and gut. The atlas does provide an
169 opportunity to review the expression of markers thought to distinguish microglia from other
170 primary macrophages. TMEM119, for example, is largely restricted to primary or PSC-derived
171 microglia, although the (Abud et al., 2017) samples have low expression of this marker
172 (Supplementary Figure 2B). P2RY12 is variably expressed across all microglial samples, but its
173 expression is also evident in different tissue-resident samples including those derived from gut
174 and synovial tissues (Figure 3D).

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176 Dendritic cells and macrophages are known professional antigen-presenting cells. The initiation
177 of adaptive immune responses requires presentation of antigen through major histocompatibility
178 complex I or II. The majority of *in vitro*-derived macrophages have low expression of HLA
179 relevant genes (Supplementary Table 2) including CIITA, a known master regulator of MHCII
180 gene expression, which suggests poor maturation in regard to their antigen presentation.
181 Nevertheless, some *in vitro*-derived macrophages cultured with stimulating factors
182 (Supplementary Figure 2C) do show inducible CIITA expression, demonstrating that they have
183 the capacity to express antigen-presenting machinery. It is also worth noting that there are *in*
184 *vitro*-derived macrophages, and microglia, (Abud et al., 2017; Honda-Ozaki et al., 2018) that do
185 appear to have high CIITA expression without stimulating factors (Supplementary Figure 2C).
186 For microglia samples (Abud et al., 2017), this may be an impact of long term culture conditions
187 in addition to supplementation of CX3CL1 and CD200, the latter confirming the authors'
188 observations. In regards to macrophage samples (Honda-Ozaki et al., 2018), this may be an
189 impact of immortalization of progenitor cells.

190

191 **PSC-derived macrophages display transcriptional hallmarks of efferocytosis**

192 There has been growing interest in the importance of metabolic reprogramming in macrophage
193 responses, so we asked whether media supplementation could explain the spread of PSC-
194 derived macrophages on the iMAC atlas. All PSC-derivation protocols supplement media with
195 fatty- or amino-acids, including L-Glutamine, non-essential amino acids (NEAA), Linoleic and
196 Linolenic acids. Some methods add fetal bovine or calf serum, but there was no obvious
197 correlation between serum addition and without. Overall, factors are so ubiquitously used that
198 supplementation alone could not explain the differences between PSC and cultured primary
199 macrophages.

200

201 Lipid homeostasis is an important role for resident tissue macrophages. A high proportion of
202 genes differentially expressed between *in vitro*-derived macrophages/microglia/kupffer cells and
203 tissue resident cells are involved in lipid transport, catabolism and in buffering the cells from
204 concomitant stresses associated with lipid turn-over. For example, reduced expression of
205 ABCA6 is consistent with high efflux of cholesterol from these macrophages (Supplementary
206 Figure 3A). Higher levels of mitochondrial acyl-CoA dehydrogenase ACADM (Supplementary
207 Figure 3B) and phosphatidate phosphatase LPIN3 (Supplementary Figure 3C) suggests high
208 lipid turnover.

209

210 Efferocytosis, or apoptotic cell clearance, has broad immunomodulatory effects (Reviewed by
211 (Elliott et al., 2017)) and active engulfment and clearance of cells by PSC-macrophages is
212 clearly observed in the absence of any inflammatory activation (Supplementary Video).
213 Efferocytosis modulates macrophages from a pro-inflammatory phenotype to one with resolving
214 qualities (Yamaguchi et al., 2014), consistent with the patterns of gene expression observed in
215 cultured macrophages. A study (Cao et al., 2019) demonstrated higher lipid uptake in PSC-
216 macrophages compared to peripheral blood MDMs, concordant with higher expression of
217 efferocytosis-related genes including S1PR1 and MERTK. We confirm that MERTK is generally
218 highly expressed in PSC-derived macrophages, but that there is also a tissue-resident
219 distribution of MERTK expression, with very low levels observed in primary alveolar

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220 macrophages, and highest levels observed in human fetal microglia (Supplementary Figure 2).
221 Tissue-resident macrophages are known first-responders to tissue damage and are key in
222 orchestrating inflammation and its subsequent resolution. This appears to be a phenotype that
223 is selected for in cultured macrophages and may be an inevitable consequence of derivation
224 that strives for high cell yield.

225

226 **PSC-derived macrophages do not recapitulate a developmental hematopoietic ontogeny.**

227 Many PSC-derived systems recapitulate fetal, rather than adult phenotypes, so it is no surprise
228 that others have argued that PSC-derivation protocols mimic primitive rather than definitive
229 myeloid biology. This is largely based on discriminating MYB expression in progenitor cells,
230 which is associated with definitive hematopoiesis and has high expression in hematopoietic
231 progenitor cells. It is clear that MYB is not required for PSC-derived myelopoiesis as
232 macrophages are derived in MYB-KO embryonic stem cells (Buchrieser et al., 2017).
233 Nevertheless, MYB is highly and ubiquitously expressed in PSC-myeloid progenitors, including
234 common myeloid progenitors and hemogenic endothelium, and is retained at high levels in
235 some PSC-derived microglia (Supplementary Figure 3D).

236

237 In comparison to primary cell datasets, PSC-macrophage studies that were overlaid onto the
238 iMAC atlas grouped broadly with cultured monocytes and tissue-resident macrophages. Despite
239 arguments on recapitulation of fetal origin, PSC-macrophages, including PSC-microglia and
240 PSC-Kupffer cells, form an extended group associated with high expression of the human
241 homologue of the F4/80 antigen, ADGRE1, as well as high expression of lipid-scavenging
242 receptors such as SCARB1 (Supplementary Table 2, 3 and 5). Furthermore, MAF expression is
243 indistinguishable in macrophages of different origin (Supplementary Figure 3E). Interestingly,
244 LIN28B, appears to be highly expressed in *in vitro*-derived macrophages compared to *in vivo*
245 and *ex vivo* cells (Supplementary Figure 3F), which may point to incomplete silencing of the
246 Let7 microRNA pathway and maintenance of a fetal state (Grant Rowe et al., 2016; Zhou et al.,
247 2017), but is also characteristic of myeloid leukemias.

248

249 Some other phenotypes previously attributed to ontogeny in PSC-derived cells may rather
250 reflect a more general culture context. For example, ADGRE1 (F4/80) expression has been
251 attributed to yolk-sac derived myeloid cells in mouse (Schulz et al., 2012). While high on PSC-
252 derived cells, ADGRE1 is also clearly upregulated in culture. This is exemplified through primary
253 human microglia that have low expression of ADGRE1 in comparison to *ex vivo* culture or PSC-
254 derived cells (Supplementary Table 5).

255

256 **PSC-Macrophages express high levels of collagen**

257

258 Collagen production and deposition alongside extracellular matrix remodeling are processes
259 involved in wound healing and scarring. Macrophages are instrumental in instructing tissue
260 repair, particularly through the production of growth factors such as TGF- β , IGF1 and PDGF
261 (Shook et al., 2018). Secreted growth factors drive fibroblasts and endothelial cells to produce
262 extracellular matrix components, promoting keloid formation as well as angiogenesis. This
263 model has macrophages influencing collagen deposition by neighboring stromal cells, however,

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264 in some instances are capable of contributing to collagen deposition as demonstrated in mouse
265 and zebrafish injury models (Simões et al., 2020). Gene-set enrichment analysis of the genes
266 that are most correlated with *in vitro*-derived macrophages moving away (Figure 4A) from the
267 tissue resident populations revealed that the most significant pathways in these cells involved
268 collagen synthesis and production (Table 1). A STRING protein-protein interaction network
269 (Figure 4B) shows that this phenotype is significantly enriched for highly connected matrix
270 remodeling, collagen deposition and cadherin-mediated cell-cell and cell-matrix interactions.

271
272 Perhaps pluripotent stem cell-derived cells are being driven to adopt a pro-fibrotic phenotype
273 through the derivation or culture contents. Initial observations on analysis of myeloid-,
274 pluripotent stem cell- and hematopoietic progenitor-derived cells, highlight higher expression of
275 collagen genes in pluripotent stem cell-derived cells (Figure 4C). To investigate whether this
276 phenotype is due to culture impact, we isolated peripheral blood monocytes and derived human
277 PSC-progenitors and cultured these cells with the same culture media in the presence of M-
278 CSF for 5 days to drive macrophage differentiation. On day 5, cells were either stimulated with
279 LPS for 2 hours before extraction or extracted as control samples for sequencing analysis.
280 Analysis of sequencing samples showed the same enriched collagen and cadherin networks
281 with high expression of these genes observed in PSC-macrophages, with little or no expression
282 in monocyte-derived macrophages regardless of whether cells were stimulated or not (Figure
283 4D). To determine this at a protein level, we carried out intracellular flow cytometry analysis of
284 type I collagen in PSC- and monocyte-derived cells (Figure 4E). Compared with staining
285 controls, we observed higher levels of type I collagen in PSC-macrophages. Collectively, our
286 results suggest that although culture media can impact cell phenotype, it is not the main driver
287 for this pro-fibrotic phenotype in PSC-macrophages in the final stages of differentiation.

288

289 **Concluding Remarks**

290 Human macrophage biology is integral to the development homeostasis and disease
291 mechanisms in every tissue in the body, but our understanding of human myeloid biology is
292 limited by the quality by the models available to us. Here we describe an integrated myeloid
293 transcriptome atlas as a novel resource to identify myeloid cells in single cell datasets and to
294 benchmark *in vitro* models of *in vivo* biology. Implementation in Stemformatics enables users to
295 upload their own data to benchmark cell types against the atlas for rapid and intuitive cell-
296 classification.

297

298 By benchmarking curated public data of PSC-macrophages and their precursor cells against the
299 atlas, it is apparent that these represent neither definitive nor primitive myelopoiesis, or rather,
300 that they imperfectly recapitulate aspects of both. PSC-conditions clearly do not mimic the
301 developmental time-frame nor tissue niche of yolk-sac, fetal liver or bone marrow. In our hands
302 PSC-macrophages display transcriptional hallmarks of efferocytosis and surprisingly collagen
303 production, which may suggest that the derivation process of these cells are driving a pro-
304 fibrotic phenotype. Perhaps the ontogeny question is less interesting than understanding the
305 molecular networks that can be co-opted in stem cell derived myeloid models, to deliver specific
306 phenotypic properties outside of the constraints of development.

307

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318

319 **Author Contributions**

320 Conception NR, JC, CAW; Experimental Investigation and Interpretation NR, VJ, JG, CAW;
321 Experimental Resources ALL, CAW; Methodology PWA, JC, YD; Data provider NR, MKS, SM;
322 Curation NR, MR, CMP, CAW; Statistical analysis YD, KALC, PWA; Writing – original draft NR,
323 CAW; Writing - review and editing NR, PWA, SM, ALL, KALC, JC, CAW; Supervision CAW,
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325

326 **Declaration of Interests**

327 None

328

329 **References**

- 330 Abud, E.M., Ramirez, R.N., Martinez, E.S., Healy, L.M., Nguyen, C.H.H., Newman, S.A.,
331 Yeromin, A. V., Scarfone, V.M., Marsh, S.E., Fimbres, C., et al. (2017). iPSC-Derived Human
332 Microglia-like Cells to Study Neurological Diseases. *Neuron* *94*, 278-293.e9.
- 333 Alasoo, K., Rodrigues, J., Mukhopadhyay, S., Knights, A.J., Mann, A.L., Kundu, K., Hale, C.,
334 Dougan, G., and Gaffney, D.J. (2018). Shared genetic effects on chromatin and gene
335 expression indicate a role for enhancer priming in immune response. *Nat. Genet.* *50*, 424–431.
- 336 Angel, P.W., Rajab, N., Deng, Y., Pacheco, C.M., Chen, T., Le Cao, K.-A., Choi, J., and Wells,
337 C. (2020). A simple, scalable approach to building a cross-platform transcriptome atlas. *BioRxiv*
338 2020.03.09.984468.
- 339 Bagger, F.O., Sasivarevic, D., Sohi, S.H., Laursen, L.G., Pundhir, S., Søndery, C.K., Winther,
340 O., Rapin, N., and Porse, B.T. (2016). BloodSpot: A database of gene expression profiles and
341 transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res.* *44*,
342 D917–D924.
- 343 Buchrieser, J., James, W., and Moore, M.D. (2017). Human Induced Pluripotent Stem Cell-
344 Derived Macrophages Share Ontogeny with MYB-Independent Tissue-Resident Macrophages.
345 *Stem Cell Reports* *8*, 334–345.
- 346 Cao, X., Yakala, G.K., van den Hil, F.E., Cochrane, A., Mummery, C.L., and Orlova, V. V.
347 (2019). Differentiation and Functional Comparison of Monocytes and Macrophages from hiPSCs
348 with Peripheral Blood Derivatives. *Stem Cell Reports* *12*, 1282–1297.
- 349 Choi, J., Pacheco, C.M., Mosbergen, R., Korn, O., Chen, T., Nagpal, I., Englart, S., Angel, P.W.,
350 and Wells, C.A. (2019a). Stemformatics: visualize and download curated stem cell data. *Nucleic*
351 *Acids Res.* *47*, D841–D846.

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- 352 Choi, J., Baldwin, T.M., Wong, M., Bolden, J.E., Fairfax, K.A., Lucas, E.C., Cole, R., Biben, C.,
353 Morgan, C., Ramsay, K.A., et al. (2019b). Haemopedia RNA-seq: a database of gene
354 expression during haematopoiesis in mice and humans. *Nucleic Acids Res.* *47*, D780–D785.
- 355 Dutertre, C.A., Becht, E., Irac, S.E., Khalilnezhad, A., Narang, V., Khalilnezhad, S., Ng, P.Y.,
356 van den Hoogen, L.L., Leong, J.Y., Lee, B., et al. (2019). Single-Cell Analysis of Human
357 Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating
358 Inflammatory Dendritic Cells. *Immunity* *51*, 573-589.e8.
- 359 Elliott, M.R., Koster, K.M., and Murphy, P.S. (2017). Efferocytosis Signaling in the Regulation of
360 Macrophage Inflammatory Responses. *J. Immunol.* *198*, 1387–1394.
- 361 Fabregat, A., Jupe, S., Matthews, L., Sidiropoulos, K., Gillespie, M., Garapati, P., Haw, R.,
362 Jassal, B., Korninger, F., May, B., et al. (2018). The Reactome Pathway Knowledgebase.
363 *Nucleic Acids Res.* *46*, D649–D655.
- 364 Gautier, E.L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A.,
365 Elpek, K.G., Gordonov, S., et al. (2012). Gene-expression profiles and transcriptional regulatory
366 pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat. Immunol.*
367 *13*, 1118–1128.
- 368 Gosselin, D., Skola, D., Coufal, N.G., Holtman, I.R., Schlachetzki, J.C.M., Sajti, E., Jaeger, B.N.,
369 O'Connor, C., Fitzpatrick, C., Pasillas, M.P., et al. (2017). An environment-dependent
370 transcriptional network specifies human microglia identity. *Science* (80-.). *356*, 1248–1259.
- 371 Grant Rowe, R., Wang, L.D., Coma, S., Han, A., Mathieu, R., Pearson, D.S., Ross, S., Sousa,
372 P., Nguyen, P.T., Rodriguez, A., et al. (2016). Developmental regulation of myeloerythroid
373 progenitor function by the Lin28b-let-7-Hmga2 axis. *J. Exp. Med.* *213*, 1497–1512.
- 374 Hay, S.B., Ferchen, K., Chetal, K., Grimes, H.L., and Salomonis, N. (2018). The Human Cell
375 Atlas bone marrow single-cell interactive web portal. *Exp. Hematol.* *68*, 51–61.
- 376 Honda-Ozaki, F., Terashima, M., Niwa, A., Saiki, N., Kawasaki, Y., Ito, H., Hotta, A., Nagahashi,
377 A., Igura, K., Asaka, I., et al. (2018). Pluripotent Stem Cell Model of Nakajo-Nishimura
378 Syndrome Untangles Proinflammatory Pathways Mediated by Oxidative Stress. *Stem Cell*
379 *Reports* *10*, 1835–1850.
- 380 Huang, E., and Wells, C.A. (2014). Environmental Influences Interface of Genetic, Epigenetic,
381 and Responsiveness Is Determined at the The Ground State of Innate Immune Downloaded
382 from. *J Immunol J. Immunol. J. Immunol.* *193*, 13–19.
- 383 Jones, J.C., Sabatini, K., Liao, X., Tran, H.T., Lynch, C.L., Morey, R.E., Glenn-Pratola, V.,
384 Boscolo, F.S., Yang, Q., Parast, M.M., et al. (2013). Melanocytes derived from transgene-free
385 human induced pluripotent stem cells. *J. Invest. Dermatol.* *133*, 2104–2108.
- 386 Joshi, K., Elso, C., Motazedian, A., Labonne, T., Schiesser, J. V., Cameron, F., Mannering, S.I.,
387 Elefanty, A.G., and Stanley, E.G. (2019). Induced pluripotent stem cell macrophages present
388 antigen to proinsulin-specific T cell receptors from donor-matched islet-infiltrating T cells in type
389 1 diabetes. *Diabetologia* *62*, 2245–2251.
- 390 Kelly, A., Gunaltay, S., McEntee, C.P., Shuttleworth, E.E., Smedley, C., Houston, S.A., Fenton,
391 T.M., Levison, S., Mann, E.R., and Travis, M.A. (2018). Human monocytes and macrophages
392 regulate immune tolerance via integrin $\alpha\beta 8$ -mediated TGF β activation. *J. Exp. Med.* *215*,
393 2725–2736.
- 394 Kong, W., Fu, Y.C., and Morris, S.A. (2020). Capybara: A computational tool to measure cell
395 identity and fate transitions. *BioRxiv* 2020.02.17.947390.

Rajab et al.

- 396 Lee, C.Z.W., Kozaki, T., and Ginhoux, F. (2018). Studying tissue macrophages in vitro: are
397 iPSC-derived cells the answer? *Nat. Rev. Immunol.* 18, 716–725.
- 398 Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a
399 recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell
400 differentiation as spin embryoid bodies. *Nat. Protoc.* 3, 768–776.
- 401 Rajab, N., Rutar, M., Laslett, A.L., and Wells, C.A. (2018). Designer macrophages: Pitfalls and
402 opportunities for modelling macrophage phenotypes from pluripotent stem cells. *Differentiation*
403 104, 42–49.
- 404 Regev, A., Teichmann, S.A., Lander, E.S., Amit, I., Benoist, C., Birney, E., Bodenmiller, B.,
405 Campbell, P., Carninci, P., Clatworthy, M., et al. (2017). The Human Cell Atlas. *Elife* 6.
- 406 Schmidl, C., Renner, K., Peter, K., Eder, R., Lassmann, T., Balwierz, P.J., Itoh, M., Nagao-Sato,
407 S., Kawaji, H., Carninci, P., et al. (2014). Transcription and enhancer profiling in human
408 monocyte subsets. *Blood* 123, e90–e99.
- 409 Schroder, K., Irvine, K.M., Taylor, M.S., Bokil, N.J., Le Cao, K.-A., Masterman, K.-A., Labzin,
410 L.I., Semple, C.A., Kapetanovic, R., Fairbairn, L., et al. (2012). Conservation and divergence in
411 Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages.
412 *Proc. Natl. Acad. Sci. U. S. A.* 109, E944-53.
- 413 Shook, B.A., Wasko, R.R., Rivera-Gonzalez, G.C., Salazar-Gatzimas, E., López-Giráldez, F.,
414 Dash, B.C., Muñoz-Rojas, A.R., Aultman, K.D., Zwick, R.K., Lei, V., et al. (2018). Myofibroblast
415 proliferation and heterogeneity are supported by macrophages during skin repair. *Science* (80-
416). 362, eaar2971.
- 417 Simões, F.C., Cahill, T.J., Kenyon, A., Gavriouchkina, D., Vieira, J.M., Sun, X., Pezzolla, D.,
418 Ravaud, C., Masmanian, E., Weinberger, M., et al. (2020). Macrophages directly contribute
419 collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nat.*
420 *Commun.* 11, 1–17.
- 421 Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M.,
422 Doncheva, N.T., Morris, J.H., Bork, P., et al. (2019). STRING v11: protein–protein association
423 networks with increased coverage, supporting functional discovery in genome-wide
424 experimental datasets. *Nucleic Acids Res.* 47, D607–D613.
- 425 Turlach, B.A., and Weingessel, A. (2019). quadprog: Functions to Solve Quadratic
426 Programming Problems. R package version 1.5-8.
- 427 Vijayan, D., Radford, K.J., Beckhouse, A.G., Ashman, R.B., and Wells, C.A. (2012). Mincle
428 polarizes human monocyte and neutrophil responses to *Candida albicans*. *Immunol. Cell Biol.*
429 90, 889–895.
- 430 Vijayan, V., Pradhan, P., Braud, L., Fuchs, H.R., Gueler, F., Motterlini, R., Foresti, R., and
431 Immenschuh, S. (2019). Human and murine macrophages exhibit differential metabolic
432 responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biol.* 22, 101147.
- 433 Villani, A.C., Satija, R., Reynolds, G., Sarkizova, S., Shekhar, K., Fletcher, J., Griesbeck, M.,
434 Butler, A., Zheng, S., Lazo, S., et al. (2017). Single-cell RNA-seq reveals new types of human
435 blood dendritic cells, monocytes, and progenitors. *Science* (80-). 356.
- 436 Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D., Burovski,
437 E., Peterson, P., Weckesser, W., Bright, J., et al. (2019). SciPy 1.0--Fundamental Algorithms for
438 Scientific Computing in Python. *Nat. Methods* 17, 261–272.
- 439 Vlahos, K., Sourris, K., Mayberry, R., McDonald, P., Bruveris, F.F., Schiesser, J. V., Bozaoglu,

Rajab *et al.*

- 440 K., Lockhart, P.J., Stanley, E.G., and Elefanty, A.G. (2019). Generation of iPSC lines from
441 peripheral blood mononuclear cells from 5 healthy adults. *Stem Cell Res.* **34**, 101380.
- 442 Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M.,
443 Komiya, S., Baron, R., and Yoshimura, A. (2001). Spred is a Sprouty-related suppressor of Ras
444 signalling. *Nature* **412**, 647–651.
- 445 Watkins, N.A., Gusnanto, A., de Bono, B., De, S., Miranda-Saavedra, D., Hardie, D.L.,
446 Angenent, W.G.J., Attwood, A.P., Ellis, P.D., Erber, W., et al. (2009). A HaemAtlas:
447 characterizing gene expression in differentiated human blood cells. *Blood* **113**, e1-9.
- 448 Yamaguchi, H., Maruyama, T., Urade, Y., and Nagata, S. (2014). Immunosuppression via
449 adenosine receptor activation by adenosine monophosphate released from apoptotic cells. *Elife*
450 **3**.
- 451 Yurchenko, M., Skjesol, A., Ryan, L., Richard, G.M., Kandasamy, R.K., Wang, N., Terhorst, C.,
452 Husebye, H., and Espevik, T. (2018). SLAMF1 is required for TLR4-mediated TRAM-TRIF–
453 dependent signaling in human macrophages. *J. Cell Biol.* **217**, 1411–1429.
- 454 Zhou, J., Chan, Z.L., Bi, C., Lu, X., Chong, P.S.Y., Chooi, J.Y., Cheong, L.L., Liu, S.C., Ching,
455 Y.Q., Zhou, Y., et al. (2017). LIN28B activation by PRL-3 promotes leukemogenesis and a stem
456 cell-like transcriptional program in AML. *Mol. Cancer Res.* **15**, 294–303.

457

458 **Figure Titles and Legends**

459

460 **Figure 1: A reference atlas for human myeloid biology**

461 (A) iMAC atlas with samples coloured by cell type. Navy blue - monocytes, blue - macrophages,
462 aqua -Dendritic cells, dark green - CD141+ DC, light green - CD1c+ DC, yellow - pDC, brown –
463 granulocytes, pink stem and progenitor cells, hemogenic endothelium. (B) iMAC atlas coloured
464 by platform: red various microarray platforms, black RNAseq platforms. (C) Validation with
465 Haemopedia RNAseq myeloid samples: diamond shape – monocytes, circle granulocytes, cross
466 DC. (D) Single cell projections of Villani *et al.* (2017) and Dutertre *et al.* (2019) samples onto
467 iMAC atlas (F) Heatmap derived from Copybara analysis of Villani *et al.* (2017) and Dutertre
468 *et al.* (2019) samples compared to iMAC cell types. Colour gradients reflect similarity of single cell
469 clusters to iMAC atlas cell types (dark least similar, to light most similar).

470

471 **Figure 2: Monocytes acquire a culture phenotype**

472 (A) Schematic of rolling monocytes, highlighting cultured cells mimic many of the features of a
473 monocyte after extravasation (B) Cultured monocytes form a distinct cluster away from *in vivo*
474 monocytes along PC3 (C) STRING-DB network of top-ranked genes differentially expressed
475 between peripheral blood (*in vivo*, n=107) and cultured monocytes (*ex vivo*, n=171) indicating
476 upregulation of cytoskeletal proteins and down regulation of endothelial-adhesion proteins (D)
477 Ranked expression (Y-axis) of gene involved in endothelial adhesion, SELL, comparing
478 cultured monocytes (n=171) with monocytes directly profiled from blood (*in vivo*, n=107). Grey
479 stripe indicates variance attributable to platform. P-value: Mann-Whitney-Wilcoxon rank sum
480 test (E) Ranked expression (Y-axis) of gene involved in the regulation of RAS/RAF signaling
481 comparing cultured monocytes (n=171) with monocytes directly profiled from blood (*in vivo*,
482 n=107). Grey stripe indicates variance attributable to platform. P-value: Mann-Whitney-Wilcoxon
483 rank sum test (F) iMAC atlas coloured by ranked expression of IL-6 (Scale bar: high ranked

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484 expression (dark red) to low ranked expression (grey) indicating axis of activation) (G) Ranked
485 expression (Y-axis) of Chemokine CCL2 comparing cultured monocytes (n=171) with
486 monocytes directly profiled from blood (*in vivo*, n=107). Grey stripe indicates variance
487 attributable to platform. P-value: Mann-Whitney-Wilcoxon rank sum test.

488

489

490 **Figure 3: Cultured and *in vitro*-derived dendritic cells do not capture aspects *in vivo***
491 **myeloid biology**

492 (A) iMAC atlas coloured by cell source to highlight origin of dendritic cells contributes to
493 clustering. Dark green samples are profiled directly from blood or tissue (*in vivo*); mid-green are
494 cultured primary cells (*ex vivo*); light green are differentiated *in vitro* from cord blood or iPSC
495 progenitors (*in vitro*). (B and C) Ranked expression (Y-axis) of (B) receptor CX3CR1 and (C)
496 cel-fusion protein DC-STAMP *in vivo* dendritic cells (n=145), *ex vivo* dendritic cells (n=17) and *in*
497 *vitro*-derived dendritic cells (n=57). Grey stripe indicates variance attributable to platform. P-
498 value: Mann-Whitney-Wilcoxon rank sum test. (D) iMAC atlas coloured by ranked expression of
499 P2RY12. (Scale bar: high ranked expression (dark red) to low ranked expression (grey)).

500

501 **Figure 4: *in vitro*-derived macrophages do not capture developmental ontogeny**

502 (A) iMAC atlas coloured by cell source to highlight *in vitro*-derived macrophages used for
503 regression testing (B) STRING_DB Protein-Protein network of *in vitro*-derived macrophages
504 highlights enrichment of collagen, growth factor and cadherin networks. Line color indicates the
505 type of interaction evidence. Light blue solid lines indicate known interactions from curated
506 databases, solid pink line indicates known interactions that have been experimentally
507 determined, bright green lines indicate gene neighborhood predicted interactions, red lines
508 indicate gene fusions predicted interactions, dark blue lines indicate gene co-occurrence
509 predicted interactions, yellow/green lines indicate textmining, black lines indicate co-expression,
510 and light purple lines indicate protein homology. (C) Violin plots of myeloid- (n=584), pluripotent
511 stem cell- (n=116) and hematopoietic progenitor- (n=201) derived cells comparing expression
512 of collagen genes (COL1A1 and COL4A2)(D) mRNA-seq gene expression from human
513 peripheral blood monocyte-derived macrophages (HMDM) (n=3) and human pluripotent stem
514 cell derived macrophages (PSCM) (n=2) samples (C= control, S = stimulated) (E) Intracellular
515 flow cytometry analysis of human peripheral blood monocyte-derived macrophages (HMDM)
516 and human pluripotent stem cell derived macrophages (PSCM), representative of 2
517 experimental repeats (n=2 HMDM, n=2 PSCM). Red = no primary antibody control, Black =
518 isotype control, Purple = Type I Collagen stain.

519

520 **Supplementary Figure Titles and Legends**

521

522 **Supplementary Figure 1: iMAC as a reference atlas and annotation tool**

523 (A) Pre-gene filtering (left) and Post-gene filtering (right). Different platforms highlighted by
524 different colours. (B) Kolmogorov–Smirnov (KS) statistics (y-axis) to assess the difference in
525 gene expression distribution between pseudo-bulk single cells DC6 and bulk sample
526 plasmacytoid dendritic cells from the atlas, with respect to the number of single cells that are
527 aggregated (x-axis). Each line indicates one of thirty random sub-samplings with replacement

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528 trial. KS statistics are calculated on each gene and averaged across all genes. A minimum KS
529 statistic is obtained when aggregating 8 cells. (C) iMAC atlas cell types before single cell
530 projection (D) Single cell projection of (Dutertre et al., 2019) and (Villani et al., 2017) samples
531 onto the iMAC atlas where an 8 cells are aggregated based on (B) cells.

532

533

534

535 **Supplementary Figure 2: Tissue Macrophages**

536 iMAC atlas coloured by ranked expression of (A) MERTK (B) TMEM119 (Scale bar: high ranked
537 expression (dark red) to low ranked expression (grey) (C) Ranked expression (Y-axis) of Class
538 II transactivator (CIITA) in vivo versus in vitro-derived macrophages (gut, synovial, kupffer,
539 microglia, macrophage). Red – activated, yellow – (Abud et al., 2017) microglia samples, khaki-
540 Honda-Ozaki et al. (2018) macrophage samples.

541

542 **Supplementary Figure 3: *in vitro*-derived macrophages do not capture developmental 543 ontogeny**

544 Ranked expression (Y-axis) of genes comparing *in vivo*, *ex vivo* and *in vitro*-derived
545 macrophages (gut, synovial, kupffer, microglia, macrophage) (A, B, C) Lipid turnover (A)
546 cholesterol efflux (B) mitochondrial acyl-CoA dehydrogenase (C) phosphatidate phosphatase.
547 (D) iMAC atlas coloured by ranked expression of MYB (Scale bar: high ranked expression (dark
548 red) to low ranked expression (grey). (E) Comparison of MAF expression from *in vivo* (n=61), *ex*
549 *vivo* (n=26) and *in vitro*- (n=96) derived macrophages (gut, synovial, kupffer, microglia,
550 macrophage). Grey stripe indicates variance attributable to platform. (E) Comparison of LIN28B
551 expression from *in vivo* (n=61), *ex vivo* (n=26) and *in vitro*- (n=96) derived macrophages (gut,
552 synovial, kupffer, microglia, macrophage). Grey stripe indicates variance attributable to platform.
553 P-value MannWhitney-Wilcoxon rank sum test.

554

555

556 **Tables with Titles and Legends**

557 **Table1: Gene-Set Enrichment Analysis**

558 Table of the top 10 Reactome pathways enriched in genes highly correlated with *in vitro*-derived
559 macrophage spread. Enrichment: number of genes in the list/number of genes in that pathway
560 (False Discovery Rate-value). Genes: multiple entries assigned to the same gene indicated by
561 underlining of gene symbol with UniProt accessions in brackets.

562

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Table 1		
Pathway	Enrichment	Genes
Collagen biosynthesis and modifying enzymes	11/76 (3.76e-09)	ADAMTS3, COL1A2, COL4A2, SERPINH1, COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1
Extracellular matrix organization	18/329 (4.48e-09)	ADAMTS1, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, LTBP1, PTPRS, ADAMTS3, COL1A2, COL4A2, KDR, LUM, SERPINH1, COL14A1, COL3A1, COL4A5, LAMB1, MFAP4
Collagen chain trimerization	9/44 (4.81e-09)	COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, COL1A2, COL4A2
Collagen formation	11/104 (2.39e-08)	ADAMTS3, COL1A2, COL4A2, SERPINH1, COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1
ECM proteoglycans	10/79 (2.39e-08)	COL1A1, COL4A1, COL5A1, PTPRS, COL1A2, COL4A2, LAMB1, COL3A1, COL4A5, LUM
Non-integrin membrane-ECM interactions	9/61 (3.83e-08)	COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, COL1A2, COL4A2, LAMB1, COL3A1, COL4A5
Integrin cell surface interactions	10/86 (3.83e-08)	COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, COL1A2, COL4A2, KDR, COL3A1, COL4A5, LUM
Assembly of collagen fibrils and other multimeric structures	9/67 (6.95e-08)	COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, COL1A2, COL4A2
Collagen degradation	9/69 (7.93e-08)	COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1, COL1A2, COL4A2
Degradation of the extracellular matrix	11/148 (3.73e-07)	ADAMTS1, COL1A2, COL4A2, LAMB1, COL14A1, COL3A1, COL4A5, COL1A1, <u>COL4A1(P02462, Q03692)</u> , COL5A1

563

564 **Supplementary Tables and Legends**

565

566 **Supplementary Table 1: *in vivo* vs. *ex vivo* monocytes**

567 Comparison of gene expression of *ex vivo* (n=171) and *in vivo* (n=107) monocytes. Columns
 568 refer to gene symbols, P-values re-calculated by Mann-Whitney-Wilcoxon rank-sum test, mean
 569 and standard deviation.

570

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571 **Supplementary Table 2: *in vivo* vs. *in vitro* macrophages**

572 Comparison of gene expression of *in vivo* (n=61) and *in vitro*- (n=96) derived macrophages (gut,
573 synovial, kupffer, microglia, macrophage). Columns refer to gene symbols, P-values
574 recalculated by Mann-Whitney-Wilcoxon rank-sum test, mean and standard deviation.

575

576 **Supplementary Table 3: *in vivo* vs. *ex vivo* vs. *in vitro* macrophages**

577 Comparison of gene expression of *in vivo* (n=61), *ex vivo* (n=26) and *in vitro*- (n=96) derived
578 macrophages (gut, synovial, kupffer, microglia, macrophage). Columns refer to gene symbols,
579 P-values re-calculated by Mann-Whitney-Wilcoxon rank-sum test, mean and standard deviation.

580

581 **Supplementary Table 4: dendritic cells**

582 Comparison of gene expression of *in vivo* (n=145), *ex vivo* (n=17) and *in vitro*- (n=57) derived
583 dendritic cells. Columns refer to gene symbols, P-values re-calculated by Mann-
584 WhitneyWilcoxon rank-sum test, mean and standard deviation.

585

586 **Supplementary Table 5: *in vivo* vs. *ex vivo* vs. *in vitro* microglia**

587 Comparison of gene expression of *in vivo* (n=10), *ex vivo* (n=21) and *in vitro*- (n=43) derived
588 microglia. Columns refer to gene symbols, P-values re-calculated by Mann-Whitney-Wilcoxon
589 rank-sum test, mean and standard deviation.

590

591 **Supplementary Table 6: Datasets and samples to compile iMAC atlas and single cell
592 projection**

593 Tissue resident macrophages and dendritic cells from peripheral blood, spleen, thymus, joint,
594 lung, gut, brain and liver. Samples also included monocytes from peripheral and cord blood, as
595 well as *in vitro* differentiated DCs from cord blood progenitors or monocyte-derived
596 macrophages. Columns include dataset accession ID, platform, Stemformatics Dataset ID,
597 number of samples, tier categorization, cell type and relevant tissue/organism part.

598

599 **Additional Supplementary Material**

600

601 **Supplementary Video**

602 Active engulfment and clearance of cells by pluripotent stem cell-derived macrophages.

603

604 **Methods**

605 Atlas formation was developed as described in (Angel et al., 2020) and is comprised of 44
606 datasets, 901 samples and 3757 genes. Mapping, and analysis of microarray and RNA
607 sequencing datasets were conducted in the Stemformatics platform. Scripts are available for
608 download from the Stemformatics BitBucket (Choi et al., 2019a)). All datasets and relevant
609 samples (Supplementary Table 6) passed stringent quality control checks required for hosting
610 on the stemformatics platform. Quality control checks include evaluation of library quality, and
611 inclusion of replicates associated with experimental design. These datasets were either already
612 hosted on stemformatics, or were downloaded from public depositories and processed through
613 the stemformatics pipeline for inclusion.

614

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615 Platform Effect Analysis and Gene Selection for PCA

616 This method assesses each gene independently for a dependence upon experimental platform.
617 The initial step is to transform expression values from RNA Sequencing and Microarray into
618 percentile values. The second step uses a univariate estimate of gene platform dependence
619 and then selects genes with a small ratio of platform dependent variance to total variance.
620 These genes are used to run the PCA (3757 genes passed this cut). The threshold for gene
621 selection is empirically determined by using the Kruskal Wallis H Test to assess the difference
622 in platform expression distribution for each principal component. A platform variance fraction of
623 0.2 is found to remove the platform effect for the first three principal component. For further
624 details, please refer to (Angel et al., 2020).

625

626 Quantification and Statistical Analysis

627 P-values were re-calculated using the Mann-Whitney-Wilcoxon rank-sum test (two-sided). This
628 was implemented via the python (version 3.7.5) SciPy package (version 1.3.1)(Virtanen et al.,
629 2019). Multiple testing over the set of genes was accounted for with Bonferroni correction
630 implemented in the statsmodels package (Seabold and Perktold, 2010)

631

632 Pseudo-bulk samples from Villani's Single Cell Data

633 Single cells were aggregated to form pseudo-bulk samples to mitigate library size differences
634 between single cell and bulk data, and to project samples onto the atlas. Each group of (known)
635 cell type in the single cell data was randomly sampled for k single cells with replacement.
636 Aggregation consisted in summing up their expression profiles. k was determined by the
637 number of sampling (i.e. how many pseudo-bulk samples for each cell type) as half of the cell
638 type's population size. The optimum aggregation size k was investigated by evaluating the
639 similarity in distribution between the atlas' plasmacytoid dendritic cells and the aggregated
640 pseudo-bulk DC6 samples using Kolmogorov–Smirnov (KS) statistic D averaged across all
641 genes. KS statistic measured the difference between the empirical cumulative distribution
642 functions of two groups of samples; the smaller the value, the closer the aggregated DC6
643 resembled the reference transcriptional profiles from the pDC in the bulk atlas. A minimum D
644 value was obtained for $k = 8$ across 30 iterations (Supplementary Figure 1). Similar results were
645 obtained for other cell types. Thus, every pseudo-bulk samples were aggregated from 8 single
646 cells.

647

648 Capybara Cell Score

649 Capybara (Kong et al., 2020) cell scores was used to measure cell identities continuum of the
650 pseudo-bulk samples using iMac as the reference. Capybara cell scores were calculated by
651 performing restricted linear regression of reference iMac samples on each of the pseudo-bulk
652 samples' expression profiles. Denote y_i the expression profile of the i^{th} pseudo-bulk sample of
653 length G , where G represents the total number of genes in the data, and X a $(G \times T)$ the
654 reference iMac matrix, where T represents the number of known cell types of interest. We
655 considered 5 cell types: Dendritic cell, Monocyte, CD141+ dendritic cell, CD1c+ dendritic cell
656 and Plasmacytoid dendritic cells. X is obtained by averaging the expression profiles of the iMac
657 samples according to their cell types. Capybara solves the optimization problem

658

$$\operatorname{argmin}_{\beta} (y_i - X\beta_i)^T (y_i - X\beta_i)$$

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659 under the constraint that

$$\beta_{it} > 0 \quad \forall t \in \{1, 2, \dots, T\}, \quad \sum_t^T \beta_{it} < 1$$

660
661 where β_{it} is a regression coefficient, or cell score, for each pseudo-bulk sample i and each
662 iMac cell type t . The cell score is obtained using quadratic programming implemented with R
663 (version 3.6.2) package *quadprog* (version 1.5-8)(Turlach and Weingessel, 2019).

664 Enrichment analysis and Protein-Protein Network

665 An enrichment analysis was conducted on the top 92 genes ranked by Pearson correlation
666 (≥ 0.7) along the upward axis including invitro-derived cells. Enriched pathways were identified
667 using these genes at Reactome (Fabregat et al., 2018) and significance ranked by p-value/false
668 discovery rate. A protein-protein network was generated using the top 92 genes on STRING-DB
670 (Szklarczyk et al., 2019). Disconnected nodes are not shown.

671 Pluripotent Stem Cell Differentiation

672 Stem cell work was carried out in accordance with The University of Melbourne ethics
673 committee HREC (approval 1851831). Stem cell lines used were PB001.1 (Vlahos et al., 2019),
674 a kind gift from the Stem Cell Core Facility at the Murdoch Children's Research Institute, and
675 HDF51 (Jones et al., 2013) was kindly provided to ALL by Prof. Jeanne Loring (The Scripps
676 Research Institute, CA, USA). Human pluripotent stem cells were differentiated into
677 macrophages based on protocol described by (Joshi et al., 2019; Ng et al., 2008), with
678 modifications. Modifications were as follows: embryoid bodies were kept in rotational cultures
679 without transference to matrigel plates for adherence, and the collection of progenitors from
680 week 2 were immediately re-suspended in RPMI-1640 containing L-Glutamine (Life
681 Technologies) and 10% Fetal Bovine Serum for macrophage differentiation (see macrophage
682 differentiation).

683 Monocyte isolation from peripheral blood

684 Buffy Coat was obtained from the Australian Red Cross Blood Service in accordance with The
685 University of Melbourne ethics committee HREC (approval 1646608). The blood was diluted
686 with PBS at a 1:3 dilution and underlayered with Ficoll-Hypaque. The underlayered blood samples
687 were centrifuged at 350g for 30 minutes at 24°C with no brake. Peripheral blood mononuclear
688 cells were isolated from the interphase and washed twice by using MACs buffer (DPBS, 0.5%
689 heat inactivated Fetal Bovine Serum, 2mM EDTA) and centrifuging at 400g for 5 minutes at
690 4°C. Cells were centrifuged at 400g for 5 minutes at 4°C and resuspended in 40µl MACs buffer
691 per 107 cells. Monocytes were positively selected by a magnetic field using Human
692 CD14 MicroBeads (MACS Miltenyi Biotec) and LS Columns (Miltenybiotec). These cells were
693 plated for macrophage differentiation (see Macrophage differentiation

694 Macrophage differentiation

695 Monocytes/progenitor cells were cultured in tissue-culture treated 6 well plates. Cells were
696 cultured in RPMI-1640 medium containing L-Glutamine (Life Technologies) with 10% Fetal
697 Bovine Serum and 100ng/ml recombinant Human M-CSF (R&D Systems; 216-MC) for 5 days.
698 Media changes were carried out on day 4.

699 Flow Cytometry

700 HMDM and PSCM were collected and centrifuged at 400g for 5 minutes. Supernatant was
701 aspirated and 5µl mouse serum was added to 'dry' pellets for 5 minutes on ice. Cells were

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706 resuspended in FACS Buffer (Hanks Balanced Salt Solution, 0.5% Human Serum Albumin) and
707 stained with CD14 or matched isotype control antibodies on ice for 20 minutes then washed
708 twice (3ml FACS Buffer, spun at 400g, 5 minutes). Resuspended cells were fixed with 4%
709 paraformaldehyde (PFA) for 15 minutes at room temperature. PFA was washed out and cells
710 rinsed twice in FACS buffer before resuspending in PBS and stored overnight at 4°C. Fixed
711 cells were permeabilized with 0.1% Triton X-100 (in 1XPBS) for 10 minutes at room temperature
712 and washed twice. Blocking buffer (0.3M glycine buffer, 10% Goat Serum, 1XPBS) was added
713 to 'dry' pellets on ice for 1 hour. Cells were stained with antibodies to Type I Collagen or
714 matched isotype control on ice for 20 minutes. Cells were washed twice then incubated with
715 secondary antibody for 20 minutes on ice in the dark. Cells were then washed twice and
716 resuspended for analysis on a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA) using
717 CytExpert acquisition software. Post-acquisition analysis was performed with FCS Express 7
718 flow cytometry software.
719

Flow Cytometry antibodies	
CD14	Brilliant Violet 421™ anti-Human CD14 (BioLegend: Cat. No. 325628)
CD14 Isotype	Brilliant Violet 421™ Mouse IgG1 (BioLegend: Cat. No. 400157)
Type I Collagen Primary antibody	Rabbit Anti-Collagen I antibody (Abcam: Cat. No. ab264074)
Secondary Antibody	Goat Anti-Rabbit IgG H&L (Alexa Fluor®488) (Abcam: Cat. No. ab150077)
Type I Collagen Isotype	Rabbit IgG monoclonal Isotype Control (Abcam: Cat. No. 172730)

720

721 Stimulation Assay

722 On day 5 of differentiation, one well containing peripheral blood monocyte- or human pluripotent
723 stem cell-derived macrophages were stimulated with 10ng/ml Lipopolysaccharide (LPS) (Sigma-
724 Aldrich; *Salmonella enterica* serotype minnesota) for 2 hours. After stimulation period, media
725 was aspirated, and the wells were washed twice with PBS (Ca²⁺+Mg²⁺ free). before cell lysis
726 using 2-mercaptoethanol (Sigma-Aldrich) and RNeasy Plus Lysis Buffer (Qiagen). Samples
727 were placed into Eppendorf's and stored at -80°C before RNA extraction.
728

729

729 RNA extraction

730 Total RNA was isolated using the RNeasy® Plus Mini Kit (Qiagen) according to manufacturer's
731 instructions. In summary: for the removal of genomic DNA, samples were placed into gDNA
732 columns and centrifuged for 30 seconds at 8000g. Ethanol (70%) was mixed with the flow
733 through and samples were transferred to RNeasy spin columns. The columns were centrifuged
734 for 15 seconds at 8000g. Buffer RW1 was then added to the columns and columns were
735 centrifuged for 15 seconds at 8000g. Buffer RPE was added to the columns and columns were
736 centrifuged for 15 seconds. Buffer RPE was again added to the columns with centrifugation at
737 8000g for 2 minutes. Columns were then placed into new collection tubes and centrifuged at full
738 speed for 1 minute to dry the membrane. RNase-free water was then added directly onto the
739 column membrane and columns placed into Eppendorf's and centrifuged at 8000g for 1 minute
740 to collect RNA. RNA quality and quantity were determined using a TapeStation (Agilent
741 Technologies 2200). Samples were stored at -80°C. Zymo Research RNA Clean &

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742 Concentrator-25 Kit was used to pool replicates (from the same donor) together and elute in
743 elute into smaller volume with maximum concentration.

744

745 RNA sequencing

746 RNA samples were processed by the Ramaciotti Centre for Genomics (University of New South
747 Wales; Sydney). Illumina Novaseq_6000 was used for mRNA-sequencing.

748

749 Data and Code Availability

750 mRNA-sequencing data is available through accession GSE150893. All public accessions are
751 listed in Supplementary Table 6. Stemformatics code is publicly available
752 at bitbucket.org/stemformatics. Atlas code is available
753 at bitbucket.org/stemformatics/s4m_pyramid/src/master/scripts/atlas.py.

754

755 Graphing software and Illustration

756 Graphs for mRNA-seq gene expression were generated using Graphpad Prism. Violin plots
757 were generated through the www.stemformatics.org platform. Schematic Figure illustration
758 created with BioRender.com

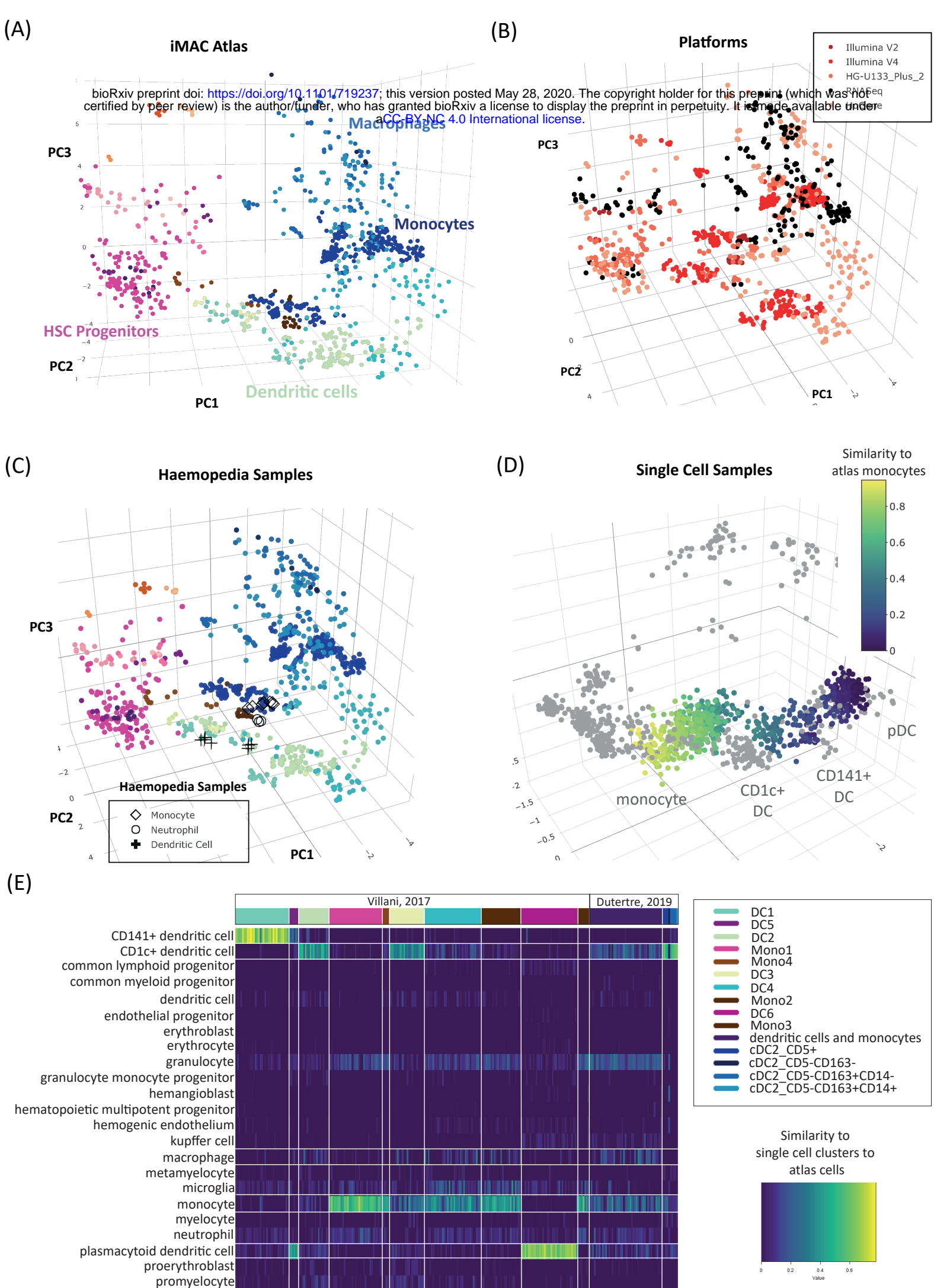


Figure 1: A reference atlas and annotation tool for human myeloid biology

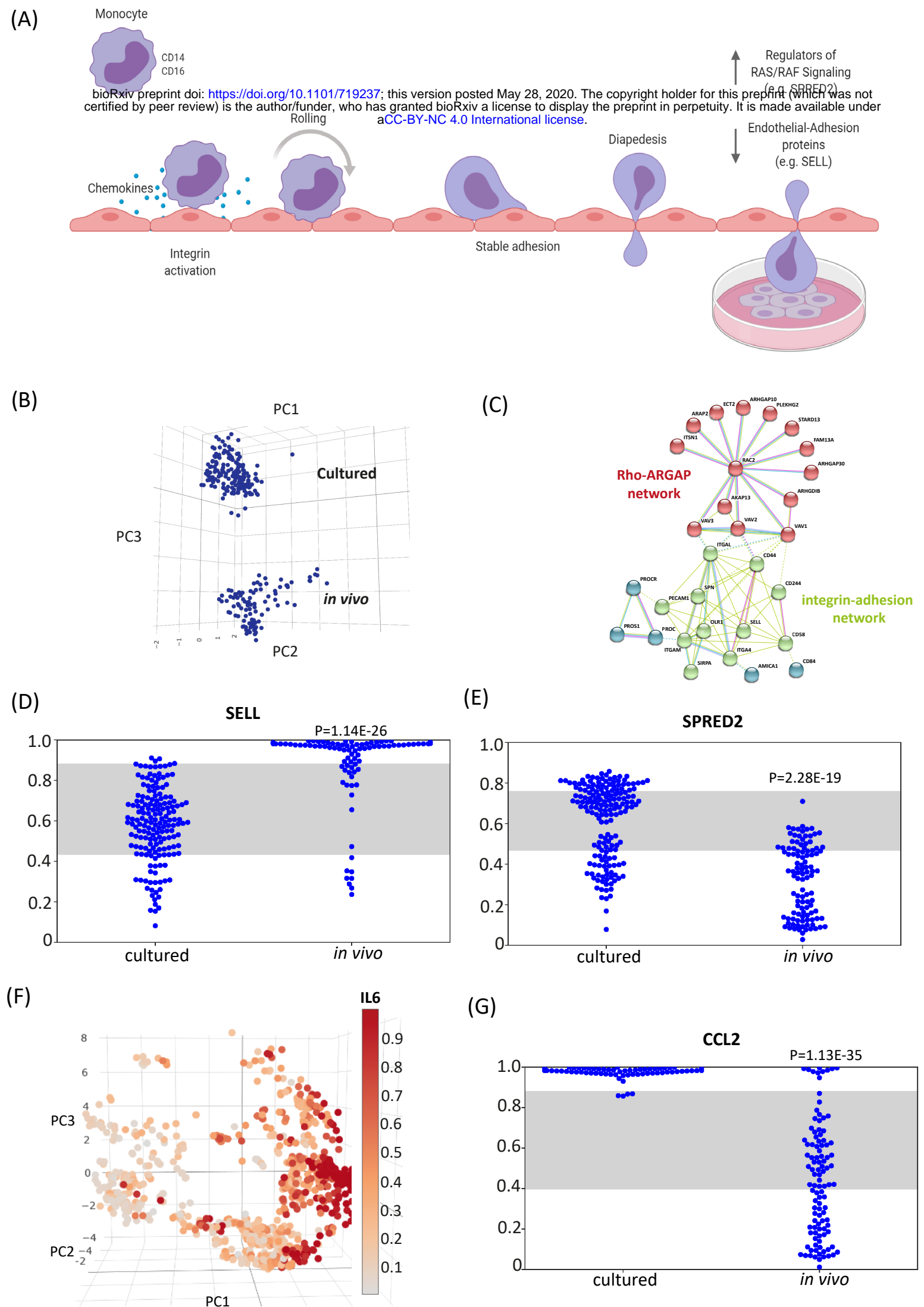


Figure 2: Monocytes acquire a culture phenotype

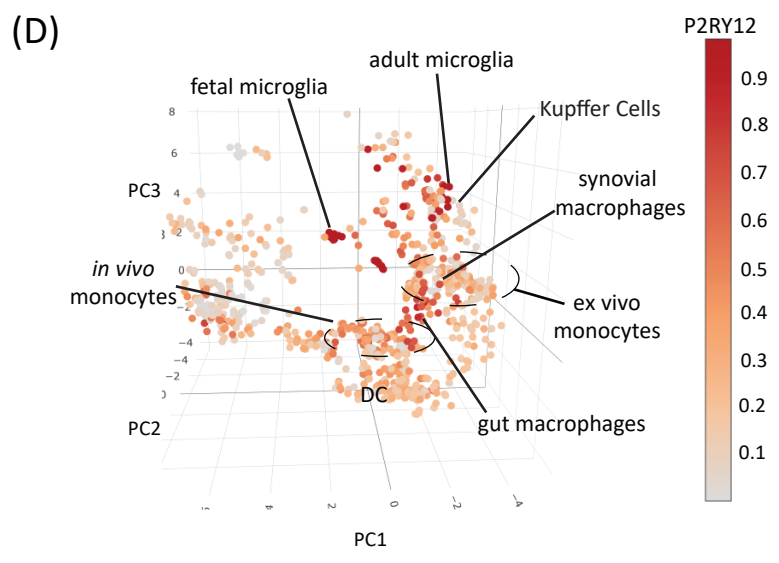
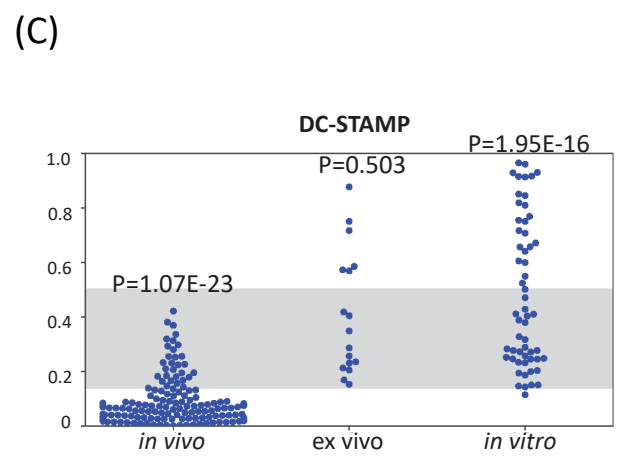
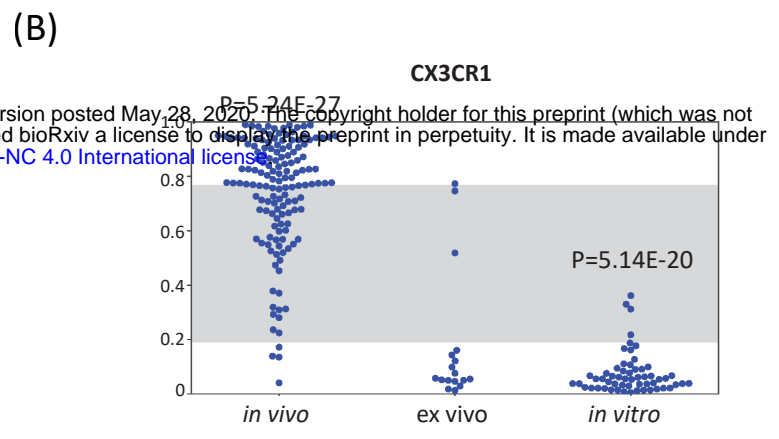
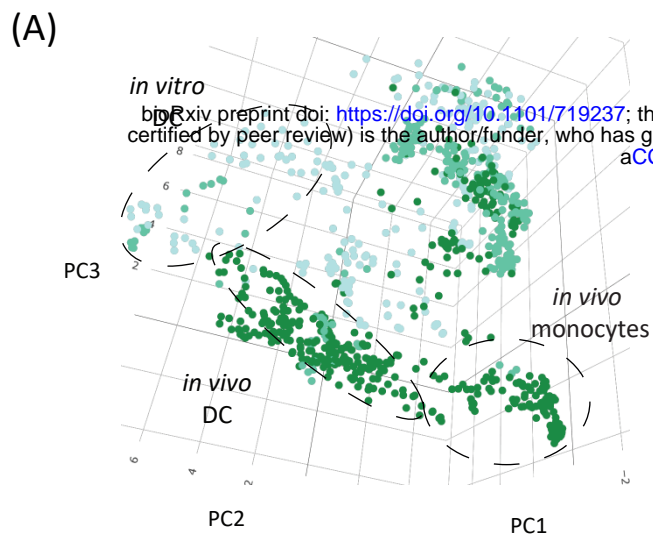


Figure 3: Cultured and *in vitro*-derived dendritic cells do not capture aspects of *in vivo* myeloid biology

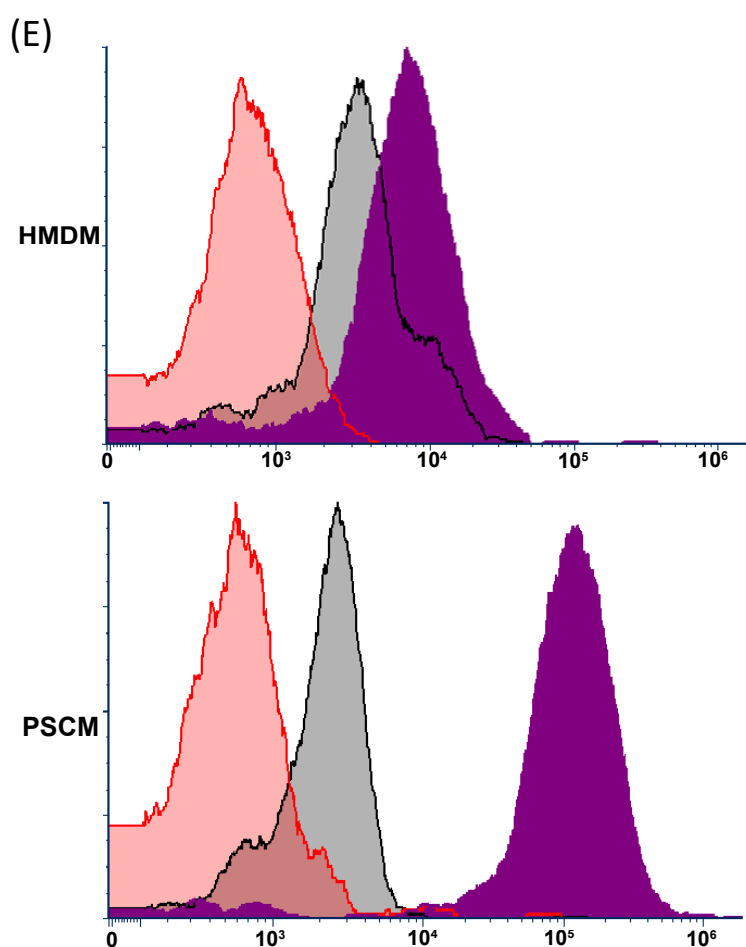
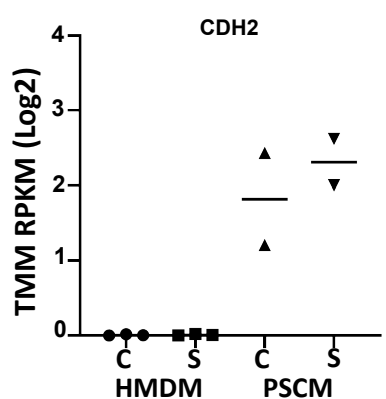
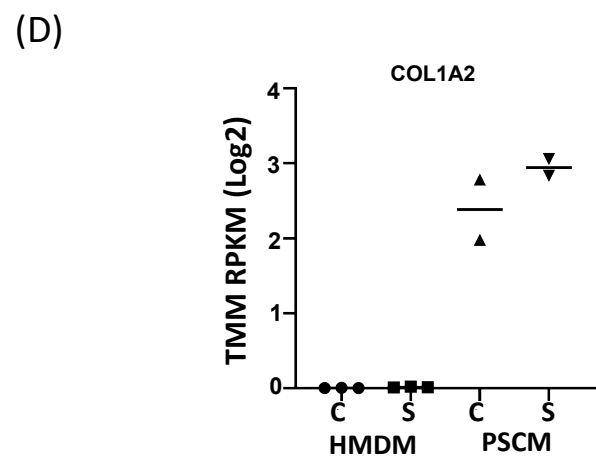
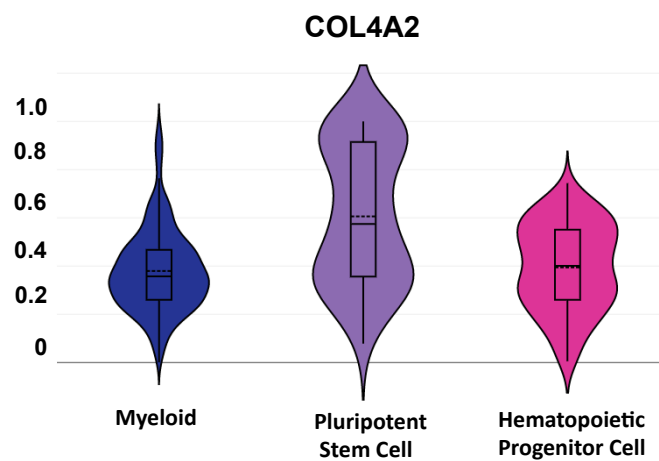
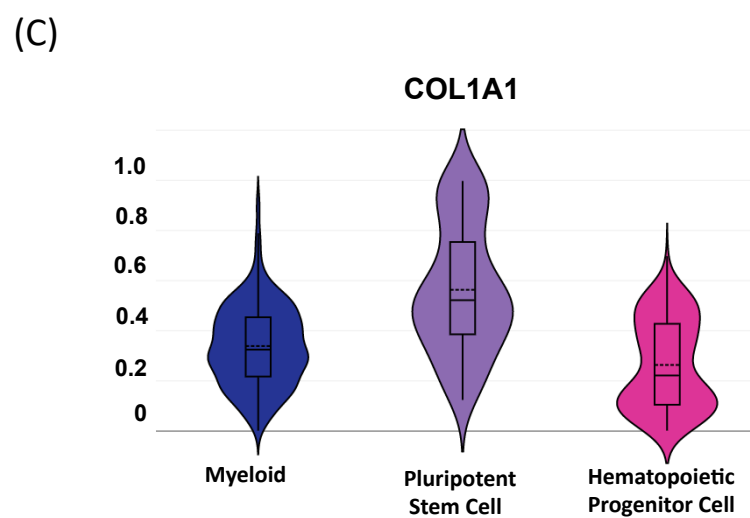
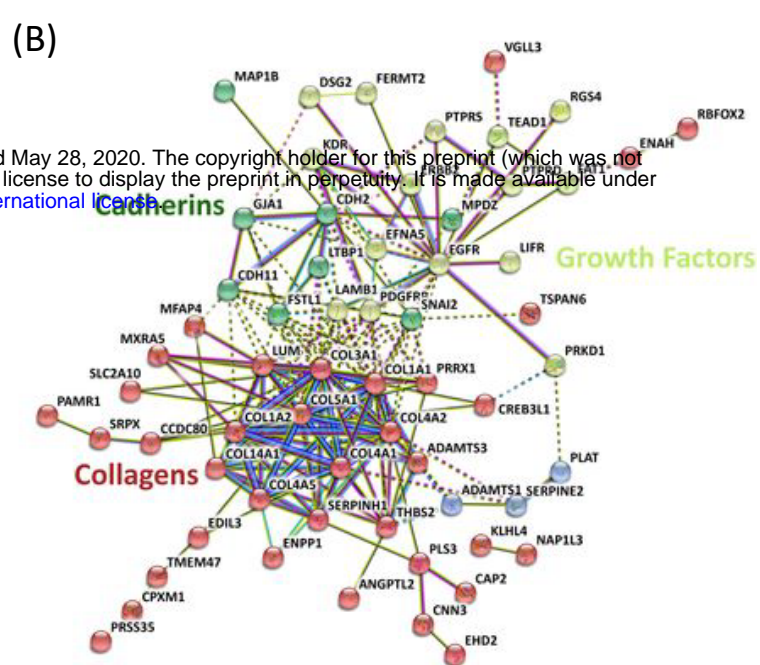
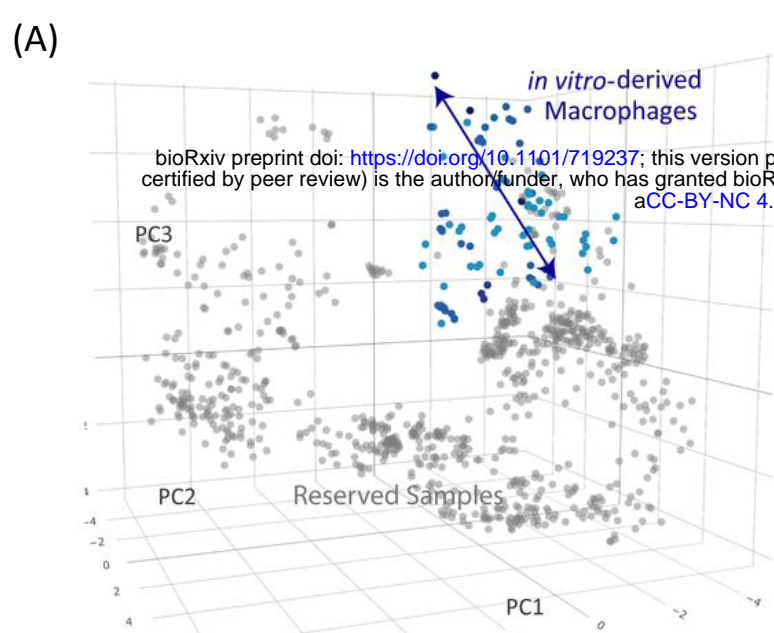


Figure 4: PSC-Macrophages display phenotype enriched for collagen production