Single-cell analysis of ovarian myeloid cells identifies aging associated changes in macrophages and signaling dynamics

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17 **Summary**

18 The aging of mammalian ovary is accompanied by an increase in tissue fibrosis and heightened

19 inflammation. Myeloid cells, including macrophages, monocytes, dendritic cells, and

20 neutrophils, play pivotal roles in shaping the ovarian tissue microenvironment and regulating

21 inflammatory responses. However, a comprehensive understanding of the roles of these cells in

22 the ovarian aging process is lacking. To bridge this knowledge gap, we utilized single-cell RNA

23 sequencing (scRNAseq) and flow cytometry analysis to functionally characterize CD45$^+$ CD11b$^+$

24 myeloid cell populations in young (3 months old) and aged (14-17 months old) murine ovaries.

25 Our dataset unveiled the presence of five ovarian macrophage subsets, including a

26 *Cx3cr1*^{low}*Cd81*^{hi} subset unique to the aged murine ovary. Most notably, our data revealed

27 significant alterations in ANNEXIN and TGFβ signaling within aged ovarian myeloid cells, which

28 suggest a novel mechanism contributing to the onset and progression of aging-associated

29 inflammation and fibrosis in the ovarian tissue.

30

31 **Key words**

32 Ovary, aging, microenvironment, myeloid cells, macrophages, inflammation, Anxa1, TGFβ,

33 single-cell RNA sequencing

Introduction

34

35   It has been well-established that the reproductive potential of women diminishes

36   decades earlier than the deterioration of many of their other physiological systems. The

37   average life expectancy for women is 79.1 years [1] while the menopause takes place at an

38   average age of 51[2]. Furthermore, the quality of oocytes produced by women significantly

39   decreases in their late 30s due to an increase in aneuploidy [3, 4]. This age-associated decline in

40   female fertility has been attributed to a diminishing ovarian reserve and the alterations in the

41   ovarian tissue microenvironment.

42   In both humans and mice, ovarian aging is associated with excessive deposition, as well

43   as altered organization of collagen and hyaluronan in the ovarian stroma, signifying tissue

44   fibrosis [5-8]. Furthermore, levels of pro- and anti-inflammatory cytokines/chemokines,

45   including interferon-γ (IFNγ), tumor necrosis factor α (TNFα), interleukin (IL)-6, IL-10, C-C Motif

46   Chemokine Ligand 2 (Ccl2), Ccl5, IL-4, and IL-13, have been shown to be significantly increased

47   in aged mouse ovaries compared to that in the young ones [7, 9], indicating an increase in

48   chronic inflammation in the tissue. These changes result, in part, in a stiff tissue texture and a

49   dysregulated signaling environment that have been linked to impairment of follicle growth [5,

50   7], ovulation [10] and oocyte quality [11], as well as potentially pathological events, including

51   the development of ovarian cancers [12, 13]. Understanding the etiology and progression of

52   these aging-associated changes in the ovarian tissue environment is crucial for the

53   advancement of health and reproductive longevity in women.

54   Immune cells are pivotal in the surveillance and regulation of the ovarian tissue

55   microenvironment [14-16]. Myeloid cells are a group of immune cells that derive from a

56   common myeloid progenitor in the bone marrow[17] and consist of innate immune cells,

57   including granulocytes, monocytes, macrophages and dendritic cells[17]. They play essential

58   roles in tissue maintenance and remodeling, as well as the orchestration of inflammatory

59   response [17, 18]. In the mammalian ovary, macrophages are the most abundantly present

60   myeloid cells, and are functionally indispensable to the tissue organization, vasculature

61   development, follicle growth, and corpus luteum formation [14, 16, 19, 20].

62   Ovarian myeloid cell populations undergo dynamic changes over the course of aging.

63   Studies in mice and human have reported aging-associated changes in the number and

64   activation status of macrophages that accompany the onset of inflammation [9, 21-24], though

65   discrepancies exist concerning the specific directions of these changes. While increased

66   macrophage presence was observed to accompany ovarian aging in some mouse studies [9,

67   22], others have reported an decreased macrophage presence in aged mouse or human ovaries

68   compared to that in the young [21, 23, 24]. Additionally, aged murine ovarian stroma also

69   harbors a higher number of eosinophils and Ly6C$^+$ monocytes compared to that in the young [8,

70   21]. Considering that myeloid cells are major producers of pro- and anti-inflammatory

71   cytokines, these findings underscore the potential involvement of myeloid cells in the initiation

72   and progression of the aging-associated inflammation.

73   In the past decade, advancements in single-cell RNA sequencing (scRNAseq) techniques

74   have re-defined transcriptomic research. This powerful technique has greatly benefitted the

75   study of ovarian aging and enabled the profiling of transcriptomic changes in aging ovarian

76   cells, including the oocytes, granulosa cells, theca cells, and stromal cells [25, 26]. However, due

77   to the relative scarcity of myeloid cells within the ovarian tissue, a comprehensive high-

78   resolution analysis of their population heterogeneity and transcriptomic changes in the aging

79   process remained largely unexplored.

80   In this study, we used fluorescence-assisted cell sorting (FACS) in combination with

81   scRNAseq to obtain a focused view of myeloid cells in young (3 months old) and aged (14-17

82   months old) murine ovarian tissue. These cells mainly consists of macrophages, monocytes,

83   dendritic cells (DCs), and neutrophils. Our dataset identified five distinct macrophage subsets in

84   the murine ovarian tissue, including a Cx3cr1^{low} Cd81^{hi} subset that is almost exclusively present

85   in the aged ovary. Our data further revealed elevated ANNEXIN and TGFβ signaling among the

86   ovarian myeloid cells at advanced reproductive age and highlighted the potential role of

87   neutrophils in the onset of aging-associated inflammation. Our findings provide a detailed atlas

88   of macrophages and other myeloid cell populations in ovarian aging, laying the foundation for

89   understanding their roles in diminished ovarian functions.

90   **Methods**

91   **Animals**

92   Virgin female C57BL/6J mice that were 2-3 months or 14-17 months of age, as well as

93   *Cx3cr1^{CreER}* and *Rosa26$^{floxed-tdTomato}$* mice, were purchased from The Jackson Laboratory.

94   Prior to each experiment, the estrus stage of all mice was examined using vaginal cytology.

95   Mice at the diestrus stage were used for all experiments. Euthanasia of the animals was

96   performed by CO_2 inhalation. All procedures on live animals were conducted in accordance with

97   the protocol reviewed and approved by the American Association for Laboratory Animal

98   Science (IACUC).

99   **Sample processing and dissociation**

100   Ovaries were harvested from mice aged 2-3 months old and 14-17 months. For the 2-3 months

101   old age group, ovaries from 6 animals were assigned to 3 replicates (ovaries from 2 animals per

102   replicate). For the 14-17 months old age group, ovaries from 5 animals were assigned to 3

103   replicates (2 replicates with ovaries from 2 animals and 1 replicate with ovaries from 1 animal).

104   All samples were dissociated into a single-cell suspension in digestion buffer (1x PBS, 5% FBS,

105   200U collagenase IV, 200U DNaseI) using a gentleMacs^{TM} dissociator with the program

106   37C_m_LDK1. The suspensions were washed with 5 mL of 1x PBS with 5% FBS and 2 nM EDTA,

107   and passed through a 100µm cell strainer to remove debris.

108   **Single-cell RNA sequencing and data analysis**

109   The ovarian cell suspensions were stained with AmCyan Live/Dead stain at a 1:500 dilution in 1x

110   PBS for 15 minutes and BioLegend TotalSeq^{TM} HTO B series hashtags at a concentration of 10

111   µg/mL concentration for 15 minutes. The cells were then pooled and stained with

112   fluorochrome-conjugated primary antibodies BV650-CD45.2 (BioLegend® 109835) and

113   PerCP/Cy5.5-CD11b (BioLegend® 101228) in 1x PBS with 5% FBS for 20 minutes. Stained cells

114   were then washed with 1xPBS with 5% FBS and resuspended in 1x PBS with 5% FBS. FACS was

115   performed by the Flow Core at UAMS using the BD FACSAria^{TM} III cell sorter, and single live cells

116   with positive BV650 and PerCP/Cy5.5 staining were collected into FBS.

117   The encapsulation of the sorted cells and single-cell library preparation were performed

118   by the UAMS Genomics Core using the 10x Chromium Controller and 10x Chromium NextGEM

119   Single-Cell 3' v3.1 kit with Featured Barcode Technology for Cell Surface Proteins following the

120   standard protocol provided by 10x Genomics. The quality of the libraries were examined using

121 the Agilent Fragment Analyzer capillary electrophoresis system, and the libraries were

122 sequenced using Illumina NextSeq® 500 platform.

123 Quality control and alignment of the raw scRNAseq data were performed by the Genomics Core

124 using Cell Ranger v 7.0.0, and the count matrices were generated for the young and aged

125 libraries. The analysis of the data was performed using R 4.2.0 [27], with R package Seurat v.4

126 [28]. In brief, the young and aged datasets were combined, normalized, scaled, and clustered

127 using UMAP as a reduction method [29]. Gene set enrichment analysis (GSEA) results were

128 generated using GSEA v.4.2.1 [30] with pre-ranked gene lists produced using Seurat and R 4.2.0.

129 Pseudotime trajectory analysis was performed using the TSCAN package v4.3 [31]. Cell-cell

130 communication analysis was performed using CellChat package v1.6.0[32].

131 **Flow cytometry**

132 The ovarian cell suspensions were stained with eBioscience™ Fixable Viability Dye eFluor™506

133 at a 1:500 dilution in 1x PBS for 15 minutes. Cells were then washed and stained with

134 fluorochrome-conjugated primary antibodies at the recommended concentration for 20

135 minutes at room temperature. Monoclonal antibodies specific to mouse CD45 (BD Bioscience®

136 566073), CD11b (BioLegend® 101267,), F4/80 (BioLegend® 123149), CD68 (BioLegend®

137 137023), Ly6C (BioLegend® 128017), CX3CR1 (Biolegend® 149027), CD81 (BioLegend® 104911),

138 MHCII (BioLegend® 107605), CD206 (Biolegend® 141731) and CD11c (BioLegend® 117365) were

139 purchased from commercial manufacturers. The Foxp3/Transcription Factor Staining buffer set

140 was used for Ki67 staining. Cells were resuspended in 200 µL of 1x PBS for subsequent analysis.

141 Flow cytometry was performed by the Flow Core at UAMS using the Cytek Northern Lights™

142 Full Spectrum Flow Cytometer. Measurements were taken on biologically independent samples.

143 The analysis of the data was performed using SpectroFlo®, Flowjo v10.7 and Flowjo plugins

144 UMAP v3.1. For UMAP projections of young and aged ovarian macrophages,

145 CD45$^+$F4/80$^+$CD11b$^+$ cells were concatenated to one FCS file before creating UMAP

146 projections. UMAP projections were performed using default parameters.

147 **Fate mapping**

148 To induce Cre recombinase activity for tracing CX3CR1+ macrophages, female

149 *Cx3cr1^{CreERT}/Rosa26^{tdTomato}* mice were generated by crossing *Cx3cr1^{CreER}* and *Rosa26$^{floxed-tdTomato}$*

150 mice. These mice were aged until they reach either 2-3 months or 14-17 months of age. To

151 induce the recombination, tamoxifen was dissolved in corn oil and administered via oral gavage

152 at a dose of 4 mg/day for three consecutive days. Following the last tamoxifen treatment, the

153 mice were sacrificed at three different time points: 3 days, 10 days, and 24 days. Ovarian tissue

154 was collected from the sacrificed mice for further analysis.

155 **Statistical analysis**

156 The statistical analyses in this study were conducted using GraphPad Prism 9 software.

157 Student's t-test (two-sided) was employed to determine the statistical significance of the flow

158 cytometry data. For the identification of differentially expressed genes between the two age

159 groups, the criteria for statistical significance were defined as a minimum log fold change of 0.3

160 and an false discovery rate adjusted p-value lower than 0.05.

161

162 **Results**

163 **Single-cell transcriptomic analysis determines the complex landscape of myeloid cells in**

164 **young and aged murine ovaries.**

165   To examine the aging-related transcriptomic changes in ovarian myeloid cells at single-

166   cell resolution, scRNAseq with FACS-sorted CD45$^+$ CD11b$^+$ cells from the ovaries of young and

167   aged C57BL/6J virgin females was conducted. To ensure the reliability and reproducibility of our

168   findings, 3 biological replicates for each age group were included and barcoded with hashtag

169   oligoes (HTO).

170   A total of 5769 cells from the two age groups passed our quality control criteria. These

171   cells segregated into 15 clusters based on their transcriptomic profiles (Figure 1B). The UMAP

172   cell cluster patterns within each age group were found to be similar among the HTO-labeled

173   replicates, indicating high robustness and reproducibility of the results (Supplemental Figure

174   S1).

175   We annotated the clusters based on their transcriptomic signatures, and identified a

176   total of eight cell types. The most abundant cell types include macrophages (*Adgre1*^{hi} *C1qa*^{hi}),

177   monocytes (*Adgre1*^{low} *C1qa*^{low}*Cx3cr1*^{hi}), dendritic cells (DC) (*Adgre1*^{low}, *Cd11c*^{hi}), and neutrophils

178   (*S100a9*^{hi} *S100a8*^{hi}). The dataset also captured small numbers of natural killer cells (NK)

179   (*Gzma*^{hi}, *Nk7g*^{hi}), B cells (*Cd79a*, *Ly6d*), fibroblasts (*Col3a1*, *Col1a2*), and endothelial cells (*Cldn5*,

180   *Cdh5*) (Figure 1C and D). Among these cell types, macrophages further segregated into five

181   distinct subsets (referred to as Mac_1 to Mac_5 based on decreasing population size),

182   monocytes consisted of two subsets distinguished by *Ly6c2* expression (*Ly6c2*^{low} Monocyte_1

183   and *Ly6c2*^{hi} Monocyte_2), and DCs consisted of three subsets distinguished by *Cd209a*, *Xcr1* and

184   *Ccr7* expression (*Cd209a*^{hi} DC_1, *Xcr1*^{hi} DC_2 and *Ccr7*^{hi} DC_3) (Figure 1C and 1D).

185

186   **Ovarian macrophage population consists of multiple functionally distinct subsets.**

187   Our initial analysis focused on the ovarian macrophages since it is the most abundant

188   among all cell types and displayed great heterogeneity in their transcriptional profiles. The

189   dataset uncovered the presence of five macrophage subsets in the ovarian tissue (Figure 2A),

190   each exhibits unique expression signatures indicative of different origins and functional

191   specializations (Figure 2B).

192   Notably, Mac_5 displayed a high expression of *Gata6* and *Tgfb2* (Figure 2B), which

193   aligned with the signature of a peritoneal macrophage population [33].

194   Among the four ovarian macrophage subsets (Mac_1, Mac_2, Mac_3, and Mac_4), gene

195   sets enrichment analysis (GSEA) indicates a significant enrichment of cell cycle regulation-

196   related genes in the transcriptomic signatures of Mac_4 (Figure 2B and 2C), indicating its

197   identity as a cluster of proliferating macrophages. Mac_1 was highly associated with the classic

198   macrophage function of antigen processing/presentation, while Mac_2 was associated with

199   ECM organization. Mac_3 was associated with activation of immune response and antigen-

200   receptor-mediated signaling, suggesting its role in regulating immune response and

201   inflammation. The results of the gene set enrichment were validated and visualized with the

202   module scores for the relevant gene sets (Supplemental Figure S2). Consistently, the terms

203   "antigen presentation", "actin and ECM organization", "activation of immune response", and

204   "cell cycle" scored high in Mac_1, Mac_2, Mac_3, and Mac_4, respectively (Supplemental

205   Figure S2).

206   A closer examination of the transcriptomic signatures of the macrophage subsets

207   revealed that the most abundant three macrophage subsets, Mac_1, Mac_2 and Mac_3, could

208   be distinguished based on their expression of the chemokine receptor *Cx3cr1* and the

209   tetraspanin *Cd81* (Figure 2D). Mac_1 exhibited high expression of both *Cx3cr1* and *Cd81*

210   (Cx3cr1^{hi}, Cd81^{hi}), Mac_2 displayed low expression of both markers (*Cx3cr1*^{low}, *Cd81*^{low}), and

211   Mac_3 showed low *Cx3cr1* and high *Cd81* expression (*Cx3cr1*^{low}, *Cd81*^{hi}) (Figure 2C).

212   Since the functions of macrophages are heavily influenced by their activation status, we

213   examined the expression pattern of markers that are commonly associated with classical (M1)

214   and alternative (M2) macrophage activation status among the ovarian macrophage subsets

215   [34]. Interestingly, a mixture of M1 and M2-associated markers was found to be expressed in all

216   macrophage subsets (Supplemental Figure S3). Thus, none of the ovarian macrophage

217   subpopulations show obvious bias towards M1 or M2 polarization.

218

219   **Cx3cr1^{hi}Cd81^{hi} Mac_1 represents a self-maintained low turnover ovarian macrophage subset.**

220   To validate the scRNAseq findings, we performed flow cytometry analysis with CD68^{+}

221   F4/80^{+} ovarian macrophages (Supplemental Figure S4). Consistent with the sequencing data,

222   ovarian macrophages segregate into distinct CX3CR1^{hi}CD81^{hi}, CX3CR1^{low}CD81^{low} and

223   CX3CR1^{low}CD81^{hi} subsets that correspond to Mac_1, Mac_2 and Mac_3 (Figure 3A).

224   Additionally, the analysis captured a CX3CR1^{hi} CD81^{low} macrophage subset not observed in the

225   scRNAseq data (Figure 3A), which may represent a transitional population.

226   The characterization of these ovarian macrophage subsets using dimensional flow

227   cytometry analysis led to several interesting observations. First, CX3CR1^{hi}CD81^{low} and

228   CX3CR1^{low}CD81^{low} (Mac_2) subsets exhibited high expression of Ly6C (Figuire 3B) and lower

229   levels of F4/80 and CD68, which is consistent with macrophages that are newly differentiated

230   from monocytes [35]. Secondly, CX3CR1^{low}CD81^{hi} (Mac_3) cells and CX3CR1^{hi}CD81^{hi} (Mac_1)

231 cells showed high expression of F4/80 and CD206 (Figure 3B), suggesting their identities as

232 tissue-resident macrophages [35]. Thirdly, although Ki67 expression was observed in all

233 macrophage subsets, the highest Ki67 expression mostly overlap with CX3CR1^{hi}CD81^{hi} (Mac_1)

234 (Figure 3B), which suggests Mac_1 as a self-maintained macrophage population with a high

235 number of proliferative cells [36]. The expression patterns of markers observed in the flow

236 cytometry analysis largely aligned with those observed in the scRNAseq dataset (Supplemental

237 Figure S5). Taken together, our data indicated that Mac_1 represents a mature, self-maintained

238 ovarian macrophage subset, while Mac_2 and CX3CR1^{hi}CD81^{low} macrophages appeared to have

239 more recently differentiated from monocytes.

240 To verify the relationships between the ovarian macrophage subsets, we generated a

241 pseudotime trajectory incorporating the four ovarian macrophage subsets and the *Ly6c2*+

242 monocyte subset (Monocyte_2) using the TSCAN package [31]. Monocyte_2 was set as the root

243 of the trajectory, as it represents the least differentiated state. The pseudotime model

244 identified Mac_3 and Mac_4 as two end point clusters, inferred as derivatives of the Mac_1

245 subset (Figure 3C). Mac_2 was identified as an intermediate state between Monocyte_2 and

246 Mac_1 (Figure 3C). Overall, the pseudotime analysis provided further evidence supporting the

247 close association of Mac_1 with Mac_3 and the proliferative macrophage population (Mac_4).

248 To validate the self-maintaining status of Mac_1, we conducted fate-mapping analysis of

249 the CX3CR1/CD81-defined ovarian macrophage subsets. All ovarian macrophages express

250 CX3CR1 and thus can be labeled in the Cx3cr1^{CreERT} inducible fate mapping mouse model, where

251 a tamoxifen-inducible Cre recombinase is expressed under the control of the endogenous

252 Cx3cr1 promoter (Figure 3D). To track all ovarian macrophage subsets and measure their

253 turnover rate in aged mice, the *Cx3cr1*[CreERT] mouse was crossed to a *Rosa26*[tdTomato] reporter

254 mouse and a pulse-chase experiment was conducted. For the four macrophage subsets, we

255 calculated the ratio of % tdTomato+ cells at the later time points to % tdTomato+ cells at day 3

256 post tamoxifen treatment. Thus, a reduced % tdTomato retention indicates a decrease in

257 tdTomato+ cells over time. Notably, while all four macrophage subsets were labeled by

258 tdTomato, the % tdTomato retention decreased dramatically in both the CX3CR1^{low}CD81^{low}

259 (Mac_2) and the CX3CR1^{hi}CD81^{low} subsets. However, the CX3CR1^{low}CD81^{hi} (Mac_3) and

260 CX3CR1^{hi}CD81^{hi} (Mac_1) subsets displayed much slower decreases in % tdTomato retention

261 (Figure 3E). These data indicate that the four ovarian macrophage subsets exhibited differences

262 in turnover properties, with Mac_1 and Mac_3 subsets being relatively long-lived in the aged

263 ovary, supporting the stable, self-maintaining property of Mac_1 and the relationship between

264 Mac_1 and Mac_3.

265 **The CX3CR1^{low} CD81^{hi} Mac_3 macrophage subset drastically expands in aged murine ovaries.**

266 The comparison of scRNAseq data between the two age groups unveils that, while the

267 presence of Mac_1, Mac_2, Mac_4 and Mac_5 did not show statistically significant differences

268 across the two age groups, the Mac_3 subset was almost exclusively present in the

269 reproductively old tissue (Figure 4A). This observation was confirmed by the flow cytometry

270 data, as the proportion of CX3CR1^{low}CD81^{hi} subset in total macrophage population increased

271 significantly in aged ovarian tissue (Figure 3A and 4B).

272 To gain insights into the properties of the Mac_3 subset, we analyzed its transcriptomic

273 profile using GSEA[30]. This analysis revealed significant downregulation of genes related to

274 chemotaxis, and a simultaneous upregulation of genes associated with antigen receptor

275   signaling, immune response activation and phagocytosis (Figure 4C). These results suggest

276   Mac_3 as a subset of low-mobility macrophages with increased phagocytic activities.

277   Remarkably, Mac_3 exhibits high expression of *Pparg* (Supplemental Figure S3), a transcription

278   regulator essential to promote lipid metabolism in macrophages [37]. This suggests that Mac_3

279   may engage in a metabolic program that enables efficient lipid utilization.

280   To explore the influence of Mac_3 within the tissue microenvironment, we inspected its

281   interactions with other cell clusters within the dataset. Employing the CellChat R package, we

282   inferred the signaling activities to and from Mac_3 in the aged myeloid cell populations based

283   on ligand-receptor interactions [32]. Intriguingly, the analysis reveals that the outgoing signals

284   from Mac_3 are predominantly that of the CCL pathway, while TGFβ, vascular endothelial

285   growth factor (VEGF), and CXCL are also among the top outgoing signaling pathways with

286   relatively high information flow. Conversely, Mac_3 mainly receives signaling through the

287   GALECTIN, TGFβ, and COMPLEMENT pathways (Figure 4D). A closer examination of the CCL

288   signaling from Mac_3 indicates that the subset mainly produces *Ccl6*, *Ccl3* and *Ccl4*. And the

289   strongest communication takes place between Mac_3 and the neutrophils through *Ccl6-Ccr1*

290   and *Ccl3-Ccr1* interactions (Figure 4E). GALECTIN signaling received by Mac_3 predominantly

291   originates from other macrophage subsets and Monocyte_2, featured with *Lgals9*-CD45

292   interactions (Figure 4E).

293   **Aged ovarian myeloid cells are featured with intensified ANNEXIN and TGFβ signaling.**

294   To explore the temporal changes in the ovarian myeloid cells and elucidate their roles in

295   the ovarian aging process, we analyzed and compared the cell-cell communication network in

296   the two age groups based on the single-cell transcriptomic data. Overall, we observed an

297   increase in both the number and the strength of cell-cell interactions among myeloid cells in

298   the aged ovary compared to that in the young, with an exception being the outgoing signaling

299   activities from Mac_2 (Figure 5A).

300   Pathways that are present in the signaling network of both age groups may exhibit

301   significant functional differences due to alterations in the senders and receivers of the signaling

302   and the predominant ligand-receptor pairs[32]. We ranked pathways shared between the two

303   age groups based on their functional deviations in the young and aged cell-cell communication

304   networks. Strikingly, the most prominent age-associated alterations were observed in ANNEXIN

305   and TGFβ pathways (Figure 5B). Additionally, BMP, VEGF, VISFATIN, TNF pathways also ranked

306   among the most functionally distinct between the two age groups (Figure 5B). Interestingly, all

307   these markedly altered pathways exhibited significantly intensified signaling activities in the

308   older age group (Figure 5C).

309   We delineated the most significantly altered ANNEXIN and TGFβ signaling pathways

310   within young and aged cells, and found drastic changes in both the sources and targets of these

311   signaling interactions (Figure 5D). Among young ovarian myeloid cells, ANNEXIN signaling

312   originates from multiple sources, including Mac_2, Mac_4, DC_1, DC_2, monocytes, and

313   neutrophils, with neutrophils being the sole target. In aged ovarian myeloid cells, however,

314   Mac_1 and Mac_5 emerged as additional sources of ANNEXIN signaling, while the target cell

315   types expanded to include neutrophils, Mac_3, Mac_5 and Monocyte_1 (Figure 5D). Regarding

316   the TGFβ pathway, there was a marked increase in overall signaling activities and a drastic

317   expansion of incoming signaling to macrophage subsets including Mac_1, Mac_2 and Mac_3

318   (Figure 5D).

319   Upon Examination of the relevant ligand and receptor expression, it became evident

320   that the changes in ANNEXIN signaling activities were mainly a result of increased expression of

321   Anxa1 in aged Mac_1 and Mac_5, and upregulation of *Anxa1* receptor *Fpr2* in Monocyte_1,

322   Mac_3 and Mac_5 (Figure 5E). Changes in TGFβ signaling can be attributed to a general

323   elevation in *Tgfb1* expression across macrophage subsets, along with increased expression of

324   *Tgfbr1* and *Tgfbr2* within the macrophages (Figure 5E).

325   Notably, Anxa1 encodes Annexin A1 (ANXA1), an anti-inflammatory protein that

326   orchestrate the resolution of inflammation. ANXA1 is a chemoattractant of monocytes[38-40].

327   Flow cytometry analysis showed that the aging-associated increase in ANNEXIN signaling to

328   Monocyte_1 is accompanied by significant increase in both the number and proportion of

329   Monocyte_1 in aged ovary (Supplemental Figure S4, Figure 5F), providing further evidence for

330   the elevated signaling activities.

331

Discussion

333   In this study, five distinct ovarian macrophage subpopulations have been identified

334   through single-cell transcriptomic analysis. Based on the expression of Ly6C, CD81 and CD11b,

335   as well as their turnover rates in the aging ovarian tissue, the CX3CR1^{hi} CD81^{hi} Mac_1 and

336   CX3CR1^{low} CD81^{low} Mac_2 subsets may correspond to the long-lived F4/80^{hi} CD11b^{int} and short-

337   lived F4/80^{int} CD11b^{hi} ovarian macrophage subtypes identified by Li et al using flow cytometry

338   in a recent publication[41]. Mac_1 was characterized as a mature, self-maintaining macrophage

339   subset that resembles tissue-residential macrophages.

340   Interestingly, our data revealed a unique subset of CX3CR1^{low}CD81^{hi} macrophages in

341   aged ovarian tissue. The high expression of Pparg in this subset indicates a distinct adaptation

342   to increased lipid metabolism. Aging and obesity often result in a similar inflammatory milieu in

343   the ovarian tissue, where aging is associated with increased visceral adipose accumulation and

344   altered lipid profiles in and around the ovary [42, 43]. It is thus plausible that the upregulation

345   of Pparg expression in this macrophage subset signifies an adaptation to the ovarian tissue

346   environment at advanced reproductive age. Moreover, it is known that PPARγ activation can

347   enhance Fc-receptor-mediated phagocytosis in macrophages [44-47]. Aligning with this, genes

348   associated with the regulation of phagocytosis are found to be upregulated in this specific

349   macrophage subpopulation, suggesting increased phagocytic activities. Another intriguing

350   discovery about the CX3CR1^{low}CD81^{hi} macrophage subset is the predominance of CCL pathway

351   in its signaling output. This subset expresses high level of *Ccl3* and *Ccl4*, known as macrophage

352   inflammatory proteins (MIPs) that can stimulate the production of pro-inflammatory cytokines,

353   including TNFα, IL-1β, and IL-6, within macrophages[48]. Additionally, it expresses *Ccl6*, a

354   chemoattractant for monocytes and macrophages[49, 50] that has been linked to pulmonary

355   inflammation and fibrosis[51, 52]. These findings suggest a plausible role for the

356   CX3CR1^{low}CD81^{hi} macrophage subset in the development of age-related chronic inflammation

357   and fibrosis within ovarian tissue. It is conceivable that this macrophage subset might be

358   associated with the F4/80+ group of multi-nuclei giant cells identified through histological

359   studies in aged ovarian tissue [7, 53]. However, further investigations will be imperative to

360   ascertain the relationship between these two subsets.

361   In addition to the emergence of the CX3CR1^{low}CD81^{hi} macrophage subset, one of the

362   most conspicuous changes associated with aging in ovarian tissue is the profound alterations

363   observed in the cell-cell communication networks, particularly within the ANNEXIN and TGFβ

364   pathways. Studies into the mechanisms and progression of inflammation suggested a potential

365   interconnection between the upregulation of these two pathways. ANXA1, a pivotal player in

366   the resolution of inflammation, performs crucial roles during infection and wound healing. It is

367   released by infiltrating myeloid cells, particularly neutrophils and macrophages[54], to

368   attenuate further neutrophil infiltration, recruit monocytes, and activate the expression of anti-

369   inflammatory cytokines and TGFβ1 in macrophages [54, 55]. Our data aligned with this concept,

370   revealing an age-related increase in the presence of Monocyte_1 (Figure 5F), and a high

371   expression of *Tgfb1* in Mac_3 (Figure 5E), both of which are targets of the heightened ANNEXIN

372   signaling in aged ovarian tissue. Given that TGFβ signaling promotes the alternative activation

373   of macrophages, the increased ANNEXIN signaling may contribute to the rise of M2-like

374   macrophages in the reproductively aged ovary [9, 21]. Consequently, our findings cast ANNEXIN

375   signaling as a compelling avenue warranting further investigation and offering novel

376   therapeutic potentials.

377   Furthermore, neutrophils emerge as a major source of ANXA1 during wound healing

378   [54]. Our single-cell data not only reflects an increase in the presence of neutrophil in aged

379   ovarian tissue (Supplemental Figure S6A), but also indicates an elevation in the intensity of both

380   incoming and outgoing ANNEXIN signaling within aged neutrophils (Supplemental Figure S6B).

381   In each ovarian cycle, the release of the egg leads to wounding of the ovarian surface and

382   subsequent wound healing that recruit neutrophils. The inability to effectively clear neutrophils

383   during this process could potentially lead to the accumulation of these granulocytes and their

384   detrimental contents in the ovarian tissue over time, which may contribute to the onset of

385   chronic tissue inflammation and fibrosis. Our data underscores the role of neutrophils as a

386   previously overlooked cellular cohort that might be intricately linked to ovarian aging.

387   Finally, our scRNAseq dataset also captured a small GATA6+ macrophage subset

388   resembling the peritoneal macrophages. Intriguingly, GATA6+ peritoneal macrophages have

389   been shown to be recruited for tissue repair in other organs within the peritoneal cavity, such

390   as the liver and intestine [56, 57]. Considering that the mammalian ovary undergoes tissue

391   damage and repair in each ovarian cycle, it is possible that the peritoneal macrophages are

392   recruited and play a functional role in the ovarian tissue repair process. Further investigation

393   into the recruitment and functional importance of peritoneal macrophages in ovarian tissue

394   repair would be an interesting area of research.

395   In addition to aging, immunological changes in ovarian tissue have been increasingly

396   associated with various pathological conditions, such as diminished ovarian reserve [22],

397   ovarian cancers [13], and polycystic ovary syndrome [58]. The scRNAseq dataset generated in

398   this study has the potential to provide valuable insights into the understanding of these

399   diseases as well. The findings of this study may have significant translational value if they can be

400   validated in ovarian specimens from human patients.

401

409

410   Authors' contribution

411   ZZ is responsible for the design and execution of the experiments, data analysis, and the writing

412   of the manuscript. LB advised the design of the experiments and writing of the manuscript. LH

413   and MB supervised the study, and advised the design/execution of the experiments and the

414   writing of the manuscript.

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535

536

537   **Figure legends**

538

539   **Figure 1. Single-cell transcriptomic analysis captured the landscape of myeloid cells in young and aged murine ovaries. A:** Schematic drawing illustrating the scRNAseq experimental set up. **B:** The clustering of CD45+CD11b+ ovarian myeloid cells with UMAP as the dimension reduction method. The plot includes cells from all age and HTO groups. **C:** The relative expression of the representative markers of each cluster shown in the combined clustering plot. The high and low expression are represented by blue and gray colors respectively. **D:** Dotplot showing the expression of markers used for the identification of each cluster.

546   **Figure 2. Ovarian macrophage population consists of multiple functionally distinct subsets. A:** The clustering of ovarian macrophages that are extracted from the CD45+CD11b+ ovarian myeloid dataset. **B:** The expression of the top15 represented markers for each macrophage subpopulations ranked by fold change. **C:** The top relevant gene sets enriched in markers that are uniquely upregulated in each cluster. The color of the data point represent the Z-score of the net enrichment score (NES) of the gene set. Orange represent high value, while blue represents low value. **D:** the violin plot showing the expression of Cd81 and Cx3cr1 in the ovarian macrophage subpopulations.

553

554   **Figure 3. CX3CR1^{hi}CD81^{hi} macrophages represents a stable self-maintaining ovarian macrophage subset. A:** Representative flow cytometry plot of CX3CR1 and CD81 expression in

556 ovarian macrophages in young and aged murine ovary. **B:** Clustering of pooled CD68^{+} F4/80^{+}

557 according to the expression of Ki67, CX3CR1, CD68, CD206, F4/80, MHCII, CD81, and Ly6C.

558 UMAP was used as the dimension reduction method. The individual plots showed the four

559 CX3CR1/CD81 defined macrophage subsets, and the expression of Ki67, CX3CR1, CD68, CD206,

560 F4/80, MHCII, CD81, and Ly6C respectively. **C:** Pseudotime trajectory model calculated based on

561 the clustering of Monocyte_2, Mac_1, Mac_2, Mac_3 and Mac_4. **D:** Schematic drawing

562 illustrating the fate mapping experiment with CX3CR1^{CreERT}/Rosa26^{tdTomato} mice. **E**: Percentage

563 of total tdTomato positive cells in the CX3CR1/CD81 defined macrophage subsets 3 days (n = 3),

564 10 days (n = 7) and 24 days (n = 6) post TAM treatment.

565

566 **Figure 4. A CX3CR1^{low} CD81^{hi} macrophage subset is uniquely present in aged murine ovaries.**

567 **A:** The clustering of ovarian macrophages split by age, shown with UMAP as the reduction

568 method. **B:** The quantification of the CX3CR1^{hi}CD81^{low}, CX3CR1^{hi}CD81^{hi}, CX3CR1^{low}CD81^{hi},

569 CX3CR1^{low}CD81^{low} macrophage subpopulations in young (n = 5) and aged (n = 7) murine ovaries

570 based on the flow cytometry analysis. For CX3CR1^{low}CD81^{hi} macrophages, p = 0.00006 (aged vs

571 young). **C:** The top relevant terms in the GSEA results of the expression signatures of the Mac_3

572 macrophage subset compared to Mac_1, Mac_2, Mac_4 and Mac_5. **D:** The top pathways in

573 the outgoing and incoming signaling from and to the Mac_3 subset. The ranking was performed

574 based on information flow. Higher value indicates higher overall signaling strength. **E:** Bubble

575 plot showing the relative strength of specific ligand-receptor interaction within the outgoing

576 CCL and incoming GALECTIN pathways from and to the Mac_3 subset. The color of the bubble

577 represent the relative communication probability.

578

579 **Figure 5. Elevated ANNEXIN and TGFβ signaling are observed among aged ovarian myeloid**

580 **cells. A:** The relative changes of the number and strength of signaling communication between

581 different cell clusters in aged vs young myeloid cells. The color of the cells represent the

582 direction of changes. Red represents increase while blue represent decrease. The bars at the

583 end of columns and rows represents the column or row sums respectively. **B:** The ranking of

584 pathways that are present in the communication network among both the young and aged

585 myeloid cells based on is functional distinction between the two age groups. Higher value

586 indicate greater changes in strength, sender/receiver identification, and dominant ligand-

587 receptor pairs. **C:** Relative information flow of the top ranked pathways in panel B in young and

588 aged communication network. The length of the bar represents relative signaling strength. **D:**

589 The chord plot that visualize the sender and receivers of ANNEXIN and TGFβ signaling in young

590 and aged communication networks. The color of the outer and inner bars represents the

591 identity of the sender and receiver of the signaling respectively. The size of the bars represents

592 the signal strength. **E:** The violin plot that shows the expression of ligand and receptors relevant

593 to the ANNEXIN and TGFβ pathways in all cell clusters split by age. **F:** The quantification of the

594 count and proportions of Ly6C^{hi} and Ly6C^{low} monocytes in young (n = 8) and aged (n = 8) murine

595 ovary based on the flow cytometry analysis. Adjusted p-values for the comparisons from left to

596 right are: Ly6C-hi monocyte absolute count aged vs young, p = 0.028127; Ly6C-low monocyte

597 absolute count aged vs young, p = 0.000466; Ly6C-hi monocyte as %Monocyte aged vs young, p

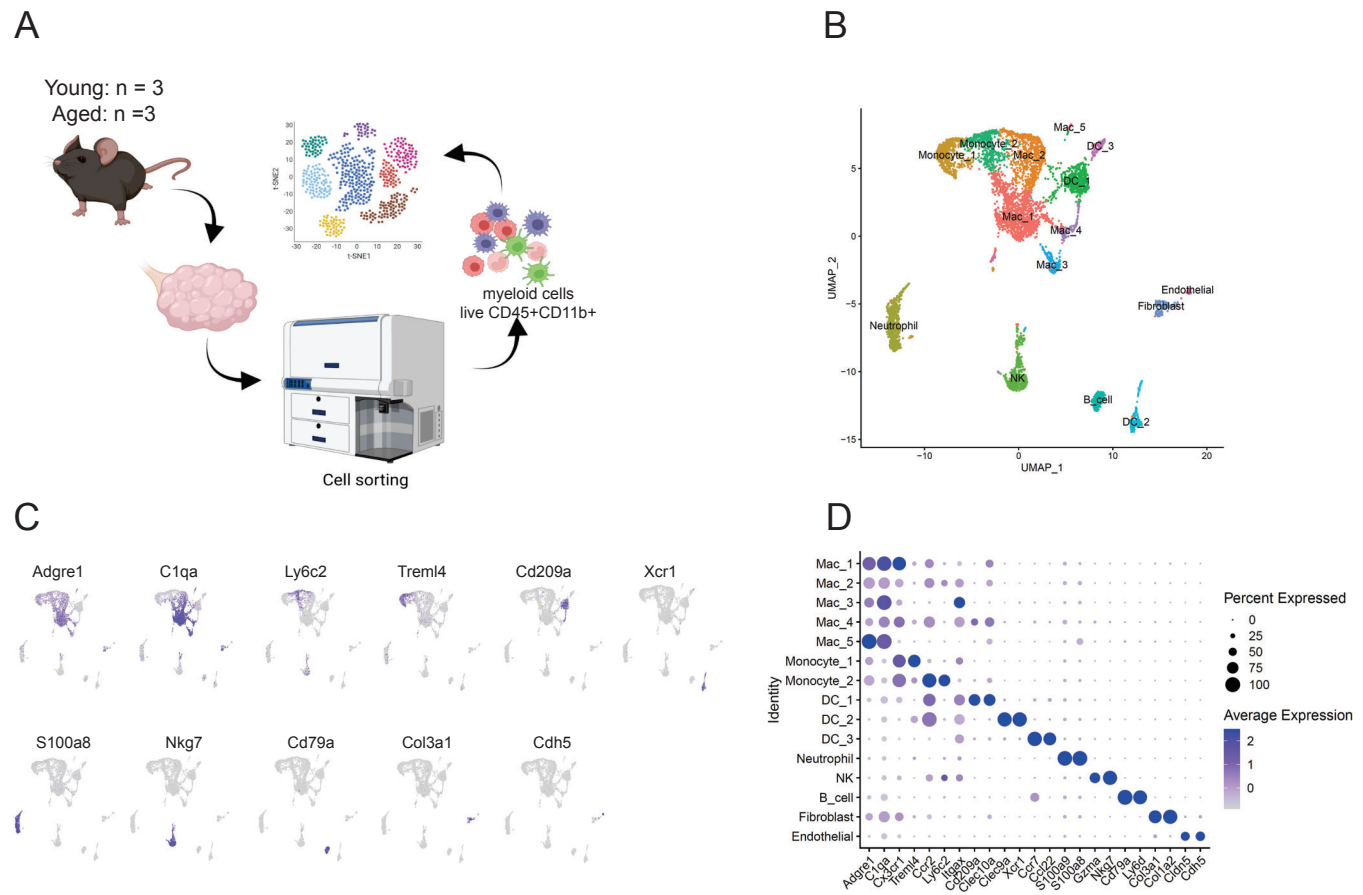598 = 0.000155; Ly6C-low monocyte as %Monocyte aged vs young, p = 0.000155.
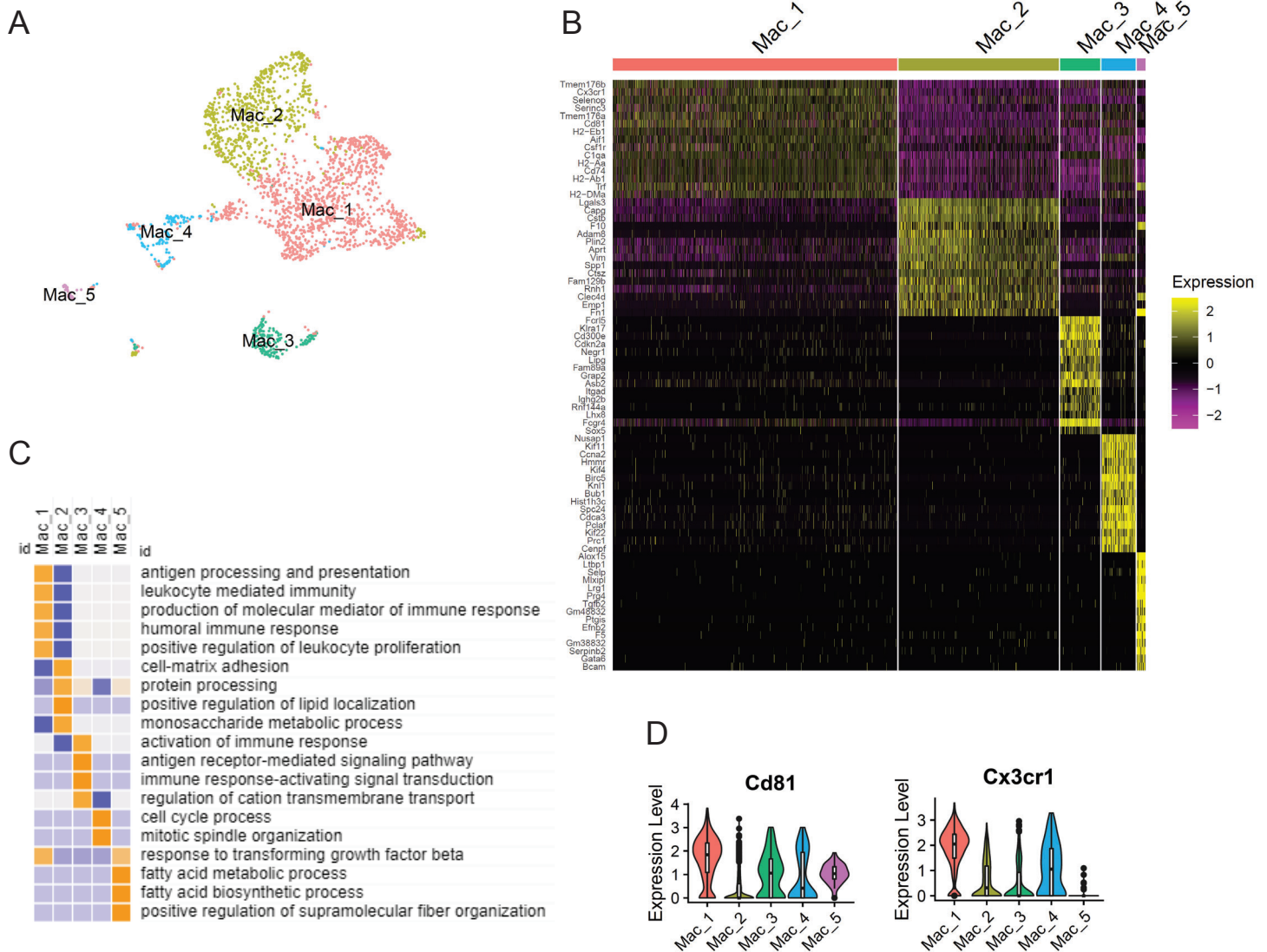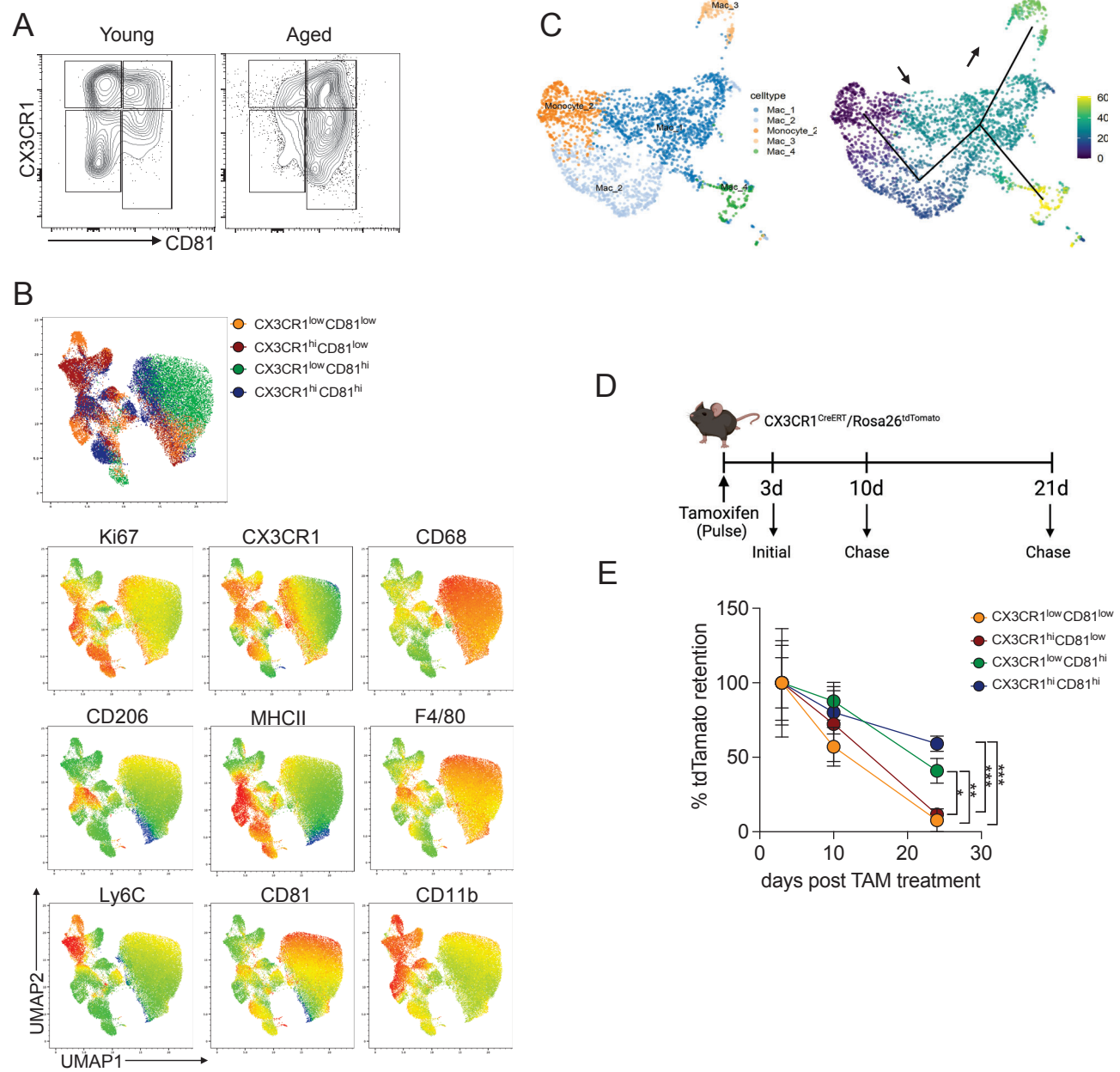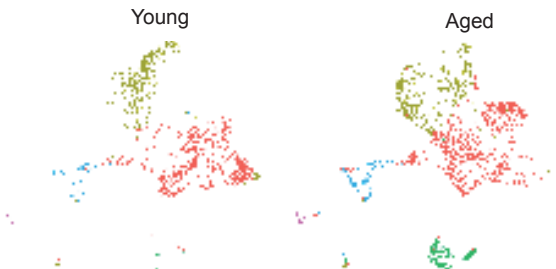
599

Figure 1

A

Young: n = 3
Aged: n =3

Cell sorting

myeloid cells
live CD45+CD11b+

B

C

D

Figure 2

A

B

C

D

Figure 3

A

Young   Aged

CX3CR1 / CD81

B

- CX3CR1^{low}CD81^{low}
- CX3CR1^{hi}CD81^{low}
- CX3CR1^{low}CD81^{hi}
- CX3CR1^{hi}CD81^{hi}

Ki67   CX3CR1   CD68

CD206   MHCII   F4/80

Ly6C   CD81   CD11b

UMAP2 / UMAP1

C

celltype
- Mac_1
- Mac_2
- Monocyte_2
- Mac_3
- Mac_4

Mac_3
Monocyte_2
Mac_1
Mac_2
Mac_4

60 40 20 0

D

CX3CR1^{CreERT}/Rosa26^{tdTomato}

3d   10d   21d

Tamoxifen
(Pulse)

Initial   Chase   Chase

E

- CX3CR1^{low}CD81^{low}
- CX3CR1^{hi}CD81^{low}
- CX3CR1^{low}CD81^{hi}
- CX3CR1^{hi}CD81^{hi}

% tdTamato retention

days post TAM treatment

*   **   ***   ***

Figure 4

A

Young   Aged

B

C

D

Outgoing signals   Incoming signals

E

Outgoing CCL signals

Incoming GALECTIN signaling

Figure 5