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1 **Origin and microenvironment contribute to the sexually dimorphic**
2 **phenotype and function of peritoneal macrophages.**

3

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1 **Abstract**

2

3 Macrophages reside in the body cavities where they maintain serosal homeostasis and provide immune
4 surveillance. Peritoneal macrophages are implicated in the aetiology of pathologies including
5 peritonitis, endometriosis and metastatic cancer thus understanding the factors that govern their
6 behaviour is vital. Using a combination of fate mapping techniques, we have investigated the impact of
7 sex and age on murine peritoneal macrophage differentiation, turnover and function. We demonstrate
8 that the sexually dimorphic replenishment of peritoneal macrophages from the bone marrow, which is
9 high in males and very low in females, is driven by changes in the local microenvironment that arise
10 upon sexual maturation. Population and single cell RNAseq revealed striking dimorphisms in gene
11 expression between male and female peritoneal macrophages that was in part explained by differences
12 in composition of these populations. By estimating the time of residency of different subsets within the
13 cavity and assessing development of dimorphisms with age and in monocytopenic *Ccr2*^{-/-} mice, we
14 demonstrate that key sex-dependent features of peritoneal macrophages are a function of the differential
15 rate of replenishment from the bone marrow while others are reliant on local microenvironment signals.
16 Importantly, we demonstrate that the dimorphic turnover of peritoneal macrophages contributes to
17 differences in the ability to protect against pneumococcal peritonitis between the sexes. These data
18 highlight the importance of considering both sex and age in susceptibility to inflammatory and
19 infectious disease.

20

21

22

1 Introduction

2

3 Macrophages are present in every tissue of the body, where they provide immune protection
4 and orchestrate tissue repair following insult or injury. Peritoneal macrophages are arguably
5 the most studied population of macrophages in the body, having been used extensively as a
6 convenient source of macrophages for *ex vivo* analyses for decades. Despite this, the
7 heterogeneity of peritoneal macrophages and much of the biology that governs their
8 development, differentiation and function remains unclear. Macrophages in the peritoneal
9 cavity are programmed for ‘silent’ clearance of apoptotic cells, maintenance of innate B1 cells
10 through secretion of CXCL13, and for immune surveillance of the cavity and neighbouring
11 viscera¹⁻⁴. However, they are also implicated in many pathologies, including peritonitis,
12 endometriosis, post-surgical adhesions, pancreatitis and metastatic cancer⁵⁻¹⁵, although the
13 exact role(s) they play in these processes is not fully understood.

14 Under physiological conditions, at least two macrophage populations are present in the
15 murine peritoneal cavity, with those expressing high levels of F4/80, CD11b and CD102
16 outnumbering their F4/80^{lo}MHCII⁺ counterparts by approximately 10-fold. F4/80^{hi}CD102⁺
17 macrophages (sometimes referred to as ‘large’ peritoneal macrophages¹⁶) rely on the
18 transcription factors C/EBP β and GATA6 for their differentiation and survival¹⁷⁻²⁰, with the
19 latter under the control of retinoic acid proposed to derive, in part, from the omentum¹⁹. In
20 contrast, F4/80^{lo}MHCII⁺ macrophages (sometimes referred to as ‘small’ peritoneal
21 macrophages) rely on IRF4 for their differentiation and can be further defined by their
22 expression of CD226 and the immunomodulatory molecule RELM α ^{21,22}. Notably, recent
23 studies employing lineage tracing techniques have established that F4/80^{lo}MHCII⁺
24 macrophages arise postnatally, are short-lived and replaced by Ly6C^{hi} classical monocytes in
25 a CCR2-dependent manner²⁰⁻²³. In contrast, F4/80^{hi}CD102⁺ macrophages are longer-lived
26 cells that originally derive from embryonic sources, but are subsequently replaced by cells of
27 haematopoietic stem cell (HSC) origin^{21,24}. Importantly, we have recently shown that unlike
28 resident macrophages in numerous other tissues the turnover of peritoneal F4/80^{hi}CD102⁺
29 macrophages from the bone marrow is highly sex-dependent, with high and low rates in male
30 and female mice respectively²¹. We have also shown that long-lived macrophages can be
31 identified by their expression of the phagocytic receptor, Tim4, whereas most recent
32 descendants of BM-derived cells amongst the F4/80^{hi}CD102⁺ macrophage compartment are
33 Tim4⁻²¹. Indeed Tim4 expression has been shown to a feature of long-lived macrophages in
34 other tissues²⁵⁻²⁹. However, it remains unclear if further heterogeneity exists amongst these

1 broadly-defined populations and if sexually-dimorphic turnover influences the composition
2 and function of the F4/80^{hi}CD102⁺ macrophage population in other ways.

3 Sex is a variable often overlooked in immunological research ³⁰ despite strong sex
4 biases in many pathologies including autoimmune disorders and infection susceptibility ³¹.
5 Notably, sex dimorphisms in the immune system are present a diverse range of species from
6 insects, bird, lizards and mammals ³¹, demonstrating this is an evolutionary conserved
7 phenomenon. It is therefore essential to understand how intrinsic factors such as sex control
8 the behaviour of innate immune effector cells. Specifically, sex has been proposed to affect
9 macrophage behaviour, such as influencing the differentiation of brain microglia ³²⁻³⁴ and sex
10 hormones appear able to directly regulate gene expression ³⁵ and proliferation ³⁶ of
11 macrophages. While previous studies have considered the effects of sex on peritoneal
12 macrophage behaviour, many of these have focussed on *in vitro* functional assessments using
13 macrophages elicited by injection of an irritant or inflammatory agent ³⁷, or have not
14 appreciated the complexity of the peritoneal macrophage compartment ^{36,38}.

15 Here we have used a combination of fate-mapping techniques together with population-
16 level and single cell RNA sequencing (scRNAseq) to dissect the role of sex in the composition,
17 environmental imprinting and function of peritoneal macrophages. We show that the
18 F4/80^{hi}CD102⁺ macrophage population is heterogeneous and that dimorphic turnover is
19 associated with divergence in the heterogeneity of this compartment with age. Specifically, we
20 demonstrate that the sexual dimorphism in replenishment from the bone marrow and phenotype
21 arise following sexual maturation. Furthermore, we provide examples of transcriptional and
22 functional dimorphisms that arise due to sex differences in turnover versus those arising
23 directly from sex differences in the peritoneal microenvironment. Importantly, we identify the
24 C-type lectin receptor CD209b (also known as Specific ICAM3-grabbing nonintegrin-related
25 1; SIGN-R1) as a marker whose expression is determined by replenishment that becomes
26 increasingly dimorphic with age, and show that sex-dependent resistance to pneumococcal
27 peritonitis arises, in part, due to dimorphic expression of CD209b.

28

29 **Results**

30 **Environment drives sexual dimorphism in macrophage replenishment in the peritoneal cavity**

31 We first set out to determine if the dimorphic effects in peritoneal macrophage replenishment were due
32 to the peritoneal environment or to cell-intrinsic differences in the ability of male and female monocytes
33 to generate F4/80^{hi}CD102⁺ macrophages at this site. To this end, we generated sex-mismatched, tissue-
34 protected bone marrow (BM) chimeric mice to measure the turnover of peritoneal F4/80^{hi}CD102⁺
35 macrophages from the BM (**Figure 1a**) and assess the role of sex in this process. Wild type (CD45.1/.2⁺)
36 mice were irradiated, with all but the head and upper torso protected with lead to prevent direct exposure

1 to ionising radiation (**Figure 1b**), before being reconstituted with sex-matched (female > female, male
2 > male) or sex-mismatched (female > male, male > female) BM. Following at least 8 weeks
3 reconstitution, the non-host chimerism was measured in peritoneal macrophages. Consistent with our
4 previous work ²¹, only low levels of non-host chimerism could be detected amongst peritoneal
5 F4/80^{hi}CD102⁺ macrophages from female > female BM chimeric mice, whereas high levels were
6 detected in their male > male counterparts (**Figure 1c&d**), confirming marked sex dimorphism in
7 macrophage turnover. Importantly, this dimorphism was specific to F4/80^{hi}CD102⁺ peritoneal
8 macrophages, as all other leukocyte subsets showed identical replenishment in male and female BM
9 chimeric mice (**Supplementary Figure 1a**). Strikingly, F4/80^{hi}CD102⁺ peritoneal macrophages from
10 sex mismatched (female > male) chimeras had similar levels of chimerism to male > male chimeras
11 (**Figure 1c&d**), demonstrating that female and male monocytes have equal ability to generate
12 F4/80^{hi}CD102⁺ macrophages in the male peritoneal cavity. Female recipients rejected male BM and
13 thus chimerism in this group could not be determined.

14 The omentum has been implicated in the differentiation of F4/80^{hi} macrophages in the
15 peritoneal cavity, potentially acting as site of macrophage maturation ^{19,39,40}. Indeed, CD102⁺
16 macrophages that co-express GATA6, can be detected amongst omental isolates ¹⁹, together with
17 CD102⁻MHCII⁺ macrophages and a population of Ly6C⁺ CD11b⁺ cells similar to monocytes (**Figure**
18 **1e** and **Supplementary Figure 1b-c**). To determine if the dimorphic replenishment of peritoneal
19 F4/80^{hi}CD102⁺ macrophages arises in the omentum, we assessed non-host chimerism in the
20 macrophage populations within this site. While this showed clear differences in the turnover of CD102-
21 defined macrophage populations from BM, with higher replenishment in the CD102⁻ fraction, no sex
22 dimorphism was detected in any monocyte/macrophage population within the omentum (**Figure 1f**).
23 Furthermore, the chimerism of omental and peritoneal CD102⁺ macrophages in male recipients was
24 identical, rather than showing the gradation that would have been expected if omental macrophages
25 were intermediate precursors between monocytes and cavity CD102⁺ cells (**Supplementary Figure**
26 **1d**). Thus, the sexual dimorphism in peritoneal F4/80^{hi}CD102⁺ macrophage replenishment is driven by
27 factors present in the local environment.

28

29 **Sexual dimorphism in peritoneal macrophage replenishment occurs following sexual maturity**

30 To extend these findings and to assess macrophage turnover at different stages of maturity, we next
31 used a genetic fate mapping approach. Adoptive transfer experiments suggest F4/80^{lo}MHCII⁺
32 macrophages in the peritoneal macrophage compartment act, in part, as precursors of F4/80^{hi}CD102⁺
33 macrophages ²⁰ and we have recently shown that this differentiation can be mapped by exploiting their
34 expression of CD11c ²¹. Thus, in CD11c^{Cre}.*Rosa26*^{LSL-eYFP} mice (**Figure 2a**), in whom active or historic
35 expression of CD11c leads to irreversible labelling with eYFP, labelled cells accumulate with age in
36 the F4/80^{hi}CD102⁺ macrophage compartment, despite these cells themselves not actively expressing
37 CD11c ²¹. We therefore used CD11c^{Cre}.*Rosa26*^{LSL-eYFP} mice to compare the rate of eYFP⁺ cell
38 accumulation in peritoneal F4/80^{hi}CD102⁺ macrophages from male and female mice. In

1 juvenile/prepubescent mice (4 weeks of age), the extent of eYFP labelling was relatively similar
2 between male and female peritoneal F4/80^{hi}CD102⁺ macrophages and indeed was marginally higher in
3 female mice (**Figure 2b**). By 16 weeks of age, the frequency of eYFP⁺ cells amongst F4/80^{hi}CD102⁺
4 macrophages had increased in both male and female mice compared with their 4-week-old counterparts.
5 However, although there was no difference in CD11c protein expression by male and female
6 F4/80^{hi}CD102⁺ macrophages (**Figure 2c**), significantly higher levels of eYFP labelling were detected
7 amongst male peritoneal macrophages, consistent with more rapid accumulation of newly differentiated
8 macrophages in male mice (**Figure 2b**). Consistent with our previous findings made using tissue-
9 protected BM chimeras²¹, the sexual dimorphism in eYFP labelling was not detected in F4/80^{hi}CD102⁺
10 macrophages from the pleural cavity (**Figure 2d**), where both male and female pleural cells exhibited
11 high levels of labelling that were equivalent to those seen in the male peritoneal cavity by 16 weeks .
12 Collectively, these data confirm that the peritoneal environment controls macrophage turnover and
13 suggest that dimorphisms arise in this site following sexual maturity.

14

15 **Ovariectomy leads to increased macrophage replenishment**

16 The onset of sexually dimorphic turnover of peritoneal macrophages following sexual maturation and
17 the uniquely slow replenishment of female peritoneal macrophages suggested that factors involved in
18 female reproductive function may drive this dimorphism. Therefore, we next assessed macrophage
19 turnover in females after ovariectomy (OVX). Thus, female > female tissue protected BM chimeric
20 were generated and after 8 weeks reconstitution, both ovaries were surgically removed (bilateral OVX),
21 before measuring non-host chimerism after another 8 weeks. To account for the potential effects of
22 surgery on macrophage replenishment, BM chimeric mice receiving sham surgery or unilateral OVX
23 were used as controls, together with unmanipulated BM chimeric mice. As expected, the cessation of
24 ovarian estradiol production caused by bilateral OVX led to complete atrophy of the uterine horn; this
25 was not seen in mice with unilateral OVX, or in other control groups (**Figure 3a**). Bilateral OVX had
26 no effect on the numbers of F4/80^{hi}CD102⁺ and CD102⁻MHCII⁺ macrophages in the peritoneal cavity
27 when compared with the control groups (**Figure 3b**). Although bilateral OVX led to increased
28 proportions and absolute numbers of eosinophils, these differences did not attain statistical significance
29 and the opposite pattern was found with B1 cells (**Supplementary Figure 2**). Importantly and in
30 striking contrast to the very low levels of chimerism (~1%) detected in unmanipulated control chimeras
31 (**Figure 3c**), sham surgery and unilateral OVX led to significant increases the level of chimerism
32 compared with unmanipulated chimeric mice, demonstrating that minimally-invasive laparotomy itself
33 appears to have long term effects on the dynamics of peritoneal macrophages in female mice.
34 Nevertheless, complete removal of the ovaries further elevated macrophage turnover, with chimerism
35 reaching approximately 12%. No difference was found between the chimerism seen after sham surgery
36 with or without unilateral OVX, indicating that the OVX procedure itself does not exaggerate the effects
37 of laparotomy and that it is the complete loss of ovarian function that underlies the further elevation in
38 macrophage turnover that results from bilateral OVX. Consistent with these results, significantly more

1 Tim4⁻CD102⁺ macrophages were present in the cavity of mice that received bilateral OVX than any
2 other group (**Figure 3d**), further supporting the idea of elevated macrophage replenishment from BM.
3 Notably, no differences in chimerism or in Tim4-defined subsets could be detected amongst
4 F4/80^{hi}CD102⁺ macrophages from the pleural cavity, again confirming that the effect of surgery and
5 ovariectomy on macrophage turnover are specific to the peritoneal cavity (**Figure 3b, c**).

6 Estrogens are the prototypical female sex steroid hormones which are ablated by OVX. To
7 assess if estrogen influences macrophage replacement, we repeated the OVX experiment with an
8 additional group of bilateral OVX mice receiving exogenous estradiol (E2). However, while this
9 treatment reversed OVX-mediated atrophy of the uterine horns and peritoneal eosinophilia, it had no
10 effect on the heightened rate of replenishment of F4/80^{hi}CD102⁺ peritoneal macrophages in OVX mice,
11 suggesting that estradiol is not directly responsible for generating the sex dimorphism in peritoneal
12 macrophage turnover (**Figure 3e**). As males exhibit much greater levels of adipose tissue in the
13 peritoneal cavity (**Supplementary Figure 3**) and a common feature of ovariectomy/oophorectomy in
14 mice and humans is increased adiposity^{41,42}, something we also noted in our experiments (data not
15 shown), we combined a high fat diet (HFD) with our BM chimeric system to reveal if changes in
16 adiposity affect replenishment of peritoneal F4/80^{hi}CD102⁺ macrophage. However, replenishment was
17 not affected by diet in either males or females, despite the expected increase in body weight and adipose
18 tissue seen in mice on an HFD (**Supplementary Figure 3**). Hence, sexual maturation controls
19 dimorphic turnover in the peritoneal cavity through a mechanism controlled at least in part by the female
20 reproductive system, but independently of estrogen and local fat deposition.

21
22

23 **Sex determines the transcriptional signature of peritoneal macrophages**

24 The difference in macrophage replenishment prompted us to assess the wider effects of sex on the
25 imprinting of peritoneal macrophage identity and function. We therefore first performed population-
26 level RNASeq on peritoneal F4/80^{hi}CD102⁺ macrophages FACS-purified from unmanipulated 10-12
27 week old male and female mice (**Supplementary Figure 4**). To limit potential confounding effects of
28 estrous cycle, the stage of each female mouse was confirmed by vaginal cytology and samples pooled
29 to include cells from all stages of the cycle. Furthermore, to limit the effects of circadian influence,
30 mice in each biological replicate were euthanised at the same time each day. Unbiased clustering was
31 then used to group the populations based on sex, with sex explaining 81% of the variance within the
32 datasets (**Figure 4a**) and differential gene expression analysis revealed that 486 mRNA transcripts were
33 differentially expressed (>1.5fold) between female and male peritoneal CD102⁺ macrophages (**Figure**
34 **4b & Supplementary Table 1**). Analysis of the 148 mRNA transcripts more highly expressed in female
35 peritoneal macrophages revealed that a large proportion was associated with immune function,
36 including the C-type lectin receptors *Clec4g*, *Cd209a* and *Cd209b*, the complement components *C4b*,
37 *C1qa*, and *C3* the immunoregulatory cytokine *Tgfb2*, the B cell chemoattractant *Cxcl13*, and as expected
38 the phagocytic receptor *Timd4* (**Figure 4c & Supplementary Table 1**). Consistent with this, ‘immune

1 response' and 'immune system processes' were among the top pathways identified by gene-set
2 enrichment analysis in genes up-regulated in female cells (**Supplementary Table 2**). Transcripts for
3 the apolipoproteins *ApoE*, *Saa2*, *Saa3* and *ApoC1* were also expressed more highly in female cells.
4 Notably, in contrast to previous work that assessed basal gene expression by total peritoneal cells across
5 the sexes³⁸, we did not detect any dimorphism in expression of toll-like receptors (TLRs), the TLR
6 adaptor molecule MyD88 or CD14 (**Supplementary Figure 5**). Moreover, the dimorphic cassette of
7 genes we identified is distinct from that recently shown to be sexually dimorphic in microglia
8 (**Supplementary Figure 5**).

9 We used flow cytometry to confirm higher expression of *Cd209b*, *Cxcl13*, and *ApoC1* by female
10 macrophages, as these were the most differentially expressed non-X-linked genes with mapped read
11 counts greater than 10. This analysis revealed unexpected heterogeneity within resident peritoneal
12 macrophages. For instance, only a proportion of male and female CD102⁺ macrophages expressed
13 CD209b, although the frequency of these was greater in females than in males (35% and 20%
14 respectively). Moreover, CD209b was expressed at a higher level on a per cell basis by female CD102⁺
15 macrophages compared with their male counterparts (**Figure 4c**), a finding consistent across different
16 strains, including *Rag1*^{-/-} mice, and mice from different housing environments (**Supplementary Figure**
17 **6**). Due to the unavailability of commercial antibodies for CXCL13 and ApoC1, we used PrimeFlow
18 technology to measure CXCL13 and ApoC1 mRNA at a single cell level using flow cytometry. Again,
19 this revealed that a greater proportion of female CD102⁺ macrophages expressed mRNA for CXCL13
20 and ApoC1 than their male counterparts, and CXCL13 mRNA was also higher on a per cell basis in
21 female cells (**Figure 4d, e**). In contrast, PrimeFlow measurement of mRNA for ApoE, the most highly
22 expressed of all differentially expressed genes by female cells by RNAseq, revealed that all peritoneal
23 macrophages expressed ApoE irrespective of sex, but that expression was higher in female cells on a
24 per cell basis. Hence, the transcriptional differences seen at population level appear to result from
25 differential gene expression at a single cell level but also from different frequencies of gene-expressing
26 cells amongst the CD102⁺ population.

27 The majority of genes more highly expressed by male peritoneal CD102⁺ macrophages were
28 associated with cell cycle, including *Cdk1*, *E2f2*, and *Mki67* (**Figure 4b & Supplementary Table 1**).
29 Pathway analysis also revealed that at least 162 of the 338 genes differentially up-regulated in male
30 CD102⁺ macrophages were associated with proliferation, and cell cycle-related processes predominated
31 among the significantly enriched pathways (**Supplementary Table 2**). Short-term BrdU pulse-chase
32 experiments confirmed that male CD102⁺ macrophages have elevated levels of *in situ* proliferation
33 compared with their female counterparts (**Figure 4g**). These analyses also identified that *Relna*, which
34 encodes the immunomodulatory cytokine RELM α and is expressed specifically by those resident
35 peritoneal macrophages that are most recently-derived from monocytes²¹, was differentially expressed
36 between sexes, with higher expression by male cells at both the mRNA and protein level (**Figure 4h**).

37 Of note, a number of genes previously reported to distinguish long-lived, embryonically-
38 derived macrophages from those of recent BM origin in the lung and liver were more highly expressed

1 in females. These included receptors involved in phagocytosis and immunity (i.e. *Timd4*, *Colec12*, and
2 *Cd209* family members), *Apoc1*, as well as the bone morphogenic receptor *Bmpr1a* (**Supplementary**
3 **Table 1**). Lowering the stringency of selection of differentially-expressed genes identified additional
4 genes within the female-specific cluster that have been associated with embryonically-derived or long-
5 lived macrophages, including *Marco* and *Cd163* that also encode phagocytic receptors (data not shown).
6 Furthermore, to discern systemic from local effects of sex, we compared gene expression by CD102⁺
7 macrophages from female peritoneal cavity to pleural CD102⁺ macrophages from both sexes. This
8 analysis identified a module of 18 genes that was uniquely upregulated by female peritoneal
9 macrophages, and that included *Apoc1*, *Cd209b*, and *Colec12*, as well as *Saa3*, *C4b*, and *Tgfb2*
10 (**Supplementary Table 3**). Conversely, the 86 genes uniquely downregulated by female peritoneal
11 macrophages compared with the other CD102⁺ populations were highly enriched for cell cycle related
12 genes and pathways (**Supplementary Table 3&4**). Thus, the more limited proliferative activity of
13 female peritoneal macrophages and their expression of numerous immune-related genes appear either
14 related to their slower replenishment from the bone-marrow or regulated directly by the unique signals
15 present within the female peritoneal microenvironment.

16

17 **scRNAseq analysis reveals dimorphic macrophage heterogeneity**

18 We next applied single cell RNA sequencing (scRNAseq) to determine whether the transcriptional
19 differences seen in our population-level data were the result of gene differences at a single cell level or
20 if dimorphism was a reflection of differential subset composition between the sexes. A broad approach
21 was used to capture all CD11b⁺ cells depleted of granulocytes and B1 B cells to allow both CD102⁻
22 F4/80^{lo}MHCII⁺ macrophages and resident CD102⁺ cells to be examined. These cells were FACS-
23 purified from age-matched 12-week-old male and female mice and droplet-based scRNASeq performed
24 using the 10X Genomics platform. 10,000 sorted cells of each sex were sequenced and following quality
25 control, analysis was performed on 4341 and 2564 cells from female and male respectively.

26 Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction analysis
27 revealed 6 clusters that were present in both male and female cells (**Figure 5a, b**). Given that the starting
28 population of CD11b⁺ cells is known to be phenotypically heterogeneous, containing resident
29 CD102⁺F4/80^{hi} macrophages, CD102⁻F4/80^{lo} MHCII⁺ macrophages and CD11c⁺MHCII⁺ cDC2²⁰⁻²³,
30 we first used a panel of known markers to validate subset identity (**Figure 5b**). 3 clusters of resident
31 macrophages (3-5) could be identified on the basis of their high expression of *Adgre1* (F4/80) and *Icam2*
32 (CD102). As expected, these were clearly distinct from short-lived CD102⁻F4/80^{lo}MHCII⁺
33 macrophages and cDC2, which were found in clusters 1 and 2 respectively, and expressed *Ccr2* (**Figure**
34 **5c, d**)²¹. However, CD102⁻F4/80^{lo}MHCII⁺ macrophages and cDC2 could be distinguished from one
35 another on the basis of expression of the DC markers *Cd209a* and *Napsa*^{27,43}, and of *Retnla* and *Fcrls*,
36 which we and others have shown to be signature markers of cavity CD102⁻F4/80^{lo}MHCII⁺ macrophages
37^{21,22,44}. Cluster 6 was defined by genes associated with cell cycle, such as *Mki67* and *Birc5*, suggesting
38 this cluster represents proliferating cells. In both sexes, the majority of cells was in cluster 5 (**Figure**

1 **5e)**, which was characterised by markers of resident F4/80^{hi}CD102⁺ macrophages including *Icam2*,
2 *Prg4* and *Tgfb2* (**Figure 5c, d & Supplementary Table 5**) that form part of the core peritoneal
3 macrophage-specific transcriptional signature⁴⁵; cluster 5 cells also expressed markers of long-lived
4 macrophages, including *Timd4* and *Apoc1*, confirming the findings above. Although the cells in cluster
5 3 expressed *Icam2*, they also expressed a number of genes that were highly expressed by the CD102⁻
6 F4/80^{lo}MHCII⁺ macrophages in cluster 2, such as *Retnla*, *H2.Aa* and *Ccr2*, suggesting a common origin
7 of these clusters, or a close relationship between them. This analysis also identified genes uniquely
8 expressed by cluster 3, including *Folr2*, which encodes the beta subunit of the folate receptor (FR β).
9 Although cluster 4 showed a distinct pattern of gene expression, such as high expression of *ApoE*, it
10 also shared features with cluster 3 and cluster 5, suggesting it may contain differentiation intermediates.
11 Consistent with our earlier analysis, we found that the *Timd4*-expressing cluster 5 was more abundant
12 amongst female cells, whereas more male cells were found within clusters 1, 2, 3 and 6 (**Figure 5c**).

13 We next used flow cytometry to determine if we could validate the additional heterogeneity
14 uncovered by our scRNAseq analysis. Given that MHCII-associated genes appeared to define
15 heterogeneity amongst Tim4⁻CD102⁺ macrophages (i.e. clusters 3 & 4), we assessed expression of
16 MHCII by Tim4-defined subsets of CD102⁺ macrophages. This confirmed that a proportion of Tim4⁻
17 CD102⁺ macrophages expressed MHCII, albeit at lower levels than CD102⁻F4/80^{lo}MHCII⁺
18 macrophages, whereas Tim4⁺ macrophages had negligible MHCII expression (**Figure 5f**). Thus, we
19 used a combination of Tim4 and MHCII to identify macrophage subsets and assessed expression of
20 other subset defining markers from the scRNAseq analysis. Consistent with the analysis above, we
21 found that ApoC1 expression was essentially exclusive to Tim4⁺MHCII⁻ macrophages (**Figure 5g**),
22 whereas both MHCII-defined Tim4⁻ macrophages lacked ApoC1 expression. Expression of CXCL13
23 was also highest amongst Tim4⁺MHCII⁻ cells, although interestingly, the proportion of CXCL13⁺ cells
24 increased progressively from Tim4⁻MHCII⁺ to Tim4⁻MHCII⁻ to Tim4⁺MHCII⁻ CD102⁺ macrophages
25 (**Figure 5g**). Although not identified as a cluster defining gene in our scRNAseq analysis due to low
26 coverage, we found that CD209b displayed the same pattern of expression as CXCL13 (**Figure 5g**).
27 Consistent with the idea that they may derive from CD102⁻F4/80^{lo}MHCII⁺ macrophages, the expression
28 of RELM α and CX3CR1 was highest on Tim4⁻MHCII⁺ macrophages and was essentially absent from
29 Tim4⁺MHCII⁻ CD102⁺ macrophages. The majority of Tim4⁻MHCII⁺ macrophages expressed high
30 levels of FR β , whereas all other populations had little or no expression, consistent with our scRNAseq
31 analysis. While *ApoE* was proposed to define cluster 4 in our scRNAseq analysis, consistent with our
32 analysis above, we found it was expressed by all CD102⁺ macrophages, although, in females, most
33 highly expressed by Tim4⁻MHCII⁺ and the level decreased progressively to Tim4⁺MHCII⁻ CD102⁺
34 macrophages. Finally, we returned to using CD11c^{Cre}.*Rosa26*^{LSL-eYFP} mice to assess if MHCII/Tim4-
35 defined subsets showed differential levels of replenishment. Notably, we found that MHCII-expressing
36 Tim4⁻CD102⁺ macrophages showed equivalent labelling to CD102⁻F4/80^{lo}MHCII⁺ macrophages in
37 female CD11c^{Cre}.*Rosa26*^{LSL-eYFP} mice, indicative of more recent derivation from CD102⁻
38 F4/80^{lo}MHCII⁺ cells. Consistent with their intermediate transcriptional profile, Tim4⁻CD102⁺

1 macrophages that had lost MHCII expression showed intermediate labelling when compared with their
2 MHCII⁺Tim4⁻ and Tim4⁺ counterparts. No difference in eYFP labelling between Tim4-defined subsets
3 was noted in male mice, consistent with more rapid replenishment of all subsets of macrophages in this
4 environment.

5 Collectively these data show that excluding proliferating cells, resident peritoneal macrophages
6 comprise three main clusters, with Tim4⁻ macrophages displaying an intermediate phenotype compared
7 with F4/80^{lo}MHCII⁺ macrophages and Tim4⁺ macrophages.

8

9 **Differential replenishment and environmental signals drive the dimorphic features of peritoneal** 10 **macrophages**

11 To dissect the dimorphic features of CD102⁺ macrophages that could be related to longevity from those
12 more directly controlled by dimorphic environmental signals, we next assessed expression of these in
13 *Ccr2*^{-/-} mice in whom macrophage replenishment is markedly reduced due to severe monocytopenia
14 ^{46,47}. Strikingly, the frequency of Tim4⁻ macrophages, as well as those expressing RELM α , FR β or
15 MHCII were markedly reduced in *Ccr2*^{-/-} mice compared with *Ccr2*^{+/+} mice irrespective of sex,
16 confirming these cells to be recently derived from monocytes (**Figure 6a & Supplementary Figure**
17 **7**). In males, CCR2 deficiency also led to reduced expression of ApoE and emergence of an ApoE⁻
18 subset of CD102⁺ macrophages (**Figure 6b**). In contrast, a higher proportion of CD102⁺ macrophages
19 in *Ccr2*^{-/-} mice expressed CD209b and ApoC1, markers that are characteristic of the Tim4⁺MHCII⁻
20 subset, suggesting these markers may be expressed selectively by long-lived macrophages (**Figure 6b**).
21 Consistent with this, Tim4⁺ macrophages expressing CD209b displayed the lowest level of replacement
22 by donor cells in BM chimeras when compared with all other CD209b/Tim4-defined macrophages,
23 even in male mice where overall replenishment from the bone marrow is markedly higher (**Figure 6c**).
24 The low levels of replacement of peritoneal CD209b⁺Tim4⁺ macrophages does not reflect derivation
25 from yolk sac progenitors, as, unlike microglia in the brain, these cells are not labelled in male or female
26 *Cdh5*^{Cre-ERT2}.*Rosa*^{LSL-tdT} mice, which allow tracing of cells arising from yolk sac haematopoiesis ⁴⁸.
27 Similar results were obtained with CD209b⁺Tim4⁺ macrophages in the pleural cavity (**Figure 6d**).
28 Hence, despite being long-lived, CD209b⁺Tim4⁺ macrophages derive from conventional
29 haematopoiesis in both sexes. Importantly, temporal analysis revealed that while little difference in
30 abundance of CD209b-expressing CD102⁺ macrophages was seen in pre-pubescent (4-5-week-old)
31 male and female mice, these cells accumulated progressively in the cavity of female mice following
32 sexual maturation. This did not occur in male mice, consistent with their higher rate of replenishment
33 from the bone marrow and indicating that acquisition of CD209b expression appears to be associated
34 with time-of-residency in the female cavity (**Figure 6e**).

35 Not all dimorphic features of peritoneal CD102⁺ macrophages were influenced by their rate of
36 replenishment. For instance, the intrinsically higher expression of CXCL13 by female macrophages
37 was not altered by CCR2 deficiency (**Figure 6f**). In parallel, although we confirmed previous findings
38 of a clear dimorphism in the numbers of B1 cells between adult male and female mice ¹⁵ and this

1 developed gradually following sexual maturation (**Supplementary Figure 8**), this phenomenon
2 remained in *Ccr2*^{-/-} mice (**Figure 6g**). Similarly, while the higher levels of proliferation by male
3 CD102⁺ macrophages developed following sexual maturation (**Figure 6h**), this was unaffected by
4 CCR2 deficiency (**Figure 6i**). This evidence that certain dimorphic features are driven by
5 environmental factors, independent of cell replenishment was supported further by the fact that
6 macrophages derived from female BM in the cavity of chimeric male showed levels of proliferation
7 that were identical to those of male BM derived macrophages in the male cavity and were higher than
8 those of female BM derived macrophages in female cavity (**Figure 6i**). Thus, the differential
9 proliferation of female and male macrophages is not due to cell-intrinsic differences in their
10 proliferative activity.

11 Taken together these data demonstrate that both local imprinting and differential turnover
12 contribute to the sexual dimorphisms seen in peritoneal macrophages.

13
14

15 **Differential CD209b expression confers an advantage on female macrophages in the setting of** 16 **pneumococcal peritonitis**

17 We postulated that differential expression of key pattern recognition receptors such as CD209b might
18 endow female macrophages with an enhanced ability to deal with bacterial infection. To test this idea,
19 we examined the acute peritonitis caused by the gram-positive bacterium *Streptococcus pneumoniae*
20 (**Figure 7a**), a localised model of infection in which resident macrophages, and in particular CD209b,
21 are indispensable for protective immunity^{49,50} whereas recruitment of neutrophils is not required⁴⁹ and
22 hence avoids any confounding effects of systemic sex-dependent effects on innate immune responses
23 that have been reported previously⁵¹. As CD209b is expressed exclusively by CD102⁺ macrophages in
24 the peritoneal cavity (**Supplementary Figure 6**), this model allowed us to directly assess the
25 importance of differential CD209b expression by CD102⁺ macrophages in bacterial elimination.
26 Strikingly, females showed enhanced capability to control *S. pneumoniae* infection, with significantly
27 lower levels of bacteria in peritoneal fluid of female mice compared with their male counterparts 20hrs
28 after inoculation (**Figure 7b**). Fewer neutrophils and Ly6C^{hi} monocytes were present in the female
29 cavity compared with male mice (**Figure 7c**), consistent with a model in which resident macrophages
30 control infection⁴⁹. In contrast, while the well-documented macrophage ‘disappearance reaction’⁵²
31 occurred in both male and female mice after infection (**Figure 7d**), significantly higher numbers of
32 CD209b-expressing macrophages persisted in the female cavity (**Figure 7d**). Administration of an anti-
33 CD209b blocking antibody (22D1)⁵³ led to increased levels of bacteraemia in female mice, although
34 this did not attain statistical significance due to variance in bacterial counts in some mice in whom the
35 macrophage ‘disappearance reaction’ was more pronounced (**Figure 7e**).

36 Thus, dimorphic expression of key immune receptors and molecules leads to differential ability
37 to handle local bacterial infection.

38

1 Discussion

2 Understanding the extrinsic and intrinsic factors that govern tissue macrophage differentiation
3 is a key goal in the field of macrophage biology. Here we reveal a striking effect of sex on the
4 phenotypic and transcriptional identity of resident peritoneal macrophages and demonstrate that this
5 contributes to the sex-dependent resistance of mice to bacterial peritonitis. Moreover, we show that this
6 arises through a combination of dimorphic microenvironmental signals and sex-dependent differences
7 in the rate of macrophage renewal from the bone marrow.

8 Using classical defining markers such as F4/80, CD11b and CD102, we found peritoneal
9 macrophages from male and female mice to be phenotypically identical. Furthermore, while some
10 studies have reported that the number of peritoneal macrophages is greater in females^{38,51}, we did not
11 routinely detect significant differences in the number of peritoneal macrophages between the sexes.
12 However, mRNA sequencing revealed marked dimorphism in the transcriptional fingerprint of resident
13 peritoneal macrophages under homeostatic conditions. Importantly, this showed that female CD102⁺
14 macrophages express higher levels of genes associated with lipid uptake and transport as well as
15 immune defence/response, including those encoding complement components, the chemoattractant
16 CXCL13, and numerous receptors involved in recognition and uptake of pathogens and apoptotic cells.
17 In contrast, the signature of male peritoneal macrophages was dominated by cell cycle associated genes
18 consistent with their elevated levels of proliferation, a dimorphism we have reported previously²¹.
19 Although others have reported dimorphic expression of TLRs and CD14 by peritoneal macrophages,
20 no consistent pattern was observed in these studies^{37,38} and we found no significant difference in mRNA
21 transcripts of the adaptor protein MyD88 or any TLRs, consistent with more recent analysis of surface
22 protein expression⁵¹. We have also found no sex differences in CD14 expression at the gene or protein
23 level. These discrepancies may relate to differences in the nature of the cells being analysed, with one
24 study using peritoneal macrophages elicited by incomplete Freund's adjuvant³⁷ and the other assessing
25 gene expression by total peritoneal cells³⁸. In contrast, our analyses involved minimal handling and
26 used rigorously characterized resident macrophages.

27 Our further analyses revealed that many of the sexually dimorphic features of macrophages
28 arose following sexual maturation, including the higher expression of CD209b and Tim4 by female
29 macrophages. Furthermore, the enhanced accumulation of peritoneal B1 cells found in females was also
30 age-dependent, suggesting that the higher levels of CXCL13 production by female macrophages may
31 also be driven by sexual maturation. Dimorphic differences in the turnover of CD102⁺ resident
32 peritoneal macrophages also appeared to largely arise following sexual maturation. Notably, the
33 kinetics of labelling in CD11c^{cre}.*Rosa26*^{LSL-eYFP} mice and a significant reversal in the autonomy of
34 female peritoneal macrophages in BM chimeras following ovariectomy suggest this aspect of
35 dimorphism in normal mice reflects a switch from replenishment to self-maintenance in females. These
36 results are consistent with recent monocyte fate mapping using *Ms4a3*^{Cre}.*Rosa26*^{LSL-tdTomato} mice
37 showing that while monocytes contribute to the maintenance of peritoneal macrophages during the
38 perinatal and adolescent period, this process wanes during adulthood in female mice (⁵⁴ & F. Ginhoux,

1 personal communication). Hence, sexual maturation leads to dimorphic changes in replenishment,
2 proliferation and gene expression by peritoneal macrophages, at least some of which are driven by the
3 female peritoneal environment.

4 Despite a significant degree of transcriptional difference at the population level, single cell
5 mRNA sequencing showed that male and female resident macrophages encompassed very similar
6 transcriptionally-defined clusters of cells. However, the relative abundance of these clusters differed
7 between sexes. In this regard, CD102⁺ peritoneal macrophages could be divided into three predominant
8 transcriptionally-distinct clusters. Of these, the cells in cluster 3 expressed CD102 together with
9 MHCII, RELM α and CX3CR1, all of which are key markers of F4/80^{lo}MHCII⁺ peritoneal
10 macrophages, suggesting that cluster 3 may be recently derived from the F4/80^{lo}MHCII⁺ macrophage
11 population that is derived from blood monocytes in adult mice²¹⁻²³. As cells in cluster 4 shared features
12 with both the MHCII-defined cluster 3 and the dominant cluster 5 population of Tim4⁺ macrophages,
13 these may represent a further intermediate differentiation state. Consistent with this idea, in female mice
14 Tim4⁻MHCII⁻ CD102⁺ macrophages, which likely represent those in cluster 4, displayed an
15 intermediate degree of replenishment from the bone marrow in our fate mapping studies between that
16 of the rapidly replenished Tim4⁻MHCII⁺ CD102⁺ macrophages and slowly replenished Tim4⁺MHCII⁻
17 cells. Furthermore, all Tim4⁻ cells were largely ablated in female *Ccr2*^{-/-} mice. A linear-developmental
18 relationship that culminates at cluster 5 would be consistent with the greater abundance of cells in this
19 cluster in females, given the slower entry of bone-marrow-derived cells into the female CD102⁺
20 macrophage pool. However, it seems unlikely that such a linear developmental relationship between
21 clusters exists in males, as Tim4 and MHCII defined subsets were found to be replenished at similarly
22 high rates. Hence, what dictates cluster identity in males remains unclear.

23 Given that the rate of replenishment from BM was markedly different between the sexes, this
24 raised the possibility that transcriptional differences could reflect different ontogenies of male and
25 female peritoneal macrophages. Indeed, a number of the genes we found to be expressed more highly
26 by female peritoneal macrophages, including *Colecl2*, *Cd163*, *Bmpr1a*, *Cdc42bpa*, *Timd4*, *Apoc1*, and
27 members of the *Cd209* family have been reported to be expressed by embryonically, but not monocyte-
28 derived macrophages in other tissues^{25,55}. This could reflect an intrinsic property of their embryonic
29 origin, or that such cells are likely to have resided in the tissue for a long period. However the fact that
30 a proportion of BM-derived peritoneal macrophages can acquire the expression of at least some of these
31 “embryonic” signature markers (e.g. Tim4, CD209b) in the setting of tissue-protected BM chimeras,
32 suggests that this is more likely related to their time-of-residency rather than rigid differences related
33 to origin. Consistent with this, co-expression of Tim4 and CD209b identifies the longest-lived
34 macrophages in the peritoneal cavity irrespective of sex. The concept that macrophages require
35 prolonged residence within the tissue to acquire their characteristic features is consistent with work
36 from the Williams lab showing that acquisition of Tim4 expression by monocyte-derived cells that
37 engraft in the liver following deletion of endogenous Kupffer cells increases with time²⁵. Notably, of
38 the two populations of peritoneal macrophages that have been described in ascites fluid from patients

1 with decompensated cirrhosis, the subset that aligns with mouse resident F4/80^{hi} macrophages exhibits
2 significantly higher expression of *TIMD4*, *CD209*, *COLEC12*, *CD163* and *APOC1*⁵⁶, suggesting these
3 may represent phylogenetically conserved markers of long-lived macrophages.

4 We also identified features that mark newly differentiated CD102⁺ macrophages and are
5 inversely related to time-of-residency, such as ApoE. This finding is consistent with repopulation
6 studies showing that microglia of monocyte origin express higher levels of ApoE⁵⁷⁻⁵⁹. While the
7 association between *ApoE* and recent arrival seems to be at odds with the higher level of ApoE
8 expression by female peritoneal macrophages, *ApoE* expression may also be controlled partly by
9 estrogen, as the *ApoE* gene contains an estrogen response element, and its expression is reduced in
10 inflammatory peritoneal macrophages by macrophage-specific deletion of the estrogen receptor alpha
11³⁵. Understanding how ApoE expression is regulated by tissue-residency and hormonal control may be
12 important in many diseases in which it is a known genetic risk factor and that exhibit strong sex-
13 biases in risk, such as Alzheimer's and cardiovascular disease⁶⁰, but also in healthy aging where *APOE*
14 variants are among the strongest predictors of human longevity⁶¹.

15 Importantly, we found the dimorphism in proliferation and CXCL13 expression by peritoneal
16 macrophages to be regulated independently of macrophage replenishment kinetics, consistent with
17 previous data showing that the proliferative capacity of macrophages is determined by signals in the
18 local microenvironment rather than their origin^{25,62}. Although these dimorphisms only developed
19 following sexual maturation, it seems unlikely that estradiol levels are responsible for the lower
20 proliferation of female peritoneal macrophages, as estradiol is reported to increase rather than inhibit
21 proliferation of these cells³⁶. Furthermore, exogenous estradiol did not rescue the elevated turnover of
22 female macrophages we found in ovariectomised mice. Similarly, exogenous estradiol does not
23 influence CXCL13 expression by peritoneal macrophage³⁶, nor did it rescue the loss of B1 cells that
24 occurred after ovariectomy (data not shown). While estradiol has been reported to upregulate the B1
25 cell regulators *Tnfrsf13b*, *Tnfrsf13*, and *Il10*¹⁵ by female peritoneal macrophages *in vitro*, none of these
26 genes were differentially expressed by macrophages in our study. Moreover, genes highly expressed by
27 male peritoneal macrophages, such as *Arg1* and *Chil3*, are increased by estradiol³⁶. Although
28 expression of receptors for progesterone and androgens did not differ between the sexes, we cannot rule
29 out a role for these steroids in generating sex dimorphisms. Thus, the exact local factor(s) driving the
30 sex dimorphisms identified here remain to be elucidated. Interestingly, pathway analysis of our
31 transcriptomic data identified 'Interferon Gamma Response' and 'Interferon Alpha Response' as gene
32 sets enriched within female macrophages, and interferons are known to be hormonally regulated⁶³.
33 Whether interferon receptor signalling plays a role in dimorphic characteristics of peritoneal
34 macrophages is the focus of ongoing work.

35 The incidence and severity of sepsis and post-surgical infections are profoundly lower in
36 women than men⁶⁴, but the mechanisms underlying these differences remain unclear. Our finding that
37 female mice are more resistant to *S. pneumoniae* peritonitis is consistent with previous work on group
38 B streptococcal peritonitis³⁸. However, while others attributed this to other elements of innate immune

1 responses, such as neutrophil recruitment ⁵¹, our data suggest that the resistance of females is at least,
2 in part, due to differences in resident peritoneal macrophages, such as elevated expression of CD209b.
3 Dimorphic expression of CXCL13 may also contribute, as it plays a central role in recruiting B1 cells
4 that produce the natural IgM ² that protects against multiple forms of infectious peritonitis ^{65,66}. As
5 activation of complement is essential for innate resistance to against *S.pneumoniae* }⁶⁵ and both CD209b
6 and natural IgM can activate the classical pathway of complement fixation during *S.pneumoniae*
7 infection ⁶⁵⁻⁶⁷, peritoneal macrophages may play several, overlapping roles in protective immunity
8 against this infection. Indeed *Clq*, *C3*, and *C4b*, as well as *Cfb*, which encodes factor B and is essential
9 for the alternative pathway of complement fixation during *S.pneumoniae* infection, were also all
10 expressed more highly by female peritoneal macrophages. We propose that this heightened barrier
11 function in the female peritoneum may have evolved to mitigate the risk of sexually transmitted
12 infection disseminating from the lower female reproductive tract ⁶⁸ or to protect against puerperal
13 peritonitis. Our findings also have wider implications for understanding peritoneal macrophage
14 behaviour following a local mechanical or inflammatory insult, when tissue resident macrophages may
15 be replaced by monocyte-derived cells that may require prolonged residence in the tissue before
16 acquiring the full profile of resident macrophages with protective functions ^{55,69-71}. The potential risks
17 in this process have been highlighted in the context of viral meningitis, where a failure of newly elicited
18 macrophages to rapidly acquire CD209b expression led to impaired neutrophil recruitment to
19 subsequent intra-cranial immune challenge ⁷², and could explain why animals exposed to sterile
20 peritoneal inflammation are more susceptible to *S. pneumoniae* peritonitis for at least several months
21 ⁷³.

22 Our studies highlight the importance of taking age and sex into account when understanding
23 the peritoneal response to disease and implicate time-of-residency as an underlying determinant of
24 resident macrophage function. Further work is needed to understand the molecular processes that
25 underlie the requirement for time-of-residency on expression of these genes and to identify the local
26 signals that govern this process. Beyond the cavity, our findings also have wider implications for the
27 molecular mechanisms that drive dimorphic production of natural IgG by peritoneal B1 cells that
28 provides women and infants with heightened resistance to blood-borne bacterial infections, particularly
29 as these antibodies are lost in the absence of peritoneal macrophages ¹⁵.

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1 **Materials and methods**

2

3 ***Animals and reagents.***

4 Wild type C57BL/6J CD45.2⁺, congenic CD45.1⁺CD45.2⁺ mice and *Ccr2*^{-/-} mice were bred and
5 maintained in specific pathogen-free facilities at the University of Edinburgh, UK. In some
6 experiments, C57BL/6J (CrI) mice were purchased from Charles River, UK. *Itgax*^{Cre} ⁷⁴ (referred here
7 to CD11c^{Cre}) mice were crossed with *Rosa26*^{LSL-YFP} mice (a gift from Dr. Megan Mcleod, University of
8 Glasgow, UK) and maintained at the University of Glasgow. For *Cdh5*^{Cre-ERT2} fate mapping, WT
9 females aged 6-10 weeks were subjected to timed matings
10 with *Cdh5*^{CreERT2+/-} or *Cdh5*^{CreERT2+/+} *Rosa26*^{tdT/tdT} males. Successful mating was judged by the presence
11 of vaginal plugs the morning after, which was considered 0.5days post conception. For induction of
12 reporter recombination in the offspring, a single dose of 4-hydroxytamoxifen (4OHT; 1.2mg) was
13 delivered by i.p. injections to pregnant females at E7.5. To counteract adverse effects of 4OHT on
14 pregnancy, 4OHT was supplemented with progesterone (0.6mg). In cases when females could not give
15 birth naturally, pups were delivered by C-section and cross-fostered with lactating CD1 females. All
16 experimental mice were age and sex matched. To perform estrous staging, vaginal lavage was
17 performed and cellular content examined following haematoxylin and eosin staining, as previously
18 described ⁷⁵. For high fat diet (HFD) experiments, tissue protected BM chimeric mice were placed on
19 HFD (58 kcal% fat and sucrose, Research Diet, D1233li) for 8 weeks starting 4 weeks post
20 reconstitution. Experiments performed at UK establishments were permitted under license by the UK
21 Home Office and were approved by the University of Edinburgh Animal Welfare and Ethical Review
22 Body or the University of Glasgow Local Ethical Review Panel.

23

24 ***Surgery.*** Ovariectomy/oophorectomy was performed on 6-week-old wild type (C57BL/6J) or tissue
25 protected BM chimeras 8 weeks post-reconstitution (16 weeks of age). Briefly, dorsal unilateral or
26 bilateral ovariectomy (OVX) was performed and mice allowed to recover for up to 8 weeks. Sham
27 surgery was performed to control for the effects of surgery on the peritoneal environment. This involved
28 identical surgery except for the excision of the ovary/ovaries. Surgery was performed under isoflurane
29 anaesthesia followed by a postoperative analgesic, buprenorphine (0.1 mg/kg), for pain management.
30 In some experiments following 7 days of recovery, mice received exogenous estradiol in the form of
31 E2 valerate (E2; 0.01 mg/kg) s.c. thrice weekly for 3 weeks.

32

33 ***Tissue-protected BM chimeric mice.*** Anaesthetised 6-12 week old C57BL/6J CD45.1⁺CD45.2⁺ animals
34 were exposed to a single dose of 9.5 Gy γ -irradiation, with all but the head and upper thorax of the
35 animals being protected by a 2 inch lead shield. Animals were subsequently given 2-5 \times 10⁶ BM cells
36 from sex matched or mismatched congenic CD45.2⁺ C57BL/6J animals by i.v. injection. Unless

1 specified, mice were left for a period of at least 8 weeks before analysis of chimerism in the tissue
2 compartments.

3

4 ***BrdU injection.*** For labelling of proliferating cells, mice were injected s.c. with 100µl of 10mg/ml
5 BrdU (Sigma) in Dulbecco's PBS 2hr before culling.

6

7 ***Preparation of single cell suspensions.*** Mice were sacrificed by CO₂ inhalation or by terminal
8 anaesthesia followed by exsanguination. Peritoneal and pleural cavities were lavaged with RPMI
9 containing 2mM EDTA and 10mM HEPES (both Invitrogen) as described previously⁷⁶. Any samples
10 with excessive erythrocyte contamination were excluded from analysis. Omental tissue was excised,
11 chopped finely and digested in 0.5ml pre-warmed collagenase D (1mg/ml; Roche) in RPMI 1640 media
12 supplemented with 2% FCS for 15 minutes in a shaking incubator at 37°C. Following disaggregation
13 with a P1000 Gilson, omental tissue was digested for a further 20mins before being placed on ice. 2.5µl
14 of 0.5M EDTA was added to each sample to inhibit enzymatic activity. Cell suspensions were passed
15 through an 100µm filter and centrifuged at 1700rpm for 10mins. The resulting cell suspension was
16 subsequently passed through a 40µm strainer prior to cell counting. All cells were maintained on ice
17 until further use. Cellular content of the preparations was assessed by cell counting using a Casey TT
18 counter (Roche) in combination with multi-colour flow-cytometry.

19

20 ***Flow cytometry.*** Equal numbers of cells were blocked with 0.025 µg anti-CD16/32 (2.4G2; Biolegend)
21 and 1:20 heat-inactivated mouse serum (Invitrogen), and then stained with a combination of the
22 antibodies detailed in Supplementary Table 2. Where appropriate, cells were subsequently stained with
23 streptavidin-conjugated fluorochromes. Dead cells were excluded using DAPI, 7-AAD or Zombie Aqua
24 fixable viability dye (Biolegend). Fluorescence-minus-one controls confirmed gating strategies, while
25 discrete populations within lineage⁺ cells were confirmed by omission of the corresponding population-
26 specific antibody. Erythrocytes in blood samples were lysed using 1x RBC Lysis buffer (Biolegend),
27 as per the manufacturer's guidelines. For intracellular staining, cells were subsequently fixed and
28 permeabilized using FoxP3/Transcription Factor Staining Buffer Set (eBioscience), and intracellular
29 staining performed using antibodies detailed in Supplementary Table 2. For the detection of BrdU, cells
30 were fixed as above and incubated with 3µg DNaseI (Sigma) for 30-60mins, before being washed in
31 PermWash (eBioscience) and then incubated with anti-BrdU antibody for 30mins at RT. Samples were
32 acquired using a FACS LSRFortessa or AriaII using FACSDiva software (BD) and analyzed with
33 FlowJo software (version 9 or 10; Tree Star). Analysis was performed on single live cells determined
34 using forward scatter height (FCS-H) versus area (FSC-A) and negativity for viability dyes. For analysis
35 of macrophage proliferation, Ki67 expression was used to determine the frequency of all
36 CD102⁺/F4/80^{hi} cells in cycle, whereas a 2h BrdU pulse before necropsy combined with Ki67
37 expression was used to identify cells in S phase, as described previously⁷⁷. mRNA was detected by
38 flow cytometry using PrimeFlow technology (ThermoFisher) using probes against ApoE (probe type

1 10; AF568), ApoC1 (probe type 4; AF488) and CXCL13 (probe type 6; AF750) according to the
2 manufacturer's guidelines.

3

4 ***Transcriptional Analysis.***

5 **Bulk RNAseq**

6 CD102⁺F4/80^{hi} cells were FACS-purified from the peritoneal and pleural cavities of unmanipulated
7 male and female mice. For each population, 25,000 cells were sorted into 500µl RLT buffer (Qiagen)
8 and snap frozen on dry ice. RNA was isolated using the RNeasy Plus Micro kit (Qiagen), at which point
9 triplicates of 25,000 cells for each population were pooled. 10 ng of total RNA were amplified and
10 converted to cDNA using Ovation RNASeq System V2 (Nugen). Sequencing was performed by
11 Edinburgh Genomics using the Illumina HiSeq 4000 system (75PE). Raw map reads were processed
12 with the R package DESeq2⁷⁸ to generate the differentially expressed genes, and the normalized count
13 reads to generate and visualize on heat maps generated by the R package pheatmap. Samples with >5%
14 of reads mapped to ribosomal RNA were removed from analysis. DEG were determined using at least
15 a 1.5-fold difference and adjusted $p < 0.01$, for each of the six pairwise comparisons. Pathway
16 enrichment analysis was performed using the GSEA online database and the R package gskb (Gene Set
17 data for pathway analysis in mouse) which makes predictions between each of the six pairwise
18 comparisons, incorporating in the analysis the statistically significant differences in gene expression.
19 The R package gskb was used to determine the chromosomal location of each of the genes and
20 transcription factors. All R code is available upon request.

21

22 **Single-cell RNAseq**

23 10K cells for male and female sorted cells were loaded in Chromium 10x in parallel. Libraries
24 were prepared as per manufacturer's protocol and sequenced on Illumina Novaseq S1. Initial
25 processing was done using Cellranger (v2.1.1) mkfastq and count (aligned to mouse assembly
26 mm10). ***Preparation of analysis ready data:*** For each dataset (filtered data from Cell Ranger
27 pipeline), we filtered out potentially low quality cells using dataset-specific thresholds based on
28 the trend of the number of genes per cell versus number of housekeeping genes per cell and
29 number of genes per cell versus percentage of mitochondrial genes per cell curves as follows.
30 More specifically, for the female data, we retained 4341 cells that have between 300 and 5000
31 genes, at least 65 housekeeping genes and percentage of mitochondrial genes over the total
32 number of expressed genes below 2%. For the male data, we retained 2564 cells that have
33 between 300 and 6000 genes, at least 70 housekeeping genes and percentage of mitochondrial
34 genes over the total number of expressed genes below 2%. Finally, we filtered out genes that
35 were expressed in less than 1% of the cells from each dataset. ***Clustering analysis of the data:***
36 Clustering and data merging using CCA was done using Seurat (v3.1.0). We used default
37 parameters and 20 principal components for aligning and clustering the data. We next removed

1 a very small cluster that lay far from all other clusters on the UMAP projection, indicating it
2 could be either contamination or doublets and constructed a phylogenetic tree of the remaining
3 clusters to understand the distances and relationship between them. Clusters that were closely
4 grouped together and did not show unique markers, were merged together. The final result
5 consists of 6890 cells grouped into 6 clusters. **Identification of differentially expressed genes**
6 **in CCA aligned clusters:** We used MAST (v1.10) as implemented in the Seurat package and
7 with default parameters to identify differentially upregulated genes between the identified
8 clusters. To overcome the bias of batch effect, we found differentially upregulated genes within
9 each dataset separately and retained the intersection of markers (conserved markers).
10 **Identification of differentially expressed genes between female and male cells:** We used
11 Student's t-test as implemented in the Seurat package between equivalent female and male
12 cells to identify differentially upregulated genes between male and female cells. We only
13 retained genes with adjusted p-value based on Bonferroni correction below 0.05. Genes that
14 were identified as differentially expressed for more than four out of the six clusters were
15 selected as global DE genes and were removed from the cluster-specific differences.
16 **Pseudotemporal ordering:** We used monocle (v2.12.0) with default parameters to build
17 pseudotemporal trajectories of the female and the male data separately. **Functional annotation**
18 **of genesets:** We used DAVID to obtain enriched Gene Ontology Biological Process (GO-BP)
19 and KEGG pathway terms for each extracted geneset. Given a list of genes upregulated in a set
20 of cells (e.g. cluster) as target and a list of all genes observed in the respective dataset as
21 background, we downloaded a list of GO-BP terms (GOTERM_BP_FAT) and a list of KEGG
22 pathway terms that were significantly enriched in the target list as a functional annotation chart.
23 We kept only terms with Benjamini adjusted p-values less than 0.05 and sorted them by
24 decreasing Fold Enrichment.
25

26 ***S. pneumoniae peritonitis.*** *S. pneumoniae* were cultured overnight on blood agar plates (5% CO₂, 95%
27 air, 37 C), inoculated into Brain Heart Infusion broth, cultured for 3 h, washed, and resuspended at 10⁴
28 CFU/ ml (estimated by OD₅₉₅) in sterile PBS. Their concentration was verified by serial dilution and
29 culture on blood agar plates. Groups of male and female, age-matched C57Bl/6 mice (8–14 wk of age)
30 were inoculated intraperitoneally with 100 µl of PBS containing 10³ CFU *S. pneumoniae* (capsular type
31 2 strain D39). Mice were culled 20 h later and peritoneal lavage performed using sterile PBS. 100 µl of
32 lavage fluid was cultured for bacterial growth for 24 h. The remaining lavage fluid was centrifuged at
33 400g for 5 mins and the resulting cells counted and prepared for flow cytometric analysis.

1 **Statistics.** Statistics were performed using Prism 7 (GraphPad Software). The statistical test used in
2 each experiment is detailed in the relevant figure legend.

3

4 **Accession codes.**

5

6 **Data availability.** Data that support the findings of this study are available from the corresponding
7 authors upon reasonable request.

8

9

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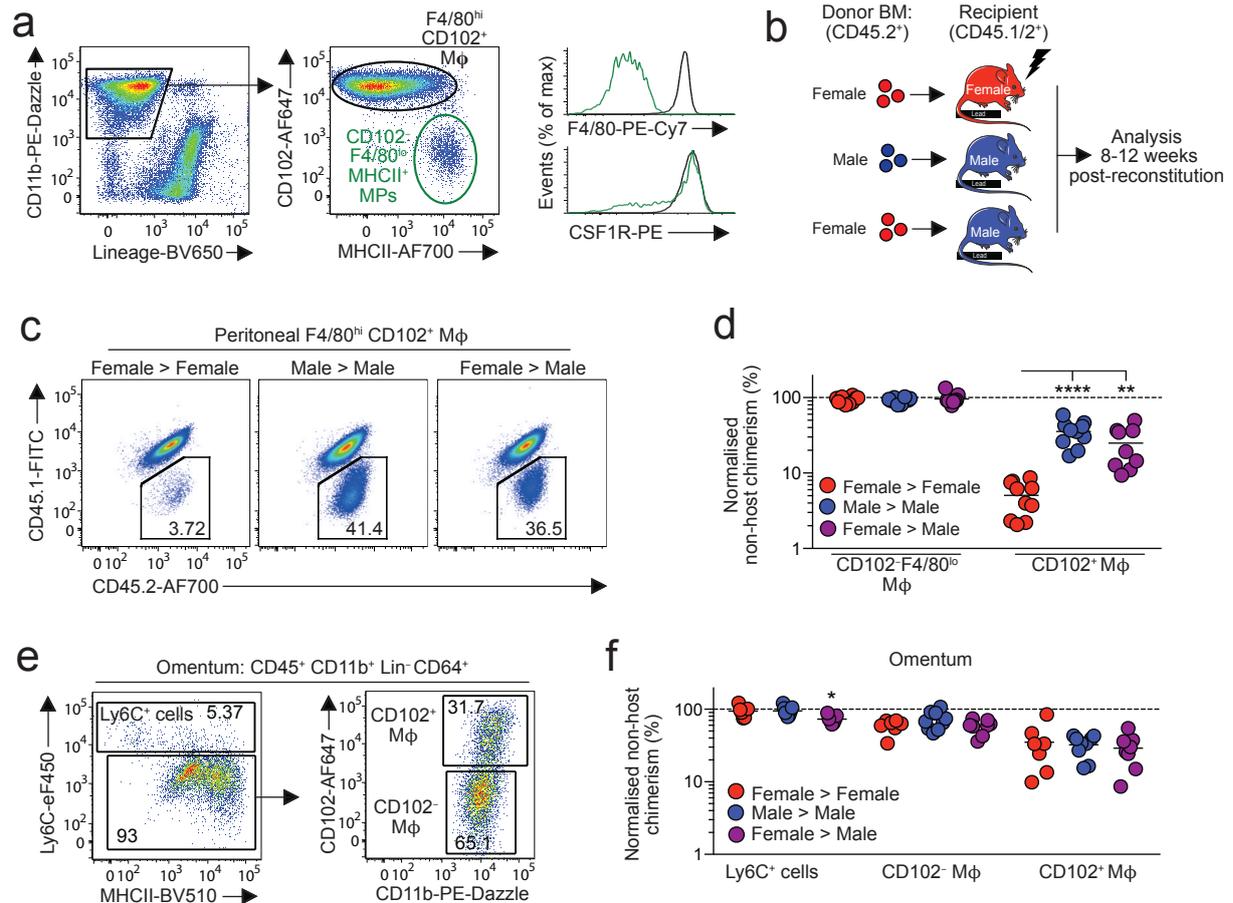
16

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21

22 **Author Contributions**

23 C.C.B. conceived and performed most of the experiments, analysed and interpreted the data, wrote the
24 manuscript and provided funding. D.A.G. designed and performed experiments and edited the
25 manuscript. N.S. performed transcriptomic analysis (population level RNAseq) and figure generation.
26 K.B. performed single cell RNAseq analysis and figure generation. P.L. performed experiments for
27 generation of scRNAseq data. C.D. provided technical assistance for the design and execution of
28 infection experiments. R.G. performed *Cdh5*-fate mapping experiments. M.M-P. helped with the design
29 and execution of high fat diet experiments. C.B. helped with the design and execution of high fat diet
30 experiments. M.B. provided access to *Cdh5* fate-mapper mice. D.D. helped with the design and
31 execution of infection experiments. P.T.K.S. provided advice for the design and interpretation of
32 experiments and edited the manuscript. N.B. performed the scRNAseq analysis, provided advice on the
33 interpretation of these data, edited the manuscript and provided funding. S.J.J. conceived and performed
34 experiments, analysed and interpreted the data, wrote the manuscript, obtained funding and supervised
35 the project.



1
2 **Figure 1. Environment drives sexual dimorphism in macrophage replenishment in the peritoneal**
3 **cavity**

4 (a) Expression of CD11b and CD3, CD19, Ly6G and SiglecF ('Lineage') by live CD45⁺ peritoneal
5 leukocytes (*left*) and expression of CD102 and MHCII by CD11b⁺ Lin⁻ cells (*centre*) from adult
6 C57Bl/6 female mice. Histograms show expression of F4/80 and CSF1R by CD102⁺ and CD102⁻
7 MHCII⁺ cells.

8 (b) Experimental scheme for the generation of sex mis-matched, tissue-protected bone marrow (BM)
9 chimeric mice.

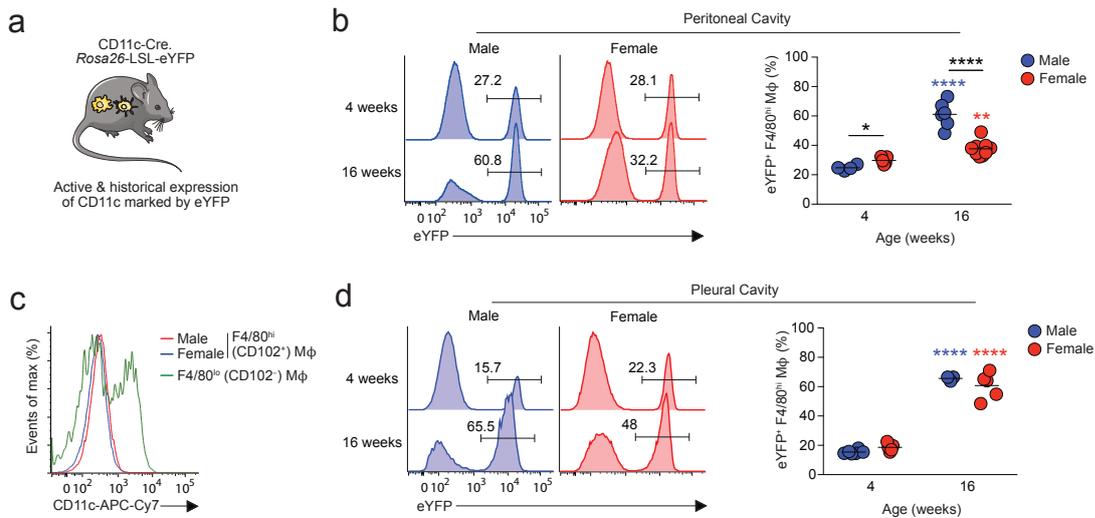
10 (c) Representative expression of CD45.1 and CD45.2 by peritoneal F4/80^{hi}CD102⁺ macrophages from
11 sex matched or mismatched tissue protected BM chimeric mice 8-12 weeks post-reconstitution.

12 (d) Normalized non-host chimerism of peritoneal F4/80^{hi}CD102⁺ macrophages from sex matched or
13 mismatched tissue-protected BM chimeric mice 8-12 weeks post-reconstitution. Data are normalised to
14 the non-host chimerism of Ly6C^{hi} monocytes. **P<0.01, ****P<0.0001 One-way ANOVA.

15 (e) Gating strategy to identify macrophages amongst omental isolates. Expression of Ly6C and MHCII
16 by CD11b⁺ Lin⁻CD64⁺ cells and expression of CD102 by Ly6C⁻ cells to identify CD102⁺ and CD102⁻
17 macrophages.

- 1 (f) Normalized non-host chimerism of omental Ly6C⁺ monocytes and CD102⁺ and CD102⁻
- 2 macrophages from mice in (d). Data are normalised to the non-host chimerism of Ly6C^{hi} monocytes.
- 3 *P<0.05. One-way ANOVA.
- 4 Symbols represent individual animals and horizontal bars represent the mean. Data represent 9
- 5 (female > male) or 10 (sex matched) mice per group pooled from two independent experiments.
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3 **Figure 2. Sexual dimorphism in peritoneal macrophage replenishment occurs following sexual**
4 **maturity**

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6 (a) Experimental scheme of CD11c^{Cre}.Rosa26^{LSL-eYFP} fate-mapping mice.

7 (b) Representative expression of eYFP by peritoneal F4/80^{hi}CD102⁺ macrophages from male and
8 female CD11c^{Cre}.Rosa26^{LSL-eYFP} fate-mapping mice at 4 and 16 weeks of age. Right, frequency of eYFP⁺
9 cells amongst F4/80^{hi}CD102⁺ macrophages in male and female mice at the indicated ages. Symbols
10 represent individual animals and horizontal bars represent the mean. Data represent 4 (male 4 weeks),
11 5 (female 4 weeks), 6 (male 16 weeks) or 9 (female 16 weeks) mice per group pooled from two
12 independent experiments.

13 (c) Expression of CD11c by peritoneal F4/80^{hi}CD102⁺ macrophages from male and female and CD102⁻
14 MHCII⁺ cells from female mice.

15 (d) Representative expression of eYFP by pleural F4/80^{hi}CD102⁺ macrophages from male and female
16 CD11c^{Cre}.Rosa26^{LSL-eYFP} fate-mapping mice at 4 and 16 weeks of age. Right, frequency of eYFP⁺ cells
17 amongst pleural F4/80^{hi}CD102⁺ macrophages in male and female mice at the indicated ages. Symbols
18 represent individual animals and horizontal bars represent the mean. Data represent 3 (male 16 weeks),
19 5 (female 4 & 16 weeks) or 6 (male 4 weeks) per group pooled from two independent experiments.

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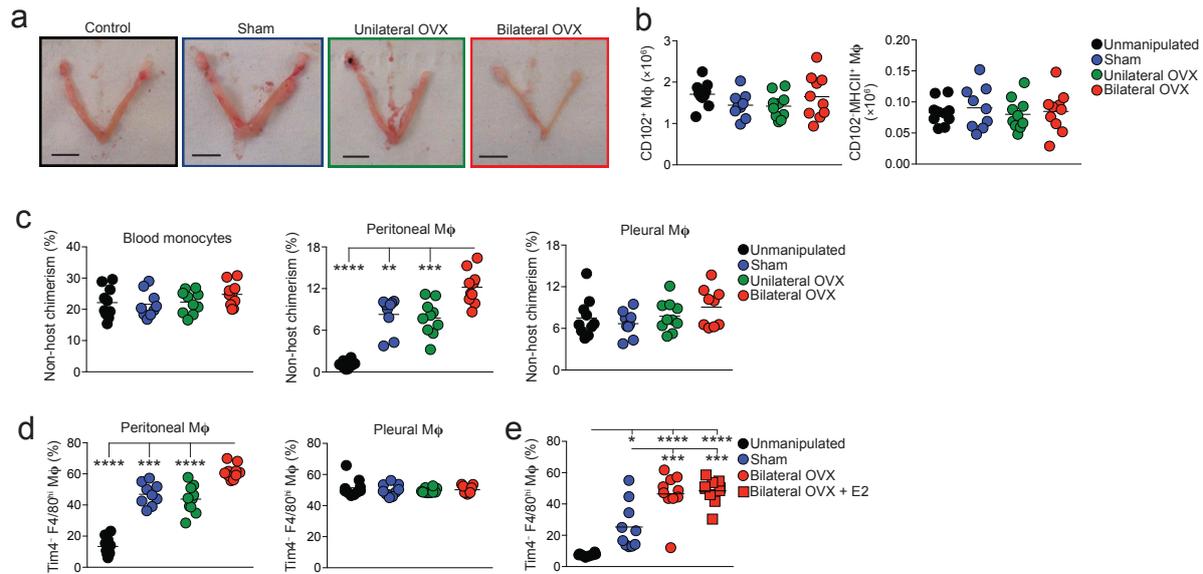


Figure 3. Ovariectomy leads to increased macrophage replenishment

(a) Representative images of the uterine horns of tissue-protected BM chimeric mice that had received unilateral or bilateral oophorectomy (OVX), sham surgery or were completely unmanipulated (control).

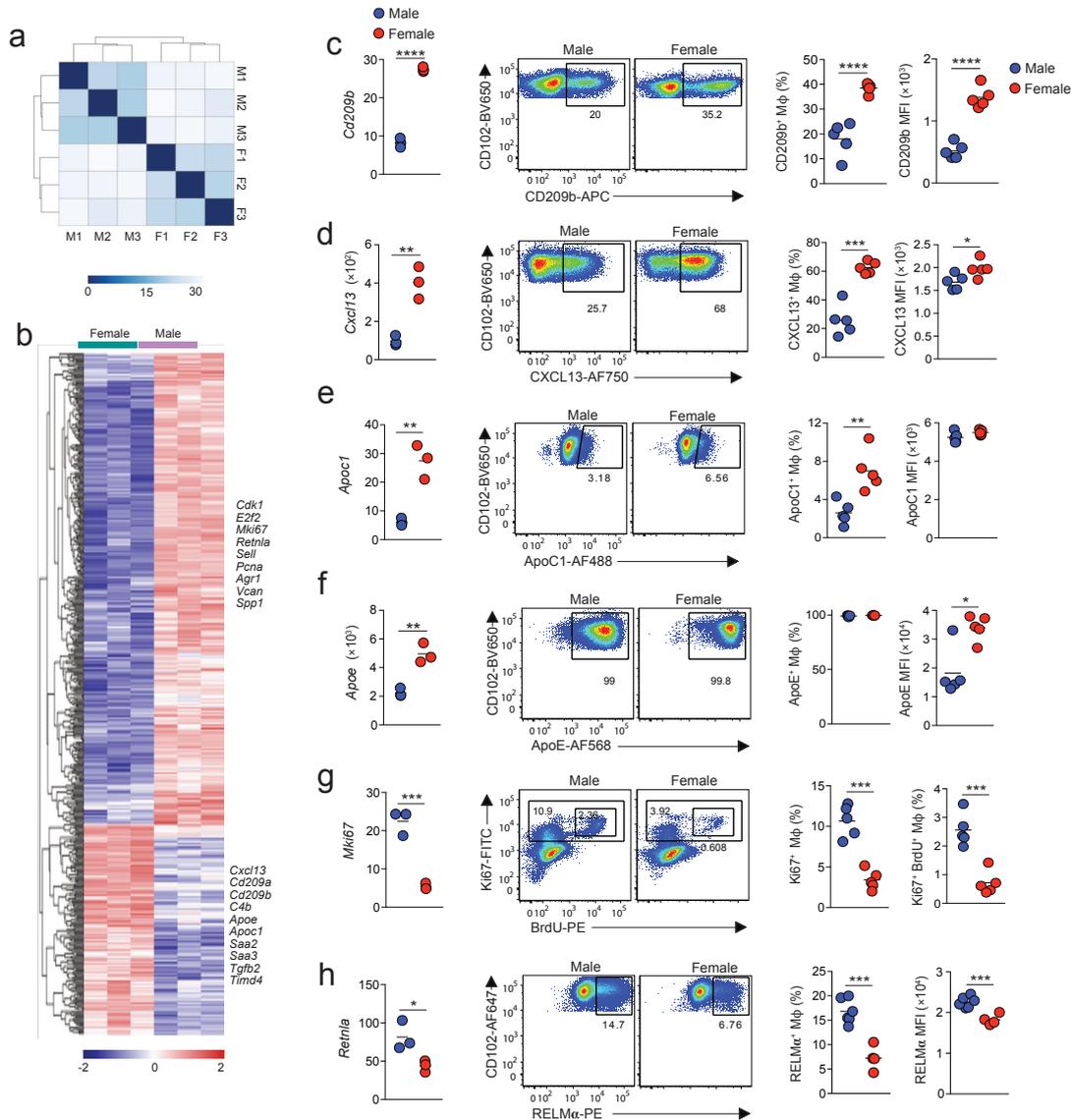
(b) Absolute number of F4/80^{hi}CD102⁺ macrophages and CD102⁻MHCII⁺ cells obtained from the peritoneal cavity of tissue-protected BM chimeric mice that had received surgery 8 weeks earlier. Symbols represent individual animals and horizontal bars represent the mean. Data represent 9 (sham) or 10 (control, unilateral, bilateral) mice per group pooled from two independent experiments.

(c) Non-host chimerism of blood Ly6C^{hi} blood monocytes (*left*) and F4/80^{hi}CD102⁺ macrophages obtained from the peritoneal (*centre*) or pleural (*right*) cavity of tissue-protected BM chimeric mice that had received surgery 8 weeks earlier. Symbols represent individual animals and horizontal bars represent the mean. Data represent 9 (sham) or 10 (control, unilateral, bilateral) mice per group pooled from two independent experiments. **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's multiple comparisons test.

(d) Frequency of Tim4⁻ cells amongst F4/80^{hi}CD102⁺ macrophages obtained from the peritoneal (*centre*) or pleural (*right*) cavity of mice in (b). ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's multiple comparisons test.

(e) Frequency of Tim4⁻ cells amongst F4/80^{hi}CD102⁺ macrophages obtained from the peritoneal cavity of unmanipulated C57Bl/6 female mice (controls) or age-matched females that received bilateral OVX or sham surgery 4 weeks earlier. One group received exogenous estradiol (E2) thrice weekly for 3 weeks. Symbols represent individual animals and horizontal bars represent the mean. Data represent 8 (control) or 10 (sham, bilateral, bilateral + E2) mice per group pooled from two independent

- 1 experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's
- 2 multiple comparisons test.
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3 **Figure 4: Sex determines the transcriptional signature of peritoneal macrophages**

4 (a) Heatmap showing distance between samples of male (M) and female (F) CD102⁺F4/80^{hi}
5 macrophages FACS-purified from the peritoneal cavity of 10-12 week old mice.

6 (b) Gene expression profile of the 148 differentially expressed (>1.5 fold) genes between male and
7 female peritoneal macrophages with selected genes highlighted.

8 (c) Expression of *Cd209b* from RNAseq (FPKM; left panel), representative expression of CD209b
9 protein (middle panels) and frequency of CD209b⁺ cells amongst CD102⁺F4/80^{hi} peritoneal
10 macrophages obtained from 10-12-week-old male or female C57BL/6 mice (right panel) and the mean
11 fluorescence intensity (MFI) of CD209b expression by these cells (far right panel). Symbols represent
12 individual animals and horizontal bars represent the mean. RNAseq data represent 3 mice per group

1 and protein analysis represents 5 mice per group from one of five independent experiments.
2 ****P<0.0001. Student's *t* test.

3 (d) Expression of *Cxcl13* from RNAseq (FPKM; *left panel*), representative expression of CXCL13
4 mRNA (*middle panels*) and frequency of CXCL13⁺ cells amongst CD102⁺F4/80^{hi} peritoneal
5 macrophages obtained from 10-12-week-old male or female C57BL/6 mice (*right panel*) and the mean
6 fluorescence intensity (MFI) of CXCL13 mRNA expression by these cells (*far right panel*). Symbols
7 represent individual animals and horizontal bars represent the mean. RNAseq data represent 3 mice per
8 group and flow cytometric analysis represents 5 mice per group from one of three independent
9 experiments. *P<0.05, ***P<0.001. Student's *t* test.

10 (e) Expression of *ApoC1* from RNAseq (FPKM; *left panel*), representative expression of ApoC1 mRNA
11 (*middle panels*) and frequency of ApoC1⁺ cells amongst CD102⁺F4/80^{hi} peritoneal macrophages
12 obtained from 10-12-week-old male or female C57BL/6 mice (*right panel*) and the mean fluorescence
13 intensity (MFI) of ApoC1 mRNA expression by these cells (*far right panel*). Symbols represent
14 individual animals and horizontal bars represent the mean. RNAseq data represent 3 mice per group
15 and flow cytometric analysis represents 5 mice per group from one of three independent experiments.
16 *P<0.05, ***P<0.001. Student's *t* test.

17 (f) Expression of *ApoE* from RNAseq (FPKM; *left panel*), representative expression of ApoE mRNA
18 (*middle panels*) and frequency of ApoE⁺ cells amongst CD102⁺F4/80^{hi} peritoneal macrophages
19 obtained from 10-12-week-old male or female C57BL/6 mice (*right panel*) and the mean fluorescence
20 intensity (MFI) of ApoE mRNA expression by these cells (*far right panel*). Symbols represent
21 individual animals and horizontal bars represent the mean. RNAseq data represent 3 mice per group
22 and flow cytometric analysis represents 5 mice per group from one of three independent experiments.
23 *P<0.05, ***P<0.001. Student's *t* test.

24 (g) Expression of *Mki67* from RNAseq (FPKM; *left panel*), representative expression of Ki67 protein
25 and BrdU incorporation (*middle panels*) and the frequency of BrdU⁺Ki67⁺ cells amongst
26 CD102⁺F4/80^{hi} peritoneal macrophages obtained from 10-12-week-old male or female C57BL/6 mice.
27 Symbols represent individual animals and horizontal bars represent the mean. Data represent 5 mice
28 per group from one of two experiments. ***P<0.001. Student's *t* test.

29 (h) Expression of *Retnla* from RNAseq (FPKM; *left panel*), representative expression of RELM α
30 protein (*middle panels*) and the frequency of RELM α ⁺ cells amongst CD102⁺F4/80^{hi} peritoneal
31 macrophages obtained from 10-12-week-old male or female *Rag1*^{-/-} C57BL/6 mice. Symbols represent
32 individual animals and horizontal bars represent the mean. *P<0.05, ***P<0.001. Student's *t* test.

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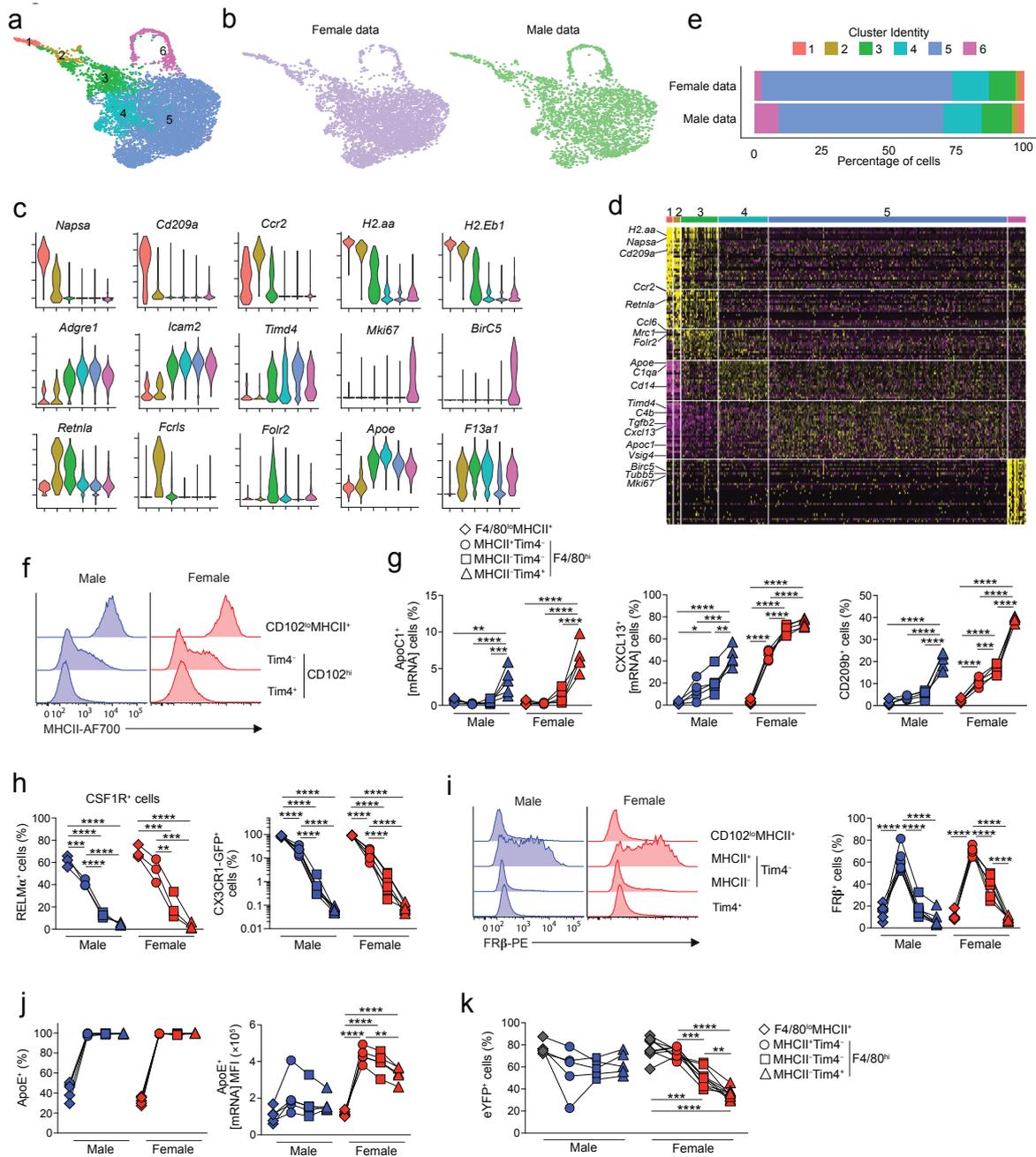


Figure 5: scRNAseq analysis reveals dimorphic macrophage heterogeneity

(a) UMAP dimensionality reduction analysis of 4341 and 2564 number of cells from the peritoneal cavity of 19 week-old male or female mice identifying 6 clusters.

(b) UMAP profile of female and male peritoneal cells.

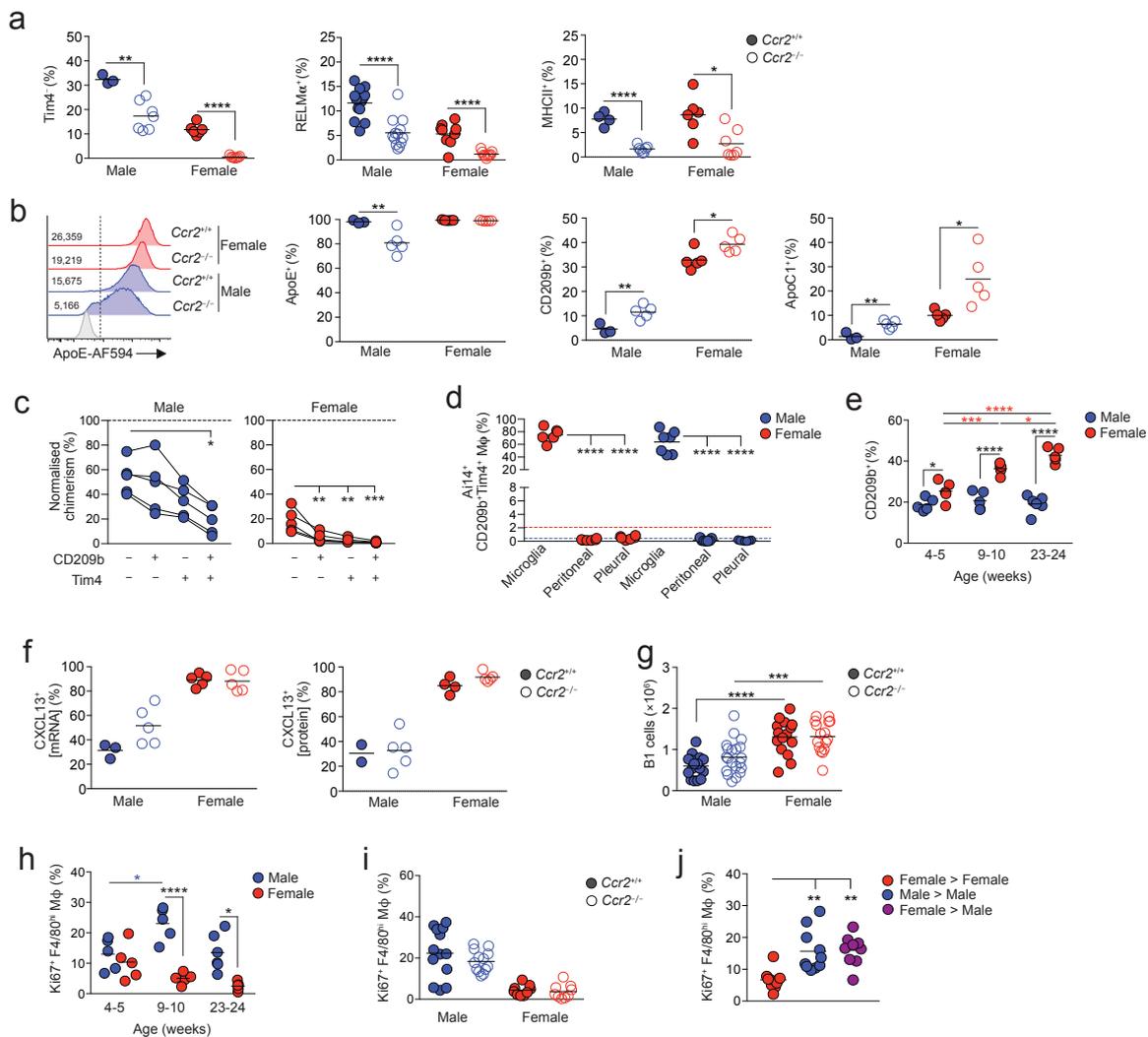
(c) Feature plots displaying expression of individual genes by merged female/male cells.

(d) Heatmap displaying the 10 most differentially expressed genes by each cluster from **a** (select genes highlighted).

(e) Relative frequency of each cluster in the female and male dataset.

- 1 (f) Representative expression of MHCII by CD102^{lo}MHCII⁺ and Tim4/MHCII-defined CD102⁺
2 peritoneal macrophages from 10-12 week old male or female C57BL/6 mice.
- 3 (g) Expression of Apoc1 (mRNA), CXCL13 (mRNA) and CD209b protein by CD102^{lo}MHCII⁺ and
4 Tim4/MHCII-defined CD102⁺ peritoneal macrophages from 10-12 week old male or female C57BL/6
5 mice. Data represent 5 mice per group from one of three independent experiments. *P<0.05, **P<0.01,
6 ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's multiple comparisons test.
- 7 (h) Expression of RELM α and CX3CR1-GFP by CD102^{lo}MHCII⁺ and Tim4/MHCII-defined CD102⁺
8 peritoneal macrophages from 10 week old (RELM α) or 15 week old (CX3CR1-GFP) male or female
9 C57BL/6 mice. Data represent 3 (RELM α), 5 (CX3CR1-GFP, female) or 7 (CX3CR1-GFP, male) mice
10 per group from one of at least 5 independent experiments (RELM α) or from one experiment (CX3CR1-
11 GFP). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's multiple
12 comparisons test.
- 13 (i) Histograms show representative expression of FR β by CD102^{lo}MHCII⁺ and Tim4-defined
14 CD102⁺ peritoneal macrophages from 10-12 week old male or female C57BL/6 mice. Scatter plot
15 show frequency of FR β ⁺ cells amongst CD102^{lo}MHCII⁺ and Tim4/MHCII-defined CD102⁺ peritoneal
16 macrophages. Data represent 6 (female) or 7 (male) mice per group pooled from two independent
17 experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA with Tukey's
18 multiple comparisons test.
- 19
- 20 (j) Frequency of ApoE⁺ (mRNA) cells amongst CD102^{lo}MHCII⁺ and Tim4/MHCII-defined CD102⁺
21 peritoneal macrophages (*left*) and mean fluorescence intensity of ApoE by these subsets (*right*) from
22 10-12 week old male or female C57BL/6 mice. Data represent 5 mice per group pooled from one of 3
23 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA with
24 Tukey's multiple comparisons test.
- 25 (k) Frequency of eYFP⁺ cells amongst F4/80, MHCII and Tim4-defined macrophages obtained from
26 16-week-old male and female CD11c^{Cre}.*Rosa26*^{LSL-eYFP} mice. Symbols represent individual animals and
27 horizontal bars represent the mean. Data represent 5 (male) or 9 (female) mice per group pooled from
28 two independent experiments.

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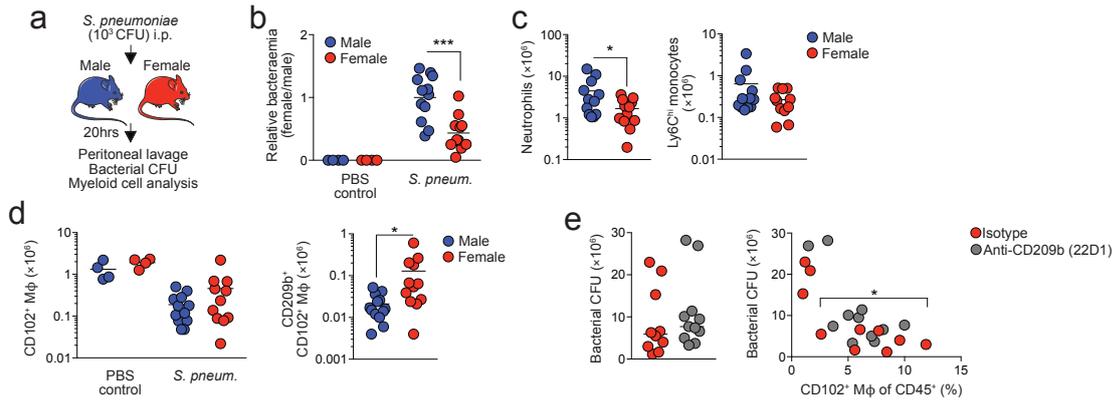
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Figure 6: Differential replenishment and environmental signals drive the dimorphic features of peritoneal macrophages

6 (a) Frequency of Tim4⁺, RELM α ⁺ and MHCII⁺ cells amongst CD102⁺ macrophages from the peritoneal
7 cavity of unmanipulated age-matched *Ccr2*^{+/+} or *Ccr2*^{-/-} mice. Symbols represent individual animals
8 and horizontal bars represent the mean. Data are pooled from two independent experiments. Tim4 data
9 represents 3 (*Ccr2*^{+/+} males), 6 (*Ccr2*^{+/+} females) or 7 (*Ccr2*^{-/-}) 22-28 week old mice per group. RELM α
10 data represent with 13 male and 9 female 14-18 week old mice per group. MHCII data represent 4
11 (*Ccr2*^{+/+} males), 6 (*Ccr2*^{+/+} females) or 7 (*Ccr2*^{-/-}) 22-28 week old mice per group. *P<0.05, **P<0.01,
12 ****P<0.0001. Student's *t* test with Holm-Sidak correction.

13 (b) Representative expression of ApoE by CD102⁺ macrophages (histograms) and frequency of ApoE⁻
14 , CD209b⁺ and ApoC1⁺ cells from the peritoneal cavity of unmanipulated age-matched *Ccr2*^{+/+} or *Ccr2*^{-/-}
15 ^{-/-} mice. Symbols represent individual animals and horizontal bars represent the mean. Data are pooled

- 1 from two independent experiments and represents 3 ($Ccr2^{+/+}$ males), 6 ($Ccr2^{+/+}$ females) or 7 ($Ccr2^{-/-}$)
2 22-28 week old mice per group. * $P < 0.05$, ** $P < 0.01$. Student's t test with Holm-Sidak correction.
- 3 (c) Normalized non-host chimerism of CD209/Tim4-defined subsets of CD102⁺ macrophages from the
4 peritoneal cavity of sex matched tissue-protected BM chimeric mice 8 weeks post-reconstitution. Data
5 are normalised to the non-host chimerism of Ly6C^{hi} monocytes. Data represent 5 mice per group from
6 one experiment. * $P < 0.05$, *** $P < 0.001$. Paired Student's t test.
- 7 (d) Proportion of tdTomato⁺ (Ai14) cells amongst microglia, peritoneal and pleural macrophages from
8 15-week-old $Cdh5^{Cre-ERT2}.Rosa26^{LSL-Ai14}.Cx3cr1^{+/gfp}$ mice administered 4-hydroxytamoxifen at E7.5.
9 Data represent 6 (female) or 7 (male) mice per group from one experiment. **** $P < 0.0001$. One-way
10 ANOVA followed by Tukey's multiple comparisons test.
- 11 (e) Frequency of cells expressing CXCL13 mRNA (*left*) or CXCL13 protein (*right*) amongst CD102⁺
12 macrophages obtained from the peritoneal cavity of unmanipulated 22-28 week old $Ccr2^{+/+}$ or $Ccr2^{-/-}$
13 mice. Symbols represent individual animals and horizontal bars represent the mean. CXCL13 mRNA
14 data represents 3 ($Ccr2^{+/+}$ males), 5 ($Ccr2^{+/+}$ females) or 5 ($Ccr2^{-/-}$) mice per group. CXCL13 protein
15 data represents 2 ($Ccr2^{+/+}$ males), 4 ($Ccr2^{+/+}$ females) or 5 ($Ccr2^{-/-}$) mice per group.
- 16 (f) The absolute number of B1 cells obtained from the peritoneal cavity of unmanipulated age matched
17 14-28 week old $Ccr2^{+/+}$ or $Ccr2^{-/-}$ mice. Data represent 15 ($Ccr2^{+/+}$ females), 16 ($Ccr2^{-/-}$ females), 17
18 ($Ccr2^{+/+}$ males) or 20 ($Ccr2^{-/-}$ females) mice per group pooled from four independent experiments.
- 19 (g) Frequency of Ki67⁺ cells amongst peritoneal F4/80^{hi} macrophages obtained from the peritoneal
20 cavity of unmanipulated 14-18 week old $Ccr2^{+/+}$ or $Ccr2^{-/-}$ mice. Data represents 15 ($Ccr2^{+/+}$ females),
21 16 ($Ccr2^{-/-}$ females), 17 ($Ccr2^{+/+}$ males) or 20 ($Ccr2^{-/-}$ females) mice per group pooled from 2
22 experiments.
- 23 (h) Frequency of Ki67⁺ cells amongst peritoneal F4/80^{hi} macrophages obtained from sex matched or
24 mismatched tissue protected BM chimeric mice 8-12 weeks post-reconstitution. Data represent 9
25 (female > male) or 10 (sex matched groups) mice per group pooled from one of two independent
26 experiments. ** $P < 0.01$. One-way ANOVA followed by Tukey's multiple comparisons test.
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3 **Figure 7: Differential CD209b expression confers an advantage on female macrophages in the**
 4 **setting of pneumococcal peritonitis**

5 (a) Experimental scheme for induction of peritonitis. Male and female mice (9-10 weeks old) were
 6 inoculated with 10^3 CFU type 2 *Streptococcus pneumoniae* (D39) and bacterial counts and assessment
 7 of the peritoneal myeloid compartment assessed after 20hrs.

8 (b) Relative bacteraemia in the peritoneal cavity of male and female mice infected 20hrs earlier (female
 9 CFU/male CFU). Symbols represent individual animals and horizontal bars represent the mean. Data
 10 represent 4 (PBS), 11 (female *S. pneumoniae*) or 12 (male *S. pneumoniae*) mice per group pooled from
 11 three independent experiments. *** $P < 0.001$. Student's *t* test.

12 (c) Absolute numbers of Ly6G⁺ neutrophils and Ly6C^{hi} monocytes in the peritoneal cavity 20hrs after
 13 inoculation with 10^3 CFU type 2 *Streptococcus pneumoniae* (D39) or in mice that received PBS.
 14 Symbols represent individual animals and horizontal bars represent the mean. Data represent 4 (PBS),
 15 11 (female *S. pneumoniae*) or 12 (male *S. pneumoniae*) mice per group pooled from three independent
 16 experiments. * $P < 0.05$. Student's *t* test.

17 (d) Absolute numbers of CD102⁺ macrophages and CD209b⁺CD102⁺ macrophages in the peritoneal
 18 cavity 20hrs after inoculation with 10^3 CFU type 2 *Streptococcus pneumoniae* (D39) or in mice that
 19 received PBS. Symbols represent individual animals and horizontal bars represent the mean. Data
 20 represent 4 (PBS), 11 (female *S. pneumoniae*) or 12 (male *S. pneumoniae*) mice per group pooled from
 21 three independent experiments. * $P < 0.05$. Student's *t* test.

22 (e) Numbers of bacteria in the peritoneal cavity of male and female mice infected 20hrs earlier and pre-
 23 treated with anti-CD209b (22D1) or a matched isotype control (Ham IgG1) 30mins prior to inoculation
 24 (left). Right, bacteraemia versus the frequency of CD102⁺ macrophages in the peritoneal cavity of mice
 25 above. Symbols represent individual animals and horizontal bars represent the mean. Data represent 10
 26 (isotype control) or 11 (anti-CD209b) mice per group pooled from two independent experiments.

27

1 **Supplementary Figure 1**

2 (a) Normalized non-host chimerism of peritoneal F4/80^{hi}CD102⁺ macrophages and indicated
3 leukocytes from sex matched tissue-protected BM chimeric mice 8-12 weeks post-reconstitution. Data
4 are normalised to the non-host chimerism of Ly6C^{hi} monocytes. Symbols represent individual animals
5 and horizontal bars represent the mean. Data represent 10 mice per group pooled from two independent
6 experiments. ****P<0.0001. Student's *t* test with Holm-Sidak correction. (b) Gating strategy for the
7 identification of omental macrophage subsets. (c) Expression of MHCII by CD102-defined macrophage
8 subsets and CD11b⁺ DC from omental digests. (d) Normalized non-host chimerism of peritoneal and
9 omental CD102⁺ macrophages from sex matched and sex mismatched tissue-protected BM chimeric
10 mice 8-12 weeks post-reconstitution. Data are normalised to the non-host chimerism of Ly6C^{hi}
11 monocytes. Symbols represent individual animals and horizontal bars represent the mean. Data
12 represent 9 (sex mismatched) or 10 (sex matched) mice per group pooled from two independent
13 experiments. **P<0.01. Student's *t* test with Holm-Sidak correction.

14 **Supplementary Figure 2**

15 (a, b) Frequency of F4/80^{hi}CD102⁺ macrophages (a) and CD102⁻MHCII⁺ cells (b) of total CD45⁺ cells
16 obtained from the peritoneal cavity of tissue-protected BM chimeric mice that had received surgery 8
17 weeks earlier. (c-f) Frequency (*left panels*) and absolute number of eosinophils (c), B1 cells (d), B2
18 cells (e) and T cells (f) from the peritoneal cavity of tissue-protected BM chimeric mice that had
19 received surgery 8 weeks earlier. Symbols represent individual animals and horizontal bars represent
20 the mean. Data represent 9 (sham) or 10 (control, unilateral, bilateral) mice per group pooled from two
21 independent experiments.

22 **Supplementary Figure 3**

23 (a) Experimental scheme for the generation of sex matched, tissue-protected bone marrow (BM)
24 chimeric mice and high-fat diet regimen. (b) Mass of abdominal adipose tissue in male and female mice
25 fed control or high fat diet for 4 weeks and rested for 8 weeks. (c) Bodyweight of male and female mice
26 fed control or high fat diet for 4 weeks and rested for 8 weeks (8) paired to starting bodyweight (0). (d)
27 Normalized non-host chimerism of peritoneal F4/80^{hi}CD102⁺ macrophages from mice in a. Symbols
28 represent individual animals and horizontal bars represent the mean. Data represent 5 (b) mice from
29 one experiment or 10 (c, d) mice per group pooled from two independent experiments.

30 **Supplementary Figure 4**

31 (a) Gating strategy for the identification and FACS-purification of CD102⁺ macrophages for
32 population-level RNAseq.

33 (b) Expression of F4/80 by peritoneal CD102-defined macrophages.

34 (c) Representative post-sort purity of CD102-defined macrophages.

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1 **Supplementary Figure 5**

2 (a) Expression of indicated TLRs and TLR adaptor molecules by male and female peritoneal
3 macrophages in the population-level RNAseq data set. (b) Expression of selected genes by male and
4 female peritoneal macrophages that were identified by Villa et al. (Cell Reports) to be expressed in a
5 sexually dimorphic fashion by microglia.

6

7 **Supplementary Figure 6**

8 (a) Representative expression of CD102 and CD209b by peritoneal all CD45⁺ leukocytes obtained from
9 age-matched male and female C57BL/6 mice. (b) Representative expression of Tim4 and CD209b by
10 peritoneal CD102⁺ macrophages obtained from age-matched male and female C57BL/6 mice housed
11 at the indicated institutions. (c) Representative expression of Tim4 and CD209b by peritoneal CD102⁺
12 macrophages obtained from age-matched male and female *Rag1*^{-/-} mice (*left*) and frequency of
13 CD209b⁺Tim4⁺ macrophages, the mean fluorescence intensity (MFI) of CD209b and frequency of
14 Tim4⁻ macrophages in each sex. Data are from one experiment with 4 (female) or 6 (male) mice per
15 group. ** P<0.01, ***P<0.001, ****P<0.0001. Student's *t* test. (d) Representative expression of
16 CD209b, Tim4 and Ki67 by peritoneal CD102⁺ macrophages obtained from age-matched male and
17 female BALB/c mice (*left*) and a summary of replicate data showing the frequency of CD209b⁺, Tim4⁺
18 and Ki67⁺ cells as well as MFI of expression. Data are from one experiment with 5 (female) or 6 (male)
19 mice per group. *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001. Student's *t* test.

20

21 **Supplementary Figure 7**

22 (a) Absolute number of FRβ⁺ CD102⁺ macrophages obtained from the peritoneal cavity of
23 unmanipulated 22-28 week old *Ccr2*^{+/+} or *Ccr2*^{-/-} mice. Symbols represent individual animals and
24 horizontal bars represent the mean. Data are from one experiment with 2 (*Ccr2*^{+/+} males), 4 (*Ccr2*^{+/+}
25 females) or 5 (*Ccr2*^{-/-}) mice per group. (b) Absolute number of B1 cells obtained from the peritoneal
26 cavity of unmanipulated male and female mice at 6-8 weeks and 14-16 weeks. Symbols represent
27 individual animals and horizontal bars represent the mean. Data are pooled from four experiments with
28 9 (females, both time points), 8 (male, 6-8 weeks) or 13 (male, 14-16 weeks) mice per group. Data for
29 the 14-16 week time point taken from data in Figure 6g. ****P<0.0001. Two-way ANOVA.

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