INTRODUCTION

Macrophages are present in every tissue of the body, where they provide immune protection and orchestrate tissue repair after insult or injury. Peritoneal macrophages are arguably the most studied population of macrophages in the body, having been used extensively as a convenient source of macrophages for ex vivo analyses for decades. In spite of this body of knowledge, the heterogeneity of peritoneal macrophages and much of the biology that governs their development, differentiation, and function remains unclear. Macrophages in the peritoneal cavity are programmed for “silent” clearance of apoptotic cells, maintenance of innate B1 cells through secretion of CXCL13, and immune surveillance of the cavity and neighboring viscera (1–4). However, they are also implicated in many pathologies, including peritonitis, endometriosis, postsurgical adhesions, pancreatitis, and metastatic cancer (5–15), although the exact roles they play in these processes are not fully understood.

Rate of replenishment and microenvironment contribute to the sexually dimorphic phenotype and function of peritoneal macrophages

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Macrophages reside in the body cavities where they maintain serosal homeostasis and provide immune surveillance. Peritoneal macrophages are implicated in the etiology of pathologies including peritonitis, endometriosis, and metastatic cancer; thus, understanding the factors that govern their behavior is vital. Using a combination of fate mapping techniques, we have investigated the impact of sex and age on murine peritoneal macrophage differentiation, turnover, and function. We demonstrate that the sexually dimorphic replenishment of peritoneal macrophages from the bone marrow, which is high in males and very low in females, is driven by changes in the local microenvironment that arise upon sexual maturation. Population and single-cell RNA sequencing revealed marked dimorphisms in gene expression between male and female peritoneal macrophages that was, in part, explained by differences in composition of these populations. By estimating the time of residency of different subsets within the cavity and assessing development of dimorphisms with age and in monocytopenic Ccr2−/− mice, we demonstrate that key sex-dependent features of peritoneal macrophages are a function of the differential rate of replenishment from the bone marrow, whereas others are reliant on local microenvironment signals. We demonstrate that the dimorphic turnover of peritoneal macrophages contributes to differences in the ability to protect against pneumococcal peritonitis between the sexes. These data highlight the importance of considering both sex and age in susceptibility to inflammatory and infectious diseases.

Under physiological conditions, at least two macrophage populations are present in the murine peritoneal cavity, with those expressing high levels of F4/80, CD11b, and CD102 outnumbering their F4/80loMHCII+ counterparts by approximately 10-fold. F4/80hiCD102+ macrophages [sometimes referred to as “large” peritoneal macrophages (16)] rely on the transcription factors C/EBPβ and GATA6 for their differentiation and survival (17–20), with the latter under the control of retinoic acid proposed to derive, in part, from the omentum (19). The F4/80hiMHCII+ compartment is heterogeneous, comprising both macrophages and conventional dendritic cells (DCs) (21–24). F4/80hiMHCII+ macrophages (sometimes referred to as “small” peritoneal macrophages or monocyte-derived DC) rely on interferon regulatory factor 4 (IRF4) for their differentiation and can be further defined by their expression of CD226 and the immunomodulatory molecule RELMα (21, 23, 24). Recent studies using lineage tracing techniques have established that F4/80hiMHCII+ macrophages that arise postnatally are short-lived and replaced by Ly6C+ classical monocytes in a CCR2-dependent manner (20–23). In contrast, F4/80loCD102+ macrophages are longer-lived cells that derive from embryonic sources but are subsequently replaced by cells of hematopoietic stem cell origin (21, 25). We have recently shown that, unlike resident macrophages in numerous other tissues, the turnover of peritoneal F4/80loCD102+ macrophages from the bone marrow (BM) is highly sex dependent, with high and low rates in male and female mice, respectively (21). We have also shown that long-lived macrophages can be identified by their expression of the phagocytic receptor, Tim4, whereas most recent descendants of BM-derived cells among the F4/80loCD102+ macrophage compartment are Tim4− (21). Consistent with this, Tim4 expression has been shown to be a feature of long-lived macrophages in other tissues (26–30).
RESULTS

Environmental factors drive sexual dimorphism in peritoneal macrophage replenishment

We first set out to determine whether the dimorphic effects in peritoneal macrophage replenishment were due to the peritoneal environment or to cell-intrinsic differences in the ability of male and female monocytes to generate F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages at this site. To this end, we generated sex-mismatched, tissue-protected BM chimeric mice to measure the turnover of peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages from female > female BM chimeric mice, whereas high levels were detected in their male > male counterparts (Fig. 1, C and D, and fig. S1B), confirming marked sex dimorphism in macrophage turnover. This dimorphism was specific to F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages, as all other leukocyte subsets in the peritoneal cavity showed identical replenishment in male and female BM chimeric mice (fig. S1C). F4/80<sup>hi</sup>CD102<sup>+</sup> peritoneal macrophages from sex-mismatched (female > male) chimeras had similar levels of chimerism to male > male chimeras (Fig. 1, C and D), demonstrating that female and male monocytes have equal ability to generate F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages in the male peritoneal cavity. Female recipients rejected male BM, and thus, chimerism in this group could not be determined.

The omentum has been implicated in the differentiation of F4/80<sup>hi</sup> macrophages in the peritoneal cavity, potentially acting as site of macrophage maturation (19, 40, 41). CD102<sup>+</sup> macrophages that coexpress GATA6 can be detected among omental isolates (19), together with CD102<sup>+</sup>MHCII<sup>+</sup> macrophages and a population of Ly6C<sup>+</sup>CD11b<sup>+</sup> cells similar to monocytes (Fig. 1E and fig. S1, D and E). To determine whether the dimorphic replenishment of peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages arises in the omentum, we assessed nonhost chimerism in the macrophage populations within this site. Although this showed clear differences in the turnover of CD102-defined macrophage populations from BM, with higher replenishment in the CD102<sup>-</sup> fraction, no sex dimorphism was detected in any monocyte/macrophage population within the omentum (Fig. 1F). Furthermore, the chimerism of omental and peritoneal CD102<sup>+</sup> macrophages in male recipients was identical, rather than showing the gradation that would have been expected if omental macrophages were intermediate precursors between monocytes and cavity CD102<sup>+</sup> cells (fig. S1F). Thus, the sexual dimorphism in peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophage replenishment is driven by factors present in the local environment.

Sexual dimorphism in peritoneal macrophage replenishment occurs after sexual maturity

To extend these findings and to assess macrophage turnover at different stages of maturity, we next used a genetic fate mapping approach. Adoptive transfer experiments suggest that F4/80<sup>hi</sup>MHCII<sup>+</sup> macrophages in the peritoneal macrophage compartment act, in part, as precursors of F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages (20), and we have recently shown that this differentiation can be mapped by exploiting their expression of CD11c (21). Thus, in CD11c<sup>Cre</sup>Rosa26<sup>SL-eYFP</sup> mice (Fig. 2A), in whom active or historic expression of CD11c leads to irreversible labeling with enhanced yellow fluorescent protein (eYFP), labeled cells accumulate with age in the F4/80<sup>hi</sup>CD102<sup>+</sup> macrophage compartment, despite these cells themselves not actively expressing CD11c (21). We therefore used CD11c<sup>Cre</sup>Rosa26<sup>SL-eYFP</sup> mice to compare the rate of eYFP<sup>+</sup> cell accumulation in peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages from male and female mice. In juvenile/pubescent mice (4 weeks of age), the extent of eYFP labeling was relatively similar between male and female peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages and was marginally higher in female mice (Fig. 2B and fig. S2A). By 16 weeks of age, the frequency of eYFP<sup>+</sup> cells among F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages had increased in both male and female mice compared with their 4-week-old counterparts. However, although there was no difference in CD11c protein expression by male and female F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages (Fig. 2C), higher levels of eYFP labeling were detected among male peritoneal monocytes to generate F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages at this site. To this end, we generated sex-mismatched (female > male, male > female) BM.

Sex is a variable often overlooked in immunological research (31) despite strong sex biases in many pathologies including autoimmune disorders and infection susceptibility (32). Sex dimorphisms in the immune system are present in a diverse range of species from insects, bird, lizards, and mammals (32), suggesting that this may be an evolutionarily conserved phenomenon. It is therefore essential to understand how intrinsic factors, such as sex, control the behavior of innate immune effector cells. Specifically, sex has been proposed to affect macrophage behavior, such as influencing the differentiation of brain microglia (33–35), and sex hormones appear able to directly regulate gene expression (36) and proliferation (37) of macrophages. Whereas previous studies have considered the effects of sex on peritoneal macrophage behavior, many of these have focused on in vitro functional assessments using macrophages elicited by injection of an irritant or inflammatory agent (38), or they have not appreciated the complexity of the peritoneal macrophage compartment (37, 39).

Here, we have used a combination of fate-mapping techniques together with population-level and single-cell RNA sequencing (scRNA-seq) to dissect the role of sex in the composition, environmental imprinting, and function of peritoneal macrophages. We show that the F4/80<sup>hi</sup>CD102<sup>+</sup> macrophage population is heterogeneous, and that dimorphic turnover is associated with divergence in the heterogeneity of this compartment with age. Specifically, we demonstrate that the sexual dimorphism in replenishment from the BM and phenotype arise after sexual maturation. Furthermore, we provide examples of transcriptional and functional dimorphisms that arise because of sex differences in turnover versus those arising directly from sex differences in the peritoneal microenvironment. We identify the C-type lectin receptor CD209b [also known as specific ICAM3-grabbing nonintegrin-related 1 (SIGN-R1)] as a marker whose expression is determined by replenishment that becomes increasingly dimorphic with age and show that sex-dependent resistance to pneumococcal peritonitis arises, in part, due to dimorphic expression of CD209b.

However, it remains unclear whether further heterogeneity exists among these broadly defined populations and whether sexually dimorphic turnover influences the composition and function of the F4/80<sup>hi</sup>CD102<sup>+</sup> macrophage population in other ways.
OVX leads to increased macrophage replenishment

The onset of sexually dimorphic turnover of peritoneal macrophages after sexual maturation and the uniquely slow replenishment of female peritoneal macrophages suggested that factors involved in female reproductive function may drive this dimorphism. Therefore, we next assessed macrophage turnover in females after ovariectomy (OVX). Thus, female > female tissue-protected BM chimeric were generated, and after 8 weeks of reconstitution, both ovaries were surgically removed (bilateral OVX), before measuring nonhost chimerism after another 8 weeks. To account for the potential effects of surgery on macrophage replenishment, BM chimeric mice receiving sham surgery or unilateral OVX were used as controls, together with unmanipulated BM chimeric mice. As expected, the cessation of ovarian estradiol production caused by bilateral OVX led to complete atrophy of the uterine horn; this was not seen in mice with unilateral OVX or in other control groups (Fig. 3A). Bilateral OVX had no effect on the numbers of F4/80<sup>+</sup>CD102<sup>−</sup> and CD102<sup>−</sup>MHCII<sup>+</sup> macrophages in the peritoneal cavity when compared with the control groups (Fig. 3B). Although bilateral OVX led to increased proportions and absolute numbers of eosinophils, these differences did not attain statistical significance and the opposite pattern was found with B1 cells (Fig. S2, B to I). Importantly and in marked contrast to the very low levels of chimerism (~1%) detected in unmanipulated control chimeras (Fig. 3C), sham surgery and unilateral OVX led to marked increases in the level of chimerism compared with unmanipulated chimeric mice, demonstrating that minimally invasive laparotomy itself appears to have long-term effects on the dynamics of peritoneal macrophages in female mice. Nevertheless, complete removal of the ovaries further elevated macrophage turnover, with chimerism reaching approximately 12%. No difference was found between the chimerism seen after sham surgery with or without unilateral OVX, indicating that the OVX procedure itself does not exaggerate the effects of laparotomy and that it is the complete loss of ovarian function that underlies the further elevation in macrophage turnover that results from bilateral OVX. Consistent with these results, significantly more Tim4<sup>−</sup>CD102<sup>−</sup> macrophages were present in the cavity of mice that received bilateral OVX than any other group (Fig. 3D), further supporting the idea of elevated macrophage replenishment from BM. No differences in chimerism or in Tim4-defined subsets could be detected among F4/80<sup>+</sup>CD102<sup>−</sup> macrophages from the pleural cavity, again confirming that the effect of surgery and OVX on macrophage turnover is specific to the peritoneal cavity (Fig. 3, B and C).

Estrogens are the prototypical female sex steroid hormones, which are ablated by OVX. To assess whether estrogen influences macrophage replacement, we repeated the OVX experiment with an additional group of bilateral OVX mice receiving exogenous estradiol. However, while this treatment reversed OVX-mediated atrophy of the uterine horns and peritoneal eosinophilia, it had no effect on the heightened rate of replenishment of F4/80<sup>+</sup>CD102<sup>−</sup> peritoneal macrophages in OVX mice, suggesting that estradiol is not directly

**Fig. 1. Environment drives sexual dimorphism in macrophage replenishment in the peritoneal cavity.** (A) Expression of CD11b and CD3, CD19, Ly6G, and SiglecF (“Lineage”) by live CD45<sup>+</sup> peritoneal leukocytes (left) and expression of CD102 and MHCII by CD11b<sup>+</sup>Lin<sup>−</sup> cells (center) from adult C57BL/6 female mice. Histograms show expression of F4/80 and CSF1R by CD102<sup>−</sup> and CD102<sup>−</sup>MHCII<sup>+</sup> cells (also see fig. S1A). (B) Scheme for the generation of sex-mismatched, tissue-protected BM chimeric mice. (C and D) Representative chimerism (C) and normalized non-host chimerism (D) of peritoneal F4/80<sup>+</sup>CD102<sup>−</sup> macrophages from sex-matched or sex-mismatched tissue-protected BM chimeric mice. As ex-
Sex determines the transcriptional signature of peritoneal macrophages

The difference in macrophage replenishment prompted us to assess the wider effects of sex on the imprinting of peritoneal macrophage identity and function. We therefore first performed population-level RNA-seq on peritoneal F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages fluorescence-activated cell sorting (FACS)-purified from unmanipulated 10- to 12-week-old male and female mice (fig. S4). To limit potential confounding effects of the estrous cycle, the stage of each female mouse was confirmed by vaginal cytology and samples pooled to include cells from all stages of the cycle. Furthermore, to limit the effects of circadian influence, mice in each biological replicate were euthanized at the same time each day. Unbiased clustering was then used to group the populations based on sex, with sex explaining 81% of the variance within the datasets (Fig. 4A), and differential gene expression analysis revealed that 486 mRNA transcripts were differentially expressed (>1.5-fold) between female and male peritoneal CD102<sup>+</sup> macrophages (Fig. 4B and table S1). Analysis of the 148 mRNA transcripts more highly expressed in female peritoneal macrophages revealed that a large proportion was associated with immune function, including the C-type lectin receptors Clec4g, C209n, and C209h; the complement components C4b, C1qa, and C3; the immunoregulatory cytokine Tgfb2; the B cell chemotactant Cxcl13; and, as expected, the phagocytic receptor Timd4 (Fig. 4C and table S1). Consistent with this, “immune response” and “immune system processes” were among the top pathways identified by gene set enrichment analysis (GSEA) in genes up-regulated in female cells (table S2). Transcripts for the apolipoproteins Apeo, Saa2, Saa3, and Apeo1 were also expressed more highly in female cells. In contrast to previous work that assessed basal gene expression by total peritoneal cells across the sexes (39), we did not detect any dimorphism in expression of Toll-like receptors (TLRs), the TLR adaptor molecule MyD88, or CD14 (fig. S5). Moreover, the dimorphic cassette of genes we identified is distinct from that recently shown to be sexually dimorphic in microglia (fig. S5, A and B), consistent with previous reports that transcriptional differences between sexes in macrophages are largely tissue specific (44).

We used flow cytometry to confirm higher expression of Cd209b, Cxcl13, and Apeo1 by female macrophages, as these were the most differentially expressed non–X-linked genes with mapped read counts greater than 10. This analysis revealed unexpected heterogeneity within resident peritoneal macrophages. For instance, only a proportion of
Fig. 3. OVX 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), had received sham surgery, or were completely unmanipulated (control). (B) Absolute number of F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages and CD102<sup>-</sup>MHCII<sup>−</sup> 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) Nonhost chimerism of blood Ly6Chi blood monocytes (left) and F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages obtained from the peritoneal (center) 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<sup>−</sup> cells among F4/80<sup>hi</sup>CD102<sup>+</sup> macrophages obtained from the peritoneal (center) or pleural (right) cavity of mice in (B). ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparisons test. For representative FACS plots, see fig. S2C. (E) Frequency of Tim4<sup>−</sup> cells among F4/80<sup>hi</sup>CD102<sup>+</sup> 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 thrice weekly for 3 weeks. Symbols represent individual animals, and horizontal bars represent the mean. Data represent 8 (control) or 10 (sham, bilateral, bilateral + estradiol) mice per group pooled from two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparisons test.

Male and female CD102<sup>+</sup> macrophages expressed CD209b, although the frequency of these was greater in females than in males (35 and 20%, respectively). Moreover, CD209b was expressed at a higher level on a per-cell basis by female CD102<sup>+</sup> macrophages compared with their male counterparts (Fig. 4C), a finding consistent across different strains, including Rag1<sup>−/−</sup> mice, and mice from different housing environments (fig. S6). Because of the unavailability of commercial antibodies for CXCL13, ApoE, and ApoC1, we used PrimeFlow technology to measure mRNA of these genes at a single-cell level but also from different frequencies of gene-expressing cells among the CD102<sup>+</sup> population. In contrast, PrimeFlow measurement of mRNA for ApoE, the most highly expressed of all differentially expressed genes (DEGs) by female cells by RNA-seq, revealed that all peritoneal macrophages expressed ApoE irrespective of sex, but that expression was higher in female cells on a per-cell basis. Hence, the transcriptional differences seen at population level appear to result from differential gene expression at a single-cell level but also from different frequencies of gene-expressing cells among the CD102<sup>+</sup> population.

Most of the genes more highly expressed by male peritoneal CD102<sup>+</sup> macrophages were associated with cell cycle, including Cdk1, E2f2, and Mki67 (Fig. 4B and table S1). Pathway analysis also revealed that at least 162 of the 338 genes differentially up-regulated in male CD102<sup>+</sup> macrophages were associated with proliferation, and cell cycle–related processes predominated among the enriched pathways (table S2). Short-term 5-bromo-2′-deoxyuridine (BrdU) pulse-chase experiments confirmed that male CD102<sup>+</sup> macrophages have elevated levels of in situ proliferation compared with their female counterparts (Fig. 4G). These analyses also identified that Retina, which encodes the immunomodulatory cytokine RELMγ, is expressed specifically by those resident peritoneal macrophages that are most recently derived from monocytes (21), was differentially expressed between sexes, with higher expression by male cells at both the mRNA and protein level (Fig. 4H).

A number of genes previously reported to distinguish long-lived, embryonically derived macrophages from those of recent BM origin in the lung and liver were more highly expressed in females. These included receptors involved in phagocytosis and immunity (i.e., Timd4, Colec12, and Cd209 family members), Apoc1, as well as the bone morphogenic receptor Bmpr1a (table S1) (26, 45, 46). Lowering the stringency of selection of DEGs identified additional genes within the female-specific cluster that have been associated with embryonically derived or long-lived macrophages, including Cd163 that also encodes a phagocytic receptor (26, 46). Furthermore, to discern systemic from local effects of sex, we compared gene expression by CD102<sup>+</sup> macrophages from female peritoneal cavity to pleural CD102<sup>+</sup> macrophages from both sexes. This analysis identified a module of 18 genes that was uniquely up-regulated by female peritoneal...
Fig. 4. Sex determines the transcriptional signature of peritoneal macrophages. (A) Heatmap showing distance between samples of male (M) and female (F) CD102+F4/80hi macrophages FACS-purified from the peritoneal cavity of 10- to 12-week-old mice. (B) Gene expression profile of the 148 differentially expressed (>1.5-fold) genes between male and female peritoneal macrophages with selected genes highlighted. (C) Expression of Cd209b from RNA-seq (fragments per kilobase of transcript per million mapped reads (FPKM); left panel), representative expression of CD209b protein (middle panels), and frequency of CD209b+ cells among CD102+F4/80hi peritoneal macrophages obtained from 10- to 12-week-old male or female C57BL/6 mice (right panel) and the mean fluorescence intensity of CD209b expression by these cells (far right panel). Symbols represent individual animals, and horizontal bars represent the mean. RNA-seq data represent three mice per group, and protein analysis represents five mice per group from one of five independent experiments. ****P < 0.0001, Student’s t test. For gating strategy, see fig. S5C. (D) Expression of Cxcl13 from RNA-seq (FPKM; left panel), representative expression of CXCL13 mRNA (middle panels), and frequency of CXCL13+ cells among CD102+F4/80hi peritoneal macrophages obtained from 10- to 12-week-old male or female C57BL/6 mice (right panel) and the mean fluorescence intensity of CXCL13 mRNA expression by these cells (far right panel). Symbols represent individual animals, and horizontal bars represent the mean. RNA-seq data represent three mice per group, and flow cytometric analysis represents five mice per group from one of three independent experiments. *P < 0.05, ***P < 0.001, Student’s t test. (E) Expression of ApoC1 from RNA-seq (FPKM; left panel), representative expression of ApoC1 mRNA (middle panels), and frequency of ApoC1+ cells among CD102+F4/80hi peritoneal macrophages obtained from 10- to 12-week-old male or female C57BL/6 mice (right panel) and the mean fluorescence intensity of ApoC1 mRNA expression by these cells (far right panel). Symbols represent individual animals, and horizontal bars represent the mean. RNA-seq data represent three mice per group, and flow cytometric analysis represents five mice per group from one of three independent experiments. *P < 0.05, ***P < 0.001, Student’s t test. (F) Expression of Retnla from RNA-seq (FPKM; left panel), representative expression of RELMα protein (middle panels), and the frequency of RELMα+ cells among CD102+F4/80hi peritoneal macrophages obtained from 10- to 12-week-old male or female C57BL/6 mice. Symbols represent individual animals, and horizontal bars represent the mean. Data represent five mice per group from one of two experiments. ***P < 0.001, Student’s t test.
macrophages and that included Apoc1, Cd209b, and Cole12, as well as Saa3, C4b, and Tgfb2 (table S3). Conversely, the 86 genes uniquely down-regulated by female peritoneal macrophages compared with the other CD102+ populations were highly enriched for cell cycle–related genes and pathways (tables S3 and S4). Thus, the more limited proliferative activity of female peritoneal macrophages and their expression of numerous immune-related genes appear either related to their slower replenishment from the BM or regulated directly by the unique signals present within the female peritoneal microenvironment.

**scRNA-seq reveals equivalent cluster identities in male and female peritoneal macrophages, but these exhibit dimorphisms in abundance and gene expression**

We next applied scRNA-seq to determine whether the transcriptional differences seen in our population-level data were the result of gene differences at a single-cell level or whether dimorphism was a reflection of differential subset composition between the sexes. A broad approach was used to capture all CD11b+ cells depleted of granulocytes and B1 B cells to allow both CD102 F4/80MHCII+ mononuclear phagocytes and resident CD102+ cells to be examined. These cells were FACS-purified from age-matched 19-week-old male and female mice, and droplet-based scRNA-seq was performed using the 10x Genomics platform. Ten thousand sorted cells of each sex were sequenced, and after quality control, analysis was performed using the 10× Genomics platform. Ten thousand sorted cells of each sex were sequenced, and after quality control, analysis was performed using the 10× Genomics platform. Ten thousand sorted cells of each sex were sequenced, and after quality control, analysis was performed using the 10× Genomics platform.

Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction analysis revealed six clusters that were present in both male and female cells (Fig. 5, A and B). Given that the starting population of CD11b+ cells is known to be phenotypically heterogeneous, containing resident CD102+ F4/80hi macrophages, CD102− F4/80lo MHCII+ macrophages, and CD11c+ MHCII+ cDC2 (20–23), we first used a panel of known markers to validate subset identity (Fig. 5B). Three clusters of resident macrophages (3 to 5) could be identified on the basis of their high expression of Adgre1 (F4/80) and Icam2 (CD102). As expected, these were distinct from short-lived CD102+ F4/80lo MHCII+ macrophages and cDC2, which were found in clusters 1 and 2, respectively, and expressed Ccr2 (Fig. 5, C and D) (21). However, CD102− F4/80lo MHCII+ macrophages and cDC2 could be distinguished from one another on the basis of expression of the DC markers Cd209a and Napsa (28, 47), and of Retnla and Fcrls, which we and others have shown to be signature markers of cavity CD102− F4/80lo MHCII+ macrophages (21, 23, 48). Cluster 6 was defined by genes associated with cell cycle, such as Mki67 and Birc5, suggesting that this cluster represents proliferating cells. In both sexes, the majority of cells was in cluster 5 (Fig. 5E), which was characterized by markers of resident F4/80hi CD102+ macrophages including Icam2, Prg4, Vsig4, and Tgfb2 (Fig. 5, C and D, and table S5) that form part of the core peritoneal macrophage-specific transcriptional signature (49); cluster 5 cells also expressed markers of long-lived macrophages, including Timd4 and Apoc1, confirming the findings above. Although the cells in cluster 3 expressed Icam2, they also expressed a number of genes that were highly expressed by the CD102− F4/80lo MHCII+ macrophages in cluster 2, such as Retnla, H2.Aa, and Ccr2, suggesting a common origin of these clusters or a close relationship between them. This analysis also identified genes expressed more highly by cluster 3, including Folr2, which encodes the beta subunit of the folate receptor (FRβ). Although cluster 4 showed a distinct pattern of gene expression, such as high expression of Apoe, it also shared features with clusters 3 and 5, suggesting that it may contain differentiation intermediates. Consistent with our earlier analysis, we found that the Timd4+expressing cluster 5 was more abundant among female cells, whereas more male cells were found within clusters 1, 2, 3, and 6 (Fig. 5C).

We next used flow cytometry to validate the additional heterogeneity uncovered by our scRNA-seq analysis. Consistent with the unbiased clustering and our previous study (21), macrophages expressing Tim4 (corresponding to cluster 5) were more abundant in female than in male mice (Fig. 5, F and G). Moreover, using this strategy, we validated high expression of ApoC1, V-set immunoglobulin domain–containing 4 (Vsig4), and CXCL13 by this subset of cells (Fig. 5H and fig. S7A). Although not identified as a cluster-defining gene in our scRNA-seq analysis because of low coverage, we found that CD209b displayed the same pattern of expression as CXCL13 (Fig. 5H). Again, confirming our scRNA-seq, we found the Tim4− fraction of CD102+ macrophages to be more abundant in males. Macrophages lacking Tim4 expression could be divided into MHCII+ and MHCII− subsets, which corresponded to clusters 3 and 4 in our scRNA-seq analysis (Fig. 5, F and G). Consistent with this, we found that FRβ was highly and selectively expressed at protein level by Tim4+ MHCII− CD102+ macrophages (Fig. 5I), and these expressed RELMα at a higher level than all other CD102+ macrophages (Fig. 5J). Whereas Apoe was proposed to define cluster 4 in our scRNA-seq analysis, we found that it was expressed by all CD102+ macrophages (Fig. 4F); although, in females, it was most highly expressed by Tim4+ MHCII+ CD102+ macrophages (Fig. 5I). To test this directly, we reanalyzed our data from adult C11cCre.Rosa26SL-eYFP mice to assess whether MHCII/Tim4–defined subsets showed differential levels of replenishment. We found that MHCII+expressing Tim4− CD102+ macrophages showed equivalent labeling to CD102+ F4/80lo MHCII+ macrophages in female C11cCre.Rosa26SL-eYFP mice, indicative of more recent derivation from CD102+ F4/80+ MHCII+ cells (Fig. 5M). Although CD102+ macrophages that had lost MHCII expression showed intermediate labeling when compared with their MHCII−Tim4+ and Tim4− counterparts. No difference in eYFP labeling between Tim4–defined subsets was noted in male mice, consistent with more rapid replenishment of all subsets of macrophages in this environment. Collectively, these data show that excluding proliferating cells, resident peritoneal macrophages comprise three main clusters, with Tim4− macrophages displaying an intermediate phenotype compared with F4/80lo MHCII+ macrophages and Tim4+ macrophages. Furthermore, although equivalent clusters of CD102+ peritoneal macrophages are found in males and females, our analysis suggests that sexual dimorphisms in gene expression in CD102+ cells identified by population-level RNA-seq arise, in part, because of differences in abundance of these clusters.

Last, we performed differential gene expression analysis on our scRNA-seq datasets to distinguish dimorphisms that arise because of differential gene expression across all CD102+ cells irrespective of cluster identity from those that only occur within individual clusters and potentially reveal sex-dependent gene expression not detected...
Fig. 5. scRNA-seq 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 six 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 DEGs by each cluster from (A) (select genes highlighted). (E) Relative frequency of each cluster in the female and male dataset. (F) Representative expression of Ki67 and BrdU incorporation by CD102+ macrophages (top panels), expression of Tim4 by Ki67−CD102+ macrophages (middle panels), and expression of MHCII by Tim4−CD102+ peritoneal macrophages from 10- to 12-week-old male or female C57BL/6 mice. (G) Relative frequency of each cluster as a proportion of all CD11b+ cells determined by flow cytometry in (F). (H) Expression of Apol1 (mRNA), CXCL13 (mRNA), and CD209b protein by CD102+/MHCII+ and Tim4/MHCII-defined CD102+ peritoneal macrophages from 10- to 12-week-old male or female C57BL/6 mice. Data represent 8 (male) and 10 (female) mice per group pooled from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparisons test. (I) Histograms show representative expression of FRβ by CD102+/MHCII+ and Tim4-defined CD102+ peritoneal macrophages from 10- to 12-week-old male or female C57BL/6 mice. Scatter plot show frequency of FRβ+ cells among CD102loMHCII+ and Tim4/MHCII-defined CD102+ peritoneal macrophages. Data represent 6 (female) or 7 (male) mice per group pooled from two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey's multiple comparisons test. (J) Expression of RELMα by CD102+/MHCII+ and Tim4/MHCII-defined CD102+ peritoneal macrophages from 10-week-old male or female C57BL/6 mice. Data represent three mice per group from one of at least five independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey's multiple comparisons test. (K) Frequency of ApoE+ cells among CD102loMHCII+ and Tim4/MHCII-defined CD102+ peritoneal macrophages (left) and mean fluorescence intensity of ApoE by these subsets (right) from 10- to 12-week-old male or female C57BL/6 mice. Data represent 8 (male) and 10 (female) mice per group pooled from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey's multiple comparisons test. (L) Expression of CX3CR1-GFP by CD102+MHCII+ and Tim4/MHCII-defined CD102+ peritoneal macrophages from 15-week-old male or female Cx3cr1+/-/gfp mice. Data represent five (female) or seven (male) mice per group pooled from three independent litters. **P < 0.01, ****P < 0.0001, one-way ANOVA with Tukey's multiple comparisons test. (M) Frequency of eYFP+ cells among F4/80-, MHCII-, and Tim4-defined macrophages obtained from 16-week-old male and female CD11cCre.Rosa26LSL-eYFP mice. Symbols represent individual animals and horizontal bars represent the mean. Data represent five (male) or nine (female) mice per group pooled from two independent experiments. (N) Relative mean fluorescence intensity of Tim4 expression by Tim4+CD102+ peritoneal macrophages from 10- to 12-week-old male or female C57BL/6 mice. Data represent 8 (male) or 11 (female) mice per group pooled from three independent experiments. *P < 0.05, Mann-Whitney test.
by our population-level RNA-seq analysis. Excluding X- and Y-link
from stress induced during cell isolation, this identified 51 and 116
genes expressed more highly by female and male cells, respectively
(36). Almost 50% of these gene differences were validated by
our population-level RNA-seq dataset when the 1.5 log-fold cutoff
limit was removed to allow detection of subtle differences (36).
For instance, in keeping with our PrimeFlow analysis (5, H and K,
and table S7), Cxcl13 and Apeo were found to be more highly expressed
in female cells of all clusters of CD102+ macrophages (table S6).
Similarly, Saa3, C4b, and Timp2 were found to be more highly expressed by female CD102+ macrophages irrespective of cluster
identity, whereas Vim, Arg1, S100 family genes, and SerpinB2 and
Serpbin B1a were among those genes expressed more highly by
male CD102+ cells irrespective of cluster. However, more than 50%
of DEGs were either unique to a single cluster of CD102+ macro-
phages or shared across only 2 of the 3 populations (table S6).
For example, in spite of being a defining marker of cluster 5, Timd4 was
identified as being differentially expressed within this cluster
between the sexes. Consistent with this, female Tim4+ macrophages
were among those genes expressed more highly by
cell-intrinsic higher expression of CXCL13 by female macrophages
in the female cavity (Fig. 6E). Similarly, enrichment of cells expressing
other markers of cluster 5, such as VSIG4, became apparent in
females with age (fig. S7B), suggesting that numerous cluster 5 genes
may be regulated by time of residency.

Not all dimorphic features of peritoneal CD102+ macrophages
were influenced by their rate of replenishment. For instance, the
intrinsic higher expression of CXCL13 by female macrophages
was not altered by CCR2 deficiency (Fig. 6F). In parallel, although
we confirmed previous findings of a clear dimorphism in the numbers
of B1 cells between adult male and female mice (15) and this develop-
ed gradually after sexual maturation (fig. S7C), this phenomenon
remained in Ccr2−/− mice (Fig. 6G). Similarly, while the higher levels of
proliferation by male CD102+ macrophages developed after sexual
maturation (Fig. 6H), this was unaffected by CCR2 deficiency (Fig. 6I).
This evidence that certain dimorphic features are driven by envi-
ronmental signals, independent of cell replenishment, was supported
further by the fact that macrophages derived from female BM in the
mammalian genital cavity were higher than those of male BM-derived macro-
phages in the peritoneal cavity (fig. S6A), this model allowed us to directly assess
the importance of differential CD102+ expression by CD102+ macro-
phages in bacterial elimination. Females showed enhanced capability

Differential replenishment and environmental signals drive
the dimorphic features of peritoneal macrophages
To dissect the dimorphic features of CD102+ macrophages that
could be related to longevity from those more directly controlled by
dimorphic environmental signals, we next assessed expression of these
in Ccr2−/− mice in whom macrophage replenishment is markedly
reduced because of severe monocytopenia (50, 51). The frequency of Tim4+ macrophages, as well as those expressing RELMα, FRα, or
MHCII, was markedly reduced in Ccr2−/− mice compared with Ccr2+/+
mice irrespective of sex, confirming these cells to be recently derived
from monocytes (Fig. 6A). In males, CCR2 deficiency also led to
reduced expression of ApoE and emergence of an ApoE− subset of
CD102+ macrophages (Fig. 6B). In contrast, a higher proportion of
CD102+ macrophages in Ccr2−/− mice expressed CD209b and
ApoC1, markers that are characteristic of the Tim4+ MHCII+ subset,
suggesting that these markers may be expressed selectively by long-
ived macrophages (Fig. 6B). Consistent with this, Tim4+ macrophages
expressing CD209b displayed the lowest level of replacement by
donor cells in BM chimeras when compared with all other CD209b/
Tim4-defined macrophages, even in male mice where overall re-
plenishment from the BM is markedly higher (Fig. 6C). The low
levels of replacement of peritoneal CD209b+Tim4+ macrophages
does not reflect derivation from yolk sac progenitors, as, unlike
microglia in the brain, these cells are not labeled in male or female
Cdh5Cre-ERT2,Rosa26Lsl-tdTom mice (Fig. 6D), which allow tracing
of cells arising from yolk sac hematopoiesis (52). Similar results
were obtained with CD209b+Tim4+ macrophages in the pleural
cavity. Hence, despite being long-lived, CD209b+Tim4+ macro-
phages derive from conventional hematopoiesis in both sexes.
Temporal analysis revealed that while little difference in abundance
of CD209b-expressing CD102+ macrophages was seen in prepubescent
(4- to 5-week-old) male and female mice, these cells accumulated
progressively in the cavity of female mice after sexual maturation.
This did not occur in male mice, consistent with their higher rate of
replenishment from the BM and indicating that acquisition of
CD209b expression appears to be associated with time of residency
in the female cavity (Fig. 6E). Similarly, enrichment of cells express-
ing other markers of cluster 5, such as VSIG4, became apparent in
females with age (fig. S7B), suggesting that numerous cluster 5 genes
may be regulated by time of residency.

Differential CD209b expression confers an
advantage on female macrophages in the setting
of pneumococcal peritonitis
We postulated that differential expression of key pattern recognition
receptors such as CD209b might endow female macrophages with an
enhanced ability to deal with bacterial infection. To test this idea,
we examined the acute peritonitis caused by infection with the
Gram-positive bacterium Streptococcus pneumoniae (Fig. 7A).
At low doses, resident macrophages, and in particular CD209b, are
indispensable for protective immunity in this model (33, 54), whereas
recruitment of neutrophils is not required (53) and hence avoids any
confounding effects of systemic sex-dependent effects on innate immune responses that have been reported previously (55). As
CD209b is expressed exclusively by CD102+ macrophages in the
peritoneal cavity (fig. S6A), this model allowed us to directly assess
the importance of differential CD209b expression by CD102+ macro-
phages in bacterial elimination. Females showed enhanced capability
Fig. 6. Differential replenishment and environmental signals drive the dimorphic features of peritoneal macrophages. (A) Frequency of Tim4+ RELMα+, MHCII+, and FRα+ cells among CD102+ macrophages from the peritoneal cavity of unmanipulated age-matched Ccr2+/+ or Ccr2−/− mice. Symbols represent individual animals, and horizontal bars represent the mean. Data are pooled from two independent experiments. Tim4 data represent three (Ccr2+/+ males), six (Ccr2−/− females), or seven (Ccr2−/−) 22- to 28-week-old mice per group. RELMα data represent with 13 male and 9 female 14- to 18-week-old mice per group. MHCII data represent four (Ccr2+/+ males), six (Ccr2−/− females), or seven (Ccr2−/−) 22- to 28-week-old mice per group. FRα data represent six (Ccr2+/+ males), eight (Ccr2−/− females), or nine (Ccr2−/− females and Ccr2+/+ males) 13-week-old mice per group. †P < 0.05; ‡P < 0.01; ¶P < 0.001, Student’s t test with Holm-Sidak correction. (B) Representative expression of ApoE mRNA by CD102+ macrophages (histograms) and frequency of ApoE+, CD209b+, and ApoC1+ cells from the peritoneal cavity of unmanipulated age-matched Ccr2+/+ or Ccr2−/− mice. Symbols represent individual animals, and horizontal bars represent the mean. Data are pooled from two independent experiments and represents three (Ccr2−/− males), six (Ccr2−/− females), or seven (Ccr2−/−) 22- to 28-week-old mice per group. †P < 0.05; ‡P < 0.01, Student’s t test with Holm-Sidak correction. (C) Normalized nonhost chimerism of CD209/Tim4-defined subsets of CD102+ macrophages from the peritoneal cavity of sex-matched tissue-protected BM chimeric mice 8 weeks after reconstitution. Data are normalized to the nonhost chimerism of Ly6C+ monocytes. Data represent five mice per group from one experiment. *P < 0.05; **P < 0.001, paired Student’s t test. (D) Proportion of tdTomato+ (Ai14) cells among microglia, peritoneal, and pleural macrophages from 15-week-old Cdh5lox/lox.Cx3cr1YFPYFP mice administered 4OHT at E7.5. Data represent six (Ccr2+/+ females), or seven (Ccr2−/− males) 22- to 28-week-old mice per group. FRα data represent six (Ccr2+/+ males), eight (Ccr2−/− females), or nine (Ccr2−/− females and Ccr2+/+ males) 13-week-old mice per group. *P < 0.05; **P < 0.01, Student’s t test with Holm-Sidak correction. (E) Frequency of cells expressing CD209b among CD102+ macrophages obtained from the peritoneal cavity of unmanipulated C57BL/6 mice of indicated ages. Symbols represent individual animals, and horizontal bars represent the mean. *P < 0.05; **P < 0.01; ***P < 0.001, Student’s t test with Holm-Sidak correction. (F) Frequency of Ki67+ cells among F4/80+ cells obtained from the peritoneal cavity of unmanipulated age-matched Ccr2+/+ or Ccr2−/− mice. Symbols represent individual animals, and horizontal bars represent the mean. *P < 0.05; **P < 0.01; ***P < 0.001, two-way ANOVA with Tukey’s multiple comparisons test. (G) Absolute number of B1 cells obtained from the peritoneal cavity of unmanipulated age-matched 14- to 28-week-old Ccr2+/+ or Ccr2−/− mice. Data represent 15 (Ccr2+/+ females), 16 (Ccr2−/− females), 17 (Ccr2−/− males), or 20 (Ccr2−/− females) mouse per group pooled from four independent experiments. (H) Frequency of cells expressing K667 among F4/80+ macrophages obtained from the peritoneal cavity of unmanipulated C57BL/6 mice of indicated ages. Symbols represent individual animals, and horizontal bars represent the mean. *P < 0.05; **P < 0.0001, two-way ANOVA with Tukey’s multiple comparisons test. (I) Frequency of Ki667+ cells among peritoneal F4/80+ macrophages obtained from the peritoneal cavity of unmanipulated 14- to 18-week-old Ccr2+/+ or Ccr2−/− mice. Data represents 15 (Ccr2+/+ females), 16 (Ccr2+/+ females), 17 (Ccr2+/+ males), or 20 (Ccr2−/− females) mouse per group pooled from two experiments. (J) Frequency of K667+ cells among peritoneal F4/80+ macrophages obtained from sex-matched or sex-mismatched tissue-protected BM chimeric mice 8 to 12 weeks after reconstitution. Data represent 9 (female > male) or 10 (sex-matched groups) mice per group pooled from one of two independent experiments. **P < 0.01, one-way ANOVA followed by Tukey’s multiple comparisons test.
Fig. 7. Differential CD209b expression confers an advantage on female macrophages in the setting of pneumococcal peritonitis. (A) Experimental scheme for induction of peritonitis. Male and female mice (9 to 10 weeks old) were inoculated with 10^3 CFU of type 2 S. pneumoniae (D39), and bacterial counts and assessment of the peritoneal myeloid compartment were assessed after 20 hours. i.p., intraperitoneally. (B) Relative bacteremia in the peritoneal cavity of male and female mice infected 20 hours earlier (female CFU/male CFU). Symbols represent individual animals, and horizontal bars represent the mean. Data represent 4 (PBS), 11 (female S. pneumoniae), or 12 (male S. pneumoniae) mice per group pooled from three independent experiments. *P < 0.001, Student’s t test. (C) Absolute numbers of Ly6C^hi neutrophils and Ly6C^lo monocytes in the peritoneal cavity 20 hours after inoculation with 10^3 CFU of type 2 S. pneumoniae (D39) or in mice that received PBS. Symbols represent individual animals, and horizontal bars represent the mean. Data represent 4 (PBS), 11 (female S. pneumoniae), or 12 (male S. pneumoniae) mice per group pooled from three independent experiments. *P < 0.05, Student’s t test. (D) Absolute numbers of CD102^+ macrophages and CD209b^+ CD102^+ macrophages in the peritoneal cavity 20 hours after inoculation with 10^3 CFU of type 2 S. pneumoniae (D39) or in mice that received PBS. Symbols represent individual animals, and horizontal bars represent the mean. Data represent 4 (PBS), 11 (female S. pneumoniae), or 12 (male S. pneumoniae) mice per group pooled from three independent experiments. *P < 0.05, Student’s t test. (E) Numbers of bacteria in the peritoneal cavity of male and female mice infected 20 hours earlier and pretreated with anti-CD209b (22D1) or a matched isotype control (Ham IgG1) 30 min before inoculation (left). Right: Bacteremia versus the frequency of CD102^+ macrophages in the peritoneal cavity of mice above. Symbols represent individual animals, and horizontal bars represent the mean. Data represent 10 (isotype control) or 11 (anti-CD209b) mice per group pooled from two independent experiments.


to control S. pneumoniae infection, with lower levels of bacteria in peritoneal fluid of female mice compared with their male counterparts 20 hours after inoculation (Fig. 7B). Fewer neutrophils and Ly6C^hi monocytes were present in the female cavity compared with male mice (Fig. 7C and Fig. S8), consistent with a model in which resident macrophages control infection (53). In contrast, while the well-documented macrophage “disappearance reaction” (56) occurred in both male and female mice after infection (Fig. 7D), higher numbers of CD209b- expressing macrophages persisted in the female cavity (Fig. 7D). Administration of an anti-CD209b blocking antibody (22D1) (57) led to increased levels of bacteremia in female mice, although this did not attain statistical significance because of variance in bacterial counts in some mice in whom the macrophage disappearance reaction was more pronounced (Fig. 7E). Thus, dimorphic expression of key immune receptors and molecules leads to differential ability to handle local bacterial infection.

**DISCUSSION**

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

Using classical defining markers such as F4/80, CD11b, and CD102, we found peritoneal macrophages from male and female mice to be phenotypically identical. However, mRNA sequencing revealed marked dimorphism in the transcriptional fingerprint of resident peritoneal macrophages under homeostatic conditions. Female CD102^+ macrophages expressed higher levels of genes associated with lipid uptake and transport as well as immune defense/response. In contrast, the signature of male peritoneal macrophages was dominated by cell cycle–associated genes consistent with their elevated levels of proliferation, a dimorphism we have reported previously (21). Although others have reported dimorphic expression of TLRs and CD14 by peritoneal macrophages (38, 39), we found no significant difference in mRNA transcripts of the adaptor protein MyD88, CD14, or any TLRs, consistent with more recent analysis of mRNA (44) and protein expression (55).

Despite a significant degree of transcriptional difference at the population level, single-cell mRNA sequencing showed that male and female resident CD102^+ macrophages encompassed three transcriptionally defined clusters of cells. Of these, the cells in cluster 3 expressed CD102 together with MHCII, RELMα, and CX3CR1, all of which are key markers of F4/80^hiMHCII^+ peritoneal macrophages, suggesting that cluster 3 may be recently derived from the F4/80^hiMHCII^+ macrophage population that is derived from blood monocytes in adult mice (21–23). As cells in cluster 4 shared features with both the MHCII-defined cluster 3 and the dominant cluster 5 population of Tim4^+ macrophages, these may represent a further intermediate differentiation state. Consistent with this idea, Tim4^-
cells were largely ablated in monocyticen Ccr2−/− mice. A linear developmental relationship that culminates at cluster 5 would be consistent with the greater abundance of cells in this cluster in females, given the slower entry of BM-derived cells into the female CD102+ macrophage pool. However, it seems unlikely that such a linear developmental relationship between clusters exists in males, as Tim4 and MHCII defined subsets were found to be replenished at similarly high rates. Hence, what dictates cluster identity in males remains unclear.

Given that the rate of replenishment from BM was markedly different between the sexes, a finding consistent with recent monocyte fate mapping using MslaC3CreRosa26LSL-tdTomato mice (58), this raised the possibility that transcriptional differences could reflect different ontogenies of male and female peritoneal macrophages. A number of the genes we found to be expressed more highly by female peritoneal macrophages in other tissues (26, 59). However, the fact that a proportion of BM-derived peritoneal macrophages can acquire the expression of at least some of these “embryonic” signature markers (e.g., Tim4 and CD209b) in the setting of tissue-protected BM chimeras suggests that this is more likely related to their time of residency rather than rigid differences related to origin. Consistently, coexpression of Tim4 and CD209b identifies the longest-lived macrophages in the peritoneal cavity irrespective of sex. The concept that macrophages require prolonged residence within the tissue to acquire their characteristic features is consistent with work from the Guilliams laboratory showing that acquisition of Tim4 expression by monocyte-derived cells that engraft in the liver after deletion of endogenous Kupffer cells occurs with time (26). Human peritoneal macrophages that align with mouse F4/80hi macrophages exhibit high expression of TIMD4, CD209, COLEC12, CD163, and APOC1 (60), suggesting that these may represent phyleogenically conserved markers of long-lived macrophages. Certain dimorphisms (e.g., proliferation and CXCL13 expression) appeared to be regulated independently of replenishment kinetics, consistent with previous data showing that the proliferative capacity of macrophages is determined by signals in the local microenvironment rather than their origin (21, 26, 61). Although our OvX studies implicate the ovaries and/or their products in controlling lower levels of replenishment in females, it seems unlikely that estradiol plays a key role, as administration of exogenous estradiol failed to rescue the effects of OvX. In addition, estradiol is unlikely to be responsible for the lower proliferation of female peritoneal macrophages, as estradiol is reported to increase rather than inhibit proliferation of these cells (37). Nevertheless, although expression of receptors for progesterone and androgens did not differ between the sexes, we cannot categorically rule out a role for these steroids in generating sex dimorphisms. Thus, the exact local factor(s) driving the sex dimorphisms identified here remains to be elucidated.

The incidence and severity of sepsis and postsurgical infections are profoundly lower in women than in men (62), but the mechanisms underlying these differences remain unclear. Our finding that female mice are more resistant to S. pneumoniae peritonitis is consistent with previous work on group B streptococcal peritonitis (39). However, whereas others attributed this to other elements of innate immune responses, such as neutrophil recruitment (55), our data suggest that the resistance of females is, at least in part, due to differences in resident peritoneal macrophages, such as elevated expression of CD209b. However, we cannot rule out other mechanisms, such as dimorphic expression of CXCL13, which plays a central role in recruiting natural immunoglobulin M (IgM)–producing B1 cells (2) (63, 64), or elevated expression of complement components C1q, C3, and C4b, as well as C3b that are essential for innate resistance against S. pneumoniae (63–65). Nonetheless, we propose that this heightened barrier function in the female peritoneum may have evolved to mitigate the risk of sexually transmitted infection disseminating from the lower female reproductive tract (66) or to protect against pneumoperitoneum.

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

MATERIALS AND METHODS

Study design

We performed phenotypic, transcriptomic, and functional analysis of peritoneal macrophages to identify sexual dimorphisms that may exist. We used tissue-protected BM chimeric mice and genetic fate mapping to assess the replenishment kinetics under normal physiological conditions. To assess the role for sex hormones in generating/maintaining sex dimorphisms, we performed OvX in C57BL/6 mice. To assess macrophage function in the context of infection, we used a model of low-dose S. pneumoniae infection (51).

Experimental animals

WT C57BL/6J CD45.2+, congenic CD45.1.CD45.2+ mice, Ccr2−/−, Rag1−/−, and Balb/c mice were bred and maintained in specific pathogen–free facilities at the University of Edinburgh, United Kingdom. In some experiments, C57BL/6J (Crl) mice were purchased from Charles River, United Kingdom, or bred and maintained at the University of Manchester, whereas Ccr2−/−, Rag1−/−, and control mice were bred and maintained at the University of Glasgow. ItgaxCre (67) (referred to here as CD11cCre) mice were crossed with Rosa26LSL-YFP mice (a gift from M. Mcleod, University of Glasgow, United Kingdom) and maintained at the University of Glasgow. For Cdh5CreERT2 fate mapping, WT females aged 6 to 10 weeks were subjected to timed matings with Cdh5CreERT2/− or Cdh5CreERT2+/− Rosa26Rd2Flx;Cx3cr1F/+/F males (experiments performed at CIML, Marseille, France). Successful mating was judged by the presence of vaginal plugs the morning after, which was considered 0.5 day after conception. For induction of reporter recombination in the offspring, a single dose of 4-hydroxytamoxifen (4OHT; 1.2 mg) was delivered by intraperitoneal injections to pregnant females at E7.5. To counteract adverse effects of 4OHT on pregnancy, 4OHT was supplemented with progesterone (0.6 mg). In cases when females could not give birth naturally, pups were delivered by C-section and cross-fostered with lactating CD1 females. All experimental mice were age- and sex-matched. To perform estrous staging, vaginal lavage was performed and cellular content was examined after hematoxylin and eosin staining, as...
previously described (68). For HFD experiments, tissue-protected BM chimeric mice were placed on HFD (58 kcal% fat and sucrose, Research Diet, D12331J) for 8 weeks starting 4 weeks after reconstitution. Experiments performed at U.K. establishments were permitted under license by the UK Home Office and were approved by the University of Edinburgh and University of Manchester Animal Welfare and Ethical Review Body’s or the University of Glasgow Local Ethical Review Panel.

**Surgery**

OVX was performed on 6-week-old WT (C57BL/6) or tissue-protected BM chimeras 8 weeks after reconstitution (16 weeks of age). Briefly, dorsal unilateral or bilateral OVX was performed and mice were allowed to recover for up to 8 weeks. Sham surgery was performed to control for the effects of surgery on the peritoneal environment. This involved identical surgery except for the excision of the ovary/ovaries. Surgery was performed under isoflurane anesthesia followed by a postoperative analgesic, buprenorphine (0.1 mg/kg), for pain management. In some experiments, after 7 days of recovery, mice received exogenous estradiol in the form of E2 valerate (E2; 0.05 mg/kg) before being placed on ice.

**Tissue-protected BM chimeric mice**

Anaesthetized 6- to 12-week-old C57BL/6] CD45.1©CD45.2© animals were exposed to a single dose of 9.5-Gy γ-irradiation, with all but the head and upper thorax of the animals being protected by a 0.05-m lead shield. Animals were subsequently given 2 x 10^8 to 5 x 10^8 BM cells from sex-matched or sex-mismatched congenic CD45.2© C57BL/6] animals by intravenous injection. Unless specified, mice were left for a period of at least 8 weeks before analysis of chimerism in the tissue compartments.

**BrdU injection**

For labeling of proliferating cells, mice were subcutaneously injected with 100 μl of BrdU (10 mg/ml) (Sigma) in Dulbecco’s phosphate-buffered saline (PBS) 2 hours before culling.

**Preparation of single-cell suspensions**

Mice were sacrificed by CO₂ inhalation or by terminal anesthesia followed by exsanguination. Peritoneal and pleural cavities were lavaged with RPMI containing 2 mM EDTA and 10 mM Hepes (both Invitrogen) as described previously (69). Any samples with excessive erythrocyte contamination were excluded from analysis. Omental tissue was excised, chopped finely, and digested in 0.5-ml prewarmed collagenase D (1 mg/ml; Roche) in RPMI 1640 media supplemented with 2% fetal calf serum for 15 min in a shaking incubator at 37°C. After disaggregation with a P1000 Gilson, omental tissue was digested for a further 20 min before being placed on ice. EDTA (2.5 μl, 0.5 M) was added to each sample to inhibit enzymatic activity. Cell suspensions were passed through a 100-μm filter and centrifuged at 1700 rpm for 10 min. The resulting cell suspension was subsequently passed through a 40-μm strainer before cell counting. All cells were maintained on ice until further use. Cellular content of the preparations was assessed by cell counting using a Cassey TT counter (Roche) in combination with multicolor flow cytometry.

**Flow cytometry**

Equal numbers of cells were blocked with 0.025 μg of anti-CD16/32 (2.4G2; BioLegend) and 1:20 heat-inactivated mouse serum (Invitrogen) and then stained with a combination of the antibodies detailed in table S8. Where appropriate, cells were subsequently stained with streptavidin-conjugated fluorochromes. Dead cells were excluded using 4′,6-diamidino-2-phenylindole (DAPI), 7-amino-actinomycin D (7-AAD), or Zombie Aqua fixable viability dye (BioLegend). Fluorescence-minus-one (FMO) controls confirmed gating strategies, and discrete populations within lineage® cells were confirmed by omission of the corresponding population-specific antibody. Erythrocytes in blood samples were lysed using 1× RBC Lysis buffer (BioLegend), as per the manufacturer’s guidelines. For intracellular staining, cells were subsequently fixed and permeabilized using FoxP3/Transcription Factor Staining Buffer Set (eBioscience), and intracellular staining was performed using antibodies detailed in table S2. For the detection of BrdU, cells were fixed as above and incubated with 3 μg of deoxyribonuclease I (Sigma) for 30 to 60 min before being washed in PermWash (eBioscience) and then incubated with anti-BrdU antibody for 30 min at room temperature. Samples were acquired using FACS LSRII or AriaII using FACS Diva software (BD) and analyzed with FlowJo software (version 9 or 10; Tree Star). Analysis was performed on single live cells determined using forward scatter height (FCS-H) versus area (FSC-A) and negativity for viability dyes. For analysis of macrophage proliferation, Ki67 expression was used to determine the frequency of all CD102©/F4/80 hi cells in cycle, whereas a 2-hour BrdU pulse before necropsy combined with Ki67 expression was used to identify cells in S phase, as described previously (70). miRNA was detected by flow cytometry using PrimeFlow technology (Thermo Fisher Scientific) using probes against ApoE (probe type 10; AF568, ApoC1 (probe type 4; AF488), and CXCL13 (probe type 6; AF750) according to the manufacturer’s guidelines. For staining controls in PrimeFlow analysis, the Target Probe Hybridization step was omitted with all other steps identical to samples.

**Transcriptional analysis**

**Bulk RNA-seq**

CD102©F4/80 hi cells were FACs-purified from the peritoneal and pleural cavities of unmanipulated male and female mice. For each population, 25,000 cells were sorted into 500 μl of RLT buffer (Qiagen) and snap-frozen on dry ice. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen), at which point triplicates of 25,000 cells for each population were pooled. Ten nanograms of total RNA was amplified and converted to complementary DNA (cDNA) using Ovation RNA-Seq System V2 (Nugen). Sequencing was performed by Edinburgh Genomics using the Illumina HiSeq 4000 system (75PE). Raw map reads were processed with the R package DESeq2 (71) to generate the DEGs, and the normalized count reads to generate and visualize on heatmaps generated by the R package heatmapp. Samples with >5% of reads mapped to ribosomal RNA were removed from analysis. DEGs were determined using at least a 1.5-fold difference and adjusted P < 0.01, for each of the six pairwise comparisons. Pathway enrichment analysis was performed using the GSEA online database and the R package gskb (gene set data for pathway analysis in mouse), which makes predictions between each of the six pairwise comparisons, incorporating in the analysis the statistically significant differences in gene expression. The R package gskb was used to determine the chromosomal localization of each of the genes and transcription factors. All R code is available upon request.

**Single-cell RNA-seq**

Ten thousand cells for male and female sorted cells were loaded in Chromium 10× in parallel. Libraries were prepared as per the
manufacturer’s protocol and sequenced on Illumina Novaseq S1. Initial processing was done using Cell Ranger (v2.1.1) mkfastq and count (aligned to mouse assembly mm10). Preparation of analysis ready data: For each dataset (filtered data from Cell Ranger pipeline), we filtered out potentially low-quality cells using dataset-specific thresholds based on the trend of the number of genes per cell versus number of housekeeping genes per cell and number of genes per cell versus percentage of mitochondrial genes per cell curves as follows. More specifically, for the female data, we retained 4341 cells that have between 300 and 5000 genes, at least 65 housekeeping genes, and percentage of mitochondrial genes over the total number of expressed genes below 2%. For the male data, we retained 2564 cells that have between 300 and 6000 genes, at least 70 housekeeping genes, and percentage of mitochondrial genes over the total number of expressed genes below 2%. Last, we filtered out genes that were expressed in less than 1% of the cells from each dataset. Clustering analysis of the data: Clustering and data merging using canonical correlation analysis (CCA) was done using Seurat (v3.1.0). We used default parameters and 20 principal components for aligning and clustering the data. We next removed a very small cluster that lay far from all other clusters on the UMAP projection, indicating that it could be either contamination or doublets, and constructed a phylogenetic tree of the remaining clusters to understand the distances and relationship between them. Clusters that were closely grouped together and did not show unique markers were merged together. The final result consists of 6890 cells grouped into six clusters. Identification of DEGs in CCA aligned clusters: We used MAST (v1.10) as implemented in the Seurat (v3.1.0) to identify differentially up-regulated genes between the identified clusters. To overcome the bias of batch effect, we found differentially up-regulated genes within each dataset separately and retained the intersection of markers (conserved markers). Identification of DEGs between female and male cells: We used Student’s t test as implemented in the Seurat package with default parameters to identify differentially up-regulated genes between female and male cells. We only retained genes with adjusted P value based on Bonferroni correction below 0.05. For definition of cluster-specific DEGs, overlap with expression in cluster 6 was ignored given that this cluster was defined by proliferation and hence comprised cells from across other clusters.

**S. pneumoniae peritonitis**

*S. pneumoniae* were cultured overnight on blood agar plates (5% CO₂, 95% air, 37°C), inoculated into Brain Heart Infusion broth, cultured for 3 hours, washed, and resuspended at 10⁵ colony-forming units (CFU)/ml [estimated by OD₆₉₅ (optical density at 595 nm)] in sterile PBS. Their concentration was verified by serial dilution and culture on blood agar plates. Groups of male and female, age-matched C57BL/6 mice (8 to 14 weeks of age) were inoculated intraperitoneally with 100 μl of PBS containing 10⁸ CFU of *S. pneumoniae* (capsular type 2 strain D39). Mice were culled 20 hours later, and peritoneal lavage was performed using sterile PBS. One hundred microliters of lavage fluid was cultured for bacterial growth for 24 hours. The remaining lavage fluid was centrifuged at 400g for 5 min, and the resulting cells were counted and prepared for flow cytometric analysis.

**Statistics**

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

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/5/48/eabc4466/DC1

Fig. S1. Replenishment kinetics of peritoneal and omental leukocytes in BM chimeric mice

Fig. S2. Gating strategies and effects of OVX on peritoneal leukocytes (associated with Figs. 1 and 5).

Fig. S3. Effects of HFD on peritoneal macrophage replenishment kinetics.

Fig. S4. Strategy for the purification of CD102+ macrophages for RNA-seq analysis (associated with Fig. 4).

Fig. S5. Additional RNA-seq analysis.

Fig. S6. Sexual dimorphisms are present in mice from different housing environments and across strains (associated with Fig. 4).

Fig. S7. Clustering of leukocytes in peritoneal fluid during S. pneumoniae–induced peritonitis (associated with Fig. 7).

Table S1. DEGs between male and female CD102+ peritoneal macrophages.

Table S2. Pathway analysis from population-level RNA-seq of male and female CD102+ peritoneal macrophages.

Table S3. Genes uniquely up-regulated by female CD102+ peritoneal macrophages compared with male peritoneal and male and female CD102+ pleural macrophages.

Table S4. GSEA from population-level RNA-seq of male and female CD102+ peritoneal macrophages.

Table S5. Cluster-defining genes from scRNA-seq.

Table S6. Cluster-specific sexually dimorphic genes identified by scRNA-seq.

Table S7. Additional statistics for data in Fig. 5.

Table S8. List of antibodies used in this study.

Data file S1. Raw data.

**REFERENCES AND NOTES**


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Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials, and RNA-seq data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus public database (www.ncbi.nlm.nih.gov/geo/). Population-level RNA-seq (accession code: 149014) and scRNA-seq [primary accession code: GSE139999 (male: GSM4151330; female: GSM4151331)].
Rate of replenishment and microenvironment contribute to the sexually dimorphic phenotype and function of peritoneal macrophages


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Sex, age, and the macrophage

Peritoneal macrophages are known to contribute to the pathology of peritonitis, endometriosis, and cancer, but the effects of age and sex on the biology of these cells are not well understood. Here, Bain et al. show that replenishment of peritoneal macrophages from the bone marrow is much higher in males compared with females. This results in marked sexual dimorphisms in the phenotypic identity of peritoneal macrophages between the sexes, including differential CD209b expression. Age- and sex-associated differences in macrophage turnover also affect resistance to pneumococcal peritonitis. These findings provide insight into the effects of sex and age on peritoneal inflammation and infection.