Macrophages can either inhibit or enhance endometriosis depending on their origin

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Abstract

Macrophages are intimately involved in the pathophysiology of endometriosis, a chronic inflammatory disorder characterized by the growth of endometrial-like tissue (lesions) outside the uterus. By combining genetic and pharmacological macrophage depletion strategies we determined the ontogeny and function of macrophages in a mouse model of induced endometriosis. We demonstrate that lesion-resident macrophages are derived from eutopic endometrial tissue, infiltrating large peritoneal macrophages (LpM) and monocytes. Endometriosis triggers continuous recruitment of monocytes that contribute to LpM and small peritoneal macrophage (SpM) pools. Depletion of eutopic endometrial macrophages results in smaller endometriosis lesions. Constitutive inhibition of monocyte recruitment significantly reduces peritoneal macrophage populations and increases the number of lesions. We propose a putative model whereby endometrial macrophages and embryo-derived LpM are 'pro-endometriosis', whilst monocyte-derived LpM are 'anti-endometriosis'. These observations highlight the importance of monocyte-derived macrophages in limiting disease progression.

Introduction

Macrophages are exceptionally diverse cells present in all tissues of the body that perform functions vital for immunity, development, tissue homeostasis and repair following injury. They modify their role depending on signals received from their local microenvironment and accordingly exhibit high degrees of transcriptional and phenotypic heterogeneity and tissue-specific function[1, 2]. Macrophages differ in their ontogeny. Whilst early studies suggested macrophages were continually replaced by circulating blood monocytes, more recent lineage-tracing experiments demonstrated that most tissue-resident macrophages (exceptions include gut, dermis and heart) are derived from embryonic precursors that seed tissues prior to birth and are maintained by self-renewal or longevity[3, 4, 5]. Tissue-resident and monocyte-derived macrophages play distinct roles both in health and disease[6]. Usually, tissue-resident macrophages play a homeostatic role, whilst monocyte-derived macrophages that are recruited to tissues during inflammation secrete pro-inflammatory cytokines and stimulate the immune system[6]. Thus, in pathological situations, macrophages are a heterogenous population. For example, hepatic macrophages (Kupffer cells) that usually maintain homeostasis become activated during acute injury and recruit monocytes that differentiate into disease-modified inflammatory macrophages that promote the progression of liver injury[7]. In pancreatic cancer, both tissue-resident and monocyte-derived macrophages populate the tumor and increase in density as the cancer progresses. The two populations are transcriptionally diverse and depletion studies revealed that tissue-resident macrophages are responsible for driving tumor progression whereas monocyte-derived macrophages act as innocent bystanders[8]. This highlights the paradigm that under disease-modified conditions tissue-resident macrophages can become adapted such that they promote disease.

The peritoneal cavity hosts two main macrophage populations: a predominant population expressing high levels of EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1 / F4/80) and low levels of Major histocompatibility class II (MHCII) known as large peritoneal macrophages (LpM), and a less abundant population that are F4/80^{lo}, MHCII^{hi} (small peritoneal macrophages; SpM). LpM are considered to be tissue macrophages and are largely embryonically derived, however it is now understood that they are gradually replaced by monocytes over time in a sexually dimorphic manner, that occurs much quicker in males[9, 10]. The SpM population are considered pro-inflammatory and are a

65 heterogeneous population[9]. Inflammatory challenge in the peritoneal cavity can result in an increase in SpM and a loss in LpM (the so-called macrophage disappearance reaction (MDR)), with the exception of helminth infections which are characterized by elevated levels of Interleukin 4 (IL-4) and a type 2 inflammatory response[11 12, 13, 14].

Endometriosis is a chronic inflammatory condition where tissue similar to the
70 endometrium, grows ectopically usually in the peritoneal cavity as 'lesions'[15]. The condition impacts an estimated 190 million women worldwide and is associated with debilitating pelvic pain and infertility[16]. Currently, therapeutic options are very limited, with the gold-standard treatments being surgical removal of lesions or suppression of ovarian hormones. Surgery is associated with high recurrence rates and ovarian suppression is contraceptive
75 and often has unwanted side effects. A high abundance of macrophages is reported both in the peritoneal cavity and in lesions of women with endometriosis[17]. It is clear that macrophages are intrinsically linked with the pathophysiology of endometriosis where they enhance establishment, proliferation and vascularisation of lesions[18, 19]. They are also critical in promoting innervation of lesions and concomitant sensitization of nerve fibres, thus
80 contributing to pain in the condition[20, 21]. Evidence from a syngeneic mouse model of induced endometriosis indicates that donor endometrial macrophages as well as host-derived macrophages can be identified in endometriosis lesions[22]. However, the exact origins of the host-derived macrophages and specific functions of the different populations remains to be determined.

85 Since macrophages play such a key role in many aspects of the pathophysiology of endometriosis they represent an attractive therapeutic target. However, the development of a viable immune-therapy targeting 'disease-promoting' macrophages or enhancing the function of 'protective' macrophages requires a comprehensive understanding of the origin and function of lesion-resident and associated peritoneal macrophages. In this study we
90 have characterized the origin of endometriosis lesion-resident macrophages and examined the dynamics of peritoneal cavity macrophage populations. Finally, we have used a combination of transgenic and pharmacological approaches to selectively deplete different populations of macrophages to assess their impact on development of endometriosis lesions.

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Results

Endometriosis lesion-resident macrophages have different origins. We induced endometriosis in wild-type C57BL/6 mice by injecting 'menses'-like endometrium from MacGreen (*Csf1r-eGFP*, macrophages are GFP+)[23] donor mice into the peritoneal cavity as previously described[22]. Two weeks following tissue injection, endometriosis lesions were collected, digested and analysed by flow cytometry. Among Cluster of differentiation (CD)45+, lineage- (CD3, CD19, CD335, Sialic acid-binding immunoglobulin-type lectin F (SIGLEC-F)), Lymphocyte antigen 6 complex, locus G6D (Ly6G)-, Integrin alpha M (CD11b)+ cells, GFP+ macrophages could be detected (Fig.1A). These data indicate that macrophages derived from the donor endometrium reside within lesions and verifies our previous findings using immunodetection[22]. Endometrial-derived macrophages (GFP+) represented 16% (standard deviation (SD) ± 8.58) of lesion-resident macrophages, whilst the remaining 84% (GFP-; SD ± 8.43) were host derived infiltrating populations (Fig.1B). Next, we sought to determine the origin of the host-derived infiltrating cells. We investigated infiltration of LpM into endometriosis lesions using dual immunodetection for F4/80 (red) and the transcription factor GATA binding protein 6 (GATA6; LpM marker, green; Fig.1C). Quantification of dual positive cells revealed that only 4% of lesion-resident cells appeared to be derived from LpM (Fig.1D). To verify these findings, we performed adoptive transfer of GFP+ LpM isolated by fluorescent activated cell sorting (FACS) from MacGreen mice (LpM and SpM were determined based on expression of F4/80 and MHCII; see below). GFP+ macrophages could be easily detected in lesions (Fig.1E) suggesting that LpM infiltrate endometriosis lesions and lose expression of GATA6 consistent with a change in phenotype within the lesion microenvironment. Conversely, GFP+ macrophages were not detected in lesions following adoptive transfer of SpM isolated from MacGreen mice, suggesting that trafficking of peritoneal macrophages to lesions was restricted to the LpM population (Fig.1F). To assess infiltration of monocytes into lesions we used dual immunodetection for F4/80 (red) and Lymphocyte antigen 6 complex, locus C (Ly6C; green). We identified Ly6C positive lesion-resident monocytes and a population of dual positive cells (yellow) were also detected indicating that monocytes infiltrated lesions and differentiated *in situ,* into macrophages (Fig.1G).

Continuous recruitment and contribution of monocytes to peritoneal macrophage populations was evident in mice with induced endometriosis. Next, we investigated

macrophage populations present in the peritoneal cavity of mice with induced endometriosis. From CD45+, Ly6G-, lineage- cells, LpM and SpM were determined based on expression of F4/80 and MHCII (Fig.2A). At one-week post tissue injection LpM (F4/80^{hi}, MHCII^{lo}) numbers were significantly higher (48399 vs 18967 cell / µl, SEM ± 7361) in sham mice (mice ovariectomised and supplemented with estradiol valerate; PBS instead of tissue injected intra-peritoneally (i.p); Fig.2B; $p<0.01$) compared to naïve animals. At 3 weeks, LpM numbers in sham animals had decreased compared to week 1 ($p<0.05$). No significant differences were found in the LpM population in mice with endometriosis. SpM (F4/80^{lo}, MHCII^{hi}) numbers were consistent between all groups of animals and time points. Ovariectomy alone can have striking impacts on macrophage pools present in the peritoneal cavity and leads to increased macrophage replenishment[10]. We repeated our results using intact recipient mice and confirmed that in this minimally invasive model, there was no loss of LpM and / or increased numbers of SpM consistent with an absent MDR (Fig.S1A). Monocytes in the peritoneal cavity were identified by expression of Ly6C (detection of classical monocytes; Fig.2C) and numbers were significantly increased in mice with induced endometriosis at 1-week post tissue injection compared to naïve mice (Fig.2D; $p<0.01$). Monocyte numbers remained consistent between weeks 1 and 3 in mice with induced endometriosis, suggesting continuing recruitment to the peritoneal cavity as a consequence of the presence of endometriotic lesions. To begin to investigate the fate of monocytes recruited to the peritoneal cavity of mice with induced endometriosis, we evaluated expression of C-C chemokine receptor type 2 (CCR2; mediates monocyte chemotaxis/ recruitment of monocytes) by F4/80^{hi} (LpM) macrophages in the peritoneal cavity using flow cytometry. Significantly elevated numbers of F4/80+, CCR2+ macrophages were recorded in mice with induced endometriosis (Fig.2E-F), suggesting that increased numbers of monocyte-derived LpM are evident in mice with endometriosis. Long-lived embryo-derived peritoneal macrophages express T-cell immunoglobulin and mucin domain containing 4 (TIM4), whilst monocyte-derived LpM do not[9], thus to further validate our hypothesis we ascertained TIM4 expression in F4/80^{hi} LpM in intact mice. Contrary to our expectation there was no difference in the ratio of TIM4+ and TIM4- LpM as analysed by flow cytometry at 1-, 2- or 3-weeks post tissue injection (Fig.S1B). We did observe a significant reduction in the proportion of F4/80^{lo}, MHCII-, CD11b+ cells between week 1 and week 3-post endometrial tissue injection (Fig.S1C-D). Only a minor proportion of these cells express Ly6C

(less than 2% of CD11b cells), indicating that this population is a differentiation intermediate (no longer a monocyte or SpM, but not yet an LpM): however they do not appear to contribute to the TIM4- F4/80^{hi} population, or if they do a mechanism exists that prevents the accumulation of significantly increased numbers of Tim4- cells in female mice using this model.

Endometrial macrophage depletion reduced endometriotic lesion size. To begin to dissect the role of macrophages with different ontogenies in endometriosis we used a number of different depletion strategies. To deplete endometrial macrophages, doxycycline was administered to iCsf1r-KO donor mice from day 15-19 (Fig.3A), such that we could achieve macrophage depletion because of the inducible ablation of the floxed allele of the *Csf1r* but maintain a decidual response which is essential for generating 'menses-like' endometrium in donor mice. The F4/80^{hi} population was significantly depleted (Fig.3B; $p<0.05$). Following transfer of macrophage-depleted donor endometrial tissue to wild-type recipients, there was no difference in the number of lesions recovered after two weeks (Fig.3C); however the lesions recovered were significantly smaller than those from mice receiving wild-type endometrium (Fig.3D; $p<0.05$). In-line with this, studies depleting peritoneal macrophages using clodronate liposomes or an F4/80 antibody have also demonstrated reduced number and size of lesions[18], suggesting that both endometrial and peritoneal (LpM) derived macrophages act to promote lesion establishment and growth. Notably, these depletion strategies do not discriminate between embryo-derived and monocyte derive LpM.

'Monocytopenic' mice with induced endometriosis had an increased number of lesions. We aimed to prevent recruitment of monocytes to the peritoneal cavity and lesions using *Ccr2* null mice. *Ccr2-/-* mice have no Ly6C^{hi} monocytes in the periphery because they cannot extravasate from the bone marrow. In *Ccr2*-/- mice with induced endometriosis (Fig.4A), LpM, SpM and monocytes were significantly depleted in the peritoneal lavage fluid (Fig.4B-E; fold change reduction of 2.3, 3.4, and 3.2 respectively). This supports the concept that in our mouse model of endometriosis monocytes are continually recruited and contribute to the LpM and SpM pools. *Ccr2*-/- mice had significantly more lesions ($p<0.05$) compared to wild-type mice (Fig.4F). Lesion size was not different between the two groups (Fig.4G). To validate these findings, we repeated the experiment in Chemokine (C-C motif) ligand 2 null (*Ccl*2-/-) mice. Although there was no difference in monocyte numbers recovered from the peritoneal lavage of *Ccl2*-/- mice with endometriosis (Fig.5D), LpM and SpM were depleted

compared to wild-type mice (Fig.5A-C; $p<0.05$). We observed a concomitant increase in the number of lesions recovered from *Ccl2*-/- mice (Fig.5E; $p<0.05$). Strikingly, *Ccr2*-/-and *Ccl2*-/-
195 mice with induced endometriosis were still able to recruit Ly6C+ monocytes from the bone marrow to the peritoneal cavity (monocytes were absent in the peritoneal lavage of naïve and sham mice; Fig.S2) and in lesions recovered from *Ccr2*-/- and *Ccl2*-/- mice we could still detect monocytes using immunodetection (Fig.4H-I and Fig.5G-H) indicating that there is some redundancy in the CCL2-CCR2 axis in the presence of endometriosis lesions and
200 suggests that monocytes may be recruited directly from the bone marrow via another mechanism.

Transient depletion of monocytes did not impact establishment of endometriotic lesions. Next, we sought to deplete monocytes but leave the SpM pool unmodified by using a function blocking CCR2 monoclonal antibody (MC21) injected into the peritoneal cavity of
205 mice with induced endometriosis (Fig.6A). Compared to a control IgG antibody (MC67), MC21 led to an increase in LpM (Fig.6C; $p<0.05$), did not modify SpM (Fig.6D) and significantly reduced the number of monocytes in the peritoneal cavity (Fig.6B and E; $p<0.05$). In mice with induced endometriosis treated with MC21 there was no difference in lesion number or size compared to mice treated with control MC67 (Fig.6F-G).

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Discussion

The pathophysiology of endometriosis remains enigmatic[15]. Although immune cell dysfunction is intrinsically linked with the disorder, our understanding of macrophage origins and respective function remains limited compared with other diseases, such as
215 cancer[24]. Macrophages are critical for endometriotic lesion establishment and survival, as indicated by early depletion experiments[18]. They play a key role in promoting growth and vascularization of ectopic tissue[19] and in promoting nerve growth, activation of nerves and pain generation in endometriosis[20, 21]. As the ontogeny of macrophages in diseased tissue is a key determinant of how they respond and contribute to pathogenesis, it is necessary to
220 understand how macrophages derived from different sources impact lesion development. This will provide a greater appreciation of how 'disease-promoting' or 'protective' macrophages could be targeted in the future as a potential therapy. The use of a syngeneic mouse model of induced endometriosis[22] has allowed intricate exploration of macrophage ontogeny and functional consequences of depleting specific populations that is impossible

225 to perform in women. In this study, we have used a model that aims to recapitulate the process of 'retrograde menstruation' by generating 'menses'-like endometrium in donor mice and injecting the tissue into the peritoneal cavity of recipient mice. We have demonstrated that lesion-resident macrophages are derived from the donor endometrium, from infiltrating LpMs and from monocytes. We used different depletion strategies to
230 explore the impact of macrophages from different origins on lesion development. The most striking result was revealed by constitutively limiting monocyte recruitment (using both *Ccr2*-/- and *Ccl2*-/- mice) and subsequently reducing both LpM and SpM replenishment, which resulted in mice with induced endometriosis developing significantly more lesions. This suggests that monocyte-derived macrophages act as guardians against the
235 development of endometriosis and raises the question as to whether monocytes are defective in women with endometriosis. The contribution of embryonic-derived and monocyte-derived macrophages to the 'tissue-resident' population is different in each tissue[3, 4, 5]. We previously demonstrated that macrophages present in the endometrium can be detected in lesions recovered from our model of induced endometriosis[22]. However,
240 little is known regarding the ontogeny of macrophages in the endometrium. In a mouse model of menstruation, utilizing MacGreen (*Csf1r-EGFP*) mice, Cousins et al demonstrated that three populations could be distinguished in the endometrium using dual staining for F4/80 and GFP: (1) a population of GFP+, F4/80- cells likely to be infiltrating monocytes, (2) a population of GFP+, F4/80+ cells suggestive of monocyte-derived macrophages, and finally
245 (3) a population of putative (but not confirmed with lineage tracing) 'tissue-resident' macrophages that are GFP-, F4/80+. The cells were localized to areas of breakdown, repair and remodelling, respectively[25]. The 'menses-like' endometrium that we recover from donor mice for transfer into recipient mice is collected at the initiation of the 'break-down' phase and is most likely to consist of monocytes and monocyte-derived macrophages. Moreover,
250 the tissue collected is the 'decidual' mass only and does not include the compartment of the uterus where putative 'tissue-resident' macrophages are located. Using an inducible *Csf1r*-knockout to generate donor endometrium we achieved depletion of macrophages (F4/80^{hi}, Ly6C^{lo}) and limited the number of monocytes (F4/80^{lo}, Ly6C^{hi}) in the tissue transferred to recipient mice. We did not observe any difference in the number of lesions formed between
255 recipient mice that received wild type or macrophage depleted endometrium, however we did find that the lesions recovered were significantly smaller in mice receiving macrophage-

depleted endometrium. The breakdown phase of the (donor) endometrium is analogous to the initial inflammatory phase of the wound healing process where pro-inflammatory macrophages play a vital role prior to wound repair[26]. If we consider endometriosis lesions as chronic wounds that do not fully resolve their inflammation, we may presume that the initial inflammatory phase begins as the endometrium breaks down during menstruation and the repair of the translocated endometrium occurs in the peritoneal cavity resulting in the formation of lesions. Importantly, reduced wound closure and delayed repair response is observed when monocytes and macrophages are depleted during the initial inflammatory phase of skin repair[27]. We suggest that a similar process occurs in mice receiving endometrium-depleted of monocytes and macrophages and this may explain reduced lesion size in recipient mice.

The ontogeny of peritoneal macrophages is better characterized. LpM are embryo-derived, long-lived and undergo self-renewal, however monocytes do continually enter the peritoneal cavity via CCR2 where they differentiate into SpM and differentiate into LpM, such that over time these monocyte-derived LpM eventually displace the embryo -derived population[9]. This occurs in a sexually dimorphic manner, occurring much quicker in male compared to female mice who retain their embryo-derived LpM for much longer; by 17 weeks of age approx. 30% of LpM in male mice are monocyte-derived whilst only approx. 15% of LpM are monocyte-derived in females. Although monocyte-derived LpM mostly phenocopy embryo-derived LpM, transcriptomically they exhibit some differences. For example, embryo-derived LpM express *Timd4*, whilst monocyte-derived LpM do not[9, 10]. The sexually dimorphic replenishment of LpM from monocytes is driven specifically by changes that occur in the peritoneal cavity upon sexual maturation in female mice and importantly, this contributes to their ability to protect against pneumoccal peritonitis; female mice have an enhanced ability to control *s.pneumoniae* infection. Of particular interest is the fact that although the MDR occurred in both males and females after infection with *s.pneumoniae*, significantly more LpM persisted in female mice[10]. In the current study, we did not observe any difference in the ratio of LpM to SpM in mice with induced endometriosis, 1-, 2- and 3-weeks after injection of endometrial tissue. It takes 12 days for LpM to be replenished following depletion of peritoneal macrophages (S. Jenkins unpublished observation), indicating that if any shift in ratio (consistent with the MDR) occurs prior to the first time-point analysed (1 week after injection) a significant amount of LpM must be retained in our

model. The number of monocytes and CCR2+ LpM was significantly elevated in the
290 peritoneal cavity of mice with induced endometriosis suggesting monocytes are continually recruited and they contribute to LpM pools. Interestingly, the number of TIM4+ and TIM4- macrophages did not change between weeks 1- and 3-post tissue injection, despite detection of a significant number of differentiation intermediates at week 1 and 2 that are neither monocytes, SpM or F4/80^{hi} cells, suggesting that a mechanism exists to control the
295 proportion of embryo-derived vs monocyte-derived cells in the female peritoneal cavity as consistent with macrophage niche models described elsewhere[28, 29].

We determined that LpM but not SpM enter endometriosis lesions using adoptive transfers; we suggest that these cells change phenotype in lesions very rapidly because only a few cells positive for both F4/80 and GATA6 were identified using dual staining. Peritoneal
300 macrophages perform important functions in other visceral organs in response to inflammation; following sterile liver injury, mature F4/80^{hi}, GATA6+ LpM traffic directly to the damaged tissue via direct recruitment across the mesothelium. Once in the liver, the macrophages undergo local proliferation, and rapid phenotype switching such that they exhibit a pro-repair profile and activate pathways to restore the injury site[30]. Our data
305 suggest that in endometriosis LpM trafficking to lesions may play a similar role, perceiving the ectopic tissue as a wound and activating repair processes. This is supported by the finding that depletion of LpM using an anti-F4/80 antibody prior to and following endometrial tissue injection results in the recovery of significantly lighter lesions[18]. Interestingly, our data suggest that SpM may have a neutral role in the pathophysiology of
310 endometriosis since they never exhibit an increase in number and appear to serve as a differentiation intermediate for monocytes transitioning into LpM.

Our data indicates a key role for monocyte recruitment to the peritoneal cavity and ectopic tissue in endometriosis. We detected lesion-resident monocytes as well as cells that were double positive for both Ly6C and F4/80 indicating that monocytes rapidly
315 differentiate in lesions. Moreover, monocyte numbers are elevated in the peritoneal cavity of mice with induced endometriosis and when monocytes are constitutively depleted this impacts significantly on LpM and SpM pools in the peritoneal cavity and results in increased lesions. Thus, we suggest that monocyte-derived macrophages act to protect the peritoneal cavity and can limit the development of lesions (Fig.7). Previous studies have demonstrated
320 infiltration and accumulation of bone marrow derived CD11b+ cells and TEK receptor

tyrosine kinase positive (TIE2+) macrophages in lesions[19, 31] supporting our findings that monocytes are recruited to lesions, not just from the periphery but perhaps also directly from the bone marrow. Our findings show that, although monocytes were depleted in *Ccr2-/-* mice with induced endometriosis, they were not completely absent, as we observed in naïve and sham animals, supporting this concept. Recruitment of Ly6C^{hi} monocytes from the bone marrow to lesions may occur via another chemokine receptor in mice with induced endometriosis. For example in atherosclerosis, monocytes are recruited to plaques by both CCR2 and CCR5[32].

Definition of the macrophage populations that reside in diseased tissues is vital for understanding macrophage-driven pathology, particularly for endometriosis where little is known about the lesion macrophage niche. In cancer models monocyte recruitment can drive tumour growth[33 34]. Alternatively, tissue resident macrophages have also been attributed to promoting tumourigenesis [8]**.** Intriguingly, these scenarios do not mirror the results of our studies that demonstrate a protective role for monocyte-derived macrophages within the peritoneal cavity of mice with induced endometriosis. This may be due to the artificial nature of the model and we may be observing a similar response to what could naturally occur in women that do not suffer from endometriosis, when the peritoneal cavity is challenged with refluxed endometrial tissue. In the future it may be possible to harness the protective properties of monocyte-derived macrophages as a potential therapy for women with endometriosis. Our findings suggest that monocytes and monocyte-derived macrophages should be explored in more depth in women with the condition. Thus, we propose a putative model that in endometriosis, macrophages derived from 'other' tissues (i.e. endometrium-derived macrophages and embryo-derived LpM) exhibit 'pro-endometriosis' functions, perhaps as they act to repair the ectopic tissue and promote establishment and development of lesions. Conversely, monocyte-derived LpM originating from monocytes recruited directly to the site of inflammation have an 'anti-endometriosis' role and are protective against the persistence of ectopic endometrial tissue. We propose that monocyte-derived LpM do not have the tissue 'memory' that embryo-derived LpM and endometrial macrophages may possess and can recognize ectopic tissue as inappropriately placed and activate pathways to clear the material. Further studies are required to validate this.

Collectively, we have demonstrated multiple origins for endometriotic lesion-resident macrophages and a key role for monocyte-derived macrophages in protecting the peritoneal cavity when challenged with ectopic endometrial tissue. Thus, we have opened up new avenues and possibilities for how macrophages can be targeted or harnessed as a therapeutic option in the treatment of endometriosis.

Materials and Methods

Animals and reagents. Wild-type C57BL/6JOIaHsd female mice were purchased from Harlan (Harlan Sprague Dawley Inc, Bicester, UK) at 8-12 weeks of age. All transgenic lines used in this study were on the C57BL/6 background, and were bred and maintained at the University of Edinburgh or the University of Warwick. All animal work was licensed and carried out in accordance with the UK Home Office Animal Experimentation (Scientific Procedures) Act 1986 and the work licensed under PPL 70/8731 (E.G). Mice had access to food and water ad libitum and were kept at an ambient temperature and humidity of 21°C and 50% respectively. Light was provided 12 hours a day from 7am-7pm. *ROSA26-rtTA:tetO-Cre:Csf1rflox/flox*[35] (colony stimulating factor 1 receptor (Csf1r) conditional knock out) allows deletion of *Csf1r* following treatment with the tetracycline analog doxycyclinehyclate (2µg/ml in 5% sucrose water; Merck) causing CSF1R expressing macrophage populations to be depleted. *B6.Cg-Tg(Csf1r-EGFP)1Hume/J* (MacGreen) express enhanced green fluorescent protein (EGFP) under control of the *Csf-1r* promoter[36]. We used a number of strategies to selectively deplete different monocyte-derived populations in the peritoneal cavity and in lesions; 1) *B6.129S4-Ccr2tm1Ifc/J (C-C chemokine receptor type 2 (Ccr2 -/-)* mice have a homozygous mutation in the *Ccr2* gene[37]. They have reduced monocytes, monocyte-derived macrophages and small peritoneal macrophages in the peritoneal cavity in addition to a low number of circulating Ly6C^{hi} monocytes due to an inability for monocytes to extravasate from the bone marrow and from blood vessels. 2) *B6.129S4-Ccl2tm1Rol/J (Chemokine (c-c motif) ligand 2 (Ccl2) -/-)* mice possess a mutation in the *SCYA2* gene encoding the CCL2 ligand. *Ccl2-/-* mice have normal peritoneal macrophage numbers but reduced recruitment of monocytes and monocyte-derived macrophages into the peritoneal cavity under inflammatory conditions[38]. 3) The monocyte depleting rat anti-mouse CCR2 mAb (clone MC21) isotype IgG2b[39] was used for monocyte depletion experiments. Mice received a daily intraperitoneal injection of 20µg per mouse of MC21 to

prevent infiltration of monocytes into the peritoneal cavity. Isotype-matched rat IgG2b
385 control antibody (MC67) was used as a control.

Mouse model of induced endometriosis. Endometriosis was induced in mice using a syngeneic model as previous described[22]. The model aims to mirror the process of 'retrograde menstruation'. In brief, donor mice were induced to undergo a 'menses'-like event by removing the ovaries and exposing the mice to a hormonal schedule similar to a
390 truncated menstrual cycle and a stimulus that causes the endometrial stromal cells to undergo decidualization[40]. Following P4 withdrawal the endometrial lining begins to shed. 4-6hrs after withdrawal of P4 the 'menses'-like endometrium is collected and injected into ovariectomized mice supplemented with estradiol valerate[40]. Lesions are recovered that contain stoma +/- epithelial cells and immune cell influx[22]. Unless otherwise stated lesions
395 were collected 2 weeks following tissue injection. For clarity, individual experiments using different combinations of transgenic and wild-type mice are described: *Experiment 1: Endometrial macrophage incorporation into lesions.* Endometrium from MacGreen donors was injected i.p into wild-type C57BL/6 recipients (n=6 mice). *Experiment 2: Incorporation of peritoneal macrophages into lesions.* LpM (F4/80^{hi}, MHCII^{lo}) or SpM (F4/80^{lo}, MHCII^{hi}) were
400 isolated from MacGreen mice using fluorescent activated cell sorting (FACs) and adoptively transferred into the peritoneal cavity of recipient mice (n=12 per population) at the same time as donor endometrium, both donor and recipient were wild-type C57BL/6. Incorporation was evaluated using immunodetection of GFP. *Experiment 3: Impact of endometrial macrophage depletion on lesion formation.* Macrophages were depleted in
405 donor endometrium by administering doxycycline to *Csf1r*-cKO mice between days 15-19 of the 'menses' protocol (Fig.4A). Macrophage depleted endometrium was injected i.p into wild-type recipients (n=9). For comparison wild-type endometrium was transferred into wild-type recipients (n=10). *Experiment 4: Depletion of monocytes in the peritoneal cavity (i).* Donor endometrium from wild-type C57BL/6 mice was injected i.p into wild-type
410 (control; n=7) or *Ccr2*-/- recipients (n=8). *Experiment 5: Depletion of monocytes in the peritoneal cavity (ii).* Wild-type donor endometrium was injected i.p into wildtype (controls; n=5) or *Ccl2*-/- recipients (n=5). In some experiments we used recipients that had not been ovariectomized (intact). Endometrial tissue was generated and injected in the same way as the standard model. Recipient mice did not receive any hormonal manipulation. In both
415 models, mice were culled 14 days post tissue injection (unless otherwise stated) and

endometriosis lesions and peritoneal lavage were collected. Peritoneal lavage was recovered by injecting 7 ml ice-cold DMEM into the peritoneal cavity followed by gentle massage and recovery. Lesions were either collected into neutral-buffered formalin for paraffin embedding and immunohistochemical analysis or DMEM for flow cytometry analysis.

Flow cytometry. Endometrium or lesions were dissected, pooled from each mouse and placed in 2ml ice-cold DMEM. Tissues were cut into small pieces using a scalpel and digested with 1 unit of Liberase DL, 1 unit of Liberase TL and 0.6 mg DNAse enzymes. The tissue and enzymes were incubated for 45 minutes at 37°C, with vortexing every 5 minutes. Following digestion samples were filtered through 100µM filters. Red blood cells were lysed from peritoneal lavages and cells derived from the endometrium or lesions, and approx. 10^6 cells per sample were blocked with 0.025mg anti-CD16/32 (clone 93; BioLegend, San Diego, CA, USA) and then stained with a panel of antibodies shown in Table 1. Brilliant™ violet stain buffer was included when required. Fluorescence minus one (FMO) and unstained controls were used to validate gating strategies. Just prior to analysis on the flow cytometer, DAPI and 123count eBeads (Thermo Fisher Scientific) were added to samples. Samples were analysed using an LSRFortessa with FACSDiva software (BD Biosciences) or FACSMelody with Chorus software and analysed with FlowJo v.9 software (FlowJo, Ashland, OR, USA). Analysis was performed on single, live cells determined using forward scatter height vs. area and negativity for live/ dead (DAPI or alternative viability dye). For fluorescent activated cell sorting red blood cell lysis, Fc blocking and fluorescent staining was performed as previously described and samples sorted into pure cell populations based on cell surface marker expression using a FACS Fusion (BD Biosciences).

Immunofluorescence. Immunofluorescence was performed as previously described[20, 21, 41]. In brief, sections were antigen retrieved with heat and pressure (buffers pH 6.0 or pH 9.0) or trypsin tablets dissolved in dH2O (for F4/80 antibody; Sigma) and incubated with sections for 20 min at 37°C. Sections were blocked for endogenous peroxidase (6% H_2O_2 in methanol) and nonspecific epitopes (species-specific serum diluted 1:5 in Tris-buffered saline and 5% bovine serum albumin, or blocking serum from species specific ImmPRESS® kit; Vector Laboratories) and incubated with primary antibody (Table 2) at 4°C overnight. Antibody detection was performed using a secondary antibody conjugated to horseradish peroxidase, often from an ImmPRESS® polymer detection kit followed by colour

development using a tyramide signal amplification system kit with cyanine (Cy)3 or fluorescein (1:50 dilution; PerkinElmer, Waltham, MA, USA). For detection of the second antigen in dual immunofluorescence, sections were boiled in citrate buffer, and the second primary antibody applied overnight and detected as above. Nuclei were stained with DAPI and sections mounted in Permafluor (Thermo Fisher Scientific). Images were captured on a LSM710 confocal microscope and AxioCam camera (Carl Zeiss). Mouse uterus was used as a positive control tissue, and negative controls had omission of the primary antibody

Fiji analysis. For cell counting of F4/80, GATA6 dual immunofluorescent stains, 4 random images at x63 objective were taken from each lesion and images quantified using Fiji plugin 'Cell Counter'. Total nuclei were counted, as well as cells positive for respective markers. Values were expressed as % of total DAPI+ cells. The area of H&E stained lesions captured using 2.5x magnification was measured in Fiji by setting the scale to a known size (198 pixels = 500μM) and then drawing around boundary of the lesion (excluding peritoneal and adipose tissue) and using the 'measure' function.

Definiens analysis. Ly6C, F4/80 dual immunofluorescence was automatically quantified using slide scanning and machine learning. Stained tissue sections were imaged on a Zeiss Axioscan.Z1 (Carl Zeiss AG, Oberkochen, Germany) at 20x using fluorescence filters configured for DAPI, FITC, and Cy3. Whole-slide .czi files were imported into TissueStudio 2.4 (Definiens AG, Munich, Germany) for automated tissue detection followed by manual correction of ROIs to delineate endometriosis lesion, peritoneum, haemorrhage, and adipose tissue. Tissue studio's built-in nuclear segmentation, using the DAPI channel, was applied within these regions to identify cell objects and these objects were then classified as positive or negative for each channel based on intensity thresholds which were used across all samples.

Statistical analysis. Statistical analysis was carried out in GraphPad Prism 7.02. Data was first analysed for normality using an Anderson Darling normality test. If data were normally distributed, either an ANOVA with a Tukey's post-hoc test (more than 2 samples) or a t-test (2 samples) was performed. If data were not normally distributed, non-parametric tests were used; either Kruskal-Wallis with a Dunn's post hoc test (more than 2 samples) or a Mann-Whitney U test (2 samples). Statistical significance was reported at $p<0.05$.

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Author contributions

E.G conceived experiments, analysed data and wrote manuscript; C.H conceived and performed experiments and analysed data. P.D and M.R performed experiments. M.M provided reagents and critical feedback. D.S performed image analysis; J.P provided critical discussion and advice on manuscript. S.J provided advice on experimental design, interpretation and critical feedback. A.W.H provided feedback on experimental design and manuscript preparation.

Figures and legends

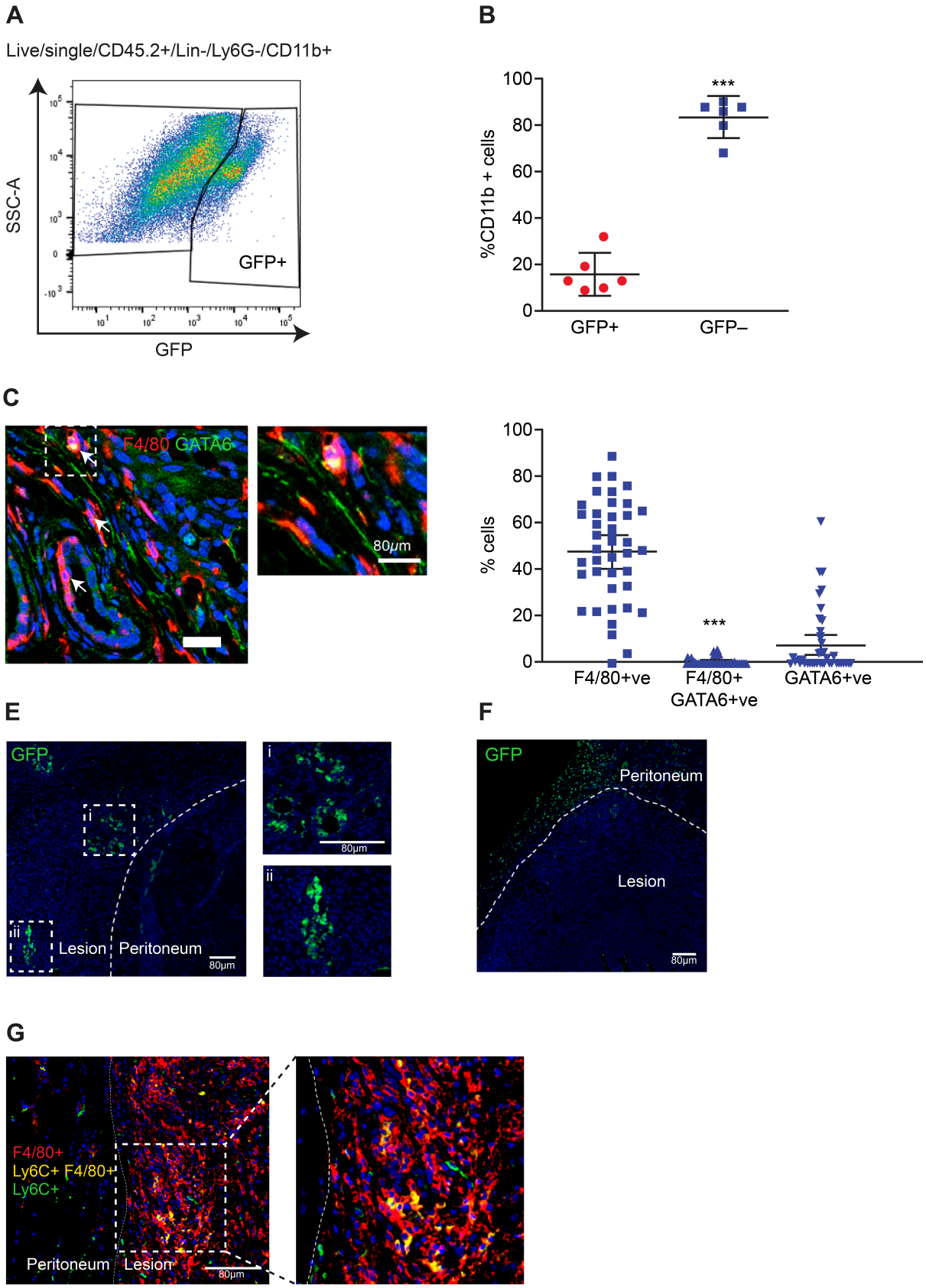

Figure 1: Lesion-resident macrophages have different origins.

510 A) Expression of GFP by lesion-resident macrophages recovered from MacGreen (donor) to wild-type (recipient) endometrial transfers (n=6).

B) Quantification of donor endometrial-derived (GFP+) macrophages vs recipient derived (GFP-) macrophages.

C) Dual immunodetection for F4/80 (red) and GATA6 (green; n=7 mice (10 lesions)). Thick
515 arrows indicate dual positive cells and thin arrows indicate GATA6- macrophages.

D) Quantification of F4/80+, dual positive and GATA6+ cells in lesions. Only 4% of cells were dual positive for F4/80 and GATA6.

E-F) Immunofluorescence for GFP on lesions collected following adoptive transfer of approx. $1x10^6$ LpM (E) or SpM (F) isolated from MacGreen mice. Curved dotted line indicates the
520 boundary between peritoneal and lesion tissue.

G) Dual immunodetection for F4/80 (red) and Ly6C (green) performed on mouse lesions.

Data are presented as mean with 95% confidence intervals. Statistical significance was determined using a Student's t-test. ***;$p<0.001$.

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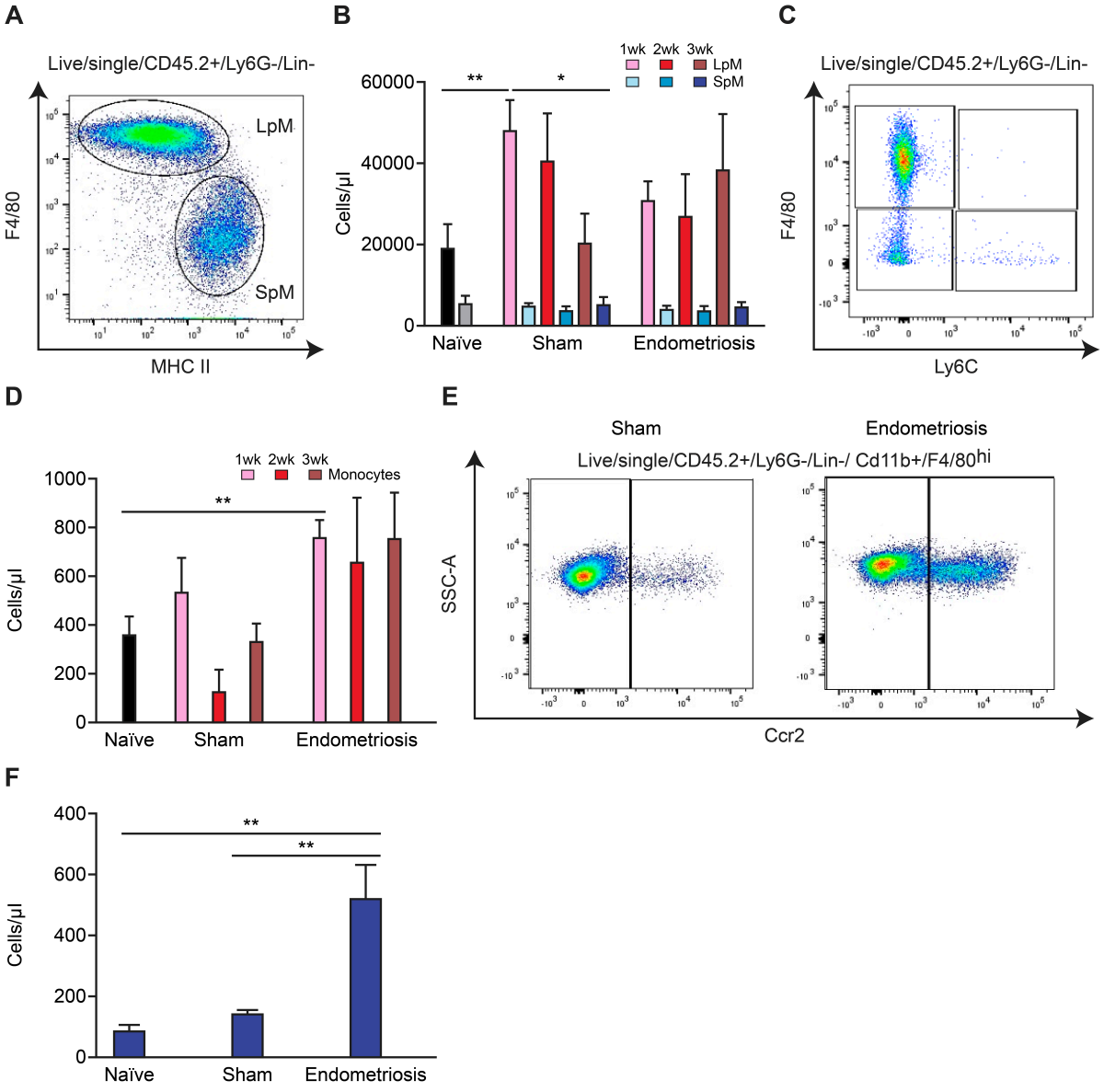

Figure 2: Monocyte recruitment and replenishment of peritoneal macrophage pools in mice with induced endometriosis.

A) LpM (F4/80^{hi}, MHCII^{lo}) and SpM (F4/80^{lo}, MHCII^{hi}) populations in the peritoneal fluid of mice.

B) Quantification of LpM and SpM of sham (n=6-8 each timepoint) and endometriosis mice at 1 (n=6), 2 (n=8) and 3 weeks (n=16) post endometrial tissue injection compared to naïve mice (n=12).

C) Flow plot indicating gating of monocytes (F4/80^{lo}, Ly6C^{hi}) in peritoneal lavage fluid.

D) Quantification of monocyte numbers of sham and endometriosis mice at 1, 2, and 3 weeks post tissue injection.

E) Flow plot demonstrating expression of CCR2 on F4/80^{hi} macrophages in peritoneal lavage fluid from sham vs endometriosis mice.

F) Quantification of CCR2 expression on F4/80^{hi} from endometriosis mice (n=5) compared to sham (n=4) and naïve (n=4) mice.

Data are presented as mean ± SEM. Statistics where determined using a one-way ANOVA and a Tukey post-hoc test, *;$p<0.05$, **;$p<0.01$.

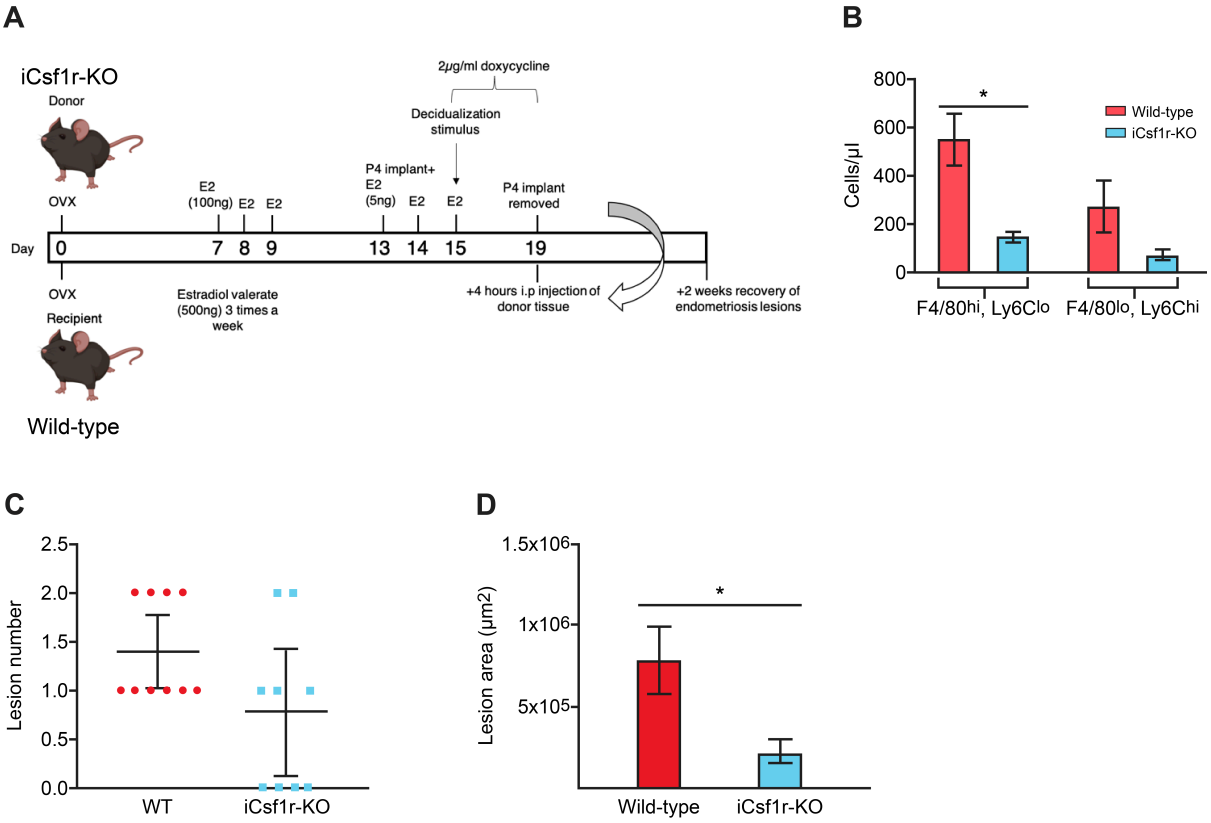

Figure 3: Endometrial macrophage depletion impacts lesion size.

A) Schematic demonstrating timing of doxycycline administration to i*Csf1r*-KO donor mice.

B) Quantification of F4/80^{hi}, Ly6C^{lo} macrophages and Ly6C^{hi}, F4/80^{lo} monocytes in donor endometrium from wild-type and iCsf1r-KO mice.

C) Number of lesions recovered from wild-type recipient mice receiving either wild-type (n=10) or i*Csf1r*-KO endometrium (n=9).

D) Area of lesions recovered from mice receiving either wild-type or i*Csf1r*-KO endometrium. Data are presented as mean ± SEM or 95% confidence intervals (C). Statistical significance was determined using a Student's t-test. *;$p<0.05$.

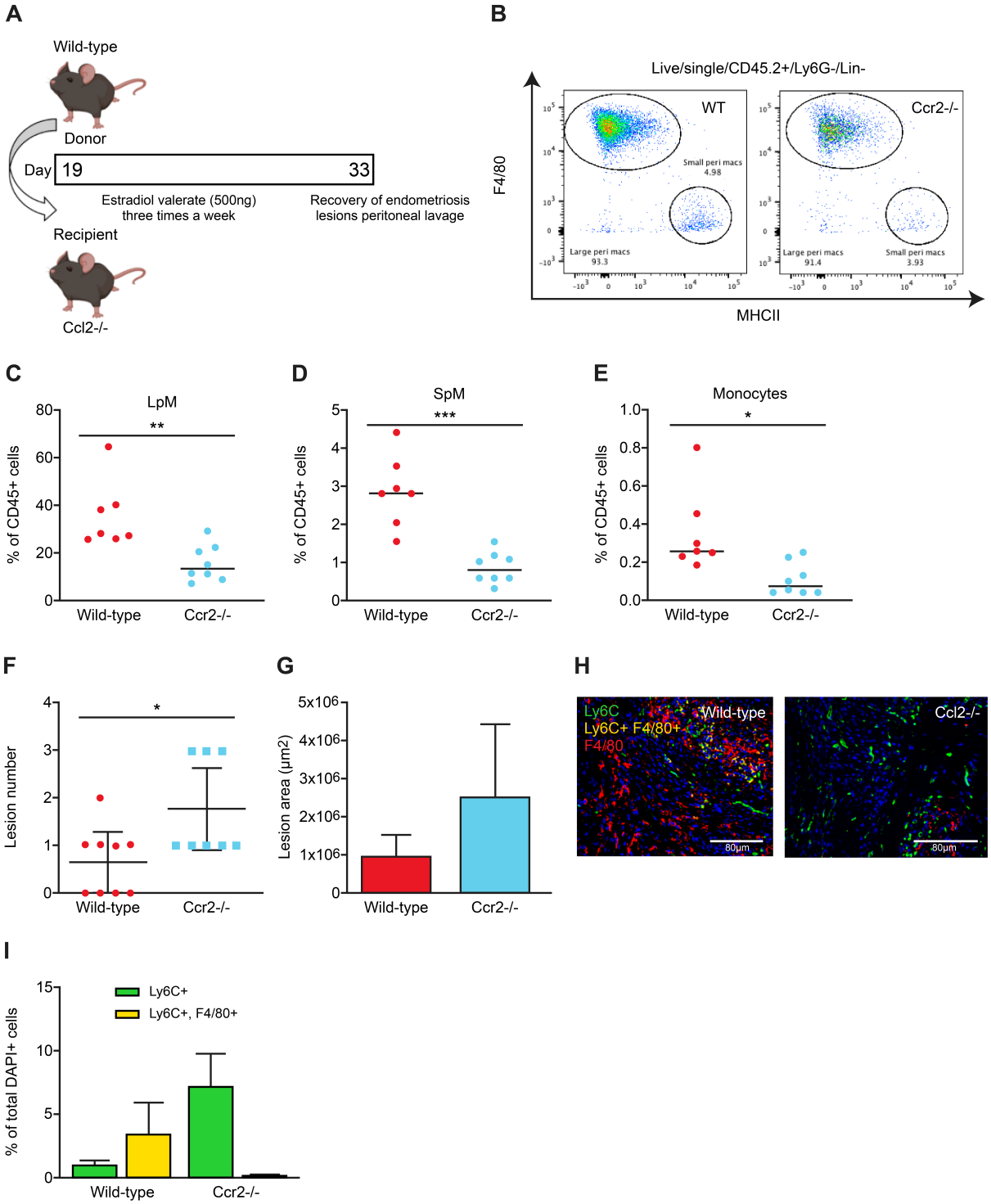

Figure 4: 'Monocytopenic' mice with induced endometriosis establish more lesions.

A) Schematic demonstrating experimental design.

605 B) Flow plot indicating gating and number of LpM (F4/80^{hi}) and SpM (MHCII^{hi}) in peritoneal lavage fluid recovered from wild-type (n=7) and *Ccr2-/-* mice (n=8) with induced endometriosis.

C-E) Quantification of (C) LpM, (D) SpM, and (E) monocytes (Ly6C^{hi}) in peritoneal lavage fluid.

610 F) Number of lesions recovered from wild-type and *Ccr2*-/- mice with induced endometriosis.

G) Size of lesions recovered from wild-type and *Ccr2*-/- mice with induced endometriosis.

H) Dual immunodetection for Ly6C (green) and F4/80 (red) on lesions recovered from wild-type and Ccr2-/- mice

615 I) Quantification of monocytes (Ly6C+; yellow bars) and monocyte-derived macrophages (Ly6C+, F4/80+; green bars) in lesions recovered from wild-type and *Ccr2*-/- mice.

Data are presented as mean ± SEM or 95% confidence intervals (F). Statistical significance was determined using a Student's t-test or Mann-Whitney test. *;p<0.05, **;p<0.01, **;p<0.001.

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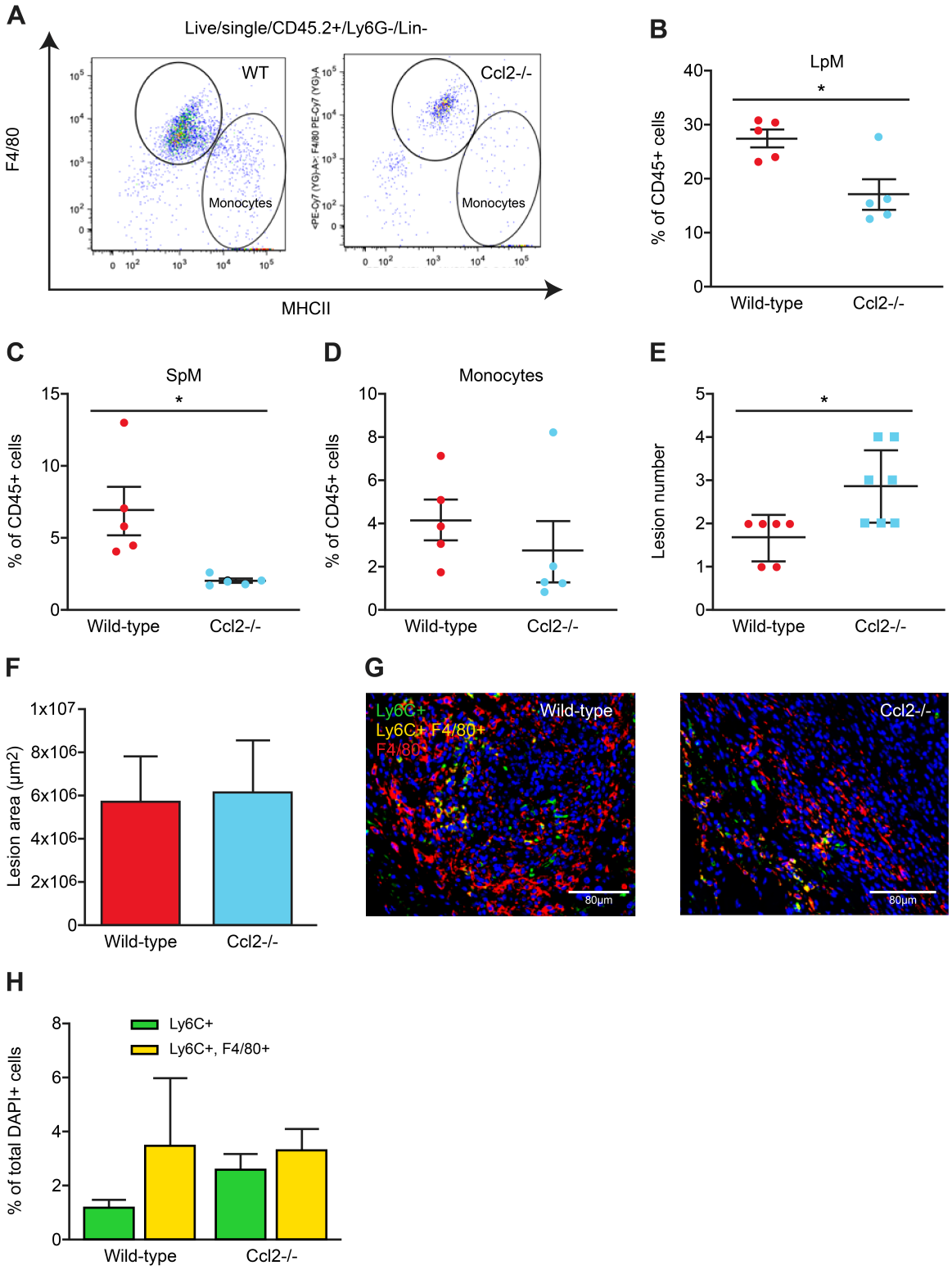

Figure 5: More lesions are evident in *Ccl2-/-* mice.

A) LpM and SpM populations in peritoneal lavage fluid from wild-type and *Ccl2-/-* mice with induced endometriosis.

(B- D) Quantification of (B) LpM,(C) SpM, and (D) monocytes in peritoneal lavage fluid from wild-type (n=5) and *Ccl2-/-* (n=5) mice with induced endometriosis.

E) Number of lesions recovered from wild-type and *Ccl2*-/- mice.

F) Size of lesions recovered from wild-type and *Ccl2*/- mice with induced endometriosis.

G) Dual immunodetection for Ly6C (green) and F4/80 (red) on lesions recovered from wild-type and *Ccl2*-/- mice

650 H) Quantification of monocytes (Ly6C+) and monocyte-derived macrophages (Ly6C+, F4/80+) in lesions recovered from wild-type and *Ccl2*-/- mice.

Data are presented as mean ± SEM. Statistical significance was determined using a Student's t-test or Mann-Whitney test. *;$p<0.05$.

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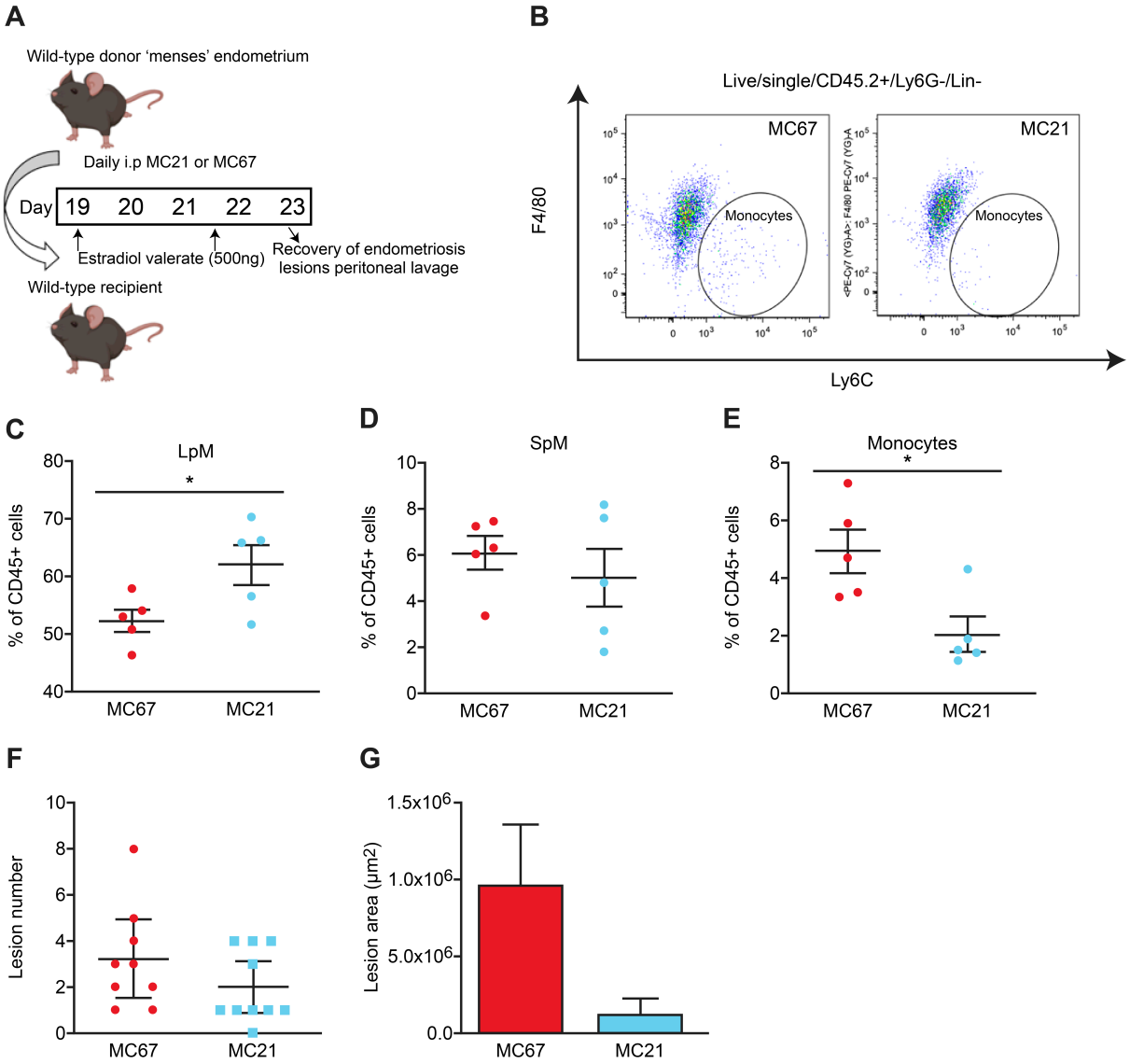

Figure 6: A function-blocking Ccr2 mAb reduces monocyte numbers without significant impact on lesion number.

A) Schematic showing the experimental design; mice with induced endometriosis were treated with a control IgG (MC67; n=9) or a function blocking CCR2 mAb (MC21; n=10).

B) Flow plot demonstrating the numbers of Ly6C^{hi} monocytes in mice with induced endometriosis treated with MC67 or MC21.

C-E) Quantification of (C) LpM, (D) SpM, (E) monocytes in the peritoneal lavage fluid of mice with induced endometriosis.

F) Number of lesions recovered from mice treated with MC67 or MC21.

G) Size of lesions recovered from mice treated with MC67 or MC21.

Data are presented as mean ± SEM. Statistical significance was determined using a student's t-test or a Mann-Whitney test. *;p<0.05.

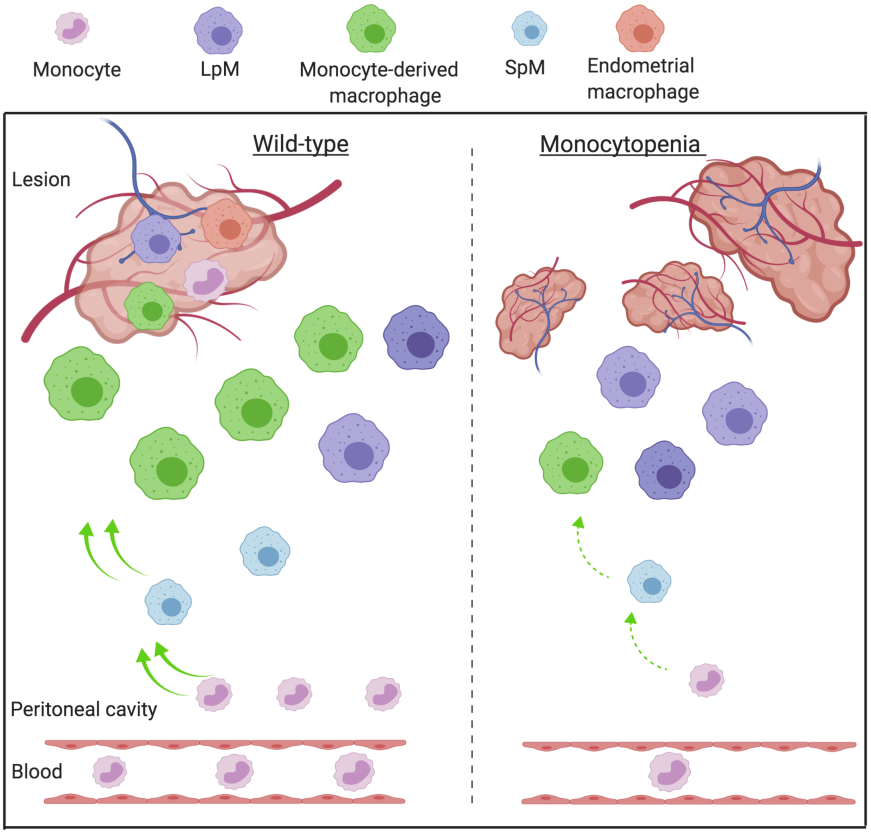

Figure 7: Monocyte-derived macrophages are guardians of the peritoneal cavity in mice with induced endometriosis.

Lesion-resident macrophages are a heterogenous population constituted by macrophages that have different origins; endometrium, peritoneum, and recruited monocytes that differentiate into macrophages in lesions. Wild-type mice with induced endometriosis have increased monocyte recruitment and replenishment of SpM and LpM pools by monocyte-derived cells. In mice where monocyte recruitment is constitutively limited (*Ccr2*-/- or *Ccl2*-/-), LpM and SpM pools are significantly reduced suggesting that the majority of LpM in the peritoneal cavity are embryo-derived. Elevated numbers of lesions develop in mice with constitutively reduced monocyte numbers suggesting that monocyte-derived macrophages protect the peritoneal cavity when challenged with ectopic endometrial tissue. We propose a putative model where endometrial and embryo-derived LpM promote lesion development, whilst monocyte-derived macrophages (possibly monocyte-derived LpM) protect the peritoneal cavity against lesion development.

Tables

Table 1. Flow cytometry antibodies			
Antibody	**Fluorochrome**	**Source**	**Dilution (v/v)**
Tim-4	PE/Cy7	Biolegend	1:200
MHC II	PE/Cy5	Biolegend	1:300
MHCII	AF700	Biolegend	1:400
CD11b	PECF594/ PE/Dazzle	Biolegend	1:300
CD45	PERCP/Cy5.5	Biolegend	1:200
CD3	FITC	Biolegend	1:500
CD19	FITC	Biolegend	1:500
CD335	FITC	Biolegend	1:500
SIGLEC F	FITC	Biolegend	1:500
Ly6G	FITC	Biolegend	1:500
Ly6G	BV650	Biolegend	1:200
Ly6C	PE	Biolegend	1:200
Ly6C	BV711	Biolegend	1:400
F4/80	APC/Cy7	Biolegend	1:500
F4/80	PECy7	Biolegend	1:200
F4/80	PERCP-Cy5.5	Biolegend	1:200
FVS660	APC	Biolegend	1:1000
Dapi			1:10,000

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Table 2. Antibodies for immunodetection						
Antibody	**Source**	**Cat number**	**Target cell**	**Species raised**	**Dilution (v/v)**	**Secondary used**
F4/80	eBioscience	14-4801	Macrophage	Rat	1:600	ImmPRESS® HRP-conjugated anti-rat antibody
GATA6	Cell Signalling Technology	5851S	LpM	Rabbit	1:3000	ImmPRESS® HRP-conjugated anti-rabbit antibody

Ly6C	Abcam	Ab15627	Monocytes	Rat	1:100	ImmPRESS® HRP-conjugated anti-rat antibody
GFP	Abcam	Ab6556	Macrophages labelled with EGFP	Mouse	1:2000	ImmPRESS® HRP-conjugated anti-mouse antibody

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