Macrophages inhibit and enhance endometriosis depending on their origin

Macrophages inhibit and enhance endometriosis depending on their origin

Chloe Hogg[1], Kavita Panir[2], Priya Dhami[2], Matthew Rosser[2], Matthias Mack[3], Daniel Soong[1], Jeffrey W Pollard[1], Stephen J Jenkins[4], Andrew W Horne[1], Erin Greaves[2]*

1. Medical Research Council (MRC) Centre for Reproductive Health, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom, EH16 4TJ
2. Centre for Early Life, Warwick Medical School, University of Warwick, Coventry, United Kingdom, CV2 2DX
3. Department of Internal Medicine II – Nephrology, University Hospital Regensburg, Regensburg, Germany, 93042
4. Centre for Inflammation Research, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom, EH16 4TJ

Corresponding author: Dr Erin Greaves

Email: Erin.Greaves@Warwick.ac.uk

Tel: 024 7696 8640

Classification: Biological Sciences, Immunology and Inflammation

Key words: Lesion, phenotype, ontogeny

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 monocyte and 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. Furthermore, we found endometriosis to trigger continuous recruitment of monocytes and expansion of CCR2+ LpM. Depletion of eutopic endometrial macrophages results in smaller endometriosis lesions, whereas constitutive inhibition of monocyte recruitment significantly reduces peritoneal macrophage populations and increases the number of lesions. Re-programming the ontogeny of peritoneal macrophages such that embryo-derived LpM are replaced by monocyte-derived LpM decreases the number of lesions that develop. We propose a putative model whereby endometrial macrophages are 'pro-endometriosis' whilst newly-recruited monocyte-derived macrophages, possibly in LpM form, are 'anti-endometriosis'. These observations highlight the importance of monocyte-derived macrophages in limiting disease progression.

Significance statement

Endometriosis is a chronic, incurable inflammatory disorder impacting 190 million women worldwide. Immune cells called macrophages are implicated in promoting endometriosis. Macrophages have different origins and their origin can dictate function. In this study we demonstrate that endometriotic lesion-resident macrophages are derived from the uterine lining (endometrium), the abdominal (peritoneal) cavity and from recruited bone-marrow precursors (monocytes). Endometriosis triggers continuous recruitment of monocytes that differentiate into macrophages that differ from those usually present within the peritoneal cavity. By depleting different populations, we demonstrate that endometrial macrophages are 'pro-endometriosis' whilst monocyte-derived peritoneal macrophages are 'anti-endometriosis' acting to protect the cavity from lesion establishment. In the future immune-based therapies may allow targeting of pro-disease macrophages and / or harnessing of anti-endometriosis macrophages in endometriosis.

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(3-5). Tissue-resident and monocyte-derived macrophages play distinct roles both in health and disease(6). Usually, tissue-resident macrophages play tissue specific homeostatic roles as well as core functions such as clearance of dying cells. On the other-hand monocyte-derived macrophages that are recruited to tissues during inflammation secrete pro-inflammatory cytokines, help clear infection and regulate the immune response(6). Thus, in pathological situations, macrophages are a heterogenous population. For example, during acute liver injury hepatic resident macrophages (Kupffer cells) become activated and recruit monocytes that initially promote liver injury, but then subsequently differentiate into inflammatory macrophages and help resolve injury and drive regeneration(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 only tissue-resident macrophages are responsible for driving tumor progression(8). These findings also highlight that under disease-modified conditions tissue-resident macrophages can become adapted such that they promote disease. Conversely, in other cancers (e.g. breast), monocytes are recruited to the tumor and differentiate into tumor-associated macrophages that drive disease (1).

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)(9). LpM are considered to be tissue

resident 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 more quickly in males(10, 11). The transcription factor GATA6 is highly expressed by all LpM in response to retinoic acid and drives a significant proportion of the tissue specific transcriptional signature of these cells(12). The SpM population is a more heterogeneous population comprising monocyte-derived macrophages and dendritic cells that are continually replenished from the blood(10, 13). Inflammatory challenge in the peritoneal cavity can result in recruitment of large numbers of inflammatory macrophages that are transcriptionally distinct from SpM(14), and a loss in LpM (the so-called macrophage disappearance reaction (MDR)), which results from formation of cell aggregates(15) or cell death(16). An exception to this is helminth infections which are characterized by expansion of LpM in response to local Th2 cytokine production(17) (18-20) (21).

Endometriosis is a chronic inflammatory condition where tissue similar to the endometrium grows ectopically, usually in the peritoneal cavity as 'lesions'(22). The condition impacts an estimated 190 million women worldwide and is associated with debilitating pelvic pain and infertility(23). 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 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(24). It is clear that macrophages are intrinsically linked with the pathophysiology of endometriosis where they enhance establishment, proliferation and vascularisation of lesions(25, 26). They are also critical in promoting innervation of lesions and concomitant sensitization of nerve fibres, thus contributing to pain in the condition(27, 28). 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(29). However, the exact origins of the host-derived macrophages and specific functions of the different populations remains to be determined.

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 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.

Results

Endometriosis lesion-resident macrophages have multiple origins. We induced endometriosis in wild-type C57BL/6 mice by injecting 'menses-like' endometrium from MacGreen donor mice (*Csf1r-eGFP*, macrophages are GFP+)(30) into the peritoneal cavity as previously described(29). In MacGreen mice all monocytes and macrophages in the shed menses-like endometrium are GFP+(31). Two weeks following tissue injection, endometriosis lesions were collected, digested and analysed by flow cytometry. GFP+ macrophages could be detected 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)+ lesion cells (Fig.1A). These data indicate that macrophages derived from the donor endometrium reside within lesions and verifies our previous findings using immunodetection(29). Endometrial-derived macrophages (GFP+) represented 16.0% (standard deviation (SD) ± 8.6) of lesion-resident macrophages, whilst the remaining 84.0% (GFP-; SD ± 8.4) 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 less than 1.0% of lesion-resident cells were 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, very few GFP+ macrophages were detected in lesions following adoptive transfer of SpM

isolated from MacGreen mice, suggesting that significant trafficking of peritoneal macrophages to lesions was restricted to the LpM population (Fig.1F). Interestingly, GFP+ SpM were instead observed located to the peritoneum adjacent to lesions. Quantification of GFP immunofluorescence revealed that a mean of 27.4% of cells in lesions were derived from LpM and a mean of 3.6% were derived from SpM (Fig.1G). 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.1H).

Continuous recruitment and contribution of monocytes to peritoneal macrophage populations 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 in sham mice (ovariectomized and treated with estradiol valerate and subject to intra-peritoneal (i.p) injection of PBS instead of endometrial tissue) compared to naïve mice (mean ± SEM = 48399 ± 7361 cells/ µl in sham and 18969 ± 5989 cells/ µl in naïve; Fig.2B; $p<0.01$). At three weeks, LpM numbers in sham animals had decreased compared to one-week ($p<0.05$). In contrast, no significant differences were found in the LpM population in mice with endometriosis compared to naïve and sham animals or within the endometriosis group at different timepoints. However, a trend can be observed indicating a moderate decrease in LpM numbers at one-week post-tissue injection in endometriosis mice compared to sham. This suggests that, compared to sham controls, there may be some loss of LpM following transfer of endometrial tissue, and this could be attributed to LpM trafficking into lesions or possible clotting of LpM as seen in peritonitis models(32). By three weeks the trend toward decreased LpM in endometriosis mice compared to sham is no longer evident and is consistent with our previous reports demonstrating an increase in LpM at 3 weeks in mice with endometriosis(27). 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(11). We repeated our results using intact recipient mice and confirmed that in this minimally invasive model, there was no

distinct shift in the ratio of SpM to LpM, suggesting that transfer of endometrial tissue does not disrupt the normal balance of peritoneal macrophages (SI Appendix, 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 one-week post tissue injection compared to naïve mice (Fig.2D; $p<0.01$). Monocyte numbers remained elevated between weeks one and three 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. In steady state conditions, long-lived embryo-derived peritoneal macrophages express T-cell immunoglobulin and mucin domain containing 4 (TIM4), whilst recently monocyte-derived LpM do not(10), thus to further validate our hypothesis we ascertained TIM4 expression in F4/80^{hi} LpM in intact mice. We found a significant reduction in TIM4^{hi} LpM at week two and three compared to week one (Fig.S1B; $p<0.05$) and a concomitant increase in TIM4^{lo} LpM at week two ($p<0.05$) and three ($p<0.01$) compared to week one (SI Appendix, Fig.S1B). This finding is consistent with heightened monocyte input into the LpM pool in endometriosis. We also observed a significant reduction in the proportion of F4/80^{lo}, MHCII- cells within CD11b+ peritoneal population between week one and week three post endometrial tissue injection (SI Appendix, Fig.S1C-D). Only a minor proportion of these cells express Ly6C (less than 2% of CD11b cells), indicating that this population may be a transitory state between monocyte and LpM.

Endometrial macrophage depletion leads to reduced endometriotic lesion size. To dissect the role of macrophages with different ontogenies in endometriosis we used a number of depletion strategies. To deplete endometrial macrophages, doxycycline was administered to i*Csf1r*-KO donor mice from day 15-19 post ovariectomy (Fig.3A), such that we could achieve macrophage depletion by inducibly ablating expression of *Csf1r*(33) . Pre-transfer analysis revealed that the endometrium was significantly depleted of F4/80^{hi} macrophages (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). These data suggest that macrophages within endometrial tissue promote the growth of endometriosis lesions but do not significantly impact the attachment of endometrial tissue to the peritoneal lining.

'Monocytopenic' mice with induced endometriosis had an increased number of lesions. Previous studies have inferred a role for recipient peritoneal macrophages in lesion development since continual i.p delivery of clodronate liposomes or anti-F4/80 antibody throughout the growth phase resulted in smaller lesions(25). Further, i.p transfer of bone marrow (BM)-derived macrophages could enhance or inhibit growth dependent on polarisation of macrophages *in vitro* prior to transfer(25). However, in addition to embryo-derived resident peritoneal macrophages, it is possible these methods also deplete endometrial macrophages (in transferred endometrial tissue) and recruited monocyte-derived cells. Notably, these studies showed that liposome-mediated depletion of embryonic resident peritoneal macrophages prior to endometrial transplantation did not significantly effect lesion development(25), suggesting the role of embryonic LpM is, at best redundant. Hence, we next aimed to prevent recruitment of host monocytes to the peritoneal cavity and lesions using *Ccr2* null monocytopenic recipient mice. LpM, SpM and monocytes were all significantly reduced in the peritoneal lavage fluid of *Ccr2-/-* mice with induced endometriosis compared to wild-type mice with endometriosis (a reduction of 56.4%, 70.5% and 69.0% in mean values respectively; Fig.4A-E and SI Appendix, Fig.S2A-C). This supports the concept that in our mouse model of endometriosis monocytes are continually recruited and contribute to the LpM and SpM pools. Furthermore, while lesion size was not different between the two groups (Fig.4G), *Ccr2*-/- mice had significantly more lesions (p<0.01) compared to wild-type mice (Fig.4F). This indicates that monocytes or monocyte-derived macrophages normally protect against establishment of lesions. To validate these findings, we repeated the experiment in Chemokine (C-C motif) ligand 2 null (*Ccl2*-/-) mice. In *Ccl2*-/- mice with endometriosis, LpM and SpM were significantly depleted compared to wild-type mice with endometriosis (a reduction of 37.9% and 69.8% in mean values respectively; Fig.5A-C; p<0.05 and SI Appendix, Fig.S2D-F). We observed a concomitant increase in the number of lesions recovered from *Ccl2*-/- mice (Fig.5E; p<0.05).

Strikingly, *Ccr2*-/-and *Ccl2*-/- mice with induced endometriosis were still able to recruit Ly6C+ monocytes from the bone marrow to the peritoneal cavity despite monocytes being absent in the peritoneal lavage of naïve and sham monocytopenic mice (SI Appendix, Fig.S3). Moreover, 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 redundancy in the CCL2-CCR2 axis in the presence of endometriosis lesions and suggests that monocytes may also be recruited via another mechanism.

Transient depletion of monocytes did not impact establishment of endometriotic lesions. Next, we sought to transiently deplete monocytes to ascertain the role of undifferentiated monocytes in lesion development whilst leaving the peritoneal macrophage populations relatively unaltered. We depleted monocytes using a function blocking CCR2 monoclonal antibody (MC21) injected into the peritoneal cavity of mice with induced endometriosis. Mice were injected with MC21, 6 hours prior to transfer of endometrial tissue, and every following day for 4 days (Fig.6A). Compared to a control IgG antibody (MC67), MC21 triggered an expansion of 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). These results rule out the possible contribution of recently recruited monocytes to the 'anti-endometriosis' function of monocyte-derived cells observed in Fig.4-5.

Re-programming the ontogeny of macrophages in the peritoneal cavity of mice with induced endometriosis leads to reduced lesion size. Next, we aimed to confirm the protective role of monocyte-derived LpM against establishment of lesions by reprogramming the ontogeny of macrophages in the peritoneal cavity. Seven days following ovariectomy we depleted peritoneal macrophages using liposomal clodronate and allowed 19 days (34) for replenishment of the niche from monocytes prior to injection of donor endometrial tissue (Fig.7A). Compared to control mice with induced endometriosis, those with re-programmed cavities exhibited elevated LpM and SpM (Fig.7C-D, $p<0.05$ and $p<0.001$ respectively). Moreover, endometriosis mice with re-programmed cavities were significantly depleted of TIM4^{hi} LpM (a reduction of 82.7% in mean values compared to control mice with endometriosis; Fig.7E; $p<0.01$) consistent with replacement of embryo-derived LpM with monocyte-derived LpM(10)(34). In mice with re-programmed cavities, the

number of lesions that developed was significantly reduced, with 5 mice developing no lesions at all ($p<0.05$; Fig.7F). There was no difference in lesion area in mice with reprogrammed cavities compared to control mice (Fig.7G). Collectively, these data strongly support the concept that monocyte-derived LpM act to protect the peritoneal cavity when challenged with ectopic endometrial tissue.

Discussion

The pathophysiology of endometriosis remains enigmatic(22). 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 cancer(35). As the ontogeny of macrophages in diseased tissue is a key determinant of how they respond and contribute to pathogenesis, it is necessary to understand how macrophages derived from different sources impact lesion development. In the current study we have determined, in a mouse model of induced endometriosis, that lesion-resident macrophages are derived from the eutopic endometrium, infiltrating LpM and monocytes. We have demonstrated that endometriosis triggers continuous recruitment of monocytes to the peritoneal cavity and heightened monocyte input into the LpM pool. We show that endometrial macrophages promote the growth but not the establishment of endometriotic lesions, whereas monocyte-derived LpMs play a protective role against lesion development.

The contribution of embryonic-derived and monocyte-derived macrophages to the 'tissue-resident' population is different in each tissue(3-5). We previously demonstrated that macrophages present in the endometrium can be detected in lesions recovered from our model of induced endometriosis(29). However, 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 (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(31). 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, 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 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, indicating that endometrial macrophages play a critical role in growth of lesions. 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(36). If we consider endometriotic 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 (or within the established lesion during cyclical remodelling) and the repair of the translocated endometrium occurs in the peritoneal cavity resulting in the formation of lesions. The subsequent phase of the tissue repair process is the proliferative phase: in mouse models of skin injury, macrophage depletion during this phase resulted in granulation tissue that had very few blood vessels and proliferative cells and a significant reduction in wound closure. This indicates that macrophages support this phase by promoting endothelial cell survival and vascularization which facilitates proliferation(37). 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. Further, previous studies have demonstrated that macrophages do not regulate survival or proliferation of uterine epithelial, stromal or vascular endothelial cells during the estrus cycle or following exogenous supplementation with estradiol and progesterone(38). This evidence supports the concept that macrophages are implicated in tissue repair after hormone withdrawal or injury (e.g. following endometrial shedding and in endometriosis), as opposed to having a trophic role under steady-state conditions.

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 continually replenish the SpM compartment and infrequently differentiate into LpM(10). Such replenishment of LpM occurs in a sexually

dimorphic manner, occurring more quickly in male compared to female mice who retain their embryo-derived LpM for much longer(10, 16), and it is likely that in adult female mice of the age used in our studies (10-12 wks) only 10-30% of peritoneal LpM would be derived from adult monocytes(11, 16). In this study we used ovariectomy and estradiol supplementation of recipient animals to allow optimal lesion development. Peritoneal surgery increases the contribution of monocytes to the peritoneal LpM however; the embryonic component would still be expected to comprise approximately 50% of the population at the time of injection of endometrial material. Although monocyte-derived LpM mostly phenocopy embryo-derived LpM, transcriptomically they exhibit some differences. For example, embryo-derived LpM express *Timd4*, whilst in steady state many monocyte-derived LpM do not, or take significant time to do so(10, 11). Notably, the number of monocytes and CCR2+ LpM was significantly elevated in the peritoneal cavity of mice with induced endometriosis, above levels seen in ovariectomised controls, suggesting monocytes are continually recruited and contribute to LpM pools during lesion development.

Consistent with a significant input of monocytes to LpM in endometriosis, our 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. When left unmanipulated, female *Ccr2*-/- mice normally exhibit equivalent numbers of LpM to wild-type controls(10). Thus, we suggest that monocyte-derived macrophages act to protect the peritoneal cavity and can limit the establishment of lesions (Fig.8).

In monocytopenic mice with endometriosis, a reduction in SpM, LpM as well as monocytes was observed. Monocytopenic mice also exhibited significantly more lesions, indicating that monocyte-derived cells are 'anti-endometriosis', although to which population of monocyte-derived cells this function can be attributed remains uncertain. As transient antibody-mediated depletion of monocytes during the establishment phase failed to increase the number of lesions but also left SpM and LpM populations intact, it seems likely that either SpM or LpM provide a dominant anti-endometriosis effect.

We determined that a significant proportion of LpM (27.4% of lesion resident cells were GFP+ following adoptive transfer of LpM) but not SpM enter endometriosis lesions using adoptive transfers; we suggest that these LpM change phenotype in lesions very

rapidly because only a few lesion cells positive for both F4/80 and GATA6 (<1%) were identified using dual staining, whereas LpM in the cavity are almost entirely positive for GATA6(12). Aside from identifying peritoneal LpM as a source of macrophages in endometriosis lesions, these findings are important because they show that LpM are able to re-programme and survive in ectopic tissue, a topic of significant controversy in the field of tissue-resident macrophage biology(39, 40). Indeed, our results are consistent with the reversible expression of GATA6 by LpM in the absence of sustained retinoic acid receptor signalling(12) and mirror recent findings that pericardial cavity GATA6+ macrophages lose expression of GATA6 following recruitment to areas of ischemic heart disease(41). In the same manner, mature F4/80^{hi} GATA6+ peritoneal LpM are reported to traffic directly across the mesothelium into the liver following sterile injury. Once in the liver, the macrophages undergo local proliferation, and up regulation of markers of alternative activation, such as Arginase 1 (*Arg1*) and *Retnla*. In the absence of peritoneal macrophages, healing was significantly delayed(42). Our data suggest that in endometriosis LpM trafficking to lesions may play a similar role, perceiving the ectopic tissue as a wound and activating repair processes. Interestingly, GATA6 positive macrophages that invade the epicardium from the pericardial space following experimental myocardial infarction were anti-fibrotic, despite a rapid loss of GATA6 expression(41). Fibrosis is a consistent feature of endometriotic lesions(43). Whilst eutopic endometrium is able to undergo scar-free healing to restore full tissue functionality, when the tissue is translocated to the peritoneal environment fibrotic 'repair' occurs to form lesions. The mechanisms responsible for this are yet to be fully resolved but pro-repair macrophages have been implicated in the process(44). Thus, it seems unlikely that LpMs trafficking into lesions contribute to fibrotic repair, however, the ontogeny of macrophages contributing to fibrogenesis in endometriosis remains to be determined. Interestingly, our data suggest that SpM (which likely include both classical dendritic cells (cDC)1 and cDC2, with the flow cytometry gates used in our study) may have a neutral role in the pathophysiology of endometriosis since they neither increased in number nor appeared to significantly contribute to the lesion-resident population. Moreover, in our final experiment we sought to re-program the peritoneal cavities of mice such that embryo-derived LpM were replaced by monocyte-derived LpM. In endometriosis mice with reprogrammed cavities significantly fewer lesions developed. Hence, we suggest it is the monocyte-derived LpM that are protective against development of endometriosis.

One limitation of the current study is the inter-experiment variation in lesion number and size in wild-type mice. The mouse model of induced endometriosis used in the study relies on spontaneous attachment of the donor endometrial tissue to the peritoneal lining of recipient mice. This avoids the additional inflammation triggered by suturing (used in a number of other models). We carefully control the amount of tissue used to inoculate recipient mice, and any donor mice that do not mount the appropriate decidual response are excluded. One possible cause that may impact the efficacy of endometrial tissue attachment is a discrete difference in the immune status of batches of mice. Future studies will determine ontogenetic and phenotypic differences in peritoneal macrophages recovered from mice with varying degrees of endometrial tissue attachment.

Our data indicates a key role for monocyte recruitment to the peritoneal cavity *and* ectopic tissue in endometriosis. We readily detected lesion-resident monocytes as well as cells that were double positive for both Ly6C and F4/80, but whether this indicates that monocytes rapidly differentiate in lesions or that differentiated monocyte-derived macrophages are recruited from the peritoneal cavity remains unclear. We speculate that monocyte-derived cells represent the largest lesion-resident population. However, we cannot currently conclude whether these are monocyte-derived LpM or monocytes recruited directly to the lesion. If the latter, we do not know whether these come via the cavity or through the newly formed vasculature associated with the lesion.

In a previous study, Bacci et al depleted peritoneal macrophages using liposomal clodronate in a mouse model of endometriosis. Depletion of peritoneal macrophages prior to transfer of endometrial tissue had no significant impact on lesion establishment and growth(25) implying that embryonic-derived LpM are redundant in lesion development. Continuous depletion during establishment and growth resulted in significantly smaller lesions, allowing the authors to conclude that the dominant role for macrophages in endometriosis is to promote the development of lesions. It may be presumed that this approach has the potential to deplete all monocytes and macrophages including those in the transferred endometrial tissue. Thus, in light of our findings that donor endometrial macrophages play a significant role in facilitating lesion growth we suggest the findings of Bacci et al are a consequence of global macrophage depletion. In contrast, our study used depletion strategies that target macrophages from different origins. We demonstrate a protective role for monocyte-derived macrophages (presumably in LpM form). Similarly,

further experiments by Bacci et al demonstrated that i.p transfer of bone marrow (BM)-derived macrophages could enhance or inhibit growth dependent on polarisation of macrophages *in vitro* prior to transfer(25). Further studies will aim to elucidate the exact phenotype and mechanism of monocyte-derived LpM in endometriosis.

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 the future it may be possible to harness the protective properties of monocyte-derived macrophages as a potential therapy for women with endometriosis. We propose a putative model that in endometriosis, macrophages derived from the endometrium exhibit 'pro-endometriosis' functions and facilitate growth of endometriotic lesions, whereas monocyte-derived cells, possibly in the form of LpM from the cavity have an 'anti-endometriosis' role and are protective against persistence of ectopic tissue and establishment of lesions.

Collectively, we have demonstrated multiple origins for endometriotic lesion-resident macrophages, a key role for monocyte-derived macrophages in protecting the peritoneal cavity when challenged with ectopic endometrial tissue and a pathological role for endogenous endometrial macrophages. Our findings imply that monocyte recruitment or monocyte-derived macrophages may be defective in women with endometriosis, and this should be explored in more depth in women with the condition. 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*(33) (colony stimulating factor 1 receptor (Csf1r) conditional

knock out) allows deletion of *Csf1r* following treatment with the tetracycline analog doxycycline (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(45). 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(46). 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(47). 3) The monocyte depleting rat anti-mouse CCR2 mAb (clone MC21) isotype IgG2b(48) was used for monocyte depletion experiments (gifted by Prof M. Mack, University Hospital Regensberg). 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 control antibody (MC67) was used as a control. To reprogram the peritoneal cavities of mice such that embryo-derived LpM were replaced with monocyte-derived LpM we administered liposomal clodronate i.p (Encapsula NanoSciences; 0.0625mg/mouse) and allowed replenishment of the niche from monocytes (see below for further details).

Mouse model of induced endometriosis. Endometriosis was induced in mice using a syngeneic model as previous described(29). 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 truncated menstrual cycle and a stimulus that causes the endometrial stromal cells to undergo decidualization(49). 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(49). Lesions are recovered that contain stoma +/- epithelial cells and immune cell influx(29). Unless otherwise stated lesions were collected 2 weeks following tissue injection. For clarity, experiments using different

pharmacological / transgenic approaches to deplete monocyte / macrophage populations 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 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 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). The data from 2 independent experiments are presented. *Experiment 4: Constitutive depletion of monocytes in the peritoneal cavity (i).* Donor endometrium from wild-type C57BL/6 mice was injected i.p into wild-type (control; n=11) or *Ccr2*-/- recipients (n=13). Data from 3 independent experiments are presented. *Experiment 5: Constitutive depletion of monocytes in the peritoneal cavity (ii).* Wild-type donor endometrium was injected i.p into wildtype (controls; n=6) or *Ccl2*-/- recipients (n=7). Data from 2 independent experiments are presented. *Experiment 6: Transient depletion of monocytes in the peritoneal cavity*. Six hours prior to endometrial transfer recipient C57BL/6 mice received i.p injection of a control IgG (MC67; n=9) or a function blocking CCR2 mAb (MC21; n=10). Syngeneic donor endometrium was then injected i.p into recipients. Mice received daily injections of MC67 or MC21 until day 5 post-tissue injection when mice were culled. Data from 2 independent experiments are presented. *Experiment 7*: *Reprogramming macrophage ontogeny in the peritoneal cavity*. Seven days post ovariectomy C57BL/6 recipient mice received an i.p injection of liposomal clodronate (0.0625mg/mouse, n=10) to deplete peritoneal macrophages. We then waited 19 days to achieve replenishment of the LpM population from monocytes(34). Syngeneic endometrial tissue was injected i.p and lesions recovered on day 5 post tissue injection. Non-depleted control mice (n=10) were ovariectomised at the same time but left unmanipulated until endometriosis tissue transfer. Data from 1 independent experiment is presented. 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 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^{TM} 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). For data where peritoneal populations are expressed as cells/ µl, absolute counts were calculated using 123 eBeads and the equation: absolute count (cells/ µl) = (cell count / eBead count) x eBead batch concentration. Final volume for cytofluorimetric analysis was 300 µl. Thus for example, 400 cells / µl is equal to 1.2 x10^5 cells per cavity

Immunofluorescence. Immunofluorescence was performed as previously described(27, 28, 50). 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 $p<0.05$.

Acknowledgements

We thank the QMRI flow cytometry and cell sorting facility technicians (University of Edinburgh) for advice on panel design, and Fiona Ballantyne for technical assistance. We thank Ronnie Grant (University of Edinburgh) and Rebecca Doust for help with figure preparation. We thank Professor Jan J Brosens and Dr Andrew Blanks for critical feedback on the manuscript. This work was supported by a Medical Research Council (MRC) Career Development Award (MR/M009238/1; to E.G), an MRC Project Grant (MR/S002456/2; E.G), an MRC Centre studentship to C.H, a Wellcome Trust Senior Investigator Award (101067/Z/12/Z; J.W.P) and an MRC Centre Grant (MR/N022556/1; J.W.P). The authors declare no conflict of interest.

Author contributions

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

References

1. J. W. Pollard, Trophic macrophages in development and disease. *Nat Rev Immunol* **9**, 259-270 (2009).
2. T. A. Wynn, A. Chawla, J. W. Pollard, Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455 (2013).
3. F. Ginhoux, S. Jung, Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol* **14**, 392-404 (2014).
4. F. Ginhoux, J. L. Schultze, P. J. Murray, J. Ochando, S. K. Biswas, New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat Immunol* **17**, 34-40 (2016).

5. C. Schulz *et al.*, A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* **336**, 86-90 (2012).
6. L. C. Davies, S. J. Jenkins, J. E. Allen, P. R. Taylor, Tissue-resident macrophages. *Nat Immunol* **14**, 986-995 (2013).
7. C. Ju, F. Tacke, Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cellular & molecular immunology* **13**, 316-327 (2016).
8. Y. Zhu *et al.*, Tissue-Resident Macrophages in Pancreatic Ductal Adenocarcinoma Originate from Embryonic Hematopoiesis and Promote Tumor Progression. *Immunity* **47**, 597 (2017).
9. C. C. Bain, S. J. Jenkins, Isolation and Identification of Murine Serous Cavity Macrophages. *Methods Mol Biol* **1784**, 51-67 (2018).
10. C. C. Bain *et al.*, Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Commun* **7**, ncomms11852 (2016).
11. C. C. Bain *et al.*, Origin and microenvironment contribute to the sexually dimorphic phenotype and function of peritoneal macrophages. *bioRxiv* 10.1101/837336, 837336 (2019).
12. Y. Okabe, R. Medzhitov, Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* **157**, 832-844 (2014).
13. C. Goudot *et al.*, Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages. *Immunity* **47**, 582-596 e586 (2017).
14. E. L. Gautier, S. Ivanov, P. Lesnik, G. J. Randolph, Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* **122**, 2714-2722 (2013).
15. X. Zhang *et al.*, Type I collagen or gelatin stimulates mouse peritoneal macrophages to aggregate and produce pro-inflammatory molecules through upregulated ROS levels. *International immunopharmacology* **76**, 105845 (2019).
16. Z. Liu *et al.*, Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. *Cell* **178**, 1509-1525.e1519 (2019).
17. S. J. Jenkins *et al.*, Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **332**, 1284-1288 (2011).
18. E. E. Ghosn *et al.*, Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A* **107**, 2568-2573 (2010).
19. E. L. Gautier, S. Ivanov, P. Lesnik, G. J. Randolph, Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* **122**, 2714-2722 (2013).
20. L. Boring *et al.*, Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *The Journal of Clinical Investigation* **100**, 2552-2561 (1997).
21. S. J. Jenkins *et al.*, IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med* **210**, 2477-2491 (2013).
22. A. W. Horne, P. T. K. Saunders, SnapShot: Endometriosis. *Cell* **179**, 1677-1677 e1671 (2019).
23. K. T. Zondervan, C. M. Becker, S. A. Missmer, Endometriosis. *N Engl J Med* **382**, 1244-1256 (2020).
24. J. Halme, M. G. Hammond, J. F. Hulka, S. G. Raj, L. M. Talbert, Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol* **64**, 151-154 (1984).

25. M. Bacci *et al.*, Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol* **175**, 547-556 (2009).
26. A. Capobianco *et al.*, Proangiogenic Tie2(+) macrophages infiltrate human and murine endometriotic lesions and dictate their growth in a mouse model of the disease. *Am J Pathol* **179**, 2651-2659 (2011).
27. R. Forster *et al.*, Macrophage-derived insulin-like growth factor-1 is a key neurotrophic and nerve-sensitizing factor in pain associated with endometriosis. *Faseb j* **33**, 11210-11222 (2019).
28. E. Greaves *et al.*, Estradiol is a critical mediator of macrophage-nerve cross talk in peritoneal endometriosis. *Am J Pathol* **185**, 2286-2297 (2015).
29. E. Greaves *et al.*, A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium. *Am J Pathol* **184**, 1930-1939 (2014).
30. R. T. Sasmono, E. Williams, Generation and characterization of MacGreen mice, the Cfs1r-EGFP transgenic mice. *Methods Mol Biol* **844**, 157-176 (2012).
31. F. L. Cousins, P. M. Kirkwood, P. T. Saunders, D. A. Gibson, Evidence for a dynamic role for mononuclear phagocytes during endometrial repair and remodelling. *Sci Rep* **6**, 36748 (2016).
32. N. Zhang *et al.*, Expression of factor V by resident macrophages boosts host defense in the peritoneal cavity. *The Journal of experimental medicine* **216**, 1291-1300 (2019).
33. J. Li, K. Chen, L. Zhu, J. W. Pollard, Conditional deletion of the colony stimulating factor-1 receptor (c-fms proto-oncogene) in mice. *Genesis* **44**, 328-335 (2006).
34. P. A. Louwe *et al.*, Recruited macrophages that colonise the post-inflammatory peritoneal niche convert into functionally divergent resident cells. *bioRxiv* 10.1101/2020.11.30.404988, 2020.2011.2030.404988 (2020).
35. C. Hogg, A. W. Horne, E. Greaves, Endometriosis-Associated Macrophages: Origin, Phenotype, and Function. *Frontiers in Endocrinology* **11** (2020).
36. T. A. Wynn, K. M. Vannella, Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity* **44**, 450-462 (2016).
37. T. Lucas *et al.*, Differential roles of macrophages in diverse phases of skin repair. *J Immunol* **184**, 3964-3977 (2010).
38. A. S. Care, W. V. Ingman, L. M. Moldenhauer, M. J. Jasper, S. A. Robertson, Ovarian steroid hormone-regulated uterine remodeling occurs independently of macrophages in mice. *Biol Reprod* **91**, 60 (2014).
39. Y. Lavin *et al.*, Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell* **159**, 1312-1326 (2014).
40. L. van de Laar *et al.*, Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages. *Immunity* **44**, 755-768 (2016).
41. J. F. Deniset *et al.*, Gata6(+) Pericardial Cavity Macrophages Relocate to the Injured Heart and Prevent Cardiac Fibrosis. *Immunity* **51**, 131-140 e135 (2019).
42. J. Wang, P. Kubes, A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. *Cell* **165**, 668-678 (2016).
43. P. Vigano *et al.*, Time to redefine endometriosis including its pro-fibrotic nature. *Hum Reprod* **33**, 347-352 (2018).

44. J. Duan, X. Liu, H. Wang, S. W. Guo, The M2a macrophage subset may be critically involved in the fibrogenesis of endometriosis in mice. *Reprod Biomed Online* **37**, 254-268 (2018).
45. R. T. Sasmono *et al.*, A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* **101**, 1155-1163 (2003).
46. L. Boring *et al.*, Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* **100**, 2552-2561 (1997).
47. B. Lu *et al.*, Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* **187**, 601-608 (1998).
48. M. Mack *et al.*, Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J Immunol* **166**, 4697-4704 (2001).
49. F. L. Cousins *et al.*, Evidence from a mouse model that epithelial cell migration and mesenchymal-epithelial transition contribute to rapid restoration of uterine tissue integrity during menstruation. *PLoS One* **9**, e86378 (2014).
50. E. Greaves *et al.*, Estrogen receptor (ER) agonists differentially regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. *Endocrinology* **155**, 4015-4026 (2014).

Figures legends

Figure 1: Lesion-resident macrophages have different origins.

Donor endometrial tissue from MacGreen mice was injected into the peritoneal cavity of wild-type recipient mice to assess incorporation of endometrial macrophages into lesions. Lesions were collected at two weeks post tissue injection in each of the separate studies presented in this figure.

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.

Dual immunodetection for identification of large peritoneal macrophages in lesions.

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

D) Quantification of F4/80+, dual positive and GATA6+ cells in lesions. Less than 1% of cells were dual positive for F4/80 and GATA6.

Adoptive transfers of MacGreen peritoneal macrophages into wild-type mice with GFP immunodetection to assess incorporation of LpM and SpM into lesions.

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 boundary between peritoneal and lesion tissue. In E (i) and (ii) show magnified images, in F (i) shows a negative control.

G) Quantification of GFP+ LpM and SpM in lesions.

Dual immunodetection for Ly6C+ monocytes in lesions.

H) 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.

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 (2 weeks post tissue injection).

F) Quantification of CCR2+, F4/80^{hi} cells 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. Donor endometrium was generated in i*Csf1r*-KO mice, with doxycycline administered to donor mice from day 15-19 to deplete endometrial macrophages prior to recovery of endometrium and i.p transfer to wild-type recipients. Lesions were recovered 2 weeks post-tissue injection.

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) (from 2 independent experiments).

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. Wild-type donor endometrium was generated as previously shown (Fig.3A) and injected i.p into ovariectomised *Ccr2*-/- recipients. Wild-type recipients were also used as controls. Lesions were recovered 2 weeks post-tissue injection.

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.

F) Number of lesions recovered from wild-type (n=11) and *Ccr2*-/- (n=13) mice with induced endometriosis (from 3 independent experiments).

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

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.

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

Wild-type donor endometrium was generated as previously shown (Fig.3A) and injected i.p into ovariectomised *Ccl2*-/- recipients as in the previous figure. Wild-type recipients were also used as controls. Lesions were recovered 2 weeks post-tissue injection.

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 (n=6) and *Ccl2*/- (n=7) mice with induced endometriosis (from 2 independent experiments).

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

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 or 95% confidence intervals (E). Statistical significance was determined using a Student's t-test or Mann-Whitney test. *;p<0.05.

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, from 2 independent experiments)), 6 hours prior to endometrial tissue injection and daily for an additional 4 days. Lesions were recovered 5 days post tissue injection.

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 or 95% confidence intervals (F). Statistical significance was determined using a student's t-test or a Mann-Whitney test. *;p<0.05.

Figure 7: Reprogramming the ontogeny of peritoneal macrophages leads to establishment of fewer lesions.

A) Schematic showing the experimental design; 7 days post ovariectomy experimental mice were administered i.p with liposomal clodronate to deplete all peritoneal macrophages (n=10). Nineteen days were allowed for replenishment of the niche prior to transfer of

endometrial tissue on day 26. Lesions were recovered 5 days post tissue injection. Control mice (n=10) did not receive liposomal clodronate (single independent experiment).

B) Flow plot demonstrating the numbers of TIM4^{hi} and TIM4^{lo} LpM in control mice with induced endometriosis or mice with re-programed cavities with induced endometriosis.

C-E) Quantification of (C) LpM, (D) SpM, (E) TIM4^{hi} LpM in the peritoneal lavage fluid of mice with induced endometriosis.

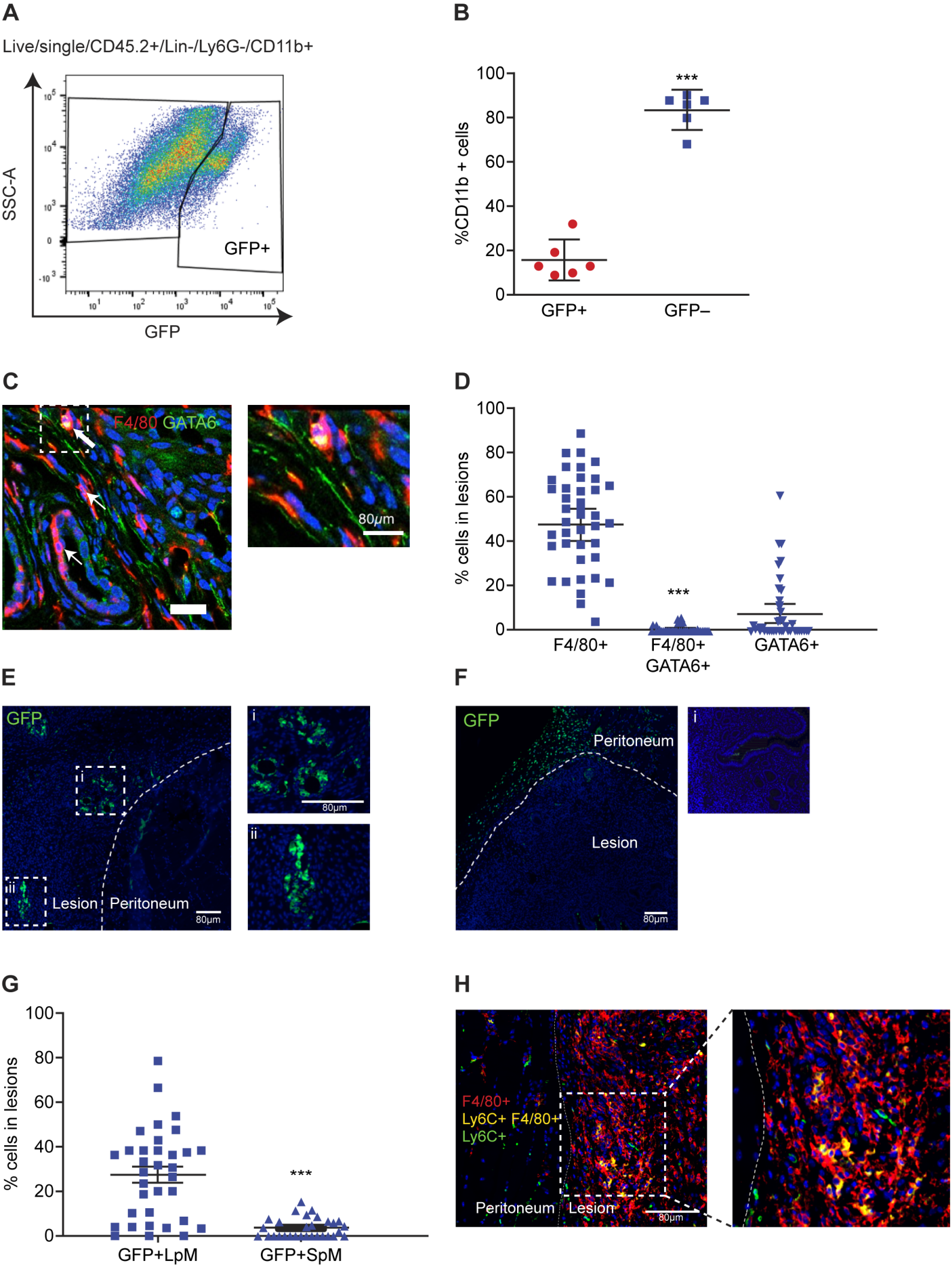
F) Number of lesions recovered from control mice and those with re-programed cavities.

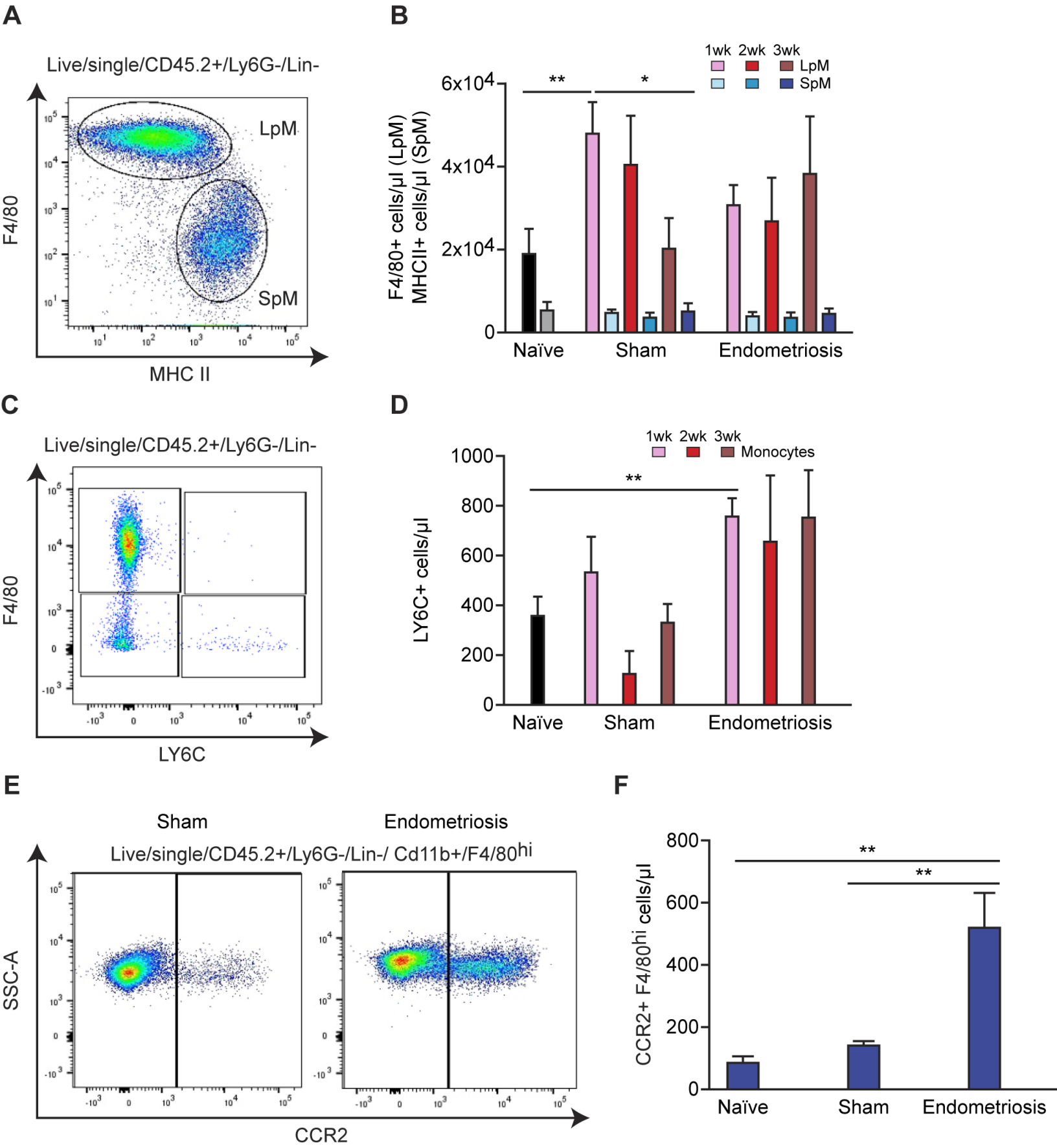
G) Size of lesions recovered from control mice and those with re-programed cavities.

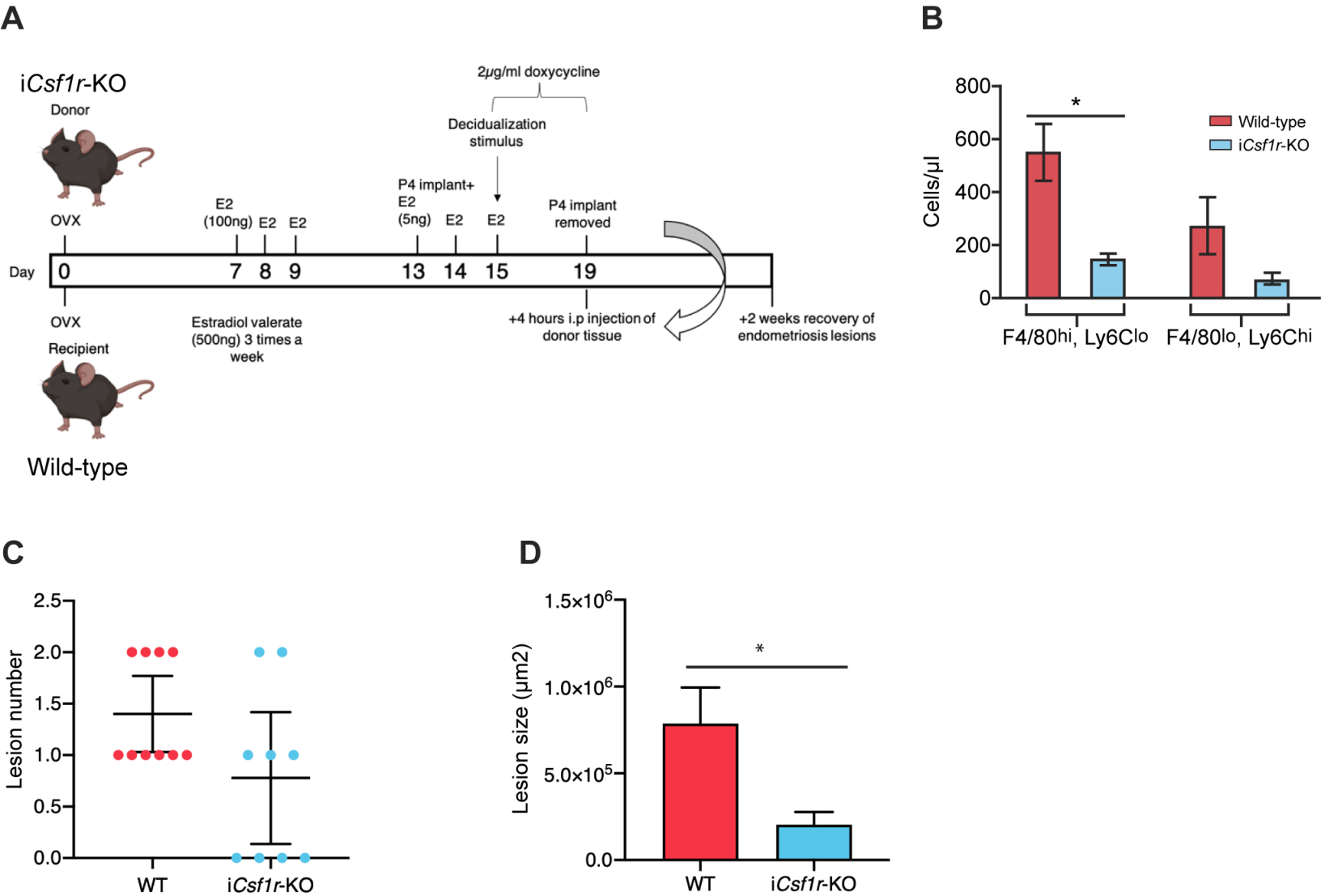
Data are presented as mean ± SEM or 95% confidence intervals (F). Statistical significance was determined using a student's t-test or a Mann-Whitney test. *;p<0.05, **;p<0.01.

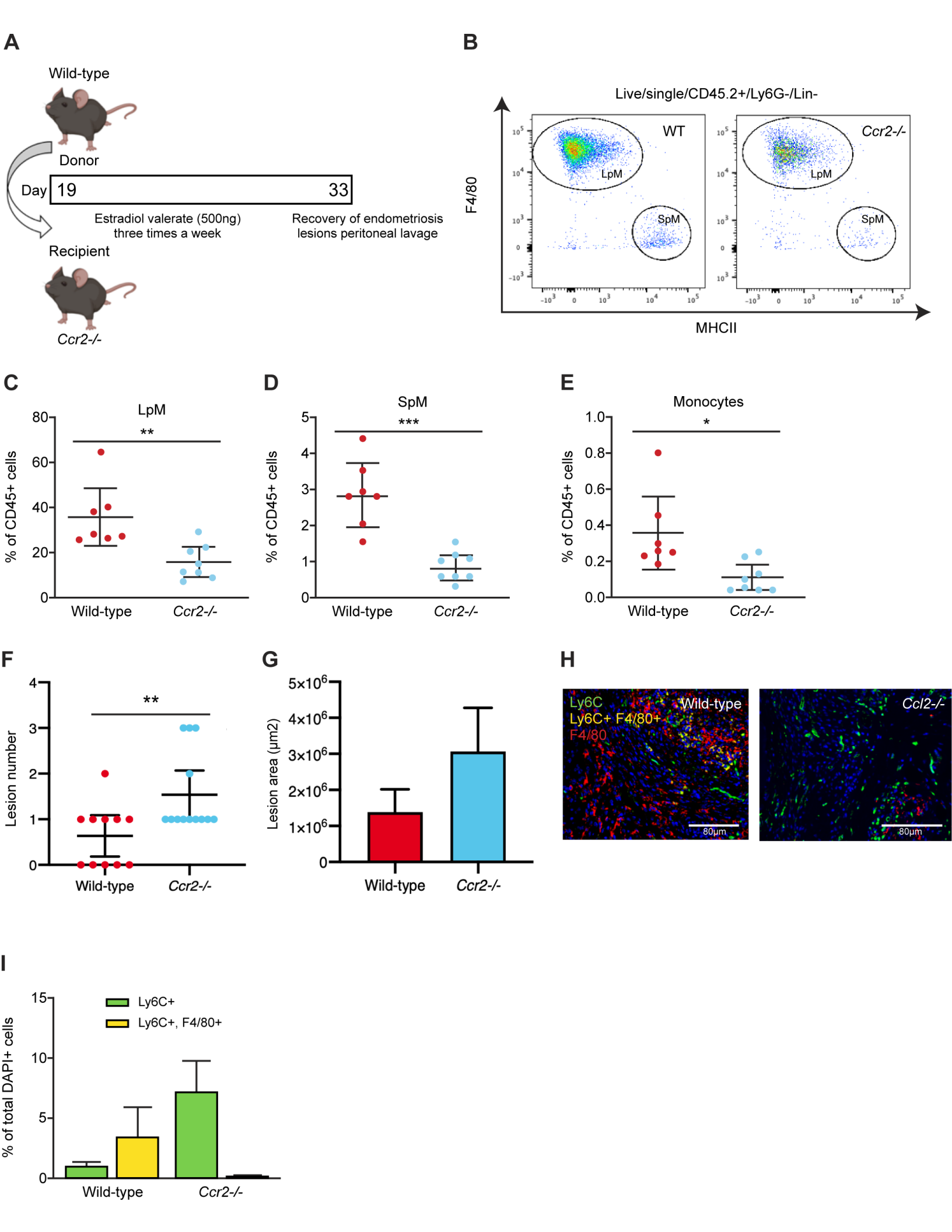
Figure 8: Monocyte-derived macrophages are guardians of the peritoneal cavity in mice with induced endometriosis. Created with BioRender.com.

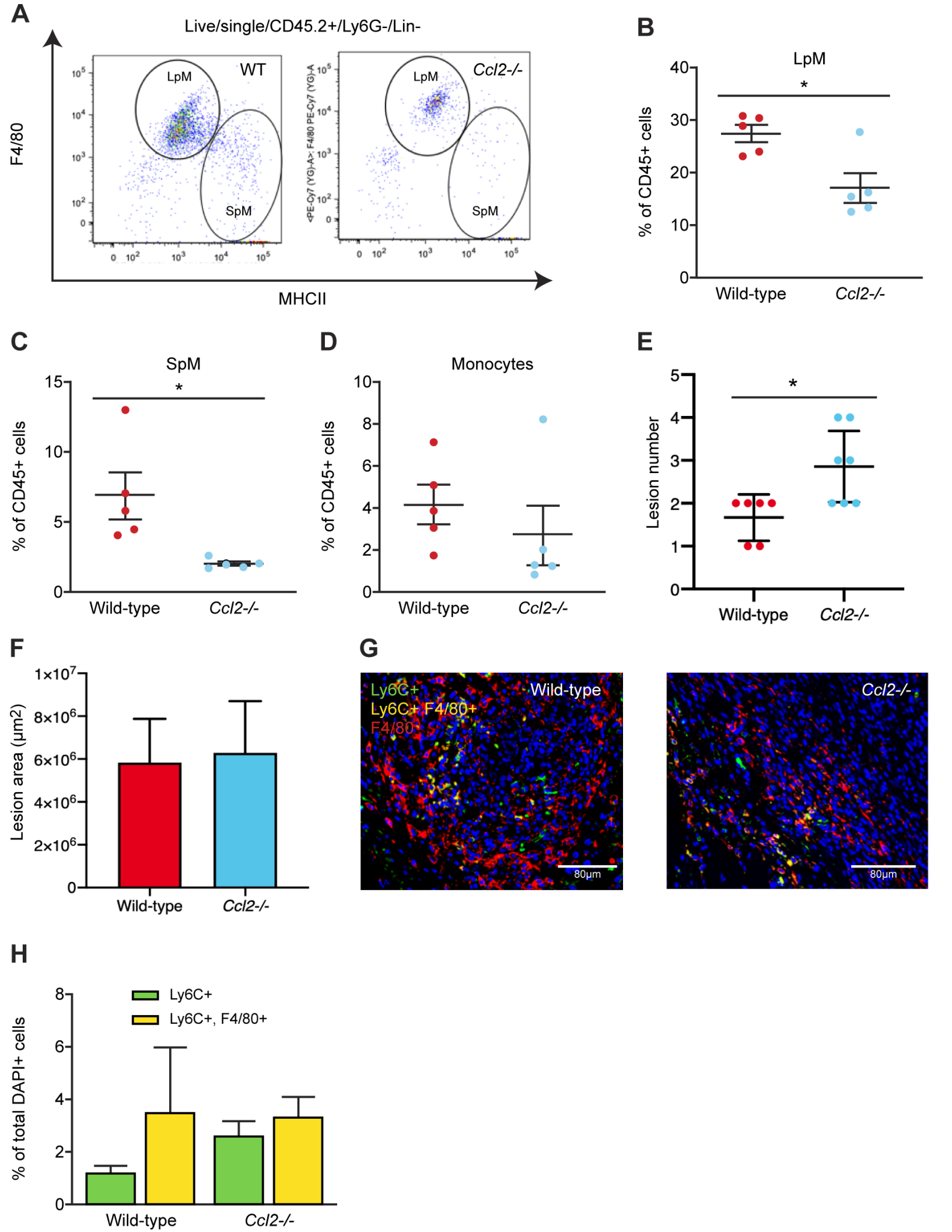
Lesion-resident macrophages are a heterogenous population constituted by macrophages that have different origins; endometrial, peritoneal (LpM), and recruited monocytes that differentiate into macrophages in lesions. Wild-type mice with induced endometriosis exhibit increased monocyte recruitment and replenishment of LpM pools from monocytes. In mice where monocyte recruitment is constitutively limited (*Ccr2*-/- or *Ccl2*-/-), LpM and SpM pools are significantly reduced, consistent with the majority of LpM in the peritoneal cavity being embryo-derived. In these (monocytopenic) mice, more lesions develop. Mice with ontogenetically reprogrammed peritoneal cavities (embryo-derived LpM depleted using liposomal clodronate followed by a 19-day replenishment window), develop significantly fewer lesions. Collectively, these data suggest that monocyte-derived LpM protect the peritoneal cavity when challenged with ectopic endometrial tissue. We propose a putative model where endometrial macrophages promote lesion growth, whilst monocyte-derived macrophages (possibly monocyte-derived LpM) protect the peritoneal cavity against establishment of lesions.

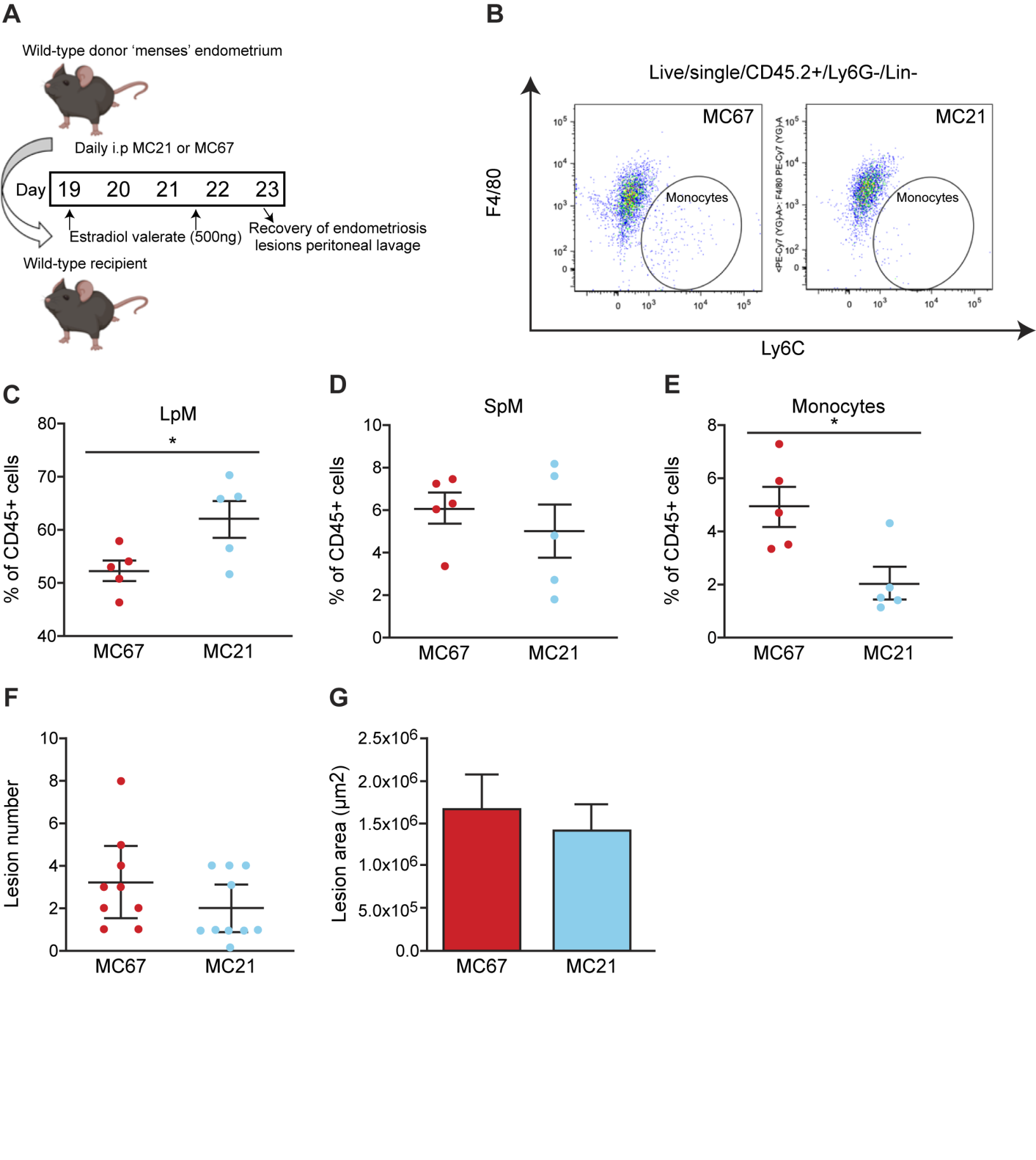

A
Live/single/CD45.2+/Lin-/Ly6G-/CD11b+
SSC-A
GFP
GFP+
B
%CD11b + cells
100
80
60
40
20
0
***
GFP+
GFP–
C
F4/80 GATA6
80μm
D
% cells in lesions
100
80
60
40
20
0
***
F4/80+
F4/80+
GATA6+
GATA6+
E
GFP
i
ii
iii
Lesion
Peritoneum
80μm
80μm
F
GFP
Peritoneum
Lesion
i
80μm
G
% cells in lesions
100
80
60
40
20
0
***
GFP+LpM
GFP+SpM
H
F4/80+
Ly6C+ F4/80+
Ly6C+
Peritoneum
Lesion
80μm

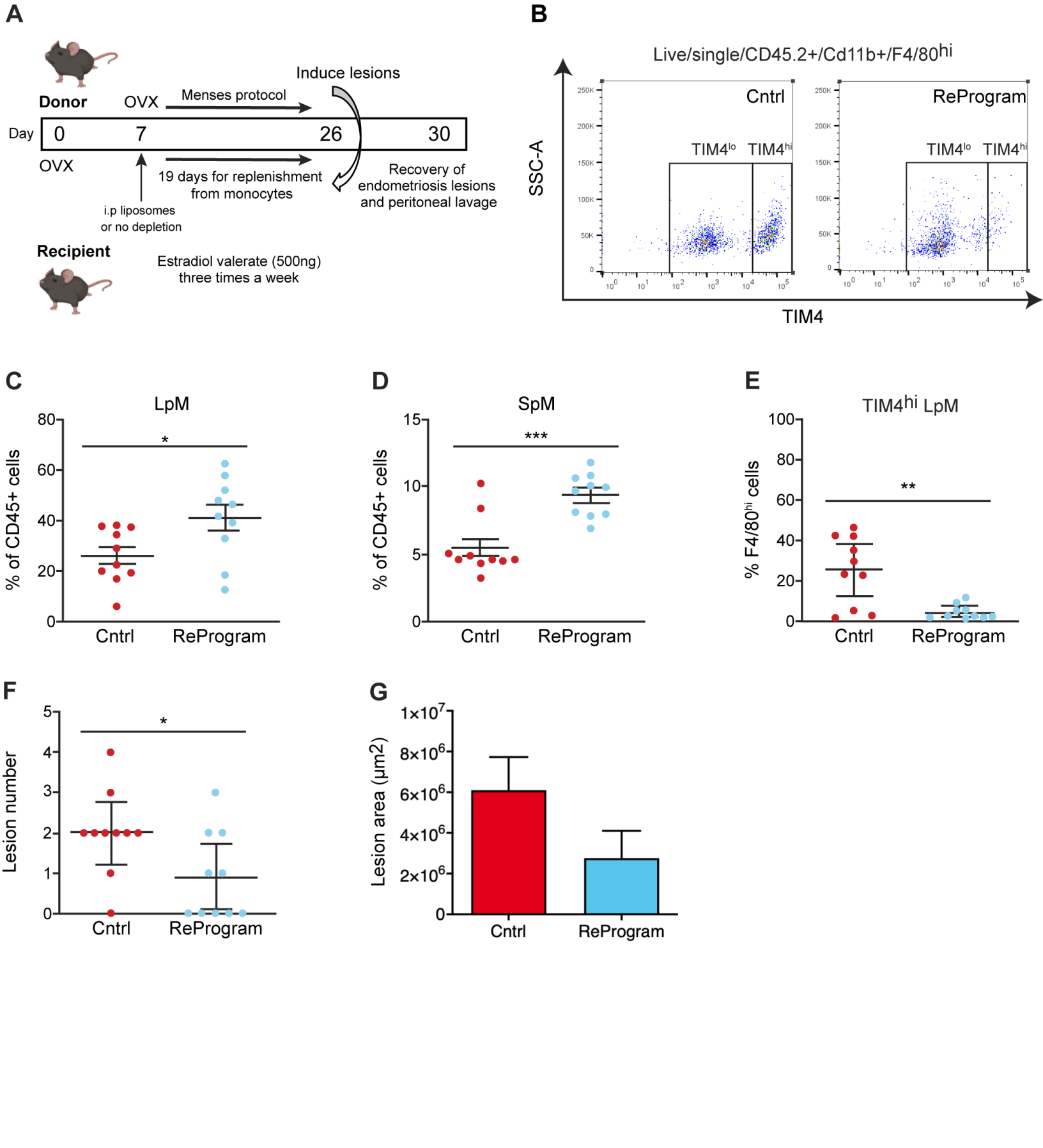

A
Live/single/CD45.2+/Ly6G-/Lin-
LpM
SpM
F4/80
MHC II
B
1wk 2wk 3wk
LpM
SpM
6x10^4
4x10^4
2x10^4
0
**
*
F4/80+ cells/µl (LpM)
MHCII+ cells/µl (SpM)
Naïve
Sham
Endometriosis
C
Live/single/CD45.2+/Ly6G-/Lin-
F4/80
LY6C
D
1wk 2wk 3wk
Monocytes
1000
800
600
400
200
0
**
LY6C+ cells/µl
Naïve
Sham
Endometriosis
E
Sham
Endometriosis
Live/single/CD45.2+/Ly6G-/Lin-/ Cd11b+/F4/80hi
SSC-A
CCR2
F
800
600
400
200
0
**
**
CCR2+ F4/80hi cells/µl
Naïve
Sham
Endometriosis

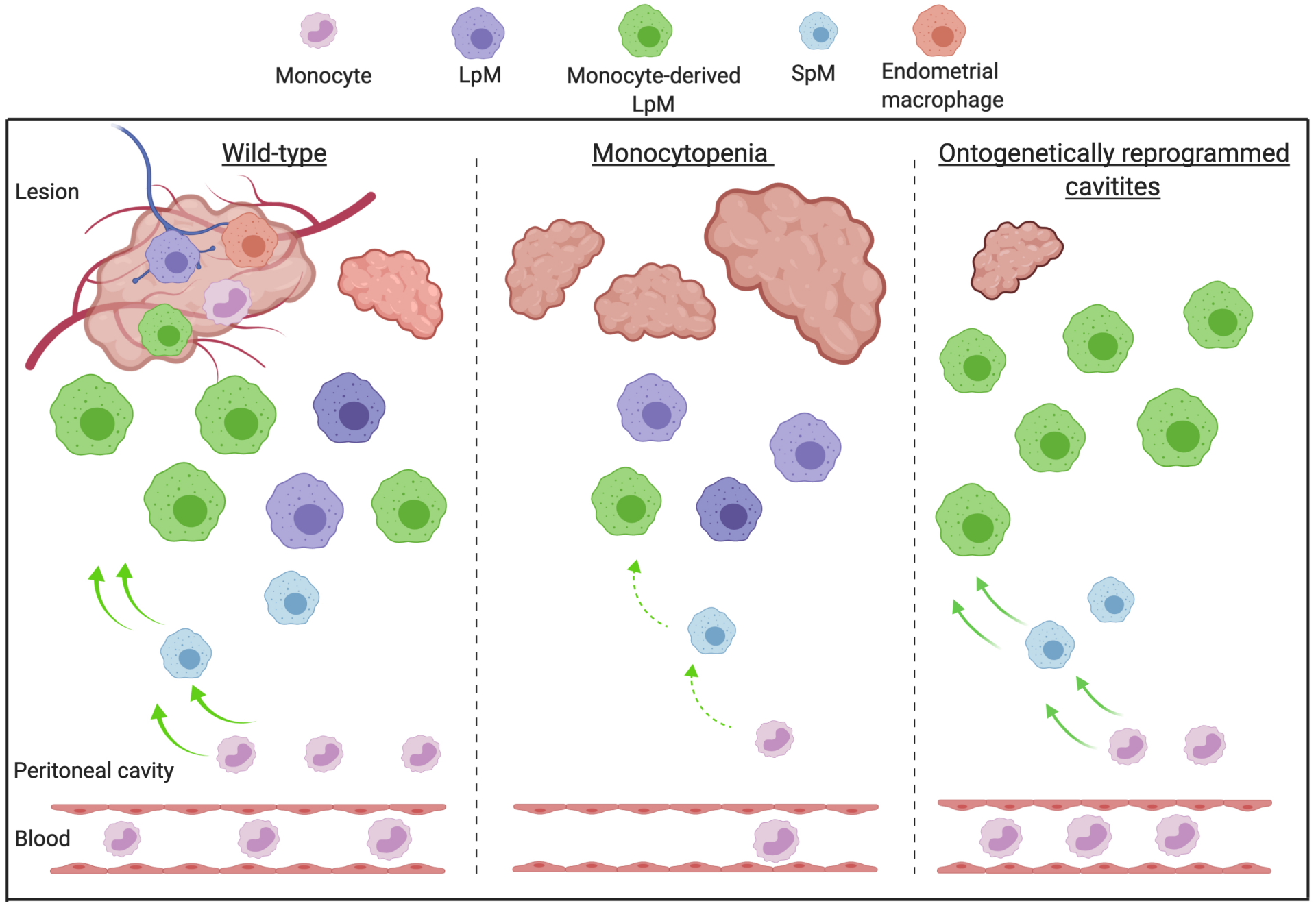

A
iCsf1r-KO
Donor
OVX
E2
(100ng) E2 E2
P4 implant+
E2
(5ng) E2 E2
2μg/ml doxycycline
Decidualization
stimulus
P4 implant
removed
Day
0 7 8 9 13 14 15 19
OVX
Recipient
Estradiol valerate
(500ng) 3 times a
week
+4 hours i.p injection of
donor tissue
+2 weeks recovery of
endometriosis lesions
Wild-type
B
800
600
400
200
0
Cells/μl
*
Wild-type
iCsf1r-KO
F4/80hi, Ly6Clo
F4/80lo, Ly6Chi
C
2.5
2.0
1.5
1.0
0.5
0.0
Lesion number
WT
iCsf1r-KO
D
1.5x10^6
1.0x10^6
5.0x10^5
0.0
Lesion size (μm2)
*
WT
iCsf1r-KO


A
Wild-type
Donor
Day 19 33
Estradiol valerate (500ng) three times a week
Recovery of endometriosis lesions peritoneal lavage
Recipient
Ccr2-/-
B
Live/single/CD45.2+/Ly6G-/Lin-
WT
Ccr2-/-
LpM
SpM
F4/80
MHCII
C
LpM
**
% of CD45+ cells
Wild-type
Ccr2-/-
D
SpM
***
% of CD45+ cells
Wild-type
Ccr2-/-
E
Monocytes
*
% of CD45+ cells
Wild-type
Ccr2-/-
F
**
Lesion number
Wild-type
Ccr2-/-
G
Lesion area (µm2)
Wild-type
Ccr2-/-
H
Ly6C
Ly6C+ F4/80+
F4/80
Wild-type
Ccl2-/-
80µm
80µm
I
Ly6C+
Ly6C+, F4/80+
% of total DAPI+ cells
Wild-type
Ccr2-/-


A
Live/single/CD45.2+/Ly6G-/Lin-
F4/80
MHCII
WT
Ccl2-/-
LpM
SpM
B
LpM
% of CD45+ cells
Wild-type
Ccl2-/-
C
SpM
% of CD45+ cells
Wild-type
Ccl2-/-
D
Monocytes
% of CD45+ cells
Wild-type
Ccl2-/-
E
Lesion number
Wild-type
Ccl2-/-
F
Lesion area (µm2)
Wild-type
Ccl2-/-
G
Ly6C+
Ly6C+ F4/80+
F4/80
Wild-type
Ccl2-/-
80µm
H
Ly6C+
Ly6C+, F4/80+
% of total DAPI+ cells
Wild-type
Ccl2-/-


A
Wild-type donor 'menses' endometrium
Daily i.p MC21 or MC67
Day 19 20 21 22 23
Estradiol valerate (500ng)
Recovery of endometriosis lesions peritoneal lavage
Wild-type recipient
B
Live/single/CD45.2+/Ly6G-/Lin-
MC67
MC21
Monocytes
F4/80
Ly6C
C
LpM
% of CD45+ cells
MC67
MC21
D
SpM
% of CD45+ cells
MC67
MC21
E
Monocytes
*
% of CD45+ cells
MC67
MC21
F
Lesion number
MC67
MC21
G
Lesion area (µm2)
2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0.0
MC67
MC21


A
Donor
OVX
Menses protocol
Induce lesions
Day
0
7
26
30
OVX
19 days for replenishment from monocytes
Recovery of endometriosis lesions and peritoneal lavage
i.p liposomes or no depletion
Recipient
Estradiol valerate (500ng) three times a week
B
Live/single/CD45.2+/Cd11b+/F4/80hi
Cntrl
ReProgram
TIM4lo
TIM4hi
SSC-A
TIM4
C
LpM
% of CD45+ cells
Cntrl
ReProgram
D
SpM
% of CD45+ cells
Cntrl
ReProgram
E
TIM4hi LpM
% F4/80hi cells
Cntrl
ReProgram
F
Lesion number
Cntrl
ReProgram
G
Lesion area (µm2)
Cntrl
ReProgram


Monocyte
LpM
Monocyte-derived LpM
SpM
Endometrial macrophage
Wild-type
Monocytopenia
Ontogenetically reprogrammed cavitites
Lesion
Peritoneal cavity
Blood

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

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 and monocyte-derived macrophages	Rat	1:100	ImmPRESS® HRP-conjugated anti-rat antibody

Macrophages inhibit and enhance endometriosis depending on their origin
MS# 2020-13776RR

Response to Editor and Reviewers

We thank the editor, editorial board members and reviewers for their consideration of the manuscript. Specifically, we thank the editor and the reviewers for their gracious comments about the study and reviewer #1 for further recommendations to improve the manuscript. We have now amended the manuscript according to these additional suggestions (highlighted in green).

Reviewer #1

Significance statement: The second sentence "macrophages promote endometriosis" is very firm and given it is based on data from animal models, I think might be better written as "Macrophages are implicated in promoting endometriosis". The 'slash' on the last line would be better as 'and/or'

We have now amended this as the reviewer suggests at line 23 and 32, respectively.

Line 61: missing word 'that'

Now corrected.

Line 64: lower case 'm' on 'Major'

Corrected.

Line 107: should be 'menses-like' not 'menses'-like

Corrected.

Line 113: lower case 'c' on 'Cluster'

Corrected.

Line 119: 'host derived' should have a hyphen here and throughout, for consistency

Corrected and checked throughout.

Line 124: delete 'appeared to be' and replace with 'were'

Corrected.

Line 145: statement of data is not clear - would be better to write 'mean + SEM = xxxx + xxx in control and xxx + xxx in estradiol valerate-treated females respectively' or similar

We thank the reviewer for pointing this out. We have now corrected this.

Line 215: all percentage values here and elsewhere should have the same number of decimal places

Corrected here and throughout.

Line 230: remove 'some'

Deleted.

Line 262: 'act as guardians' is unscientific - better as 'act to guard against' or 'act to protect against'

Amended at line 258.

Line 311: remove hyphen in 'endometrium-depleted'

Corrected at line 305.

Discussion: It would be helpful to add a sentence to acknowledge the high degree of between experiment variation as limitation of the model, and to suggest potential reasons for the high variance between experimental runs.

We have added an additional paragraph to the discussion at line 375 that highlights the limitation and discusses a possible reason for this and future directions.

Discussion lines 295-305: Reviewer #2 raises the question of possible trophic roles of macrophages in eutopic endometrium. This possibility has been examined by Care et al., "Ovarian Steroid Hormone-Regulated Uterine Remodeling Occurs Independently of Macrophages in Mice" Biology of Reproduction, Volume 91, Issue 3, 1 September 2014, 60, 1-12, https://doi.org/10.1095/biolreprod.113.116509 and other publications from the same authors. Using a CD11b-dtr macrophage depletion model, the authors found macrophages do not regulate survival or proliferation in uterine epithelial, stromal or vascular endothelial cells in the estrous cycle or after decidualisation. This might be cited as additional evidence to support the inference in lines 295-305 that macrophages are implicated in tissue recovery and remodelling after hormone withdrawal or injury, as opposed to tissue generation and homeostasis.

We thank the reviewer for this suggestion which links nicely in the discussion. We have added details and citation to line 307.