Journal of Reproductive Immunology 132 (2019) 1–8

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Macrophages infiltrating endometriosis-like lesions exhibit progressive phenotype changes in a heterologous mouse model

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ARTICLE INFO

Keywords:
Endometriosis
Macrophage polarisation
Macrophage
Tissue remodelling
MacGreen mouse

ABSTRACT

Endometriotic lesion development involves complex interactions between endometrial tissue, the peritoneum and immune cells. Macrophages are essential in this process; however their precise roles are not defined. To investigate whether infiltrating macrophages acquire functionally different phenotypes during lesion development, human endometrial tissues were grafted into immunodeficient mice expressing macrophage-specific green fluorescent protein (GFP). Although the numbers of GFP-positive macrophages were similar in lesions 4, 7, 10 and 14 days after grafting, their surface markers changed over time. Inflammatory markers MHC class II (MHC II) and iNOS were present on 36% and 41% of macrophages respectively early in lesion development at day 4, whereas abundance of tissue remodelling markers peaked later, with arginase 1 most highly expressed on 57% of macrophages at day 7 and scavenger receptor A (CD204) on 66% of macrophages at day 14. This is consistent with a transition from classical M1 macrophage activity to an alternate M2 profile, which correlates to histological hallmarks of initially acute inflammation followed by tissue remodelling during lesion development. This progressive shift in phenotype is likely to be relevant to the mechanisms by which macrophages are central players in endometriosis-like lesion development.

1. Introduction

Endometriosis is a debilitating disease causing pelvic pain and infertility in 10–15% of women of reproductive age (Vigano et al., 2004). Endometriosis is believed to arise from endometrial fragments that pass retrogradely through the fallopian tubes into the pelvic cavity, during menstruation (Sampson, 1927). Studies in rat and mouse models of disease indicate that development of endometriotic lesions involves a scripted series of cellular events that include attachment, tissue breakdown, inflammation, tissue remodelling, neovascularisation and neural regeneration (Flores et al., 2007; Hull et al., 2008). Histological comparisons (Bruner-Tran et al., 2002; Grummer et al., 2001) and functional global analyses of transcriptomes in human lesions indicate these are authentic models reflecting similar changes in human endometriotic lesions (Eyster et al., 2007; Flores et al., 2007; Hull et al., 2008; Wu et al., 2006). However, little is understood of the critical interactions between endometrial tissue, the peritoneum and immune cells, which are necessary for disease initiation. This limits the development of new treatment options and preventative interventions for endometriosis.

Macrophages play a diverse number of roles in immune system responses, tissue remodeling and the maintenance of tissue homeostasis. Macrophage infiltration is critical for endometriotic lesion development and macrophages are found in high numbers in endometriotic tissues and the peritoneal fluid from women with endometriosis (Bacci et al., 2009; Capobianco et al., 2011). Different macrophage functions are reflected in the expression of different surface antigens. Inflammatory macrophages display classical activation (M1) markers such as MHC class II (MHC II), while alternatively activated (M2) macrophages display alternate immune markers such as scavenger receptor (CD204) (Capobianco and Rovere-Querini, 2013). Although it is over-simplistic to describe complex phenotypic variance in a binomial way, M1 and M2

Abbreviations: AFS, American Fertility Society; *Cfms*, macrophage colony-stimulating factor-1 (CSF1) gene; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; H&E, Haematoxylin and Eosin; iNOS, inducible nitric oxide synthase; MHC II, class II major histocompatibility complex (MHC); PFA, paraformaldehyde; *Prkdc*, protein kinase, DNA activated, catalytic polypeptide; rASRM, Revised American Society for Reproductive Medicine; *Scid*, severe combined immunodeficiency; TGFB1, transforming growth factor beta 1; αSMA, alpha smooth muscle actin

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https://doi.org/10.1016/j.jri.2019.01.002
Received 22 May 2018; Received in revised form 18 December 2018; Accepted 21 January 2019

activation status markers give insight into the functionality of macrophages located in tissues at a particular point in time.

Most macrophages located within endometriotic lesions are systemically-derived and infiltrate the lesion from the peripheral blood or peritoneal cavity during lesion development, but a small proportion may originate from the ectopic endometrial fragment (Greaves et al., 2014). Both endogenous and recruited macrophages are likely to exert different functions at progressive stages of lesion development. Their phenotypes once recruited into lesions would be determined by cytokines and other regulatory factors produced within the lesion. One such factor is transforming growth factor beta 1 (TGFB1), which is secreted by peritoneal mesothelial and immune cells including macrophages (Young et al., 2014) and upregulated in peritoneal fluid and serum from women with endometriosis. We have reported that TGFB1 deficiency is associated with reduced ectopic endometrial lesion weight and significantly lower numbers of macrophages, raising the possibility that TGFB1 regulates lesion development via effects on macrophage phenotype and function (Hull et al., 2012).

Little is known about the phenotype of macrophages in endometriosis and how phenotype may change during lesion development. In a tumour microenvironment, distinct subgroups of macrophages with different activation markers have been identified that play opposing roles that ultimately facilitate tumour development (Movahedi et al., 2010; Wang et al., 2011). We hypothesised that macrophages in endometriotic tissues also undergo dynamic changes in phenotype and function over the course of lesion development. To address this hypothesis, we utilised a transgenic mouse model wherein the macrophage specific *Cfms* promoter drives expression of green fluorescent protein (GFP), crossed onto a background of severe combined immunodeficiency (*Cfms-Gfp/Prkdc*^{scid} mice), in order to identify and characterise host-derived macrophages infiltrating ectopic human xenografts over the course of lesion development.

2. Materials and methods

The use of human endometrial tissues for this study was approved by the University of Adelaide Human Research Ethics Committee (HREC) and the Children, Youth & Women's Health Service (CYWHS) HREC. All animal procedures were approved by the University of Adelaide Animal Ethics Committee (AEC), and the CYWHS-AEC. The GMO Dealing Authorisation was obtained from the Institutional Biosafety Committee at the University of Adelaide. All animal experiments were conducted in accordance with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes (7th edition, 2004).

2.1. Collection of endometrial tissue

Endometrial biopsies were collected using Pipelle suction curettes (Pipelle de Cornier, Laboratoire C.C.D, France) either during laparoscopic surgery (n = 4) or in an outpatient setting (n = 1). All 5 women were between 32 and 39 years of age (median age, 35 years), had regular menstrual cycles (28–30 days) and used no medication in the 3 months prior to tissue collection. One patient had rASRM Stage III endometriosis, three were surgically defined as being endometriosis-free whereas one patient did not have her peritoneal cavity visualised (Table 1). The eutopic endometrium was taken at the proliferative (n = 2), secretory (n = 2) and early menstrual (n = 1) phases of the cycle as confirmed by Noyes criteria (Noyes et al., 1975).

Biopsies were immediately placed in pre-warmed phenol-red free DMEM/F12 culture media (Sigma-Aldrich Co. Ltd, Australia) with 1% Insulin-Transferrin-Selenium-X Supplement (ITS) (GIBCO™, Invitrogen Corporation), 1% penicillin/streptomycin, 0.1% EX-CYTE® Growth Enhancement Supplement (Celliance, Kankakee, IL) and 10 nM of 17β-estradiol (SigmaAldrich). The tissues were aseptically sectioned into 1 mm^3 fragments and stored in groups of 15-18 in tissue culture inserts in an incubator at 37 °C before being aspirated into a syringe for injection into experimental mice.

Table 1
Clinical information of patients.

Patient	Age	Stage of cycle	Revised AFS score
1	32	Proliferative	No endometriosis
2	35	Proliferative	Asymptomatic (Pelvis was not visualised)
3	32	Late Secretory	No endometriosis
4	35	Early Menstrual	Stage III
5	39	Late Secretory	No endometriosis

2.2. Cfms-Gfp mouse model of endometriosis

Mouse colonies were maintained under controlled light (12L: 12D photoperiod) and temperature, in pathogen-free conditions in individually ventilated cages and fed *ad libitum* with a standard diet and sterilised water. All experimental procedures were performed in a laminar flow hood using sterile instruments.

A colony of *Cfms-Gfp* mice was established from breeding pairs expressing enhanced green fluorescent protein (GFP) under the macrophage-specific promoter cfms (Sasmono et al., 2003) kindly provided by David Hume (Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia). Male *Cfms-Gfp* mice were mated with female mice homozygous for the *Prkdc*^{scid} (SCID) mutation (Laboratory Animal Services, Adelaide, Australia). The presence of the *Cfms-Gfp* transgene was detected by PCR using the forward and reverse primers 5′GAC GTA AAC GGC CAC AAG TT and 5′GAA CTT CAG GGT CAG CTT GC (Geneworks Pty Ltd, Thebarton, Australia), respectively. FACS analysis was used to confirm the phenotype of the resultant *Cfms-Gfp/Prkdc*^{scid} mice by detecting green fluorescent light emission in macrophages and the absence of lymphocytes in the scatter plot (Supplemental Figure S1).

2.3. Induction of endometriosis in mice

Mice were anaesthetised using isoflurane (Veterinary Companies of Australia Pty Ltd, Australia) before all invasive procedures. Six-week old mice were bilaterally ovariectomised and a slow-release estradiol pellet (1.5 mg, 60-day; Innovative Research, Sarasota, FL) was inserted subcutaneously in the lower left flank. Six days after ovariectomy, 15–18 of the 1 mm^3 fragments of human endometrial tissue were randomly allocated for subcutaneous injection at a ventral midline site as previously described (Hull et al., 2008). Following surgery, 0.02% Mepivacaine (Nature Vet Pty Ltd, Australia) local anaesthetic was applied to the incision site.

Cfms-Gfp mice receiving tissues from the same patient were divided into four groups destined for euthanasia at four different time points, days 4, 7, 10 and 14. Five experimental replicates were performed with 11 mice in the day 4, 14 mice in the day 7, 13 mice in the day 10 and 13 mice in the day 14 time point groups. From these mice a total of 11 lesions were collected at each of days 4 and 7, 9 lesions at day 10 and 12 at day 14.

Xenografted lesions from *Cfms-Gfp* mice were excised after euthanasia and fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, followed by an overnight incubation in 18% sucrose at 4 °C before were embedded in Tissue-Tek® OCT compound (ProSciTech, Thuringowa, Australia).

2.4. Morphometric analysis

Every 10th microscope section of the xenografts was stained using Haematoxylin and Eosin (H&E). Only lesions with endometrial glands were used for subsequent immunofluorescent staining. For each lesion, 5 H&E stained slides were scanned using the Nanozoomer 2.0

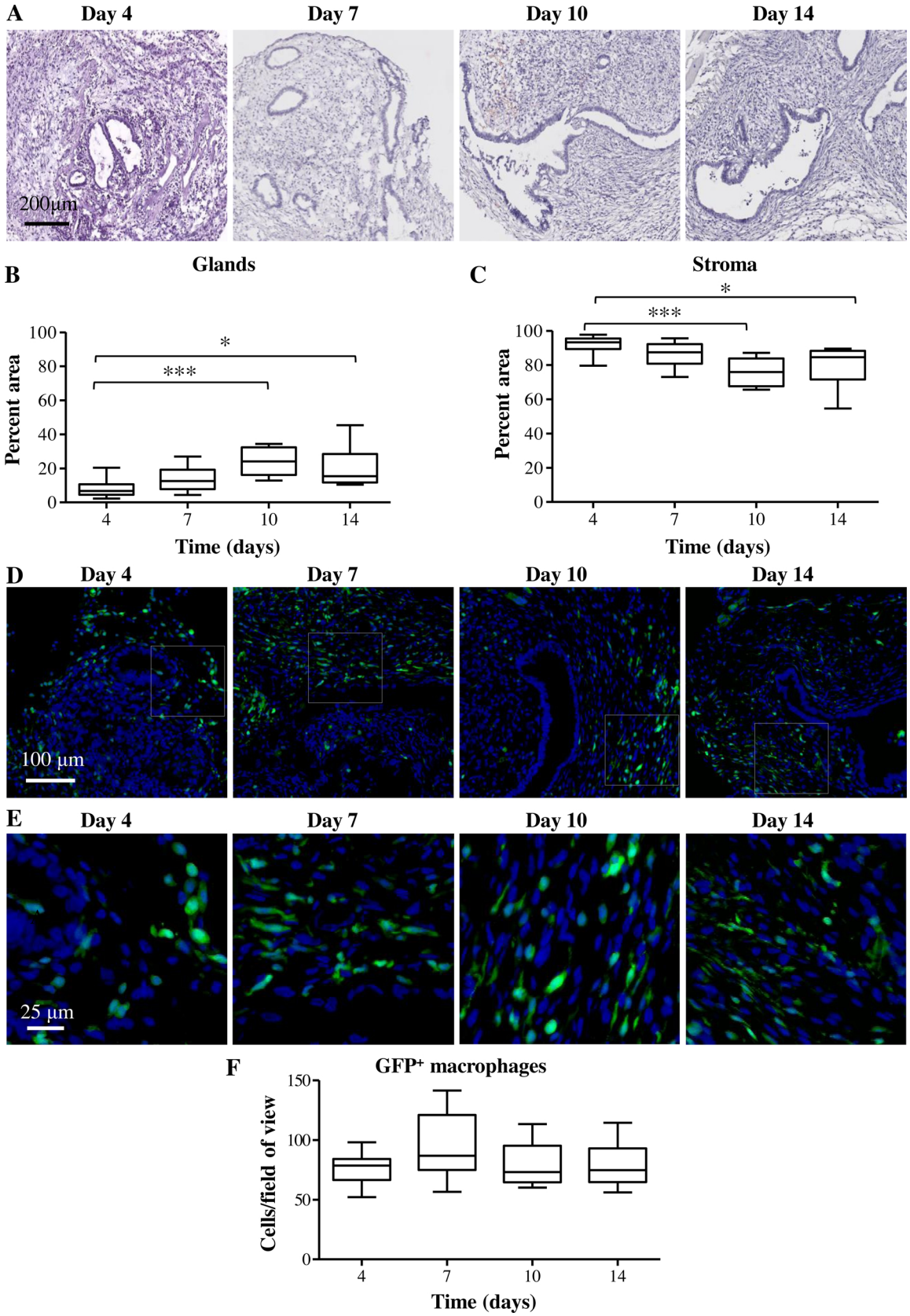

Fig. 1. Development of endometriosis-like lesions in *Cfms-Gfp/Prkdc*^{scid} mice. (A) Gross morphology of the lesions stained using Haematoxylin and Eosin (top panel). (B and C) The changes in lesion morphology were reflected on the percentage of glandular and stromal areas. (D–E) Macrophage recruitment into the lesion was visualised using GFP marker. (F) The recruitment of these cells was determined by the quantification of cells/field for each lesion. The median for each group was compared using Kruskal Wallis test, followed by multiple comparison tests and significance inferred at $p < 0.05$ (* $p < 0.05$ and *** $p < 0.001$, versus Day 4). Boxes represent the 25th and 75th percentiles, with median line; error bars represent minimum and maximum values.

(Hamamatsu, Japan) and analysed with NDP software (Hamamatsu). The tracing tool outlined and measured the percentage of the lesions occupied by the endometrial glands (glandular area fraction). The stromal area fraction was determined as the percentage of the lesion which was outside the glandular area fraction.

2.5. Macrophage marker analysis in endometriosis-like lesions

Air-dried PFA-fixed frozen sections were rinsed in phosphate-buffered saline (PBS) before blocking in 10% host secondary antibody serum (Sigma-Aldrich) for 30 min. After blocking, the following primary antibodies were then incubated at 4 °C overnight: rat anti-mouse class II major histocompatibility complex (MHC II) (Ia/Ie) (TIB120, undiluted hybridoma supernatant; ATCC, Rockville, MD), polyclonal rabbit anti-mouse iNOS (NOS2) (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal goat anti-arginase 1 (10 μg/ml; Abcam, Cambridge, UK) and rat anti-mouse scavenger receptor type I/II (CD204) (clone 2F8, 10 μg/ml; AbD Serotec, Oxford, UK) to identify macrophage markers. Rabbit anti-mouse collagen 1 (AB765 P, 12.5 μg/ml; Chemicon, EMD Millipore Corporation, Temecula, CA) antibody was used to detect type 1 collagen.

Sections were incubated with secondary antibodies and fluorescent conjugates at room temperature for one hour. Visualisation of anti-iNOS and anti-collagen 1 antibodies was achieved using Alexa Fluor 594-conjugated goat anti-rabbit IgG (at 1:250 and 1:200, respectively; Invitrogen). Anti- MHC II and anti-CD204 antibody binding was detected using Alex Fluor 594 -conjugated rabbit anti-rat IgG at 1:500 (F4/80 and MHC II) and 1:1000 dilutions (CD204). Meanwhile, anti-arginase 1 antibodies lesions were localised using biotinylated rabbit anti-goat IgG (1:500; Chemicon) and detected using a Streptavidin/Alexa Fluor 594 conjugate (1:200; Invitrogen). All sections were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D9542; Sigma-Aldrich) and mounted in fluorescence mounting medium (Dako Australia, North Sydney, Australia).

Control human endometrial and mouse uterine histology sections were included in each staining run to test the species specificity of primary antibody. Isotype-matched immunoglobulins (IgGs) were used to delineate the background signal for each antibody staining condition (Supplemental Figure S2).

Three random fields from both peripheral and central aspects of each lesion were assessed for macrophage markers and collagen staining. Image analysis was performed using a Nikon TE inverted microscope, using three colour settings for DAPI, GFP and Alexa-Fluor 594, at 20x magnification. The fluorescent images were merged using ImageJ software (Wayne Rasband, National Institute of Health) and analysed using cell counter (Plugins > Analyse > Cell Counter).

2.6. Statistical analysis

Data from histologic and immunohistologic analyses were averaged to provide a single result for each endometrial tissue injection in a mouse (i.e. when 2 lesions incidentally developed from one injection the data were averaged) and presented as medians and ranges for each dataset. For each marker, the results are given as the percentage of positive cells. The non-parametric Kruskal Wallis and Mann Whitney U tests (PRISM 5; GraphPad Software, Inc., San Diego, CA) were used to evaluate differences attributable to treatments in both experiments, with post-hoc Dunn's multiple comparison test and significance inferred at $p < 0.05$.

3. Results

3.1. Longitudinal changes in lesion structure

To evaluate the phenotype of infiltrating macrophages over the course of endometriosis-like lesion development, human endometrial tissue fragments were instilled subcutaneously to the *Cfms-Gfp* recipient mice, and recipients were then killed 4, 7, 10 or 14 days later. The majority of *Cfms-Gfp* recipient mice exhibited lesion development (82% in day 4, 71% in day 7, 77% in day 10 and 92% in day 14 groups). The median weight and size of xenografts harvested from host *Cfms-Gfp* mice at day 4, 7, 10 and 14 post-implantation did not change substantially over time.

Histochemical analysis showed that endometrial tissue fragments grafted into *Cfms-Gfp* mice developed into endometriosis-like lesions with distinct glandular structures and stromal compartments (Fig. 1A). The proportion of lesions comprising glands increased progressively from day 4 until day 10 and then stabilised, differing significantly across the four time-points ($p = 0.001$; Fig. 1B). This reflected an expanding epithelial surface, with a 3.6-fold increase in the glandular epithelium fraction between day 4 and day 10 ($p < 0.001$) and a further 2.3-fold increase ($p < 0.05$) by day 14. The proportion of stromal tissue varied in a reciprocal pattern to glands ($p = 0.01$), declining from day 4 to day 10 and then remaining constant at day 14 (Fig. 1C).

3.2. Longitudinal changes in macrophage phenotype

Recipient-derived GFP^+ macrophages were present in the outermost (peripheral) stroma region of endometriosis-like lesions from day 4 (Fig. 1D and E). Macrophages predominated in the centrally-located stroma from day 7 to day 14. There was no difference in the number of GFP^+ macrophages per field across the four time points (Fig. 1F; $p > 0.05$).

Macrophages expressing MHC II (GFP^+ MHC II^+) were present in the peripheral stroma of endometriosis-like lesions across the four time points (Fig. 2A). There was variation across the four time points ($p = 0.03$) with median values declining from 36% of macrophages at day 4 to 23% at day 7 ($p < 0.005$), then stabilising at 36% and 34% on days 10 and 14, respectively (Fig. 3A).

Macrophages expressing iNOS (GFP^+ $iNOS^+$) demonstrated variation across the course of lesion development ($p < 0.0001$) (Figs. 2B and 3 B). Median values of GFP^+ $iNOS^+$ macrophages declined from 41% and 48% of macrophages at day 4 and day 7, respectively to 24% at day 10 and 20% at day 14 ($p < 0.05$ and $p < 0.001$, respectively).

The percentage of macrophages expressing arginase 1 (GFP^+ $Arg1^+$) also varied across the time course ($p = 0.006$) (Fig. 2C). The population of GFP^+Arg1^+ increased by 1.3-fold from 43% at day 4 to 57% of macrophages at day 7 ($p < 0.05$), remained steady at day 10, before reducing to 38% at day 14 ($p < 0.05$ vs day 7) (Fig. 3C).

Scavenger receptor A, CD204-positive macrophages (GFP^+ $CD204^+$) were present in all lesions and the median for percentage of GFP^+ $CD204^+$ cells differed across the four time points ($p = 0.02$; Fig. 2D). The proportion of $CD204^+$ macrophages was steady from 53% at day 4 to 51% at day 7 before increasing to 66% at day 14 ($p < 0.05$) (Fig. 3D).

3.3. Longitudinal changes in collagen type 1

Collagen type 1 (collagen 1) infiltration of endometriosis-like lesions was noted at day 4 and throughout lesion development to day 14 (Fig. 2E). Stromal areas positive for collagen 1 were mostly in the periphery and in some lesions, collagen was also present in the areas proximate to the epithelium. At day 4, macrophages (GFP^+) were restricted to the periphery of lesions and collagen 1 staining was minimal. At day 7 to day 14, collagen 1 became progressively more abundant, and was localised in the vicinity of macrophages as they advanced into the centre of lesions within the collagen-positive stroma. Differences in collagen 1 staining were evident between the four time points ($p = 0.02$) (Fig. 3E). The percent positive area of collagen 1 in lesions progressively increased over the time course and significantly increased by 1.7-fold from 22% at day 4 to 37% at day 14.

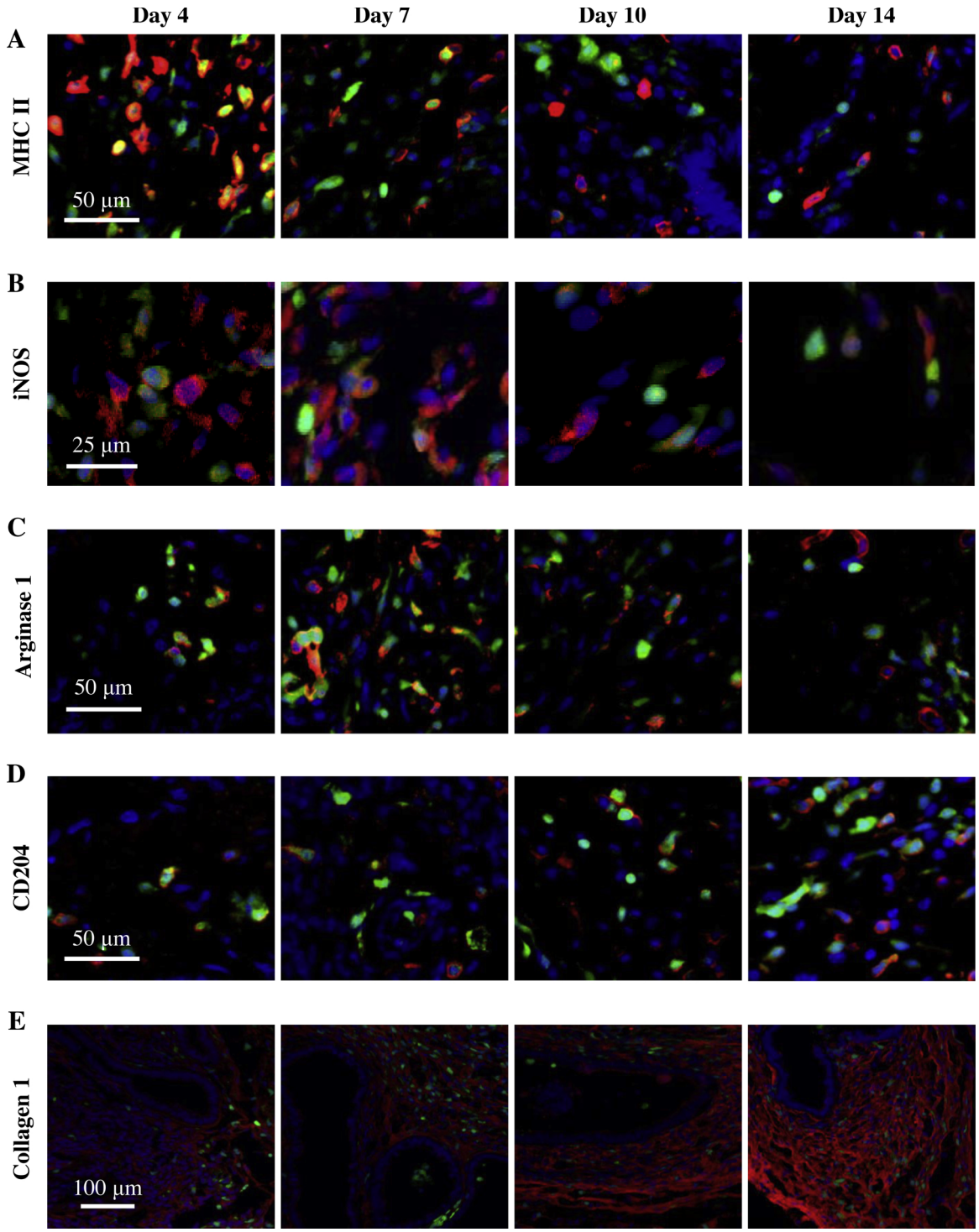

Fig. 2. Expression of different macrophage phenotype markers at four different time points in endometriosis-like lesions from *Cfms-Gfp/Prkdc*^{scid} mice. GFP-expressing macrophages (green) were labelled with antibodies to phenotype markers, MHC II (A), iNOS (B), arginase 1 (C), CD204 (D), and collagen 1 (E) (red). Changes in macrophage phenotype are demonstrated by the density of expression of the co-localised marker at each time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

To explore changes in macrophage activation status in ectopic human endometrial derived lesions, we developed an immunodeficient mouse model of endometriosis whereby recipient mice expressed GFP specifically in macrophages. Using confocal fluorescent microscopy we were able to accurately establish the co-localisation of surface markers associated with classical or alternative phenotype to GFP-labelled macrophages during lesion development. Our findings indicate a progressive change in the proportions of macrophages expressing a range of markers during endometriosis-like lesion development, transitioning from patterns associated with an inflammatory to a reparative phenotype. This raises the possibility that alterations in macrophage activity could exert effects on endometriosis disease progression. For example a weak macrophage-driven inflammatory response may fail to degrade and clear ectopic tissues leading to the establishment of disease.

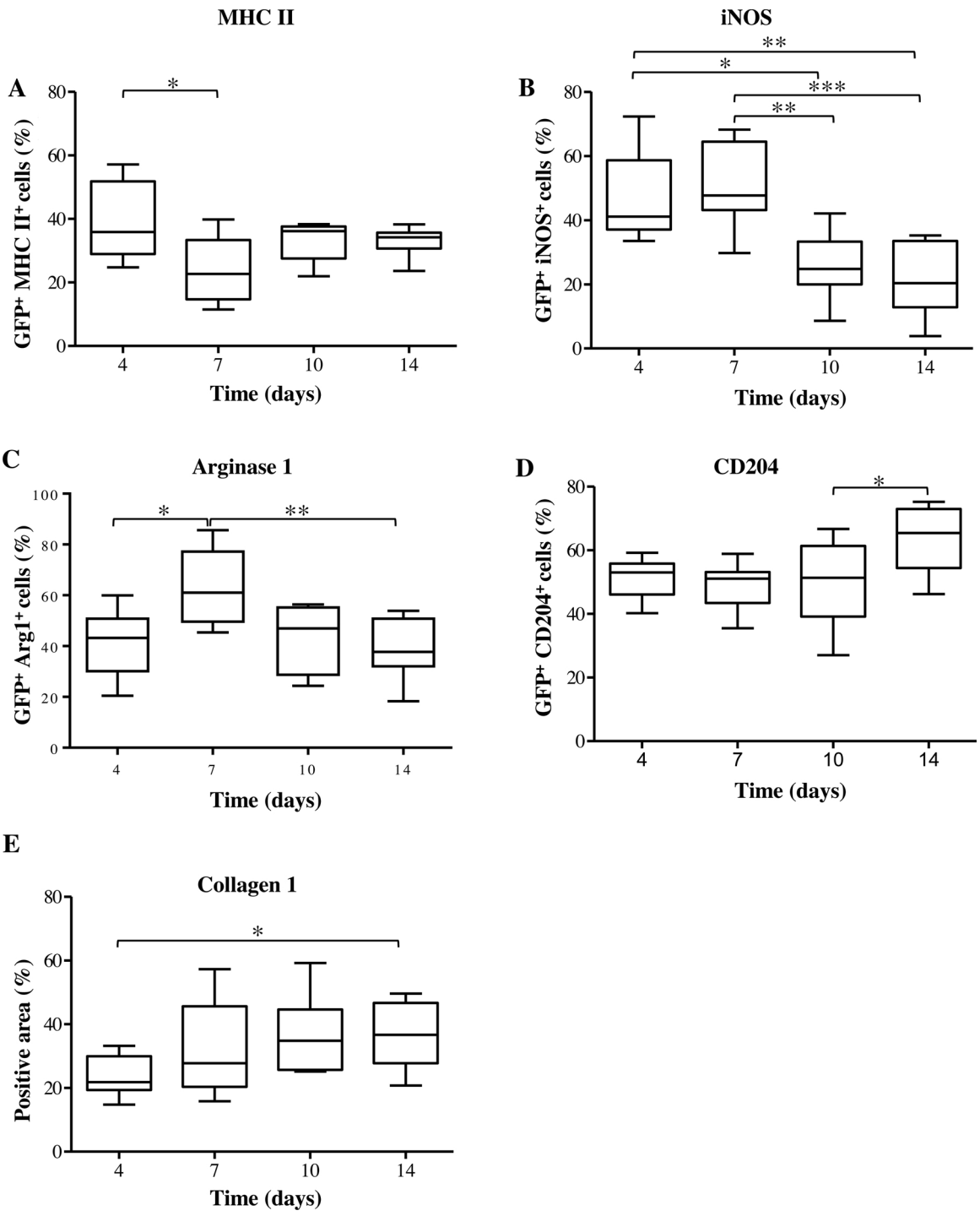

Fig. 3. Quantification of macrophage activation marker co-localisation and collagen 1 in endometriosis-like lesions in *Cfms-Gfp/Prkdc*^{scid} mice. Percent of GFP-positive cells expressing MHC II (A), iNOS (B), arginase 1 (C) and CD204 (D) were quantified from six different fields representing form periphery and centre of the tissues. Collagen 1-positive area (E) was determined using Image J. Median for the percentage of GFP-expressing activated macrophage markers and collagen-positive area for each group was compared using Kruskal Wallis test, followed by multiple comparison tests and significance inferred at $p < 0.05$ (* $p < 0.05$ and 472 **$p < 0.01$ and *** $p < 0.001$). Boxes represent the 25th and 75 percentiles, with median line; error 473 bars represent minimum and maximum values.

Alternatively macrophages with exaggerated reparative properties may enhance the growth and development of endometriosis.

In women, sampling endometriotic tissues at one time point during surgery, obscures dynamic changes in lesion development. Furthermore, high ethical and cost barriers preclude the use of non-human primates as the only natural model of endometriosis (D'Hooghe et al., 2009). Given the complexity of the cellular interactions within endometriosis lesions, murine models are the only way of exploring the first few weeks of endometriosis-lesion development but there are caveats regarding their ability to represent human disease. Murine endometrium differs from human tissue in that it remodels without shedding in an oestrous cycle, and requires the presence of a blastocyst for decidualisation (Cousins et al., 2014).

These distinct properties explain poor results from direct engraftment of murine endometrium. In heterologous mouse models of endometriosis, to avoid interspecies graft vs host disease, human

endometrial tissue is engrafted into recipient mice rendered immunosuppressed by genetic manipulation to induce T and B cell deficiency (Hull et al., 2012). As the adaptive immune compartment is absent in SCID mice, the interaction between the murine immune response and human endometrial tissue only approximates the human endometriosis condition. However the endometrial component of the resultant xenograft is very representative of human disease and macrophage responses resemble those seen in human endometriosis as the innate immune system is intact. Use of this heterologous, immunocompromised mouse model of endometriosis allowed us to specifically explore the innate host macrophage response to human ectopic tissues. GFP fluorescent labelling readily identified host-derived macrophages infiltrating ectopic human xenografts over the course of lesion development. This ensured that positive staining for macrophage activation markers was attributed to host-derived, infiltrating GFP positive macrophage populations, as opposed to resident macrophages within ectopic endometrial tissues.

In *Cfms-Gfp* mice, we found that macrophages recruited into the donor human endometrial tissue fragments transitioned from a predominantly M1 phenotype characterised by the expression of MHC II and iNOS to a predominantly M2 phenotype with the expression of arginase 1 and then CD204 over the first 14 days of lesion development. This shift is likely to represent a functional change from pro-inflammatory, immunogenic (M1) activity to tissue remodelling, reparative (M2) activity.

The ectopic endometrial lesions were examined longitudinally to define the macrophage contribution to distinct morphological stages described previously (Hull et al., 2008). The chosen time points represent an early inflammatory stage (day 4), the transition from an inflammatory to a remodelling structure (day 7), the peak time for tissue remodelling (day 10) and finally, the stable established lesion (day 14) (Hull et al., 2008; Lin et al., 2006). This pattern of morphological progression is believed to reflect changes that cyclically recur in women in response to the reproductive hormone fluctuations of the menstrual cycle (Deligdisch, 2000).

The variation observed in lesion size is typical of the heterogeneity inherent to immunocompromised, xenograft models of endometriosis. Transplanted endometrial fragments were prepared after collection from different women at various stages of the cycle, with varying disease status, which might have differentially affected tissue capacity to recruit macrophages, resistance to apoptosis, induction of a reparative response and establishment of blood supply after attachment. However there was little evidence of effects of donor cycle phase, since engraftment of ectopic endometrium into the mice occurred 80% of the time, regardless of the cycle phase in which the human endometrium was obtained, consistent with previous studies (Hull et al., 2008, 2012). The number of infiltrating GFP-positive macrophages did not change longitudinally during ectopic endometrial tissue development, however there were significant alterations in their spatial distribution within the lesion, with a progressive movement from the periphery to the centre of the lesion, and altered expression of informative surface markers.

MHC II-expressing macrophages were more abundant at the early stages of lesion development in *Cfms-Gfp* mice, in harmony with the function of MHC II as an activation marker and antigen presenting molecule commonly expressed on macrophages within inflammatory environments, including endometriosis (D'Hooghe et al., 2001). iNOS is a typical inflammatory M1 macrophage marker which is elevated in macrophages associated with endometriosis in women (Osborn et al., 2002; Wu et al., 1999) and this was recapitulated in *Cfms-Gfp* lesions. iNOS expression was most pronounced at day 4 and day 7, consistent with the acute inflammatory morphological appearance of lesions at this time. The iNOS level then declined as the lesion developed histological changes indicative of tissue remodelling at day 10 and 14.

Arginase 1 is associated with tissue remodelling activity and $Arg1^+$ macrophages are identified as pro-angiogenic in tumour (Movahedi et al., 2010) and wound healing models (Daley et al., 2010). *Arg1* gene expression is upregulated in endometriosis-like lesions three days after transplantation into mice (Pelch et al., 2010), concurring with the predominance of $Arg1^+$ macrophages during the mid-phase of xenograft development on day 7.

Macrophage scavenger receptor (CD204) facilitates the clearance of apoptotic cells during tissue repair (Todt et al., 2008) and can enhance the expansion of endometrial stromal cells (Itoh et al., 2013). Peritoneal macrophages from women with endometriosis displayed reduced levels of human scavenger receptor (CD36) and thus appeared to be less efficient at clearing apoptotic cells than macrophages from disease-free women (Chuang et al., 2009). Consistent with the remodelling function of CD36-positive macrophages, there was a trend to higher CD204 expression at day 14, when tissue repair peaks compared to earlier time points.

The increase in collagen 1 deposition over time in *Cfms-Gfp* endometriotic tissues synergises with the progressive accumulation of M2 macrophages in the later stages of lesion development. Unlike M1 cells, M2 macrophages promote collagen synthesis by virtue of their augmented arginase 1 and related factors (Mantovani et al., 2013). Collagen deposition appears to be a progressive process eventually resulting in fibrosis in endometriotic tissues. Collagen type 1 A (*Col1 A2*) was identified as a differentially regulated transcript in endometriosis-like xenografts in nude mice and histochemical staining reveals high levels of collagen in human and experimental ectopic endometrial tissues (Hull et al., 2008).

In conclusion, this study describes a zenith of inflammatory M1 type macrophage markers early in endometriosis-like lesion development, followed by a later shift to M2 reparative marker expression. Reasonably this would be accompanied by a transition in macrophage function from acute inflammatory activities such as phagocytosis and chemotaxis to tissue remodelling and repair roles such as extracellular matrix synthesis and angiogenesis, although this remains to be confirmed. This data adds to previous work to strengthen the evidence that macrophages are critical regulators of ectopic endometrial lesion development (Capobianco and Rovere-Querini, 2013). Future experiments will investigate the identity of specific cytokines and growth factors that control the progressive change in infiltrating macrophage phenotype. TGFB1 is one candidate factor that likely contributes to the shift from M1 to M2 macrophage phenotype, given its known function as a driver of M2 macrophage differentiation. Targeting TGFB1 or other relevant chemokines, cytokines or epigenetic modulators such as microRNAs identified as modulators of macrophage phenotype warrants further investigation as a strategy to tackle disease development in women with endometriosis.

Author contribution

M.Z.J. performed and designed experiments, analysed data and wrote the manuscript. W.V.I., S.A.R. and M.L.H. supervised the research and contributed to development of the study and writing the manuscript. All authors provided final approval of the version to be published.

Acknowledgements

Supported by Ballentyne Medical and Surgical Research Grant from the A.J. and J.S. Ballentyne Trust (M.L.H.), New Zealand and National Health and Medical Research Council project grant APP1013950 (M.L.H.), Australia. M.Z.J. was the recipient of Adelaide Graduate Fee Scholarship from The University of Adelaide, Australia. We thank the patients who kindly provided endometrial tissue for this study, surgeons and nurses at Women's and Children's Hospital Adelaide, Australia.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2019.01.002.

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