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Associations between endometrial macrophages and persistence of endometritis in postpartum dairy cows

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ABSTRACT

Cows can spontaneously recover from postpartum endometritis. An early predictive diagnosis could avoid unnecessary treatment of recovery cows thus limiting the emergence of antimicrobial resistance, and provide timed treatment to persistent cases. This study first characterised the presence, proportion and phenotype of endometrial macrophages (**Mφ**) in postpartum dairy cows (n = 173) with distinct endometrial health status. Neutrophils (**PMN**) and Mφ counts were evaluated in Diff-Quickstained uterine cytology slides at 21 and 42 days postpartum (**DPP**). At 21 DPP, the Mφ proportion was higher in cows later recovering than in cows with persistent endometritis until 42 DPP, and the PMN:Mφ ratio was higher in persistent than in recovery cows. Immuno-labelling showed that at 21 DPP, recovery cows had higher M2 (CD163+) counts and lower M1:M2 ratio than cows with persistent endometritis, whereas M1 (CD86+) counts were not different. Immuno-labelling of tissue sections from biopsy samples collected at 42 DPP showed that total Mφ and M2 counts were higher in cows with persistent endometritis than in recovery cows. In conclusion, results evidence that the persistence of endometritis is associated with a retarded/failure of Mφ-driven, namely M2-driven, pro-resolving mechanisms. This suggests that endometrial Mφ content at 21 DPP shows the potential to assist in predicting the recovery or persistence of postpartum endometritis in dairy cows, and prompts for comprehensive validation studies.

Implications

Postpartum endometritis stands as a serious burden of the dairy industry globally. Endometritis may show spontaneous recovery or evolve into a subclinical persistent form. An early prognostic tool would avoid unnecessary treatment of recovery cows and the emergence of antimicrobial resistance, and allow timed treatment of cows prone to endometritis persistence. This study first evaluates the role of endometrial macrophages in the recovery or persistence of postpartum endometritis in dairy cows. Results evidence that a lack or delay in macrophage polarisation to M2 phenotype is associated to the persistence of endometritis. This suggests macrophage proportion and/or phenotype as potential markers of endometritis persistence, and prompts for comprehensive validation studies.

Introduction

Postpartum uterine disease, affecting up to 50% of high-yielding dairy cows, remains a serious constraint to the dairy industry, impairing animal health, welfare, fertility and milk production (Bradford et al., 2015; Bromfield et al., 2015; LeBlanc, 2023). After parturition, ascending bacterial invasion and contamination of the uterus are unavoidable, which leads to uterine inflammation and potential infection (Sheldon et al., 2008; Pascottini and LeBlanc, 2020). This inflammatory reaction is essential for the clearance of pathogens and lochia, but if uncontrolled, may persist at levels that subsequently affect fertility. Although a robust but temporary and controlled inflammatory response is required for endometrial repair (Gilbert and Santos, 2016; Sheldon et al., 2019), homeostasis should be restored by 6 weeks postpartum (≈ 42 days), requiring

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resolution of inflammation by this time (Barański et al., 2012; Gilbert and Santos, 2016). Therefore, in the pathogenesis of postpartum uterine disease, bacterial load *per se* is not decisive, since failure in host resolution mechanisms leads to uncontrolled inflammation persisting even after elimination of pathogens, maintaining clinical or subclinical disease (Sheldon et al., 2006; Pereira et al., 2022).

The inflammatory cascade initiates with the activation of pattern recognition receptors on endometrial epithelial cells by bacteria and damaged cells (Sheldon et al., 2017). In an ideal outcome, this is successively followed by infiltration of plasmatic fluids (oedema), influx of polymorphonuclear neutrophils (**PMN**) and recruitment of monocytes, these inflammatory cells being chemoattracted to the endometrium to phagocytise and kill bacteria (Gilbert and Santos, 2016; Chiang and Serhan, 2017). The influx of PMN into the uterine lumen constitutes the first and most significant component of the cellular immune response, phagocytising bacteria and synthesising cytokines to attract other immune cells (Mateus et al., 2002; Ahmadi et al., 2005; Kim et al., 2014), namely monocytes (Hussen et al., 2016), which differentiate into macrophages (**Mφ**). After killing bacteria, PMN undergo apoptosis and are phagocytised and removed from the uterus by Mφ or by reverse migration to the bone marrow (Pascottini and LeBlanc, 2020).

Postpartum clinical metritis and clinical endometritis are easily diagnosed by their clinical signs, which allow for early appropriate treatment. However, persistent subclinical endometritis without any recognisable clinical sign frequently evolves from the clinical forms even following appropriate therapy (Lima et al., 2014). This form requires invasive, time-consuming and expensive procedures for its diagnosis, and has currently no effective treatment (LeBlanc et al., 2002; Barlund et al., 2008). Uterine cytology, evaluating the proportion of PMN in slides assembled from a uterine swab, emerged as a standard technique for the diagnosis of endometritis, named cytological endometritis, although the sampling time and PMN cut-off are still subject to debate (Kasimanickam et al., 2004; Johnson et al., 2015; Wagener et al., 2017; Druker et al., 2022). Several uterine lumen swabbing techniques were described, including various cytobrush tools, providing reliable sensitivity and specificity, despite a lack of capacity to detect cellular infiltration in deeper layers of the endometrium (Helfrich et al., 2020). Endometrial biopsy allows assessment of the deeper endometrial layers (Chapwanya et al., 2010), but the sampled tissue may not be representative of the total endometrium, and its potential effects on pregnancy risk were pointed out (Pascottini et al., 2016).

Although the postpartum endometrial PMN populations are well–characterised in both cytology and biopsy samples, the presence and significance of Mφ remain elusive (De Boer et al., 2014; Kusaka et al., 2020). In humans and lab species, upon differentiation, non-activated (M0 phenotype) Mφ are activated (polarised) either in pro-inflammatory (M1, CD86+) or anti-inflammatory (M2, CD163+) phenotypes, contributing to tissue destruction or regeneration, depending on functional tissue-derived signals (reviewed by Yao et al., 2019; Yan et al., 2024). The M1 phenotype is typically activated by IFNγ or LPS stimuli and synthetise pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-18, IL-12), whereas the M2 phenotype is typically activated after exposure to IL-4, IL-10 and IL-13 and synthetise anti-inflammatory cytokines (IL-4, IL-10, TGFβ). In the cow, uterine caruncular Mφ phenotype was related to placental retention (Nelli et al., 2019), but the significance of Mφ phenotype on the development of endometritis is unknown.

Cows with endometritis can spontaneously recover (Mateus et al., 2002; Pereira et al., 2022), and an early predictive diagnostic method discriminating endometritis recovery and persistence cases could avoid unnecessary treatment of recovery cases and the emergence of antimicrobial resistance while providing timed treatment to persistent cases. Diagnosis of endometritis based on uterine cytology PMN counts has not provided useful predictive discriminant information on the recovery or persistence nature of inflammation. Therefore, identification of markers of endometritis recovery/persistence would be of relevance for the management of postpartum endometritis. Previous work (Pereira et al., 2020) identified adipokines (adiponectin and chemerin) as molecular markers of endometritis persistence. However, this approach involves laboratory analyses of blood and/or uterine fluid samples. The aim of this study was to characterise the presence, proportion, phenotype and potential prognostic and diagnostic value of Mφ in endometrial cytology and biopsy samples in postpartum endometritis in dairy cows. The hypothesis to be tested was that Mφ, as relevant cell mediators of the inflammatory resolving mechanisms, could be associated to the path of postpartum endometritis, and emerge as potential markers for the prediction of endometritis recovery or persistence.

Material and methods

Study design

For this observational retrospective cohort study, the field work was performed in a commercial 750 milking Holstein-Friesian dairy cow herd, with thrice-a-day milking and a milk yield average of 13 800 kg/cow at 305 days in milk. A Total Mixed Ratio adjusted to maintenance and milk yield was distributed just before every time cows returned from the milking parlour. First artificial insemination was performed after a voluntary waiting period of 70 days. The study comprised a follow-up from calving to 42 days postpartum (**DPP**). In this follow-up, until 20 DPP, cows were daily monitored for clinical signs of puerperal disease, including measurement of rectal temperature, assessment of feed intake and recording of milk yield. Every other day, the vaginal discharge was evaluated visually using a Metricheck® device (EndoControl Sampler, Minitube, La Selva del Camp, Spain), and twice a week, the uterine size and tone were evaluated through palpation *per rectum*. Cows exhibiting fetid reddish-brown vaginal discharge, hyperthermia (≥ 39.5 °C) and a drop in milk yield and feed consumption, associated to an enlarged uterus were diagnosed with metritis. The mammary gland was inspected at each milking and once a week, a milk sample of each mammary gland was evaluated by the Californian Mastitis Test and for somatic cell count. Clinical mastitis was diagnosed by clinical signs and Californian Mastitis Test positivity, whereas subclinical mastitis was diagnosed by somatic cell count ≥ 200.000 cells/mL, in the absence of clinical signs and Californian Mastitis Test positivity. Sampling for somatic cell count was continued weekly until 42 DPP. Exclusion criteria included cases of twin calving, dystocia, retention of foetal membranes, metritis, mastitis (clinical and subclinical), lameness, clinical metabolic disease (hypocalcemia, ketosis, displacement of abomasum), and any other disease or any treatment arising until 20 DPP or thereafter until 42 DPP.

At 21 DPP (range 20–22 DPP), all cows not previously excluded were submitted, sequentially and in the following order, to a vaginal discharge evaluation using a Metricheck® device, scored on a 0–3 scale (Williams et al., 2005), an ultrasound examination (7.5 MHz rectal probe, ExaGo, Echo Control Medical, France), an endometrial swabbing for uterine cytology, and a uterine low-volume flush of the previously gravid uterine horn. At 42 DPP (range 41–43 DPP), all these procedures except the uterine low-volume flush were repeated, which was followed by a uterine biopsy of the previously gravid horn. At 21 DPP, cows with uterine cytology proportion of PMN ≥ 18% were diagnosed with Endometritis (Kasimanickam et al., 2004; Pereira et al., 2020). At

42 DPP, Endometritis cows with endometrial cytology PMN proportion < 5% were considered to have spontaneously recovered, whereas cows with PMN proportion ≥ 5% were considered to have a persistent Endometritis (Gilbert et al., 2005; Pereira et al., 2020). A vaginal discharge with score 1–3 was considered positive. This evaluation allowed to identify a correspondence between the presence of vaginal discharge and cytological endometritis at 21 and 42 DPP.

A total of 173 cows (68 primiparous and 105 multiparous) were enrolled in the study and allocated into three groups of distinct endometrial health status, including a control group Healthy (**H**, n = 85; without endometritis), a group Endometritis Recovered (**E-R**, n = 56) and a group Endometritis Persistent (**E-P**, n = 32). In all cows, the proportion of PMN and Mφ was evaluated in uterine cytology slides stained with Diff-Quick, and the PMN:Mφ ratio was calculated. To confirm the identification of Mφ in Diff-Quick –stained slides and to evaluate Mφ phenotype at 21 DPP, a subset of cows was selected for the labelling of protein markers of total Mφ (CD68+), M1 (CD86+), and M2 (CD163+) by immunocytochemistry (**ICC**, n = 36). This also allowed to assess the agreement between Diff-Quick staining and ICC for identifying Mφ populations. A second subset of cows was selected to evaluate the protein abundance of Mφ markers by Western Blot (**WB**, n = 35) in the cellular pellet of the low-volume uterine flushing. This allowed to correlate the counts of total Mφ, M1 and M2 with the abundance of the respective protein markers within the uterine lumen. Finally, a larger subset of cows, including the previous subsets was selected to evaluate the Mφ content and phenotype in biopsy samples at 42 DPP by immunohistochemistry (**IHC**, n = 50). This approach was chosen to identify the presence of Mφ within the deep endometrial layers, even if their presence could no longer be detected in the luminal compartment, therefore giving further insight into the persistence of endometritis. Blood samples were collected weekly, from 2 to 42 DPP, for measurements not considered in this study.

Uterine cytology

Uterine cytology was performed at *corpus uteri* using an adapted cytobrush technique (Pereira et al., 2020). Each swab was rolled along 2 microscope slides, which were sprayed with a fixator (M-FIX®, Merck Millipore, Darmstadt, Germany), stained with a modified Wright-Giemsa® (Diff-Quick, MAIM SL, Barcelona, Spain) and examined using a 400x magnification in a microscope (Olympus, BX51) by two independent technicians. Populations of epithelial cells, PMN and Mφ were counted and their proportion in a total of 400 cells (200 cells in each slide) was calculated. The Mφ were identified as microvacuolated cells with abundant cytoplasm (foamy Mφ).

Immunocytochemistry (ICC)

In a subset of cows (H = 12, E-R = 12, E-P = 12), three additional cytobrush-derived slides were used to evaluate the Mφ phenotype by ICC. Fixed slides were washed in 70% ethanol solution for 2 min to remove fixator's polyethylene glycol, hydrated with deionised water for 5 min, and immersed in a 3% hydrogen peroxide solution for 15 min at room temperature to quench endogenous peroxidase activity. After a 5 min PBS wash, slides were microwaved for 3 min three times in an antigen unmasking solution, according to antibody (CD68 and CD86 – 10 mM Tris with 1 mM EDTA buffer pH 9.0; CD163 – 10 mM citrate buffer pH 6.2), left to cool to room temperature, and washed 5 min in PBS. Non–specific binding was blocked with 5% bovine serum albumin (A7906; Sigma-Aldrich), for 1 h in a humid chamber at room temperature. Each slide was probed either with monoclonal antibody anti-CD68 (Total Mφ, 1:25; monoclonal mouse anti-bovine CD68, ED1, MA5-16654, Thermo Fisher Scientific), anti-CD86 (M1,1:50; monoclonal mouse anti-bovine CD86, IL-A190, MCA2437GA, BioRad) and anti-CD163 (M2, 1:200; monoclonal mouse anti-bovine CD163, EDHu-1, MCA1853, BioRad) diluted in 5% bovine serum albumin, and incubated overnight at 4 °C in a humid chamber. After 3 times of PBS washing, each for 5 min, slides were incubated with the peroxidase conjugate polyclonal goat anti-mouse secondary antibody (1:100; A2554, Sigma-Aldrich) for 1 h in a humid chamber at room temperature. Following another 3 times PBS washing, each for 5 min, immunoreactivity was revealed by incubation with DAB chromagen, according to manufacturer instructions (ImmPACT® DAB Substrate Kit, SK-4105, Vector Laboratories). Finally, slides were counterstained with Meyers haematoxylin (Merck), dehydrated and mounted with Entellan® (Merck). Stainings without the primary antibody and with the mouse IgG1 isotype control (02-6100, Thermo Fisher Scientific) were used as negative controls. Proportions of CD68, CD86 and CD163 positive cells were calculated by counting a total of 400 cells using a microscope (Olympus, BX51).

Low-volume uterine flushing

A sample of uterine fluid was obtained by flushing the previously gravid uterine horn with PBS, using a silicone-coated latex Foley catheter (Rusch Gold, Rusch, Perak, Malaysia), aseptically mounted in a metal stylet and covered with a sanitary sheath. The catheter was introduced into the proximal third of the uterine horn, the cuff inflated, and 50 mL of sterile PBS introduced into the uterine horn, which was subsequently massaged prior to collecting the flush into a 50–mL centrifuge tube (Corning™ 430829). The sample was refrigerated at 5 °C until centrifugation (2 000xg, 15 min), and the pellet was collected into 2 mL Eppendorf tubes with 1 mL RNA Later and stored at –80 °C until assayed. The supernatant was also collected and stored for assays not considered in this article.

Western blot (WB) analysis of uterine fluid pellet

In a subset of cows (H = 19, E-R = 11, E-P = 5), the pellet sample was washed three times with PBS by centrifugation at 13 000xg for 5 min, to obtain total protein extract. The pellet was then resuspended in 1 mL of Qproteome Mammalian Protein Prep Kit (37901, Quiagen) with protease inhibitor (A32953, Thermo Fisher Scientific) and 1 µl of benzonase (Thermo Scientific), homogenised on ice, and disrupted in TissueLyser II (Quiagen, Hilden, Germany) 3 cycles of 25 Hz, 30 s each, and finally centrifuged at 14 000xg for 10 min at 4 °C to recover the supernatant. The protein concentration in the supernatant fraction was measured at 595 nm in a spectrometer, using Bradford reagent (Bio-Rad, Hercules, CA, USA). For SDS-PAGE, 40 µg (CD68) or 80 µg (CD163) of total protein were mixed with Laemmli Buffer (M11701, Nzytech) (ZN112, NZYthec), denatured at 95 °C for 8 min, cooled in ice for 5 min, then loaded into a 8% separating polyacrylamide gel, and proteins separated by SDS-PAGE, at a constant voltage of 75 V, for 2 h, in 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Proteins were transferred to 0.45 µm Invitrolon™ PVDF membranes (LC2005, Invitrogen) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 70 min. After blocking in blocking solution 5% non-fat milk with 0.1% Tween 20, for 2 h at room temperature, membranes were incubated with the primary antibodies anti-CD163 (1:1000; monoclonal mouse anti-bovine CD163, EDHu-1, MCA1853, BioRad) and anti-CD86 (1:2000; monoclonal mouse anti-bovine CD86, IL-A190, MCA2437GA, BioRad) diluted in blocking solution and incubated overnight at 4 °C. To normalise protein load, mouse monoclonal antibody against β-actin (1:10 000,

AB20272, Abcam) was added in each assay run. Membranes were washed 3 times with PBS containing 0.1% Tween 20 for 5 min, then with PBS to remove Tween 20, and incubated with secondary antibody (1:20 000, Goat Anti-Mouse IgG1, 1070-05, SouthernBiotech) for 1.5 h at room temperature. To each membrane, chemiluminescent substrate (SuperSignal™ West Pico Plus, 34580, Thermo Fisher Scientific) was applied for 60 s prior to imaging with a ChemiDoc MP system and Image Lab 4.1 software XRS + (BioRad, Hercules, CA). Exposure time depended on antibody (CD86 – 5 min; CD163 – 10 min; ß-actin – 15 s). Anti-CD86 antibody detects the protein isoforms of 80 kDa (Yang et al., 2022). Anti-CD163 antibody detects the expected protein isoforms of 130–140 kDa (datasheet MCA1853, BioRad). To normalise the bands on each membrane, after subtracting the background, the total pixel counts for CD86 or CD163 bands were divided by the respective total pixel counts of β-actin bands, and results expressed in arbitrary densitometry units.

Endometrial biopsy

The endometrial biopsy was performed in the previously pregnant uterine horn, following an epidural anaesthesia, using a modified Kervokian-Younge biopsy instrument (Alcyon, Paris, France), as previously described (Pereira et al., 2020). Briefly, the sterile biopsy instrument, guarded in a protective sheath, was introduced into the vagina and advanced to the external cervical orifice, where the protective sheath was ruptured. The biopsy instrument was then guided into the first third of the uterine horn, where biopsy jaws (length × width: 1.2 × 0.55 cm, jaws with a rounded polished border) were opened, and an endometrial sample of about 1 cm^2 was collected. The endometrial tissue was fixed in a 4% paraformaldehyde solution and later embedded in paraffin.

Immunohistochemistry (IHC)

In a subset of biopsy samples (H = 20, E-R = 18, E-P = 12), paraffin–embedded endometrial samples were serially sectioned at a thickness of 4 μm, deparaffinised and hydrated. Quenching of endogenous peroxidase activity, antigen retrieval and non–specific background elimination were performed similarly to above–described ICC procedure. Sections were incubated overnight at 4 °C with primary antibodies anti-CD68 (1:50; monoclonal mouse anti-bovine CD68, ED1, MA5-16654, Thermo Fisher Scientific) and anti-CD163 (1:200, MCA1853, BioRad) diluted in PBS. Sections were then washed 3 times in PBS for 5 min and incubated at room temperature for 1 h, with the secondary antibody (1:100, A2554, Sigma-Aldrich). After washing 3 times in PBS for 5 min, immunoreactivity was revealed by incubation with DAB chromagen/DAB buffer at room temperature, according to manufacturer instructions (ImmPACT® DAB Substrate Kit, SK-4105, Vector Laboratories). Finally, the sections were counterstained with Meyers haematoxylin (Merck), dehydrated and mounted with Entellan® (Merck). Sections were acquired using a microscope (Olympus, BX51) equipped with a digital camera (Olympus, DP21). In each section, stained cells were counted in 20 fields at 400x magnification.

Statistical analysis

The experimental unit was the cow. Data were recorded in Excel 2013 (Microsoft Corp.) spreadsheets and imported into SAS (version 9.4, SAS Institute Inc.) for statistical analysis. ANOVA (One-way ANOVA) (PROC GLM) was used to compare PMN, Mφ and PMN:Mφ ratio at 21 and 42 DPP between groups and used the Tukey post hoc adjustment for multiple tests. Parity (primiparous versus multiparous; lactation number) was included in the models as a fixed effect and kept if $P < 0.10$. Differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.05 < P \leq 0.10$. The assumption of homoscedasticity was evaluated visually using the plots of studentised residuals against predicted values, and the assumption of normality of residuals was evaluated using the Q-Q plots (diagnostic plots in Supplementary Figure S1). Cohen's kappa statistic was used to assess the agreement between two observers in counting Mφ in Diff-Quick–stained cytology samples.

Spearman Correlation Coefficient (PROC CORR) was used to determine correlations between uterine cytology Diff-Quick –stained Mφ proportion, and CD68, CD86 and CD163 positive cells counted in ICC, or CD86 and CD163 protein abundance detected by WB (scatter plots in Supplementary Figure S2). A canonical discriminant analysis evaluated PMN, Mφ and PMN:Mφ ratio at 21 DPP for a predictive model of endometritis persistence. For this purpose, an interactive forward stepwise analysis (PROC STEPDISC) selected the variables with discriminant capacity, and cross-validation was conducted (PROC DISCRIM). Predictive accuracy was also evaluated by Receiver Operating Characteristic (**ROC**) curve analysis, which coordinates allowed Youden Index calculation, the determination of the optimal cut-off, odds ratio, sensitivity, specificity, positive predictive value and negative predictive value. Given the small dataset, model calibration was assessed using the calibration intercept and slope derived from out-of-fold predictions obtained through 5-fold cross-validation. The dataset was randomly partitioned into five equal-sized folds. For each iteration, a logistic regression model was trained on four folds and used to predict probabilities on the held-out fold. This process ensured that each observation received a predicted probability from a model that was not trained on that observation (i.e., out-of-sample prediction). The predicted probabilities from all five iterations were combined, and a secondary logistic regression was performed using the logit of the predicted probabilities as the sole predictor of the observed outcome.

To determine the required number of cows to include in the subsets, a power analysis was performed posthoc based on previous work, in which a CV of 92% was obtained when comparing the means of Mφ proportions in cytology samples of endometritis cows with spontaneous recovery or persistent inflammation. In the present study, a difference of 78% between means was observed, indicating that a difference of at least 78% is expected between these two groups of cows. The following formula was applied, considering a Student's t-value of 2.0 ($P = 0.05$): $n = 2t^2 \times (CV)^2 \div \text{difference}\%^2$. This analysis indicated a minimum of 11 cows per group. This was considered for the cow subsets for ICC and IHC. However, there was no previous knowledge on the CV between groups, of the protein abundance of Mφ markers; therefore, the same number determined for ICC and IHC was chosen. In the cow subset for WB, the number of E-P cows was 5, probably resulting in an underpowered analysis for the comparison of group means of protein abundance.

Results

Postpartum endometritis prevalence

At 21 DPP, cows without previous puerperal disease (n = 173) had an endometritis prevalence of 51% (88/173). In these 88 Endometritis cows, the spontaneous recovery between 21 and 42 DPP was 64% (56/88; Endometritis Recovered cows; E-R cows), whereas in 32 cases (36%), inflammation persisted until 42 DPP (Endometritis Persistent cows; E-P cows). In 85 cows (49%), no cytological endometritis was detected at both 21 and 42 DPP

(Healthy cows; H cows). Apart from the 173 enrolled cows, during the follow-up, three cows without previous puerperal disease and without endometritis at 21 DPP presented endometritis at 42 DPP. These three cows were excluded from the study.

At 21 DPP, a score 1–3 vaginal discharge was observed in 25% (8/32) of E-P cows and in 4% (2/56) of E-R cows. Interestingly, at 21 DPP, also 4% (3/85) of H cows showed score 1–2 vaginal discharge, although with very low PMN counts (≈ 1%), potentially reflecting vaginitis or remains of lochia still present in the vagina. In all remaining cows (160/173, 93%), vaginal discharge was either absent or of score 0. Therefore, at 21 DPP, most Endometritis cows (78/88, 89%) presented only a cytological (subclinical) form. At 42 DPP, in all 173 cows, vaginal discharge was either absent or of score 0. Therefore, all 32 E-P cows presented a cytological (subclinical) form of the disease.

The fixed effect of parity, either as primiparous versus multiparous ($0.627 < P < 0.150$) or as lactation number ($0.992 < P < 0.1476$), was not significant in any model, so it was excluded from all analyses.

Endometrial cytological findings

Fig. 1 illustrates representative images of Diff-Quick–stained uterine cytology slides of cows allocated to groups with distinct endometrial health status, at 21 and 42 DPP. The proportion of PMN in uterine cytology slides naturally reflects the retrospective allocation of cows to endometrial health status groups, based on PMN cut-off values (Table 1). Of interest, at 21 DPP, PMN proportion was higher ($P < 0.01$) in E-P than in E-R cows. At 42 DPP, E-P cows still showed high PMN proportion (12.4%), whereas this proportion was lower than 2% in H and E-R cows.

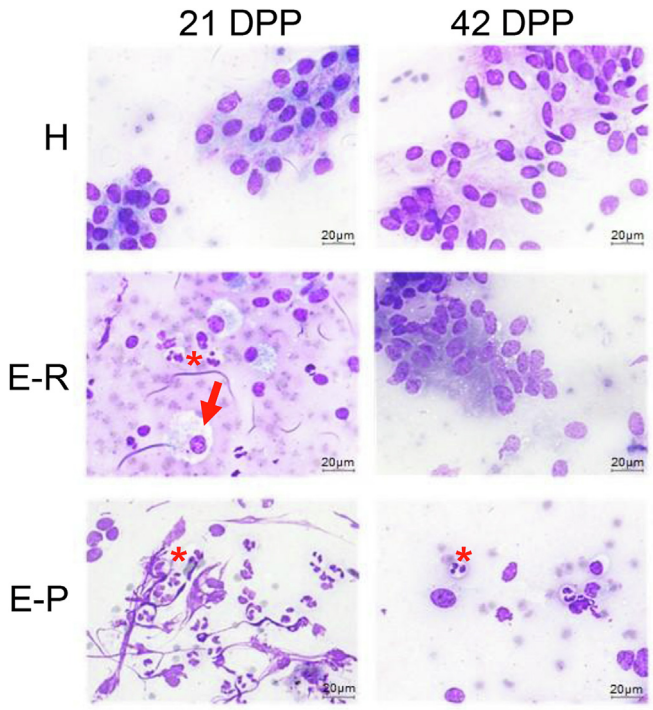

Fig. 1. Representative images of cytological findings in cows with distinct endometrial health status at 21 and 42 DPP (400x). Images were acquired using a microscope (Olympus, BX51) equipped with a digital camera (Olympus, DP21). Healthy (H) cows - mostly epithelial cells and low PMN and Mφ counts; Endometritis recovered (E-R) cows – high PMN and Mφ counts at 21 DPP, but low counts at 42 DPP; Endometritis Persistent (E-P) cows – high PMN and low Mφ counts at 21 DPP, but still high PMN counts at 42 DPP. Abbreviations: DPP = days postpartum; PMN = polymorphonuclear neutrophils; Mφ = macrophages. * points to PMN; ↑ points to Mφ.

Table 1
Uterine cytological findings of cows with distinct uterine health status.

Groups	H	E-R	E-P
n	85	56	32
PMN 21 DPP (%)	3.4 + 1.5^{a}	32.5 + 1.9 ^{b,x}	43.2 ± 2.5 ^{c,x}
PMN 42 DPP (%)	1.4 + 0.7^{a}	1.4 ± 0.9 ^{a,y}	12.4 ± 1.2 ^{b,y}
Mφ 21 DPP (%)	2.4 ± 0.4 ^{a,x}	5.4 ± 0.5 ^{b,x}	1.4 ± 0.6 ^{a}
Mφ 42 DPP (%)	0.7 ± 0.1 ^{a,y}	0.8 ± 0.2 ^{a,y}	1.5 ± 0.2 ^{b}
PMN:Mφ 21 DPP	4.6 ± 4.5 ^{a}	13.1 ± 5.5 ^{a}	63.0 ± 7.3 ^{b,x}
PMN:Mφ 42 DPP	3.3 ± 0.9 ^{a}	2.9 ± 1.1 ^{a}	16.3 ± 1.5 ^{b,y}

Abbreviations: DPP = days postpartum; PMN = polymorphonuclear neutrophils; Mφ = macrophages; values are Mean ± SEM, except otherwise stated; values with different superscripts differ significantly (between columns within a row: ^{abc} $P < 0.05$; between rows within a column: ^{xy} $P < 0.05$). Groups: H = Healthy; E-R = Endometritis Recovered; E-P = Endometritis Persistent.

At 21 DPP, the proportion of Mφ was higher ($P < 0.0001$) in E-R than in H and E-P cows. In contrast, at 42 DPP, the proportion of Mφ was higher ($P < 0.05$) in E-P than in H and E-R cows. At both 21 and 42 DPP, the PMN:Mφ ratio was higher in E-P than in H ($P < 0.0001$) and E-R ($P < 0.001$) cows. There was a strong agreement between the two technicians on the identification of Mφ in Diff-Quick–stained slides at 21 DPP (Cohen's Kappa = 0.853; $P < 0.001$) and 42 DPP (Cohen's Kappa = 0.892; $P < 0.001$).

Regarding the Diff-Quick–stained slides, the intra-assay agreement for PMN counts (in twin slides) showed a CV = 8.6% (r = 0.993; $P < 0.0001$). The intra-assay agreement for Macrophage counts (in twin slides) showed a CV = 9.9% (r = 0.979; $P < 0.0001$). An inter-assay agreement for Diff-Quick–stained slides was not available as all slides were only observed in the sampling day by the two technicians.

The discriminant analysis showed that Mφ at 21 DPP was able to differentiate between persistence and recovery of endometritis. Fig. 2 represents the ROC analysis of Mφ at 21 DPP, to discriminate cows with persistent endometritis at 42 DPP. This ROC analysis showed that Endometritis cows with a Mφ proportion below 3.5% at 21 DPP had a higher risk of persistent endometritis by 42 DPP (Area under the curve = 0.9023; $P < 0.001$; Sensitivity = 0.97, Specificity = 0.71, Positive predictive value = 0.66, Negative predictive value = 0.98).

Calibration assessment based on out-of-fold predictions revealed an intercept of –0.0336 and a calibration slope of 0.8579. The intercept, being close to zero, indicates that the model does not systematically over- or underpredict across the probability range. The slope, ideally equal to 1 in a perfectly calibrated model, was slightly below this ideal. This suggests that the model's predicted probabilities were slightly underconfident. The calibration plot is shown in Supplementary Figure S3.

Macrophage immunocytochemistry (ICC) at 21 days postpartum

Representative images of ICC–stained Mφ are illustrated in Fig. 3, and Table 2 shows the Spearman correlations between Diff-Quick and ICC–stained Mφ at 21 and 42 DPP. Diff-Quick and ICC staining of total Mφ showed a strong correlation at 21 DPP (r = 0.966; $P < 0.0001$) and 42 DPP (r = 0.911; $P < 0.0001$). At 21 DPP, Diff-Quick and ICC staining showed a strong correlation for M2 (r = 0.851; $P < 0.0001$) but only moderate (r = 0.522; $P < 0.05$) for M1. The M1 proportion was similar in the three groups, whereas the M2 proportion was higher ($P < 0.01$) in E-R than in H and E-P cows. The M1:M2 ratio was higher ($P < 0.01$) in E-P than in H and E-R cows (Fig. 4).

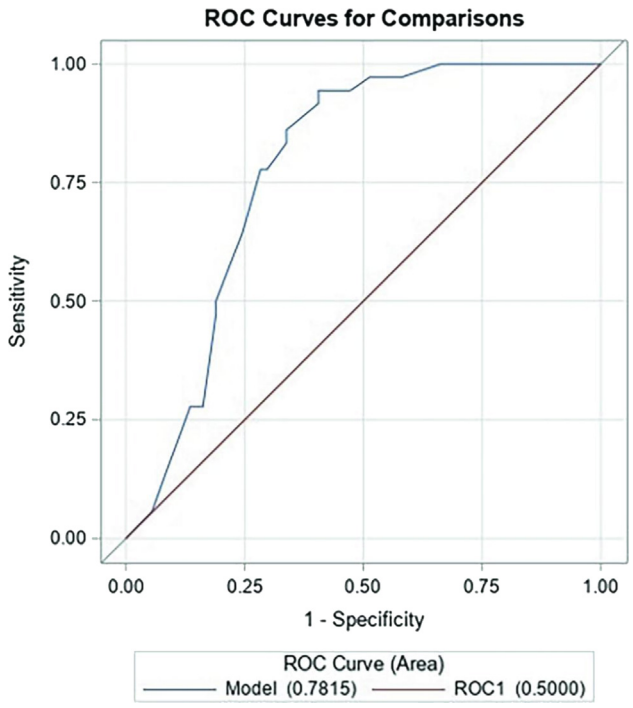

Fig. 2. Receiver Operating Characteristic (ROC) curve analysis of Mφ proportion at 21 days postpartum to discriminate cows with persistent endometritis at 42 days postpartum: Endometritis cows with macrophages proportion below 3.5% at 21 days postpartum had a higher risk of persistent endometritis by 42 days postpartum (Area under the curve = 0.9023; $P < 0.001$; Sensitivity = 0.97, Specificity = 0.71, Positive predictive value = 0.66, Negative predictive value = 0.98).

Immunohistochemistry (IHC) macrophage detection in biopsy-derived endometrial tissue at 42 days postpartum

Histological analysis (haematoxylin-eosin staining) of endometrial samples collected through a biopsy of the previously gravid uterine horn at 42 DPP did not show any distinctive gross differences between the three groups of cows, namely in the infiltration of inflammatory cells in the endometrial layers. Regarding the endometrial infiltration of total Mφ and M2, Fig. 5A shows representative images of IHC labelled Mφ in endometrial tissue at 42 DPP. The counts of total Mφ and M2 were higher in E-P than in E-R cows ($P < 0.05$) (Fig. 5B), although only low counts were present in the endometrial layers at this postpartum time.

Western blot (WB) analysis of macrophage phenotype protein markers

Densitometry analysis (Fig. 6A and B; Supplementary Figures S4 and S5) showed that whereas CD86 (M1 phenotype) protein abundance was similar in the three groups, CD163 (M2 phenotype) protein bands had higher intensity ($P < 0.05$) in E-R than in H and E-P cows. An inter-assay agreement of CV = 14.9% (r = 0.991; $P < 0.0001$) was determined by running samples in more than one membrane, but an intra-assay agreement was not possible as samples were not repeated in the same membrane.

Discussion

This study firstly evaluated the presence, proportion, phenotype and potential prognostic and diagnostic value of Mφ populations identified in endometrial samples of postpartum dairy cows with distinct uterine health status, and namely the Mφ relevance for the recovery or persistence of endometritis.

The observed prevalence of postpartum endometritis (51%) is in accordance with data reported in the literature for high-yielding postpartum dairy cows (LeBlanc, 2008; Barański et al., 2012; Pérez-Báez et al., 2021), without differences between primiparous and multiparous cows (Galvão et al., 2011; Cheong et al., 2011). Most Endometritis cows showed a subclinical (only cytological) form of the disease, without a positive vaginal discharge score, as also reported by others (Salah and Yimer, 2017; Kusaka et al., 2022). This accounted for 89% of Endometritis cows at 21 DPP and for 100% of cows with persistent endometritis at 42 DPP. In Endometritis cows, without any treatment, spontaneous recovery of endometrial PMN counts to physiological values occurred in 64% of cows by 42 DPP. This indicates that a large proportion of Endometritis cows is apparently able to resolve endometrial inflammation, and any treatment is potentially unnecessary. In fact, assessment of postpartum endometritis treatments, either with prostaglandin F2alpha or cephapirin, revealed inconsistent results (Haimerl et al., 2013; Barański et al., 2022). However, as evidenced by Pereira et al. (2022), compared to healthy cows, Endometritis cows that exhibit endometrial histological recovery by 42 DPP still have differential endometrial transcription of genes at this stage, especially those related to cell adhesion and IFN-dependent or IFN-stimulated genes, and this may be potentially responsible for carry over effects in reproductive performance.

The postpartum PMN influx into the endometrium is essential for bacterial clearance. However, puerperal PMN phagocytic activity is impaired (Mateus et al., 2002), and circulating PMN of transition cows are often exposed to high concentrations of lipid- and inflammation-derived metabolites. This might influence the function of these immune cells before they reach the uterine lumen (Leblanc, 2020; Pascottini et al., 2023). Such an effect could be detrimental to recovery as the rapid resolution of inflammation requires a strong initial recruitment of inflammatory cells into the uterus (Gilbert and Santos, 2016). In the present study, uterine cytology PMN proportions were higher in E-P than in E-R cows at 21 and 42 DPP, which support the concept that persistence of endometritis is not caused by a decreased influx of PMN into the uterus, but potentially by the presence of less functional PMN, contributing to an impaired inflammatory response.

Macrophages are major actors involved in the inflammatory response. However, the presence and relevance of Mφ populations in the persistence of cow postpartum uterine inflammation were unknown (Oruc et al., 2015; Kusaka et al., 2020; Cengiz et al., 2021). Results of this study showed that E-R cows had higher Mφ proportions in uterine cytology-stained slides at 21 DPP than H and E-P cows. This evidences that the spontaneous recovery is associated with an early endometrial Mφ influx. In contrast, in E-P cows, early Mφ recruitment to the uterus was impaired, which potentially resulted in failure of inflammatory pro-resolving mechanisms. In H cows, the low proportion of Mφ at 21 DPP may be due to the low intensity of inflammatory stimuli or to an earlier Mφ recruitment response, i.e. before the sampling time point used in the present study. A decrease of Mφ between 21 and 42 DPP was observed in both H and E-R cows, coincident with a time associated to endometrial recovery from inflammation (Dadarwal et al., 2019). The above results evidence that uterine cytology–derived Mφ counts at 21 DPP can potentially assist in predicting the persistence or recovery from endometritis at 42 DPP.

The PMN:Mφ ratio at 21 DPP was higher in E-P than in E-R cows, suggesting that chemotaxis of PMN and Mφ was unbalanced and contributed to impair the pro-resolving response (Foley et al., 2012). In early postpartum healthy cows, there is a switch from a

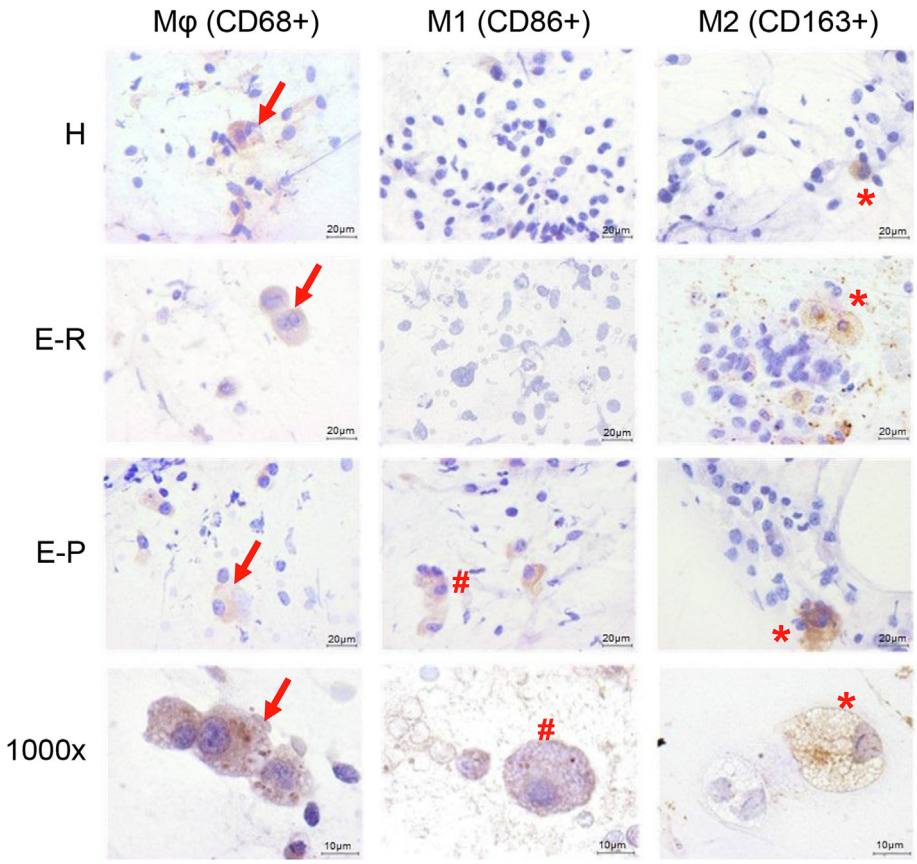

Fig. 3. Representative images of immunocytochemistry in uterine cytology smears of total macrophages (CD68+) and of the M1 (CD86+) and M2 (CD163+) phenotypes of cows with distinct health status, at 21 days postpartum (400x; last row – 1 000x). Images were acquired using a microscope (Olympus, BX51) equipped with a digital camera (Olympus, DP21). Labelled macrophages: CD68+ (↑), CD86+ (#) and CD163+ (*). Cow groups: H – Healthy; E-R – Endometritis Recovered by 42 days postpartum; E-P - Endometritis Persistent by 42 days postpartum.

Table 2
Spearman's correlations between uterine cytology smears' macrophage counts observed in Diff-Quick staining and immunocytochemistry (with CD68, CD163 and CD86) at 21 and 42 DPP in postpartum dairy cows.

Item	Diff-Quick 21DPP	CD68+ 21DPP	CD86+ 21DPP	CD163+ 21DPP	Diff-Quick 42DPP	CD68+ 42DPP
Diff-Quick 21DPP	–	0.966 ***	0.522 *	0.851 ***	–0. 033	0.006
CD68+ 21DPP		–	0.615 **	0.851 ***	0.180	0.237
CD86+ 21DPP			–	0.196	0.524	0.440
CD163+ 21DPP				–	–0.305	–0.313
Diff-Quick 42DPP					–	0.911***

*$P < 0.05$ **$P < 0.01$ and ***$P < 0.001$.
Abbreviations: CD68+ cells = total macrophages (Mφ); CD86+ cells = Mφ of phenotype M1; CD163+ cells = Mφ of phenotype M2; DPP = days postpartum.

pro-inflammatory to an anti-inflammatory pro-resolving gene expression profile and cytokine production (Kasimanickam et al., 2013; Bradford et al., 2015; Dadarwal et al., 2019). This shift seems to occur later in E-P cows, explaining the presence of PMN, Mφ and the high PMN:Mφ ratio at 42 DPP, and suggesting that a delayed pro-resolving process remains still active at this later stage.

In the Diff-Quik staining session runs, variations in sample quality, potential staining duration and reagent consistency could have contributed to systematic non-differential misclassification bias. Lee et al. (2018) reported that, at 4 weeks postpartum and using a 14% PMN threshold in endometrial cytology, the sensitivity and specificity of PMN count for the classification of endometritis were 31.3 and 81.7%, respectively. Druker et al. (2022) reported a sensitivity of 42% and a specificity of 68.6% when using a 4% PMN threshold at 30–40 days in milk, based on pregnancy risk at 180 DIM. Therefore, the Diff-Quick counts were the greatest bias concern for systematic non-differential misclassification of endometritis cows. However, these risks were mitigated through the implementation of standardised protocols for sample collection, staining and slide preparation, along with comprehensive rater training prior to the start of the study. Also, as a qualitative bias assessment, such misclassification typically biases results towards the null, potentially underestimating true differences. However, due to the dependence of PMN and macrophage counts in endometrial cytology, the possibility of bias in either direction cannot be excluded (Dohoo, 2014).

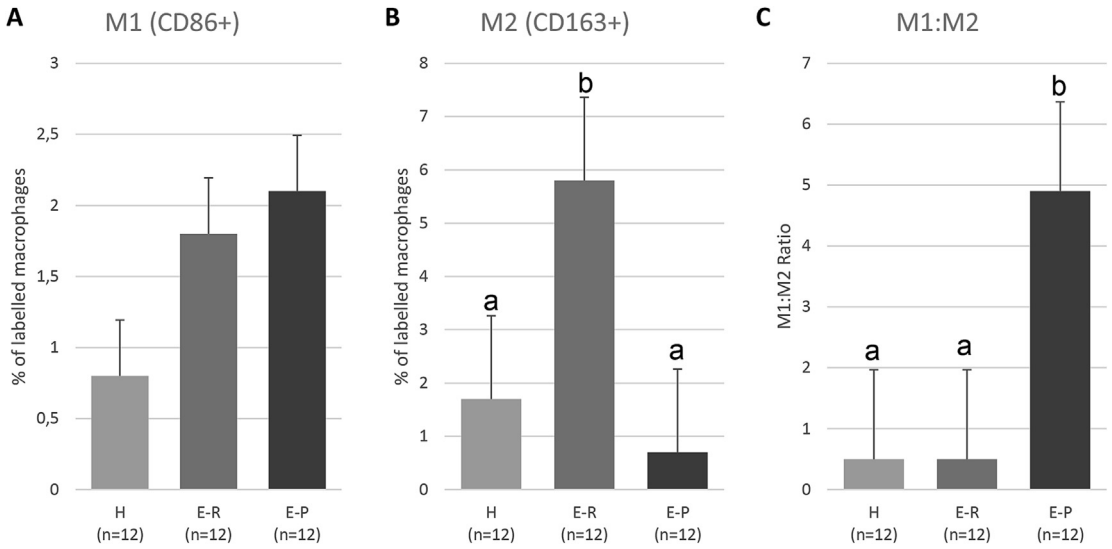

Fig. 4. Characterisation of macrophage (Mφ) phenotype by immunocytochemistry (ICC) of uterine cytology slides of cows with distinct uterine health status at 21 days postpartum: H – Healthy; E-R - Endometritis Recovered; E-P - Endometritis Persistent. Proportions of Mφ M1 phenotype (A), Mφ M2 phenotype (B) and M1:M2 ratio (C). Different letters indicate a significant difference at $P < 0.05$.

Identification of Mφ populations in uterine cytology Diff-Quick –stained slides was based on morphology, and therefore, only foamy Mφ were counted. To confirm Mφ identification and evaluate Mφ phenotype, ICC was also performed in uterine cytology slides using Mφ protein markers. Total Mφ counts in Diff-Quick– and ICC–stained slides were strongly correlated at 21 and 42 DPP, illustrating that Diff-Quick staining is an accurate technique to determine Mφ counts, and these cells can be evaluated together with PMN in routine uterine cytology procedures. The ICC labelling allowed for the differentiation between M1 and M2 phenotypes. A strong correlation was also found between Diff-Quick– and ICC –stained M2 at 21 and 42 DPP, but these correlations were only moderate, although significant, with M1 cells. The pro-resolution M2 phenotype has a "foamy" morphology, extended cytoplasm, higher nucleocytoplasmic ratio and is larger than the pro-inflammatory M1 phenotype (Ehmedah et al., 2019 ; Lerouge et al., 2023). Therefore, Diff-Quick staining is probably more efficient in the identification of phenotype M2 than M1.

At 21 DPP, E-R cows showed higher M2 proportions than H and E-P cows, and E-P cows presented a higher M1:M2 ratio than H and E-R cows. As the M2 phenotype is actively involved in inflammatory pro-resolution mechanisms, a lack of polarisation into the M2 phenotype or an excessive polarisation towards the pro-inflammatory M1 phenotype in early postpartum could predispose to persistence of inflammation. The M2 phenotype is activated following an inflammatory stimulus (e.g. bacteria, PMN, damaged cell, etc.), generating pro-resolution lipid metabolites and vacuoles (Grajchen et al., 2018).

Swabbing for uterine cytology is usually obtained from *corpus uteri* and concerns were raised that immune cells' distribution might not be representative of the whole uterus (Pascottini et al., 2016). The pellet of uterine fluid collected through a low-volume flushing of the previously gravid uterine horn provides a representative sample of cells/protein of the postpartum uterus. Therefore, the protein content of CD86 (M1) and CD163 (M2) was evaluated through WB analysis. Although this analysis was underpowered due to a lower representation of E-P cows, densitometry analysis showed that E-R cows had CD163 bands with higher intensity than bands from H and E-P cows. These findings give further evidence that the abundance of the anti-inflammatory macrophage phenotype M2 seems to be crucial to the resolution of uterine inflammation.

The Mφ counts in uterine cytology samples at 42 DPP were low. However, uterine cytology does not allow evaluation of immune cell population dynamics in deeper layers of the endometrium. To evaluate Mφ presence and phenotype within the endometrium at 42 DPP, biopsy-derived sections were processed for IHC. The counts of M2 were higher in E-P than in E-R cows, denoting a later presence of Mφ in the endometrium and a delayed pro-resolving path, associated with the persistence of endometritis.

In conclusion, this is a foundational study on the role of macrophages on the spontaneous resolution or persistence of endometritis in postpartum dairy cows. Results evidence that an early postpartum endometrial influx of macrophages is associated to recovery, whereas an absent or late influx is associated to persistence of endometritis. Results also evidence a link between endometrial macrophage phenotype and recovery or persistence of endometritis. A predominant M2 phenotype favours recovery, whereas a high M1:M2 ratio favours persistence, suggesting a link between an absent or delayed macrophage polarisation and persistence of inflammation. These results prompt for studies to unveil the macrophage-driven pro-resolving mechanisms at the cellular and molecular levels and their interaction with other players of uterine inflammation. In cows with endometritis at 21 DPP, the proportion of Mφ in uterine cytology Diff-Quick–stained slides was able to discriminate between cows that spontaneously recovered and those where endometritis persisted until 42 DPP. However, further studies are needed to validate the role of Mφ and the phenotype M2 as markers of postpartum endometritis recovery or persistence, considering large-scale evaluations in different production systems, cow breeds and climatic conditions. This could be of high relevance for therapeutic decision-making at diagnosis of endometritis (21 DPP), avoiding unnecessary antibiotic treatment and emergence of antimicrobial resistance, or allowing immediate treatment of cows prone to persistence of endometritis. Although routine Diff-Quick–stained uterine cytology slides were equally suitable for the counting of PMN and macrophages, identifying these latter cells requires some training. Therefore, it is important to standardise macrophage evaluation, enabling the training of veterinarians to perform this evaluation across multiple farms worldwide.

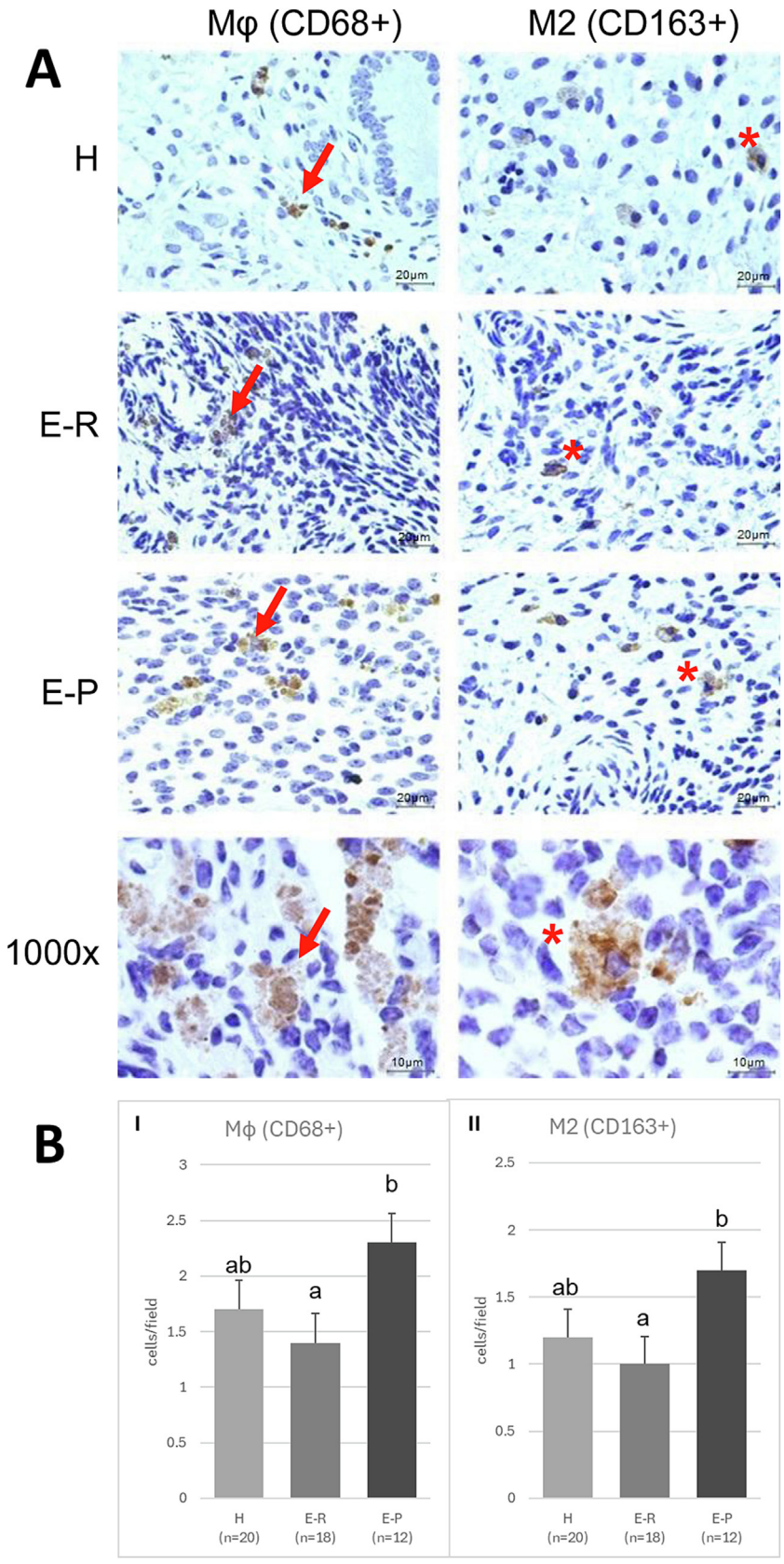

Fig. 5. A - Representative photomicrographs of immunohistochemistry (IHC) labelled total macrophages (Mφ; CD68+) and M2 phenotype (CD163+) in endometrial sections from biopsies of cows with distinct uterine health status (400x; last row – 1 000x). Images were acquired using a microscope (Olympus, BX51) equipped with a digital camera (Olympus, DP21). Labelled Mφ CD68+ (↑) and M2 CD163+ (*) phenotype. B - IHC counts of total Mφ (I) and M2 (II) at 42 days postpartum. Different letters indicate a significant difference at $P < 0.05$. Cow groups: H – Healthy; E-R – Endometritis Recovered; E-P – Endometritis Persistent.

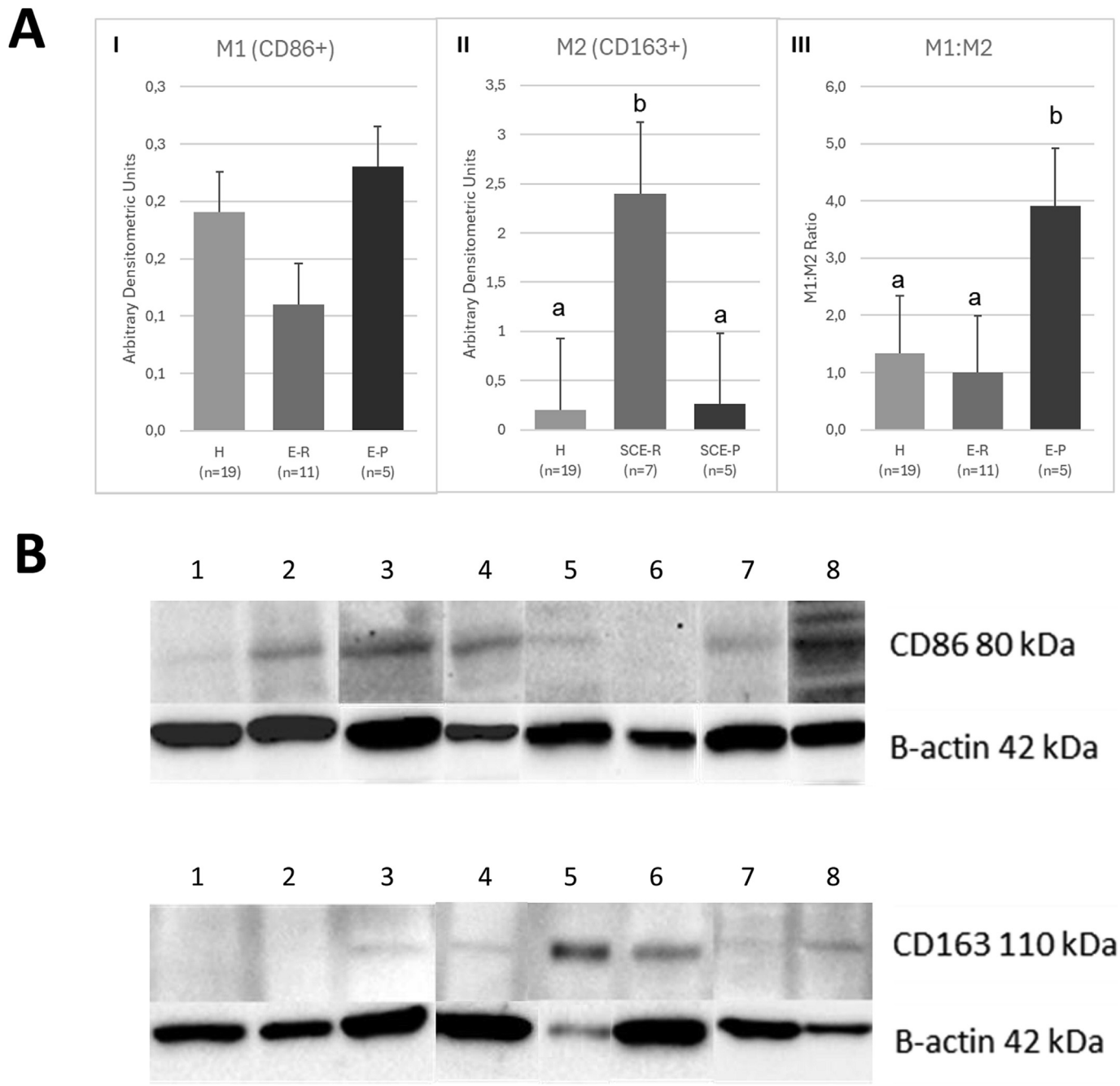

Fig. 6. A - Western blot (WB) analysis of cellular pellet of low-volume flushing of uterine fluid (I-III). WB protein abundance analysis: macrophage phenotype M1 (CD86+) (I), macrophage phenotype M2 (CD163+) (II) and M1:M2 ratio (III); different letters (a,b) indicate a significant difference at $P < 0.05$. B – Representative blots from groups of cows: H (Healthy; lanes 1–3), E-R (Endometritis Recovered; lanes 4–6) and E-P (Endometritis Persistent; lanes 7–8). Upper row – CD86 (total macrophages); lower row – CD163 (M2 macrophages). Image acquisition with ChemiDoc MP system and Image Lab 4.1 software XRS + (BioRad, Hercules, CA). Original WB in Supplementary Figure S4 (I-IV) and S5 (I-II).

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101581) can be found at the foot of the online page, in the Appendix section.

Ethics approval

The project was approved by the Institutional Animal Care and Use Committee (CEIE: Comissão de Ética para a Investigação e Ensino, Lisboa, Portugal; Reference CEIE n°36/2020). All clinical procedures were conducted in compliance with the European Union legislation to use animals for experimental purposes (Directive 2010/63/UE).

Data and model availability statement

The data/models were not deposited in an official repository. Information can be made available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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