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Research paper

Impact of tissue enzymatic digestion on analysis of immune cells in mouse reproductive mucosa with a focus on γδ T cells

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ABSTRACT

Mucosal tissues are enriched in γδ T lymphocytes, which maintain epithelial homeostasis, however, the homeostatic mechanisms are still incompletely understood. To elucidate their role in the tissue integrity governance within the female genital mucosa we employed flow cytometry, which is a powerful tool used for the characterization of tissue-resident immune cells, however, often requiring cell release upon tissue enzymatic disaggregation. Here, we analyzed the impact of various proteolytic enzymes in their ability to effectively isolate viable immune cells from the reproductive system of non-pregnant mice. Murine vaginas and uteri were digested using commercially available enzyme blends (liberases) and single enzymes (dispase II and collagenase IV). Among tested enzymes, liberases released the highest number of cells from digested tissues while dispase II and collagenase IV led to a significant decrease in the number of isolated live cells. Also, liberases had only minor detrimental effects on cell viability and detection of CD45, CD3ε, γδ TCR and CD11c positive cells. We found that a single liberase blend called Liberase TL was the most suited for the analysis of γδ T cells in the reproductive tract. By examining two distinct phases of the estrous cycle – estrus and diestrus, characterized by high and low epithelial stratification, respectively, we showed that higher numbers of γδ T lymphocytes were present in the latter cycle phase in vagina and uterus. Interestingly, the diestrus-associated increase in γδ T lymphocyte number was also observed in reproductive tract draining lumbar lymph nodes but not in more distant, inguinal lymph nodes. Our data indicate that enzymes used for reproductive mucosa digestion have profound effects on the cell viability and isolation efficiency, which consequently influence the phenotypic and quantitative analysis of immune cells.

1. Introduction

Mucosal tissues remain under strict surveillance by the immune system in order to protect the organism against any harmful insults. One of the most important immune cell populations within mucosa are intraepithelial T lymphocytes (IELs) and their subset expressing a T cell receptor with γ and δ chains (γδ TCR) (Nielsen et al., 2017). The biology and functions of γδ T cells still remain largely unclear, however, published studies show that mice deficient in this particular cell population have defects in regulation of epithelial functions, such as increased carcinogenesis, diminished wound healing or increased permeability of epithelia (Nielsen et al., 2017). Within vaginal epithelium prevail IELs with Vγ6-Vδ1 TCR (Vγ4-Vδ1 according to Garman's nomenclature (Garman et al., 1986)) (Rakasz et al., 1996) and it is suggested that they play a role in epithelium self-renewal mechanisms and in antiviral responses since viral infections were shown to induce proliferation of γδ T cells but not αβ TCR-expressing lymphocytes in the vaginal epithelium (Nishimura et al., 2004; Rakasz et al., 1999). Using tissue clearing and microscopy methods we have previously shown that vaginal γδ T cells respond to sex hormone-induced epithelial changes during the estrous cycle as their number during epithelium thinning in diestrus increased with concomitant re-distribution toward apical epithelial layers (Mikolajewicz and Chodaczek, 2019).

Clearing-based novel microscopy techniques are very powerful in determining cell localization, however, they require the use of transgenic fluorescent reporter mice for analysis of selected cell populations. Moreover, since multiple reporter mice are not common, typically a visualization of only a single cell type is possible. Also, immunolabeling is not always compatible with clearing, especially for weakly expressed antigens (Richardson and Lichtman, 2015). By contrast, using

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multicolor flow cytometry, many surface and intracellular markers can be examined simultaneously in non-transgenic mice without the time-consuming and expensive breeding of reporter mice. However, preparation of tissue for analysis requires a reliable digestion protocol that balances efficient cell liberation with tissue degradation, enhances cell yields and protects cell surface markers from digestion. While mechanical disintegration of spleen or lymph nodes is sufficient for isolation of lymphocytes (Autengruber et al., 2012), release of stromal and dendritic cells from mucosal tissues requires a combination of mechanical dissection and enzymatic digestion (Fidel Jr. et al., 1996). Thus, the characterization of immune cell populations from the reproductive tract is challenging in particular. This is due to a lower number of immune cells compared to epithelial and stromal cells, especially in the estrus phase, when the epithelium thickens and becomes stratified (White et al., 2000). Most published protocols rely on mechanical dispersion with or without prior digestion of tissues. Collagenase or dispase in combination with DNase are commonly used enzymes in processing and isolation of immune cells from mouse uterus (Pinget et al., 2016; Kaushic et al., 1998; Blaisdell and Erlebacher, 2014). However, there is no standard protocol for the digestion of the vagina. In this study, in addition to standard collagenase IV and dispase II preparations, we chose liberases, which are enzyme blends consisting of highly purified type I and II collagenases and a neutral protease (thermolysin or dispase).

The goal of the present study was to establish a protocol for the isolation of immune cells from uterus and vagina with a focus on γδ T cells. To our knowledge, this is the first study comparing the effectiveness of enzymatic digestion on cell release in reproductive mucosa during the estrous cycle. We performed qualitative and quantitative cellular analysis in the vagina and uterus during the two most dominant and distinct phases of the cycle, estrus and diestrus. Our data demonstrate that liberases, and Liberase TL in particular, excel other tested enzyme preparations and enable a reliable quantitative analysis of γδ T cells in the reproductive tract.

2. Materials and methods

2.1. Mice

Female 8 weeks old C57BL/6 J mice were purchased from the Experimental Medicine Center, Medical University of Bialystok (Bialystok, Poland). Tcrd-H2BeGFP transgenic mice (Prinz et al., 2006) were a kind gift of Dr. Immo Prinz (Institute of Immunology, Hanover Medical School, Hanover, Germany). The mice were housed at the PORT Polish Center for Technology Development in Wroclaw, Poland in individually ventilated cages in 12:12 h light-dark cycle under specific pathogen-free conditions with water and food available *ad libitum*. All experiments were in accordance with the Local Ethics Committee for Experiments on Animals at Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw (No 58/2015).

2.2. Hormone treatment

Female mice were used for experiments in estrus or diestrus phase after performing vaginal smears using a cotton swab (Cora et al., 2015; Gonzalez, 2016). The smears were stained with a Cytocolor kit (Merck, Germany) as previously described (Maj et al., 2014) (Supplementary Fig. 1). Also, to induce a synchronized estrus or diestrus stage mice were treated with fertility hormones. Mice were subcutaneously injected with 3 mg of medroxyprogesterone acetate (Lanza et al., 2010) (Depogeston, Biowet Pulawy, Pulawy, Poland) to induce diestrus or 100 ng of estradiol benzoate (O'Brien et al., 2006) (Cayman Chemical Company, Ann Arbor, MI, USA) in sesame oil (MP Biomedicals, Warsaw, Poland) to induce estrus.

2.3. Reproductive tract digestion

After confirmation of the estrous cycle phase, mice were euthanized by cervical dislocation. Vaginas were rinsed three times with PBS (50 µl) to remove cornified epithelium in estrus and mucus in diestrus (Supplementary Fig. 1). Reproductive tracts were dissected, divided into vagina and uterus (cervix and uterine horns) and placed on ice in PBS (ThermoFisher Scientific, Waltham, MA, USA). Six different enzymes were used for tissue digestion: Liberase DH, Liberase TL, Liberase TM, Liberase TH (Roche, Mannheim, Germany; 5.2 U/mg), Collagenase IV (BioShop, Burlington, ON, Canada; > 125 U/mg) and Dispase II (Roche, ≥ 0.8 U/mg). Liberases are named according to the thermolysin or dispase content: TH - thermolysin high, TM - thermolysin medium, TL - thermolysin low or DH - dispase high. All enzymes were prepared at 1 mg/ml concentration in Hank's Balanced Salt Solution (HBSS, ThermoFisher Scientific) supplemented with 30 µg/ml DNase I (grade II, from bovine pancreas, Roche). Vagina and uterus were minced separately into small fragments in a 2 ml Eppendorf tube using curved dissection scissors and incubated with enzymes or in PBS (undigested) at 37 °C for 1 h with shaking (Eppendorf ThermoMixer C, 800 rpm). After digestion, tissues were passed through a 40 µm cell strainer (Corning, Corning, NY, USA) placed in a 50 ml conical tube, washed in PBS and centrifuged at 300 ×*g* for 5 min at 4 °C. The pellet was resuspended in 3 ml of RPMI 1640 (Sigma Aldrich, Saint Louis, MO, USA), transferred to 3 ml of Lympholyte-M Cell Separation Media (1.875 ± 0.001 g/ml, Cedarlane Laboratories, Burlington, NC, USA) in a 15 ml conical tube and centrifuged at 300 ×*g* for 20 min at RT (accel 2, brake 1, Eppendorf 5810R Series Centrifuge, Rotor A-4-62). The cells at the interface were carefully removed using a serological pipette, transferred into a new tube and washed with PBS. Single-cell suspensions were then stained and analyzed by FACS (fluorescence-activated cell sorting). Typically, the preparation of cells from 5 mice took approximately 3 h and the FACS staining lasted additional 1.5 h.

2.4. Preparation of lymph nodes

Isolated lumbar and inguinal lymph nodes were pressed through a 40 µm cell strainer (Corning), washed in PBS and centrifuged at 300 ×*g* for 5 min at 4 °C. Single-cell suspensions were then stained and analyzed by FACS.

2.5. Flow cytometry

The cell suspensions were incubated with Fixable Viability Dye eFluor 780 (1:1000, eBioscience, Waltham, MA, USA) for 30 min at 4 °C to determine cell viability. Next, the cells were washed two times with PBS and stained with fluorochrome-labelled antibodies for 30 min at 4 °C in 100 µl of staining buffer (PBS + 2% FBS (Sigma Aldrich) + 2 mM EDTA (Gibco, Waltham, MA, USA)). Fluorochrome-conjugated anti-mouse antibodies used for extracellular staining included: Alexa Fluor 647 CD3ε (1:200, clone 145-2C11, Biolegend, San Diego, CA, USA), Pacific Blue CD45.2 (1:200, clone 104, Biolegend), PE TCR γδ (1:400, clone GL3, Stemcell Technologies, Vancouver, Canada), Alexa Fluor 488 CD11c (1:200, clone N418, Biolegend) or PE CD3 (1:300, clone 17A2, Biolegend), Pacific Blue CD45.2 (1:200, clone 104, Biolegend) and Alexa Fluor 647 TCR γδ (1:300, clone GL3, Biolegend). After incubation, the cells were washed twice in PBS and then fixed in 1% PBS-buffered formaldehyde overnight. Before FACS analysis samples were washed twice in PBS. Absolute counts in each sample were determined by CountBright absolute counting beads (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Appropriate isotype controls were used to determine specific staining. Compensations beads were used to perform the compensation (UltraComp eBeads, Invitrogen). Samples were analyzed using LSR Fortessa (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Gating strategies for cells identification are presented in Supplementary Fig. 2 and Supplementary Fig. 3.

2.6. Statistical analysis

Experiments were conducted at least two times with similar results. Figures show representative results. Statistical analysis was performed using the non-parametric Mann-Whitney *U* test (two-tailed) as detailed in figure captions. The *p*-value < 0.05 was considered statistically significant. All statistical analysis was performed with GraphPad Prism v6 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Effects of enzymatic digestion on isolation efficiency and cell viability

To characterize the vaginal and uterine immune cells, we first had to optimize the protocol for liberating immune cells from the genital tract taking into account the estrous cycle phase. As mentioned earlier, sex hormones modulate the metabolic status of epithelial cells and change the number of epithelial cell layers, which may lead to different live cell yields in respective cycle phases. Isolated and devoid of mucus vaginas and uteri from mice in estrus or diestrus, were first mechanically minced with scissors, then incubated with or without tested enzymes and, finally, pushed through a 40 µm cell strainer. Cell suspensions from undigested and enzyme-treated organs were then compared by analyzing the absolute number of cells obtained from a single tissue. The cell number in each sample was determined by CountBright absolute counting beads. As shown in Fig. 1, the phase of the estrous cycle greatly affected the number of isolated cells. Except for collagenase IV and dispase II, we were able to receive more cells in diestrus than in estrus. Also, processing of uteri led to release of higher number of cells compared to vaginas. Surprisingly, the mechanical dispersion alone produced more cells than digestion with collagenase IV and dispase II.

Knowing that tissue processing can significantly affect cell viability we decided to determine how many live cells can be obtained using isolation protocols that we were testing. Viable cells were identified by gating on cells that were not stained with Fixable Viability Dye eFluor 780, used for dead cell exclusion (Supplementary Fig. 2). Fig. 2a, b demonstrates that the method of tissue preparation strongly influenced the proportion of live cells, which ranged from 52% to 95% in the vagina and from 56% to 98% in the uterus.

Cells obtained from tissues subjected only to mechanical disaggregation were characterized by high viability (median value was 79%). To better visualize the effects of enzymes on the cell status we normalized results to the undigested group (Fig. 2c, d). Crude collagenase IV and dispase II treatment substantially reduced the cell viability, which was particularly evident in estrus. Interestingly, treatment of vaginas and uteri with liberases led to significantly higher viability of isolated cells, but only in diestrus.

3.2. Effects of enzymatic digestion on cellular composition of the reproductive tract

Following the enzymatic treatment, we performed FACS analysis of isolated cells stained for the most common markers of immune cells that are frequently found in the reproductive tract, such as CD45 (leukocytes), CD3ε (T lymphocytes), CD11c (dendritic cells, macrophages, granulocytes) and γδ TCR (γδ T cells). The number of isolated cells from a particular population after enzyme treatment was compared to the corresponding cell number from undigested tissues, which was arbitrarily set to 100%. The color-coded heat map in Fig. 3 demonstrates that, in general, liberases enhanced the accessibility of antibodies to their cognate antigens on cells with some exceptions in diestrus. Collagenase IV and dispase II treatment markedly reduced the number of $CD45^+$ leukocytes and their subpopulations, especially in diestrus. Surprisingly, we did not observe this detrimental activity in case of γδ TCR marker, except for collagenase IV in the vagina.

When we looked directly at proportions of isolated leukocyte populations we found that the percentage of $CD45^+$ cells was lower in vagina than in uterus and it changed during the estrous cycle (Fig. 4a, c). In vagina, the proportion increased in diestrus while we did not observe any significant changes in the percentage of leukocytes in uterine cells between estrus and diestrus. Moreover, $CD11c^+$ cells dominated $CD45^+$ population in the vagina during estrus, less so in diestrus (Fig. 4b). We also found that in diestrus there was a clear increase in the proportion of vaginal γδ T lymphocytes and other $CD3^+$ cells, irrespective of the enzyme type (at least in case of $CD3^+$ marker). Surprisingly, after treatment with Liberase TM and TH in diestrus and Liberase TH and collagenase IV in estrus, the vaginal γδ T lymphocyte population was not visible at all. In mechanically dispersed uteri there was a large proportion of $CD11c^+$ cells which decreased after enzymatic processing leaving $CD45^+$ $CD11c^-$ $CD3^-$ cells as a dominant population irrespective of the cycle phase (Fig. 4b, d). The precise phenotypic characterization of these cells is a subject of our future studies. We did not see similar changes in uterine lymphocyte proportions as in vagina.

3.3. Effects of enzymatic digestion on analysis of vaginal γδ T cells during the estrous cycle

Consistent with Fig. 5a-c, we found that isolation and numbers of detected γδ T cells varied depending on tested enzymes, reproductive tract compartment, and the cycle phase. All liberases were able to

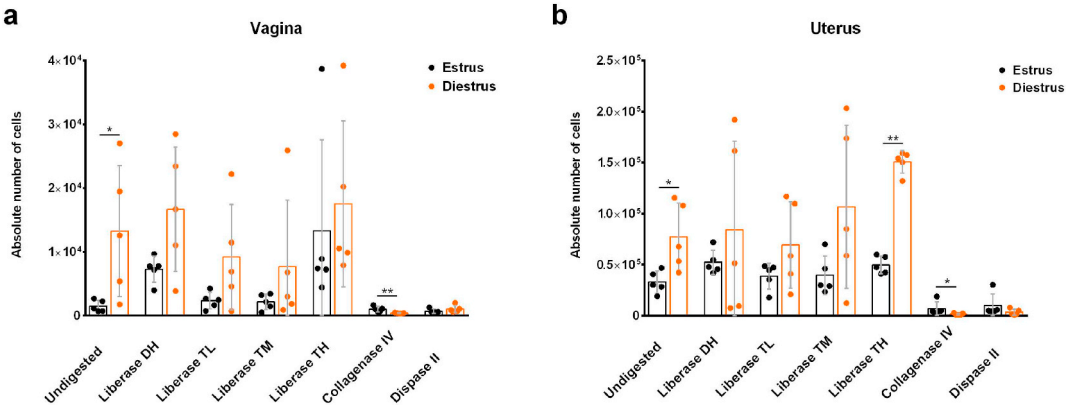

Fig. 1. Efficiency of cell release using different isolation protocols. Graph shows absolute number of cells after digestion of vagina (a) and uterus (b) (see Supplementary Fig. 1, the "Cells" gate). Each bar represents a median (with 25%–75% percentile range) from 5 mice in estrus (black spots and bars) and diestrus (orange spots and bars). $*p < 0.05$, $**p < 0.01$, Mann-Whitney *U* test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

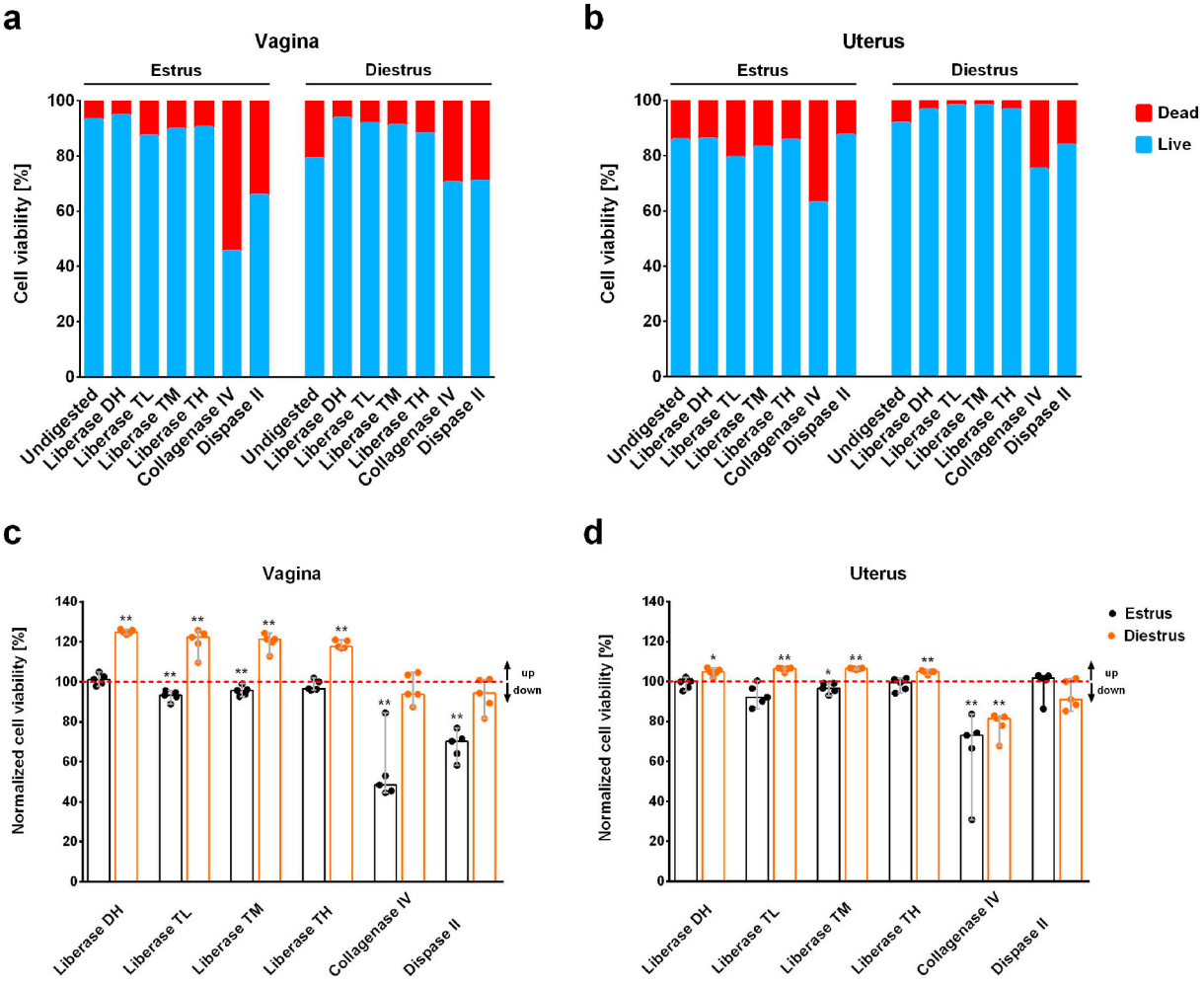

Fig. 2. Effects of the isolation protocol and the estrous cycle phase on the cell viability. Graphs show the cell viability in (a) vagina and (b) uterus based on the number of live single cells ("live cells" gate) with regard to the number of objects in the "Cells" gate (see Supplementary Fig. 1). Each bar represents a median from 5 mice. (c, d) Cell viability was normalized to undigested samples. Results are represented as a median (with 25%–75% percentile range) of the normalized number of live single cells from vagina and uterus in estrus (black spots and bars) and diestrus (orange spots and bars). $*p < 0.05$, $**p < 0.01$, Mann-Whitney U test (comparisons with a respective undigested condition). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

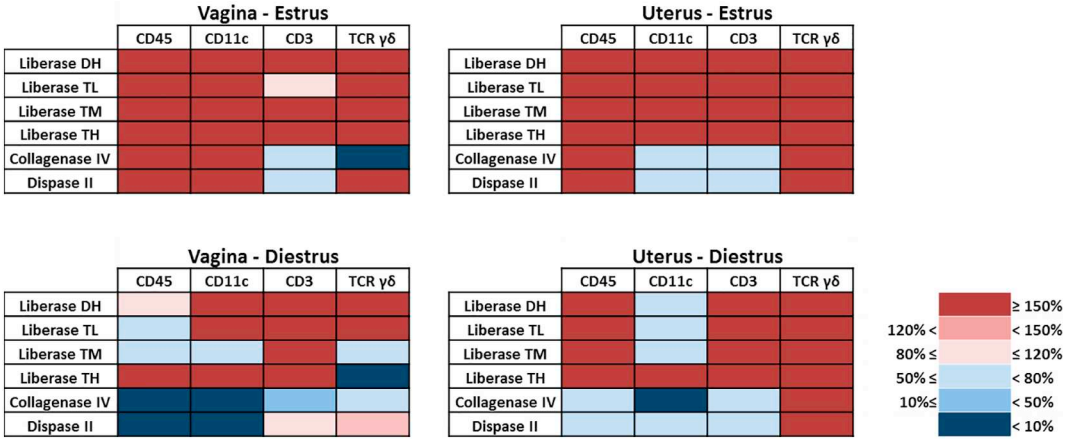

Fig. 3. Effects of tissue enzymatic digestion on the number of released live immune cells from the reproductive tract. Vaginas and uteri were disaggregated using different protocols and isolated cells were stained with antibodies specific for selected surface markers and analyzed by FACS. The number of positive cells obtained after enzymatic digestion was compared to the cell number from undigested vaginas or uteri, which was arbitrarily set to 100%. Each block in the map is based on a mean value from 5 mice. Red hue indicates elevated surface level following the enzymatic digestion, blue indicates reduced surface level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

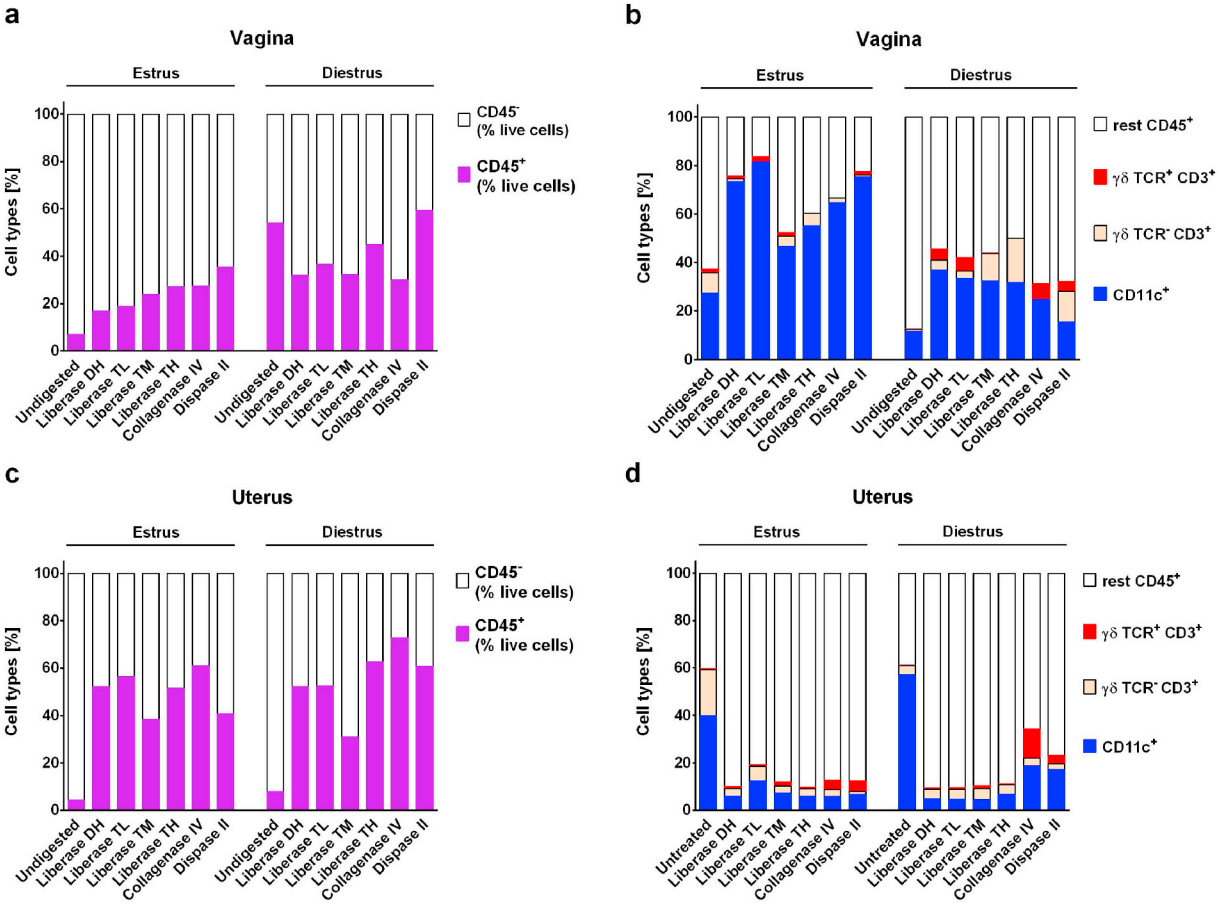

Fig. 4. Cellular composition after enzymatic digestion of vagina and uterus. Bar graphs (a) and (c) show the percentage of vaginal and uterine $CD45^+$ cells in the "live cells" gate, respectively. Bar graphs (b) and (d) show the percentage of $CD11c^+$, γδ TCR^- $CD3^+$ and γδ TCR^+ $CD3^+$ cells in the "$CD45^+$ cells" gate. The medians from 5 mice for each treatment are shown.

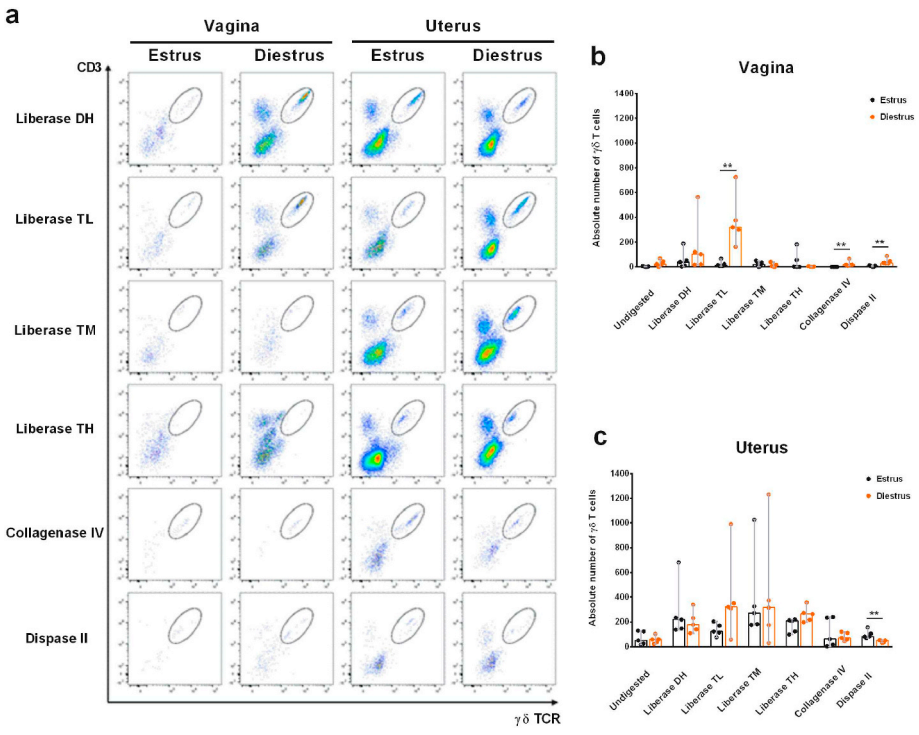

Fig. 5. Changes in γδ T cell population in vagina and uterus during the estrous cycle after enzymatic digestion. (a) Representative flow cytometry dot plot graphs for all tested enzymes showing the γδ T cell populations. Graphs (b) and (c) show the absolute number of γδ T cells in vagina and uterus of C57BL/6 J mice, respectively. Each spot represents an individual mouse. Results are represented as a median (with 25%–75% percentile range) in estrus (black) and diestrus (orange). $^{**}p < 0.01$, Mann-Whitney *U* test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

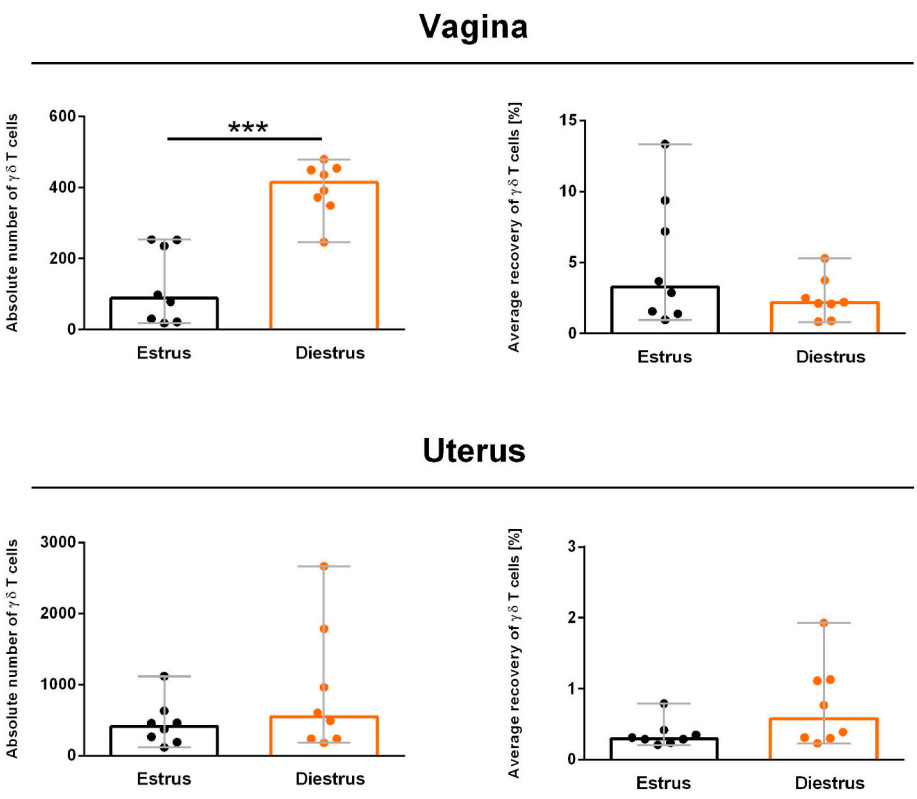

Fig. 6. Comparison of γδ T cell population in vagina and uterus during the estrous cycle after Liberase TL digestion. Results are represented as a median (with 25%–75% percentile range) of the absolute number of γδ T cells or the average recovery of γδ T cells (the percentage of cells in the "live cells" gate) in vagina and uterus. Each spot represents an individual Tcrd-H2BeGFP mouse in estrus (black) and diestrus (orange). ***$p < 0.001$, Mann-Whitney U test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

release the cells much better than collagenase IV and dispase II in the uterus, while in vagina only Liberase TL was comparably efficient, however, only in diestrus. The number of vaginal γδ T lymphocytes increased during diestrus reaching statistical significance in case of Liberase TL, collagenase IV and dispase II.

We also took advantage of Tcrd-H2BeGFP mice, which are γδ T cell fluorescent reporters on the C57BL/6 J background with green fluorescent protein (GFP) expressed in nuclei of γδ T lymphocytes (Prinz et al., 2006). Supplementary Fig. 3 confirms that almost all GFP-expressing cells in the reproductive tract express γδ TCR. We wanted to compare γδ T cell yields based on TCR staining and GFP signal. GFP-based detection led to slightly higher γδ T cell counts when we used Liberase TL as the most efficient enzyme blend (Fig. 6). We were able to obtain 88 γδ T cells (median value; 23–248 – 25%-75% percentiles, respectively) from a single vagina in estrus and 414 γδ T cells (median value; 355–453 – 25%-75% percentiles, respectively) in diestrus. In uterus upon Liberase TL treatment, we observed a similar trend in γδ T cell number changes, but the difference between the cycle stages was not statistically significant (Fig. 6). The recovery of γδ T cells was in the range of 0.3% to 4% of isolated live cells.

To verify whether the hormone-induced changes in γδ T cell number pertain only to the reproductive tract, we also analyzed $CD45^+$ and γδ T cell populations in lymph nodes (Supplementary Fig. 4). Based on the comparison of lumbar lymph nodes, which drain the reproductive tract, and more distant inguinal lymph nodes, we found that the number of γδ T cells increased in the former in diestrus, whereas in the latter it remained unchanged during the estrous cycle. The number of $CD45^+$ leukocytes increased in both lymph node sites in diestrus.

4. Discussion

The goal of this study was to establish a protocol for immune cell isolation from uterus and vagina enabling analysis of γδ T cells. Numerous enzymatic digestion-based protocols for cell release from various tissues have been described in the literature, however, there are large differences between enzyme combinations, working concentrations, incubation times, target tissues and analyzed markers, making it difficult to compare results and make general conclusions. In our studies, we decided to test the activity of a series of commercially available enzyme blends (liberases) and commonly used in tissue processing enzymes such as collagenase IV and dispase II. Mechanical disaggregation with scissors and a cell strainer was used as a control procedure to avoid potential digestion of surface markers on cells of interest. We analyzed separately two compartments of the reproductive tract: vagina and uterus, both consisting of stroma and epithelium. Similarly to the epidermis, vaginal epithelium is stratified, while in uterus there is only a single layer of cells. We also investigated how cell isolation efficiency is affected by sex hormones, which regulate the status of epithelium during the estrous cycle. In the estradiol-dominated estrus phase, epithelial cells proliferate, become stratified and form a cornified layer, which is apically positioned. In diestrus, when estradiol level is low, cornified cells are shed and the epithelium becomes thin being approximately seven cells thick (Mikolajewicz and Chodaczek, 2019).

Our data show that the phase of the estrous cycle greatly affected the number of obtained cells. In spite of the larger tissue thickness in estrus, the cell yield was much lower compared to diestrus and the reference enzymes (collagenase IV and dispase II) were even less effective than mechanical processing. This could be possibly explained by stronger cellular connections within stratified and keratinized layers and their insensitivity to collagenase and dispase. Alternatively, endogenous matrix metalloproteinases secreted during diestrus could explain the overall larger number of cells being recovered in undigested control uteri (Curry Jr and Osteen, 2001). Isolation method described by Itohara et al. (Itohara et al., 1990), which utilized 0.5% trypsin and 30% Percoll gradient for digestion of vagina in the diestrus phase, yielded approximately 1×10^4 cells from each vagina (Rakasz et al., 1999). Using liberases (Liberase DH and TH, in particular) we obtained $> 1.5 \times 10^4$ cells from a single vagina in diestrus, which was almost two times more cells compared to estrus.

We suspected that proteolytic enzymes can differently affect the release of various populations of cells, thus, we carried out surface staining of cells obtained from tissues after mechanical disaggregation

or after enzymatic digestion. We compared three common markers such as CD45, CD3ε, CD11c and also γδ TCR, which is present on γδ T lymphocytes. We found that collagenase IV and dispase II treatment markedly reduced the number of detected $CD45^+$ leukocytes in both reproductive tract compartments, especially in diestrus. These findings are consistent with previous reports, where dispase digestion of rat central nervous system (Ford et al., 1996) and mouse spleens (Autengruber et al., 2012) substantially reduced the detectable levels of a number of leukocyte cell surface markers. We did not observe any significant changes in the percentage of leukocytes among uterine cells in estrus and diestrus. This was in contrast to a work by Diener et al. (Diener et al., 2016) who reported that the population of leukocytes within uterus changed in number during the estrous cycle, with maximum numbers observed in the estrus. Interestingly, the proportion of vaginal $CD11c^+$ cells decreased in diestrus. This could be explained by an influx of unidentified yet subpopulation of $CD45^+$ cells or migration of $CD11c^+$ from the vagina to the closest draining lymph node, which would change cell proportions in the tissue. Also, compared to estrus, we noticed an increase in T lymphocyte content in diestrus and the highest absolute number of isolated γδ T cells was found upon tissue processing with Liberase TL. Surprisingly, liberases with medium and high thermolysin content were much less effective in γδ T cell release or γδ TCR marker preservation.

Our finding of γδ T cell number fluctuations during the estrous cycle stays in agreement with our previous microscopic study of the optically cleared reproductive tract (Mikolajewicz and Chodaczek, 2019). Using two-photon microscopy and dedicated image analysis we found that during the diestrus phase the number of γδ T cells in the vaginal wall increased compared to estrus. The proportion of γδ T lymphocytes residing in epithelium and stroma remained constant, however, irrespective of the cycle phase, and was close to 3:1, respectively. In this work, we also noticed a moderate increase in γδ T cell number in the uterus in diestrus, albeit not statistically significant. This was also supported by others who showed that the proportion of γδ T cells in the non-pregnant uterus was the highest in diestrus and the lowest in proestrus (Pinget et al., 2016). In contrast, another study revealed that relative abundance of leukocytes within the uterine tissue changed over the course of the estrous cycle, with maximum numbers observed in estrus (Diener et al., 2016). Differences between these studies could probably result from different isolation protocols based on collagenase D (Pinget et al., 2016) and collagenase I (Diener et al., 2016).

The sex hormone-induced changes in γδ T cells were also shown in lumbar lymph nodes, which drain the reproductive tract. The cell preparation did not involve any enzymatic processing, thus, any potential digestion of surface markers can be excluded. At this point, however, it is not clear whether the increase in the number of γδ T lymphocytes in these nodes in diestrus resulted from their local proliferation, migration out of vagina or accumulation of peripheral blood γδ T cells. Future studies focusing on the analysis of γδ TCR repertoire in the reproductive tract will hopefully address the true identity of γδ T lymphocytes.

5. Conclusions

Altogether our data indicate that enzymatic digestion of mucosal tissues such as vagina and uterus can have profound effects on the cell isolation efficiency and surface marker availability with direct consequences for phenotypic FACS analysis. We found that Liberase TL is the most suitable enzyme for the analysis of the lymphocyte population in the vagina and uterus in both examined hormonal stages. Collagenase IV and dispase II were much less effective in the reproductive tract digestion than all tested liberases. We believe that presented in this paper protocol can contribute to a better understanding of the biology of immune cells in the reproductive tract.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112665.

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