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Technical Note

Optimized isolation of renal plasma cells for flow cytometric analysis

Sandra Schaffer[a,c], Andrea Maul-Pavicic[a], Reinhard E. Voll[a,b,*,1], Nina Chevalier[a,*,1]

[a] Department of Rheumatology and Clinical Immunology, Medical Centre - University of Freiburg, Faculty of Medicine, 79106 Freiburg, Baden-Wuerttemberg, Germany
[b] Center of Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, Germany
[c] Faculty of Biology, University of Freiburg, Freiburg, Germany

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ABSTRACT

Plasma cells (PCs) secrete antibodies and play an essential role in protective immunity, but also in pathogenesis of antibody-mediated diseases. Physiologically, PCs mainly reside within bone marrow and spleen. In autoimmune diseases such as systemic lupus erythematosus (SLE) autoantibody-producing PCs can also be found at sites of inflammation, e.g. in nephritic kidneys. Therefore, efficient methods are required to reliably analyze and compare PCs at different sites. Flow cytometry and ELISpot analyses are frequently employed for PC characterization and require the preparation of single cell suspensions. To that end, enzymatic digestion is commonly used to isolate immune cells from solid organs like kidneys, occasionally also from lymphoid organs. In this study we show that enzymatic digestion using collagenase may lead to a loss of certain surface markers, e.g. the PC markers CD138 and CD267 (TACI). Therefore, we established an optimized protocol for preparing renal single cells by merely applying mechanical tissue disruption. Omitting enzymatic digestion, this method enables a reliable characterization of viable renal PCs by flow cytometry and cell sorting. We further show that mechanic cell preparation is favorable for lymphocytic immune cell enrichment, while enzymatic disruption improves the yield of digitating or stroma cell populations.

1. Introduction

Analysis and purification of immune cells from lymphoid but also non-lymphoid organs by flow cytometry or bead-based methods are routinely used in immunology. In autoimmune diseases, immune cells can infiltrate and damage non-lymphoid organs. For instance, autoantibody-secreting plasma cells (PCs) can be detected within inflamed kidneys of SLE patients and murine lupus models such as NZB/W F1 and MRL/lpr mice (Starke et al., 2011; Sekine et al., 2004; Espeli et al., 2011). To investigate their role, infiltrating immune cells need to be released from organ tissue. This is often achieved by combined mechanical and enzymatic disruption, in some cases followed by enrichment using Percoll density gradients (Starke et al., 2011; Espeli et al., 2011; Moore et al., 2013; Pisitkun et al., 2012; Berry et al., 2017; Kruger et al., 2004). In the literature, various enzymatic approaches including different types and concentrations of collagenase can be found. Enzymatic digestion is generally performed at 37 °C for 20 up to 60 min. A few protocols have been described for enzyme-free release of immune cells from fibrous organs (Nistala et al., 2016; Ascon et al., 2006). Such approaches are of particular importance when enzymatic digestion adversely affects detection of cell surface markers. Here, we show that the commonly used procedure of enzymatic digestion with collagenase can impair detection of the common PC markers CD138 and CD267 (TACI) (Pracht et al., 2017). For this reason, we developed an exclusively mechanical tissue disruption protocol allowing reliable detection, isolation and analysis of PCs by flow cytometry. We further compared different mechanic versus enzymatic tissue dissection techniques in terms of cell viability, yield of total cells, leukocytes and immune cell populations.

2. Materials and methods

2.1. Animals

Female, proteinuric MRL/lpr or NZB/W F1 mice were used in this study. The study was performed in accordance to institutional guidelines for animal care and was approved by the Regierungspräsidium Freiburg.

2.2. Preparation of renal single cell suspensions

Animals were sacrificed and perfused with PBS. Subsequently,

* Corresponding authors.
E-mail address: nina.chevalier@uniklinik-freiburg.des (N. Chevalier).
[1] Equal contribution.

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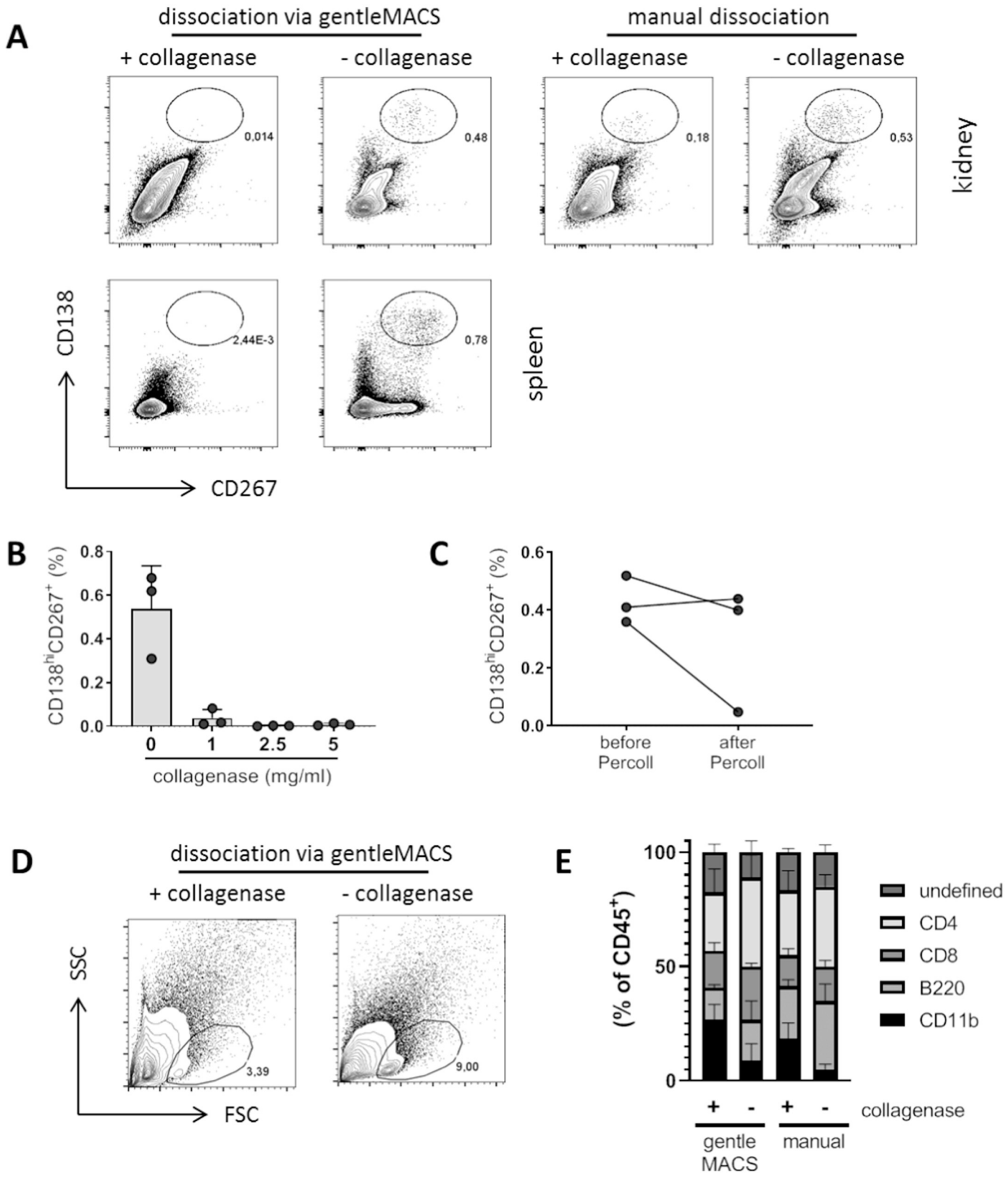

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Fig. 1. Collagenase treatment impairs detection of plasma cell (PC) markers CD138 and CD267 and improves the release of non-lymphocytic cells.
(A, D, E) Flow cytometric analysis of renal and splenic tissue, which was exclusively mechanically disrupted (− collagenase) or in combination with enzymatic digestion (+ collagenase) using 1 mg/ml collagenase. Single cell suspensions were prepared either manually or using the gentleMACS™ Dissociator. Data are representative of at least 3 mice. (A) Representative flow cytometry plots of renal and splenic single cells (doublets excluded). PCs are commonly defined as $CD138^{hi}CD267^{+}$ cells of viable Zombie-negative lymphocytes. (B) Kidneys were digested or not with 1, 2.5 or 5 mg/ml collagenase and were disrupted using the gentleMACS™ Dissociator. Shown are scatter plots of the percentage of $CD138^{hi}CD267^{+}$ cells/ viable lymphocytes (doublets excluded). Results are depicted as mean + SD with each symbol representing one mouse. (C) Renal tissue was disrupted exclusively mechanically using the gentleMACS™ Dissociator. Part of the cells was further purified by Percoll gradient and expression of $CD138^{hi}CD267^{+}$ on viable Zombie-negative lymphocytes was determined before and after gradient purification. (D) Representative flow cytometry plots of renal cell suspensions showing the percentage of lymphocytes/ whole cells. Note that the lymphocyte gate is larger than usual, since PCs are larger than e.g. B cells and also exhibit higher granularity. (E) Stacked bars showing the mean percentage + SD of $CD4^{+}$, $CD8^{+}$, $B220^{+}$ and $CD11b^{+}$ cells from viable $CD45^{+}$ kidney-infiltrating leukocytes.

kidneys were removed, decapsulated, cut into 4 equal pieces (from two kidneys per donor), were minced using a scalpel and transferred into 4 ml R10 (RPMI 1640 with 10% FCS and 50 U/ml penicillin together with 50 µg/ml streptomycin) supplemented or not with 1–5 mg/ml Collagenase IV (Life Technologies). Tissue dissection was either performed using C Tubes (Miltenyi) and the gentleMACS™ Dissociator (Miltenyi) or manually by crushing non-digested or enzymatically digested (30 min at 37 °C) minced kidney or spleen pieces using a syringe plunger. For mechanical kidney disruption with C Tubes, the following programs were applied at room temperature (RT): m_lung_01_02 (duration: 37 s, rounds per run: 168) two to three times, followed by m_lung_02_01 (duration: 38 s, rounds per run: 2083) and m_spleen_04_01 (duration: 60 s, rounds per run: 1270). For enzymatic kidney dissection with C Tubes, the program m_lung_01_02 was run two to three times prior to collagenase digestion followed by the program m_spleen_04_01 after digestion. In both cases, cell suspensions were passed through a 70 µm followed by a 40 µm cell strainer (Greiner Bio One). After one washing step with PBS/2% FCS, cells were counted using a Neubauer chamber. Where indicated, leukocytes were further enriched using Percoll gradient (Easycholl, Biochrom) separation.

2.3. Flow cytometry

Up to 2.5×10^6 cells were first incubated with anti-CD16/32 (Clone: 93, BioLegend) to block unspecific binding to Fcγ receptors, followed by light protected staining of surface markers for 30 min at 4 °C. We performed cell analysis using BD LSR Fortessa and FlowJo software (Treestar).

2.4. Antibodies and reagents for flow cytometry

For flow cytometric staining the following antibodies were used: BV421-conjugated anti-CD138 (clone: 281–2, BioLegend), PE-conjugated anti-CD267 (clone: ebio8F10–3, eBioscience), FITC-conjugated anti-CD4 (clone: GK 1.5, BD), PerCP conjugated anti-CD8a (clone: 53–6.7, BioLegend), eFluor 506-conjugated anti-CD45 (clone: 30-F11, eBiosciences), PE-conjugated anti-CD11b (clone: M1/70, BioLegend), Pacific Blue-conjugated anti-CD45R/B220 (clone: RA3-6B2, BioLegend). 123count eBeads (affymetrix, eBioscience) and Zombie NIR (BioLegend) were employed according to manufacturer's instructions for absolute flow cytometry based counting of cells and dead cell discrimination, respectively.

2.5. Enzyme-linked immuno spot assay (ELISpot)

For detection of IgG secreting cells, ELISpot plates ($MultiScreen_{HTS}$-IP, Merck) were pre-wet with 70% ethanol for 1 min followed by three washing steps. All washing steps were performed with PBS. Subsequently, plates were coated with goat anti-mouse IgG antibodies (H + L specific, polyclonal, Jackson ImmunoResearch) diluted in PBS overnight at 4 °C and were washed four times the next day. Unspecific binding sites were blocked using PBS/2% FCS for at least 1 h at RT and cells were incubated at appropriate cell numbers for 2 h at 37 °C with 5% CO_2. Following five washing steps, bound secreted IgG molecules were detected with an Fc γ-chain specific goat anti-IgG antibody coupled to horseradish peroxidase molecules (polyclonal, Jackson ImmunoResearch) diluted in PBS/2% FCS for 1 h at RT. After four washing steps, the precipitating substrate TMB (KPL) was added to make spots visible. The enzyme-substrate reaction was stopped by multiple washing steps with H_2O from both membrane sides. Plates were dried overnight protected from light and were scanned using an ELISpot Reader (C.T.L. ImmunoSpot®).

3. Results

3.1. Impaired PC detection upon dissection of kidney tissue with collagenase

We compared detectability of renal PCs by flow cytometry using combined CD138 and CD267 staining (Pracht et al., 2017) applying different kidney dissection strategies. Strikingly, enzymatic digestion with collagenase led to strongly impaired CD138 and CD267 staining (Fig. 1A/B). Notably, manual dissociation markedly reduced CD138 and CD267 detectability, which was completely lost when the gentleMACS™ Dissociator technique was applied (Fig. 1A, upper panel). We could confirm impaired staining of PC surface markers upon collagenase treatment also in splenic tissue (Fig. 1A, lower panel). Furthermore, by digesting renal tissue with collagenase concentrations ranging from 1 to 5 mg/ml (Fig. 1B), we could demonstrate that even low concentrations of collagenase strongly impair PC detection. We also examined the influence of Percoll gradient separation on PC detectability as that is often employed for PC purification from solid organs. This additional step did not influence staining of CD138 and CD267, but led to significant cell loss (Fig. 1C, S1C).

3.2. Influence of tissue dissection methods on cell viability and cell counts

We further compared the influence of the different tissue dissection methods on cell viability, whole cell counts as well as immune cell subset retrieval. With regard to total cell counts, collagenase digestion combined with dissociation by the gentleMACS™ Dissociator led to a 2.8-fold increase of total cells, which was not observed when collagenase digestion was combined with manual dissociation (Fig. S1A, left). In contrast, collagenase treatment only slightly increased the number of total leukocytes ($CD45^{+}$) both for dissociation with the gentleMACS™ Dissociator as well as for manual dissociation (Fig. S1A, middle), while frequencies of lymphocytes were increased upon mechanical compared to enzymatic disruption (Fig. 1D, S2).

In case of sole mechanical dissection, manual versus gentleMACS-based generation of single cell suspensions increased total cell counts, whereas there was no difference between these two techniques when combined with enzymatic digestion (Fig. S1B, left). In contrast, there was no difference regarding the yield of $CD45^{+}$ viable leukocytes when manual versus gentleMACS-based tissue dissociation was performed (Fig. S1B, middle).

Generally, neither tissue dissection method significantly affected the viability of cells as determined by the use of Zombie dyes (BioLegend) for live/dead cell discrimination (Fig. S1A/B, right). Conversely, leukocyte enrichment by Percoll gradient reduced cell viability (Fig. S1C,

right) and total as well as total viable CD45$^+$ cell counts (Fig. S1C, left and middle). The latter was even more pronounced for CD45$^+$ cell counts and cell viability when using the gentleMACS™ Dissociator without enzymatic digestion.

We also analyzed how the different dissection methods influence retrieval of the most important immune cell populations among CD45$^+$ leukocytes, including B220$^+$ B cells, CD4$^+$ and CD8$^+$ T cells as well as CD11b$^+$ cells, the latter comprising mainly innate immune cells such as monocytes, dendritic cells and neutrophils (Fig. 1E, S2). We observed a consistent relative increase of CD11b$^+$ cells in collagenase-treated kidneys, which was accompanied by a relative decrease of especially T and to a lesser extent B lymphocytes. This effect was more pronounced after cell isolation using the gentleMACS™ Dissociator.

4. Discussion

The investigation of PCs at different sites requires standardized isolation and staining protocols. Our newly established protocol is based on mere mechanical, non-enzymatic disruption of renal tissue. It allows in a fast and economical way to reliably detect CD138^{hi}CD267$^+$ PCs by flow cytometry.

In our study, we could clearly show that enzymatic digestion with collagenase impairs the detection of the PC markers CD138 and CD267, which are critical for PC identification and purification. We assume this can be most likely explained by cleavage within the extracellular part of the surface molecules and removal of the epitopes recognized by the antibodies upon collagenase digestion. We can exclude that collagenase treatment alters PC viability or susceptibility to apoptosis (Fig. S1A, right). This is further emphasized by the fact that collagenase-treated PCs are still able to secrete immunoglobulins, independent of the collagenase concentration (Fig. S3).

Comparison of cells from different sites requires equal dissection protocols. Commonly, PCs from lymphoid organs such as spleen or bone marrow are isolated without enzymatic digestion. Therefore, avoiding collagenase is mandatory for reliable comparison of renal PCs to those from lymphoid organs. Moreover, renouncing collagenase is less cost-intensive and reduces processing time. Likewise, we did not see any advantage in employing time-consuming purification by Percoll gradient separation as it leads to higher variability and significant cell loss.

While problematic for PC detection, collagenase treatment may be beneficial when CD11b$^+$ leukocytes or stroma cells are to be isolated. The fact that collagenase treatment increased the number of total cells, but not that of viable CD45$^+$ leukocytes, suggests that enzymatic digestion particularly improves the release of stroma and tubular cells, which are usually tightly attached to the matrix. Likewise, within CD45$^+$ leukocytes the yield of digitate CD11b$^+$ cells was improved over lymphocytic cells (Fig. 1E, S2). The yield of total cells and these immune cell populations could be further increased when enzymatic tissue dissection by gentleMACS™ Dissociator was employed (Fig. 1E, S1A, S2). We assume this may be due to standardized tissue disruption prior enzymatic digestion and thereby improved collagenase accessibility and efficiency. Likewise, poorer enzymatic tissue digestion in manually compared to gentleMACS-processed tissues may also account for the observed complete loss versus reduction of CD138/CD267 staining for PCs (Fig. 1A).

In summary, preparing single cell suspensions from kidneys with the above described protocol is a gentle and much faster method than such involving enzymatic digestion and allows valid and accurate cytometric analysis and purification. Generally, sole mechanical disruption should be applied whenever sensitive surface markers are used to analyze or isolate/ purify cells, either by flow cytometric cell sorting or magnetic bead-assisted cell isolation. Using devices such as the gentleMACS™ Dissociator have the additional advantage of providing a user-independent, standardized procedure for preparing single cells.

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Declaration of Competing interests

The authors declare no commercial or financial conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.019.

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