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Research paper

Phenotypic characterization and functional analysis of human tumor immune infiltration after mechanical and enzymatic disaggregation

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ABSTRACT

Multi-parametric flow cytometry analysis is a reliable method for phenotypic and functional characterization of tumor infiltrating immune cells (TIIC). The isolation of infiltrating leukocytes from solid tumors can be achieved through various methods which can be both enzymatic and mechanical; however, these methods may alter cell biology. The aim of this study was to compare the effects of three tissue disaggregation techniques on TIIC biology in breast, kidney and lung tumor specimens. We therefore compared two enzymatic treatments using either collagenase type IA alone or in combination with collagenase type IV and DNase I type II, and one mechanical system (Medimachine™). We evaluated the impact of treatments on cell viability, surface marker integrity and proliferative capacity. We show that cell viability was not significantly altered by treatments. However, enzymatic treatments decreased cell proliferation; specifically collagenases and DNase provoked a significant decrease in detection of surface markers such as CD4, CD8, CD45RA and CD14, indicating that results of phenotypic studies employing these techniques could be affected. In conclusion, mechanical tissue disaggregation by Medimachine™ appears to be optimal to maintain phenotypic and functional TIIC features.

1. Introduction

Solid tumors are frequently infiltrated by immune cells such as T and B lymphocytes, natural killer (NK) cells, NKT cells, macrophages, neutrophils, mast cells, dendritic cells (DC), myeloid-derived suppressor cells (MDSC) and eosinophils (de Visser et al., 2006; Grivennikov et al., 2010). These immune cells may contribute favorably or detrimentally to tumor outcome depending on their number and proportion, heterogeneity, maturation state and localization (Galon et al., 2006; Talmadge et al., 2007; Pages et al., 2010). Phenotypic and functional characterization of immune cells within tumors enables us to characterize the crosstalk between the immune cells and tumor cells. Such characterization may also serve as a prognostic marker depending on the cell types

Abbreviations: CFSE, carboxy-fluorescein succinimidyl ester; DC, dendritic cells; Mab, monoclonal antibody; MFI, mean fluorescence intensity; NK, natural killer cells; MDSC, myeloid-derived suppressor cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; TIIC, tumor infiltrating immune cells; TIL, tumor infiltrating lymphocytes.

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present in the tumor environment (Galon et al., 2006). Multiparametric flow cytometry analysis is a powerful method to perform comprehensive characterization of immune cells through evaluation of the co-expression of several surface and intracellular proteins (Moloney and Shreffler, 2008). This approach is aptly applied to cells in suspension. However, its utilization for the characterization of tumor infiltrating immune cells (TIIC) requires the extraction of the immune cells from solid tumor masses. The first tumor disaggregation protocols generating viable cell suspensions were described in the late 1970s (Hamburger and Salmon, 1977; Salmon et al., 1978; Rasey and Nelson, 1980); since then, various protocols have been elaborated (Cerra et al., 1990; Cunningham, 2010). These protocols usually involve mechanical or enzymatic disaggregation, either alone and/or in combination. Enzymatic digestion commonly involves collagenase, hyaluronidase, dispase, trypsin or DNase. Mechanical methods of tissue dissociation are based on aspiration, vortexing, scraping or tissue pressing (Cornacchiari et al., 1995; Ottesen et al., 1996; Singh, 1998; Vos et al., 2003). However, these tissue treatments could affect immune cell biology or cell epitopes, thereby causing changes in cell phenotype and altering flow cytometry profile (Engelholm et al., 1985; Stoeck et al., 1990; Sutherland et al., 1992; Mulder et al., 1994; Abuzakouk et al., 1996; Flynn et al., 1999; Novelli et al., 2000; Van Damme et al., 2000).

In this paper, we have investigated the optimal mechanical and enzymatic tissue disruption method to isolate TIIC that would allow optimal phenotypic and functional analyses. We compared two enzymatic digestion protocols, using collagenase type IA 0.03% (Col I) alone or in combination with collagenase type IV 0.1% and DNase I type II 0.001% (Col I + IV), and a mechanical disruption system using a Medimachine™ (Medi) (Novelli et al., 2000; Godin-Ethier et al., 2009) on solid tumors from human kidney, breast and lung cancer specimens. We determined that mechanical disaggregation by Medimachine™ was optimal for viability, cell surface marker integrity maintenance and T cell proliferation studies.

2. Materials and methods

2.1. Tumor and blood sample collection

Breast, kidney and lung tumor samples from patients who had recently undergone radical or partial mastectomies, nephrectomies and lung resection respectively, were collected and briefly stored in Iscove's Modified Dulbecco's Medium (Gibco, Invitrogen; Carlsbad, CA, USA). Surgery was performed at the Centre Hospitalier de l'Université de Montréal, Québec, Canada (CHUM). Breast tumor samples were provided by the CHUM/Fonds de la Recherche en Santé du Québec (FRSQ) breast cancer tissue bank of the FRSQ Cancer Network.

Peripheral blood samples were obtained from healthy donors recruited by the Division of Hematology and Immunodeficiency at the Royal Victoria Hospital (Montréal, Québec, Canada) and from cancer patients. This study was approved by the Ethics Committee of the CHUM. Informed consent was obtained from each donor or patient prior to the collection of the tumor and blood samples.

2.2. Preparation of tumor cell suspensions and TIIC extraction

All tumor specimens were kept on ice and processed within 2 h of resection. Each tumor sample was placed in a Petri dish on ice, carefully dissected free of surrounding normal and necrotic tissue and sliced into small pieces of approximately 2 mm^3 with a scalpel. To control for tumor infiltration heterogeneity, tumor fragments were mixed before separation into three portions. The first portion was mechanically disrupted while the two others were enzymatically digested. Mechanical disruption was performed using a Medimachine™ (DAKO, Cytomation; Glostrup, Denmark). Tumor fragments were placed in a sterile microblade-equipped polyethylene chamber (Medicon, BD Biosciences, San Jose, CA, USA) with 1–2 ml of RPMI 1640 medium (Wisent; St-Bruno, Québec, Canada) and dissociated 3 to 4 times for 30 s at a constant speed of 100 rpm as described previously (Novelli et al., 2000). The cell suspension was filtered through a porous polyester membrane (Filcons, BD Biosciences). After filtration, cells were washed twice with 1× Phosphate Buffer Solution, pH 7.0 (PBS, Wisent) at 300 g. Enzymatic digestions were performed on the other two pools of tumor pieces in RPMI 1640 medium. The first protocol used 0.03% of type IA collagenase (Sigma-Aldrich, Oakville, Ontario, Canada) (200 U/ml) while the second used 0.1% type IV collagenase (Sigma-Aldrich) (530 U/ml), 0.001% type II DNase I (Sigma-Aldrich) (20 U/ml) and 0.03% type IA collagenase. Generally, 5 ml of enzyme solution was required per 1 g (or less) of tissue. Digestions were performed at 37 °C for 2 h. The single-cell suspension obtained by enzymatic digestion was then passed through a 40 μm (BD Biosciences) sterile sieve to remove undigested tissue fragments and washed twice with PBS at 300 g for 5 min.

To obtain TIIC-enriched samples free of erythrocytes, tumor cells and cell debris from the mechanical disruption and enzymatic digestion, cell suspensions were purified by centrifugation over a Leukocyte Separation Medium cushion (Wisent) at 300 g for 30 min. TIIC were recovered from the interface, washed twice with PBS and stained for flow cytometry analysis.

2.3. Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated from heparinized whole blood samples of cancer patients or normal donors using Leukocyte Separation Medium (Wisent). Leukocyte separation medium (Wisent) cushion was carefully added to the bottom of the test tube containing whole blood diluted 1/2 with PBS. The mixture was then centrifuged at 300 g for 30 min and PBMC were recovered from the interface, washed twice and stained for flow cytometry analysis.

2.4. Flow cytometry analysis

Cells were first stained with the Live/Dead Fixable Dead Cell Stain Kit (Invitrogen; Carlsbad, CA, USA) to discriminate between live and dead cells. Cells were stained with the following fluorescent dye-conjugated monoclonal antibodies (Mab) from BD Biosciences: CD3 Alexa-700, CD4 Fluorescein isothiocyanate (FITC), CD8 Allophycocyanin-H7 (APC-H7), CD19 Alexa-700, CD14 Pacific Blue (PB), CD45RA Phycoerythrin

(PE), CD45RO Allophycocyanin (APC), and HLA-DR Phycoerythrin Cy7 (Pe-Cy7). Corresponding IgG isotypes were used as controls to account for non-specific binding (BD Biosciences). Before cell staining, non-specific binding sites were blocked with human gamma globulin (Jackson ImmunoResearch; West Grove, PA, USA). Cell surface markers were stained for 30 min at 4 °C, washed with staining buffer (PBS containing 1% FBS and 0.1% $NaNO_3$) and then fixed in 2% paraformaldehyde for 10 min at 4 °C. Cells were washed and resuspended in staining buffer. Flow cytometry data were acquired using an LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star). The mean fluorescence intensity (MFI) was calculated on cells stained positively with the specific conjugated Mab.

2.5. T cell proliferation

After PBMC or TIIC preparation, 8×10^5 leukocytes were washed and resuspended in serum-free RPMI 1640 medium before staining for 15 min at 37 °C with 5 μM of Carboxyfluorescein succinimidyl ester (CFSE; Molecular Probe, Invitrogen). Cells were washed and resuspended in 200 μl of complete medium consisting of Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 7.5% heat-inactivated AB serum (Gemini Bio-Products, Calabasas, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10 μg/ml gentamicin (all from Wisent), 300 IU/ml of recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA) and incubated 4 days in a plate previously coated overnight with 5 μg/ml of anti-CD3 (OKT3; eBiosciences, San Diego, CA, USA). Following incubation, cells were harvested by pipetting and proliferation was assessed by flow cytometry analysis as described above.

2.6. Statistical analysis

Statistical analyses were performed with general linear models using SPSS software version 18.0 for Windows (LEAD Technologies, Chicago, IL, USA). Multiple range comparisons of paired means were done using a Least Significant Difference (LSD) test with Bonferroni adjustment. Level of significance was set at $p<0.05$. Data is reported as mean ± SEM. Comparison between treated and untreated cells in marker and proliferation quantification was statistically analyzed by unpaired sample Student *t* test with a significance level of $p<0.05$.

3. Results

3.1. Mechanical and enzymatic disaggregation methods similarly affect cell viability

Our first objective was to evaluate TIIC viability following tumor disaggregation by either mechanical disruption using the Medimachine™ (Medi), or two different enzymatic treatments: 1) collagenase type IA (Col I); and 2) collagenase types IA and IV with DNase I type II (Col I + IV). Tumor specimens were obtained from 10 lungs, 5 breasts and 6 kidneys of cancer patients. After gating on $CD3^+$ T lymphocytes from TIIC prepared from lung (Fig. 1A), breast (Fig. 1B) and kidney tumors (Fig. 1C), no significant difference in the percentage of dead cells stained with respect to the method of tumor disaggregation ($p>0.05$) or the type of tumor ($p>0.05$) was observed. Tumor disaggregation routinely induced around 15 to 30% cell mortality (Fig. 1) but showed no statistical variation between methods. Similar observations were noted with $CD19^+$ B lymphocytes and $CD14^+$ monocytes (data not shown).

3.2. Tumor disaggregation procedures differently decrease detection of selected markers

We then attempted to evaluate the effects of tissue disaggregation directly on immune cells within PBMC, which lack the 3-dimensional architecture of a solid tumor. Consequently, these were used as genuine untreated controls for each experiment, which served as baseline for all parameters. PBMC from 5 normal donors were treated either mechanically by Medimachine™ or digested enzymatically for 30, 60, 90 or 120 min with methods previously described. The presence of eight commonly used lymphocyte and monocyte cell surface markers for PBMC identification was analyzed by flow cytometry before and after exposure to each disaggregation treatment. These markers included CD3, CD4 and CD8 (T lymphocytes), CD45RO (antigen-experienced T lymphocytes), CD45RA (naive and terminally-differentiated T lymphocytes (Arlettaz et al., 1999)), HLA-DR (activated cells), CD19 (B lymphocytes), and CD14 (monocytes/macrophages). Mechanical treatment by Medimachine™ did not induce any changes in the surface levels of any of these markers when compared to freshly isolated untreated cells ($p>0.05$; Fig. 2). The mean percentage expression of PBMC surface markers at each time point within each enzymatic digestion condition was compared to undigested samples (Fig. 2). The relative integrity of CD3 and CD45RO was not affected by the duration of digestion with Col I and Col I + IV ($p>0.05$; data not shown). Likewise, Col I treatment did not affect CD45RA ($p>0.05$; Fig. 2). However, digestion of PBMC with Col I resulted in fading of CD14 and HLA-DR to 67% and 77% of undigested controls over the first 30 min, respectively, and was time-dependent ($p<0.05$; Fig. 2). Fading was also observed for CD4 after 60 min of digestion and for CD8 and CD19 after 90 min of treatment. Globally, Col I + IV treatment provoked a decrease in surface-detected CD4, CD8, HLA-DR, CD45RA, CD14 and CD19 within 30 min ($p<0.05$; Fig. 2). Markers such as CD4, CD45RA and CD14 were more sensitive to this treatment and lost nearly 80% of initial marker levels after 120 min of incubation. Furthermore, this treatment caused a 50% decrease in CD4 T cell marker detection after 30 min of incubation. Effect on CD8, HLA-DR and CD19 was moderate but reached 65%, 67% and 76% of initial levels of control samples, respectively.

Similar experiments were performed on TIIC prepared from 10 lung (Supplementary data Fig. 1A), 5 breast (Supplementary data Fig. 1B) and 10 kidney tumors (Supplementary data Fig. 1C). Origin of TIIC (kidney, breast or lung) had no influence on the modulation of cell surface marker detection (CD3, CD4, CD19, CD14, CD45RO, CD45RA and HLA-DR) induced by the enzymatic disaggregation protocols ($p>0.05$; results summarized in Fig. 3). Reported decreases in cell surface markers were similar regardless of tissue origin. Because of variability between individual cell levels of specific markers, the collected data from each profile were normalized to the levels obtained from TIIC isolated by mechanical disaggregation. A value of 100% was assigned to TIIC isolated by Medimachine™

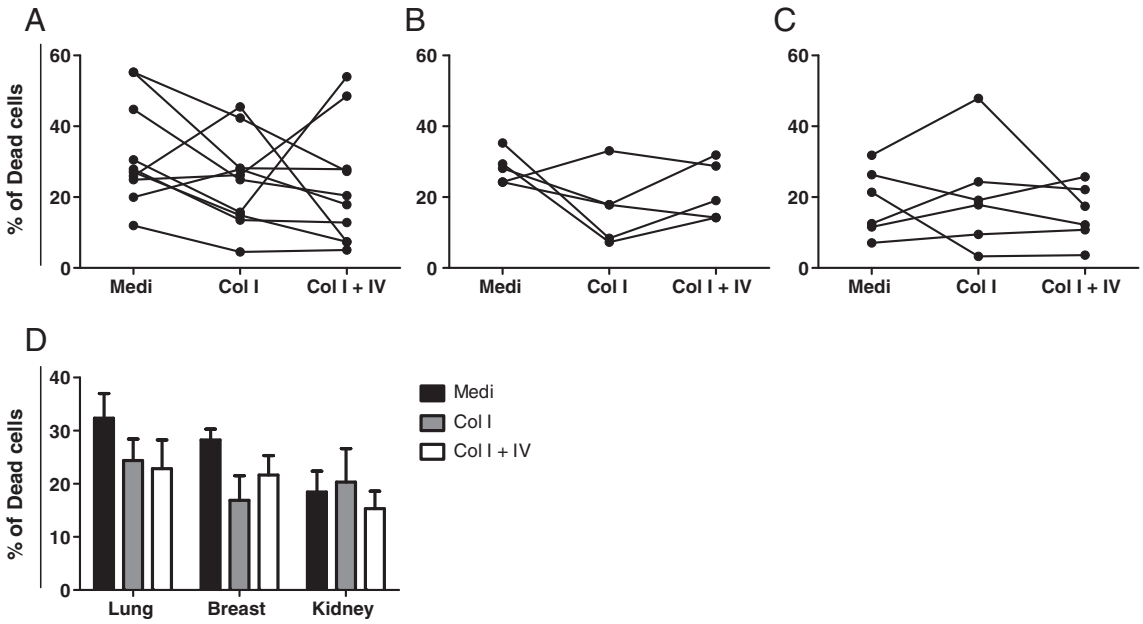

Fig. 1. Effect of disaggregation methods on cell viability. The percentage of dead lymphocytes ($CD3^+$/Dead dye^+ cells) was determined in TIIC following 3×30 s of grinding using a Medimachine™ (Medi) or 2 h of tissue digestion using two enzymatic digestion protocols (Col I or Col I + IV). Observations for 10 lung cancer patients (A), 5 breast cancer patients (B) and 6 kidney cancer patients (C) are shown. Samples from the same patient are connected by solid lines. (D) Summary of observations (mean ± SEM). Statistical significance was evaluated by the LSD test with *P<0.05.

treatment based on results from PBMC where mechanical treatment had the slightest effects on surface markers. The CD3, CD45RO, CD19, HLA-DR and CD14 markers were not significantly affected by any of the three isolation protocols ($p>0.05$; Fig. 3). Col I treatment provoked a 20% decrease in CD4 detection on TIIC compared to Medimachine™ treatment in lung tumors ($p<0.05$; Fig. 3). This represented the only significant effect noted for this digestion protocol. On the other hand, Col I + IV treatment caused stronger fading of surface markers, particularly CD4, CD8 and CD45RA ($p<0.05$; Fig. 3). This protocol caused a decrease of nearly 65% for CD4 and 40% for CD45RA. Occasionally, this digestion protocol completely abolished the detection of these markers (Supplementary data Fig. 1 panel A CD4 and panel C CD45RA). Levels of CD8 in lung and kidney tumors were also reduced by nearly 20% with Col I + IV treatment. In summary, mechanical treatment by Medimachine™ had minimal effects on cell surface markers in all samples, while enzymatic treatments, particularly Col I + IV, caused a pronounced decrease of markers such as CD4, CD8 and CD45RA.

3.3. Enzymatic treatments alter T lymphocyte proliferation

In order to evaluate disaggregation protocols' effects on cell proliferation, T lymphocytes were isolated from PBMC in heparinized whole blood samples collected from 6 normal donors and activated by CD3 stimulation in presence of IL-2. Cell proliferation was determined following 4 days of culture. We first demonstrated that the rate of cell proliferation was unchanged after mechanical treatment compared to untreated T cells, whereas enzymatic digestion, specifically Col I + IV treatment ($p<0.05$) decreased cell proliferation by 47% (Fig. 4 A, B). We also noted a decreased number of cell divisions (Fig. 4 C, D), which indicates that enzymatic treatment decreased T cell proliferation potential. Similar observations were made for T lymphocytes isolated from two kidney tumors where mechanical isolation was associated with increased T cell proliferation compared to enzymatic treatments (Supplementary data 2).

4. Discussion

In the present study, TIIC single cell suspensions were prepared from lung, breast and kidney tumors by mechanical and enzymatic tissue disaggregation. Using flow cytometry, we obtained higher and preserved cell surface marker expression with a mechanical disaggregation protocol using the Medimachine™ allowing better cellular phenotypic characterization compared to enzymatic treatments which decreased CD4, CD8, CD45RA, CD14, HLA-DR and CD19 detection.

Previous studies reported the effects of disaggregation protocols on immune cell biology from different normal tissues (Flynn et al., 1999; Van Damme et al., 2000; Van Landuyt et al., 2010) or tumors (Novelli et al., 2000) but none of the above adapted their protocols to the various tumor tissue origins. In their study, Novelli et al. studied mechanical disaggregation with and without collagenase pre-treatment in regards to phenotype of lymphocytes from cutaneous cancers (Novelli et al., 2000), but did not evaluate the effect of collagenase alone. Here, we chose to evaluate protocol consequences separately.

Enzyme treatments are widely used to obtain cell suspensions from tissues; however, mechanical methods present numerous advantages. First, the procedure takes only minutes to perform compared to enzymatic digestion which can take several hours. To truly reflect *in vivo* conditions, cells must be analyzed promptly following

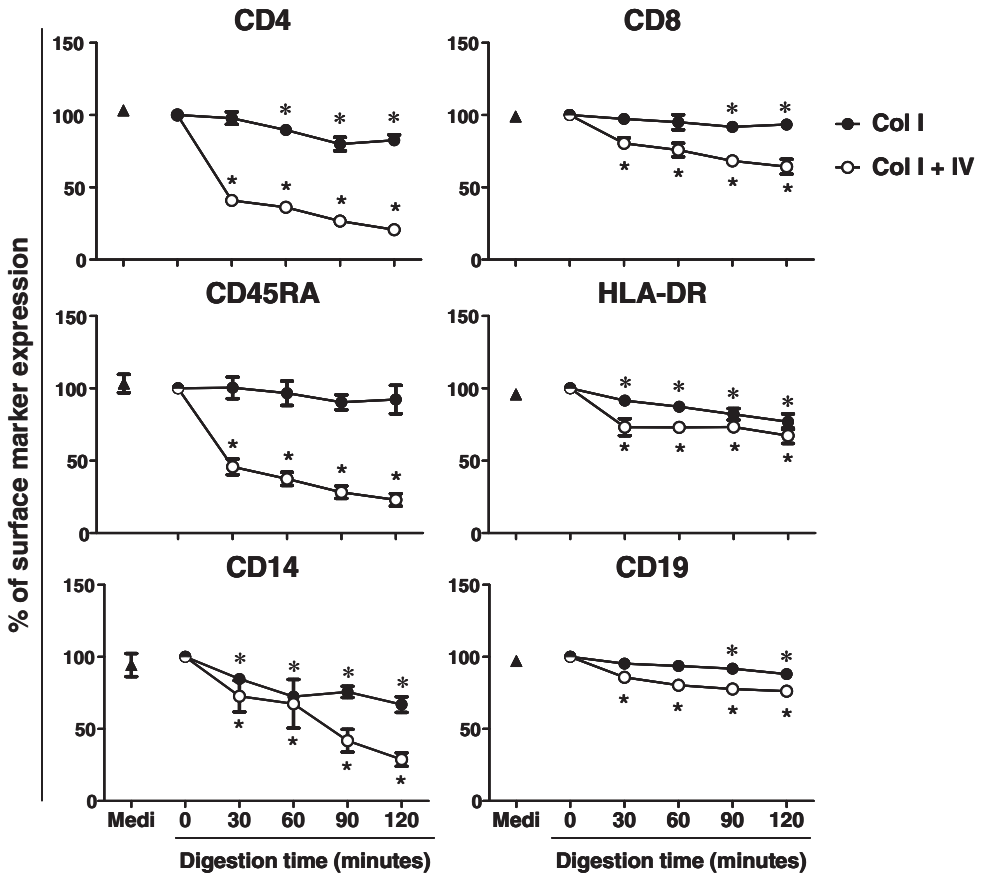

Fig. 2. Evaluation of cell surface marker detection after enzymatic treatment of PBMC. PBMC from 5 normal donors were treated by two enzymatic treatments (Col I or Col I + IV). Mean Fluorescence Intensity of surface markers was evaluated for each marker before and after treatment. Mean ± SEM is calculated on percentage of surface marker expression compared to basal expression of untreated cells. Statistical significance was evaluated by the Student *t* test with $P<0.05$ considered as statistically significant (* for Col I and * for Col I + V).

surgery to encompass original state and characteristics and minimize modifications due to lengthy manipulation. Moreover, mechanical disaggregation is independent of tissue structure and characteristics; tumors possess a wide range of physical peculiarities depending on their stage and their tissue origin. As shown in the present study, mortality induced by mechanical treatment was similar to enzymatic digestion protocols selected, irrespectively of tissue origin and cell type ($CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, $CD14^+$ monocytes).

Based on results obtained with PBMC, MFI of cell surface markers were unchanged following mechanical disaggregation with the Medimachine™ compared to untreated cells. In addition, TIIC biology appeared to be minimally affected by Medimachine™ as opposed to enzymatic treatments. Moreover, Medimachine™-treated lymphocytes obtained from PBMC were able to respond to proliferative signals to levels reminiscent of untreated cells. However, some of these observations were made only with PBMC due to a limiting number of TIIC recovered from the three isolation techniques in all experiments. Evidently, treatment of PBMC already in suspension cannot be directly compared to TIIC isolated from a complex tissue such as a tumor, however in absence of a better control, PBMC were used as the reference point. Nevertheless, results obtained for cell surface markers and proliferation with TIIC were generally similar compared with PBMC. Immunohistochemistry of paraffin-embedded clinical samples could appear as an alternative, but certain limitations prevent its use as a control of basal surface marker expression. Specifically, a slide represents a single plane of the complex tissue which cannot reflect the heterogeneity of the immune infiltrate. Therefore, extrapolations from these slides to reflect the entire TIIC population are impossible.

However, it has been demonstrated that enzymatic treatments result in better extraction yield (Novelli et al., 2000), making them attractive for cell isolation from solid tissues, especially to optimize recovery of tumor infiltrating lymphocytes (TIL) for culture suitable for adoptive transfer (Yannelli et al., 1996). Accordingly, we generally obtained more than three times the number of TIIC by enzymatic tissue digestion with Col I + IV compared to Medimachine™ but not with collagenase I alone (data not shown). This could be explained by the absence of DNase in the medium: during enzymatic digestion, release of DNA by dead cells can cause cell aggregation, decreasing the number of available TIIC. Yield can also vary with tissue composition; this parameter should thus be considered when choosing an optimal enzymatic cocktail for given tissues.

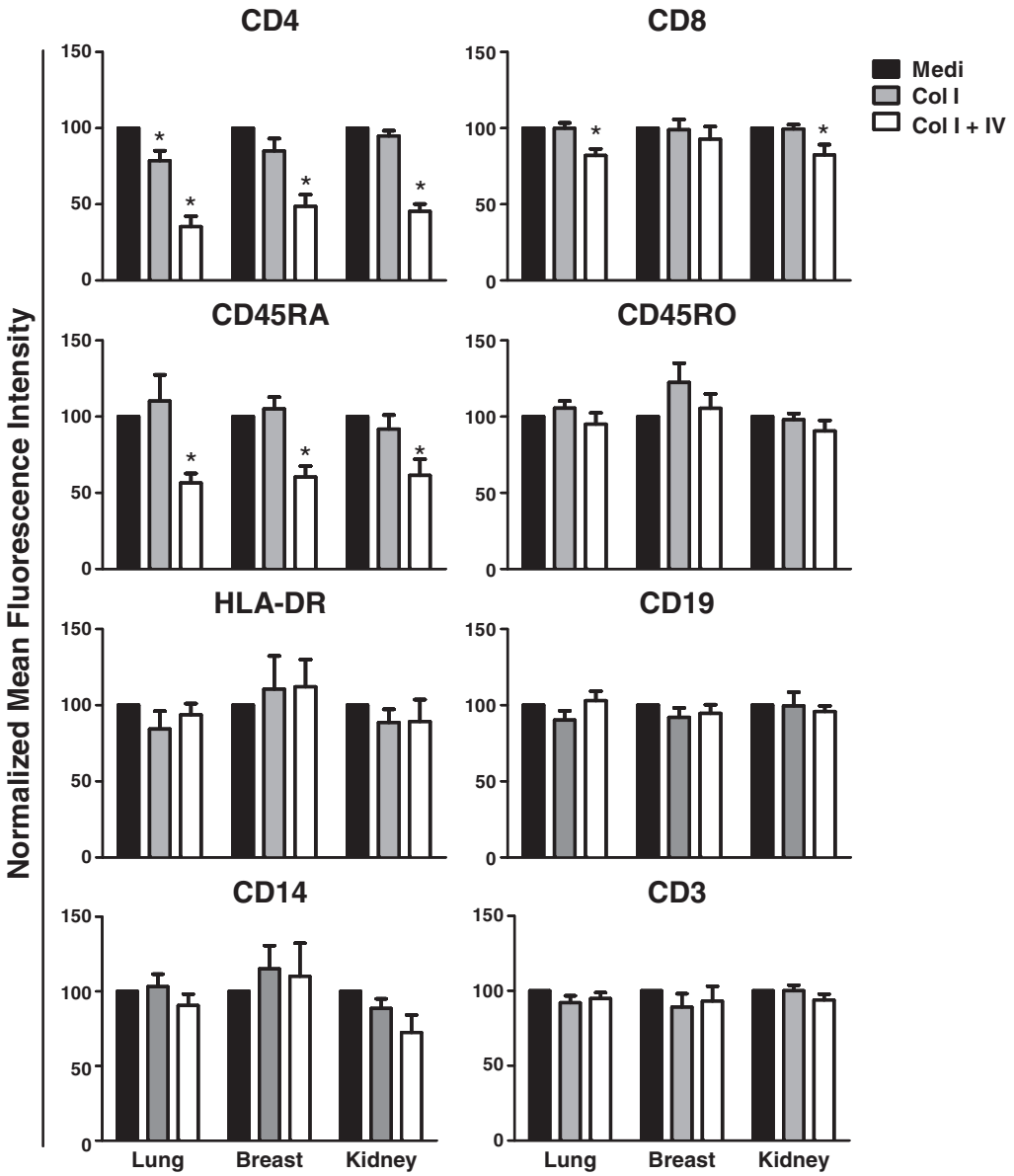

Fig. 3. Effect of disaggregation methods on TIIC surface marker detection. Tumor tissues from 10 lung, 5 breast, and 10 kidney tumors were disaggregated mechanically by Medimachine™ (Medi; black bars) or enzymatically (Col I gray bars; or Col I + IV white bars). Detection of 8 TIIC surface markers was evaluated by flow cytometry. Mean Fluorescence Intensity (MFI) was determined and normalized with mechanical disaggregation (100% of marker expression). Transformed data are represented as mean ± SEM. Statistical significance was evaluated by the Bonferroni LSD test with * $P<0.05$.

Enzymatic tissue treatment by Col I + IV induced a greater loss of marker expression, associated with a decrease in the markers' MFI, compared to Col I treatment. Differences between these two enzymatic mixtures consisted of the presence of type IV collagenase and type II DNase I. Mulder et al. (1994) and Flynn et al. (1999) previously reported that these enzymes alter cell surface markers. In their study, Mulder and colleagues reported that commercial DNase preparations are frequently contaminated with proteolytic enzymes such as proteases that recognize the amino-acid sequence of surface markers. This signal loss can be attenuated with highly purified DNase preparation. This could explain the cell surface marker loss we observed. To discriminate whether this marker loss was induced by the presence of DNase or type IV collagenase, it would be necessary to perform tissue disaggregation with a combination of type I and IV collagenase without DNase, particularly considering Van Damme et al.'s demonstration that DNase affects CD8 but not CD4 (Van Damme et al., 2000). Extensive optimization was required to establish the optimal enzyme concentration used in our study.

Given the robust nature of flow cytometry, the quality of phenotypic analysis is dependent on cell surface receptor integrity. The fading of surface markers induced by enzymatic treatments can be countered by *in vitro* culture of cells (Stoeck et al., 1990) frequently done for TIIC. This culture period has proven sufficient to fully restore basal levels of all

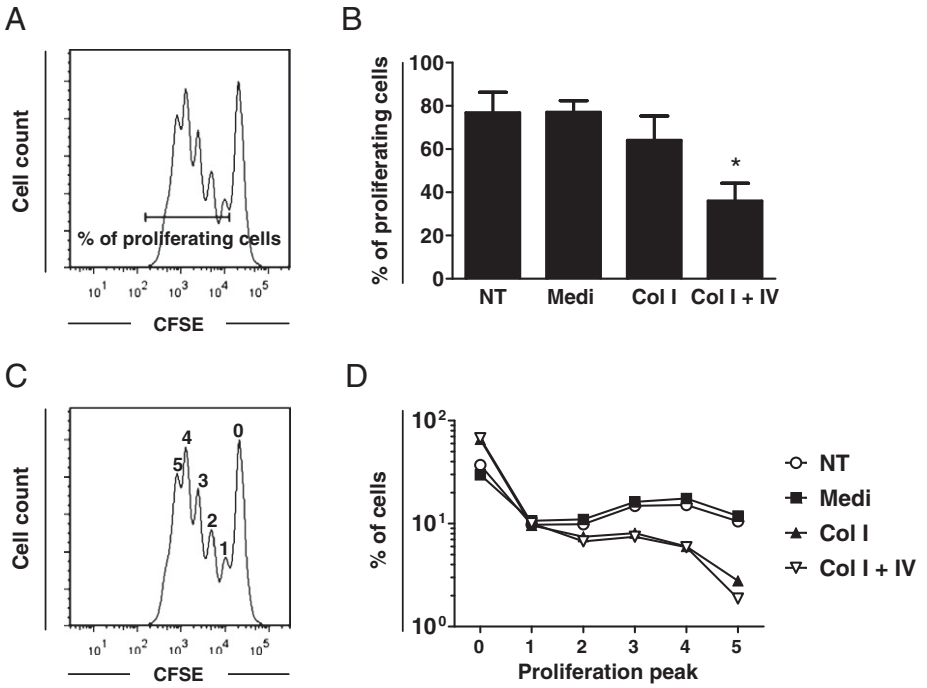

Fig. 4. Effect of disaggregation methods on T lymphocyte proliferation. PBMC from 6 normal donors were stained by CFSE and treated either by grinding using Medimachine™ (Medi) or by enzymatic digestion for 2 h using either Col I or Col I + IV, while the control cells were not treated (NT). Aliquots for each of the four treatments were treated with either IL-2 alone, or IL-2 combined with OKT3. The percentage of proliferating lymphocytes was determined by flow cytometry after 5 days in culture for each donor (A — representative graph from one donor). Percentage of proliferation for the 6 patients was then determined and the data presented as the mean ± SEM with the level of significance set at * $P<0.05$ (B). The cell proliferative capacity for each donor was determined by the percentage of cells in each division peak (C, peak 0 represented unproliferated cells). D is representative of one donor.

affected surface makers (Mulder et al., 1994) but has the disadvantage of inducing cell biology modifications due to culture. Consequently, it is critical to develop tools to analyze TIIC as soon as possible after enrichment.

In conclusion, our data highlight the fact that Medimachine™ disaggregation is an efficient method for single-cell preparation regardless of tumor origin. Although this mechanical method has its strengths and weaknesses, enzymatic tissue treatment could induce artefactual results. It would therefore be recommended that all studies using enzymes for cell isolation be minimally tested on PBMC to evaluate the treatment's impact on chosen markers.

Supplementary materials related to this article can be found online at doi:10.1016/j.jim.2011.07.002.

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