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IMPACT OF ENZYMATIC TISSUE DISINTEGRATION ON THE LEVEL OF SURFACE MOLECULE EXPRESSION AND IMMUNE CELL FUNCTION

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Immunological characterization of immune cells that reside in specific anatomic compartments often requires their isolation from the respective tissue on the basis of enzymatic tissue disintegration. Applying enzymatic digestion of primary splenocytes, we evaluated the impact of collagenase and dispase, two enzymes that are commonly used for the liberation of immune cells from tissues, on the detectability of 48 immunologically relevant surface molecules that are frequently used for flow cytometric identification, isolation, and characterization of immune cell subsets. Whereas collagenase treatment had only minor effects on surface expression of most molecules tested, dispase treatment considerably affected antibody-mediated detectability of the majority of surface markers in subsequent FACS analyses. This effect was long lasting and, in case of high-dose dispase treatment, evident for the majority of surface molecules even after 24 h of *in vitro* culture. Of note, high-dose dispase treatment not only affected surface expression of certain molecules but also impaired antigen-specific proliferation of $CD4^+$ and $CD8^+$ T cells. Together, our data indicate that enzymatic tissue disintegration can have profound effects on the expression of a variety of cell-surface molecules with direct consequences for phenotypic analysis, FACS- and MACS-based target cell isolation, and immune cell function in cell culture experiments.

Keywords: dispase, collagenase, T cell proliferation, surface molecules, enzymatic digestion, flow cytometry

Introduction

The immune system is highly compartmentalized, and the specific milieu existing in a given tissue greatly influences the phenotype and function of immune cells in the respective anatomic compartment. For instance, immune cells residing in the intestinal immune system may largely differ from those located in the peripheral blood, the skin, or the respiratory tract [1]. Therefore, the functional and molecular characterization of cellular subsets often necessitates investigation of immune cells obtained directly from tissues of particular interest. Whereas mechanical treatments are generally sufficient for isolation of cells from classical immunological tissues such as the blood, spleen, lymph nodes or thymus, high yield purification of vital cells from mucosal or other tissues often deserve the combined use of mechanical dissection and enzymatic tissue disintegration [2–7]. More recently, the immunological function of non-classical immune cells such as epithelial and endothelial cells has become increasingly appreciated. Of note, protocols to isolate these resident cells from tissues do often require even harsher conditions with respect to the nature or concentration of the enzyme to be used for efficient liberation of cells from tissues than those used for the isolation of lymphocytes or monocytes [8–10].

Enzymes typically used for tissue disintegration are collagenase and dispase I, often in combination with DNase, EDTA, and DTT to minimize cell clumping and to support cell dissociation. Although there is some evidence that these enzymes may affect the expression of surface antigens and immune cell function [6, 11, 12], many experimental approaches require cell numbers that cannot be obtained in sufficient quantity and quality by mechanical approaches alone. Enzyme-mediated reduced surface abundance of certain molecules may be a possible reason for the often observed discrepancy between data obtained by immunohistochemical staining of intact tissue, gene expression data, and data obtained by flow cytometry following surface staining of cells isolated on the basis of enzymatic tissue digestion. Moreover, since surface molecules mediate key immunological functions such as cell adhesion, pathogen recognition, and outside-in-signaling, lack of or reduced surface expression of certain molecules

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due to enzymatic cleavage may also impinge on immune cell functions giving rise to misleading results in subsequent *in vitro* cell culture assays.

Multicolor flow cytometry analysis with an increasing panel of commercially available antibodies and fluorochromes has developed a robust and reliable method for both the phenotypic characterization and the isolation of immune cell subsets from different tissues. Working with pre-clinical mouse models for intestinal and pulmonary inflammation, we routinely apply enzymatic digestion protocols for the isolation of cellular subsets from the gut and the lung [3, 7, 9, 13]. We observed that the experimental procedures applied for mucosal T cell isolation as well as for the isolation of alveolar type II epithelial cells from the murine lung, in particular dispase I treatment, may have a considerable impact on the expression level of certain surface molecules. In this study, we therefore systematically evaluated the impact of two commonly used enzymes, collagenase D and dispase I, on the detectability of as many as 48 different surface markers that are frequently used for the flow cytometric identification, isolation, and characterization of immune cell populations and/or exhibit important immunological functions. To further investigate the immunological consequences of enzyme-mediated reduction of surface marker expression, we performed *in vitro* T cell stimulation assays to directly test the impact of collagenase and dispase I treatment on T cell receptor-mediated antigen recognition and proliferation.

Materials and methods

Mice

BALB/c mice were obtained from Harlan (Borchen, Germany). Transgenic mice expressing a T cell receptor (TCR) specific for the MHC class II-restricted influenza hemagglutinin (HA) peptide 110–120 on $CD4^+$ T cells (TCR/HA) [14] or the MHC class I-restricted HA-peptide 512–520 on $CD8^+$ T cells (CL4) [15] were bred in our animal facility at the Helmholtz Centre for Infection Research, Braunschweig, and were kept under specific pathogen-free (SPF) conditions.

Cell preparation and enzymatic digestion

Spleens were rinsed with PBS to obtain single cell suspensions followed by erythrocyte lysis. Clumps and remaining tissue were removed by transfer through a 70-μm cell strainer. Splenocytes were digested with collagenase D from *Clostridium histolyticum* or dispase I from *Bacillus polymyxa* as specified in *Table 1*.

Following enzymatic digestion, cells were collected by centrifugation, enzyme containing supernatant was discarded, and cells were resuspended in IMDM supplemented with 10% FCS, 1% Pen/Strep, and 0.25 mM ß-mercaptoethanol (IMDM complete).

FACS analysis

Spleen cells (5×10^5) were stained either immediately after enzymatic digestion or after a 24 h recovery phase in IMDM complete at 37°C and 5% CO_2. Splenocytes were stained for 10 min at 4°C either directly with fluorescent-labeled antibodies or indirectly with biotin-conjugated antibodies followed by a secondary staining step with streptavidin-PE. Subsequently, cells were washed twice with PBS, fixed with 2% paraformaldehyde for 20 min at room temperature, and finally analyzed for cell surface expression of selected markers indicated in *Table 2*. Positively stained cells were quantified using a BD FACS Canto and analyzed with FlowJo software. Gates were set on single, living cells, according to their forward and sideward scatter characteristics. Percent marker positive enzyme-treated samples were normalized to marker positive untreated control samples, referred to as % expression. Unless otherwise stated, experiments were repeated three times, and one representative result out of three independent measurements is shown.

Proliferation assay

Collagenase- or dispase-treated splenocytes (5×10^5) from TCR/HA transgenic mice were plated in 96-well, round-bottom plates in a total volume of 200 μl IMDM supplemented with 10% FCS, 1% penicillin/streptomycin and 0.25 mM ß-mercaptoethanol. $CD4^+$ T cells were stimulated with HA peptide 110–120 and cultured for 24 h at 37°C. Proliferation was detected following the addition of ^{3}[H]-thymidine and culture for another 16 h at 37°C. Incorporation of ^{3}[H]-thymidine was measured by scintillation counting. Cell proliferation is presented as mean counts per minute (cpm) of triplicate wells, and one representative result out of three independent experiments with similar results is shown. In case $CD8^+$ T cell prolifera-

Table 1. Conditions for enzymatic digestion of splenocytes

Enzyme	Manufacturer	Experimental conditions
Dispase I (high dose)	BD Bioscience	200 U (50 U/ml, pre-warmed) for 45′ at room temperature
Dispase I (low dose)	Roche Diagnostics	4 U (0.8 U/ml) for 45′ at 37°C in IMDM + 5% FCS
Collagenase D	Roche Diagnostics	0.221 U (0.044 U/ml) for 45′ at 37°C in IMDM + 5% FCS

tion was analyzed, 1×10^5 collagenase- or dispase-treated splenocytes obtained from CL4 transgenic mice were stimulated with the respective peptide HA 512–520 as described above and cultured for 24 h at 37°C, and ^{3}[H]-thymidine was measured by scintillation counting 12 h after the addition of ^{3}[H]-thymidine HA-specific CD8$^+$ T cell proliferation was evaluated in triplicates; the experiment was repeated twice with similar results.

Statistical analysis

Statistical analysis was performed using GraphPadPrism Software using unpaired, two-tailed *t*-test. Statistical significance is shown as p value of < 0.05 indicated as *, $p < 0.01$ as ** and $p < 0.001$ as ***.

Results and discussion

Enzymatic digestion affects expression level of surface molecules on immune cells

To get a focused starting point for deciphering the specific impact of different enzymes on surface molecule expression on immune cells, we performed broadly based digestion experiments. To this end, freshly isolated mouse splenocytes were incubated *in vitro* with collagenase D or dispase I as indicated in *Table 1*. Experimental conditions were adapted to established protocols routinely used for the liberation of lymphocytes and epithelial cells from tissues. Following enzymatic treatment, surface staining of selected molecular markers (refer to *Table 2*) was performed, and surface expression was determined by FACS analysis. Molecules analyzed included, upon others, typical T cell (CD3, CD4, CD8), B cell (B220, CD19), granulocyte (Gr-1), or NK cell (NK1.1, Pan-NK) markers, molecules commonly expressed on antigen presenting cells such as macrophages or dendritic cells (F4/80, CD11b, CD11c), molecules involved in antigen presentation and co-stimulation (MHC classes I and II, CD28, CD80, CD86, 4-1BB, PD1), cytokine receptors (IL-10R, CD25), integrins (CD103, CD11b, CD11c), and several other immunologically relevant surface molecules. As depicted in *Figure 1* this initial screening of 48 different surface molecules revealed that dispase treatment markedly reduces the expression of numerous molecules on the surface of immune cells, including CD11c, ICAM-1, CD40L, CD162, CD8, CD25, CD69, and PD-1L both at high and low enzyme concentrations. Of note, collagenase treatment of splenocytes did not reduce surface expression of most tested molecules, but in contrast induced surface expression of certain markers such as CD11c, CD40, F4/80, and MHC class I *(Fig. 1)*.

Of the 48 pre-screened surface molecules, 18 markers exhibiting pronounced sensitivity to enzymatic digestion were selected for further analysis. Following enzymatic digestion, splenocytes were surface stained and analyzed by flow cytometry as described before. As depicted in *Figure 2*, immune cell digestion with high dispase concentrations resulted in dramatic decrease in surface expression of CD4, CD8, CD19, CD162, CD62L, CD25, CD27, ICOS-L, and PD-1L. Diminished surface expression of most of these markers on splenocytes was also evident following low-dose dispase digestion although to a lower extend *(Fig. 2)*. As observed before, only a minor impact on surface marker expression was found in case of collagenase digestion. Whereas *Figure 2a* shows representative histograms obtained in one selected experiment, *Figure 2b* summarizes data obtained in three independent digestion experiments, scrutinizing significant reduction of immunologically relevant molecules from the surface of immune cells especially following dispase treatment.

Recovery of surface molecule expression on immune cells after in vitro *culture*

In order to test whether surface molecule expression may be restored after short-term *in vitro* culture, splenocytes that underwent enzymatic digestion were cultured for 24 h before reassessment of surface marker expression by flow cytometry. As depicted in *Figure 3*, surface expression to baseline level was found for CD162, CD62L, CD25, CD27, CD80, and ICOS-L after *in vitro* culture following low dispase treatment. Expression of other markers such as CD4, CD8, CD40L, and PD-1L was only partially restored and remained below the surface expression level of untreated cells. However, 1 day of culture was sufficient to restore >50% surface expression for all markers analyzed. In contrast to this, high-dose dispase treatment rendered the cells almost completely negative for surface expression of CD8, CD19, CD162, CD25, CD27, ICOSL, and PD-1L even 24 h after the initial treatment. Other markers such as CD4, CD11b, CD62L, and CD80, which were fully restored 24 h after low dispase digestion, still did not reach surface expression level found in untreated control cells. Of note, surface molecule expression pattern of splenocytes treated with collagenase largely resembled that of undigested immune cells following 24 h *in vitro* culture.

High dose dispase treatment impairs antigen specific CD4+ and CD8+ T cell proliferation

Since surface molecules play a fundamental role in cell–cell interaction and as part of the immunological synapse are crucially involved in the transmission of positive or negative signals from one immune cell to another, we studied the impact of enzymatic digestion on the outcome of antigen-specific T cell proliferation. To this end, splenocytes were isolated from TCR-HA and CL4 transgenic mice, respectively, that harbor a certain population of hemagglutinin (HA)-specific CD4$^+$ (TCR-HA) or CD8$^+$ (CL4) T cells within their T cell repertoire. Splenocytes were subjected to collagenase, and high- and low-dose dis-

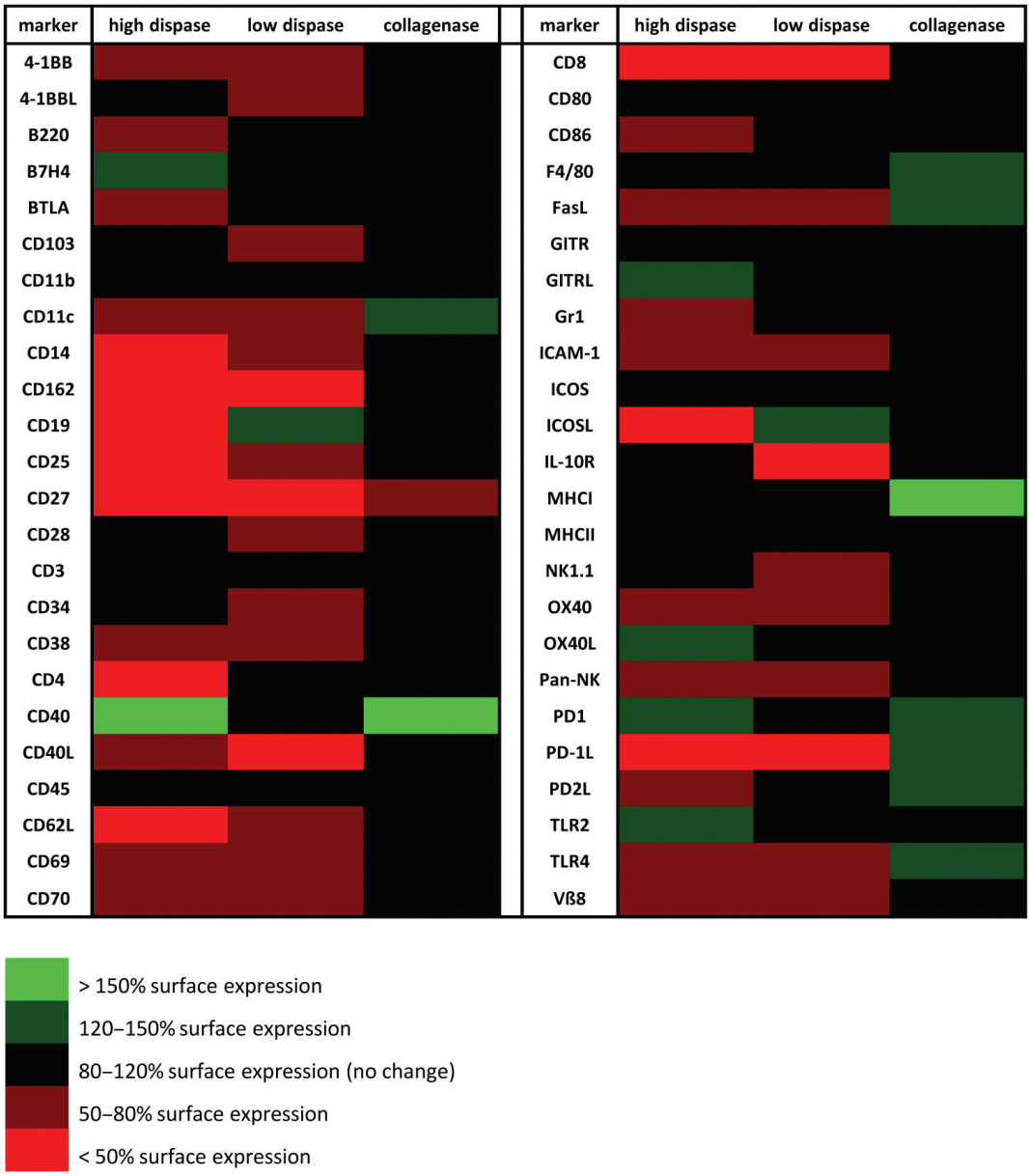

Fig. 1. Enzymatic digestion broadly affects surface marker expression. BALB/c splenocytes were digested with collagenase D (0.044 U/ml) or high (50 U/ml) or low (0.8 U/ml) concentrations of dispase I, respectively, followed by antibody staining of 48 selected surface markers and FACS analysis. Percent positive cells obtained after enzymatic digestion was normalized to surface marker expression on untreated splenocytes which was arbitrarily set to 100%. Green color indicates elevated surface expression following enzymatic digestion, red color indicates reduced surface expression; black indicates no change in surface expression

pase treatment followed by *in vitro* culture in the presence of the corresponding MHC class I or MHC class II HA-peptide. As depicted in *Figure 4*, high dispase treatment of splenocytes drastically reduces the proliferative capacity of $CD4^+$ as well as of $CD8^+$ T cells. In contrast and consistent with the far less pronounced impact of collagenase treatment on surface molecule expression, collagenase digestion of immune cells does not impair antigen-specific proliferation of $CD4^+$ T cells *(Fig. 4a)* but even elevates proliferation in case of antigenic stimulation of HA-specific $CD8^+$ T cells *(Fig. 4b)*. Together, these data indicate that isolation of cellular subsets by means of enzymatic tissue disintegration may have sustained effects on surface expression of a broad range of immunologically relevant molecules with direct consequences for functionality of immune cell subsets in subsequent *in vitro* test assays.

The observation that the use of enzymes to isolate cellular subsets from complex tissues may have side effects with respect to surface marker expression and cell function is not entirely new. Consistent with our data, dispase treatment has been shown before to dramatically reduce CD4 and CD25 surface expression on murine T cells [11], whereas collagenase/DNase digestion of human peripheral blood leukocytes had only marginal impact on expression level of surface molecules such as MHC class II, CD3, CD8, and interleukin-2 receptor on human peripheral blood leukocytes [16]. Whereas we found sustained effect of dispase treatment on CD4 and CD8 surface ex-

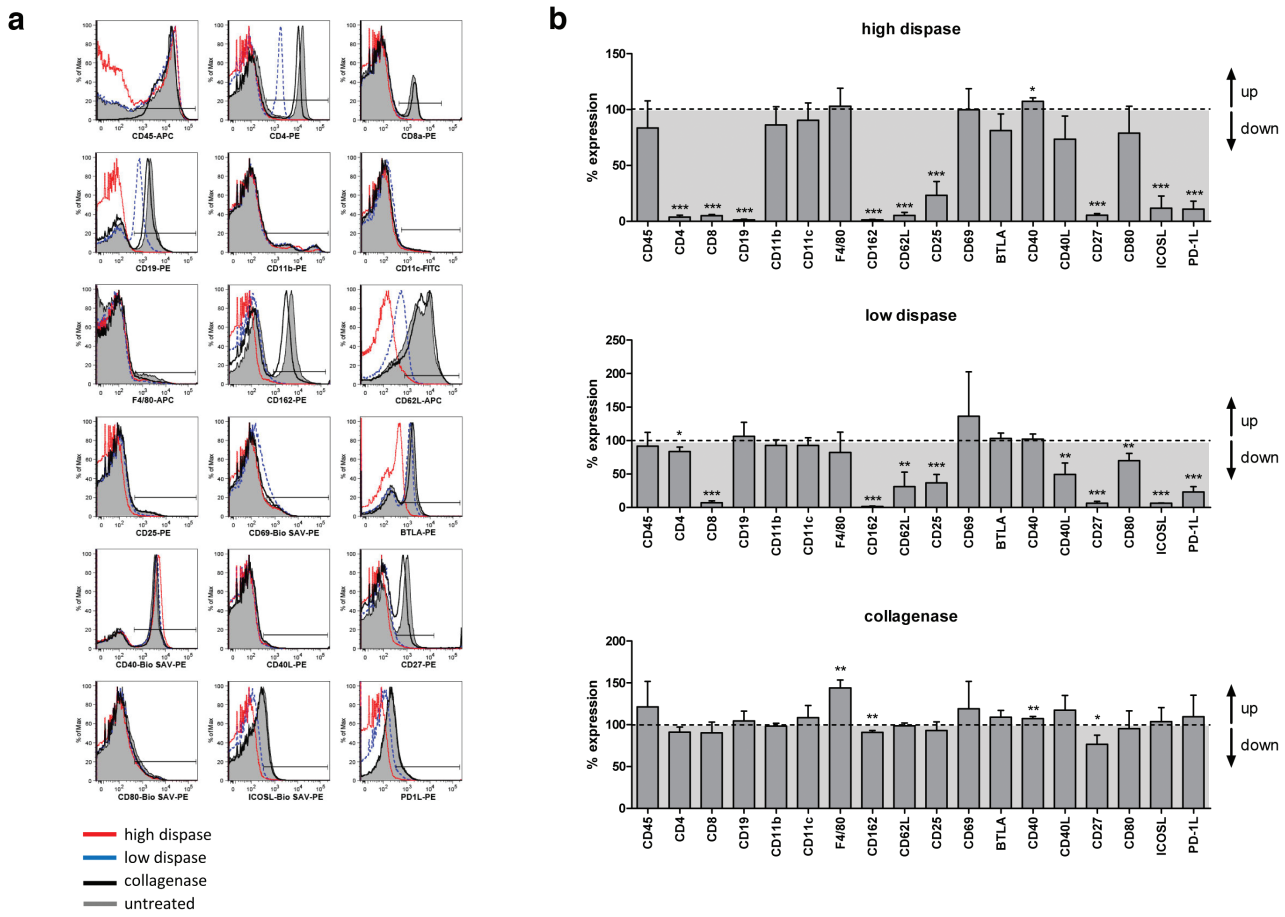

Fig. 2. Quantification of the impact of collagenase and dispase treatment on surface expression of selected markers. (a) Splenocytes were digested with either collagenase D or different dispase I concentrations as described in Materials and methods, followed by antibody staining of selected surface molecules and FACS analysis. Untreated splenocytes served as internal control. Histograms show representative results obtained in one out of three independent experiments. (b) Data obtained from three independent digestion experiments were combined to quantify the impact of enzymatic treatment on surface expression levels of all 18 markers tested. For every marker analyzed, percent expression after enzymatic treatment was normalized to marker expression on untreated splenocytes which was defined as 100%. *p* values were calculated using *t*-test, and $p<0.05$ is indicated as *, $p<0.01$ is indicated as **, and $p<0.001$ is indicated as ***

pression even 24 h after treatment, White and colleagues demonstrated recovery of CD4 and CD8 expression after overnight culture following treatment of human female reproductive tract cells with an enzyme cocktail composed of pancreatin, hyaluromidase, and collagenase [12]. Since many different protocols exist with, in part, great variations regarding enzyme concentrations, combination of enzymes used for the isolation of cells, target cells/tissue, incubation times, nature of markers analyzed, etc., existing data are difficult to compare or to generalize. We were facing the problem that enzymatic digestion may have profound effects on surface marker expression first when we applied the standard protocol routinely used in our laboratory for the isolation of lung $CD4^+$ T cells [3] to pulmonary $CD8^+$ T cells. Consistent with *Figure 2*, we found that the CD8 molecule appears to be extremely vulnerable to the enzymatic activity of dispase I, even at low concentrations, whereas collagenase treatment does not affect CD8 expression and is at the same time sufficient for isolation of high lymphocyte numbers from the murine lung (data not shown). It would therefore be highly recommended to set up optimized experimental conditions with respect to the enzyme(s) used for liberation of cells dependent on the cell type of interest for subsequent magnetic or flow cytometric isolation or FACS analysis to avoid difficulties in the detection of selected markers applying flow cytometric analysis or to prevent low yield of cells when applying classical cell isolation protocols such as FACS or MACS that are based on positive selection of antibody-stained target cells.

Enzymatic removal of surface molecules may also account for the potential discordance between FACS data and data obtained by gene expression analysis. In order to isolate primary alveolar type II epithelial cells (AECII) from the murine lung, we are generally applying a protocol that is based on high dispase treatment [13]. Whereas gene expression profiling using Affymetrix GeneChips and quantitative real-time RT-PCR revealed in part dramatic differences in the expression level of several surface molecules between AECII isolated from the lung of healthy mice and mice suffering from autoimmune-mediated lung inflammation [13], we were not able to confirm differen-

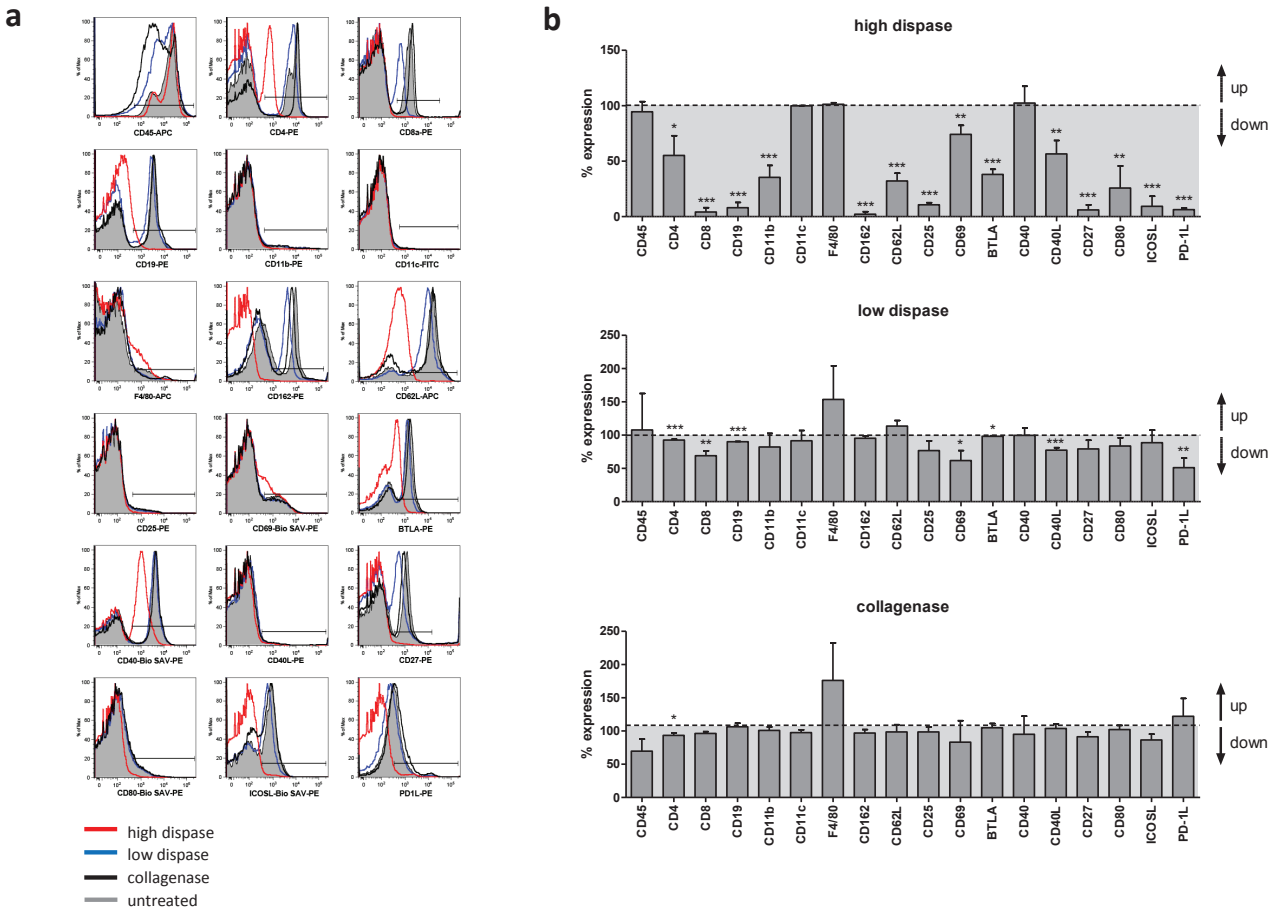

Fig. 3. Partial recovery of surface molecule expression on immune cells following 24 h *in vitro* culture after enzymatic digestion. (a) Splenocytes were digested with either collagenase D or different dispase I concentrations as described in Materials and methods, followed by 24 h *in vitro* culture in IMDM compl. at 37°C and 5% CO_2. Cells were harvested and stained for subsequent FACS analysis with the antibodies indicated on the *x* axis of the histograms. Untreated splenocytes served as internal control. Histograms show representative results obtained in one out of three independent experiments. (b) Data obtained from three independent digestion experiments were combined to quantify the impact of enzymatic treatment on surface expression levels of all 18 markers tested. For every marker analyzed, percent expression after enzymatic treatment was normalized to marker expression on untreated splenocytes which was defined as 100%. *p* values were calculated using *t*-test; $p < 0.05$ is indicated as *, $p < 0.01$ is indicated as ** and $p < 0.001$ is indicated as ***

tial expression for all of the markers tested on the protein level by applying antibody-mediated surface staining and FACS analysis (unpublished data). Thus, to circumvent this obvious technical limitation of enzyme-mediated tissue disintegration, which is on the one hand the prerequisite for the liberation of AECII from the lung tissue but at the same time destroys potential surface marker epitopes needed for subsequent antibody staining and FACS-based characterization, we are currently establishing protocols to apply iterative chip-based cytometry (iCBC) to whole lung tissue slices. Like multi-epitope-ligand cartography (MELC) described by Schubert et al., iCBC represents a microscopic robot technology for high-throughput protein co-localization studies that runs cycles of fluorescence tagging, imaging, and bleaching *in situ* and thereby allows for the analysis of virtually unlimited numbers of intracellular and surface markers even on living immune cells [17, 18]. Automated single-cell recognition software provides numerous options for marker combination analysis and strategic experimental conception, and thus MELC and iCBC may represent powerful alternatives for the characterization of cells within tissues in case their liberation would require experimental conditions being incompatible with subsequent flow cytometric analysis of surface markers of particular interest.

In addition to the obvious obstacle that proteolytic enzymes diminish expression levels of certain key cell surface molecules, and thereby complicate identification of cellular subsets by FACS-based approaches and their isolation using magnetic beads or flow cytometry, these molecular alterations obviously can also affect immune cell functions. Holt and colleagues investigated the impact of collagenase/DNase digestion procedure of human lung parenchyma on polyclonal T cell proliferation, antigen presentation, IL-1 production, and NK cell activity [16]. Consistent with the relatively minor effect of the enzyme combination on the expression of T cell, B cell, monocyte, and macrophage markers, the cells behaved almost normally in all functional assays [16]. Interestingly, background proliferation of T cells and spontaneous IL-1 production tend to be elevated in enzyme-treated cells, which may be indicative for their transient activation during collagenase

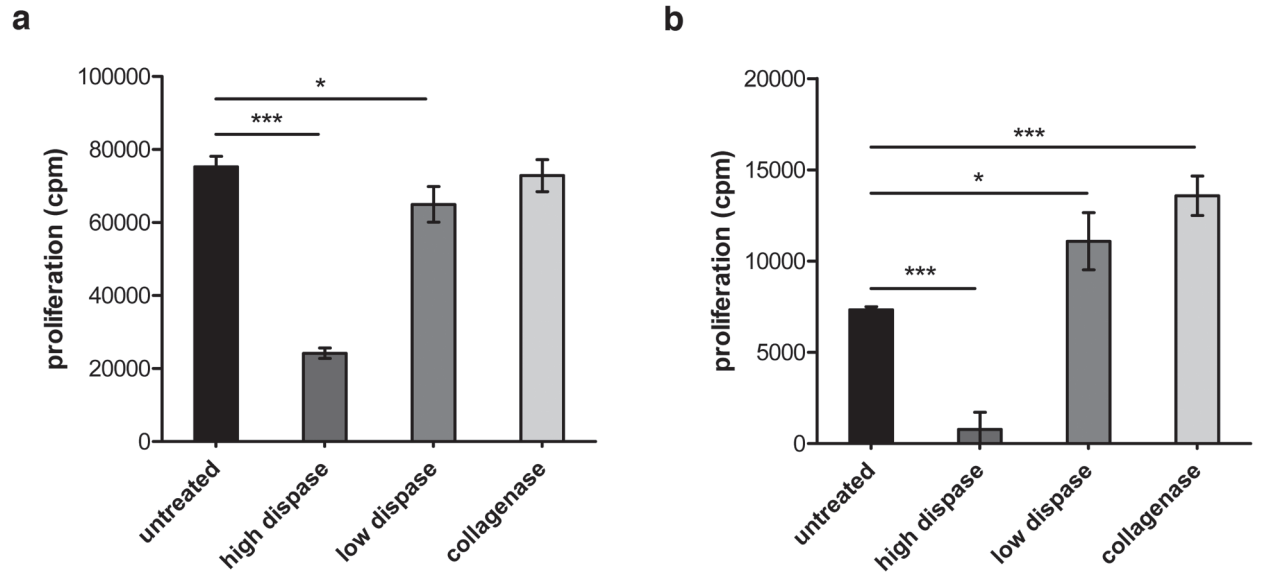

Fig. 4. Impact of enzymatic digestion on proliferative capacity of $CD4^+$ and $CD8^+$ T cells. (a) Splenocytes were isolated from TCR-HA mice and treated with collagenase or dispase as described in the section Materials and methods. Subsequently, 5×10^5 enzyme-treated spleen cells and untreated control cells were seeded per well of a 96-well microtiter plate and stimulated *in vitro* with 0.01 μg/ml of the corresponding HA peptide 110–120 for 24 h. ^{3}[H]-thymidine was added for another 16 h of culture before harvesting the cells and scintillation counting to determine antigen-specific $CD4^+$ T cell proliferation. (b) 1×10^5 splenocytes isolated from CL4 mice and treated with the respective enzymes were stimulated *in vitro* with 0.001 μg/ml of the corresponding HA peptide 512–520 for 24 h before the addition of ^{3}[H]-thymidine and further culture for 12 h. Radioactively labeled cells were harvested, and antigen-specific $CD8^+$ T cell proliferation was determined by scintillation counting. *p* values were calculated using *t*-test; $p < 0.05$ is indicated as *, and $p < 0.001$ is indicated as ***

digestion. This is in line with our observation that of the 48 markers tested, nine were found to be induced rather than removed from the surface of immune cells *(Fig. 1)*. Of note, not only enzymatic but also mechanical cell isolation techniques can affect immune cell function in subsequent *in vitro* studies. In this context, Bland and colleagues have shown that isolation of human large bowel lymphoid cells on the basis of mechanic processes stimulated the synthesis of prostaglandin E2, which directly suppressed cytotoxic activity in mechanically-liberated lymphocytes. This inhibitory effect was not observed when using a protocol based on collagenase digestion [19].

Using a well-defined experimental setup based on transgenic HA-specific T cells, we could show that treatment of immune cells with high dispase concentration drastically reduces antigen-specific proliferation of both $CD4^+$ and $CD8^+$ T cells *(Fig. 4)*. This was not unexpected, since dispase-mediated cleavage of key surface molecules including the two co-receptors CD4 and CD8 on T cells may directly hamper optimal formation of the immunological synapse, thereby interfering with T cell receptor-mediated recognition of MHC/peptide complexes on the surface of antigen presenting cells [20]. In addition, molecules such as CD27 and the high-affinity IL-2 receptor CD25 (and possibly other important receptors not included in this survey) were found to be extremely sensitive to dispase treatment, which may have direct effects on the expansion and survival of activated T cells [21, 22]. Interestingly, whereas high-dose dispase digestion dampens *in vitro* T cell proliferation, collagenase treatment even seems to promote $CD8^+$ T cell activation *(Fig. 4)*. This is well in line with our observation that collagenase does not reduce surface expression of molecular markers on immune cells *(Figs 1 and 2)*, and moreover, expression level of MHC class I molecules is even increased in collagenase-treated cells, suggesting improved MHC class I mediated antigen display in support of improved $CD8^+$ T cell proliferation.

Together our data indicate that enzymatic tissue disintegration can have profound effects on the expression of a variety of cell-surface molecules with direct consequences for phenotypic analysis, FACS- and MACS-based target cell isolation, and immune cell function in cell culture experiments. This underscores the importance of designing adequate pre-experiments to decipher the specific impact of a particular enzyme cocktail used for the liberation of immune cells from a tissue on their phenotype and function, and thereby to prevent drawing invalid conclusions.

Acknowledgements

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Table 2. Antibodies and 2nd step reagents used for flow cytometry

Antibody	Clone	Fluorochrome	Manufacturer
Anti-B7H4	clone 9	PE	BioLegend
Anti-CD3ε	145-2C11	PE	eBioscience
Anti-CD4	GK1.5	PE	eBioscience
Anti-CD8a	53-6.7	PE	BD Pharmingen
Anti-CD11b	M1/70	PE	eBioscience
Anti-CD11c	HL3	FITC	BD Pharmingen
Anti-CD14	Sa2-8	PE	eBioscience
Anti-CD19	1D3	PE	eBioscience
Anti-CD25	PC61	PE	BD Pharmingen
Anti-CD27	LG.3A10	PE	BD Pharmingen
Anti-CD28	37.51	PE	BD Pharmingen
Anti-CD34	RAM34	PE	eBioscience
Anti-CD38	90	PE	BD Pharmingen
Anti-CD40	Mar-23	Biotin	BD Pharmingen
Anti-CD45	30-F11	APC	BD Pharmingen
Anti-CD45R (B220)	RA3-6B2	APC	eBioscience
Anti-CD49b (Pan-NK)	DX5	FITC	BD Pharmingen
Anti-CD54 (ICAM-1)	YN1/1-7	Bio	Kindly provided by S. Weiss
Anti-CD62L	MEL-14	APC	BD Pharmingen
Anti-CD69	H1.2F3	PE	BD Pharmingen
Anti-CD70	FR70	PE	eBioscience
Anti-CD80 (B7.1)	16-10A1	Bio	eBioscience
Anti-CD86 (B7.2)	GL1	Bio	BD Pharmingen
Anti-CD103	2E7	FITC	BD Pharmingen
Anti-CD134 (OX40)	OX-86	PE	eBioscience
Anti-CD137 (4-1BB)	17B5	PE	eBioscience
Anti-CD137L (4-1BBL)	TKS-1	Bio	eBioscience
Anti-CD154 (CD40L)	MR1	PE	BD Pharmingen
Anti-CD162 (PSGL-1)	2PH1	PE	BD Pharmingen
Anti-CD178 (FasL)	MLF3	PE	BD Pharmingen
Anti-CD210 (IL-10R)	1B1.3a	PE	BD Pharmingen
Anti-CD252 (OX40L)	RM134L	PE	BioLegend
Anti-CD272 (BTLA)	6F7	PE	eBioscience
Anti-CD273 (PD-2L)	TY25	PE	BD Pharmingen
Anti-CD274 (PD-1L)	MIH5	PE	eBioscience
Anti-CD275 (ICOSL)	HK5.3	PE	BioLegend
Anti-CD278 (ICOS)	7E.17G9	PE	eBioscience
Anti-CD279 (PD-1)	J43	PE	BD Pharmingen

Table 2. *Continued*

Antibody	Clone	Fluorochrome	Manufacturer
Anti-CD282 (TLR4)	6C2	PE	eBioscience
Anti-CD284 (TLR2)	UT41	PE	eBioscience
Anti-F4/80	BM8	APC	eBioscience
Anti-GITR	DTA-1	PE	eBioscience
Anti-GITRL	YGL 386	PE	BioLegend
Anti-Ly6G (Gr-1)	RB6-8C5	PE-Cy7	eBioscience
Anti-MHCI (H-2Kb)	AF6-88.5	FITC	BD Pharmingen
Anti-MHCII	M5/114.15.2	APC	eBioscience
Anti-NK1.1	PK136	PE	BD Pharmingen
Anti-Vß8	F23.1	PE	BD Pharmingen
Streptavidin		PE	BD Pharmingen

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