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Insight into the Leukemia Microenvironment and Cell-cell Interactions Using Flow Cytometry

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Additional information is available at the end of the chapter

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Abstract

Cancer cells, including leukemia cells, reside in a complex microenvironment, which influences biology and activity of the cells. The protective role of bone marrow stromal cells is already commonly recognized. Remodeling of stroma cell functions by leukemia cells is also well documented. In this respect, different routes of interactions were defined, such as direct cell-cell interactions or indirect cross talk, by release of soluble factors or vesicular particles containing proteins, RNAs and other molecules. Since intercellular communication seems to play a role in various biological processes, it might be important to conduct studies in co-culture systems, which at least mimic partially more physiological conditions, and enables this intercellular exchange to occur. Thus, it is crucial to improve analytical methods of investigation of co-cultured cells, to study their interactions and so to understand biology of leukemia in order to understand molecular mechanisms and offer novel therapeutic strategies. The present chapter outlines the importance of modern, multiparameter flow cytometry methods, which allow to analyze interactions between different types of cells within the leukemia microenvironment. Importantly, the proposed experimental setups can be easily transformed to study different cell types and different biological systems.

Keywords: co-culture, cell-cell interactions, cell tracking, tracking dyes, proliferation dyes, viability dyes, barcoding, cancer microenvironment, stroma, regulatory T cells, extracellular vesicles (EVs), tunneling nanotubes (TNTs)

1. Introduction

Cancer cells, including leukemia cells, reside in a microenvironment, which influences biology and activity of these cells. The importance of microenvironment in leukemia cells biology is widely accepted. Leukemia microenvironment is a complex and dynamic network, comprising different types of cells [1]. There is a direct and indirect cross talk between these cells, which modulates cell function, signal transduction and response to therapy. This intercellular interactions can contribute to leukemogenesis [2]. Thus, unraveling of interplay between all types of cells and the microenvironment is crucial for understanding the biology of leukemia.

Because of its key regulatory role, the cross talk between leukemia and bystander cells is of interest as possible therapeutic target [3]. It is critically important to take such cell-cell interactions into account in the studies of potential therapeutic strategies, novel therapeutic targets and novel treatment regimes. To partially mimic the physiological conditions, an increasing number of the basic research as well as preclinical studies in the cancer biology area use the *in vitro* cell co-culture systems, in which two or more types of cells are cultured together [4]. This allows natural interactions to occur between different types of cells, which normally coexist within the microenvironment.

Therefore, it becomes crucial to improve analytical methods of investigation of co-cultured cells to study their interactions and so to understand biology of leukemia in order to offer novel therapeutic strategies. The principle of flow cytometry is separation of cells/events based on their fluorescence signals. Using different types of cell tracking techniques, combined with modern, multiparameter flow cytometry methods, it has become possible to analyze interactions between different types of cells within the leukemia microenvironment. Flow cytometry allows for gating cells of interest from the whole cell mixture based on the fluorescence parameter to study different cell features, cellular processes and signaling in separated subpopulations.

2. Bone marrow niche in leukemia

The concept of “niche” has been introduced in 1978 by Schofield to describe the possibility of association of hematopoietic stem cell (HSC) with its surroundings that regulates and determines HSC fate, such as self-renewal, differentiation, proliferation and survival [5]. Since then, many evidences appeared showing its composition and function. The bone marrow microenvironment (BMM) is complex and consists of the extracellular matrix and multiple cell populations, including different types of endothelial cells, osteolineage cells, osteoclasts, adipocytes, sympathetic neurons, non-myelinating Schwann cells, mesenchymal cells and macrophages [1, 6]. Interactions between leukemia cells and their niche are bi-directional. They represent either direct interactions by cell-cell contact or indirect interactions by secreted soluble factors and extracellular vesicles.

Over the years, our understanding of the BMM role in leukemogenesis has greatly increased. Bone marrow microenvironment supports proleukemic features of cells. Regulation of leukemia stem/progenitor cells (LS/PC) strongly depends on the signals from the bone marrow microenvironment [3, 7]. They promote leukemia progression as well as development of resistance

to therapy. Bone marrow microenvironment is a key player in the pathogenesis and leukemia development by creating conditions which promote survival and proliferation of leukemic blasts [8].

There are many evidence confirming protective role of the bone marrow stroma. Several mechanisms leading to chemoresistance in leukemia cells mediated by BMM have been described. Among these, stroma fibroblasts represent one of the main population within the bone marrow niche, strongly supporting leukemogenesis. Interaction of leukemia cells with stroma fibroblasts results in the increased resistance to cell death in response to drug treatment, changes in metabolism and decreased clonogenic potential [9, 10]. Leukemia cells can also shape the bone marrow microenvironment. We have observed that leukemia cells participate in the remodeling of stroma extracellular matrix [11]. Due to secretion of stroma-modifying enzymes, leukemia cells support disarrangement in the leukemic bone marrow. Another aspect of the leukemia microenvironment is development and promotion of the inflammatory microenvironment [12, 13]. Activation of intrinsic factors like NF- κ B as well as extrinsic components including cytokines, chemokines and adhesion molecules leads to complex responses promoting proinflammatory state. In general, this supports the process of transformation, survival and proliferation of leukemia cells.

Altogether, due to their importance and key role, targeting the leukemia-stroma interactions has gained big interest as a novel and attractive strategy for anti-leukemia treatment. Currently, our understanding of the function and importance of the bone marrow niche in hematological neoplasms has increased due to improved murine models and development of imaging tools [14–16]. Nevertheless, the *in vitro* studies are still necessary to investigate mechanisms and signaling between leukemia and stromal cells as well as a proof of concept experiments in the drug designing and testing of novel therapeutic strategies.

Our group is involved in the studies of leukemia-stroma interactions and remodeling of stroma cell functions mediated by leukemia cells. We have used experimental setups to investigate leukemia cells *in vitro*, either cell lines or primary cells from patients, in the conditions mimicking the bone marrow milieu, thus hypoxia and co-culture with stroma fibroblasts. Herein we present and discuss some of the experimental systems utilized to study the role of the cross talk between leukemia and stromal cells to which multiparameter flow cytometry can be adapted. Importantly, these experimental approaches can be transferred to other cell types and different biological systems.

3. Fluorescent cell tracking to follow cell type, function, and state in co-cultures

Upon co-culture conditions, when different types of cells are cultured together, the key component of the experimental strategy enabling cell analysis is to separate both cell types, either physically by sorting or by flow cytometry gating. For this, cells have to express some fluorescent protein or have to be stained and tracked by fluorescent dye. This allows to separate signals from each type of cell based on the different fluorochromes using flow cytometry. The scheme and typical histograms to analyze tracked cells in co-culture are presented in **Figure 1**.

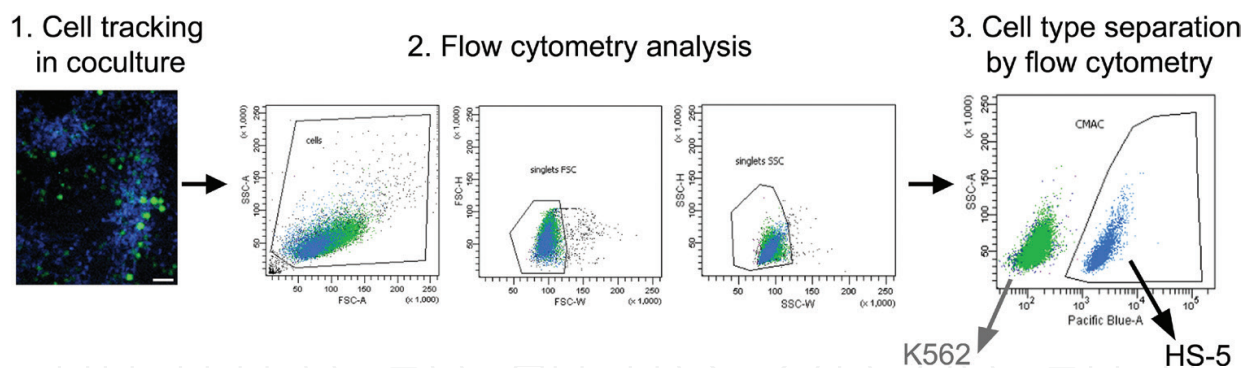


Figure 1. Scheme of the cell type separation by fluorescent tracking followed by flow cytometry gating.

3.1. Fluorescent cell trackers

Cell trackers have been successfully used for years in multiple studies, starting from immunology, which intensively utilized flow cytometry techniques [17–19]. Today, there is a broad spectrum of commercially available tracking dyes, which simultaneously allow to track cells and study proliferation and/or viability. Cell tracking dyes vary widely in their chemistries and fluorescence properties but the great majority fall into one of two classes based on their mechanism of cell labeling. They are represented by “membrane dyes,” which are highly lipophilic dyes that go stably but noncovalently into cell membranes or “protein dyes,” which are amino-reactive dyes that form stable covalent bonds with cell proteins [20]. They are safe and can be used in viable cells even in the long-term cultures. Big variability allows excitation by any available laser and usage in combination with other fluorochromes in multiparameter studies. **Table 1** presents the tracking dyes currently available for different lasers. These dyes can be used separately or combined due to their different excitation signals. It is important to mention that due to its nature, the fluorescent signal of labeled population is decreasing, depending on the growth rate of cells. So, quickly dividing cells will lose tracing signal faster than cells with longer cell cycle. This feature, on the one hand, enables monitoring of cell proliferation rate in co-culture setups but on the other hand limits the time frame within which the subpopulation of dye-labeled cells can be distinguished. **Figure 2** presents an example of the time-course of CMAC fluorescence measured by flow cytometry, when different concentrations of CMAC dye were used. After 72 h, two populations were not clearly separated anymore when the lower concentration of CMAC was used. Therefore, also concentration of the dye used for labeling of different types of cells should be chosen accordingly to the level of fluorescence detected at the end time point of planned experiment and verified in the toxicity assay.

Another strategy to track cells is expression of fluorescent proteins. Green fluorescent protein (GFP) has been purified in the 60-ties from *Aequorea victoria* [21]. This has started a new era of studies, in which original or engineered GFP as well as other fluorescent proteins were utilized to tag proteins of interest. Today, GFP derivatives, yellow fluorescent protein (YFP) and a big family of red fluorescence proteins (RFP), which are derivatives of DsRed isolated from *Discosoma* sp. [22], are commonly used in many different studies. The advantage of this strategy is stable fluorescent signal; however, the stable expression of fluorescent proteins has some limitations, requires transfection of cells and is connected with the nonphysiological

Function	Excitation laser [nm]	Dye	Emission (nm)
Proliferation	405 (violet)	CellTrace violet cell proliferation kit	450
		Violet proliferation dye 450	450
	488 (blue)	CFSE	520
		EdU proliferation kit (iFluor 488)	520
	530 (yellow-green)	CellTrace yellow cell proliferation kit	579
	560 (yellow)	CellTrace yellow cell proliferation kit	579
Viability	355 (UV)	CellTrace calcein blue	425
		Hoechst 33258	450
		DAPI	470
	405 (violet)	CellTrace calcein violet	452
		SYTOX blue dead cell stain	480
	488 (blue)	CellTrace calcein green	515
		SYTOX green dead cell stain	523
		TO-PRO™-1 iodide	530
		SYTOX orange dead cell stain	570
		Propidium iodide	617
		7-AAD (7-aminoactinomycin D)	647
		7-AAD (7-aminoactinomycin D)	647
	530 (yellow-green)	SYTOX orange dead cell stain	570
		Propidium iodide	617
		7-AAD (7-aminoactinomycin D)	647
	560 (yellow)	Propidium iodide	617
		7-AAD (7-aminoactinomycin D)	647
	633/640 (red)	TO-PRO™-3 iodide	661
Fixable viability	355 (UV)	Live/dead fixable dead cell stain kit (450)	450
		Live/dead fixable dead cell stain kit (450)	450
		Fixable viability stain 450	450
	405 (violet)	Fixable viability stain 510	510
		Live/dead fixable dead cell stain kit (525)	525
		Live/dead fixable dead cell stain kit (575)	575
		Fixable viability stain 575 V	575
		Fixable viability stain 520	520
		Live/dead fixable dead cell stain kit (530)	530
		Live/dead fixable dead cell stain kit (585)	585
	488 (blue)	Fixable viability stain 520	520
		Live/dead fixable dead cell stain kit (530)	530
		Live/dead fixable dead cell stain kit (585)	585

Function	Excitation laser [nm]	Dye	Emission (nm)
Cell tracking	530 (yellow-green)	Fixable viability stain 620	620
	560 (yellow)	Fixable viability stain 570	570
	633/640 (red)	Fixable viability stain 660	660
		Live/dead fixable dead cell stain kit (660)	660
		Fixable viability stain 700	700
		Live/dead fixable dead cell stain kit (780)	780
	355 (UV)	Hoechst 33342	450
		CytoPainter blue cell tracking staining kit	455
		CellTracker™ blue CMF2HC	464
		CellTracker™ blue CMAC	466
	405 (violet)	CellTracker™ violet BMQC	516
		CytoPainter green cell tracking staining kit	520
	488 (blue)	CellTracker™ green CMFDA	517
	530 (yellow-green)	CytoPainter orange cell tracking staining kit	560
	560 (yellow)	CellTracker™ CM-DiI	570
		CellTracker™ orange CMRA	575
		CytoPainter red cell tracking staining kit	600
		CellTracker™ CMTPX	602
	633/640 (red)	CellTracker™ deep red	650
		CytoPainter deep red cell tracking staining kit	650

Table 1. Tracking dyes used for fluorescent cell staining; function, excitation, and emission spectra as well as full name are shown.

overexpression of novel form of protein. On the other hand, the signal from the expressed fluorescent protein is relatively stable over time. Thus, this attitude might have advantage over cell tracking in case of long-term experiments.

Recently, a highly advanced methodology based on the lentiviral fluorescent genetic barcoding system for flow cytometry-based multiplex cell tracking has been used to follow clonal subpopulations in the real time [23]. Cells transduced with the lentiviral vectors are individually marked by a highly characteristic pattern of insertion sites inherited by all their progeny. The system allows creation of 26, 14, or 6 unique labels, and the color-coded populations can be tracked for up to 28 days.

Another strategy to perform high-content multiplex analysis is based on the fluorescent cell barcoding (FCB) technique [24, 25]. In FCB, each sample is labeled with a unique fluorescent signature (barcode), of different fluorescence intensity (due to dilutions) and/or emission wavelength, mixed together with other samples, then stained with antibodies or probes and analyzed by flow cytometry as a single sample. Today, there is a variability of FCB dyes

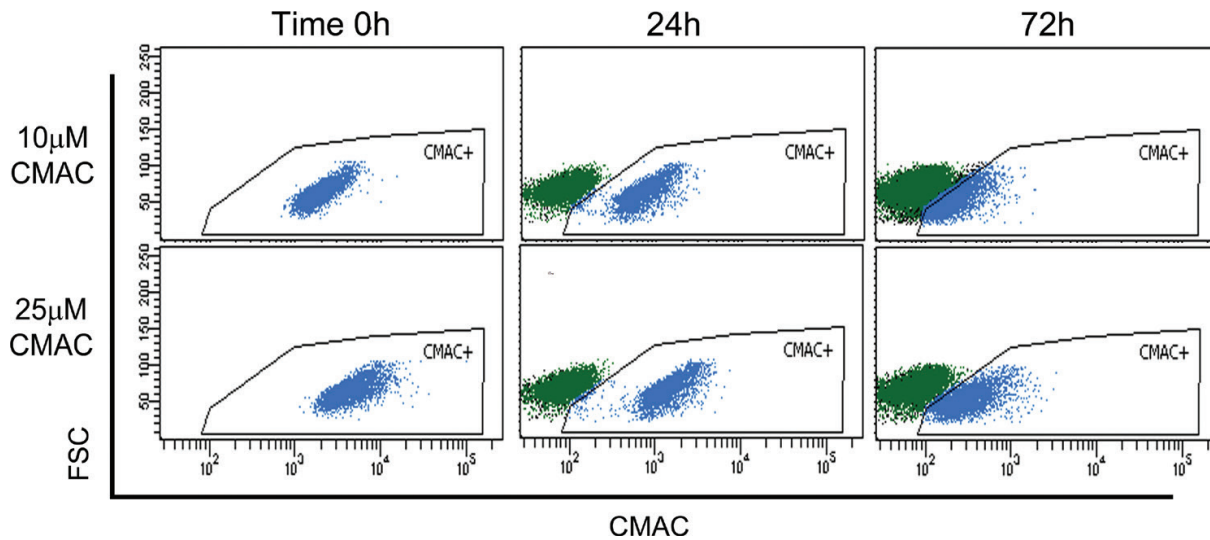


Figure 2. The time-course of CMAC fluorescence measured by flow cytometry upon conditions when different concentrations (10 and 25 μ M) of CMAC dye were used. CMAC-positive (CMAC+, blue) subpopulation is gated. Green subpopulation represents CMAC-negative cells in co-culture.

which can be utilized, such as CBD500, Pacific Orange NHS ester, DyLight 350 NHS ester, and DyLight 800 NHS ester, Pacific Blue dyes, Alexa dyes, eFluor, and others. Using this technique allows to perform multiplate assays in relatively short time and with economical use of reagents, as mixing sample prior to staining, antibody consumption is reduced 10- to 100-fold. Importantly, either viable or fixed samples can be barcoded, thus giving broad possibilities to combine FCB with other multiparameter protocols. Such strategy can be used to analyze samples under treatments, co-cultured, or stimulated with different agents. FCB has been successfully utilized for different studies, including drug screening and cell signaling profiling studies [26], intracellular cytokine production [27], immunophenotyping [28], and others.

3.2. Cell proliferation dyes

Quantitative assessment of proliferation is an important parameter in leukemia and other cancer studies. Especially, when co-culture with stroma provides protective signaling, proliferation is analyzed as one of the major parameters to estimate cytostatic effects. Moreover, proliferation dyes can also be utilized to fluorescently track cells in co-culture. So, in addition to detection and quantifying cell divisions, these dyes can be used to distinguish one type of cells from the other. The measurement, independently on the tracking dye used, relies on the same mechanism, namely permanent labeling of cells without affecting morphology or physiology to trace generations or divisions *in vivo* or *in vitro*. Dilutions of the dye equally distributed to daughter cells as a result of cell divisions can be measured by flow cytometry [17, 20]. The scheme of CFSE dilution with each cell division and flow cytometry measurement is presented in **Figure 3**.

For many years, CFSE dye (carboxyfluorescein succinimidyl ester) was a gold standard for tracking cell divisions [29]; however, today there is a variety of tracking dyes allowing to measure the proliferation index. Proliferation dyes are available for the UV, violet, blue, yellow, and red lasers, giving more flexibility in multiplexed experiments [29, 30] (see **Table 1**, Proliferation). Compared to the classical CFSE, modern proliferation trackers are less cytotoxic

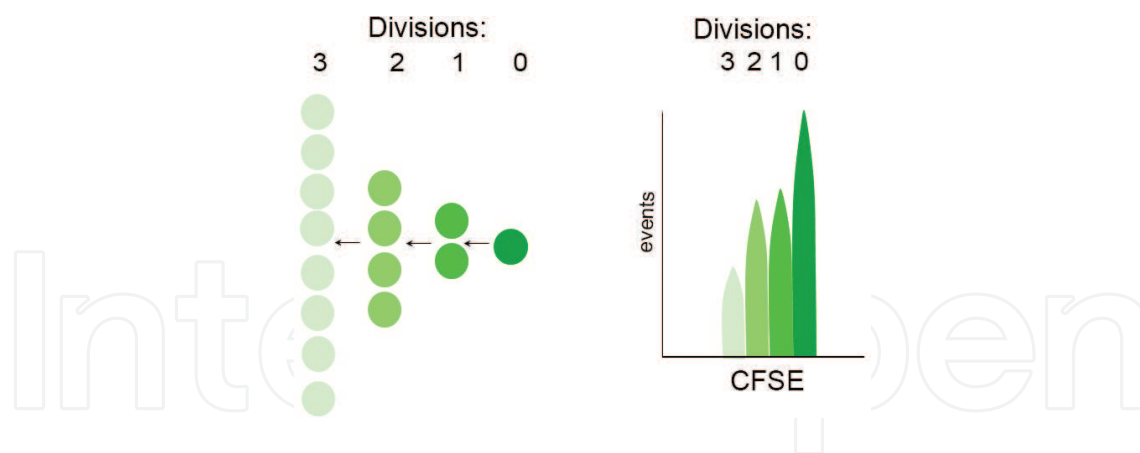


Figure 3. The scheme of tracking cells with proliferation dye. Dilution of CFSE with cell divisions (left panel) and CFSE detection by flow cytometry (right panel) are shown.

and more stable. Importantly, what has been already mentioned above, decrease of the fluorescence signal resulting from dye dilution with every division has to be considered when tracking cells with proliferation dyes.

In our studies, in which GFP-expressing CML cells have been co-cultured with stroma fibroblasts labeled with CMAC dye, tracking of subpopulations has been combined with analysis of proliferation (**Figure 4**). To do so, subpopulations have been gated based on the fluorescent signal of the cell tracker, either GFP (A) or CMAC (B), followed by separate analysis of cell divisions in each subpopulation. Such strategy allowed to investigate the impact of co-culture conditions on the proliferation index of either leukemia or stromal cells. This protocols can also be utilized in co-cultures under treatment with anticancer drugs to verify the protective effect of stroma. Similar strategy has been used to investigate interactions of lymphocytes with mesenchymal stem cells [31] as well as in the co-cultures of healthy or malign breast tissue-derived stromal cells with human breast adenocarcinoma cell line MCF-7 [32]. Altogether, proliferation dyes represent a very potent group of tracking dyes analyzed with a broad range of available lasers and cytometers.

3.3. Multiparameter studies

Modern flow cytometry, due to development of multiple laser systems, provides possibility for polychromatic analysis of many parameters in single cells. The key to success, particularly in the studies, in which multiple dyes are used to track different types of cell, is therefore to understand the critical issues enabling optimal use of different tracking dyes. It has to be noticed that some limitations appear when using tracking dyes in co-culture experiments. Each class of commercially available cell trackers—lipophilic “membrane” dyes and amino-reactive “protein” dyes possess advantages and limitations. Thus, there is a need for optimization of different proliferation tracking dyes especially when combined together [33]. Recently, a novel edition of protocols to study cell proliferation by dye dilution, alone or in combination with other methods, has been published [34]. The authors discuss currently available tracking dyes, with suppliers and new spectral properties, as well as describe evaluations to be performed when selecting one for use in multicolor proliferation monitoring. The protocols presented by the authors address the critical issues related

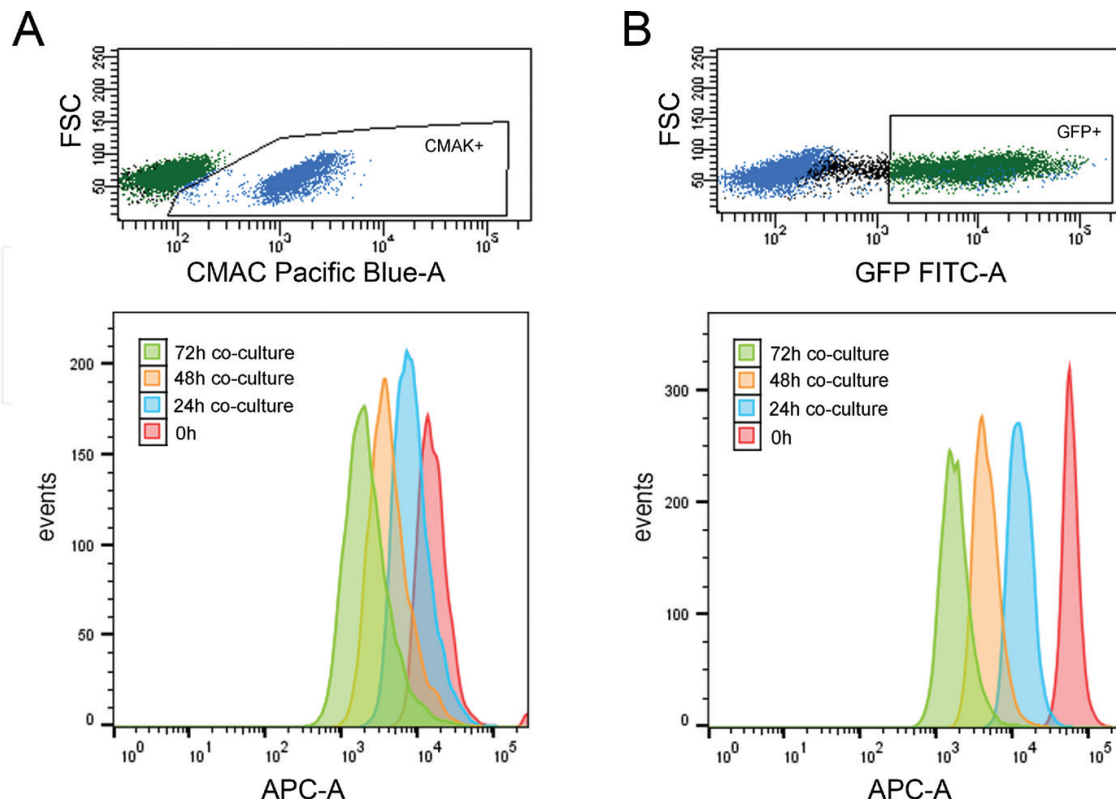


Figure 4. Analysis of cell proliferation estimated upon tracking with CMAC, combined with cell-type gating in co-culture of leukemia and stromal cells. (A) Leukemia cells expressed GFP protein (GFP+) and (B) stromal cells were tracked with CMAC (CMAC+). Labeling with CMAC allowed for cell-type gating and simultaneous analysis of cell divisions. CMAC was detected in the APC channel; histograms show CMAC dilution at different time points of co-culture.

to suboptimal tracking combinations, reproducible labeling, combining of cell tracking dyes with fluorescent antibodies staining and others. Briefly, the recommendations include evaluating the dye's spectral profile and available cytometers/lasers to optimize compatibility with other fluorochromes and to minimize compensation problems, evaluating the effect of labeling on cell growth rate, testing cell divisions and dye dilutions to determine the maximum number of generations to be included when using dye dilution profiles, and verifying that relevant cell functions remain unaltered by tracking dye labeling.

As proliferation is tracked in living cells, it enables to combine analysis of cell proliferation with other live-cell applications, such as immunophenotyping, cell sorting, cell cycle analysis, mitochondrial potential, ROS studies, and others. This possibility has been utilized, for example, in the studies in which myelin-phagocytosing macrophages were co-cultured with CFSE-labeled T lymphocytes followed by analysis of surface receptors and role of nitric oxide [35]. In other studies, cell tracker has been used to investigate immune cells proliferation and cytotoxicity [36].

Another group of trackers is represented by the fixable viability dyes, enabling the post-fixation discrimination of dead cells, which lost plasma membrane integrity before fixation (see **Table 1**, Viability). This gives a novel opportunity to combine them with staining procedures which require fixation. Thus, analysis of internal targets, like as intracellular protein levels or γ H2AX, within the population of cells which was alive prior fixation, was possible. In our

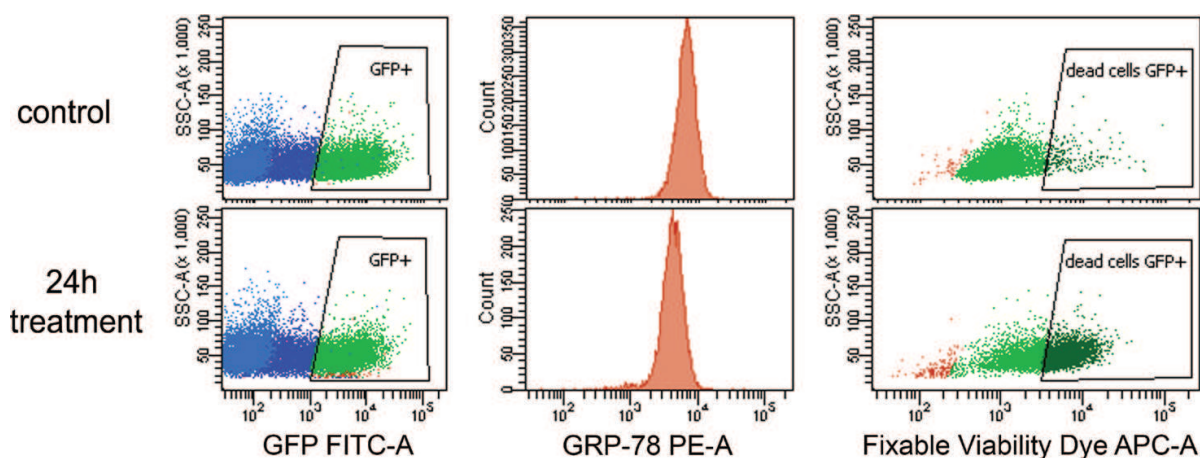


Figure 5. Combination of cell tracking by expression of GFP protein (GFP+, green), with analysis of apoptosis by using fixable viability dye and estimation of intracellular level of Grp78 protein (red histograms). Subpopulation expressing high level of GFP was gated (GFP+) and further analyzed. Cells were untreated (control) or treated with antileukemic drug for 24 h.

studies, we have combined cell tracking with GFP expression together with analysis of apoptosis by fixable viability dye and detection of intracellular level of Grp78 protein (**Figure 5**). Cells were co-cultured and the subpopulation expressing high level of GFP (GFP+) was gated and further analyzed. Additionally, as cells were treated with anti-leukemia drug, this procedure enabled to estimate sensitivity to treatment by detection of dead cells.

4. Application of flow cytometry in the studies of cell-cell cross talk by extracellular vesicles

4.1. Extracellular vesicles

In recent years, extracellular vesicles (EVs) have emerged as an important mean of intercellular communication, both in physiological conditions and in disease. They seem to play a crucial role in the cell-cell communication between cancer cells and the neighboring cells and they have been shown to be important for the development of cancer [37, 38]. Extracellular vesicles are released by most cell types and contain lipids, proteins, and different nucleic acids—mRNAs, ncRNAs (including miRNAs), and fragments of DNA. EVs are classified depending on their origin: released from multivesicular bodies (exosomes, 30–100 nm), shed from plasma membrane (microvesicles, usually 50–500 nm), or formed during apoptosis (apoptotic bodies, about 1 μ m) [39, 40]. EVs can influence targeted cells both locally and in distant tissues. They regulate multiple biological processes, such as cell differentiation, tissue homeostasis, immune response, and cell signaling, and can contribute to disease progression, for example, by affecting tumor microenvironment [41]. Recently, the role of EVs in tissue repair, immunotherapy, and drug delivery has also been evaluated.

Extracellular vesicles play a significant role in pathology of hematological malignancies, including chronic myeloid leukemia (CML) [42]. It has been demonstrated that exosomes produced by

CML cells support leukemic cell growth and survival in an autocrine manner, both *in vitro* and *in vivo* [43]. Moreover, factors facilitating leukemic transformation have been identified within vesicles released by leukemia cells [44]. Extracellular vesicles not only directly supported leukemia cell growth but also potentially modified components of CML microenvironment to further promote leukemia progression. CML-derived exosomes activated EGFR signaling in stromal cells, which led to IL-8 production by stroma bone marrow fibroblasts and which in turn stimulated leukemia cell adhesion to stromal cells [45, 46]. CML exosomes have been widely studied in terms of their influence on neovascularization process, which has previously been shown to support progression of leukemia [47]. Our own unpublished data suggest that extracellular vesicles released by murine BCR-ABL-expressing progenitor cells may also influence function of cells in distant tissues. Altogether, this supports the notion that leukemic EVs constitute an important mechanism of disease progression both by direct influence on CML cells and by indirect modification of leukemic niche components and immune cells. Moreover, EVs components, such as proteins and microRNAs, have recently become of interest as biomarkers in hematological malignancies [42, 48, 49]. Thus, analysis of EVs number, size, and composition may soon be used to diagnose, stage, and even analyze relapse of hematological malignancies in the clinical setting.

4.2. Analysis of EVs uptake and binding by flow cytometry

Multiple studies underline functional relevance of different extracellular vesicles. Thus, a significant aspect of research is analysis of fluorescently labeled EVs uptake or binding to different cells, both in *in vitro* and *in vivo* experiments. It allows to confirm biological relevance of experiments and exclusion of indirect effects on target cells. Different analytical techniques are used to study extracellular vesicles, as there is no one single method which can be proposed to analyze these particles [50]. Confocal microscopy is widely used to analyze EVs uptake by cells [51]. However, the flow cytometry provides a very good and fast method for quantification of EVs uptake or binding to cells.

Flow cytometry allows to quickly assess if association of EVs with target cells is dose- and time-dependent. This may incline biologically active doses of EVs and thus help choose appropriate amounts of EVs for further functional studies. Multicolor flow cytometry allows to monitor association of EVs to different subpopulations of cells, especially *in vivo* or in *ex vivo* cultures of, for example, immune cells. This is doable if a combination of fluorescent EVs labeling is used in parallel with staining of protein markers of subpopulations with fluorochrome-conjugated antibodies. Moreover, flow cytometry analysis enables correlation of EVs uptake with various biological parameters, such as cell viability, apoptosis, proliferation, and expression of signaling molecules. Such approach allows more precise and direct observation of functional effects of EVs. On the other hand, opposed to confocal microscopy, flow cytometry does not distinguish between uptake and binding of extracellular vesicles to target cells, but rather gives information on association of EVs with target cells. Such information may be relevant in terms of mechanism of influence on target cells, as solely binding to surface of target cell suggests regulation through mediators (mostly proteins) on EVs surface rather than by, for example, non-coding RNAs in the lumen.

Various methods and reagents may be used to fluorescently label extracellular vesicles for analysis using flow cytometry (Table 2). EVs staining can either lead to labeling of all types of

Function	Excitation laser (nm)	Dye	Emission (nm)
Lumen labeling	488 (blue)	CFDA-SE (CFSE)	517
		CalceinAM	515
Membrane labeling	488 (blue)	PKH67	504
		DiO	501
		PKH26	567
	560 (yellow)	Dil	565
		DiD	665
	633/640 (red)		

Table 2. Fluorescent dyes used to fluorescently label extracellular vesicles for analysis by flow cytometry; function, excitation, and emission spectra as well as full name are shown.

vesicles or any of the specific populations, like exosomes or microvesicles. To stain EVs lumen, widely used dyes such as CFSE and CalceinAM may be used. Those dyes in non-fluorescent form passively enter EVs and due to esterase activity in the EV lumen are transformed into fluorescent molecules. Gray et al. demonstrated that CalceinAM stains only intact EVs, which may be advantageous for its use in both EVs uptake studies and direct flow cytometry of EVs [52]. CFSE staining was applied to quantitatively show differential association of cancer EVs with myeloid cells depending on their differentiation into various stages of monocyte and dendritic cell development [53]. In our experiments, we have used CFSE labeling to study the dose-dependent uptake of EVs released by murine BCR-ABL-expressing cells by thymocytes (**Figure 6**). It is clearly visible that increasing amounts of fluorescently labeled EVs correlate with increased fluorescence of CFSE signal detected in acceptor thymocytes.

Membrane staining is also widely used to label EVs with the use of dyes such as PKH67, PKH26, DiD, Dil, and DiO. They allow a wider choice of fluorescence spectra but, as they are lipophilic, can form various aggregates and micelles. Thus, in this case, EVs need to be carefully washed to avoid adding unbound dye which may nonspecifically label cells. PKH67

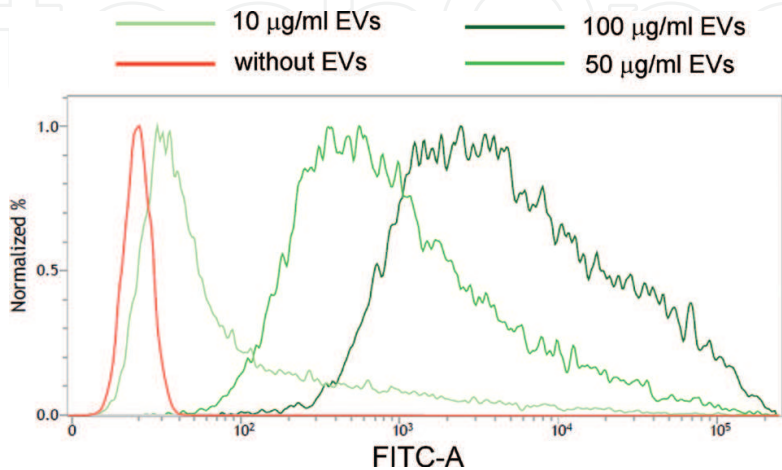


Figure 6. CFSE labeling to study uptake of EVs. Leukemia-released EVs were labeled with CFSE and different amounts of EVs were added to thymocytes. Fluorescence of CFSE in thymocytes was detected by flow cytometry in FITC channel.

was used for EVs labeling to track time-dependent uptake of mantle cell lymphoma-derived exosomes by different immune cells and cell lines and to investigate their possible therapeutic potential. Lai et al. took another approach on staining of EV membrane by fusing GFP at N-terminus with palmitoylation signal and thus directing it to plasma and EV membrane. This enabled more specific staining than with the use of PKH67 dye. Additionally, it allows effective *in vivo* tracking of EVs released by tumors [51]. Furthermore, fusion of specific EV-enriched proteins (such as tetraspanins—CD9, CD63, CD81; ESCRT proteins—Tsg101; proteins widely expressed on cells producing EVs—e.g. HLA proteins) with GFP/RFP or other fluorescent proteins can be applied. CD63-GFP and CD81-GFP have already been used by different groups to track exchange of EVs using flow cytometry [54].

4.3. Flow cytometry analysis of extracellular vesicles

Flow cytometry has emerged as one of the tools for analysis of extracellular vesicles and their composition. As detection limit of conventional flow cytometers is around 500 nm, bead capture of EVs has been used to facilitate, for example, analysis of proteins expressed on their surface. However, direct flow cytometry of extracellular vesicles has also been widely performed [55]. As light scatter triggering itself is not sufficient to distinguish nanoscale vesicles, fluorescence triggering has been used to directly identify EVs on flow cytometers. Commercially available flow cytometer (BD Influx) has been adapted for high-throughput analysis of fluorescently labeled vesicles. PKH67 staining of EVs allowed fluorescence-based thresholding of events, and by modifying cytometer elements, the authors were also able to observe size distribution of exosomes in the FSC channel. The described technique allowed to identify molecules expressed on the surface of EVs [56] and then apply the method to perform quantitative and qualitative analyses of dendritic cell-released EVs upon different stimulations and culture conditions [57]. Direct flow cytometry of fluorescently labeled EVs using conventional flow cytometers has been applied to study mesenchymal stromal cell markers (CD90, CD44, CD73) on EVs released by HS-5 and K562 cells as well as to analyze surface molecules expressed on microvesicles isolated from patient sera with hematological tumors (CD20, CD38, CD30) [48].

However, direct flow cytometry of EVs needs to be used cautiously, as different sample parameters and cytometer settings may affect measurements. It was demonstrated that due to high concentration of very small molecules in suspension, the measurement may not be precise, due to analysis of multiple events as one. Flow cytometry is emerging as a tool for direct analysis of EVs and their surface markers, allowing fast and precise measurements. Various aspects of EVs analysis by FACS are demonstrated in a special issue of Cytometry Part A Journal “Measurement of Extracellular Vesicles and Other Submicron Size Particles by Flow Cytometry” [58]. It is noteworthy that due to small size of EVs precise fluorescent labeling needs to be applied and conventional flow cytometer settings need to be cautiously optimized, for example, by using fluorescent beads of different sizes (e.g. 200 and 100 nm beads).

4.4. Fluorescent tracking and flow cytometry analysis of direct, intercellular transfer of vesicular cargo

Recently, a novel way of intercellular communication has been described. This is mediated by the long intercellular connections called tunneling nanotubes (TNTs), which link two cells

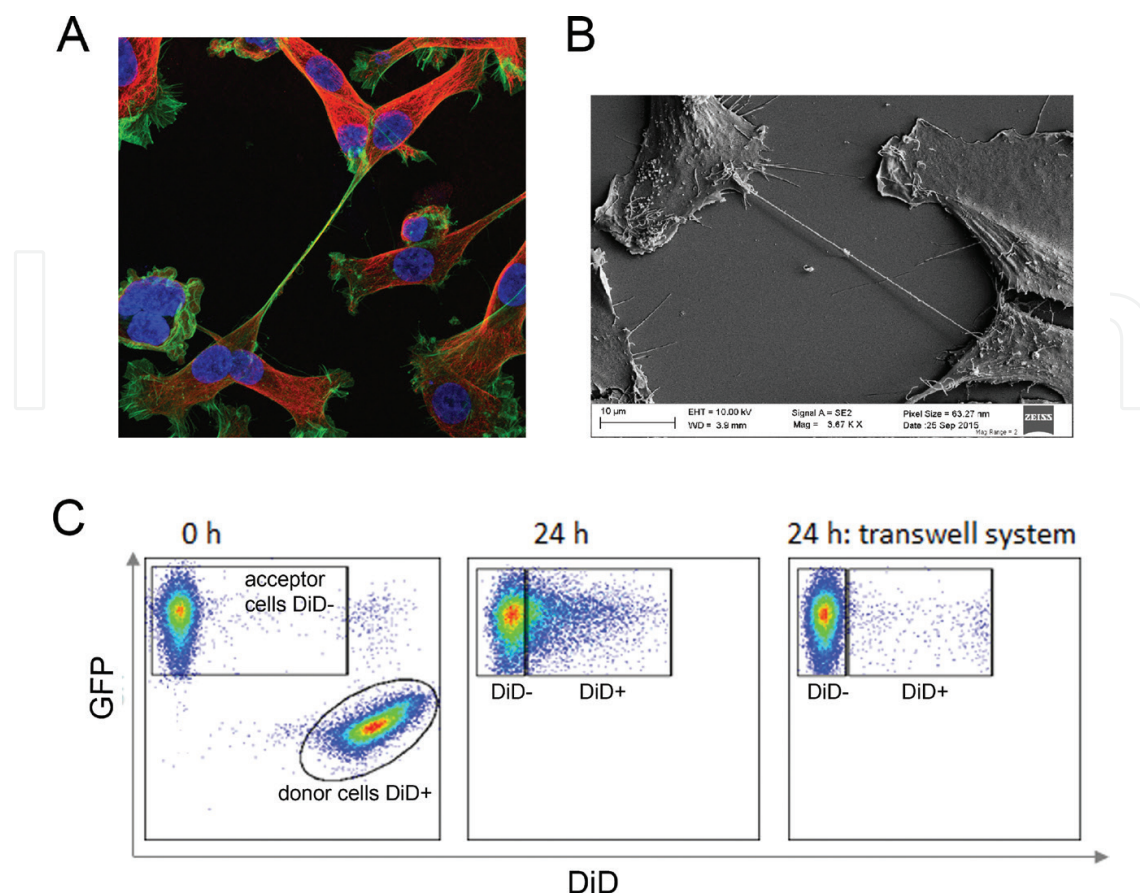


Figure 7. (A) Overlay of confocal images presenting formation of tunneling nanotube (TNT); actin—green, microtubules—red, nuclei—blue; (B) SEM micrograph of a TNT formed between two distant cells; (C) flow cytometry dot plots showing the gating strategy to separate the donor and acceptor populations upon labeling of vesicular cargo by DiD (0 h), the uptake of vesicular cargo visible as appearance of DiD+ acceptor cells (24 h), and the absence of uptake upon donor and acceptor populations physical separation in control experiment (24 h: transwell system).

and enable long-range, direct communication between distant cells [59]. Formation of this structure has been observed between different types of cells by different groups including ours [60–63] and **Figure 7A** and **B**. They have been shown to mediate the cell-to-cell transfer of vesicles, organelles, electrical stimuli, and small molecules [64–66]. The proposed functions of TNTs and mechanism are still not well understood; however, there is growing evidence that cell-cell communication *via* TNTs might play an important role in the cancerogenesis. They are actively formed between leukemia cells and bone marrow-derived mesenchymal stromal cells to promote viability and chemoresistance of leukemia cells [67].

Vesicular cargo can be actively transferred by TNTs between two distinct cells. For now, the flow cytometry is one of the best methodologies to track and analyze cells, which obtained vesicles from donor cells [68–70]. For this, methods to label vesicles has been adapted (**Table 2**) and combined with cell type tracking to distinguish donor and acceptor cells. First, vesicles in donor cells are labeled with lipophilic dyes, such as DiD, DiO, and others, and mixed with acceptor cell population. Flow cytometry allows to separate both subpopulations—positive population of donor cells (DiD+) and negative population of acceptor cells (DiD-), together with additional combination with cell-type tracking with GFP (**Figure 7C**, time 0 h). After

incubation, the fluorescence signal appears in the acceptor cells (GFP+, DiD+), indicating uptake of the vesicular cargo transferred from donor cells (**Figure 7C**, time 24 h). To confirm that vesicles were transferred by direct cell-cell contact, not secreted into the medium, the trans-well control has been performed. In this case, the vesicle uptake has not been observed (**Figure 7C**, transwell). Such methodology has been already successfully used in different cellular systems [60, 69, 71] to analyze direct transfer of vesicles between cells.

5. Interactions of leukemia cells with immunosuppressive regulatory T cells

Immune responses against hematological cancers are less characterized than those against solid tumors. Among them, chronic myeloid leukemia (CML) is not an exception [72]. Little is known about mechanisms of innate and acquired immunity exerted against CML. Although, the involvement of CD4+ and CD8+ T cell as well as NK cells and humoral response in the immunity against CML is widely accepted. Recently, CML-specific antigens exerting cellular and humoral response have been identified [73, 74]. The growing body of evidences is pointing the role of cytotoxic CD8+ T cell as a central player in the anti-CML response. [3, 8] Moreover, CML is considered as one of the diseases which are most sensitive to immunological manipulation [75].

Analysis of blood of untreated patients diagnosed with CML reveals decreased number of NK-cells, which are antitumor effector cells [76]. Moreover, the increased number of inhibitory cells such as T regulatory (T_{reg}) cells and myeloid-derived suppressor cells were present [77]. Also, it has been shown that CML cells (including CD34+ leukemia stem cells) express programmed death receptor ligand 1 (PD-L1). Binding of PD-L1 to the PD-1 receptor expressed on T cells suppresses their effector function.

Immune evasion is a major obstacle for effective anticancer therapy. T regulatory (T_{reg}) cells are recognized as one of the most promising targets for therapy, which could reverse the unresponsiveness of the immune system during malignancy [73]. T_{reg} cells are essential for maintaining homeostasis of the immune system during the steady state and inflammation. The balance between immune activation and tolerance mediated by T_{reg} cells is crucial for maintaining proper responses. The suppression exerted by T_{reg} cells to different types of immune cells has been extensively studied. Activity of T_{reg} cells is beneficial for maintaining self-tolerance, during resolution of inflammation, and for limiting inadequate immune responses. However, their ability to suppress different types of immune cells may also restrain beneficial responses by limiting the anti-tumor immunity. T_{reg} cells constitute a major component of the tumor-infiltrating lymphocytes in human malignancies as well as in mouse experimental models of cancer. Moreover, the increased number of T_{reg} cells among tumor-infiltrating lymphocytes correlates with a poor prognosis and an increased risk of recurrence in majority of solid tumors. In CML patients, the increased number of T_{reg} cells is linked to limited anti-tumor immune effector responses [78, 79]. Data suggest that a balance between the effector and suppressor arms of the immune system could be important in mediating a successful and treatment-free remission (TFR). However, a major goal in CML treatment is to identify the uppermost target to maximize beneficial immune response and promote TFR success [74]. In this context, the study of T regulatory cells is critical.

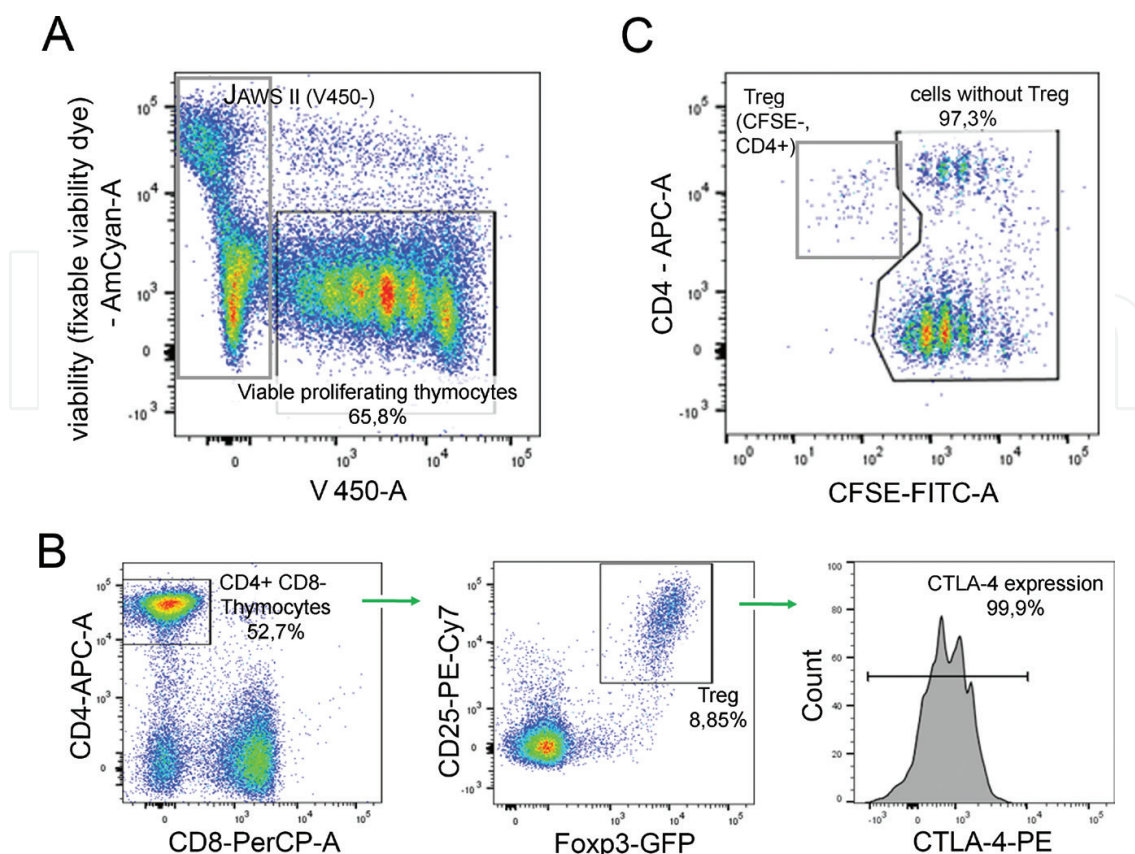


Figure 8. Flow cytometry analysis of T regulatory (T_{reg}) cells differentiation and function. (A) Analysis of cell viability and proliferation (AmCyan channel—fixable viability stain eFluor 506, V450—violet proliferation dye 450). V450 staining allows to track thymocyte proliferation and distinguish them from JAWS II cells (V450-). (B) Identification of T regulatory cells by sequential gating and analysis of CTLA-4 expression. (C) CFSE staining allows to track proliferation of responder cells in an *in vitro* suppression test. An example of combination with CD4-APC staining, which helps eliminate remaining Treg cells (CFSE-, CD4+) in analysis, is presented.

To explore the CML-derived extracellular vesicles impact on T regulatory cell development and activity, we adjust two *in vitro* assays, both based on the flow cytometry.

5.1. Flow cytometry analysis of T regulatory (T_{reg}) cells differentiation and function

To study the effect of CML extracellular vesicles on the differentiation of thymic regulatory T cells (tT_{reg}), we have employed a previously described *in vitro* assay of tT_{reg} differentiation on JAWS II immature dendritic cells [80]. Briefly, during the step of preactivation of thymocytes on α CD3-coated plates, we added extracellular vesicles to allow their uptake or binding solely by thymocytes. Thymocytes were additionally stained with a violet proliferation dye 450, which would allow to track their proliferation and distinguish them from JAWS II cells. After 24 h of preactivation, thymocytes were washed and co-cultured with JAWS II cells. Afterwards, tT_{reg} differentiation using multicolor flow cytometry was analyzed. This analysis allowed to track viability, proliferation (**Figure 8A**), differentiation of tT_{reg} , as well as their phenotype (**Figure 8B**). Extracellular vesicles can also potentially influence suppressive activity of mature, already differentiated tT_{reg} . To study this phenomenon, we sorted tT_{reg} and cultured them, first with CML extracellular vesicles for 24 h and afterwards with conventional T cells (responder cells) stimulated for proliferation, to observe differences in suppressive activity manifested by decreased

proliferation. Labeling of responder cells with CFSE allowed to track their proliferation after co-culture with tT_{reg} , which have been preincubated with either CML-derived or control extracellular vesicles. This in turn allowed to assess differences in tT_{reg} suppressive activity due to influence of EVs. Combination with additional staining of surface receptors such as CD4 or CD8 enabled to distinguish between suppression towards helper (CD4+) or cytotoxic (CD8+) T cells (**Figure 8C**).

6. Summary

Many studies already confirmed that leukemia cells behave differently whether they are cultured alone or in co-culture with stromal cells. This mimics some elements of the bone marrow microenvironment and represents more physiological conditions. Also, the cross talk between leukemia and immune cells is an example showing importance of the interactions within the leukemia microenvironment. Thus, in our opinion, analysis of cell signaling in the co-culture conditions is highly informative and might have some therapeutic implications.

Studies of leukemia cells *in vitro/ex vivo*, including analysis of primary cells and stem/progenitor subpopulations, represent an important step in development of novel therapeutic strategies. Different parameters, such as viability, proliferation, DNA damage, clonogenic potential, and others, are investigated after treatment with drugs or drug candidates. Pretreatment with investigated drugs can also be followed by mice Xenograft *in vivo* studies to investigate their therapeutic potential. All these studies are critically important as a first step in analysis of potential therapeutic strategies.

Nevertheless, we propose that in the next step or simultaneously, some of these studies might be performed also upon co-culture conditions to add an essential element of the cross-talk with stroma components, which normally exist *in vivo*. These conditions allow to verify whether the treatment is still effective even upon protective impact of stroma. This fulfills, at least partially, the big gap between *in vitro* experiments and *in vivo* conditions.

In this chapter, we described and discussed flow cytometry applications, which can be used to perform co-culture studies to analyze some signaling elements within the leukemia microenvironment. This strategy enables to distinguish between the cell types and to investigate the cross talk between cancer and surrounding cells, signaling pathways regulated by the cell-cell interactions, as well as sensitivity to treatment with anticancer drugs in the stroma-mimicking conditions. Utilizing modern flow cytometry and a broad spectrum of currently available dyes/trackers allows to perform highly informative studies not only because of the use of multiparameter cytometry but also because of more complex cellular context, which is taken under consideration. Moreover, even if the described flow cytometry applications rely on the leukemia microenvironment studies, they are uniform and can be broadly applied into another biological context.

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Conflict of interest

The authors declare no conflict of interest.

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