Video Article

Rapid and Robust Analysis of Cellular and Molecular Polarization Induced by Chemokine Signaling

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Abstract

Cells respond to chemokine stimulation by losing their round shape in a process called polarization, and by altering the subcellular localization of many proteins. Classic imaging techniques have been used to study these phenomena. However, they required the manual acquisition of many cells followed by time consuming quantification of the morphology and the co-localization of the staining of tens of cells. Here, a rapid and powerful method is described to study these phenomena on samples consisting of several thousands of cells using an imaging flow cytometry technology that combines the advantages of a microscope with those of a cytometer. Using T lymphocytes stimulated with CCL19 and staining for MHC Class I molecules and filamentous actin, a gating strategy is presented to measure simultaneously the degree of shape alterations and the extent of co-localization of markers that are affected by CCL19 signaling. Moreover, this gating strategy allowed us to observe the segregation of filamentous actin (at the front) and phosphorylated Ezrin-Radixin-Moesin (phospho-ERM) proteins (at the rear) in polarized T cells after CXCL12 stimulation. This technique was also useful to observe the blocking effect on polarization of two different elements: inhibition of actin polymerization by a pharmacological inhibitor and expression of mutants of the Par6/atypical PKC signaling pathway. Thus, evidence is shown that this technique is useful to analyze both morphological alterations and protein redistributions.

Video Link

The video component of this article can be found at https://www.jove.com/video/52140/

Introduction

Chemokines are small soluble proteins that attract cells to specialized locations[1]. Therefore, they participate in the correct positioning of cells in tissues, a crucial function in development and physiology. The immune system is no exception to this rule as it relies on the action of many different cell types which act in concert to mount an effective immune response. By controlling the specific location of one immune cell type in a given state, chemokines are pre-required before foreign antigens can be detected and neutralized.

In T lymphocytes in particular, chemokines bind to specific surface receptors which, upon engagement, elicit many intracellular signals (calcium rise, ERK phosphorylation, Rho GTPases activation, increase in integrins affinity and cytoskeletal alterations) that favor T cell motility[2,3]. At the cellular level, one can observe morphological alterations elicited upon chemokine stimulation. These changes in cell shapes are especially dramatic in T cells: resting T cells have a bead-like round morphology when travelling in the blood stream. However, the sensing of the presence of chemokines in inflammatory sites or at proximity of lymphoid organs is going to change the shape of T cells which now adopt a typical “hand-mirror” morphology consisting of a bipolar form: a leading edge at the front and a trailing edge, or uropod, at the back[4]. In addition, intracellular components can segregate into these two opposite regions of a polarized T cell to sustain migration. For example, actin filaments polymerization increases upon chemokine stimulation[5] and polymerized actin accumulates at the front of a polarized T cell[2]. On the other hand, several proteins, such as phosphorylated proteins of the Ezrin-Radixin-Moesin (ERM) family which link the plasma membrane to the cortical F-actin cytoskeleton, re-localize at the uropod of polarized T cells[6]. Interestingly, we and others have shown that this polarization process is required for T cell migration. Indeed, any treatment that interferes with polarization will inhibit cellular motility. For instance, inhibition of the activity of the members of the atypical protein kinases C (PKC) family, PKCζ and PKCι blocks T cell polarization and their migratory scanning process of dendritic cells[7]. T cell polarization is also regulated by Rho GTPases. We have shown that the modulation of RhoA activity by the recently described Fam65b protein interferes with T cell changes in morphology and their capacity to migrate in a Transwell assay[6]. As polarization is a prerequisite step for cellular motility, it is thus crucial to be able to quantify it as a main readout of chemokine responses. Cell shape alterations were previously measured manually[8]. However, this kind of quantification is very time-consuming, so that usually only a few tens of cells are taken into account.

Here, a new method is presented to rapidly quantify the degree of shape alterations of T lymphocytes exposed to chemokine stimulation. An imaging flow cytometry technology (see **Table of Specific Reagents/Equipment**) is used, which combines the advantages of a flow cytometer and a microscope[9] to quantify efficiently the amount of polarized cells in different conditions of chemokine stimulation. In addition to

the quantification of the morphological alterations that one can measure robustly with this technology, it is also possible to evaluate changes in the subcellular localization of some proteins upon chemokine signaling.

Protocol

1. Preparation of T Lymphocytes

1. Prepare primary mouse or human T cells as described[10,11].
2. Cultivate human T cells in complete RPMI medium with 10% human serum AB at a density of 2 - 3 x 10^6 cells/ml.
   NOTE: The use of fetal calf serum (FCS) instead of human serum can elicit spontaneous polarization of a large fraction of human T cells in culture. Keep these cells in culture for 4 - 5 days and further process them at any time during this period.
3. Maintain mouse T cells in complete RPMI medium supplemented with 10% FCS at a similar cell density. Use right away or the next day, after an O/N culture at 37 °C in the presence of 10 ng/ml IL7.
4. Cultivate the CEM human T cell line in complete RPMI supplemented with 10% FCS.

2. Chemokine Stimulation

1. Wash 5 x 10^5 cells per experimental condition in warm HBSS medium supplemented with 10 mM Hepes. For some experiments, co-transfect primary human T cells the day before with 2 µg pmaxGFP and 8 µg pcDNA3.1$^+$ (empty vector, control), PKCζ kinase dead, or N-terminal Par6 plasmids.
2. Resuspend the cell pellet in warm HBSS-Hepes medium (use 0.3 ml x number of experimental conditions).
3. Distribute the cells in 1.5 ml microcentrifuge tubes.
   NOTE: Therefore, one tube corresponding to a single experimental condition will contain 5 x 10^5 cells in 0.3 ml HBSS-Hepes medium. For some experiments, add 500 nM Latrunculin A or vehicle (DMSO) and incubate the cells for 30 min before chemokine stimulation.
4. Add 10 to 500 ng/ml CCL19 or CXCL12 chemokine in each tube.
   NOTE: We usually use 10 - 200 ng/ml chemokine for human T cells. CEM cells do not express the CCR7 receptor that binds CCL19. Therefore, CEM cells will only be able to respond to a CXCL12 stimulation. Moreover, use higher chemokine concentrations (300 - 500 ng/ml) to polarize mouse T cells.
5. Flip the tubes several times and incubate them in a 37 °C water bath for 8 or 10 min for primary T cells or CEM, respectively.
6. Stop the polarization process by adding to each tube 0.3 ml of a warm solution containing 2% paraformaldehyde (PFA) and 10 mM Hepes in PBS. Flip the tubes and incubate them at 37 °C for 5 min.

3. Stainings

1. Transfer the cells from the microcentrifuge tubes to flow cytometry tubes.
2. Fill the tubes completely with a RT solution containing 1% bovine serum albumin (BSA), 0.5 mM EDTA and 10 mM Hepes in PBS.
3. Centrifuge the tubes at 460 x g for 4 min.
4. Discard the supernatant and resuspend the cells in 100 µl of a RT solution containing 5% FCS in PBS.
5. Add 5 µl of FITC-anti-HLA-ABC in each tube, vortex them, and incubate them for 30 min at RT, away from light.
6. Wash cells once with PBS containing 5% FCS, and once with PBS only.
7. At RT: add 500 µl 1% PFA, incubate for 10 min, then wash cells with a solution containing 1% BSA, 0.5 mM EDTA and 10 mM HEPES in PBS.
8. Discard the supernatant, fill the tubes completely with a solution of PBS containing 0.2% BSA, 0.1% saponin, 0.5 mM EDTA and 10 mM Hepes (permeabilization buffer).
9. Wash cells twice in this medium.
10. Flip the tubes to remove the medium and vortex them to dissociate the cell pellet.
11. Add 0.25 µg/ml TRITC-Phalloidin and/or a 1:100 dilution of anti-P-ERM antibody to each tube, vortex them and incubate for 30 min at RT.
12. Wash cells with the permeabilization buffer. Incubate with a fluorescent secondary antibody if necessary.
13. Resuspend the cells in 200 µl PBS and transfer them to microcentrifuge tubes.
    NOTE: Cells are ready for acquisition. Alternatively, while keeping a maximum total volume of 200 µl per tube, add concentrated PFA to a final 1% concentration in order to preserve the stainings if the cells are not to be used the same day for further processing.

4. Images Acquisition and Analysis

1. Switch on the apparatus (see **Table of Specific Reagents/Equipment**) and let it calibrate itself according to the manufacturer's instructions.
   NOTE: The INSPIRE software was used with a 40X objective (0.75 NA). The IDEAS 3.0 software was used for all the reported quantifications.
2. Prepare a series of acquisition windows which can be saved in a template for future experiments. Draw histograms that quantify the RMS gradient values. From the "In Focus" population, selected on the RMS gradient histogram, delimitate the "Single cell" population on a histogram of the Area. Once the microcentrifuge tube has been inserted in the machine, adjust the laser power, gate on individual cells and acquire 5,000 T lymphocytes.
3. In the Ideas software, open the acquisition file and create a histogram that will show the value of the gradient RMS value for bright field images (Channel 1) obtained for each individual cell. Draw a gate on the RMS gradient histogram that considers cells exhibiting RMS gradient value above 57.
   NOTE: The RMS gradient allows taking into account in the analysis only the T lymphocytes that are in the focal plane. We have determined empirically that individual cells exhibiting a RMS gradient value superior to 57 are in the focal plane, and can be accurately considered.

4. In the Analysis/Masks menu, create a new mask, called Erode(M01,2) from the morphology mask on the brightfield images M01, eroded of 2 pixels. Then, in the Analysis/Features menu, create a new feature of the Area, calculated on the Erode(M01,2) mask. From the cells selected in the previous gate, open a histogram showing the Area of cells using this newly created mask.
   NOTE: The morphology masks that are available by default are much bigger than the actual size of the cell. Thus, it is more precise to erode it up to 2 pixels.
5. Draw a gate on the individual T cells, excluding the calibration beads and debris (small Area) and the doublets (large Area). Adjust this gate at each acquisition.
   NOTE: Variations in cell size will affect the position of the gate. As mouse T cells are smaller than human T cells which are themselves smaller than CEM cells, the area of each cell type will be proportional to its size.
6. Considering the single, in-focus cells that were gated at the previous steps, open a dot plot consisting of the Raw Max Pixel on the HLA-ABC staining (Channel 2, x axis) as a function of the Raw Max Pixel on the phalloidin staining (Channel 4, y axis). Create a gate to exclude the unstained or saturated fluorescent events. Open two histograms showing the Raw Max Pixel for HLA-ABC (Channel 2) and phalloidin (Channel 4) stainings.
7. In the Analysis/Masks menu, create a new mask, called Erode(M02,2) from the morphology mask of the HLA-ABC stained cells (Channel 2), eroded of 2 pixels. Then, in the Analysis/Features menu, create a new feature consisting of the Circularity parameter, calculated on the Erode(M02,2).
   NOTE: This parameter measures the deviation of the cell shape from a circle. Therefore, a perfectly round T cell will have a high circularity value whereas elongated polarized cells will exhibit a low circularity index.
8. Subsequently, create another histogram that will report the values of the Circularity feature from the previously gated cells. Use this histogram to directly plot the distribution of an individual, in-focus cell population according to the value of the Circularity for each individual cell.
9. Looking at the shape of the histogram for non-stimulated cells, draw a gate for polarized cells, starting at the lowest circularity value up to the circularity limit value where most of the non-stimulated cells are plotted. Look at the statistics display for the percentage of polarized cells among the gated population (%Gated).
   NOTE: We have set this gate for Circularity index values below 13.
10. In order to look at the polarization of the actin staining in the cells, plot a histogram for the Bright Detail Similarity R3 value, comparing the HLA-ABC staining (Channel 2) and the phalloidin staining (Channel 4).
    NOTE: The bigger the value of this feature, the more superimposed the two stainings are.
11. Using the same gating strategy as before, plot a gate on the non-stimulated cells for the segregated staining that shows the percentage of cells with polarized actin staining.
12. When completed, the percentage of cells exhibiting a segregation in the distribution of the MHC Class I and phalloidin stainings, the percentage of polarized cells together with the mean values of the Circularity and Similarity indexes of all the cells ± SD are shown in the corresponding statistics panels.

Representative Results

The first example chosen here concerns the use of human primary T lymphocytes stimulated with CCL19. However, the same strategy can be used with primary mouse T cells, T cell lines or any other cell type responsive to chemokine stimulation. The gating strategy presented here includes a series of windows that select the focused events, then the single cells. Finally the range of fluorescence intensity is followed here as an example on two markers: the MHC Class I HLA-ABC and filamentous actin for resting (**Figure 1**) or CCL19-stimulated (**Figure 2**) T lymphocytes.

First, the Circularity index is measured in untreated (**Figure 1**) or CCL19-stimulated (**Figure 2**) T lymphocytes. The fact that resting cells have a major single peak with a high Circularity index value indicates that most cells are close to round. However, it is clear that CCL19 stimulation elicits the appearance of a second subpopulation of cells with a low Circularity index. This subpopulation encompasses cells that have adopted a polarized morphology. Also, when representing the Bright Detail Similarity index between HLA-ABC and F-actin staining, one can observe that some cells exhibit a drop in the value of this index upon CCL19 treatment, indicating that both markers show a lower degree of co-localization. Interestingly, from the dot-plots presented previously, one can position the curser on any particular dot to get a picture of the cell consisting of a brightfield image and an image for each of the stainings performed. **Figure 3** thus presents examples of CCL19 stimulated T lymphocytes that fall in the gate of non polarized (**Figure 3A**) or polarized (**Figure 3B**) cells. One can clearly visualize the differences in morphology between both subpopulations. From the statistics tables below the Circularity histograms from **Figures 1** and **2**, one can plot the percentage of cells that fall in the polarized gate in the absence or presence of CCL19 (**Figure 3C**). As expected, it is clear that chemokine stimulation increases the percentage of polarized T cells (from 11.2% to 50.6%). One can also plot the mean Circularity index of the whole cell population obtained in both conditions (**Figure 3D**). CCL19 stimulation elicits a drop in this index. However, as only half the cells are polarized, the change in the mean Circularity index observed upon CCL19 stimulation is small.

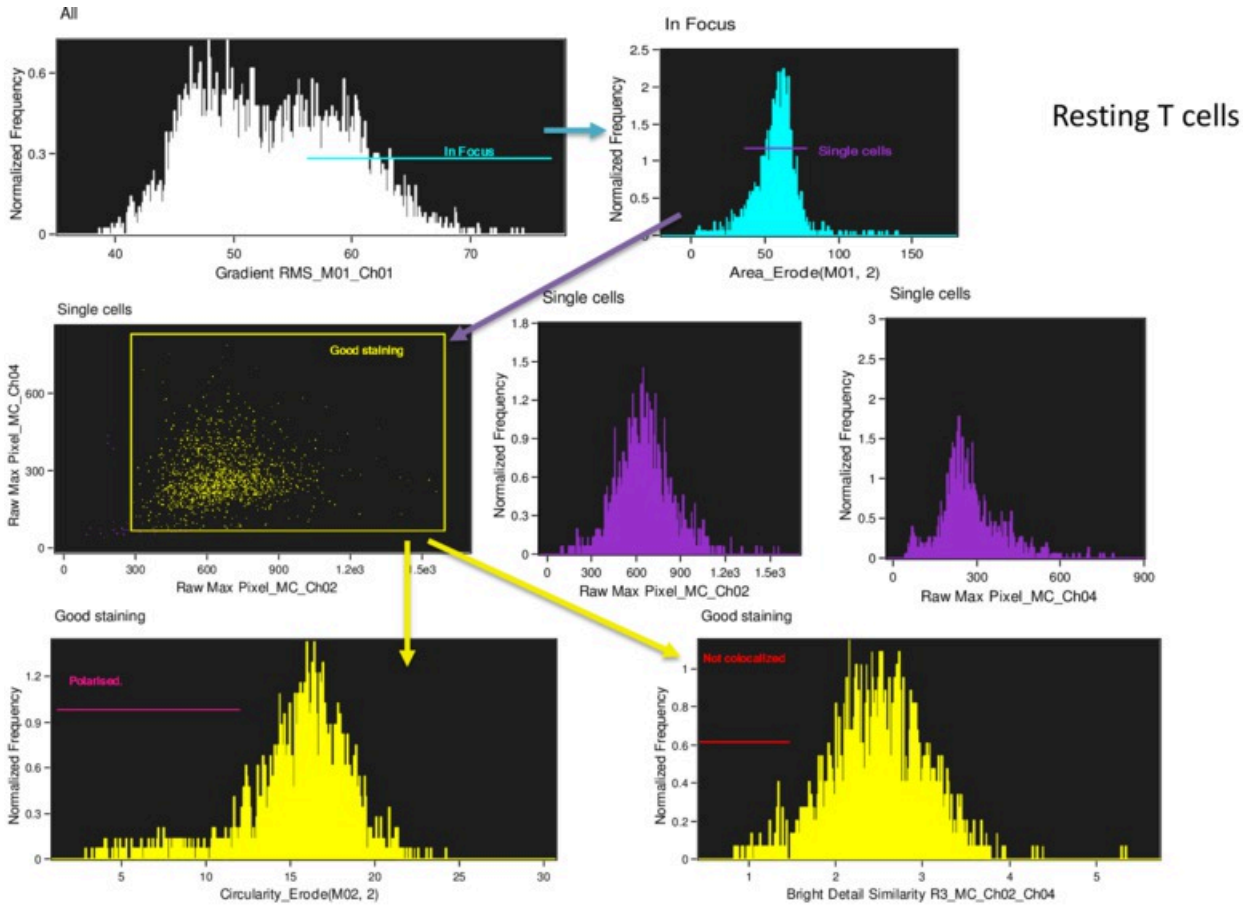
Similarly, **Figure 4** represents examples of CCL19-stimulated cells, in which both the HLA-ABC and the F-actin stainings do (**Figure 4A**) or do not colocalize (**Figure 4B**). From the statistics tables below the Bright Detail Similarity histograms shown in **Figures 1** and **2**, one can obtain the proportion of cells that exhibit an index value below 1.5, representing the cells that exhibit a very low degree of co-localization between HLA-ABC and F-actin. **Figure 4C** shows that the fraction of cells that falls in this gate increases upon CCL19 stimulation (from 3.4% to 13.9%). Collecting the values of the index for each individual cell, one can also plot the mean Bright Detail Similarity index of the whole cell population obtained in both conditions (**Figure 4D**). CCL19 elicits a drop in the index, however, for the same reasons as previously, changes are small. By looking at the images of the corresponding cells, it appears that this drop in HLA-ABC – F-actin co-localization is mainly due to the clustering of F-actin in one pole of the cell. Conversely, the subcellular distribution of HLA-ABC MHC Class I molecules does not seem to be grossly affected by CCL19 stimulation.

That is the reason why we next decided to test further the methodology by analyzing two markers (F-actin and phospho-ERM) known to segregate in opposing poles of chemokine-stimulated T cells. Human T cells stimulated with CXCL12 polarized strongly, as quantified in **Figure 5A** with the same gating strategy than before (from 5.95% to 79.10% polarized cells after 8 min of stimulation with 50 ng/mL CXCL12). In these conditions, there is a strong decrease in the mean Bright Detail Similarity index as shown in **Figures 5B** and **5D**, meaning that F-actin and

phospho-ERM colocalize less after CXCL12 stimulation. As expected, the percentage of cells exhibiting a segregation of the markers chosen here is higher (48%, **Figure 5C**) compared to the ones previously studied (14%, **Figure 4C**). Accordingly, the drop in the mean Similarity index of each condition is also more obvious (**Figure 5D**). Therefore, the imaging flow cytometry technique presented here is suitable to quantify the degree of co-localization of two stainings in different settings.

Finally, we decided to illustrate to what extent the perturbation of some cytoskeletal element or signaling pathway known to affect changes in T cell morphology upon chemokine stimulation, could be measured by imaging flow cytometry. Control T cells were first compared with some pretreated with the actin-disrupting drug latrunculin A (**Figure 6A**). Cells were stimulated with CXCL12, stained with fluorescent phalloidin and analyzed by imaging flow cytometry. Histograms showing the Raw Max Pixel intensity of the F-actin staining are shown for both cell populations (**Figure 6A**, top). As expected from the severing activity of latrunculin A towards actin filaments, the intensity of the phalloidin staining is lower in these cells (right panel) compared to control T lymphocytes treated with the vehicle DMSO (left panel). Moreover, latrunculin A-treated T cells exhibit a larger Circularity index indicating that they remain rounder than control cells (**Figure 6A**, bottom). This can be quantified by a gate set on the histograms to measure the percentage of cells that exhibit morphological alterations. Although 32% of control cells get polarized in this condition, only 10% of latrunculin A –treated T lymphocytes exhibit any shape changes (**Figure 6B**). Therefore, it is shown here that the imaging flow cytometry technology is suitable to measure differences in morphological modifications when one studies the potential effect of some cytoskeletal elements in this phenomenon.

Secondly, the acquisition of a large number of cells with this method also opens the possibility to analyze rapidly small subsets of cells present as a minority. In **Figure 7**, an example is presented where our analysis is specifically restricted to the behavior of T cells co-transfected with GFP together with plasmids encoding for dominant negative mutants of the polarity complex Par6/PKCζ. The histogram corresponding to the non-transfected cells has allowed us to gate specifically on the relevant GFP^+ T cells (**Figure 7A**, left). The intensity of GFP measured as histograms that quantify the distribution of the fluorescence Intensity of the channel 2 indeed shows that only a small fraction of the whole cell population has been transfected (**Figure 7A**). The degree of morphological polarization of these transfected cells was then quantified by measuring only the Circularity index of the GFP^+ cells (**Figure 7B**). An additional gate for T cells exhibiting low values of Circularity allowed estimation of the percentage of polarized cells in each condition (**Figure 7C**). The results show that disruption of the function of the Par6/PKCζ signaling pathway inhibited T cell polarization induced by both CCL19 and CXCL12 chemokines, in line with our previous findings[7].



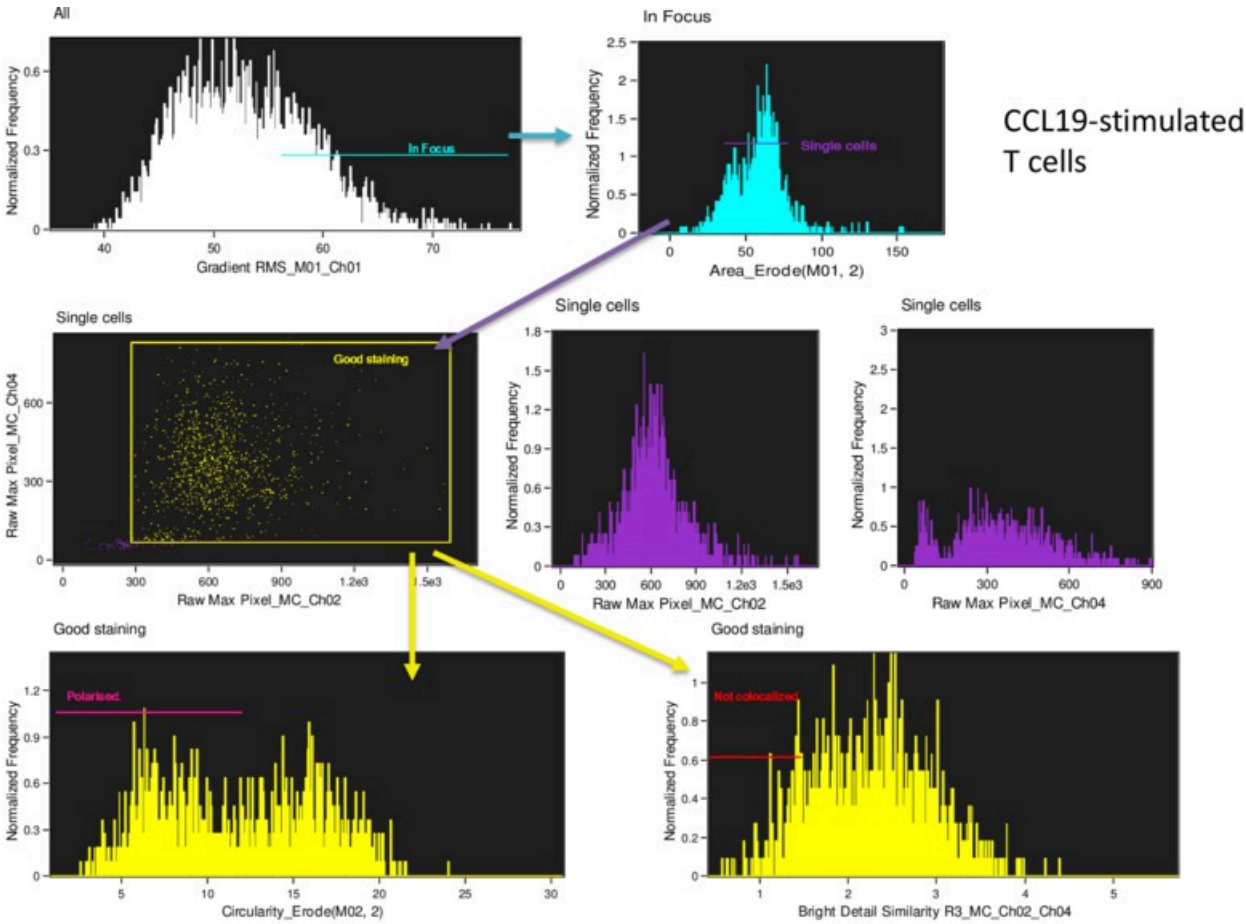
Circularity_Erode(M02, 2)

Population	Count	%Total	%Gated	Mean	Std. Dev.
Good staining & Single cells & In Focus	1469	29.4	100	15.43	3.245
Polarised. & Good staining & Single cells & In Focus_1	164	3.28	11.2	8.552	2.559

Bright Detail Similarity R3_MC_Ch02_Ch04

Population	Count	%Total	%Gated
Good staining & Single cells & In Focus	1469	29.4	100
Not colocalized & Good staining & Single cells & In Focus	50	1	3.4

Figure 1. Representative non-stimulated staining results on primary human T cells. The upper left panel shows the histogram repartition of the Gradient RMS calculated with the M01 mask on the bright field images (Channel 1). The upper right panel shows the Area of the M01 mask eroded of 2 pixels, based on the brightfield images (Channel 1) of the In Focus cells gated from the previous plot. The middle panels show the Raw Max Pixel values of both the HLA-ABC and Phalloidin stainings (Channels 2 and 4, respectively) either as a dot plot (left panel) or as histograms (middle and right panels) for the individual, in-focus cells selected at the previous step. From this population of individual, in-focus cells with correct staining, is calculated the Circularity feature on the M02 mask eroded of 2 pixels of the HLA-ABC staining (lower left panel) and the Bright Detail Similarity R3 feature (lower right panel) showing the degree of similarity between the HLA-ABC and the Phalloidin stainings. For these two latest plots, statistics indicating the repartition of the population (% gated, mean of the feature, S.D.) are indicated in a table below.
Please click here to view a larger version of this figure.



Circularity_Erode(M02, 2)

Population	Count	%Total	%Gated	Mean	Std. Dev.
Good staining & Single cells & In Focus	1102	22	100	11.83	4.685
Polarised. & Good staining & Single cells & In Focus_1	558	11.2	50.6	7.749	2.144

Bright Detail Similarity R3_MC_Ch02_Ch04

Population	Count	%Total	%Gated
Good staining & Single cells & In Focus	1102	22	100
Not colocalized & Good staining & Single cells & In Focus	153	3.06	13.9

Figure 2: Representative staining results on primary human T cells stimulated with 200 ng/ml CCL19 for 8 min. The panel layout and gating strategy are identical to **Figure 1**. Please click here to view a larger version of this figure.

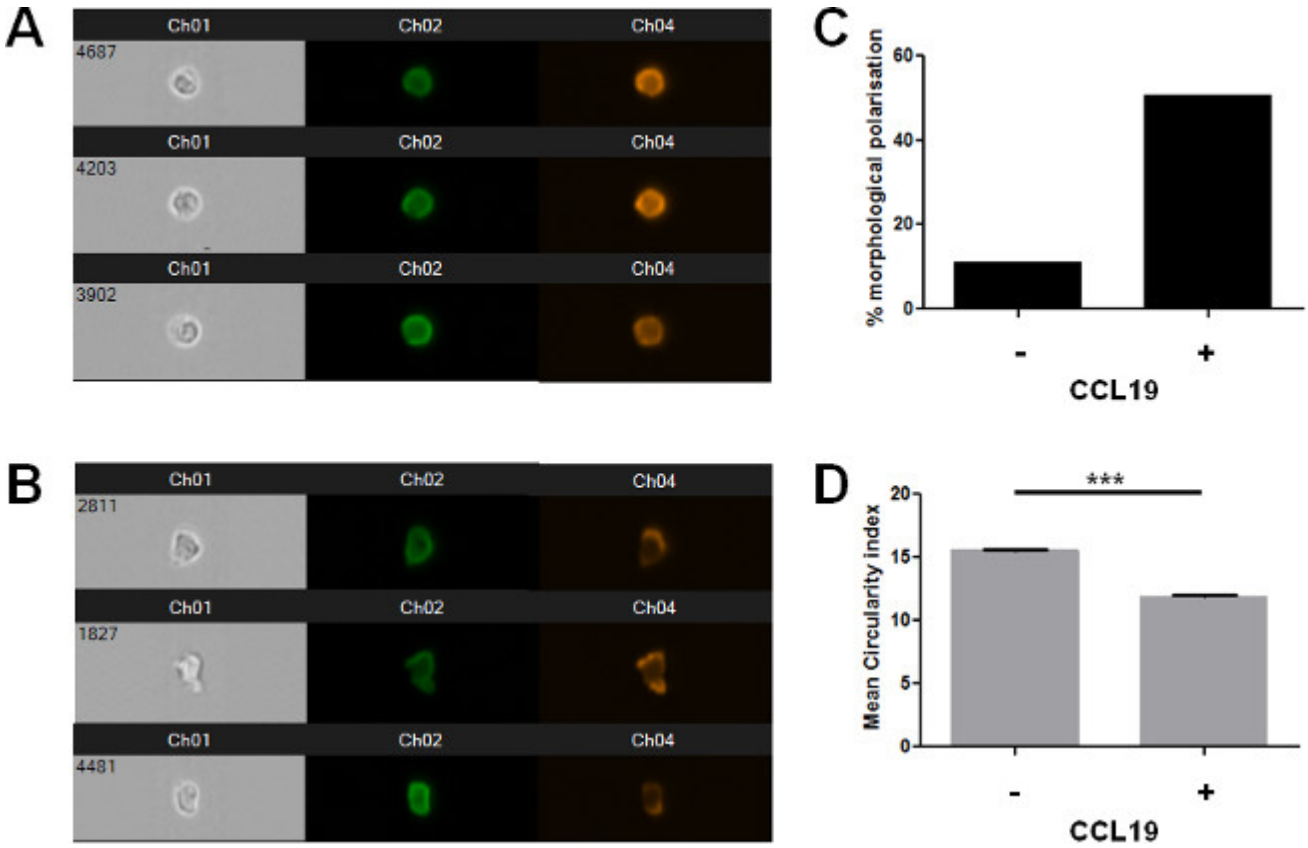


Figure 3: Quantification of the morphological status of T lymphocytes stimulated by CCL19. (A,B) Examples of cells from **Figure 2** outside or in the "polarized" gate, respectively. **(C)** Histogram showing the percentage of polarized T cells obtained in the absence of CCL19 (**Figure 1**, -) or with 200 ng/ml CCL19 (**Figure 2**, +). **(D)** Histogram representing the mean values of the circularity index ± SE in the individual, in-focus cell population in the absence (-) or presence (+) of CCL19. *** $p < 0.001$. Please click here to view a larger version of this figure.

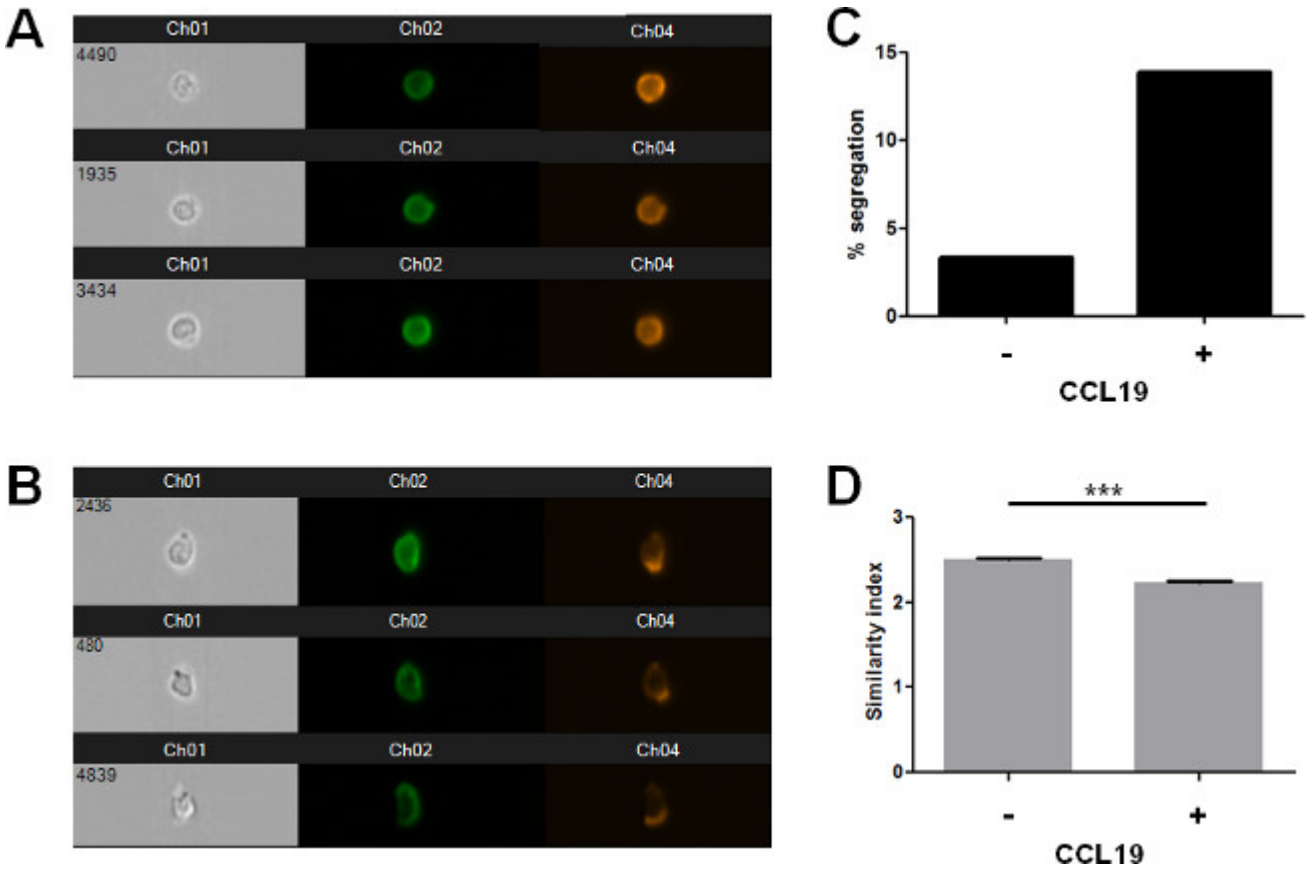


Figure 4. Quantification of the degree of co-localization between the HLA-ABC and phalloidin stainings. (A,B) Examples of cells from **Figure 2** outside or in the "not colocalized" gate from the Bright Detail Similarity histogram, respectively. **(C)** Histogram showing the percentage of T cells exhibiting no co-localization between both markers in un-stimulated (-) or CCL19-stimulated (+) conditions. **(D)** Histogram representing the mean values of the Similarity index ± SE in the individual, in-focus cell population in the absence (-) or presence (+) of CCL19. *** $p < 0.001$. Please click here to view a larger version of this figure.

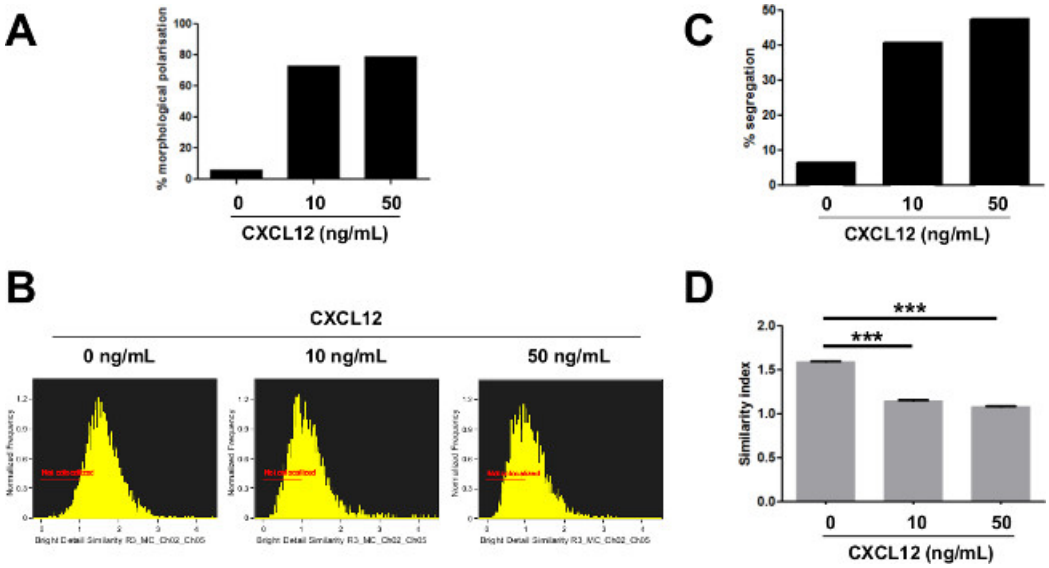


Figure 5. Analysis of F-actin – phospho-ERM exclusion upon CXCL12 stimulation of human primary T cells. (A) Percentage of polarized T cells in the absence or presence of CXCL12 (10 or 50 ng/mL). **(B)** Imaging flow cytometry histograms showing the repartition of the individual, in-focus cell population according to the Bright Detail Similarity index that compares the extent of co-localization between the phospho-ERM and phalloidin stainings, in the absence of CXCL12 or with 10 ng/ml or 50 ng/ml CXCL12. **(C)** Quantification of the proportion of cells exhibiting no co-localization of the pERM and the phalloidin stainings in T cells stimulated or not with CXCL12. **(D)** Quantification of the mean values of the Similarity index ± SE in each condition. *** $p < 0.001$. Please click here to view a larger version of this figure.

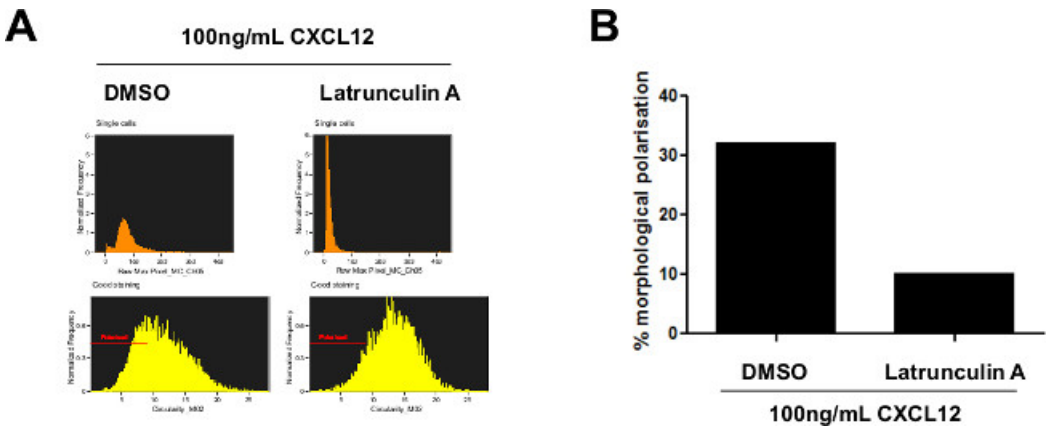


Figure 6. Effect of actin polymerization on T cell polarization. (A) F-actin staining intensity (upper panels) and Circularity (lower panels) of primary human T cells treated with Latrunculin A (500 nM, 30 min) or DMSO and then stimulated with 100 ng/ml CXCL12 for 8 min. **(B)** Histogram showing the percentage of polarized T cells treated with DMSO or Latrunculin A after 8 min of stimulation with CXCL12. Please click here to view a larger version of this figure.

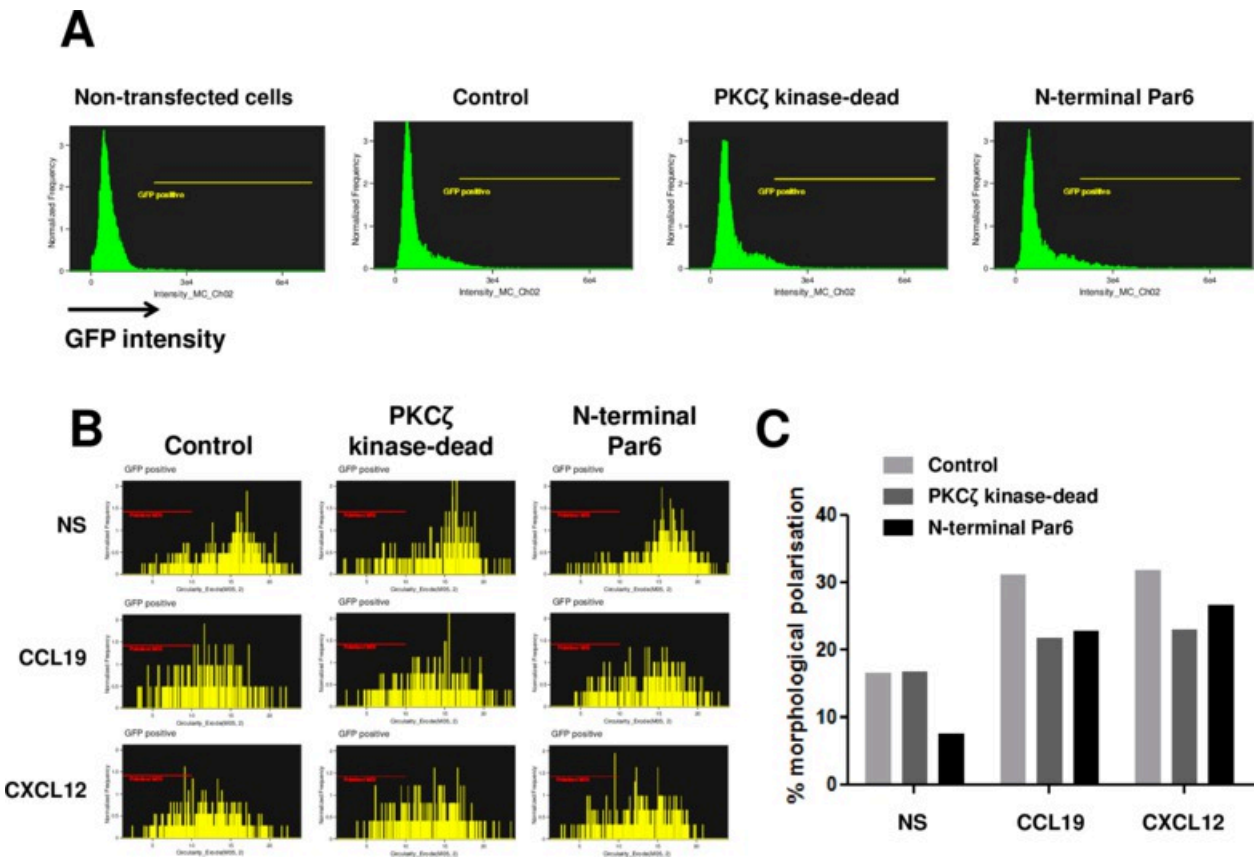


Figure 7. Expression of the PKCζ kinase-dead mutant or the N-terminal mutant of Par6 blocks T cell polarization after CCL19 or CXCL12 treatment. (A) Imaging flow cytometry histograms showing the expression intensity of GFP, in non-transfected primary T cells or primary T cells co-transfected with GFP and an empty vector (Control) or the kinase-dead mutant of PKCζ or the N-terminal part of Par6. **(B)** Imaging flow cytometry histograms representing the Circularity index of the GFP-positive cell population expressing the different constructs at the basal level (NS: No Stimulation) or after CCL19 or CXCL12 stimulation (100 ng/ml for 8 min). **(C)** Quantification of the percentage of morphologically polarized T cells expressing the different constructs at the basal level or after CCL19 or CXCL12 stimulation. Please click here to view a larger version of this figure.

Discussion

Using a recent technology of imaging flow cytometry, a rapid and informative gating strategy to analyze cellular and molecular events induced by chemokine stimulation is presented. From a single experiment, one can obtain two main types of information: the changes in cell morphology induced by chemokine stimulation and the subcellular distribution of different proteins during the polarization process. Interestingly, evidence is also provided for the possibility to analyze a large number of cells, which allows statistical robustness and relevant analysis of a small fraction

of the whole cell population. As reported, this technique can also be used for analyzing molecular redistribution events in the context of the immunological synapse[12]. In addition, a similar set up has already been used for SDF1-stimulated T cells but no analysis of the degree of co-localization between two markers has been provided[13].

Although this technique can thus be suitable for many cell types, adherent cells cannot be directly analyzed and cells in suspension need to be fixed prior the acquisition. In addition to these intrinsic limitations of the machine, it is critical to keep the cells at 37 °C when stimulating them. Temperature is indeed key to achieve proper T cell polarization since cytoskeletal changes, such as actin polymerization that drives shape changes, are very dependent on the temperature. Our experiments with latrunculin A treated cells confirm that proper actin remodeling induced by chemokine stimulation is indeed crucial for a cell to achieve its fully polarized state. In addition to the data provided here with this drug, we also tested the imaging flow cytometry technology with human primary T lymphocytes transfected with mutant proteins that inhibit the Par6/PKCζ signaling pathway. Consistently with our previously published results[7], it is shown here that this polarity complex favors T cell polarization.

Upon chemokine stimulation, T cells lose their round morphology to adopt a polarized shape. We have found the Circularity parameter to be the most accurate to quantify those morphological alterations. However, other parameters describing shape changes are proposed by the analysis software, such as the Aspect Ratio or the Elongatedness. Therefore, one can easily test the contribution of a particular signaling pathway or a specific protein in this process.

In this study, we have always observed that the percentage of cells exhibiting a morphological polarization is higher than the one for molecular polarization. This indicates that a fraction of cells that adopt a change in morphology do not present large molecular redistributions of the markers studied. This could arise from the fact that some cells lose their round shape so they exhibit a lower Circularity index although they do not clearly show a leading edge and a uropod. These partially polarized T cells could still fall in the gate of morphologically polarized T cells without presenting obvious molecular redistributions of typical markers. Moreover, differences in the chemokine signaling threshold that could be higher for markers redistribution than for shape alterations could also explain these differences.

It is also shown here that the HLA-ABC marker is not relocalized in polarized T cells and there is no change in the intensity of this staining upon CCL19 stimulation. Conversely, a confirmation is provided here that F-actin staining increases upon CCL19 treatment (**Figures 1** and **2**) as already known, since chemokine stimulation activates actin polymerization[5]. Moreover, it is shown that actin filaments get relocalized at the front of polarized cells. Consequently, the Bright Detail Similarity index that measures the extent of co-localization between both markers tends to decrease upon CCL19 stimulation. As a comparison, such an analysis is provided here for the degree of F-actin co-localization with phospho-ERM proteins that get excluded from the cell front. The percentage of chemokine-stimulated T cells exhibiting F-actin – MHC Class I segregation is much lower than the one for F-actin – phospho-ERM (14% *vs.* 48%, respectively) since F-actin relocalizes at the front and phospho-ERM proteins at the rear of polarized T cells, whereas the repartition of MHC Class I molecules is not affected by chemokine stimulation. This Bright Detail Similarity index can thus be systematically used to compare the distribution of two markers in polarized T cells. Therefore, this technique is very appropriate to quantify the degree of co-localization or co-exclusion of two different markers on a large number of cells or, potentially, on rare events present in a broad cell population.

Disclosures

The authors declare that they have no competing financial interests.

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