Video Article

Preparation of CD4$^+$ T Cells for Analysis of GD3 and GD2 Ganglioside Membrane Expression by Microscopy

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Abstract

The methods described herein for activation of naïve CD4$^+$ T cells in suspension and their adherence in coverslips for confocal microscopy analysis allow the spatial localization and visualization of gangliosides involved in CD4$^+$ T cell activation, that complement expression profiling experiments such as flow cytometry, western blotting or real-time PCR. The quantification of ganglioside expression through flow cytometry and their cellular localization through microscopy can be obtained by the use of anti-ganglioside antibodies with high affinity and specificity. Nonetheless, an adequate handling of cells in suspension involves the treatment of culture plates to promote the necessary adherence required for fluorescence or confocal microscopy acquisition. In this work, we describe a protocol for determining GD3 and GD2 ganglioside expression and colocalization with the TCR during naïve CD4$^+$ T cell activation. Also, real-time PCR experiments using <40,000 cells are described for the determination of the GD3 and GM2/GD2 synthase genes, demonstrating that gene analysis experiments can be performed with a low number of cells and without the need of additional low input RNA kits.

Video Link

The video component of this article can be found at https://www.jove.com/video/54569/

Introduction

The CD4$^+$ T cells orchestrate the immune response through their effector functions after activation by antigen presenting cells[1]. The study of the cellular mechanisms that are modulated during activation allows insight into a basic process of immune function. However, the study of naïve CD4$^+$ T cells can be complicated because they represent a very small population of cells in the blood periphery[2].

Through fluorescence microscopy several reports have studied the localization of different molecules involved in CD4$^+$ T cell activation, mainly proteins associated to the plasma membrane[3]. The gangliosides are sialic acid containing glycosphingolipids and although they have been extensively studied in nerve cells where they are abundant, other cells such as immune cells also express gangliosides with biologically relevant functions[4,5]. We previously reported that during activation of human naïve CD4$^+$ T cells there is an upregulation of the α2,8 sialyltransferase ST8Sia 1 (GD3 synthase) and the GM2/GD2 synthase, that induce the significant surface neoexpression of GD2 and the upregulation of GD3 ganglioside[6]. Further study of GD3, GD2 and other gangliosides in immune cells is necessary to complement a protein-based partial view of immune function.

Commonly, the study of ganglioside expression is based on techniques such as Thin Layer Chromatography (TLC)[7], but this technique does not allow the spatial localization of gangliosides at the plasma membrane or in subcellular compartments, limiting biological analysis.

In this work, we describe a protocol for the antibody-mediated identification and localization of GD3 and GD2 gangliosides in human naïve CD4$^+$ T cells and PBMCs after anti-CD3/anti-CD28 activation. With this protocol it is also possible to analyze the gene and molecular expression of gangliosides in a low number of cells in suspension, with acquisition of high quality images[6], considering the small size of lymphocytes (9 µm).

Protocol

Peripheral blood from healthy male donors was obtained with informed consent and approval of the Bioethics Committee of the Centro de Investigaciòn en Dinámica Celular- Universidad Autònoma del Estado de Morelos.

1. Isolation and Activation of Human Naïve CD4$^+$ T Cells

1. Collect 2 ml of peripheral blood derived from healthy human donors through informed consent and dilute with 2 ml of sterile PBS-EDTA (1x Phosphate Buffered Saline (PBS), 2 mM EDTA, pH 7.4). Add the diluted blood slowly on top of 3 ml of sucrose solution with 1.077 density as previously described[8].
   Note: Differential migration during centrifugation caused by differences in density will cause erythrocytes to sediment completely and a layer of less dense mononuclear cells will form above. 2 ml of peripheral blood will render approximately 0.5 x 10^6 naïve CD4$^+$ T cells after the purification steps.
2. Centrifuge 30 min at 250 x g at 21 °C (use rotor for round bottom tubes with fixed angle of 30 mm) with no break to generate the gradient of peripheral blood mononuclear cells (PBMCs). After centrifugation, the PBMCs can be clearly observed as a white ring. Collect the PBMCs with a pipette and transfer to sterile tube for washing.
3. After three washes with PBS-EDTA solution at 250 x g for 10 min proceed to the purification of naïve CD4$^+$ T cells. Sorting methods or several types of purification kits are available for this purpose. Preferably, use >1 x 10^7 PBMCs for purification.
   1. Purify naïve CD4$^+$ T cells by negative selection with magnetic beads. Perform negative selection with anti CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a (glycophorin A) antibodies. Assess the percentage of purity by determining CD4+CD45RA+ cells through flow cytometry.
      Note: Optionally, proceed to incubation of PBMCs overnight at 37 °C and 5 % CO_2 with a density of 1 x 10^6 cells/ml in 10 ml of supplemented medium to promote monocyte adherence and continue with naïve CD4$^+$ T cell purification. The efficient recovery of naïve CD4$^+$ T cells from peripheral blood depends largely on the purification system.
4. Prepare 24-well cell culture plates by adding 250 µl per well of PBS with 5 µg/ml anti CD3 monoclonal antibody and incubate for at least 2 hr at 37 °C.
   Note: Plates with 96 wells can be used when a smaller number from naïve CD4$^+$ T cells are used (*e.g.,* 2 x10^4-1 x 10^5).
5. Remove the anti CD3 antibody dilution from the 24 well or 96 well plates and wash the plates twice using sterile 1x PBS.
6. Dilute the naïve CD4$^+$ T cells with >95 % of purity (CD4+CD45RA+) to a density of 1 x 10^6 cells/ml in advanced RPMI 1640 medium supplemented with 3 % of fetal bovine serum (or RPMI supplemented with 10 % of serum), 2 mM glutamine and antibiotics (1 U/ml penicillin, 1 µg/ml streptomycin).
   Note: If ganglioside localization is required in PBMCs, follow the same protocol for dilution and stimulation as described below.
7. Add 500 µl of the cell dilution to anti CD3 coated wells and to non-coated wells (control cells) in the 24 well plate. Alternatively, add 100 µl of the cell dilution to the wells of the 96 well plate.
8. Complete the stimulation conditions of naïve CD4$^+$ T cells in anti CD3 coated wells by adding 1 µg/ml of anti CD28 antibody.
9. Keep the resting CD4$^+$ T cells as control in the non-coated wells without addition of anti CD28 antibodies. Incubate the resting and activated CD4$^+$ T cell for 0 to 72 hr under standard conditions of 37 °C and 5% CO_2.
10. To verify an adequate activation evaluate by flow cytometry the expression of the CD69 early activation marker (16 hr post-activation) or CD25 late activation marker (48 hr post-activation) in resting and activated naïve CD4$^+$ T cells incubated at 37 °C and 5% CO_2 in absence or presence of anti CD3/CD28 antibodies[9,10].

2. Adherence of Resting and Activated Naïve CD4$^+$ T Cells

1. Sterilize 12 mm round coverslips by autoclaving and UV light exposure for at least 15 min.
2. Place coverslips in sterile 24-well cell culture plates.
   Note: For cultures with less than 1 x 10^5 cells it is preferable to use slide chambers with adapted small wells to prevent dispersion of cells in the coverslip.
3. Coat coverslips (or slide chamber) with 200 µl of poly-L-lysine (M.W. 150-300 KDa) 0.1% (w/v) solution and incubate for 5 min at room temperature (RT), remove and dry for at least 1 hr at RT.
4. Mix carefully and collect the resting and activated naïve CD4$^+$ T cells from the 24-well plates. Count the cells and if necessary adjust with fresh supplemented medium to transfer 1 x 10^5 cells to the poly-L-lysine coated coverslips. Incubate the cells for a minimum of 6 hr at 37 ^oC and 5% CO_2.

3. Collecting and Fixation of Resting and Activated Naïve CD4$^+$ T Cells

1. Carefully remove the medium from cultured resting and activated naïve CD4$^+$ T cells.
2. Add 500 µl of RT sterile PBS slowly.
   Note: Careful addition and removal of solutions from wells prevents the detachment of cells from the coverslip.
3. Discard the PBS and add another 500 µl PBS to thoroughly remove culture media.
4. Fix the cells by addition of 500 µl filtered 4% paraformaldehyde (filtration removes microparticles that can interfere with microscopy analysis) and incubate for 30 min at RT (preferred) or overnight at 4 °C.
   Caution: Paraformaldehyde is a toxic and volatile compound. It is known to be a human carcinogen. Use carefully with an appropriate safety protocol.
5. Remove the fixative and wash at least twice with PBS at RT.
6. Proceed to the next step or maintain the plate wells with 500 µl of PBS at 4 °C for several days until staining.
   Note: Sterility during this process helps avoid microorganism growth.

4. Staining, Mounting and Visualization by Confocal Microscopy

1. Remove the PBS from the wells. Add 500 µl of cold blocking buffer (1x PBS, 0.5% standard grade bovine serum albumin, 1% fetal bovine serum pH 7.4). Incubate for 20 min on ice.

2. Remove the blocking buffer carefully and add the primary antibodies (anti gangliosides GD3 clone R24, GD2 clone 14G2a), PE-conjugated anti CD25 and APC-conjugated anti TCR and isotypes controls diluted in blocking buffer to 2.5 µg/ml.
3. Incubate 2 hr on ice or overnight at 4 °C.
   Note: Slow orbital agitation is preferable.
4. Remove the antibody carefully and add slowly 500 µl of PBS. Repeat twice.
5. Add the secondary antibodies (FITC conjugated anti IgG3 for R24 anti GD3, Alexa Fluor 488-conjugated or Alexa Fluor 647-conjugated anti IgG2a for 14G2a anti GD2) diluted in blocking buffer to 0.1 µg/ml.
6. Incubate for 1 hr on ice in the dark without shaking.
7. Wash three times in PBS as described in 4.4). Keep the wells with enough PBS to avoid the dehydration of samples.
8. Add Hoechst 333258 stain diluted in PBS to 0.1 µg/ml.
9. Incubate 15 min at RT and remove. Wash three times in PBS.
10. Place the slides on a paper towel and add 20 µl of mounting solution (50% glycerol in PBS) or commercial mounting solutions to avoid fading and quenching from samples.
11. Carefully pick up the coverslip with fine tweezers and place it on the mounting solution. Ensure that the side of the coverslip where cells are attached is in contact with the solution. Seal the coverslips with nail polish and analyze using fluorescence or confocal microscopy.

5. Isolation of RNA

1. Prepare anti CD3 antibody (5 µg/ml) coated 96-well plates. Incubate 4 x 10^4 naïve $CD4^+$ T cells/well in 100 µl of supplemented culture medium with anti CD28 antibody (1 µg/ml) to complete the stimulus. Incubate at 37 °C and 5% CO_2 for 0 to 72 hr.
2. After different post-activation times place the cells into a microcentrifuge tube.
3. Add 500 µl of PBS and centrifuge at 250 x g for 5 min at RT.
4. Remove the supernatant and add 1ml Trizol reagent.
5. Incubate for 5 min at RT and keep the sample at -80 °C for at least 24 hr. Proceed with the RNA isolation when needed.
   Note: Leaving the sample at -80 °C for at least 24 hr improves RNA yield. Also, use of hand gloves is essential to avoid RNA degradation by RNases.
6. Defrost the sample by incubation at 37 °C for 2 min in a water bath.
7. Add 200 µl chloroform and shake vigorously by hand for 30 sec (to avoid aggressive mixing of RNA by vortexing). Incubate 5 min at RT.
8. Centrifuge 15 min at 12,000 x g at 4 °C.
9. Recover the aqueous phase into a new microcentrifuge tube.
10. Add 500 µl isopropanol and invert the tube three times.
11. Incubate 10 min at RT and centrifuge for 10 min at 12,000 x g at 4 °C.
12. Discard the supernatant by inversion.
13. Add 1 ml of ice cold 75% ethanol and centrifuge 10 min at 12,000 x g at 4 °C.
14. Completely discard the supernatant and dry the RNA pellet by leaving the cap of the tube open.
    Note: At this point the pellet is not clearly visible. Proceed anyway.
15. Resuspend the pellet in 20 µl of molecular grade water. Calculate the concentration and purity of nucleic acids by measuring the 260 nm and 280 nm absorbance using nanodrop.
16. Proceed carefully to synthesis of cDNA by using any conventional reverse transcriptase kit and following the manufacturer´s protocol.
    Note: Approximately 0.5-2 µg RNA is obtained from 4 x 10^4 naïve $CD4^+$ T cells. cDNA can be synthetized from 100 ng of RNA and used in real-time PCR reactions without the need for low RNA input kits.

Representative Results

The protocol described in this manuscript renders a good quality of cultured and adhered resting and activated human naïve $CD4^+$ T cells (**Figure 1**). The activated $CD4^+$ T cells show the characteristic proliferative profile (**Figure 1B**) in comparison to the resting condition (**Figure 1A**). The CD25 late activation marker is useful to evaluate the efficient activation at 72 hr observed by confocal microscopy (**Figure 1C**). The CD69 marker is currently used as early activation marker by flow cytometry or microscopy. Adherence of resting and activated naïve $CD4^+$ T cells to a coverslip coated with poly-L-lysine are useful to study the expression and localization of GD3 and TCR showed by a double labeling with anti GD3 and anti TCR antibodies (**Figure 2**). As we reported previously, activation is accompanied by remodeling of GD3 and GD2 gangliosides in the cells surface[6]. Additionally, gene expression quantification of the GD3 and GM2/GD2 synthases is performed from 4 x 10^4 cells (**Figures 2** and **3**). The neo-expression of the GD2 ganglioside and TCR colocalization is adequately observed by confocal microscopy, demonstrating a possible role of GD2 in TCR clustering during activation (**Figure 3**). **Figure 4** shows the localization of the GD3 and GD2 ganglioside stained simultaneously in activated $CD4^+$ T cells. Additionally, activated PBMCs were stained with anti GD3 and anti GD2 antibodies showing that this protocol can be used in other immune cell populations found in PMBCs, particularly GD2 that was found to be expressed in all the activated PMBCs (**Figure 5**).

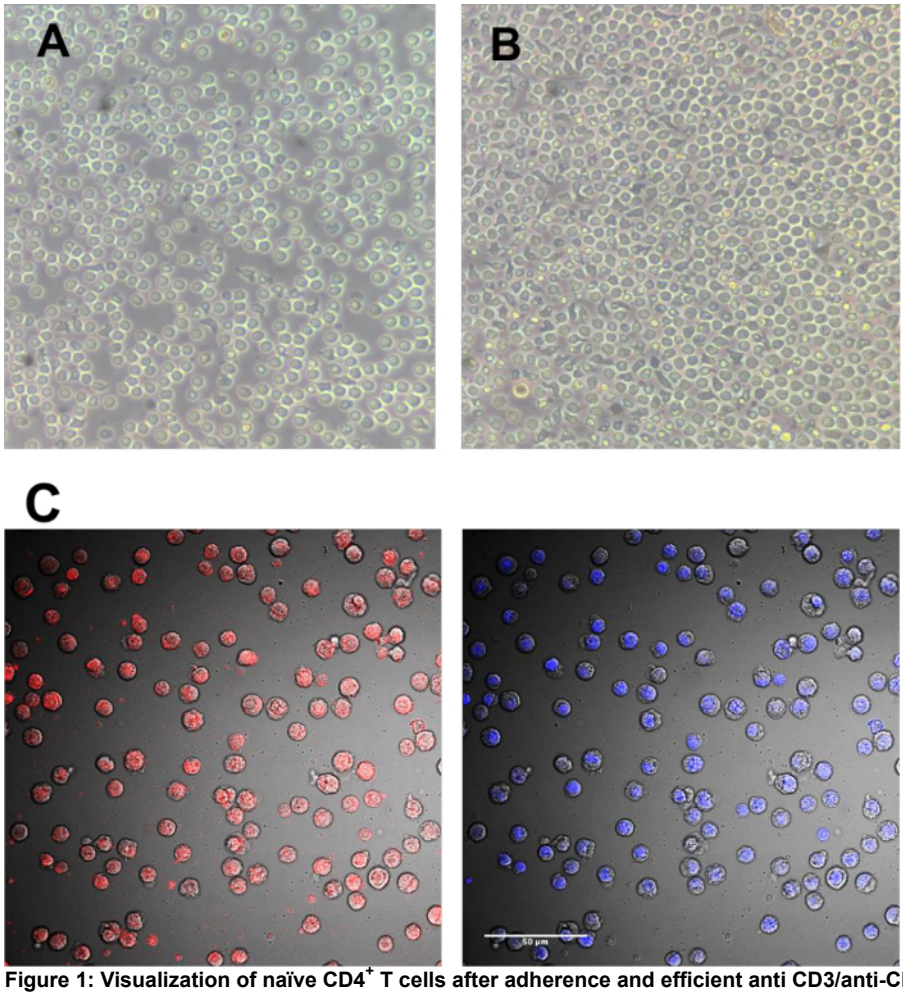

Figure 1: Visualization of naïve CD4$^+$ T cells after adherence and efficient anti CD3/anti-CD28 activation. (**A**) Resting CD4$^+$ T cells at 72 hr post-activation attached on poly-L-lysine treated coverslip are observed by brightfield microscopy. (**B**) Activated CD4$^+$ T cells at 72 hr attached on poly-L-lysine treated coverslip. (**C**) CD25 marker expression (red) and Hoechst 333258 stained nuclei (blue) of anti CD3/anti CD28 activated CD4$^+$ T cells at 72 hr post-activation. Scale bar = 50 µm. Images were obtained by confocal microscopy with a 60X S/1.3 oil objective with 2X digital zoom. Please click here to view a larger version of this figure.

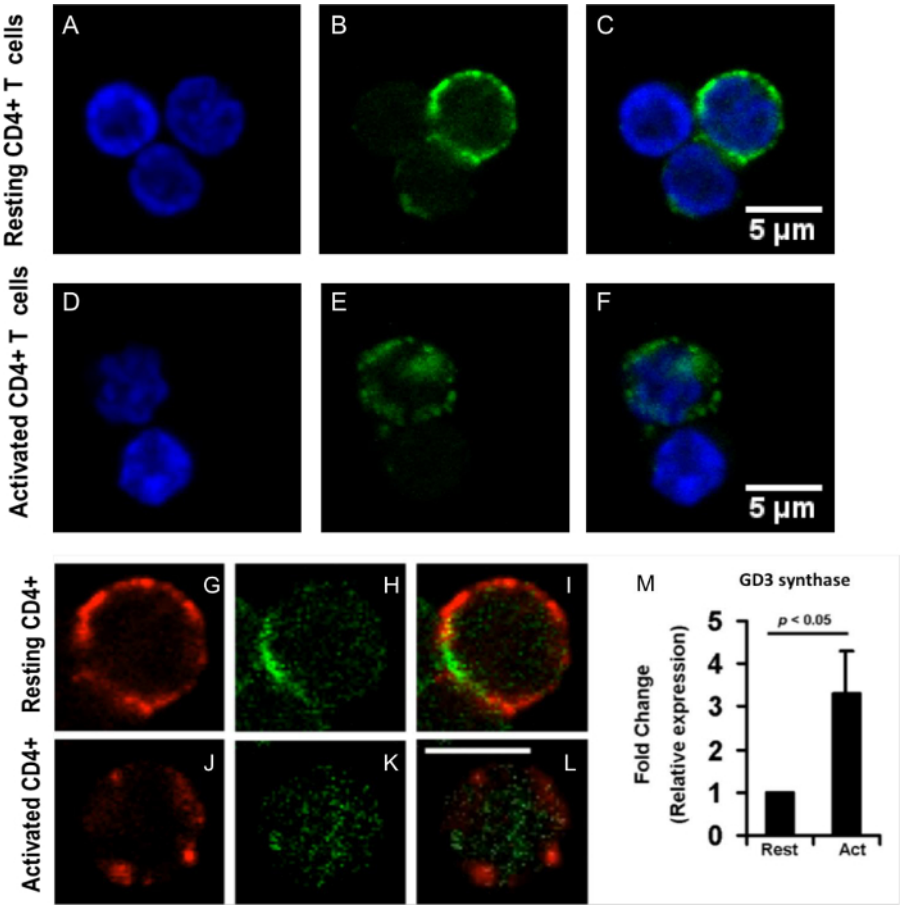

Figure 2: GD3 ganglioside and TCR stain and microscopy localization in resting and activated naïve CD4$^+$ T cells at 72 h post-activation. Resting CD4$^+$ T cells with (**A**) nuclei stained with Hoechst 333258, (**B**) GD3 ganglioside localization, (**C**) Merge of nuclei and GD3 ganglioside. Activated CD4$^+$ T cells with (**D**) nuclei stained with Hoechst 333258, (**E**) GD3 ganglioside localization and (**F**) Merge of nuclei and GD3 ganglioside. (**G**) and (**H**) show TCR and GD3 ganglioside in resting naïve CD4$^+$ T cells. (**J** and **K**) corresponds to TCR and GD3 ganglioside staining in activated CD4$^+$ T cells. Confocal images of TCR (red) and GD3 ganglioside (green) localization in resting naïve CD4$^+$ T cells (**I**) and anti CD3/CD28 Activated naïve CD4$^+$ T cells 72 hr post-activation (**L**). The GD3, TCR and nuclei stain was assessed by confocal microscopy from one stack with a 60X S/1.3 oil objective with 2X digital zoom. (**M**) The graph shows the gene expression for GD3 synthase determined by real-time PCR expressed as Fold change from 4 x 10^4 resting (Rest) and activated (Act) naïve CD4$^+$ T cells at 72 hr post-activation. Data are the mean ± S. D. from three independent donors. Please click here to view a larger version of this figure.

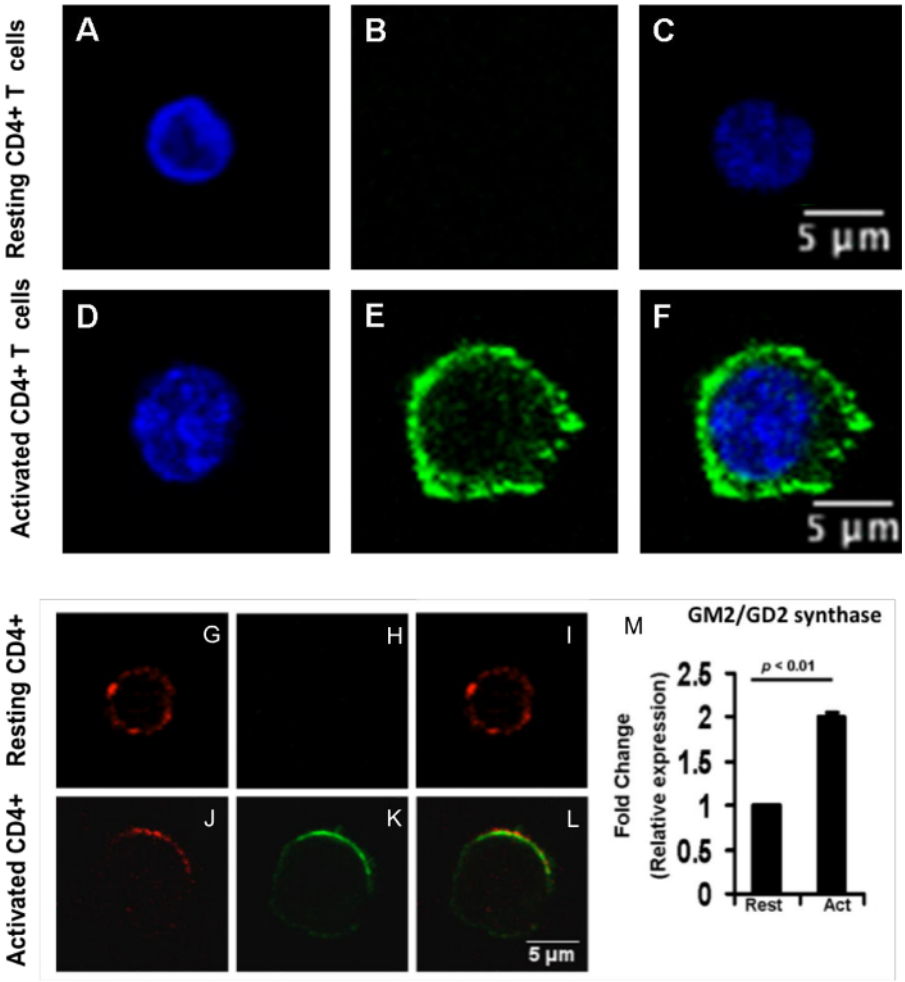

Figure 3: GD2 ganglioside and TCR stain and microscopy localization in resting and activated naïve CD4+T cells at 72 h post-activation. Resting $CD4^+$ T cells with (**A**) nuclei stained with Hoechst 333258, (**B**) GD2 ganglioside localization, (**C**) Merge of nuclei and GD2 ganglioside. Activated $CD4^+$ T cells with (**D**) nuclei stained with Hoechst 333258, (**E**) GD2 ganglioside localization and (**F**) Merge of nuclei and GD2 ganglioside. (**G** and **H**) show TCR and GD2 ganglioside in resting naïve $CD4^+$ T cells. (**J** and **K**) corresponds to TCR and GD2 ganglioside staining in activated $CD4^+$ T cells. Confocal images of TCR (red) and GD2 ganglioside (green) localization in resting naïve $CD4^+$ T cells (**I**) and anti CD3/CD28 Activated naïve $CD4^+$ T cells 72 hr post-activation (**L**). The GD2, TCR and nuclei stain was assessed by confocal microscopy from one stack with a 60X S/1.3 oil objective with 2X digital zoom. (**M**) The graph shows the gene expression for GM2/GD2 synthase determined by real-time PCR expressed as Fold change from 4 x 10^4 resting (Rest) and activated (Act) naïve $CD4^+$ T cells at 72 hr post-activation. Data are the mean ± S. D. from three independent donors. Please click here to view a larger version of this figure.

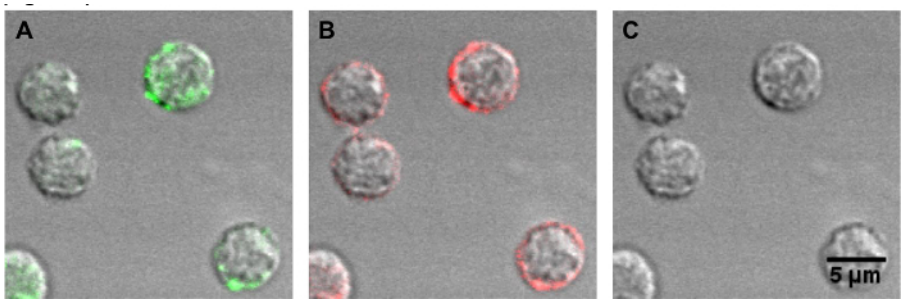

Figure 4: Double stain with anti-GD3 and anti-GD2 antibodies for GD3 and GD2 gangliosides localization. (**A**) GD3 ganglioside identified with R24 anti GD3 antibody in activated $CD4^+$ T cells shows intracellular and membrane localization. (**B**) GD2 ganglioside identified with 14G2a anti GD2 antibody in activated $CD4^+$ T cells shows only plasma membrane localization. (**C**) Brightfield microscopy from activated $CD4^+$ T cells. The GD3 and GD2 stain was assessed by confocal microscopy from one stack with a 60X S/1.3 oil objective with 2X digital zoom. Please click here to view a larger version of this figure.

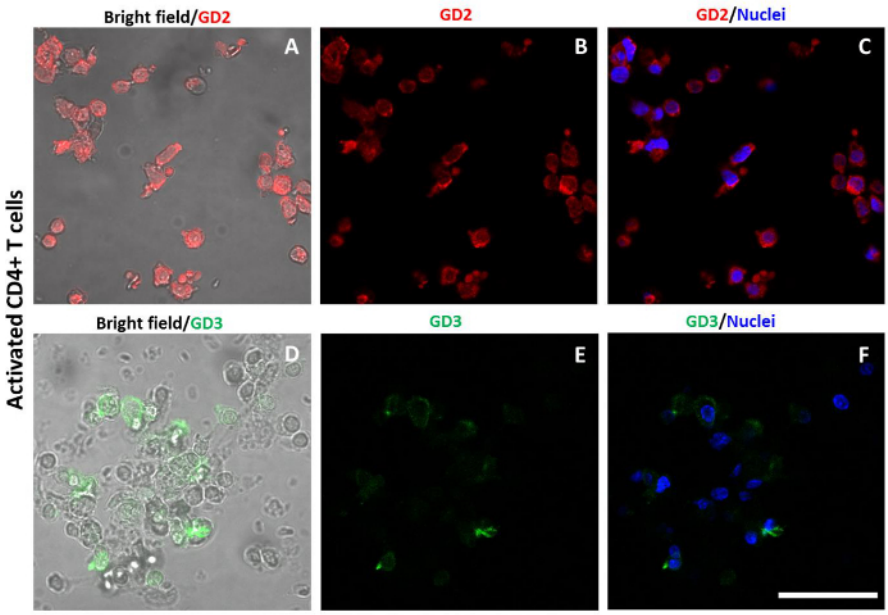

Figure 5: GD3 and GD2 ganglioside localization in activated PBMCs. (**A**) GD2 ganglioside was detected with 14G2a anti GD2 antibody in anti CD3/anti CD28 72 hr post-activated PBMCs overlay with brightfield microscopy, (**B**) GD2 ganglioside expression and (**C**) GD2 ganglioside and nuclei merge. (**D**) GD3 ganglioside stained with R24 anti GD3 antibody overlay with brightfield, (**E**) GD3 ganglioside expression and (**F**) GD3 ganglioside and nuclei merge. The GD3, GD2 and nuclei stain was assessed by confocal microscopy from one stack with a 60X S/1.3 oil objective with 2X digital zoom. Scale bar = 45 µm. Please click here to view a larger version of this figure.

Discussion

The described protocol can be used to localize gangliosides or proteins in cell suspensions of $CD4^+$ T cells or other immune cells (*e.g.*, PMBCs, **Figure 5**) starting from a small number of cells. Because of the small size of T cells and non-adherent properties, the acquisition of fluorescence microscopic images results in poor information or low quality if the cells are not correctly adhered.

This protocol combined with a good quality confocal microscopy analysis is a key advantage over the use of techniques such as thin layer chromatography for detection of gangliosides because it reveals the spatial localization and dynamics of gangliosides during treatment (*e.g.,* T cell activation), leading to acquisition of biologically relevant data[11,12,13]. Also, this protocol allows the evaluation of ganglioside expression starting from a small number of $CD4^+$ T cells (2×10^4) that is useful for optimizing the number of replicates or different assays[6].

The careful treatment during washes and antibody staining after adhesion is crucial to maintain cell number and integrity. Because of the weak binding of $CD4^+$ T cells to the coverslip, it is also very important to carefully add and remove solutions; otherwise a reduction in cell number and increased fluorescent debris will be encountered. When assessed through confocal microscopy, the immunostaining of gangliosides confers additional information such as spatial localization or co-localization with other molecules in the plasma membrane or intracellular compartments. Subcellular localization requires the use of subcellular markers[14,15]. In addition, fresh and not fixed $CD4^+$ T cells need to be used for staining when determining ganglioside expression restricted to the cell surface. Although fixation of $CD4^+$ T cells helps preserve the sample for at least 5 days, it carries the risk of permeabilization and intracellular staining.

However, because of the structural similarity between gangliosides, it is always advisable to take into account the specificity of antibodies used. Several of the commercially available anti-ganglioside monoclonal antibodies can be used and for some of them the specificity has been probed by ELISA, TLC, *etc.*[16,17,18].

Previous works demonstrate that it is possible to obtain information regarding localization and expression of GD3 and GD2 gangliosides from 2×10^4 cells/per condition[6]. Also, this protocol describes the capability of performing gene profiling experiments from 4×10^4 cells, allowing optimization of silencing experiments such as those based in lentiviral particles[6].

Disclosures

The authors have nothing to disclose.

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