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Expresión de los puntos de control inmunológico en el inmunoma intestinal humano y en sus precursores sanguíneos

Expression of immune checkpoint molecules in the human intestinal immunome and its blood precursors

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RESUMEN

La enfermedad inflamatoria intestinal (EII) es una patología crónica de rápido crecimiento en países industrializados a nivel mundial que engloba la enfermedad de Crohn y la colitis ulcerosa. Es una enfermedad de etiología multifactorial en la que interaccionan factores genéticos, microbiológicos, ambientales e inmunológicos. Uno de los mecanismos implicados es una respuesta inmunitaria frente a la microbiota intestinal. Los puntos de control immunitario juegan un papel fundamental en la regulación de la respuesta immunitaria y en el mantenimiento de la homeostasis intestinal, y están relacionados con la EII al haberse visto una desregulación en pacientes con esta patología. En este trabajo se ha realizado la puesta a punto de un panel de citometría spectral de 39 marcadores para el estudio de la expresion de los puntos de control inmunitarios en sangre periférica y en tejido. Además, se caracterizó la expresión en sangre periférica de PD-1, PD-L1, VISTA, TIGIT, LAG-3 y CTLA-4 en 4 poblaciones celulares (monocitos, células T, células B y células NK). Gracias a la puesta a punto del panel , seremos capaces del estudio de diversas subpoblaciones celulares, permitiendo un inmunofenotipado detallado, y el análisis de la expresión de 6 puntos de control inmunitarios simultáneamente en una misma muestra. Es el primer paso necesario para la utilización de este panel en muestras de pacientes con EII, que permitirá estudiar en detalle la expresión de los principals puntos de control inmunitarios a la vez en los distintos segmentos del tracto intestinal, sangre periférica y en distintos subgrupos de la enfermedad.

Palabras clave

Enfermedad inflamatoria intestinal, Citometría de flujo espectral, Puntos de control inmunes, Inmunofenotipado, Optimización de panel de citometría

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic pathology with a rapid growth in industrialized countries worldwide including Crohn´s disease and ulcerative colitis. It is a disease of multifactorial etiology involving genetic, microbial, environmental and immunological factors. One of the key mechanisms involved is an immune response against intestinal microbiota. Immune checkpoints play a crucial role in the regulation of the immune response and in maintaining the intestinal homeostasis, and they are related with IBD as their dysregulation has been observed in patients with this disease. A 39-marker spectral flow cytometry panel was optimized to study the immune checkpoint expression in peripheral blood and tissue. Besides, the

expression of PD-1, PD-L1, VISTA, TIGIT, LAG-3 and CTLA-4 was characterized in peripheral blood across 4 main cell populations (monocytes, T cells, B cells and NK cells). This panel optimization will allow the study of several cell populations, allowing a detailed immunophenotyping, and the analysis of the expression of 6 immune checkpoints simultaneously within the same sample. This is the first essential step to use this panel in samples of patients with IBD, and will allow a detailed analysis of the main immune checkpoints expression in different segments of the intestinal tract, peripheral blood and in different subtypes of the disease.

Keywords

Inflammatory Bowel Disease, Spectral flow cytometry, Immune Checkpoints, Immunophenotyping, Flow cytometry panel optimization

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GLOSSARY OF ABBREVIATIONS

IBD: Inflammatory bowel disease

UC: Ulcerative colitis

CD: Crohn´s disease

PD-1: Programmed cell death 1

CTLA-4: Cytotoxic T lymphocyte-associated protein 4

PD-L1: programmed cell death ligand 1

TCR: T cell receptor

TIGIT: T-cell immunoreceptor with Ig and ITIM domains

VISTA: V-domain Ig suppressor of T cell activation

LAG-3: Lymphocyte activation gene 3

Tregs: Regulatory T cells

NK: Natural Killer

DCs: Dendritic cells

ImC: Immune-mediated colitis

IELs: Intraepithelial lymphocytes

LPMCs: Lamina propria mononuclear cells

PBMCs: Peripheral blood mononuclear cells

FMO: Fluorescence minus one

APC: Antigen presenting cell

SF: Spectral flow cytometry

1. Introduction

Inflammatory bowel disease (IBD) is a chronic and severe disease of intestinal inflammation with a complex pathogenesis (1) IBD can be divided in two main subtypes: Ulcerative colitis (UC) and Crohn´s disease (CD). The prevalence of IBD is increasing rapidly with over 2.5 million patients in Europe and 1 million in the US, and it is a growing problem in industrialized countries, not only Europe and the US but also in emerging nations in Asia, South America and Middle East (2,3). In Spain, the incidence of the disease ranges from 9.6 to 44.3 cases per 100.000 inhabitants and has increased in recent years. In addition, it has high associated costs, between 4.6 and 5.6 billion euros per year in Europe (4).

The main subtypes of IBD present different characteristics. On the one hand, UC is characterized by a diffuse mucosal inflammation that affects the colonic part of the gastrointestinal tract, with depletion of goblet mucin and presence of a high number of neutrophils in the lamina propria and crypts. On the other hand, CD can affect any segment of the gastrointestinal tract, and there is an aggregation of macrophages and granulomas (5). In CD the most common type of inflammation is transmural, while in UC it is found in the mucosa (6). The origin of the disease is multifactorial. The epithelial barrier function and the adaptative immune response are crucial for maintaining the homeostasis in the gut. In healthy conditions, there is a mucus layer secreted by the goblet cells, that prevents intestinal epithelial cells from interacting with bacteria. The common immunoglobulin present in mucosas, IgA, is also present here, and together with antimicrobial peptides secreted by Paneth Cells protect from bacteria. There is a balance maintained to avoid immune cells acting against microbiota but at the same time attacking pathogens and excesive entry of luminal microbiota. When there is a disfunction in those mechanisms a chronic inflamation can arise, leading to tissue damage (6,7). In the recent years, the role of microbiota in several diseases has lead to investigation in IBD. Bacteria, fungi and viruses not only exert a beneficial effect in IBD releasing beneficial molecules and metabolites that helps to repair the epithelial barrier function but also can produce negative metabolites, such as toxins, that lead to an increase of inflammation (8). Genetics can contribute to this disfunction too, with some susceptibility genes described in the recent years such as NOD-2 or MST-1 (9,10). The role of genetics in IBD is more prominent in CD than in UC (6). Environmental factors have an important role in the development of the disease. Smoking increases the risk of developing CD, physical activity seems to reduce the risk of developing IBD, and pollution is also related, as people living in urban areas has an increased risk of developing IBD, while rural population has a decreased risk. Changes in diet or even antibiotic use can contribute to developing the disease (11,12).

In relation with this work, immune checkpoints are crucial pathways for maintaining self-

tolerance and regulating the amplitude of the immune response. The majority of immune checkpoints are initiated by ligand-receptor interactions (13). The programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are two of the most well-known immune checkpoints. PD-1 joins to its ligand programmed cell death ligand 1 (PD-L1), expressed in the surface of cells, mechanism frequently used by tumor cells to stop the immune response. CTLA-4 has a inhibitory role in T cells, it is an intracellular protein in unactivated T cells that translocates to the cell membrane in response to a T cell receptor (TCR) engagement and a costimulatory signal. This translocation avoid CD28 from joining to costimulatory molecules and mediates an inhibitory signal (14). There are other immune checkpoints proteins, such as T-cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA) and lymphocyte activation gene 3 (LAG-3), that are potential targets for future therapies (15). TIGIT has different ligands, present in different tissues and in the case of his ligand CD155, it is expressed in dendritic cells, T cells, B cells and macrophages. TIGIT itself is expressed on lymphocytes (Regulatory T cells (Tregs), memory and natural killer (NK) cells), and there is an upregulation when those cells are activated. The result of the union is an overall immune suppression response in cells. LAG-3 has an structure highly homologous to CD4, and it is expressed on activated T and B cells, NK and dendritic cells (DCs), and when it is activated it leads to a downregulation of T cells functions (16). VISTA is a protein mainly expressed on hematopoietic cells, with a highly regulated expression on antigen-presenting cells and T cells. The overexpression of VISTA in antigen-presenting cells leads to inhibitory signals for both $CD4^+$ and $CD8^+$ T cells (17). Some immune checkpoints are important in maintaining intestinal homeostasis. For instance, PD-1:PD-L1 interactions prevent $CD8^+$T cell mediated autoimmunity against intestinal self antigens in mice (18).

Despite the success of immune chekpoints inhibitors in diseases such as cancer, there is a well described adverse effect called immune-mediated colitis (ImC). This is a condition developed by some patients in response to immune checkpoint inhibitor therapy. The risk of developing this condition is lowest after anti-PD-1/PD-L1 treatment, followed by anti- CTLA-4 treatment. It has been observed that endoscopic appearance is very similar to classic IBD, but histology differs. The impact of ImC is significant, affecting negatively morbidity, quality of life and, in some cases, it can lead to death. A precisse diagnosis of ImC is essential to prevent from severe complications. ImC often requieres the use of a immunosuppressive therapy, such as corticosteroids in the first-line and infliximab or vedolizumab in the second line, with negative effects in tumor progression (19,20).

In order to study the activity of certain proteins, flow cytometry is useful to analyze the expression of different molecules in cells. It not only allows the identification of multiple cell populations from blood and tissue, but also can sort cells for future analysis. They have 3 main components: fluidics, optics and electronics. Flow cytometers consists on a group of different

lasers that work as light sources to produce signals that are read by detectors. The signals are converted into files with the extension (.fcs). Each cell pass through the lasser, with a sheath fluid that is pressurized to transport the sample into the laser intercerpt where the sample is analyzed. Light can be measured in two directions, forward and side. The first one correlates with cell size, while side scatter correlates with granularity. New flow cytometres have emerged, such us spectral analyzers, flow cytometers that can measure the entire fluorescent emission spectra, that solves the problem of compensation, removing spectral overlap (21,22). It is a useful technique that have shown potential in IBD, improving the current knowledge in immune-phenotyping comparing blood and intestinal tissue from patients with IBD (23).

2. Hypothesis and objectives

The hypothesis of this work is that immune checkpoints play a relevant role in IBD, and are altered in patients with this condition.

The main objective is to set up a flow cytometry panel for both human blood and intestinal samples in order to a future study of the expression of various immune checkpoints across different cell populations. To achieve this main objective, it has been divided into 3 objectives:

- Titration of antibodies in order to find the optimal concentration to be used in the panel.
- Identification of specific immune cell populations across blood and tissue.
- Analysis of the expression patterns of 6 immune checkpoints molecules in peripheral blood.

3. Materials and methods

3.1. REAGENTS AND SOLUTIONS

TABLE 1. Reagents used.

REAGENT	COMPANY
HBSS: Hank´s Balanced Salt Solution	Gibco
PBS: Phospate Buffered Saline	Cytiva Hyclone
RPMI: RPMI Medium1640	Gibco
Paraformaldehyde	Protocol
Bovine Serum Albumin (BSA) Fraction V Ig free	Gibco
FBS: Fetal Bovine Serum	Thermofisher
NaN_3: Sodium azide	Sigma-Aldrich
EDTA	Sigma-Aldrich
DTT: Dithiothreitol	Sigma-Aldrich
Collagenase D	Sigma Aldrich
Liberase TL Research Grade	Merck
Benzonase	ThermoFisher Scientific
Dimethyl Sulfoxide: DMSO	MP Biomedicals
Trypan Blue Stain (0.4%)	Gibco

REAGENT	COMPOSITION
FACS BUFFER	PBS, 2% FCS filtered + 3mM NaN3 + 1mM EDTA
WASH BUFFER	PBS + 0.2% BSA + 0.089% NaN_3
DTT + EDTA	HBSS, DTT 1mM, EDTA 0.5mM
COLAGENASE + LIBERASE + BENZONASE	RPMI + 1mg/ml Collagenase D + 20 µg/ml Liberase + 26U/ml Benzonase
CRYOPRESERVATION MEDIA	90% FCS filtered + 10% DMSO

3.2. MATERIAL

TABLE 2. Material used.

MATERIAL	COMPANY
Pasteur pipette	Fisherbrand
Micropipettes	Gilson
Cytometry Tubes	Falcon-A Corning Brand
Falcon Tubes 15 ml	Corning
Falcon Tubes 50 ml	Corning
100 µm and 70 µm Cell Strainers	Fisherbrand
Cryovials	Fisherbrand
Cytek Aurora (5 laser) cytometer	Cytek
Counting chamber improved Neubauer	Blaubrand

3.3. PATIENT COHORTS

Samples were taken during normal colonoscopies of the colorectal cancer screening program at Hospital Clínico Universitario in Valladolid, Spain. Blood samples were taken from healthy controls at the same hospital. Written informed consent was obtained from all patients (Ethics approval PI-24-556 by the Ethics Committee from Valladolid Este).

3.4. BIOLOGICAL SAMPLES

3.4.1 LAMINA PROPRIA MONONUCLEAR CELLS AND INTRAEPITHELIAL LYMPHOCYTES ISOLATION FROM BIOPSES

Cells were obtained from intestinal biopsies. Six biopsies were taken from three parts of the digestive tract: terminal ileum, right colon and left colon. Biopsies were obtained by colonoscopy, and collected in RPMI. The samples were taken to the laboratory for processing.

Once the biopsies were in the laboratory, one from each segment of the digestive tract was used for culture. The rest were processed so they were transferred to a 15 ml tube containing 5 ml of DTT+EDTA solution. One tube was needed for each segment and patient. Tubes were incubated at 37 ºC for 30 minutes in a shaker (200 rpm). During this process the tissue was degraded. DTT is a reagent that destroys the mucus layer, and EDTA is a calcium chelator that breaks the enterocyte junctions, releasing intraepithelial lymphocytes (IELs). After 30 minutes, the solution was transferred to a 50 ml tube with a 100 µm cell strainer. It is important to avoid transferring tissue into the strainer; if this occurs, it is taken with a sterile Pasteur pipette and transferred back to the 15 ml tube. The procedure is repeated again. After the second incubation with DTT+EDTA, the solution was added to the 50 ml tube again, with the same 100 µm cell strainer. The tube was centrifuged for 10 minutes at 400 x g and 4 ºC and the supernatant aspirated. The cells remained at the bottom of the tube. Two milliliters of freezing medium were added and cells resuspended and divided into two cryotubes. The cryotubes were transferred into a freezing container and rapidly transported to the -80ºC freezer, where they remain until needed.

The next step was obtaining lamina propia mononuclear cells (LPMCs). The residual tissue was incubated in a solution that contained three enzymes: collagenase, liberase and benzonase. The enzymes degrade the lamina propria and release the LPMCs. Each tube was incubated with 5 ml of this solution for 30 minutes at 37ºC in a shaker. After 30 minutes the procedure is the same as before: the solution is transferred into a new 50 ml tube with a 100 µm cell strainer. This is repeated again. After the second incubation, the solution was transferred to the 50 ml tube and 1ml of sterile RPMI was added to stop the reaction. With the residual tissue a third incubation was performed. Once LPMCs were in the 50 ml tube, the tubes were centrifuged for 10 minutes at 400 x g and 4 ºC and the supernatant was aspirated. Cells were resuspended in 2 ml of freezing media and divided into 2 cryotubes, that were cryopreserved in a freezing container at -80ºC.

3.4.2. PERIPHERAL BLOOD MONUNUCLEAR CELLS ISOLATION

Cells were obtained from blood samples collected in EDTA and heparin tubes. They were isolated using Cytiva Ficoll-Plaque™ PLUS. For the procedure, one 15 ml Falcon tube was needed per tube of blood. Each 15 ml tube had 3 ml of Ficoll, and the blood was slowly added to the tube without mixing (8-9 ml of blood per tube). The tubes were centrifuged at 800 x g during 30 minutes at 4 Cº without brake. After centrifugation, peripheral blood mononuclear cells (PBMCs) were collected with a sterile Pasteur pipette from the layer between plasma and Ficoll, and added to a tube that contained 1ml of RPMI. Tubes were centrifuged at 400 x g during 5 minutes, the supernatant was removed and the pellet was resuspended in 2 ml of freezing medium, which was distributed equally in 2 cryotubes and rapidly transported to the -80ºC freezer in a freezing container. Cryotubes were moved to liquid nitrogen until use. All procedures were performed in a laminar flow hood, and all the material used is sterile, to avoid contamination.

3.5. ANTIBODY STAINING AND SPECTRAL CYTOMETRY ACQUISITION

A full stain was performed, for that, PBMCs from healthy donors were taken and washed with 1 ml of PBS. After centrifugation (5 minutes, 4 ºC, 400 x g), cells were resuspended in 1 ml of PBS. Four microlitres of the sample were taken and mixed with 36 µl of Trypan Blue Stain. Ten microlitres were transferred to the Neubauer Chamber, and cell counting was performed. Cells were divided into 2 cytometry tubes, one unstained and 1 full staining. The next step was add 10 µl of BD Horizon Brilliant Stain Buffer Plus™ (Cat. 566385, BD Biosciences, California, EE. UU.) and 5 µl of True Stain Monocyte Blocker™(Cat. 426103, BioLegend®, San Diego, EE. UU) to the full staining tube. After that, 1.25 µl of TCRyd was added, and incubated 10 minutes in the dark at room temperature. Antibody mix containing the rest of antibodies was prepared, and added to the cytometry tube after the 10 minutes of incubation. It was incubated for 30 minutes in the dark at room temperature. One mililitre of Wash buffer was added, and the tube centrifugated for 5 minutes at 400 x g and 4ºC. Supernatant was removed. The final step was sample fixation with paraformaldehyde 1%. Ten minutes incubation in the dark at room temperature. Finally, 1 ml of wash buffer was added, centrifugated for 5 minutes at 400 x g and 4ºC and supernatant removed. Cells were resuspended in wash buffer and preserves at 4ºC until spectral cytometry acquisition.

3.6. ANTIBODY TITRATION

An important step to optimice a flow cytometry panel that includes a high number of antibodies is titration, in order to obtain the optimal concentrations, with the lowest amount of antibody. Out of the 39 markers included in the panel, 13 required titration (PD-1, Pdpn, LAG3, EpCAM, CD49b, PECAM-1, CD10, TIGIT, PSGL-1, PDGFRa, CD90.2, PD-L1 and CD69). Eight of them

can be detected in peripheral blood (PD-1, LAG-3, CD49b, CD10, TIGIT, PSGL-a, PD-L1 y CD69) and 5 of them were detected in biopsies (Pdpn, EpCAM, PECAM-1, PDGFRa and CD90.2). The rest of the markers included in the panel were already titrated.

The first step was to ensure an adequate number of cells to make the titration. At least 250.000 cells were needed per tube. To count, a Neubauer counting chamber and a microscope were used. The procedure consisted on washing the cells with 2 ml of PBS, centrifugating 5 minutes at 400 x g and 4 ºC, and aspirating the supernatant. They were resuspended in 1 ml of PBS, and 4 µl of the solution was taken, mixed with 36 µl of trypan blue and homogenized. Ten microlitres of the mix were transferred to the Neubauer counting chamber and viable cells were counted in the four quadrants to calculate an average. The number of cells per tube was extrapolated.

For each antibody tritation 7 cytometry tubes were used: 5 for antibody dilutions (1/50, 1/100, 1/200, 1/400 y 1/800), 1 tube for the fluorescence minus one (FMO), and 1 tube for the unstained. To make the antibody dilutions 5 eppendorf tubes were used. Three hundred microlitres of wash buffer in the first tube, 150 µl in the rest. Six microlitres of the antibody were added to the first tube, mixed and 150 µl transferred to the second tube. This process was repeated until all five dilutions were prepared.

Cells were now distributed in 2 tubes. 1 tube is the “unstained”, in which no antibody was added. In the other tube there was a pool of cells used together for the general titration process, and later divided when the staining with the antibody was performed. The first step was the viability staining, with 5 µl of Viability Blue. Fiveteen minutes of incubation in dark, room temperature. The tubes are washed, adding 1 ml of wash buffer and centrifugating 5 minutes at 400 x g and 4 ºC. The supernatant is aspirated. The next step was adding 10 µl of BD Horizon Brilliant Stain Buffer Plus™ (Cat. 566385, BD Biosciences, California, EE. UU.) and 5 µl of True Stain Monocyte Blocker™(Cat. 426103, BioLegend®, San Diego, EE. UU). This reagents help to avoid unspecific unions. Then, the staining with CD3 and CD45 was performed. One hundred microlitres of the mix previously prepared was added to the tube. The maximum number of cells that can be stained with this quantity of mix is 2 million, so this must be taken into account. The mix was prepared adding 5 µl of CD3, 2.5 µl of CD45 and 92.5 µl of wash buffer. Twenty minutes incubation in the dark at room temperature. One mililitre of wash buffer was added and centrifuged 5 minutes at 400 x g and 4 ºC. The supernatant was aspirated. Now the antibodies dilutions were added. Cells were equally divided into 5 cytometry tubes, and dilutions were added. Thirty minutes incubation in the dark. After that, 1 ml of Wash Buffer was added to each tube and centrifugated for 5 minutes at 400 x g and 4ºC. Supernatant was aspired. The last step was sample fixation using 300 µl of 1% paraformaldehyde. The sample was incubated for 10 minutes in the dark. After the 10 minutes incubation, 1 ml of wash buffer was added and the sample was centrifuged for 5 minutes at 400 x g. The supernatant was aspirated. The pellet was resuspended

in 300 µl of wash buffer, vortexed and maintained at 4 ºC until flow cytometry analysis.
For PDGFRa titration fibroblasts were used. The procedure was similar, but a different staining mix was used. Instead of CD3 and CD45, markers more suitable for fibroblasts, CD90 and CD45 antibodies, were used.
Stimulation was needed in order to make the titration of some markers. One microlitre of anti-CD3 and 99 µl of sterile PBS were added to each well in a plaque. It was sealed with parafilm and incubated overnight at 4 ºC. After the incubation cells were prepared. Supernatant was retired from each stimulated well and cells added. Two microlitres of anti-CD28 were added, and incubated overnight at 37ºC. Titration was made as previously explained, without adding CD3 antibody.

3.7. REFERENCE CONTROLS

Reference controls were made using beads. The procedure consisted on adding 1 drop of positive beads to one cytometry tube containing 100 µl of FACS buffer . Two microlitres of antibody was added to the tube and incubated 20 minutes in the dark at room temperature. After the incubation, 1 ml of FACS buffer was added, and the tube was centrifuged 5 minutes at 400 x g and 4 ºC. The supernatant was aspirated, and the pellet was resuspended in 500 µl of FACS buffer. After that, 1 drop of negative beads was added to the tube, and the sample fixation was done adding 300 µl of 1% paraformaldehyde. 10 minutes incubation. The tube was washed again adding 1 ml of FACS buffer, centrifugating 5 minutes at 400 x g and 4 ºC and aspirating the supernatant. The pellet was resuspended in FACS, and transported to the flow cytometer. Once in the flow cytometre, the spectrum of each marker was compared to the one provided by the company.

3.8. DATA ANALYSIS

Flow cytometry files were analyzed with the OMIQ software from Dotmatics (www.omiq.ai). Cell populations were separated according to the expression of certain markers. Debris was removed and singlets selected. Viable cells were taken by the expression on LIVE DEAD Blue-A viability marker. After that, cell populations characterization was made by checking markers expression (FIGURES 3 and 4)

4. Results

4.1. TITRATION IN BLOOD

Eight antibodies were titrated in peripheral blood (PD-1, LAG-3, CD49b, CD10, TIGIT, PSGL-a, PD-L1 y CD69). CD69, PD-L1 and LAG-3 didn´t show a good discrimination capacity in any of the dilutions. The rest of antibodies showed good discrimination capacity in some dilutions. The optimal dilutions of those antibodies were 1/50 in PD-1 (FIGURE A1), 1/400 in CD49b (FIGURE 1), 1/50 in CD10 (FIGURE A2), 1/50 in TIGIT (FIGURE A3) and 1/800 in PSGL-1 (FIGURE A4). After stimulating CD69, PD-L1 and LAG-3 with anti-CD3 and anti-CD28, the dilution chosen was 1/50 for CD69 (FIGURE A5), 1/100 for LAG-3 (FIGURE A6) and 1/50 for PD-L1 (FIGURE A7). In the FIGURE 1 a representative image of titration in peripheral blood is shown. FMO showed cells not expressing CD49b. After adding the antibody in different dilutions, the separation of both poblations was similar in every dilution, without changes between 1/100, 1/200 and 1/400. Histograms were used to set the line between $CD49b^+$ and $CD49b^-$ cells.

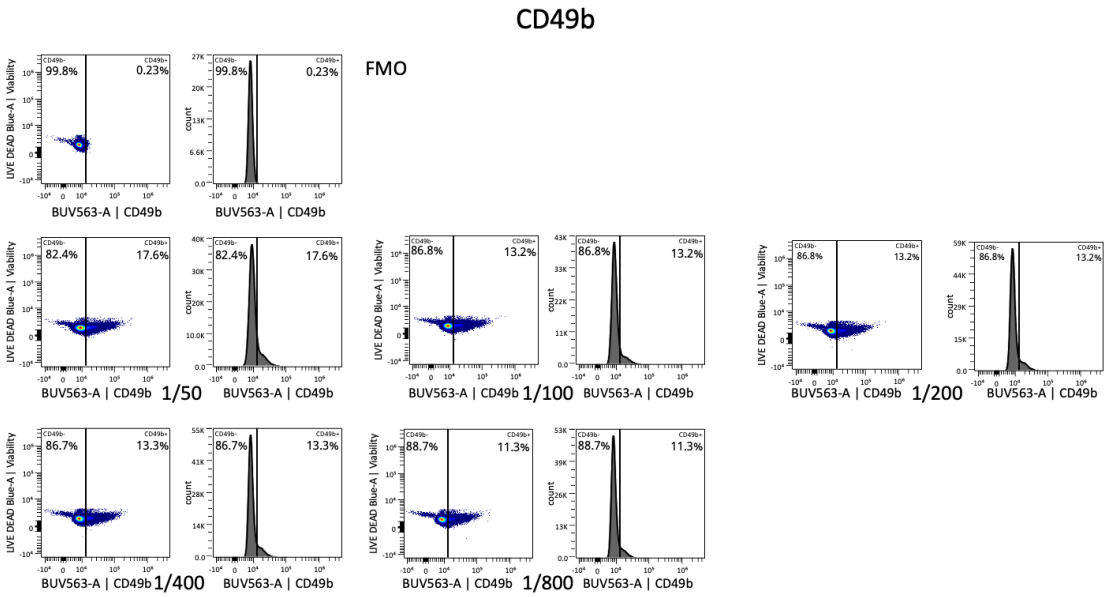

FIGURE 1. Representative image of antibody titration in peripheral blood. *Five dilutions were tested to choose the optimal quantity of antibody needed. In the y axis viability was shown. In the x axis each antibody expression is shown. A scatterplot and a histogram were analyzed to choose the dilution. FMO sample is also shown in this figure, in order to separate populations of cells expressing CD49b and the ones that don´t express it. The optimal dilution selected was 1/400.*

4.2. TITRATION IN GUT

Five antibodies were titrated in tissue (EpCAM, PECAM, PdPn, CD90.2 and PDGFRa). EpCAM, PdPn, CD90.2 and PDGFRa didn´t show a good discrimination capacity in any of the dilutions. PECAM showed good discrimination capacity in all of the dilutions. The optimal dilutions of those antibodies were 1/50 in EpCAM (FIGURE 2), 1/800 in PECAM (FIGURE A8), 1/50 in PdPn (FIGURE A9), 1/50 in CD90.2 (FIGURE A10) and 1/50 in PDGFRa (FIGURE A11). In the FIGURE 2 a representative image of titration in tissue is shown. FMO showed cells not expressing EpCAM. After adding the antibody in different dilutions, the separation of both populations was better in the 1/50 dilution.

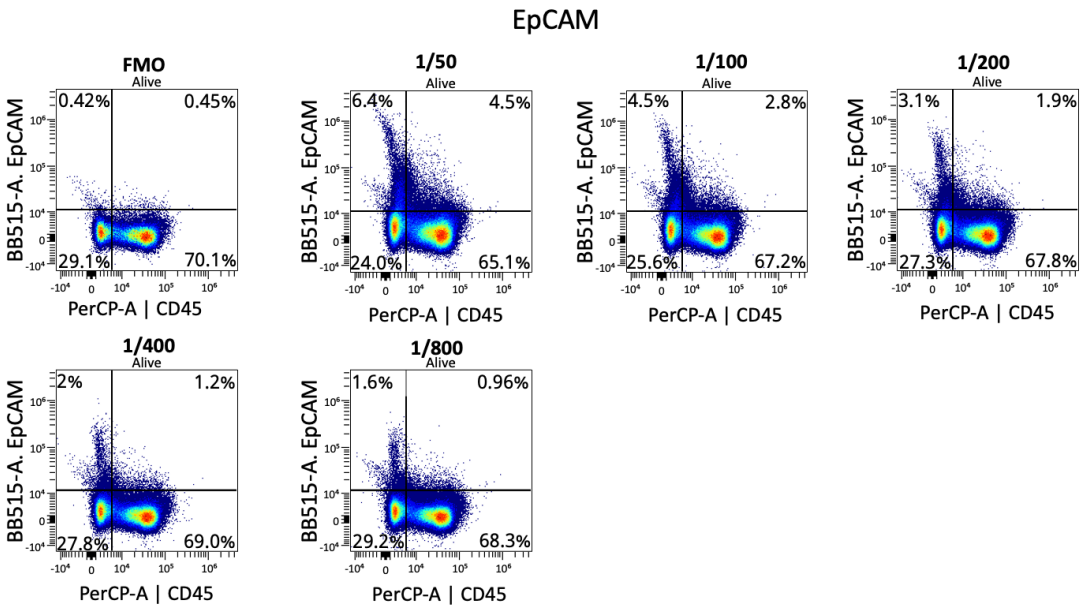

FIGURE 2. Representative image of antibody titration in intestinal tissue. *The same 5 dilutions were tested, and FMO was analyzed. The marker CD45 in the x axis and EpCAM in y axis. Four quadrants were separated, with 4 different populations of cells. The porcentages of each quadrant were similar between 1/100, 1/200, 1/400 and 1/800, and the optimal dilution was in 1/50.*

4.3. PANEL

TABLE 3. Composition of the flow cytometry panel*. Thirty nine markers were used in this panel. Fluorochrome, clon, company and concentrations are shown.*

Marker	Fluorochrome	Clon	Company	(µl/100 µl)	Titration
PD-1 (CD279)	Alexa Fluor 647	NAT105	BioLegend	2	1/50
Pdpn	APC	NC-08	BioLegend	2	1/50
LAG3 (CD223)	APC/FIRE810	11C3C65	BioLegend	1	1/100
CD27	APC/H7	M-T271	BD	5	1/20
CD127	APC/R700	HIL-7R-M21	BD	5	1/20
EpCAM (CD326)	BB 515	EBA-1	BD	2	1/50
CD45RA	BUV 395	5H9	BD	1.25	1/80
CD16	BUV 496	3G8	BD	0.625	1/160
CD49b	BUV 563	AK-7	BD	0.25	1/400
PECAM-1 (CD31)	BUV 615	WM59	BD	0.125	1/800
VISTA	BUV 661	MIH65	BD	2	1/50
CD56	BUV 737	NCAM16.2	BD	1.25	1/80
CD8	BUV 805	SK1	BD	0.625	1/160
CCR7 (CD197)	BV 421	C043H7	BioLegend	5	1/20
IgD	BV 480	IA6-2	BD	0.625	1/160
CD3	BV 510	SK7	BioLegend	5	1/20
IgM	BV 570	MHM-88	BioLegend	3.125	1/32
CD10	BV 605	HI10a	BioLegend	2	1/50
TIGIT	BV 650	TgMab-2	BD	2	1/50
CCR6 (CD196)	BV 711	G034E3	BioLegend	1.25	1/80
PSGL-1 (CD162)	BV 750	KPL-1	BD	0.125	1/800
CD103	BV 785	Ber-ACT8	BioLegend	2	1/50
CD4	cFluor YG584	SK3	Cytek	0.5	1/200
CD11c	eFluor 450	3.9	eBioscience	5	1/20
IgA	FITC	GOXHU	Thermo Fisher	1.25	1/80
CD20	Pacific Orange	HI47	Invitrogen	5	1/20
PDGFRa (CD140a)	PE	16A1	BioLegend	2	1/50
CD24	PE/Alexa 610	SN3	Invitrogen	2.5	1/40
CD25	PE/Alexa 700	-	Life Technologies	2.5	1/40
CD90.2	PE/Cy5	5-E10	BioLegend	2	1/50
CXCR3 (CD183)	PE/Cy7	G025H7	BioLegend	5	1/20
CTLA4	PE/Dazzle594	BNI3	BioLegend	2	1/50
HLA-DR	PE/Fire810	L243	BioLegend	5	1/20
CD45	PerCP	2D1	BioLegend	2.5	1/40
PD-L1 (CD274)	PerCP/Cy5.5	29E.2A3	BioLegend	2	1/50
TCRyd	PerCP/eFluor710	B1.1	eBioscience	1.25	1/80
CD14	Spark blue 550	63D3	BioLegend	2.5	1/40
CD19	Spark NIR 685	HIB19	BioLegend	1.25	1/80
CD69	Super Bright 436	H1.2F3	Invitrogen	2 µl	1/50

4.4. GATING STRATEGY

Main immune cells were identified by the expression of specific markers in blood (FIGURE 3)

4.4.1. PERIPHERAL BLOOD

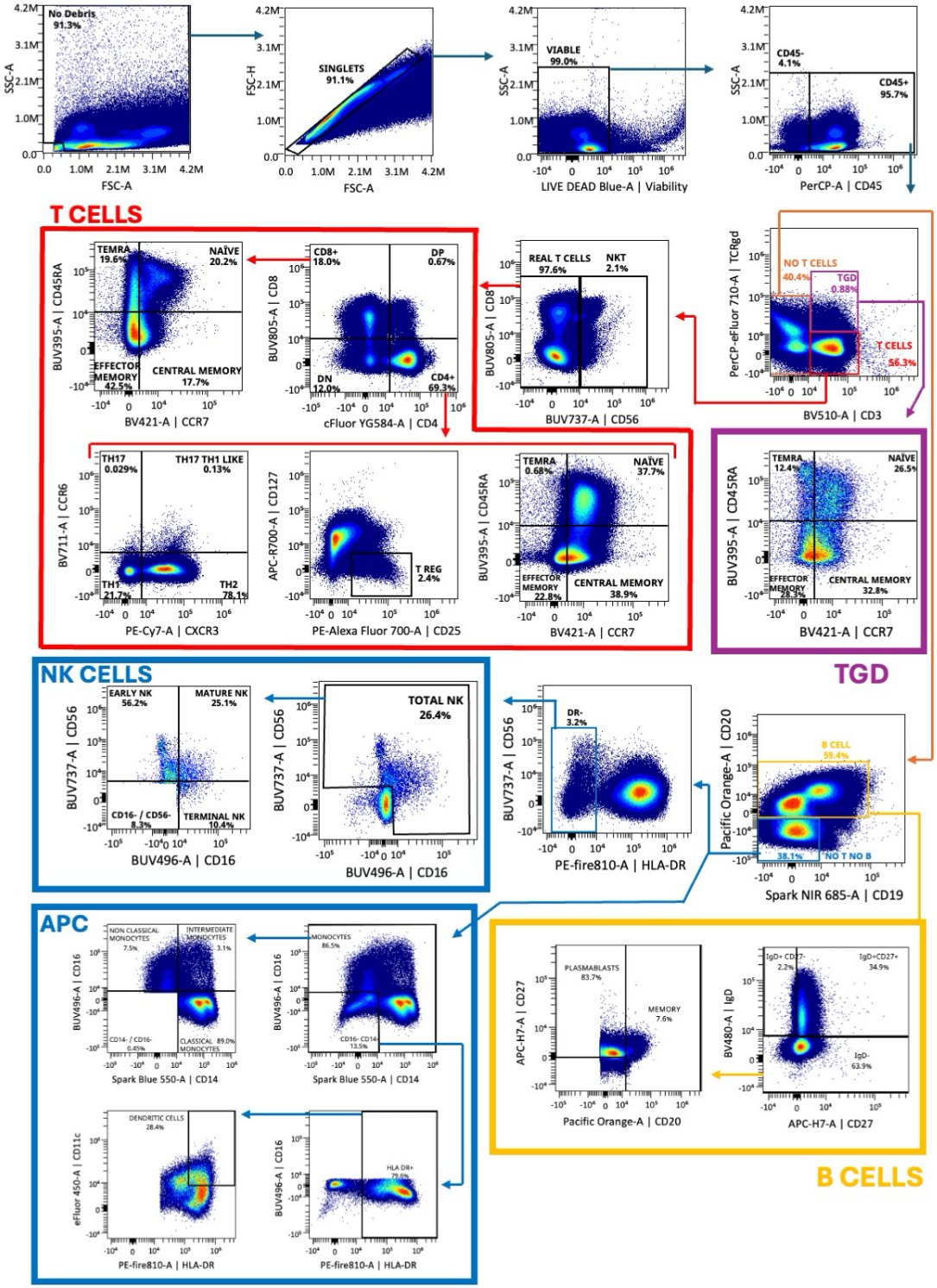

FIGURE 3. Gating strategy in peripheral blood*. Different cell populations were identified following this gating. Debris were eliminated, and only singlets were taken. Non-vible cells were eliminated. From viable cells CD45⁺ cells were selected. Three main divisions were made by looking for CD3 and TCRgd expression: T cells, No T cells and Tgd cells. T cells were divided in NKT cells and Real T cells, and inside Real T cells different subsets were analysed. No T cells were divided into B cells and no T no B cells, and this last group divided into NK cells, monocytes and dendritic cells.*

4.4.2. GUT

Main immune cells were identified by the expression of specific markers in tissue (FIGURE 4)

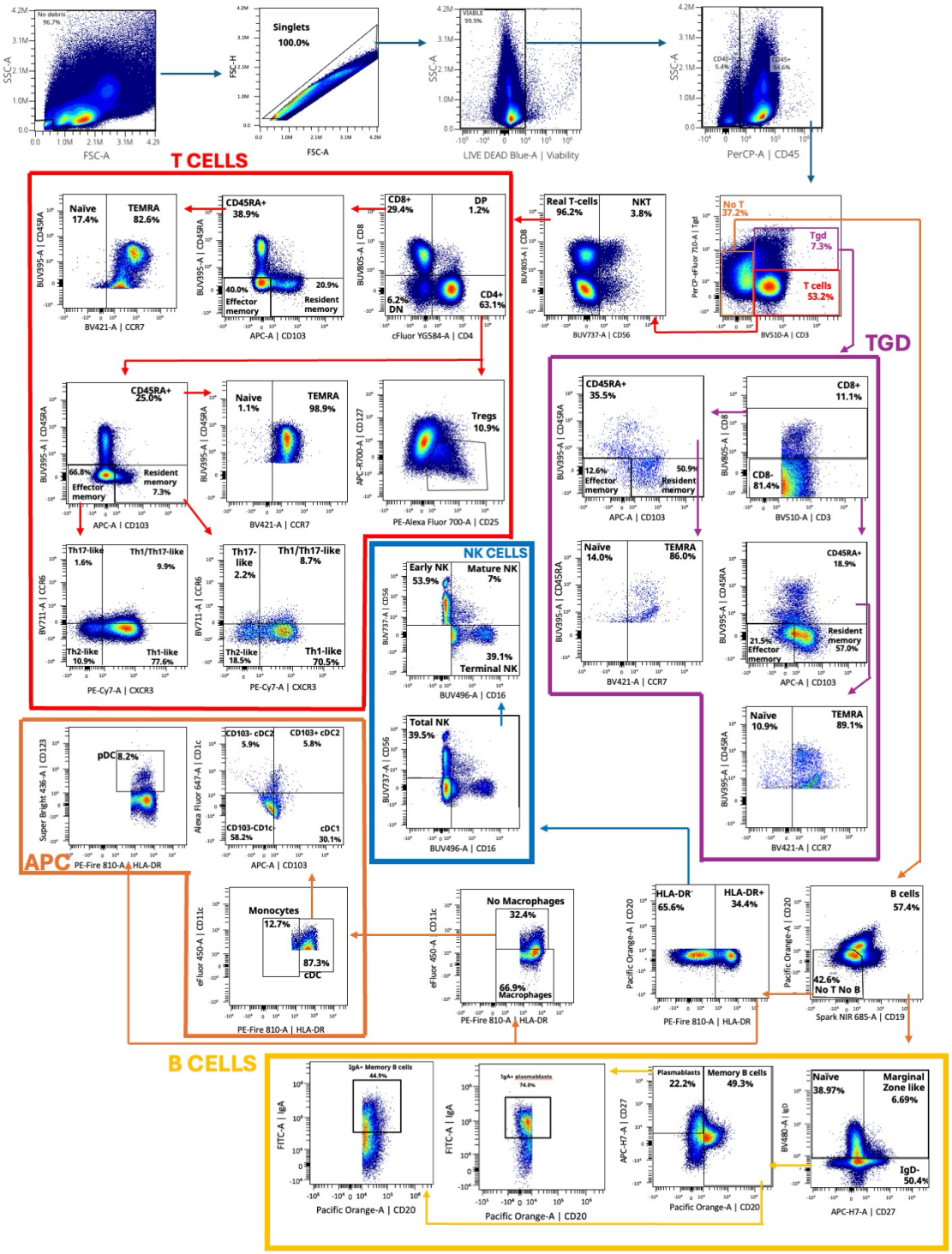

***FIGURE 4. Gating strategy in gut**. Different cell populations were identified following this gating. Debris were eliminated, and only singlets were taken. Non-vible cells were eliminated. From viable cells CD45^{+} cells were selected. Three main divisions were made by looking for CD3 and TCRgd expression: T cells, No T cells and Tgd cells. T cells were divided in NKT cells and Real T cells, and inside Real T cells different subsets were analysed. No T cells were divided into B cells and no T no B cells, and this last group divided into NK cells, monocytes and dendritic cells (conventional dendritic cells and plasmacytoid dendritic cells). Tgd cells were divided into subgroups according to CD8 expression.*

4.5. IMMUNE CHECKPOINTS EXPRESSION

Expression of immune checkpoints was studied in peripheral blood (FIGURE 5). 4 main cell populations were analyzed: T cells, B cells, natural killer cells and monocytes. T cells expressed mainly PD-L1, CTLA-4 and TIGIT. B cells expressed TIGIT and CTLA-4. NK cells had a higher expression of PD-1 and TIGIT. Monocytes expressed mainly PD-L1.

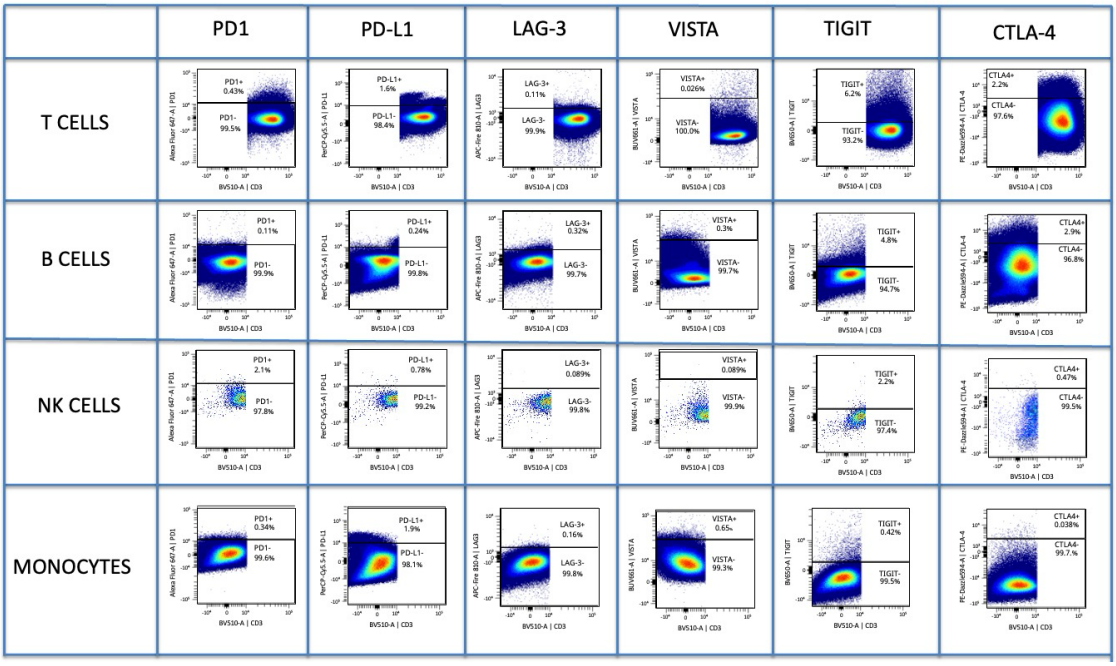

FIGURE 5. Immune checkpoints expression in peripheral blood. *Expression of PD1, PD-L1, LAG-3, VISTA, TIGIT and CTLA-4 across 4 main immune cells populations: T cells, B cells, NK cells and Monocytes. CD3 was selected for the x axis.*

5. Discussion

Traditionally, IBD has been a disease more prevalent in westernized countries of North America, Europe and Asia, but it has become a global disease affecting newly industrialised countries and with an incidence that is increasing every year (24). This growing burden underscores the importance of understand the pathophysiology of the disease, and search for new treatments, not only more effective but also cheaper for public health systems.

The aim of this work was to establish and optimize a flow cytometry panel to identify immune cell populations and the expression of certain immune checkpoint proteins in both peripheral blood and intestinal biopsy samples. Developing a strong and reproducible panel has a big relevance in research, in order to improve the characterization of IBD patients. Spectral flow cytometry (SF) was used. The main difference between SF and coventional flow cytometry is the number of cell markers. SF can analyze at the same time a large number of markers at the single-cell level, and despite using the same principles than conventional flow cytometry, SF can collect high-resolution spectral measurements, capturing the full spectrum of emission throughout all spectral wavelengths. The main problem about using such a big number of antibodies is fluorescence spillover, and it is important to find a panel without critical overlap in the major peaks (25). This technique allows analyzing at the same time the main immune checkpoints across the most important cell subsets. This is a huge advantage, as we can observe the coexpression of certain markers.

Immune checkpoints has been widely studied in the recents year due to their role in cancer. The tumor microenvironment is infiltrated with immune cells that have a decreased activity. This is mainly due to signaling and metabolic suppression. For example, tumors can upregulate immune checkpoint ligands in order to create a inhibitory signal that suppresses the immune response. The antibodies designed to block this molecules have been a revolution in cancer treatment, but with some severe side effects such as autoimmune diseases (26). This relation between immune checkpoints, inflammation and autoimmune diseases has lead to further investigation in other diseases such as infectious diseases or autoimmune diseases (27,28) Some researchers have demostrated the role of immune checkpoints in IBD in which most of them are paradoxically overexpressed. There is an altered expression of PD-1 and PD-L1, with an increased expression in IBD patients that results in the disregulation of Th1/Th2 responses and overall interferon gamma production, leading to inflammation (29). Beswick et al described an increased expression of PD-L1 in UC and a decrease in CD, and the downregulation of PD-L1 led to a more extensive colitis (30). CTLA-4 has also been associated with IBD, expressed in colonic innate lymphoid cells (tissue resident innate lymphocytes important in the mucosal barrier and the defense against pathogens), and the role of CTLA-4 as an important controller of the host response against microbiota has been suggested. The blockage or deletion of this molecule is associated with a

exacerbated disease both in animals models and humans (31). In contrast, TIGIT expression has been described as downregulated in $CD4^+$ and $CD8^+$ T cells of pedriatic IBD patients. This downregulation is related with the activity of the disease, and it has also been observed in some specific cell subsets such as $CD38^+$ effector T cells isolated from peripheral blood, which lose the expression of TIGIT in IBD patients (32,33). VISTA has not been studied specifically in IBD but his role as a key regulator of immune response is known, as it maintains peripheral tolerance and controls immune responses. It has been proposed as a potential treatment for autoimmune disease, as its depletion in animals models has shown progression of autoimmune diseases (34). Finally, LAG-3 is found overexpressed in the inflammed mucosa, specially on activated effector memory T cells, and surprisingly correlates with disease activity in UC patients. This suggests that blocking LAG-3 could lead to a remission of the disease (35). Despite these findings, few studies have assessed the co-expression of multiple immune checkpoints across immune cell subsets at the same time. Our panel addresses this gap by enabling simultaneous detection of PD-1, PD-L1, CTLA-4, TIGIT, LAG-3, and VISTA. This will allow us to explore whether checkpoint expression is coordinated within specific cell types and how this may relate to immune exhaustion or ineffective regulation. Interestingly, we would hypothesize a reduction in checkpoint expression in inflamed tissues due to exhaustion, but in some cases, a paradoxical overexpression has been observed. This raises important questions: Is checkpoint upregulation insufficient to suppress inflammation? Or does it reflect a compensatory but ineffective immune response?

Our results reveal distinct patterns of immune checkpoint expression across T and B lymphocytes, NK cells and monocytes in healthy controls.

PD1 is expressed on T cells consistent with its well-established role in regulating T cell activation, preventing autoimmunity and contributing to T cell-exhaustion in chronic inflammation and cancer. Interestingly, its expression is also prominent on NK cells. Although PD-1 expression on NK cells is tipically low under under homeostatic conditions, a small percentage of mature NK cells that express PD-1 in peripheral blood has been described (36). One study analyzed 200 buffy coats obtained from healthy donors by using cytofluoromtric analysis and characterized a subset of PD-1 positive cells in about 25% of the donors analyzed, and it was restricted to a specific subset on $CD56^{dim}$ NK cells (37). A induced expression of PD-1 has also been described in healthy patients infected by cytomegalovirus (38). The expression of PD-1 in our results is probably due to the biological expression of PD-1 in NK cells from healthy donors.

PD-L1, the ligand of PD-1, is predominantly expressed in APCs, and its binding to PD-1 leads to a decrease of immunity. In our results PD-L1 is mainly expressed in monocytes, with some expression in T cells and NK cells. The expression of PD-L1 in NK cells is usually low in basal conditions, but it has been described an increase under certain conditions such as inflammation and tumoral microenvironment (38). The expression in T cells has been described, specially in tumor-infiltrating lymphocytes, and the stimulation of T cells is needed to increase it (39). The

expression in our NK cells could be due to the sample processing.

CTLA-4 is detected mainly in T cells, as expected. However, there is also expression in B cells. While less well-characterized, CTLA-4 expression in B cells has been reported in both mice and humans, specifically in B1 and B2 cells, and may contribute to the regulation of B cell activation and antigen presentation (40).

LAG-3 and **VISTA** exhibited minimal expression across all analyzed populations. Both of them are inducible checkpoints, so their expression are typically upregulated in response to a repetitive antigen stimulation (41) (15,42).

TIGIT show a high expression in T cells, B cells and NK cells highlighting its key role in immune regulation. TIGIT is expressed on lymphocites and NK cells, and its expression is upregulated when the activation of these cells occurs. In addition, it can be expressed on regulatory B cells, and it is essential for effective immune regulation (16,43).

This study has several limitations. The most significant relates to the samples used. While the samples used to optimize the panel were from healthy donors, the immune checkpoints analysis was conducted using cells from just one patient, so the results should be taken as preliminary data. Nevertheless, these findings validate the use of this spectral flow cytometry panel in analyzing the expression of six immune checkpoints simultaneously. It serves as a starting point for exploring how the patterns are altered in inflammatory diseases such as IBD, and how they could be used as a therapeutic strategy beyond oncology.

6. Conclusions

The optimization of the flow cytometry panel constitutes an important initial step in the development and application of the high-dimensional flow cytometry panel in immune cells populations to characterize immune checkpoint expression. By the simultaneous analysis of six immune checkpoints (PD-1, PD-L1, CTLA-4, TIGIT, LAG-3 and VISTA) in blood the potential of the panel has been demonstrated. It can help to study the situation of the immune system in IBD. In addition, the panel can also be used in fibroblasts.

Our premilimary data reveals an immune ckeckpoint activity consistent with existing literature while also revealing unexpected findings, such as elevated PD-1 expression on NK cells and low expression of CTLA-4 in B cells. These observations reveals how complex immune checkpoints are. Despite the limitations of using one single patient sample for checkpoint analysis, this work sets a solid basis for future studies to understand how immune checkpoint expression is altered in IBD and other inflammatory diseases. Ultimately, this panel may sarve as a valuable tool for identifying immunological biomarkers and guiding the development of more targeted and effective immunotherapies.

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APPENDIX

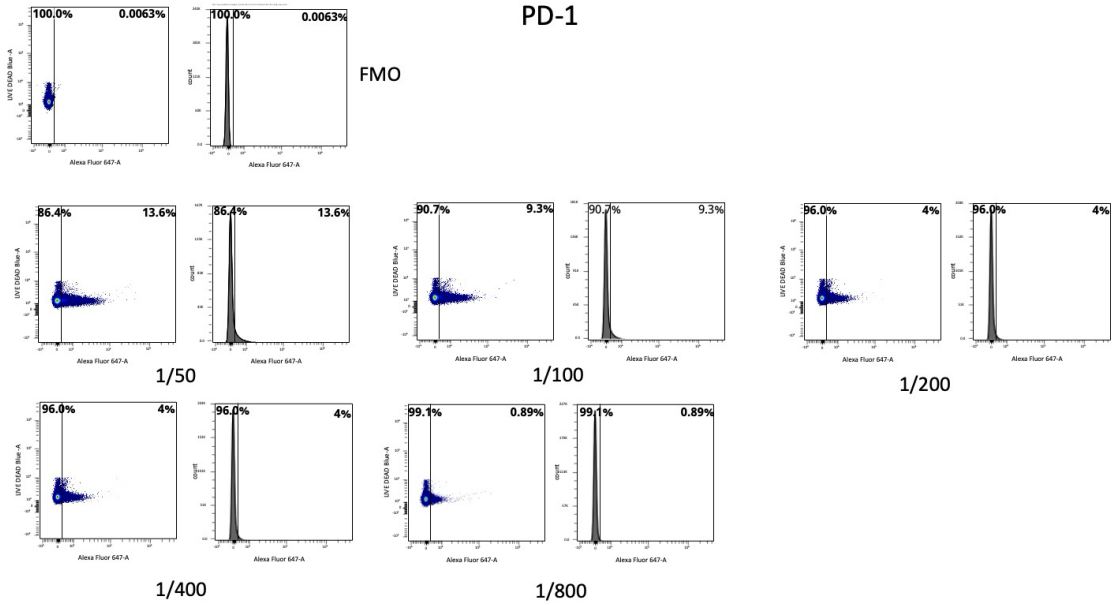

FIGURE A1. Titration of PD-1 in PBMCs. *The optimal concentration was 1/50.*

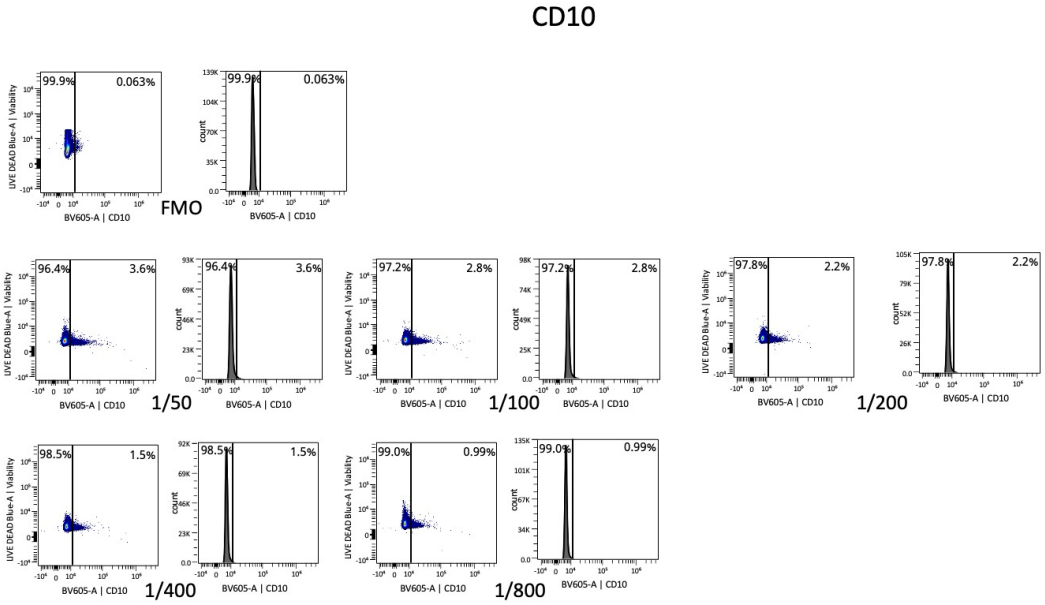

FIGURE A2. Titration of CD10 in PBMCs. *The optimal concentration was 1/50.*

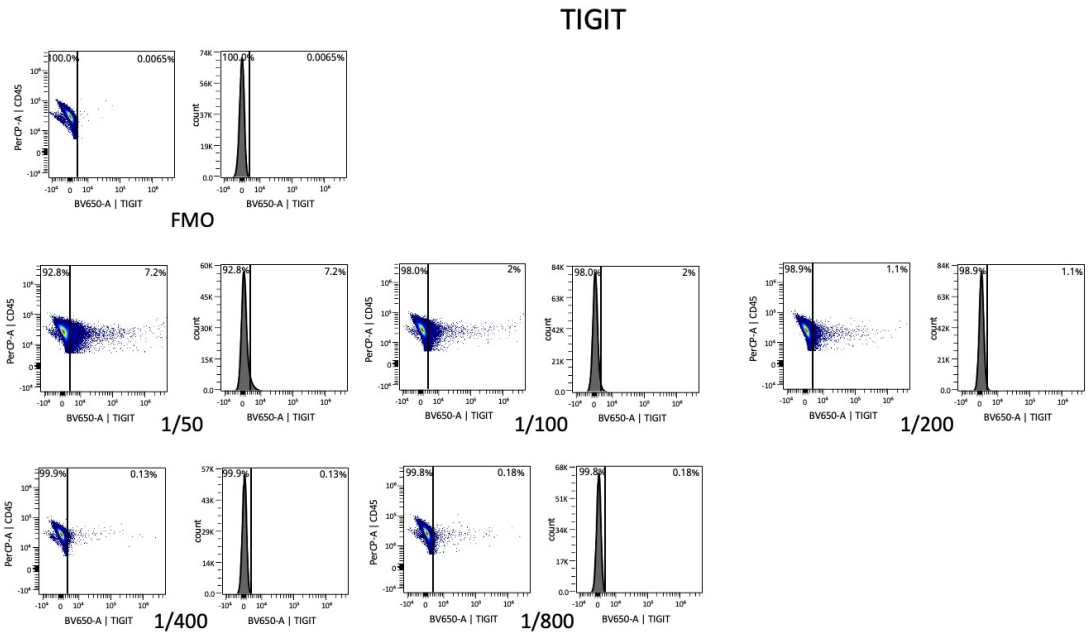

FIGURE A3. Titration of TIGIT in PBMCs. *The optimal concentration was 1/50.*

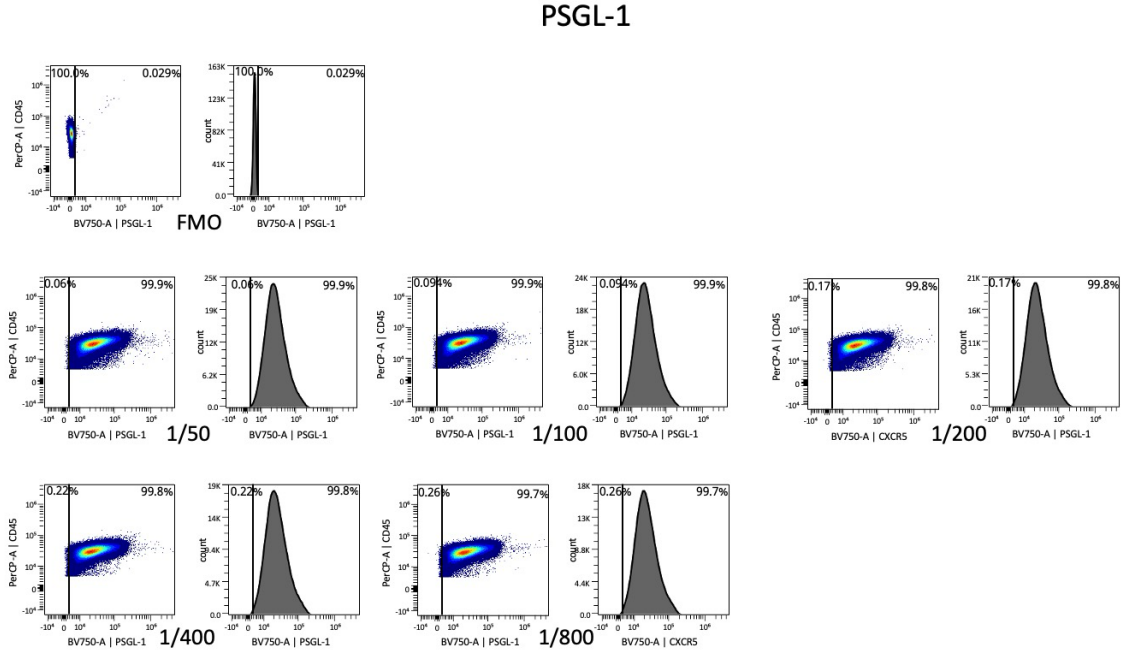

FIGURE A4. Titration of PSGL-1 in PBMCs. *The optimal concentration was 1/50.*

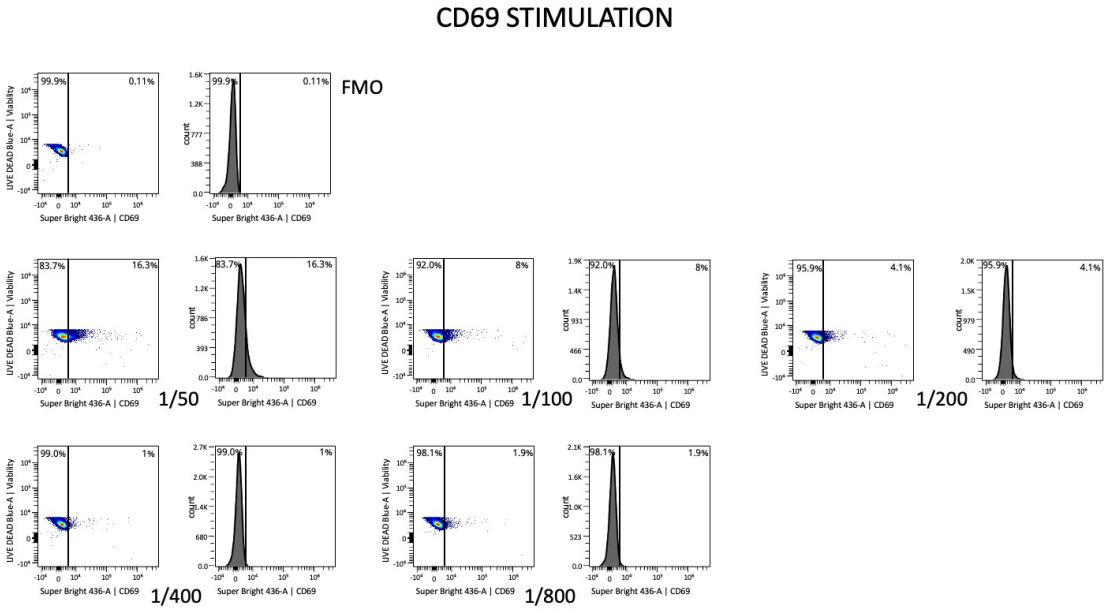

FIGURE A5. Titration of CD69 after stimulation, in PBMCs. *The optimal concentration was 1/50.*

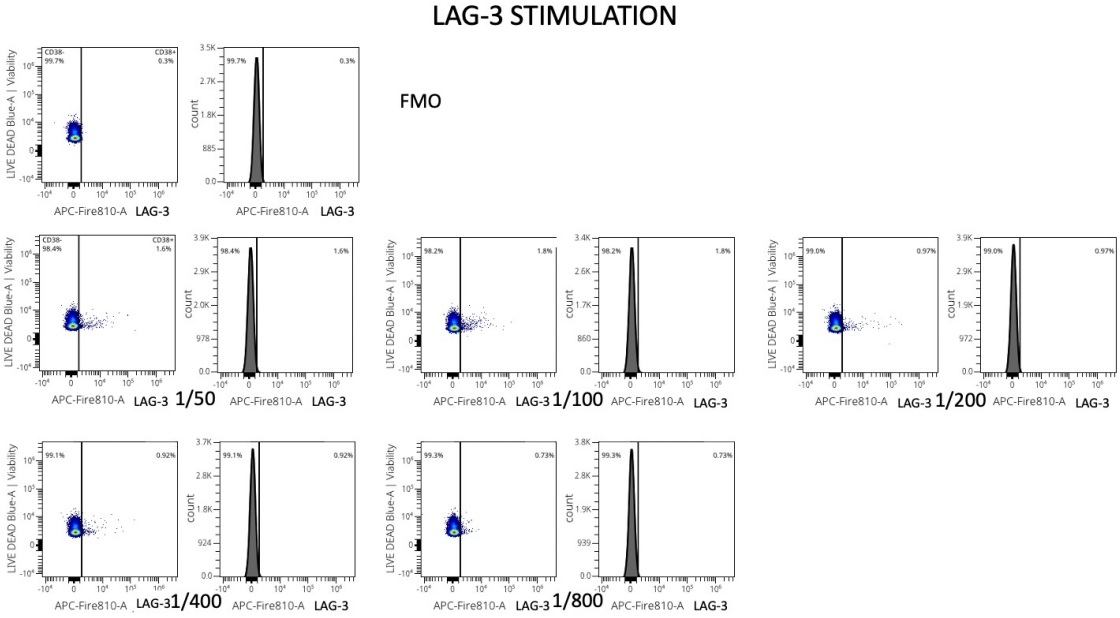

FIGURE A6. Titration of LAG-3 after stimulation, in PBMCs. *The optimal concentration was 1/100.*

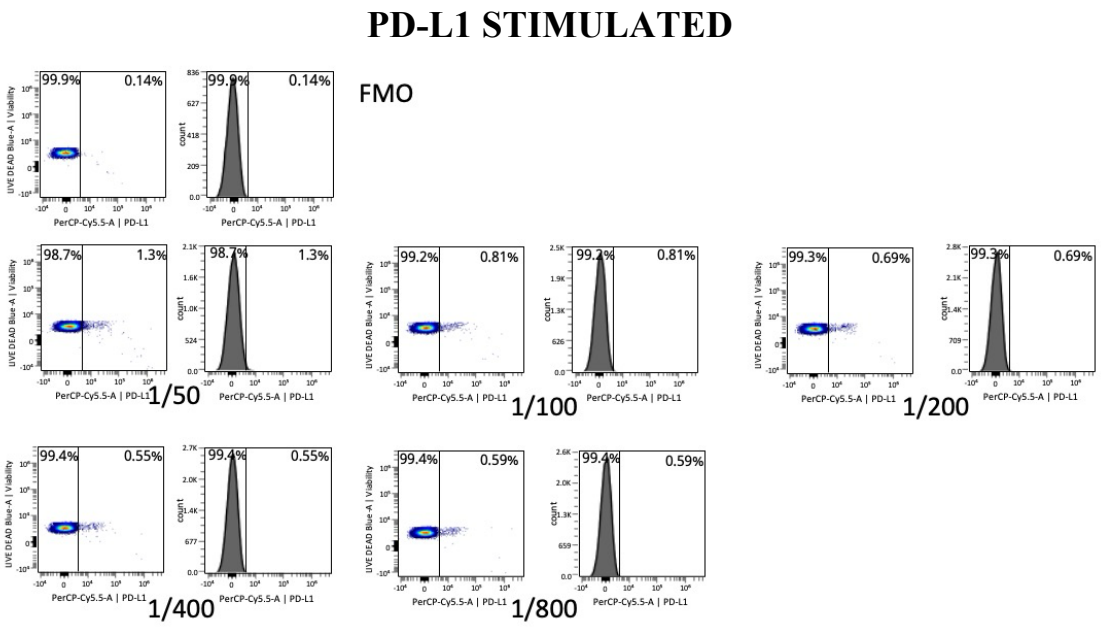

FIGURE A7. Titration of PD-L1 after stimulation, in PBMCs. *The optimal concentration was 1/50.*

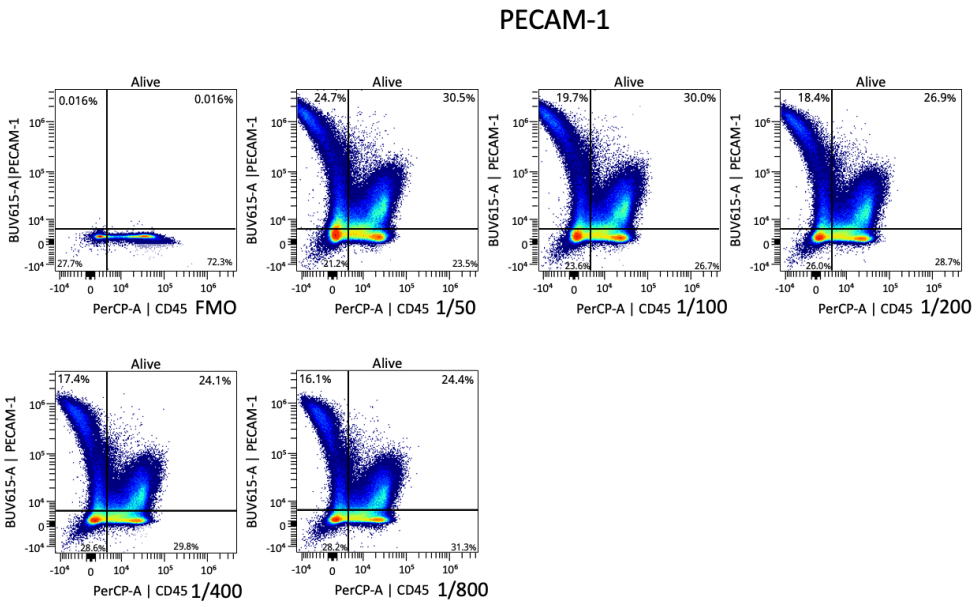

FIGURE A8. Titration of PECAM-1 in LPMCs. *The optimal concentration was 1/800.*

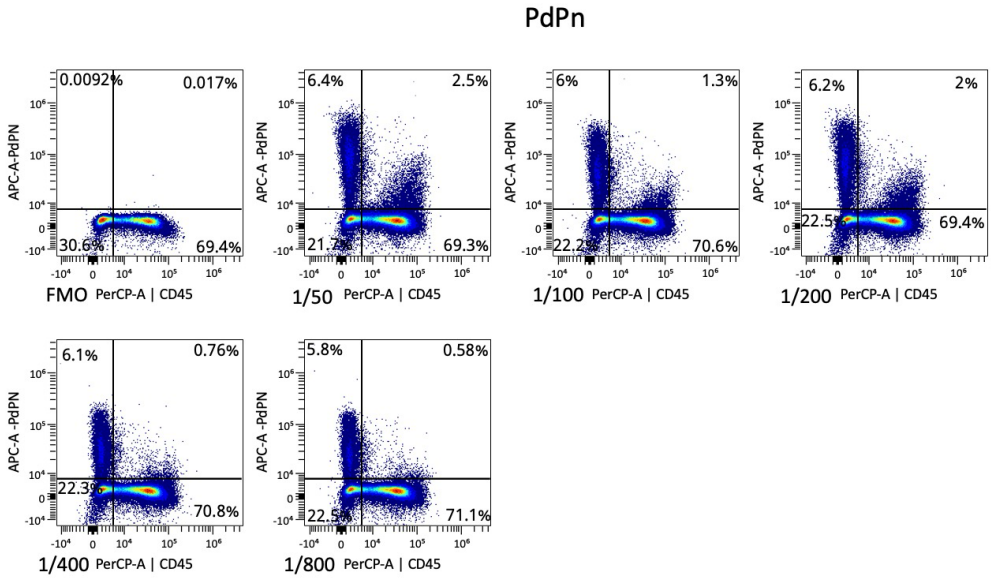

FIGURE A9. Titration of PdPn in LPMCs. *The optimal concentration was 1/50.*

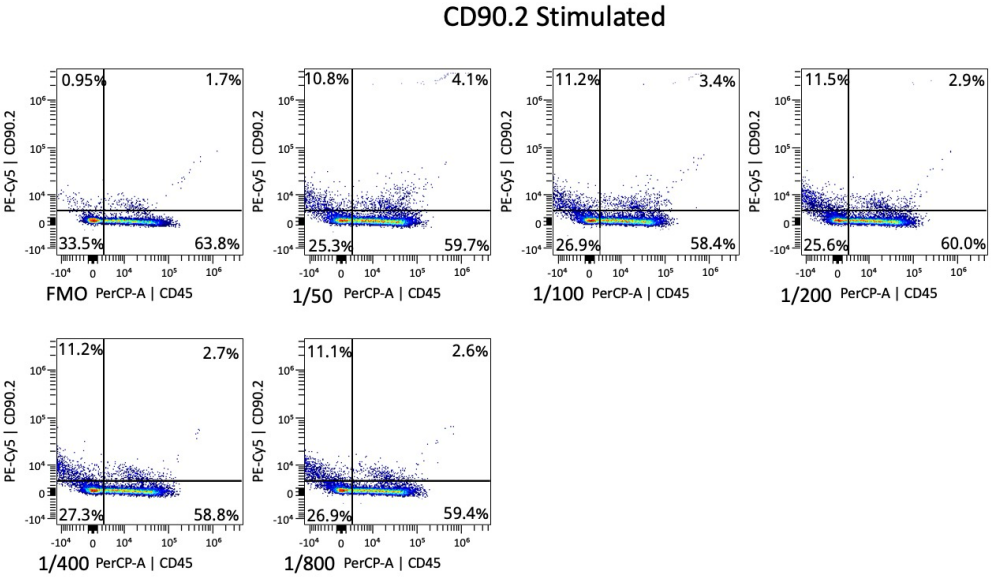

FIGURE A10. Titration of CD90.2 in LPMCs after stimulation. *The optimal concentration was 1/50.*

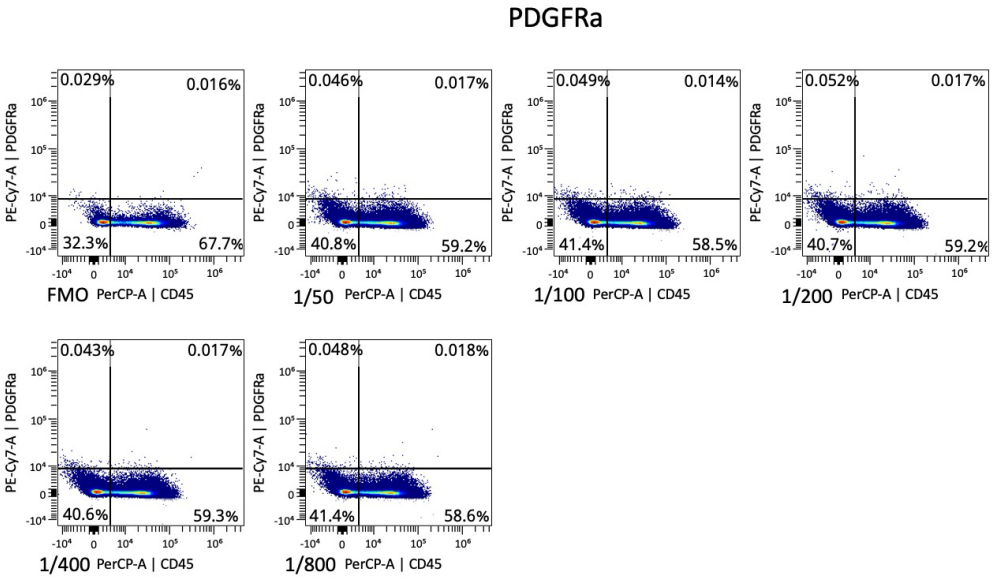

FIGURE A11. Titration of PDGFRa in LPMCs. *The optimal concentration was 1/50.*