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DETERMINATION OF CD26 MARKER ON HUMAN LYMPHOCYTES, PREVIOUSLY TREATED WITH CYTOKINES

DOLOČANJE OZNAČEVALCA CD26 NA PREDHODNO S CITOKINI STIMULIRANIH ČLOVEŠKIH LIMFOCITIH

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I performed the experimental part of my master's thesis at the Faculty of Pharmacy, the Department of Biochemistry and Molecular Biology, University of Santiago de Compostela, as a part of Erasmus student exchange, under the mentorship of prof. dr. Borut Božič, mag. pharm., spec. med. biochem. and co-mentorship of prof. dr. Rubén Varela-Calviño, Ph.D.

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Statement

I declare that I carried out my master's thesis work independently under the mentorship of prof. dr. Borut Božič, mag. pharm., spec. med. biochem. and co-mentorship of prof. dr. Rubén Varela-Calviño, Ph.D.

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ABSTRACT

Introduction: Dipeptidyl peptidase IV (DPPIV), also known as CD26, is a multifunctional membrane glycoprotein, expressed on cell surface of various cells, including T lymphocytes. Its surface expression on T lymphocytes is closely related to cell activation and differentiation. There's an up-regulation of the marker on Th17 and Th1 cells and a down-regulation on Th2 cells.

Research aim: The aim of this study was to determine DPPIV surface and intracellular expression on different helper T subsets (Th0, Th1, Th2, Th17) and find a correlation between them. Our objective was also to determine the amount of enzyme released to the medium during the incubation and the necessity of $CD4^+$ isolation step.

Methods: PBMCs for our experiments were obtained form 9 healthy individuals. For the determination of CD26 expression on different Th subtypes, cells were stained with antibodies against CD3, CD4, CD45RO and CD26 for surface analysis and anti-CD26 antibodies for intracellular analysis. Analysis of DPPIV expression on lymphocytes was done using flow cytometry. The presence of the enzyme in the cultivation medium after a 3-day cultivation was determined with ELISA and its activity with enzyme activity test.

Results and discussion: The highest CD26 surface expression was observed on Th17 population, followed by Th1, Th0, and Th2 subtypes, with a significant difference between Th17 and Th2 subsets ($p<0.05$). The highest CD26 intracellular expression was observed on Th1 subtype, followed by Th0, Th2, and Th17 with no significant differences between them. The isolation step was much more important for intracellular analysis, where cells were stained only with antibodies against CD26, compared to surface analysis, where our target population was selected based on the presence of other membrane markers. The presence and the activity of the enzyme in the cultivation medium after a 3-day incubation were not high enough to be detected by any of the methods in most of our samples.

Conclusion: Although we did not manage to find a correlation between CD26 surface and intracellular expression on different helper T subsets, we solved some other questions, among them the importance of $CD4^+$ isolation step for intracellular analysis. It was evident that at the time of maximum surface expression of CD26, its leaking to the medium was still negligible.

Key words: dipeptidyl peptidase IV (DPPIV) / CD26, helper T cells, flow cytometry, cluster of differentiation (CD)

RAZŠIRJEN POVZETEK

Uvod: Dipeptidil peptidaza IV (DPPIV) ali označevalec CD26 je membranski glikoprotein, ki je prisoten tako na površini epitelijskih, endotelijskih in acinarnih celic različnih tkiv, kot tudi krvnih celic, predvsem limfocitov T. Ima pomembno vlogo v regulaciji diferenciacije in rasti limfocitov T, udeležen pa je tudi pri signalizaciji. Izražanje označevalca CD26 je na limfocitih T v mirujočem stanju nizko, se močno poveča po njihovi aktivaciji in doseže maksimum po treh dneh. Pomembno vlogo pri regulaciji izražanja imajo citokini. Njihova prisotnost v okolju namreč vpliva na to, v kateri tip celic pomagalk se bodo razvile naivne $CD4^+$ celice. Vplivajo tudi na regulacijo izražanja označevalca CD26 na površini limfocitov T. Izražanje je zmanjšano na celicah Th2 in povečano na celicah Th1 in Th17, z največjim izražanjem na Th17 populaciji.

Namen: Namen študije je bil določiti izražanje označevalca CD26 na površini in v notranjosti različnih podvrst celic T pomagalk (Th0, Th1, Th2, Th17). Ker so bile študije o izražanju označevalca CD26 na površini različnih celic T pomagalk že narejene, je bil naš glavni namen najti povezavo med membranskim in znotrajceličnim izražanjem. Med zastavljenimi cilji je bila tudi ugotovitev, ali je izolacija $CD4^+$ celic za tovrstno analizo potrebna, in določitev izločanja encima z membrane v medij s testom ELISA ter testom encimske aktivnosti.

Metode: Celice smo pridobili 1) iz periferne krvi treh zdravih prostovoljcev z izolacijo perifernih mononuklearnih celic (PBMC) s pomočjo gradientnega centrifugiranja s fikolom oz. 2) s postopkom odmrznitve že izoliranih PBMC šestih zdravih prostovoljcev, pridobljenih iz celične banke laboratorija. V primeru izolacije celic iz krvi smo izvedli tudi magnetno pogojeno ločevanje $CD4^+$ celic, v primeru odmrznitve celic pa tega ločevanja nismo izvedli. Po izolaciji celic smo te suspendirali v gojitvenem mediju in jim dodali anti-CD3/anti-CD28 aktivator limfocitov T ter citokine za diferenciacijo: IL-12 in protitelesa za nevtralizacijo IL-4 za Th1 diferenciacijo; IL-4 in protitelesa za nevtralizacijo IL-12 za Th2 diferenciacijo; IL-1β, IL-23 in protitelesa za nevtralizacijo IL-4 ter IFNγ za Th17 diferenciacijo. V primeru Th0 diferenciacije v medij nismo dodali citokinov. Celice smo inkubirali 3 dni pri temperaturi 37 °C in atmosferi s 5 % CO_2. Po inkubaciji smo celice pripravili za označevanje s protitelesi. Za analizo prisotnosti označevalca CD26 na površini celic smo celice označili s primarnimi s

fluorokromi označenimi protitelesi proti CD3 (označena s PerCP), CD4 (označena z APC), CD45RO (označena s FITC) in CD26 (označena s PE). Za analizo prisotnosti označevalca CD26 v notranjosti celic smo te najprej fiksirali in permealizirali ter jih nato označili s primarnimi s fluorokromi označenimi protitelesi proti označevalcu CD26 (označena s FITC). Za vsak vzorec smo pripravili tudi kontrolo, v katero nismo dodajali protiteles. Tako pripravljene suspenzije celic smo analizirali s pretočnim citometrom s štirimi fluorescenčnimi detektorji. Prisotnost encima v gojitvenem mediju smo določili s sendvič testom ELISA z encimskim zaznavnim sistemom hrenove peroksidaze, encimsko aktivnost pa z uporabo substrata Gly-Pro-p-nitroanilida, ki ob prisotnosti DPPIV hidrolizira v Gly-Pro in p-nitroanilin (p-NA), ta pa absorbira pri valovni dolžini 405 nm. Encimska aktivnost je bila podana kot količina nastalega produkta (p-NA) na minuto.

Rezultati in razprava: Celice smo analizirali s pretočno citometrijo, s pomočjo katere smo izmerili intenziteto fluorescence, ki je odvisna od količine označenih protiteles, vezanih na ciljne označevalce, in posledično prisotnost označevalcev na površini celic oz. v njihovi notranjosti. Pri analizi označevalca CD26 na površini celic T pomagalk smo limfocite analizirali glede na prisotnost membranskih označevalcev CD3, CD4, CD45RO in CD26, pri znotrajcelični analizi pa le glede na prisotnost označevalca CD26. Povprečno 47,25 % aktiviranih celic T pomagalk je izražalo označevalec CD26 na površini pri Th2 tipu celic, 37,51 % pri Th0 tipu celic, 29,96 % pri Th17 tipu celic in 17,85 % pri Th1 tipu celic, brez statistično značilnih razlik med njimi. Mediana intenzitete fluorescence je bila najvišja pri Th17 populaciji in najnižja pri Th2 populaciji, s statistično značilno razliko med njima, kar se je skladalo z dosedanjimi študijami. V primerjavi s celicami brez dodanih citokinov (Th0), Th1 celice niso izražale povečanega izražanja označevalca na površini, kot je bilo dokazano v dosedanjih študijah, ampak je bilo izražanje nižje. Pri znotrajcelični analizi deležev $CD26^{+}$ limfocitov nismo mogli primerjati med seboj zaradi različnih izolacijskih postopkov. Mediana intenzitete fluorescence znotrajceličnega označevalca CD26 je bila najvišja pri podvrsti Th1, kateri sta sledili podvrsti Th0 in Th2. Najnižje izražanje označevalca je bilo opaženo pri populaciji Th17. Rezultati se niso ujemali z našimi pričakovanji in niso podprli teorije, da bo imela podvrsta celic pomagalk z največjim membranskim izražanjem, zaradi večjega prenosa proteina na celično membrano, najmanjše znotrajcelično izražanje. To se je pokazalo le pri

celicah Th17, ki so imele največje površinsko in najmanjše znotrajcelično izražanje označevalca CD26. Za vse ostale podvrste celic T pomagalk to ni veljalo.

Ugotovili smo, da pri analizi površinskega izražanja označevalca izolacija $CD4^+$ celic ni bila ključnega pomena, saj smo celice analizirali tudi glede na površinske označevalce CD3, CD4 in CD45RO ter na ta način določili tarčno populacijo (aktivirane celice T pomagalke). Pri znotrajcelični analizi je bila izolacija veliko pomembnejša, saj ostalih označevalcev nismo določali in je bil zato pri neizolirani populaciji delež $CD26^+$ celic veliko manjši v primerjavi z deležem pri izolirani populaciji. Zaradi različnih izolacijskih postopkov je bila primerjava deleža $CD26^+$ celic med podvrstami celic T pomagalk nemogoča.

Prisotnost DPPIV v gojitvenem mediju je bila pod mejo detekcije v večini vzorcev tako pri testu ELISA, kot tudi pri testu encimske aktivnosti. Ti rezultati nakazujejo, da je bilo izločanje encima v medij po treh dneh od aktivacije limfocitov T zanemarljivo. Razlog za to je lahko v relativno kratki inkubaciji celic. Po treh dneh doseže izražanje označevalca na površini celic maksimum, zato predvidevamo, da v tej fazi še ne pride do velikega izločanja označevalca CD26 z membrane v medij.

Zaključek: Pod danimi pogoji nam povezave med membranskim in znotrajceličnim izražanjem označevalca CD26 ni uspelo najti, smo pa kljub temu med raziskavo prišli do drugih pomembnih ugotovitev. Ugotovili smo, da je za znotrajcelično analizo označevalca pomembna izolacija $CD4^+$ populacije in da je po treh dneh od aktivacije limfocitov T, ko je izražanje označevalca CD26 na membrani največje, izločanje le-tega v medij zanemarljivo. Zaradi statistično neznačilnih razlik v izražanju znotrajceličnega označevalca CD26 med posameznimi podvrstami celic T pomagalk, in ker rezultati nakazujejo na vpliv intraindividualnih razlik v izražanju označevalca CD26, bi večje število prostovoljcev, vključenih v študijo, pomembno vplivalo na rezultate. Možno pa je tudi, da je dinamika sinteze in izražanja označevalca CD26 drugačna, kot smo predpostavljali.

Ključne besede: dipeptidil peptidaza IV (DPPIV) / označevalec CD26, celice T pomagalke, pretočna citometrija, označevalec pripadnosti (CD)

LIST OF ABBREVIATIONS

ADA	adenosine deaminase
APC	allophycocyanine
AgPC	antigen presenting cell
BSA	bovine serum albumin
CD	cluster of differentiation
DPPIV/CD26	dipeptidyl peptidase IV
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FI	fluorescence intensity
FSC	forward scatter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
HSA	human serum albumin
IFN	interferon
IL	interleukin
LOD	limit of detection
LOQ	limit of quantification
MACS	magnetic-activated cell sorting
MFI	median fluorescence intensity
MHC	major histocompatibility complex
NK	natural killer
OPD	o-phenylenediamine dihydrochloride
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PFA	paraformaldehyde

PMT	photomultiplier tube
p-NA	p-nitroaniline
PSG	penicillin, streptomycin, glutamine
SD	standard deviation
SSC	side scatter
Tc	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	helper T cell
TNF	tumor necrosis factor
Treg	regulatory T cell

1 INTRODUCTION

1.1 IMMUNE SYSTEM

The immune system protects the body from harmful foreign substances from the environment, like bacteria and viruses, and is essential for survival (1). The immune system has evolved a wide variety of defense mechanisms to protect against potential invaders that could harm the host. But at the same time it must be sophisticated enough to distinguish the individual's own cells from harmful invading organisms and not attack its own cells or flora in the gut, skin and other tissues. A variety of cells and soluble molecules that these cells secrete are involved in the immune response (Figure 1). Although leucocytes play a central role in all immune responses, other cells also participate. They are involved in signaling to the lymphocytes and responding to the cytokines released by T cells and macrophages (2).

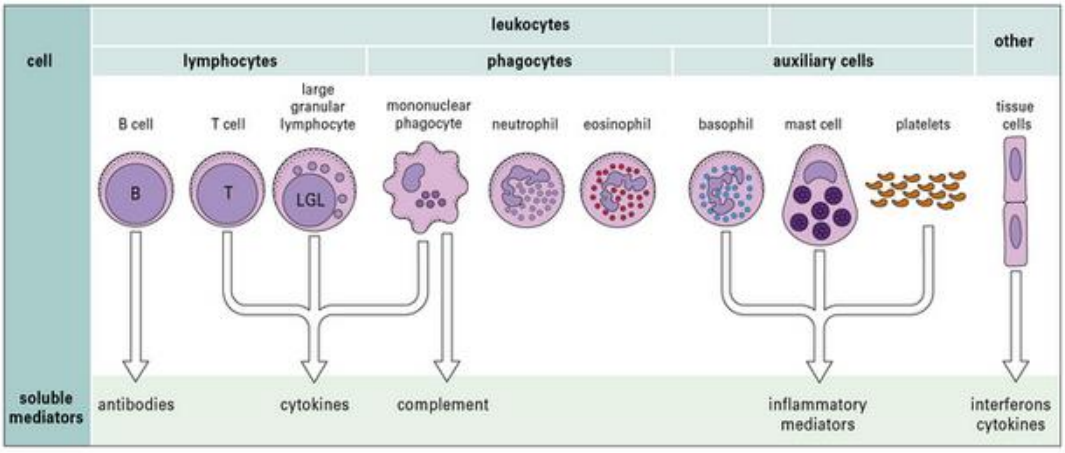

Figure 1: *Components of the immune system and the mediators they produce (2)*

First line of the host's defense is called *innate immune response*, which is activated upon the first exposure to a new pathogen and causes inflammation. It is mediated by phagocytes, including macrophages and dendritic cells (3). The innate immune response plays an important role in the activation of adaptive immune response. T lymphocytes can be activated by macrophages that have phagocytosed bacteria and more importantly by the dendritic cells, which are specialized for presenting antigens to T cells (4). *Adaptive immune response* is a

much more sophisticated defense, highly specific to a particular pathogen. All substances that provoke an adaptive immune response in the organism are called antigens (5). It is mediated by lymphocytes, with B and T cells being the major components. B cells are derived and developed in bone marrow (*b*one marrow-derived lymphocytes), while T cells are derived from bone marrow, but developed in thymus (*t*hymus-derived lymphocytes) (2, 3).

1.2 T LYMPHOCYTES

T cell or T lymphocyte is a type of leukocyte that is a part of the adaptive immune response. They are derived from hematopoietic stem cells in the bone marrow, but they mature in the thymus, where they can differentiate into different T cell subtypes (5):

1) *Regulatory T cells (Treg):* they express a CD4 marker on their surface and perform mostly suppressive functions, which include prevention of autoimmune diseases, suppression of allergies, asthma, and pathogen-induced immunopathology, they control the magnitude of immune response by effector Th cells and induce maternal tolerance to the fetus (6).
2) *Helper T cells (Th):* they also express a CD4 marker on their surface and participate in adaptive immune response by helping activate B cells and cytotoxic T cells (7).
3) *Cytotoxic T cells (Tc):* they express a CD8 marker on their surface and are involved in killing infected target cells (8).

1.2.1 HELPER T CELLS

Th cells are a very important part of the adaptive immune system. They have multiple functions, including helping activate Tc cells to kill infected cells, B cells to secrete antibodies and macrophages to digest microbes. But Th cells themselves can only function when activated into effector cells. In order to transform themselves into effector cells they need to be activated by antigen presenting cells (AgPCs). When there is an infection present in the body, microbes stimulate dendritic cells to produce cytokines. Dendritic cells then migrate to a peripheral lymphoid organ and activate naïve T cells. Into which type of effector cell they will transform depends on the cytokines in their environment, produced by dendritic cells (7).

Th cells can be further divided into different groups based on their cytokine profiles:

1) *Type 1 helper cells (Th1 cells)*: they interact with mononuclear phagocytes, helping them destroy intracellular pathogens (2). They secrete IL-2, IFN-γ, and TNF-α, that play a major role in cell-mediated immune responses (9).
2) *Type 2 helper cells (Th2 cells):* they mostly defend the organism against extracellular pathogens by interacting with B cells, stimulating them to divide, proliferate, and produce antibodies and thus providing humoral immunity (2). They secrete IL-4, IL-5, IL-6, and IL-10, that are involved in the proliferation and differentiation of B cells (9).
3) *Type 17 helper cells (Th17):* they are involved in the elimination of extracellular bacteria and fungi. They also promote activation of macrophages and neutrophils by producing IL-17A (10). They secrete IL-17A, IL-17F and IL-22 (11).
4) *Type 0 helper cells (Th0):* they produce cytokines of both, Th1 and Th2 cell subtypes, and are thought to be their precursors (12, 13).

Th subtypes, their function, secreting cytokines, and cytokines involved in their differentiation are presented in Table I.

Table I: Different Th subtypes and cytokines involved in their differentiation, cytokines that they produce and their function in immune system (11, 14)

	Th1	**Th2**	**Th17**
Differentiation	IL-12	IL-4	IL-23, IL-1β
Production	IL-2, IFN-γ, TNF-α	IL-4, IL-5, IL-6, IL-10, IL-13	IL-17A, IL-17F, IL-22
Functions	Cellular immunity (intracellular infections with microorganisms)	Humoral immunity; stimulation of B cell proliferation (extracellular infections, allergic immune reactions)	Protection in certain infections, helping eliminate bacteria and fungi

1.3 LEUKOCYTE SURFACE MARKERS AND THEIR IMPORTANCE

All leukocytes express a large number of different functionally important molecules (markers) on their surface, which can be used to distinguish different cell subsets. The presence of surface molecules of different cell populations allows them to be identified using fluorescent antibodies. These can be applied to tissue sections for the identification of cell populations or used in flow cytometry for the separation and enumeration of cells in suspensions, based on their size and fluorescent staining. The major function of marker molecules is the communication between lymphocytes and their environment. They play a major role in cell trafficking, adhesion, and activation (15).

1.3.1 LEUKOCYTE CD MARKERS

Cluster of differentiation (CD) molecules are antigen markers present on the surface of leukocytes and other cells of the immune system and are useful for their identification, characterization, and isolation (16).

Some of the CD markers:

- CD3 – expressed on T cells and thymocytes. It plays an essential role in the expression of T cell receptor (TCR) and TCR signal transduction (16).
- CD4 – expressed on major histocompatibility complex (MHC) class II-restricted T cells, some thymocytes, macrophages and monocytes. It works as a co-receptor for MHC class II-restricted T cell activation and thymic differentiation marker for T cells (16).
- CD45 – expressed on all hematopoietic cells except erythrocytes and platelets. Its expression is especially high on lymphocytes. It is involved in the regulation of variety of cellular processes, including cell growth, differentiation and mitotic cycle. It also plays an important role in T and B cell antigen-receptor-mediated activation (16).
- CD45RO – expressed on activated and memory T cells, B cell subsets, immature thymocytes, activated monocytes, macrophages, dendritic cell subsets and granulocytes. It is involved in receptor-mediated signaling and cell activation (16).
- CD26 – expressed on activated T cells, mature thymocytes, B cell subsets, macrophages, natural killer (NK) cells, some epithelial cells and lymphatic endothelial

cells. It is an exopeptidase, cleaving dipeptides from protein N-termini. It has T-cell co-stimulation functions and may be involved in lymphatic vessel adhesion (16).

1.3.2 IMMUNOPHENOTYPING

Immunophenotyping is a technique that uses antibodies to identify cells based on the expression of their markers. Cell markers have become very useful in the identification of specific cell populations. But they are often expressed on more than one cell type, thus two or more different antibodies for different markers can be used. Each of these antibodies is coupled with a different fluorochrome, and the signals they emit are then measured with a flow cytometer. Many of these markers are CD markers, which are present on immunological cells. They are often used for detection in flow cytometry (17).

1.4 DIPEPTIDYL PEPTIDASE IV OR CD26

1.4.1 GENERAL PROPERTIES OF DPPIV

Dipeptidyl peptidase IV (DPPIV), also known as CD26, is a multifunctional integral membrane protease, with a catalytic site in its extracellular domain (18, 19). It is a cell surface glycoprotein, widely expressed on epithelial, endothelial and acinar cells of various tissues and blood cells; mainly on T cells, but also on B and NK cells (18, 20). It is also found in a soluble form in serum. The soluble form might originate from endothelial or epithelial cells and also from leukocytes (19). The origin of DPPIV from T lymphocytes is supported by the fact that their isoforms are analogous and they both bind adenosine deaminase (ADA) with similar specificity and affinity (21). DPPIV belongs to a prolyl oligopeptidase family, a group of atypical serine proteases that are able to hydrolyse the prolyl bond. It preferentially cleaves X-proline or X-alanine dipeptides from N-terminal end of polypeptides (20). It can also cleave dipeptides with hydroxyproline, serine, glycine, valine or leucyne in the penultimate position, but less efficiently (18).

1.4.2 DPPIV FUNCTIONS

Data from several groups have shown that DPPIV plays a central role in the regulation of differentiation and growth of T lymphocytes and in TCR-mediated signal transduction (22). Its

functions include ADA binding, peptidase activity, it acts as a receptor and a co-stimulatory protein, adhesion molecule for collagen and fibronectin, and is involved in apoptosis (20, 23). It is also involved in differentiation of cells, preventing the cells to convert into a malignant phenotype (23). As a receptor, CD26 binds ADA, which catalyzes the hydrolysis of extracellular adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine (19). ADA is important for the development and function of lymphoid tissue. Adenosine and deoxyadenosine both suppress T and B cell activation and are degraded by ADA. Binding of ADA to CD26 reduces adenosine concentrations and leads to the induction of T cell proliferation (23). As a protease, DPPIV cleaves several peptides present in the body, including neuropeptide Y, peptide YY, substance P, β-casomorphin, endomorphin-1, endomorphin-2, kentsin, glucagon-like peptide 1, glucagon-like peptide 2, glucose-dependent insulinotropic polypeptide, gastrin-releasing peptide, glucagon, vasoactive intestinal peptide, and peptide histidine methionine (19). It plays a major role in the immune response; abnormal expression is found in autoimmune diseases, cancer, and HIV related diseases (23).

1.4.3 DPPIV EXPRESSION

Although other blood cells express CD26 as well, it is predominantly expressed on T lymphocytes (24). Resting lymphocytes show low levels of CD26, but its expression is strongly up-regulated following activation, up to 10-fold (20). Surface expression of CD26 on T cells reaches its maximum after three days after their activation (23).

CD26 is expressed on activated $CD4^+$ and $CD8^+$ T lymphocytes and the expression on Th cells is restricted to $CD4^+$ memory cells ($CD45RO^+/CD29^+$) (23). Membrane DPPIV expression is down-regulated on Th2 cells and up-regulated on Th1 cells and Th17 cells, latter showing the highest expression (20, 25). Cytokines play an important role in the regulation of expression, interleukin-12 (IL-12) increasing it and IL-4 decreasing it (20, 23).

1.4.4 DPPIV AS A BIOMARKER – ABNORMAL DPPIV EXPRESSION

DPPIV serves as an early predictive biomarker in several diseases such as multiple sclerosis, diabetes, HIV, and different types of cancer (26).

1.4.4.1 Elevated DPPIV levels

An increased expression of a CD26 marker on T cells from peripheral blood is present in patients with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and Grave's disease, inflammatory diseases such as bowel disease and also in patients with type 2 diabetes (23). Altered levels of CD26 in the microenvironments of specific tumors have been associated with various types of cancer. Elevated expression and/or activity are found in patients with thyroid follicular carcinoma, T and B lymphomas and leukemias, astrocytic tumors, and gastrointestinal stromal tumors (27).

1.4.4.2 Decreased DPPIV levels

A decreased expression of a CD26 marker on T cells is present in immunodeficiency diseases. T cells of patients with leukemia show lower CD26 expression or a complete absence of the marker (28). Patients infected with HIV-1 show a decrease in CD26-positive T cells, followed by a general reduction in the number of $CD4^+$ cells (20). Decrease and loss of DPPIV expression in tumors' microenvironments are found in patients with melanoma, prostatic, endometrial, colorectal, hematological and renal cancers, along with lung and skin squamous cell carcinomas (27).

1.5 DIFFERENTIATION OF $CD4^+$ T CELLS BY CYTOKINE STIMULATION

The lymphocyte population is composed mostly of T cells, B cells, and NK cells. T lymphocytes, responsible for cellular immunity, together with B cells, responsible for humoral immunity, form adaptive immunity. T lymphocytes mature in the thymus and undergo differentiation in the peripheral lymphoid organs when encountered with antigens (29).

1.5.1 IN VIVO STIMULATION AND DIFFERENTIATION

$CD4^+$ cells play an important role in adaptive immune response. Naïve $CD4^+$ T cells are activated by interacting with antigens, presented to the cells by an AgPCs. Following activation, cells are proliferated and differentiated into a specific subtype of effector cell. Multiple factors influence lineage-specific differentiation, like cytokine microenvironment,

concentration of antigens, type of AgPCs, as well as co-stimulatory molecules. Naïve T cells can differentiate into several types of Th cells, including Th1, Th2, Th17, or Treg cells; each of them characterized with a specific cytokine profile (29).

1.5.2 IN VITRO STIMULATION AND DIFFERENTIATION

Traditional in vitro activation is achieved by incubating T cells in the presence of mitogenic lectins such as phytohemaglutinin or concanavalin A. Another approach, which is a better imitation of *in vivo* stimulation by AgPCs, uses beads coated with anti-CD3 and anti-CD28 (30). For the differentiation of cells appropriate cytokines are added to the cell suspension, which is then incubated for 3 – 5 days at 37 °C (10). Different cytokines can be used for the differentiation of naïve $CD4^+$ T cells into different Th subsets:

1) *Th1 cells:* Th1 in vitro differentiation is induced when IL-12 alone or in combination with IL-2 is added to the culture and IL-4 is neutralized (31, 32).
2) *Th2 cells:* Th2 in vitro differentiation can be obtained in the presence of IL-2 and IL-4 (34). Neutralizing antibodies to interferon-γ (IFN-γ) also induce Th2 polarization (32).
3) *Th17 cells:* Th17 in vitro differentiation can be obtained in the presence of IL-2, IL-6, IL-1β, IL-23, and transforming growth factor-β (TGF-β), although IL-2, IL-6, and TGF-β are not necessary for the differentiation. IL-4, IFN-γ, and IL-27 suppress Th17 differentiation (11, 32).

Once the polarization of a T cell population begins, the cells start producing the cytokines that are needed to reinforce polarization. At the same time these cytokines suppress the development of any other T cell subsets (33).

1.6 ISOLATION AND IDENTIFICATION OF CELLS OF THE IMMUNE SYSTEM

We can evaluate immune responses using human peripheral blood mononuclear cells (PBMCs), which consist of about 70 – 90 % lymphocytes (T cells, B cells and NK cells), 10 - 20 % monocytes, and 1 – 2 % dendritic cells. Within the lymphocyte population, there are 70

– 85 % $CD3^+$ T cells, 5 – 10 % B cells, and 5 – 20 % NK cells. The $CD3^+$ population is composed of 2/3 $CD4^+$ cells and 1/3 $CD8^+$ cells (34).

1.6.1 ISOLATION OF PBMCs FROM HUMAN BLOOD USING DENSITY GRADIENT CENTRIFUGATION

The most common method for isolation of PBMCs from blood is density centrifugation using Ficoll (35). Ficoll is a high density solution, composed of polysucrose and sodium diatrizoate with adjusted density (36). Its density is greater than that of lymphocytes, but lower than the density of red blood cells and granulocytes. When blood is centrifuged together with Ficoll, red blood cells and granulocytes pass through Ficoll to the bottom, while lymphocytes stay at the interface of the medium and Ficoll (Figure 2). Plasma has the lowest density and remains at the top (15).

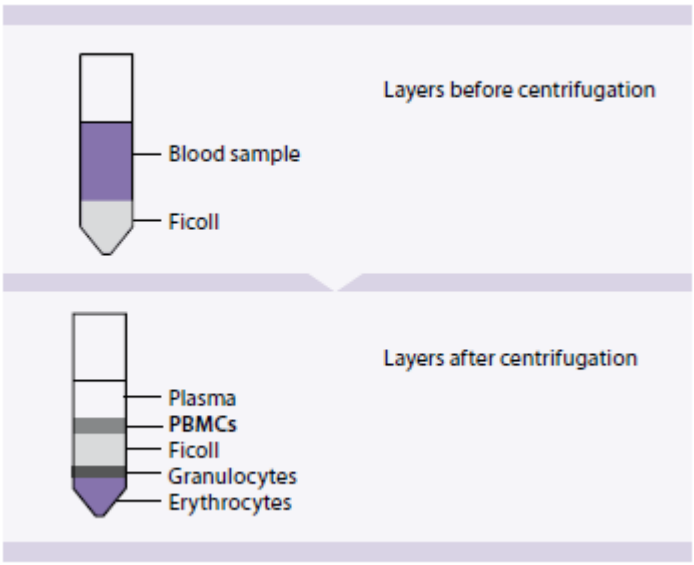

Figure 2: *Layers before and after density gradient centrifugation (37)*

1.6.2 CELL SEPARATION BY IMMUNOMAGNETIC MICROBEADS WITH INDIRECT METHOD

This method can be used for the separation of cell populations, based on the expression of different CD markers on their surface. It is a two-step method, involving binding of primary monoclonal biotinylated antibody and secondary antibody-coated magnetic beads (beads coated with monoclonal antibiotin antibodies). Cells bound to the magnetic beads bind to the

column, and the rest of the cells are washed away. If we need the bound fraction, we need to wash them from the column for collection (15).

1.6.3 FLOW CYTOMETRY

Flow cytometry is used for the analysis of multiple characteristics of the cells. It can measure cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular molecules (38). It is also used for measuring fluorescence intensity (FI) produced by fluorescent-labeled antibodies that bind to target proteins or ligands in the cell (39).

1.6.3.1 General Principles

When a cell suspension is applied to the cytometer, cells are hydrodynamically focused through a measuring device one cell at a time, causing a beam of light to scatter, as showed in Figure 3. The scattered light is then detected with two different types of detectors, one placed in front of the light beam (forward scatter – FSC), indicating cell size, and the other placed at the side (side scatter – SSC), indicating internal complexity/granularity of a cell. We can separate cells into different populations based on their FSC and SSC. Large cells produce a high FSC, while more granular cells produce a high SSC (39).

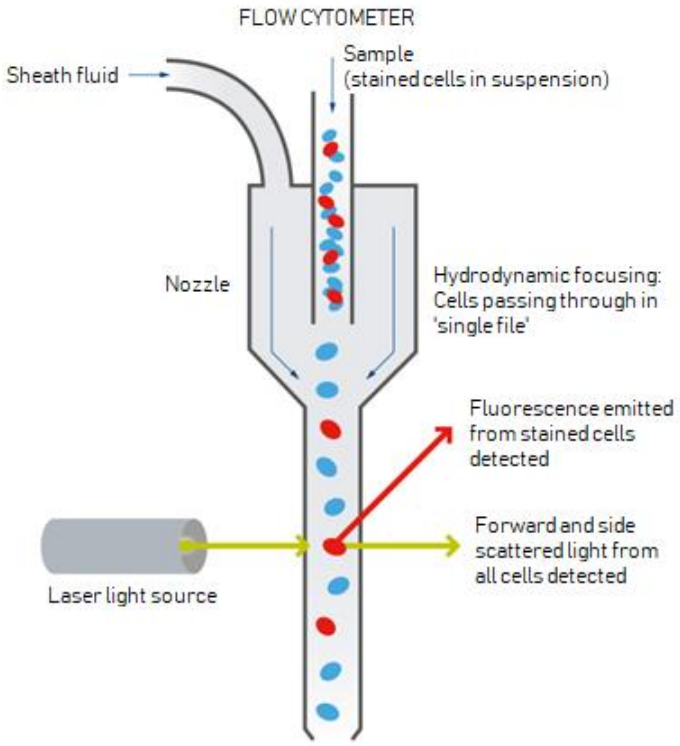

Figure 3: *Principles of flow cytometry (39)*

1.6.3.2 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting can separate a cell population into sub-populations with a help of fluorescent labeling. Each cell has specific proteins/markers on their surface and molecules such as DNA and cytokines in the interior. We can use those to separate them into different groups by staining them with fluorophore-conjugated antibodies. Fluorophores are fluorescent markers which absorb light at a certain wavelength and re-emit it at a longer one. The first process is called excitation and the second one emission, also known as fluorescence. Fluorophores are excited with a laser of a specific wavelength and their emission is measured with fluorescence detectors (40).

FSC, SSC and emitted fluorescent light are split into different wavelengths and channeled within the flow cytometer. The fluorescent light is filtered in a way that only specific wavelengths are transmitted to the detectors – photomultiplier tubes (PMTs), as shown in Figure 4 (39).

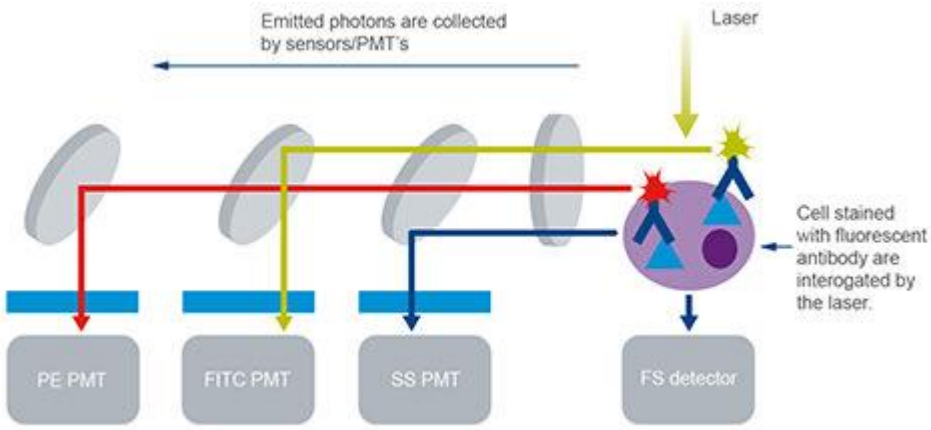

Figure 4: *Detection of FSC, SSC, and fluorescence by PMTs (39)*

Some of the fluorophores used in flow cytometry:

- **Fluorescein isothiocyanate (FITC)** – it is a fluorescein derivate, commonly used as a fluorescent reagent for biological researches (41).
- **Phycoerythrin (PE)** – it is a phycobiliprotein, isolated from red algae (42).
- **Allophycocyanine (APC)** – it is a phycobiliprotein, isolated from red algae (43).
- **Peridinin chlorophyll protein complex (PerCP)** – it is a carotenoid pigment (44).

Their excitation and emission maximums are presented in Table II.

Table II: Commonly used fluorophores and their excitation and emission maximums (41-44)

Fluorochrome	Excitation maximum	Emission maximum
FITC	490 nm	525 nm
PE	565 nm	578 nm
APC	650 nm	660 nm
PerCP	488 nm	675 nm

1.6.3.3 Immunofluorescence antibody staining

1) *Direct method*

   It is a one-step method using a single antibody. Fluorophore-conjugated antibodies are added to the cell suspension and bound directly to the target molecules. This method

has the advantages of including only one antibody incubation step and the possibility of non-specific binding of the secondary antibody is excluded (39).

2) *Indirect method*

This method includes binding of two antibodies. First, cells are incubated with unlabeled primary antibodies, and after, fluorophore-labeled secondary antibodies are added. These antibodies have specificity for the primary antibodies. The avidin-biotin system can also be used, where an antibody is conjugated to biotin and detected with fluorophore-labeled avidin (39). The advantages of this method are its flexibility, because different secondary antibodies can be used, and its sensitivity, since several secondary antibodies bind to the primary antibody, causing the signal to amplify. But cross-reactivity of secondary antibodies may occur, causing false positive or higher results (45). Visual representation of direct and indirect staining is shown in Figure 5.

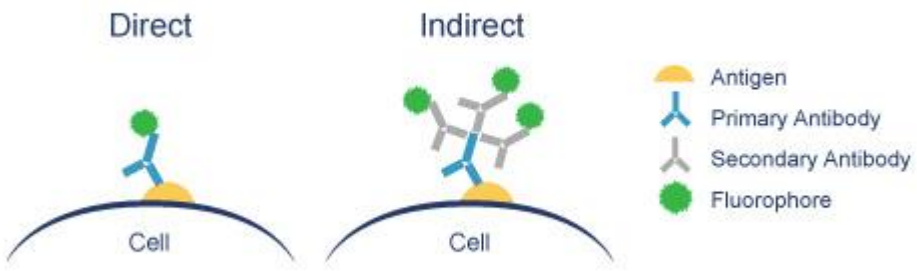

Figure 5: *Direct and indirect staining (45)*

1.6.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a method used for a detection and/or quantitative determination of a specific protein in a complex suspension. We can analyze protein samples immobilized to microplate wells using specific antibodies (46).

1.6.4.1 Sandwich ELISA

Two different antibodies are used in sandwich ELISA. They both bind specifically to the antigen, but each reacting with a different epitope. The first antibody binds to the microtiter

plate and it is called capture or coating antibody, and the second one is called detection antibody. Sandwich ELISA is more sensitive than traditional (direct) ELISA and it provides higher signals (46).

1.6.4.2 Biotin-avidin sandwich ELISA

It is a multiple step assay, designed to detect a certain antigen in a complex protein mixture. It uses biotinylated detection antibodies and enzymes horseradish peroxidase (HRP) or alkaline phosphatase conjugated to avidin or streptavidin, which bind very strongly and specifically to the biotin molecule. Steps of biotin-avidin sandwich ELISA are presented in Figure 6. Several substrates that are catalyzed by HRP into a coloured or fluorescent compound can be used, for instance o-phenylenediamine dihydrochloride (OPD). The reaction between a substrate and HRP is presented in Figure 7. Coloured product accumulates over time relative to the amount of enzyme present in the sample. The product signal is measured spectrophotometrically or fluorometrically (46).

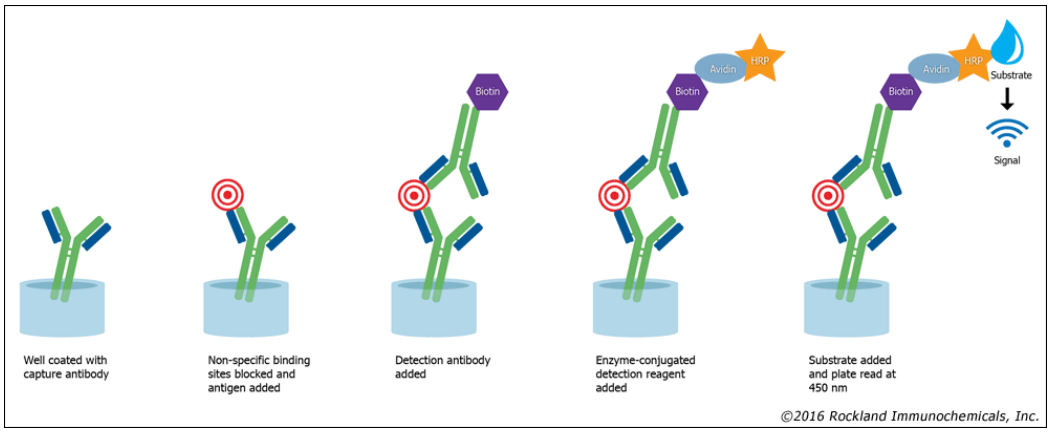

Figure 6: *Steps of biotin-avidin sandwich ELISA (47)*

1.6.4.3 Steps of biotin-avidin/streptavidin sandwich ELISA

1) COATING: The microtiter plate is coated with antigen-specific antibodies (capture antibodies).
2) PLATE BLOCKING: Blocking agent is applied to the microtiter plate to prevent non-specific binding of antigens and antibodies to the wells of a microtiter plate.

3) ANTIGEN IMMOBILIZATION: Sample is added and antigens from the mixture bind specifically to the capture antibodies.
4) ADDITION OF DETECTION ANTIBODIES: Biotinylated detection antibodies are added and they bind to antigens specifically.
5) ADDITION OF AVIDIN/STREPTAVIDIN-HRP: When avidin/streptavidin-HRP is added, it binds specifically to biotin.
6) DETECTION: Substrate solution (OPD and H_2O_2) is added.
7) SIGNAL MEASUREMENT: The signal is detected with a microplate reader set to 450 nm.

(46)

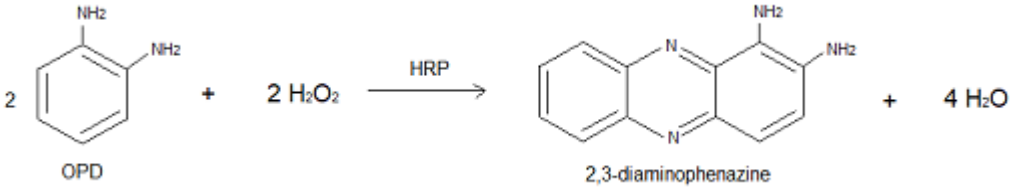

Figure 7: *The reaction between OPD and hydrogen peroxide in the presence of HRP (48)*

2 RESEARCH AIM

The aim of this study is to determine surface and intracellular expression of CD26 marker on Th0, Th1, Th2, and Th17 cell subtypes of healthy individuals. Since surface expression of CD26 on different Th subsets has been studied before, our main goal is to find a correlation between surface and intracellular CD26 expression. Our objective is also to determine the amount of the enzyme released to the medium during a 3-day incubation following T lymphocyte activation, and assess the necessity of $CD4^+$ isolation step.

We will analyze cell populations, obtained from healthy individuals' blood, previously activated and differentiated into various Th subtypes with cytokines, using flow cytometry. Cells will be analyzed using fluorescent labeling for different markers on their surface or in their interior. The amount of the enzyme released to the medium will be evaluated with ELISA and an enzyme activity test.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 BIOLOGICAL MATERIAL

Biological material	Acquired
Blood of healthy individuals	Seemingly healthy individuals, selected from staff members of the Faculty of Pharmacy, University of Santiago de Compostela
Frozen PBMCs	Cell bank of the Faculty of Pharmacy, Department of Biochemistry and Molecular Biology, University of Santiago de Compostela

3.1.2 MEDIA AND CHEMICALS

Media and chemicals	Company
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Anti-biotin MicroBeads	Miltenyi Biotec
BD Cytofix/Cytoperm	BD Biosciences
BD Perm/Wash 10x	BD Biosciences
CD45RO FITC/ CD26 PE/ CD3 PerCP/ CD4 APC Mouse Monoclonal Antibody to human	Immunostep
Dynabeads, Human T-Activator CD3/CD28	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA) 0.5 M	Sigma Aldrich
Fetal calf serum (FCS)	Invitrogen
Ficoll Paque Plus	Sigma Aldrich
FITC Mouse Anti-Human CD26	BD Biosciences
Gly-Pro-4-Nitroanilide solution (20 µg/mL)	Sigma-Aldrich
Human DPPIV Antibody (Capture antibody)	R&D Systems
Human DPPIV Biotinylated Antibody (Detection antibody)	R&D Systems

Human DPPIV/CD26	R&D Systems
Human Serum Albumin (HSA)	Sigma Aldrich
IL-12	PeproTech
IL-1β	PeproTech
IL-23	PeproTech
IL-4	Miltenyi Biotech
Naive $CD4^+$ T Cell Biotin-Antibody Cocktail II, human	Miltenyi biotec
OPD Peroxidase Substrate	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	Gibco
Paraformaldehyde (PFA)	Sigma Aldrich
Penicillin-Streptomycin-Glutamine (PSG)	Invitrogen
Purified NA/LE Mouse Anti-Human IFNγ, 1.0 mg/mL	BD Biosciences
Purified NA/LE Mouse Anti-Human IL-12, 1.0 mg/mL	BD Biosciences
Purified NA/LE Rat Anti-Human IL-4, 1.0 mg/mL	BD Biosciences
RPMI Medium 1640	Gibco
Sodium azide	Sigma Aldrich
Streptavidin-HRP	Sigma-Aldrich
Tris 1 M	Sigma-Aldrich
Trypan blue	R&D Systems
Tween 20 for ELISA Wash Buffer	Sigma-Aldrich

3.1.3 PREPARED SOLUTIONS

Prepared solution	Component
0.1 M Tris	1 M Tris diluted with Mili-Q water
2 mM EDTA	0.5 M EDTA diluted with Mili-Q water to 2 mM EDTA
Buffer for magnetic separation	5 % BSA in 2 mM EDTA

Fixation solution for cytometry	2 % FCS, 1 % HSA, 1 % BSA, 0.1 % sodium azide in PBS
Paraformaldehyde fixation solution for cytometry	2 % PFA in PBS
R10 Medium for cell cultivation	10 % FCS and 1 % PSG (Penicillin, Streptomycin, Glutamine) in RPMI Medium
Reagent Diluent for ELISA	1 % BSA in PBS
Substrate Solution for ELISA	1 OPD tablet and 1 urea hydrogen peroxide/buffer tablet dissolved in 20 mL of deionized water (Mili-Q)
Wash Buffer for ELISA	0.05 % Tween 20 in PBS

3.2 EQUIPMENT

3.2.1 MACHINES

Name	Type	Company
Balance	CB Junior	COBOS Precisión
Centrifuge	Allegra X-12R centrifuge	Beckman Coulter
Flow Cytometer	FACScalibur	Becton Dickinson
Eppendorf microcentrifuge	Beckman Coulter microfuge 18 Centrifuge	Beckman Coulter
Freezer (-20°C)	/	Zanussi
CO_2 Incubator	Sanyo MCO-20AIC	Sanyo
Liquid nitrogen freezer	Air Liquide ARPEGE40	Air Liquide
Microplate reader	BioRad Model 680	BioRad
Microscope	Nikon Eclipse TS100	Nikon
Precision weight (with calibration weights)	Mettler PM200	Mettler Toledo
Refrigerator	/	Liebherr
Water bath	P Selecta Tectron	Selecta
Water purification system	Sartorius ARIUM611DI	Sartorius

3.2.2 LABORATORY EQUIPMENT

Name	Type	Company
Automatic pipettes	2 µL, 5 µL, 10 µL, 100 µL, 200 µL, 500 µL, 1000 µL	LabMate, Eppendorf
Cell culture microplates	48-, 96-well plates	Falcon
Centrifuge tubes	Conical centrifuge tubes: 10 mL, 15 mL, 50 mL	Falcon
Cold resistant gloves	Flex Shield BOA Actifresh	JUBA
Counting chamber (Neubauer)	Bright-Line	Hausser Scientific
Eppendorf tubes	Polypropylene: 0.5 mL, 1.5 mL	Sarstedt
Graduated cylinders	Graduated glass cylinders 50-1000 mL	Simax
MS column	Column for magnetic separation	Miltenyi Biotec
Multichannel pipette	8 x 200 µL	Eppendorf
Pipette controller (for aspiration pipettes)	IBS Pipetboy acu	Integra Biosciences
Self-standing centrifuge tubes	Self-standing graduated centrifuge tube with a screw cap, 50mL	Falcon
Serological pipettes	5 mL, 10 mL, 25 mL, 50 mL	FisherBrand
Plastic disposable transfer pipette	2 mL	FisherBrand
Tubes for cytometry	Round-bottom polystyrene tubes with a snap cap, 5 mL	Falcon

3.2 METHODS

Cells were obtained from 9 healthy individuals using two different procedures: 1) density gradient centrifugation of peripheral blood obtained from 3 healthy donors and 2) thawing of frozen PBMCs of 6 healthy individuals obtained from a cell bank of the Faculty of pharmacy, USC.

3.2.1 ISOLATION OF PBMCs FROM HUMAN BLOOD BY DENSITY GRADIENT CENTRIFUGATION

PBMCs isolation from human blood was performed for the following experiments: 10/3, 20/3, 24/3.

All the following steps were performed in a laminar flow hood:

1) We transferred 15 mL of blood in one 50 mL tube and diluted it with PBS in a V/V ratio 1:1, so we got a total of 30 mL of diluted blood.
2) Ficoll solution was left at room temperature for about 30 min and then divided in two 50 mL conical tubes, with 7.5 mL in each. 15 mL of diluted blood was carefully layered over each Ficoll solution in a V/V ratio of Ficoll : diluted blood = 1 : 2.
3) We centrifuged blood with Ficoll at 400xg for 30 min at 25 °C with the brake set off.
4) We aspirated the upper layer (plasma) using a transfer pipette, leaving the mononuclear cell layer below undisturbed. Mononuclear cell layers from both conical tubes were carefully transferred to a new, 50 mL tube, using a new transfer pipette.
5) We diluted the PBMCs with PBS to 50 mL and centrifuged again, this time at 300xg for 10 min at 25 °C with the brake on.
6) We completely removed the supernatant and resuspended cell pellet in 10 mL of PBS to count the cells with Neubauer Chamber. This was done by mixing 10 µL of cell suspension with 10 µL of Trypan Blue on each side of the chamber and placing the tip of a pipette at the open end of the chamber, letting the fluid enter by capillary action. Trypan Blue helps us detect dead cells, which appear blue under the microscope. We placed the chamber on a microscope and counted only clear, living cells. Total number of cells in total was calculated by Equation *1*.

Equation 1:

$$\text{N of cells} = \frac{N1+N2}{\text{N of squares x Vsq x dilution}} \text{ x V (mL)}$$

N – number

N1 – counted number of cells in the first square

N2 – counted number of cells in the second square

Vsq – volume of one square (0.1 x 1 x 1 mm^3) = 10^{-4} mL

V – total cell suspension volume (mL)

7) The cell suspension was centrifuged under the same conditions as before and the supernatant was aspirated completely. We continued with magnetic separation of obtained PBMCs.

3.2.2 MAGNETIC SEPARATION

Magnetic separation of PBMCs was performed for the following experiments: 10/3, 20/3, 24/3. Isolation was made by immunomagnetic negative selection using Naïve $CD4^+$ T cell biotin-antibody cocktail II.

3.2.2.1 Magnetic labeling

1) We resuspended the cell pellet in 40 µL of buffer for magnetic separation per 10^7 cells and added 10 µL of Naive $CD4^+$ T cell biotin-antibody cocktail II per 10^7 cells. Naïve $CD4^+$ T cell biotin-antibody cocktail II is composed of biotin-conjugated monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a (Glycophorin A). The cell suspension was mixed well and incubated in a refrigerator (2 – 8 °C) for 10 min.
2) The cells were washed with 1 – 2 mL of buffer for magnetic separation per 10^7 cells and centrifuged for 10 min, at acceleration 300xg and 4 °C.
3) We aspirated the supernatant completely and resuspended the cell pellet in 80 µL of buffer for magnetic separation per 10^7 cells and 20 µL of anti-biotin MicroBeads per 10^7 cells were added. After we mixed the suspension, we put it in the refrigerator for additional 15 min.
4) The cells were washed with 1 – 2 mL of buffer for magnetic separation per 10^7 cells, centrifuged under the same conditions as before and the supernatant was aspirated completely.
5) We resuspended the cell pellet in 500 µL of buffer for magnetic separation for up to 10^8 cells.

3.2.2.2 Magnetic separation with magnetic-activated cell sorting (MACS)

1) We placed the magnetic separation column in the magnetic field of the MACS separator. In order to prepare the column for the magnetic separation, 3 times 500 µL of buffer for magnetic separation had to be rinsed through first.
2) When the entire buffer for magnetic separation went through, cell suspension was applied onto the column and the fraction was collected in a flask. We washed the column with 500 µL of buffer for magnetic separation and repeated it two times. Collected sample represented naïve $CD4^+$ T cells.
3) We counted cells with a Neubauer chamber and centrifuged them for 10 min, at acceleration 300xg and temperature 4 °C and aspirated the supernatant completely. The cells were resuspended in 1 – 4 mL of cultivation medium, depending on the number of the cells (to final cell concentration about 1 – 4 x 10^6 cells/mL).

3.2.3 THAWING PROCEDURE

When a blood donor was not available we used frozen PBMCs, which were thawed by the thawing procedure. Frozen PBMCs were used in the following experiments: 28/4, 8/5, 12/5, 19/5, 2/6, 8/6. The thawing procedure is stressful to frozen cells and thus must be performed quickly. The freezing medium contains a cryoprotectant dimethyl sulfoxide, which is toxic to cells above 4 °C, therefore it is essential that cells are thawed as quickly as possible and diluted in culture medium to minimize the toxic effects (49).

The thawing procedure was performed in the following steps:

1) We collected a vial of frozen PBMCs from liquid nitrogen storage wearing appropriate equipment (laboratory coat, cryogenic gloves, and safety goggles) and slightly loosened the cap of the vial to release any trapped liquid nitrogen out. The vials must be handled with care since they can explode upon warming due to the expansion of trapped liquid nitrogen.
2) We retightened the cap and immediately transferred the vial to a 37 °C water bath, holding it with tweezers, and not letting it immerse completely to prevent contamination. We let the cells thaw in a water bath for approximately 1 – 2 min, until there was only a tiny ice-crystal left.

3) From this point on, aseptic technique was used and all the work was done in a laminar flow hood. When we removed the vial from the water bath, we wiped it with 70 % ethanol and put it in a laminar flow hood, where we slowly added pre-warmed growth medium R10 (1 drop/s), filling the entire vial.
4) The cell suspension was transferred to a sterile conical tube containing 10 – 12 mL of warm R10 medium and centrifuged at 300xg for 10 min at 25 °C.
5) We removed the supernatant completely using a sterile transfer pipette and resuspended the cell pellet in 10 mL of room-temperature PBS. We counted the cells using Neubauer Chamber and centrifuged them under the same conditions as before.
6) After the supernatant was removed completely, the cell pellet was resuspended in R10 medium to about 10^6 cells per 1 mL. Cells were incubated at 37 °C for 3 – 5 h with a slightly opened cap and in a slightly inclined position.
7) After incubation, the cell suspension was centrifuged at 300xg for 10 min at 25°C. We completely removed the supernatant and resuspended cell pellet in 10 mL PBS. We counted the cells with a Neubauer Chamber and centrifuged suspension under the same conditions as before. We resuspended the cells in 1 – 4 mL of R10 medium, depending on the number of the cells (to final cell concentration about 1 – 4 x 10^6 cells/mL).

Further magnetic separation was not made in case of thawed PBMCs, all cells were used for differentiation.

3.2.4 DIFFERENTIATION

After cells were isolated from blood or thawed, different cytokines were added to their cultivation medium in order to differentiate naïve $CD4^+$ cells into specific Th effector cell types. First, the appropriate amount of Dynabeads Human T activator CD3/CD28 was added (6.25 µL per 10^6 cells) to the cell suspensions of all the experiments for the activation and expansion of human T cells. The suspension was divided into 2 – 4 wells of a 48-well plate and the calculated volumes of certain cytokines were added to each well for the differentiation:

- Th0: nothing
- Th1: IL-12 (2 ng/mL), anti-IL-4 (100 ng/mL)

- Th2: IL-4 (25 ng/mL), anti-IL-12 (2 μg/mL)
- Th17: IL-1β (10 ng/mL), IL-23 (10 ng/mL), anti-IL-4 (1 μg/mL), anti-IFNγ (1 μg/mL)

The cells were incubated at optimal growth conditions (37 °C and 5 % CO_2) for a period of 3 days.

For each of the 9 experiments that we performed, cells were under different polarizing conditions: distinct cytokines were added in order to differentiate naïve $CD4^+$ cells into specific Th effector cell types (Table III).

Table III: Differentiation of $CD4^+$ lymphocytes based on in vitro polarizing conditions for each experiment

Date	**10/3**	**20/3**	**24/3**	**28/4**	**8/5**	**12/5**	**19/5**	**2/6**	**8/6**
Polarizing conditions	Th1	Th0, Th1	Th0, Th1	Th0, Th2	Th0, Th2	Th0, Th2	Th0, Th2	Th0, Th17	Th0, Th17

3.2.5 STAINING PROCEDURE

After 3 days of incubation, we transferred the cells to Eppendorf tubes (each cell type to a separate one) and then centrifuged them in a microcentrifuge at 800xg for 10 min. The supernatant was removed and stored in a freezer for later analysis and the pellet was resuspended in 100 μL PBS. Each tube was divided in 4, with 25 μL in each. One was for extracellular staining, one for intracellular staining and a control for each.

3.2.5.1 Extracellular staining

Direct staining was carried out by adding 5 μL of fluorescent-labeled primary antibodies against CD3, CD4, CD45RO, and CD26 (Anti-human CD45RO FITC/CD26 PE/CD3 PerCP/CD4 APC Mouse Monoclonal Antibodies) to the cell suspension and incubated it in a refrigerator for 15 min. The cells were washed with 1 mL of PBS and centrifuged under the same conditions as before. We completely removed the supernatant and resuspended the pellet in 500 μL PBS. We transferred cell suspension to a tube for flow cytometry and put it on ice.
Preparation of control: The same procedure, but no antibodies were added.

3.2.5.2 Intracellular staining

For intracellular staining, 50 µL of Cytofix/Cytoperm was added first for fixation and permeabilization of cells. The suspension was incubated in the dark, for 20 min, at room temperature. We washed the cells with 1 mL of diluted Perm/Wash buffer (Perm/Wash : PBS V/V ratio = 1 : 9), which is a permeabilizing agent, and then centrifuged them in a microcentrifuge at 800xg for 10 min. The supernatant was completely removed and cells were resuspended in 25 µL of Perm/Wash. 5 µL of fluorescent-labeled primary antibodies against CD26 (FITC Mouse Anti-Human CD26 monoclonal antibodies) was added to the suspension, which was then incubated in a refrigerator for 15 min. After the incubation, we washed the cells with 1 mL of Perm/Wash and centrifuged them under the same conditions as before. The supernatant was completely removed and cells were resuspended in 500 µL of Perm/Wash, transferred to a tube for cytometry and put on ice.

Preparation of control: The same procedure, but no antibodies were added.

3.2.6 FIXATION OF CELLS

When it was not possible to pass stained cells through the flow cytometer within an hour, we had to fix them. Cells were fixed for a day in 28/4 assay and for three days in 8/5 assay.

Instead of PBS/Perm/Wash, cells were resuspended in 100 µL of 2 % PFA and incubated in the dark, for 15 min, at room temperature. The cells were washed with 1 mL PBS and centrifuged at 800xg for 10 min. The supernatant was completely removed and cells were resuspended in 500 µL of PBS with 2 % fetal FCS, 1 % HSA, 1 % BSA, and 0.1 % sodium azide and stored in the refrigerator overnight.

3.2.7 FLOW CYTOMETRY ANALYSIS

After the cells were stained with different antibodies, we analyzed them with a tetra-colour flow cytometer, using lymphocyte gate. Expression of different markers was determined by measuring fluorescence of fluorophore-conjugated antibodies. FITC, PE, and PerCP fluorophores were excited by a red laser (488 nm) and APC was excited by a blue laser (640 nm). The emission was measured with four different fluorescence detectors:

- FL-1 (FITC): 533/30

- FL-2 (PE): 585/40
- FL-3 (PerCP): 670
- FL-4 (APC): 675/25

3.2.8 DETERMINATION OF CD26 IN THE CULTIVATION MEDIUM

The presence and activity of DPPIV in a cultivation medium was determined with ELISA and an enzyme activity test.

3.2.8.1 ELISA

Plate preparation

1) We diluted the Capture Antibody (360 µg/mL) in PBS to a concentration 2 µg/mL and coated a 96-well microplate with it, 100 µL per well. We sealed the plate and incubated it overnight at room temperature.
2) We inverted the plate and blotted it against clean towels to remove the liquid from wells and then washed them with 200 µL of Wash Buffer, repeating the process three times, for a total of four washes. We completely removed the liquid from all wells during and after washing by blotting it against clean paper towels.
3) We blocked plates by adding 300 µL of Reagent Diluent to each well and incubated for 1 hour at room temperature.
4) We repeated the wash in step two.

Assay procedure

1) We added 300 µL of sample (diluted in Reagent Diluent in a V/V ratio 1:1) and standards per well. Reagent Diluent was used as a negative control (blank). All of them were carried out in a duplicate. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature.
2) We repeated the wash from step 2 of Plate preparation.
3) We added 100 µL of Detection Antibody, diluted in Reagent Diluent, to each well, covered the plate with an adhesive strip and incubated it for 2 hours at room temperature.

4) We repeated the wash from step 2 of Plate preparation.
5) We added 100 µL of the working dilution of Streptavidin-HRP to each well, covered the plate and incubated it in the dark, for 20 min, at room temperature
6) We repeated the aspiration from step 2 of Plate preparation.
7) We added 100 µL of Substrate solution to each well and incubated the plate in the dark, at room temperature.
8) We determined the optical density after 20, 25, 30 and 40 min with the same microplate reader at 450 nm and with a wavelength correction 570 nm.

Reagent preparation

We warmed all reagents to a room temperature half an hour in advance and prepared all working dilutions right before use.

- **Streptavidin-HRP:** HRP-conjugated streptavidin needs to be diluted with Reagent Diluent in a V/V ratio 1:200, as specified on the vial. We mixed 28 µL of Streptavidin-HRP and 5.6 mL of Reagent Diluent.
- **Human DPPIV Capture Antibody:** The concentration of monoclonal rat anti-human DPPIV antibody, when reconstituted with 1 mL PBS, was 360 µg/mL. We diluted it to a working concentration of 2 µg/mL by mixing 31 µL of the solution with 5.6 mL PBS.
- **Human DPPIV Detection Antibody:** The concentration of biotinylated polyclonal goat anti-human DPPIV antibody, when reconstituted with 1 mL of Reagent Diluent, was 36 µg/mL. We diluted it to a working concentration of 200 ng/mL by mixing 31 µL of the solution with 5.6 mL Reagent Diluent.
- **Human DPPIV Standard:** Standards were prepared from recombinant human DPPIV. Each vial contained 80 ng/mL of recombinant human DPPIV when reconstituted with 0.5 mL of Reagent Diluent. A five-point standard curve using 2-fold serial dilutions in Reagent Diluent was made with a high standard of 2000 pg/mL and a low standard of 125 pg/mL (Figure 8).
- **Substrate solution:** We dissolved 1 OPD tablet and 1 urea hydrogen peroxide/buffer tablet in 20 mL of deionized water, providing a concentration of 0.4 mg/mL for OPD,

0.4 mg/mL for urea hydrogen peroxide, and 0.05 M for phosphate-citrate. The final solution had a pH=5.

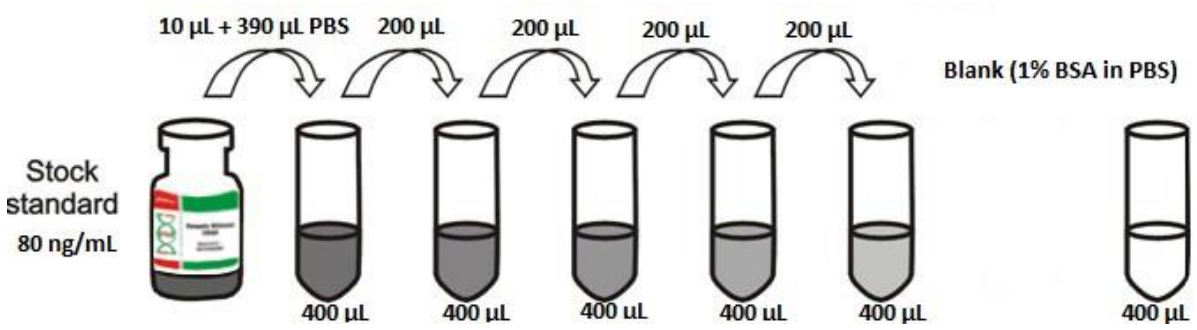

Figure 8: *DPPIV Standard preparation using 2-fold serial dilutions (51)*

3.2.8.2 Enzyme activity

To determine DPPIV activity we performed an enzyme activity test using Gly-Pro-p-Nitroanilide as a substrate, which is cleaved by DPPIV into Gly-Pro and p-Nitroaniline (p-NA), as shown in Figure 9. p-NA absorbs strongly at 405 nm and can be measured spectrophotometrically. The pH for optimal DPPIV activity is 7.4 – 8.7 (50).

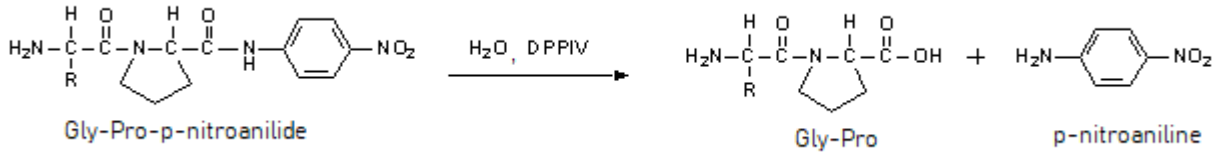

Figure 9: *The reaction between a substrate and DPPIV (50)*

Enzyme activity was performed in a 96-well plate. We put 100 µL of sample in each well and added 100 µL of 0.2 µg/mL substrate solution for assays and 100 µL of 0.1 M Tris for control. For the determination of product quantity, a five-point standard curve using 2-fold serial dilutions of p-NA in 0.1 M Tris was made, with a high standard of 0.8 mM and a low standard of 0.05 mM. The assays, controls, and standards were all carried out in a duplicate.

- **ASSAY:** 100 µL sample + 100 µL 0.2 µg/mL DPPIV substrate (Gly-Pro-p-Nitroanilide)

- **CONTROL:** 100 µL sample + 100 µL 0.1 M Tris

The original concentration of a substrate solution was 20 µg/mL. We diluted it to a concentration of 0.2 µg/mL with 0.1 M Tris, which was prepared by mixing 1 M Tris (pH=8.2) and Mili-Q water in a ratio 1:9. The new solution was still in the optimal pH range.

The plate was incubated at 37 °C protected from light and the absorbances were measured at 405 nm at 30 and 60 min, using BioRad Model 680 microplate reader. The enzyme activity was defined as the amount of product (p-NA) per minute per litre (U/L), cleaved by DPPIV at pH 7.44 and 37 °C.

3.2.9 STATISTICAL ANALYSIS

Studen's t-test was used for establishing significant differences between two sets of data. We used it to determine differences in median fluorescence intensities (MFIs) and percentages of $CD26^+$ cells between control and samples, as well as for comparison of samples between each other.

3.2.9.1 Comparison of analyzed populations with control

For the comparison of samples with control we used:

- *One-tailed t-test:* to compare samples with control, one-tailed t-test was used because we were testing the statistical significance in only one direction.
- *Paired t-test:* we used paired t-test because our measurements were linked.

This test was used to determine if there was a significant difference in intracellular expression of CD26 marker between control and samples by comparing their MFIs.

3.2.9.2 Comparison of different analyzed populations with each other

For the comparison of different analyzed populations between each other we used:

- *Two-tailed t-test:* we used a two-tailed t-test because we wanted to test a significant difference in both directions.

- *Unpaired t-test:* we used unpaired t-test because our measurements were independent of each other.

This test was used to determine whether there is a significant difference in 1) intracellular CD26 expression between different Th subsets by comparing their MFIs, 2) the fraction of $CD26^+$ cells in effector memory $CD4^+$ lymphocyte population between different Th subsets, and 3) expression levels of CD26 on effector memory $CD4^+$ lymphocytes between different Th subsets by comparing their MFIs.

4 RESULTS AND DISCUSSION

4.1 FLOW CYTOMETRY ANALYSIS

After isolation and incubation cells were stained with antibodies against CD3, CD4, CD45RO and CD26 as indicated in Methods, and analyzed by flow cytometry. Two main sets of experiments were performed: 1) PBMCs isolation from peripheral blood followed by magnetic separation of $CD4^+$ T lymphocytes and 2) use of whole PBMCs thawed from liquid nitrogen with no further separation. All lymphocytes were identified by light scatter profiles (FSC/SSC) and further analyzed based on the expression of CD3, CD4, CD45RO, and CD26 markers for superficial expression and CD26 marker for intracellular expression. We analyzed our data with flow cytometry data analysis software: Flowing Software 2, which allows making dot plots, histograms, and overlay histograms by applying different parameters. The flow cytometry analysis for all the experiments is attached in Supplements. In the first three experiments (10/3, 20/3, 24/3) PBMCs were isolated from blood and they were further separated by magnetic separation where only $CD4^+$ T cells were collected. In all other experiments (28/4, 8/5, 12/5, 19/5, 2/6, 8/6) PBMCs were thawed and no further separation was made. Stained cells were fixed with 2 % PFA for a day in 28/4 assay and for three days in 8/5 assay. To determine the extent of CD26 expression on analyzed populations, we focused on two main parameters: fraction of cells expressing the marker and CD26 MFIs.

4.1.1 SURFACE ANALYSIS OF CD26 EXPRESSION ON T LYMPHOCYTES UNDER POLARISING CONDITIONS

Four different antibodies were used for the surface analysis: antibodies against CD3, CD4, CD45RO, and CD26. For the identification of positive population, a negative gate was set for each experiment with a help of control. This allowed us to determine the level of background fluorescence and autofluorescence. The gate was the same for all the experiments for CD3/CD4 population (10/10), but it varied from experiment to experiment for CD45RO/CD26 population. Surface flow cytometry analysis for all the experiments is attached in Supplements.

Our data is shown in a homogenous population that is concentrated around mean and median values. We decided to represent the data with median fluorescence intensity rather than mean fluorescence intensity because it is less affected by outliers or skewed data. FI increases logarithmically, making it more sensitive to outliers and causing the mean to drift in the direction of skew data and becoming less accurate for the generalization of population. The dispersion of the data was represented with minimum and maximum FIs, which were determined from the histograms and are approximate.

Cells were presented in FSC/SSC dot plot, and R-1 region was created based on lymphocyte aggregation. The R-1 population was divided into 4 populations based on CD3/CD4 marker expression: 1) $CD3^{-}CD4^{-}$ population, composed of NK and B cells, 2) $CD3^{+}CD4^{-}$ population, composed of cytotoxic T cells, 3) $CD3^{-}CD4^{+}$ population, composed of accessory cells, which play a role in organizing lymphoid tissue during development and support T cell help for B cells (52), and 4) $CD3^{+}CD4^{+}$ population, composed of helper and regulatory T cells. $CD3^{+}CD4^{+}$ population was gated and further identified with CD26 and CD45RO expression. The gating strategy is represented in Figure 10. The expression of CD26 marker on effector/memory ($CD45RO^{+}$) cells was of our interest. CD26 marker was present on both, $CD45RO^{+}$ and $CD45RO^{-}$ populations, but the surface expression was not higher on $CD45RO^{+}$ population as expected, since based on previous studies, CD26 surface expression is up-regulated on activated lymphocytes compared to resting lymphocytes (19).

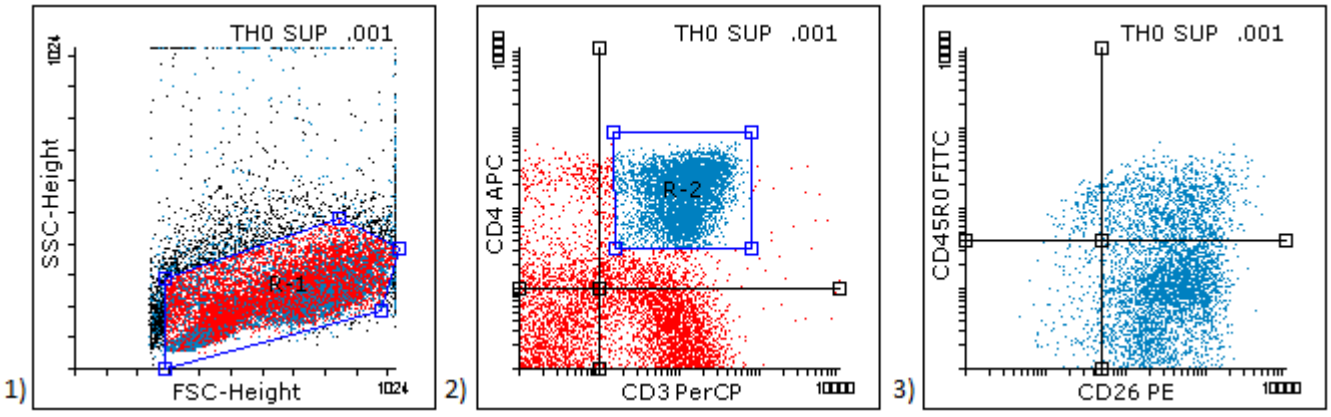

Figure 10: *Flow cytometry gating strategy and analysis of CD26 surface expression by $CD4^{+}$ T lymphocytes under polarizing conditions: From left to right: 1) lymphocytes represented in a FSC/SSC dot plot and R-1*

population gated manually, 2) R-1 population defined by surface CD3 and CD4 expression and $CD3^+CD4^+$ population gated as R-2, 3) R-2 population defined by surface CD26 and CD45RO expression.

We determined the percentage of $CD3^+CD4^+$ cells, $CD45RO^+CD26^+$ cells and CD26 MFI for $CD45RO^+CD26^+$ population for each experiment and calculated average values for every cell subtype. In case of 3 or more repetitions, standard deviation (SD) was calculated by Equation *2*.

Equation 2:

$$SD = \sqrt{\frac{\sum(\mathrm{xi} - \bar{x})}{N - 1}}$$

xi – individual value

$\bar{x}$ – mean value

N – number of all values

4.1.1.1 Comparison of surface CD26 expression between different Th subsets

The analysis of surface CD26 expression on different Th subsets is presented in Tables IV-VII. In a case of Th0 subtype, two average percentages of $CD3^+CD4^+$ cells were calculated due to different isolation protocols. $CD4^+$ cells were used in all the experiments for Th1 analysis, and PBMCs were used in the experiments for Th2 and Th17 analysis.

Table IV: Fractions of $CD3^+CD4^+$ and $CD26^+CD45RO^+$ cells in Th0 population and surface CD26 expression on $CD4^+$ effector/memory population represented by MFIs and minimum/maximum FIs for each experiment

Th0					
Date	**Fraction of $CD3^+CD4^+$ (%)**	**Fraction of $CD26^+CD45RO^+$ (%)**	**CD26 MFI**	**Min. FI**	**Max. FI**
24/3	76.91	31.60	89.77	20	2000
28/4	43.50	49.71	72.34	10	4000
12/5	36.36	34.20	218.70	20	7000
19/5	43.90	52.84	81.31	10	2000
2/6	54.64	31.31	406.79	40	10000

8/6	38.16	25.38	441.09	50	10000
Average	**76.91/43.31***	**37.51**	**218.33**	**25**	**5833.33**
SD	**7.13****	**11.09**	**168.38**	**16.43**	**3710.35**

*average fraction of $CD3^+CD4^+$ cells for $CD4^+$ population/all PBMCs

**SD calculated for the experiments from 28/4 to 8/6

Table V: Fractions of $CD3^+CD4^+$ and $CD26^+CD45RO^+$ cells in Th1 population and surface CD26 expression on $CD4^+$ effector/memory population represented by MFIs and minimum/maximum FIs for each experiment

Th1					
Date	**Fraction of $CD3^+CD4^+$ (%)**	**Fraction of $CD26^+CD45RO^+$ (%)**	**CD26 MFI**	**Min. FI**	**Max. FI**
10/3	84.55	1.90	171.54	20	1500
24/3	75.20	33.80	85.05	20	2000
Average	**79.88**	**17.85**	**128.30**	**20**	**1750**

Table VI: Fractions of $CD3^+CD4^+$ and $CD26^+CD45RO^+$ cells in Th2 population and surface CD26 expression on $CD4^+$ effector/memory population represented by MFIs and minimum/maximum FIs for each experiment

Th2					
Date	**Fraction of $CD3^+CD4^+$ (%)**	**Fraction of $CD26^+CD45RO^+$ (%)**	**CD26 MFI**	**Min. FI**	**Max. FI**
28/4	43.49	48.99	82.05	10	1500
12/5	37.26	43.51	187.69	20	4000
19/5	43.43	49.25	83.54	10	1500
Average	**41.39**	**47.25**	**117.76**	**13.33**	**2333.33**
SD	**3.58**	**3.24**	**60.57**	**5.77**	**1443.38**

Table VII: Fractions of $CD3^+CD4^+$ and $CD26^+CD45RO^+$ cells in Th17 population and surface CD26 expression on $CD4^+$ effector/memory population represented by MFIs and minimum/maximum FIs for each experiment

Th17					
Date	**Fraction of $CD3^+CD4^+$ (%)**	**Fraction of $CD26^+CD45RO^+$ (%)**	**CD26 MFI**	**Min. FI**	**Max. FI**

2/6	53.54	34.28	429.35	40	10000
8/6	37.58	25.63	392.42	40	10000
Average	**45.56**	**29.96**	**410.89**	**40**	**10000**

From tables IV-VII we can see that $CD3^+CD4^+$ cell fractions were the highest in the experiments 10/3-24/3 (between 75 and 85 %) and much lower in experiments 28/4-8/6 (between 37 and 54 %). The reason for that is in different isolation protocols. In the first three experiments $CD4^+$ isolation was performed and in all other experiments all PBMCs were used. The fraction of $CD3^+CD4^+$ cells for PBMCs population was in the expected range, since typically lymphocytes present 70 – 90 % of PBMCs population, 70 – 85 % of which are $CD3^+$ T cells and $CD3^+$ cells are composed of 2/3 $CD4^+$ cells and 1/3 $CD8^+$ cells (34). Because of different isolation protocols, the average $CD3^+CD4^+$ percentages are not representative for each subset. We focused on the fraction of cells expressing CD45RO/CD26, and surface expression of CD26 on $CD45RO^+CD26^+$ population. Subset with the highest fraction of $CD45RO^+CD26^+$ cells in their population was Th2 with an average of 47.25 %, followed by Th0 (37.51 %), Th17 (29.96 %), and Th1 (17.85 %), as presented in Figure 11. The highest level of CD26 membrane expression was identified on Th17 cells, which was in line with previous studies (25), with an average MFI of 410.89. The surface expression on Th1 cells (average MFI=128.30) was lower compared to Th0 population (average MFI=218.33), although previous studies have shown up-regulation of CD26 marker on this subset (20, 53). There was also a down-regulation of the marker on Th2 cells, which showed the lowest CD26 levels (average MFI=117.76), as we predicted based on previous studies (20, 53). Average surface MFIs for each subset are presented in Figure 12.

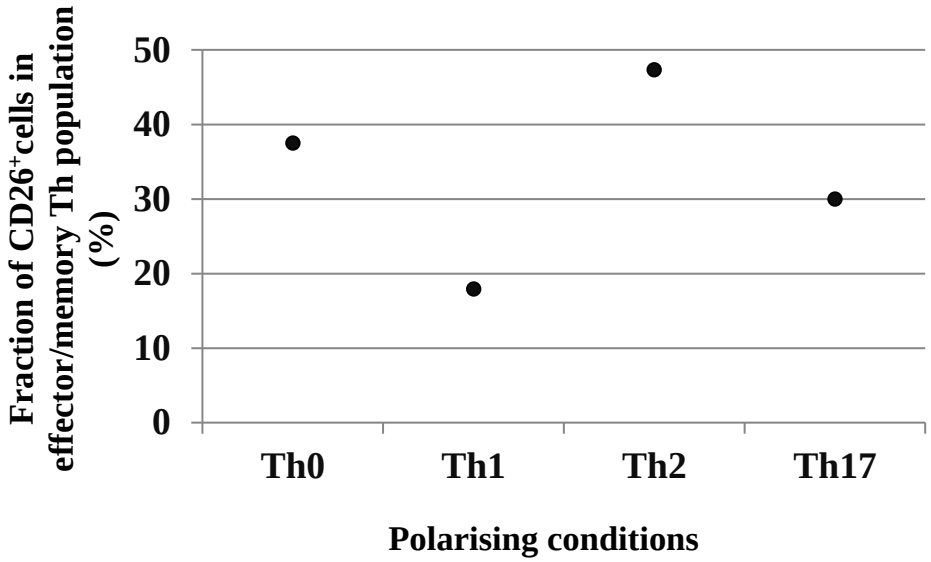

Figure 11: *A fraction of CD45RO$^+$CD26$^+$ cells in CD3$^+$CD4$^+$ population: cells with the highest extent of CD26 expression were Th2 (47.25 %), followed by Th0 (37.51 %), Th17 (29.96 %), and Th1 (17.85 %).*

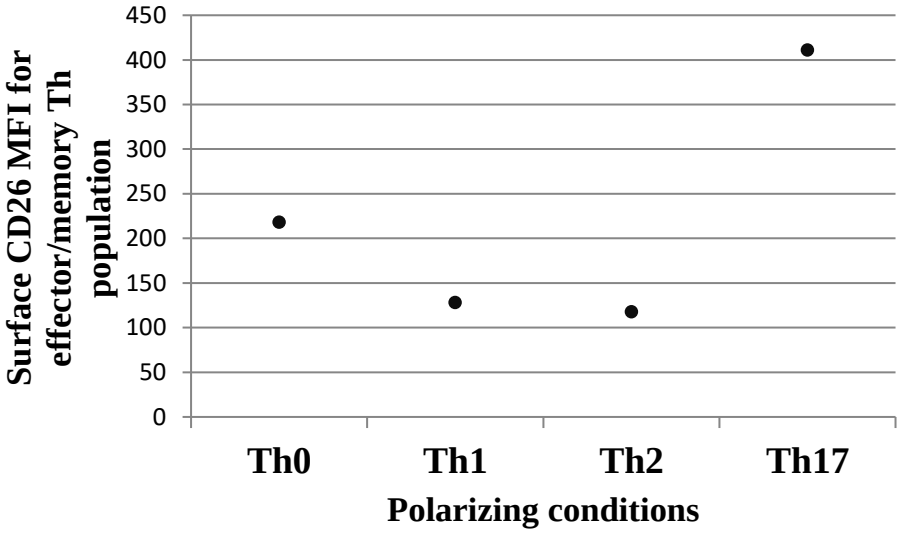

Figure 12: *Average surface CD26 MFIs for CD45RO$^+$CD26$^+$ population of different Th subsets: cells with the highest expression were Th17 (410.89), followed by Th0 (218.33), Th1 (128.30), and Th2 (117.76).*

STATISTICAL ANALYSIS

We used student's t-test to determine whether there is a significant difference in fractions of $CD26^+$ cells and the expression levels of CD26 on effector/memory $CD4^+$ lymphocytes between different Th subsets.

1) $CD45RO^+CD26^+$ fraction comparison between Th subsets

Regarding fractions of effector/memory $CD4^+$ lymphocytes expressing CD26 on their surface, no significant differences were found between Th subsets. The results are presented in Table VIII.

Table VIII: P values comparing fractions of $CD45RO^+CD26^+$ cells between different Th subsets

$CD45RO^+CD26^+$ fraction comparison	P value
Th0 / Th1	0.424
/ Th2	0.091
/ Th17	0.302
Th1 / Th2	0.313
/ Th17	0.584
Th2 / Th17	0.113

2) Comparison of CD26 surface expression between Th subsets

This was done by comparing CD26 MFIs of analyzed populations. There was an up-regulation of the marker on IL-17 producing cells and down-regulation on Th1 and Th2 cells. The lowest CD26 levels were observed on Th2 population. Compared to PBMCs where no cytokines were added (Th0), only Th17 cells showed significantly higher CD26 surface expression. The difference in the expression of the marker was not significant comparing Th1 and Th2 subsets to Th0. Comparing different subsets between each other, there was a significant difference in CD26 expression levels only between the highest expressing subset (Th17) and a subset with the lowest expression (Th2), where $p<0.05$. P values are presented in Table IX.

Table IX: P values comparing CD26 MFIs of $CD45RO^{+}CD26^{+}$ population between different Th subsets

Subset MFI comparison	P value
Th0 / Th1	0.314
/ Th2	0.190
/ Th17	0.038
Th1 / Th2	0.762
/ Th17	0.061
Th2 / Th17	0.001

4.1.2 INTRACELLULAR ANALYSIS OF CD26 EXPRESSION IN T LYMPHOCYTES UNDER POLARIZING CONDITIONS

Intracellular CD26 expression was determined by measuring FIs of samples stained with fluorophore-conjugated antibodies against CD26 and comparing them with control, where no antibodies were added. We determined the fraction of $CD26^{+}$ cells and their MFIs.

In the first, FSC/SSC dot plot, we created a region of lymphocytes based on their aggregation and marked it as R-1. Only cells from the R-1 region were shown in the second dot plot, with CD26 expression on the abscissa and SSC on the ordinate. First we analyzed control samples, where no antibodies were added. We determined a limit for positive CD26 expression with the help of control, as shown in Figure 13. Intracellular flow cytometry analysis for all the experiments is attached in Supplements.

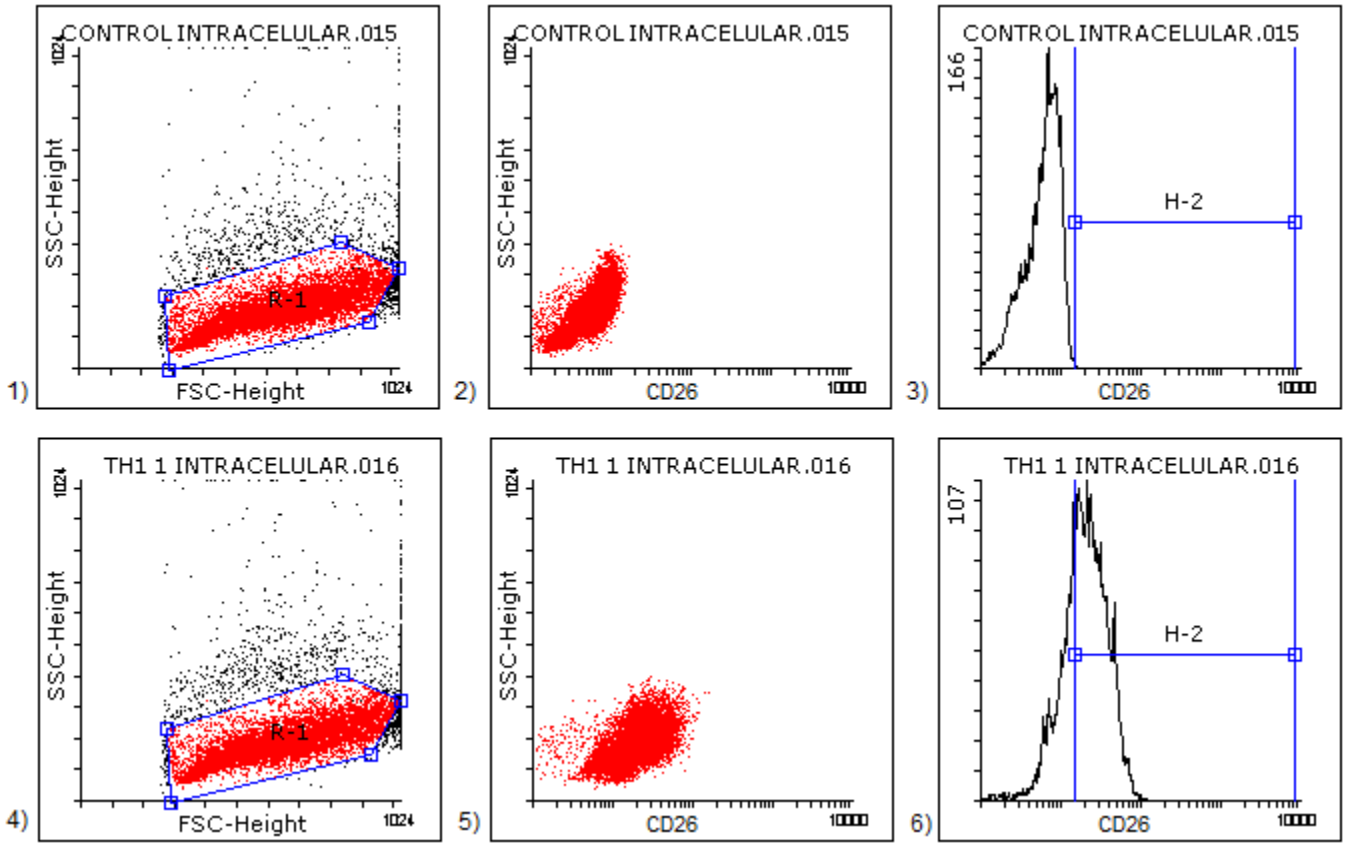

Figure 13: *Cytometry gating strategy and analysis of CD26 intracellular expression for control (1-3) and analyzed population (4-6) by their FSC/SSC profile (1, 4), CD26/SSC profile (2, 5) and intracellular CD26 FI (3, 6).*

Our data is shown in a homogeneous population that is concentrated around mean and median values. Since these two values did not differ a lot in our experiments, we decided to present CD26 expression with only one parameter, that is median fluorescence intensity. We compared CD26 expression in samples containing antibodies and control samples with overlay histograms, as shown in Figure 14. Most of the stained samples showed higher FIs than control, which indicates the presence of CD26 marker.

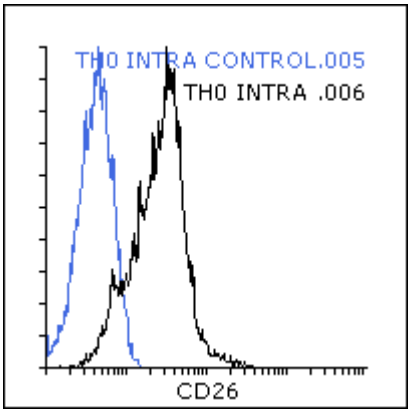

Figure 14: *Overlay histogram comparing Th0 intracellular CD26 FI of control (blue line) and sample (black line).*

4.1.2.1 Comparison of intracellular CD26 expression between control and samples

The difference in intracellular CD26 expression between control and samples was determined by comparing CD26 MFIs of the entire R-1 population (Figure 15). We represented the dispersion of our data with minimum and maximum values, which were determined from the histograms and are approximate. The results are presented in Tables X-XIII.

Table X: Comparison of MFIs and minimum/maximum FIs between control and samples for Th0 subtype experiments

	Th0					
	Control			***Samples***		
Date	**MFI**	**Min. FI**	**Max. FI**	**MFI**	**Min. FI**	**Max. FI**
20/3	6.04	0	30	31.62	1	550
24/3	4.37	0	18	27.14	1	400
28/4	4.37	0	15	3.92	0	18
8/5	5.73	0	70	7.77	0	70
12/5	9.31	0	380	13.46	0	380
2/6	11.14	0	40	17.15	0	70
8/6	3.59	0	15	5.94	0	25
Average	**6.36**	**0**	**81.14**	**14.57**	**0.29**	**216.14**
SD	**2.82**	**0**	**133.22**	**10.70**	**0.49**	**220.09**

Table XI: Comparison of MFIs and minimum/maximum FIs between control and samples for Th1 subtype experiments

Th1						
	Control			***Samples***		
Date	**MFI**	**Min. FI**	**Max. FI**	**MFI**	**Min. FI**	**Max. FI**
10/3	6.49	0	20	20.17	0	120
20/3	5.23	0	20	15.40	0	380
24/3	4.57	0	20	30.51	1	450
Average	**5.43**	**0**	**20**	**22.03**	**0.33**	**316.67**
SD	**0.98**	**0**	**0**	**7.72**	**0.58**	**173.88**

Table XII: Comparison of MFIs and minimum/maximum FIs between control and samples for Th2 subtype experiments

Th2						
	Control			***Samples***		
Date	**MFI**	**Min. FI**	**Max. FI**	**MFI**	**Min. FI**	**Max. FI**
28/4	4.91	0	15	4.18	0	18
8/5	5.38	0	42	7.77	0	60
12/5	8.74	0	380	15.82	0	380
Average	**6.34**	**0**	**145.67**	**9.26**	**0**	**152.67**
SD	**2.09**	**0**	**203.39**	**5.96**	**0**	**197.99**

Table XIII: Comparison of MFIs and minimum/maximum FIs between control and samples for Th17 subtype experiments

Th17						
	Control			***Samples***		
Date	**MFI**	**Min. FI**	**Max. FI**	**MFI**	**Min. FI**	**Max. FI**
2/6	10.94	0	50	17.47	0	70
8/6	3.85	0	18	6.38	0	25
Average	**7.40**	**0**	**34**	**11.93**	**0**	**47.5**

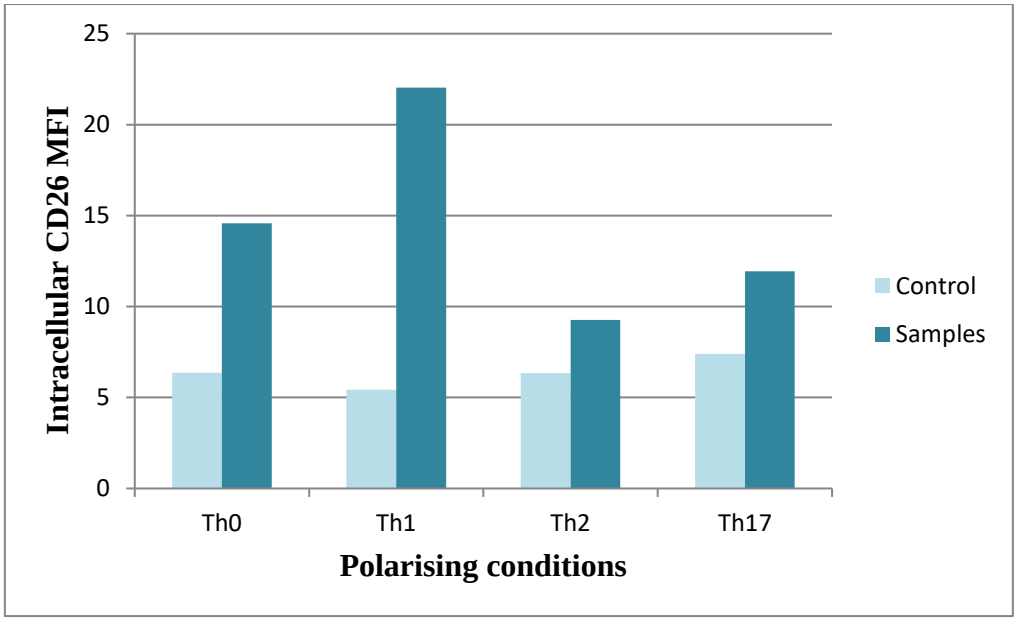

Figure 15: *Intracellular CD26 MFIs of control and samples for entire R-1 population*

If there is a shift to the right present in the peak's maximum of the analyzed population compared to control, it indicates a presence of CD26 marker. In most of the experiments, MFI of analyzed population was higher compared to control. The biggest shift in the peak's maximum was observed in 24/3 Th0 and 24/3 Th1 experiments where the analyzed populations expressed about 10 times more CD26 marker than control. The expression was approximately 7 times higher in 20/3 Th0 experiment and about 4 times higher in 10/3 Th1 experiment. There was a minimum shift in 20/3 Th1, 8/5 Th2, 12/5 Th0, 12/5 Th2, 2/6 Th0, 2/6 Th17, 8/6 Th0, and 8/6 Th17 experiments, where the expression of CD26 marker on analyzed population was higher for a factor 2 or less compared to control. No shift was present in the 28/4 Th0, 28/4 Th2, and 8/5 Th0 experiments, which indicates the absence of intracellular marker in these populations. The dispersion of the data was generally higher in analyzed populations compared to control. The shift was greater in the experiments where $CD4^+$ cells were isolated (10/3, 20/3, 24/3) compared to other experiments. We noticed that in the experiments where cells were fixed for a period of 1 – 3 days before flow cytometry analysis, the control and analyzed populations were overlapping (28/4 Th0, 28/4 Th2, 8/5 Th0) or there was a minimum shift present in the analyzed population (8/5 Th2), as shown in Figure 16. This suggests that the brightness of fluorophores was reduced during the incubation. But

the possibility that cells did not express any marker in their interior in these experiments cannot be excluded.

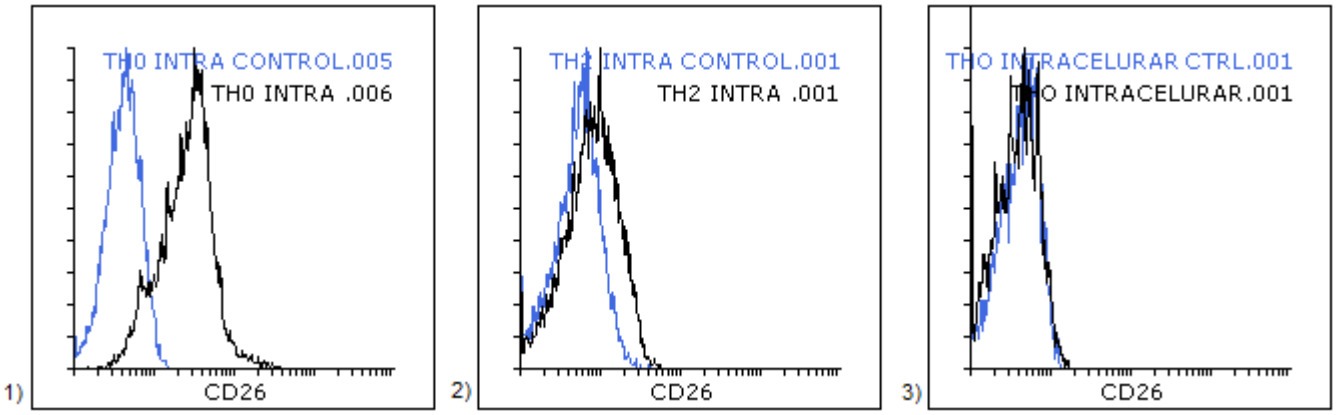

Figure 16: *Overlay histograms comparing CD26 FIs of control and samples: 1) a visible shift of the analyzed population (24/3 Th0 experiment); 2) a minimum shift of the analyzed population (8/5 Th2 experiment); 3) no shift present in the analyzed population (28/4 Th0 experiment).*

We noticed that the expression varied a lot depending on the date of the experiment. Cells from the same patients showed very similar results in the histogram shape as well as in the shift of the analyzed population compared to control (Figure 17), independent of their subtype. Since we used the same isolation protocol, cell stimulation, and flow cytometry analysis for all the experiments, the results indicate intraindividual differences between the donors.

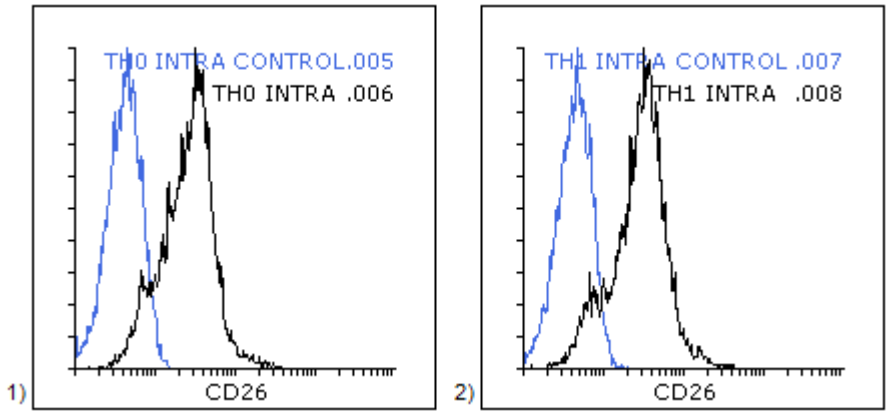

Figure 17: *Overlay histograms comparing CD26 FI of control and analyzed population for Th0 (1) and Th1 (2) subtype of the same experiment (20/3).*

STATISTICAL ANALYSIS

We used student's t-test to determine whether there is a significant difference in intracellular CD26 expression between control and samples for different subsets. This was done by comparing CD26 MFIs of control populations, where no antibodies against CD26 were added, and samples, stained with labeled anti-CD26 antibodies. The expression of the marker was significantly higher for Th0 and Th1 analyzed populations compared to control ($p<0.05$), but the difference was not significant between these two populations for Th2 and Th17 subsets ($p<0.05$). Calculated P values are presented in Table XIV.

Table XIV: P values comparing intracellular CD26 MFIs between control and samples for different Th subsets

	Th0	Th1	Th2	Th17
P value	0.034	0.037	0.164	0.132

4.1.2.2 Comparison of intracellular CD26 expression between different Th subsets

The extent of CD26 expression for different Th subtypes was determined with fractions of $CD26^+$ cells and its intracellular density with MFIs of $CD26^+$ populations. Average values for both parameters were calculated for each Th subset. The results are presented in Tables XV-XVIII. Because of different isolation protocols, fractions of $CD26^+$ cells were not comparable between the experiments and consequently the subsets. In case of Th0 subtype, two average percentages of $CD26^+$ cells were calculated due to different isolation protocols. $CD4^+$ cells were used in all experiments for Th1 analysis, and PBMCs were used in Th2 and Th17 experiments.

Table XV: Fractions of $CD26^+$ cells and intracellular CD26 MFIs of $CD26^+$ population for Th0 subtype experiments

Th0		
Date	**Fraction of $CD26^+$ cells (%)**	**MFI for $CD26^+$ cells**
20/3	62.05	49.58

24/3	71.45	34.60
28/4	0.57	16.11
8/5	5.23	33.38
12/5	4.92	39.60
2/6	7.73	35.55
8/6	8.52	11.76
Average	**66.75/5.39***	**31.51**
SD	**3.11****	**13.22**

*average % of $CD26^+$ cells for $CD4^+$ population/all PBMCs

**SD calculated for the experiments from 28/4 to 8/6

Table XVI: Fractions of $CD26^+$ cells and intracellular CD26 MFIs of $CD26^+$ population for Th1 subtype experiments

Th1		
Date	**Fraction of $CD26^+$ cells (%)**	**MFI for $CD26^+$ cells**
10/3	69.34	25.95
20/3	41.14	42.94
24/3	72.82	37.86
Average	**61.10**	**35.58**
SD	**17.37**	**8.72**

Table XVII: Fractions of $CD26^+$ cells and intracellular CD26 MFIs of $CD26^+$ population for Th2 subtype experiments

Th2		
Date	**Fraction of $CD26^+$ cells (%)**	**MFI for $CD26^+$ cells**
28/4	0.18	19.28
8/5	5.93	26.66
12/5	7.63	37.86
Average	**4.58**	**27.93**
SD	**3.90**	**9.36**

Table XVIII: Fractions of $CD26^+$ cells and intracellular CD26 MFIs of $CD26^+$ population for Th17 subtype experiments

Th17		
Date	**Fraction of $CD26^+$ cells (%)**	**MFI for $CD26^+$ cells**
2/6	7.70	35.55
8/6	12.44	11.97
Average	**10.07**	**23.76**

If we compare different subsets between each other we see that the subtype with the highest intracellular expression was Th1 (MFI=35.58), followed by Th0 (MFI=31.51) and Th2 (MFI=27.93). Cells with the lowest CD26 expression were Th17 (MFI=23.76). Average intracellular CD26 expressions for different subsets are presented in Figure 18. Because of different isolation procedures between the experiments, we could not compare fractions of cells expressing CD26 marker for different subsets. But we can say that the majority of $CD4^+$ cells were expressing CD26 marker in their interior in the experiments where the isolation was performed (10/3, 20/3, 24/3), with an average of 63.36 %. The percentages are much lower in the rest of the experiments, where the average fraction of $CD26^+$ cells in PBMCs population was 6.09 %.

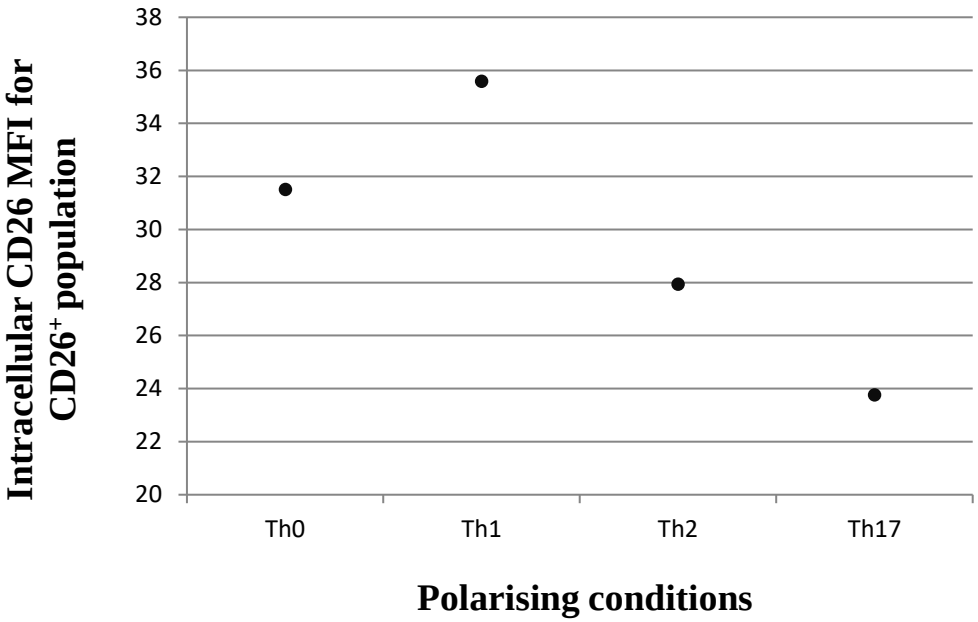

Figure 18: *Average intracellular CD26 MFIs for CD26$^+$ population on different Th subsets: cells with the highest expression were Th1 (35.58), followed by Th0 (31.51), Th2 (27.93), and Th17 (23.76).*

STATISTICAL ANALYSIS

To determine whether there is a significant difference in intracellular CD26 expression between different Th subsets, we compared CD26 MFIs of CD26$^+$ lymphocytes between each other. The results are presented in Table XIX. Student's t-test showed no significant difference in the intracellular expression of the marker between Th subsets.

Table XIX: P values comparing intracellular CD26 MFIs of CD26$^+$ population between different Th subsets

Comparison (CD26 MFI)	P value
Th0 / Th1	0.251
/ Th2	0.323
/ Th17	0.728
Th1 / Th2	0.090
/ Th17	0.279
Th2 / Th17	0.726

4.1.3 THE CORRELATION BETWEEN SURFACE AND INTRACELLULAR CD26 EXPRESSION

Since the expression of CD26 marker on the surface of Th subtypes has been studied before, our aim was to find a correlation between a surface and intracellular expression of the marker. Superficial and intracellular CD26 MFIs for different Th subsets going from highest to lowest, obtained in our experiments, are presented below:

Superficial: Th17 (553.73) > Th0 (330.20) > Th1 (193.88) > Th2 (181.66)
Intracellular: Th1 (30.58) > Th0 (20.53) > Th2 (14.43) > Th17 (12.48)

We were expecting the subtype with the highest membrane expression to have the lowest intracellular expression, since membrane-bound proteins are synthesized in the interior of the cell and then transferred to the membrane. Th17 subtype showed the highest surface expression and the lowest intracellular expression, but other subtypes did not follow the pattern. The reason for that might be in the process of transferring CD26 to the surface. Our study was cross-sectional and even though the maximum expression of CD26 marker on T cells reaches a maximum 3 days after their activation, as stated in previous studies (23), it is possible that the process has not been finished yet and was still ongoing in some cells. We can see that the intracellular CD26 MFIs did not differ a lot and that differences between Th subsets were not significant. Because of low number of individuals involved in this study and the inraindividual differences' influence, our results might not accurately capture the variation in the broader population and cannot be generalized.

4.1.4 THE INFLUENCE OF DIFFERENT ISOLATION PROCEDURES ON THE RESULTS

In some of our experiments we isolated naïve $CD4^+$ T cells using magnetic separation and in others, all PBMCs were used. The purpose of different isolation protocols was to observe the influence of the isolation step on the results and to determine whether the isolation step is necessary.

In Figure 19 we can see that in case of PBMCs without further separation there were all 4 populations present: $CD3^+CD4^+$, $CD3^+CD4^-$, $CD3^-CD4^+$, and $CD3^-CD4^-$. Isolated $CD4^+$ cells only had two main populations on the other hand: $CD3^+CD4^+$ and $CD3^-CD4^-$, while the other two disappeared. Since $CD4^+$ isolation was performed by negative selection, there is a possibility of $CD4^-$ population present. For surface analysis we stained cells with CD3, CD4, CD45RO, CD26 antibodies, so we automatically eliminated the rest of the cells when choosing only those of our interest ($CD3^+CD4^+$ cells; R-2 region) for further analysis, meaning that CD4 T cell isolation did not influence our analysis that much. But for intracellular analysis where we stained the cells for CD26 marker only, the isolation step was much more important.

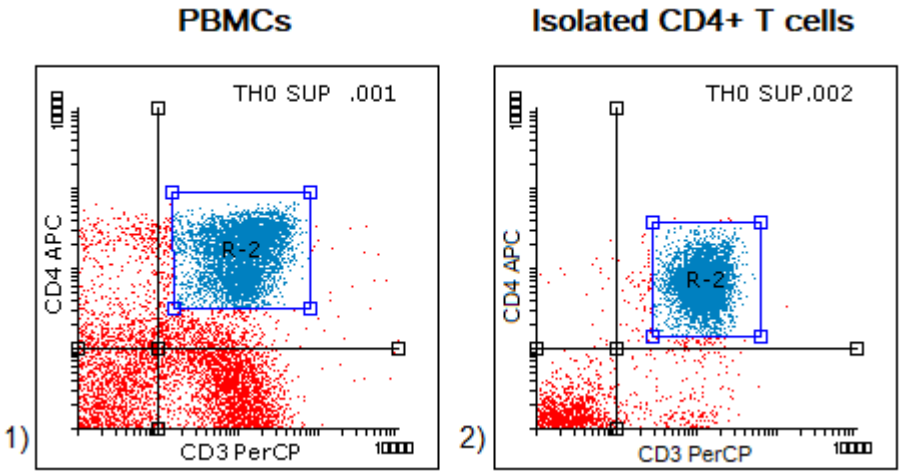

Figure 19: *The comparison of CD3/CD4 dot plots of surface analysis in a case 1) when no separation was performed and all PBMCs were used, and 2) when a magnetic separation of naïve CD4+ T cells was performed*

For intracellular analysis, cells were labeled only with fluorescent antibodies against CD26. In Figure 20 we can see that there is much bigger shift present in a population where $CD4^+$ T cells were isolated compared to the population where all PBMCs were used. We observed significantly higher shifts in the analyzed population compared to control where $CD4^+$ T cell separation was performed. Therefore, it is much easier to analyze data and determine the extent of CD26 expression in case of intracellular analysis if $CD4^+$ T cells are isolated. Due to different isolation protocols it was impossible to compare the percentages of cells expressing the marker, so consistency is important.

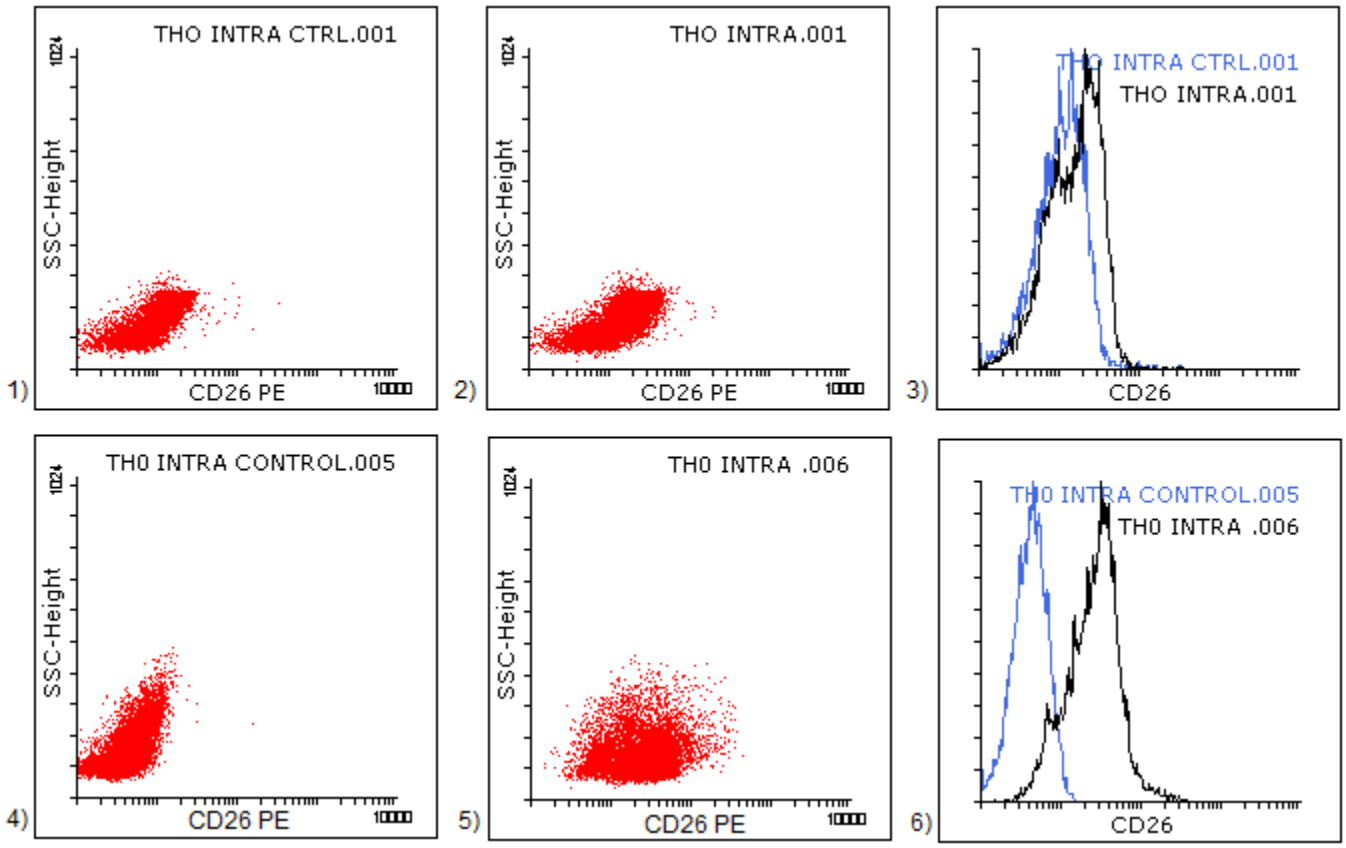

Figure 20: *The comparison of intracellular CD26 expression on PBMCs (1-3) and isolated CD4$^+$ T cell population (4-6); 1) dot plot representing CD26 expression for control, 2) dot plot representing CD26 expression for sample, 3) overlay histogram comparing intracellular CD26 FI of control (blue line) and sample (black line), 4) dot plot representing CD26 expression for control, 5) dot plot representing CD26 expression for sample, 6) overlay histogram comparing intracellular CD26 fluorescence intensity of control (blue line) and sample (black line).*

To summarize, when we were performing surface analysis of CD26 marker, the isolation step was not necessary since we labeled the cells with antibodies against different markers in order to obtain target population. For intracellular analysis on the other hand, isolation step was of great importance, because we did not use antibodies against other markers and the comparison had to be made on total lymphocyte population. There is a much smaller part of Th cells present in that case and the shift is not as visible as on a purified CD4$^+$ T cell population.

4.2 THE PRESENCE OF CD26 IN THE CULTIVATION MEDIUM

We determined the presence and activity of CD26 in a medium after 3-day cultivation with ELISA test and enzymatic activity. We wanted to see if the enzyme is released to the medium and in what quantities.

4.2.1 ELISA

Sandwich ELISA was performed according to the manufacturer's protocol (DuoSet ELISA, R&D Systems). The signal was measured after 20, 25, 30 and 40 min. The absorbances were increasing with time and were the highest after 30 min of incubation. A total of 20 samples were collected and a concentration of DPPIV in each was determined after 30 min of incubation.

We determined the unknown concentration of DPPIV in our samples with the help of a calibration curve, shown in Figure 21. Absorbances of 5 different known concentrations (standards) were measured in duplicates and an equation was established using linear regression. Standards' average absorbances are presented in Table XXI. We noticed that values varied a lot between the duplicates of standard. The absorbances in the first column were significantly higher compared to their duplicates in the second column ($p<0.05$), as presented in Table XX. There was a significant difference between the duplicates only comparing first two columns with each other. We believe the reason for that could be an error in pipetting with a multichannel pipette, and consequently lower volumes and higher concentrations in the first column. Due to small volumes, any deviations in the medium volumes across the plate are significant. Because of that we decided to discard the first column of the plate. With only one repetition we could not calculate SD and consequently a limit of detection (LOD) and a limit of quantification (LOQ), so we determined them from the graph (Figure 21):

LOD = 125 pg/mL

LOQ = 250 pg/mL

We decided to use the lowest measured concentration (125 pg/mL), which showed a slightly higher signal than blank, as LOD, and second lowest concentration (250 pg/mL) as LOQ. A blank correction was made for all the absorbancies, with a blank value of 0.159.

Table XX: Absorbances measured after 30 min of incubation without a blank correction: first 5 rows in columns 1 and 2 representing standards in duplicates going from highest to lowest concentration; first two columns in a 6th row representing blank; and samples representing the rest of the plate

	1	*2*	*3*	*4*	*5*	*6*	*7*
1	0.344	0.293	0.167	0.116	0.179	0.132	0.152
2	0.294	0.263	0.177	0.146	0.173	0.129	0.157
3	0.191	0.192	0.217	0.213	0.118	0.110	0.130
4	0.191	0.184	0.275	0.278	0.118	0.117	0.151
5	0.191	0.164	0.154	0.173	0.094	0.114	0.113
6	0.222	0.159	0.179	0.172	0.103	0.097	0.005
7	0.253	0.128	0.119	0.088	0.114	0.090	0.119
8	0.286	0.180	0.136	0.112	0.167	0.101	0.145

Table XXI: Absorbances of standards measured after 30 min of incubation without (A) and with a blank correction (A - A_{blank})

Standard concentration [pg/mL]	**A**	**A - A_{blank}**
125	0.164	0.005
250	0.184	0.025
500	0.192	0.033
1000	0.263	0.104
2000	0.293	0.134

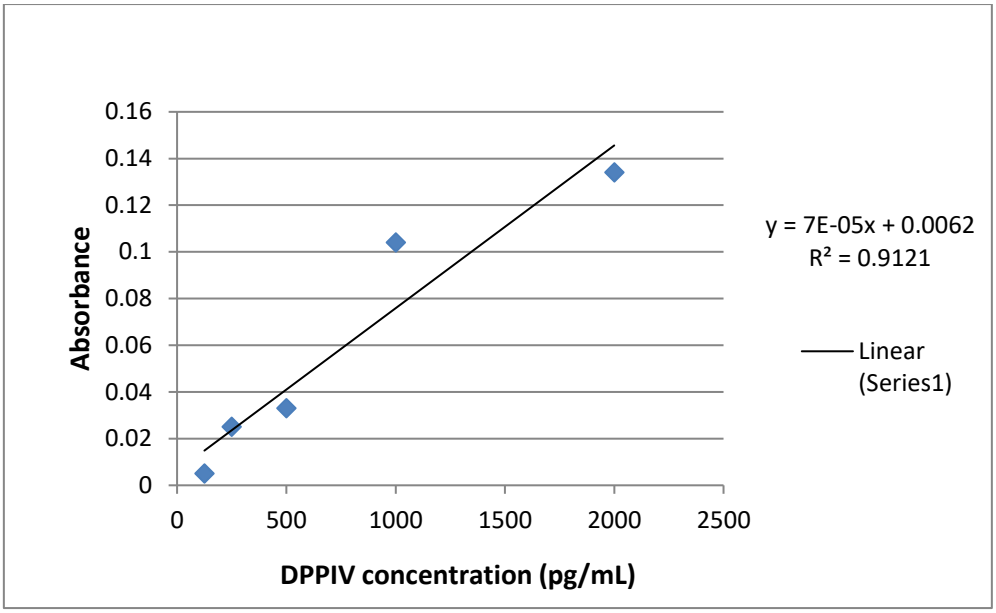

Figure 21: *Calibration curve of standard solution*

Absorbences of five different known concentrations (standards) were measured and Equation *3* was established using linear regression.

Equation 3:

$$y = 7 * 10^{-5}\mathrm{x} + 0.006$$

We calculated average values of duplicate measurements and transformed them to DPPIV concentrations using the equation. The calculated values are presented in Table XXII. A blank correction was made for all the absorbances (blank was substracted from all the measurements). LOD of the method was 125 pg/mL and LOQ was 250 pg/mL.

Table XXII: Sample absorbances with a blank correction transformed into product concentrations

Experiment	Absorbance – blank	Average absorbance	Product concentration [pg/mL]
10/3 Th1	-0.031	-0.031	[1]UDL
10/3 Th1	0.021	0.021	214 ([2]UQL)
20/3 Th0	0.008	-0.0175	[1]UDL
	-0.043		

20/3 Th1	0.018	0.0025	[1]UDL
	-0.013		
24/3 Th0	0.058	0.056	714
	0.054		
24/3 Th1	0.116	0.1175	1593
	0.119		
28/4 Th0	-0.04	-0.0555	[1]UDL
	-0.071		
28/4 Th2	-0.023	-0.035	[1]UDL
	-0.047		
8/5 Th0	0.02	-0.0035	[1]UDL
	-0.027		
8/5 Th2	0.014	-0.008	[1]UDL
	-0.03		
12/5 Th0	-0.041	-0.045	[1]UDL
	-0.049		
12/5 Th2	-0.041	-0.0415	[1]UDL
	-0.042		
19/5 Th0	-0.065	-0.055	[1]UDL
	-0.045		
19/5 Th2	-0.056	-0.059	[1]UDL
	-0.062		
2/6 Th0	-0.045	-0.057	[1]UDL
	-0.069		
2/6 Th0	0.008	-0.025	[1]UDL
	-0.058		
2/6 Th17	-0.007	-0.0045	[1]UDL
	-0.002		
2/6 Th17	-0.029	-0.0185	[1]UDL
	-0.008		
8/6 Th0	-0.046	-0.1	[1]UDL
	-0.154		
8/6 Th17	-0.04	-0.027	[1]UDL
	-0.014		

[1]UDL – under detection limit

[2]UQL – under quantification limit

The measured absorbances were negative in 28 out of 38 samples, meaning that the measured signal was lower compared to the blank. The presence of DPPIV in most of our samples was not high enough to be detected. The concentration of the product was under detection limit in

17 out of 20 samples and under quantification limit in 1 out of 20 samples. Only two samples were above quantification limit and their concentrations were determined. Before performing ELISA, our samples were diluted in a V/V ratio 1:1, so the final concentrations had to be multiplied by two. DPPIV concentration in Th0 cultivation medium for 24/3 was 1428 pg/mL and for Th1 cells of the same date was 3186 pg/mL.

We could achieve a more accurate evaluation of variation within the assays by more repetitions of the blank measurements and by repeating standard measurements, since we had to eliminate one duplicate due to mistake. This would provide enough data for SD calculation and would lead to more accurate LOD and LOQ values.

4.2.2 ENZYME ACTIVITY

Absorbances of five standards with known p-NA quantity were measured and an equation was established using linear regression (Figure 22). We calculated average absorbances of duplicate measurements and transformed them to product (p-nitroaniline) quantities using standard curve equation, which is presented with Equation *4*. A blank correction was made for all the absorbances.

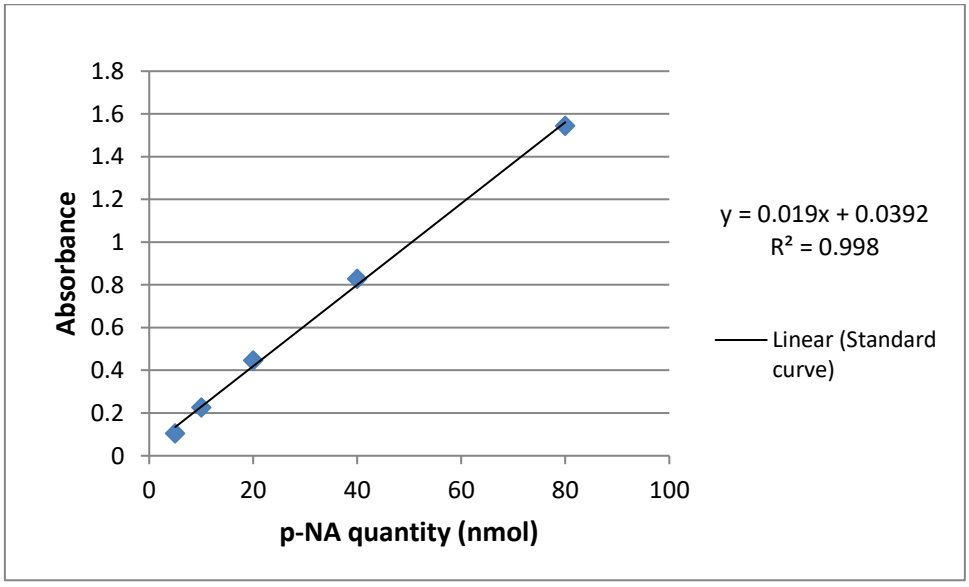

Figure 22: *Calibration curve of p-NA*

Equation 4:

$$y = 0.019x + 0.039$$

LOD and LOQ are normally calculated using standard deviation of low concentrations of the standard. Since we only had two repetitions of each concentration, we used a difference in absorbances of the lowest measured p-NA quantity (5 nmol) instead of SD:

A_1 (5 nmol) = 0.149

A_2 (5nmol) = 0.155

A_2 - A_1 = 0.006

LOD was calculated by Equation *5* and LOQ by Equation *6*.

Equation 5:

$$LOD = \frac{3 \text{ x } \delta}{S} = \frac{3 \text{ x } 0.006}{0.019} = 0.95\ nmol$$

δ – standard deviation or in our case the difference in absorbances

S – slope of the calibration line

Equation 6:

$$LOQ = \frac{10 \text{ x } \delta}{S} = \frac{10 \text{ x } 0.006}{0.019} = 3.2\ nmol$$

A total of 20 samples were collected and their enzyme activity was determined. The results of the enzyme activity after 30 min of incubation are presented in Table XXIII.

Table XXIII: Product quantities, enzyme activities and enzyme activities given per L and number of cells calculated for different samples

Assay	Product quantity [nmol]	Enzyme activity [µU]	N of cells*10^9/L	mU/L/10^9 cel.
10/3 Th1	[1]UDL	/	3	/
	[1]UDL	/	3	/
20/3 Th0	[2]UQL	/	4.2	/

20/3 Th1	[2]UQL	/	4.2	/
24/3 Th0	[1]UDL	/	6.65	/
24/3 Th1	[2]UQL	/	6.65	/
28/4 Th0	[1]UDL	/	2.925	/
28/4 Th2	[1]UDL	/	2.925	/
8/5 Th0	[1]UDL	/	1.55	/
8/5 Th2	[1]UDL	/	1.55	/
12/5 Th0	[1]UDL	/	2.5	/
12/5 Th2	[1]UDL	/	2.5	/
19/5 Th0	[2]UQL	/	1.575	/
19/5 Th2	[1]UDL	/	1.575	/
2/6 Th0	[2]UQL	/	1.8	/
	4.98	166.08	1.8	923
2/6 Th17	[1]UDL	/	1.8	/
	[2]UQL	/	1.8	/
8/6 Th0	[2]UQL	/	2.025	/
8/6 Th17	[2]UQL	/	2.025	/

[1] – Under detection limit

[2] – Under quantification limit

The enzyme activity is represented with enzyme unit (U), which is defined as the amount of the enzyme that catalyzes the transformation of 1 μmol of substrate per minute.

The presence of DPPIV or its enzyme activity in most of our samples was not high enough to produce a signal which could be reliably detected. The quantity of a product was under detection limit in 11 out of 20 of our samples and under quantification limit in 8 out of 20 of our samples. Only one sample was above quantification limit and we determined its enzyme activity using Equation *7*.

Equation 7:

$$EA = \left(\frac{(\text{A medium sample} - \text{A medium blank}) - \ 0.039}{0.019} \right) / 30$$

4.2.3 THE RELEASE OF DPPIV INTO THE MEDIUM

The presence of DPPIV in cultivation medium was not detectable in most of the samples, which means that the cells were not releasing the enzyme or were releasing it very low quantities. It is evident from our results that at the time of maximum surface expression of CD26, its leakage to the medium was still negligible. The expression of DPPIV on the surface of resting T lymphocytes is low (20) and reaches a maximum 3 days after their activation (23), which suggests that the enzyme is not released to the medium during that period. Since our cells were incubated for a period of 3 days, it is possible that the release of DPPIV was not happening yet, at least not in high quantities.

5 CONCLUSION

The results in this study showed up-regulation of CD26 surface expression on effector/memory Th17 lymphocytes and down-regulation on Th1 and Th2 lymphocytes, with the lowest expression on Th2 subtype and a significant difference between Th17 and Th2 subsets, although previous studies have shown up-regulation of the marker on Th1 population (20, 53). Subtype with the highest intracellular CD26 expression was Th1, followed by Th0, Th2, and Th17, with no significant differences between the subsets. Three days after the activation of T cells, the differences in intracellular expression of CD26 were not significant between studied Th subsets and did not show any correlation with surface expression of the marker. The obtained results disagree with our hypothesis, according to which the subset with the highest CD26 expression should have the lowest intracellular expression and vice versa. Nevertheless we solved some methodological questions, like the importance of $CD4^+$ isolation step. Contrary to the surface analysis, where the isolation step did not influence the results, $CD4^+$ isolation step was of great importance for intracellular analysis. Regarding the secretion of the enzyme to the medium, it was evident that at the time of maximum surface expression of CD26, its leakage to the medium was still negligible, since it was not detected in most of our samples neither by ELISA nor enzyme activity assay.

Experimental work for this master thesis was part of comprehensive research project and because of limited time of our research we did not manage to find a correlation between surface and intracellular expression of CD26 marker on different Th subsets. As it is evident from our results, the differences in intracellular CD26 expression between Th subsets 3 days after T cell activation were not significant, and the analysis of CD26 expression was indicating intraindividual differences within the donors. Because of that, our results might not accurately capture the variation in the broader population and cannot be generalized. Taking all of that into consideration, we believe that increasing the number of individuals participating in the study would greatly contribute to more representative results and thus the achievement of our objective. It is also possible that the dynamic of synthesis and expression of DPPIV is different than we predicted.

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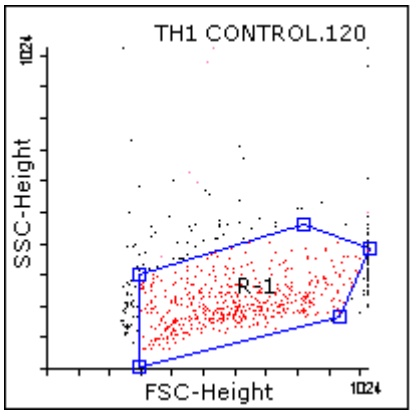
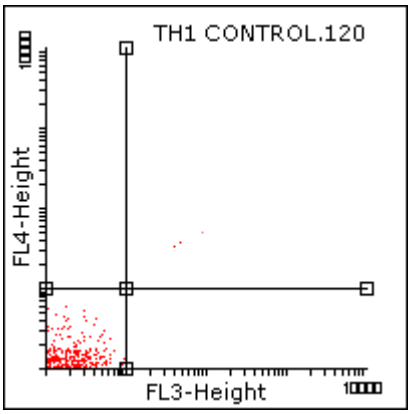
7 SUPPLEMENTS

7.1 SURFACE EXPRESSION ANALYSIS

10/03

- *Th1 control*

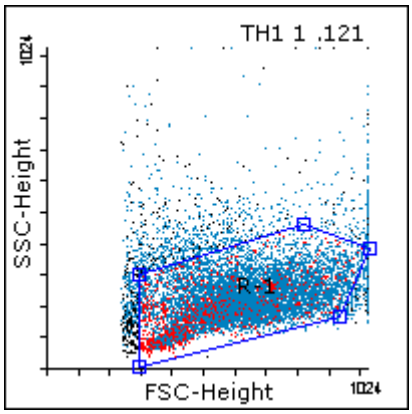


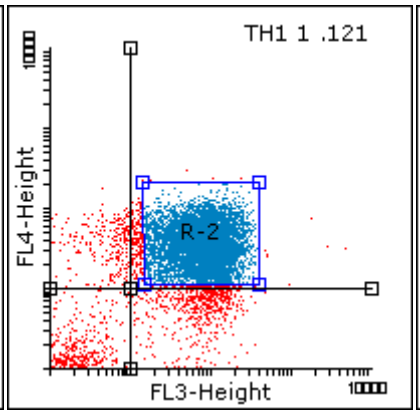


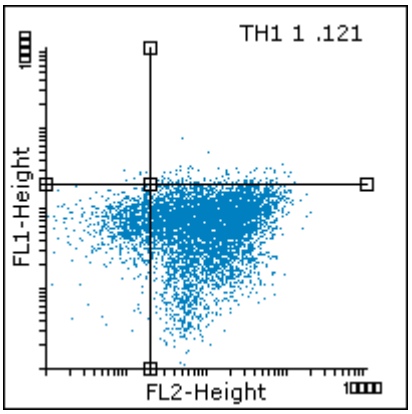
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	645	100.00	601.24	248.53	558.21	224.94	578.00	220.00
R-1	496	76.90	604.36	214.86	575.99	202.67	592.00	208.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	496	100.00	2.42	1.75	1.78	1.43	1.54	1.29
Left Bottom	492	99.19	2.06	1.52	1.73	1.40	1.54	1.29
Right Bottom	1	0.20	10.00	1.00	10.00	1.00	0.00	0.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	3	0.60	58.27	39.94	55.07	39.48	48.26	36.85

- *Th1*







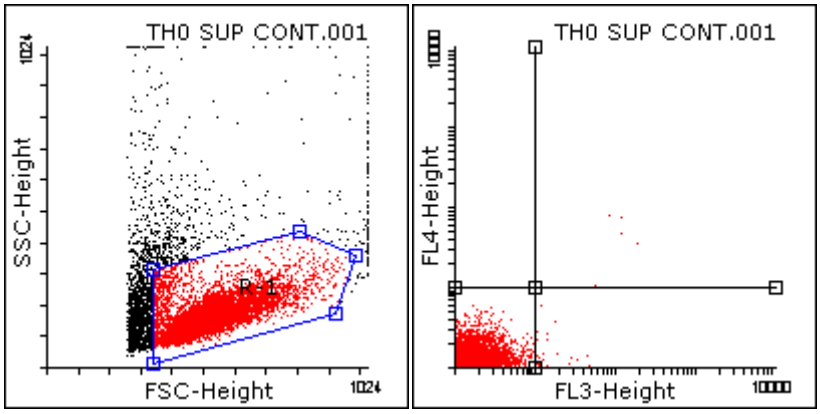
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	616.44	244.58	581.65	223.28	599.00	227.00
R-1	8,218	82.18	610.48	218.97	586.53	205.47	601.00	215.00
R-1 AND R-2	6,634	66.34	629.00	226.47	607.12	214.73	621.00	222.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,218	100.00	84.82	37.25	54.77	26.51	72.99	33.38
R-2	6,634	80.73	93.29	42.12	76.68	36.51	80.58	37.52
R-1 AND R-2	6,634	80.73	93.29	42.12	76.68	36.51	80.58	37.52
Left Bottom	470	5.72	2.59	1.98	2.12	1.65	1.96	1.37
Right Bottom	547	6.66	89.68	6.16	71.06	5.34	79.86	6.55
Left Top	246	2.99	5.24	44.09	4.20	37.07	5.57	39.60
Right Top	6,948	84.55	92.79	41.86	73.18	35.91	78.44	37.18

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	6,634	100.00	145.46	73.71	82.35	56.22	86.60	66.71
R-1 AND R-2	6,634	100.00	145.46	73.71	82.35	56.22	86.60	66.71
Left Bottom	773	11.65	11.32	70.21	9.76	60.89	11.65	66.12
Right Bottom	5,724	86.28	161.13	70.11	108.70	53.72	104.60	65.52
Left Top	11	0.17	12.56	234.57	11.04	232.34	12.52	220.67
Right Top	126	1.90	268.23	244.74	156.86	239.47	171.54	224.68

24/03

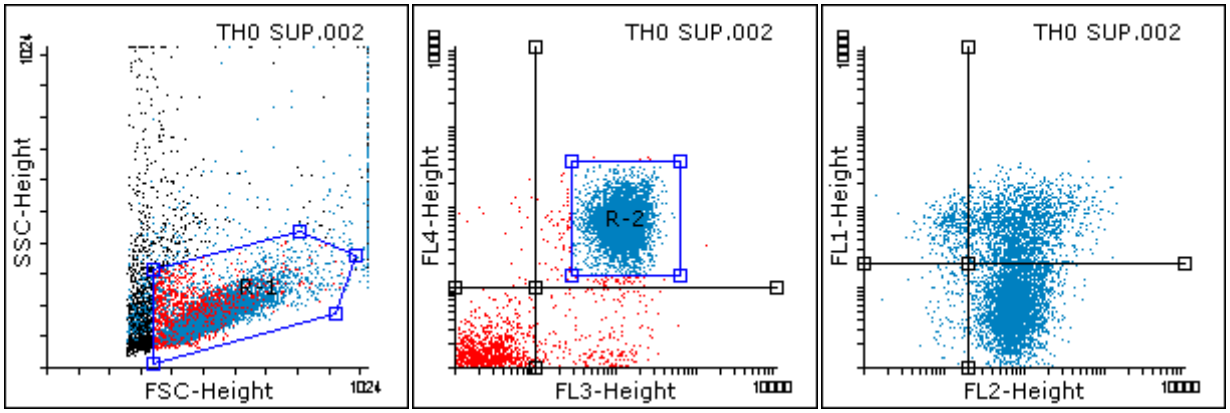
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	480.50	184.99	462.81	162.19	457.00	151.00
R-1	8,057	80.57	504.88	157.00	494.73	147.72	474.00	142.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,057	100.00	1.78	1.40	1.47	1.31	1.24	1.25
Left Bottom	8,017	99.50	1.63	1.37	1.45	1.31	1.24	1.25
Right Bottom	35	0.43	19.56	1.49	17.11	1.33	14.20	1.09
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	5	0.06	113.38	49.02	104.79	39.95	85.05	35.87

- *Th0*

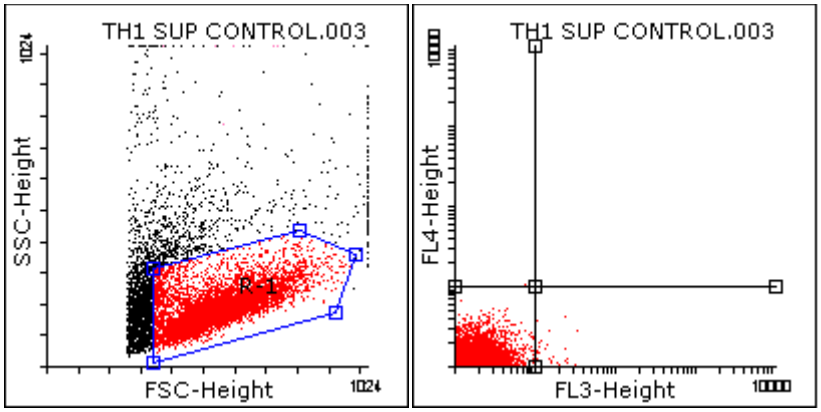


	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	487.44	189.64	467.79	161.79	456.00	148.00
R-1	8,073	80.73	496.80	157.05	487.57	147.52	468.00	140.00
R-1 AND R-2	6,085	60.85	503.29	153.05	494.18	144.22	469.00	134.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,073	100.00	104.44	54.17	56.63	26.14	106.50	51.40
R-2	6,085	75.37	127.76	69.88	118.17	61.58	120.79	60.43
R-1 AND R-2	6,085	75.37	127.76	69.88	118.17	61.58	120.79	60.43
Left Bottom	1,395	17.28	2.80	1.71	2.27	1.52	2.15	1.37
Right Bottom	444	5.50	117.54	1.91	72.13	1.46	86.60	1.00
Left Top	24	0.30	4.93	79.01	3.80	50.20	5.38	67.93
Right Top	6,209	76.91	126.74	69.60	115.85	60.70	120.79	59.89

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	6,085	100.00	95.27	29.97	71.74	12.80	74.32	10.00
R-1 AND R-2	6,085	100.00	95.27	29.97	71.74	12.80	74.32	10.00
Left Bottom	103	1.69	12.62	7.73	11.65	5.80	12.41	5.99
Right Bottom	3,760	61.79	85.47	6.66	75.79	5.33	74.32	5.33
Left Top	299	4.91	12.63	64.32	11.88	58.23	12.63	60.98
Right Top	1,923	31.60	131.70	71.41	93.95	58.53	89.77	59.35

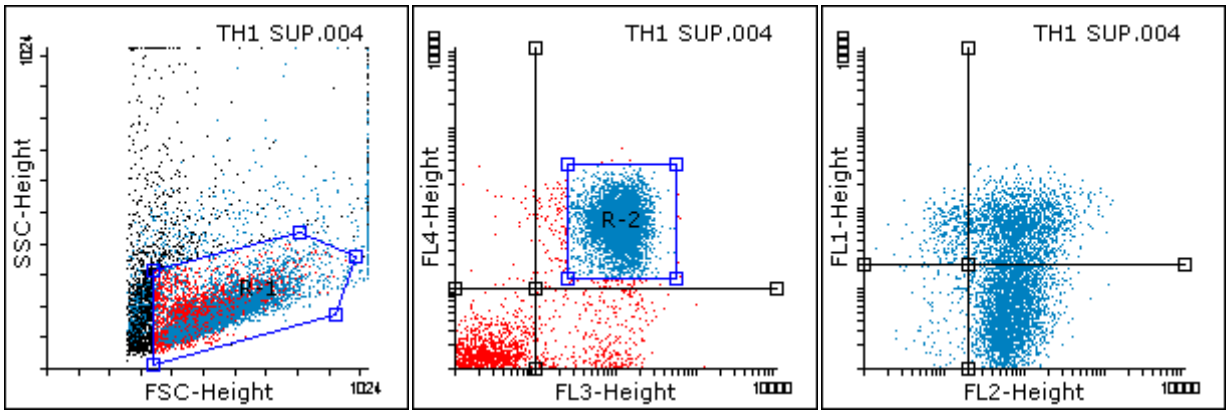
- *Th1 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	490.04	209.17	469.19	183.32	467.00	177.00
R-1	7,589	75.89	522.90	176.88	510.98	166.52	498.00	167.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	7,589	100.00	1.85	1.38	1.58	1.32	1.37	1.25
Left Bottom	7,552	99.51	1.78	1.38	1.56	1.32	1.37	1.25
Right Bottom	36	0.47	16.42	1.62	15.27	1.48	14.20	1.27
Left Top	1	0.01	7.50	10.55	7.50	10.55	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th1*



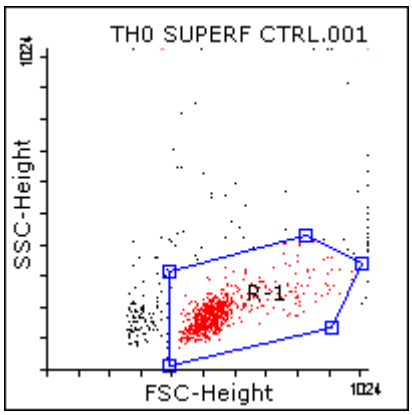
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	497.90	208.92	474.80	179.15	466.00	171.00
R-1	7,677	76.77	513.67	173.04	502.35	162.31	488.00	161.00
R-1 AND R-2	5,621	56.21	526.09	170.68	514.93	160.71	499.00	156.00

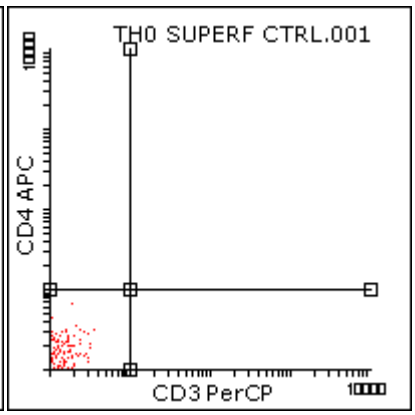
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	7,677	100.00	99.14	53.14	55.82	24.41	100.90	47.83
R-2	5,621	73.22	122.18	69.50	111.71	59.71	117.57	58.29
R-1 AND R-2	5,621	73.22	122.18	69.50	111.71	59.71	117.57	58.29
Left Bottom	1,221	15.90	2.89	1.74	2.35	1.55	2.23	1.38
Right Bottom	639	8.32	93.70	2.06	70.93	1.54	82.79	1.01
Left Top	43	0.56	5.24	85.90	3.90	58.22	5.23	71.69
Right Top	5,773	75.20	120.80	69.43	108.32	58.97	116.52	57.77

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,621	100.00	87.56	30.86	66.12	12.63	67.32	11.14
R-1 AND R-2	5,621	100.00	87.56	30.86	66.12	12.63	67.32	11.14
Left Bottom	109	1.94	11.44	7.52	9.96	5.31	11.24	5.94
Right Bottom	3,352	59.63	77.76	6.48	68.18	4.84	65.52	4.91
Left Top	260	4.63	12.28	62.95	11.36	55.98	12.30	59.35
Right Top	1,900	33.80	119.52	70.80	88.83	58.75	85.05	59.89

28/04

- *Th0 control*

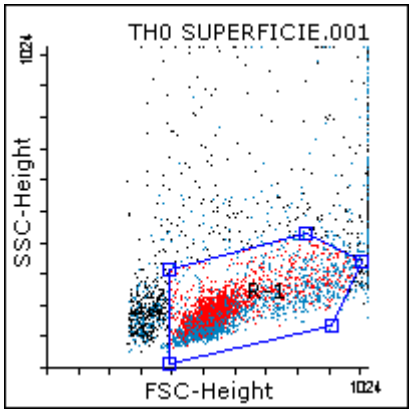


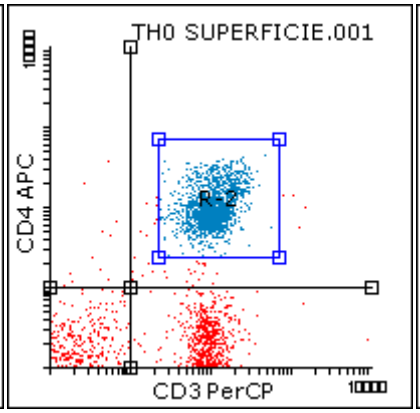


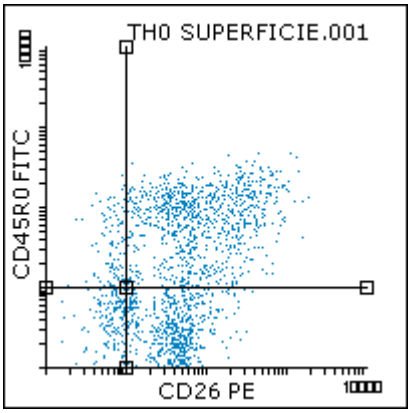
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	930	100.00	537.14	207.30	517.60	186.61	520.00	178.00
R-1	752	80.86	551.16	182.36	544.01	175.29	527.00	176.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	752	100.00	1.33	1.26	1.24	1.18	1.01	1.00
Left Bottom	750	99.73	1.30	1.26	1.23	1.18	1.01	1.00
Right Bottom	2	0.27	13.05	1.00	12.69	1.00	10.00	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th0*





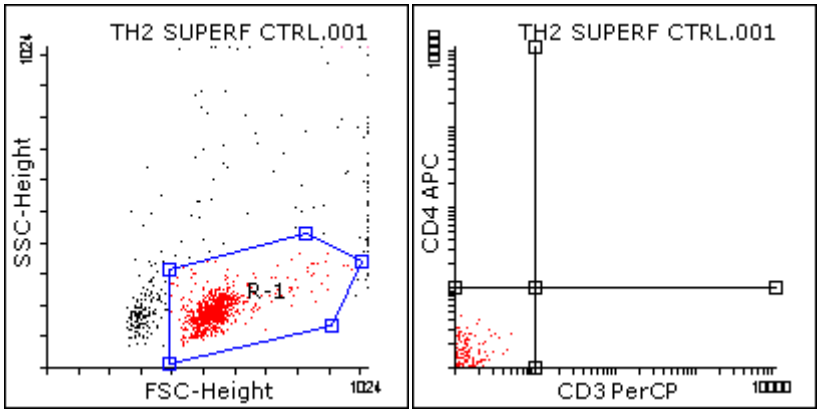


	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,565	100.00	606.28	250.72	574.41	207.61	533.00	181.00
R-1	4,078	73.28	567.20	179.48	556.35	170.33	529.00	168.00
R-1 AND R-2	1,738	31.23	578.89	169.09	564.62	157.59	526.00	152.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	4,078	100.00	90.69	49.69	47.85	8.76	87.38	3.02
R-2	1,738	42.62	115.56	112.90	104.53	98.15	106.50	89.77
R-1 AND R-2	1,738	42.62	115.56	112.90	104.53	98.15	106.50	89.77
Left Bottom	717	17.58	2.37	1.65	1.97	1.38	1.81	1.00
Right Bottom	1,571	38.52	102.45	1.61	88.69	1.35	89.77	1.00
Left Top	15	0.37	3.09	86.33	2.48	43.40	2.62	44.11
Right Top	1,774	43.50	116.68	111.41	103.11	95.55	105.54	88.96

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,738	100.00	102.48	53.05	44.73	16.24	45.32	14.59
R-1 AND R-2	1,738	100.00	102.48	53.05	44.73	16.24	45.32	14.59
Left Bottom	162	9.32	6.79	4.47	6.33	3.70	6.98	4.07
Right Bottom	603	34.70	52.28	3.41	41.19	2.69	43.32	2.64
Left Top	105	6.04	6.95	50.28	6.60	34.53	7.10	33.68
Right Top	864	49.71	167.24	97.33	86.37	68.66	72.34	83.54

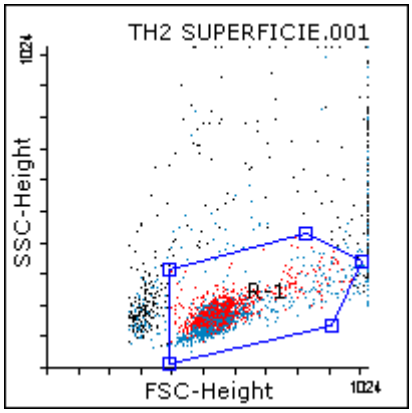
- *Th2 control*

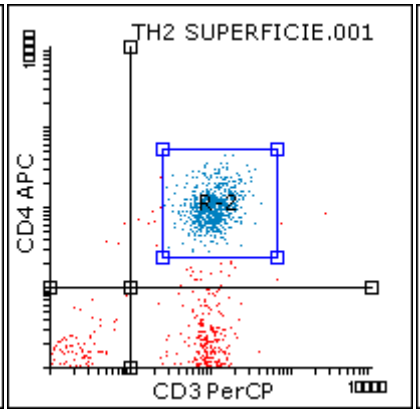


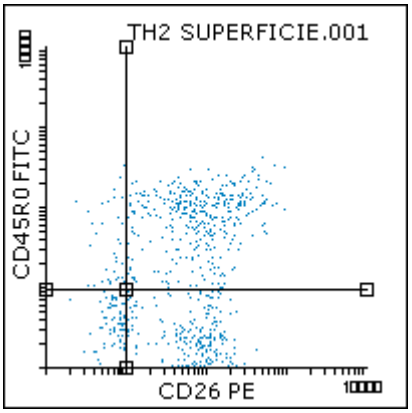
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,155	100.00	536.57	225.33	515.82	198.19	526.00	182.00
R-1	889	76.97	540.31	183.31	535.77	177.73	531.00	179.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	889	100.00	1.28	1.23	1.22	1.16	1.00	1.00
Left Bottom	889	100.00	1.28	1.23	1.22	1.16	1.00	1.00
Right Bottom	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th2*







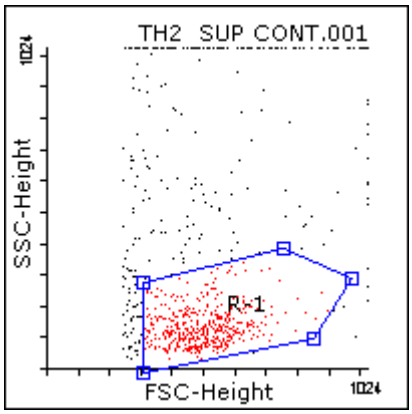
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	2,325	100.00	586.10	225.48	559.28	194.08	541.00	174.00
R-1	1,766	75.96	565.68	171.59	557.69	164.81	541.00	166.00
R-1 AND R-2	739	31.78	571.51	160.22	561.23	151.79	537.00	153.00

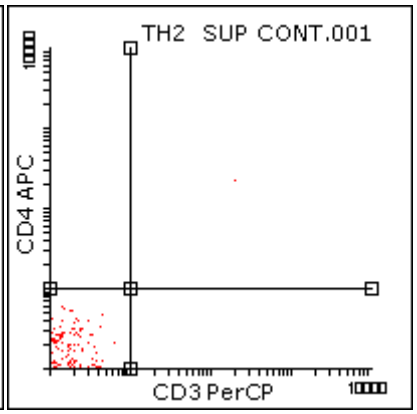
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,766	100.00	91.99	47.86	50.07	8.37	91.40	2.46
R-2	739	41.85	115.76	110.60	106.31	99.00	108.43	97.34
R-1 AND R-2	739	41.85	115.76	110.60	106.31	99.00	108.43	97.34
Left Bottom	291	16.48	1.94	1.49	1.66	1.30	1.46	1.00
Right Bottom	702	39.75	99.79	1.53	92.39	1.27	93.06	1.00
Left Top	4	0.23	5.35	37.17	4.13	31.55	4.87	36.52
Right Top	768	43.49	119.48	107.88	105.35	94.01	107.46	95.60

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	739	100.00	98.84	51.91	51.22	14.78	68.54	12.41
R-1 AND R-2	739	100.00	98.84	51.91	51.22	14.78	68.54	12.41
Left Bottom	87	11.77	7.14	4.40	6.85	3.79	7.04	3.89
Right Bottom	254	34.37	91.49	2.77	70.57	2.16	85.82	2.04
Left Top	36	4.87	6.71	59.56	6.34	33.41	7.10	23.08
Right Top	362	48.99	135.19	97.04	81.66	72.84	82.05	89.77

08/05

- *Th2 control*

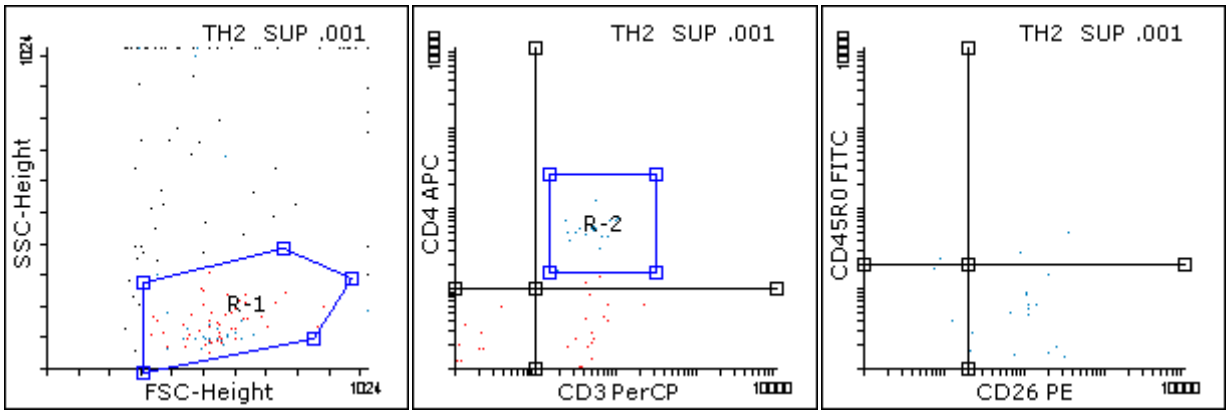




	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	840	100.00	550.01	388.17	510.47	240.27	498.00	177.00
R-1	484	57.62	497.42	135.27	487.23	123.16	494.00	122.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	484	100.00	1.94	1.89	1.39	1.29	1.11	1.00
Left Bottom	483	99.79	1.51	1.42	1.37	1.28	1.11	1.00
Right Bottom	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	1	0.21	205.35	226.71	205.35	226.71	0.00	0.00

- *Th2*



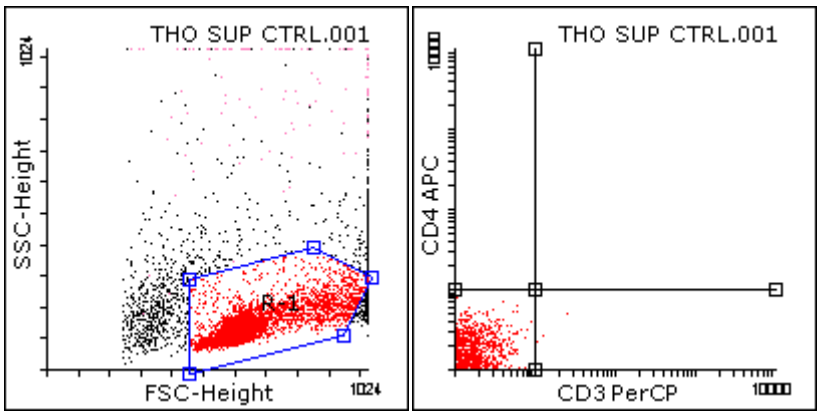
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	180	100.00	624.82	547.98	571.88	364.69	550.00	392.00
R-1	73	40.56	543.86	140.47	534.55	129.71	528.00	126.00
R-1 AND R-2	22	12.22	525.59	105.23	520.72	102.23	518.00	98.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	73	100.00	35.53	20.03	11.89	5.53	30.78	2.84
R-2	22	30.14	61.13	59.97	54.03	56.03	54.74	56.74
R-1 AND R-2	22	30.14	61.13	59.97	54.03	56.03	54.74	56.74
Left Bottom	29	39.73	1.42	2.06	1.32	1.67	1.00	1.26
Right Bottom	20	27.40	55.10	2.86	47.25	2.25	44.91	2.17
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	24	32.88	60.45	56.07	53.86	49.62	54.74	56.23

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	22	100.00	119.53	10.78	75.33	6.09	108.43	5.52
R-1 AND R-2	22	100.00	119.53	10.78	75.33	6.09	108.43	5.52
Left Bottom	2	9.09	10.06	10.85	9.78	8.47	7.70	4.07
Right Bottom	15	68.18	131.57	4.84	105.23	3.55	109.41	4.70
Left Top	1	4.55	8.90	23.29	8.90	23.29	0.00	0.00
Right Top	4	18.18	156.78	29.89	101.82	27.82	88.96	21.10

12/05

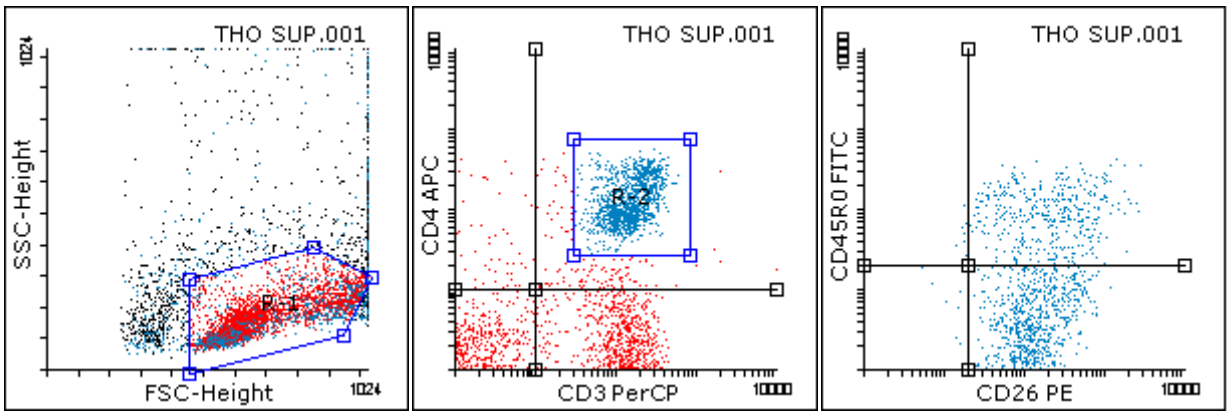
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,190	100.00	742.07	238.26	701.40	199.31	696.00	194.00
R-1	3,138	60.46	710.92	166.05	694.14	154.05	651.00	156.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,138	100.00	1.76	1.43	1.51	1.29	1.27	1.00
Left Bottom	3,130	99.75	1.73	1.43	1.51	1.29	1.27	1.00
Right Bottom	8	0.25	13.59	1.86	12.98	1.53	10.94	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th0*

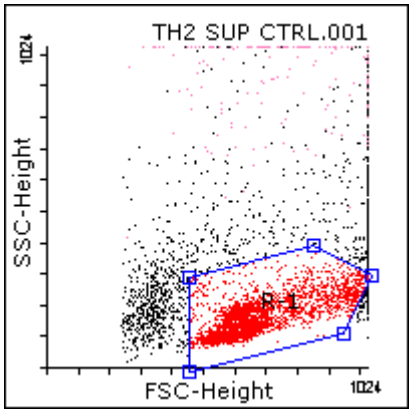


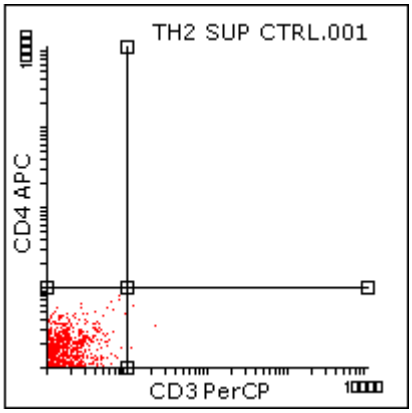
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,685	100.00	748.69	253.20	710.60	208.39	700.00	203.00
R-1	3,523	61.97	708.33	172.25	690.53	159.36	644.00	163.00
R-1 AND R-2	1,155	20.32	711.55	159.47	689.55	144.21	624.00	134.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,523	100.00	115.81	53.76	40.22	7.83	99.10	2.92
R-2	1,155	32.78	165.10	149.49	144.13	123.25	147.22	108.43
R-1 AND R-2	1,155	32.78	165.10	149.49	144.13	123.25	147.22	108.43
Left Bottom	859	24.38	2.34	1.98	1.87	1.60	1.65	1.15
Right Bottom	1,300	36.90	138.12	2.05	113.71	1.57	122.98	1.00
Left Top	80	2.27	2.77	74.47	2.06	35.22	1.75	22.27
Right Top	1,281	36.36	176.46	139.77	131.92	105.17	140.75	100.90

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,155	100.00	230.52	40.73	147.94	10.57	137.00	7.70
R-1 AND R-2	1,155	100.00	230.52	40.73	147.94	10.57	137.00	7.70
Left Bottom	10	0.87	13.95	6.25	12.92	4.18	14.86	2.81
Right Bottom	743	64.33	176.33	5.15	134.82	3.51	128.64	3.37
Left Top	7	0.61	17.34	55.12	17.26	50.03	18.11	49.14
Right Top	395	34.20	341.70	108.28	194.67	83.88	218.70	87.38

- *Th2 control*

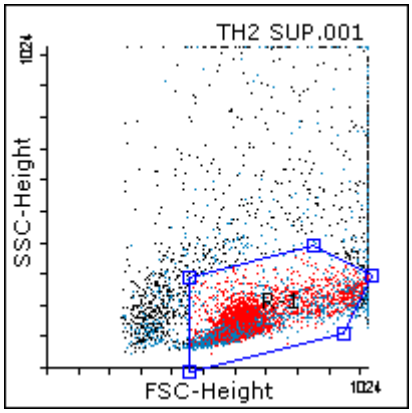


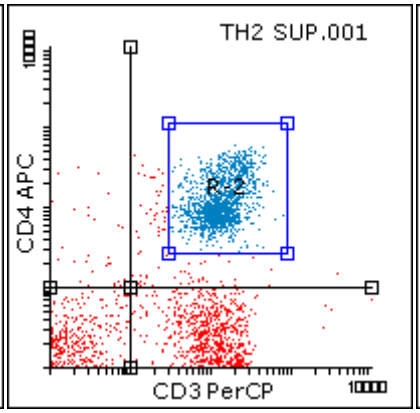


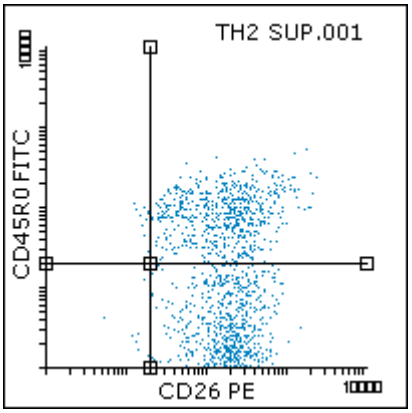
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,565	100.00	744.35	266.79	701.73	219.22	683.00	219.00
R-1	3,108	55.85	701.52	171.07	684.20	158.30	637.00	157.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,108	100.00	1.69	1.39	1.47	1.26	1.22	1.00
Left Bottom	3,103	99.84	1.67	1.39	1.47	1.26	1.22	1.00
Right Bottom	5	0.16	17.54	2.62	16.82	2.04	12.19	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th2*







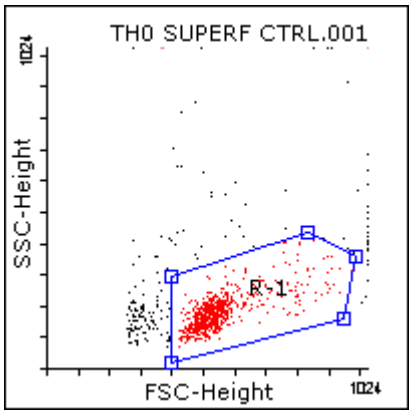
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	6,810	100.00	756.06	253.78	717.55	210.87	688.00	207.00
R-1	3,935	57.78	694.78	168.41	678.11	156.50	634.00	157.00
R-1 AND R-2	1,386	20.35	697.89	157.90	677.02	143.14	622.00	128.00

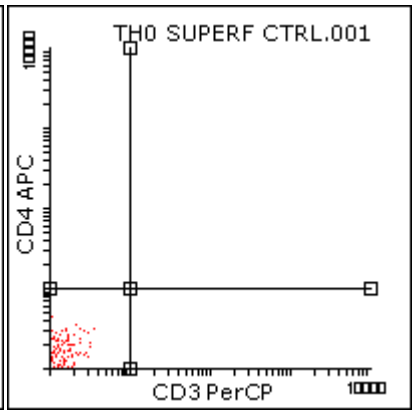
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,935	100.00	107.36	55.07	39.91	7.99	90.58	2.89
R-2	1,386	35.22	148.55	146.25	129.59	119.08	127.49	101.82
R-1 AND R-2	1,386	35.22	148.55	146.25	129.59	119.08	127.49	101.82
Left Bottom	938	23.84	2.12	1.88	1.73	1.55	1.45	1.12
Right Bottom	1,485	37.74	138.51	2.04	105.79	1.59	106.50	1.00
Left Top	44	1.12	3.02	79.79	2.25	38.52	2.05	22.07
Right Top	1,466	37.26	146.40	142.15	121.03	111.54	125.21	99.10

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,386	100.00	252.84	48.62	177.52	12.47	201.69	11.14
R-1 AND R-2	1,386	100.00	252.84	48.62	177.52	12.47	201.69	11.14
Left Bottom	8	0.58	13.52	3.75	12.81	2.90	13.82	2.50
Right Bottom	765	55.19	242.17	4.22	194.38	2.72	209.08	2.23
Left Top	10	0.72	16.48	76.36	16.34	71.11	16.40	79.86
Right Top	603	43.51	273.47	105.08	170.45	85.13	187.69	86.60

19/05

- *Th0 control*

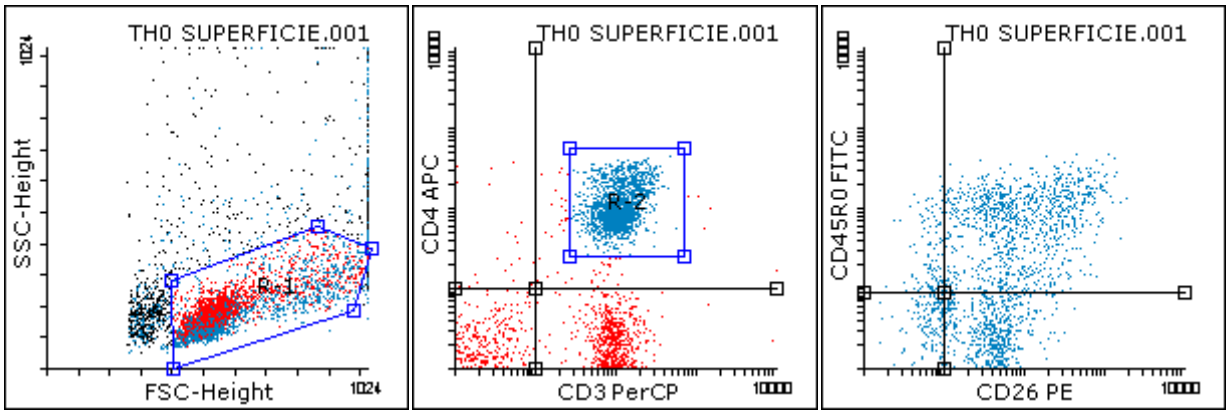




	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	930	100.00	537.14	207.30	517.60	186.61	520.00	178.00
R-1	747	80.32	551.44	182.32	544.53	175.33	528.00	176.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	747	100.00	1.33	1.26	1.23	1.17	1.01	1.00
Left Bottom	745	99.73	1.29	1.26	1.23	1.17	1.01	1.00
Right Bottom	2	0.27	13.05	1.00	12.69	1.00	10.00	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th0*

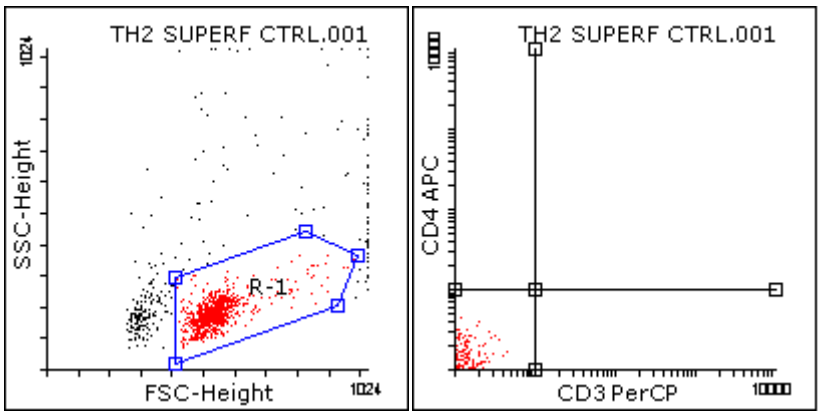


	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,565	100.00	606.28	250.72	574.41	207.61	533.00	181.00
R-1	4,221	75.85	586.01	186.49	571.15	175.31	532.00	170.00
R-1 AND R-2	1,798	32.31	598.55	177.51	580.28	163.36	530.00	155.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	4,221	100.00	95.51	53.18	49.16	9.19	88.17	3.25
R-2	1,798	42.60	118.75	119.20	107.12	102.44	108.43	92.22
R-1 AND R-2	1,798	42.60	118.75	119.20	107.12	102.44	108.43	92.22
Left Bottom	720	17.06	2.36	1.66	1.97	1.39	1.83	1.00
Right Bottom	1,621	38.40	105.02	1.63	90.41	1.36	90.58	1.00
Left Top	26	0.62	3.48	100.27	2.58	46.19	2.62	23.71
Right Top	1,853	43.90	124.64	117.65	104.90	99.57	106.50	91.40

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,798	100.00	114.15	55.90	48.35	17.28	47.83	15.96
R-1 AND R-2	1,798	100.00	114.15	55.90	48.35	17.28	47.83	15.96
Left Bottom	146	8.12	6.83	4.03	6.40	3.43	6.98	3.65
Right Bottom	585	32.54	52.03	3.22	41.21	2.60	43.32	2.57
Left Top	117	6.51	6.95	45.56	6.61	29.49	7.10	22.47
Right Top	950	52.84	182.10	97.58	93.02	66.64	81.31	82.79

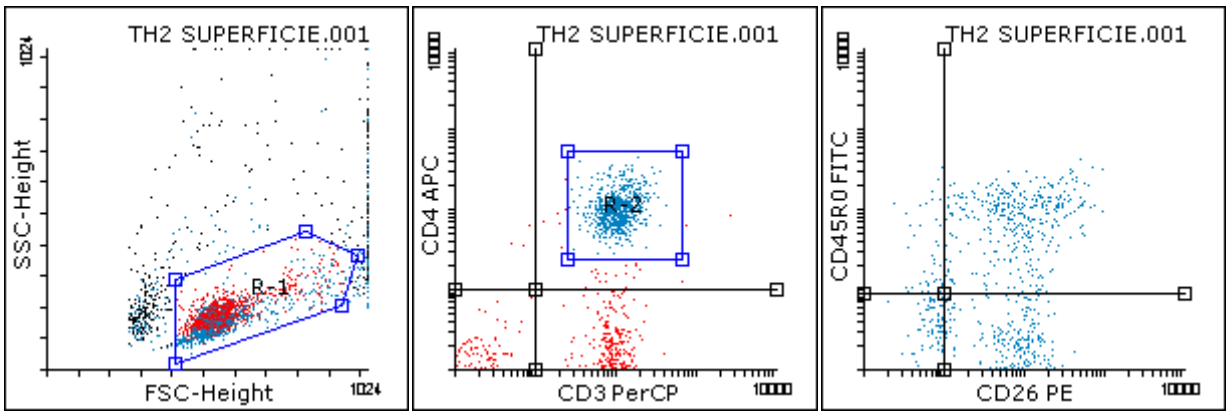
- *Th2 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,155	100.00	536.57	225.33	515.82	198.19	526.00	182.00
R-1	878	76.02	541.19	182.18	536.92	176.80	531.00	179.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	878	100.00	1.28	1.23	1.21	1.16	1.00	1.00
Left Bottom	878	100.00	1.28	1.23	1.21	1.16	1.00	1.00
Right Bottom	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th2*



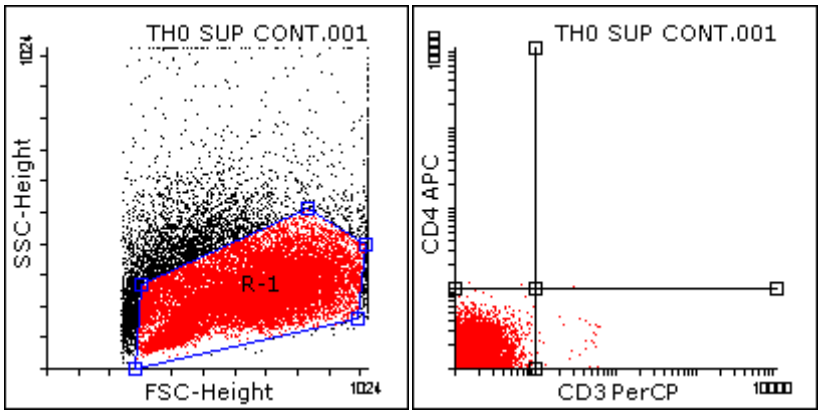
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	2,325	100.00	586.10	225.48	559.28	194.08	541.00	174.00
R-1	1,750	75.27	565.97	171.35	558.33	164.65	541.00	166.00
R-1 AND R-2	733	31.53	571.07	159.78	561.15	151.58	537.00	153.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	1,750	100.00	91.88	47.70	49.96	8.34	91.40	2.39
R-2	733	41.89	115.55	110.20	106.17	98.90	108.43	97.34
R-1 AND R-2	733	41.89	115.55	110.20	106.17	98.90	108.43	97.34
Left Bottom	289	16.51	1.92	1.48	1.65	1.29	1.46	1.00
Right Bottom	696	39.77	99.62	1.52	92.35	1.27	93.06	1.00
Left Top	4	0.23	5.35	37.17	4.13	31.55	4.87	36.52
Right Top	760	43.43	119.51	107.66	105.53	94.18	108.43	95.60

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	733	100.00	99.33	52.13	51.31	14.72	67.93	12.19
R-1 AND R-2	733	100.00	99.33	52.13	51.31	14.72	67.93	12.19
Left Bottom	83	11.32	7.20	4.17	6.91	3.63	7.10	3.59
Right Bottom	249	33.97	91.29	2.67	70.36	2.11	85.82	2.00
Left Top	40	5.46	6.64	54.53	6.27	29.40	7.04	20.54
Right Top	361	49.25	136.32	97.01	82.59	71.77	83.54	89.77

02/06

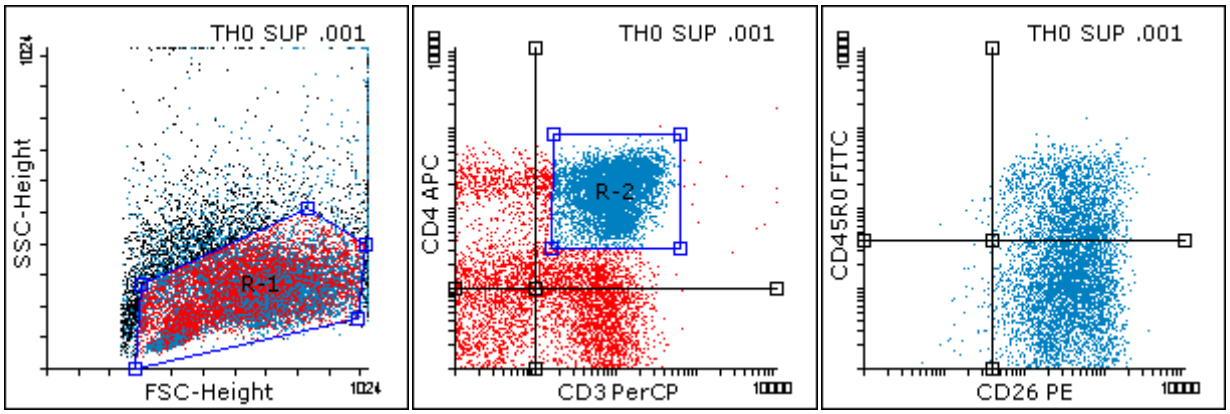
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	22,455	100.00	621.33	269.86	582.20	240.25	632.00	252.00
R-1	17,377	77.39	628.35	233.04	601.35	214.52	652.00	232.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	17,377	100.00	1.93	1.43	1.61	1.29	1.39	1.00
Left Bottom	17,331	99.74	1.84	1.42	1.59	1.29	1.39	1.00
Right Bottom	43	0.25	39.26	1.88	34.01	1.63	46.14	1.45
Left Top	2	0.01	4.17	11.46	3.19	11.44	1.49	10.94
Right Top	1	0.01	29.16	10.55	29.16	10.55	0.00	0.00

- *Th0*

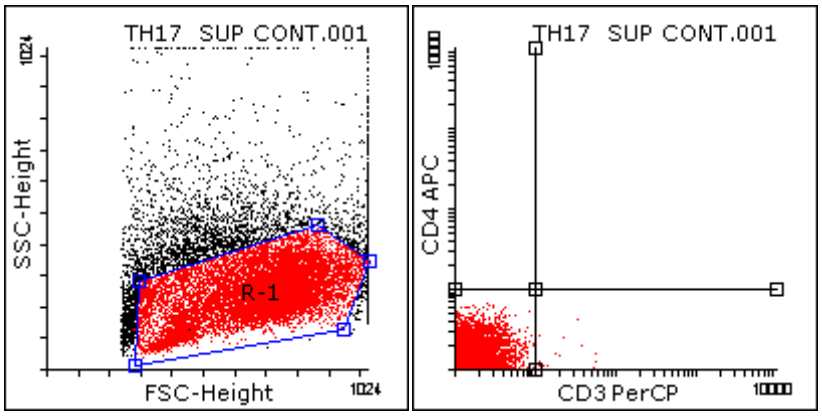


	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	16,665	100.00	651.91	286.36	613.58	252.27	652.00	259.00
R-1	12,584	75.51	635.60	234.85	610.03	217.45	650.00	235.00
R-1 AND R-2	5,797	34.79	646.64	228.43	620.11	209.93	671.00	231.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	12,584	100.00	77.37	112.55	28.44	33.73	55.23	62.08
R-2	5,797	46.07	114.77	196.39	92.00	165.27	94.75	186.01
R-1 AND R-2	5,797	46.07	114.77	196.39	92.00	165.27	94.75	186.01
Left Bottom	1,660	13.19	2.74	3.96	2.05	3.04	1.68	3.28
Right Bottom	2,299	18.27	81.87	3.74	63.40	2.75	68.54	2.84
Left Top	1,730	13.75	2.98	119.34	2.13	56.00	1.64	36.85
Right Top	6,876	54.64	112.75	173.72	79.13	123.09	85.82	162.53

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,797	100.00	488.22	55.89	353.29	16.90	368.47	15.12
R-1 AND R-2	5,797	100.00	488.22	55.89	353.29	16.90	368.47	15.12
Left Bottom	40	0.69	25.58	7.48	23.19	4.92	26.18	5.88
Right Bottom	3,925	67.71	478.31	11.06	353.10	6.94	358.66	7.99
Left Top	17	0.29	30.84	97.51	29.30	88.82	32.49	75.67
Right Top	1,815	31.31	524.14	153.50	384.44	117.10	406.79	106.50

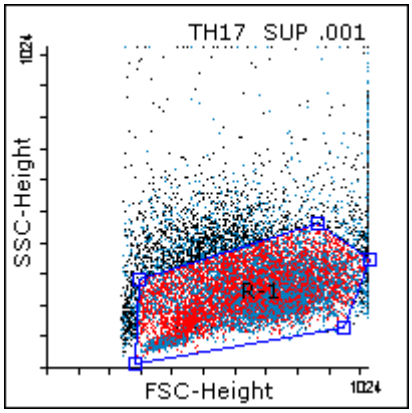
- *Th17 control*

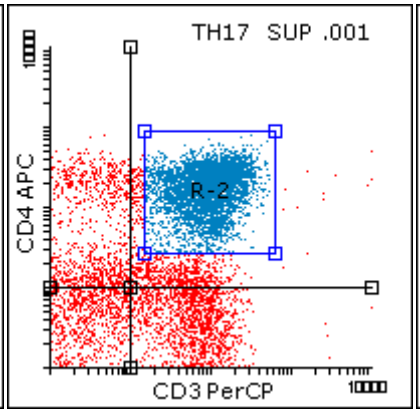


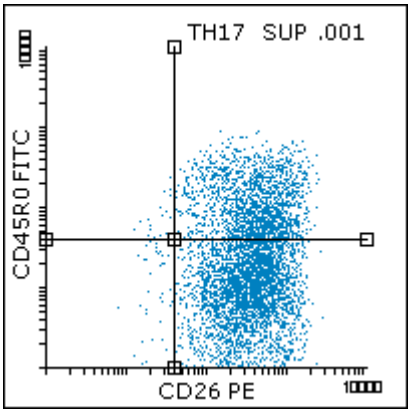
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	14,160	100.00	654.14	288.86	614.06	260.43	676.00	267.00
R-1	10,355	73.13	660.22	242.57	633.35	227.97	691.00	243.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,355	100.00	1.97	1.42	1.66	1.29	1.45	1.00
Left Bottom	10,329	99.75	1.91	1.42	1.65	1.29	1.45	1.00
Right Bottom	26	0.25	25.58	1.38	21.57	1.27	19.81	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

- *Th17*







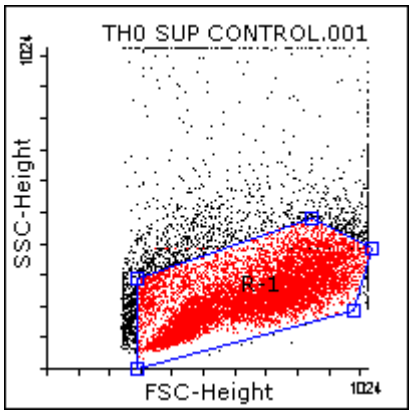
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	13,605	100.00	665.27	283.02	629.05	255.90	671.00	262.00
R-1	10,212	75.06	655.99	238.38	631.02	223.63	674.00	239.00
R-1 AND R-2	4,775	35.10	673.81	233.01	650.13	217.54	696.00	233.00

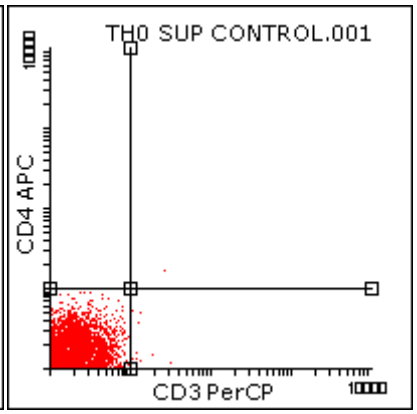
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,212	100.00	76.14	114.07	26.85	33.70	53.76	66.12
R-2	4,775	46.76	110.96	197.68	89.86	168.04	94.75	187.69
R-1 AND R-2	4,775	46.76	110.96	197.68	89.86	168.04	94.75	187.69
Left Bottom	1,443	14.13	2.69	4.27	1.98	3.26	1.50	3.75
Right Bottom	1,863	18.24	88.02	3.53	61.09	2.57	66.12	2.57
Left Top	1,425	13.95	2.82	123.16	2.01	57.77	1.32	45.73
Right Top	5,468	53.54	110.70	178.60	79.48	130.77	87.38	173.09

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	4,775	100.00	478.80	60.35	342.62	19.93	365.17	17.78
R-1 AND R-2	4,775	100.00	478.80	60.35	342.62	19.93	365.17	17.78
Left Bottom	43	0.90	24.49	13.87	23.13	7.87	24.36	8.20
Right Bottom	3,076	64.42	455.75	11.80	334.80	7.85	342.89	8.82
Left Top	19	0.40	26.83	85.12	25.60	73.20	26.42	66.71
Right Top	1,637	34.28	539.29	152.49	395.79	115.84	429.35	103.66

08/06

- *Th0 control*

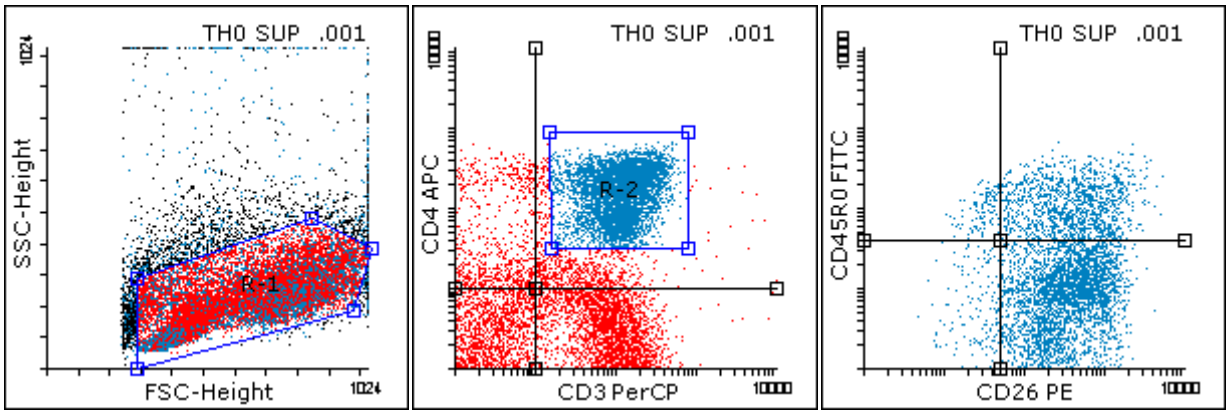




	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,815	100.00	622.61	267.13	582.01	232.09	615.00	240.00
R-1	8,859	81.91	626.73	226.69	595.02	207.17	629.00	223.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,859	100.00	2.17	1.47	1.80	1.31	1.60	1.00
Left Bottom	8,836	99.74	2.14	1.46	1.79	1.31	1.60	1.00
Right Bottom	22	0.25	13.16	1.67	12.68	1.40	11.86	1.00
Left Top	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Right Top	1	0.01	26.42	16.70	26.42	16.70	0.00	0.00

- *Th0*

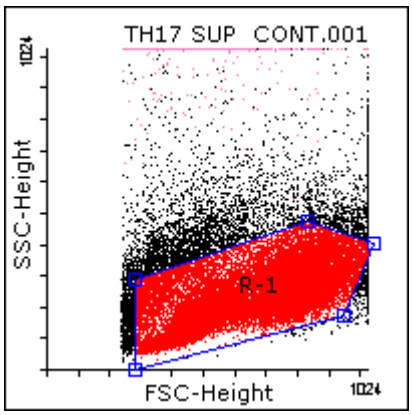


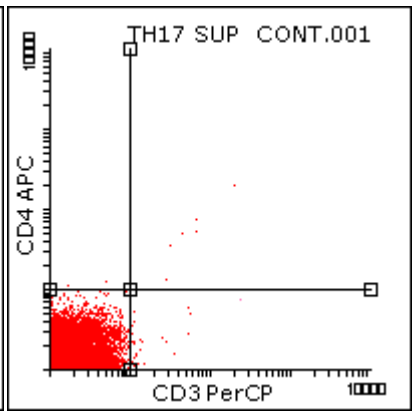
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	16,935	100.00	626.71	255.01	584.56	222.99	615.00	231.00
R-1	14,053	82.98	623.57	221.09	590.44	202.10	620.00	217.00
R-1 AND R-2	4,676	27.61	630.30	208.69	593.16	186.60	659.00	209.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	14,053	100.00	100.01	74.84	29.07	12.45	66.12	8.43
R-2	4,676	33.27	143.05	185.20	113.72	148.52	117.57	164.00
R-1 AND R-2	4,676	33.27	143.05	185.20	113.72	148.52	117.57	164.00
Left Bottom	2,969	21.13	2.63	2.95	2.00	2.17	1.67	1.81
Right Bottom	4,347	30.93	143.87	2.41	95.32	1.76	100.90	1.02
Left Top	1,359	9.67	2.67	99.03	1.86	42.90	1.02	23.71
Right Top	5,362	38.16	143.26	167.44	98.60	117.38	108.43	143.30

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	4,676	100.00	492.36	44.09	306.31	14.47	336.78	12.19
R-1 AND R-2	4,676	100.00	492.36	44.09	306.31	14.47	336.78	12.19
Left Bottom	156	3.34	29.65	9.07	26.12	5.47	30.23	5.09
Right Bottom	3,215	68.76	479.28	10.22	338.67	6.69	342.89	8.13
Left Top	118	2.52	32.58	108.26	30.73	98.43	32.49	98.22
Right Top	1,187	25.38	634.30	134.04	405.38	109.97	441.09	108.43

- *Th17 control*

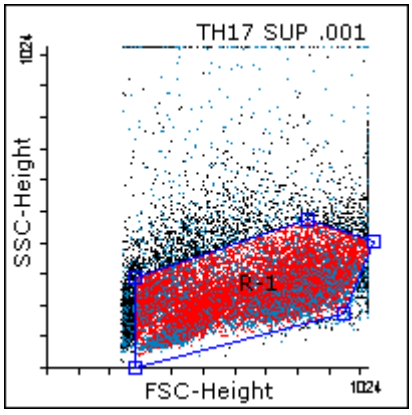


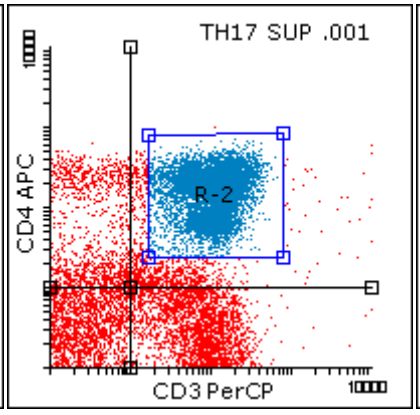


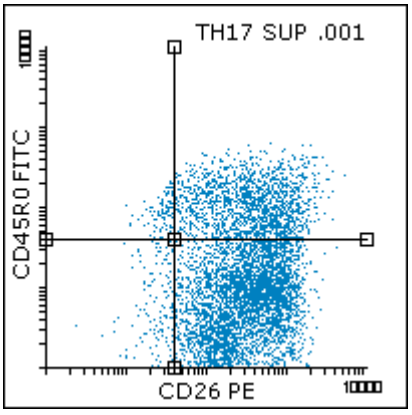
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	29,932	100.00	631.32	275.66	588.13	239.69	629.00	251.00
R-1	23,547	78.67	635.99	230.68	602.18	210.51	652.00	229.00

	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	23,547	100.00	2.10	1.48	1.72	1.31	1.49	1.00
Left Bottom	23,483	99.73	2.04	1.46	1.71	1.31	1.49	1.00
Right Bottom	53	0.23	21.79	2.06	15.48	1.64	11.44	1.20
Left Top	4	0.02	2.41	11.56	2.04	11.52	1.68	11.14
Right Top	7	0.03	63.56	62.02	44.91	41.10	44.11	50.48

- *Th17*







	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	21,615	100.00	624.29	260.93	579.28	229.97	606.00	237.00
R-1	17,428	80.63	622.06	226.18	587.17	208.17	612.00	222.00
R-1 AND R-2	5,767	26.68	619.22	209.08	581.62	188.55	633.00	208.00

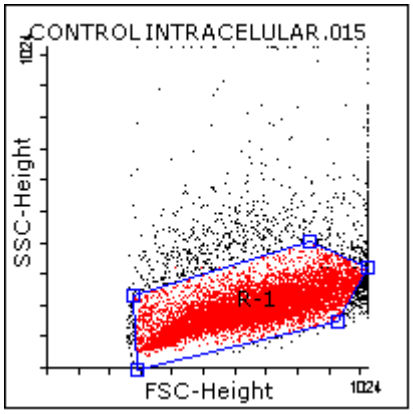
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	17,428	100.00	85.70	77.00	23.64	13.76	53.28	9.65
R-2	5,767	33.09	124.64	180.87	100.29	146.22	106.50	165.48
R-1 AND R-2	5,767	33.09	124.64	180.87	100.29	146.22	106.50	165.48
Left Bottom	3,798	21.79	2.47	3.39	1.88	2.52	1.45	2.46
Right Bottom	4,997	28.67	121.07	2.45	83.21	1.77	91.40	1.00
Left Top	2,058	11.81	2.67	109.63	1.88	48.31	1.09	25.95
Right Top	6,550	37.58	133.32	166.56	87.63	118.95	97.34	148.55

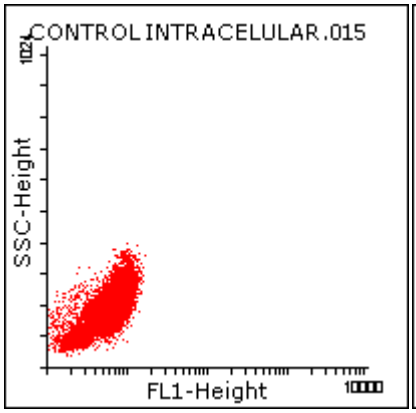
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,767	100.00	459.75	43.35	287.04	13.11	316.23	10.75
R-1 AND R-2	5,767	100.00	459.75	43.35	287.04	13.11	316.23	10.75
Left Bottom	145	2.51	24.80	9.97	22.53	5.62	24.58	4.78
Right Bottom	4,028	69.85	448.02	9.42	309.58	5.85	319.08	6.38
Left Top	116	2.01	29.57	97.14	28.53	90.27	30.23	86.60
Right Top	1,478	25.63	568.16	134.86	359.37	110.17	392.42	106.50

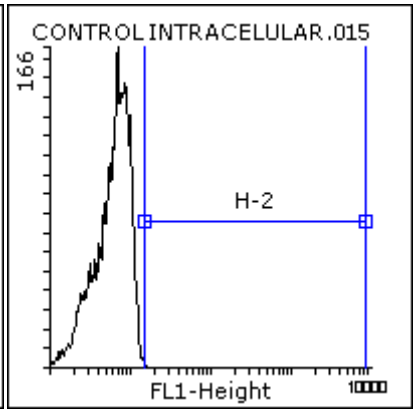
7.2 INTRACELLULAR EXPRESSION ANALYSIS

10/03

- *Th1 control*



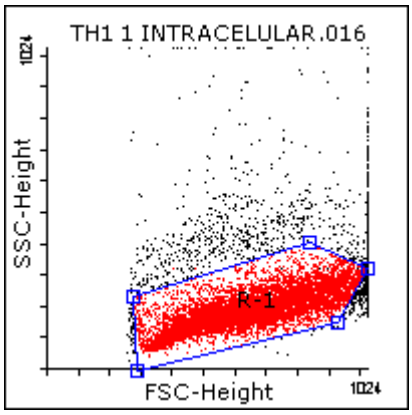


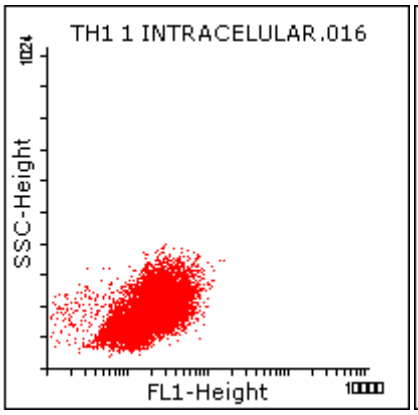


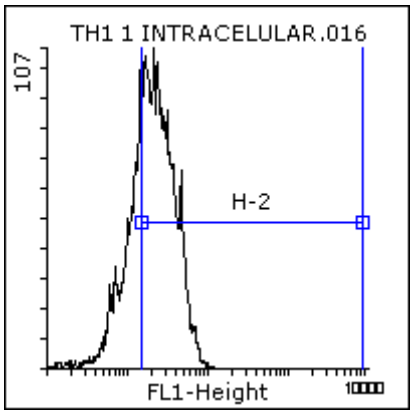
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	676.50	221.48	642.11	199.26	669.00	199.00
R-1	8,275	82.75	639.71	187.90	612.80	177.18	640.00	185.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,275	100.00	6.45	5.77	6.49	42.47
H-2	5	0.06	15.92	15.91	15.40	3.85

- *Th1*



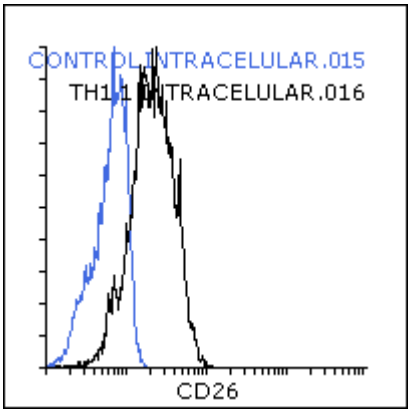




	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	679.54	224.23	646.61	202.01	671.00	201.00
R-1	8,261	82.61	644.17	189.55	618.50	179.22	643.00	187.00

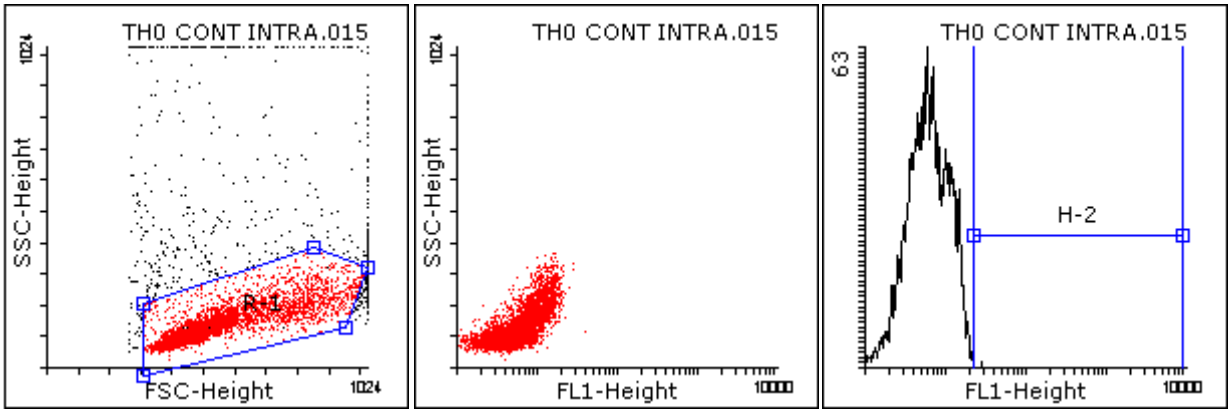
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,261	100.00	23.77	19.55	20.17	62.37
H-2	5,728	69.34	29.87	27.36	25.95	46.23

- *Overlay histogram: Th1 control vs. Th1*



20/03

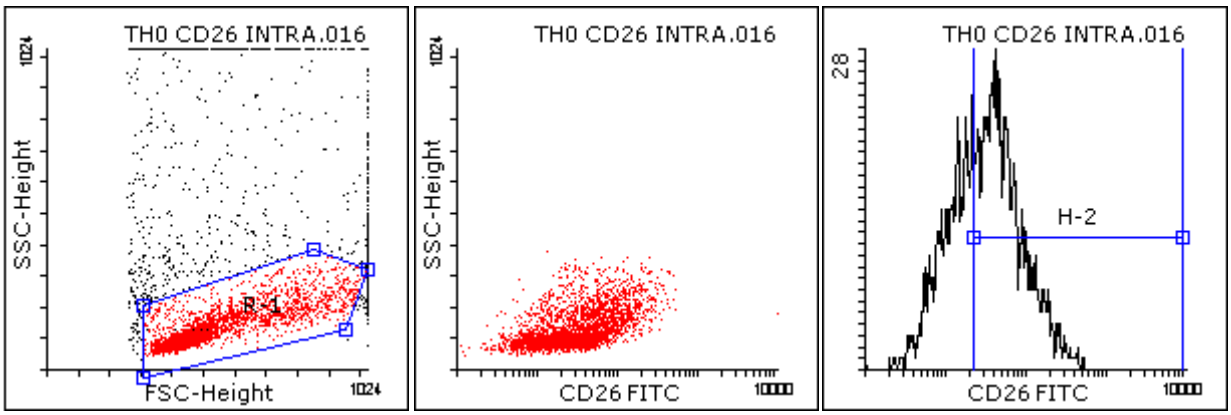
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,115	100.00	573.42	194.78	541.76	150.00	488.00	130.00
R-1	4,300	84.07	542.36	134.89	520.76	123.43	477.00	114.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	4,300	100.00	7.10	6.04	6.04	56.77
H-2	4	0.09	29.88	29.43	27.38	18.10

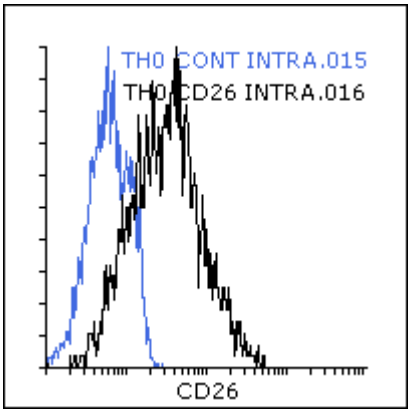
- *Th0*



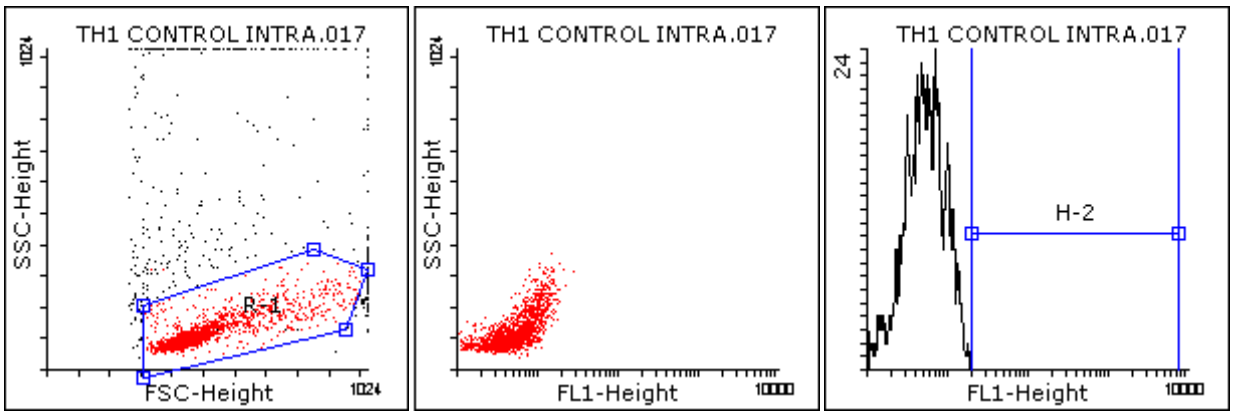
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	4,110	100.00	568.86	268.42	532.53	179.92	478.00	147.00
R-1	2,983	72.58	531.31	131.77	510.15	120.05	468.00	108.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	2,983	100.00	53.38	30.64	31.62	355.95
H-2	1,851	62.05	73.05	56.87	49.58	89.28

- *Overlay histogram: Th0 control vs. Th0*



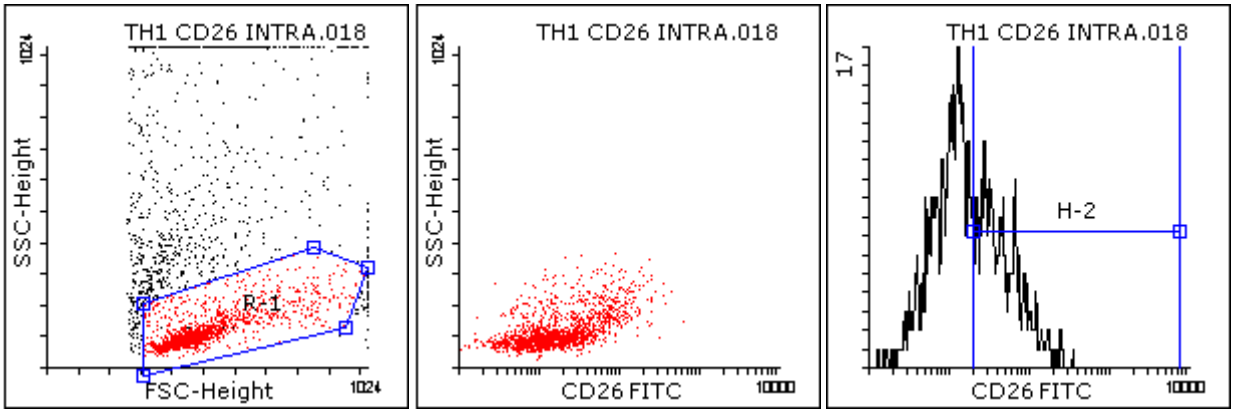
- *Th1 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	2,085	100.00	536.76	219.89	508.70	153.05	471.00	121.00
R-1	1,650	79.14	516.18	123.04	499.17	113.35	468.00	105.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	1,650	100.00	6.06	5.15	5.23	56.92
H-2	3	0.18	24.42	24.22	22.88	13.19

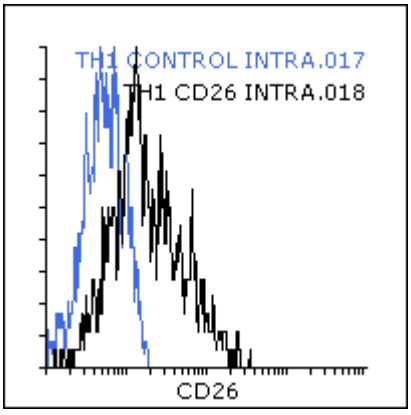
- *Th1*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	2,610	100.00	514.46	332.45	484.45	206.84	457.00	165.00
R-1	1,546	59.23	504.25	118.92	488.85	108.09	462.00	99.00

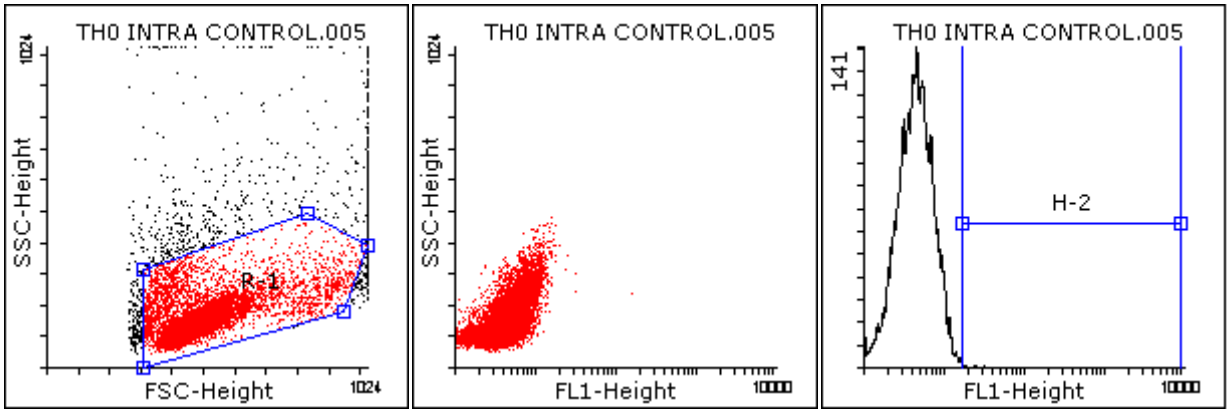
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	1,546	100.00	31.24	17.92	15.40	142.20
H-2	636	41.14	61.33	48.14	42.94	92.65

- *Overlay histogram: Th1 control vs. Th1*



24/03

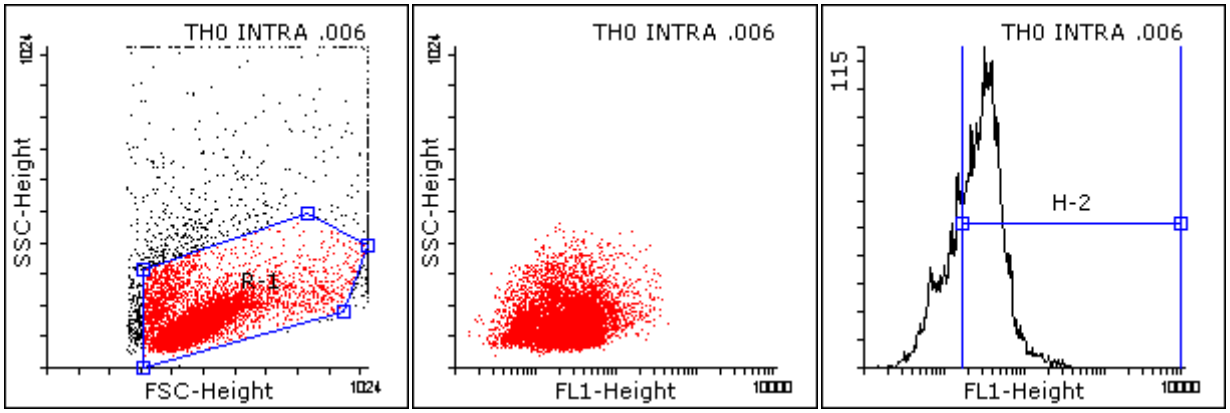
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	520.67	193.92	495.68	159.51	458.00	142.00
R-1	8,758	87.58	488.81	151.77	475.63	139.19	452.00	132.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,758	100.00	4.87	4.28	4.37	61.06
H-2	11	0.13	34.30	25.82	20.17	112.69

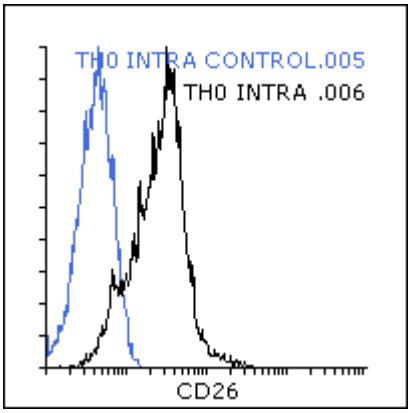
- *Th0*



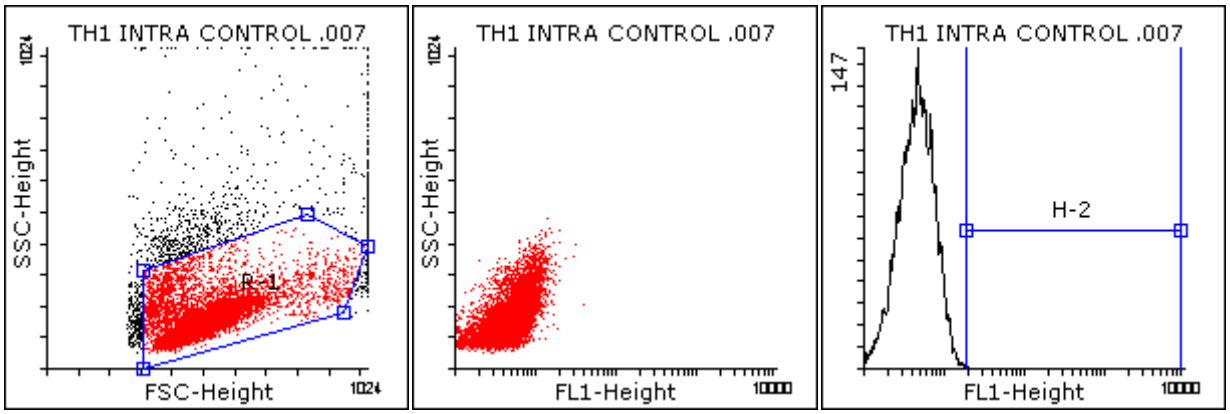
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	493.12	183.94	474.14	150.43	449.00	135.00
R-1	8,918	89.18	475.61	144.91	464.90	132.99	447.00	126.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,918	100.00	33.00	24.77	27.14	92.90
H-2	6,372	71.45	42.03	36.21	34.60	76.12

- *Overlay histogram: Th0 control vs. Th0*



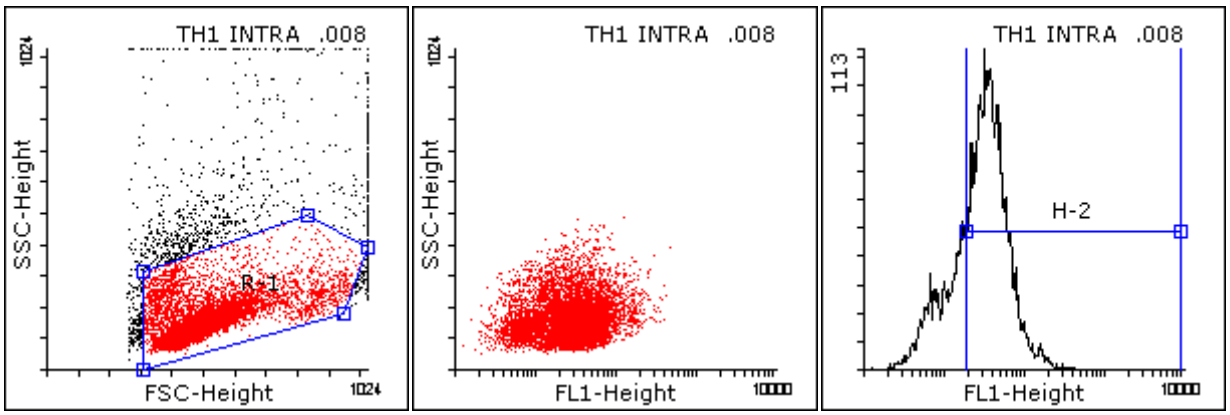
- *Th1 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	513.72	192.89	492.88	163.72	470.00	152.00
R-1	8,761	87.61	494.37	157.33	482.36	145.08	467.00	143.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,761	100.00	5.03	4.44	4.57	51.40
H-2	9	0.10	24.88	24.07	19.99	27.24

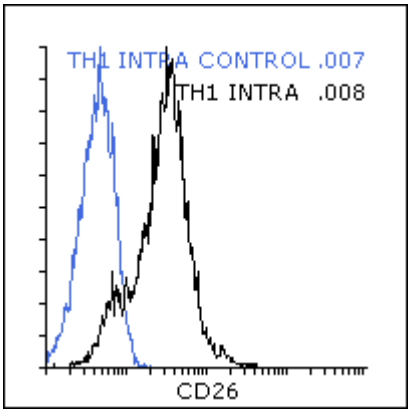
- *Th1*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,000	100.00	527.30	202.13	503.62	166.47	475.00	153.00
R-1	8,645	86.45	500.44	155.60	486.91	143.45	469.00	142.00

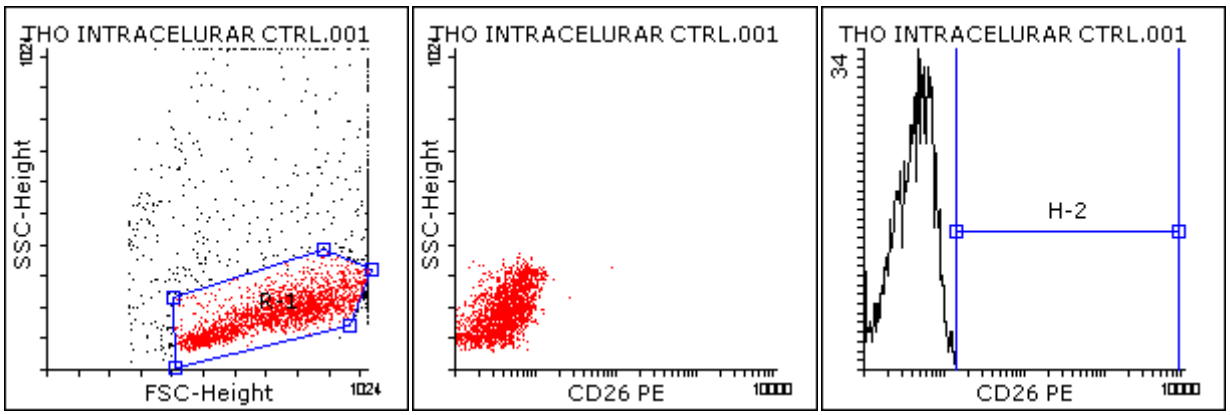
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	8,645	100.00	36.73	27.36	30.51	87.06
H-2	6,295	72.82	46.43	40.35	37.86	69.83

- *Overlay histogram: Th1 control vs. Th1*



28/04

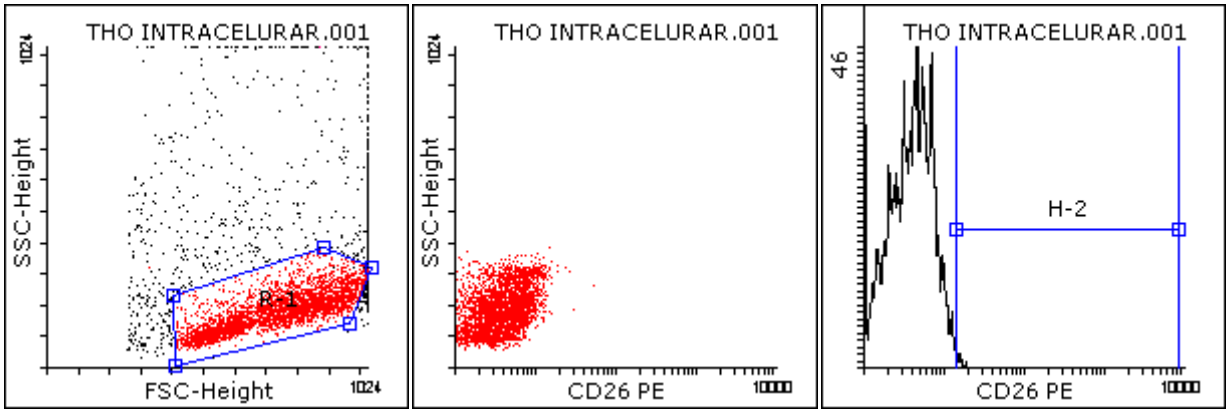
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,375	100.00	788.27	286.45	755.22	234.59	819.00	227.00
R-1	2,204	65.30	741.29	188.31	719.72	175.61	749.00	185.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	2,204	100.00	4.74	4.05	4.37	64.89
H-2	4	0.18	36.96	27.45	16.40	84.64

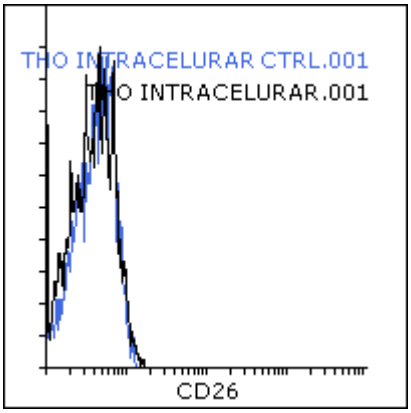
- *Th0*



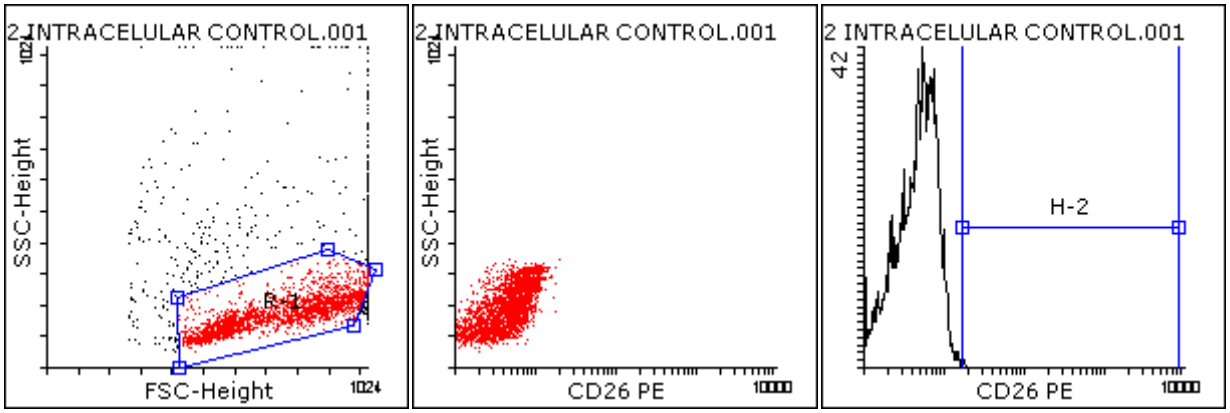
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	5,145	100.00	792.53	280.77	758.21	232.72	830.00	227.00
R-1	3,339	64.90	745.61	189.88	723.72	177.24	762.00	187.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	3,339	100.00	4.45	3.65	3.92	65.12
H-2	19	0.57	19.41	18.16	16.11	46.22

- *Overlay histogram: Th0 control vs. Th0*



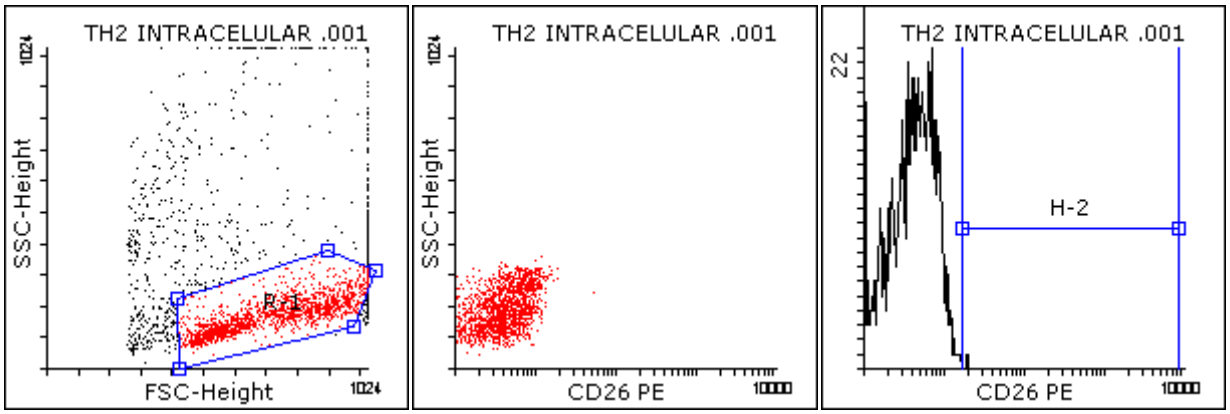
- *Th2 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	3,720	100.00	822.82	252.06	790.93	218.43	888.00	215.00
R-1	2,686	72.20	794.06	194.09	769.03	182.20	830.00	188.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	2,686	100.00	5.11	4.36	4.91	52.43
H-2	5	0.19	17.93	17.91	17.47	4.46

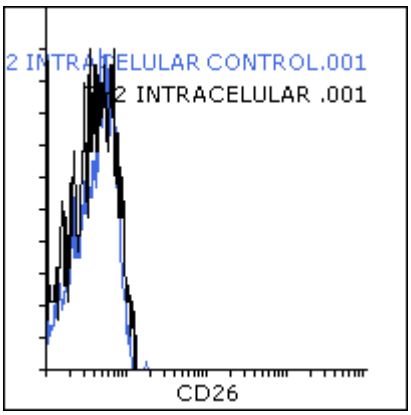
- *Th2*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	2,730	100.00	757.44	290.79	709.66	234.09	794.00	220.00
R-1	1,652	60.51	770.88	190.09	745.56	178.47	782.00	184.00

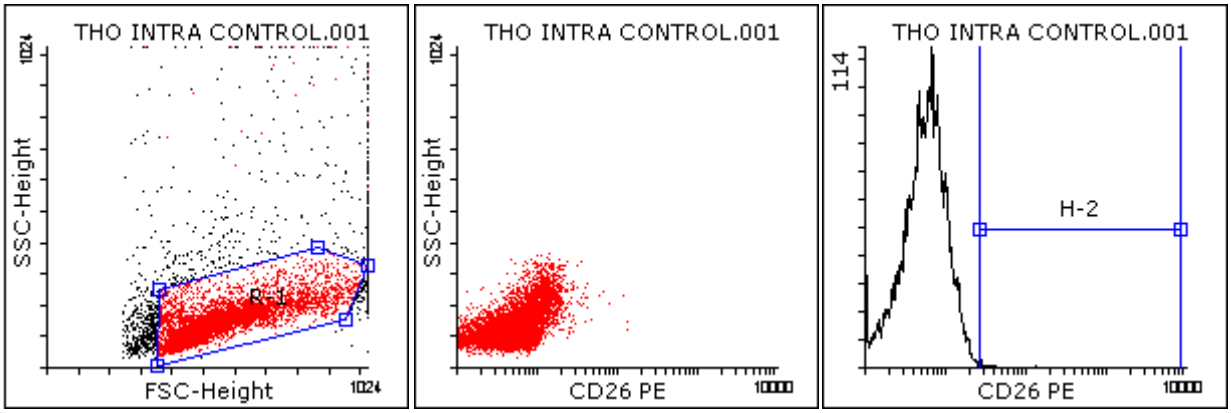
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	1,652	100.00	4.71	3.83	4.18	65.35
H-2	3	0.18	30.72	27.06	19.28	53.05

- *Overlay histogram: Th2 control vs. Th2*



08/05

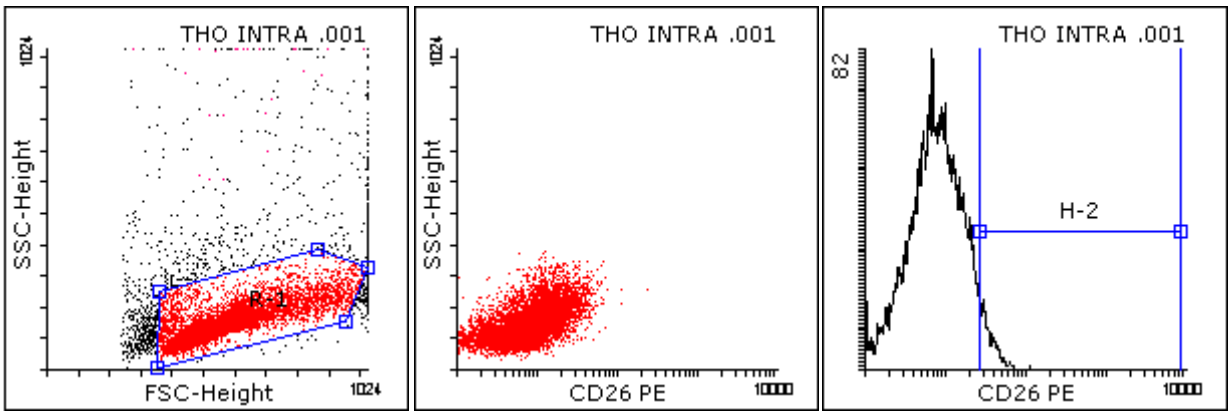
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,835	100.00	554.15	165.57	529.61	139.69	494.00	128.00
R-1	7,551	85.47	531.74	134.61	518.54	125.40	492.00	121.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	7,551	100.00	6.56	5.34	5.73	76.22
H-2	34	0.45	44.99	39.64	34.60	63.57

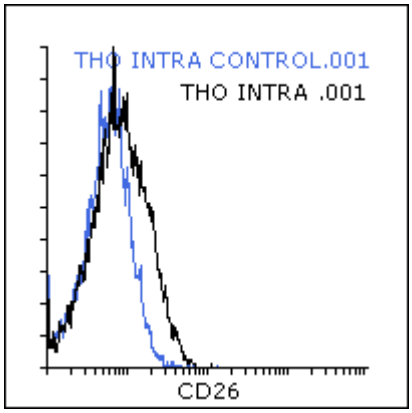
- *Th0*



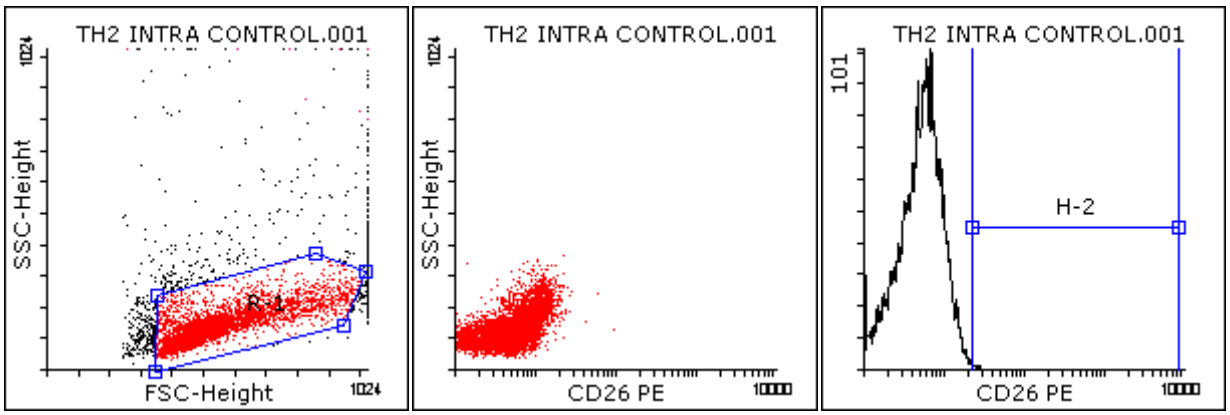
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,475	100.00	600.26	189.55	568.44	158.43	525.00	147.00
R-1	6,809	80.34	564.30	147.24	547.32	136.47	514.00	135.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	6,809	100.00	10.32	7.59	7.77	88.35
H-2	356	5.23	37.06	35.52	33.38	40.19

- *Overlay histogram: Th0 control vs. Th0*



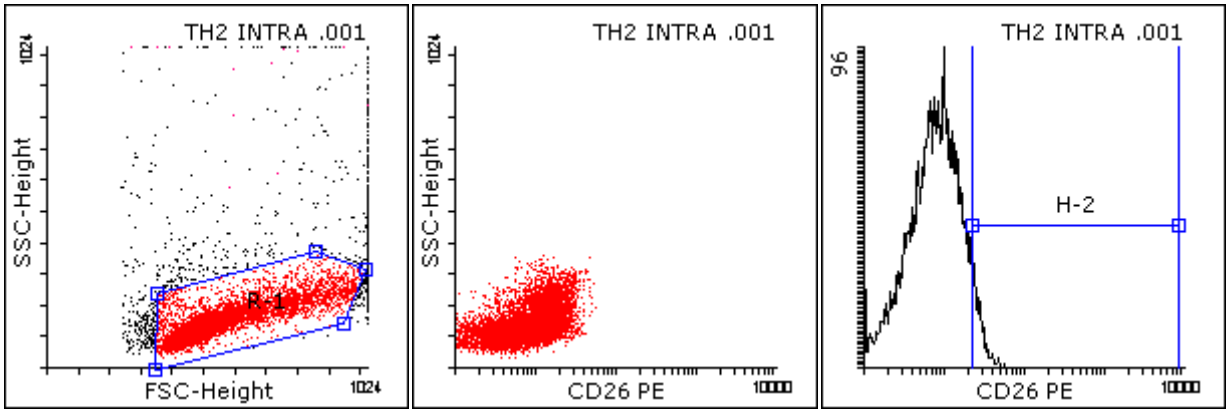
- *Th2 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	7,755	100.00	546.05	154.50	521.47	133.24	481.00	122.00
R-1	6,740	86.91	516.69	127.81	503.90	119.97	478.00	117.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	6,740	100.00	5.93	4.90	5.38	63.28
H-2	14	0.21	32.88	29.58	24.36	57.90

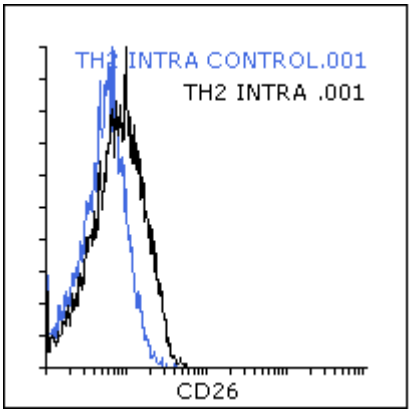
- *Th2*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	9,135	100.00	582.92	176.26	553.22	149.01	496.00	132.00
R-1	7,736	84.69	540.21	138.98	524.26	129.60	488.00	123.00

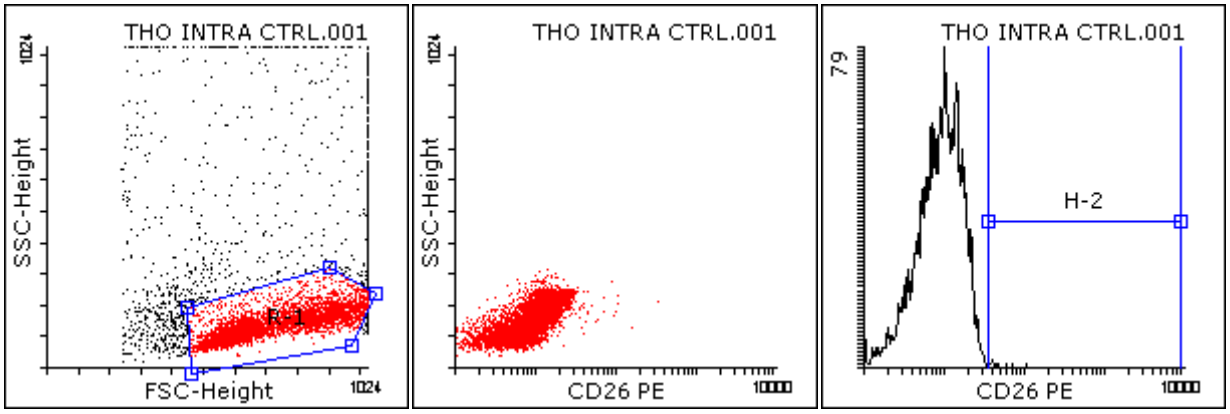
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	7,736	100.00	9.54	7.31	7.77	72.90
H-2	459	5.93	28.21	27.71	26.66	20.64

- *Overlay histogram: Th2 control vs. Th2*



12/05

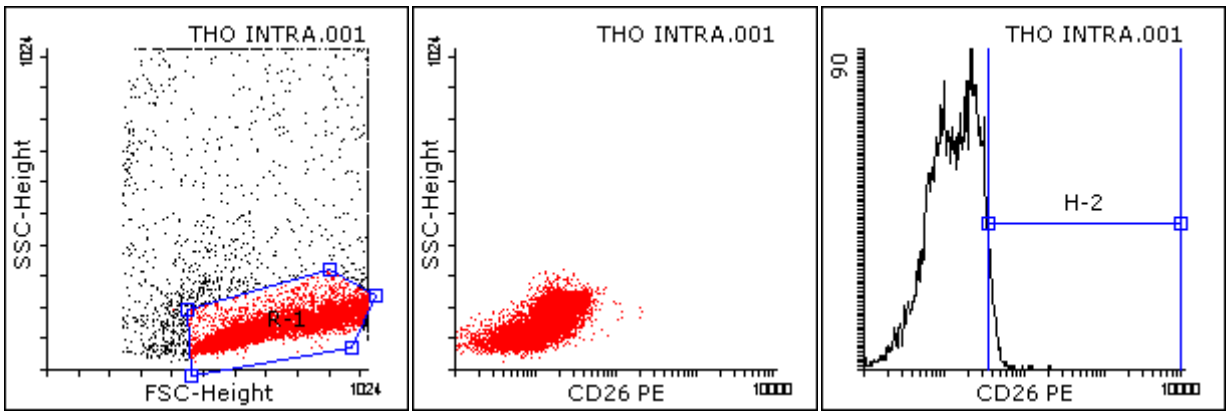
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	7,800	100.00	800.84	228.43	766.70	183.63	849.00	175.00
R-1	5,385	69.04	775.18	152.11	752.87	142.62	754.00	147.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,385	100.00	10.49	8.54	9.31	78.81
H-2	25	0.46	72.78	60.36	44.91	82.72

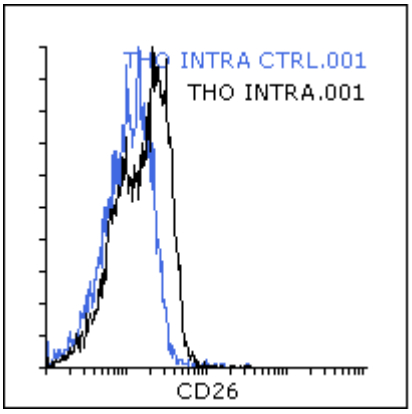
- *Th0*



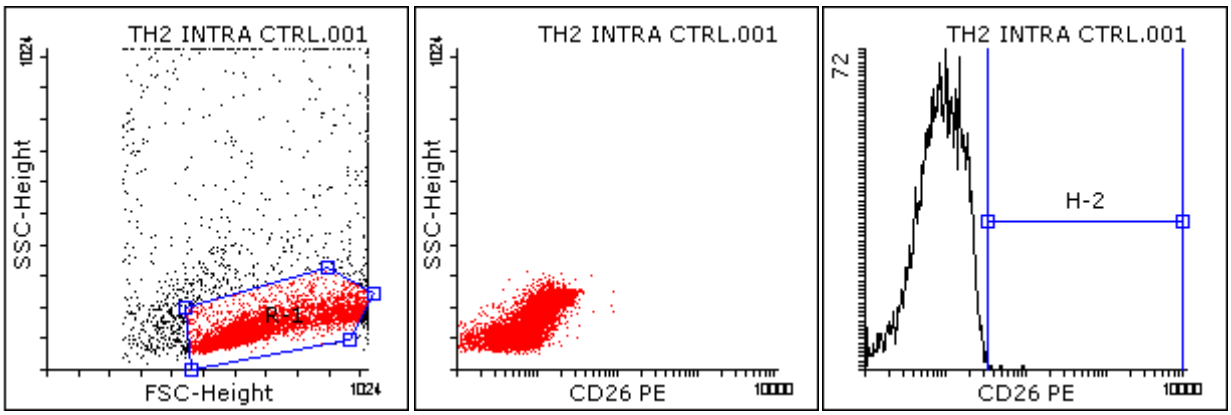
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	11,280	100.00	799.42	232.85	766.21	184.87	839.00	176.00
R-1	7,872	69.79	775.36	151.28	752.92	141.54	757.00	146.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	7,872	100.00	16.08	12.63	13.46	70.12
H-2	387	4.92	44.12	42.43	39.60	40.98

- *Overlay histogram: Th0 control vs. Th0*



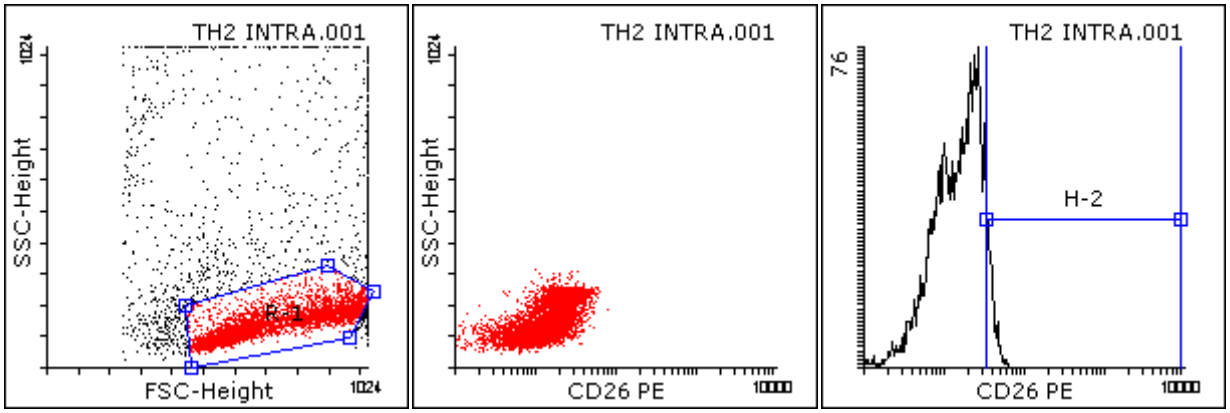
- *Th2 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,560	100.00	834.29	245.66	799.88	203.76	987.00	207.00
R-1	5,978	56.61	762.66	157.40	738.81	146.77	694.00	148.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,978	100.00	10.16	8.20	8.74	63.31
H-2	11	0.18	52.58	48.42	38.20	43.48

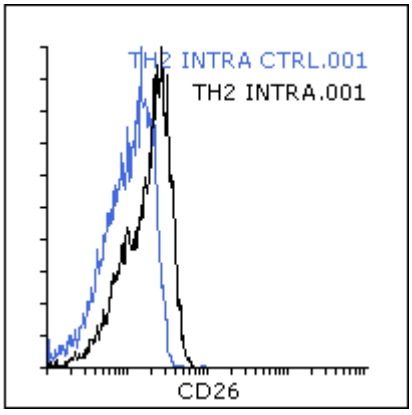
- *Th2*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,425	100.00	850.25	260.30	816.29	213.64	1,023.00	215.00
R-1	5,698	54.66	784.44	161.86	759.79	151.02	767.00	156.00

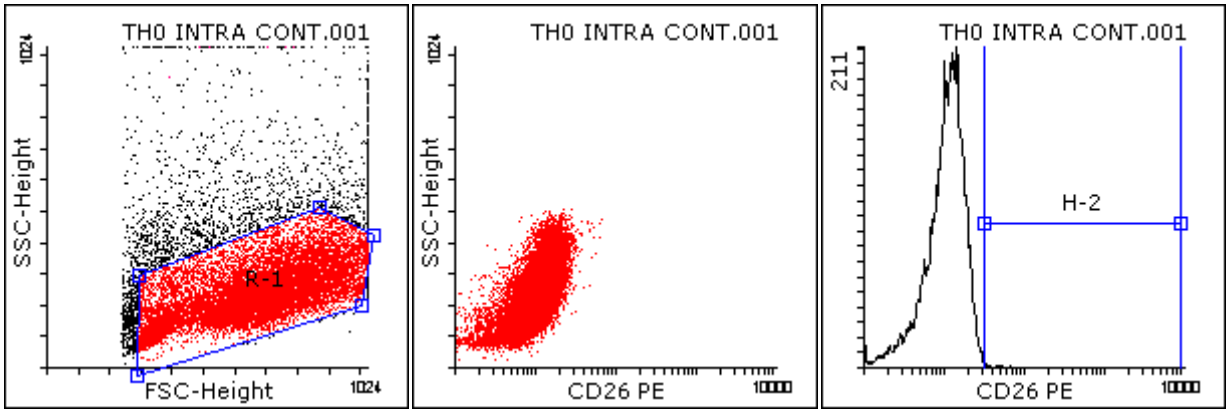
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,698	100.00	17.41	14.10	15.82	59.57
H-2	435	7.63	39.50	39.13	37.86	14.76

- *Overlay histogram: Th2 control vs. Th2*



02/06

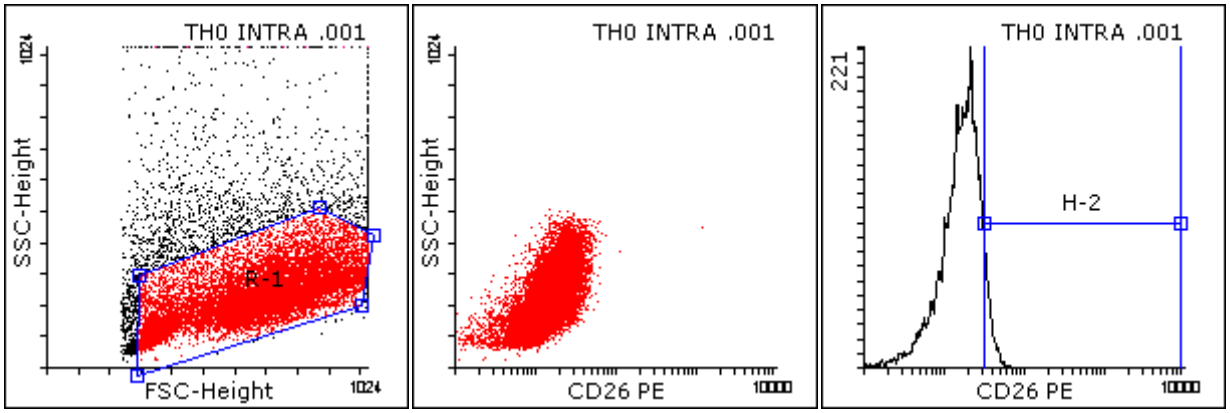
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	14,685	100.00	658.27	278.30	615.64	244.47	676.00	250.00
R-1	12,293	83.71	665.35	241.47	630.41	223.13	684.00	235.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	12,293	100.00	11.53	9.96	11.14	49.01
H-2	33	0.27	41.57	40.40	36.85	25.04

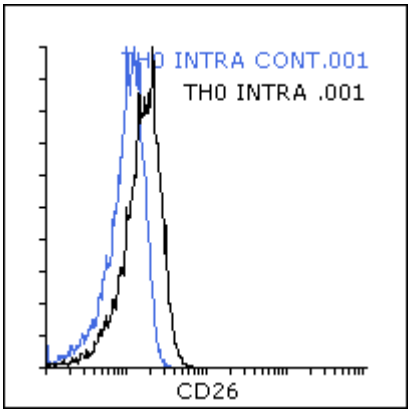
- *Th0*



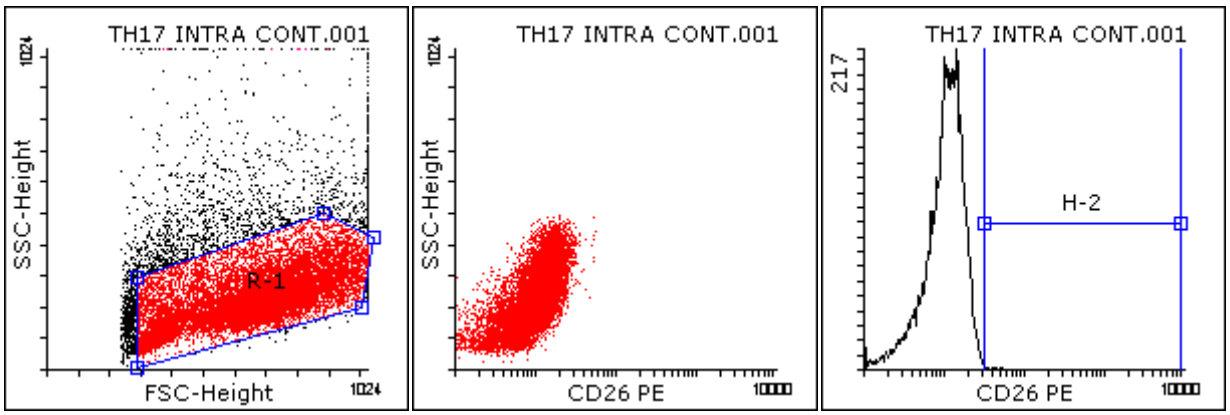
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	14,850	100.00	664.45	274.20	624.57	242.19	682.00	246.00
R-1	12,584	84.74	670.45	238.24	637.74	221.44	688.00	233.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	12,584	100.00	18.13	15.53	17.15	76.41
H-2	973	7.73	38.65	37.16	35.55	97.51

- *Overlay histogram: Th0 control vs. Th0*



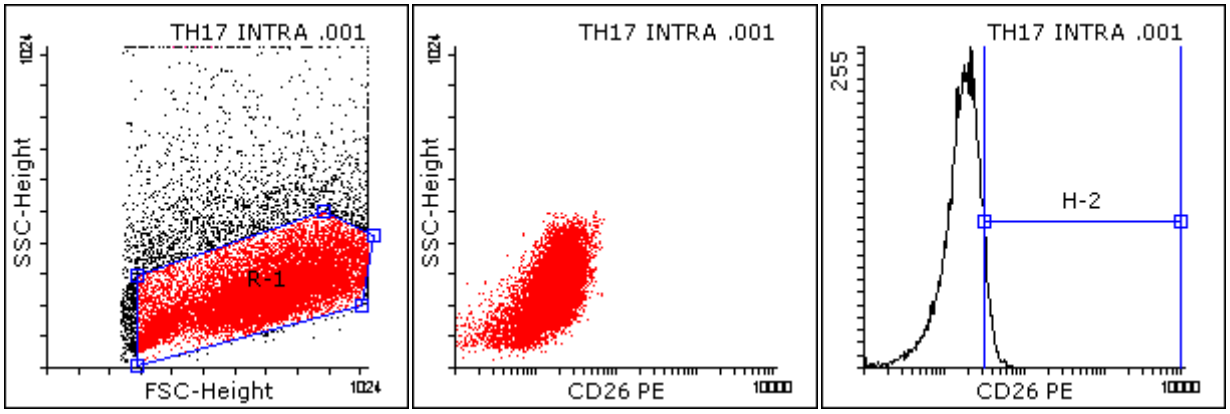
- *Th17 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	15,045	100.00	652.66	266.30	610.68	236.64	670.00	242.00
R-1	12,678	84.27	661.08	235.88	626.94	218.80	679.00	231.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	12,678	100.00	11.31	9.78	10.94	48.43
H-2	32	0.25	39.09	38.32	34.91	20.85

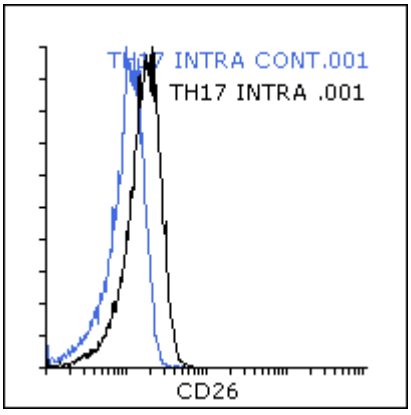
- *Th17*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	18,015	100.00	683.53	284.76	646.03	253.64	700.00	255.00
R-1	15,086	83.74	690.55	246.57	660.76	231.05	705.00	241.00

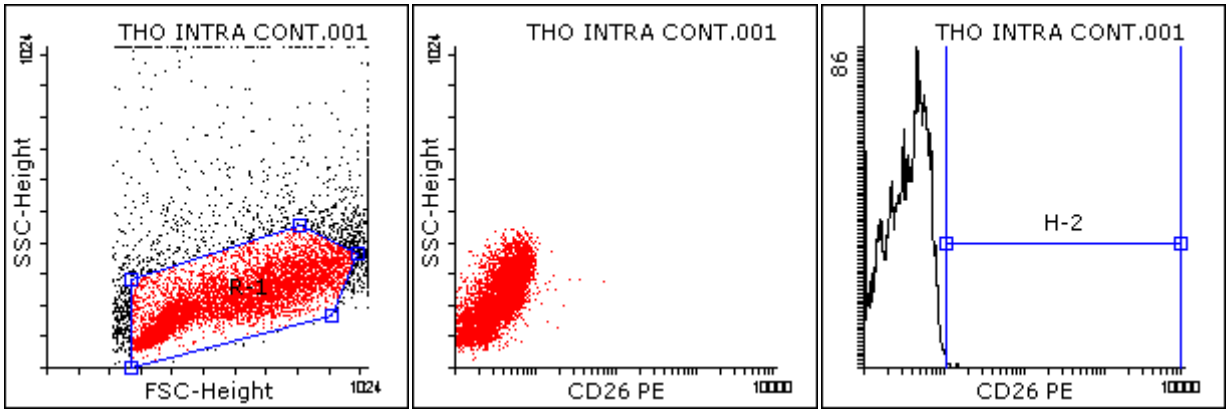
	Events	% of Vis	Mean	GeoMean	Median	CV
All events	15,086	100.00	18.44	16.07	17.47	47.71
H-2	1,161	7.70	37.33	36.91	35.55	16.38

- *Overlay histogram: Th17 control vs. Th17*



08/06

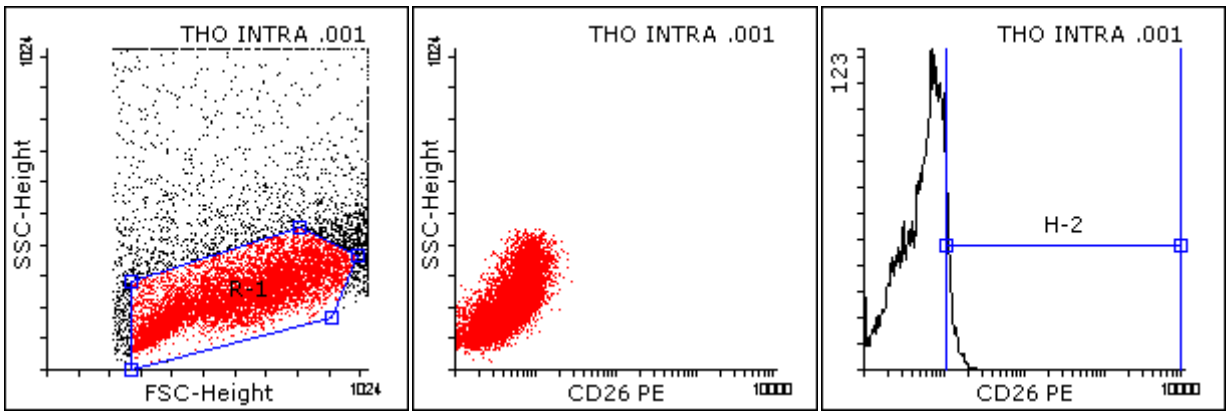
- *Th0 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	7,335	100.00	618.00	278.84	567.11	239.31	615.00	249.00
R-1	5,553	75.71	585.15	221.76	549.23	203.63	581.00	221.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,553	100.00	3.87	3.27	3.59	60.27
H-2	19	0.34	18.84	16.28	14.46	72.14

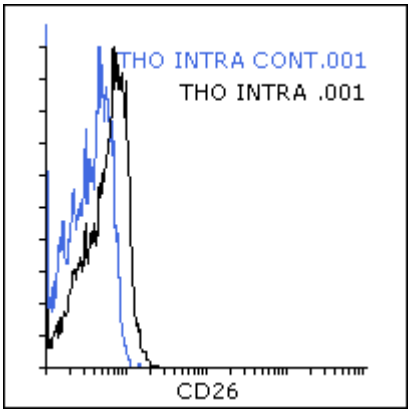
- *Th0*



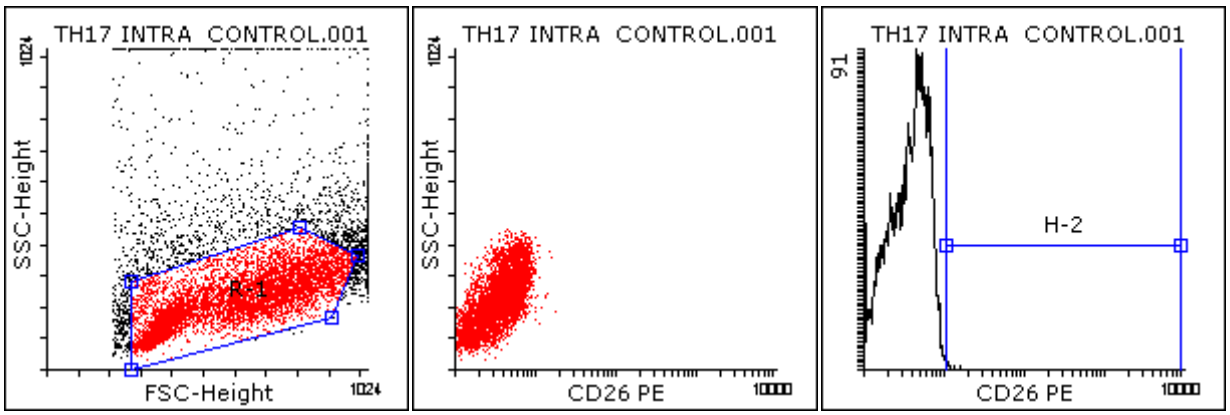
	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	10,740	100.00	630.59	317.75	582.44	268.58	631.00	270.00
R-1	7,852	73.11	586.51	232.63	552.94	215.37	594.00	231.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	7,852	100.00	6.10	5.12	5.94	54.07
H-2	669	8.52	12.61	12.44	11.76	18.21

- *Overlay histogram: Th0 control vs. Th0*



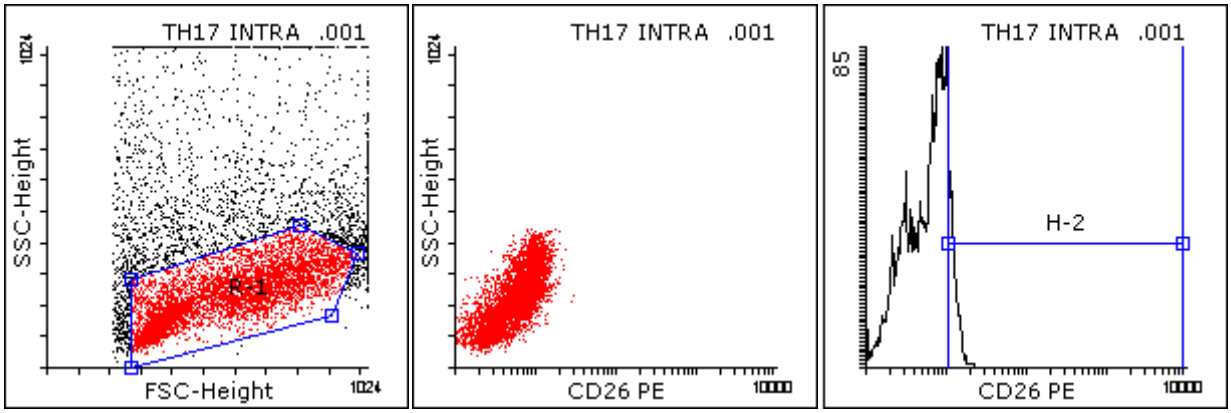
- *Th17 control*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,160	100.00	639.42	302.17	587.99	258.38	651.00	267.00
R-1	5,823	71.36	600.56	227.51	564.19	210.02	616.00	226.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,823	100.00	4.03	3.48	3.85	50.57
H-2	14	0.24	12.77	12.60	11.34	16.67

- *Th17*



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	8,145	100.00	583.73	343.34	532.83	278.50	564.00	278.00
R-1	5,370	65.93	564.22	225.44	528.59	206.72	558.00	224.00

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	5,370	100.00	6.51	5.48	6.38	53.91
H-2	668	12.44	12.61	12.47	11.97	16.54

- *Overlay histogram: Th17 control vs. Th17*